Interorgan protein and glutamine metabolism in the tumor bearing rat

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INTERORGAN

**PROTEIN AND GLUTAMINE METABOLISM
IN THE TUMOR BEARING RAT**

**INTERORGAN PROTEIN AND GLUTAMINE
METABOLISM IN THE TUMOR BEARING RAT**

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan
de Universiteit Maastricht
op gezag van de Rector Magnificus, Prof. mr. M.J. Cohen,
volgens besluit van het College van Decanen
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Aan mijn ouders

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GENERAL INTRODUCTION

1.1 CANCER CACHEXIA

Weight loss and malnutrition have long been associated with a poor clinical outcome after surgery (1). Anorexia, muscle wasting and progressive depletion of adipose tissue have often been described in cancer patients and the clinical syndrome associated with these symptoms is referred to as cancer cachexia (2,3). Cachexia is derived from the Greek word *kakos* meaning ugly or bad and *hexis* meaning circumstances or condition. Cachexia is associated with several diseases e.g. tuberculosis, malaria, AIDS and cancer. A broad spectrum of symptoms, physical findings and laboratory findings have been attributed to the syndrome (Table 1) (4). The condition of the cachectic cancer patient differs from simple malnutrition due to fasting or starvation because host intermediary metabolism (carbohydrate, lipid and protein metabolism) is abnormal making nutritional therapy less successful (3,5).

Reports on the incidence of cancer cachexia at the time of presentation in a clinical setting vary between 3% and more than 80% (4,6-10). The variation is first of all caused by the fact that different cancer populations from and in different socio-economical settings are studied. Moreover, there appears to be a lack of convenient indicators to assess nutritional depletion. Most surveys have concentrated on weight loss, lean body mass, depletion of fat mass or serum protein levels (1,8,11). These parameters, however, are not only a reflection of the nutritional status but are also influenced by the disease state of the patient (12,13). To improve the nutritional assessment of patients, several nutritional indices were introduced to better identify patients at risk for nutritional related complications and to assess the efficiency of nutritional interventions (8,11,12,14,15). Most of these indices, however, remain more dependent on the disease status than on nutritional status.

In our hospital approximately 60% of the patients admitted to the surgical ward for gastrointestinal cancer are nutritionally depleted. In a multivariate analysis blood loss and age but not nutritional depletion, were associated with the development of postoperative complications (12). Nutritional depletion was only associated with the severity of a complication once a complication was present. This emphasizes that impact of the nutritional status only mildly affects the risk of complications compared to other factors and that a nutritional intervention will have limited impact.

Although the prevalence of cancer cachexia decreased over the years (8,16), nutritional intervention did not improve the nutritional status as much as first expected (17). Nutritional support to cancer patients was greatly improved in the seventies with the introduction of Total Parenteral Nutrition (TPN). Although it was first thought that TPN could completely reverse protein catabolism and improve clinical outcome of cachectic cancer patients (18), this was not reaffirmed in later studies (17,19). This was mostly attributed to the abnormal metabolic response to nutrients by cancer patients and the inability to reverse metabolic defects by artificial nutrition. In an elegant study Wolfe et al showed with ^{14}C [urea] that whole body protein anabolism, which can be achieved when giving parenteral nutrition to unstressed depleted patients with benign disease, cannot be achieved in cancer patients, whether depleted or not (13,20). The ongoing catabolism could only be blunted with parenteral nutrition and, for that matter, more in depleted than in non-depleted cancer patients. In general, TPN may only be effective in a subgroup of patients who are at risk (13,15,16). Thus, advantages of TPN must be weighed against the risk of complications of parenteral nutrition.

A point of controversy remains whether nutritional support influences tumor growth in cancer patients. Most evidence for nutritional support associated stimulation of tumor growth has arisen from early animal studies (21,22), although a recent human study also suggests that tumor protein synthesis and tumor growth increase with nutritional interventions (23,24). Most clinical studies and other animal studies, however, show that tumor growth is relatively independent of the nutritional status of the host and nutritional interventions are only of benefit for the host (25-27). Norton et al suggest that nutritional stimulation of cancer is only present when the tumor burden exceeds 10% of the body weight (4,7). In man, the tumor burden rarely exceeds 1% of the body mass and therefore may be uninfluenced by these mechanisms. Furthermore, Bozetti recently showed that good nutritional status of the host was associated with a lowered tumor cell proliferation in patients with non-Hodgkin's lymphoma (28).

In conclusion, perioperative parenteral nutrition only improved clinical outcome of a limited subgroup of patients with severe malnutrition at high risk for the development of postoperative complications (8,15,16). Protein anabolism cannot be

Table 1. Clinical features of cancer cachexia (4).

Symptoms	Physical findings	Laboratory findings
Weakness	Weight loss	Anemia
Fatigue	Skeletal muscle atrophy	Hypoproteinemia
Malaise	Adipose tissue loss	Hyperglycemia
Anorexia	Myopathy	Deficiency states
		Electrolytes
		Vitamins
		Minerals
		Energy

achieved in cancer patients with TPN or enteral nutrition, in contrast to patients with benign disease (3,13,28,29). To further improve the efficacy of nutritional support in cancer patients it became clear in the early nineteen-eighties that fundamental research investigating the underlying changes in intermediary metabolism due to the presence of cancer was needed to appropriately counter these changes and improve clinical outcome (17,19).

Metabolic features

Persistent weight loss despite nutritional support which is observed in cachectic cancer patients has been attributed to abnormalities in carbohydrate, lipid, protein and amino acid metabolism. Most of these findings have been demonstrated to occur before any signs of weight loss and anorexia are present, suggesting that these metabolic changes are a cause rather than an consequence of cancer cachexia (30-36).

Increased energy expenditure

It has repeatedly been demonstrated that both resting and non-resting energy expenditure is elevated in cancer patients and it has been suggested that this contributes to weight loss in patients with solid tumors (20,37), lung cancer patients (32,38) and patients with pancreatic cancer (39). However, unchanged and hypometabolic responses have also been described in gastrointestinal and hepatobiliary cancer patients when compared with patients with weight loss of a

similar magnitude due to benign disease or with weight stable cancer patients (20,32,40,41). It is, however, clear that resting energy expenditure reduces in normal patients during fasting or starvation and that this normal response appears to be blunted in malnourished cancer patient (42-44).

Carbohydrate metabolism

Both in man and in animal tumor models severe abnormalities in carbohydrate metabolism are observed. As early as 1925, Cori and Cori demonstrated decreased glucose levels and increased lactate levels across sarcoma bearing chicken wings when compared with the opposite wing suggesting high glycolytic activity in solid tumors (45). The early observation that malignant cells had high anaerobic glycolysis and increased lactate production also suggested that tumor cells could influence carbohydrate metabolism in cancer patients (46). Increased uptake of glucose with concomitant increased lactate release has been demonstrated in a variety of tumors transplanted on a vascular pedicle (47). Finally, Norton et al showed that human sarcoma bearing limbs also had increased glucose consumption and lactate release compared to the non-tumor bearing limb (48). This first suggested that the tumor interfered with glucose metabolism by acting as a "glucose trap" (31). However, in a study with esophageal cancer patients Burt et al observed increased glucose uptake and lactate release from forearm muscle suggesting that humoral factors induced by the presence of cancer also increase glycolysis of non-tumor tissue of the host (49).

Lactate produced by glycolysis can either be oxidized or be regenerated to glucose via gluconeogenesis in the kidney or liver (50). Increased gluconeogenesis has been observed *in vitro* and *in vivo* in both cancer patients and in tumor bearing animals (31,51-55). Peripheral glycolysis with a concomitant visceral gluconeogenesis has been referred to as Cori cycling and is considered to be an energy wasting process. In animal studies glucose recycling via the Cori cycle increased to approximately 52% in large tumor bearing animals (54). Increased Cori cycling has been postulated to be at the basis of cachectic energy depletion of cancer patients (52,56,57). However, energy wasting by gluconeogenic loss accounts for only 20-40% of the increased energy expenditure (9 kcal/kg/day) (7,58). Furthermore, Young et al estimated that if 85% of lactate production would be used to produce glucose and only 15% would be completely oxidized, there would be no energy loss (59). It is therefore generally not accepted that the increased Cori cycle is fully responsible for the energy depletion in cancer cachexia but rather plays a minor role. It has, however, been proven that gluconeogenesis increases during cancer and that, certainly in advanced cancer, it apparently does not respond to usual homeostatic mechanisms. Shaw et al showed with isotopically labeled glucose

that glucose infusions do not suppress endogenous glucose production in advanced cancer patients as observed in healthy controls (51).

Increased gluconeogenesis has also been observed from glycerol (58) and alanine (30,31,54,60,61). The contribution, however, of these substrates to gluconeogenesis is of relatively minor importance compared to the contribution of lactate (20,30,54,58).

Blocking gluconeogenesis in cancer patients has been successfully tried with hydrazine sulfate which inhibits the enzyme phosphoenol pyruvate carboxykinase. Chlebowski et al showed that hydrazine sulfate improves glucose tolerance and reduces fasting glucose production in cancer patients (62). The same group further showed in a double blind trial with lung cancer patients that one month of hydrazine sulfate treatment reduced whole body protein breakdown by approximately 27% which was accompanied with a maintenance of serum albumin concentrations. Iatrogenic ketosis has also proposed to reduce glucose supply to the tumor with a simultaneous preservation of host energy reserves (63). Although a 7 days ketogenic diet decreased arterial glucose concentrations of cachectic cancer patients, it had no effect on nitrogen balance or whole body protein turnover (63).

Another important phenomenon in carbohydrate metabolism of the cancer bearing state is decreased glucose tolerance (64,65). Reduced peripheral sensitivity to insulin has been observed in cancer patients and several rodent cancer models (64-67), although in a few cases also increased glucose sensitivity has been observed in cancer patients and tumor bearing animals (49,64,67). Reduced hepatic insulin sensitivity (64) and reduced pancreatic release in response to feeding has been observed and may be responsible for the decreased glucose tolerance (2,54,66). Interestingly, protein metabolism of incubated muscle cells of both cancer patients and tumor bearing mice has a normal (anabolic) response to insulin (64,66) and two recent studies by Brennan in cancer patients showed that insulin supplementation increases whole body and regional muscle protein synthesis of cancer patients to a similar extent as in control patients (68,69).

In conclusion, carbohydrate metabolism in cancer is characterized by increased glucose turnover, increased gluconeogenesis and insulin resistance for glucose.

Lipid metabolism

A great proportion of the weight loss in cancer patients is fat loss (32,37,70) which in animals can reach 80% of the fat stores (71,72). Although depletion of fat reserves in cancer patients is partly due to the reduced caloric intake, several specific changes in fat metabolism are attributed to the presence of cancer. Depletion of fat stores in the progress of cancer is associated with hypertriglyceridemia (20,72). The latter may be of particular importance for the immune response of the host to cancer

as increased lipid concentrations have long been recognized as being immunosuppressive (2,73,74).

Depletion and hyperlipidemia in cancer have first of all been attributed to increased lipolysis (20,72). Using stable isotopes, Shaw and Wolfe demonstrated that weight losing cancer patients had increased glycerol and free fatty acid turnover and release into plasma (20,75). However, not in all studies increased glycerol and fatty acid turnover rates were observed (58,76). Furthermore, decreased plasma clearance of free fatty acids and VLDL-triglycerides has also been described (33,77). Lipoprotein lipase is the protein responsible for triglyceride clearance and decreased mRNA and activity of the enzyme have been observed in both cancer patients (78) and animals (33,79). In addition, decreased tolerance to enteral and parenteral lipid infusions has been observed with disproportionately increased free fatty acid concentrations indicating impaired lipogenesis in cancer (80). In summary, depletion of fat stores, hyperlipidemia and increased turnover of glycerol and fatty acids are common findings in both cancer patients and tumor bearing animals.

Whole body protein metabolism

Whole body protein turnover measurements reflect protein synthesis and breakdown of several organs. Increased turnover rates of one organ may be accompanied by decreased turnover rates of other organs and thus not influence whole body turnover rates. However, protein turnover is an energy expensive process which accounts for approximately 10-20% of the basal metabolic expenditure (81). Therefore, whole body protein turnover measurements in cancer patients inform us of the energy expenses involved in changes in protein metabolism but do not effectively inform us on protein metabolism of specific organs (81).

Most studies on whole body protein kinetics suggest an increased protein turnover in cancer patients and tumor bearing animals (40,51,69,82-89). In a few studies whole body protein turnover remained unchanged (90,91) and it was suggested that this was observed because it was performed in the fed state (91). Norton et al similarly showed in an animal study that a period of fasting-refeeding increased whole body protein turnover in normal rats but had no effect on whole body protein turnover of tumor bearing rats (87). However, in a study by Melville and Garlick, lung cancer patients had increased whole body protein turnover rates in the fasted state which remained so during feeding (85). Therefore, the differences in whole body protein turnover are generally explained by the fact that small patient groups are studied, different control groups are used, different methods are used to measure whole body protein turnover and, most important, the fact that whole body protein turnover is a compilation of several turnover rates of individual organs as stated above (see also chapter 2).

Regional protein turnover

Muscle tissue is the largest protein pool in the body and has been suggested to account for approximately 50% of the whole body protein turnover under normal circumstances (4). Despite the observed increased whole body protein turnover in tumor bearing rats, muscle protein synthesis decreased in the same animals (87). Decreased protein synthesis rates have also been observed in Yoshida and Novikoff tumor bearing rats (35,92). However, increased protein degradation rates are held equally or more responsible for the loss of muscle proteins in cancer (34,92-94). The protein loss in muscle of tumor bearing rats appears to be limited to myofibrillar proteins (95). In this study sarcoplasmatic and extracellular proteins increased by approximately 25-30% compared to the myofibrillar proteins. Despite these changes, the performance of muscle over a wide range of stimulation frequencies was not affected by the presence of cancer (96).

Lundholm showed that *in vitro* muscle protein synthesis rates were decreased in cancer patients (97,98). However in another study Lundholm et al showed that, after an overnight fast, cancer patients had no significant increase in amino acid efflux from the leg compared to malnourished non-cancer patients (99) suggesting that peripheral nitrogen conservation after fasting remains unchanged. In response to enteral nutrition, however, the non-cancer patients decreased their amino acid efflux whereas cancer patients had an unchanged amino acid efflux (99).

Increased protein fractional synthetic rates in cancer patients have also been observed (100). As nitrogen loss occurred in muscle, this would implicate that protein degradation increased even further. Heber et al showed that urinary 3-methylhistidine/creatinine excretion rates were elevated in lung cancer patients (101). This was not confirmed in another study (102) and, thus, data on protein breakdown in human cancer cachexia remain conflicting when using 3-methylhistidine effluxes from muscle and urinary excretion ratios. Previous studies by Lundholm showed increased lysosomal protease activity in cancer patients and mice (97). Increased tissue protease's have also been found by others and increased protein breakdown thus appears to be a major determinant of muscle wasting during cancer cachexia (103,104). It is noteworthy, however, that another proteolytic pathway has recently been discovered which possibly has a much greater impact on muscle wasting during catabolic diseases (105,106). Muscle contains three major proteolytic pathways: the classical lysosomal pathway (cathepsins B, H, L and D), the cytosolic Ca^{2+} -dependent pathway (μ - and m-calpains) (107) and the cytosolic ATP-ubiquitin-dependent proteolytic pathway (108,109). The latter was first believed to degrade abnormal and short-lived proteins but is now also recognized to be responsible for the degradation of long-lived proteins during sepsis (110), acidosis

(111), glucocorticoid induced protein breakdown (105,112,113) and in cancer (114,115). A coordinated activation of all three proteolytic pathways seems to prevail in different wasting conditions to eliminate different classes of proteins (105,112).

In vivo protein breakdown rates have always been difficult to measure. Most data on protein breakdown are indirectly measured or studied *ex vivo*. In 1987 Barret and Gelfand developed an *in vivo* two compartment model to calculate protein breakdown rates across an organ (116). This model was also shown to be valid in humans (117). The model has recently been extended to three compartments by Biolo and Wolfe which not only made it possible to simultaneously calculate *in vivo* membrane transport rates but also appeared to improve the accuracy of intracellular protein turnover data (118,119). Methods and models to calculate protein turnover are more extensively discussed in chapter 2.

Studies on regional protein turnover of other organs are generally limited to visceral organs. In early research tumor growth was associated with an increased liver mass (120). Both *in vitro* and *in vivo* studies showed that hepatic protein synthesis increases (35,87,121). Warren et al showed that in MCA tumor bearing rats, liver protein synthesis increases proportionally to the tumor load and that this applies equally for secretory and structural proteins of the liver (122). This was, however, not confirmed in a human study which showed that the synthesis rates of fixed hepatic proteins decreased whereas the synthesis of acute phase export proteins increased (89). Emery et al also showed in a very aggressive tumor model that synthesis of export proteins is more affected by the presence of cancer than synthesis of fixed proteins (94). Acute phase protein production has a central role in the development of cancer cachexia (*vide infra*).

Spleens of tumor bearing animals also have increased protein contents, whereas visceral organs like the kidney and heart remain unaffected by the presence of a tumor (120). This was also reflected by unchanged protein synthesis rates in tumor bearing animals of most visceral organs, e.g. lung, heart and kidney (22,97). Tumor bearing rats also had intestines which weighed approximately 10% less than those of paired controls (123). This was accompanied by a 26% decrease in villus height in the same tumor bearing animals (123). However, the fractional synthetic rate of jejunal protein in tumor bearing rats reaches approximately 260-330% (124) which is almost twice as high as the normal jejunal protein synthetic rate (125,126). This suggests that protein breakdown must increase more than twofold in tumor bearing animals but this has not been measured yet.

The contribution of the individual tissues to whole body protein kinetics was estimated by Kawamura et al (88). In this study the major contribution to the increased whole body protein turnover came from the tumor. The relative

contribution of liver and muscle tissue decreased whereas that of other organs remained relatively unchanged.

Glutamine metabolism

Glutamine is the most abundant free amino acid in the body (127). It is biochemically a non-essential amino acid of five carbons and two nitrogen side chains (an α -amino group and a δ -amide group). It can be synthesized from glutamate and ammonia by an ATP dependent reaction catalyzed by glutamine synthetase (EC 6.3.1.2) which activity is present in brain, liver, kidney, muscle, spleen and lungs (128-130). Muscle contributes to approximately 40-50% of the body mass and it has been estimated that as much as 70-80% of the total body free amino acid pool is within skeletal muscle (131,132). Excluding taurine, glutamine contributes to approximately 61% (human) (131,132) or 20-30% (rat) (133-135) of the free amino acid pool in muscle and several studies have shown that the majority of the total body glutamine is synthesized in and released from skeletal muscle (136,137).

Glutamine has several important biological functions. It first of all serves as a non-toxic nitrogen shuttle to transport ammonia from peripheral tissues to visceral organs (liver and kidney) where ammonia can be transformed to urea or directly excreted. Glutamine is also a major gluconeogenic amino acid. Although alanine is thought to be the major gluconeogenic amino acid, a recent study in human volunteers showed that glutamine is the gluconeogenic amino acid by which quantitatively the majority of carbon skeletons, derived from proteins, are transferred to the glucose pool (138). Glutamine metabolism of the liver and kidney is also closely linked to acid-base homeostasis (139-141). Furthermore, glutamine is avidly consumed by dividing cells, such as fibroblasts (142-145), lymphocytes (146-149), tumor cells (150-153) and enterocytes (154,155). These cells consume glutamine at a high rate for nucleotide production and as energy source (146,155). Finally, it has been shown that in cases of oxidant stress, glutamine supports the production of the intracellular radical scavenger glutathione (156-159).

Tumor cells are rapidly dividing cells and interfere with host glutamine metabolism. It has been suggested that intracellular glutamine concentrations of the tumor negatively correlates with tumor growth rate (153,160,161). This is attributed to the increased purine and pyrimidine biosynthesis pathways which utilize glutamine. However, glutaminase is the main glutamine utilizing enzyme in cancer cells. On further processing of glutamate controversy still exists. Further oxidation of the carbon skeleton could occur completely in the mitochondria via glutamate dehydrogenase activity but it has also been suggested that glutamine is oxidized exclusively via a pathway involving glutamate-oxaloacetate transaminase (153).

Whatever the case, tumor cells are major glutamine consumers and compete with the host for circulating glutamine (162). In several animal models the tumor appears to act as a "glutamine trap" (152,161-164). This observation was not confirmed in human tumor tissue (165,166). The presence of a tumor does, however, induce remarkable changes in host glutamine metabolism (162).

Changes in host glutamine metabolism induced by the presence of cancer result in depletion of the arterial and intracellular muscle glutamine pool (123,159,162,167,168). It has been postulated that depletion of these pools occur because during progression of cancer, the tumor and an activated immune system become major glutamine consumers whereas muscle tissue becomes exhausted over time and, in a pre-cachectic phase, loses its capacity to produce sufficient glutamine (162). This finally results in decreased functioning of several other glutamine consuming organs of the host (169-171). Within this concept, glutamine thus becomes a "conditionally" essential amino acid because the capacity of endogenous biosynthesis is exceeded by tissue utilization (159,172-176). A similar concept has been postulated to occur in cases of severe stress, infections or trauma (173,177,178).

Postulated mechanisms for the mediation of cancer cachexia

The mediation of cancer cachexia is incompletely understood. It is generally assumed that cachexia is humorally mediated by mediators of an inflammatory response (cytokines), classical hormones and other regulatory peptides. Tumor induced anorexia and weight loss was shown to be transmittable in tumor bearing rats through plasma factors (2,179). By using parabiotic cross circulation of normal and tumor bearing rats, Norton et al showed that cancer cachexia was humorally mediated (180). Humoral mediation of cancer cachexia was also confirmed in studies in which metabolic alterations of cachexia like muscle wasting could be induced with plasma of tumor bearing rats given to control rats (72) and prevention of cancer cachexia by, e.g. a splenectomy (181).

Mediators of the inflammatory response and cachexia

Cytokines such as Tumor Necrosis Factor α (TNF α) and Interleukin-1 (IL1) have been proposed to be the main mediators of cancer cachexia (182-186). TNF α was first isolated as mediator of cachexia by decreasing plasma lipoprotein lipase activity (187). *In vitro* studies also showed that TNF α suppresses lipoprotein lipase activity in adipocytes (188-190). *In vivo* studies with TNF α showed that it can give similar metabolic changes as observed in wasting and cachectic disease states: arterial

hypertriglyceremia, increased muscle protein breakdown, increased glycogenolysis and increased peripheral lactate production (182,191-194). Increased serum $\text{TNF}\alpha$ levels have been observed in several cachectic diseases, e.g. AIDS, chronic parasitic infections (195,196). Elevated $\text{TNF}\alpha$ levels have also been observed in cachectic tumor bearing rats and correlated well with the tumor burden (197). Elevated $\text{TNF}\alpha$ levels were confirmed in other tumor bearing animals but correlated not always with tumor burden (198). In several other studies serum $\text{TNF}\alpha$ was not elevated at all (199-202). Similarly, in cancer patients elevated $\text{TNF}\alpha$ levels have been observed in some studies (203,204) but not in other studies (39,205-207). Anti-cachectin antibodies attenuated cachexia induced by the implantation of a tumor in several studies (208,209). It is therefore generally assumed that $\text{TNF}\alpha$ has a regulatory role in tumor associated metabolic changes, even though no measurable plasma concentrations were observed. It must therefore act as local mediator (210,211).

The biological function of IL1 greatly overlaps that of $\text{TNF}\alpha$ (212) and infusions with IL1 also induced anorexia, weight loss and loss of body proteins in rats (186). Elevated IL1 plasma concentrations are seldom found in cancer patients (89,204) or tumor bearing animals (185,198) but antibodies against an IL1 receptor do attenuate cachectic symptoms in tumor bearing mice to a similar extent as anti- $\text{TNF}\alpha$ antibodies (185). As with $\text{TNF}\alpha$, IL1 is thought to act locally as an autocrine mediator. Local involvement of IL1 has been confirmed in a study by Strassmann (213). In this study it was shown that by systematically blocking the IL1 receptor no change in cancer cachexia was observed whereas local injection in the tumor of anti IL1 receptor antibodies cachexia could be prevented (without influencing tumor growth). Local involvement was also confirmed by increased IL1 production by the spleen of tumor bearing mice without any effect on plasma levels (211).

γ -Interferon ($\text{IFN}\gamma$) is also suggested to be a central mediator of cancer cachexia (201). Although elevated plasma levels were not detectable, repetitive intraperitoneal administration of recombinant murine $\text{IFN}\gamma$ induced anorexia and weight loss. Furthermore, polyclonal antibodies against rat $\text{IFN}\gamma$ partly attenuated cancer induced anorexia and weight loss (201). The multiple synergistic interactions of $\text{IFN}\gamma$ with $\text{TNF}\alpha$ and IL1 suggest that these mediators are all part of a final common pathway.

Another important and multifunctional cytokine involved in cancer cachexia is IL6 (214). In contrast to $\text{TNF}\alpha$ and IL1, IL6 is generally detectable in serum or plasma of cancer patients and tumor bearing animals (39,42,89,199,200,204,213). Again, IL6 blockades attenuated the anorexia and weight loss associated with tumor growth in animal models (200). This was also confirmed with the experimental drug, suramin

which inhibits the binding of IL6 with its receptor (215). Because IL6 has been reported to be increasingly produced by many tissues of the cancer bearing host, e.g. liver tissue, kidney, small intestine and tumor tissue, Lundholm postulated that IL6, unlike TNF α and IL-1, acts more in a paracrine fashion than in an autocrine fashion which is induced by other host-derived factors (210). Strassman postulated that in his tumor model the direct cellular source of IL6 are the tumor cells responding to IL1 produced by tumor infiltrating phagocytes (215).

Other studies have also confirmed that endogenous IL6 production is a more distal and direct mediator of cachexia (than e.g. IL1, TNF α) and the acute phase response during inflammation (216,217). IL6 is known to be the principal factor to elicit an acute phase protein response both in vitro (218) and in vivo (219). Furthermore, circulating IL6 levels of tumor bearing rats correlated well with tumor load and plasma acute phase proteins (220-222). Although this was not confirmed in a human study (39), an increased spontaneous IL6 release of peripheral blood monocytes was associated with a positive acute phase response.

The acute phase response refers to a series of physiologic and metabolic changes in response to tissue damage induced by injury, infection or inflammation (223). In the liver protein synthesis shifts from albumin synthesis to the production of acute phase proteins such as C-reactive protein, serum amyloid A protein, α_2 -macroglobulin and α_1 -antitrypsin (224). The role of the acute phase proteins in cancer is still subject of debate but several ideas have been postulated. It has been hypothesized that acute phase proteins inhibit tumor growth (225,226), increase tumor growth and spreading (227), bind to damaged tissue, membrane phospholipids and DNA (224) or create an inflammatory barrier around the tumor similar as during bacterial infections (223). The acute phase response is, however, mostly recognized as a relatively unspecific response to microbes and subsequent tissue destruction (220,223). Moreover, increasing evidence exists that the acute phase response has a relation with, and an etiologic role in, metabolic disturbances observed in cancer cachexia (216).

The acute phase response is not only activated but also modulated by cytokine production. Examples of cytokines with inhibitory function are IL4 and IL10 (223). IL4 is primarily released by T-helper 2 (Th2) cells and appear to modulate the acute phase response by downregulating TNF α , IL1 and IL8 release of monocytes and macrophages (223,228). It furthermore enhances apoptosis of monocytes hereby decreasing the amount of inflammatory cells. IL10, produced by macrophages and Th2 lymphocytes, also inhibits IL1, TNF α and IL6 production.

These changes in the cytokine network should be seen in the light of the extensive studies on protein energy malnutrition reporting decreased lymphocyte proliferation response to mitogens, decreased CD4⁺ T helper cells, decreased complement

activity and decreased secondary antibody response to certain antigens or vaccines (229,230). Interestingly, it was recently observed that monocytes of malnourished children produced less $\text{TNF}\alpha$ (and IL6) after stimulation with lipopolysaccharide (LPS) at the time of admission than after nutritional rehabilitation (231). Another study also reported a reduced capability of IL1 produced by monocytes of malnourished patients to produce fever in a rabbit (232). It appears that protein energy malnutrition attenuates the immune response in the non-cancer bearing state, whereas protein energy malnutrition during the cancer bearing state activates the immune system. It goes beyond the scope of this overview to unravel the entire network of cytokines related to cancer cachexia, in particular related to the relative importance of each individual cytokine but it has become increasingly evident that they have a central role in the development of cancer cachexia.

The cytokines and acute phase proteins also have a close relationship with, and partly depend on, concomitant hormonal changes. $\text{TNF}\alpha$ or IL6 infusions in human volunteers increase the stress hormone response (194,233). Anti- $\text{TNF}\alpha$ antibodies reverse the early changes in insulin and corticosteroid levels of tumor bearing animals (208). On the other hand, insulin reduces the IL1 and IL6 type cytokine effects on whereas glucocorticoids enhance most cytokine effects on the acute phase response (223). The close relationship between the acute phase response and the classical hormones was also elegantly shown by Lundholm in a study where the *in vivo* cytokine induced acute phase protein synthesis depended on intact adrenal function and plasma glucocorticoid concentrations (234).

Hormones and cachexia

Many of the metabolic changes observed in cancer have long been conceived to be mediated by the classical hormones. Hormone profiles alter rapidly after tumor transplantation in animals (235) and in malnourished cancer patients (198,236). It appears that the presence of a tumor rapidly elicits a complex endocrine response of the host (235).

Increased cortisol secretion was one of the earliest hormonal changes observed in cancer cachexia (6,104,237). In tumor bearing animals marked adrenal hypertrophy was observed (6,238) and hypophysectomy could not prevent this hypertrophy (239). Early studies also suggested that the adrenals are hyporesponsive to ACTH in the early stage of tumor growth, become hyperresponsive when the tumor grows, and become hyporesponsive again at the end stage (6,240). Increased cortisol or corticosterone has been observed in several other tumor animal models (104,198,208,241) although not in all (121). Whether increased plasma corticosterone levels are specific for cancer was doubted by Inculet and colleagues who showed that in MCA tumor bearing rats no relationship existed between plasma

corticosterone levels and tumor burden (30). Moreover, Tessitore showed that in spite of sustained elevation of plasma corticosterone concentrations, adrenalectomy did not substantially alter the general course of cachexia in tumor bearing animals (104).

In malnourished cancer patients an increased 24 hours urinary cortisol excretion compared with malnourished control patients was also observed (236). Similarly, the same study showed increased urinary excretion of adrenaline and noradrenaline in malnourished cancer patients although this elevation was still within the reference value for well-nourished individuals (236). In tumor bearing animals the increased urinary catecholamine excretion appeared to be linear and proportionally to tumor load (198). However, in another animal study increased catecholamine concentrations were only temporarily observed (235). It therefore remains uncertain what exact role corticoids and catecholamines have in the development of cancer cachexia. Despite this uncertainty, several studies have already been performed to counter these effects with β 2-agonists such as cimaterol and clenbuterol.

Cimaterol both decreased protein breakdown and increased protein synthesis of muscle of tumor bearing animals (242,243). Cimaterol also increased muscle protein content of MCA tumor bearing rats when given in conjunction with TPN. Similar effects have been observed with the β 2-agonist clenbuterol (244,245). In a recent study it was further shown that the anabolic effects of β 2-agonists are realized by decreasing the ATP-ubiquitin dependent proteolysis which, as discussed earlier, is upregulated in cancer (114).

Many of the aberrations in host glucose metabolism have also been attributed to decreased host insulin/glucagon ratios (55). This can in part be explained by increased glucagon levels which have been observed in most studies concerning tumor bearing animals and cancer patients (49,55,198,246-248). Furthermore, the rat tumor burden appears to correlate well with plasma glucagon levels (30). Hyperglucagonemia causes anorexia, insulin resistance, increased gluconeogenesis and increases nitrogen loss (249). Wolfe further showed with insulin clamping that glucose substrate cycling is dependent on plasma glucagon concentrations (250). As mentioned previously, these metabolic alterations have all been observed in cancer cachexia.

Cancer induced decreased insulin/glucagon ratio's can also be explained by decreased insulin concentrations. Although both in cancer patients and in tumor bearing animals decreased insulin levels are observed (30,49,104,198,208,246,247), it is at present not clear whether this is a primary or secondary response to cancer. Several animal studies show that the decreased insulin concentrations and concomitant insulin resistance are secondary to the decreased food intake and not the presence of a tumor per se (66,247). In one other study, even a 63% increase of

the insulin concentration was observed in tumor bearing animals secondary to increased gluconeogenesis and elevated glucose concentrations (121). Although unknown whether decreased insulin concentrations are primary or secondary, insulin supplementation is one of the most widely used therapies against cancer cachexia.

The possibility to use insulin suppletion against cancer cachexia was particularly lucrative because of the early documentation that the insulin resistance to carbohydrate metabolism did not affect protein metabolism (4). In animal studies supplementation with insulin reversed cancer induced anorexia (202,247,251,252), decreased the weight loss (55,202,246), reduced tumor glycolysis and host gluconeogenesis (246), improved nitrogen accretion of host tissue (66,202,252), and even reduced mortality rates (253). Insulin has also been given to cancer patients and this resulted in a decreased whole body protein breakdown rate in both weight stable and weight losing cancer patients (69). The anabolic effects of insulin was also confirmed in another study by measuring protein synthesis rates in forearm muscle (68). Both weight losing and non-weight losing cancer patients showed appropriate stimulation of muscle protein synthesis rates in response to insulin (68).

In a recent study, cancer cachexia was prevented with octreotide, which prevents glucagon secretion, combined with exogenously given insulin (55). Increased carcass weight, muscle and liver protein content and retardation of tumor growth compared with untreated tumor bearing controls was observed. Strangely, liver gluconeogenic enzymes were increased in the glucagon deprived rats which may be an effect of increased levels of other counter regulatory hormones. Of further interest is the fact that several studies have shown that the toxic and metabolic changes in glucose and protein metabolism induced by $\text{TNF}\alpha$ can be reversed or at least ameliorated by insulin supplementation (254,255).

Changes in several other plasma hormone concentrations have also been observed (235). Recently, much interest in literature is given to the interaction of cancer and growth hormone. Growth hormone has been given to patients with several cachectic or acute catabolic disease states (46). Growth hormone has anabolic effects in healthy volunteers (256,257), is effective against postoperative muscle protein breakdown (258) and also appears to be of benefit against the wasting during diabetes (259), AIDS (260) and sepsis (261). In MCA tumor bearing rats, human growth hormone failed to attenuate host body wasting and muscle protein loss (262). In a study with metastatic mammary adenocarcinoma, growth hormone did improve muscle weight and protein content (263). Combined with protein deprivation, growth hormone even reduced tumor growth possibly due to the rate limiting availability of amino acids (263). Furthermore, rat growth hormone appears to improve host body composition of tumor bearing rats with approximately a thousand fold less doses (264,265). In a clinical study with cancer patients human

growth hormone increased whole body protein synthesis simultaneously with whole body protein breakdown but the effects on protein synthesis were larger (266). In the same study, however, no changes in muscle protein turnover was observed.

Tumor growth stimulation with growth hormone still remains a point of concern (4) although most *in vivo* studies showed no increase in tumor progression by growth hormone (262,263). Malignancy is, however, still regarded as a contraindication to growth hormone treatment until the differential regulation of metabolic and mitogenic actions of growth hormone are better understood (46).

1.2 TUMOR MODELS TO STUDY CANCER CACHEXIA

Although metabolic changes of cancer cachexia and the possible mechanisms involved in the mediation of the syndrome have been studied widely in the past three decades, this has not yet resulted in an adequate understanding of the disturbances or in adequate nutritional therapy. Several tumor models have been utilized to study cancer induced anorexia and cachexia in animals. The clinical relevance of several of these animal models has been questioned because the tumor generally grows to a size not observed in clinical practice (20). Nonetheless, important information regarding host-tumor interactions have been acquired over the last years. In this respect, the methylcholantrene-induced sarcoma (MCA tumor) has been used by several investigators, including from our laboratory, to study host-tumor interactions related to, e.g. protein metabolism, ammonia metabolism, interorgan glutamine metabolism and several neuro-humoral changes (123,163,167,247,270-272). The use of MCA tumors has several advantages over murine models. First, the larger size of the animals makes it possible to study regional metabolism of several organs (gut, liver and hindquarter muscle). Second, the large organs and plasma pool of the rat do not require pooling of tissue or plasma from several animals. However, the major disadvantage of using rats instead of mice is that most immunological laboratory tests are suitable for mice and not in all cases for rats. Most studies on MCA tumor bearing rats report that the tumor becomes palpable at day 7-11 after tumor implantation and grows more or less exponentially (21,31,163,267). From day 14-20 food intake starts to decline until death which occurs approximately five weeks after inoculation (21,27,31,120,121,163,199,252). The tumor is malignant and locally very aggressive but does not metastasize. Metabolic changes observed in the MCA tumor bearing rat are related to alterations in carbohydrate, nitrogen and lipid metabolism summarized in Table 2.

Table 2. Changes in carbohydrate and nitrogen metabolism in MCA tumor bearing rats.

	Effect tumor	Reference
Carbohydrate metabolism		
Arterial glucose	decreased, unchanged	(31,66,72), (246,247,251)
Glucose pool size	decreased	(31)
Glucose turnover	increased	(31)
Gluconeogenesis	increased	(31,60)
Glucose tolerance	decreased	(66)
Cori cycle activity	increased	(31,60)
Protein metabolism		
Nitrogen balance	decreased	(252,267)
Protein turnover	increased	(87)
Muscle		
Protein synthesis	decreased, unchanged	(87), (242)
Protein breakdown	increased	(87)
Liver		
Protein synthesis	increased	(87,121,122)
Protein breakdown	increased	(72,122)
Alanine turnover	increased	(31)
Ammonia concentrations	increased	(247,268)
Glutamine concentrations	decreased	(123,167,246,269)
Lipid metabolism		
Triglyceride concentrations	increased	(72)
Lipoprotein lipase activity	decreased	(79)

Most disturbances in glucose metabolism which are described in the first part of this chapter, were also found in MCA tumor bearing rats. These include an increased glucose turnover, increased gluconeogenesis, decreased glucose tolerance and increased insulin resistance. Nitrogen metabolism of MCA tumor bearing rats is characterized by increased nitrogen loss, increased whole body protein turnover rates and wasting of peripheral muscle tissue with a relatively sparing of visceral organ nitrogen. Changes in amino acid metabolism have also been reported for alanine and glutamine, the major nitrogen carriers of the body. The MCA tumor model is therefore appropriate for the study of interorgan amino acid metabolism, particularly of these two amino acids. Furthermore, studies on fat metabolism displaying increased arterial triglyderide concentrations and decreased triglycerides clearance by lipoprotein lipase.

Several studies showed that MCA tumors induce an inflammatory response. These include, sometimes but not always, increased $\text{TNF}\alpha$ concentrations (197,199,201), increased systemic (199) and local IL6 (210) concentrations and an increased acute phase protein response (220,274). It is generally believed that the induction of these inflammatory mediators in MCA tumor bearing rats is essential for the altered carbohydrate and nitrogen metabolism but the exact role of each mediator and the interactions between these mediators and the neuro-hormonal response remains to be elucidated.

	Control	Small tumor burden	Large tumor burden
α_2 Macroglobulin	<50	200 $\pm$ 22	6000 $\pm$ 300
$\text{TNF}\alpha$	not detectable	not detectable	not detectable
IL6	not detectable	—	257 $\pm$ 50

Table 3. Systemic inflammatory response of MCA tumor bearing Lewis rats. α_2 Macroglobulin (IU/ml) was tested by single immuno diffusion (275). $\text{TNF}\alpha$ was tested by both ELISA using anti-murine $\text{TNF}\alpha$ monoclonal TN3 and by the WEHI 164 bio-assay (276). IL6 (pg/ml) was tested by the B9 hybridoma bioassay using the MTT detection method (277).

Figure 1. Growth curve of MCA tumors in Lewis rats (n=5). Curve fitting reveals that the tumor grows polynomial (see also chapter 5).

Figure 2 (page 27). 1A: Paraffine section of MCA tumor tissue subjected to hematoxylin and eosin staining (100x). 1B: same paraffine section as 1A at 400x. 2A: Lymphocyte staining of tumor tissue (CD3⁺ cells, 100x). 2B: same section as 2A at 400x. The tumor appears as a fibrosarcoma. Local aggressiveness is shown by the many dividing cells and aneuploid cell fractions (1A and 1B). Lymphocyte infiltration is observed at the peripheral sides of the tumor and at several spots within the tumor (2A and 2B).

Studies of the MCA tumor model are mostly derived from the MCA tumor originally induced by Grant and Wells in 1974 into Fisher 344 rats and subsequently passaged on in this strain (120,162,278). At the starting point of the work on this thesis no tumor was present in our laboratory. We therefore chemically induced a MCA tumor in rats (279). Lewis rats were used for practical reasons because they are homebred at the University (Centralized Animal Facilities, University of Limburg). MCA (10 mg, Sigma W6501, St Louis, USA) was dissolved in alcohol (1 ml, 75%), paraffine (1 ml, Merck 7174) or DMSO (1 ml, Merck 2931) and injected subcutaneously in the rats. When the tumor was approximately 5-10% of the body weight it was serially transplanted to other rats to maintain the tumor *in vivo*. Tumor tissue was further made to a cell suspension to grow *in vitro* and to maintain a stock in liquid nitrogen. The *in vitro* stock has also been tested and proven to be malignant *in vivo* again. At present approximately 15 MCA cell-lines are stored in liquid nitrogen.

Figure 3. Food intake by control and MCA-tumor bearing rats. Small tumor bearing rats had a tumor burden of 5-10% of the body weight, large tumor bearing rats a tumor burden of 15-30% of the body weight. No changes in food intake were observed in any of the experiments over the last three years.

The MCA tumor induced in Lewis rats grows rapidly, is locally very aggressive and does not metastasize. The tumor appears to grow exponentially (Fig. 1) but the curve fitting of the growth curve reveals that it grows according to a polynomial curve. This indicates that the growth of larger tumors is inhibited with increasing tumor load. Hematoxylin and eosin staining of the tumor (Fig. 2A) shows the malignant and locally very aggressive character of the tumor similar to the description in literature (280). Lymphocyte staining (anti-CD3, DAKO A452, Glostrup, Danmark) further shows that the tumor induces an immune response in the rats (Fig. 2B). The immune response was also confirmed by the systemic presence of high levels of the acute phase proteins and IL6 (Table 3). We also tested whether $TNF\alpha$ was present in serum but we could not find detectable levels using either an ELISA or bio-assay.

MCA tumors were transplanted for more than 3 years without any signs of anorexia (Fig. 3). The reason for this contrast with most studies reported in literature are unknown but the food intake of tumor bearing rats being normal made it possible to observe changes in host metabolism which were only induced specifically by tumor-host interactions. The lack of decreased food intake also induced a moderate cancer cachexia as can be seen in figure 4.

Figure 4. Carcass weight of control, small tumor bearing and large tumor bearing rats.

In contrast to several reports in literature, carcass weight of the host (total body weight minus the tumor) remained fairly constant. However, wasting of muscle tissue and redistribution of nutrients to visceral organs were observed (Fig. 5) making the model suitable to study moderate cancer cachexia as is more often seen in the clinical practice of oncology.

Figure 5. Dry weight of several organs (gastrocnemius muscle, small intestine, liver and spleen) in control, small tumor bearing and large tumor bearing rats.

1.3 OUTLINE OF THE THESIS

The thesis is based on three principal questions which arose at the starting point. The first one was whether we were able to measure *in vivo* protein synthesis and breakdown rates of muscle tissue and splanchnic organs in small laboratory animals. The second question was how the presence of a tumor affected protein and glutamine metabolism of these organs and, lastly, we wanted to know whether these organ specific changes influenced the normal metabolic response after a surgical challenge.

The first aim of this thesis was to evaluate *in vivo* intracellular protein and amino acid measurements in muscle, gut and liver tissue of the rat (chapter 2, 3 and 4). Selective catheterization of arterial and venous vessels across several organs and

constant infusions of radioactive labeled amino acids has made it possible to simultaneously measure *in vivo* protein synthesis, protein breakdown, intracellular amino acid disposal and production and amino acid membrane transport rates of several organs. Assumptions underlying the different methods are briefly outlined in chapter 2. Because these methods were previously used in large animals and humans, we tested the possibility to use the methods in small laboratory animals (chapter 3, 4). The effects of starvation on protein and amino acid kinetics were also tested in these models because decreased food intake often accompanies cancer cachexia.

As no tumor model was present in our laboratory, methylcholantrene (MCA) was used to chemically induce a subcutaneous sarcoma in rats. These tumors have previously been used by others to study metabolic changes of cancer cachexia. After induction of the tumor we first studied the effects of these tumors on food intake, weight changes, whole body and regional muscle protein and amino acid kinetics (chapter 5). Amino acid turnover was focused to glutamine as this amino acid is a specific nutrient for rapidly dividing cells, e.g. tumor cells, enterocytes and immune cells.

The effects of increasing tumor loads on gut protein and amino acid turnover are studied in chapter 6 and these changes are related to morphological and functional changes. The liver has a central role in amino acid homeostasis. Liver protein metabolism was studied in chapter 7 and related to urea production and the acute phase response. Liver glutamine kinetics during growth of cancer are discussed in chapter 8.

After a surgical trauma amino acid and protein kinetics change dramatically to supply host defense organs with nutrients. To test whether postoperative amino acid and protein metabolism were disturbed in the cancer bearing host, we also studied muscle and gut protein and amino acid turnover on the second postoperative day after a hysterectomy. These results are described and discussed in chapter 9 and 10.

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METHODS TO MEASURE *IN VIVO* AMINO ACID AND PROTEIN TURNOVER

Introduction

The fact that proteins are constantly synthesized and broken down has been described in the early works of Schoenheimer (1) and Cuthbertson (2). Since then several techniques have been developed to study protein and amino acid metabolism. Early techniques were mostly clinical descriptive assessments which provide qualitative information whereas the more recently used techniques involve isotopically labeled amino acids which provide more quantitative information on amino acid and protein turnover. This chapter of the thesis will concentrate on reviewing the latter techniques. Because we used radioactive amino acids as tracers to study amino acid and protein turnover rates, all formulas described in this chapter use the specific activity (SA, dpm/nmol) of these amino acids in different plasma and tissue pools. Although the nature of stable isotopes is different, the underlying principles and assumptions are similar to calculate amino acid and protein turnover. Isotope enrichment of amino acids is analogue to SA in the formulas (3).

Nitrogen-balance studies

Nitrogen balance studies have often been used to study whole body nitrogen metabolism (4). It is a relatively simple clinical tool to grossly evaluate nitrogen metabolism. Nitrogen loss (fecal and urinary) is subtracted from nitrogen intake and as approximately 16% of protein consist of nitrogen, net protein loss or gain can be calculated by multiplying nitrogen loss or gain with 6.25. Several limitations of nitrogen balance studies have been outlined previously (5). In general, the technique is rather gross and imprecise. Nitrogen losses are often underestimated because of incomplete collection of feces and urine. It is further underestimated by an undefined

amount of nitrogen lost through insensible skin loss. Also, nitrogen intake is often overestimated because of unconsummated nutrients (5). Therefore, this technique has been criticized by studies which compared nitrogen balance techniques with protein turnover measurements using labeled amino acids. Golden et al studied patients recovering from protein energy malnutrition with both techniques (6) and showed that nitrogen balances correlated very poorly with the actual protein synthesis and breakdown rates. Nitrogen balance studies should therefore only be seen as gross estimates to evaluate protein metabolism.

Whole body protein turnover and amino acid flux

Proteins are built of a repertoire of 20 amino acids (7). Metabolism and turnover of amino acids has been used to estimate protein turnover. The majority of the amino acids present in the body are bound to proteins and only approximately 0.5% is present in the intracellular and extracellular free amino acid pool. Fluxes and turnover rates in this pool have been used to describe protein metabolism.

Both stable (²H, ¹³C or ¹⁵N) and unstable (³H or ¹⁴C) isotopes of amino acids have been used to quantitatively evaluate whole body protein turnover. Whole body protein turnover can be calculated in an one compartment model (Fig. 1). The model evaluates the exchange of amino acids from and into the extracellular free amino acid pool. It is assumed that the infusion of a labeled amino acid and the rate of appearance (Ra) of the same (unlabeled) amino acid occur in this single pool. Thus, a primed constant infusion of a labeled amino acid is given and at steady state conditions samples are drawn from the plasma pool. At steady state (Fig. 1) the amount of tracer amino acid lost will be equal to the tracer amino acid infused and plasma Ra can be calculated as follows (3):

Tracer in	=	Tracer out or	
Ra	=	Rd	
I	=	Ra x SA	
Ra	=	I / SA,	(1)

where Ra is the rate of appearance in nmol/min, Rd the rate of disappearance (nmol/min), I is the infusion rate of the labeled amino acid (dpm/min) and SA the specific activity (dpm/nmol) of the infused amino acid measured in plasma at steady state. Infusion and sampling of labeled amino acids can in fact be performed in two ways: a venous infusion with arterial sampling (V-A mode) and an arterial infusion with central venous sampling (A-V mode). In a study by Katz et al. considerable

differences have been observed between the two methods when infusing ^{14}C or ^3H labeled lactate and alanine (8-10).

Figure 1. One compartment model to measure whole body protein turnover. After a primed constant infusion of labeled amino acid (see inlet), steady state conditions are reached (tracer infused equals tracer lost from the whole body) and samples are drawn downstream of the infusion site after complete mixing of the tracer with the amino acid pool. The inlet shows the theoretical arterial specific activity reached after adequate priming and constant infusion of the tracer.

These differences can be attributed to differences in cardiac output, the fractional turnover of the unlabeled amino acid pool (trac $\acute{e}$ e) and to the sites of utilization or production of the trac $\acute{e}$ e (3,11,12). It is generally agreed upon that if the tracer which is infused in blood, fully mixes with all the trac $\acute{e}$ e being produced by the body before uptake in tissue occurs, the whole body R_a , which is calculated with V-A mode, is valid because arterial SA is the SA at the site where the tracer/trac $\acute{e}$ e disappears. This is true for glucose, glycerol and fatty acids. However, amino acids are both released and metabolized intracellularly throughout the body. Turnover or uptake thus takes place with a SA more closely to the capillary or venous SA (11,12). The A-V mode would thus be more appropriate to calculate whole body turnover and the V-A mode underestimates the actual turnover rates. The major draw-back of the A-V mode to calculate whole body protein and amino acid turnover is, however, the fact that it is impractical to obtain the central venous samples (right atrium). Furthermore, the contribution of the lungs to whole body protein turnover is ignored. The V-A mode is therefore generally used to calculate whole body protein and amino acid turnover.

When using ^{15}N amino acid labeling, whole body protein turnover can also be assessed by determining urinary metabolite (urea or ammonia) enrichment (13). The

latter method has repeatedly been used in early studies but has several practical and methodological problems. Long infusion times (up to 30 hours) are needed to acquire isotopic steady state conditions for urea in plasma and urine. This not only induces practical problems to perform such studies but also bring in doubt the metabolic and isotopic steady state conditions which are assumptions which must be met to perform these studies. The ammonia pool is much smaller but ammonia nitrogen enrichment is derived primarily from the amide N of glutamine and thus only represents part of the total N pool (3). Thus, turnover rates calculated with ammonia instead of urea are consistently lower and have been suggested to reflect predominantly muscle protein turnover. The controversy which endproduct, ammonia or urea, appropriately reflects whole body protein turnover has not yet been resolved and the method has generally been abandoned (3,4).

Whole body amino acid turnover rates measured by the V-A mode can be determined by the dilution of labeled carbon, hydrogen and nitrogen. Dilution of ^{15}N labeled amino acids also include the movement of nitrogen between the different amino acids through the ample transamination and transamidation reactions. However, discrepancies have also been observed in some studies using different carbon and hydrogen labeled (^{14}C , ^{13}C , ^3H or ^2H) amino acids (3,9,10,14) [pp392]. It has been suggested that these differences occur because the turnover of ^3H or ^2H labeled amino acids measure replacement of the complete mass whereas ^{14}C or ^{13}C labeled amino acids also include recycling of carbons (10). For carbon and hydrogen labeled phenylalanine and leucine no differences in whole body protein turnover rates were observed using either isotopic label (15-17).

Labeled leucine and phenylalanine are currently the most commonly used amino acids to measure protein turnover. Both amino acids have the advantage that they rapidly equilibrate with the intracellular free amino acid and amino-acyl-tRNA pool (18). Leucine has the additional advantage that it is intracellularly in rapid equilibration with its intracellular transamination product, α -ketoisocaproate (KIC), which rapidly equilibrates with plasma KIC. Gut, muscle kidney and adipose tissue all have active transaminase activity and plasma KIC is thus a pooled value reflecting intracellular enrichment or specific activity of leucine in these tissues (3,19). However, KIC specific activity is not at equilibrium with the entire intracellular leucine pool and plasma KIC specific activity is generally higher than intracellular leucine specific activity (3). Muscle intracellular leucine specific activity is thus approximately 30% lower than plasma KIC specific activity (20). However, for other essential amino acids (phenylalanine, lysine) no interaction and equilibrium as for leucine-KIC is present and turnover rates calculated with these amino acids in the V-A mode also result in an underestimation of the true whole body protein turnover depending on the tracer amino acid used. Obled and colleagues (21) constantly infused several ^{14}C

labeled amino acids for six hours in rats. They showed that the whole body intracellular specific activity was 67%, 71%, 56% and 35% of the arterial specific activity for ^{14}C labeled threonine, lysine, tyrosine and leucine, respectively. The study shows that arterial leucine specific activity is not a good value to calculate whole body turnover. However, KIC specific activity underestimates the actual turnover rate to a similar extent (30-40%) as the arterial specific activity in studies with other tracer amino acids.

To determine whole body protein synthesis with leucine-KIC labeling the leucine fraction which is oxidized has to be determined from CO_2 specific activity or enrichment in breath samples. This also requires adequate priming of the bicarbonate pool which is approximately 85 times the labeled CO_2 production rate under normal conditions (3). To appropriately collect CO_2 samples metabolic chambers with isolated ventilation systems are necessary to prevent contamination of breath samples by unlabeled CO_2 from normal air (3).

Using phenylalanine as tracer amino acid has the advantage that it enables the simultaneous estimation of whole body amino acid oxidation by calculating whole body hydroxylation of phenylalanine to tyrosine (22). Hydroxylation of phenylalanine can be calculated by the relative arterial SA of phenylalanine and tyrosine at steady state, their relative concentrations in the arterial plasma pool multiplied by their relative content in protein (22). In formula:

$$\text{Hydroxylation} = \text{WBRa}_{\text{phe}} \times [\text{Prot}]_{\text{phe}}/[\text{Prot}]_{\text{tyr}} \times 1/[(\text{SA}_{\text{phe}}/\text{SA}_{\text{tyr}})-1] \quad (2)$$

where WHRa_{phe} is the whole body rate of appearance of phenylalanine, $\text{SA}_{\text{phenylalanine}}$ and $\text{SA}_{\text{tyrosine}}$ are the arterial specific activity of phenylalanine and tyrosine and $[\text{Prot}]_{\text{phe}}$, $[\text{Prot}]_{\text{tyr}}$ the relative content of both amino acids in body protein. Unless accumulation of tyrosine occurs, hydroxylation of phenylalanine also reflects oxidation rate of amino acids at steady state (22). At steady state whole body Ra is equal to whole body disappearance rate (Rd). Subtracting hydroxylation from whole body Rd results in a Rd which is used for protein synthesis because activity of other metabolic routes of phenylalanine (e.g. transamination) are considered to be low. Thus using phenylalanine as tracer gives a fairly easy and good estimation of changes in whole body protein turnover, whole body hydroxylation, whole body protein synthesis and is at present the tracer amino acid of preference in several laboratories.

Whole body protein turnover was first seen as a reflection of muscle protein turnover. Muscle accounts for approximately 50-70% of the bodies proteins and whole body protein turnover has been estimated to be determined for 50% by muscle in healthy volunteers (23). It becomes, however, more and more clear that changes in whole body protein turnover rates can be determined by changes in the smaller

but more rapidly turning-over, protein pools of other organs like the liver and gut. Fryburg et al (14) for example observed that administration of growth hormone increased muscle protein synthesis by approximately 70% whereas no effects on whole body protein turnover were observed. Similarly, Wolfe et al (24) observed that insulin did not affect whole body protein turnover in cancer patients whereas muscle protein synthesis rates increased by approximately 65%. It can be postulated that the whole body turnover measurements were not sensitive enough to detect these differences but assuming muscle to contribute to approximately 50% of the whole body turnover, an expected increase in whole body protein turnover of 30-35% should have been observed. This is more than the detection limit and it must therefore be assumed that the increase in muscle protein turnover is accompanied by decreased protein turnover rates of other organs. These data emphasize the difficulties in interpreting whole body protein turnover data and question the value of these turnover rates.

Figure 2. Schematic view of how higher protein turnover rates amplify a percentual effect of changes in protein synthesis or breakdown. If low turnover rates are present a percentual change in either protein synthesis or breakdown has a smaller effect than if higher turnover rates are present. It is generally assumed that this is of benefit to adapt more rapidly to a changing environment. However, a similar kind of metabolic process can also be described as futile resulting in energy loss.

Two other considerations concerning whole body protein turnover rates have, however, not yet been mentioned. First of all, it has been estimated that approximately 20% of the resting energy expenditure is the result of protein turnover (4). Changes in whole body protein turnover are therefore also a reflection of

changes in energy expenditure or whole body energy status. Secondly, knowledge of the level whole body protein turnover may also be of value to estimate the possibility of the whole body protein pool to adapt to environmental changes. It has been estimated that approximately 80% of the amino acids released by protein breakdown are reutilized for protein synthesis (4). Protein synthesis and breakdown are present at the same time within an organ, tissue or cell because this may improve the regulation of protein metabolism. Two opposite metabolic processes simultaneously present will give a biochemical substrate cycle which can amplify metabolic signals (7,25,26). For instance, a substrate cycle has been described for the simultaneous presence of phosphorylation and hydrolysis of fructose 6-phosphate and fructose 1,6-biphosphate. Newsholme et al (27) also postulated that the same mechanism may be the driving force of protein turnover. Increased protein turnover rates facilitate the possibility to adapt rapidly to changing conditions because the net effects of a certain change is amplified by the magnitude of the turnover rates. The concept is further explained in figure 2. The level of whole body protein turnover rates is thus an indication of its possibility to adapt to its changing surroundings. It must, however, also be kept in mind that these biological roles of substrate cycles are purely hypothetical and the presence of substrate cycles may be futile and indicate imperfectness of the regulation of metabolic routes.

Protein synthesis in tissue

Protein synthesis has also been measured at regional tissue levels. These methods can be divided into two types of assessments: the direct incorporation technique and the A-V dilution technique.

Incorporation technique

The incorporation technique was first introduced in studies Lofffield and Harris (28) and later by Waterlow (29,30) and by Haliday (23) (Fig. 3). It requires a (primed) constant infusion of a labeled amino acid and the measurement of enrichment or specific activity during a specific period in the precursor pool of protein synthesis and in the protein pool. The method thus requires biopsies of tissue and fairly straightforward measures the incorporation of a certain amino acid into proteins over a certain time period. In formula:

$$\text{FSR} = \frac{\text{SA}_{\text{prot}}(t) - \text{SA}_{\text{prot}}(0)}{\int_0^t \text{SA}_{\text{precursor}}(t) dt} \quad (3)$$

where t is the time of infusion (minutes or hours), FSR is the fractional synthetic rate (%/ minute or hour), $\text{SA}_{\text{prot}}(t) - \text{SA}_{\text{prot}}(0)$ the difference between the protein bound specific activity of tracer amino acid at time point t (end of experiment) and zero in dpm/nmol and $\int_0^t \text{SA}_{\text{precursor}}(t) dt$ the area under the curve of the precursor pool for protein synthesis (arterial or intracellular SA).

Controversy, however, still exists whether the enrichment or specific activity of the true precursor pool is measurable or whether a good estimate of the enrichment or specific activity of the precursor pool can be made. The true precursor pool enrichment or specific activity for protein synthesis is the amino-acyl-tRNA of the amino acid studied (3,19,31,32). This pool, however, is limited in size and aminoacylation of tRNA has a half-life of approximately 1 second (3,33). This obviously limits direct measurements of tRNA enrichment or specific activity and estimations of the precursor pool labeling have been derived from the arterial, venous and intracellular free amino acid pool. It has been observed that muscle tissue tRNA labeling during a constant infusion of labeled amino acids lies between the labeling of the arterial pool and the intracellular free amino acid pool (3,19,33). It has thus been proposed by Wolfe (34) to calculate upper bounds and lower bounds of protein synthesis rates. The specific activity in the arterial pool exceeds that of the tRNA pool and calculations with the arterial enrichment or specific activity will thus underestimate protein synthesis rates and can be seen as the lower bounds. The intracellular and venous specific activity is generally lower than the tRNA pool and calculations with these two pools as precursor pool labeling will overestimate the true protein synthesis rates and thus may be seen as upper bounds.

In light of this controversy, Garlick et al developed the so-called "flooding dose" technique in small animals (35,36). The underlying principle is that the differences in enrichment and specific activity of the different amino acid pools (arterial, intracellular, tRNA, venous) are all minimized by giving a very large amount of amino acid with a certain, measurable, specific activity. Thus all free amino acid pools are flooded with the amino acid with a constant specific activity during a short period of time. Although the method has given valid and reproducible results by the group of Garlick in mostly small animals (35,37-42), it remains controversial as to whether flooding the free pool of a single amino acid influences measurements of protein synthesis (19,43). The major criticism therefore is that giving such an excess of amino acids above the total body free amino acid pool, influences amino acid

metabolism and protein synthesis rates (3,19). Several studies using both techniques indicate that the flooding dose technique artificially increases protein synthesis rates whereas other studies could not confirm this (44). To make data from the flooding dose technique comparable to data using a constant infusion, Chinkes, Rosenblatt and Wolfe suggested to reanalyze the data derived from flooding dose experiments with the assumption that protein synthesis rates are stimulated proportional to the change in intracellular concentration. In this manner a lower bound of protein synthesis rates can be calculated whereas protein synthesis rates calculated by the standard flooding dose technique account for an upper bound of protein synthesis rates (43).

Protein synthesis *in vitro* is also measured by the incorporation of isotopically-labeled amino acids. Phenylalanine and tyrosine are for muscle the amino acid of choice since neither is appreciably metabolized by muscle tissue (45-47). *In vitro* protein synthesis rates of hepatocytes are generally determined by the incorporation of valine into protein as the metabolic fate of branched chain amino acids in hepatocytes is in majority into proteins (48-51). By adding an 5 mM solution of labeled valine to hepatocytes a similar situation as the flooding dose is achieved to attain a constant specific activity of the precursor pool (49,50).

Figure 3. Model to measure protein synthesis by the incorporation technique. Constant labeling of the precursor pool during a specific time results in labeling of protein and by using formula 3 protein synthesis rate can be calculated as fraction per time unit.

A-V dilution technique

In 1987, Barret and Gelfand (52) described a two compartment model to measure *in vivo* protein turnover of an extremity based on calculations performed in earlier work of Rannels and colleagues (53) (Fig 4). After a primed constant infusion of labeled phenylalanine plasma flow is measured and samples are drawn from the arterial and venous blood across an extremity. Calculations in a two compartment

model enable the simultaneous measurements of protein synthesis and breakdown rates. The technique is relatively noninvasive as no tissue biopsies are required. The model has been used to describe protein turnover of muscle and splanchnic tissue of both man and large animals (14,16,54-57).

Figure 4. Model described by Barret and Gelfand to calculate protein synthesis and breakdown in a two compartment model (52,58). The two compartments where samples are drawn are the arterial pool and the venous pool. Protein turnover is calculated by the venous dilution of labeled amino acid arriving through the arterial pool under steady state conditions. The first mathematical model (52) assumes the venous pool specific activity to be a reflection of the specific activity of the precursor pool for protein synthesis whereas the second model (58) assumes the arterial pool to give a better estimate.

In the A-V dilution model protein degradation is estimated from the venous dilution of phenylalanine specific activity and the extraction rate (ER) of both tracer and tracee; e.g. the ER of labeled phenylalanine is equal to:

$$ER = ([DPM]_{art} - [DPM]_{ven}) / [DPM]_{art} \tag{4}$$

where $[DPM]_{art,ven}$ is the amount of dpm derived from radioactive phenylalanine in the arterial and venous pool, respectively. Phenylalanine entry in the venous compartment is equal to the sum of: 1) the arterial inflow which is not extracted and, 2) phenylalanine derived from protein breakdown which is not reutilized for protein synthesis. In formula:

$$\begin{aligned} \text{Total entry in the vein} &= [PF \times [Phe]_{art} \times (1 - ER)] + [Ra \times (1 - ER)] \text{ or} \\ &= (PF \times [Phe]_{art} + Ra) \times (1 - ER) \end{aligned} \tag{5}$$

where $[Phe]_{art}$ is the arterial phenylalanine concentration ($\mu\text{mol/l}$), PF the organ plasma flow ($\text{ml} \times 100 \text{ gr. body weight}^{-1} \times \text{min}^{-1}$) and Ra the rate of appearance of

phenylalanine by protein breakdown ($\text{nmol} \times 100 \text{ gr. body weight}^{-1} \times \text{min}^{-1}$). The net uptake of tracer activity can also be calculated (assuming no net production of radioactive amino acids by tissue during the study period) with the following formula:

$$\text{Uptake tracer} = \text{PF} \times [\text{DPM}]_{\text{art}} \times (1 - \text{ER}) \quad (6)$$

where Uptake is the net uptake of phenylalanine in dpm and $[\text{DPM}]_{\text{art}}$ the amount of arterial dpm derived from radioactive phenylalanine. Dividing equation (6) by (5) results in:

$$\text{SA}_{\text{ven}} = \frac{\text{PF} \times [\text{DPM}]_{\text{art}}}{\text{PF} \times ([\text{Phe}]_{\text{art}} + \text{Ra})}$$

which can be rearranged to:

$$\text{Ra} = \text{PF} \times [\text{Phe}]_{\text{art}} \times [(\text{SA}_{\text{art}} / \text{SA}_{\text{ven}}) - 1] \quad (7)$$

Phenylalanine rate of disappearance (R_d , protein synthesis rates) can further be calculated by the difference between net balance of unlabeled phenylalanine and protein breakdown rates:

$$\text{net balance} = \text{PF} \times ([\text{Phe}]_{\text{art}} - [\text{Phe}]_{\text{ven}}) \quad (8)$$

$$R_d = \text{net balance} + \text{Ra} \quad (9)$$

It was explicitly noted that protein synthesis rates (R_d) calculated in this manner use the venous specific activity as precursor pool for protein synthesis. R_a and R_d are expressed as $\text{nmol phenylalanine} \times 100 \text{ gr. body weight} \times \text{min}^{-1}$

In 1987 Gelfand and Barret almost simultaneously published the same two compartment model, only now calculating R_a and R_d using the arterial specific activity instead of the venous specific activity as reference pool for estimating phenylalanine kinetics (58). Calculations are based on the disappearance of labeled phenylalanine. Formulas derived thus become:

$$\text{Ra} = \text{PF} \times [\text{Phe}]_{\text{ven}} \times (1 - \text{SA}_{\text{ven}} / \text{SA}_{\text{art}}) \quad (10)$$

$$R_d = \text{net balance} + \text{Ra} \quad (11)$$

In several studies using two compartment modeling, protein synthesis rates were simultaneously measured with the incorporation technique and A-V compartment

modeling and similar synthesis rates were observed which suggest the model to be valid (56,57,59,60).

In the early nineties, the two compartment model was extended to a three compartment model (34,60,61) (Fig. 5). The three compartment model enabled the simultaneous measurements of intracellular protein turnover with transmembrane transport of amino acids. Biolo and Wolfe further suggest this model to be a better reflection of protein turnover because it also includes the intracellular use of amino acids derived from intracellular protein breakdown. Membrane transport rates are calculated for the *in vivo* phenylalanine flux from the arterial pool to the intracellular muscle pool, the intracellular pool to the venous pool and for direct shunting from the arterial pool to the venous pool. Disposal and production of intracellular free phenylalanine are calculated and from these protein synthesis rates and protein breakdown rates are deduced.

Figure 5. Three compartment model to describe protein turnover and amino acid membrane transport introduced by Biolo and Wolfe (34). Amino acids enter the hindquarter via the arterial pool (F_a) and leave via the venous pool (F_{va}). Membrane transport rates from the arterial pool to the intracellular free amino acid pool ($F_{m,a}$), from the intracellular pool to the venous pool ($F_{v,m}$) and direct A-V shunting ($F_{v,a}$), are calculated as described in methods. Intracellular disposal (D) and production (P) of free amino acids are calculated simultaneously with membrane transport rates and is for phenylalanine directly related to respectively protein synthesis and breakdown.

Net balance of both phenylalanine and ^3H -phenylalanine across the organ is equal to:

$$\text{NB} = \text{PF} \times ([\text{Phe}]_{\text{art}} - [\text{Phe}]_{\text{ven}}) \quad (12)$$

and,

$$\text{nb} = \text{PF} \times (\text{SA}_{\text{art}} \times [\text{Phe}]_{\text{art}}) - (\text{SA}_{\text{ven}} \times [\text{Phe}]_{\text{ven}}) \quad (13)$$

where NB and nb are the net balances of phenylalanine ($\text{nmol} \times 100 \text{ gr. body weight}^{-1} \times \text{min}^{-1}$) and ^3H -phenylalanine ($\text{dpm} \times 100 \text{ gr. body weight}^{-1} \times \text{min}^{-1}$) across the organ, $[\text{Phe}]_{\text{art or ven}}$ are arterial or venous plasma concentrations ($\mu\text{mol/l}$), and PF is plasma flow ($\text{ml} \times 100 \text{ gr. body weight}^{-1} \times \text{min}^{-1}$). The two net balances are equal to differences between the transport rate of amino acid entering and leaving the tissue:

$$\text{NB} = F_{\text{m,a}} - F_{\text{v,m}} \quad (14)$$

$$\text{nb} = (\text{SA}_{\text{art}} \times F_{\text{m,a}}) - (\text{SA}_{\text{ven}} \times F_{\text{v,m}}) \quad (15)$$

$F_{\text{m,a}}$ and $F_{\text{v,m}}$ are plasma membrane transport rates from the arterial plasma pool to the free intracellular amino acid tissue pool and from intracellular pool to the venous plasma pool ($\text{nmol} \times 100 \text{ gr. body weight}^{-1} \times \text{min}^{-1}$). By substituting equations (12) and (13) in (14) and (15), transport rates are calculated:

$$F_{\text{m,a}} = \left[\left(\frac{\text{SA}_{\text{tissue}} - \text{SA}_{\text{ven}}}{\text{SA}_{\text{art}} - \text{SA}_{\text{tissue}}} \right) \times [\text{Phe}]_{\text{ven}} + [\text{Phe}]_{\text{art}} \right] \times \text{PF} \quad (16)$$

$$F_{\text{v,m}} = \left[\left(\frac{\text{SA}_{\text{tissue}} - \text{SA}_{\text{ven}}}{\text{SA}_{\text{art}} - \text{SA}_{\text{tissue}}} \right) \times [\text{Phe}]_{\text{ven}} + [\text{Phe}]_{\text{ven}} \right] \times \text{PF} \quad (17)$$

$$F_{\text{v,a}} = F_{\text{in}} - F_{\text{m,a}} \quad (18)$$

$F_{\text{v,a}}$ is equal to the direct arterial venous shunting and F_{in} the rate to which phenylalanine enters the arterial pool ($= \text{PF} \times [\text{Phe}]_{\text{art}}$). Assuming no de novo intracellular production of ^3H -phenylalanine during the infusion period reaching isotopic steady state, the nb of ^3H -phenylalanine equals the disposal of ^3H -phenylalanine from the intracellular pool. The NB of phenylalanine equals the difference between disposal and production of phenylalanine and therefore we can derive the following formula's:

$$D = nb / SA_{\text{tissue}} \quad (19)$$

$$P = D - NB \quad (20)$$

where D is equal to the disposal of the intracellular phenylalanine and P is the production of phenylalanine into this pool ($\text{nmol} \times 100 \text{ gr. body weight}^{-1} \times \text{min}^{-1}$). Rd calculated in the two compartment model (equations (9) and (11)) has great similarities with intracellular disposal calculated in a three compartment model. Rd in equation (9) is equivalent to:

$$Rd = nb / SA_{\text{ven}} \quad (21)$$

Rd in equation (10) is equivalent to:

$$Rd = nb / SA_{\text{art}} \quad (22)$$

Looking more closely, protein synthesis in the three proposed models are thus calculated assuming that SA_{tissue} , SA_{ven} or SA_{art} to reflect precursor pool labeling.

Similar as in the incorporation technique the true precursor pool labeling for protein synthesis rates is not measured in any of the A-V dilution models. Two compartment modeling therefore already resulted in two models to calculate protein synthesis rates. The first model uses the arterial pool to determine the disappearance rate of an amino acid (from the relative change in specific activity or enrichment in the incoming arterial plasma). The other model takes calculates the venous dilution of the specific activity or enrichment of amino acids. The first is thus like the mentioned lower bound whereas the second is thus like the upper bound. The three compartment model uses the intracellular dilution of the specific activity or enrichment and can be considered as an upper bound most close to the true value of protein synthesis.

The A-V dilution models have only been applied in large animal and human studies (14,16,54-57). Small animal studies have been used to study metabolism under a variety of circumstances but not for protein turnover studies in this manner. Potential technical problems concerning selective arterial and venous catheterization and flow measurements have been suggested to be responsible for this (18). However, a similar technical procedure has been used in several laboratories to measure interorgan glucose, lactate and amino acid metabolism (62-66). We therefore decided to study the possibility to use the A-V dilution model to measure *in vivo* protein kinetics in small laboratory animals (chapter 3 and 4).

In vitro and ex vivo techniques

Protein breakdown rates have mostly been measured *in vitro* (18). Phenylalanine or tyrosine release of incubated muscle in the presence of cycloheximide (to block protein synthesis and thus prevent re-incorporation of amino acids in protein) is generally used to measure total protein breakdown. The release of 3-methylhistidine (3MH) is also used to specifically calculate the proportion of myofibrillar protein breakdown (actin and myosin). The major problem with *in vitro* measurements is the fact that the *in vivo* humoral and physical conditions are not preserved. This obviously influences the observed protein breakdown rates and impede an understanding of its regulation (18). This shortcoming of *in vitro* breakdown measurements becomes evident by the fact that in most studies proteolysis of control muscles is quite high (46,67,68). Effects of disease or interventions are thus always tested under standard catabolic conditions.

3-Methylhistidine flux and excretions

3MH urinary excretion and extremity flux have been used to calculate *in vivo* protein breakdown (69-73). 3MH is an amino acid residue in actin and myosin and found at a 1:1 concentration ratio in these proteins (1 mol protein consists of 1 mol 3MH) (3). It is formed by posttranslational methylation of histidine present in proteins and, when released by protein breakdown, cannot be used for protein synthesis because no specific t-RNA exists for 3MH (74). 3MH is also not metabolized to any significant extent and its major metabolic fate therefore is urinary excretion. Urinary excretion of 3MH has thus been employed to evaluate skeletal protein breakdown during starvation (72), infections (73,75), trauma (70,76,77) and cancer cachexia (69,78,79). However, several studies demonstrated that urinary excretion of 3MH is not only specific for protein breakdown of skeletal muscle but may be determined for 20-50% by splanchnic protein turnover (80,81). The problem can be circumvented by the measurement of arterial-venous concentration differences across an extremity (62,70,73,82). This, however, requires an invasive procedure whereas the urinary collection provides a relatively simple, noninvasive clinical procedure to evaluate protein breakdown.

Recently, deuterium labeled 3MH has been given as a bolus to healthy volunteers to determine whole body protein breakdown rates (83). Using a three compartment model, Rathmacher et al were able to calculate intracellular 3MH turnover which correlated well with urinary 3MH excretion. Although the method has the advantage that it excludes the errors induced by quantitative urine collection, further validation in

catabolic diseases is needed, in particular related to the proportional changes in protein turnover of different tissues which determine 3MH release into plasma.

A-V dilution technique

At present, the A-V dilution model appears to be the best method to determine *in vivo* protein breakdown rates. As discussed above the technique involves selective catheterization and infusion of isotopically labeled amino acids which has been proven to be valid in humans and large animals. *In vivo* measurements of protein breakdown rates have the great advantage that measurements are made under normal physical and humoral conditions. It has thus been suggested that caution should be obtained in interpreting results obtained from *in vitro* data (3,18) and warranted a further exploration of possibilities to measure *in vivo* protein breakdown rates in small laboratory animals. The A-V dilution model was therefore tested in this thesis to calculate *in vivo* protein breakdown rates.

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MUSCLE PROTEIN AND AMINO ACID TURNOVER IN RATS *IN VIVO*: EFFECTS OF SHORT-TERM AND PROLONGED STARVATION.

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Abstract

Protein loss in muscle can be caused by decreased protein synthesis, increased breakdown or both. In small animals the tracer incorporation technique is mostly used to measure protein synthesis but for degradation measurements *in vitro* or *ex vivo* settings are required. In human and large animal studies the arteriovenous dilution technique is used because it enables the measurement of synthesis and breakdown rates simultaneously. The applicability in small animals has not yet been proven. We used a starvation model to compare both techniques.

A primed constant infusion of L-[2,6-³H]-phenylalanine was given to male Lewis rats after 16, 40, 64 and 112 hours starvation. Protein synthesis rates of the gastrocnemius muscle was measured by the incorporation technique and compared with hindquarter protein turnover calculated in a two and three compartment arteriovenous dilution model.

Whole body phenylalanine rate of appearance decreased from 456 ± 32 after 16 hours to 334 ± 34 ($\text{nmol} \times 100 \text{ g bw}^{-1} \times \text{min}^{-1}$) after 112 hours starvation. Protein synthesis rates of the gastrocnemius muscle measured by the tracer incorporation technique decreased from 3.6 ± 0.4 after 16 hours starvation to 2.2 ± 0.3 after 64 hours starvation and 1.8 ± 0.4 (%/day) after 112 hours starvation. Hindquarter protein breakdown, calculated with the tracer dilution model, increased after 112 hours starvation from 28 ± 12 to 77 ± 15 ($\text{nmol} \times 100 \text{ g bw}^{-1} \times \text{min}^{-1}$). Using the tracer dilution model, however, the calculated protein synthesis rate across the hindquarter also increased after prolonged starvation (29 ± 7 and 68 ± 16 $\text{nmol} \times 100 \text{ g bw}^{-1} \times \text{min}^{-1}$ after 16 and 112 hours, respectively). In conjunction with this, calculated bidirectional

membrane transport rates were also enhanced. Using valine and glutamine as tracer, the enhanced amino acid turnover rates were confirmed.

In conclusion, our results show that during short periods of starvation both methods give similar results. After prolonged starvation, however, an opposite change in disappearance rate and protein synthesis rate was observed. Assumptions made to calculate protein turnover using the arteriovenous model may account for the discrepancy and care must be taken with the interpretation when using only one model in anaesthetized small animals.

Introduction

The presence of disease is often associated with a spontaneous decrease of food intake. Although the exact role of the decreased food intake in the development of severe metabolic disorders such as cachexia is not well defined, some authors consider it the most important determinant (1, 2).

During starvation muscle protein is broken down to supply amino acids for increased oxidation and gluconeogenesis in the liver (3). During short periods of starvation decreased protein synthesis rates lead to the observed net muscle protein loss (4, 5, 6). When starvation is prolonged increased protein breakdown rates are suggested to be the determining factors (5, 7-10). Information on protein breakdown rates in these studies was derived from hindquarter perfusions (7, 8), incubated muscle studies (10) or from indirect measurements (5, 9) but never from direct measurements *in vivo*.

For several years the arteriovenous (A-V) dilution technique has been used to measure protein breakdown rates *in vivo* simultaneously with synthesis rates (11-13). The protein synthesis rates calculated with the A-V model correlated well with fractional synthetic rates (FSR%) measured by the incorporation model (11-15). However, the A-V dilution method has mostly been applied in human and large animal studies. In contrast to the smaller animal studies, a human study indicated that protein loss during short starvation (60 h) was mainly due to increased proteolysis with an unchanged protein synthesis rate (16). Data *in vivo* in small animals on both protein breakdown and synthesis rates using the A-V dilution model are not available but may provide a better understanding of the possible discrepancy between *in vivo* human and animal studies *ex vivo*.

Thus, the effects *in vivo* of short (16 and 40 h) and prolonged starvation (64 and 112 h) on muscle protein turnover were studied in rats. Protein synthesis rates, calculated with the A-V dilution method across the hindquarter were compared with the FSR% of the gastrocnemius muscle measured by the tracer incorporation technique. Three

compartment modelling also enabled us to examine the effects of starvation on amino acid membrane transport rates *in vivo* (14, 17).

Materials and methods

Animals.

Male Lewis rats (280-350 g) obtained from the Centralized Animal Facilities of the University of Limburg (Maastricht, The Netherlands) were used for the study. During the experiment, rats were individually housed in metabolic cages taking into account an adaptation period of four days before the experiment. Rats were given standard laboratory rat chow (SRMA 1210, Hopefarms, Woerden, The Netherlands) and water *ad libitum*. They were subject to standard 12 hour light-dark cycle periods (07:30 to 19:30 hours), whereas room temperature was maintained at 25°C. The experiments were performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals (17) and approved by the Ethics Committee of Animal Research of the University of Limburg.

Experimental groups

Rats (n=10 per group) were randomly divided into five groups. The first group fasted for 16 h (control group), the second group 40 h, the third 64 h and the fourth 112 h before sampling. A fifth group was added as a second control group for possible effects due to prolonged housing in metabolic cages. These rats were housed in metabolic cages for 112 h, but only fasted overnight before sampling.

Study procedure

Rats were randomly assigned to metabolic cages and weighed daily at 08:00 hours. All infusion and sampling experiments were performed as described previously (18-21). In brief, under ether (Chempropack BV, Dordrecht, The Netherlands) anaesthesia and at constant, pre-anaesthesia body temperature, a laparotomy was performed. The right renal vein, a tertiary branch of the mesenteric vein, the portal vein, the hepatic vein, the inferior vena cava and the aorta (just above the iliac bifurcation) were cannulated using a 25-gauge needle fixed in a Silastic tube (Silastic Medical Grade tubing 0.051 cm ID, 0.094 OD, Dow Corning Corporation, Medical Products, Midland, MO, USA) and fixed with cyanoacrylate (Cyanolit 201, Het Rubberhuis, Maastricht, The Netherlands). The right carotid artery was next catheterized (Intramedic Polyethylene Tubing PE 50, 0.051 cm ID, 0.094 cm OD, Clay Adams, Parsipany, NY, USA). The complete procedure took approximately 40 minutes. Liver, Portal Drained Viscera

(PDV) and hindquarter metabolism was measured simultaneously. Liver and PDV results are discussed in chapter 4.

For flow measurements, the indicator dilution method with *para*-aminohippuric acid (PAH) was used (pH 7.4, iso-osmolar, Sigma A 1422, St Louis, MI, USA). A primed (0.15 ml/100 g bw, 50 mmol/L) constant infusion ($0.75 \text{ ml} \times 100 \text{ g bw}^{-1} \times \text{h}^{-1}$, 5 mmol/L) of PAH was simultaneously infused in the mesenteric vein and the aorta, using a Minipuls 3 Peristaltic Pump (Gilson Med. Electr. Inc., Villiers-le-Bel, France).

To study protein turnover and amino acid membrane transport kinetics, L-[2,6- ^3H]-phenylalanine (^3H -Phe, Sigma P 6053, St Louis, USA), L-[3,4- ^3H]-glutamine (^3H -Gln, NEN DuPont, NET-551, Mechelen, Belgium) and L-[3,4- ^3H]-valine (^3H -Val, Amersham Int plc, TRK533, Buckinghamshire, UK) were used as tracers. A primed ($1 \mu\text{Ci}/100 \text{ g bw}$) constant infusion ($1 \mu\text{Ci} \times 100 \text{ g bw}^{-1} \times \text{h}^{-1}$) was given into the right renal vein.

In a pilot experiment, steady state conditions necessary to measure protein turnover were measured (Fig. 1). At 30 minutes, isotopic steady state had been reached at both 16-h and 112-h starved rats.

Figure 1. Arterial steady state conditions of ^3H -Phe during a primed constant infusion. Values are mean $\pm$ SEM. ○ After 16 hours starvation (n=4) ■ After 112 hours starvation (n=5).

Sampling procedure

Blood (1.2 ml per catheter) was sampled from the hepatic vein, portal vein, inferior caval vein and carotid artery at a maximum rate of approximately 300 μ l/min. All blood was collected in heparinized cups (Lithium-Heparin Micro-sample container CB100, Sarstedt, FRG) on ice. The right gastrocnemius muscle was then dissected free, immediately freeze clamped, placed in liquid nitrogen and stored at -80°C until further analysis.

For PAH determinations, 50 μ l of heparinized blood was added to 500 μ l trichloroacetic acid GR 10% (TCA 10% w/v, Merck 807, Darmstadt, Germany), vortexed and centrifuged at 8900 *g* at 4°C (Hettich Universac 30 RF, Tuttlingen Germany). The supernatant was frozen in liquid nitrogen and stored at -80°C until further analysis. For hematocrit determinations a micro-hematocrit tube (Modulohm 88302, Herlev, Denmark) was filled with heparinized blood and centrifuged at 10 000 *g* at room temperature (Micro Hematocrit Centrifuge, Hawksley LTD, 891481, UK). Hematocrit was read with a Micro Hematocrit Reader (Hawksley Ltd, UK). Plasma was obtained by whole blood centrifugation at 8900 *g* at 4°C for 5 minutes. For glucose and lactate determinations, 200 μ l plasma was vortexed with 20 μ l of 50% TCA, put into liquid nitrogen and stored at -80°C . For plasma amino acid determinations 200 μ l plasma was added to 8 mg 5-sulfosalicylic acid (SSA 5%, Brunswick, 10346, Amsterdam, The Netherlands) for deproteinization, vortexed, frozen in liquid nitrogen and stored at -80°C .

Biochemical analysis

Plasma glucose, lactate and PAH were determined spectrophotometrically on a Cobas Mira S (Roche Diagnostica, Hoffman-La Roche, Basel, Switzerland) by standard enzymatic methods, using commercially available kits as described previously (19, 21). Standard plasma amino acids were determined by fully automated high performance liquid chromatography (HPLC) using 2-3 mmol/L spherisorb ODS II packing material and pre-column derivatization with ortho-phthaldialdehyde as described previously (22).

To determine plasma amino acid specific activity (SA), plasma was fractionated on an ion exchange column using a technique adapted from a previously described method (23, 24). In short, deproteinized plasma (50 μ l) was passed over the cation exchange resin column (Gold-column, 200 mm-4.6 mm, Ultropac 8 resin packing, 8 mm-0.5mm, N^o 4310-142, Pharmacia LKB, Uppsala, Sweden) at a flow rate of 0.425 ml/min and a total running time of 79 minutes. A 1.0 mol/L lithium citrate buffer (pH 3.45) was used as eluent for 39 minutes. The next 6 minutes a lithium hydroxide solution (12.6 g/L) was eluted whereafter the eluent reversed to the previous for the

remaining run. The column was maintained at 59°C during the first 30 minutes, heated to 79°C in a column oven (Mistral, Spark Holland, Emmen, The Netherlands) for 10 minutes and brought back to 59°C henceforth. For detection of the amino acids, a UV-detector was used (UV-M II detector, 254 nm filter, Pharmacia LKB, Uppsala, Sweden) and the fractions (2 ml) were collected in 6 ml miniature vials (Packard Instruments BV, Groningen, The Netherlands) placed in a fraction collector (Helifrac, Pharmacia LKB). Amino acid concentrations in the fractions were determined by HPLC as described previously and no overlap between phenylalanine and tyrosine was observed (22). The recovery of each amino acid was determined and found to be >95%. Formula 989 (6 ml, NEN Research Products, N° NEF 989, Boston, USA) was added as scintillation cocktail and the sample was counted in a automatic Liquid Scintillation System (Beckmann Instruments Inc., LS 3801, USA).

For the determination of tissue free amino acid concentrations and SA, muscle tissue was pulverized using a mortar and pestle precooled in liquid nitrogen. For amino acid determinations the tissue was further homogenized and deproteinized using an Ultra-Turrax (Omni 10001, N° 10-0427, Waterbury, CT, USA). Approximately 100 mg tissue was added to 500 µl SSA 5% with norvaline 0.5 mmol/L (BDH 10346) as an internal standard. The homogenate was frozen in liquid nitrogen and stored at -80°C until further determinations.

To determine the tissue dry weight approximately 200 mg pulverized tissue was freeze dried for 24 h in a Speedvac (type SC200, Savant Instruments Inc., Farmingdale, NY, USA) connected with a refrigerated condensation trap (type RT 400, Savant Instruments Inc.).

For the determination of the amino acid composition of tissue protein and the incorporation of radioactive phenylalanine and tyrosine in protein, the pellet of the tissue homogenate was used. The pellet was washed three times with milliQ distilled water at 4°C and then freeze dried in the Speedvac. The freeze dried acid non-soluble proteins were hydrolyzed in 2 ml, 6 mol/L HCl (Merck, N° 923), at 110°C for 18 hours in a vacuum sustained tube. Protein amino acid analysis and SA determinations were analyzed as described above, after the carbon particles were removed by a 0.22 µm micro filter (Millipore, GSWP 01300, Molsheim, France).

Calculations and tracer models

Hindquarter plasma flow, substrate flux and muscle concentrations

Plasma flow across the hindquarter was measured using the PAH indicator dilution method as described previously (19-21). Hindquarter substrate fluxes are calculated by multiplying the inferior vena caval - arterial concentration differences by the mean

hindquarter plasma flow of the group and are expressed in $\text{nmol} \times 100 \text{ g bw}^{-1} \times \text{min}^{-1}$. As amino acid erythrocyte transport is known to be slow in comparison with plasma transport, plasma flow instead of blood flow was used in this study (25). A positive flux indicates net release, a negative flux reflects a net uptake. α -Amino-nitrogen (α -AN) was calculated as the sum of the individual amino acids measured (22).

Tissue concentrations of amino acids are expressed in mmol/L and derived by the multiplication of tissue homogenate amino acid concentration (mmol/kg wet weight) and the percentage tissue water content.

Figure 2. Three compartment model to describe *in vivo* membrane transport and protein kinetics in hindquarter muscle (17). Amino acids enter the hindquarter via the arterial pool (F_{in}) and leave via the venous pool (F_{out}). Membrane transport rates from the arterial pool to the intracellular free amino acid pool ($F_{M,A}$), from the intracellular pool to the venous pool ($F_{v,M}$) and direct A-V shunting ($F_{v,A}$), are calculated as described in methods. Intracellular disposal (D) and production (P) of free amino acids are calculated simultaneously with membrane transport rates and is for phenylalanine directly related to respectively protein synthesis and breakdown.

Protein turnover

To estimate whole body protein turnover, whole body rate of appearance (R_a) of phenylalanine was calculated by dividing the venous infusion rate of ^3H -Phe by the arterial SA at steady state (12). Whole body rate of appearance is expressed in $\text{nmol} \times 100 \text{ g bw}^{-1} \times \text{min}^{-1}$.

Hindquarter muscle protein turnover rates were calculated using a two compartment model as first described by Barrett et al. (11). Hindquarter rate of appearance (Ra) and rate of disappearance (Rd) are calculated by the venous dilution of ^3H -Phe and the A-V net balance of phenylalanine. Knowing the phenylalanine content in muscle protein enables to transform Ra and Rd into protein breakdown and protein synthesis rates as in muscle the metabolic fate of phenylalanine is limited to protein synthesis and breakdown (11, 12, 26).

Recently a three compartment model (Fig. 2) was introduced by Biolo and co-workers (14-17). Adding the third compartment not only makes it possible to calculate protein turnover *in vivo* but also plasma-tissue membrane transport rates and metabolic shunting of amino acids. Plasma-tissue membrane transport rates are calculated as the amino acid flux *in vivo* from the arterial pool to the intracellular muscle pool ($F_{m,a}$) and from the intracellular pool to the venous pool ($F_{v,m}$). Metabolic shunting can be derived from the flux entering the venous pool from the arterial pool bypassing the intracellular free amino acid pool ($F_{v,a}$). Protein synthesis rate is simultaneously calculated from the intracellular disposal (D) of phenylalanine and protein breakdown rate from the intracellular production (P) of phenylalanine.

Simultaneous with calculations in two or three compartment models, protein synthesis rates were also calculated by the incorporation of ^3H -Phe into acid non-soluble protein and expressed as Fractional Synthetic Rate per day (FSR%) (12, 27). This is equal to the rate of increase of SA in acid in-soluble protein divided by the area under the curve of the precursor pool SA. The area under the curve is calculated as the integration of the arterial specific activity of phenylalanine over the 40 minutes infusion time.

Labeling of the charged tRNA pool reflects the true precursor pool SA which should be used to calculate protein synthesis (12, 14). Arterial SA is an overestimate of the actual precursor pool SA and the calculated protein synthesis rates with arterial SA as precursor pool labeling will give the lower bound of protein synthesis rates. Using intracellular free phenylalanine SA in the calculation of protein synthesis underestimates the actual precursor pool SA and will give the upper bound of protein synthesis rates. Both upper and lower bounds of protein turnover are given as it is the best estimate of the true value (12, 14). For comparison upper and lower bounds of disposal and production are also calculated using intracellular and arterial SA as precursor pool labeling (14-17).

Statistical analysis

Results are presented as mean $\pm$ SEM. Effects of time was tested by Oneway ANOVA with *post hoc* individual group comparison by the Student-Newman-Keuls test (28). Significance from zero was tested by the Wilcoxon test. In the second experiment

significance was tested by Students *t*-test. Significance was considered present at $p<0.05$.

Results

All data concerning plasma and tissue concentrations and specific activities were not significantly different between control group 1 and 5. Therefore, the results of these groups were pooled to obtain one control group. Overnight fasted rats lost $5.4 \pm 0.3\%$ of the initial body weight. Weight loss increased to $10.1 \pm 0.3\%$, $12.8 \pm 0.5\%$ and $17.5 \pm 1.1\%$ after respectively 40, 64 and 112 hours starvation. The decrease in body weight seems to arrest between 40 and 64 hours starvation.

Arterial and intracellular concentrations

Glucose and lactate concentrations (Table 1) were relatively high. This has previously been described to be an effect of ether anaesthesia (29). Glucose concentrations increased slightly after prolonged starvation. Arterial concentrations of glutamine, the main plasma nitrogen transporter, and phenylalanine remained unchanged after short and prolonged starvation. Tyrosine concentrations, however, decreased after prolonged starvation. The sum of all amino acids (α -AN) remained unchanged. All data were in agreement with previously reported values (20).

Table 1. Arterial concentrations. Values are mean $\pm$ SEM. Arterial concentrations for glucose and lactate in mmol/L, for amino acids in μ mol/L. α -AN is the sum of all amino acids (21). Statistical analysis by Oneway ANOVA and Student-Newman-Keuls test for comparison of individual groups ($p<0.05$) a vs 16 h, b vs 40 h, c vs 64 h, d vs 112 h, ns= not significant.

	16 h	40 h	64 h	112 h	ANOVA p value
Glucose	11.7 $\pm$ 0.4	11.8 $\pm$ 0.4	13.7 $\pm$ 0.5 ^{a,b,d}	12.1 $\pm$ 0.7	0.007
Lactate	7.9 $\pm$ 0.7	9.5 $\pm$ 0.7	9.5 $\pm$ 0.5	9.3 $\pm$ 0.7	ns
Glutamine	634 $\pm$ 20	686 $\pm$ 37	636 $\pm$ 30	644 $\pm$ 30	ns
Tyrosine	88 $\pm$ 6	94 $\pm$ 6	74 $\pm$ 3	75 $\pm$ 4	0.03
Phenylalanine	62 $\pm$ 4	66 $\pm$ 6	58 $\pm$ 4	63 $\pm$ 4	ns
α -AN	3005 $\pm$ 175	3241 $\pm$ 219	3005 $\pm$ 179	3085 $\pm$ 117	ns

Table 2. Hindquarter plasma flow and fluxes. Values are mean $\pm$ SEM. Plasma flow is expressed in ml $\times$ 100 g $bw^{-1} \times min^{-1}$ and substrate fluxes in nmol $\times$ 100 g $bw^{-1} \times min^{-1}$, except total amino acids (α -AN) in μ mol $\times$ 100 g $bw^{-1} \times min^{-1}$. Flux was calculated by multiplying the group mean plasma flow and A-V concentration differences. Effects of time by Oneway ANOVA and Student-Newman-Keuls test for comparison of individual groups ($p < 0.05$): ^a vs 16 hours, ^b vs 40 hours, ^c vs 64 hours, ^d vs 112 hours. ns=not significant. Individual groups were tested for significance from zero by Wilcoxon test: [§] not significantly different from zero.

	16h	40h	64h	112h	ANOVA p value
Plasma flow	1.4 $\pm$ 0.3	1.8 $\pm$ 0.5	2.0 $\pm$ 1.0	1.9 $\pm$ 0.5	ns
Glucose	-1322 $\pm$ 350	-1843 $\pm$ 567	-3034 $\pm$ 813	-1083 $\pm$ 467 [§]	ns
Lactate	70 $\pm$ 57 [§]	347 $\pm$ 922 [§]	466 $\pm$ 444 [§]	2599 $\pm$ 571 ^{c, §}	ns
Glutamine	89 $\pm$ 25	73 $\pm$ 60 [§]	270 $\pm$ 43 ^{a, b}	337 $\pm$ 73 ^{a, b}	0.002
Tyrosine	-4.5 $\pm$ 3.6 [§]	-2.6 $\pm$ 5.4 [§]	4.3 $\pm$ 4.5 [§]	10.1 $\pm$ 5.0	0.05
Phenylalanine	-1.9 $\pm$ 2.5 [§]	0.9 $\pm$ 4.8 [§]	5.2 $\pm$ 3.4 [§]	9.0 $\pm$ 2.0	ns
α -AN	122 $\pm$ 120 [§]	184 $\pm$ 229 [§]	498 $\pm$ 195	857 $\pm$ 161 ^{a, b}	0.02

Table 3. Intracellular muscle tissue amino acid concentrations and water content of gastrocnemius muscle. Values are mean $\pm$ SEM. Water content is expressed as percentage water in wet weight, concentrations in μ mol/kg wet weight, except α -AN in mmol/kg wet weight. Statistical analysis by Oneway ANOVA and Student-Newman-Keuls test for comparison of individual groups ($p < 0.05$): ^a vs 16 hours, ^b vs 40 hours, ^c vs 64 hours, ^d vs 112 hours. ns=not significant.

	16h	40h	64h	112h	ANOVA p value
Water %	81.0 $\pm$ 0.5	81.0 $\pm$ 0.2	81.1 $\pm$ 0.2	80.5 $\pm$ 0.2	ns
Glutamine	2817 $\pm$ 96	3373 $\pm$ 206 ^{a, c}	2739 $\pm$ 116	3037 $\pm$ 171	0.01
Tyrosine	167 $\pm$ 5	226 $\pm$ 13 ^{a, c, d}	149 $\pm$ 6	160 $\pm$ 8	<0.001
Phenylalanine	93 $\pm$ 5	113 $\pm$ 4 ^{a, c}	90 $\pm$ 4	103 $\pm$ 4	0.006
α -AN	30.5 $\pm$ 1.4	35.5 $\pm$ 3.9	29.0 $\pm$ 1.0	34.1 $\pm$ 1.3	ns

Hindquarter plasma flow, fluxes and muscle tissue concentrations

Plasma flow (Table 2) across the hindquarter did not change significantly in time. Although glucose uptake across the hindquarter seemed to increase to a maximum at 64 h starvation, the uptake was not significantly different between the groups. Lactate release also tended to increase with a maximum at 112 h of starvation. The release of glutamine and total α -AN by the hindquarter increased progressively in time, suggestive for increased net protein breakdown. In line with this, tyrosine release also increased and phenylalanine showed a similar trend after prolonged starvation.

The tissue water content of the gastrocnemius muscle (Table 3) was not significantly different between the groups. Glutamine, phenylalanine, tyrosine and α -AN concentrations were all increased at 40 h of starvation and decreased to control values after 64 and 112 h starvation.

Table 4. Whole body Ra of phenylalanine; hindquarter Ra, Rd disposal and production of phenylalanine. Values are mean $\pm$ SEM and expressed in nmol $\times$ 100 g $bw^{-1} \times min^{-1}$. Statistical analysis by Oneway ANOVA and Student-Newman-Keuls test for comparison of individual groups ($p < 0.05$): ^a vs 16 hours, ^b vs 40 hours, ^c vs 64 hours, ^d vs 112 hours.

	16h	40h	64h	112h	ANOVA p value
<i>Whole body</i>					
Ra	456 $\pm$ 32	450 $\pm$ 31	348 $\pm$ 30	334 $\pm$ 54	0.03
<i>Hindquarter</i>					
Ra	28 $\pm$ 12	8 $\pm$ 6	77 $\pm$ 34 ^{a,b}	77 $\pm$ 15	0.03
Rd	29 $\pm$ 7	7 $\pm$ 9	71 $\pm$ 36	68 $\pm$ 16	0.05
<i>Production</i>					
Upper bound	23 $\pm$ 11	11 $\pm$ 8	72 $\pm$ 28 ^{a,b}	74 $\pm$ 14	0.01
Lower bound	16 $\pm$ 6	6 $\pm$ 5	37 $\pm$ 12 ^b	48 $\pm$ 8 ^{a,b}	0.01
<i>Disposal</i>					
Upper bound	24 $\pm$ 13	10 $\pm$ 12	66 $\pm$ 30	65 $\pm$ 15	0.06
Lower bound	17 $\pm$ 6	5 $\pm$ 8	32 $\pm$ 14	39 $\pm$ 8 ^{a,c}	0.04

Protein turnover

Whole body phenylalanine Ra (Table 4) of the control group was, per 100 g bw, in agreement with literature data (30). As observed previously, whole body Ra decreased significantly after 64 and 112 h starvation (31).

Across the hindquarter, however, the Ra and Rd of phenylalanine (Table 4) increased after 64 and 112 h starvation indicating enhancement of muscle protein breakdown and synthesis. The largest variability was observed at 64 h - suggestive of a transitional phase.

For comparison, we also calculated intracellular production and disposal of phenylalanine in a three compartment model (14-17). Lower bounds are calculated with the arterial SA as precursor pool SA, upper bounds are calculated with the intracellular free amino acid SA as precursor pool labeling (14, 17). Upper bounds of phenylalanine production and disposal gave the same pattern as Rd and Ra and were of comparable magnitude. Upper and lower bound of intracellular phenylalanine production increased with increasing duration of starvation. The lower bound of intracellular disposal also increased after prolonged starvation.

Figure 3. Protein synthesis and protein breakdown rates in hindquarter muscle after short and prolonged starvation measured with the A-V dilution model. Values are mean $\pm$ SEM and expressed % per day. $\square$ Upper bounds, $\blacksquare$ Lower bounds. The upper bounds are calculated with intracellular free SA as precursor pool SA, the lower bounds are calculated with arterial SA as precursor pool SA. Statistical analysis by Oneway ANOVA: protein synthesis upper bound: $p=0.10$, protein synthesis lower bound: $p=0.05$, protein breakdown upper bound: $p=0.02$, protein breakdown lower bound: $p=0.004$. Student-Newman-Keuls test for comparison of individual groups ($p<0.05$): ^a vs 16 hours, ^b vs 40 hours, ^c vs 64 hours, ^d vs 112 hours.

Protein synthesis and breakdown rates derived from the A-V dilution data of phenylalanine are given as fractional synthetic rate and fractional breakdown rate in Fig. 3. Both upper and lower bounds of fractional breakdown rates in muscle increased after prolonged starvation. The lower bound of the fractional synthetic rates calculated with the A-V dilution model also increased after prolonged starvation. In contrast, upper and lower bounds of fractional synthetic rates calculated by incorporation of ^3H -Phe in acid non-soluble proteins (Fig. 4), decreased significantly after 64 and 112 h starvation.

Membrane transport and A-V shunting

Using the three compartment model both inward ($F_{m,a}$) and outward ($F_{v,m}$) phenylalanine plasma-tissue membrane transport (Fig. 5) remained unchanged at 40 h, but increased significantly after 64 and 112 h starvation. Metabolic shunting ($F_{v,a}$) increased after 40 h whereafter it decreased to approximately zero after 112 h starvation.

Figure 4. Fractional synthetic rate (FSR%) of acid non-soluble proteins in gastrocnemius muscle measured by the incorporation technique. Values are mean $\pm$ SEM and expressed as % muscle protein per day. $\square$ Upper bounds, $\blacksquare$ Lower bounds. The upper bounds are calculated with intracellular free SA as precursor pool SA, the lower bounds are calculated with arterial SA as precursor pool SA. Statistical analysis by Oneway ANOVA: upper bound: $p=0.22$, lower bound: $p=0.006$. Student-Newman-Keuls test for comparison of individual groups ($p<0.05$): ^a vs 16 hours, ^b vs 40 hours, ^c vs 64 hours, ^d vs 112 hours.

Figure 5. Plasma-muscle membrane transport rates of phenylalanine after short and prolonged starvation. Values are mean $\pm$ SEM. Statistical analysis by Oneway ANOVA: $F_{m,a}$ (inward arterial-tissue membrane transport rate) $p=0.01$; $F_{v,m}$ (outward tissue-venous transport rate) $p=0.01$; $F_{v,a}$ (metabolic shunting) $p=0.04$. Student-Newman-Keuls test for comparison of individual groups ($p<0.05$): ^a vs 16 hours, ^b vs 40 hours, ^c vs 64 hours, ^d vs 112 hours.

Table 5. Hindquarter Ra and Rd glutamine and valine after 16 hours and 112 hours starvation. In a second experiment glutamine, valine and phenylalanine turnover were tested simultaneously. Values are mean $\pm$ SEM and expressed in nmol $\times$ 100 g bw⁻¹ $\times$ min⁻¹. Statistical analysis by Students *t*-test.

	16 h	112h	p value
<i>Glutamine</i>			
Ra	354 $\pm$ 19	1068 $\pm$ 88	0.001
Rd	276 $\pm$ 29	753 $\pm$ 60	<0.001
<i>Valine</i>			
Ra	33 $\pm$ 9	94 $\pm$ 8	0.001
Rd	41 $\pm$ 6	90 $\pm$ 8	0.001
<i>Phenylalanine</i>			
Ra	21 $\pm$ 4	53 $\pm$ 7	0.003
Rd	16 $\pm$ 3	39 $\pm$ 5	0.004

Hindquarter amino acid turnover

To test whether the increased turnover rates after prolonged starvation was only specific for phenylalanine, we also measured turnover rates of glutamine and the branched chain amino acid valine in a second experiment (Table 5). Hindquarter muscle turnover of glutamine and valine both increased after prolonged starvation. It appears that prolonged starvation induces an increased turnover of most amino acids. In the second experiment prolonged starvation increased phenylalanine turnover to a similar extent as in the first experiment, thus making the results reproducible.

Discussion

The A-V dilution model was introduced to measure protein synthesis and breakdown rates simultaneously and proven to be comparable to the incorporation technique in human and large animal studies under several circumstances (11, 12, 14, 15). The technique is similar to the one we and others use in hindquarter A-V substrate flux studies in small animals (19, 21, 29, 32-35). We therefore tested the applicability of the model and the effects of short and prolonged starvation in rats undergoing standard procedures used in flux measurements.

Owing to a net protein breakdown of muscle, phenylalanine and total amino acid efflux increased when starvation proceeded. The increased net protein breakdown of muscle during starvation should be reflected by the difference between Ra and Rd or production and disposal. The concentration of phenylalanine in muscle protein is approximately 230 nmol/mg (data not shown) and rat hindquarter dry weight is approximately 3300 mg/100 g bw (35). Assuming whole body weight loss to be proportional to hindquarter protein loss, an 18% weight loss as we observed after 112 h starvation, should lead to an efflux of approximately 20 nmol phenylalanine $\times$ 100 g $\text{bw}^{-1} \times \text{min}^{-1}$. Net fluxes of phenylalanine calculated after 40, 64 and 112 h starvation, however, only became significant from zero in the 112 h starved group with a maximum of 9.0 nmol $\times$ 100 g $\text{bw}^{-1} \times \text{min}^{-1}$. Although part of the weight loss can be accounted for by loss of lipids and carbohydrates not resulting in a release of phenylalanine, the flux calculated was far below the theoretical release. Also, the coefficient of variance of Ra and Rd (or production and disposal) was 12-20% and too large to allow this size of differences between protein breakdown and synthesis to become significant. We therefore conclude that flux and protein turnover measured by the A-V dilution model can only be applied to evaluate acute metabolic changes which exceed protein loss of at least 4% per day or changes in Ra and Rd of at least 20%.

Protein breakdown rates we calculated with this model tripled after prolonged starvation and are similar to indirectly estimated breakdown rates described previously (37). The rapid enhancement of proteolysis observed after 4-5 days' starvation in the current study has also been observed in *ex vivo* studies. Prolonged starvation of rats induces secondary lysosome formation with marked autophagy of muscle after 4-6 days (37). This increase has also been referred to as being an emergency response of an end-stage starvation (7, 37).

In this end-stage starvation (112 h starved group) protein synthesis rates measured by the incorporation method decreased with approximately 50%, in agreement with literature data (5, 7). In contrast, protein synthesis rates measured by the A-V dilution model almost tripled. This increase was independent of the use of a two- or a three-compartment model. Protein synthesis rates were similar in the post-absorptive group using either one of the models but diverged after prolonged starvation. Because the dilution of labeled phenylalanine by protein breakdown seen during prolonged starvation was not reflected by a similar increase of the net phenylalanine efflux, phenylalanine produced by protein breakdown must have been reused according to A-V modelling. Thus, the incorporation technique and the A-V dilution model in rats do not seem to measure the same metabolic events after extreme starvation. The reason for this discrepancy is not known but may be the result of the different assumptions underlying both models.

The tracer incorporation method is straightforward and measures synthesis of muscle proteins which precipitate in TCA. The A-V dilution model measures the turnover of phenylalanine across the hindquarter which is presumed to be for 85-90% a reflection of muscle protein turnover (14). Although stripping the skin had no influence on amino acid flux in hindquarter perfusion studies (38), various other studies have shown that skin and bone have high protein turnover rates and that the non-muscle component of the hindquarter is more active in protein synthesis than is muscle (39, 40). However, 2 days' starvation also reduced protein synthesis rates of skin and bone (measured by the tracer incorporation technique) (40) and therefore protein synthesis of non-muscle tissue in hindquarter cannot explain the increased Rd (and disposal) after prolonged starvation.

Phenylalanine is used as tracer because in muscle this amino acid is only used for protein synthesis and production only originates from protein breakdown (11, 12, 26). Alternative routes are unknown in muscle. The discrepancy of FSR% and Rd may therefore be that each method measures synthesis of different protein pools. The acid insoluble protein pool measured by the incorporation model contains the larger structural proteins in muscle. Smaller peptides remain soluble and are not part of the measurement (6, 41). In contrast, protein synthesis rate reflected by the A-V dilution model measures synthesis of all peptides and proteins across the hindquarter.

However, not only is the acid soluble pool approximately 100-fold smaller than the acid insoluble pool, but it has also been observed previously that the turnover of proteins is generally proportionally to its size (42). The only proteins with a rapid turnover are those which are incorrectly made (42). In line with this is the observation that 40% of newly synthesized collagen proteins are degraded before being released (43). It is however, unlikely that the turnover of this small, acid soluble, incorrectly made, protein pool is responsible for the tripled disappearance rate of phenylalanine. The increased turnover of glutamine and valine observed in the second experiment suggest that the turnover rate of all amino acids calculated with the A-V dilution model increased regardless of the rate of protein synthesis. It appears that in the rat after prolonged starvation and under the experimental conditions used, muscle protein synthesis is not measurable with the A-V dilution model.

Using the three compartment model plasma-tissue membrane transport rates *in vivo* were measured. The observed magnitude of membrane transport *in vivo* was in the same range reported previously as initial transport rates in rat muscle (44) and in perfused rat hindquarter estimated at physiological amino acid concentrations (45). This suggests that the *in vivo* method we used to measure amino acid membrane transport is in agreement with the scarce literature data.

Short starvation of 40 hours led to no significant change in metabolic shunting or bidirectional membrane transport rates of phenylalanine. When starvation proceeded, shunting of phenylalanine reduced to approximately zero and bidirectional membrane transport rates of phenylalanine significantly increased. These changes indicate that the increased muscle amino acid turnover was not only an increased intracellular turnover but was also related to an enhancement of membrane transport rates. Although analysis *via* which transport system and how this occurred was not subject of the present study, we must assume that the increased phenylalanine membrane transport rates is System L carrier mediated (45). System L is a high-capacity, low-affinity carrier system (45). Its activity *in vitro* has been linked to the availability of leucine for protein synthesis (46). In our study arterial leucine did not change significantly during starvation but intracellular leucine concentration in the 64 h starved rats was almost half of the concentration observed in control rats (data not shown). Thus, *in vivo*, leucine availability may have been the initiating factor to increase System L amino acid membrane transport activity. Prolonged starvation increases glucagon, glucocorticoid, growth hormone and thyroxine (T_4) levels and decreases insulin and triiodothyronine (T_3) concentrations (3, 9). System L transport system is *in vitro* relatively insensitive for hormonal changes (except for thyroid hormones) (45, 46) but has recently been shown *in vivo* to be sensitive to e.g. insulin (47). *In vivo* changes in

the sensitivity to these hormones or other plasma factors, may thus be involved in the regulation of amino acid membrane transport rates (3).

In conclusion, our results show that the A-V dilution model is applicable to measure protein turnover and amino acid plasma-tissue membrane transport in muscle in overnight fasted and briefly starved rats. However, after prolonged starvation, end-stage proteolysis resulted in a discrepancy of amino acid disappearance rate into muscle tissue and the actual protein synthesis rate measured by the incorporation technique. Care must be taken with the interpretation when using only one model in anaesthetized small animals.

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IN VIVO AMINO ACID METABOLISM OF GUT AND LIVER DURING SHORT AND PROLONGED STARVATION

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Abstract

During starvation splanchnic organs are proportionally more affected by protein loss than other organs. Amino acid membrane transport is one of the regulating mechanisms of protein turnover but until now *in vivo* data are lacking. To study *in vivo* phenylalanine and tyrosine membrane transport and protein turnover in splanchnic organs, a primed continuous infusion of L-[2,6-³H]Phenylalanine was given to control rats (postabsorptive) and after short (40 hours) and prolonged (112 hours) starvation. Data were analyzed using a three compartment model previously used in muscle membrane transport studies.

Inward and outward amino acid plasma-tissue membrane transport rates in both the liver and gut were upregulated after prolonged starvation. Metabolic shunting of phenylalanine and tyrosine increased in the gut but decreased to zero in the liver after prolonged starvation. In conjunction with this, gut and liver protein turnover increased after prolonged starvation. In the liver the net uptake of gluconeogenic precursors also increased, indicative for increased gluconeogenesis. The observed changes in amino acid metabolism in both splanchnic organs after prolonged starvation, may reflect an adaptation of the gut and liver to nutritional deprivation and could be of benefit during refeeding.

Introduction

During starvation net protein loss in splanchnic organs (gut and liver) is substantially more severe than in muscle tissue (19, 40). The effects of starvation on splanchnic organs seems contradictory to the observed functional adaptations related to amino

acid and protein metabolism. In the gut, active transport of nutrients in and out of the lumen increase after starvation (3, 18). In the liver, starvation also leads to an upregulation of metabolic processes aimed at enhanced ketogenesis and gluconeogenesis (34, 36, 37). This appears to be related to enhanced amino acid membrane transport by hepatocytes (14, 20, 21, 28, 39). However, all previously mentioned membrane transport studies of the gut and liver were performed *in vitro* or *ex vivo* because manipulation can be performed under controlled conditions. In addition, *in vivo* studies are more difficult to perform (39). Recently, several studies in large animals were published in which *in vivo* membrane transport rates in hindquarter muscle and gut were measured using a primed constant infusion of radioactive (or stable isotope) tracers (4, 7, 10, 44). Similar operative and sampling procedures have been used by our group and others to measure amino acid interorgan metabolism in rats (12, 13, 22, 38). We therefore aimed to study the applicability and validity of this tracer model to measure *in vivo* gut and liver membrane transport activity in rats and relate these parameters to protein turnover and the net uptake or release of gluconeogenic precursors. The effects of short and prolonged starvation were studied as these circumstances often accompany disease and pathophysiological events.

Materials and Methods

Animals

Male Lewis rats (200-300 g) obtained from the Centralized Animal Facilities (University of Limburg, Maastricht, the Netherlands) were used for this study. During the experiment, rats were individually housed in metabolic cages after an adaptation period of 4 days prior to the experiment. Water intake was *ad libitum*. They were subject to standard 12 hour light-dark cycle periods (7:30 A.M. to 7:30 P.M.), whereas room temperature was maintained at 25°C. The experiments were performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals (17) and approved by the Ethical Committee of Animal Research of the University of Limburg.

Experiment 1

The first experiment was performed to study metabolic steady state conditions necessary for *in vivo* membrane transport studies. As prolonged starvation may influence vital organ functioning and stability of steady state conditions, rats (n=10) were studied in control conditions (overnight fasted) and in conditions of extreme fasting (112 hours).

Experiment 2

In the second experiment rats ($n=40$) were randomly divided into four groups. The first group fasted overnight (16 hours) as control group. A second group fasted for 40 hours (short starvation) and a third group 112 hours (prolonged starvation) prior to sampling. A fourth group was added as a second control group for possible effects due to prolonged housing in metabolic cages. These rats were housed in metabolic cages for 112 hours, yet only fasted overnight prior to sampling.

Study and sampling procedures

The experimental conditions were similar in both experiments. Rats were randomly put in metabolic cages and weighted daily at 8:00 A.M. All infusion and sampling experiments were performed as described previously (12, 13, Chapter 3). In brief, under ether (Chempropack BV, Dordrecht, NL) anesthesia and at constant, pre-anesthesia body temperature, a laparotomy was performed. The right renal vein, a tertiary branch of the mesenteric vein, the portal vein, the hepatic vein, the inferior caval vein and the aorta were cannulated using a 25 gauge needle fixed in a Silastic tube (Silastic Medical Grade tubing 0.051 cm ID, 0.094 OD, Dow Corning Corporation, Medical Products, Midland, MO, USA) and fixed with cyano-acrylate (Cyanolit 201, Het Rubberhuis, Maastricht, The Netherlands). Next, the right carotid artery was catheterized (Intramedic Polyethylene Tubing PE 50, 0.051 cm ID, 0.094 cm OD, Clay Adams, Parsipany, NY, USA). The complete procedure took approximately 40 minutes. Liver, Portal Drained Viscera (PDV) and hindquarter metabolism were measured simultaneously. The results of hindquarter metabolism are discussed in Chapter 3.

For flow measurements, the indicator dilution method with *para*-aminohippuric acid (PAH) was used as described in detail in chapter 3. To study *in vivo* plasma-tissue membrane transport and protein kinetics, a primed constant infusion of L-[2,6- ^3H]Phenylalanine (^3H -Phe) (Sigma P 6053, St Louis, USA) was given in the renal vein as described in chapter 3.

In the first experiment we verified whether the infusion time of 40 minutes was sufficient to reach isotopic steady state concentrations for ^3H -Phe and L-[2,6- ^3H]Tyrosine (^3H -Tyr). Arterial blood (0.6 ml per sample) was sampled 20, 30 and 40 minutes after the bolus of ^3H -Phenylalanine was given. In the second experiment blood (1.2 ml per catheter) was sampled from the hepatic vein, portal vein, inferior caval vein and carotid artery at a maximum rate of approximately 300 $\mu\text{L}/\text{min}$. Withdrawing this amount of blood simultaneously will cause hypovolemia and therefore precludes measuring amino acid and protein metabolism at multiple time points as in the first experiment. All blood was collected in heparinized cups (Lithium-Heparin Micro-sample container CB100, Sarstedt, FRG) on ice. Hereafter, a 1 cm piece of

jejunum, approximately 1 cm distal of Treitz ligament, and the left liver lobe were dissected free, directly freeze-clamped, put in liquid nitrogen and stored at -80°C until further analysis.

For PAH determinations, 50 μL heparinized blood was added to 500 μL Trichloroacetic acid GR 10% (w/v) (TCA, Merck 807, Darmstadt, FRG), vortexed and centrifuged at 8,900 g at 4°C (Hettich Universac 30 RF, Tuttlingen, Germany). The supernatant was frozen in liquid nitrogen and stored at -80°C until further analysis. For hematocrit determinations a micro-hematocrit tube (Modulohm 88302, Herlev, Denmark) was filled with heparinized blood and centrifuged at 10,000 g at room temperature (Micro Hematocrit Centrifuge, Hawksley LTD, 891481, UK). Hematocrit was read with a Micro Hematocrit Reader (Hawksley Ltd, UK). Plasma was obtained by whole blood centrifugation at 8,900 g at 4°C for 5 minutes. For glucose, lactate, urea and ammonia determinations, 200 μL plasma was vortexed with 20 μL TCA 50%, put into liquid nitrogen and stored at -80°C . For plasma amino acid determinations 200 μL plasma was added to 8 mg 5-Sulfosalicylic acid (SSA 5%) (Brunschwig, 10346, Amsterdam, NL) for deproteinization, vortexed, frozen in liquid nitrogen and stored at -80°C .

Biochemical analysis

Acid base homeostasis, Po_2 and Pco_2 were immediately determined using a Automatic Blood Gas System (AVL List, 993, Graz, Austria). Alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), γ -glutamyl transferase (γGT) and creatinine were determined in serum using standard procedures. Plasma glucose, lactate, ammonia, urea and PAH were determined spectrophotometrically on a Cobas Mira S (Roche Diagnostica, Hoffman-La Roche, Basel, Switzerland) by standard enzymatic methods, using commercially available kits as described previously (12, 13). Standard plasma amino acids were determined by fully automated high performance liquid chromatography (HPLC) using 2-3 μM spherisorb ODS II packing material and pre-column derivatization with o-phthalaldehyde as described previously (42). To determine plasma phenylalanine and tyrosine specific activity (SA), plasma was isolated on an ion exchange column using a technique adapted from a method described previously (16, see also chapter 3).

For the determination of tissue amino acid concentrations and SA, tissue was pulverized using a mortar and pestle precooled in liquid nitrogen. For amino acid determinations the tissue was further homogenized and deproteinized using an Ultra-Turrax (Omni 10001, N $^{\circ}$ 10-0427, Waterbury, CT, USA). Approximately 100 mg tissue was added to 500 μL SSA 5% with norvaline 0.5 mM (BDH 10346) as an internal standard. The homogenate was frozen in liquid nitrogen and stored at -80°C until further determinations.

To determine the tissue dry weight approximately 200 mg pulverized tissue was freeze dried for 24 hours in a Speedvac (type SC200, Savant Instruments Inc., Farmingdale NY, USA) connected with a refrigerated condensation trap (type RT 400, Savant Instruments Inc., Farmingdale NY, USA).

Calculations

Plasma flow, substrate fluxes and intracellular concentrations

Plasma flow across the PDV and liver was calculated using PAH in the indicator dilution method as has been extensively described previously (12, 13). PDV substrate fluxes are calculated by multiplying the portal venous-arterial concentrations differences with the mean PDV plasma flow of the group and are expressed in nmol/100 g bw/min. Splanchnic organ flux was calculated by multiplying the hepatic plasma flow with hepatic vein-arterial concentration differences. Liver flux was calculated by subtracting PDV flux from the splanchnic flux as described previously (12, 13). A positive flux indicates net release, a negative flux reflects a net uptake. α -Amino-nitrogen (α -AN) was calculated as the sum of the individual amino acids measured (42). Tissue concentrations of amino acids are expressed in μ M and derived by dividing the tissue homogenate amino acid concentration (μ mol/kg wet weight) by the percentage tissue water.

Membrane transport rates and protein turnover

Three compartment modelling was used to calculate plasma-tissue membrane transport rates and protein turnover (4, 7, 10, 44). The model divides an organ in two vascular compartments and a tissue compartment (see chapter 2 pp 56-58 and figure 5; chapter 3, figure 2). Inward plasma-tissue membrane transport rates ($F_{t,a}$) are calculated as the *in vivo* amino acid flux from the afferent vascular pool into the intracellular free amino acid pool. Outward plasma-tissue membrane transport rates ($F_{v,t}$) are calculated as flux from the intracellular pool to the efferent venous pool. Metabolic shunting ($F_{v,a}$) of amino acids is described as the substrate flux from the arterial pool entering the organ straight into the venous pool. Across the PDV both 3 H-Phe and 3 H-Tyr were used to calculate protein turnover. PDV production (P) of phenylalanine and tyrosine reflects the intracellular amount of amino acid produced by protein breakdown and by intraluminal absorption of amino acids. Disposal (D) of phenylalanine and tyrosine in the gut reflects protein synthesis.

Similar as in flux calculations, liver membrane transport values are derived from subtracting PDV values from splanchnic organ membrane transport rates. Disposal of phenylalanine in the liver reflects protein synthesis and hydroxylation to tyrosine (41).

Disposal was calculated by dividing tracer net balance (nb) across the liver by the intracellular specific activity of phenylalanine. Tracer net balance can be calculated by subtracting PDV tracer net balance from splanchnic organ net balance. Phenylalanine hydroxylation to tyrosine was calculated from the liver phenylalanine net uptake multiplied by the ratio of hepatic venous tyrosine SA and the SA of phenylalanine entering the liver (9). This has to be corrected for the dilution of hepatic venous tyrosine SA by liver protein breakdown. Subtraction of hydroxylation from liver disposal (D) reflects the phenylalanine used for protein synthesis only. Liver phenylalanine production (P) only originates from protein breakdown and can be calculated by subtracting liver phenylalanine tracee net balance from liver phenylalanine disposal.

Statistical analysis.

Results are presented as mean $\pm$ SEM. For statistical analysis the Mann-Whitney *U* test (non-parametric) was used to compare group differences (33). Significance was considered present at $p < 0.05$.

Results

Experiment 1

ALAT, ASAT, γ GT and creatinine (Table 1) were all within normal ranges (27). The serum enzyme activities indicate that liver and kidney functioning were normal after both 16 and 112 hours starvation. It confirms previous studies that the starvation period used in our model does not induce major organ failure (15, 26). This was also confirmed by the maintenance of acid base homeostasis between 20 and 40 minutes of the operative procedure (Figure 1). Arterial pH, bicarbonate and base excess were maintained at steady state and were not different between rats starving for 16 hours or 112 hours. Also, arterial P_{CO_2} , P_{O_2} and O_2 hemoglobin saturation remained constant during the procedure in both groups of rats.

As illustrated in Table 1, metabolic and isotopic steady state conditions were present between 20 and 40 minutes after the start of the procedure. Arterial concentrations and specific activity of phenylalanine and tyrosine were constant between 20 and 40 minutes indicating conditions that allow the calculation of amino acid and protein turnover across the gut and the liver. Whole body protein turnover, calculated by the whole body Appearance Rate of phenylalanine, was significantly lower measured at multiple time points. The variation of the calculated turnover was similar at all three time points in both 16 hours and 112 hours starved rats. Moreover, the difference between whole body protein turnover of 16 hours and 112 hours starved rats was reproducible at the three time points.

Table 1. Liver and kidney functioning and steady-state conditions. Values are mean $\pm$ SEM. ALAT, ASAT, γ GT concentrations are in IU/l, creatinine, phenylalanine and tyrosine concentrations in mM, specific activity is in dpm/nmol, protein turnover in nmol $\times$ 100 g bw $\times$ minute. Blood was sampled 20, 30 and 40 minutes after the start of the operative procedure to determine metabolic and isotopic steady state and the reproducibility of amino acid concentration and specific activity measurements in 16- and 112-h starved rats. Liver and kidney functioning was determined by serum enzymatic activity of ALAT, ASAT, γ GT and creatinine.

<i>Blood sampling time:</i>	20 min		30 min		40 min	
	16 h	112 h	16 h	112 h	16 h	112 h
<i>Rats starved for:</i>						
ALAT	-	-	-	-	29 $\pm$ 4	38 $\pm$ 5
ASAT	-	-	-	-	73 $\pm$ 5	74 $\pm$ 5
γ GT	-	-	-	-	2.4 $\pm$ 0.5	3.4 $\pm$ 0.5
Creatinine	-	-	-	-	75 $\pm$ 3	88 $\pm$ 3
Phenylalanine						
Concentration	60 $\pm$ 2	57 $\pm$ 3	61 $\pm$ 3	55 $\pm$ 3	61 $\pm$ 3	59 $\pm$ 3
Specific Activity	194 $\pm$ 9	207 $\pm$ 12	195 $\pm$ 9	217 $\pm$ 12	202 $\pm$ 9	228 $\pm$ 14
Tyrosine						
Concentration	76 $\pm$ 6	61 $\pm$ 4	79 $\pm$ 8	59 $\pm$ 4	76 $\pm$ 6	59 $\pm$ 5
Specific Activity	17 $\pm$ 1	25 $\pm$ 1	18 $\pm$ 1	25 $\pm$ 2	19 $\pm$ 1	26 $\pm$ 2
Whole Body Protein turnover	256 $\pm$ 10	226 $\pm$ 14	254 $\pm$ 11	215 $\pm$ 12	245 $\pm$ 9	205 $\pm$ 13

Figure 1. Acid base homeostasis, arterial pO₂, pCO₂ and hemoglobin O₂ saturation to determine steady state conditions after 16 hours (O) and 112 hours (>) starvation. Multiple samples were taken 20, 30 and 40 minutes after the start of the operative procedure.

Experiment 2

Plasma and tissue amino acid concentrations and specific activities were not significantly different between control group 1 and 4 and the results of these groups were pooled.

Rat body weight decreased $5.4 \pm 0.3\%$ after 16 hours starvation, $10.1 \pm 0.3\%$ after 40 hours and $17.5 \pm 1.1\%$ after 112 hours starvation. Arterial concentrations of ammonia (Table 2) did not change in time whereas urea concentrations decreased

only slightly after prolonged starvation. Both arterial glucose and lactate concentrations were relatively high due to the effects of ether anaesthesia as described previously (22). Glucose concentrations did not change during starvation but lactate concentrations tended to increase after short and prolonged starvation. Total α -AN, the main plasma nitrogen transporters glutamine and alanine, and phenylalanine concentrations remained unchanged over time. Tyrosine concentrations, however, decreased after prolonged starvation.

Intracellular concentrations

Water content (Table 3) of the jejunum increased slightly after prolonged starvation. The intracellular tyrosine concentration increased significantly after short starvation. Other amino acid concentrations showed a similar trend. Liver intracellular water content remained unchanged. Intracellular amino acid concentrations showed no significant changes except for glutamate that increased.

Table 2. Arterial substrate concentrations after 16 hours, 40 hours and 112 hours starvation. Values are mean $\pm$ SEM. Arterial concentrations for glucose, lactate and urea in mM, for ammonia and amino acids in μ M. α -AN is the sum of all amino acids (42). Mann-Whitney U: vs 16 hours: * $p < 0.05$.

	Time after starvation, h					
	16		40		112	
Ammonia	317	$\pm$ 26	360	$\pm$ 48	293	$\pm$ 37
Urea	5.0	$\pm$ 0.2	5.2	$\pm$ 0.3	4.0	$\pm$ 0.3*
Glucose	11.7	$\pm$ 0.4	11.8	$\pm$ 0.4	12.1	$\pm$ 0.7
Lactate	7.9	$\pm$ 0.7	9.5	$\pm$ 0.7	9.3	$\pm$ 0.7
Glutamine	634	$\pm$ 20	686	$\pm$ 37	644	$\pm$ 30
Alanine	408	$\pm$ 38	419	$\pm$ 42	388	$\pm$ 29
Tyrosine	88	$\pm$ 6	94	$\pm$ 6	75	$\pm$ 4*
Phenylalanine	62	$\pm$ 4	66	$\pm$ 6	63	$\pm$ 4
α -AN	3005	$\pm$ 175	3241	$\pm$ 219	3085	$\pm$ 117

Table 3. Intracellular jejunal and liver tissue amino acid concentrations and water content. Values are mean $\pm$ SEM. Water content is expressed as percentage water in wet weight, concentrations in $\mu\text{mol/kg}$ wet weight, except $\alpha\text{-AN}$ in mmol/kg wet weight. Mann-Whitney *U*: vs 16 hours: ^a $p<0.05$, ^b $p<0.01$, ^c $p<0.001$, vs 40 hours ^d $p<0.05$, ^e $p<0.01$, ^f $p<0.001$.

	Time after starvation, h		
	16	40	112
<i>Jejunum</i>			
Water %	75.8 $\pm$ 0.3	75.9 $\pm$ 0.8	78.5 $\pm$ 0.5 ^{c,e}
Glutamate	3611 $\pm$ 295	4761 $\pm$ 535 ^a	3906 $\pm$ 552
Glutamine	1171 $\pm$ 56	1235 $\pm$ 79	1007 $\pm$ 110
Alanine	2244 $\pm$ 149	2659 $\pm$ 286	2273 $\pm$ 182
Tyrosine	206 $\pm$ 17	314 $\pm$ 44 ^b	194 $\pm$ 26
Phenylalanine	155 $\pm$ 22	198 $\pm$ 46	171 $\pm$ 28
$\alpha\text{-AN}$	26.0 $\pm$ 1.7	29.0 $\pm$ 1.7	26.9 $\pm$ 2.3
<i>Liver</i>			
Water %	71.1 $\pm$ 0.3	70.3 $\pm$ 0.3	70.7 $\pm$ 0.5
Glutamate	3048 $\pm$ 200	3378 $\pm$ 417	3566 $\pm$ 304 ^d
Glutamine	3418 $\pm$ 209	4062 $\pm$ 291	3123 $\pm$ 407
Alanine	3554 $\pm$ 272	3421 $\pm$ 219	4347 $\pm$ 371
Tyrosine	101 $\pm$ 11	65 $\pm$ 25	68 $\pm$ 17
Phenylalanine	90 $\pm$ 4	80 $\pm$ 9	93 $\pm$ 5
$\alpha\text{-AN}$	26.3 $\pm$ 0.9	24.5 $\pm$ 1.5	26.1 $\pm$ 1.4

PDV and liver plasma flow and substrate fluxes

Plasma flow (Table 4) of both the gut and liver remained unchanged. Ammonia production by the PDV increased after short and prolonged starvation, albeit not significantly because of a concomitant increase of the variation. Ammonia net uptake by the liver increased in conjunction with increased urea production.

Gut glucose consumption increased, although not significantly. In the liver glucose production tended to increase and lactate consumption increased during short and prolonged starvation indicating increased gluconeogenesis.

The PDV flux of both nitrogen carriers glutamine and alanine remained consistent; glutamine being net consumed and alanine being net produced. In the liver both amino acids were already consumed in the postabsorptive control rats. Alanine uptake, a known gluconeogenic amino acid, almost doubled after short and prolonged starvation.

The small uptake of phenylalanine and tyrosine by the gut in the postabsorptive state remained similar after short and prolonged starvation. Across the liver phenylalanine and tyrosine consumption increased during starvation. Total α -AN flux of the PDV remained unchanged during the starvation periods but again showed a greater variation when starvation proceeded. In the liver, however, total α -AN uptake was higher at 40 and 112 hours of starvation.

Table 4. PDV and Liver plasma flow and substrate fluxes. Values are mean $\pm$ SEM. Plasma flow is expressed in ml/100 g bw/min, substrate fluxes in nmol/100 g bw/min. Flux was calculated by multiplying the group mean plasma flow and A-V concentration differences. Mann-Whitney U: vs 16 hours: ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$, vs 40 hours ^d $p < 0.05$, ^e $p < 0.01$, ^f $p < 0.001$.

	Time after starvation, h					
	16		40		112	
<i>PDV</i>						
Plasma flow	1.9 ±	0.3	1.6 ±	0.7	2.5 ±	0.8
Ammonia	69 ±	80	516 ±	294	590 ±	458
Glucose	-639 ±	627	-2303 ±	715	-2261 ±	858
Lactate	-1972 ±	885	-2860 ±	1094	-1913 ±	977
Glutamate	9 ±	10	63 ±	92	93 ±	53
Glutamine	-427 ±	71	-437 ±	42	-454 ±	86
Alanine	260 ±	57	451 ±	115	480 ±	123
Tyrosine	-15 ±	4	7 ±	13	-10 ±	10
Phenylalanine	-10 ±	3	13 ±	14 ^a	-1 ±	15
α-AN	-199 ±	109	544 ±	457	385 ±	444
<i>Liver</i>						
Plasma flow	2.5 ±	0.3	3.1 ±	0.7	3.3 ±	0.8
Ammonia	-244 ±	103	-1273 ±	328 ^b	-883 ±	625
Urea	504 ±	224	672 ±	203	1895 ±	884
Glucose	809 ±	1236	4899 ±	1260	2116 ±	1811
Lactate	-2797 ±	746	-9580 ±	1594 ^b	-5688 ±	2384
Glutamate	115 ±	59	107 ±	84	-214 ±	116 ^{b,d}
Glutamine	-98 ±	80	-219 ±	54	-262 ±	84
Alanine	-203 ±	95	-569 ±	155 ^a	-502 ±	256
Tyrosine	-9 ±	8	-42 ±	12 ^a	-34 ±	13
Phenylalanine	-6 ±	8	-40 ±	15	-51 ±	13 ^b
α-AN	-561 ±	246	-1806 ±	414 ^a	-2239 ±	756 ^a

Table 5. Gut plasma-tissue membrane transport rates of phenylalanine and tyrosine. Values are mean $\pm$ SEM and are expressed in nmol/100 g bw/min. $F_{t,a}$: arterial-tissue transport rate; $F_{v,t}$: tissue-portal vein transport rate; $F_{v,a}$: metabolic shunting of amino acid. Mann-Whitney U: vs 16 hours: ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$, vs 40 hours ^d $p < 0.05$, ^e $p < 0.01$, ^f $p < 0.001$.

	Time after starvation, h		
	16	40	112
<i>Phenylalanine</i>			
$F_{t,a}$	36 $\pm$ 7	39 $\pm$ 6	75 $\pm$ 12 ^{b,d}
$F_{v,t}$	37 $\pm$ 7	56 $\pm$ 13	94 $\pm$ 10 ^{c,d}
$F_{v,a}$	61 $\pm$ 7	61 $\pm$ 3	76 $\pm$ 3 ^e
<i>Tyrosine</i>			
$F_{t,a}$	42 $\pm$ 4	60 $\pm$ 11	72 $\pm$ 10 ^b
$F_{v,t}$	31 $\pm$ 4	58 $\pm$ 3 ^b	62 $\pm$ 9 ^b
$F_{v,a}$	89 $\pm$ 13	94 $\pm$ 8	126 $\pm$ 11 ^{a,d}

Table 6. Liver plasma-tissue membrane transport rates of phenylalanine and tyrosine. Values are mean $\pm$ SEM and are expressed in nmol/100 g bw/min. $F_{t,a}$: arterial and portal venous-tissue transport rate; $F_{v,t}$: tissue-hepatic vein transport rate; $F_{v,a}$: metabolic shunting of amino acid. Mann-Whitney U: vs 16 hours: ^a $p < 0.05$, ^b $p < 0.01$, vs 40 hours ^c $p < 0.05$.

	Time after starvation, h		
	16	40	112
<i>Phenylalanine</i>			
$F_{t,a}$	15 $\pm$ 9	19 $\pm$ 12	47 $\pm$ 17
$F_{v,t}$	4 $\pm$ 9	-12 $\pm$ 9	4 $\pm$ 23
$F_{v,a}$	26 $\pm$ 8	58 $\pm$ 13	-11 $\pm$ 15 ^{a,c}
<i>Tyrosine</i>			
$F_{t,a}$	23 $\pm$ 7	72 $\pm$ 20 ^b	78 $\pm$ 12 ^b
$F_{v,t}$	8 $\pm$ 6	40 $\pm$ 20	44 $\pm$ 18 ^b
$F_{v,a}$	37 $\pm$ 9	57 $\pm$ 20	-23 $\pm$ 11 ^{b,c}

Gut and liver amino acid membrane transport

The plasma-tissue membrane transport rates (Table 5) of phenylalanine and tyrosine in the gut increased progressively in both inward ($F_{t,a}$) and outward ($F_{v,t}$) direction during starvation. In the gut metabolic shunting of tyrosine also increased after prolonged starvation. Shunting of phenylalanine ($F_{v,a}$) increased after prolonged starvation when compared to short starvation.

In the liver phenylalanine inward and outward plasma-tissue membrane transport rates (Table 6) remained unchanged although inward transport rates tended to increase after prolonged starvation. Tyrosine membrane transport rates in the liver increased in both directions after 40 hours and 112 hours starvation. Shunting of phenylalanine and tyrosine in the liver showed a similar pattern with a rapid drop to zero after prolonged starvation.

Gut and liver protein turnover

Production (protein breakdown) and disposal (protein synthesis) of phenylalanine and tyrosine (Table 7) in the PDV increased after 112 hours of starvation. Liver phenylalanine production and total intracellular disposal (Table 8) also increased after prolonged starvation. The hydroxylation of phenylalanine to tyrosine by the liver did not change during short and prolonged starvation and varied between the 26 (112 hours) to 43% (16 hours) of total disposal. Therefore, phenylalanine disposal used for protein synthesis had to be increased after 112 hours starvation.

Table 7. PDV disposal (protein synthesis) and production (protein breakdown) of phenylalanine and tyrosine. Values are mean $\pm$ SEM and expressed in nmol/100 g bw/min. Mann-Whitney U; vs 16 hours: ^a $p < 0.05$, ^b $p < 0.01$.

	Time after starvation, h		
	16	40	112
<i>Phenylalanine</i>			
Disposal	81 $\pm$ 16	80 $\pm$ 41	189 $\pm$ 64 ^b
Production	82 $\pm$ 16	90 $\pm$ 35	207 $\pm$ 59 ^b
<i>Tyrosine</i>			
Disposal	101 $\pm$ 14	126 $\pm$ 37	158 $\pm$ 33
Production	89 $\pm$ 14	123 $\pm$ 30 ^a	148 $\pm$ 37 ^a

Table 8. Liver phenylalanine disposal (protein synthesis and hydroxylation), production (protein breakdown) and hydroxylation rate of phenylalanine into tyrosine. Values are mean $\pm$ SEM and expressed in nmol/100 g bw/min. Mann-Whitney U: vs 16 hours: ^a $p < 0.01$, vs 40 hours ^b $p < 0.05$.

	Time after starvation, h		
	16	40	112
Disposal	28 $\pm$ 10	69 $\pm$ 30	126 $\pm$ 351 ^{a,b}
Production	16 $\pm$ 10	38 $\pm$ 25	82 $\pm$ 28 ^a
Hydroxylation	12 $\pm$ 6	21 $\pm$ 10	34 $\pm$ 16

Discussion

During starvation the lack of supply of nutrients through the enteral route will force the gut to extract a substantial greater proportion of amino acids from arterial plasma. Liver metabolism must adapt to starvation by supplying other organs with glucose and specific amino acids during a state of overall nutrient scarcity. Glucose is produced by an increased liver gluconeogenesis (34, 36) and for this process amino acids originate from intracellular protein breakdown or from an increased amino acid uptake from the plasma pool.

All studies on amino acid membrane transport are either performed *in vitro* or in *ex vivo* perfusion models. We used a three compartment model (4, 7, 10, 44) to calculate *in vivo* plasma-tissue membrane transport rates. Steady state conditions necessary to use the model were verified in the first experiment. The operative procedure has been used for organ flux measurements before (12, 13, 22, 38) and we confirmed the presence of both isotopic and metabolic steady state conditions between 20 and 40 minutes after the start of the operative procedure. In addition, the experiment showed that amino acid concentrations and specific activity measurements were not affected by the experimental procedure itself. The small size of the animals preclude repeated measurements and left us with single point observations in the second experiment. The reproducibility in the first experiment, however, suggest the second experiment to yield valid results.

The second experiment shows an increase in phenylalanine and tyrosine plasma-tissue membrane transport rates of the gut after prolonged starvation. In conjunction with this, gut protein turnover also increases. In the liver prolonged starvation induces an increase of tyrosine membrane transport rates in both directions. Phenylalanine membrane transport rates remain unchanged although the inward membrane transport

rate tended to increase after prolonged starvation. Liver protein turnover also increased. Similarly, we observed an increase in liver ureagenesis and an increase in the uptake of gluconeogenic precursors. Although in both the gut and liver membrane transport rates were increased, no increase in the net release of phenylalanine was observed.

Amino acids are transported across the cell membrane by several transport systems with overlapping substrate specificity (11, 21). Phenylalanine and tyrosine are mainly transported by System L (L1 and L2) (21, 35). Most amino acid membrane transport systems are responsive to hormonal changes and to deprivation of amino acids but System L is generally considered not to be responsive to nutritional deprivation (1, 11, 20). We observed decreased arterial tyrosine concentrations in relation to increased membrane transport rates of both phenylalanine and tyrosine. The decreased arterial tyrosine concentrations could have upregulated the System L transport of both phenylalanine and tyrosine. However, *in vitro* studies have also shown that the System L amino acids can use other transport systems to enter the cell (1). The increase in amino acid membrane transport observed in our study may be the result of other transport system activity more sensitive to amino acid deprivation. The use of an *in vivo* three compartment model cannot differentiate between different transport systems but measures the total transport rate of amino acids irrespective of the carrier system used.

The maximum *in vitro* transport rates ($V_{\max}$) in hepatocytes ranges from 0.6 nmol/mg protein/min for L1 to 3 nmol/mg protein/min for L2 (35). This would give *in vivo* maximum transport rates of 300 to 1600 nmol/100 g bw/min assuming the rat liver to contain 500 mg protein/100 g bw in rats (25). The intracellular and arterial concentrations of phenylalanine observed in our study are approximately 5% of the K_m values of L1 and L2. The theoretical transport rates deducted from *in vitro* data would thus be between 15 and 80 nmol/100 g bw/min, which is in the same range we observed. The transport rates are also in the same range as were found in muscle tissue (4). The three compartment model therefore appears to be applicable to measure *in vivo* membrane transport rates in the gut and liver.

Protein turnover of both organs increased simultaneously with amino acid membrane transport. A net protein loss of gut and liver which is generally observed during starvation (19, 40) should be reflected in the difference between the production and the disposal or in the net release of phenylalanine or tyrosine by these organs. In our study production and disposal of the liver and gut were not significantly different in any group and were in balance at any specific moment. In the gut our measurement of protein breakdown may be confounded by the intraluminal digestion of protein from bacteria, exfoliated cells and secreted enzymes. Protein breakdown will therefore be overestimated in this model as suggested previously by Biolo et al (7). Estimation of

protein synthesis is not affected by intraluminal digestion and absorption of proteins because the specific activity of the precursor pool (intracellular free amino acid pool) is measured directly. However, it also appears that the A-V dilution model in the rat is not accurate enough to measure the difference between protein synthesis and breakdown in splanchnic organs. In the assumption that the rat liver contains 500 mg protein/100 g bw ($\approx 175 \times 10^3$ nmol phenylalanine) (43), a net protein loss of the liver of 20% per day (8, 31) will produce an estimated release of 25 nmol phenylalanine/min. Although the difference between production and disposal using the A-V dilution model increased from 12 (16 hours starvation) to 44 nmol phenylalanine/100 g bw/min after 112 hours starvation, this difference did not become significant using the A-V dilution model due to the observed variance. Therefore, production and disposal should each be considered as independent, separate parameters during the different periods of starvation. The variation in the *in vivo* data is too large to make the model applicable to detect net differences between protein synthesis and breakdown during starvation.

The *in vivo* protein breakdown rates in both splanchnic organs are in agreement with existing *in vitro* and *ex vivo* literature data (23, 24, 31). Rat liver perfusion and perfused hepatocyte studies have shown that starvation induces non-specific (intralysosomal) breakdown of mainly long lived structural proteins (29, 30, 31). The (extralysosomal) breakdown of short lived and abnormal proteins which normally account for approximately one third of total proteolysis, however, is not influenced by starvation (29). Therefore, when starvation proceeds the short lived protein pool will become more prominent and may have a substantially greater part in the total turnover of proteins. We hypothesize that the increase in total liver protein turnover we measured in our *in vivo* study may reflect a high turnover of these proteins.

Contradiction exists with one other *in vivo* study on effects of starvation on protein synthesis in the gut and liver of the rat (25). In this study the incorporation of a radiolabeled amino acid in structural proteins (mostly acid non-soluble) was studied to calculate protein synthesis. A possible explanation could be that the smaller, short lived, soluble proteins of both splanchnic organs are excluded using incorporation studies. In addition, the liver produces mostly secretory proteins. Although this problem is thought to be circumvented by shortening the infusion period of the tracer, it is possible that only part of these proteins are measured by the incorporation technique (25). A-V dilution models measure the intracellular production and disposal of amino acids and reflect the turnover of all proteins. This makes these models, in respect to protein synthesis, not fully comparable with incorporation models especially in metabolic stress, as good correlations have been observed in muscle under physiological conditions (5, 6). Further studies will be necessary to test whether both methods measure protein synthesis of the same proteins in splanchnic organs.

In conclusion, our results show that under steady state conditions the three compartment model is applicable to study *in vivo* amino acid membrane transport and protein turnover in the rat liver and gut. Starvation leads to enhanced *in vivo* membrane transport of phenylalanine and tyrosine in the gut and of tyrosine in the liver. This occurs in conjunction with an increased splanchnic protein turnover. The increased amino acid membrane transport and protein turnover could be an *in vivo* adaptive process to nutritional deprivation which then should be of benefit during refeeding as previously suggested by Newsholme (32). In view of this hypothesis, higher turnover rates of proteins amplify the control of protein metabolism and make metabolic processes more efficient when food is no longer restricted.

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INCREASED WHOLE BODY PROTEIN AND GLUTAMINE TURNOVER IN ADVANCED CANCER IS NOT MATCHED BY AN INCREASED MUSCLE PROTEIN AND GLUTAMINE TURNOVER

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Submitted

Abstract

In the progress of cancer major disturbances in protein and glutamine metabolism have been observed. Muscle is the major protein pool and glutamine source in the body. Aim of this study was to investigate whether changes in whole body protein and glutamine turnover, induced by cancer, are matched by similar changes in regional muscle metabolism.

A MCA sarcoma was implanted subcutaneously in female Lewis rats. Rats were studied bearing small (5-15% of body weight) or large (15-30% of body weight) tumor loads and compared with sham implanted free fed and paired controls. Body composition was determined by the distribution of an i.p. bolus of $^3\text{H}_2\text{O}$. Under anesthesia a primed constant infusion of L-[2,6- ^3H]phenylalanine and L-[3,4- ^3H]glutamine was given and at steady state, whole body, hindquarter muscle and tumor protein and glutamine turnover were calculated using compartment modeling.

No anorexia occurred and a small decrease in host carcass weight was observed mainly by decreased fat mass. Whole body protein turnover increased from 115 ± 14 (nmol $\times$ 100 gr. body weight $^{-1} \times$ min $^{-1}$) in free fed control rats to 239 ± 29 in the large tumor bearing rats. Net tumor protein synthesis accounted for 28 ± 1 and 49 ± 1 nmol $\times$ 100 gr. body weight $^{-1} \times$ min $^{-1}$. Muscle protein turnover increased in the small tumor bearing group but decreased again in the large tumor bearing rats. Whole body glutamine turnover remained unchanged in the small tumor bearing animals (2481 ± 248 and 1996 ± 268 nmol $\times$ 100 gr. body weight $^{-1} \times$ min $^{-1}$ in control and small tumor bearing rats, respectively) and increased by 25% in the large tumor bearing animals. In contrast, muscle glutamine turnover more than doubled in the small tumor bearing group but returned to control values in the large tumor bearing animals.

The current study shows that in the presence of a small tumor whole body protein turnover increases and that this was in part related to protein turnover of the tumor. Muscle protein breakdown increased in these rats with a concomitant increase in glutamine production from the hindquarter. In animals bearing larger tumors whole body glutamine turnover increased. This increase, however, was only for a small part caused by tumor metabolism. Muscle glutamine turnover even decreased. Therefore, the increase in whole body glutamine turnover appears to be caused by increased turnover in visceral organs.

Introduction

Glutamine, a nonessential amino acid, is the most abundant free amino acid in the body (1). In case of severe illness, e.g. trauma and sepsis, glutamine pools become depleted (2,3). Recent studies suggest that glutamine supplementation in depleted patients is of benefit to the functioning of vital organs thereby improving clinical outcome (4-6). It has been hypothesized that one of the pathophysiological mechanisms of protein catabolism may be that under specific conditions the amount of glutamine needed becomes greater than the body can produce (3,7). Glutamine thus becomes "conditionally" essential.

The major source for glutamine production is skeletal muscle. Muscle contains approximately half of the free amino acid pool and this amino acid pool consists for more than 60% of glutamine (8). In muscle, glutamine is produced from protein breakdown and from ammonia and glutamate (9). It serves as a non-toxic nitrogen shuttle to carry nitrogen from peripheral tissue to gut and liver (10). Gut and liver can hydrolyze the terminal amide group of glutamine and use the remaining α -amino group for the production of various amino acids or use the carbon skeleton as energy source (11). The liver can also use the carbon skeleton for gluconeogenesis (11).

During growth of a malignant tumor, interorgan glutamine metabolism becomes disturbed. This has been related to the development of cancer cachexia (11). Glutamine consumption of tumor cells (12,13) and activation of the immune system (14) have been suggested to lead to decreased arterial glutamine concentrations. These studies, however, have also shown that although glutamine production from hindquarter muscle increases during tumor growth, arterial glutamine concentrations decrease (15). It has therefore been suggested that the increased glutamine release by muscle was insufficient and that this may be one of the regulating factors of protein catabolism in other organs typical of cancer cachexia.

To test this hypothesis we stoichiometrically compared *in vivo* whole body glutamine turnover with the actual *in vivo* glutamine turnover of muscle and tumor tissue in a well established cancer model. In addition, whole body, muscle and tumor

protein turnover were determined to relate changes in glutamine metabolism to protein catabolism.

Materials and methods

Animals

After an adaptation period of 3 days, female Lewis rats (200-225 g, Centralized Animal Facilities, University of Limburg, Maastricht, NL) were individually housed in metabolic cages to monitor body weight and food intake. Rats were given standard laboratory rat chow (SRMA 1210, Hopefarms, Woerden, NL) and subjected to standard 12 hour light-dark cycle periods (7:30 A.M. to 7:30 P.M.) with room temperature maintained at 25°C. The experiments were performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals (16) and approved by the Ethical Committee of Animal Research of the University of Limburg.

Tumor and study groups

The tumor was induced by injecting 10 mg methylcholanthrene (MCA, Sigma W6501) subcutaneously (17). Afterwards, the tumor was maintained *in vivo* by serially transplanting tissue suspensions through a 18G needle. MCA tumors are often used in metabolic studies in Fisher 344 rats (12,15,18) and are known for their rapid growth, invasion of local tissue and absence of metastatic potential (19). Small tumor bearing rats (ST) were studied when the tumor was 5-15% of the host body weight, large tumor bearing rats (LT) when the tumor was 15-30% of the host body weight. To avoid effects of time (e.g. duration of stay in metabolic cages), tumor tissue was transplanted bilateral on the back in the ST group, whereas it was quadruple implanted in the LT group. Control rats were sham implanted with saline. Tumor growth was monitored by measuring two orthogonal dimensions and by calculating the volume assuming the tumor to be a prolate spheroid (20). Although a pilot study indicated that food intake remained unchanged in tumor bearing rats, studies by others have repeatedly shown that MCA tumors induce anorexia when the tumor becomes large (19,21). We therefore included a free fed and pairfed control group.

Experimental procedures

In a first experiment changes in body composition were determined. Postabsorptive rats (n=30) were given an intraperitoneal bolus of $^3\text{H}_2\text{O}$ (Amersham, UK, 0.1 $\mu\text{Ci}/\text{ml}$, 1 ml/100 gr. body weight). After 120 minutes blood was sampled from the orbital plexus. Plasma $^3\text{H}_2\text{O}$ was determined as described previously (22). The distribution volume of $^3\text{H}_2\text{O}$ (Total Body Water, TBW) was computed for each rat

as the ratio between the dose injected and the activity (dpm/ml plasma water) of $^3\text{H}_2\text{O}$ at steady state. Lean body mass (LBM) of carcass and tumor was calculated from TBW divided by 0.83 (23-25). Fat free mass was determined by subtracting LBM from total body weight. Subtracting tumor weight from LBM will give an estimation of carcass lean body mass.

In a second experiment protein and glutamine metabolism were studied in free fed control, paired control, small tumor bearing and large tumor bearing rats ($n=40$) as described previously (26,27, chapter 2, 3, 4). In brief, postabsorptive, under ether anesthesia and at constant body temperature, a laparotomy was performed. The right renal vein, the inferior caval vein and the aorta were cannulated with Silastic tube (0.051 cm inner diameter, 0.094 outer diameter). The right carotid artery was catheterized with a PE canula (0.051 cm ID, 0.094 cm OD). For flow measurements, the indicator dilution method with *para*-aminohippuric acid (PAH) was used. A primed constant infusion was given in the distal aorta just above the bifurcation. The priming dose was 0.15 ml/100 gr. body weight (50 mM) and the infusion rate was $0.75 \text{ ml} \times 100 \text{ gr. body weight}^{-1} \times \text{h}^{-1}$ (5 mM). To study whole body and hindquarter muscle protein and glutamine turnover, a primed constant infusion of L-[2,6- ^3H]phenylalanine (Sigma P 6053, St. Louis, USA) and L-[3,4- ^3H]glutamine (NEN, NET 551, Groningen, The Netherlands) was given in the renal vein (priming dose: $1 \mu\text{Ci}/100 \text{ gr. bw}$; infusion rate: $1 \mu\text{Ci} \times 100 \text{ gr. bw}^{-1} \times \text{min}^{-1}$). The time to reach isotopic steady state conditions was first determined in a pilot study.

Sampling procedure

Blood (1.5 ml per catheter) was simultaneously sampled after 40 minutes infusion from the inferior caval vein and carotid artery at a rate of approximately 500 $\mu\text{l}/\text{min}$. This is the minimal infusion period previously shown to be necessary to reach isotopic steady state conditions (28,29, chapter 3, 4)). All blood was collected in heparinized cups (Lithium-Heparin, CB100, Sarstedt, FRG) on ice. Hereafter, the right gastrocnemius muscle was dissected free, directly freeze-clamped, put in liquid nitrogen and stored at -80°C until further analysis. For PAH determinations, 50 μl heparinized blood was added to 500 μl TCA (10% w/v, Merck 807, Darmstadt, FRG), vortexed and centrifuged at 8,900 g at 4°C . The supernatant was frozen in liquid nitrogen and stored at -80°C until further analysis. For hematocrit determinations a micro-hematocrit tube was filled with heparinized blood and centrifuged at 10,000 g at room temperature. Hematocrit was read with a Micro Hematocrit Reader (Hawksley Ltd, UK). Plasma was obtained by whole blood centrifugation at 8,900 g at 4°C for 5 minutes. For ammonia determinations, 500 μl plasma was vortexed with 50 μl TCA 50%, put into liquid nitrogen and stored at -80°C . For plasma amino acid

determinations 300 μ l plasma was added to 12 mg 5-sulfosalicylic acid (Brunschwig, 10346, Amsterdam, NL) for deproteinization, vortexed, frozen in liquid nitrogen and stored at -80°C.

Biochemical analysis

Plasma ammonia and PAH were determined spectrophotometrically on a Cobas Mira S (Roche Diagnostica, Hoffman-La Roche, Basel, Switzerland) by standard enzymatic methods, using commercially available kits as described previously (26,27). Standard plasma amino acids were determined by fully automated HPLC (30). To determine plasma phenylalanine, glutamine and tyrosine specific activity (SA), plasma was isolated on a cation exchange resin column as described previously (31). For the determination of tissue ammonia, polyamines, amino acid concentration and specific activity, tissue was pulverized using a mortar and pestle precooled in liquid nitrogen. The tissue was further homogenized and deproteinized in a Mini-Beadbeater (Biospec products, Bartlesville USA). Approximately 100 mg tissue was added to 400 μ l SSA 5%, with 300 gr. glass beads (diameter 1 mm, Biospeck Products) and beaten for 30 seconds. The homogenate was centrifuged at 4°C at 11,000 g and the supernatant frozen in liquid nitrogen and stored at -80°C until further determinations. To determine tissue dry weight and water content approximately 200 mg pulverized tissue was freeze dried for 24 hours in a Speedvac (SC200, Savant Instruments Inc., Farmingdale NY, USA) connected with a refrigerated condensation trap (type RT 400, Savant Instruments Inc.). Polyamine concentrations in the tumor were determined on HPLC as described previously (32). Phenylalanine content in tumor proteins were determined by gas phase hydrolysis of tumor proteins by HCl for 6 hours at 150°C.

Calculations

Hindquarter plasma flow and substrate fluxes

Plasma flow across the hindquarter was calculated using PAH in the indicator dilution method described previously (26,27). Substrate fluxes are calculated by multiplying the venous-arterial concentration differences with the mean hindquarter plasma flow of the group and are expressed in $\text{nmol} \times 100 \text{ g body weight}^{-1} \times \text{min}^{-1}$. A positive flux indicates net release, a negative flux reflects a net uptake. α -Amino-nitrogen (α -AN) was calculated as the sum of the individual amino acids measured, excluding taurine and α -amino butyric acid (30). Branched chain amino acids (BCAA) are the sum of valine, leucine and isoleucine. Tissue concentrations of amino acids

are expressed in $\mu\text{mol/l}$ and derived by dividing tissue homogenate amino acid concentration ($\mu\text{mol/kg}$ wet weight) by tissue water content.

Glutamine and protein turnover

Whole body glutamine and phenylalanine rate of appearance (R_a) were calculated from the arterial dilution of the tracer amino acid at steady state (33, chapter 2) and is expressed in $\text{nmol} \times 100 \text{ gr. body weight}^{-1} \times \text{min}^{-1}$. The turnover of the essential amino acid phenylalanine is used to reflect whole body protein turnover. Hydroxylation (HDX) of phenylalanine can be calculated from the relative SA of phenylalanine and tyrosine and their relative content in whole body protein (33, chapter 2) and reflects, in steady state conditions, the oxidation rate of amino acids (33). In steady state whole body R_a equals whole body R_d and by subtracting HDX from whole body R_d phenylalanine whole body R_d used for protein synthesis ($R_{d_{\text{protein synthesis}}}$) is obtained. Glutamine, being a non-essential amino acid, can be produced by protein breakdown and, non-protein derived, by the transamidation of glutamate. Knowing the relative amino acid content of phenylalanine and glutamine in body proteins (34) makes it possible to calculate glutamine R_a derived from protein breakdown and non-protein derived glutamine R_a .

Hindquarter muscle phenylalanine and glutamine turnover are calculated from the arterial-venous dilution of the tracer amino acids across the hindquarter calculated in a two compartment model (35, chapter 2). This will give phenylalanine and glutamine Rate of Appearance (R_a) and Disappearance (R_d) across the hindquarter. R_a of phenylalanine reflects protein breakdown and R_d protein synthesis as the only fate of phenylalanine in muscle is protein (33,35). Hindquarter R_a and R_d are expressed as $\text{nmol} \times 100 \text{ gr. carcass weight}^{-1} \times \text{min}^{-1}$ because hindquarter mass is more related to carcass weight than total body weight (which exists for 0-30% of tumor mass). The hindquarter consists of several tissue compartments but muscle generally accounts for 80-90% of total hindquarter protein turnover (36). To relate the hindquarter muscle compartment with the whole body muscle compartment we considered that hindquarter muscle comprises approximately 50% of the total muscle pool of the body (37).

Tumor protein and glutamine turnover

Because no single artery and vein are present to measure glutamine and protein turnover across the tumor only estimates can be made. Net protein synthesis of the tumor in both small tumor bearing animals (double implantation) and large tumor bearing animals (quadruple implantation) is calculated after a polynomial curve fit was made to characterize tumor growth (Fig. 1). The tumor growth rate 1 day prior to

the measurements was determined per minute and multiplied by the amount of phenylalanine present in tumor proteins determined after hydrolysis of tumor tissue:

$$\text{Protein synthesis} = \Delta T \times \text{Prot}_{\text{phe}} \times \left(\frac{1}{24 \times 60} \right) \times \left(\frac{100}{\text{bw}} \right)$$

where ΔT is increase in tumor mass from day 19-20 (mg dry weight), Prot_{phe} the phenylalanine content in tumor protein (nmol/mg dry weight), bw the body weight. Tumor protein synthesis is thus expressed as nmol phenylalanine $\times 100 \text{ gr. body weight}^{-1} \times \text{min}^{-1}$. It is emphasized that this is net protein synthesis in the tumor and that the actual turnover of proteins in the tumor is higher.

Figure 1. Growth curve of MCA tumor which was double implanted ($\circ$) in the small tumor bearing group (ST) or quadruple implanted ($\bullet$) in the large tumor bearing group (LT). Linear least square regression analysis was used to determine the following coefficients in polynomial curves:
 ST: $Y=0.65 - 1.3X + 0.46X^2$, Square root of coefficient of determinations (Rval) = 0.99;
 LT: $Y=1.10 - 2.16X + 0.76X^2$, Rval = 0.99.

Upper and lower bounds of tumor glutamine turnover were determined. After starting the primed constant infusion of ^3H -Gln, the SA of intracellular glutamine can change by two possible mechanisms: 1) if the arterial plasma pool exchanges rapidly with the intracellular tumor pool an immediate steady state will be present of which the magnitude will depend on the arterial steady state SA and the (unlabeled) intracellular glutamine production; 2) if the exchange with the intracellular pool is relatively low, or not present at all, the intracellular SA will increase from zero at a slow rate during constant infusion as steady state is not obtained. In the first situation glutamine turnover can be determined by the relative change in the SA of glutamate, the product of intracellular glutamine breakdown, in relation to the intracellular SA of glutamine. Glutamine turnover is thus calculated as the percentage of glutamine metabolized to glutamate. This is similar to the incorporation technique used to determine protein synthesis and will give the fractional turnover of glutamine. Multiplied with the total intracellular glutamine pool of the tumor, glutamine turnover is calculated in nmol/min. In formula:

$$\text{Glutamine turnover} = \frac{\Delta \text{SA}_{\text{glutamate}}}{\Delta \text{SA}_{\text{glutamine}}} \times \frac{C_{\text{tumor}} \times T}{\Delta t}$$

$\Delta \text{SA}_{\text{glutamate, glutamine}}$ are increases in intracellular tumor specific activity of glutamate and glutamine (dpm/nmol) during the infusion period, Δt the infusion time (minutes), C_{tumor} the intracellular glutamine concentration (nmol/gr. wet weight) and T the tumor mass in gram. Glutamine turnover is expressed in nmol/min. However, this will only be true if the intracellular glutamine SA was immediately almost similar as the activity measured at the end of the experiment. Glutamine turnover thus underestimates the actual turnover and the calculated value can be considered a lower bound of glutamine turnover in the tumor.

If the second mechanism is true, the SA of intracellular ^3H -Gln starts at zero and rises linearly during the experiment depending on the relative glutamine turnover and the constant arterial steady state SA. The fractional turnover can thus be determined by dividing the ratio of arterial and intracellular SA of ^3H -Gln by the infusion time and multiplying this with the total intracellular glutamine pool. In formula:

$$\text{Glutamine turnover} = \frac{\text{SA}_{\text{glutamine, intra}}}{\text{SA}_{\text{glutamine, art}}} \times \frac{C_{\text{tumor}} \times T}{\Delta t}$$

$\Delta SA_{\text{glutamine, art}}$ is the increase in arterial glutamine SA at steady state (dpm/nmol) and $SA_{\text{glutamine, intra}}$ the intracellular glutamine SA (dpm/nmol). Glutamine turnover is thus calculated as uptake by tumor tissue from the arterial blood and the turnover calculated in this manner can be considered an upper bound of intracellular glutamine turnover of the tumor.

Statistical analysis

Results are presented as mean $\pm$ SEM. Oneway ANOVA was used to test significance. Individual group comparison was performed by *post hoc* testing with Student-Newman-Keuls. Significance from zero was tested by the Wilcoxon test. Significance was considered present at $p < 0.05$. Data were analyzed using the Statistical Package for Social Sciences (SPSS, (38)).

Results

Food intake and body composition

Food intake of small and large tumor bearing rats did not decrease during the study period and was not different from free fed or paired control rats (Fig. 2). Carcass weight (total body weight minus tumor weight) were equal in all three groups (Table 1). Fat mass was reduced in large tumor bearing rats.

Arterial concentrations

Arterial ammonia (Table 2) was not significantly different in both tumor bearing groups. Glutamine concentrations decreased with increasing tumor load whereas glutamate concentrations remained unchanged. The concentration of alanine, phenylalanine, branched chain amino acids and total amino acid concentration increased in the small tumor bearing animal. Alanine and total arterial amino acid concentrations were also increased in large tumor bearing animals.

Whole body protein and glutamine turnover

In a pilot experiment steady state conditions were determined. Steady state conditions were present after 30 minutes infusion of ^3H -glutamine and ^3H -phenylalanine (Fig. 3). During the infusion of ^3H -phenylalanine, steady state conditions were also achieved for ^3H -tyrosine. For further studies a minimal infusion time of 40 minutes was employed.

Figure 2. Food intake of free fed control (upper left), pairfed control (upper right), small tumor bearing (lower left) and large tumor bearing (lower right) rats. No significant changes in food intake between different groups.

Table 1. General characteristics of control and tumor bearing rats of the first experiment. Values are mean $\pm$ SEM (grams). Statistical analysis by Oneway ANOVA with *post hoc* testing by Student-Newman-Keuls: ^a $p < 0.05$ versus free fed control group; ^b $p < 0.05$ versus small tumor bearing group.

	FF control	Small tumor	Large tumor
Body composition			
Body weight	190 $\pm$ 3	196 $\pm$ 5	226 $\pm$ 5
Tumor weight	-	16 $\pm$ 2	43 $\pm$ 3 ^b
% of bw	-	8 $\pm$ 1	16 $\pm$ 1
Carcass weight	190 $\pm$ 3	181 $\pm$ 5	184 $\pm$ 4 ^a
FFM carcass	159 $\pm$ 2	159 $\pm$ 3	166 $\pm$ 4
Fat mass carcass	30 $\pm$ 2	26 $\pm$ 3	18 $\pm$ 2 ^a

Table 2. Arterial concentrations of ammonia and amino acids. Values are mean $\pm$ SEM ($\mu\text{mol/l}$). Statistical analysis by Oneway ANOVA with *post hoc* testing by Student-Newman-Keuls: ^a $p < 0.05$ versus free fed control group; ^b $p < 0.05$ versus pairfed control group; ^c $p < 0.05$ versus small tumor bearing group.

	FF control	PF control	Small tumor	Large tumor
Ammonia	186 $\pm$ 23	204 $\pm$ 13	232 $\pm$ 35	205 $\pm$ 17
Glutamine	643 $\pm$ 13	642 $\pm$ 33	605 $\pm$ 24	567 $\pm$ 16 ^a
Glutamate	107 $\pm$ 10	99 $\pm$ 2	130 $\pm$ 23	117 $\pm$ 6
Alanine	226 $\pm$ 43	221 $\pm$ 23	385 $\pm$ 63 ^a	464 $\pm$ 58 ^{a,b}
Phenylalanine	53 $\pm$ 2	54 $\pm$ 1	75 $\pm$ 4 ^{a,b}	74 $\pm$ 3 ^{a,b}
BCAA	271 $\pm$ 18	236 $\pm$ 9	349 $\pm$ 23 ^{a,b,c}	293 $\pm$ 13
α -AN	2655 $\pm$ 72	2489 $\pm$ 32	3253 $\pm$ 226 ^{a,b}	3018 $\pm$ 94 ^b

Table 3. Whole body phenylalanine and glutamine turnover. Values are mean $\pm$ SEM and in $\text{nmol} \times 100 \text{ gr. body weight}^{-1} \times \text{min}^{-1}$. Statistical analysis by Oneway ANOVA with *post hoc* testing by Student Newman Keuls; ^a $p < 0.05$ versus free fed control group; ^b $p < 0.05$ versus pair fed control group; ^c $p < 0.05$ versus small tumor bearing group.

	FF control	PF control	Small tumor	Large tumor
<i>Phenylalanine</i>				
Ra	115 $\pm$ 14	132 $\pm$ 15	202 $\pm$ 50	239 $\pm$ 29 ^a
Hydroxylation	18 $\pm$ 43	26 $\pm$ 5	37 $\pm$ 16	56 $\pm$ 16 ^a
Rd (protein synthesis)	98 $\pm$ 13	106 $\pm$ 15	176 $\pm$ 40	184 $\pm$ 25 ^a
<i>Glutamine</i>				
Ra (total)	2481 $\pm$ 248	2073 $\pm$ 264	1996 $\pm$ 268	3081 $\pm$ 215 ^{a,b,c}
Ra (protein derived)	356 $\pm$ 29	323 $\pm$ 39	498 $\pm$ 122	586 $\pm$ 73 ^a
Ra (non-protein derived)	2125 $\pm$ 228	1750 $\pm$ 243	1498 $\pm$ 285	2528 $\pm$ 177 ^{a,b,c}

groups. Phenylalanine and BCAA concentrations increased in both tumor bearing groups, whereas intracellular alanine concentration increased in the large tumor bearing rats. The total intracellular amino acid concentration was not significantly different between the groups.

Hindquarter plasma flow (Table 5) did not differ significantly between the groups although the mean values were higher in both tumor bearing groups. Ammonia efflux tended to increase in the small tumor bearing group but this did not become significant. The net release of glutamine, phenylalanine and the sum of all amino acids increased in the small tumor bearing group and decreased again in the large tumor bearing rats.

Hindquarter Ra and Rd were not significantly different between the groups (Fig.4, upper panel). Changes in Ra and Rd between the groups are within the detection limits of the A-V dilution method to measure protein turnover rates as previously described by us (28). However, the difference between phenylalanine Ra and Rd increased in the small tumor bearing group indicating an increased net protein breakdown. In the large tumor bearing group the difference between Ra and Rd decreased to control values, indicating no net, measurable, protein breakdown.

Hindquarter glutamine Ra increased significantly compared to hindquarter Rd resulting in an increased net glutamine release in the small tumor bearing animals (Fig 4, lower panel).

Table 4. Gastrocnemius water content and amino acid concentrations. Values are mean $\pm$ SEM and expressed as $\mu\text{mol/l}$. Statistical analysis by Oneway ANOVA with post hoc testing by Student-Newman-Keuls: ^a $p < 0.05$ versus free fed control group; ^b $p < 0.05$ versus pair fed control group.

	FF control	PF control	Small tumor	Large tumor
%H ₂ O	74.0 $\pm$ 1.0	72.7 $\pm$ 1.0	74.5 $\pm$ 0.4	75.1 $\pm$ 0.4
Glutamine	4617 $\pm$ 132	4949 $\pm$ 281	3484 $\pm$ 188 ^{a,b}	3882 $\pm$ 357 ^b
Glutamate	691 $\pm$ 108	742 $\pm$ 84	537 $\pm$ 56	829 $\pm$ 137
Alanine	965 $\pm$ 178	787 $\pm$ 58	1253 $\pm$ 135	1326 $\pm$ 135 ^b
Phenylalanine	108 $\pm$ 6	106 $\pm$ 4	146 $\pm$ 11 ^{a,b}	152 $\pm$ 5 ^{a,b}
BCAA	555 $\pm$ 78	418 $\pm$ 33	696 $\pm$ 64 ^b	663 $\pm$ 42 ^b
α -AN	24317 $\pm$ 588	23430 $\pm$ 736	21246 $\pm$ 757	21363 $\pm$ 1087

Figure 3. Arterial specific activities of amino acids after a primed constant infusion of ^3H -glutamine and ^3H -phenylalanine. Steady state conditions were reached within 30 minutes after the priming bolus was given. Note that the phenylalanine hydroxylation product ^3H -tyrosine also reaches steady state conditions.

Whole body phenylalanine R_a (Table 3) increased in the small tumor bearing animals although this did not become significant. In the large tumor bearing animals whole body phenylalanine R_a increased. Hydroxylation and whole body protein synthesis rate ($R_{d_{\text{protein synthesis}}}$) were also increased in the large tumor bearing rats.

Whole body glutamine R_a remained unchanged in the small tumor bearing rats but increased by approximately 25% in the large tumor bearing group compared to the free fed controls. Compared to the pair fed controls the increase was approximately 48%. In all groups most of the glutamine appearance rate was non-protein derived. The increase in whole body glutamine turnover in large tumor bearing rats was also mostly non-protein derived.

Hindquarter muscle metabolism

Intracellular water of muscle (Table 4) remained unchanged. The intracellular concentration of glutamine was decreased to a similar extent in small and large tumor bearing rats. Intracellular glutamate was not significantly different between the

In the large tumor bearing group glutamine Ra decreased to control values. However, glutamine Rd was reduced to zero resulting in a net efflux of glutamine from the hindquarter.

Tumor growth and metabolism

Intracellular water of tumor tissue was higher than of muscle but similar in small and large tumors (Table 6). Intracellular ammonia concentration increased in large tumors compared to small tumors. Intracellular glutamine concentrations in tumor tissue was much lower than in muscle whereas intracellular glutamate concentrations were higher than in muscle, indicating net consumption of glutamine and production of glutamate by tumor cells. Alanine concentrations were also remarkably high in tumor tissue compared to muscle tissue. High BCAA concentrations were also observed in tumor tissue. Glutamine, glutamate, alanine and total amino acid concentrations were decreased in the large tumors compared to the smaller tumors.

Between day 19 and 20 small tumors and large tumors increased by 3.7 ml and 6.1 ml, respectively (Table 7, Fig.1). Small tumors had an estimated protein synthesis rate of 28 ± 0.4 nmol phenylalanine $\times$ 100 gr. body weight⁻¹ $\times$ min⁻¹ whereas large tumors had an approximate synthesis rate of 49 ± 1.2 nmol phenylalanine $\times$ 100 gr. body weight⁻¹ $\times$ min⁻¹.

Table 6. Tumor ammonia and amino acid concentrations. Values are mean $\pm$ SEM (μ mol/l). Statistical analysis between small and large tumors by Mann Whitney U test: ^a p<0.05, ^b p<0.01.

	Small tumor			Large tumor		
H ₂ O %	86.0	$\pm$	1.1	89.7	$\pm$	0.5
Ammonia	2298	$\pm$	320	2871	$\pm$	567
Glutamine	2084	$\pm$	149	1516	$\pm$	99 ^b
Glutamate	3870	$\pm$	235	2923	$\pm$	203 ^a
Alanine	6540	$\pm$	445	4860	$\pm$	521 ^a
Phenylalanine	192	$\pm$	12	196	$\pm$	30
BCAA	1347	$\pm$	106	1187	$\pm$	144
α - AN	12837	$\pm$	811	11135	$\pm$	817

Table 7. Calculated tumor protein and glutamine turnover. Values are mean $\pm$ SEM. Protein turnover is calculated from the growth curve (Fig 1). Maximal glutamine turnover is determined by the arterial and intracellular glutamine SA ratio, the minimal turnover rate by the ratio of the intracellular SA of glutamine and glutamate. Statistical analysis between small and large tumors by Mann Whitney U test: ^a $p < 0.05$, ^b $p < 0.001$.

	Small tumor	Large tumor
Protein turnover		
Tumor weight (gr.)	26 $\pm$ 3	52 $\pm$ 4 ^b
Tumor growth (ml/day)	3.7	6.1
Concentration (nmol Phe/mg dw)	187 $\pm$ 5	187 $\pm$ 5
Turnover Phe (nmol/100 gr.bw/min)	28 $\pm$ 0.5	49 $\pm$ 1.2
Glutamine turnover		
Glutamine SA (dpm/nmol)	6.1 $\pm$ 2.7	1.8 $\pm$ 0.3 ^a
Glutamate SA (dpm/nmol)	0.6 $\pm$ 0.1	0.4 $\pm$ 0.1
Glutamine pool (μ mol)	53 $\pm$ 6	84 $\pm$ 10
Upper bound		
Glutamine turnover (%)	2.0 $\pm$ 0.8	1.0 $\pm$ 0.2
Glutamine turnover (nmol/min)	1016 $\pm$ 295	779 $\pm$ 196
Turnover (nmol/100 gr. bw/min)	471 $\pm$ 14	364 $\pm$ 9
Lower bound		
Glutamine turnover (%)	0.51 $\pm$ 0.01	0.60 $\pm$ 0.01
Glutamine turnover (nmol/min)	296 $\pm$ 87	471 $\pm$ 112
Turnover (nmol/100 gr. bw/min)	132 $\pm$ 39	219 $\pm$ 51

Figure 5. Intracellular polyamine concentrations in tumor tissue. Values are mean $\pm$ SEM and expressed as μ mol/L. Statistical analysis by Mann Whitney U: * $p < 0.05$, ** $p < 0.01$. Right axis: spermine and spermidine, left axis: putrescine.

Table 5. Hindquarter plasma flow and ammonia and amino acid fluxes. Values are mean $\pm$ SEM. Flow in ml $\times$ 100 g carcass weight⁻¹ $\times$ min⁻¹, flux in nmol $\times$ 100 gr. carcass weight⁻¹ $\times$ min⁻¹. A negative flux reflects net uptake and a positive flux reflects net release. Statistical analysis by Oneway ANOVA with *post hoc* individual group comparison by Student-Newman-Keuls: ^ap<0.05 versus free fed control group; ^bp<0.05 versus pair fed control group; ^cp<0.05 versus large tumor bearing group. Wilcoxon test: [§] p<0.05, *not* significant from zero.

	FF control	PF control	Small tumor	Large Tumor
Plasma flow	1.2 $\pm$ 0.4	1.6 $\pm$ 0.4	2.0 $\pm$ 0.7	2.0 $\pm$ 0.8
Ammonia	70 $\pm$ 10	94 $\pm$ 12	159 $\pm$ 44	114 $\pm$ 14
Glutamine	128 $\pm$ 15	109 $\pm$ 27	289 $\pm$ 64 ^b	170 $\pm$ 43
Glutamate	-31 $\pm$ 9	-27 $\pm$ 4	-22 $\pm$ 15 [§]	-47 $\pm$ 12
Alanine	24 $\pm$ 20 [§]	8 $\pm$ 19 [§]	11 $\pm$ 65 [§]	32 $\pm$ 41 [§]
Phenylalanine	1.3 $\pm$ 2.0 [§]	0.7 $\pm$ 1.3 [§]	16 $\pm$ 5 ^{a,b,c}	6 $\pm$ 2
BCAA	-29 $\pm$ 12 [§]	-40 $\pm$ 9	5 $\pm$ 26 [§]	23 $\pm$ 7
α -AN	186 $\pm$ 56	230 $\pm$ 39	832 $\pm$ 247 ^{a,b,c}	333 $\pm$ 89

Figure 4. Hindquarter protein and glutamine turnover in control (free fed and paired), small tumor bearing and large tumor bearing rats. Data are mean $\pm$ SEM. $\square$ Ra, $\blacksquare$ Rd

Top: protein turnover expressed as nmol phenylalanine/100 gr. carcass weight/min.

Bottom: glutamine turnover expressed in nmol glutamine/100 gr. carcass weight/min.

Statistical Analysis between different groups by Oneway ANOVA: no significant changes. Mann Whitney U for significant difference between Ra and Rd within group: [#] p<0.05.

Phenylalanine

Figure 6A. Relative effects of small and large tumors on whole body protein turnover. Whole body protein turnover is subdivided in the turnover of three compartments, tumor, muscle and other. All values are in $\text{nmol} \times 100 \text{ gr. body weight}^{-1} \times \text{min}^{-1}$. Whole body muscle compartment is calculated by assuming hindquarter muscle to be 50% of total muscle mass of the body (6,54). Control values are the averages of the free fed and pairfed control groups. For further explanation see text results.

The upper bound of glutamine turnover in small tumors was $1016 \pm 295 \text{ nmol/min}$ whereas the lower bound was $296 \pm 87 \text{ nmol/min}$. Although large tumors had twice the mass the upper and lower bounds were in the same range (upper bound: $779 \pm 196 \text{ nmol/min}$, lower bound: $471 \pm 112 \text{ nmol/min}$) suggesting that these tumors are metabolically relatively less active per gram tissue. This was confirmed by the intracellular polyamine concentrations. Polyamine concentrations are indicators for cellular proliferation and differentiation (39,40). Putrescine, spermine and spermidine concentrations all decreased in the large tumors compared to the smaller ones (Fig. 5).

A summary of the contributions of tumor and muscle tissue to whole body protein and glutamine metabolism is given in Fig. 6A and 6B. For these calculations hindquarter muscle is assumed to be approximately 50% of the total body muscle mass and both muscle and tumor turnover rates are all adjusted to turnover rates per

Figure 6B. Relative effects of small and large tumors on whole body glutamine turnover. Whole body glutamine turnover is subdivided in the turnover of three compartments, tumor, muscle and other. All values are in $\text{nmol} \times 100 \text{ gr. body weight}^{-1} \times \text{min}^{-1}$. Whole body muscle compartment is calculated by assuming hindquarter muscle to be 50% of total muscle mass of the body (6,54). Control values are the averages of the free fed and pairfed control groups. For further explanation see text results.

100 gr. body weight. Control data are the average of free fed and pairfed data. Muscle tissue decreasingly contributes to whole body protein turnover of large tumor bearing rats (Fig. 6A). The contribution of non-muscle non-tumor tissue increased in small tumor bearing rats and increased further when the tumor became large. Tumor tissue protein synthesis determines 16% and 27% of whole body protein synthesis in small tumor bearing and large tumor bearing rats, respectively.

Changes in glutamine turnover are to a great extent similar (Fig. 6B). Muscle contributes approximately 16% to whole body turnover in control rats. Although whole body glutamine turnover remains the same in small tumor bearing rats (Table 3), the relative contribution of muscle increases to approximately 45%. In large tumor bearing rats, when whole body glutamine turnover increased by 25-48% compared to free fed and pairfed controls (Table 3), the relative contribution of muscle decreased to 7% and other organs predominate whole body glutamine Ra.

To understand the alterations in glutamine and protein metabolism observed in cancer, turnover studies were performed at whole body and at regional muscle levels. The rat MCA tumor model was used because this model has been used previously to study cancer metabolism related to anorexia and cachexia (12,14). The tumor did not induce anorexia in our rats. Therefore, the metabolic changes observed can only be attributed to the presence of the tumor. However, as nutrient intake remained the same, nutrient delivery to, and metabolism of non-tumor tissues must have been changed.

Protein metabolism

Tumor bearing rats had increased whole body protein turnover rates, thus mimicking observations in other tumor bearing rat models (41,42) and cancer patients (43,44). However, the current study shows that changes in whole body protein turnover in animal models are in part caused by tumor protein turnover. In the current study net protein synthesis of the tumor was calculated. The actual turnover of tumor proteins is probably higher. An indication of the actual protein turnover can be derived from the intracellular specific activity of phenylalanine in tumor. This was approximately 2-3% of the arterial specific activity (data not shown). In muscle tissue this was approximately 8-10% of arterial specific activity. This indicates that the intracellular dilution of ^3H -phenylalanine by protein breakdown at steady state was approximately 4 time higher than in muscle and that protein turnover is also higher than in muscle. However, for accurate measurements plasma flow across the tumor is needed.

In small tumor bearing rats protein turnover rates of non-tumor, non-muscle tissue increased and this increased further in the large tumor bearing animals. Organs responsible for the increased protein turnover in MCA tumor bearing rats are visceral organs like the liver, spleen and immune cells. These organs and cells are known to increase their protein synthesis rates due to the acute phase response (21,45,46). In the large tumor bearing rats, part of the increased turnover of phenylalanine was due to an increased hydroxylation rate indicating increased amino acid oxidation in these

animals. Increased oxidation of amino acids could have occurred in visceral organs of the host but possibly also to a large extent in tumor tissue.

Regional measurements of muscle protein turnover in the hindquarter showed increased turnover rates in small tumor bearing rats resulting in net protein breakdown. Muscle wasting of tumor bearing rats is characterized by increased loss of the non-soluble myofibrillar proteins whereas the amount of sarcoplasmatic soluble proteins even increases slightly (47). The A-V dilution method measures protein turnover of both the soluble sarcoplasmatic and the myofibrillar protein pool in muscle. Therefore, changes in the higher turnover rate of the soluble pool in muscle may have overshadowed changes in the myofibrillar compartment and the effect of increasing tumor loads on myofibrillar proteins may have been underestimated. However, irrespective of the different turnover rates of the different protein pools in muscle, an increased net breakdown of myofibrillar proteins should also result in a net phenylalanine release. The observed increase in net release of phenylalanine in the small tumor bearing rats is thus probably a reflection of net protein breakdown of myofibrillar proteins.

Protein turnover and net breakdown returned to control values in large tumor bearing rats. This is consistent with a previous study by Chen et al (15). It therefore appears that muscle tissue was exhausted in the large tumor bearing rats as was postulated previously by Souba (14). This exhaustion appears to be of particular importance for glutamine because it is the major amino acid released by muscle protein breakdown (48).

Glutamine metabolism

The importance of glutamine was also shown in the current study by the 25-48% increase in whole body glutamine turnover in large tumor bearing rats. The increased whole body glutamine turnover indicates that both production and consumption are increased. Figure 5 shows that tumor tissue is only for a small part responsible for the increased glutamine consumption. The uptake of glutamine by tumor tissue has previously been estimated to be approximately $15 \text{ nmol} \times \text{gr. tumor}^{-1} \times \text{min}^{-1}$ which is in the same range as measured in the current study (49). Although tumor mass was approximately double in large tumor bearing rats compared to small tumor bearing rats, glutamine turnover did not increase as much. We therefore also measured tissue polyamine concentrations as indicator for tissue proliferation. The decreased

polyamine concentrations and, per gram tissue, decreased glutamine turnover of tumor tissue in large tumor bearing rats, indicate that these tumors have become less metabolically active. This could be related to the size of the tumors. The growth rate of the quadruple implanted tumors were only 164% of the double implanted and not twice the rate as expected. The quadruple implanted tumors in larger tumor bearing rats appear to exhaust the host more rapidly and hereby inhibit its own nutrient delivery.

Other major glutamine consuming organs are the kidney, gut and immune cells. However, previous studies in MCA tumor bearing rats showed that net glutamine consumption by both the kidney and gut did not increase but remained unchanged and decreased, respectively (15,50,51). Glutamine consumption of lymphocytes has been estimated to be $200\text{--}300 \text{ nmol} \times 100 \text{ gr. body weight}^{-1} \times \text{min}^{-1}$ (52). Increased amounts of leukocytes and activation of the immune cells, which increase glutamine consumption by approximately 50% (53), can thus theoretically explain a major part of the increased whole body glutamine consumption.

Glutamine turnover and production of hindquarter muscle increased in small tumor bearing rats but this decreased in the large tumor bearing rats relative to the increased whole body glutamine turnover. The liver and lungs are both other major contributors to glutamine production. Studies with MCA tumor bearing rats also showed that the liver can become an organ of net glutamine release as the tumor grows (54). In another study by the same group, however, very large tumors appear to induce an increased uptake of glutamine rather than release by the liver (14,55). The effects of increasing tumor loads on lung glutamine metabolism have not been described but previous studies have shown increased glutamine synthetase activity in lungs with concomitant net glutamine production in cases of metabolic stress (56,57). It therefore seems reasonable to assume that whole body glutamine metabolism is increasingly affected by liver and lung glutamine production as the tumor grows.

Tumor growth was associated with decreased intracellular glutamine concentrations in muscle. The production of glutamine from protein breakdown can only account for a small amount of the total glutamine efflux. Assuming the ratio of phenylalanine and glutamine present in protein to be approximately 1:3 (34), the net glutamine production from protein breakdown in the small tumor bearing group would be $132 \text{ nmol} \times 100 \text{ gr. body weight}^{-1} \times \text{min}^{-1}$. Thus, most of the observed increase in

glutamine production observed in small tumor bearing rats, is a result of increased amidation of glutamate. The uptake of glutamate remained unchanged in the small tumor bearing group. Besides protein breakdown, muscle can produce glutamate by the transamination of α -ketoglutarate or branched chain amino acids (valine, leucine and isoleucine) (10). The increased intracellular branched chain amino acid concentrations and unchanged glutamate concentrations indicate that there was no shortage of glutamine precursors. It thus appears that the decreased intracellular glutamine concentrations are not as much the resultant of decreased glutamine synthesis from its precursors but more of an increased glutamine release from muscle tissue. This is in agreement with the hypothesis postulated by Rennie that decreased intracellular glutamine concentration associated with catabolic diseases, such as sepsis and diabetes, is not regulated by its production but by increased membrane transport rates (58). It is further in agreement with a previous study demonstrating an increased glutamine efflux from muscle of tumor bearing rats associated with the presence of increased intracellular glutamine synthetase mRNA (15).

Glutamine membrane transport and glutamine efflux from the hindquarter are to a great extent hormonally controlled by glucocorticoids (59-61). Furthermore, hypercortisolemia, induced in healthy volunteers, increases whole body glutamine turnover by approximately 40% which is in the same range as observed in the current study. Increased glucocorticoid concentrations and increased urinary excretion of cortisol have been observed in cancer patients (62) and in several tumor bearing animals (63,64) and may thus have regulated the metabolic changes observed in our tumor bearing rats. However, increased concentrations of most other stress hormones have also been observed in MCA tumor bearing rats in conjunction with increased levels of several catabolic cytokines (46,65,66). The complex interplay of these hormones and cytokines in respect to the regulations of the metabolic disturbances of the tumor bearing rat is beyond the scope of the current study.

The increased whole body glutamine turnover rates should also be seen in the perspective of other metabolic pathways. Glutamine has a key role in intermediary metabolism as non-toxic interorgan ammonia carrier but also as a major gluconeogenic precursor (67). Moreover, the interaction goes both ways as approximately 13% of glutamine carbon skeletons are derived from glucose (68). If we further consider that the glucose pool and turnover is approximately a six fold

higher than glutamine turnover and glucose turnover increases by 55% in large MCA tumor bearing rats (69), it seems plausible that this also affects glutamine turnover. However, the exact quantitative influence of changes in glucose metabolism on glutamine metabolism of tumor bearing rats was not part of the current study and needs further research.

In summary, the current study shows that in the presence of a small tumor whole body protein turnover increases and that this was in part related to protein synthesis of the tumor. Muscle protein breakdown increased in these rats with a concomitant increase in glutamine production from the hindquarter. In animals bearing larger tumors whole body glutamine turnover increased. This increase, however, was only for a small part caused by tumor metabolism. Muscle glutamine turnover even decreased. Therefore, the increase in whole body glutamine turnover appears to be caused by increased turnover in visceral organs.

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GLUTAMINE DEPLETION AND INCREASED GUT PERMEABILITY IN NON-ANORECTIC, NON-WEIGHT LOSING TUMOR BEARING RATS

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Abstract

Glutamine is an essential amino acid for rapidly dividing cells such as enterocytes. The progress of cancer is associated with a decrease of arterial and muscle glutamine concentrations. Objectives were to test whether increasing tumor loads affect gut intracellular glutamine handling, protein turnover and gut absorptive and barrier function. Tumor bearing rats were studied with a tumor load of 5-15% or 15-30% of body weight. Portal drained visceral net uptake or release of energy substrates, amino acids and intestinal protein turnover were studied. Gut absorptive capacity and permeability was assessed by the urinary recovery of 3-O-methyl-D-glucose or Lactulose/Rhamnose (L/R) ratio after an oral gavage.

In tumor bearing rats the net uptake of energy substrates (ketones, glutamine) and net protein synthesis increased across the portal drained viscera while mucosal glutamine concentrations decreased. Absorptive capacity remained unchanged in both tumor bearing groups. The L/R ratio, however, increased with increasing tumor load indicating loss of gut barrier function. This was not related to changes in villus height, crypt depth or changes in mucosal cell populations but to decreased intracellular polyamine concentrations.

In conclusion, the presence of a MCA tumor leads to altered mucosal glutamine metabolism and loss of gut barrier function possibly related to disturbed proliferation or differentiation of enterocytes.

In cancer disease wasting of body mass negatively affects clinical outcome of therapeutic interventions (1). Anorexia and changes in host intermediary metabolism result in progressive weight loss and, if left unresolved, inevitably result in host death (2). In this respect, gut functioning has a central role in the cancer bearing host. Adequate digestion and absorption of nutrients is necessary to supply the host. Moreover, due to its high turnover of cells, the gut must retain specific nutrients for its own use. The amino acid glutamine is a preferential energy substrate for enterocytes and is essential for nucleotide production (3,4). If glutamine is insufficiently available, the gut appears to lose its barrier function against luminal bacteria, endotoxins and other toxic products (5). In extreme catabolic diseases, e.g. burn injury or sepsis, loss of gut barrier functioning has been suggested to become the axis of an amplification loop with ongoing catabolism and death as final result (6-11).

The progress of cancer has been associated with depletion of arterial and muscle intracellular glutamine pools (12,13). Furthermore, decreased net glutamine uptake by portal drained viscera was observed in cancer bearing rodents (14) which was associated with a fall in mucosal glutaminase activity (15). It appears that the gut is compromised in the utilization of its major energy substrate and this may influence its functioning. Objectives of the present study are to test whether increasing tumor loads affect intracellular glutamine handling and protein turnover in portal drained viscera and whether they also affect gut absorption capacity or barrier function.

Materials and methods

Animals and tumor model

Female Lewis rats (200-225 g, Centralized Animal Facilities, University of Limburg, Maastricht, NL) were individually housed in metabolic cages during the experiment after an adaptation period of 3 days. Rats were given standard laboratory rat chow (SRMA 1210, Hopefarms, Woerden, NL) and subjected to standard 12 hour light-dark cycle periods (7:30 A.M. to 7:30 P.M.) with room temperature maintained at 25°C. The experiments were performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals (16) and approved by the Ethical Committee of Animal Research of the University of Limburg.

A subcutaneous methylcholanthrene (MCA) induced fibro sarcoma (12, 17, 18, 19) was used as cancer model (17). MCA tumors are often used in metabolic studies and are known for its rapid growth, invasion of local tissue and absence of metastatic potential (12, 18,19). Rats (n=40) were randomly divided into four groups:

a small tumor bearing group (ST), a large tumor bearing group (LT), a paired control group and a control group fed ad libitum. In the ST group the tumor was allowed to grow until it reached 5-15% of the host body weight. In the LT group the tumor grew to 15-30% of the host body weight. Tumor tissue was transplanted bilaterally on the back in the ST group, whereas it was quadruple implanted in the LT group. Control rats were sham implanted with saline. Tumor growth was monitored by measuring two orthogonal dimensions and by calculating the volume assuming the tumor to be a prolate spheroid (20). Tumor size, body weight and food intake were monitored daily.

Experiment 1

Metabolic experiments were performed as described previously (21,22, see in detail chapter 2 and 4). In brief, under ether anesthesia, the right renal vein, a tertiary branch of the mesenteric vein, the portal vein and the carotid artery were cannulated with a silastic or PE tube and fixed with cyano-acrylate. For flow measurements, the indicator dilution method with *para*-aminohippuric acid infused in the mesenteric vein was used. To study protein and glutamine turnover, a primed constant infusion of L-[2,6-³H]phenylalanine and L-[3,4-³H]glutamine given in the right renal vein. The priming dose and infusion rate were $1 \mu\text{Ci} \times 100 \text{ g bw}^{-1}$ and $1 \mu\text{Ci} \times 100 \text{ g bw}^{-1} \times \text{h}^{-1}$ respectively. Blood was simultaneously sampled from the portal vein and carotid artery and collected in heparinized cups on ice. Hereafter, 1 cm jejunal tissue approximately 10 cm beyond Treitz ligament was dissected free, directly freeze-clamped, put in liquid nitrogen and stored at -80°C until further analysis.

For PAH determinations, 50 μL heparinized blood was added to 500 μL TCA GR 10%, vortexed, centrifuged at 8,900 g at 4°C , the supernatant stored at -80°C after snap freezing with liquid nitrogen. For hematocrit determinations a micro-hematocrit tube was filled with heparinized blood and centrifuged at 10,000 g at room temperature. Hematocrit was read with a Micro Hematocrit Reader. Plasma was obtained by whole blood centrifugation at 8,900 g at 4°C for 5 minutes. For ammonia, urea, glucose, lactate and ketone (β -hydroxybutyrate, acetoacetate) determinations, 500 μL plasma was vortexed with 20 μL TCA 50%, put into liquid nitrogen and stored at -80°C . For plasma amino acid determinations and amino acid specific activities, 300 μL plasma was added to 12 mg 5-sulfosalicylic acid for deproteinization, vortexed, frozen in liquid nitrogen and stored at -80°C .

Experiment 2

Gut absorptive and barrier functioning was examined in a second experiment. As no anorexia was observed in the first experiment, all groups were freely fed. On day 0, 6, 12, and 18 after tumor implantation an oral dose (1 ml, iso-osmolar) of lactulose

(292 mmol/L, Centrafarm 15211381, Etten-Leur, NL), L-rhamnose (10 mmol/L, Janssen Chimica 1740845, Geel, Belgium) and 3-O-methyl-D-glucose (5.5 mmol/L, Sigma M4879, Geel, Belgium) was given at 8:00 AM. The dosage of lactulose was relatively low compared to previous studies because the sensitivity of the HPLC determination is very high (23). Using this dosage no diarrhea was observed. Urine was collected for 10 hours in a flask containing 6N HCl to prevent bacterial overgrowth. Previous to the oral gavage, 12 hour urine was collected to test for the presence of sugars in native urine. No lactulose, L-rhamnose or 3-O-methyl-D-glucose was present. Urinary recovery of the three sugars was calculated as follows: the volume of urine collected was multiplied with the concentration of the sugar. This product was divided by the oral intake. Permeability of the gut was assessed by the ratio of the recovery of lactulose and L-rhamnose. This has previously been shown to be a better parameter than using lactulose recovery alone. Incomplete doses, reduced recovery, altered intestinal dilution, altered transit time, altered body distribution and renal clearance have similar effects on both recoveries and thus do not influence the ratio (24).

After sacrifice, small parts of the jejunum (10 cm beyond Treitz ligament) were excised and fixed in formaldehyde for 24 hours. Tissue was embedded in paraffin and oriented so that transverse sections of the jejunum were obtained. Sections (5 μ m) were cut using a microtome (Leica J1130, Rijswijk, NL) and stained with standard haematoxyline-eosine. Villus height and crypt depth were measured using a Jandel Video Analysis System (Erkath, Germany) as described previously (5). Only crypt-villus units were included which had a continuity from the crypt bottom to villus tip. Means of villus heights and crypt depths were obtained after at least 10 measurements each of three different jejunal tissue specimens. Measurements were performed by two independent observers and all slides were coded so that the observer was unaware from which group of rats the sections originated. Intraobserver and interobserver variance for these measurements were 3% and 7%, respectively (5).

Intraepithelial lymphocytes were stained in formalin fixed, paraffin embedded tissue sections with anti-CD3 polyclonal antibodies (DAKO A452, Glostrup, Danmark) as described previously (25). Intraepithelial CD3⁺ positive cells seen in the epithelial morphological regions of the villus-crypt were counted in ten high powerfield of each section and screened for the relative number of CD3⁺ positive cells and enterocytes respectively (25). CD3⁺ positive cells are expressed as percentage of enterocytes.

Biochemical analysis

Plasma ammonia, urea, glucose, lactate, β -hydroxybutyrate, acetoacetate and PAH were determined spectrophotometrically on a Cobas Mira S (Roche Diagnostica, Hoffman-La Roche, Basel, Switzerland) by standard enzymatic methods, using commercially available kits as described previously (21,26). Standard plasma amino acids were determined by fully automated high performance liquid chromatography (HPLC) as described previously (27). To determine plasma phenylalanine and glutamine specific activity (SA), plasma was isolated on a cation exchange resin column as described previously (28). For the determination of tissue ammonia, polyamine and amino acid concentrations, tissue was pulverized using a mortar and pestle precooled in liquid nitrogen. The tissue was further homogenized and deproteinized in a Mini-Beadbeater (Biospec products, Bartlesville USA). Approximately 100 mg tissue was added to 400 μ l SSA 5%, with 300 mg glass beads (diameter 1 mm, Biospeck Products) and beaten for 30 seconds. The homogenate was centrifuged at 4°C at 1,100 g and the supernatant frozen in liquid nitrogen and stored at -80°C until further determinations. To determine tissue dry weight and water content approximately 200 mg pulverized tissue was freeze dried for 24 hours in a Speedvac (SC200, Savant Instruments Inc., Farmingdale NY, USA) connected with a refrigerated condensation trap (type RT 400, Savant Instruments Inc.).

Concentrations of intestinal polyamines putrescine, spermidine and spermine were determined by HPLC (29). Urine lactulose, L-rhamnose and 3-O-methyl-D-glucose were determined by routine HPLC after derivatization with FMOC-hydrazine (23).

Calculations

Plasma flow across the portal drained viscera (PDV) was calculated using PAH in the indicator dilution method described previously (21,26, chapter 4). Substrate fluxes are calculated by multiplying the venous-arterial concentration differences with the mean portal plasma flow of the group and are expressed in $\text{nmol} \times 100 \text{ g bw}^{-1} \times \text{min}^{-1}$. A positive flux indicates net release, a negative flux reflects a net uptake. Fractional extraction of amino acids were calculated by dividing the difference between arterial and portal venous concentrations by arterial concentrations. It is expressed as percentage of the arterial concentration. α -Amino-nitrogen was calculated as the sum of the individual amino acids measured (27), branched chain amino acids (BCAA) as the sum of valine, leucine, isoleucine and ketones as the sum of β -hydroxybutyrate and acetoacetate. Tissue concentrations of amino acids are expressed in $\mu\text{mol/L}$ and derived by dividing the tissue homogenate amino acid concentration ($\mu\text{mol/kg}$ wet weight) by tissue water content.

PDV glutamine and phenylalanine turnover can be calculated from the arterial-venous dilution of the tracer amino acids across the PDV calculated in a three compartment model (22,30, see in detail chapter 2). Intracellular phenylalanine and glutamine disposal (D) and production (P) can thus be calculated together with *in vivo* membrane transport rates and metabolic shunting of amino acids. Intracellular disposal of phenylalanine is a reflection of protein synthesis and intracellular production of phenylalanine reflects protein breakdown as the gut has very low hydroxylase activity (31).

Statistical analysis

Results are presented as mean $\pm$ SEM. The Mann Whitney *U* test corrected with the Bonferroni procedure was used to test significance between control and tumor bearing groups (32). Multiple time parameters in experiment 2 were analyzed with two-way ANOVA in which time and the presence of a tumor were the main effects. Significance was considered present at $p < 0.05$.

Results

Food intake of tumor bearing rats did not decrease during the study period and was not different from control rats (33, see also chapter 5, fig. 2). Therefore, the results of the paired and the freely fed control groups in the first experiment were pooled.

Table 1. Arterial ammonia and amino acid concentrations in control, small and large tumor bearing rats. Values are presented in $\mu\text{mol/L}$ (mean $\pm$ SEM). Mann Whitney *U* nonparametric test versus control group: ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$.

	Control	Small tumor	Large tumor
Ammonia	195 $\pm$ 13	232 $\pm$ 35	205 $\pm$ 17
Glutamine	642 $\pm$ 17	605 $\pm$ 24	567 $\pm$ 16 ^b
Glutamate	109 $\pm$ 6	130 $\pm$ 23	117 $\pm$ 6
Citrulline	43 $\pm$ 2	41 $\pm$ 2	42 $\pm$ 2
Alanine	224 $\pm$ 24	385 $\pm$ 63 ^b	464 $\pm$ 58 ^b
Phenylalanine	53 $\pm$ 2	75 $\pm$ 4 ^c	74 $\pm$ 3 ^c
BCAA	255 $\pm$ 11	349 $\pm$ 23 ^b	293 $\pm$ 13 ^a
α -Amino-nitrogen	2727 $\pm$ 51	3598 $\pm$ 269 ^b	3420 $\pm$ 101 ^c

Table 2. Mucosal concentrations of ammonia and amino acid. Values in $\mu\text{mol/L}$ (mean $\pm$ SEM) except H_2O in %. Mann Whitney U nonparametric test versus control group: ^a $p<0.05$, ^b $p<0.01$, ^c $p<0.001$.

	Control	Small tumor	Large tumor
H_2O (%)	72.6 $\pm$ 0.7	74.5 $\pm$ 1.0	75.0 $\pm$ 0.6 ^a
Ammonia	3942 $\pm$ 314	3478 $\pm$ 336	3042 $\pm$ 196 ^b
Glutamine	1796 $\pm$ 110	1410 $\pm$ 97 ^a	1209 $\pm$ 102 ^c
Glutamate	5611 $\pm$ 285	4806 $\pm$ 169 ^b	4129 $\pm$ 240 ^c
Aspartate	896 $\pm$ 59	701 $\pm$ 78 ^a	596 $\pm$ 91 ^b
Citrulline	251 $\pm$ 10	223 $\pm$ 14	182 $\pm$ 10 ^c
Alanine	2487 $\pm$ 154	2378 $\pm$ 223	2042 $\pm$ 184 ^a
Phenylalanine	906 $\pm$ 131	681 $\pm$ 98	481 $\pm$ 86 ^b
BCAA	3703 $\pm$ 479	2811 $\pm$ 442	2042 $\pm$ 383 ^b
α -Amino-nitrogen	38359 $\pm$ 1501	34578 $\pm$ 1470	30021 $\pm$ 1152 ^c

Intestinal metabolism

Most arterial amino acids increased in both tumor bearing rats with the exception of glutamine which decreased (Table 1). In contrast, mucosal amino acid concentrations of the gut decreased dramatically with increasing tumor load (Table 2). The mean plasma flow across the portal drained viscera, which under normal conditions reflects intestinal flow, increased substantially but not significantly ($p=0.08$, Table 3). This increase may have remained undetected by the variation (type II error). Glucose, lactate, ketones and glutamine are the major energy substrates of the gut. The uptake of glucose and lactate remained unchanged whereas the uptake of ketones and glutamine increased in the large tumor bearing rats. Ammonia, alanine and citrulline are major end products of gut glutamine consumption. The release of ammonia and alanine increased confirming the increased use of glutamine by portal drained organs. Citrulline release tended to increase ($p=0.08$). To look more specifically into intestinal glutamine turnover and *in vivo* membrane transport rates ^3H -glutamine was infused. In the large tumor bearing animals, both the ingoing (F_{in}) and outgoing (F_{out}) amount of glutamine increased in the portal drained viscera. This resulted in a doubling of both inward and outward glutamine membrane transport rates (Table 4). Intracellular glutamine turnover (disposal and production) also increased which resulted in the increased flux (uptake) of glutamine by portal drained organs in large tumor bearing rats.

Protein kinetics of portal drained viscera are calculated by the intracellular disposal of phenylalanine (protein synthesis) and production (protein breakdown). Although in small tumor bearing rats more phenylalanine entered the ingoing pool of

Table 3. PDV plasma flow, ammonia and amino acid fluxes. Values are presented in $\text{nmol} \times 100 \text{ g bw}^{-1} \times \text{min}^{-1}$ (mean $\pm$ SEM). (-) reflects net uptake, (+) reflects net release of a substrate. Mann Whitney *U* nonparametric test versus control group: ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$.

	Control	Small tumor	Large tumor
Flow	0.9 $\pm$ 0.2	1.3 $\pm$ 0.4	2.8 $\pm$ 0.7
Glucose	-659 $\pm$ 174	-550 $\pm$ 200	-770 $\pm$ 279
Lactate	-596 $\pm$ 160	603 $\pm$ 361 ^c	-739 $\pm$ 515 ^d
Ketones	-63 $\pm$ 10	-99 $\pm$ 18	-219 $\pm$ 49 ^b
Ammonia	154 $\pm$ 45	184 $\pm$ 35	352 $\pm$ 72 ^b
Glutamine	-284 $\pm$ 31	-299 $\pm$ 29	-574 $\pm$ 77 ^c
Glutamate	15 $\pm$ 6	5 $\pm$ 5	-22 $\pm$ 13 ^c
Citrulline	29 $\pm$ 5	30 $\pm$ 4	51 $\pm$ 9
Alanine	149 $\pm$ 29	174 $\pm$ 32	274 $\pm$ 52 ^a
Phenylalanine	-4 $\pm$ 2	-2 $\pm$ 3	-10 $\pm$ 4 ^a
BCAA	2 $\pm$ 7	-0.5 $\pm$ 14	-19 $\pm$ 30
α -Amino-nitrogen	-119 $\pm$ 62	-148 $\pm$ 85	-456 $\pm$ 151 ^a

Table 4. PDV glutamine extraction, turnover and intracellular kinetics. All data are presented in $\text{nmol glutamine} \times 100 \text{ g bw}^{-1} \times \text{min}^{-1}$ except extraction % of arterial concentration (mean $\pm$ SEM). F_{in} is the amount of glutamine entering the PDV through arterial plasma as described in chapter 2 (Fig. 5, page 56, equation 12-20), F_{out} is the amount of glutamine leaving PDV by the portal vein, $F_{j,a}$ is inward directed plasma tissue membrane transport, $F_{p,j}$ is outward directed plasma tissue membrane transport, $F_{p,a}$ is metabolic shunting, disposal of glutamine reflects intracellular glutamine consumption whereas production reflects intracellular production. Mann Whitney *U* nonparametric test versus control group: ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$.

	Control	Small tumor	Large tumor
Glutamine extraction	39 $\pm$ 4	38 $\pm$ 4	36 $\pm$ 3
F_{in}	732 $\pm$ 52	784 $\pm$ 30	1587 $\pm$ 44 ^c
F_{out}	448 $\pm$ 47	485 $\pm$ 43	1015 $\pm$ 36 ^c
$F_{j,a}$	848 $\pm$ 146	639 $\pm$ 121	1691 $\pm$ 306 ^b
$F_{p,j}$	565 $\pm$ 154	339 $\pm$ 106	1119 $\pm$ 320 ^a
$F_{p,a}$	-116 $\pm$ 130	145 $\pm$ 146	-102 $\pm$ 320
Disposal	476 $\pm$ 59	435 $\pm$ 36	968 $\pm$ 88 ^c
Production	192 $\pm$ 46	136 $\pm$ 8	395 $\pm$ 77 ^a
Flux	-284 $\pm$ 31	-299 $\pm$ 29	-574 $\pm$ 77 ^c

Table 5. PDV protein turnover and intracellular kinetics. All data are presented in nmol glutamine $\times$ 100 g $\text{bw}^{-1} \times \text{min}^{-1}$ (mean $\pm$ SEM). F_{in} is the amount of glutamine entering the PDV through arterial plasma, F_{out} is the amount of glutamine leaving PDV by the portal vein, $F_{\text{I},\text{a}}$ is inward directed plasma tissue membrane transport, $F_{\text{P},\text{I}}$ is outward directed plasma tissue membrane transport, $F_{\text{P},\text{a}}$ is metabolic shunting, disposal of glutamine reflects intracellular glutamine consumption whereas production reflects intracellular production 2 (see in detail, chapter 2, page 56, fig 5, equation 12-20). Mann Whitney U nonparametric test versus control group: ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$.

	Control	Small tumor	Large tumor
F_{in}	61 $\pm$ 4	98 $\pm$ 6 ^c	207 $\pm$ 7 ^c
F_{out}	57 $\pm$ 4	96 $\pm$ 9 ^c	197 $\pm$ 7 ^c
$F_{\text{I},\text{a}}$	25 $\pm$ 3	29 $\pm$ 7	79 $\pm$ 11 ^c
$F_{\text{P},\text{I}}$	22 $\pm$ 3	29 $\pm$ 8	69 $\pm$ 11 ^c
$F_{\text{P},\text{a}}$	35 $\pm$ 3	69 $\pm$ 6 ^c	128 $\pm$ 10 ^c
Disposal	299 $\pm$ 52	177 $\pm$ 37	168 $\pm$ 36 ^a
Production	295 $\pm$ 52	176 $\pm$ 38	158 $\pm$ 36 ^a
Flux	-4 $\pm$ 2	-2 $\pm$ 3	-10 $\pm$ 4 ^b

the portal drained viscera (F_{in}), this did not result in increased membrane transport rates into the cell but did increase metabolic shunting of phenylalanine (Table 5).

In large tumor bearing rats the ingoing (F_{in}) and outgoing phenylalanine increased (F_{out}) compared to control and small tumor bearing rats. This resulted in increased inward membrane transport rates, outward membrane transport rates and metabolic shunting. Protein turnover (both breakdown and synthesis) decreased in the large tumor bearing rats. Phenylalanine flux (difference protein breakdown and synthesis), however, increased in the large tumor bearing animals indicating net protein breakdown.

Intestinal function

3-O-Methyl-D-glucose recovery in urine was not significantly different over time between the different groups. Lactulose recovery of small and large tumor bearing rats increased compared to control rats. L-Rhamnose recovery was similar in all groups at the different time points. The lactulose/L-rhamnose ratio increased in both tumor bearing rats compared to control rats indicating gut barrier loss with increasing tumor loads (Fig. 1).

To test whether this was due to villus atrophy we measured villus height and crypt depth (Table 7). Both villus height and crypt depth, however, remained unchanged in both tumor bearing rats. The increased permeability may also be explained by

alterations in mucosal cell populations (24), in particular increasing amounts of inflammatory cells or its mediators. Therefore the relative amount of intraepithelial lymphocytes were counted. The percentage intraepithelial lymphocytes, however, remained unchanged in the small tumor bearing rats and decreased in the large tumor bearing rats.

Finally, we measured intestinal polyamine concentrations (Table 8) as an indicator of enterocyte cell proliferation and differentiation (34). Intracellular putrescine, spermidine and spermine concentrations decreased significantly in the large tumor bearing rats. Furthermore, the molar ratio of spermidine to spermine, an indicator of cell growth under a variety of circumstances (35), decreased in the large tumor bearing rats.

Figure 1. Ratio of urinary recovery of lactulose and L-rhamnose after an oral iso-osmolar dose (mean $\pm$ SEM). $\square$ Control group, $\square$ Small tumor bearing group, $\blacksquare$ Large tumor bearing group. Statistical analysis by two-way ANOVA (effects of group): Control versus small TB group: * $p < 0.001$; Control versus large TB group: # $p < 0.001$. ND=not determined

Table 6. Urinary recovery of an oral dose of lactulose, rhamnose and 3-O-methyl-D-glucose in control, small and large tumor bearing animals. Values are urinary recovery of an iso-osmolar oral dose after 12 hours and expressed in % of the oral dose (mean $\pm$ SEM). nd: not determined. Statistical analysis by two way ANOVA: control versus small tumor bearing rats: * $p < 0.001$; control versus large tumor bearing rats: # $p < 0.001$.

	Control	Small tumor	Large tumor
3-O-methyl-D-Glucose			
Day 0	76 $\pm$ 3	62 $\pm$ 14	67 $\pm$ 12
Day 6	83 $\pm$ 3	84 $\pm$ 5	75 $\pm$ 9
Day 12	71 $\pm$ 5	92 $\pm$ 12	81 $\pm$ 8
Day 18	72 $\pm$ 10	nd	67 $\pm$ 10
Lactulose			
Day 0	0.15 $\pm$ 0.01	0.22 $\pm$ 0.07	0.16 $\pm$ 0.04
Day 6	0.18 $\pm$ 0.02	0.28 $\pm$ 0.05	0.23 $\pm$ 0.03
Day 12	0.14 $\pm$ 0.01	0.32 $\pm$ 0.08 *	0.27 $\pm$ 0.05
Day 18	0.26 $\pm$ 0.08	nd	0.50 $\pm$ 0.09 #
L-Rhamnose			
Day 0	7.8 $\pm$ 0.4	10.7 $\pm$ 1.3	9.1 $\pm$ 1.0
Day 6	10.3 $\pm$ 0.6	11.2 $\pm$ 1.1	9.6 $\pm$ 1.5
Day 12	7.8 $\pm$ 0.5	7.1 $\pm$ 0.8	6.5 $\pm$ 0.6
Day 18	6.8 $\pm$ 0.9	nd	7.5 $\pm$ 0.7

Table 7. Villus height, crypt depth and percentile intra-epithelial lymphocytes in control, small and large tumor bearing animals. Villus height, crypt depth are in μm (mean $\pm$ SEM). IEL are the relative intraepithelial lymphocytes in the gut villus as percentage of enterocytes. Mann Whitney *U* nonparametric test versus control group: # $p < 0.05$.

	Control	Small tumor	Large tumor
Villus height	418 $\pm$ 12	409 $\pm$ 26	432 $\pm$ 18
Crypt depth	194 $\pm$ 12	197 $\pm$ 14	199 $\pm$ 14
IEL	21 $\pm$ 2	22 $\pm$ 2	16 $\pm$ 2 #

Table 8. Intracellular polyamine concentrations in the jejunum. Values are in $\mu\text{mol/L}$ except spermidine/spermine which is the ratio of the two polyamines (mean $\pm$ SEM). Mann Whitney *U* versus control group: ^a $p < 0.05$, ^b $p < 0.01$.

	Control	Small tumor	Large tumor
Putrescine	163 $\pm$ 40	153 $\pm$ 19	126 $\pm$ 12
Spermidine	3816 $\pm$ 537	3368 $\pm$ 545	2095 $\pm$ 237 ^b
Spermine	1708 $\pm$ 337	1439 $\pm$ 252	1077 $\pm$ 141
Spermidine/spermine	2.3 $\pm$ 0.1	2.4 $\pm$ 0.1	1.9 $\pm$ 0.1 ^a

Discussion

Glutamine is an important respiratory fuel and nucleotide precursor for the gastrointestinal tract (3,4,36). Disturbances in glutamine metabolism have been observed previously in MCA tumor bearing rats (12,37-39). In particular depletion of arterial and intracellular muscle glutamine pools have been observed (12,38) which were associated with decreased gut glutaminase activity in tumor bearing rats (15). In the current study arterial and intestinal intracellular glutamine also decreased with increasing tumor loads. In contrast, inward directed glutamine membrane transport rates and intracellular glutamine disposal increased in the portal drained viscera.

Endproducts of glutamine metabolism in the gut are ammonia (38%), citrulline (28%) and alanine (24%) (3,36). Net release of these products increased simultaneously with the increased intracellular disposal of glutamine suggesting enhanced metabolism of glutamine by the gut. However, the increased consumption of glutamine by portal drained viscera also includes uptake by other organs drained by the portal vein, e.g. the spleen. Lymphocytes present in the spleen are also avid consumers of glutamine and in cancer bearing rats both growth of the spleen as activation of lymphocytes have been observed (40-43). However, the metabolic endproducts of glutamine by rat lymphocytes are glutamate and aspartate instead of alanine and citrulline (36,44). We therefore postulate that the increased glutamine turnover in PDV in the large tumor bearing rats is a reflection of enhanced intracellular glutamine metabolism in enterocytes.

Protein turnover measured by the A-V dilution of ^3H -phenylalanine across the portal drained viscera has several methodological problems (22,45). It is recognized that intracellular disposal reflects protein synthesis but the intracellular production of

phenylalanine is a compilation of intracellular protein breakdown and the breakdown of proteins in the gut lumen (exfoliated cells, enzymes, bacterial proteins) (22,45). We observed decreased intestinal protein turnover and decreased protein synthesis rates in large tumor bearing rats. The decreased protein turnover did not result in villus atrophy or cryptal changes but was related to decreased mucosal polyamine concentrations.

Decreased mucosal polyamine concentrations have previously been associated with disturbed maturation at the villus tip (34,46). Moreover, polyamine concentrations are associated to intestinal cell growth. In this respect, polyamines have been shown to regulate the expression of growth related genes and have been related to the stabilization of cell membranes (34). Therefore, reduced polyamine concentrations could be related to loosening of tight junctions between enterocytes which could have caused the decreased gut barrier function we observed in cancer bearing rats.

Loss of gut barrier function became apparent in both tumor bearing rats by the increased lactulose/L-rhamnose ratio. The ratio of urinary recovery of lactulose and L-rhamnose reflects the ability of two macromolecules with different sizes to permeate the gut barrier. Although the precise anatomic intestinal permeation route of lactulose and L-rhamnose is unclear (47), L-rhamnose can permeate the mucosal barrier at a higher rate than lactulose due to better accessibility and size differences (47-49). The changes in the lactulose/L-rhamnose ratio observed in cancer were mostly due to increased recoveries of lactulose with no significant changes in L-rhamnose recoveries. This is in agreement with the observed unchanged villus height and crypt depth which would have decreased L-rhamnose (and 3-O-methyl-D-glucose) recovery (24,50). It indicates that the tight junctions may be dynamic and have opened as a (patho)physiological response in these tumor bearing rats (49). A similar response has been suggested to cause increased permeability during intestinal inflammation (49). Urinary lactulose recovery in control rats was slightly lower than the normal range of results (0.4%-1%) found by others (51,52), possibly due to the fact that we used iso-osmolar solutions instead of the hyperosmolar solutions used by others.

Bacterial translocation has been described in tumor bearing rats and mice (15,39). This has previously been related to increased permeability of macromolecules such as lactulose (6,7,53). The suggested relation is questionable as bacteria and endotoxins are far too large to pass through the same pores as lactulose. Only peptides, either bacterial or nutritional, of the same size (maximal 1.1 nm) can be expected to pass the intestinal barrier in the tumor bearing rats. Increased permeability for these macromolecules is, however, generally assumed to be pathophysiological and harmful for host body function (24).

Decreased gut barrier function has also been related to intestinal inflammation (54-57), atrophy of intestinal villi (58,59) and decreased availability of arterial glutamine to the gut (5,48). We observed no increase in inflammatory cells, no villus atrophy but depletion of arterial and intracellular glutamine was present in tumor bearing rats. In line with this, addition of glutamine to parenteral nutrition has previously been shown to improve intestinal barrier function, in both rats and man, as measured by the permeability of sugar probes (5,48). Also, in rats, parenteral glutamine suppletion reversed bacterial translocation and reversed the decreased gut IgA production induced by TPN (60). Impaired glutamine turnover in tumor bearing rats may thus be at the basis of the reduced gut barrier function.

Both the increased glutamine turnover and decreased mucosal polyamine concentrations could be related to the functional loss of the intestine. Recently, *in vitro* enterocyte polyamine production was also found to be glutamine concentration dependent (61). Therefore, we hypothesize that in cancer bearing rats, the increased glutamine turnover resulted in depletion of glutamine pools with concomitant decreased intestinal polyamine production which consequently induced loss of cell membrane stability with loss of gut barrier function for macromolecules.

In conclusion, this study shows that the presence of a MCA tumor leads to and an increased intracellular glutamine turnover with intracellular glutamine depletion. Although absorption remains intact, the gut loses its barrier function without morphological changes in villus height and crypt depth. Increased loss of gut barrier function is possibly related to disturbed growth or differentiation of enterocytes. The chronic loss of gut barrier to these macromolecules could be of harm to the cancer bearing host and be an important factor in the etiology of, gut associated, morbidity observed in cancer.

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HEPATIC AMINO ACID AND PROTEIN METABOLISM IN MODERATE CACHECTIC TUMOR BEARING RATS

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Abstract

Cancer cachexia is characterized by loss of lean body mass. Under this condition peripheral proteins are broken down and their amino acids are transferred to visceral organs and the tumor. The liver is the principle organ in the regulation of protein and amino acid metabolism but liver amino acid kinetics in cancer are unclear. Therefore, we examined the effects of increasing tumor loads on hepatic protein turnover and amino acid handling.

A MCA-induced sarcoma was implanted subcutaneously in Lewis rats (± 200 gr.). Rats were studied when the tumor was 5-15% or 15-30% of body weight. Control rats were sham implanted. Under anesthesia a primed constant infusion of *para*-aminohippuric acid and L-[3,4-³H]-valine was given to calculate hepatic substrate fluxes and protein turnover. Serum α_2 -macroglobulin concentration was measured to determine the acute phase response.

Carcass weight decreased approximately 10% in large tumor bearing rats ($p < 0.001$). Liver wet weight increased from 5.5 ± 0.1 (gr.) to 5.9 ± 0.2 in the small tumor bearing group and 7.3 ± 0.3 ($p < 0.001$) in the large tumor bearing group with minimal changes in water content. Serum α_2 -macroglobulin concentration, essential and gluconeogenic amino acid uptake by the liver increased in large tumor bearing animals. This contrasted with reduced liver ammonia uptake and unchanged urea production in tumor bearing rats. In the small tumor bearing group liver protein synthesis increased whereas protein breakdown remained unchanged. In the large tumor bearing group protein synthesis also increased but protein breakdown decreased to zero.

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The study shows that in tumor bearing rats, liver uptake of essential and gluconeogenic amino acids without significant increases in urea or glucose production. Synthesis of both structural and export proteins, e.g acute phase proteins, increases suggesting that the liver becomes a more efficient nitrogen sparing and active protein synthesizing organ during the growth of a malignant tumor.

Introduction

Cancer cachexia is a clinical syndrome characterized by excessive nitrogen loss and increased protein breakdown (1). Peripheral proteins are broken down and amino acids are mobilized and transferred to visceral organs and the tumor (2,3). The liver is a central organ in maintaining amino acid homeostasis (4). The liver is capable to take up amino acids for the production of defense peptides and proteins, gluconeogenesis and ureagenesis (4). Defense peptides and proteins produced in response to cancer are glutathion, acute phase proteins and immune related proteins of the reticulo endothelial system, e.g. Kupffer cells (5-8). In addition, previous studies also showed that synthesis of structural proteins of the liver raised proportionally to the tumor burden to maintain and enlarge liver protein mass (9).

It has long been recognized that hepatic gluconeogenesis increases in the progress of cancer (10-14). Augmented glucose production in cancer has been demonstrated to occur from glycerol, lactate and the amino acid alanine (11,12,15). Recently, glutamine has been recognized to contribute to an even larger extent to the glucose molecule carbon skeletons than alanine (16). Increased gluconeogenesis thus may be related to the depletion of glutamine observed in several other studies (5). Increased gluconeogenesis has also been held responsible for decreased plasma concentrations of other gluconeogenic amino acids, e.g. serine and threonine (17). If the carbon skeleton of amino acids is used to produce glucose, the amino group is processed to urea by the liver to be removed from the body by the kidney. For this reason, increased urea synthesis in the liver may occur in cancer explaining a large part the nitrogen loss observed. Contradictory results, however, have been reported in several animal studies. In tumor bearing mice decreased urea excretion (18,19) and increased nitrogen retention (20) is observed. In addition, decreased plasma concentrations of amino acids of the urea cycle were observed and suggested to reflect a diminished urea cycle activity (17,21).

The present study was undertaken to evaluate the effects of increasing tumor loads in rats on liver amino acid handling. We specifically wanted to evaluate how net amino acid uptake by the liver of the tumor bearing host contributed to protein turnover, urea synthesis and net glucose release.

Animals

Female Lewis rats (200-225 g, Centralized Animal Facilities, University of Limburg, Maastricht, NL) were individually housed in metabolic cages during the experiment after an adaptation period of 3 days. Rats were given standard laboratory rat chow ad libitum (SRMA 1210, Hopefarms, Woerden, NL) and subjected to standard 12 hour light-dark cycle periods (7:30 A.M. to 7:30 P.M.) with room temperature maintained at 25°C. The experiments were performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals (22) and approved by the Ethical Committee of Animal Research of the University of Limburg.

Study procedure

Methylcholanthrene (MCA) induced sarcomas were implanted subcutaneously as described previously in chapter 5 and 6. MCA tumors are often used in metabolic studies and are known for its rapid growth, invasion of local tissue and absence of metastatic potential (13, 23, 24, 25, 26).

Two studies were performed. In both experiments rats were randomly divided into three groups: a small tumor bearing group (ST), a large tumor bearing group (LT) and a control group fed ad libitum. In the ST group the tumor was allowed to grow until it reached 5-15% of the host body weight. In the LT group the tumor grew to 15-30% of the host body weight. To avoid effects of time, tumor tissue was transplanted bilaterally on the back in the ST group, whereas it was quadruple implanted in the LT group. Control rats were sham implanted with saline. Tumor growth was monitored by measuring two orthogonal dimensions and by calculating the volume assuming the tumor to be a prolate spheroid (27). Rats were housed in metabolic cages and tumor size, body weight and food intake were monitored daily.

In a first experiment (n=10 per group) the acute phase response was determined by measuring serum α_2 -macroglobulin (28). Blood was obtained under ether anaesthesia by puncture of the retrobulbar venous plexus.

In a second experiment (n=16 per group) liver metabolism was examined as described in chapter 4 (29,30). In brief, under ether anesthesia and at constant, pre-anesthesia body temperature, a laparotomy was performed. The right renal vein, the mesenteric vein, the portal vein, the hepatic vein and the aorta were cannulated using a 25 gauge needle fixed in a Silastic tube (Silastic Medical Grade tubing 0.051 cm ID, 0.094 OD, Dow Corning Corporation, Midland, MO, USA) and fixed with

cyano-acrylate. For flow measurements, the indicator dilution method with *para*-aminohippuric acid (PAH) was used (pH 7.4, iso-osmolaric, Sigma A 1422, St Louis, MI, USA). A primed ($0.15 \text{ ml} \times 100 \text{ g bw}^{-1}$, 50 mol/L) continuous infusion ($0.75 \text{ ml} \times 100 \text{ g bw}^{-1} \times \text{h}^{-1}$, 5 mmol/L) of PAH was infused in the mesenteric vein, using a Minipuls 3 Peristaltic Pump (Gilson Med. Electr. Inc., Villiers-le-Bel, France). A primed ($1 \text{ } \mu\text{Ci} \times 100 \text{ g bw}^{-1}$) constant infusion ($1 \text{ } \mu\text{Ci} \times 100 \text{ g bw}^{-1} \times \text{h}^{-1}$) of L-[3,4- ^3H]valine ($^3\text{H-Val}$, Amersham Int plc, TRK533, Buckinghamshire, UK) and L-[3,4- ^3H]glutamine ($^3\text{H-Gln}$, NEN NET-551, Mechelen, Belgium) was given in the right renal vein. The results concerning hepatic glutamine turnover are discussed in a chapter 8. A minimum infusion time of 30 minutes was needed to reach steady state concentrations for PAH, $^3\text{H-Val}$ and $^3\text{H-Gln}$ (30). In experiment 2 the whole procedure took 40 minutes before blood was sampled.

Sampling procedure

In the second experiment 1.0 ml blood was simultaneously sampled from the portal vein, hepatic vein and aorta. All blood was collected in heparinized cups on ice. Hereafter, the liver was dissected free, directly freeze-clamped, put in liquid nitrogen and stored at -80°C until further analysis. For PAH determinations, $50 \text{ } \mu\text{l}$ heparinized blood was added to $500 \text{ } \mu\text{l}$ TCA 10% (Merck 807, Darmstadt, Germany), vortexed and centrifuged at $8,900 \text{ g}$ at 4°C (Universac 30RF, Hettich, Tuttlingen, Germany). The supernatant was frozen in liquid nitrogen and stored at -80°C until further analysis. For hematocrit determinations a micro-hematocrit tube was filled with heparinized blood and centrifuged at $10,000 \text{ g}$ at room temperature. Hematocrit was read with a Micro Hematocrit Reader. Plasma was obtained by whole blood centrifugation at $8,900 \text{ g}$ at 4°C for 5 minutes. For ammonia, urea, glucose and lactate determinations, $200 \text{ } \mu\text{l}$ plasma was vortexed with $20 \text{ } \mu\text{l}$ TCA 50%, put into liquid nitrogen and stored at -80°C . To determine plasma amino acid specific activities and concentrations, 8 mg 5-sulfosalicylic acid (SSA) was added to $200 \text{ } \mu\text{l}$ plasma, vortexed, frozen in liquid nitrogen and stored at -80°C .

Analysis

Plasma ammonia, urea, glucose, lactate and PAH were determined spectrophotometrically on a Cobas Mira S (Roche Diagnostica, Hoffman-La Roche, Basel, Switzerland) by standard enzymatic methods, using commercially available kits as described previously (29,31). Plasma amino acid concentrations and specific activity were determined by HPLC as described previously (32). Serum α_2 -macroglobulin was determined by single immunodiffusion using mono specific antibodies against rat α_2 -macroglobulin (28). For the determination of tissue ammonia, amino acid concentrations and specific activities, liver tissue was

pulverized using a mortar and pestle precooled in liquid nitrogen. The tissue was further homogenized and deproteinized in a Mini-Beadbeater (Biospec products, Bartlesville USA). Approximately 100 mg tissue was added to 400 μ l SSA 5%, with 300 mg glass beads (diameter 1 mm, Biospeck Products) and beaten for 30 seconds. The homogenate was centrifuged at 4°C at 1,100 g and the supernatant frozen in liquid nitrogen and stored at -80°C until further determinations.

To determine tissue dry weight and water content approximately 200 mg pulverized tissue was freeze dried for 24 hours in a Speedvac (SC200, Savant Instruments Inc., Farmingdale NY, USA) connected with a refrigerated condensation trap (Savant Instruments Inc.).

Calculations

Hepatic plasma flow, flux and intracellular concentrations

Plasma flow across liver was calculated using PAH in the indicator dilution method as described previously (33,34). Splanchnic flux was calculated by multiplying the mean hepatic plasma flow with hepatic vein-arterial concentration differences. Portal drained viscera (PDV) flux is the mean PDV plasma flow multiplied with portal vein-arterial concentration difference. Liver flux was calculated by subtracting PDV flux from the splanchnic flux (33,34). A positive flux indicates net release, a negative flux reflects a net uptake. α -Amino-nitrogen was calculated as the sum of the individual amino acids measured (32). Essential, non-essential and gluconeogenic amino acids are defined as described previously (35). Tissue concentrations of amino acids are expressed in μ mol/L and derived by dividing the tissue homogenate amino acid concentration (μ mol/kg wet weight) by the tissue water content.

Hepatic protein turnover

Liver *in vivo* protein turnover was calculated in a three compartment model as described in detail in chapter 2 and 4 (30,36). The three compartments involved are the following: 1) vascular afferent pool (hepatic artery and portal vein); 2) intracellular free amino acid pool; 3) vascular efferent pool (hepatic vein). Disposal and production of valine are calculated out of and into the intracellular free amino acid pool. Disposal of valine was used to estimate liver protein synthesis as the liver has low valine transaminase activity (37). Similar, valine production can only originate from protein breakdown. Valine is used both *in vitro* and *in vivo* to measure hepatocyte protein turnover (38,39).

Membrane transport rates are calculated as the amino acid flux from the afferent pool to the intracellular pool (F_{LA}), the amino acid flux from the intracellular pool into

the hepatic vein (F_{LH}) and the amino acid flux shunting from afferent vessels into the hepatic vein (F_{HA}).

Statistical Analysis

Results are presented as mean $\pm$ SEM. The Mann Whitney *U* test corrected with the Bonferroni procedure was used to test significance between tumor bearing groups and control group (40). Significance was considered present at $p<0.05$.

Results

General response

The presence of MCA tumors did not reduce food intake (Figure 1) while carcass weight decreased only moderately in the large tumor bearing animals (Table 1). Liver weight was unchanged in the small tumor bearing animals and increased approximately 30% in the large tumor bearing animals after 24 days. Serum α_2 -macroglobulin concentration raised with increasing tumor loads. In the large tumor bearing rats α_2 -macroglobulin concentrations were elevated comparable to those seen in response to severe acute inflammatory stimuli such as turpentine or surgical trauma (28,41).

Figure 1. Food intake of control, small tumor bearing and large tumor bearing rats. All rats were fed ad libitum. No significant changes in food intake between different groups.

Table 1. Tumor weight, carcass weight, liver wet weight, dry weight, intracellular water content and serum α_2 -macroglobuline concentrations. Values are grams (mean $\pm$ SEM) except α_2 -macroglobuline ($\mu\text{g/ml}$) and measured at day of implantation (day 1) and day of experiment (day 24). Detection level α_2 -macroglobuline is 50 $\mu\text{g/ml}$. Statistical analysis by Mann Whitney U vs control group: ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$.

	Control		Small tumor		Large tumor	
	day 1	day 24	day 1	day 24	day 1	day 24
Tumor weight	-	-	-	-	-	-
Carcass weight	200 $\pm$ 4	200 $\pm$ 4	192 $\pm$ 3	198 $\pm$ 2	199 $\pm$ 3	181 $\pm$ 2 ^c
Liver						
Wet weight	-	5.5 $\pm$ 0.1	-	5.9 $\pm$ 0.2	-	7.3 $\pm$ 0.3 ^c
Dry weight	-	1.56 $\pm$ 0.10	-	1.54 $\pm$ 0.05	-	1.90 $\pm$ 0.08 ^c
% Water	-	71.6 $\pm$ 0.5	-	73.9 $\pm$ 0.4	-	73.7 $\pm$ 0.9
α_2 -Macroglobuline	-	<50	-	1019 $\pm$ 509 ^a	-	5300 $\pm$ 1040 ^c

Arterial concentrations

Arterial glucose concentrations diminished in the small tumor bearing animals and decreased further in the large tumor bearing animals (Table 2). Arterial lactate concentrations increased with increasing tumor load.

An arterial hyperaminoacidemia developed in both groups of tumor bearing rats (Fig. 2, Table 2). In the tumor bearing rats both essential and non-essential amino acid concentrations increased. In control rats almost 30% of non-essential amino acids consisted of glutamine which proportion concentration did not change in the presence of a tumor. Concentrations of all gluconeogenic amino acids increased in tumor bearing rats.

Liver intracellular concentrations

Intracellular ammonia did not vary between controls and tumor bearing rats (Table 3). Total α -amino acid nitrogen remained unchanged in the small tumor bearing animals but decreased in the large tumor bearing animals (Table 3, Fig. 3). However, mostly non essential amino acids decreased and of these primarily glutamine and taurine.

Table 2. Arterial glucose, lactate, ammonia, urea and amino acid concentrations in control, small and large tumor bearing rats. Values are presented in $\mu\text{mol/L}$ except glucose, lactate and urea in mmol/L (mean $\pm$ SEM). Mann Whitney *U* vs control group: ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$. BCAA: Branched Chain Amino Acids (leucine, isoleucine, valine). Essential amino acids in italics, gluconeogenic amino acids in bold (32).

	Control	Small tumor	Large tumor
Glucose	9.7 $\pm$ 0.1	8.7 $\pm$ 0.4 ^c	7.7 $\pm$ 0.2 ^c
Lactate	6.4 $\pm$ 0.3	7.4 $\pm$ 0.3 ^c	8.4 $\pm$ 0.4 ^c
Ammonia	174 $\pm$ 10	169 $\pm$ 8	161 $\pm$ 9
Urea	3.9 $\pm$ 0.2	3.9 $\pm$ 0.2	3.8 $\pm$ 0.1
Glutamine	550 $\pm$ 25	577 $\pm$ 12	539 $\pm$ 28
Glutamate	62 $\pm$ 4	82 $\pm$ 6 ^a	118 $\pm$ 10 ^c
Asparagine	33 $\pm$ 2	43 $\pm$ 1 ^c	42 $\pm$ 2 ^b
<i>Serine/Histidine</i>	189 $\pm$ 15	235 $\pm$ 7 ^a	199 $\pm$ 11
Glycine	180 $\pm$ 15	263 $\pm$ 18 ^c	276 $\pm$ 11 ^c
<i>Threonine</i>	83 $\pm$ 4	87 $\pm$ 5	70 $\pm$ 4 ^b
Citrulline	46 $\pm$ 3	52 $\pm$ 2	64 $\pm$ 17
Alanine	321 $\pm$ 18	359 $\pm$ 18	358 $\pm$ 25
Arginine	118 $\pm$ 5	161 $\pm$ 6 ^c	150 $\pm$ 8 ^c
Taurine	150 $\pm$ 9	302 $\pm$ 25 ^c	342 $\pm$ 37 ^c
α -Aminobutyric acid	22 $\pm$ 3	36 $\pm$ 2 ^c	48 $\pm$ 9 ^c
Tyrosine	61 $\pm$ 4	94 $\pm$ 4 ^c	113 $\pm$ 31 ^c
<i>Valine</i>	117 $\pm$ 7	154 $\pm$ 6 ^c	168 $\pm$ 10 ^c
<i>Methionine</i>	45 $\pm$ 2	53 $\pm$ 1 ^c	50 $\pm$ 2 ^a
<i>Isoleucine</i>	48 $\pm$ 3	70 $\pm$ 3 ^c	72 $\pm$ 4 ^c
<i>Phenylalanine</i>	47 $\pm$ 2	66 $\pm$ 2 ^c	77 $\pm$ 7 ^c
<i>Tryptophan</i>	48 $\pm$ 3	38 $\pm$ 2 ^c	33 $\pm$ 2 ^c
<i>Leucine</i>	73 $\pm$ 4	102 $\pm$ 4 ^c	98 $\pm$ 5 ^c
Ornithine	18 $\pm$ 2	37 $\pm$ 3 ^c	46 $\pm$ 5 ^c
<i>Lysine</i>	291 $\pm$ 15	409 $\pm$ 15 ^c	356 $\pm$ 25 ^b
BCAA	239 $\pm$ 13	326 $\pm$ 12 ^c	326 $\pm$ 14 ^c

Essential amino acid concentrations either increased (histidine, valine, isoleucine, phenylalanine, leucine) or remained unchanged (threonine, methionine, tryptophan, lysine) with increasing tumor loads. The sum of essential amino acids increased in the large tumor bearing rats. The sum of gluconeogenic amino acids decreased in the small tumor bearing rats and remained reduced in the large tumor bearing rats.

Table 3. Intracellular ammonia and amino acid concentrations in the liver of control, small and large tumor bearing rats. Values are presented in $\mu\text{mol/L}$ (mean $\pm$ SEM). Mann Whitney *U* vs control group: ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$. BCAA: Branched Chain Amino Acids (leucine, isoleucine, valine). Essential amino acids in italics, gluconeogenic amino acids in bold (32).

	Control	Small tumor	Large tumor
Ammonia	1681 $\pm$ 133	1801 $\pm$ 155	1691 $\pm$ 78
Glutamine	5221 $\pm$ 236	3700 $\pm$ 297 ^c	3052 $\pm$ 298 ^c
<i>Histidine</i>	408 $\pm$ 19	390 $\pm$ 22	533 $\pm$ 21
Glutamate	5128 $\pm$ 237	5066 $\pm$ 216	4818 $\pm$ 339
Asparagine	68 $\pm$ 5	135 $\pm$ 28 ^b	103 $\pm$ 14 ^b
<i>Serine</i>	404 $\pm$ 18	484 $\pm$ 45	518 $\pm$ 51 ^c
Glycine	3658 $\pm$ 128	2674 $\pm$ 111	2962 $\pm$ 137 ^c
Threonine	758 $\pm$ 63	787 $\pm$ 173	897 $\pm$ 105
Citrulline	99 $\pm$ 7	122 $\pm$ 8 ^a	128 $\pm$ 10 ^a
<i>Alanine</i>	4243 $\pm$ 376	4452 $\pm$ 294	4563 $\pm$ 322
<i>Arginine</i>	261 $\pm$ 75	407 $\pm$ 79	178 $\pm$ 62
Taurine	14006 $\pm$ 869	15962 $\pm$ 1345	11309 $\pm$ 1298 ^a
α -Aminobutyric acid	102 $\pm$ 14	165 $\pm$ 8	230 $\pm$ 23 ^c
Tyrosine	161 $\pm$ 12	163 $\pm$ 8	201 $\pm$ 11 ^a
<i>Valine</i>	238 $\pm$ 16	267 $\pm$ 15	288 $\pm$ 21 ^a
Methionine	133 $\pm$ 10	127 $\pm$ 11	146 $\pm$ 19
<i>Isoleucine</i>	118 $\pm$ 8	136 $\pm$ 8	141 $\pm$ 9 ^a
<i>Phenylalanine</i>	112 $\pm$ 4	124 $\pm$ 5 ^a	136 $\pm$ 7 ^b
Tryptophan	39 $\pm$ 2	42 $\pm$ 2	41 $\pm$ 4
<i>Leucine</i>	228 $\pm$ 14	279 $\pm$ 13 ^a	284 $\pm$ 17 ^a
Ornithine	203 $\pm$ 12	272 $\pm$ 15 ^b	326 $\pm$ 27 ^c
<i>Lysine</i>	884 $\pm$ 88	966 $\pm$ 155	830 $\pm$ 47
BCAA	584 $\pm$ 28	682 $\pm$ 37	713 $\pm$ 35 ^a

Urea cycle intermediate amino acids are ornithine, citrulline and arginine. The first two increased in tumor bearing rats but arginine concentrations decreased below control values in the large tumor bearing rats.

Hepatic plasma flow and flux

Hepatic plasma flow was similar in control and both tumor bearing animals (Table 4). Glucose release decreased in small tumor bearing rats although not significantly. Lactate uptake increased in the large tumor bearing animals. In line with this, we found an increased uptake of gluconeogenic amino acids in the large tumor bearing

Table 4. Hepatic glucose, lactate, ammonia, urea and amino acid fluxes in control, small and large tumor bearing rats. Values are presented in $\text{nmol} \times 100 \text{ g bw}^{-1} \times \text{min}^{-1}$ (mean $\pm$ SEM). Mann Whitney *U* vs control group: ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$. BCAA: Branched Chain Amino Acids (leucine, isoleucine, valine). Essential amino acids in italics, gluconeogenic amino acids in bold (32).

	Control	Small tumor	Large tumor
Flow	2.5 $\pm$ 0.5	2.6 $\pm$ 0.6	2.7 $\pm$ 0.6
Glucose	3184 $\pm$ 792	1910 $\pm$ 467	3310 $\pm$ 487
Lactate	-2940 $\pm$ 702	-3659 $\pm$ 461	-4235 $\pm$ 448 ^a
Ammonia	-310 $\pm$ 36	-160 $\pm$ 32 ^b	-177 $\pm$ 30 ^b
Urea	820 $\pm$ 169	456 $\pm$ 81	521 $\pm$ 57
Glutamine	112 $\pm$ 52	-64 $\pm$ 34 ^b	37 $\pm$ 38
Glutamate	-19 $\pm$ 15	108 $\pm$ 28 ^c	112 $\pm$ 28 ^c
Asparagine	-43 $\pm$ 5	57 $\pm$ 7	66 $\pm$ 6 ^b
<i>Serine/Histidine</i>	-115 $\pm$ 19	-166 $\pm$ 22	-155 $\pm$ 22
Glycine	-201 $\pm$ 33	-184 $\pm$ 27	-262 $\pm$ 30
<i>Threonine</i>	-57 $\pm$ 26	-63 $\pm$ 8	-68 $\pm$ 8
Citrulline	-42 $\pm$ 11	-20 $\pm$ 7	-56 $\pm$ 8 ^e
Alanine	-396 $\pm$ 79	-321 $\pm$ 63	-545 $\pm$ 52 ^d
Arginine	-53 $\pm$ 9	-97 $\pm$ 14 ^a	-114 $\pm$ 15 ^b
Taurine	-52 $\pm$ 18	-9 $\pm$ 26	-84 $\pm$ 45
α -Aminobutyric acid	-11 $\pm$ 3	-3 $\pm$ 3	-7 $\pm$ 4
Tyrosine	-22 $\pm$ 5	-18 $\pm$ 7	-31 $\pm$ 10
Valine	26 $\pm$ 8	8 $\pm$ 12	-36 $\pm$ 18 ^c
<i>Methionine</i>	1 $\pm$ 5	-22 $\pm$ 7 ^b	-28 $\pm$ 5 ^c
<i>Isoleucine</i>	13 $\pm$ 4	13 $\pm$ 6	-6 $\pm$ 6 ^a
<i>Phenylalanine</i>	-3 $\pm$ 4	-9 $\pm$ 3	-32 $\pm$ 8 ^b
<i>Tryptophan</i>	4 $\pm$ 7	3 $\pm$ 3	-3 $\pm$ 4
<i>Leucine</i>	26 $\pm$ 14	17 $\pm$ 8	-19 $\pm$ 13 ^a
Ornithine	3 $\pm$ 4	31 $\pm$ 10 ^b	-2 $\pm$ 12
<i>Lysine</i>	-136 $\pm$ 18	-161 $\pm$ 31	-208 $\pm$ 50
BCAA	64 $\pm$ 24	38 $\pm$ 24	-62 $\pm$ 34 ^b

rats (Table 4, Fig. 4). Alanine uptake accounted for 40 to 46% of the uptake of all gluconeogenic amino acids by the liver. The uptake of total α -amino acids remained unchanged in the small tumor bearing rats but tended to increase in the large tumor bearing rats. This was due to an increased uptake of essential amino acids.

The uptake of non-essential amino acids also only tended to increase. Ammonia uptake decreased in both tumor bearing rats. Urea release remained unchanged in the small and large tumor bearing animals.

Figure 2. Arterial concentrations (μmol/l) of total α-amino nitrogen (top, left), gluconeogenic amino acids (top, right), essential amino acids (bottom, left) and non essential amino acids (bottom, right).

p=0.05, * p=0.01, ^ p=0.001. TB = tumor bearing.

Figure 3. Intracellular concentrations (μmol/l) of total α-amino nitrogen (top, left), gluconeogenic amino acids (top, right), essential amino acids (bottom, left) and non essential amino acids (bottom, right).

p=0.05, * p=0.01, ^ p=0.001. TB = tumor bearing.

Figure 4. Fluxes of total α -amino nitrogen (top, left), gluconeogenic amino acids (top, right), essential amino acids (bottom, left) and non essential amino acids (bottom, right) across the liver. A negative flux reflects net uptake, a positive flux reflects net release of amino acids. ^a $p=0.05$, ^b $p=0.01$, ^c $p=0.001$. TB =tumor bearing

Figure 5. Intracellular disposal of valine (top), production of valine (middle) and flux of valine across the liver. Disposal of valine reflects protein synthesis whereas production of valine reflects protein breakdown. Valine flux is the difference between disposal and production (negative: net uptake; positive: net release). ^a $p=0.05$, ^b $p=0.01$, ^c $p=0.001$. TB = tumor bearing.

Hepatic protein turnover and valine membrane transport

Liver valine disposal (protein synthesis) increased in the small tumor bearing rats and remained increased at that level in the large tumor bearing animals (Fig. 5). Valine production (protein breakdown) was unchanged in the small tumor bearing animals but decreased to zero in the large tumor bearing animals. Liver valine flux (difference protein breakdown and synthesis) was thus reduced to zero in the small tumor bearing rats by the increased protein synthesis rates. In the large tumor bearing animals valine flux became negative (=net protein synthesis) by strongly decreased protein breakdown rates.

Valine inward membrane transport rates (F_{LA}) were unchanged in the small tumor bearing rats and increased only slightly in the large tumor bearing rats (Table 5). Outward membrane transport (F_{HL}) was not significantly different between the groups. In the large tumor bearing group valine shunting (F_{HA}) increased more than fifty percent.

To see whether plasma-amino acid membrane transport determines a large fraction of intracellular amino acid turnover, we calculated total intracellular amino acid appearance rate of valine as the sum of valine inward membrane transport plus valine production from intracellular protein breakdown (R_{ai} , Table 6). Membrane transport determines 74% of all de novo appearance of valine in the free intracellular amino acid pool of control rats. Approximately 26% of intracellular amino acid appearance in the hepatocyte is determined by protein breakdown (production). In the small tumor bearing rats protein breakdown accounted for a relatively larger amount of total valine appearance rate than in control rats, whereas this decreased to zero in the large tumor bearing rats.

Table 5. Liver valine membrane transport in control, small and large tumor bearing rats. Values are presented in nmol valine $\times$ 100 g $bw^{-1} \times min^{-1}$ (mean $\pm$ SEM). F_{LA} : inward directed membrane transport rates; F_{HL} : outward directed membrane transport; F_{HA} : metabolic shunting. * $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$ vs control group.

	Control	Small tumor	Large tumor
F_{LA}	88 $\pm$ 31	87 $\pm$ 50	108 $\pm$ 33 ^a
F_{HL}	105 $\pm$ 37	95 $\pm$ 56	71 $\pm$ 42
F_{HA}	201 $\pm$ 31	301 $\pm$ 51	325 $\pm$ 37 ^b

Table 6. Total intracellular valine turnover and relative contribution of amino acid membrane transport and intracellular protein breakdown (Production). F_{LA} : inward membrane transport of valine; P: intracellular production from protein breakdown; R_a : total intracellular appearance rate (F_{LA} + production). R_a is in nmol valine $\times 100$ g $bw^{-1} \times min^{-1}$, F_{LA}/R_a and P/R_a are the fractions to which membrane transport and protein breakdown contribute to total intracellular appearance rate. Statistics: vs control group: ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$.

	Control	Small tumor	Large tumor
R_a	142 $\pm$ 40	180 $\pm$ 71	174 $\pm$ 57
F_{LA}/R_a	0.74 $\pm$ 0.07	0.58 $\pm$ 0.06 ^a	0.97 $\pm$ 0.04
P/R_a	0.26 $\pm$ 0.07	0.41 $\pm$ 0.06 ^a	0.03 $\pm$ 0.04

Discussion

The liver has a dual role in the development of cancer. It regulates a great part of the intermediary metabolism, particularly related to amino acid and glucose homeostasis. It is, however, also involved in the acute phase response. Processing of amino acids by the liver in cancer therefore both serves the production of defense proteins and the maintenance of metabolic homeostasis. In our rat cancer model no anorexia was observed. The inflammatory and metabolic changes observed can thus only be attributed to the presence of the tumor. However, the unchanged nutrient intake in the presence of a tumor of 5-15% or 15-30% of the body weight must have changed nutrient delivery to non-tumor tissues with subsequent adaptations in intermediary metabolism. Net carcass weight loss was limited in this cancer model. The current study thus appears to be a reflection of metabolic changes observed in moderate cancer cachexia, e.g. early detected cancer patients with no, or hardly any, nitrogen and weight loss.

Urea production by the liver remained unchanged in both groups of tumor bearing rats although it tended to decrease ($p = 0.07$). The decrease may have remained undetected by the variation of the data (type II error). Citrulline and ornithine are intermediary amino acids of the urea cycle. In a previous study decreased arterial concentrations of these amino acids were found indicative for decreased urea cycle activity in tumor bearing rats (17). In the current study increased concentrations of these amino acids were observed in arterial plasma and, moreover, in the liver of tumor bearing rats. We feel that this is more in agreement with decreased urea cycling as intermediate substrates of the urea cycle appear not to be used. However,

it also indicates that changes in amino acid concentrations are difficult to translate into kinetic parameters and the interpretation of only amino acid concentrations should be done with great care.

Unchanged or possibly decreased activity of the urea cycle in tumor bearing animals also explains the increased arterial amino acid concentrations. We previously showed that the presence of the tumor increases peripheral protein breakdown with an enhanced net efflux of amino acids from muscle (42). Thus, mobilization of amino acids in combination with unchanged or decreased urea cycle activity and increased arterial amino acid concentrations, directs amino acid interorgan transfer towards other tissues, e.g. immune cells and tumor tissue.

Arginine is also an intermediary amino acid of the urea cycle. The decreased intracellular arginine concentrations in large tumor bearing rats are probably not related to the urea cycle but to the production of nitric oxide: arginine is the precursor of nitric oxide and nitric oxide production by hepatocytes and Kupffer cells increases after stimulation with inflammatory mediators (8).

Two environmental stimuli are of influence on urea production in tumor bearing rats. The availability of α -amino nitrogen is a well described factor which influences liver amino acid uptake and processing (35,43). In particular low protein diets decrease the availability of amino acids for the liver (43). The uptake of amino acids by the liver thus decreases with subsequent reduced urea production. In our tumor bearing rats, however, arterial concentrations increased with increasing tumor loads suggesting a greater availability of amino acids for potential urea synthesis. Another important mechanism influencing urea synthesis is related to circulating peptides/proteins induced by the presence of the tumor. Hormones and cytokines are known to influence liver urea synthesis and protein synthesis in other stress conditions, such as burns, infection and trauma (44). Increased, decreased and unchanged insulin and glucagon levels have been observed in MCA tumor bearing rats (38,45,46). In addition, increased levels of several cytokines, e.g. $\text{TNF}\alpha$, IL1, IL6, have been observed in the cancer bearing host (47-51). These cytokines are all able to induce *in vitro* amino acid uptake by hepatocytes (52, 53). Involvement of IL6 is further in agreement with the general concept that it is one of the most important mediators of the acute phase response as observed in the current study (28,51). The positive effects of the increased arterial amino acid concentrations on the urea cycle therefore appears to be counteracted by humoral factors. However, the exact etiology of such a nitrogen sparing mechanism by the liver in the cancer bearing host needs further research.

Increased Cori cycling and increased gluconeogenesis have been demonstrated in previous studies of sarcoma bearing rats (15,46,54). In our study no changes in net glucose production were observed. This may have been caused by the negative

effect of ether anesthesia on gluconeogenesis (55). We, however, also did not measure the exact gluconeogenesis but only the net production of glucose. This will clearly underestimate liver gluconeogenesis in particular when inflammatory cells of the liver, such as Kupffer cells, are activated and consume glucose.

Lactate and amino acids are major precursors for gluconeogenesis (56). If the carbon skeleton of amino acids is transformed to glucose, the amino group is processed to urea. Amino acids first transfer their amino group to aspartate which donates one nitrogen group to urea. The second nitrogen of urea is derived from the amide group of glutamine or free ammonia. Gluconeogenic amino acid uptake in control rats is thus more or less in balance with the nitrogen transferred to urea in control rats (Table 4, 850 versus 820 nmol $\times$ 100 g bw^{-1} $\times$ min $^{-1}$). In the small tumor bearing rats the uptake of gluconeogenic amino acids exceeds the nitrogen used for urea production by 348 nmol $\times$ 100 g bw^{-1} $\times$ min $^{-1}$ and this surplus increases to 705 nmol $\times$ 100 g bw^{-1} $\times$ min $^{-1}$ in large tumor bearing rats. Thus, it appears that the liver prefers to use gluconeogenic amino acids for other processes, mainly protein synthesis. *Acute phase protein synthesis has previously been shown to increase secondary to tumor growth* (51). Serum α_2 -macroglobulin is one of the major acute phase proteins in the rat and is a broad spectrum protease inhibitor that can bind cytokines and regulates tissue damage and restructuring (28,57). Increased levels of several cytokines, damage and necrosis of tumor are features of our tumor model and may very well explain the raised increased acute phase protein synthesis.

Protein synthesis in the hepatocyte was measured by intracellular valine disposal. This was possible as the enzyme activity of the major other metabolic route, transamination, is very low in liver tissue compared to other tissues (37). Maximal transamination of valine in the rat liver is 75 nmol $\times$ 100 g bw^{-1} $\times$ min $^{-1}$ and the K_m value is 1.2-2.5 mM (58). Our *in vivo* intracellular valine concentrations are approximately one tenth of the K_m value. Estimated *in vivo* transamination would thus be approximately 4 nmol $\times$ 100 g bw^{-1} $\times$ min $^{-1}$ which comprises almost half of the intracellular disposal of valine in control rats. However, in the tumor bearing rats this was only one tenth of total disposal and most of intracellular valine disposal can thus be accounted for by protein synthesis.

In control rats protein breakdown rates exceeded protein synthesis rates resulting in a net protein loss with a net efflux of valine. The negative protein balance of the liver in control rats is related to the experimental conditions. First, all rats are studied postabsorptively. Second, previous studies, although not all (59) suggest that ether anesthesia decreases protein synthesis rates of the liver (60, 61). The increased net protein synthesis rates in tumor bearing rats should thus be interpreted relative to the control rats and may in absolute terms be an underestimation of the actual protein synthesis rates.

Liver protein synthesis is the sum of export proteins and structural proteins. Serum α_2 -macroglobulin is considered to be the most reactive acute phase protein exported by the liver (28). It has a turnover of approximately 8% per day and a Kd of 820.000 (62). Assuming plasma volume of rats to be 4% of bodyweight (62), the increased serum α_2 -macroglobulin concentrations in the large tumor bearing rats equals an additional production of 48 mg α_2 -macroglobuline proteins. This is approximately 27800 nmol valine (63) over approximately 18 days or 0.5 nmol valine $\times$ 100 gr. $\text{bw}^{-1} \times \text{min}^{-1}$. Although other acute phase proteins (e.g. haptoglobin, coeruloplasmine, α_1 acid glycoprotein) are also produced by hepatocytes in response to inflammation, the discrepancy between the calculated intracellular disposal (44 nmol $\times$ 100 gr. $\text{bw}^{-1} \times \text{min}^{-1}$) and the theoretical disposal for α_2 -macroglobuline (0.5 nmol), suggest that the increased protein synthesis in majority reflects increased synthesis of structural proteins of the liver.

The increased protein synthesis in the small tumor bearing rats was not accompanied by increased membrane transport rates of valine. This indicates that changes in membrane transport activity were not involved in the regulation of liver protein synthesis. This can also be seen in the relative contribution of amino acid membrane transport and intracellular production to total intracellular amino acid turnover (R_{ai} , Table 6). The relative contribution of intracellular production increased at the cost of membrane transport rates in the small tumor bearing rats. In the large tumor bearing rats, however, valine production decreased to almost zero and membrane transport was still sufficiently high to supply valine for protein synthesis. The unchanged valine membrane transport despite the increased need for intracellular valine in these animals possibly indicates that amino acid membrane transport of these amino acids in our rat cancer model is working at submaximal activity. These *in vivo* findings are in agreement with *in vitro* observations on hepatocyt system L membrane transport carriers. These studies show that system L membrane transport carriers demonstrate a relatively high activity in hepatocytes but do not maintain large concentration differences across cell membranes (64). Furthermore, hepatic system L has been shown not to play any significant role in liver amino acid turnover in the presence of altered amino acid availability due to high or low protein diets (43).

In conclusion, the study shows that in tumor bearing rats, liver uptake of essential and gluconeogenic amino acids increases. Glucose and urea production remain unchanged whereas protein synthesis of both structural and export proteins, e.g acute phase proteins, increase. These changes indicate that the liver becomes a more efficient nitrogen sparing and active protein synthesizing organ during the growth of a malignant tumor.

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HEPATIC GLUTAMINE KINETICS IN CANCER

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Submitted

Abstract

The liver plays a key role in glutamine homeostasis. Cancer cachexia is thought to be related to deteriorated glutamine metabolism. Aim of this study was to examine the effects of increasing tumor loads on hepatic glutamine membrane transport and intra- and inter-cellular glutamine turnover.

A MCA-induced sarcoma was implanted subcutaneously in Lewis rats (± 200 g). Rats were studied when the tumor was 5-15% or 15-30% of body weight. Control rats were sham implanted. Under anesthesia a primed constant infusion of *para*-aminohippuric acid and L-[3,4- ^3H]-glutamine was given. At steady state, glutamine turnover and membrane transport rates were calculated in a three compartment model of the liver.

Carcass weight decreased approximately 10% in large tumor bearing rats. Liver wet weight increased from 5.6 ± 0.1 (gr.) to 5.9 ± 0.2 (gr.) in the small tumor bearing group to 7.3 ± 0.3 (gr.) in the large tumor bearing group. Arterial glutamine concentrations remained unchanged whereas hepatic intracellular glutamine concentrations decreased in both tumor bearing rats. The net balance of glutamine across the liver changed from a net release in control rats to a net uptake in the small tumor bearing group. In the large tumor bearing group glutamine balance was not different from zero. Hepatic intracellular glutamine disposal (control 198 ± 87 nmol $\times 100$ g $\text{bw}^{-1} \times \text{minute}^{-1}$) increased in the small tumor bearing group (624 ± 127 nmol $\times 100$ g $\text{bw}^{-1} \times \text{minute}^{-1}$) and decreased to control values in the large tumor bearing group (266 ± 59 nmol $\times 100$ g $\text{bw}^{-1} \times \text{minute}^{-1}$). Increased intracellular glutamine turnover was accompanied by a net increased inward directed membrane transport rate. Normalized for the concentrations, both inward and outward membrane transport rates increased indicating increased transport activity in both

tumor bearing rats. The study shows that modulation of liver glutamine breakdown is an important determinant of glutamine handling in tumor bearing rats.

Introduction

Glutamine is the most abundant amino acid in the body and has many key metabolic functions (1,2). The liver is a central organ in maintaining glutamine homeostasis as it can either produce or consume glutamine (3). Periportal hepatocytes have high glutaminase activity and can deamidate glutamine resulting in the formation of ammonia and glutamate (4). Glutamine is thus a substrate for gluconeogenesis and ureagenesis in the liver. Liver glutamine synthetase activity is mostly restricted to perivenous cells (4,5). Plasma glutamate and ammonia are almost completely used for glutamine synthesis. This results in a so called intercellular glutamine cycle in the liver which normally results in neither a net uptake nor release of glutamine (6). It also makes it possible for the liver to consume or release net amounts of glutamine depending on prevailing metabolic pressure.

Plasma-tissue membrane transport represents an important step in liver glutamine metabolism. Glutamine membrane transport systems build up a plasma-tissue (cytosolic and mitochondrial) concentration gradient to enable liver glutaminase to be active near its K_m (5). In this respect, glutamine membrane transport rates were suggested to be rate controlling in hepatic glutamine synthesis and degradation handling (4,5).

Following injury, stress or in the progress of cancer, glutamine plasma and intracellular skeletal muscle pools become depleted (7,8). This has been associated with increased protein catabolism. Failure of the liver to produce sufficient glutamine was suggested to be one of the main pathophysiological mechanisms involved (2).

The present study was undertaken to evaluate the effects of an increasing tumor burden on *in vivo* liver glutamine membrane transport. A recently introduced *in vivo* model was used to simultaneously measure *in vivo* amino acid membrane transport rates and intracellular turnover (9,10). In doing so, we also wanted to compare *in vivo* membrane transport rates in cancer with intracellular glutamine turnover to test whether membrane transport rates have regulating properties *in vivo*.

Study procedure

The present study was simultaneously done with the study on hepatic metabolism of other amino acids and on hepatic protein turnover as described in chapter 7. Therefore, animals, study procedure and biochemical analysis are as described on page 151-154. In brief, a methylcholanthrene (MCA) induced tumor, which is serially transplanted in our laboratory, was used to study the effects of cancer on hepatic glutamine metabolism. Female Lewis rats (200-225 g, $n=48$) were randomly divided into three groups: a small tumor bearing group (ST), a large tumor bearing group (LT) and a control group fed ad libitum. In the ST group the tumor was allowed to grow until it reached 5-15% of the host body weight. In the LT group the tumor grew to 15-30% of the host body weight. Rats were housed in metabolic cages and tumor size, body weight and food intake were monitored daily. Food consumption of tumor bearing animals was similar to control animals until the day of measurements (see chapter 7).

In vivo hepatic glutamine membrane transport rates and liver glutamine turnover were calculated in a three compartment model (see figure 5, chapter 2). The three compartments involved are the following: 1) vascular afferent pool (hepatic artery and portal vein); 2) intracellular pool; 3) vascular efferent pool (hepatic vein). Disposal and production of glutamine is calculated out of and into the intracellular free amino acid pool. Membrane transport rates are calculated as the amino acid flux from the afferent pool to the intracellular pool ($F_{i,a}$), the amino acid flux from the intracellular pool into the hepatic vein ($F_{i,h}$) and the amino acid flux shunted from afferent vessels into the hepatic vein ($F_{h,a}$).

To compare intracellular glutamine kinetics between the groups all data were normalized per unit of glutamine concentration of the source pool (11, 12). $F_{i,a}$ was divided by the arterial concentration, $F_{v,i}$ and disposal by the intracellular concentration. These parameters describe the volume of arterial plasma or intracellular water cleared of glutamine *via* inward or outward membrane transport per unit of time. Total intracellular appearance (R_a) is defined as the sum of $F_{i,a}$ and intracellular production (P) (11). $F_{i,a}/R_a$ expresses the fractional contribution of membrane transport to intracellular glutamine turnover relative to total glutamine turnover (12). P/R_a expresses the fractional contribution of intracellular glutamine production relative to total glutamine turnover.

Statistical Analysis

Results are presented as mean $\pm$ SEM. The Mann Whitney *U* corrected with the Bonferroni procedure was used to test significance (13). Significance was considered present at $p < 0.05$.

Results

Arterial, portal and intracellular concentrations

Ammonia remained unchanged in arterial plasma of both tumor bearing rats (Table 1). Portal venous ammonia concentration decreased with increasing tumor loads. Hepatic intracellular ammonia was remained unaffected by the presence of the tumors. Glutamate concentrations increased in arterial and portal venous blood of large tumor bearing animals but remained unchanged intracellularly.

Table 1. Plasma and intracellular ammonia and amino acid concentrations in control, small and large tumor bearing rats. Values are mean $\pm$ SEM (μ M). Mann Whitney test *U* vs control group: ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$; vs small tumor bearing group: ^d $p < 0.05$, ^e $p < 0.01$, ^f $p < 0.001$.

	Control	Small tumor	Large tumor
Arterial concentrations			
Ammonia	174 $\pm$ 10	169 $\pm$ 8	161 $\pm$ 9
Glutamate	62 $\pm$ 4	82 $\pm$ 6 ^a	118 $\pm$ 10 ^{c,e}
Glutamine	550 $\pm$ 25	577 $\pm$ 12	539 $\pm$ 28
Portal concentrations			
Ammonia	332 $\pm$ 19	287 $\pm$ 11	277 $\pm$ 12 ^b
Glutamate	86 $\pm$ 5	77 $\pm$ 6	114 $\pm$ 12 ^{a,d}
Glutamine	263 $\pm$ 16	335 $\pm$ 15 ^c	326 $\pm$ 11 ^c
Intracellular concentrations			
Ammonia	1681 $\pm$ 132	1801 $\pm$ 155	1691 $\pm$ 78
Glutamate	5128 $\pm$ 237	5066 $\pm$ 216	4819 $\pm$ 338
Glutamine	5221 $\pm$ 236	3700 $\pm$ 186 ^c	3052 $\pm$ 298 ^{c,e}

Glutamine concentration remained unchanged in the arterial blood and increased in portal venous blood of both tumor bearing rats. Intracellular glutamine concentrations decreased by approximately 29% and 41% in the small and large tumor bearing animals, respectively.

Hepatic plasma flow and net flux

Hepatic plasma flow was similar in control and both tumor bearing animals (Table 2). Ammonia uptake, decreased in both tumor bearing rats. Glutamate flux changed from zero to a net release in both tumor bearing rats. Glutamine flux changed from a net release to a net uptake in small tumor bearing rats. In large tumor bearing rats glutamine flux became not significantly different from zero.

Hepatic glutamine turnover and membrane transport rates

Intracellular glutamine disposal increased threefold in small tumor bearing animals and decreased again in large tumor bearing animals (Table 3). Glutamine production showed a similar pattern as intracellular disposal, although not significantly.

In conjunction with increased glutamine turnover, liver inward membrane transport rates ($F_{i,a}$) increased in the small tumor bearing rats. Outward membrane transport ($F_{v,i}$) remained unchanged in both tumor bearing rats.

Table 2. Hepatic plasma flow and fluxes of ammonia and amino acids in control, small and large tumor bearing rats. Plasma flow in $\text{ml} \times 100 \text{ g bw}^{-1} \times \text{min}^{-1}$, flux in $\text{nmol} \times 100 \text{ g bw}^{-1} \times \text{min}^{-1}$ (mean $\pm$ SEM). Mann Whitney *U* test vs control group: ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$; vs small tumor bearing group: ^d $p < 0.05$, ^e $p < 0.01$, ^f $p < 0.001$.

	Control	Small tumor	Large tumor
Flow	2.5 $\pm$ 0.5	2.6 $\pm$ 0.6	2.7 $\pm$ 0.6
Ammonia	-310 $\pm$ 36	-160 $\pm$ 32	-177 $\pm$ 30
Glutamate	-19 $\pm$ 15	108 $\pm$ 28 ^c	112 $\pm$ 28 ^c
Glutamine	112 $\pm$ 52	-64 $\pm$ 34 ^b	37 $\pm$ 38

Table 3. Liver glutamine kinetics in control, small and large tumor bearing rats. Values are presented in $\text{nmol} \times 100 \text{ g bw}^{-1} \times \text{min}^{-1}$ (mean $\pm$ SEM). Statistical analysis by Mann Whitney *U* test vs control group: ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$; vs small tumor bearing group: ^d $p < 0.05$, ^e $p < 0.01$, ^f $p < 0.001$.

	Control	Small tumor	Large tumor
Intracellular turnover			
Disposal	198 $\pm$ 87	624 $\pm$ 127 ^a	266 $\pm$ 59 ^c
Production	314 $\pm$ 62	561 $\pm$ 130	303 $\pm$ 64
NB	112 $\pm$ 52	-64 $\pm$ 34 ^b	37 $\pm$ 38
Membrane transport rates			
$F_{l,a}$	731 $\pm$ 45	942 $\pm$ 51 ^c	911 $\pm$ 43 ^c
$F_{h,i}$	849 $\pm$ 68	879 $\pm$ 59	948 $\pm$ 49
$F_{h,a}$	184 $\pm$ 22	210 $\pm$ 57	132 $\pm$ 33

Table 4. Glutamine membrane transport system activity and intracellular disposal normalized to the concentration of source pool. Values are presented $\text{ml} \times 100 \text{ g bw}^{-1} \times \text{min}^{-1}$ (mean $\pm$ SEM). Statistical analysis by Mann Whitney *U* test vs control group: ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$; vs small tumor bearing group: ^d $p < 0.05$, ^e $p < 0.01$, ^f $p < 0.001$.

	Control	Small tumor	Large tumor
$F_{l,a}/C_{art}$	1.32 $\pm$ 0.06	1.64 $\pm$ 0.09 ^c	1.70 $\pm$ 0.08 ^c
$F_{h,i}/C_{liver}$	0.16 $\pm$ 0.01	0.24 $\pm$ 0.02 ^c	0.34 $\pm$ 0.03 ^{c,f}
Disposal/ C_{liver}	0.045 $\pm$ 0.021	0.169 $\pm$ 0.032 ^b	0.089 $\pm$ 0.019 ^d

Normalization of membrane transport rates for the concentrations in the pool of origin, gives an estimate of the ability of the transport system to clear vascular or intracellular amino acid pools (transport system activity) (11,12). Normalization of inward membrane transport activity shows that arterial clearance of glutamine by the liver increased approximately 24-28% in both tumor bearing rats (Table 4). Outward membrane transport normalized for the intracellular concentration (intracellular clearance of glutamine) increased in the small and large tumor bearing rats by more than 50% and 100%.

Table 5. Relationship between inward directed membrane transport rates, intracellular glutamine production and total intracellular glutamine appearance rate. R_{ai} is total intracellular glutamine appearance rate ($F_{1,a} + \text{Production}$) and is presented as $\text{ml} \times 100 \text{ g bw}^{-1} \times \text{min}^{-1}$ (mean $\pm$ SEM). $F_{1,a}/R_{ai}$ and $\text{Production}/R_{ai}$ are fractional rates of intracellular glutamine appearance rate. Statistical analysis by Mann Whitney U test vs control group: ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$; vs small tumor bearing group: ^d $p < 0.05$, ^e $p < 0.01$, ^f $p < 0.001$.

	Control	Small tumor	Large tumor
R_{ai}	1046 $\pm$ 94	1503 $\pm$ 175	1214 $\pm$ 95
Periportal			
$F_{1,a}/R_{ai}$	0.72 $\pm$ 0.03	0.72 $\pm$ 0.08	0.78 $\pm$ 0.04
Perivenous			
$\text{Production}/R_{ai}$	0.28 $\pm$ 0.03	0.28 $\pm$ 0.08	0.21 $\pm$ 0.04

In control rats total intracellular glutamine turnover (R_{ai}) was for 72% determined by arterial plasma-liver tissue glutamine membrane transport and for 28% determined by intracellular production of glutamine (Table 5). The latter fraction remained unchanged in small tumor bearing rats but tended to decrease in large tumor bearing rats making glutamine turnover in the liver of advanced tumor bearing rats more dependent on membrane transport rates.

Discussion

Cachexia is a common cause of death in advanced cancer (14). Nutritional interventions have been limited in their success because pathophysiological mechanisms underlying the cachectic syndrome are poorly understood. Glutamine has recently been recognized as a key amino acid in physiological interorgan amino acid exchange (1). In addition, glutamine depletion has also been associated with several catabolic disease states (7). The present study was performed to evaluate changes in hepatic glutamine handling induced by increasing tumor loads.

The tumor in the present study induced only moderate cancer cachexia (see chapter 7). No anorexia was present and carcass weight only reduced by approximately 10% in advanced cancer. Liver mass, however, increased in this rodent cancer model which has previously been described to an acute phase response to the tumor (15,16).

Furthermore, disturbed glutamine homeostasis becomes apparent in these tumor bearing rats as the bodies major glutamine pools become depleted (17). In the current study we also observed sharp decreases in intracellular glutamine concentrations in the liver of tumor bearing rats. These concentrations are highly dependent on membrane transport rates which maintain a more than tenfold plasma-tissue concentration gradient (Table 1). In the liver glutamine is exclusively and actively taken up by Na^+ -dependent System N (18). Intracellular glutamine breakdown, disposal in our model, by phosphate-dependent mitochondrial glutaminase depends on the maintenance of intracellular and intramitochondrial glutamine concentrations by System N (5).

Both disposal and System N inward membrane transport take place in the periportal hepatocytes which account for approximately 92% of all hepatocytes in the liver (5). The K_m and V_{max} of System N are 1.3 mM and $10 \text{ nmol} \times \text{mg}^{-1} \times \text{minute}^{-1}$ respectively (19) and when considering the arterial glutamine concentrations in control rats this would give a theoretical transport rate of $1.2 \text{ nmol} \times \text{mg}^{-1} \times \text{minute}^{-1}$ (approximately $1650 \text{ nmol} \times 100 \text{ g bw}^{-1} \times \text{minute}^{-1}$). Considering portal venous concentrations the theoretical membrane transport rate would be approximately $789 \text{ nmol} \times \text{mg}^{-1} \times \text{minute}^{-1}$. Membrane transport rates calculated in the three compartment model in the current study thus more closely resembles membrane transport rates from portal venous plasma into the hepatocyte.

Membrane transport rates increased in the small tumor bearing rats despite unchanged arterial glutamine concentrations. Therefore, membrane transport capacity had to increase as was shown in the normalized values (Table 4). The normalized values remained elevated in the large tumor bearing animals. Disposal of glutamine increased a threefold in the small tumor bearing rats. This is exceptional because mitochondrial glutaminase has a very high K_m (28 mM) and glutamine breakdown is thus strictly concentration dependent. The elevated glutamine breakdown could only have been possible with elevated cytosolic-mitochondrial membrane transport rates and subsequent concentration gradients between the intracellular cytosolic pool and the mitochondrial pool. In the large tumor bearing rats glutamine disposal decreased again. This may have been due to a failure of cytosolic-mitochondrial glutamine membrane transport rates due to a further decrease in intracellular glutamine concentrations (40%). Although plasma-tissue membrane transport capacity increased in the large tumor bearing rats, the actual transport rate did not increase sufficiently to maintain intracellular glutamine concentrations and subsequent glutamine breakdown.

Glutaminase produces both ammonia and glutamate from glutamine (20). Hepatic ammonia flux and intracellular ammonia concentrations remained unchanged in the

small tumor bearing rats. The ammonia derived from glutamine is preferentially used for carbamoyl phosphate (urea cycle) production in the periportal cells (4). Urea flux across the liver, however, remained unchanged in both tumor bearing rats (chapter 7, table 4). If not used for urea synthesis, ammonia could also have been transported from periportal cells to perivenous cells. Indeed, glutamine production also increased and a large part of the ammonia produced by periportal cells was probably used for perivenous glutamine synthesis. Simultaneous breakdown and synthesis of glutamine by different cell subsets of the liver has previously been called the intercellular glutamine cycle (5, 21).

In vivo intercellular glutamine kinetics and cycling calculated in the three compartment model used in the current study are summarized in figure 1. In all rats the majority of glutamine entered the intracellular pool and only a minority was directly shunted into the hepatic vein. During the increased glutamine turnover in small tumor bearing rats, glutamine membrane transport rates became of approximately the same magnitude as intracellular glutamine disposal and breakdown. However, more importantly, the model shows that a great part of glutamine entering the periportal cells (*via* System N), was also released by these cells. The latter occurs Na^+ -independent *via* System "n" which is a facilitated diffusion (22). In control rats 731 nmol entered the periportal cells whereas only 198 nmol were broken down (figure 1). Therefore, 533 nmol, which is approximately 73% of the glutamine actively taken up, left periportal cells *via* System "n".

Looking more closely in the small tumor bearing rats, this percentage decreased to 33%. This means that the increase in glutamine disposal was not as much made possible by increasing System N membrane transport rates but more by decreasing the facilitated diffusion of glutamine by System "n". In the large tumor bearing rats, approximately 71% of glutamine which entered periportal cells was also released by these cells as was seen in control rats.

In the large tumor bearing animals the decreased glutamine disposal rates also made the net glutamine flux of the liver approximately zero. In these animals glutamate release, one of the end products of glutamine breakdown, was still increased. If not originating from glutamine, glutamate must have been produced from aspartate or TCA cycle intermediates as has been shown *in vitro* (23). This would be in agreement with a recent study in which it was shown that the liver of tumor bearing rats demonstrates a progressive loss of energy generating capacity (24).

Figure 1. Glutamine kinetics in control and tumor bearing rats. Glutamine uptake of hepatocytes involves largely Na^+ -dependent System N which is mostly active in periportal hepatocytes (32). Glutamine release by hepatocytes occurs via facilitated diffusion of System "n". Glutamine disposal or breakdown is restricted to periportal cells which contain intramitochondrial phosphate dependent glutaminase, whereas glutamine synthesis is restricted to perivenous cells containing glutamine synthetase. A control rats, B small tumor bearing rats, C large tumor bearing rats.

Glutamate is the actual energy substrate of tumor cells when they consume glutamine (25). Previous studies suggested that the high uptake of glutamine by tumor cells may not be essential to cell proliferation if sufficient other energy and anabolic precursors were supplied (26). Although *in vivo* uptake of glutamate by tumor cells has been observed (27), most reports show that glutamate is taken up and oxidized much

less effectively than glutamine due to low membrane transport rates (25,28). Therefore, the reason for increased glutamate release by the liver probably lies within the liver of the tumor bearing rat. It may be related to the relatively decreased perivenous glutamine production in the large tumor bearing rats or insufficient substrate oxidation as suggested previously in hepatocytes of tumor bearing rats (24).

In conclusion, our study shows that the liver changes from an organ of net release to an organ of net glutamine uptake in the presence of a small tumor. In advanced cancer glutamine consumption and production are in balance. The major changes in liver glutamine metabolism were found in glutamine disposal which was regulated by changes periportal facilitated diffusion of glutamine via System "n" membrane transport rates.

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CANCER REDUCES THE METABOLIC RESPONSE OF MUSCLE TO SURGICAL STRESS IN THE RAT

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Submitted

Abstract

The metabolic response to surgical stress is characterized by muscle protein breakdown and mobilization of amino acids, e.g. glutamine, from peripheral tissue to visceral organs. Cancer is related to increased whole body protein breakdown which may influence the normal metabolic response after surgery. Aim was to study the effects of cancer on postoperative peripheral muscle protein and glutamine turnover. MCA-induced sarcomas were implanted subcutaneously in female Lewis rats (± 200 g). Tumor bearing rats were studied when the tumor was 5-15% of body weight. Control rats were sham implanted. Hysterectomy was performed in control and tumor bearing rats as a standardized operative procedure. At the second postoperative day a primed constant infusion of *para*-aminohippuric acid, L-[2,6- 3 H]phenylalanine and L-[3,4- 3 H]glutamine was given to study hindquarter muscle protein turnover and glutamine kinetics.

In control rats muscle protein synthesis almost doubled and protein breakdown increased a threefold after hysterectomy with concomitant increased outward amino acid membrane transport rates. Hysterectomy did not change protein synthesis or breakdown rates in tumor bearing rats. Muscle glutamine production, membrane transport and release increased after hysterectomy in control rats. Tumor bearing rats had depressed membrane transport rates and showed no surgical stress response related to muscle glutamine metabolism.

The present study shows that surgical stress induces an increased mobilization of amino acids, e.g. glutamine, from muscle which does not occur in the cancer bearing state. The reduced metabolic response to surgery in the cancer bearing host may be of particular importance for the functioning of visceral organs which utilize amino acids like glutamine at a high rate after trauma.

The metabolic response to surgical trauma is characterized by increased peripheral protein breakdown and mobilization of amino acids (1,2). Amino acids are transported to visceral organs for gluconeogenesis and the acute phase response (1,2). In this respect, glutamine appears to have a central role. It has long been recognized as a non-toxic ammonia or nitrogen carrier from muscle to visceral organs (3,4). Furthermore, glutamine is a major gluconeogenic amino acid by which new carbon skeletons derived from proteins and amino acids are transferred to the glucose pool (5). Moreover, glutamine is essential for rapidly dividing cells, e.g. immune cells and enterocytes, as energy source and as precursor for nucleotides. It was therefore hypothesized that peripheral production of glutamine may be rate limiting and thus make glutamine conditionally essential for the functioning of the immune system, gut and liver (6-9). This has been confirmed in several clinical and animal studies in which supplementation of glutamine resulted in improvement of immune, gut and liver functioning (10-14).

Cachectic cancer patients are characterized by excessive weight loss (15). Poor nutritional status has been associated with a poor outcome of surgical treatment (16,17). In animal studies, advanced cancer has been associated with decreased mobilization of glutamine suggesting that muscle tissue was exhausted in these animals (18,19). An insufficient capacity of muscle to produce glutamine may have clinical impact on functioning of glutamine consuming organs like the gut, liver and the immune system of the cancer bearing host. Decreased capacity to mobilize amino acids, in particular glutamine, can impair postoperative immune function or induce loss of gut barrier function, and make cancer patients more prone to postoperative morbidity. We wanted to test this hypothesis in the cancer bearing host undergoing mild surgery.

Aim of the present study was to measure muscle amino acid efflux, in particular that of glutamine, in tumor bearing rats after a mild surgical trauma. As muscle glutamine release is related to several intracellular metabolic processes in muscle, e.g. *de novo* production from glutamate, membrane transport rates or intracellular protein turnover, isotope labeled amino acids were used to measure intracellular glutamine and protein kinetics in hindquarter muscle.

Animals and study groups

Female Lewis rats (190-210 gr., Centralized Animal Facilities, University of Limburg, Maastricht, NL) were individually housed in metabolic cages during the experiment after an adaptation period of 3 days. Rats were given standard laboratory rat chow (SRMA 1210, Hopefarms, Woerden, NL) and subjected to standard 12 hour light-dark cycle periods (7:30 A.M. to 7:30 P.M.) with room temperature maintained at 25°C. The experiments were performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals (20) and approved by the Ethical Committee of Animal Research of the University of Limburg.

Rats (n=40) were randomly assigned to one of the following four groups:

- 1) control, sham anesthesia,
- 2) control, hysterectomized,
- 3) tumor bearing, sham anesthesia,
- 4) tumor bearing, hysterectomized.

In the tumor bearing group a methylcholantrene (MCA) sarcoma was implanted subcutaneously. The tumor is maintained in our laboratory *in vivo* by serially transplanting tissue suspensions through an 18G needle. A MCA tumor is often used in metabolic studies and is known for its rapid growth, invasion of local tissue and absence of metastatic potential (18,21,22). The tumor was allowed to grow until it reached 5-15% of the host body weight. Non-tumor bearing rats were sham implanted with saline. Tumor growth was monitored by measuring two orthogonal dimensions and by calculating the volume assuming the tumor to be a prolate spheroid (23). Tumor size, body weight and food intake were monitored daily. Carcass weight was determined by subtracting estimated tumor weight from total body weight.

Surgical procedure

All rats were anaesthetized with diethyl ether (Merck 923, Darmstadt, Germany). The surgical groups were subjected to a laparotomy. The bicornuate uterus was dissected free and ligated on both sides from the ovaries with vicryl 3-0 (Ethicon, Norderstedt, Germany). Next, the uterus was ligated at the basis with vicryl 3-0 and removed from the abdomen. The abdominal wound was closed with vicryl 3-0 in two layers. The whole procedure took approximately 10-15 minutes. This standard procedure has been described previously (24-26). It involves the removal of a non-essential organ and in patients the metabolic responses to this type of surgical trauma were found to correspond with findings after cholecystectomy (26). Non-operated rats underwent sham anesthesia with diethyl ether for 15 minutes.

Study procedures

Metabolic studies were performed postabsorptive two days after the operation or sham anesthesia. Hindquarter fluxes, protein and glutamine turnover were examined as described previously (27,28) and in chapter 2, 3 and 4. In brief, under ether anesthesia and at constant, pre-anesthesia body temperature, a laparotomy was performed. The right renal vein, the aorta and caval vein (just above the bifurcation) were cannulated using a 25 gauge needle fixed in a Silastic tube (Silastic Medical Grade tubing 0.051 cm ID, 0.094 OD, Dow Corning Corporation, Midland, MO, USA) and fixed with cyano-acrylate. For flow measurements, the indicator dilution method with *para*-aminohippuric acid (PAH) was used (pH 7.4, iso-osmolaric). A primed ($0.15 \text{ ml} \times 100 \text{ gr. body weight}^{-1}$, 50 mmol/l) constant infusion ($0.75 \text{ ml} \times 100 \text{ gr. body weight}^{-1} \times \text{h}^{-1}$, 5 mmol/l) of PAH was infused in the aorta, using a Minipuls 3 Peristaltic Pump. For turnover studies, a primed ($1 \mu\text{Ci} \times 100 \text{ gr. body weight}^{-1}$) constant infusion ($1 \mu\text{Ci} \times 100 \text{ gr. body weight}^{-1} \times \text{h}^{-1}$) of L-[3,4- ^3H]phenylalanine and L-[3,4- ^3H]glutamine was given in the right renal vein. A minimum infusion time of 30 minutes was needed to reach steady state concentrations for PAH, ^3H -phenylalanine and ^3H -glutamine as described previously (28,29). The right carotid artery was cannulated during the equilibrium period with PE 50 catheter (Intramedic, 0.051 cm ID, 0.094 cm OD, Clay Adams, Parsippany, NY, USA). The complete procedure took approximately 40 minutes. Hindquarter measurements were simultaneously done with measurements across portal drained viscera described in chapter 10.

Blood (1.0 ml per catheter) was simultaneously sampled from the caval vein and carotid artery. All blood was collected in heparinized cups on ice. Hereafter, the right gastrocnemius muscle was dissected free, weighted, directly freeze-clamped, put in liquid nitrogen and stored at -80°C until further analysis.

For PAH determinations, $50 \mu\text{l}$ heparinized blood was added to $500 \mu\text{l}$ TCA (10% w/v), vortexed and centrifuged at $8,900 \text{ g}$ at 4°C . The supernatant was frozen in liquid nitrogen and stored at -80°C until further analysis. For hematocrit determinations a micro-hematocrit tube was filled with heparinized blood and centrifuged at $10,000 \text{ g}$ at room temperature. Hematocrit was read with a Micro Hematocrit Reader. Plasma was obtained by whole blood centrifugation at $8,900 \text{ g}$ at 4°C for 5 minutes. For ammonia, urea, glucose and lactate determinations, $200 \mu\text{l}$ plasma was vortexed with $20 \mu\text{l}$ TCA 50%, put into liquid nitrogen and stored at -80°C . To determine plasma amino acid specific activities and concentrations, $300 \mu\text{l}$ plasma was added to 12 mg 5-sulfosalicylic acid (SSA, Brunschwig, 10346, Amsterdam, NL), vortexed, frozen in liquid nitrogen and stored at -80°C .

Plasma ammonia, urea, glucose, lactate and PAH were determined spectrophotometrically on a Cobas Mira S (Roche Diagnostica, Hoffman-La Roche, Basel, Switzerland) by standard enzymatic methods, using commercially available

kits (27,28). Plasma amino acid concentrations and specific activity were determined by fully automated HPLC as described previously (30). For the determination of tissue ammonia, amino acid concentrations and specific activities, muscle tissue was pulverized using a mortar and pestle precooled in liquid nitrogen. The tissue was further homogenized and deproteinized in a Mini-Beadbeater (Biospec products, Bartlesville, USA). Approximately 100 mg tissue was added to 400 μ l SSA 5%, with 300 mg glass beads (diameter 1 mm, Biospeck Products) and beaten for 30 seconds. The homogenate was centrifuged at 4°C at 11,000 g and the supernatant frozen in liquid nitrogen and stored at -80°C until further determinations.

To determine tissue dry weight and water content approximately 200 mg pulverized tissue was freeze dried for 24 hours in a Speedvac (SC200, Savant Instruments Inc., Farmingdale NY, USA) connected with a refrigerated condensation trap.

Calculations

Hindquarter plasma flow and substrate fluxes

Plasma flow across the hindquarter was calculated using PAH in the indicator dilution method (27,28). Substrate fluxes are calculated by multiplying the venous-arterial concentration differences with the mean hindquarter plasma flow of the group and are expressed in $\text{nmol} \times 100 \text{ gr. body weight}^{-1} \times \text{min}^{-1}$. A positive flux indicates net release, a negative flux reflects a net uptake. α -Amino-nitrogen was calculated as the sum of the individual amino acids measured except taurine (30). The sum of essential amino acids was calculated as described previously (31). Tissue concentrations of amino acids are expressed in $\mu\text{mol/l}$ and derived by dividing the tissue homogenate amino acid concentration ($\mu\text{mol/kg}$ wet weight) by the tissue water content (l/kg wet weight).

Protein and glutamine kinetics

Hindquarter protein and glutamine turnover were calculated by the A-V dilution of radioactively labeled phenylalanine and glutamine across the hindquarter calculated in a three compartment model (28,32,33). Intracellular disposal and production of phenylalanine and glutamine can thus be derived and reflect *in vivo* intracellular turnover of these amino acids. Production of phenylalanine reflects protein breakdown and disposal reflects protein synthesis because metabolism of phenylalanine in muscle is restricted to synthesis into and breakdown from protein (34). Furthermore, glutamine and phenylalanine membrane transport rates can simultaneously be measured: $F_{m,a}$ are plasma-tissue inward directed transport rates,

$F_{m,v}$ are plasma tissue outward directed membrane transport rates and $F_{v,a}$ reflect metabolic shunting of amino acids (32).

Glutamine, being a non-essential amino acid, can be produced by protein breakdown and, non-protein derived, by the transamination of glutamate. Knowing the relative amino acid content of phenylalanine and glutamine in protein (35) enables us to calculate glutamine production derived from protein breakdown and glutamine production which is non-protein derived (de novo synthesis).

Statistical Analysis

Results are presented as mean $\pm$ SEM. Data were analyzed using the statistical program SPSS (36). Two way analysis of variance (ANOVA) was used to detect significant effects of tumor load, surgical trauma and the interaction between tumor load and trauma. Significant interactions suggest that the effects of surgery were not the same for control (non-tumor bearing) and tumor bearing rats. Significance from zero was tested by the Wilcoxon signed-ranks test. Significance was considered present at $p < 0.05$.

Results

A hysterectomy had no influence on tumor weight (Table 1). Initial carcass weights were similar in all groups and no significant changes were observed in the preoperative period. On the second postoperative day hysterectomized control and tumor bearing rats lost approximately twice as much of their carcass weight than the sham anesthetized rats.

Food intake of control and tumor bearing rats remained unchanged in the preoperative period. Postoperative food intake decreased to approximately 20-25% in operated rats compared to sham anesthetized rats.

Arterial concentrations

Tumor bearing rats had decreased arterial glucose concentrations (Table 2). Hysterectomy had no influence on arterial glucose concentrations. The tumor bearing state and hysterectomy both increased arterial lactate concentrations while a relatively diminished increase in arterial lactate after surgery in tumor bearing rats was observed.

Surgery decreased arterial ammonia in both control rats and tumor bearing rats. Urea concentrations remained unchanged in the presence of a tumor or after hysterectomy.

	Control		Tumor bearing		p value		
	Sham	Hysterectomy	Sham	Hysterectomy	T	S	I
Tumor weight	-	-	38 ± 2	32 ± 3	<0.001	ns	ns
Carcass weight							
Initial	195 ± 3	200 ± 3	200 ± 3	204 ± 3	ns	ns	ns
Preoperative	200 ± 3	205 ± 4	198 ± 4	201 ± 4	ns	ns	ns
Postoperative (day 2)	190 ± 2	184 ± 4	191 ± 7	180 ± 4	ns	0.02	ns
Food intake							
Initial	18 ± 1	17 ± 1	17 ± 1	15 ± 2	ns	ns	ns
Preoperative	17 ± 1	15 ± 1	17 ± 1	16 ± 2	ns	ns	ns
Postoperative	16 ± 1	4 ± 2	15 ± 3	3 ± 1	ns	<0.001	ns

Table 1. Carcass weight, tumor weight and food intake during the experiment. Values are mean ± SEM. Carcass weight is in grams, food intake in gram/day except postoperative which is in total intake after hysterectomy until the metabolic study. Statistical analysis by Two-Way ANOVA: T= effects of tumor load, S = effects of surgery, I = interaction of tumor load and surgery, ns = not significant.

	Control		Tumor bearing		p value		
	Sham	Hysterectomy	Sham	Hysterectomy	T	S	I
Glucose	10.2 ± 0.3	10.7 ± 0.2	9.3 ± 0.4	9.1 ± 0.4	<0.001	ns	ns
Lactate	5.2 ± 0.4	7.7 ± 0.3	7.3 ± 0.5	8.1 ± 0.4	0.001	<0.001	0.03
Ammonia	173 ± 9	129 ± 9	147 ± 14	115 ± 5	ns	0.001	ns
Urea	3.4 ± 0.1	3.5 ± 0.2	3.6 ± 0.3	3.2 ± 0.2	ns	ns	ns
α-Amino-nitrogen	2685 ± 63	2980 ± 257	3648 ± 232	3318 ± 118	0.001	ns	ns
Essential AA	1019 ± 35	1156 ± 92	1568 ± 115	1472 ± 51	<0.001	ns	0.03
Non-essential AA	1665 ± 48	1823 ± 176	2382 ± 182	2040 ± 90	0.03	ns	ns
Glutamate	40 ± 3	77 ± 20	67 ± 5	66 ± 5	ns	ns	ns
Glutamine	628 ± 14	535 ± 23	528 ± 20	476 ± 21	<0.001	0.004	ns
Alanine	271 ± 24	480 ± 98	542 ± 76	564 ± 71	0.03	ns	ns
Branched Chain AA	227 ± 17	257 ± 23	302 ± 26	284 ± 10	0.008	ns	ns

Table 2. Arterial ammonia and amino acid concentrations in control and tumor bearing rats after hysterectomy, sham operation or pair fed to operated rats. Glucose, lactate and urea in mmol/l, ammonia and amino acids in $\mu\text{mol/l}$ (mean $\pm$ SEM). Statistical analysis by Two Way ANOVA: T = effects of tumor load, S = effects of surgery, I = interaction between tumor load and surgery, AA = amino acids.

The presence of a tumor increased arterial α -amino-nitrogen concentrations (both essential and non-essential) whereas hysterectomy had no effect. Glutamine and alanine are non-essential amino acids which represent approximately 30% of total arterial α -amino-nitrogen (37). They are the most important interorgan nitrogen carriers in the body. Arterial glutamine concentrations were decreased in tumor bearing rats and decreased after hysterectomy in both tumor bearing and control rats. In contrast, arterial alanine increased in tumor bearing rats. Hysterectomy had no effect on arterial alanine. Arterial branched chain amino acid concentrations also increased in tumor bearing rats whereas hysterectomy had no effect on these concentrations.

Hindquarter plasma flow and fluxes

Hindquarter plasma flow remained unchanged between the groups (Table 3). No significant changes in glucose uptake by the hindquarter was observed. It was, however, only significantly different from zero in the tumor bearing group. Lactate release increased after hysterectomy more than a twofold in control rats while tumor bearing rats had no increase in lactate release after hysterectomy.

Ammonia release by the hindquarter, reflecting breakdown of amino acids and nucleotides (38), was not significantly different between different groups although the release became non-significant from zero after hysterectomy in tumor bearing rats. Hindquarter α -amino-nitrogen release was increased after hysterectomy. The six-fold increase in amino acid release after surgery in control rats was attenuated by the presence of a tumor. The increased release of total amino acids after surgery was mostly due to an increased release of non-essential amino acids, again attenuated by the presence of a tumor.

Glutamine release by the hindquarter increased after hysterectomy in control rats. In tumor bearing rats glutamine release remained unchanged after surgery. Glutamate, the intracellular precursor of glutamine, was taken up in sham control rats, hysterectomized control rats and sham tumor bearing rats while only a significant release was seen in hysterectomized tumor bearing rats. Alanine release by the hindquarter increased after surgery in both control and tumor bearing rats.

Hindquarter protein and glutamine kinetics

Intracellular disposal of phenylalanine in muscle (protein synthesis) was decreased in the presence of a tumor and did not increase in response to surgery in tumor bearing rats as in control rats (Fig. 1). Protein breakdown, measured by the intracellular production of phenylalanine, increased after hysterectomy. However, in tumor bearing rats intracellular production of phenylalanine did not increase after surgery. Phenylalanine flux, the difference between protein synthesis and protein

	Control		Tumor bearing		p value		
	Sham	Hysterectomy	Sham	Hysterectomy	T	S	I
Plasma flow	2.1 ± 0.4	4.2 ± 0.9	3.2 ± 1.0	2.6 ± 1.0	ns	ns	ns
Glucose	-505 ± 508 *	-996 ± 1059 *	-1989 ± 538	-361 ± 612 *	ns	ns	ns
Lactate	2573 ± 182	6277 ± 724	4420 ± 622	3871 ± 428	ns	0.009	<0.001
Ammonia	165 ± 18	188 ± 53	189 ± 97	52 ± 27 *	ns	ns	ns
α-Amino-nitrogen	297 ± 147	1739 ± 364	897 ± 324	1310 ± 241	ns	0.007	0.05
Essential AA	38 ± 50 *	473 ± 136	181 ± 152 *	298 ± 88	ns	0.05	ns
Non Essential AA	260 ± 105	1267 ± 235	716 ± 185	1012 ± 165	ns	0.002	0.03
Glutamine	199 ± 41	595 ± 73	346 ± 39	389 ± 37	ns	0.001	<0.001
Glutamate	-13 ± 3	-43 ± 12	-42 ± 6	21 ± 5	ns	ns	0.002
Alanine	-21 ± 10 *	203 ± 70	191 ± 100 *	307 ± 110	ns	0.05	ns
BCAA	-65 ± 14	-8 ± 29 *	-31 ± 38 *	42 ± 27 *	ns	0.02	ns

Table 3. Hindquarter plasma flow, ammonia and amino acid fluxes. Values are presented in nmol x 100 gr. body weight⁻¹ x min⁻¹ except plasma flow in ml x 100 gr. body weight⁻¹ x min⁻¹ (mean ± SEM). Statistical analysis by Two-Way ANOVA: T = effects of tumor load, S = effects of surgery, I = interaction between tumor load and surgery. Wilcoxon's test: * *not* significant from zero (p<0.05). (-) reflects net uptake, (+) reflects net release of a substrate.

breakdown rates, increased after hysterectomy in control rats. This response was also absent in tumor bearing rats.

Phenylalanine plasma-tissue membrane transport rates were in agreement with the pattern of intracellular turnover (Fig. 2). Both surgery and the presence of a tumor did not significantly influence inward directed membrane transport rates ($F_{m,a}$). There was, however, an opposite change after surgery in control and tumor bearing rats: control rats increased their inward membrane transport rates while tumor bearing rats decreased their transport rates in response to hysterectomy. Outward membrane transport rates ($F_{v,m}$) were increased after surgery. However, no change was observed after surgery in tumor bearing rats. Metabolic shunting of phenylalanine in muscle tissue ($F_{v,a}$) was increased in tumor bearing rats and no effect of surgery was observed. There was, however, a relative increased shunting in control rats whereas it relatively decreased after surgery in tumor bearing rats.

Figure 1. Hindquarter muscle protein turnover (mean $\pm$ SEM). Statistical analysis by Two-Way ANOVA for effects of tumor load, hysterectomy and interaction of the two. $\square$ Sham anesthetized rats, $\blacksquare$ Hysterectomized rats. A: Intracellular disposal or protein synthesis. Effect tumor: $p=0.02$; effect hysterectomy: ns; interaction: $p=0.05$. B: Intracellular production or protein breakdown. Effect tumor: ns; effect hysterectomy: $p=0.03$; interaction $p=0.007$. C: Net phenylalanine flux. Effect tumor: ns; effect hysterectomy: $p=0.03$; interaction: $p=0.03$.

Figure 2. Phenylalanine membrane transport in muscle tissue (nmol phenylalanine $\times$ 100 gr. body weight⁻¹ $\times$ min⁻¹). □ Sham anesthetized rats, ■ Hysterectomized rats.

A: $F_{m,a}$, inward membrane transport. Effect tumor: ns; effect hysterectomy: ns; interaction: $p=0.002$.

B: $F_{v,m}$, outward membrane transport. Effect tumor: ns; effect hysterectomy: $p=0.04$; interaction: $p=0.001$.

C: $F_{v,a}$, metabolic shunting of phenylalanine. Effect tumor: $p<0.001$; effect hysterectomy: ns; interaction: $p<0.001$

Intracellular glutamine production was increased after hysterectomy in control rats (Fig. 3). Tumor bearing rats had unchanged glutamine production rates in response to surgery. No significant changes in intracellular glutamine disposal was observed after hysterectomy or due to the presence of a tumor. Glutamine release (difference between production and disposal) also increased after surgery and this response was absent in the tumor bearing state.

Glutamine inward directed membrane transport rates ($F_{m,a}$) increased after surgery (Fig. 4). Tumor bearing rats had decreased inward directed membrane transport rates. Outward membrane transport rates ($F_{v,m}$) increased after surgery and this response was completely attenuated in the tumor bearing rats.

Figure 3. Hindquarter glutamine turnover. (mean $\pm$ SEM). $\square$ Sham anesthetized rats, $\blacksquare$ Hysterectomized rats.

A: Intracellular disposal or consumption. Effect tumor: ns; effect hysterectomy: ns; interaction: ns.

B: Intracellular production. Effect tumor: ns; effect hysterectomy: $p=0.05$; interaction: $p=0.01$.

C: Net glutamine flux. Effect tumor: ns; effect hysterectomy: $p=0.001$; interaction: $p<0.001$.

Hysterectomy or the cancer bearing state did not influence metabolic shunting of glutamine separately but there was a significant interaction between the two. In control rats shunting increased whereas a decrease was observed after surgery in tumor bearing rats.

Muscle weight and intracellular concentrations

Gastrocnemius wet weight and dry weight decreased in tumor bearing rats (Table 4). Hysterectomy had no significant effect on muscle weight. Intracellular hydration of muscle was not different between the groups.

Intracellular ammonia remained unchanged. Total intracellular α -amino acid concentrations were increased in tumor bearing rats. Surgery decreased intracellular

Figure 4. Glutamine membrane transport rates in muscle tissue (mean $\pm$ SEM). $\square$ Sham anesthetized rats, $\blacksquare$ Hysterectomized rats. A: $F_{m,a}$ inward membrane transport. Effect tumor: $p < 0.001$; effect hysterectomy: $p = 0.04$; interaction: ns. B: $F_{v,m}$ outward membrane transport. Effect tumor: $p < 0.001$; effect hysterectomy: $p = 0.001$; interaction: $p = 0.002$. C: $F_{v,a}$ metabolic shunting of glutamine. Effect tumor: ns; effect hysterectomy: ns; interaction: $p < 0.001$.

α -amino acid concentrations to control values. The increased α -amino acid concentration in tumor bearing rats appears to be due to increased essential and non-essential amino acid concentrations although this was not significant when measured separately.

Intracellular glutamine concentrations decreased as an effect of surgery and as an effect of cancer. Intracellular glutamate, alanine, phenylalanine and branched amino acids increased in tumor bearing rats. Intracellular phenylalanine concentrations remained unchanged after hysterectomy in tumor bearing rats while hysterectomy increased intracellular phenylalanine in control rats.

	Control		Tumor bearing		p value		
	Sham	Hysterectomy	Sham	Hysterectomy	T	S	I
Wet weight	1.23 ± 0.02	1.15 ± 0.02	1.04 ± 0.05	1.04 ± 0.05	<0.001	ns	ns
Dry weight	0.295 ± .006	0.275 ± .006	0.252 ± .013	0.245 ± .011	<0.001	ns	ns
H ₂ O %	76.0 ± 0.3	75.9 ± 0.2	75.6 ± 0.3	76.2 ± 0.2	ns	ns	ns
Ammonia	2515 ± 266	2235 ± 333	2235 ± 308	2319 ± 239	ns	ns	ns
α-Amino-nitrogen	13421 ± 762	14526 ± 359	16345 ± 664	14646 ± 584	0.02	ns	0.01
Essential	2008 ± 321	2596 ± 188	3236 ± 211	2533 ± 343	ns	ns	ns
Non-essential	11161 ± 613	11930 ± 249	13109 ± 534	11831 ± 423	ns	ns	ns
Glutamine	4179 ± 263	3910 ± 155	3718 ± 211	3324 ± 265	0.01	0.05	ns
Glutamate	899 ± 72	949 ± 61	1322 ± 120	1226 ± 115	0.02	ns	ns
Alanine	1428 ± 229	2199 ± 297	3052 ± 493	2641 ± 207	0.02	ns	ns
Phenylalanine	105 ± 10	134 ± 7	165 ± 11	157 ± 9	0.001	ns	0.05
BCAA	333 ± 50	401 ± 38	520 ± 55	484 ± 37	0.008	ns	ns

Table 4. Gastrocnemius wet weight, dry weight, intracellular hydration, ammonia and amino acid concentrations. Weights in grams, concentrations in $\mu\text{mol/l}$ (mean $\pm$ SEM). Statistical analysis by Two-Way ANOVA: T = effects of tumor load, S = effects of surgery, I = interaction between tumor load and surgery.

Figure 5. Whole body protein turnover in nmol phenylalanine x 100 gr. body weight⁻¹ x min⁻¹. □ Sham anesthetized rats, ■ Hysterectomized rats.

A: Whole body Ra. Effect tumor: $p < 0.001$; effect hysterectomy: ns; interaction: ns.

B: Hydroxylation rate. Effect tumor: $p = 0.001$; effect hysterectomy: ns; interaction: ns.

C: Whole body Rd into protein. Effect tumor: $p = 0.001$; effect hysterectomy: ns; interaction: ns.

Whole body protein and glutamine turnover

Surgery had no significant effect on whole body phenylalanine Ra (Fig. 5). Tumor bearing rats had increased whole body protein turnover rates. Phenylalanine hydroxylation, an indication of amino acid oxidation, was increased in tumor bearing rats but remained unaffected by hysterectomy. Whole body Rd was also only increased in tumor bearing rats.

Whole body glutamine Ra decreased in control rats in response to surgery while it increased after surgery in the presence of a tumor (Fig. 6). Glutamine Ra from protein breakdown was relatively low compared to de novo synthesis. Glutamine Ra from protein breakdown increased in tumor bearing rats. Whole body de novo glutamine synthesis was not affected by hysterectomy or the presence of a tumor but there was again an opposite response to surgery in control and tumor bearing rats.

Figure 6. Whole body glutamine turnover in $\text{nmol} \times 100 \text{ gr. body weight}^{-1} \times \text{min}^{-1}$.

Sham anesthetized rats, ■ Hysterectomized rats.

A: Total whole body Ra. Effect tumor: ns; effect hysterectomy: ns; interaction: $p=0.02$.

B: Ra from protein breakdown. Effect tumor: $p<0.001$; effect hysterectomy: ns; interaction: ns.

C: Ra, de novo. Effect tumor: ns; effect hysterectomy: ns; interaction: $p=0.05$.

Discussion

The current study shows that after a surgical trauma muscle proteins are acutely broken down with subsequent amino acid release by the hindquarter. It also shows that the presence of cancer prohibits the normal postoperative response in muscle related to specific changes in muscle metabolism.

The amino acids glutamine and alanine, although biochemically non-essential amino acids, play dominant roles in interorgan amino acid exchange. Glutamine is also essential as major energy source and nucleotide precursor for rapidly dividing cells, e.g. immune cells and enterocytes and has been postulated to be conditionally essential in after severe trauma and metabolic stress (6,8,9). Hysterectomy increased glutamine release by the hindquarter in control rats by increasing muscle

glutamine production and membrane transport rates. This specific response to surgery was absent in tumor bearing rats. The K_m for glutamine membrane transport in muscle ($K_m < 0.3$ mM) normally lies far below intracellular concentrations and thus transport approaches saturation (7). Therefore, muscle glutamine membrane transport normally remains independent of changes in intracellular glutamine concentrations (7). In the current study, glutamine membrane transport increased after surgery in control rats and this could only be accomplished by increasing the amount of transport carriers. The unchanged membrane transport rates after surgery in tumor bearing rats thus indicates an inability to increase membrane transport carriers. The inability to increase membrane transport carriers is again related to the inhibited postsurgical increase in protein synthesis rates in muscle of tumor bearing rats. Intracellular muscle glutamine production also failed to increase after surgical stress in tumor bearing rats. The decreased intracellular glutamine concentrations in these rats further suggest that glutamine synthetase capacity, which normally increases after injury (39,40), had decreased. This failure by muscle to produce glutamine was also confirmed by the release of glutamate, the major precursor of glutamine in muscle, after surgery in tumor bearing rats while all other groups had an uptake of glutamate.

Glutamine release by muscle has previously been postulated to have a key role during an immune challenge as the flux generating step (7,39). An impairment of glutamine release after surgery in tumor bearing rats decreases the possibility for visceral organs to increase their glutamine uptake under surgical stress which normally occurs. For instance, visceral organs (liver, spleen, gut) and immune cells increase their uptake of glutamine for gluconeogenesis and for the production of defense proteins and peptides such as glutathione (41,42). Furthermore, lymphocytes, macrophages and natural killer cells all consume glutamine at a high rate as energy source and for nucleotide production. *In vitro* and *in vivo* activation of these cells increases the consumption of glutamine (39,43,44). Interestingly, an impaired acute phase response to surgical stress is observed in cancer patients (45). Therefore, a decreased nutrient supply by peripheral tissue, as measured in the current study, may have an etiologic role in this.

Although muscle glutamine production remained unchanged after surgery in the tumor bearing host, alanine release by the hindquarter increased. Alanine production in muscle is derived from pyruvate whereas the carbon skeletons of glutamine are mostly derived from protein breakdown. Pyruvate production increases with increased glycolysis. This should, however, also be accompanied by increased lactate production. The latter was increased after surgery in control rats but remained unchanged after surgery in the tumor bearing rats. Because lactate is released in much larger amounts than alanine, glycolysis in peripheral muscle must have

remained unchanged after surgery in tumor bearing rats but only a larger fraction of pyruvate is being channeled into alanine instead of lactate. A valid reason for this phenomenon remains to be established.

The normal catabolic response to surgical stress is mediated by a range of neuro-endocrine and immune responses (46). This response includes the sympathetic nervous system response, the release of counter regulatory hormones (glucagon, cortisol and adrenaline), insulin and several inflammatory mediators such as $\text{TNF}\alpha$ and IL-6. A more chronic release of these mediators has also been observed in tumor bearing rats (47). The lack of metabolic response after surgery in tumor bearing rats suggests that either the normal neuro-endocrine response is not present or that resistance to this response has been provoked by the chronic presence of the tumor. Resistance or tolerance to these mediators has been described in several human and animal studies (24,48-50). The regulatory role of these mediators in the attenuated response of tumor bearing rats, however, needs further research.

Previous studies concerning postoperative protein metabolism have been contradicting. Increased (51,52), unchanged (53,54) and decreased (55-57) whole body protein synthesis and breakdown rates have been observed. These differences are partly caused by differences in methods used to measure protein turnover and by the differences in populations that were studied. Moreover, whole body protein turnover appears to be a compilation of different protein synthesis and breakdown rates of several organs (58). Although muscle protein pool is by far the largest protein pool and whole body protein turnover is often seen as a reflection of muscle protein turnover, changes of organs with a rapid protein turnover rate, e.g. splanchnic organs, can have a larger impact on whole body turnover measurements than changes in muscle protein turnover (59). The discrepancy between whole body protein turnover and regional muscle protein turnover has previously been observed in several other studies (60-62). In the current study, the postoperatively increased protein breakdown rates of peripheral muscle in control rats did not affect whole body protein turnover. Other organs thus must have decreased their protein turnover rates. We therefore feel that protein turnover of the largest protein pool in the body, muscle tissue, should be measured at regional level and not derived from whole body data under these circumstances.

In conclusion, the presence of cancer induces moderate muscle protein breakdown and decreased mobilization of amino acids, in particular of glutamine, after standard surgery. This reduced metabolic response to surgery may affect functioning of other organs supposedly to take up amino acids, e.g. glutamine, for the acute phase response.

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INTESTINAL RESPONSES TO SURGERY IN TUMOR BEARING RATS

*I. de Blaauw, N.E.P. Deutz, M.F. von Meyenfeldt**Submitted***Abstract**

The early metabolic response to surgical stress is characterized by increased protein breakdown of the gut to deliver amino acids for the acute phase response. Because cancer is related to major disturbances in protein metabolism, we hypothesized that the normal metabolic response to surgery is attenuated in tumor bearing rats.

Female Lewis rats (± 200 g) were studied bearing a subcutaneous tumor or after sham implantation. Hysterectomy was performed in half of the tumor bearing and control rats as a standardized operative procedure. Postoperative an oral dose of lactulose and L-rhamnose was given and urinary recovery of the two sugar probes was determined after 12 hours to assess gut permeability. Protein and glutamine kinetics of the gut were determined using a primed constant infusion of L-[2,6- 3 H]-phenylalanine and L-[3,4- 3 H]-glutamine.

In control rats, net protein breakdown increased from 6 ± 3 to 32 ± 8 nmol phenylalanine $\times 100$ g $\text{bw}^{-1} \times \text{min}^{-1}$ after hysterectomy. This was accompanied by increased amino acid membrane transport rates and metabolic shunting. In tumor bearing rats this increased net protein breakdown was attenuated (8 ± 4 versus 17 ± 4 nmol $\times 100$ g $\text{bw}^{-1} \times \text{min}^{-1}$). Glutamine is the major energy substrate of the gut. Intracellular glutamine uptake by the gut increased after hysterectomy in control rats from 427 ± 28 to 937 ± 114 nmol $\times 100$ g $\text{bw}^{-1} \times \text{min}^{-1}$ which response was attenuated in tumor bearing rats (352 ± 40 to 613 ± 78 nmol $\times 100$ g $\text{bw}^{-1} \times \text{min}^{-1}$). The presence of tumor and surgery both increased lactulose/L-rhamnose recovery ratio indicating loss of gut barrier function induced by both events.

In conclusion, the study shows that a mild surgical trauma increases protein breakdown of the gut in conjunction with increased intracellular glutamine

consumption. In the presence of a tumor, intestinal protein turnover and glutamine uptake by the gut are chronically depressed resulting in an attenuated metabolic response to a surgical trauma with increased loss of gut barrier function.

Introduction

Cells of the gastrointestinal tract have a life span of approximately 48 hours (1) with a protein turnover of more than 100% (2,3) enabling the intestinal tract to adapt rapidly to fasting, feeding and metabolic stress (3). During metabolic stress the small intestine loses weight by increasing protein breakdown rates while protein synthesis rates remain relatively unchanged (2,4,5). The breakdown of gut proteins and subsequent release of amino acids has been postulated to be of crucial importance in the early phase, the so-called "ebb" phase (6), after severe injury or trauma (7,8). Thus, besides its absorptive and barrier function, the gut is currently recognized to have a central and regulatory role in protein and amino acid metabolism during injury, sepsis and severe metabolic stress (9,10).

Both human and animal studies have revealed that glutamine is an important nutrient for the gut (11,12). Glutamine is essential for enterocytes as energy source, as nucleotide precursor (13,14) and for polyamine production (15). We previously reported several marked changes in glutamine metabolism of the gut in the progress of cancer (16). Arterial and intracellular glutamine concentrations decreased with increasing tumor loads and this was paralleled, in the large tumor bearing state, with increased intracellular glutamine turnover rates. These disturbances in glutamine metabolism were associated with decreased polyamine concentrations and loss of the gut barrier function. Thus, similar to severe trauma and sepsis we observed that the progress of cancer disturbs glutamine metabolism in such a manner that intestinal function is diminished (17-19).

To obtain more insight as to how cancer affects the normal metabolic and functional response of the gut to a surgical insult the current study was performed. Aim of the present study was twofold: to test whether the intestine of tumor bearing rats is still capable of producing amino acids by increasing its protein breakdown rates after a mild surgical trauma. Furthermore, we wanted to examine how the cancer bearing state influences intracellular glutamine turnover and gut barrier function after a surgical trauma.

Animals and tumor model

Female Lewis rats (190-210 gr., Centralized Animal Facilities, University of Limburg, Maastricht, NL) were individually housed in metabolic cages during the experiment after an adaptation period of 3 days. Rats were given standard laboratory rat chow (SRMA 1210, Hopefarms, Woerden, NL) and subjected to standard 12 hour light-dark cycle periods (7:30 A.M. to 7:30 P.M.) with room temperature maintained at 25°C. The experiments were performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals (20) and approved by the Ethical Committee of Animal Research of the University of Limburg.

The tumor was induced by injecting 10 mg methylcholantrene (MCA, Sigma Chemie, W6501, Axel, NL) subcutaneously (21). Afterwards, the tumor was maintained *in vivo* by serially transplanting tissue suspensions through a 18G needle. MCA tumors are often used in metabolic studies and are known for its rapid growth, invasion of local tissue and absence of metastatic potential (22-24). The tumor was allowed to grow until it reached 5-15% of the host body weight. Non-tumor bearing rats were sham implanted with saline. Tumor growth was monitored by measuring two orthogonal dimensions and by calculating the volume assuming the tumor to be a prolate spheroid (25). Rats were housed in metabolic cages and tumor size, body weight and food intake were monitored daily. Carcass weight was determined by subtracting estimated tumor weight from total body weight.

Groups and surgical procedure

Rats (n=40) were randomly assigned to one of the following four groups:

- 1) control, sham anesthetized,
- 2) control, hysterectomized,
- 3) tumor-bearing, sham anesthetized,
- 4) tumor bearing, hysterectomized.

The surgical groups were subjected to a laparotomy under diethyl ether anesthesia (Merck 923, Darmstadt, Germany). The bicornuate uterus was dissected free and ligated on both sides from the ovaries with vicryl 3-0 (Ethicon, Norderstedt, Germany). The uterus was ligated at the basis with vicryl 3-0 and removed from the abdomen. The abdominal wound was closed in two layers with vicryl 3-0. The whole procedure took approximately 10-15 minutes. This standard procedure has been described previously (26-28). It involves the removal of a non-essential organ for the individual and metabolic responses to this kind of surgical trauma correspond with findings after cholecystectomy in patients (28). All non-operated rats underwent

sham anesthesia with diethyl ether for 15 minutes. Food intake and body weight were monitored daily.

Study procedures

PDV fluxes, protein and glutamine turnover were examined as described previously (3,29,30) and performed simultaneously with hindquarter measurements as described in chapter 9. In brief, postabsorptive, under ether anesthesia and at constant, pre-anesthesia body temperature, rats were subjected to a laparotomy. The right renal vein, portal vein, a tertiary branch of the mesenteric vein and aorta were cannulated using a 25 gauge needle fixed in a Silastic tube (Silastic Medical Grade tubing 0.051 cm ID, 0.094 OD, Dow Corning Corporation, Midland, MO, USA) and fixed with cyano-acrylate. For flow measurements, the indicator dilution method with *para*-aminohippuric acid (PAH) was used (pH 7.4, iso-osmolaric, Sigma A 1422, St Louis, MI, USA). A primed ($0.15 \text{ ml} \times 100 \text{ g bw}^{-1}$, 50 mmol/l) constant infusion ($0.75 \text{ ml} \times 100 \text{ g bw}^{-1} \times \text{h}^{-1}$, 5 mmol/L) of PAH was infused in the mesenteric vein, using a Minipuls 3 Peristaltic Pump (Gilson Medical Electronics Inc., Villiers-le-Bel, France). For turnover studies, a primed ($1 \mu\text{Ci} \times 100 \text{ g bw}^{-1}$) constant infusion ($1 \mu\text{Ci} \times 100 \text{ g bw}^{-1} \times \text{h}^{-1}$) of L-[3,4- ^3H]phenylalanine (NEN Dupont, NET-493, Mechelen, Belgium) and L-[3,4- ^3H]glutamine (NEN Dupont, NET-551) was given in the right renal vein. A minimum infusion time of 30 minutes was needed to reach steady state concentrations for PAH, ^3H -phenylalanine and ^3H -glutamine (3). The right carotid artery was cannulated during the equilibrium period with PE 50 catheter (Intramedic, 0.051 cm ID, 0.094 cm OD, Clay Adams, Parispany, NY, USA). The complete procedure took approximately 40 minutes.

Blood (1.0 ml per catheter) was simultaneously sampled from the portal vein and carotid artery. All blood was collected in heparinized cups (Lithium-Heparin, D51588, Sarstedt, Germany) on ice. Hereafter, the small intestine was dissected free from the pyloric sphincter to the coecal valve, flushed with ice-cold saline and weighted. A small part of the jejunum was directly freeze-clamped, put in liquid nitrogen and stored at -80°C until further analysis.

For PAH determinations, 50 μl heparinized blood was added to 500 μl TCA 10% (w/v), vortexed and centrifuged at 8,900 g at 4°C . The supernatant was frozen in liquid nitrogen and stored at -80°C until further analysis. For hematocrit determinations a micro-hematocrit tube was filled with heparinized blood and centrifuged at 10,000 g at room temperature. Hematocrit was read with a Micro Hematocrit Reader (Hawksley Ltd, UK). Plasma was obtained by whole blood centrifugation at 8,900 g at 4°C for 5 minutes. For ammonia, urea, glucose and lactate determinations, 200 μl plasma was vortexed with 20 μl TCA 50%, put into

liquid nitrogen and stored at -80°C . To determine plasma amino acid specific activities and concentrations, 300 μl plasma was added to 8 mg 5-sulfosalicylic acid, vortexed, frozen in liquid nitrogen and stored at -80°C .

Biochemical analysis

Plasma ammonia, urea, glucose, lactate and PAH were determined spectrophotometrically on a Cobas Mira S (Roche Diagnostica, Hoffman-La Roche, Basel, Switzerland) by standard enzymatic methods, using commercially available kits (3,29,30). Plasma amino acid concentrations and specific activity were determined by fully automated HPLC as described previously (31). For the determination of intestinal ammonia, amino acid concentrations and specific activities, tissue was pulverized using a mortar and pestle precooled in liquid nitrogen. The tissue was further homogenized and deproteinized in a Mini-Beadbeater (Biospec products, Bartlesville, USA). Approximately 100 mg tissue was added to 400 μl SSA 5%, with 300 mg glass beads (diameter 1 mm, Biospeck Products) and beaten for 30 seconds. The homogenate was centrifuged at 4°C at 11,000 g and the supernatant frozen in liquid nitrogen and stored at -80°C until further determinations.

To determine tissue dry weight and water content approximately 200 mg pulverized tissue was freeze dried for 24 hours in a Speedvac (SC200, Savant Instruments Inc., Farmingdale NY, USA) connected with a refrigerated condensation trap.

Calculations

Plasma flow and substrate fluxes

Plasma flow across the portal drained viscera was calculated using PAH in the indicator dilution method (3,29,30). Substrate fluxes are calculated by multiplying the venous-arterial concentration differences with the mean plasma flow of the group and are expressed in $\text{nmol} \times 100 \text{ gr. body weight}^{-1} \times \text{min}^{-1}$. A positive flux indicates net release, a negative flux reflects a net uptake. Total release of α -amino-nitrogen was calculated as the sum of the individual amino acids which are released by the PDV except taurine (31). Tissue concentrations of amino acids are expressed in $\mu\text{mol/l}$ and derived by dividing the tissue homogenate amino acid concentration ($\mu\text{mol/kg wet weight}$) with the tissue water content.

Protein and glutamine kinetics

Protein and glutamine turnover were calculated by the A-V dilution of tritium labeled phenylalanine and glutamine across the PDV calculated in a three compartment model (30,32,33). Intracellular disposal (D) and production (P) of phenylalanine and glutamine can thus be calculated and reflect *in vivo* intracellular turnover of these amino acids. Production of phenylalanine reflects protein breakdown and disposal reflects protein synthesis because intestinal metabolism of phenylalanine is restricted to synthesis into and breakdown from protein (34). Furthermore, amino acid membrane transport rates can simultaneously be measured: $F_{i,a}$ are plasma-tissue inward directed transport rates, $F_{p,j}$ are plasma-tissue outward directed membrane transport rates and $F_{p,a}$ reflects metabolic shunting of amino acids (30,32,33).

Statistical Analysis

Results are presented as mean $\pm$ SEM. Statistical analysis was performed using the statistical package SPSS (35). Two-Way ANOVA was used to test effects of tumor load, hysterectomy and an interaction between tumor load and hysterectomy. Significance from zero was tested by the Wilcoxon signed-ranks test. Significance was considered present at $p < 0.05$.

Results

Initial and preoperative carcass weights were similar in all groups (Table 1). Hysterectomized rats lost more of their body weight than sham anesthetized rats. Food intake was similar in all groups and remained unchanged in the preoperative period. Postoperative food intake decreased in hysterectomized control and tumor bearing rats to approximately 18-20% of the preoperative daily food intake.

Plasma flow and fluxes

Plasma flow across the portal drained viscera was in the same range as described previously (29,30) and increased after hysterectomy in control and tumor bearing rats (Table 2). Glucose uptake by the portal drained viscera was not influenced by the presence of a tumor or by hysterectomy. However, it decreased to levels not different from zero in the tumor bearing hysterectomized rats. Lactate release changed to an uptake after hysterectomy in control rats. In the tumor bearing state lactate release was increased whereas a hysterectomy in tumor bearing rats had no effect on lactate flux.

	Control		Tumor bearing		p value		
	sham	hysterectomy	sham	hysterectomy	T	S	I
Tumor weight	-	-	38 ± 2	32 ± 3	<0.001	ns	ns
Carcass weight							
Initial	195 ± 3	200 ± 3	200 ± 3	204 ± 3	ns	ns	ns
Preoperative	200 ± 3	205 ± 4	198 ± 4	201 ± 4	ns	ns	ns
Postoperative (day 2)	190 ± 2	184 ± 4	191 ± 7	180 ± 4	ns	0.02	ns
Food intake							
Initial	18 ± 1	17 ± 1	17 ± 1	15 ± 2	ns	ns	ns
Preoperative	17 ± 1	15 ± 1	17 ± 1	16 ± 2	ns	ns	ns
Postoperative	16 ± 1	4 ± 2	15 ± 3	3 ± 1	ns	<0.001	ns

Table 1. Carcass weight, tumor weight and food intake during the experiment. Values are mean ± SEM. Carcass weight is in grams, food intake in gram/day except postoperative which is in total intake after hysterectomy or sham anesthesia the metabolic study procedure. Statistical analysis by Two-Way ANOVA: T= effects of tumor load, S = effects of surgery, I = interaction of tumor load and surgery.

	Control		Tumor bearing		p value		
	sham	hysterectomy	sham	hysterectomy	T	S	I
Plasma flow	1.5 ± 0.9	4.9 ± 2.5	2.4 ± 0.9	4.2 ± 1.5	ns	0.05	ns
Glucose	-641 ± 274	-1961 ± 856	-983 ± 378	-296 ± 559 #	ns	ns	ns
Lactate	126 ± 78 #	-1378 ± 624	745 ± 456 #	688 ± 365 #	0.02	ns	0.02
Ammonia	256 ± 36	620 ± 91	263 ± 28	474 ± 49	ns	<0.001	0.04
Glutamine	-427 ± 28	-937 ± 114	-352 ± 48	-613 ± 78	0.04	<0.001	0.007
Glutamate	32 ± 3	24 ± 27 #	24 ± 7	22 ± 8	ns	ns	ns
Alanine	303 ± 28	807 ± 91	300 ± 56	598 ± 55	ns	<0.001	0.04
Citrulline	54 ± 9	131 ± 14	30 ± 13 #	69 ± 11	0.001	<0.001	0.03
Total release α-AN	491 ± 83	1318 ± 230	289 ± 160	877 ± 147	0.01	0.001	0.02

Table 2. PDV plasma flow, ammonia and amino acid fluxes. Values are presented in nmol x 100 gr. body weight⁻¹ x min⁻¹ (mean ± SEM). Total release α-AN is the sum of all amino acids released by the portal drained viscera. Statistical analysis by Two-Way ANOVA: T = effect of tumor, S = effect of surgery, I = effect of interaction between presence of a tumor and surgery. Significance from zero tested by Wilcoxon: # not significant from zero (p<0.05). Fluxes: (-) reflects net uptake, (+) reflects net release of a substrate.

The hysterectomy increased net glutamine uptake by the gut. In tumor bearing rats glutamine uptake was decreased and, the postoperative increase was less than in control rats. Ammonia, glutamate, alanine and citrulline are the major end products of glutamine consumption by the gut (13). Ammonia release increased after surgery. This release was higher in control rats than in tumor bearing rats. Glutamate production by the gut remained unchanged after surgery in both control and tumor bearing rats. Alanine release increased after surgery but this release increased not as much in the tumor bearing rats than in the control rats. Citrulline release decreased in tumor bearing rats. It increased after surgery, but this response was also attenuated in the tumor bearing rats. Finally, the sum of α -amino nitrogen released in the portal vein increased after hysterectomy. It was decreased in tumor bearing rats and the response to surgery was, again, attenuated.

Figure 1. Intracellular protein turnover of the PDV. Values are mean $\pm$ SEM. Intracellular protein synthesis is calculated as intracellular disposal of phenylalanine, protein breakdown as intracellular production of phenylalanine (30,32). The flux of phenylalanine is the difference between protein synthesis and breakdown: (+) net release, (-) net uptake of phenylalanine. Statistical analysis by Two-Way ANOVA for effects of tumor load, hysterectomy and interaction of the two.

□ Sham anesthetized rats, ■ Hysterectomized rats. **A:** Intracellular protein synthesis. Effect tumor: $p=0.003$; effect hysterectomy: $p=0.006$; interaction: $p=0.02$. **B:** Intracellular protein breakdown. Effect tumor: $p=0.001$; effect hysterectomy: $p<0.001$; interaction $p=0.005$. **C:** Net phenylalanine flux. Effect tumor: ns; effect hysterectomy: $p<0.001$; interaction: $p=0.05$.

Protein and glutamine kinetics

Intracellular disposal of phenylalanine (protein synthesis) and production of phenylalanine (protein breakdown) were lower in tumor bearing rats (Fig. 1). Both protein synthesis and breakdown increased after surgery but to a lesser extent in the tumor bearing rats. Protein breakdown rates increased more than synthesis rates resulting in an increased efflux of phenylalanine (net protein breakdown, Fig. 1, bottom panel) of the gut after surgery. Again, this net efflux was attenuated in the tumor bearing rats.

Phenylalanine membrane transport rates resembled intracellular protein turnover (Table 3). After the hysterectomized inward ($F_{i,a}$) and outward ($F_{p,j}$) membrane transport rates increased. In tumor bearing rats the postoperative increase of inward and outward membrane transport rates was attenuated. Metabolic shunting of phenylalanine was increased in tumor bearing rats. Surgery also increased metabolic shunting across the PDV.

Figure 2. Intracellular glutamine turnover of PDV. Values in $\text{nmol} \times 100 \text{ gr. body weight}^{-1} \times \text{min}^{-1}$. Intracellular turnover is measured as disposal (consumption) and production of glutamine (30,32). The flux is the difference between disposal and production: (+) net release, (-) net uptake. Statistical analysis by Two-Way ANOVA for effects of tumor load, hysterectomy and interaction of the two. □ Sham anesthetized rats, ■ Hysterectomized rats.

A: Intracellular disposal. Effect tumor: ns; effect hysterectomy: $p=0.001$; interaction: ns.

B: Intracellular production. Effect tumor: ns; effect hysterectomy: $p=0.002$; interaction: ns.

C: Net glutamine flux. Effect tumor: $p=0.002$; effect hysterectomy: $p<0.001$; interaction: $p=0.04$.

	Control		Tumor bearing		p value		
	sham	hysterectomy	sham	hysterectomy	T	S	I
Phenylalanine							
$F_{j,a}$	24 ± 4	102 ± 22	13 ± 13	34 ± 9	0.002	0.002	0.02
$F_{p,j}$	27 ± 4	134 ± 22	21 ± 11	51 ± 11	0.001	<0.001	0.002
$F_{p,a}$	45 ± 2	147 ± 43	135 ± 23	204 ± 11	0.007	0.003	ns
Glutamine							
$F_{j,a}$	588 ± 77	1194 ± 142	373 ± 47	776 ± 164	0.002	<0.001	ns
$F_{p,j}$	160 ± 63	256 ± 149	20 ± 33	163 ± 109	ns	ns	ns
$F_{p,a}$	458 ± 73	1531 ± 190	865 ± 62	1236 ± 139	ns	<0.001	0.01

Table 3. Phenylalanine and glutamine membrane transport rates across the PDV. Values are presented in $\text{nmol} \times 100 \text{ gr. body weight}^{-1} \times \text{min}^{-1}$ (mean $\pm$ SEM). $F_{j,a}$: Inward directed membrane transport, $F_{p,j}$: outward directed membrane transport, $F_{p,a}$: metabolic shunting across the PDV. Statistical analysis by Two-Way ANOVA: T = effect of tumor, S = effect of surgery, I = effect of interaction between presence of a tumor and surgery.

Intracellular glutamine disposal (consumption) increased after a hysterectomy (Fig. 2). Intracellular glutamine production was lower than disposal. Glutamine production increased after hysterectomy. The difference between consumption and production is reflected by the flux of glutamine (Fig. 2, Table 2). This flux (net uptake) was decreased in tumor bearing rats, increased after hysterectomy. The postoperative response was attenuated in tumor bearing rats. Glutamine inward ($F_{i,a}$) membrane transport rates were decreased in the tumor bearing rats (Table 3). Surgery increased these rates in both control and tumor bearing rats. Outward ($F_{p,j}$) membrane transport rates was not significantly different between the groups, possibly due to the relatively high variation. Metabolic shunting of glutamine across the PDV ($F_{p,a}$) increased after surgery in control rats. In tumor bearing rats, hysterectomy had a smaller effect because metabolic shunting was already elevated in the non-operated rats.

Figure 3. Urinary recovery of lactulose, L-rhamnose and its ratio to test gut permeability. An iso-osmolar oral dose of both sugar probes was given and the recovery was determined in urine after 12 hours. Gut permeability was assessed by the lactulose/L-rhamnose ratio of the urinary recovery. Values are means $\pm$ SEM and in % per 12 hours. Statistical analysis by Two-Way ANOVA for effect tumor, effect surgery and the interaction between tumor and surgery. $\square$ Sham anesthetized rats, $\blacksquare$ Hysterectomized rats.

A: Lactulose recovery: effect tumor, $p=0.03$; effect surgery, $p=0.001$; interaction, ns.
 B: L-Rhamnose recovery: effect tumor, $p=0.02$; effect surgery, $p<0.001$; interaction, $p=0.02$.
 C: Lactulose/rhamnose ratio: effect tumor, $p=0.05$; effect surgery, $p=0.01$; interaction, ns.

	control		tumor bearing		p value		
	sham	hysterectomy	sham	hysterectomy	T	S	I
H ₂ O %	74.1 ± 0.6	73.1 ± 0.8	73.5 ± 0.9	73.6 ± 0.6	ns	ns	ns
Ammonia	2284 ± 84	2073 ± 293	2027 ± 256	1528 ± 133	0.01	ns	ns
Glutamine	769 ± 54	845 ± 66	759 ± 48	748 ± 47	ns	ns	ns
Glutamate	2983 ± 184	3338 ± 201	2712 ± 208	2927 ± 131	ns	ns	ns
Alanine	2082 ± 152	2643 ± 316	2597 ± 253	2707 ± 270	ns	0.05	ns
Citrulline	218 ± 51	270 ± 54	216 ± 31	246 ± 32	ns	ns	ns
Phenylalanine	151 ± 19	191 ± 36	215 ± 21	185 ± 13	ns	ns	ns
α-Amino-nitrogen	11359 ± 156	13011 ± 1180	13946 ± 783	13118 ± 1044	ns	ns	ns

Table 4. Jejunal hydration and intracellular ammonia and amino acid concentrations. Concentrations in $\mu\text{mol/l}$ (mean $\pm$ SEM). α -Amino-nitrogen is the sum of individual amino acids measured except taurine (31). Statistical analysis by Two-Way ANOVA: T = effect of tumor, S = effect of surgery, I = effect of interaction between presence of a tumor and surgery.

Intracellular concentrations

Intracellular water remained unchanged (Table 4). Intracellular ammonia was decreased in tumor bearing rats while hysterectomy had no effect on this. Intracellular alanine increased after hysterectomy in both control and tumor bearing rats. Intracellular glutamine, glutamate, phenylalanine, citrulline and total α -amino-nitrogen remained unchanged between the different groups.

Lactulose and L-rhamnose recovery

Urinary recovery of lactulose and L-rhamnose was increased in tumor bearing rats (Fig. 3). It also increased after surgery. The lactulose/L-rhamnose ratio, indicative for gut permeability, was increased in tumor bearing rats. Surgery also increased the recovery ratio.

Discussion

The main observation in the present study is that surgical stress increases gut protein turnover and amino acid membrane transport resulting in increased net breakdown of intestinal proteins with subsequent release of amino acids. The gut is a metabolically very active organ with protein turnover rates of more than 100% per day, one of the highest in the body (2). The high turnover rates are primarily needed to make up for the intraluminal cell loss. This accounts for approximately half of the protein synthesis rates in enterocytes (2). Both increasing amounts of exfoliated cells and increased secretion of proteins have been observed during severe stress (36,37). This may partly be responsible for the increased protein turnover observed in the present study. However, high turnover rates are also of extreme importance for the gut to adapt rapidly to changing environmental conditions (3,38). It has been outlined by Abumrad that in the early "ebb" phase of a trauma, breakdown of proteins with high turnover rates is initiated to transfer amino acids to visceral organs for gluconeogenesis and the production of acute phase proteins (7). The high protein turnover rates of the intestinal tract make this organ suitable to adapt to this first phase after a trauma. In a recently published study on exercise induced metabolic stress, 60-90 minutes of exercise almost doubled gut proteolysis apparently aimed at sparing muscle protein (8). The muscle proteolytic response with subsequent amino acid release is generally thought to occur in a later phase ("flow phase") after trauma (6). The close anatomic relationship of the gut with the liver has the other advantage that the release of amino acids in the portal vein makes them almost instantly available for gluconeogenesis and the production of acute phase proteins. As in

humans direct measurements are difficult, Lund et al. calculated the intestinal release of amino acids indirectly in the portal vein from the total splanchnic amino acid uptake and the net urea production (39). They also found an increased release of amino acids into the portal vein during and in the early recovery phase after a cholecystectomy, a comparable trauma to the hysterectomy in rats (28).

The current study shows that the presence of cancer depresses intestinal protein turnover resulting in a reduced net protein breakdown and α -amino acid release. This could both have consequences for gluconeogenesis and for the early hepatic acute phase response. Arterial glucose, however, remained unchanged after surgery in the tumor bearing rats (see chapter 9, table II) suggesting no major impairment of the gluconeogenesis. Furthermore, decreased acute phase protein production by the liver has previously been observed in both cancer patients and in malnourished children (40,41). This impaired response may be related to the attenuated early mobilization of amino acids from the gut as observed in the current study.

Functioning of the gut itself was assessed by the metabolic kinetics of its major energy substrate glutamine and by gut barrier function. The mild surgical trauma we applied increased intracellular glutamine consumption, production and net uptake by the gut. This is in agreement with increased glutamine consumption by the gut following a mild surgical trauma observed in dogs (10). In a piglet study, however, we observed a decreased glutamine uptake by the gut following major abdominal surgery (42). It has been postulated that mild metabolic stress increases glutamine consumption by the gut whereas in severe metabolic stress, e.g. sepsis or endotoxemia, glutamine consumption by the gut decreases (18). Our results therefore confirm that we studied protein and glutamine kinetics after a mild surgical trauma as stated previously.

The increased net uptake of glutamine by the gut was attenuated in tumor bearing rats although this became not significant for intracellular consumption. The postoperative increased uptake of glutamine by the PDV occurred in conjunction with increased ammonia, alanine and citrulline release which are end products of glutamine consumption by the gut (13). Measurement of end products is necessary because the portal drained viscera contain several organs capable of consuming glutamine. Glutamate is an end product of lymphocyte glutamine consumption whereas alanine and citrulline are end products of enterocyte glutamine consumption. Because we observed no change in glutamate release into the portal vein it seems reasonable to assume that the increased glutamine consumption in the portal drained viscera after a mild surgical trauma was mostly located in the gut. The postoperative release of glutamine end-products was also attenuated by the

presence of a tumor, thus confirming that glutamine metabolism in the gut of tumor bearing rats was attenuated on the second postoperative day.

Increased consumption of glutamine by the gut has been related to decreased glucose consumption thus sparing glucose for tissues with an obligate glucose requirement (11,43). In our study glucose consumption decreased to a value not significantly different from zero in hysterectomized tumor bearing rats indicating that a similar phenomenon is present. Particularly, the postoperative increased glucocorticoid release has been held responsible for this mechanism enabling the host to spare glucose for reparative tissues (11). Our data could thus also indicate that the postoperative metabolic response in tumor bearing rats is of benefit for reparative tissue but this needs further research.

Intestinal function was also tested by gut barrier function. Gut barrier function was measured by the recovery ratio of lactulose/L-rhamnose. This ratio increased in tumor bearing rats as was shown previously (16). It also increased after surgery confirming previous studies which showed that metabolic stress induced by multiple trauma (44), endotoxemia (45) or shock (46) decreases gut barrier function. Increased permeability has previously been postulated to be the cause of a constant state of systemic inflammation possible the basis and driving force of systemic endotoxemia and sepsis (43). Whether this is true in tumor bearing rats remains to be elucidated. The extra increase in the postoperative days after the surgical trauma in tumor bearing rats was small and the clinical relevance remains to be determined. Combined with the protein turnover data, however, the current study shows that in the presence of cancer one of the first immune stations of splanchnic organs, the liver, has to cope with a decreased nutrient supply and an increased immune challenge in the first postoperative days.

In conclusion, the study shows that a mild surgical trauma increases protein breakdown of the gut in conjunction with increased glutamine consumption. In the presence of a tumor, intestinal protein turnover, glutamine uptake and inward directed membrane transport are chronically depressed resulting in an attenuated response to a surgical trauma with a depressed mobilization of amino acids. Gut barrier function is decreased in the presence of a tumor. Surgery also decreases gut barrier function and the effects were strongest in tumor bearing rats. The diminished delivery of nutrients to other organs, particularly the liver, after surgical stress could be the cause of the decreased acute phase response previously reported in cancer patients.

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SUMMARY AND PERSPECTIVES FOR THE FUTURE

Summary

The presence of cancer is accompanied by metabolic disturbances resulting in wasting of body mass. These events have an adverse effect on clinical outcome and, if left untreated, inevitably contributes to an earlier death (1). The changes observed in cancer cover a broad spectrum of intermediary metabolism. In this thesis tumor induced changes in protein and amino acid metabolism are studied in a rat tumor model. In addition, the consequences of these metabolic changes for the response to a surgical trauma are studied.

The thesis consists of three parts. First, a model is described to study *in vivo* protein and amino acid turnover in organs. Second, the effect of an increasing tumor load on protein and amino acid metabolism in muscle, gut and liver is studied. Finally, the effects of a surgical trauma on protein and amino acid metabolism in these organs in the cancer bearing host are studied.

Chapter 3 and 4 describe several models to measure *in vivo* amino acid and protein turnover across muscle and splanchnic organs. In these studies we showed that the A-V dilution method is applicable to measure protein turnover in small laboratory animals. The major advantage of the method is the possibility to measure *in vivo* protein synthesis, protein breakdown and amino acid plasma tissue membrane transport rates simultaneously. Compartment modelling made it possible to measure protein and amino acid kinetics across the portal drained viscera, the liver and hindquarter muscle. The results of chapter 3 and 4 further show that during short starvation protein synthesis measurements using the A-V dilution method gives similar results as the measurements using by the incorporation technique. After a prolonged starvation of 112 hours, however, both methods showed an opposite change in protein synthesis rates. It appears that the disappearance of amino acids measured by the A-V dilution method is not into the proteins which are measured by the incorporation technique (TCA-insoluble proteins) but into soluble proteins which are not measured

with the incorporation technique. These effects may have been induced by the experimental procedure which includes ether anesthesia and a laparotomy. If we want to extrapolate the results to the clinical setting, our data indicate that amino acid and protein turnover of the gut, liver and muscle increase in patients suffering from prolonged starvation and undergoing surgery. Whether this rapid turnover is a functional adaptation or is a futile cycle remains to be elucidated. We chose to measure protein and amino acid kinetics using the A-V dilution method because it allows the measurements of different parameters in the same animal at the same time.

In chapter 5, 6, 7 and 8 the effects of a MCA tumor on whole body, hindquarter muscle, gut and liver protein and amino acid metabolism was studied. MCA tumor have been used extensively to study tumor induced changes in feeding behavior and changes in carbohydrate, lipid and protein metabolism (2-5). In these studies Fisher 344 rats were used and the tumor repeatedly induced anorexia with severe cachexia resulting in death. In our laboratory, however, the presence of this type of tumor had no influence on food intake of rats during 3½ years of experiments. Moreover, we imported tissue from a MCA tumor line from the US (gift from W.T. Chance, PhD, University of Cincinnati Medical Center, Cincinnati, Ohio), which was known to induce anorexia, and implanted these in Fisher 344 rats. Surprisingly, again no anorexia occurred (unpublished results). It is beyond the scope of this thesis to explain the differences in feeding behavior but this may be related to the different microbiological status of animals, differences in components of rat chow or differences in susceptibility to cytokines as was shown in a colitis model (6,7).

The tumor model used in the experiments described in this thesis induced no anorexia and only moderate or no carcass weight loss. With a tumor burden of 5-15% or 15-30% of body weight, nutrient delivery and intermediate metabolism of the host must have adapted. If extrapolation of the tumor model had to be made, it is therefore best comparable to early detected, non-anorectic, moderately weight losing cancer patients (e.g. colon cancer).

Results of experiments described in chapter 5 show that whole body protein turnover increased with increasing tumor loads. Muscle protein turnover and net breakdown increased in the small tumor bearing rats (tumor 5-15% of body weight). In large tumor bearing rats muscle protein turnover and net breakdown decreased to control values as if muscle tissue was exhausted. In conjunction with this, glutamine turnover and glutamine release by hindquarter muscle decreased to control values. This contrasted with whole body glutamine turnover which almost doubled in large tumor bearing rats. This may be related to an increased turnover of glutamine in the immune system. It may, however, also be related to changes in glucose metabolism which is related to glutamine metabolism by transamination to glutamate and further processing to TCA cycle intermediates.

In chapter 6 results of experiments are described which show that glutamine uptake by the gut increased in the presence of a large tumor load. This was, however, accompanied with decreased intracellular glutamine concentrations and an increased gut permeability. The latter was not related with signs of villus atrophy but may be related to a decreased differentiation of the mucosal cells as assessed by decreased intracellular polyamine concentrations.

The results of experiments described in chapter 7 show how the liver adapts to the increased demand for amino acids and proteins by e.g. tumor and immune cells. The liver increases the uptake of essential and gluconeogenic amino acids. These amino acids were not used for glucose production and urea synthesis but were mostly used for protein synthesis of export and structural proteins. Serum α_2 -macroglobulin, one of the most reactive acute phase proteins in the rat, increased to a level comparable to maximum levels after a laparotomy.

In chapter 8 liver glutamine handling in response to a tumor is described. The liver has a central role in glutamine homeostasis because it has high glutaminase activity and glutamine synthetase activity with consumption at the periportal hepatocytes and production at the perivenous hepatocytes. This results in a so called "intercellular" glutamine cycling. The three compartment model made it possible to give an *in vivo* estimate of the activity of both zones. The study indicated that changes in glutaminase activity in majority determined whether the liver consumed or released glutamine in the presence of cancer. Furthermore, it was shown that in the presence of a small tumor the liver changes from an organ of net glutamine release to an organ of net uptake. In large tumor bearing rats the liver changed again in an organ of net glutamine release. This occurred in conjunction with decreasing arterial glutamine concentrations indicating for an adaptative mechanism of the liver to maintain glutamine homeostasis.

Changes in protein turnover rates of different organs in tumor bearing rats are summarized in figure 1A (disposal or protein synthesis) and B (production or protein breakdown). In control rats protein synthesis rates were highest in the gut followed by the total muscle compartment and was smallest in the liver. In tumor bearing rats muscle protein synthesis rates remained unchanged and intracellular gut protein synthesis rates decreased to a level only slightly higher than muscle. Liver protein synthesis increased and to a level similar to the muscle compartment in tumor bearing animals. In control rats intracellular protein breakdown was also highest in the gut. In small tumor bearing rats gut protein breakdown decreased to a level similar to the muscle compartment and the liver. In large tumor bearing rats protein breakdown decreased to zero whereas protein breakdown in the other two tissue compartments decreased only slightly.

Figure 1.

A: Intracellular protein synthesis in muscle, gut and liver tissue.

B: Intracellular protein breakdown in muscle, gut and liver tissue.

Data are derived from chapter 5, 6, and 7 and standardized to 100 gr. body weight. Hindquarter muscle compartment is assumed to account for approximately 50% of the whole body muscle compartment.

Changes in glutamine turnover are summarized in figure 2A (disposal) and 2B (production). Alterations in intracellular glutamine disposal predominantly occurred in the muscle compartment. Muscle glutamine turnover (both disposal and production) increased in the small tumor bearing rats. In large tumor bearing rats it decreased again. The latter was accompanied by increased glutamine disposal and production in the gut. In the liver glutamine disposal increased in small tumor bearing rats whereas it decreased again in large tumor bearing rats. Intracellular glutamine production did not significantly change in the liver of tumor bearing rats. Large tumor bearing animals therefore appear to have a decreased capacity of glutamine producing organs which results in depletion of both the intracellular and plasma glutamine pools.

Intracellular disposal

Figure 2.

Intracellular disposal (A) and production (B) of glutamine in muscle, gut and liver tissue.

Data are derived from chapter 5, 6, and 8 and standardized to 100 gr. body weight.

Intracellular production

In chapter 9 and 10 the effects of cancer on postoperative muscle and gut protein and glutamine metabolism were studied. In both studies it was shown that amino acids are mobilized from muscle and gut tissue in response to a surgical trauma. It has been postulated that in the early phase after a trauma ("ebb phase") the mobilization of amino acids occurs from protein pools with a rapid turnover, e.g. from the gut (8). In a later phase amino acids are degraded from proteins with a slower turnover, e.g. muscle. In our experiments mobilization of amino acids was observed from proteins of both the gut and muscle. This indicates that measurements were performed at a time when the mobile pool of the gut was still prone to degradation whereas degradation of structural proteins of muscle already began. The experimental conditions, however, may also have caused the simultaneous degradation of the mobile proteins and less mobile proteins. The experimental conditions require anesthesia and a laparotomy and these conditions may also be seen as a second hit (first hit: hysterectomy). This second hit (anesthesia and laparotomy) may have again induced breakdown of mobile proteins of the gut whereas breakdown of muscle proteins was induced as a late response to the first

hit, thus explaining the simultaneous presence of breakdown of gut and muscle proteins.

In the tumor bearing state, rats were unable to respond normally to a surgical trauma. This resulted in an attenuated protein breakdown of muscle with a decreased amino acid release. Glutamine is in this respect of particular importance as normally 30% of the amino acids release by muscle consists of glutamine and this release may overall be the limiting step in further metabolic pathways. This includes the obligatory increased uptake by immune cells in response to a trauma. A relatively decreased postoperative release of glutamine in cancer would thus compromise the host.

Protein breakdown and amino acid mobilization of the gut was also attenuated in the tumor bearing state. The uptake of glutamine by the gut of tumor bearing rats was relatively diminished in response to surgery. This resulted in a further decrease of gut barrier function. The cancer bearing state attenuates the normal postoperative amino acid release of both mobile and structural proteins and decreases gut barrier function. The studies suggest that the presence of cancer compromises the endogenous nutrient supply to facilitate the acute phase and immune response while, at the same moment, the antigenic load for the immune system, particularly that of the liver, increases.

Perspectives for the future

In this thesis several aspects of tumor induced changes in host protein and amino acid metabolism have been described. However, several of these descriptions resulted in more questions to be answered. Some of these questions need further fundamental research, others ask for intervention studies in humans or laboratory animals.

Prolonged starvation resulted in a discrepancy in two methods of measuring *in vivo* protein synthesis under our experimental conditions. Both methods appear to measure synthesis of two different protein pools in muscle, gut and liver tissue. One pool increases it's synthesis and turnover rates whereas the synthesis of another protein pool decreases it's turnover. To elucidate whether the high turnover of one of the pools is of benefit a better identification of the pool is needed. Furthermore, the discrepancy may also occur in disease and other experimental conditions. This warrants a careful in the interpretation of data when only one model is used. It also emphasizes the need for an adequate explanation of the phenomenon.

In chapter 4 it was shown that whole body glutamine turnover increased far more than expected to be needed for glutamine consumption only. The glucose-glutamine

cycle was discussed but needs further research. This may also be of particular importance in cancer patients because increased glucose turnover rates and decreased arterial glutamine concentrations have both been observed in the progress of cancer. Recent studies showed that glutamine is a greater carbon donor to glucose than alanine which has always been assumed to be the primary gluconeogenic amino acid (9). The existence and extent of glutamine-glucose cycling may also be of importance in several studies giving glutamine supplementation in parenteral or enteral nutrition. More fundamental research needs to be initiated on the subject.

Recent studies on muscle protein breakdown revealed that this occurs in several wasting conditions, including cancer cachexia, by the ATP-ubiquitin degradation system (10-12). This pathway was first thought to be only active in the degradation of small, soluble, often incorrectly made proteins, but is now recognized to be involved in the degradation of structural proteins as well. Future research should answer the question whether the ATP-ubiquitin pathway targets specific proteins in cancer cachexia and to what extent this occurs in different organs.

Changes in protein and glutamine metabolism of the tumor bearing rat may be prevented or treated with nutritional interventions. Several animals and clinical studies have reported benefits of enteral and parenteral glutamine suppletion. Similarly, pharmacological interventions with e.g. growth hormone or IGF-1 are promising. Interestingly, combination therapy (nutritional and hormonal) have recently shown to have synergistic effects *in vitro* and *in vivo* (13-15). Particularly, the combination of glutamine and an anabolic agent seems to have the best prospects in the near future.

Although not studied in this thesis, interventions in the immune activation should be considered in the prevention and or treatment of cancer cachexia. The immune response is however a complex response having both beneficial and deleterious aspects. Simple blockades of $\text{TNF}\alpha$, IL1 or IL6 have proven to stop and even turn back several metabolic changes of cancer cachexia (16,17). However, blockades of one central pathway have mostly shown to be of benefit in disease states involving disturbances in single biochemical or immunological pathways. The cachectic syndrome is of such a complex kind that a relatively "crude", single intervention can not really be expected to cover the complete changes in intermediary metabolism. Moreover, intervention studies directed against e.g. $\text{TNF}\alpha$, although recognized as key mediator of sepsis, worsened the clinical outcome of septic patients. Modulation of the metabolic changes, probably at several fronts, should be considered in future. This implicates that therapeutic interventions do not only have different aspects (pharmaceutical, hormonal, nutritional) but may also have to differentiate between

different patient populations depending on the pending metabolic disturbances in carbohydrate, lipid or protein metabolism.

Of particular interest in this respect is the acute phase response. Acute phase proteins are recognized as proteins to modulate the immune response to trauma, infections and possibly cancer. Whether this is true for metabolic responses, particularly in cancer, is unknown but would be an interesting field of research. Acute phase proteins can thus help modulating the metabolic response or, when produced in excess, may further deteriorate this response.

Extrapolation of experimental data of this thesis to the clinical setting are a primary goal but should be done with great care and remain difficult. Fortunately, the department of surgery in Maastricht has the possibilities to do both clinical and experimental nutritional research. In the last few years clinical research has focused on the effects of glutamine suppletion to parenteral nutrition and several beneficial aspects have been observed. Although clinical research is expected to be able to assess fundamental aspects in the human situation using stable isotopes, specific effects at organ level still remain difficult to obtain and interpret as sampling of tissue specimens can only be done in selected cases. Clinical and experimental research should therefore be well attuned to each other with two-way directed exchange of information. These conditions appear to be present in the Maastricht clinical and research setting and progress in nutritional therapy for cancer cachexia is part of promising perspectives for the future.

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In de aanwezigheid van kanker ontstaan stoornissen in de stofwisseling die leiden tot gewichtsverlies en ondervoeding. Dit heeft een negatief effect op de uitkomst van de behandeling van kanker en draagt bij, indien onbehandeld, tot een vervroegde dood. De stoornissen in de stofwisseling betreffen zowel de suiker-, vet-, aminozuur- en eiwit-stofwisseling. Doel van dit proefschrift was de door kanker geïnduceerde veranderingen in de aminozuur- en eiwit-stofwisseling te bestuderen in ratten waarbij tumoren onderhuids geïmplantéerd zijn.

Het proefschrift bestaat uit drie delen. Allereerst wordt een model geïntroduceerd om in een verdoofde, levende rat (*in vivo*) de eiwit- en aminozuur-stofwisseling te meten in verschillende organen (spier, darm en lever). Als tweede worden de effecten van een steeds groter wordende tumor op de eiwit- en aminozuur-stofwisseling van spier-, darm- en lever-weefsel bestudeerd. Tot slot wordt bestudeerd hoe de stofwisseling bij kanker-dragende ratten reageert op een standaard-operatie (chirurgisch trauma).

Deel I

Hoofdstuk 1 van het proefschrift vormt een introductie tot de algemene problematiek van de stofwisselingsstoornissen die gezien worden bij kanker en, meer specifiek, tot de eiwit- en aminozuur-stofwisselingsstoornissen bij kanker. Hoewel veel onderzoek op dit gebied reeds verricht is, heeft het nog niet geresulteerd in een goed begrip van de veranderingen en in een adequate behandeling van de verstoringen.

Hoofdstuk 2 geeft een samenvatting van de tot nu toe bekende mogelijkheden om de eiwit- en aminozuur-stofwisseling te meten. Uit de bestaande literatuur bleek dat het nog niet mogelijk was om dit *in vivo* te kunnen meten in de rat. In hoofdstuk 3 en 4 worden daarom verschillende methoden en modellen getest en gevalideerd om aanmaak en verbruik (omzetting of turnover) van eiwitten en aminozuren te meten. In deze studies tonen we aan dat de zogenaamde arteriele-veneuze verdunningsmethode bruikbaar is om dit te meten en, in gezonde omstandigheden, vergelijkbaar is als de zogenaamde incorporatie-techniek. Indien daarbij een drie compartimenten rekenmodel gebruikt wordt kan tevens de snelheid van het membraan transport waarmee aminozuren vanuit het bloed naar de weefsels gaan,

gemeten worden. In hoofdstuk 3 laten we ook zien dat na langdurig hongeren de arteriele-veneuze verdunningsmethode een verhoging van de eiwitsynthese laat zien terwijl de zogenaamde incorporatie-techniek een daling in de eiwitsynthese laat zien. Het lijkt erop dat de incorporatie-techniek alleen de synthese meet van grote, niet in zuur oplosbare eiwitten, terwijl de arteriele-veneuze verdunningsmethode de synthese meet van grote en kleine eiwitten die zowel zuur-oplosbaar als niet-zuur-oplosbaar zijn. De arteriele-veneuze verdunningsmethode laat dus zien dat na langdurig hongeren de eiwitafbraak en eiwitsynthese omhoog gaat hetgeen deels leidt tot een netto eiwit afbraak (afbraak gaat meer omhoog dan de synthese) en deels tot een verhoogde omzetting van eiwitten (zogenaamde verhoogde eiwit turnover). Of een verhoogde turnover van eiwitten van nut is of dat het een nutteloos en energie-kostend proces is, verdient nader onderzoek.

Deel II

In hoofdstuk 5,6,7 en 8 worden de effecten van een methylcholanthrene (MCA) geïnduceerde tumor op de turnover van eiwitten en aminozuren in spier, darm en lever gemeten. MCA tumoren zijn in veel onderzoeken gebruikt om stofwisselingsstoornissen bij kanker te bestuderen. In deze studies worden echter ratten van de Fisher stam gebruikt. Bij dit type rat leidt de aanwezigheid van een dergelijke tumor tot een drastische afname van de voedselinname dat gepaard gaat met een drastisch gewichtsverlies. In ons laboratorium hebben wij Lewis ratten gebruikt en, gedurende de 3½ jaar onderzoek, hebben wij nooit een afname in de voedselinname gezien en werd slechts een zeer mild verlies aan lichaamsgewicht waargenomen. Uiteindelijk hebben wij een MCA tumor uit Cincinatti (gekregen van WT Chance, Universiteit van Cincinnati) bij Fisher ratten geïmplantéerd op ons laboratorium. Hoewel deze tumor op het laboratorium te Cincinnati duidelijk veranderingen in de voedselinname induceerde, bleek dit niet het geval op ons laboratorium. Dit kan samenhangen met het verschil in het type voedsel dat in beide laboratoria gebruikt wordt, de verschillende bacteriën die in de darm aanwezig zijn bij ratten uit verschillende laboratoria, en mogelijk ook met het verschil in gevoeligheid van verschillende ratten stammen voor zogenaamde ontstekingsmediatoren (zoals ook beschreven is bij onderzoek naar de oorzaak van darmontstekingen). Het valt echter buiten de "scoop" van dit proefschrift om het gedragsverschil in voedselinname te verklaren.

Bij een tumor gewicht van 5-15% en 15-30% van het lichaamsgewicht vindt geen verandering in voedselinname plaats. Dit impliceert dat de stofwisseling wordt aangepast om met dezelfde hoeveelheid voedsel uit te komen. Indien deze

aanpassingen en veranderingen in de stofwisseling vergeleken zouden moeten worden met de kliniek zou deze het best vergelijkbaar zijn met de vroeg ontdekte, nog niet anorectische kanker patiënt met matig gewichtsverlies (b.v. patiënt met kanker van de dikke darm).

In hoofdstuk 5 wordt beschreven hoe met toenemende tumor-grootte de omzetting van eiwitten en aminozuren omhoog gaat indien deze gemeten wordt over het gehele lichaam. Normaal bepaalt de eiwitstofwisseling van spierweefsel het merendeel van de eiwit turnover van het gehele lichaam. Bij eiwitafbraak komen aminozuren vrij en bij afbraak van spiereiwit worden deze voor een groot deel omgezet tot het aminozuur glutamine. Glutamine wordt vervolgens afgegeven aan de bloedbaan en getransporteerd naar andere organen (nier, lever en darm). De eiwit turnover en eiwitafbraak van spierweefsel neemt toe bij kleine tumor-dragende ratten (5-10% van het lichaamsgewicht). Deze eiwitafbraak leidt tot een vergrootte uitscheiding van het aminozuur glutamine. Bij grote tumoren (15-30% van het lichaamsgewicht) neemt de eiwit turnover en eiwitafbraak weer af alsof de spier (metabool) uitgeput raakt. Ook de glutamine turnover daalt naar het niveau van controle ratten. Dit contrasteert met de turnover van glutamine wanneer deze over het gehele lichaam gemeten wordt. De turnover van het gehele lichaam blijft juist onveranderd bij kleine tumoren en verdubbelt bij grote tumoren. De verdubbeling bij grote tumoren kan samenhangen met een vergrootte opname van glutamine als energie substraat en als substraat om nucleotiden te maken door het immuunsysteem. De verhoogde glutamine turnover kan echter ook samen hangen met veranderingen in de glucose stofwisseling aangezien glutamine tevens omgezet kan worden tot een intermediair van de citroenzuur cyclus.

In hoofdstuk 6 wordt beschreven hoe met het groter worden van de tumor de consumptie glutamine door de darm toeneemt. Dit gebeurt in de aanwezigheid van verlaagde glutamine concentraties in de darm zelf. Tevens wordt een toegenomen doorlaatbaarheid van de darm gezien zonder tekenen van atrofie (celverlies). De toegenomen doorlaatbaarheid was wel geassocieerd met een afgenomen differentiatie (ontwikkelingsgraad) van cellen van de darm.

De resultaten van hoofdstuk 7 laten zien hoe de lever aanpast aan de toegenomen behoefte aan eiwitten en aminozuren dat ontstaat door de ontwikkeling van een tumor en de hiermee gepaard gaande activatie van afweercellen. De lever zorgt in het algemeen voor een evenwichtige verdeling van aminozuren, maakt er suiker van en gebruikt een deel voor eigen eiwitsynthese. We laten zien dat, in de aanwezigheid van kanker, aminozuren worden opgenomen door de lever voor met name de aanmaak van structurele eiwitten van de lever zelf. Daarnaast worden aminozuren opgenomen voor zogenaamde export-eiwitten. Deze export-eiwitten zijn voor een groot deel de acute fase eiwitten. We laten tevens zien dat het

zogenaamde α_2 -macroglobuline, een van de meest reactieve acute fase eiwitten in de rat, stijgt bij kanker-dragende ratten naar een niveau dat wij ook zien na bijvoorbeeld een grote operatie in de buik.

In hoofdstuk 8 wordt de stofwisseling van het aminozuur glutamine in de lever omschreven. De lever speelt een belangrijke rol in de stofwisseling van glutamine omdat het zowel glutamine kan aanmaken als afbreken. Dit resulteert normaliter in een zogenaamde "intercellulaire" glutamine cyclus tussen verschillende cel populaties in de lever. Het drie compartimenten model maakt het mogelijk om de snelheid van aanmaak en afbraak in de verschillende cel populaties *in vivo* te meten. De studie laat zien dat de afbraak van glutamine, die plaatsvindt in de zogenaamde periportale cellen, de belangrijkste determinant is die regelt of de lever glutamine netto uitstoot of opneemt. De studie laat verder zien dat de lever bij kleine tumoren de uitstoot van glutamine verandert in een opname van glutamine. Als de tumor groter wordt geeft de lever wederom netto glutamine af. Dit gebeurt in samenhang met verlaagde arteriële glutamine concentraties. Het lijkt erop dat de lever glutamine afgeeft aan de bloedbaan bij groter wordende tumoren om de concentraties in het bloed op peil te houden.

Deel III

Hoofdstuk 9 en 10 werden de effecten van een standaard operatie op de eiwit- en aminozuur-stofwisseling bij kanker bestudeerd. In beide hoofdstukken wordt aangetoond dat na een operatie (chirurgisch trauma) aminozuren gemobiliseerd worden door eiwitafbraak in de darm en in spierweefsel. In de vroege, zogenaamde "ebb fase", na een chirurgisch trauma worden de aminozuren gemobiliseerd uit de eiwitten met een hoge omzettingssnelheid. Dit zijn voornamelijk de eiwitten van de darm. In de latere fase, de zogenaamde "flow fase", komen de aminozuren van de eiwitten met een langzamere omzettingssnelheid vrij. Dit zijn voornamelijk de spiereiwitten. Kanker-dragende ratten kunnen niet normaal responderen op een chirurgisch trauma. De eiwitafbraak in spier weefsel blijft achter en er vindt met name onvoldoende mobilisatie van glutamine plaats vanuit de spier. Aangezien glutamine na een operatie nodig is voor de afweercellen, zal een verminderde beschikbaarheid van glutamine de afweer compromitteren.

De eiwitafbraak van de darm blijft ook achter bij de kanker-dragende rat. Dit gaat gepaard met een toegenomen doorlaatbaarheid van de darm voor lichaamsvreemde stoffen. Beide studies suggereren dat bij kanker de voorziening van belangrijke aminozuren achterblijft vergeleken bij de voorziening van nutrienten indien er sprake is van een gezonde conditie. Het niet kunnen aanpassen van de stofwisseling tijdens

kanker compromitteert met name de acute fase respons en de immuunrespons, terwijl op hetzelfde moment het aanbod van vreemde stoffen waartegen het immuunsysteem moet strijden toegenomen is. Dit kan verklaren waarom kanker patiënten een verhoogd risico hebben op bijkomende ziekte na operaties en is richtinggevend voor keuzes van toekomstige interventies.

Onderzoek naar ondervoeding in Nederland verzin je niet als je in Ethiopië werkzaam bent. Ondervoeding verwacht je ook niet in een overvoedde maatschappij met dierenambulances en bakkers die zich zorgen maken om hun brood als de bisschop oproept het 7e gebod te negeren. Vreemder lijkt het nog dat chirurgen zich ermee bezig houden. Toch is onderzoek naar de oorzaken van ondervoeding ook chirurgisch onderzoek. In 1955 schreven Rhoads en Alexander dat "cases of starvation in the United States at the middle of the 20th century are largely surgical" (Nutritional problems of surgical patients. Ann NY Acad Sci, 1955;63:268-275). Hiermee vertolkten ze een algemene gedachtengang die aan de basis heeft gestaan van de ontwikkeling van parenterale voeding. Tevens is het de basis van het feit dat de chirurgie er historisch gezien mee belast is. Goede chirurgen behandelen en beheersen hun complicaties. Dit doe je niet altijd alleen. Zo ook dit onderzoek. Zoals al in zoveel proefschriften is geschreven, was dit proefschrift niet tot stand gekomen zonder de medewerking van en de samenwerking met anderen. Deze wil ik allemaal bedanken, ook degenen die in het nu volgende niet direct bij naam genoemd worden.

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Tot slot, Nicole, uiteraard ben ik weer even sprakeloos als in ik ooit was aan het Zeelandse strand. Het mooiste hebben we gehad en het mooiste moet nog komen.

Ivo de Blaauw werd op 28 november 1959 geboren te Amsterdam. In 1979 behaalde hij het diploma Gymnasium β aan het stedelijk Gymnasium te Nijmegen. In 1979-1980 studeerde hij Spaanse Taal en Letterkunde aan de Katholieke Universiteit te Nijmegen. In 1980-1981 studeerde hij Geneeskunde aan de Rijksuniversiteit te Gent. In 1981 werd begonnen aan de studie Geneeskunde aan de Katholieke Universiteit te Nijmegen. In 1989 behaalde hij het doctoraal examen en in 1989 het artsexamen. In deze periode was hij gedurende 5 maanden als co-assistent werkzaam in de 1^e-lijnsgezondheidszorg te Niquinohomo (Nicaragua). Van 1989-1991 was hij werkzaam als assistent chirurgie/gynaecologie-verloskunde/interne geneeskunde in ziekenhuis de Gelderse Vallei te Ede in het kader van een tropenopleiding. In 1991 was hij werkzaam als arts in Mekele General Hospital te Tigray (Ethiopië) in het kader van een noodhulp-project van Artsen zonder Grenzen. Na terugkomst werkte hij als arts in het Asielzoekerscentrum te Udenhout, als AGNIO Chirurgie in het Rode Kruis ziekenhuis te Beverwijk en kortdurend in Lokichokio (Kenya) voor Artsen zonder Grenzen en de Verenigde Naties. De laatste werkzaamheden waren i.v.m. het opstarten en verzorgen van gezondheidszorg in een juist ontstaan Sudanese vluchtelingenkamp. Van oktober 1992 tot en met mei 1996 was hij werkzaam als onderzoeker in opleiding binnen het project "Karakterisering van het veranderd aminozuur metabolisme bij kanker en de relatie met cytokines" (N.W.O. project: 904-62-125) bij de vakgroep Algemene Heelkunde aan de Universiteit van Limburg te Maastricht hetgeen leidde tot dit proefschrift. Het onderzoek werd begeleid door Prof. dr. M.F. von Meyenfeldt en dr. N.E.P. Deutz. Sinds juni 1996 is hij werkzaam als arts-assistent in opleiding tot chirurg in het Diaconessenhuis te Eindhoven (opleider Dr. W.J. Prakken). Tot slot, de auteur is getrouwd met Nicole van de Kar.