Maastricht University

Branched chain amino acid metabolism in portal-systemic shunting : experimental studies in the rat

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Branched chain amino acid metabolism in portal-systemic shunting

Experimental studies in the rat

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Branched chain amino acid metabolism in portal-systemic shunting

Experimental studies in the rat

PROEFSCHRIFT

Ter verkrijging van de graad van doctor in de geneeskunde
aan de Rijksuniversiteit Limburg te Maastricht
op gezag van de Rector Magnificus Prof. Dr. H.C. Hemker
hoogleraar in de Faculteit der Geneeskunde,
en volgens besluit van het College van Dekanen,
in het openbaar te verdedigen in de aula van de universiteit
op vrijdag 14 december 1984 des namiddags te vier uur.

door

Jurjen Elle Geert de Boer

geboren te Amsterdam

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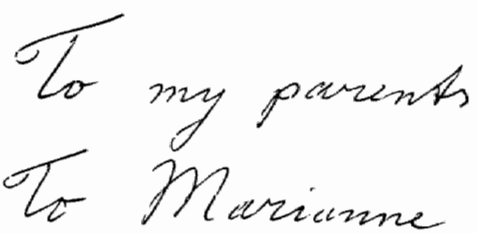
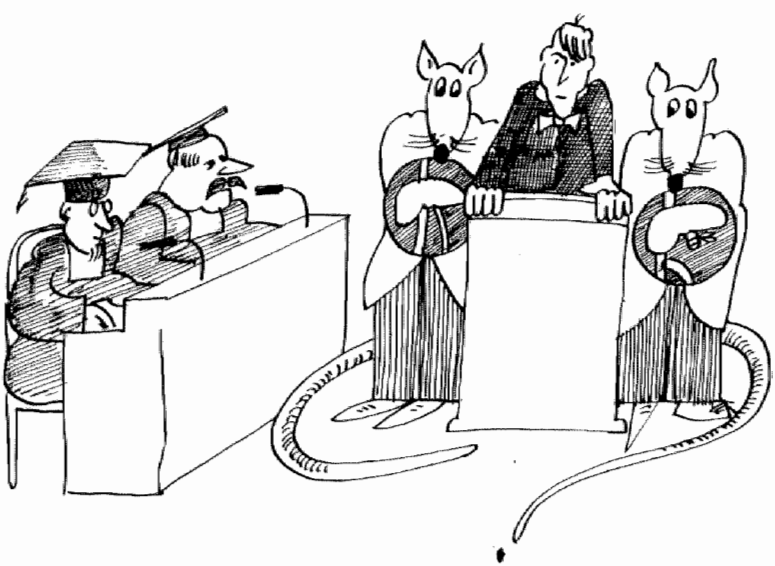
‚Wie jaagt mij voort gelijk het noodlot?'

‚De Ikzelf

die op mijn rug rijdt.'

Tagore





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Abbreviations

AA	aminoacids
AAA	aromatic aminoacids
ADP	adenosine diphosphate
AIB	α-amino isobutyric acid
ATP	adenosine triphosphate
BCAA	branched-chain aminoacids
BCOA	branched-chain oxoacids
BCOA-DH	branched-chain oxoacid dehydrogenase
CNS	central nervous system
CoA/CoASH	coenzyme A
DNA	deoxyribonucleic acid
GABA	γ-aminobutyric acid
FAD^+	flavine adenine dinucleotine
HE	hepatic encephalopathy
IRG	immunoreactive glucagon
IRI	immunoreactive insulin
α-KICA-DH	α-ketoisocaproic acid dehydrogenase
NAA	neutral aminoacids
NAD^+/NADH	nicotinamide adenine dinucleotide
PCS	porta-caval shunt(ing)
PDH	pyruvate dehydrogenase
PSS	portal-systemic shunt(ing)

CHAPTER I

GENERAL INTRODUCTION

Subject of the thesis

The subject of this thesis originates from our interest in disturbances in plasma neutral amino acids in patients and experimental animals with liver disease or liver insufficiency. The thesis will focus especially on the decrease in plasma branched chain amino acid levels in liver disease, because that decrease may be relevant for hepatic encephalopathy and for the higher rate of catabolism prevailing in severe hepatic failure. Aspects of basic and clinical interest in branched chain amino acid metabolism will be reviewed in this chapter. Diseases influencing branched chain amino acid levels will also be discussed, as well as metabolic factors influencing the degradation of branched chain amino acids at the cellular level. Finally, a short justification will be given of the experiments that were performed.

Aspects of clinical interest in branched chain amino acids (BCAA)

In recent years, much attention has been focused on several aspects of BCAA metabolism:

* BCAA may serve as alternative fuel in fasting (93), in severe catabolic states (7) and during extensive exercise (110).
* BCAA, especially leucine, may serve as a possible regulator of protein turnover (10). Leucine, in particular, has anabolic effects (11,16).

* Many clinical conditions, diseases and altered nutritional states, are accompanied by altered BCAA levels in plasma and very often also intracellularly in muscle and other tissues (2,26,49,50).
* A disturbed plasma neutral amino acid (NAA) pattern, including low BCAA levels, is clinically associated with hepatic encephalopathy (37,70,13, 122).
* Based upon a normalization of the lowered plasma BCAA levels in several disease states, the administration of BCAA-enriched solutions is advocated and used as a possible therapy for patients with hepatic encephalopathy (38,43) and/or in severe catabolic states such as sepsis (42,44) or trauma (5,41). However, there is still no real proof that these solutions, orally or parenterally administered, are more beneficial than conventional solutions.

These aspects of BCAA will be discussed briefly.

BCAA: alternative energy substrate

Extensive exercise, fasting, starvation and catabolic diseases are clinical situations with a net negative nitrogen balance. Shortly after extensive exercise, plasma BCAA levels are slightly decreased in healthy man (33). Semi-starvation and starvation are accompanied by lowered plasma BCAA levels (26,31). Severely catabolic patients or animals, e.g. after trauma or with sepsis or severe liver failure, also have low plasma BCAA levels (2,49,50,67,113). It has been suggested that, in these conditions, BCAA may serve as alternative energy substrates (13,14,54,95,117). For some of these conditions, an increased uptake and degradation of BCAA in muscle has been reported (1,103,132), and most probably, these BCAA originate mainly from skeletal muscle proteolysis (71). A muscle energy deficit has been proposed (7) and this may be explained by different mechanisms in different clinical states. It is known that during exercise, liver blood flow decreases whereas simultaneously the blood flow to the exercizing muscles increases. While transamination of the BCAA in muscle largely depends upon the delivery of these amino acids (75), in working muscle transamination and probably further degradation of BCAA may be increased simply because of the increase of blood flow. It is even suggested that, in exercise, it might be advantageous to use amino acids as energy substrate

(109). However, there is no proof that increased catabolism of BCAA is induced by exercise.

In severely catabolic patients or animals, that have a strongly negative nitrogen balance, the possible explanation is somewhat different. It has been suggested that in these subjects, BCAA may serve as alternative energy substrate when less glucose or lipids are available or when these substrates cannot be utilized. It has even been suggested that, in addition to plasma BCAA, these subjects presumably consume their own muscle protein to supply energy (7). The shortage of energy in muscle and other organs might be the result of a diminished capacity to utilize glucose and fatty acids, due to glucose intolerance and insulin resistance, which have indeed been observed after trauma and in sepsis (3a) and have recently also been reported in liver cirrhosis (6,128). The insulin resistance may be due to a decreased number of functional receptors (6) or to post-receptor defects. In addition, the raised insulin level might inhibit lipolysis resulting in a decreased release of free fatty acids from adipose tissue. However, there is no evidence for decreased lipolysis in cirrhosis, which pleads against a muscle energy deficit.

Furthermore, the contribution of BCAA degradation to overall energy metabolism in severe catabolic states can only be of minor importance. In severe disease an adult may loose 20 g nitrogen per day, which is equivalent to 120 g protein. containing 36 g BCAA. If all this would be muscle protein, these BCAA would furnish 150 cal, assuming that complete degradation occurs. Our own data (chapters III and IV) and data from Kipnis group suggest however, that degradation is incomplete and may become even less complete after PCS. This implies that the contribution of BCAA to the total energy coverage may be even less (50-100 cal per day for an adult). This is only a minute part of the total daily energy expenditure (2500-3000 cal). Apparently, the body is able to cover 95% or more of its energy needs from other energy sources, even in an extremely catabolic state. Therefore, it is unlikely that muscle protein degradation would significantly contribute to the energy requirements, especially in the presence of increased fatty acid availability.

Leucine: anabolic properties and regulator of protein turnover

A number of factors known to be important for the growth of muscle has been shown to alter in vitro the overall rates of protein degradation as well as protein synthesis in skeletal and cardiac muscle. Among these are glucose, insulin, amino acids, arachidonic acid and prostaglandins E_2 and $F_{2\alpha}$ (53,112). Insulin is undoubtedly the most potent and most important physiological factor regulating overall protein balance in skeletal muscle. A rise in insulin levels after meals stimulates the net uptake of amino acids by skeletal muscle and their incorporation into protein. The fall in insulin postabsorptively and during fasting, signals a net release of amino acids from muscle (28,116). In muscle and other tissues, insulin not only stimulates protein synthesis but also inhibits protein degradation (47,51,52,77,88,107). These two hormonal actions have complementary effects in producing net tissue protein gain. The effects on protein synthesis and degradation are also observed in the absence of exogenous glucose or amino acids and thus, are not only the result of the ability of insulin to stimulate nutrient transport (47). Glucose by itself also inhibits protein degradation without, however, affecting protein synthesis (47).

The presence of plasma amino acids stimulates protein synthesis and diminishes protein breakdown. It is evident that all amino acids or at least all essential amino acids must be present to be able to synthesize protein. However, the stimulatory effect on protein synthesis is mainly attributable to the presence of BCAA (78). This being established, the BCAA were tested on their ability to stimulate protein synthesis, both separately and in combinations. From these studies it was found that leucine in particular promotes protein synthesis and inhibits protein breakdown (10,11,47,56,78,107). No other plasma amino acid has such an influence on protein turnover. The effects of leucine on protein turnover in muscle have been illustrated in vitro at concentrations of leucine (0.1-0.5 mM) that are, at least partly, in the physiological range. Therefore, this mechanism of regulation may be active in vivo. In vitro studies demonstrated that proteolysis was progressively inhibited at high leucine concentrations (0.3-1.0 mM) (126),that can be present in plasma of patients receiving amino acid mixtures intravenously or as dietary supplements.

In vitro studies of protein synthesis and proteolysis showed that the presence of a competitive inhibitor of leucine transamination (e.g. cycloserine) dissociated the two anabolic effects of leucine. The inhibition of protein breakdown requires leucine catabolism, while the enhancement of synthesis does not (56,126). The α-keto analogue of leucine, α-ketoisocaproic acid, seems to be responsible for the inhibition of protein breakdown. Many reports have been published in which infusions of BCAA or leucine alone are claimed to reduce severe protein catabolism in patients with strongly negative nitrogen balance (5,41,42,43,44). In patients, α-ketoisocaproic acid infusions have also been claimed to improve nitrogen balance and should even be more effective than leucine in patients with renal failure (87), as α-ketoisocaproic acid may have a more rapid transport into cells than leucine (94).

Altered plasma BCAA levels

Altered BCAA levels in plasma are encountered in health and disease. In healthy subjects, fluctuations of plasma BCAA levels are relatively small and always transient, e.g. after a protein-rich meal or after an overnight fast. In disease, deviations in plasma BCAA levels are often more pronounced and persist over long periods. When normal food intake is maintained, diurnal variations in plasma BCAA are superimposed on the deviating plasma BCAA levels induced by the disease, unless (multiple) organ failure prevents "normal" processing of ingested amino acids. In several conditions with deviating plasma BCAA levels, intracellular concentrations (mostly based on intramuscular measurements) change proportionally to the plasma concentrations, as, for example, in starvation (2,26) where both plasma and muscle BCAA concentrations are increased. That this does not always occur, is demonstrated in severe injury and sepsis which are accompanied by strongly increased intramuscular concentrations of BCAA, while plasma BCAA are only slightly increased in severe injury (2,49) and are normal or even decreased in sepsis (2). Other diseases with altered plasma BCAA levels are diabetes (30,130,131), obesity (40), renal failure (74) and liver insufficiency and portal systemic shunting (67,113). The direction of change in plasma BCAA levels in several diseases will be indicated under: "Disease states influencing plasma BCAA levels".

Hepatic encephalopathy and amino acid neurotransmitter theory

Hepatic encephalopathy (HE) is a neuropsychiatric syndrome complicating acute as well as chronic hepatocellular failure and/or portal systemic shunting. Despite intensive investigations, mostly in experimental animals, and the demonstration of correlations between possibly involved agents and the mental state (grade of HE) in patients, and despite the steadily growing amount of information on metabolic changes associated with liver failure, the pathogenesis of HE is still poorly understood. Recently, Zieve mentioned 20 different abnormalities in liver failure which might conceivably contribute to HE (133).

An abnormal plasma neutral amino acid (NAA) pattern is generally found in patients with liver cirrhosis (50,67,113). This pattern consists of an increase of aromatic amino acids (AAA; phenylalanine, tyrosine and tryptophan) and decreased levels of the BCAA (leucine, isoleucine and valine). In addition, plasma methionine is often increased. All NAA are dependent on the same transport system for their passage through membranes (15); this also applies for their transport across the blood-brain barrier (68,120). Consequently, NAA compete for entry across the blood-brain barrier. Entrance of the individual NAA into the brain will be determined by their concentrations in plasma and thus, the plasma pattern will be reflected in the brain. AAA serve as precursors of both normal or physiological neurotransmitters, and abnormal or pathological neurotransmitters.The latter are also termed false neurotransmitters. Normally, tyrosine is the precursor of dopamine and norepinephrine (and also epinephrine); tryptophan is the precursor of serotonin. Deviations of normal NAA concentrations in brain, and thus, of neurotransmitter precursors, may induce an abnormal neurotransmitter pattern in brain. This theory was supported by several studies in experimental animals (21,23,69). Therefore, the distorted plasma NAA pattern in patients with severe liver disease and portal systemic shunting was associated with the occurrence of hepatic encephalopathy (39,70,90, 122). The first hypothesis which attempted to relate an abnormal neurotransmission to the pathogenesis of HE, in combination with the altered plasma NAA pattern, was the "amino acid - neurotransmitter" hypothesis or "false neurotransmitter" hypothesis, formulated by Fischer and Baldessarini (36). It was proposed that in liver failure, the "false" neurotransmitters

octopamine and β-phenylethanolamine were synthesized from tyrosine and phenylalanine respectively, in preference to dopamine and norepinephrine (70). The altered neurotransmitter pattern would result in neural inhibition due to the competition of physiological and false neurotransmitters for receptor sites. An analogous mechanism might be responsible for increased cerebral levels of serotonin, due to increased cerebral tryptophan. According to this theory, serotonin may contribute to neural inhibition in HE by blocking its own receptors and by interfering in the degradative pathway of tyrosine to octopamine and norepinephrine, that is controlled, among others, by phenylalanine, tryptophan, false neurotransmitters and serotonin (35). This amino acid neurotransmitter theory stimulated the use of BCAA enriched solutions in encephalopathic patients, with the aim to restore normal brain function. Further investigations yielded data that supported the original hypothesis, but also data that put restraints on the theory. The theory was readjusted by implicating ammonia, via formation of glutamine, as the agent promoting transport of NAA across the blood-brain barrier (70). Objections have been raised against this theory (19,34,85, 133). This leaves room for another theory: the γ-aminobutyric acid theory. Amino acid neurotransmitters are responsible for almost all neurotransmission in the central nervous system (CNS). About 90% of all neurons bear receptors for glutamate and about 40% for γ-aminobutyric acid (GABA). In mammalian tissues , GABA is produced exclusively in the brain, but gastrointestinal bacteria can also produce GABA. Normally, GABA, released into the portal vein, is almost completely cleared by the liver. In liver insufficiency, GABA might escape clearance and enter the systemic circulation. To explain a depressing effect on the brain, increased permeability of the blood-brain barrier has been postulated in liver insufficiency because amines cannot enter the brain under normal conditions. Although the theory is attractive, much work needs to be done to prove its validity. Older theories that suggested short-chain fatty acids and mercaptans as causal agents for HE (134,135), largely based on correlations between the concentrations of these agents and HE, have lost importance. However, using new techniques for the determination of mercaptans in breath and serum (124,125), a positive correlation was reported in cirrhotics with and without HE. The oldest theory, indicating ammonia as the toxic agent per se, lost its value as such, but ammonia still may play a role in ex-

plaining the existing low BCAA levels in liver failure (76) and may induce a chain of events which finally results in changes in neutrotransmission.

BCAA-enriched solutions

The amino acid neurotransmitter theory and the "peripheral muscle energy deficit" theory have advocated and initiated the use of BCAA-enriched solutions in severe liver disease and in severe catabolic states.

Providing BCAA-enriched solutions to severely injured and septic patients, as well as to liver patients would supply metabolizable energy substrates, expecting thereby to obviate the need to break down fibrillar muscle protein. Moreover, advantage could be taken of the special anabolic effect of leucine by its regulatory role in protein turnover. Infusions of BCAA solutions into laboratory animals and patients with sepsis, stress or (operative) trauma seemed successful. Freund reported an improved nitrogen balance in rats after laparotomy (44), and Blackburn had positive results in septic rats (5). In small numbers of patients, uncontrolled trials suggested an efficacious effect by improving nitrogen balance without serious complications (4,42,97). However, reliable double-blind prospective trials have not been reported. Such a trial in our institute did not demonstrate a clinical benefit of BCAA enrichment (unpublished data).

Feeding BCAA-enriched solutions (or solutions containing only BCAA) to patients with liver failure are based partially on the same arguments, serving the same purposes. However, administration of such solutions to encephalopathic or comatose patients or experimental animals is largely based on the amino acid neurotransmitter theory. The aim is the correction of the pathologic NAA pattern in plasma and consequently in brain, thereby restoring normal concentrations of physiological neurotransmitters and consequently the normal neurological state. In dogs in which encephalopathy was evoked as a consequence of surgical porta-caval shunts, infusions of BCAA-enriched solutions seemed beneficial in suppressing neurological disorders and reducing mortality (121). Many clinical trials in patients have been reported but the results are controversial, ranging from beneficial (38,96,114) to harmful (27). Most studies in this field are not completely comparable, however. The many variables include regimens, quantities of BCAA given, duration of infusion and above all, the diversity in patients.

In addition to trials with BCAA or BCAA-enriched solutions, administered orally or parenterally, trials have been started infusing solutions enriched with the α-keto acid analogues of the BCAA. It was assumed that (trans)amination occurs at sufficient rates in the body, thereby lowering the elevated ammonia levels. Especially the ornithine salts of these keto-acids have been used (64,115). Little benefit has been reported until now.

Disease states influencing plasma BCAA levels

The aspects of BCAA metabolism discussed above, receive much attention nowadays. Extension of knowledge of BCAA metabolism is necessary, especially with regard to regulatory factors in pathologic conditions. The theories mentioned (amino acid neurotransmitter theory and muscle energy deficit theory) and therapies based upon these theories, originate from the observation that plasma levels of BCAA are decreased in several pathologic conditions. However, the cause of these low levels is still largely unknown.

Malnutrition and starvation

As early as in 1963, a depression of almost all plasma amino acids, including the BCAA, has been reported in tropical kwashiorkor (65), a syndrome that is common in the Third World and that is claimed to arise from diets adequate in calories but grossly deficient in protein.

Similarly, long-term starvation results, after an initial rise in plasma BCAA levels, after 2-7 days of fasting in a decrease of plasma BCAA levels (31). This decrease becomes more pronounced as starvation progresses (31). Sherwin reported a progressive decrease in the metabolic clearance rate of leucine throughout starvation (118). From in vivo measurements, there is evidence that the initial rise in plasma BCAA levels is, at least partially, a consequence of a decreased clearance in association with unchanged rates of net release from muscle (106). The later decline in plasma BCAA levels reflects, at least partially, the fact that net release from muscle tissue falls (32) to an even greater extent than the overall rate of removal from plasma (118). In contrast to the evidence of decreased

removal from plasma in vivo throughout starvation, in vitro studies indicated an increase in leucine oxidation by muscle tissue from fasted rats (55). Seven years later, in vitro studies showed a decreased oxidation of BCAA by adipose tissue in starving rats (45). That adipose tissue had a significant capacity to catabolize BCAA, had only just been recognized (45,121). Thus, although adipose tissue has generally not been considered to be of overriding importance in BCAA metabolism, it may, in fact, be responsible for the reduced utilization of these amino acids during starvation. The regulatory mechanism is still obscure.

Diabetes

For a long time, it has been recognized that insulin plays an important role in the regulation of amino acid metabolism. In accordance with this notion, an altered exchange of amino acids in liver and muscle has been observed in diabetics in both the postabsorptive state and after a protein-containing diet. BCAA are not an exception to this (130). Fasting untreated diabetics have highly elevated plasma concentrations of BCAA (130) and even more increased levels after feeding a protein meal (131). In vitro studies have demonstrated that, in muscle from streptozotocin-induced diabetic rats, BCAA are catabolized to a greater extent, compared with controls (9; unpublished data), despite a less facilitated entry of BCAA into muscle cells by lack of insulin. These findings, in combination with the strongly elevated BCAA levels in plasma, suggest a greatly decreased uptake and utilization of these amino acids at another extrahepatic site. Obviously, adipose tissue is a likely candidate as suggested by decreased BCAA catabolism in vitro in adipose tissue from untreated streptozotocin-induced diabetic rats (60; unpublished observations). Moreover, adipose tissue metabolism of BCAA is readily influenced by lack of insulin.

Obesity

Obese subjects have normal or slightly increased plasma BCAA levels in the postabsorptive state and greatly increased levels in the fed state (40). Entrance of BCAA into peripheral tissues is hindered by the existing insulin resistance. In vitro experiments with tissues from obese Zucker

rats (fa/fa) and from their lean littermates (Fa/fa), showed a decreased oxidation of leucine in both muscle and adipose tissue in the obese group, even in the fasted state. Diminished uptake and utilization of BCAA, due to insulin resistance may therefore, at least partially, be responsible for the high circulating BCAA levels in plasma in obesity.

Sepsis and trauma

Plasma BCAA levels have been shown to be depressed in patients with sepsis (2,42) and trauma (2,26,49). In recent investigations, it was demonstrated that severe trauma and sepsis are associated with tissue depletion of energy-rich compounds and a decreased cellular energy level (79). These findings might be in accordance with the proposed energy deficit theory. In sepsis and severe injury, muscle cells may oxidize BCAA to compensate for reduced intracellular oxidation of glucose (95) and, in general, for inadequate degradation of energy substrates (117). In sepsis and trauma intracellular glucose concentrations are increased tenfold (P.Fürst, personal communication) and plasma fatty acids are also increased. This suggests an impairment of substrate use, rather than lack of fuel availability in the cell. A role of degradation of BCAA in the maintenance of the energy charge of the cell is still unclear. Therefore, low plasma BCAA levels in sepsis and trauma can not yet been explained.

Liver disease, liver insufficiency and portal-systemic shunting

Disturbances in the plasma NAA pattern have been reported in liver disease, in man (67,113) and in experimental animals with liver insufficiency and/or portal-systemic shunting (48,121). AAA, either from exogenous or from endogenous sources, are exclusively broken down by the liver, where the carbon skeleton may be degraded completely to CO_2 or may be used as substrate for gluconeogenesis. Consequently, it is not surprising that elevated plasma levels of AAA are observed in both acute and chronic forms of decreased liver function. In addition, free tryptophan levels are increased, altering the bound:free ratio. This is due to competition with increased concentrations of fatty acids for binding sites on albumin which is generally low in concentration. As a result, increased levels of free

tryptophan contribute to the increase of plasma AAA in liver failure. However, decreased plasma levels of BCAA are part of the deranged AA pattern in chronic liver failure. In acute liver failure, plasma BCAA levels are approximately normal (108,113). BCAA are unique in that they are the only essential amino acids (EAA) that are almost exclusively metabolized extrahepatically. Hepatic uptake of BCAA from portal venous blood is less than 15% (3). Muscle (55,93,119), adipose tissue (57), brain (93,119) and kidney (55,119) are able to metabolize BCAA to CO_2. The peripheral breakdown of BCAA can only explain why plasma BCAA levels are not increased in liver failure. Explanations for the observed decreased plasma BCAA levels are still speculative. As mentioned before, states of malnutrition and long-term starvation are accompanied by low plasma BCAA levels. Patients with liver disease are often malnourished and partly starved. Furthermore, it is still unclear whether lowered plasma BCAA are caused by hepatic parenchymatous disease itself or by portal-systemic shunting. It has been demonstrated in dogs (123), that porta-caval shunting induces low plasma BCAA levels 1, 2 and 4 weeks post-operatively. At these time points the dogs were happy, walking and eating dogs. Similar results will be described for rats in chapter II. This demonstrates that, at least in experimental animals, portal-systemic shunting itself can induce low plasma BCAA levels in the absence of malnutrition or starvation. In addition, parenchymatous disease is no prerequisite to cause the altered plasma AA pattern. It appears to us that a distinction between metabolic changes due to parenchymatous disease and hepatic inflow is artificial because both entities together determine total hepatic metabolic function.

The possible role of several organs and tissues in the in vivo regulation of plasma BCAA levels

In the physiological situation, plasma BCAA levels vary within narrow limits, even after ingestion of large amounts of protein or after an overnight fast. It has been briefly mentioned already, that BCAA are almost exclusively broken down extrahepatically, i.e. in muscle, adipose tissue, brain and kidney, in that quantitative order. It appears that the availability of BCAA partly determines its plasma levels. Postabsorptive plasma

BCAA levels are lower than levels after a meal. However, the total amount of free BCAA, that circulates in the plasma in excess after a meal compared to the postabsorptive state, is negligible compared to the total amount of BCAA ingested. After consumption of a protein rich Western meal, only 10-20% of the ingested BCAA is needed for protein synthesis. Consequently, large amounts of ingested BCAA must be taken up and metabolized in some way after a meal.

Adipose tissue

It has been suggested by Goodman (46,57,59) that adipose tissue, in particular, can degrade BCAA and can utilize part of the carbon skeleton for fatty acid synthesis. An excess of dietary BCAA might be partly degraded in such a way and some BCAA-derived carbon directly stored in adipose tissue.

Muscle

Muscle might be another important tissue for degradation of BCAA (55,93) but its homeostatic role in BCAA metabolism may be limited for several reasons:

* Protein synthesis cannot serve to store quantitatively important amounts of BCAA because, as mentioned, only a small part of the BCAA ingested is used for protein synthesis.
* Only degradation of the carbon skeleton may fulfil a homeostatic role because storage of BCAA-derived carbon into fatty acids in muscle is negligible (93). Degradation is inhibited when energy stores (ATP, phosphocreatine and glycogen) become saturated. This occurs readily during or after a meal when the organism switches to storage, instead of combustion.

Brain, kidney and other organs

It has been reported that brain and kidney can degrade BCAA (55,93, 119). The contribution of these organs to total BCAA catabolism in the body is, due to their small size, quantitatively unimportant. This is illustra-

ted in table I, in which the irreversible degradation of ^{14}C-(1)-L-leucine (s.a. 0.25 mCi/mmol; [leucine] = 0.1 mM) in several organs is listed. The reported activities must be viewed as approximate activities, because identical conditions, optimized for diaphragm and adipose tissue, were used for all organs. We realize that these conditions are suboptimal for skeletal muscle in which no perfect linearity of the recorded activity was measured with respect to tissue weight and reaction time, most probably indicating a limitation in the diffusion of substrate. Therefore, we made

IRREVERSIBLE LEUCINE DEGRADATION

Tissue	Activity (pmol/mg.90 min)	% of total body activity	
		based on skeletal muscle	based on diaphragm
Adipose tissue	152	57.3	23.5
Diaphragm	177		71.5
Skeletal muscle	31	29.8	
Heart muscle	55	0.6	0.2
Small intestine	18	0.4	0.2
Brain	152	2.5	1.0
Liver	39	5.9	2.3
Kidney	134	3.3	1.3

Table 1
Irreversible degradation of ^{14}C-(1)-L-leucine (s.a. 0.25 mCi/mmol;0.1 mM) by several organs of normal male Sprague-Dawley rats (approximately 290 g), determined in triplicate and expressed in pmol/mg wet weight.90 min (n=4). A rough calculation of the percent contribution of each organ is given, based on organ weights and on the assumption that adipose tissue contributes 15% to total body weight and muscle 40%. Because of the different activities that were recorded in diaphragm and skeletal muscle, two calculations have been carried out (see text).

two rough calculations of the percent contribution of the organs to the total in vitro leucine decarboxylation, based on the recorded activity in skeletal muscle, and based on the recorded activity in diaphragm, assuming that the in vivo activity with optimal substrate supply will more closely resemble the activity recorded in diaphragm (table I). Probably the truth is somewhere in between. The only purpose of this table, however, is to illustrate that organs other than muscle and adipose tissue are quantitatively less relevant with respect to the degradation of plasma BCAA.

Liver

Species differences may interfere with the exclusive extrahepatic degradation of BCAA. In rats for example, another mechanism may also operate in homeostasis. After transamination of BCAA in muscle in rats, a significant part of the resulting branched chain oxo acids (BCOA) is released into the circulation (25,80) ; further degradation can continue in the liver. In man, this is quantitatively unimportant (25).

In summary, adipose tissue and muscle are quantitatively the most important organs for the disposal of an excess of ingested BCAA or similarly, for causing decreases in plasma BCAA concentrations. It should be noted, however, that the absolute amount of BCAA that must be metabolized in excess to cause lower plasma levels, is minute (probably 2-3 ‰) compared with the amounts that are metabolized (degraded and/or stored) after a meal. Furthermore, most BCAA are located intracellularly and this intracellular pool is much greater than the plasma pool of BCAA. Obviously, it is virtually impossible to measure increased degradation necessary to decrease plasma BCAA levels, in vivo. No technique would be sensitive enough to detect these small differences. Therefore, it is more appropriate to study in vitro the ability of peripheral tissues (i.e. muscle and adipose tissue) to degrade BCAA, present at physiological concentrations. The ability to metabolize BCAA in these tissues may be influenced by several factors:

* Mechanism(s) for transport of BCAA across membranes of cells and cellular organelles.
* Activity of rate-limiting enzymes in the degradation of BCAA.

* Sensitivity of the tissues to hormones exhibiting stimulatory or inhibitory effects, e.g. stimulation of transport over cell membranes or induction of enzymes in the degradation of BCAA or influencing the ratio of active to total enzyme(s).

In vitro influence on BCAA metabolism in non-hepatic tissues; relevance for the in vivo situation

The observation in vitro that muscle is able to metabolize BCAA in significant quantities (93) has led to the hypothesis that lowered plasma BCAA levels result from increased peripheral degradation (90.122). In general, only muscle was held responsible for this increased peripheral breakdown. However, studies from Goodman (46,57,59) and from our own group (121) suggested that adipose tissue may also have a significant capacity to catabolize BCAA.

Sensitivity to hormones: influence of insulin

In the literature, several reports suggest an inverse correlation between plasma BCAA levels and plasma insulin levels. Portal-systemic shunting is accompanied by elevated insulin levels and decreased BCAA levels (67,90,113,122). Unsupplied insulin-dependent diabetes is accompanied by low insulin levels and high BCAA levels in plasma (30,130,131). In obesity, insulin levels and BCAA levels are high (29,40), but insulin resistance is present (73,98). In early sepsis (hyperdynamic phase), plasma insulin is increased and BCAA are low (42). The inverse correlation between insulin levels and BCAA levels, and the demonstration that the human forearm exhibits a net uptake of BCAA after a bolus injection with insulin (105), suggest a causal relationship between insulin and BCAA and the existence of a general mechanism to change plasma BCAA levels in several pathological conditions. Possibly plasma insulin may influence plasma BCAA levels by changing the ability of one or more peripheral tissues (i.e. muscle and adipose tissue) to degrade BCAA. In that case, low BCAA-degrading activity may be expected in such tissues in diabetes and obesity, and an increased activity in early sepsis, after trauma and after portal-syste-

mic shunting. However, the influence of insulin on muscle and adipose tissue, differs in some way in both physiological and pathological states. When these differences interfere with BCAA metabolism in these tissues, the suggested causal relationship between insulin and BCAA degrading activity may be expected to be observed only in the tissue that is predominantly responsible for the deviated plasma BCAA-levels. In this respect, it is important to note that insulin resistance has been observed with regard to the metabolism of glucose, in patients with liver disease (17,18,111). Hepatogenic diabetes has already been described before (86). More recently, this has been ascribed to insulin resistance, partly at the receptor level and partly at the postreceptor level (6,72). It has been the subject of recent studies to investigate whether insulin resistance also affects BCAA metabolism. It was shown, employing insulin-clamp techniques, that cirrhotic patients exhibited "BCAA-intolerance" after a protein meal (81,82). This suggests that indeed insulin resistance in cirrhotics also applies to BCAA, but is in contrast with the observed low postabsorptive plasma BCAA levels in liver patients, if hyperinsulinism in cirrhotics would affect plasma BCAA levels. On the other hand, insulin resistance has been demonstrated in monocytes, erythrocytes and muscle (6), but not in adipose tissue (63), considered to be important for disposal of BCAA, and thus for changes in plasma BCAA levels.

Enzymatic activity

An altered peripheral metabolism of BCAA might also be regulated at the level of the rate-limiting enzyme(s) in the degradation of BCAA. In most extrahepatic tissues tested, including muscle and adipose tissue (12,22,59,94), the rate-limiting enzyme in the catabolic pathway of BCAA is the branched chain oxoacid dehydrogenase (BCOA-DH; EC 1.2.4.4.), sometimes also indicated as branched chain oxoacid decarboxylase or named after the particular branched chain oxo acids: α-ketoisocaproic acid (keto-leucine), α-keto- β -methyl valeric acid (keto-isoleucine) and α-keto isovaleric acid (keto-valine). To avoid confusion, the term dehydrogenase is used in this thesis, because some decarboxylating activity is reported to be present in the cytosol of liver cells, catalyzed by an enzyme that is independent of some co-factors required for BCOA-DH activity (i.e. NAD^+ and CoASH), and

exclusively reacting with α-ketoisocaproic acid as substrate (61,84). The first step in the degradative pathway of BCAA is the reversible transamination of the amino group to α-ketoglutarate, catalysed by branched chain aminoacid α-oxoglutarate amino tranferase (EC 2.6.1.6.) (fig.1), producing glutamate and the corresponding branched chain oxoacids. BCOA-DH is the second enzyme involved in the degradation of BCAA. While the transaminase is rate-limiting in liver (119), the dehydrogenase appears, as already mentioned, to be rate-limiting in other tissues (12,22,59,94). BCOA-DH is located on the inner surface of the mitochondrial inner membrane, as reported for liver, kidney and muscle cells (83). BCOA-DH, not yet isolated in pure form, has many similarities with the well known pyruvate dehydrogenase (PDH) enzyme complex (20,104), which is also located at the inner surface of the inner mitochondrial membrane. Like the PDH-complex, the BCOA-DH complex has a high molecular weight (about $3x10^6$ dalton) and contains three enzymes: BCOA-DH, dihydrolipoyl transacetylase and dihydrolipoyl dehydrogenase. Both enzyme complexes, PDH and BCOA-DH, have, in partially purified preparations, the same co-factor requirements: NAD^+, CoASH, dihydrolipoic acid, FAD^+ and thiamine pyrophosphate (TPP). The similarities go even further. PDH has two regulatory enzymes: pyruvate dehydrogenase kinase which inactivates PDH in the presence of ATP and Mg^{++}, and pyruvate dehydrogenase phosphatase, which activates the enzyme in the presence of Mg^{++} or Ca^{++} (89). BCOA-DH is inactivated by phosphorylation and reactivated by dephosphorylation(66,91,92,99,101). The similar location of both enzyme complexes, and especially binding to the membrane, means that both enzymatic activities will be present together in cell free preparations and purified mitochondrial fractions. However, the BCOA-DH activity seems to be 5% or less of the PDH activity (46). The possibility that BCOA serve as alternative substrates for PDH, has been excluded by heat inactivation experiments at 44^oC, by selective inactivation experiments with antibodies directed against PDH and by competitive inhibition of BCAA (46,60). Another similarity of BCOA-DH with PDH is the fact that in vitro activity in adipose tissue can be influenced by insulin (46,60). Although only short-term influence has been reported, this is especially interesting with regard to the hyperinsulinism noted in liver patients. Goodman described an increased BCOA-DH activity in adipose tissue segments after short-term preincubation with a high concentration of insulin (1 mU/ml).

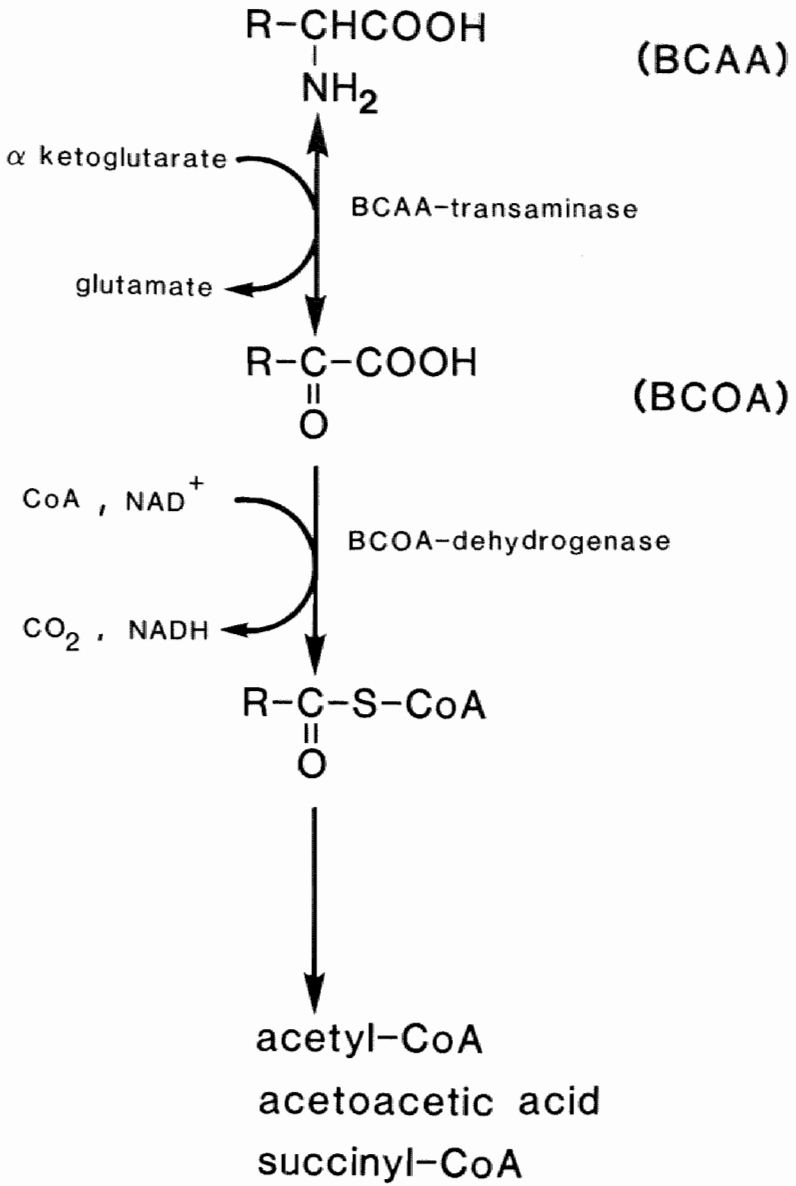

Figure 1
Schematic presentation of the first and second step in the degradation of BCAA.

This increased activity was maintained after homogenization of the tissue (60). In addition, he reported a short-term influence of insulin in cell-free preparations of adipose tissue, but these effects were only observed when the activity was assayed at substrate concentrations below 50 μM (60). Disappearance of the effect of insulin at higher substrate concentrations suggests that insulin might lower the apparent Km of the enzyme, rather than increase the Vmax. This suggestion was confirmed by a shift of the apparent Km of 101 μM to 32 μM in a cell-free preparation of adipose tissue, prepared after incubation of whole tissue segments without and with insulin (1 mU/ml). However, the insulin concentration used, was supraphysiological. In muscle and liver preparations, the enzymatic activity can be influenced by addition of carnitine during the assay (127,129). End-product inhibition (NADH, CoA-esters) and competitive substrate inhibition (α-oxoacids, but not pyruvate; some CoA-esters compete with CoASH itself) has been reported (8,100,104,129). In whole tissue, other factors (e.g. muscle work, glucose, fatty acids and Ca^{+}) may influence BCOA-DH activity by interfering with the permeability of membranes or by providing other metabolizable substrates to cover, for example, energy needs of the cells and thus depending on the type of tissue.

Transport of BCAA and BCOA

Transport of substrates of BCOA-DH have been investigated in liver- and muscle preparations and in blood cells by several authors. In red cells, BCOA transport across cell membranes takes place at rapid rates mediated by either the Cl^{-}/HCO_3^{-} anion transporter or the monocarboxylate transporter as evidenced by the inhibition by specific inhibitors of these transport systems, respectively α-cyano-β(1-phenylindol-3-yl)-acetate (UK 5099) and diïsothiocyanostilbene disulphonic acid (DIDS) (62). Inhibition by α-cyanocinnamate, an inhibitor of the monocarboxylate translocator, results in a 60% reduction of transport of oxoacids over the mitochondrial membrane (102). In contrast to pyruvate transport, the transport of the BCOA does not occur by passive diffusion. A nonmetabolizable amino acid analogue, the α-amino isobutyric acid (AIB), was used as a marker of amino acid transport over the adipocyte membrane, to explore the possibility that insulin might stimulate leucine oxidation by increasing its transport.

Leucine and AIB share a common carrier system (57,58). Adipose tissue failed to respond to insulin with an increase in the rate of transport of AIB, but AIB was readily concentrated intracellulary to about five times the extracellular concentration after 40 minutes. While increased uptake of BCAA in muscle has been reported in several physiological and pathological conditions (24,105), there are, as yet, no indications that in vitro transport of BCAA or BCOA is limited or stimulated (receptor defects and decreased receptor numbers for insulin excluded) and that transport may have influence on the reported BCOA-DH activities.

Why are plasma BCAA levels decreased in patients with liver cirrhosis and in animals with experimental portal systemic-shunting ?

It has already briefly been mentioned that plasma BCAA are thought to be decreased in patients or animals with compromised liver function due to intensified degradation in non-hepatic tissues. The hypothesis was also forwarded that insulin may be instrumental in increased peripheral degradation because hyperinsulinism is common in liver disease and because an inverse correlation between plasma BCAA and insulin can be demonstrated in several disease states (90). Neither tissue sensitivity to insulin nor transport seem to play a role in the lowering of plasma BCAA levels. Firstly, if insulin resistance with regard to glucose also applies to BCAA, increased plasma BCAA levels would be expected and secondly, no indications have been found for altered transport of substrates of the rate-limiting enzyme in BCAA-degradation influencing the activity of the enzyme. Competition with AAA for transport over cell membranes might result in decreased levels of BCAA intracellularly. Increased levels are found, however. This may be due to increased transport of all NAA as has been demonstrated for brain. Besides the proposed "insulin-BCAA" theory, it has very recently been suggested (76) that plasma ammonia levels may influence plasma BCAA levels in liver disease. According to this theory, increased ammonia levels, often noted in liver disease, might enhance the formation of glutamate from α-ketoglutarate, in the reaction coupled to the transamination of BCAA and formation of BCOA, by removing the glutamate for glutamine synthesis.

Outline of this thesis

When studying mechanisms underlying the decrease of plasma-BCAA in patients or animals with compromised liver function, an animal model is needed that exhibits both hyperinsulinism and lowered BCAA levels. In addition, this animal model should not suffer from malnutrition, starvation, diabetes or sepsis. Such an animal model (rats with PCS) is described in chapter II.

Subsequently, in vitro metabolism of BCAA in muscle and adipose tissue of such PCS rats will be studied (chapter III and IV). Incubations are performed with ^{14}C-(1)-L-leucine and ^{14}C-(U)-L-leucine with tissues from overnight-fasted and fed rats (chapter III).
Similar experiments (only fed state) are described in chapter IV with isoleucine and valine, that will also be tested in these tissues in the presence of unlabeled leucine to explore the reported stimulating effect of leucine on the degradation of the other BCAA. In addition, the fate of BCAA-derived carbon incorporated into fat, will be determined by thin layer chromatography (chapter IV).

While alterations in membrane transport of BCAA or BCOA, as well as insulin receptor or post-receptor defects, are unlikely to be the cause of the changes in in vitro BCAA-metabolism as described, most likely alterations are expected at the enzyme level. Therefore, the activity of BCOA-DH will be measured in muscle and adipose tissue from PCS- and control rats (chapter V). A possible difference in short-term stimulation by insulin of BCOA-DH activity in adipose tissue of these rats will be investigated (chapter V).

Finally the relevance of these findings for the in vivo situation will be discussed and views expressed concerning the presumed pathogenic effects of decreased branched chain amino acid levels (chapter VI).

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CHAPTER II

SEQUENTIAL METABOLIC CHARACTERISTICS FOLLOWING PORTA-CAVAL SHUNT IN RATS

Summary

A porta-caval shunt (PCS) model is frequently employed to study phenomena inherent to portal-systemic shunting of splanchnic blood. In many species, a PCS induces hepatic insufficiency, accompanied by encephalopathy. Rats tolerate a PCS better and exhibit no or only slight encephalopathy. Moreover, age and environment seem to have a large impact on the ability to tolerate a PCS. This explains the discrepancies between the results of different investigators and the varying time periods reported between the PCS operation and the optimum time for experiments. To characterize the PCS-model (button technique) in rats with respect to metabolic parameters in our field of interest, we studied three groups of male Sprague Dawley rats (non-operated (n = 12), sham-operated (n = 12) and PCS-operated (n = 13)) for four weeks following the shunt operation. Initial body weight was about 200 g. Body weight, plasma amino acid pattern, plasma immunoreactive insulin (IRI) and plasma immunoreactive glucagon (IRG) were determined before operation and weekly thereafter. Blood samples were drawn from the venous plexus of the orbita of rats in a 14-16 hr fasted state.

Body weight in the PCS group decreased for one week post-shunt and then increased at about the same rate as in control groups. Plasma IRI, IRG and aromatic amino acid (AAA) concentrations were highest one week post-shunt, and tended to normalize in the next weeks. Plasma branched chain amino acid (BCAA) concentrations were decreased in the first, second and third week post-shunt, after which normalization occurred. These data demonstrate that after three to four weeks, male Sprague Dawley rats start to recover from the metabolic disturbances caused by the PCS with regard to the parameters measured. Therefore, experiments in this area of interest, especially those relating to BCAA metabolism, should be carried out 2-3 weeks after the shunt-operation ("button" technique).

Introduction

Animals with end-to-side porta-caval shunt (PCS) have been used extensively to study the metabolic consequences of the portal-systemic shunting of blood around the liver. In most species, a portal-systemic shunt even-

tually leads to hepatic failure, encephalopathy and death. During the last 10 years, alternative theories have been proposed, linking hepatic encephalopathy to a disturbed neutral amino acid (NAA) pattern (5, 10, 12, 16, 20). This pattern has been reported in man and in several animal species and consists of decreased plasma levels of branched chain amino acids (BCAA) and increased levels of aromatic amino acids (AAA). It has been postulated that the decreased plasma BCAA levels are caused by hyperinsulinism (16), often present in portal-systemic shunting and in hepatic failure. To establish this postulated causality and to gain insight into the underlying mechanism(s), an animal model is required which exhibits the altered plasma amino acid profile and hormone level. Rats subjected to PCS are reported to fulfil these criteria (3, 11, 13, 18). Following general guidelines in the literature, we initially used rats 5-6 weeks after a PCS-operation and determined the plasma amino acid profiles, plasma immunoreactive insulin (IRI) and plasma immunoreactive glucagon (IRG). However, no convincing differences could be detected between groups of rats with PCS, sham-operated and non-operated controls with regard to the parameters measured. Apparently, after 5-6 weeks, PCS rats had, at least partly, adapted to the PCS status and had become indistinguishable in plasma BCAA and IRI levels from control rats. Therefore, it was necessary to redefine our PCS rat model in terms of changes characteristic for portal-systemic shunting (PSS). A series of relevant parameters, namely body weight, plasma glucose, plasma amino acids, plasma IRI and plasma IRG, were measured before operation and 1, 2, 3 and 4 weeks after operation in PCS rats, sham-operated rats and non-operated controls. In addition, a separate group of rats was subjected to splenoportography at 2½ and 4½ weeks after a PCS-operation and compared with control rats.

Materials and methods

Male Sprague-Dawley rats weighing 190-200 g were used. They were housed under standard laboratory circumstances with a fixed dark-light schedule (dark 19.00 - 7.00; light 7.00 - 19.00), not relocated for at least one week, and were fed regular laboratory rat chow (Hope Farms SRM-A, Woerden, The Netherlands) and water ad libitum. Thirty-seven rats were

PLASMA - A.A.	UNOPERATED CONTROLS	SHAM - OPERATED					P.C.S. - OPERATED				
	Ref. Value	week 0	week 1	week 2	week 3	week 4	week 0	week 1	week 2	week 3	week 4
Valine	167 ± 34	178 ± 27	218 ± 52	177 ± 27	157 ± 18	165 ± 60	165 ± 45	168 ± 49	135 ± 47	135 ± 13	171 ± 68
Isoleucine	85 ± 18	88 13	102 16	80 13	79 13	79 4	89 25	76 11	59 7	57 9	67 13
Leucine	125 ± 25	135 27	145 31	114 20	104 10	105 14	131 38	113 20	83 11	80 9	96 6
Threonine	268 ± 50	268 50	259 65	253 36	212 52	230 36	271 56	190 43	129 11	152 18	145 28
Serine	287 ± 49	304 43	266 69	252 69	235 31	270 54	304 58	243 29	217 22	229 16	229 31
Tyrosine	67 ± 15	69 9	74 22	68 16	60 12	61 16	72 20	101 20	80 16	81 18	85 20
Phenylalanine	45 ± 13	37 9	54 11	46 7	42 11	41 6	44 11	75 18	51 7	55 16	61 18
Tryptophan	47 ± 27	47 10	31 9	44 9	36 9	37 8	42 11	38 20	37 20	49 16	42 8
Histidine	51 ± 12	46 11	49 13	53 18	42 4	45 12	51 16	84 13	68 9	59 11	66 18
Cystine	70 ± 43	87 13	61 13	65 11	73 9	63 10	84 11	28 10	20 9	45 15	39 10
Methionine	47 ± 11	50 9	43 20	45 13	37 9	42 8	49 9	57 11	41 4	43 18	49 12
Citrulline	77 ± 61	103 11	97 29	81 27	81 25	88 26	114 13	116 13	119 27	118 22	133 24
Ornithine	40 ± 12	45 7	38 11	33 9	32 7	38 10	42 11	65 22	43 13	49 11	53 18
Arginine	136 ± 33	134 8	120 31	143 31	114 25	112 15	136 20	174 38	169 45	131 25	137 28
Asparagine	91 ± 29	93 11	83 16	92 20	82 11	85 12	87 52	102 25	77 13	96 36	99 26
Glutamine	490 ± 96	538 78	387 85	356 76	397 65	342 48	471 103	586 49	562 124	494 60	513 110
Glycine	447 ± 96	524 78	929 737	845 560	421 121	622 506	567 208	518 150	1116 977	822 822	562 146
Alanine	329 ± 78	362 74	335 99	382 96	272 40	303 48	310 90	426 134	300 29	329 83	319 46
Lysine	382 ± 107	360 47	344 101	389 105	332 103	308 28	370 83	344 58	273 22	255 16	267 26

Table I
Mean values of plasma amino acids in µmol/l (± S.D.), pre- and postoperatively, in male Sprague-Dawley rats: PCS-operated (n = 13), sham-operated (n = 12) and unoperated controls (institutional laboratory control values, n = 75). All rats were fasted for at least 14 hours.

divided into three groups: unoperated controls (n=12), sham-operated rats (n=12) and PCS rats (n=13). PCS operations were performed under ether anaesthesia according to the technique described by Lee and Fisher (14), and modified by Funovics and Fischer to give a quicker and more uniform "non-suture" or "button" technique (7). The button is made of teflon tube with an inner diameter of 1.6 and an outer diameter of 2.3 mm. The PCS operation takes about 25 minutes. The sham-operated group underwent laparotomy and had the portal vein occluded for 10 minutes while the inferior caval vein was partially occluded for 5 minutes. The control group was not treated at all.

Blood. Two-and-a-half ml of blood was drawn from rats in the fasted state by ocular puncture with hematocrit capillaries, before and 1, 2, 3 and 4 weeks after surgery. The blood was collected in heparinized tubes and cooled on ice immediately. From the freshly drawn blood, 0.6 ml was pipetted into a cooled test tube containing 60 µl of the protease inhibitor aprotinin (Trasylol^{R}) for determination of IRG. Both tubes were centrifuged for 15 minutes at 2000 g at 4^{o}C. Plasma was removed and frozen immediately at -70^{o}C until analysis.

Plasma glucose was determined enzymatically with glucose-oxidase in a Beckman-glucose analyser-2.

Plasma IRI was determined with a commercially available radioimmunoassay kit (Wellcome, Beckenham, England) with human standards and anti-human antisera. The normal range for fasted rats was: 18.2 $\pm$ 8.1 µU/ml (mean $\pm$ S.D.).

Plama IRG was measured with a commercial radioimmunoassay kit (Biolab, Brussels, Belgium) with human standards and anti-human antisera 30 K. Normal range for fasted rats was: 225 $\pm$ 79 pg/ml (mean $\pm$ S.D.).

Plasma amino acids were determined on a LKB 4400 amino acid analyzer running a physiological program with 5 Li^{+}-buffers as described by Dilley and Rocek (4). The plasma was deproteinized with sulfosalicylic acid (5% w/v) and filtered through 0.22 µm pores (Millipore filters). Normal values for rats were determined in 75 untreated male Sprague-Dawley rats of about 200-300 g (see table I).

In this study, overnight-fasted values are given for IRI, IRG and amino acids in order to eliminate interindividual variations due to differences in food intake.

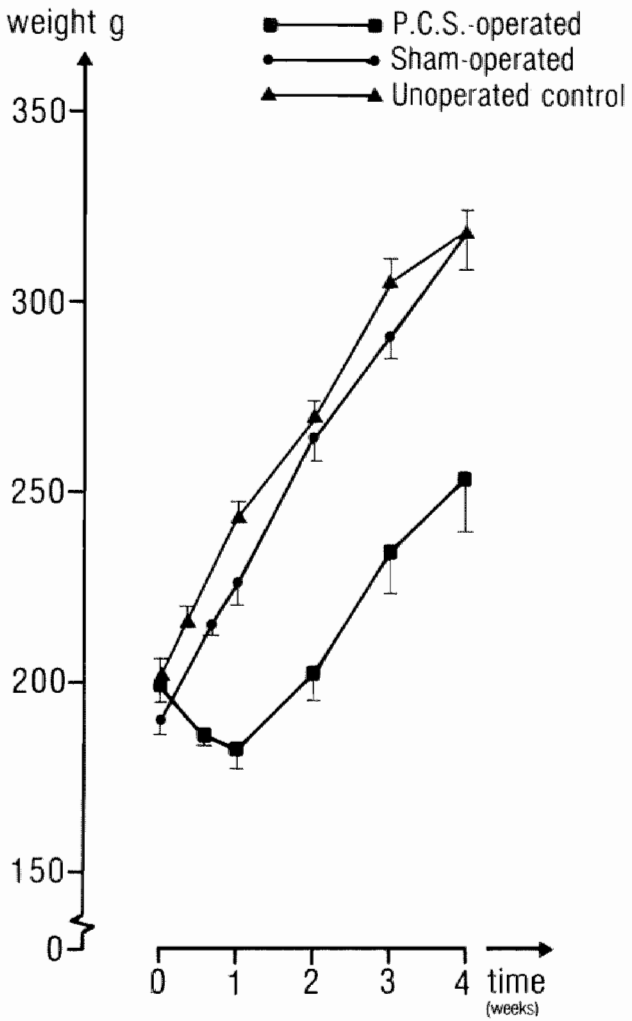


Figure 1
Postoperative body weight curves (mean ± sem) of male Sprague-Dawley rats: controls (n = 12), sham-operated rats (n = 12) and porta-caval-shunt-operated rats (n = 13), shunted according to the 'button' technique.

For statistical calculations the Wilcoxon signed-rank test for inter-weekly differences within a group and the Wilcoxon rank-sum test for differences between two groups (15) were used.

Results

Body weight

Figure 1 shows the body weight curves in PCS-rats, sham-operated rats and non-operated controls. Characteristic in the curve of PCS rats is a weight loss of 5-15% during the first week after surgery, a minimum body weight 1 week postoperatively (p.o.) and an increase thereafter at a rate comparable to that in sham-operated and non-operated control rats. The pre-operative weight of PCS rats is reached two weeks after the operation. As is clear from the standard errors in Figure 1, individual body weight curves of PCS rats exhibit a large variability at 3 and 4 weeks p.o.

Plasma IRI and IRG

Figure 2 demonstrates that postabsorptive plasma levels of insulin in PCS rats were 60% higher than the preoperative values ($p < 0.04$) and the levels of glucagon about 300% higher ($p < 0.02$) one week p.o., after which they tended to normalize. Sham-operated animals showed the same pattern but to a lesser extent, namely about 10% rise in insulin ($p < 0.05$) and about 100% in glucagon ($p < 0.02$) one week p.o. Three weeks after the sham-operation, they were completely normalized. Compared to the sham-operated rats, the PCS animals had significantly higher plasma insulin levels up to 3 weeks p.o. (44, 51 and 38% respectively after 1, 2 and 3 weeks p.o.) and significantly higher plasma glucagon levels up to at least 4 weeks p.o. (69, 100, 113 and 78% respectively in week 1, 2, 3 and 4 p.o.). The non-operated control groups had normal plasma insulin and glucagon levels throughout the experiment.

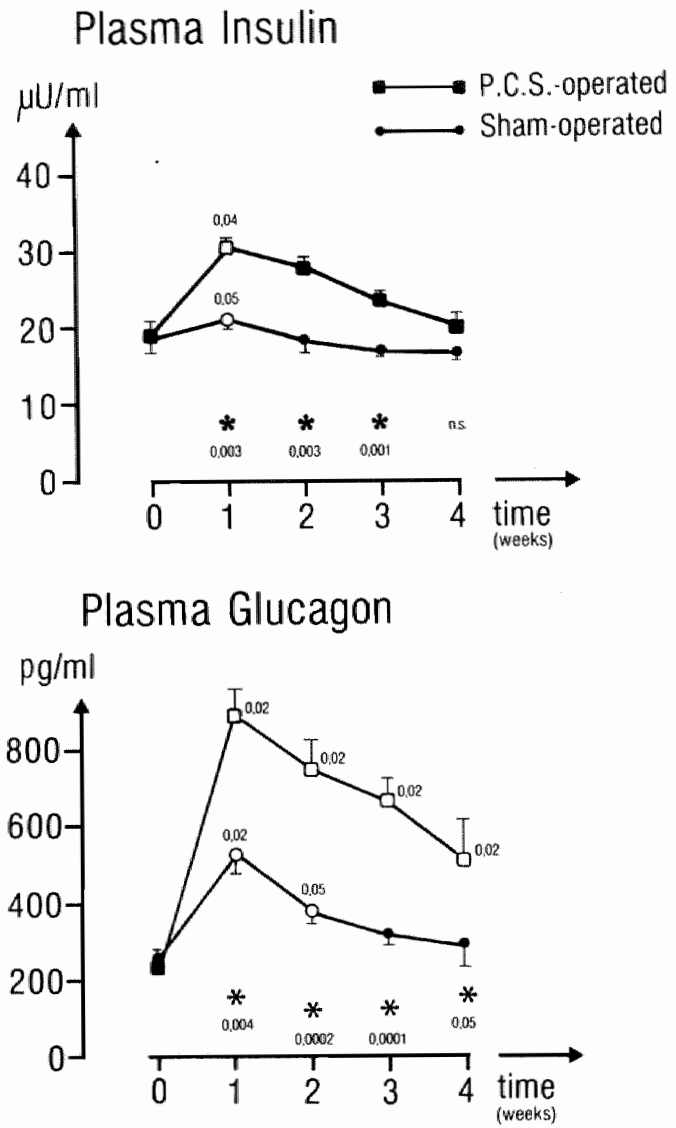


Figure 2

Plasma immunoreactive insulin (IRI) and immunoreactive glucagon (IRG) in sham- and porta-caval-shunt-operated male Sprague-Dawley rats (mean ± sem). Open symbols indicate statistical significance compared with the pre-operative value. Asterisks above the X-axis indicate statistically significant differences between PCS-rats and sham-operated rats. Unoperated controls fell within the normal range (see Materials and methods).

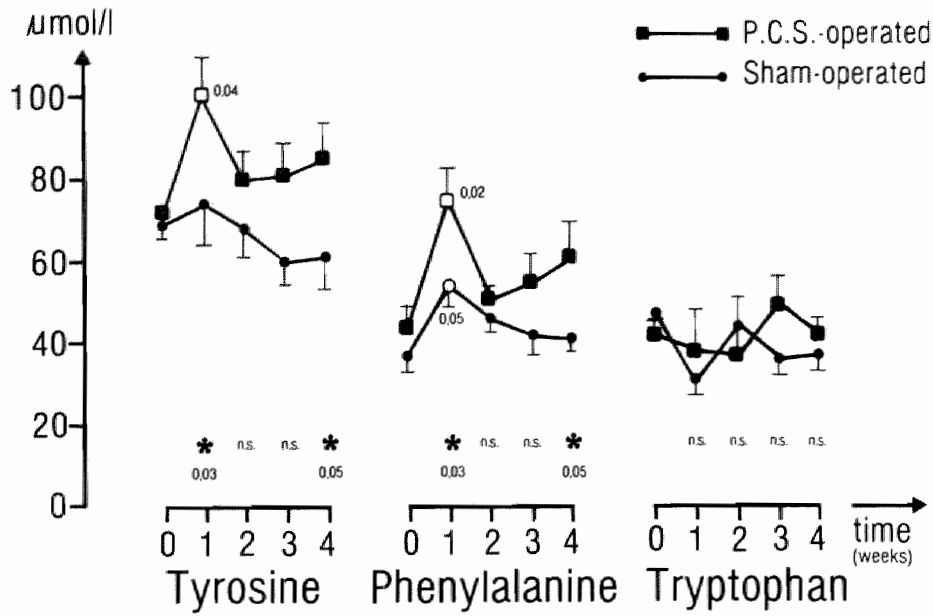


Figure 3
Plasma aromatic amino acids (AAA; mean ± sem) in male Sprague-Dawley rats; sham- and PCS operated. Unoperated controls fell within the normal range (see table I, first column). Symbols are used as indicated in fig. 2.

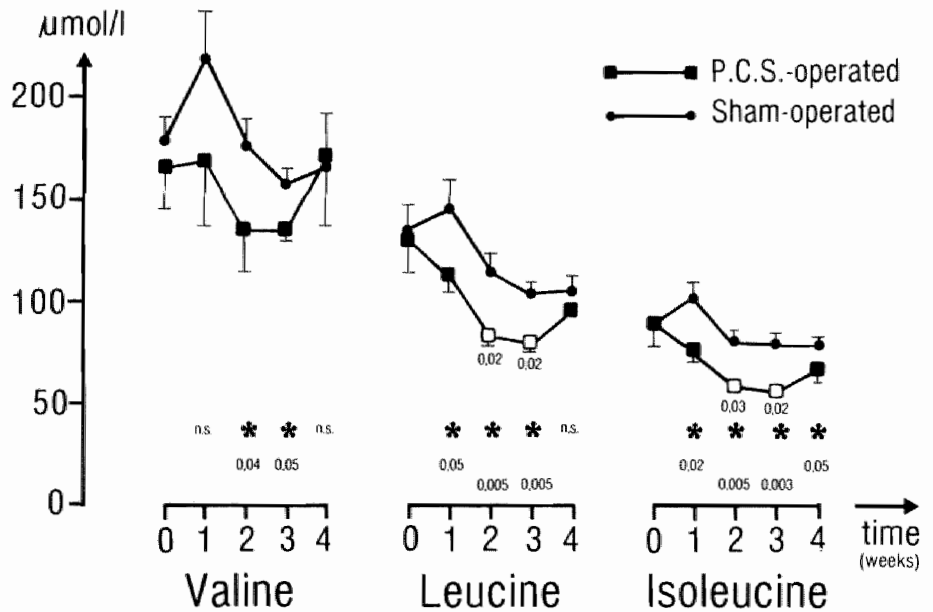


Figure 4
Plasma branched chain amino acids (BCAA; mean ± sem) in male Sprague-Dawley rats; sham- and PCS-operated. Unoperated controls fell within the normal range (see table I, first column). Symbols are used as described in the legend of Fig. 2.

Plasma Amino acid	Time p.o. (weeks)	Direction of change	Significance (p)	
			PCS vs pre-op.	PCS vs sham.
Threonine	2-3	⇩	0.02-0.02	0.002-0.002
Serine	2	⇩	0.04	N.S.
Cystine	1-2-3-4	⇩	0.04-0.02-0.04-0.04	0.003-0.002-0.005-0.005
Lysine	2-3	⇩	0.02-0.04	0.02-0.02
Histidine	1	⇧	0.04	0.003
Ornithine	1	⇧	0.05	0.01
Glutamine	1	⇧	0.04	0.002
Arginine	1	⇧	N.S.	0.03

Table II
Statistical significances of some plasma amino acids, other than AAA and BCAA, due to portacaval shunting; both compared with the pre-operative value and compared with sham-operated rats (data from table I).

Plasma amino acids

Plasma amino acid levels in the various groups are listed in table I. Normal values for overnight-fasted unoperated male Sprague-Dawley rats in our laboratory are given in the first column.

Aromatic amino acids (fig. 3)

The plasma levels of the aromatic amino acids (AAA) tyrosine and phenylalanine in the PCS group were significantly increased ($p < 0.03$) one week after the shunt operation; in the sham group, the plasma phenylalanine level was also significantly elevated ($p < 0.05$) one week p.o., but less than in the PCS-group ($p < 0.02$; fig. 3, table I). Compared with sham-

operated rats, the PCS-rats had significantly higher plasma levels of tyrosine and phenylalanine ($p < 0.03$) one week p.o. No significant difference was recorded 2 and 3 weeks p.o. between the PCS group and the sham-group, while in week 4 p.o., the difference just reached significance ($p < 0.05$). Tryptophan was not significantly altered.

Branched chain amino acids

Plasma levels of the branched chain amino acids (BCAA) valine, leucine and isoleucine, are shown in figure 4. In the PCS rats, plasma levels of all three BCAA decreased, stabilized at the low level 2-3 weeks p.o. and tended to normalize thereafter. Compared to the pre-operative values, levels of leucine and isoleucine in the PCS group 2 and 3 weeks p.o. were significantly lower (leucine $p < 0.02$ and isoleucine $p < 0.03$). The decrease in valine concentration did not reach statistical significance, due to greater individual variation; in 4 rats, the plasma valine concentration was completely unaffected.

Other amino acids than AAA and BCAA, that reached significant differences due to portacaval shunting, are listed in table II.

Splenoportography

A separate group of rats shunted according to the "button" technique was subjected to splenoportography to evaluate the patency of the shunt with respect to the time-period postoperatively. Each rat had only one splenoportography. Consequently, results from different time-periods p.o. are derived from different rats and may only give a rough indication of PCS-rats with similar body weight curves. PCS rats that gained weight like the rats used in the study described above ($n = 7$), had, 2½ weeks p.o. open shunts and no clear collaterals, while the liver was not visualized. In contrast, in rats that did not gain weight and/or were still under their initial body weight ($n = 4$), a clear obstruction of the shunt 2½ weeks p.o. was visualized as well as collaterals to the stomach and esophagus but not to the liver, evidenced by absence of contrast in the liver. All rats subjected to splenoportography 4½ weeks p.o. ($n = 5$) gained weight, including rats that did not gain weight at 2½ weeks p.o. All these rats had large collaterals and contrast in the liver, independent of an obstruction of the shunt. For representative pictures, see fig. 5.

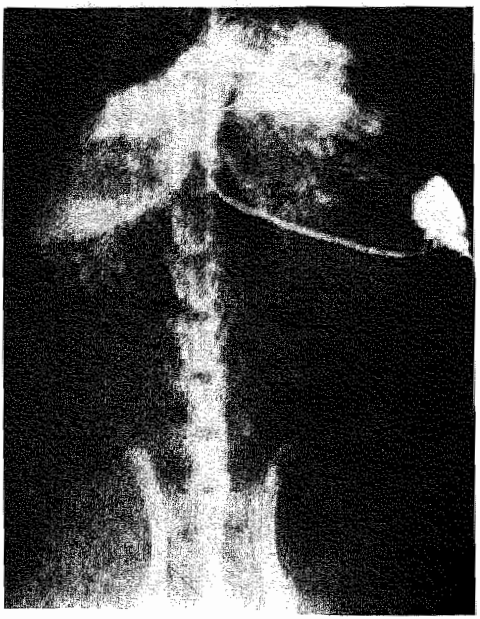
Rats, portacaval shunted according to the "suture" technique (n = 10), lost more weight (fig. 6) than "button"-shunted rats. Additionally, splenoportography was done in 5 of these rats after 2½ weeks. Hardly any visualisable collaterals were seen and two of the 5 rats had an obstructed shunt. At 4½ weeks p.o., all rats (n = 5) had an obstructed shunt. Three of these rats had developed collaterals, two of which had contrast in the liver.

Discussion

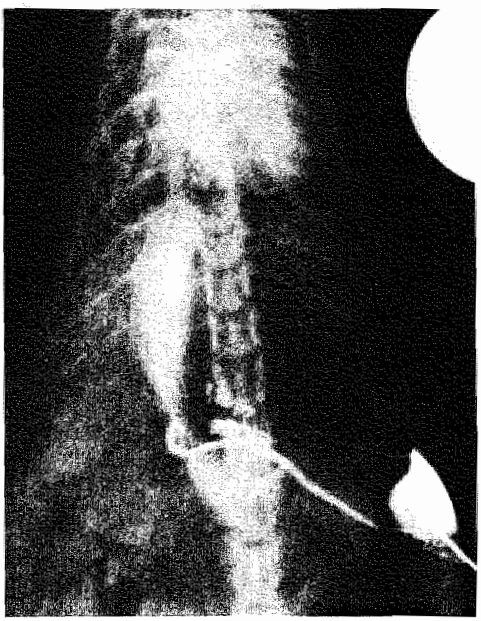
In earlier experiments, we could not confirm, 5-6 weeks p.o., the metabolic characteristics described in the literature, using the "non-suture" technique. Besides the condition of the rats and the time of shunting, other factors may have been responsible for this, including the rat species employed, the technical skill with which the operations were carried out and several environmental factors. A non-suture technique was chosen because it permits standardization of the operation time, the occlusion time of the portal vein, and within certain limits, the diameter of the anastomosis. The significance of these factors, however, cannot easily be quantified.

In order to eliminate fluctuations in plasma amino acids, plasma IRI and plasma IRG levels due to interindividual differences in food intake, blood samples in this study were taken in the overnight-fasted state. Pair-feeding might circumvent problems of differences in food intake. Rats pair-fed suboptimal amounts of food, however, exhibit an abnormal eating-behaviour, consuming the food that is granted to them within a relatively short time and starving thereafter. Thus, we decided that pair-fed rats did not form a suitable control group. We did not use post-decapitation blood because analysis of blood obtained by puncture of the retrobulbar plexus of the orbita yields more reliable and reproducible results.

Our findings lead us to conclude that all the above mentioned factors should be standardized as much as possible for a reproducible rat PCS model. Every investigator, before starting experiments, should carefully define his/her own rat model in terms of metabolic characteristics specific for the model.



a



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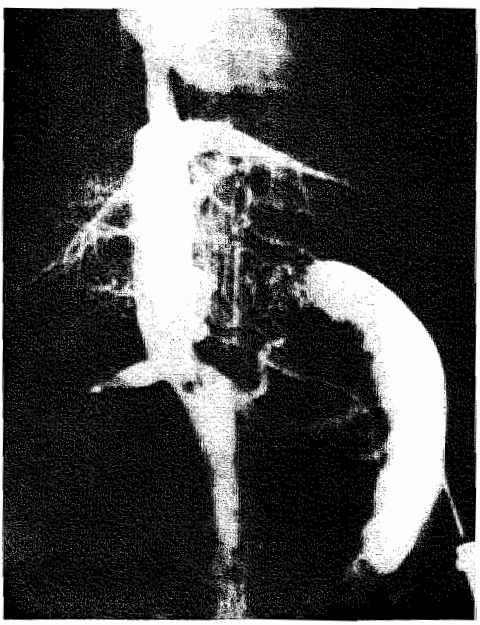

d

Figure 5

a. Splenoportography of control rat. Opafication of the spleen, lienal vein and portal vein. Subsequently filling of the whole liver and, at the right side of the picture, some superposition of liver and heart.
b. Splenoportography 2½ weeks p.o. of button-shunted rat. Opafication of spleen, lienal vein and directly filling of inferior caval vein (some narrowing due to to the button). No contrast in the liver, opafication in the heart.
c. Splenoportography 2½ weeks p.o. of button-shunted rat. Opafication of part of the spleen with subsequently filling of obstructed portal vein. Collaterals are visualized. No contrast in the liver.
d. Splenoportography 4½ weeks p.o. of button-shunted rat. Open porta-caval shunt. Opafication of the whole inferior and superior caval vein and extended collaterals resulting in the visualization of the liver.

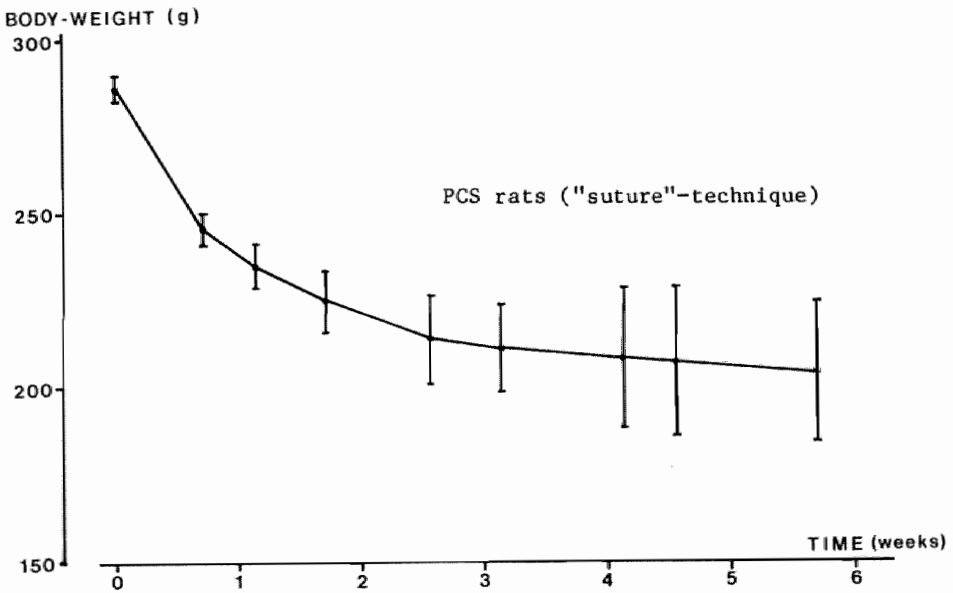

Figure 6

Postoperative body weight curves (mean ± sem) of male Sprague-Dawley rats (n = 10), shunted according the "suture" technique.

Body weight

Reproducible body weight curves were recorded in rats of about 200 grams at the time of the PCS operation. Rats with a body weight below 180 grams had a higher mortality rate and greater variations in body weight and metabolic parameters after operation. Rats above 300 grams do not tolerate a PCS operation as well as younger rats, exhibiting an increased mortality. Before undergoing the PCS operation, rats should be in optimal condition. Rats suffering from viral infections, like PPLO or Pasteurella, lose more weight during the first 2 weeks (about 25%) and do not reach their pre-operative body weight until 3-4 weeks p.o. Stress due to transport, abnormal noise, etc. in the week before the PCS operation has a large impact on growth. Pentobarbital anaesthesia, instead of ether, also influences growth. Most investigators report weight losses of up to 30% or more (3, 9, 18, 19) and lowest body weights are generally recorded 4-6 weeks p.o.. Preoperative weights are not reached within 6 weeks or even 5 months. Sham-operated rats, on the other hand, start gaining weight one week after the operation or later, which also contrasts with our results. Initial declines in body weight in the PCS-group are most probably caused by a low food intake. This is suggested from the glucose levels measured in the "fed" state which are comparable to those in fasted rats (fig. 7). The favourable body weight curves in PCS rats after 2-3 weeks warrant the conclusion that the metabolic changes observed after 2-3 weeks are specific for porta-caval shunting and are not partly due to undernutrition.

At the time of blood sampling and sacrifice, hematocrits proved to have returned to normal.

Plasma insulin and glucagon

Decreases in plasma BCAA levels have been linked to hyperinsulinism (16). It is clear that decreased plasma levels of BCAA 2 to 3 weeks p.o. in the PCS rats, compared to these in sham-operated rats and unoperated controls, are accompanied by a mild hyperinsulinemia (fig. 2) together with a three-fold increase in plasma glucagon levels. The mild hyperinsulinemia is observed in all PCS rats 2 weeks p.o., but the levels of insulin in some rats have normalized within 3 weeks. In sham-operated rats, plasma IRG was

most greatly increased 1 week p.o., probably due to operation trauma (fig. 2). High glucagon levels persisted in PCS rats for at least 4 weeks p.o.. In the PCS-operated group, peak values of insulin and glucagon were observed 1 week p.o. due to PCS and to operation trauma (see shams). This is earlier compared with suggestions in the literature that glucagon levels increase gradually in the first weeks until a stabilized high level is achieved. No reference value for 1 week p.o. could be found in the literature.

Using the "suture"-technique, Pector et al (18) reported normal plasma-insulin levels in PCS Wistar rats and in pair-fed sham-operated controls 3 weeks p.o., together with significant hyperglucagonemia in PCS rats compared with the controls. On the other hand, Rossouw et al (19), also using a "suture"-technique, found lower insulin levels in their PCS group compared to the non-operated control group, but their data are apparently derived from fed rats. As their PCS rats were virtually semi-starved, insulin levels might have been influenced by the low food intake. Basal insulin levels in a lean "marasmic" PCS rat may be expected to be low. This means that equal insulin levels in PCS rats and controls in the "fed" state may be interpreted as a relative hyperinsulinism in the PCS rats. We used the "non-suture" technique and confirmed the results of Pector e.a. (18) with respect to the hyperglucagonemia; the three-fold rise in glucagon levels was combined with a marginally elevated plasma-insulin level 3 weeks p.o. Our insulin : glucagon molar ratio at the time of stabilized altered BCAA levels does not deviate from those reported in the literature (20, 21); lowest values were recorded 2-3 weeks p.o.

Aromatic and branched chain amino acids

Our AAA measurements contrast with reports of highly significant elevations in the plasma levels of all three AAA 4 weeks p.o. and later (11, 13). Tyrosine and phenylalanine were significantly elevated 4 weeks p.o., while 5½ weeks p.o. in a separate group of rats, there was only a tendency in the AAA to be elevated (table III). No reference could be found in the literature concerning the significantly elevated plasma AAA levels that we recorded 1 week p.o. in PCS rats. Plasma BCAA levels in PCS rats were consistently depressed 2 and 3 weeks after operation, whereas these

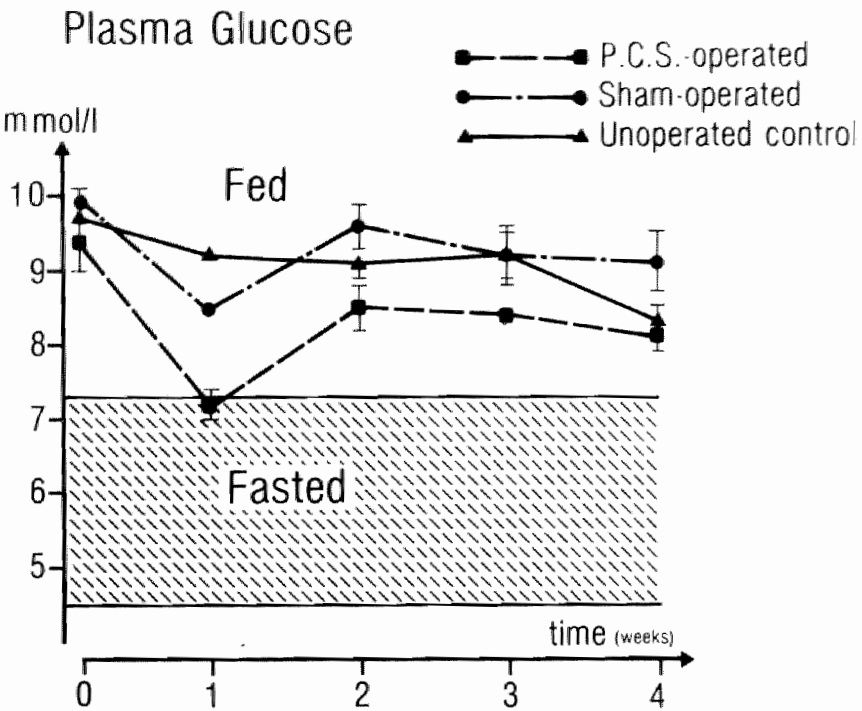

Figure 7
Plasma glucose levels (mean ± sem) in fed male Sprague-Dawley rats pre-operatively and 1, 2, 3 and 4 weeks postoperatively (p.o.): controls (n = 12), sham-operated rats (n = 12) and PCS rats (n = 13).

PLASMA NEUTRAL AMINOACIDS

Plasma AA (μmol/l)	Control (n = 10)	PCS operated (n = 13)
Valine	165 ± 23	176 ± 28
Isoleucine	90 ± 12	91 ± 15
Leucine	132 ± 14	147 ± 22
Tyrosine	61 ± 12	74 ± 9
Phenylalanine	49 ± 14	56 ± 9
Tryptophan	46 ± 14	52 ± 14

Table III
Mean (± S.D.) plasma neutral amino acids (NAA) in PCS ("button"-technique) and control rats 5½ weeks p.o.

levels were almost normalized at 4 weeks and had completely normalized at 5½ weeks p.o. (determined in another group of rats, table III). This is in contrast with reports of decreased BCAA levels 5 or more weeks p.o. (13). The elevated plasma BCAA levels in sham-operated animals and the normal to slightly decreased levels in PCS rats 1 week p.o. might be explained by operation trauma. Injury alters the plasma AA-pattern. This pattern differs from that observed in other catabolic states and includes elevated AAA-levels and elevated BCAA-levels (1). That the BCAA levels in PCS-rats are normal, or slightly increased 1 week p.o., indicates that the mechanism lowering the BCAA is already effective. Significant changes in other plasma amino acids than AAA and BCAA, due to PCS (listed in table II), are in good agreement with data from Hawkins et al (9) for "suture"- shunted rats 7-8 weeks p.o. weighing a little more than their pre-operative value, but 40% less than their controls. Noteworthy is the lowered cystine which is seldom reported.

In an analogous experiment, blood was drawn in the fed state. Determination of the plasma neutral amino acid profile demonstrated that changes in the PCS group were comparable to that of fasted PCS rats when compared with controls. Phenylalanine however, was increased 2-3 weeks p.o. Interindividual fluctuations were greater, probably due to differences in food intake. For the same reason, interpretation of the data for insulin was not possible.

BCAA : AAA ratio

Experience with the determinations of physiological plasma amino acid profiles in man and rats has proved that an interindividual range of about 15 percent is normal. Porta-caval shunts in man and rats may increase interindividual differences. This means that the ratio of BCAA to AAA has less value in shunted individuals. In a group, however, it is a worthwhile parameter for a possible correlation with impairment of the mental state. Developing encephalopathy is said to be indicated by a decreased BCAA : AAA ratio (6). For humans and dogs, the normal ratio (3 - 4) decreases to 1 in coma (20). Assuming that these normal values are also valid for rats, the sham-operated group in our experiment is within the normal range while the shunted group is significantly decreased 1-3 weeks p.o. relative to sham-

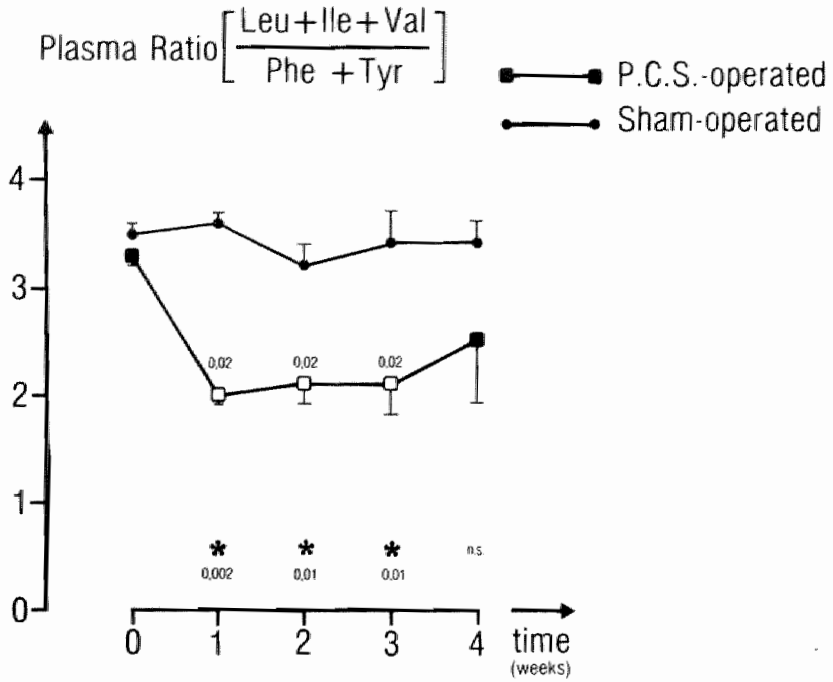

Figure 8
Mean (± sem) plasma ratio of branched chain amino acids (BCAA) and phenylalanine and tyrosine (AAA) in PCS-rats (n = 13) and sham-operated rats (n = 12). Symbols are used as described in the legend of fig. 2.

operated rats and to the preoperative value (fig. 8). Normalization occurs at 4 weeks p.o. No values below 2 were recorded and the rats exhibited no convincing signs of encephalopathy as reported in the literature (8). However, we did not try to quantitate a possible encephalopathy by psychometric tests.

Although ammonia may be one of the factors in the pathogenesis of hepatic encephalopathy, it was not determined in this study due to the limited amount of blood that could be drawn in in vivo rat experiments. In this way, we diminished the risk of rats developing anemia and thus influencing the results. From other experiments, it is clear that plasma ammonia levels are moderately elevated (1.5 - 3 fold) 2½ weeks after the shunt operation (controls 47±7 and PCS 77±12 µmol/l; mean ± S.D.).

In a study like this, it is impossible to measure all the parameters of interest in plasma in the same rats, nor is it possible to record parameters frequently over an extended period because of the limited blood volume available and the stress induced by repeated blood sampling. We took all precautions to exclude any other influences on the parameters of interest, except the shunt itself. This study was designed to characterize the PCS rat model operated with a "button" technique, but it was felt necessary to add some findings of PCS rats shunted in our laboratory with the "suture" technique. Drawing definite conclusions is very difficult. There are, in general, important differences between the "suture"- and the "non-suture" PCS rat model, when data from the same time-periods after the operation are compared. Obvious differences exist in growth, encephalopathy, collaterals from the portal area to the liver, liver weight, amino acids, glucagon and probably catecholamines. Plasma enzymes (ALT, AST, GLDH and AP) are increased after the PCS-operation in both "non-suture" and "suture"-shunted rats, but tend to normalize, only in the "button"-shunted group, after 3 (GLDH) or 4 weeks. According to the literature (22), serum IgA levels are also increased in some PCS rats, most pronounced in the "non-suture"-shunted group, indicating a diminished excretory function (2, 17). The PCS rats selected according to our criteria are relatively healthy rats, only influenced by the shunting of blood around the liver and probably slight portal hypertension which often exists in liver disease and is standardized as much as possible in our model by the use of "buttons" with a standardized internal diameter. Rats shunted with the "non-suture" technique, that did not fulfil our criteria, and were less healthy, more closely resembled the "suture"-shunted rats. "Suture"-shunted rats are, in general, depleted animals at the time they are used in experiments, compared with "button" PCS-rats fulfilling our criteria. In our opinion, it is quite possible that parameters measured in "suture"-shunted rats are influenced by other factors than the shunt itself, e.g. prolonged starvation and malnutrition. The process of normalization of metabolic disturbances of the PCS after 4 weeks in our "button" shunted rats may be due to the ability of rats to adapt when no extreme catabolic state has developed. The forming of collateral blood vessels may be crucial for the normalization, probably favoured by an optimal condition of the rats. The presence of collaterals suggests that some degree of portal hypertension must be

present, despite the fact that care was taken to employ buttons with an inner diameter that allowed the passage of a normal sized portal vein of rats weighing 200-250 grams, and despite the fact that twisting of the portal vein was prevented as much as possible. Close examination of the site of the button at sacrifice showed dense adhesions which might cause a slight stenosis. While no signs of infection or positive cultures were found, it is not excluded that slight stenosis develops as a result of a non-bacterial inflammatory reaction due to the presence of a corpus alienum (the teflon button).

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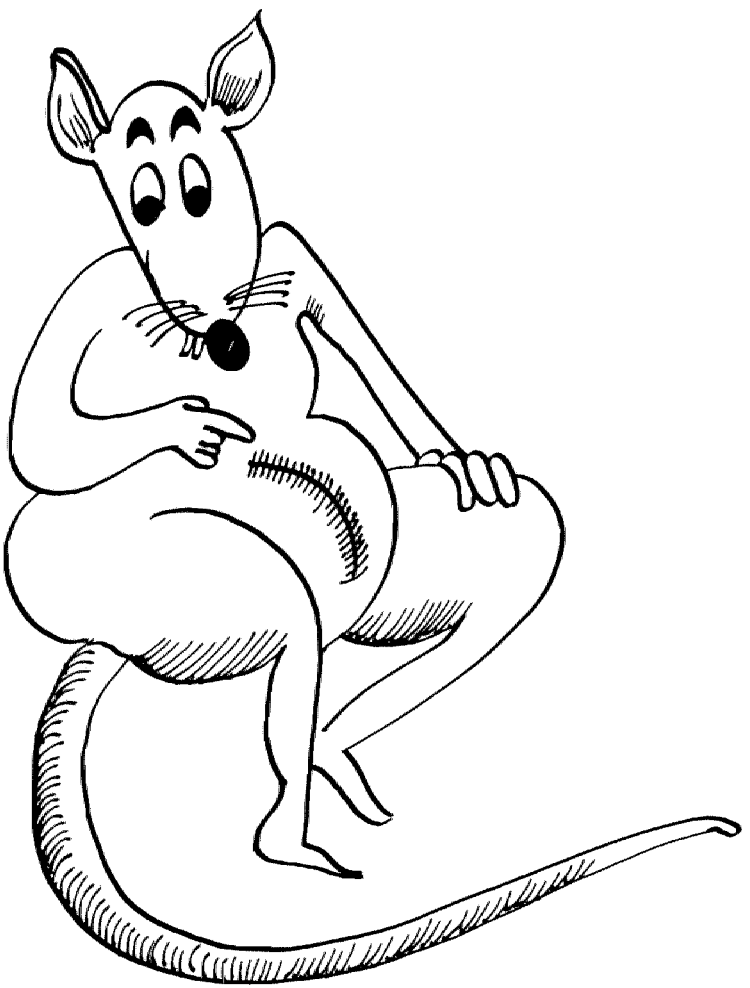


CHAPTER III

INFLUENCE OF PORTAL-SYSTEMIC SHUNTING ON LEUCINE METABOLISM IN MUSCLE AND ADIPOSE TISSUE OF FASTED AND FED RATS

Summary

Decreased levels of branched chain amino acids (BCAA) in plasma have been frequently reported in patients and experimental animals with portal-systemic shunting (PSS) and hepatic insufficiency. To study the mechanism underlying this phenomenon, in vitro incubations were carried out with samples of muscle and adipose tissue from overnight fasted and fed porta-caval shunt (PCS) operated rats, sham-operated (SO)- and non-operated controls. The incubation medium consisted of Krebs-Ringer bicarbonate buffer with (5mM) or without glucose and contained physiological concentrations (0.1mM) of ^{14}C-(U)-L-leucine or ^{14}C-(1)-L-leucine (s.a. 0.25 mCi/mmol). The incubation studies were done 2½ weeks after the operation. The results indicate that:

1. Two and a half weeks after the operations, significantly decreased levels of plasma BCAA occur in PCS rats as compared to control rats. At this time PCS rats weighed about 115% of their preoperative body weight and had significantly increased plasma insulin levels.
2. Sham-operated controls were not different from unoperated controls with respect to the parameters measured.
3. The addition of glucose increased the in vitro catabolism of ^{14}C-(U)-leucine by adipose tissue from normal and PCS operated rats. Both CO_2-production and incorporation of leucine-derived carbon into fat were increased. Leucine metabolism by muscle was influenced by glucose only in the PCS group where more leucine was incorporated into muscle protein.
4. Feeding did not significantly alter the in vitro CO_2-production from ^{14}C-(C)-leucine by muscle, but CO_2-production tended to be increased in the control groups and decreased in the PCS group. In adipose tissue, feeding changed in vitro the fate of a part of leucine-derived carbon in both the control and PCS group by diminishing the amount released as CO_2 and strongly augmenting the amount incorporated into fat.
5. PCS increased the catabolism of leucine in adipose tissue in vitro by increasing irreversible decarboxylation of the C-1 atom of leucine. In muscle, decarboxylation of the C-1 atom was not significantly altered.

6. After PCS, the carbon skeleton of leucine that is left after decarboxylation of the C-1 atom, was in vitro catabolized to CO_2 to a lesser extent compared to controls, in both muscle and adipose tissue, and was incorporated into adipose tissue to a greater extent.
7. The carbon from all leucine that is irreversibly decarboxylated in adipose tissue from both sham-operated and PCS-operated rats, is incorporated into fat or metabolized to CO_2. This indicates that other reactions are negligible. In muscle, however, a significant part of the carbon atoms from the decarboxylated leucine, cannot be traced in protein or in the CO_2 released.

After PCS, an increased catabolism of leucine can be measured in vitro in adipose tissue, independent of an overnight-fast. The role of adipose tissue in this increased catabolism after PCS seems to be extremely important. These results may explain the decreased plasma leucine levels encountered in patients and animals with PCS and hepatic insufficiency.

Introduction

In recent years, much attention has been paid to several aspects of branched chain amino acid (BCAA) metabolism. Clinically, a distorted neutral amino acid (NAA) pattern in plasma, including low BCAA levels, is associated with hepatic encephalopathy (11). BCAA might serve as alternative fuel in severe catabolic states (2,9) and during exhaustive exercise (1). Furthermore it has been suggested that leucine serves as a possible regulator of protein synthesis and turnover (4,12,26).

Patients and experimental animals with compromised liver function and/or portal-systemic shunting (PSS) have decreased plasma BCAA levels and elevated plasma insulin levels (10). It has been postulated that these two phenomena are causally related (23). Decreased plasma BCAA levels have been suggested to result from increased peripheral uptake caused by hyperinsulinemia. The exact nature of this peripheral uptake has not been specified. Less than 15% of the BCAA in the portal blood is extracted by the liver (36). Insignificant amounts of BCAA are degraded by the liver; these are probably the only amino acids whose carbon skeletons can be completely broken down peripherally. In most literature reports, only muscle is considered to be capable of fulfilling this role. However, early and more recent work by Goodman (14,15) suggests that adipose tissue may also have a significant capacity to catabolize BCAA. Our investigation was performed to determine the influence of porta-caval shunting on the in vitro metabolism of leucine-derived carbon by muscle and adipose tissue. Two studies are

described. The first one, in which ^{14}C-(U)-L-leucine serves as the substrate, deals with differences in in vitro leucine metabolism due to the post-absorptive or fed state of the rats, from which the tissues were derived, and due to the presence of glucose (5mM) in the incubation medium. In the second study described, either ^{14}C-(U)-L-leucine or ^{14}C-(1)-L--leucine served, in separate incubation flasks, as the substrate for tissues derived from the same rats (fed state only). This allows estimation of what happens with the rest of the carbon skeleton of leucine after decarboxylation.

Materials and methods

Male Sprague-Dawley rats (200-240 g) were used. They were housed under standard laboratory conditions with a fixed dark-light schedule (12 hrs dark; 12 hrs light) and were fed regular laboratory rat chow and water ad libitum. PCS operations were performed under ether anesthesia employing a "button" technique (13). The PCS operation took about 25 minutes. Mortality was 10-15% and death occurred mostly within 24 hours after operation. The sham-operated rats underwent laparotomy and portal vein occlusion for 10 minutes.

Blood was drawn under ether anesthesia by ocular puncture, before operation and before sacrifice in the study.

Plasma insulin (IRI) was determined with a commercially available radioimmunoassay kit (Wellcome, Beckenham, England) with human standards and antisera.

Plasma amino acids were determined in plasma that was deproteinized with sulfosalicylic acid (5% w/v) and filtered through 0.22 µm pore filters. Samples were analyzed on a LKB 4400 amino acid analyzer running a physiological program with 5 Li^+-buffers according to Dilley and Rocek (8).

Tissue DNA content was determined in muscle according to Burton (3) and in adipose tissue according to Rafael and Vsiansky (32).

Incubation procedure

The animals were killed by cervical dislocation; the epididymal fat pads and the diaphragm were removed quickly. Tissue samples of 30-40 mg (diaphragm or epididymal fat pad) were placed in siliconized 25 ml Erlenmeyer

flasks containing 5 ml carbogenated Krebs-Ringer bicarbonate buffer pH 7.4 and placed in a shaker-bath. After 15 minutes pre-incubation, ^{14}C-(U)-L-leucine (0.25 mCi/mmol) or ^{14}C-(1)-L-leucine (0.25 mCi/mmol) was added to a concentration of 0.1 mM. ^{14}C-(U)-leucine (340 mCi/mmol) and ^{14}C-(1)-leucine (55 mCi/mmol) were purchased from New England Nuclear Corp. (Boston, Mass.). The incubations were carried out for 90 min at 38.5° C in a shaker-bath at 60 cycles per minute. From each rat, tissue samples were incubated in triplicate for measurement of labeled CO_2 produced by muscle and adipose tissue, and in duplicate for determination of incorporation of leucine-derived carbon into protein by muscle and into fat by adipose tissue. CO_2-release and incorporation of leucine-derived carbon into muscle tissue were determined essentially according to Odessey and Goldberg (25) with some minor modifications in the chemicals used, including the tissue solubilizer Soluene^R-350 (0.5 N Quaternary Ammonium Hydroxide in Toluene; Packard-Becker BV, Chemical Operations, Groningen, The Netherlands). A major modification was the use of separate incubation flasks for the determination of CO_2-release and incorporation of leucine-derived carbon into muscle protein and into fat in adipose tissue, to avoid the loss of CO_2 released. The incorporation of ^{14}C-(U)-leucine-derived carbon into fat in adipose tissue was determined according to Goodman (14).

Results

Body weight

PCS rats lost 10% of their body weight postoperatively (p.o.), but had regained their preoperative weight within two weeks. Starting from their minimum weight (3-7 days p.o.), they showed similar growth rates compared with sham-operated rats (SO rats) and unoperated control rats. Rats that failed to gain weight p.o., or had not reached their initial body weight 2½ weeks p.o., were excluded from the study. At the time of the experiments (2½ weeks p.o.), PCS rats, SO rats and control rats, used in the first study described, weighed about 115%, 140% and 140%, respectively, of their initial weights. Rats used in the incubation study in which ^{14}C-(1)-leucine was compared with ^{14}C-(U)-leucine, weighed about 115% and 135% respectively for PCS and SO rats.

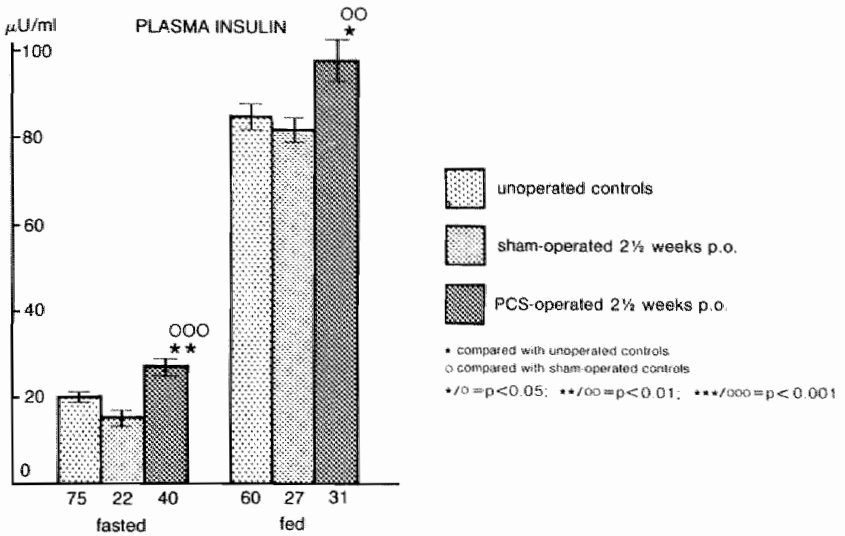


Figure 1
Plasma immunoreactive insulin (IRI) in fasted and fed unoperated, sham-operated and PCS-operated male Sprague-Dawley rats (mean ± sem).

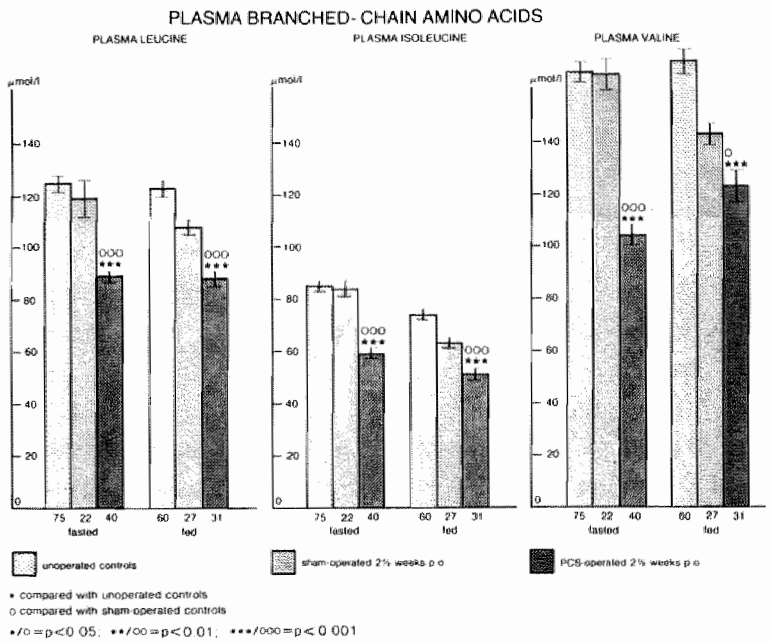


Figure 2
Plasma branched-chain amino acids in fasted and fed unoperated, sham-operated and PCS-operated male Sprague-Dawley rats (mean ± sem).

Insulin (IRI)

Fasted (14-18 hrs) and fed values of IRI in the plasma of PCS rats, SO rats and control rats were derived from separate groups of rats (fig. 1). In the fasted state, plasma IRI levels were increased in the PCS group (30-50%) as compared to both control groups. In the fed state, plasma IRI levels in the PCS group were also increased by about the same absolute amount.

Amino acid levels

In the PCS group, plasma BCAA-levels 2½ weeks p.o. (fig. 2) were consistently decreased to a 70% level as compared to the SO group and the control group. This result was obtained for both fasted and fed rats.

Incubations with ^{14}C-(U)-L-leucine; influence of feeding; influence of glucose in the incubation medium

The leucine-metabolizing activity in diaphragm and epididymal adipose tissue was measured in the presence of a physiological concentration of ^{14}C-(U)-L-leucine (0.1 mM) with and without glucose and was expressed in pmol/ µg DNA.90 min., calculated from the specific activity of leucine in the incubation medium. The measured parameters, CO_2-production by muscle and adipose tissue, incorporation into muscle protein and incorporation into fat, were linear with time to at least 120 minutes. A correction for tissue DNA-content was applied to correct for different numbers of cells in the tissues used, due to differences in cell size between PCS- and control rat-derived tissue.

Diaphragm

The amount of leucine-carbon derived CO_2, produced in vitro by diaphragm from ^{14}C-(U)-leucine, was not significantly altered by PCS (except in the fed state without glucose; $p < 0.05$), nor by the presence of glucose in the incubation medium (fig. 3), when results are expressed per µg DNA. Feeding did seem to increase CO_2-production from ^{14}C-(U)-leucine in diaphragm from unoperated and sham-operated rats (no significance reached), while this CO_2 production in diaphragm from PCS-operated rats tended to be

slightly decreased. No significant differences were noted between unoperated control rats and SO rats. When glucose was present in the incubation medium, the incorporation of ^{14}C-(U)-leucine derived carbon into protein in diaphragm, also shown in fig. 3, was increased by PCS in the fed state ($p < 0.05$). Feeding or the presence of glucose in the incubation medium did not significantly alter the incorporation of ^{14}C-(U)-leucine into muscle protein.

Epididymal adipose tissue

CO_2-production from ^{14}C-(U)-leucine by adipose tissue was not significantly affected by PCS under all measured conditions (fig. 3a, 3b, 3c and 3d). The presence of glucose in the incubation medium increased the CO_2 production by adipose tissue in all three groups ranging between 34-104% ($0.05 < p < 0.005$). In the presence of glucose (fig. 3a and 3b), feeding decreased CO_2 production in all groups between 19-34 % and was significant in the SO group ($p < 0.001$) and PCS operated group ($p < 0.01$).

When glucose was added to the incubation medium, a three-fold increase was observed in the incorporation of ^{14}C-(U)-leucine derived carbon into adipose tissue in the PCS-group (fig. 3a and 3b; $p < 0.001$). In the absence of glucose, a decrease was noted in the fasted state, when control groups were compared with the PCS group ($p < 0.01$), while in the fed state a two-fold increased incorporation in the PCS group was detected ($p < 0.05$). Feeding increased incorporation of leucine-derived carbon in the presence of glucose in all groups ($p < 0.005$). Without glucose, feeding increased leucine-carbon incorporation only in the PCS group ($p < 0.001$), and even decreased the incorporation in the control groups ($p < 0.005$). Glucose in the incubation medium strongly enhanced the level of incorporation of ^{14}C-(U)-leucine-derived carbon in all groups ($p < 0.005$).

Incubations with ^{14}C-(U)-L-leucine vs ^{14}C-(1)-L-leucine; irreversible decarboxylated leucine and degree of oxidation of the leucine carbon skeleton

These incubations were carried out in the presence of glucose (5mM) and with tissues from fed rats. ^{14}C-(U)-L-leucine and ^{14}C-(1)-L-leucine were added to a physiological concentration (0.1 mM) and had the same specific activity (0.25 mCi/mmol). The consequence of this is a sixfold

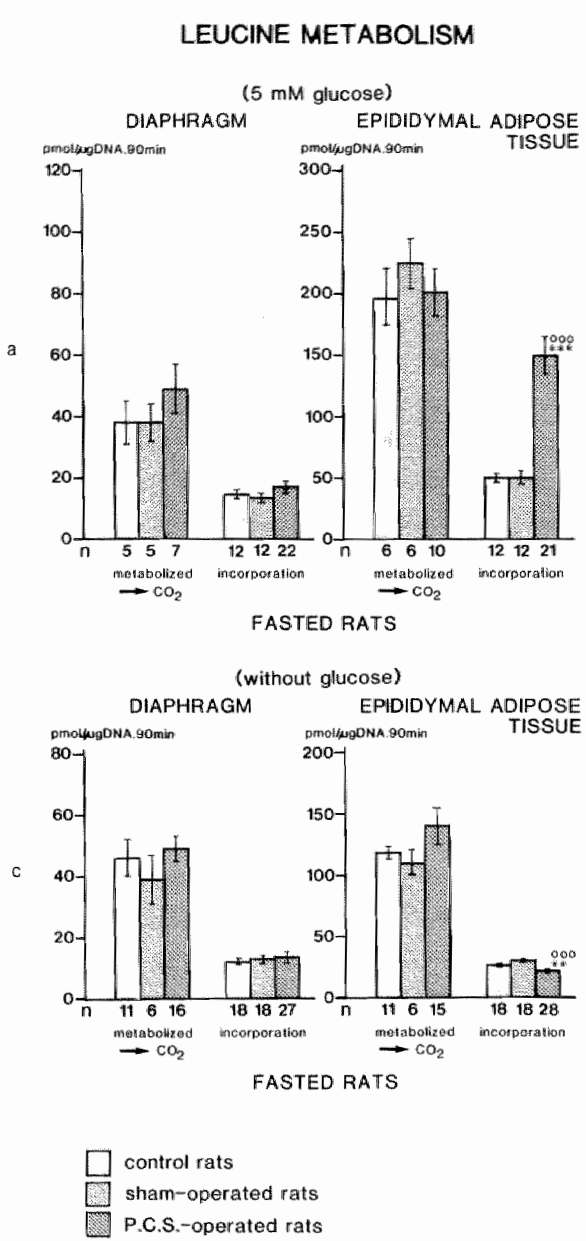
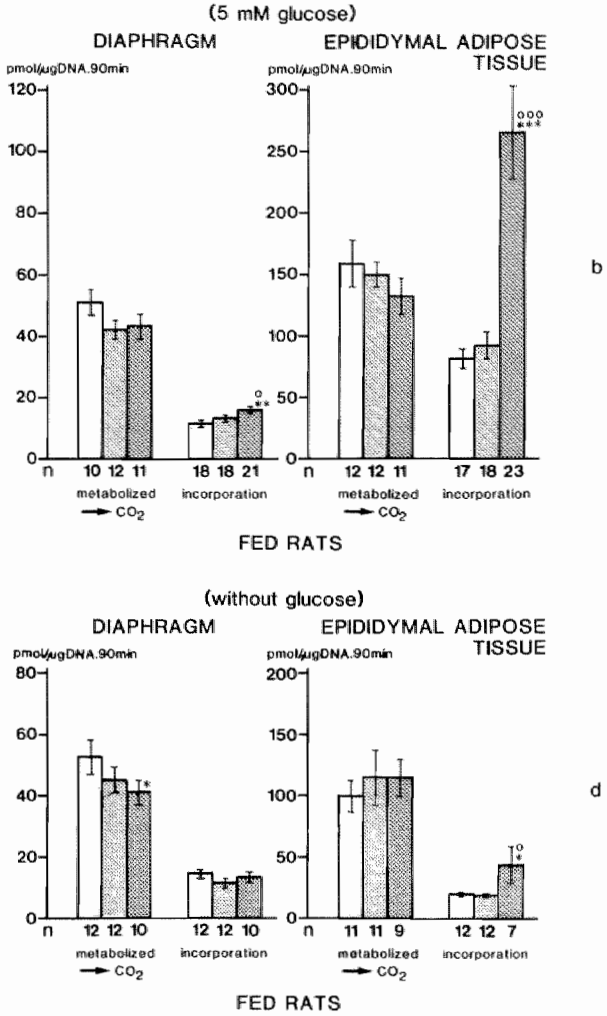


Figure 3
Leucine-metabolism in vitro in diaphragm and epididymal adipose tissue derived from fasted (a and c) and fed (b and d) unoperated, sham-operated (2½ weeks p.o.) and PCS-operated (2½ weeks p.o.) rats, measured in the presence of 0.1 mM ^{14}C-(U)-L-leucine with (a and b) and without (c and d) 5 mM glucose in the incubation medium (mean ± sem). Details are described in Materials and Methods.

LEUCINE METABOLISM



* compared with unoperated controls
o compared with sham-operated controls
*/o = $p<0.05$; **/oo = $p<0.01$, ***/ooo = $p<0.001$

Leucine-derived carbon released as CO_2 by diaphragm, incorporation of leucine-derived carbon into muscle protein, leucine-derived carbon released as CO_2 by epididymal adipose tissue and incorporation of leucine-derived carbon into fat in adipose tissue are plotted separately (from left to right, respectively). Results are expressed in pmol/µg DNA.90 min.

LEUCINE METABOLISM

^{14}C-(1)-L-LEU vs. ^{14}C-(U)-L-LEU

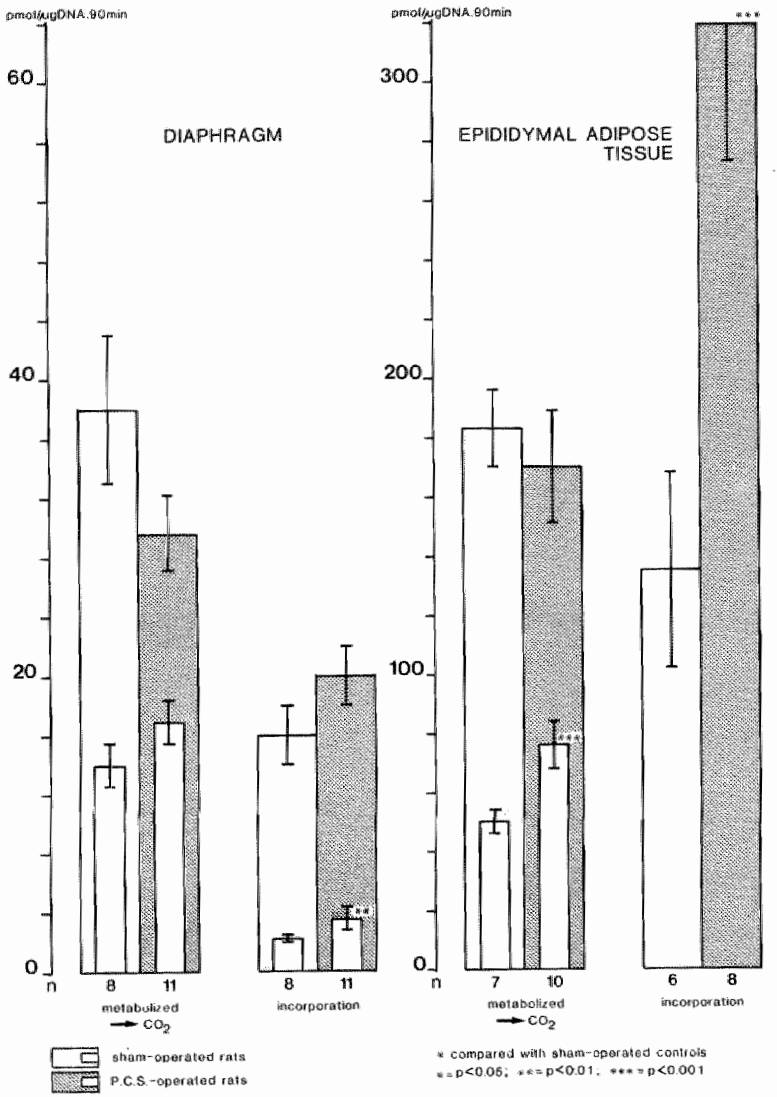


Figure 4

Leucine-metabolism in vitro in diaphragm and epididymal adipose tissue derived fr fed sham-operated (2½ weeks p.o.) and PCS-operated (2½ weeks p.o.) rats, measur in the presence of 5 mM glucose and 0.1 mM ^{14}C-(U)-L-leucine or 0.1 mM ^{14}C-(1)-leucine in the incubation medium (mean ± sem). Details are described in Materia and Methods. Leucine-derived carbon released as CO_2 by diaphragm, incorporation leucine-derived carbon into muscle protein, leucine-derived carbon released as C by epididymal adipose tissue and incorporation of leucine-derived carbon into f in adipose tissue are plotted separately (from left to right, respectively Results are expressed in pmol/ µg DNA.90 min. Note that the ordinates ha different scales for diaphragm and adipose tissue (consequence of difference tissue DNA content). The inner bars represent results derived from incubations which ^{14}C-(1)-leucine served as substrate.

higher specific activity for the C-1 carbon atom in ^{14}C-(1)-labeled leucine compared to ^{14}C-(U)-labeled leucine. Therefore, the results presented in fig. 4 were corrected for this difference to be able to compare the proportion of irreversibly decarboxylated (C-1-atom) leucine, representing the first step of leucine degradation, to the amount of leucine derived carbon originating from the rest of the leucine molecule. The results are expressed per µg DNA in the tissue.

Diaphragm

No significant differences were measured in CO_2-production from either ^{14}C-(1)- or ^{14}C-(U)-labeled leucine between tissues derived from SO-rats and PCS rats, but there is a tendency for CO_2-production from ^{14}C-(1)-leucine to be increased and from ^{14}C-(U)-leucine to be decreased ($p < 0.1$) by PCS. A small increase in incorporation of leucine into muscle protein was detected. This increase was significant when ^{14}C-(1)-leucine was used as substrate ($p < 0.01$), while p reached 0.1 when ^{14}C-(U)-leucine was added. The ratio of ^{14}C-label incorporated into protein in the SO-group and PCS operated group, starting from ^{14}C-(1)- and from ^{14}C-(U)-leucine, is exactly 1 when no correction is made for the 6 times higher specific activity of the C-1- carbon atom in ^{14}C-(1)-leucine. This indicates that leucine is incorporated as "whole" leucine.

Adipose tissue

CO_2-production from ^{14}C-(U)-leucine by adipose tissue was not altered by PCS, in contrast to the CO_2 production from ^{14}C-(1)-leucine, which was significantly increased ($p < 0.0005$). Incorporation of ^{14}C-(U)-leucine-derived carbon into fat of adipose tissue was increased two-fold in the PCS group. As expected, no ^{14}C-(1)-leucine derived carbon could be detected in fat.

Discussion

These results indicate that PCS increases the catabolism of leucine in adipose tissue in vitro by increasing irreversible decarboxylation of the C-1 atom of leucine and by increasing the incorporation of leucine degradation products (mainly acetyl-CoA) into fat. This increased degradation of leucine by adipose tissue may contribute to the decreased circulating

leucine levels in plasma after PCS. It is peculiar that these studies seem to indicate that degradation products of leucine, which is catabolized to a greater extent after PCS, serve as precursors for fatty acid synthesis. Especially in PCS rats, which normally have less muscle mass and less storage fat than normal rats, an increased irreversible decarboxylation of leucine and corresponding fatty acid synthesis in particular, seems to conflict with the desirability to retain or maintain positive nitrogen balance in these rats and with the special anabolic properties ascribed to leucine, such as stimulation of protein synthesis and regulation of protein turnover.

This study also indicates that in vitro after PCS, the carbon-skeleton of leucine that is left after decarboxylation of the C-1 atom, is catabolized to CO_2 to a lesser extent. The recorded values for CO_2-production and incorporation into fat after incubation with ^{14}C-(U)-leucine account for complete degradation of the decarboxylated leucine as estimated in incubations with ^{14}C-(1)-leucine, both in control and in PCS rats. However, in PCS rats, the remaining carbon-skeleton after decarboxylation of leucine, is degraded to CO_2 to a lesser extent and incorporated into fatty acids to a greater extent.

Careful definition of the PCS rat model showed that, in our institute, PCS rats lost some specific metabolic characteristics of PCS 3½-4 weeks after the operation (7), mainly due to the development of collateral blood vessels between the portal circulation and the liver. Therefore, experiments were carried out 2½ weeks p.o. In order to eliminate the possibility that non-specific metabolic effects of the operation would influence the results, sham-operated controls were included in the first study described, in addition to non-operated controls. Because no differences were observed between sham-operated controls and non-operated controls, the non-operated controls were omitted in the second study. The PCS rats employed in this study lost weight 3-4 days after the operation and from the fourth day exhibited normal growth rates and the metabolic characteristics that are thought to be specific for PCS including changes in plasma AA pattern and pancreatic hormone levels. Of these, decreased plasma BCAA levels and hyperinsulinemia are relevant for this study. In many studies, PCS rats failed to gain weight in the weeks following the operation (5,30,34) and

were virtually malnourished at the time of the experiments, making an exact interpretation of the data difficult.

In our experiments, the PCS-rats were slightly leaner due to a reduced food intake in the first days after the operation. Leaner rats have less adipose tissue and smaller fat cells, containing less fat and relatively more protein. Expressing the biochemical metabolic data of the incubation studies per unit wet tissue weight, implies that different numbers of cells are compared between the controls and the PCS-group. We assumed that the total number of cells in a rat does not change within 2½ weeks after the PCS operation, compared with a sham operation, and chose tissue DNA-content to correct for different numbers of cells. All recorded values in the studies described were corrected for individual tissue DNA content. However, there was also a considerable difference in DNA content between diaphragms from PCS-rats and control rats, indicating a difference in myofibrillar protein content per cell. A difference in (de)hydration of diaphragms from control rats and PCS operated rats was excluded by tissue-nitrogen determinations (chemiluminescence method; ref. 38). Mean values for tissue DNA content in diaphragm and adipose tissue of PCS- and SO rats are listed in table I. This correction for tissue DNA content influenced the interpretation of our data.

TISSUE DNA CONTENT (μg DNA/mg wet weight)

	Adipose tissue	Muscle
Sham-operated and non operated	0.31 ± 0.04	1.69 ± 0.11
PCS-operated	0.45 ± 0.07	2.33 ± 0.31

Table I

Tissue DNA content in diaphragm and epididymal adipose tissue from control rats (unoperated and SO-operated) and PCS operated rats; mean ± SD (n=50).

Our results from the tissue incubations with 0.1 mM ^{14}C-(U)-L-leucine and with ^{14}C-(1)-L-leucine (fig. 4) demonstrate that PCS increased the amount of leucine metabolized in vitro by adipose tissue. The increased CO_2-production from the C-1 labeled substrate represents increased irreversible decarboxylation of the C-1 atom of leucine. In contrast, CO_2-production from ^{14}C-(U)-leucine in the PCS group was not different from the control (SO-) group. The difference in CO_2-production between ^{14}C-(U)- and ^{14}C-(1)-leucine is the result of further metabolism of the leucine-carbon skeleton after decarboxylation of the C-1 atom, and may be difficult to interpret because of the existence of several potential routes of further metabolism of leucine degradation products; different carbon atoms may be involved differently in further metabolism. The decreased ratio between CO_2-production from ^{14}C-(U)- and ^{14}C-(1) leucine in adipose tissue in the PCS group, as compared to the SO-group, reflects a proportionally less complete catabolism of leucine (to CO_2). However, all carbon atoms derived from leucine that is irreversibly decarboxylated in vitro in adipose tissue of both PCS rats and control rats, are incorporated in fat or catabolized to CO_2. Other reactions seem to be quantitatively unimportant in this respect. This is illustrated by the fact that, in both groups, the sum of the amount of leucine-carbon degraded to CO_2 and the amount stored in fat during incubation with ^{14}C-(U)-leucine, approximately equals six times the CO_2 produced during incubation with ^{14}C-(1)-leucine (PCS-group: 488 vs 6 x 76; SO-group: 318 vs 6 x 50). The possibility that different intracellular pools of acetyl-CoA in tissues from PCS rats and control rats have influenced the results, seems to be only of theoretical importance. The increased incorporation leucine-derived carbon into fat after PCS, implies that increased fatty acid turnover must exist in PCS rats because PCS rats have less fat than controls.

In addition to the differences detected in leucine metabolism by adipose tissue, a small increase in incorporation of leucine-carbon into muscle protein was measured in vitro in diaphragm after PCS. The ratio of leucine-derived ^{14}C from ^{14}C-(U)- and ^{14}C-(1)-leucine in muscle protein was six to one. This demonstrates, as expected, that "whole" leucine molecules are incorporated into muscle protein. Leucine incorporation into muscle protein, increased by PCS, can be reutilized after proteolysis for new protein synthesis. As in PCS rats no increased growth-rate or muscle hyper-

trophy has been observed, the in vitro result in muscle suggests an increased protein-turnover rate in vivo. This finding is supported by an increased 3-methyl-histidine excretion in the urine of PCS rats. We recorded in six PCS-operated rats (about 240 g) a 3-methylhistidine excretion in the urine of 1.44 ± 0.10 μmol/24 hrs, while six controls (circa 300 g) excreted 1.18 ± 0.18 μmol/24 hrs. While 3-methylhistidine excretion does not exclusively indicate skeletal muscle protein degradation (22), our finding of increased incorporation of leucine into diaphragm in vitro after PCS strongly suggests that the increased 3-methylhistidine excretion originates from skeletal muscle rather than from the gut. In contrast to the increased incorporation of leucine into muscle protein from PCS-rats, the increased decarboxylation of leucine in adipose tissue means a definitive loss as leucine, because after deamination and decarboxylation, the rest of the molecule is metabolised to acetyl-CoA and acetoacetic acid from which leucine cannot be resynthesized.

In vitro CO_2-production, by diaphragm, from ^{14}C-(1)-leucine tended to be increased in the PCS group, while, from ^{14}C-(U)-leucine, it strongly tended to be decreased after PCS ($p < 0.1$). Therefore, irreversible degradation (decarboxylation) may have been slightly enhanced after PCS, but the rest of the leucine-molecule is less completely degraded to CO_2 in muscle. This pleads against a muscle energy deficit as the driving force for increased BCAA catabolism.

Incorporation of label into fatty acids in muscle, and into protein in adipose tissue were not measured in these experiments. It has been reported that these quantities are negligible (25).

The presence of glucose in the incubation medium did not influence CO_2-production and incorporation into protein in diaphragm of normal rats, but did influence CO_2-production and incorporation of label into fat, in adipose tissue (fig. 3). This indicates that different mechanisms are operative. The exact mechanism of action of glucose in the metabolism of leucine in adipose tissue is not known. The integrity of the cell or some cellular organelle may be concerned in the expression of this effect. The difference may be partially explained by the formation of glucose-derived glycerol which serves as the "backbone" for triglyceride synthesis. The data measured in the presence of glucose are, of course, more relevant to the in vivo situation.

The increase of the peripheral leucine metabolism after PCS in adipose tissue was observed in tissues derived from both fasted and fed rats (fig. 3). Consequently, this appeared to be no effect of fasting or feeding, accompanied by short-term changes in circulating leucine or pancreatic hormones. However, feeding changed the fate of a part of leucine-derived carbon in adipose tissue by decreasing CO_2-release and increasing the incorporation into fatty acids. This effect was most pronounced when glucose was present in the incubation medium and occurred in both normal and PCS rats. Fluctuations in circulating levels of glucose, amino acids and insulin may exert short-term control as suggested by studies of Goodman and Frick (14,16). Using tissue slices and cell-free preparations, they have shown in vitro that some aspects of the processing of leucine by adipose tissue from normal rats, can be influenced by adding these components to the incubation medium. This has been suggested to be due to activation of α-keto-isocaproic acid-dehydrogenase (α-KICA-DH), the rate-limiting enzyme in the catabolic pathway of leucine in most tissues, but not in liver. They have also shown that these tissues retain in vitro some of their characteristics with regard to the catabolism of leucine, which has been suggested to be due to the total amount of α-KICA-DH (active and inactive), induced by in vivo exposure of the tissues to insulin, for instance in tissues from diabetic rats and from rats fed diets containing variable amounts of protein (16). The increased in vitro leucine metabolism in tissues from PCS-rats must result from long-term regulation of the leucine degradative pathway because it persists after overnight fasting. As mentioned, evidence is accumulating that insulin is involved in determining in vitro the rate of breakdown of leucine in adipose tissue. Hyperinsulinemia was consistently present in our PCS rats both in the fasted and fed state, although the percentage increase in the fed state was small (fig. 2). The fact that overnight-fasting hardly influences the alterations in leucine metabolism in adipose tissue (and in muscle) after PCS, indicates that the time needed to express the effect is days or weeks rather than hours. For these reasons, it is very likely that the increased in vitro leucine metabolism in adipose tissue from PCS rats is caused by long lasting hyperinsulinism due to portal-systemic shunting. Both increased decarboxylation of leucine mediated by α-KICA-DH, and increased fatty acid synthesis may result from hyperinsulinism. The increased muscle protein synthesis after PCS may also

be the result of prevailing hyperinsulinism. In muscle, α-KICA-DH seems not to be regulated by insulin, but by ATP, redox state and muscle work (24,29). In addition, insulin is reported to stimulate uptake of BCAA in muscle (31,37) but the possible influence has not been measured in these experiments.

The cause for the hyperinsulinism in portal systemic-shunting and patients with chronic liver failure is still subject to controversy. A causal relationship between plasma insulin levels and plasma BCAA levels has often been suggested, but has yet to be proven.

The data obtained with adipose tissue suggest an extremely well fed state in PCS rats compared to controls, despite equal or even slightly decreased food intake. Even after overnight fasting, fatty acid synthesis from leucine-derived carbon (via acetyl-CoA) in PCS rats is increased compared to fed control rats (fig. 3). If this is indeed an insulin effect, it may be concluded that insulin levels are inappropriate with respect to the quantity of food consumed, inducing increased irreversible decarboxylation of leucine and incorporation of leucine-carbon into fat. It would further imply, that these increased insulin levels are effective in adipose tissue of PCS rats. It is true that insulin resistance is documented with regard to the metabolism of glucose and BCAA in cirrhotic patients (20,21), but our data suggest that this does not occur in adipose tissue. This is in accordance with the very recently demonstrated absence of insulin receptor changes in adipose tissue of cirrhotic patients by Harewood et al (17).

A problem arises considering the importance of our results for the in vivo situation; increased catabolism of leucine and other BCAA in vivo may be counterbalanced by the decreased circulating plasma BCAA-levels. However, intracellular concentrations are reported to be normal or even increased (18). It is difficult to speculate about in vivo rates from DNA-corrected data. The ratio between leucine catabolism per mg wet weight of muscle and adipose tissue in vitro, combined with the percentage of these tissues of total body weight, may however apply to the in vivo situation. Our data expressed per mg wet weight, demonstrated that in vitro leucine catabolism in muscle and adipose tissue, at a physiological leucine concentration, is roughly equal per unit weight of either tissue from PCS and control rats. In tissues from fed control rats, the in vitro leucine

catabolism in muscle is slightly higher than in adipose tissue, while in tissues from fed PCS rats, the in vitro leucine catabolism in adipose tissue is more pronounced. Assuming that muscle comprises approximately 40% of the total body weight, and adipose tissue 10%, our data suggest that in normal rats, adipose tissue may metabolize approximately 25% of total ingested and endogenously released leucine. In PCS rats, adipose tissue plays a quantitatively more important role; the contribution of adipose tissue to leucine breakdown may be estimated to be 35% or more, depending on the total amount of adipose tissue, the number of actively leucine metabolizing fat-cells and probably the size of these cells.

Ordinary rat chow contains approximately 25% protein by weight; it is estimated that approximately 20-30% of this protein consists of BCAA. The healthy rat needs only a small amount of these BCAA for protein synthesis, so that the majority must be disposed of in another way. Muscle and adipose tissue are quantitatively the main organs that metabolize BCAA. Brain and kidney are able to metabolize significant amounts of BCAA per unit weight but this is less relevant for overall metabolism due to their small size. Excess of BCAA has therefore largely to be disposed of by degradation to CO_2 in muscle and adipose tissue, and by incorporation into fatty acids.

The increased leucine catabolism after PCS, measured in vitro in the presence of glucose, may contribute to the decreased plasma leucine level in PCS-rats. In this respect, another question remains to be answered. Does the increased in vitro degradation of leucine only result in decreased plasma leucine levels in vivo which in turn counterbalance ongoing degradation, or is over-all catabolism induced due to a relative lack of leucine? In this study, we only included PCS-rats that gained weight. Growth rate was proportional to food intake. At sacrifice, PCS rats had less fat and less muscle mass than controls, as evidenced by the weights of the diaphragms and epididymal fat pads that were excised and by tissue DNA-content of muscle and adipose tissue (table I). It is noteworthy that, although we excluded results from rats with low body weights (less than 110% of the preoperative weight) in this study, for reasons mentioned, the highest incorporation rates in adipose tissue were recorded in these malnourished rats. It is, however, very unlikely that a lower growth rate and relative malnutrition may have caused the phenomena observed. While in

long-term starvation, decreased protein turnover and decreased fatty acid turnover occur and low insulin levels exist, in this study the opposite was demonstrated in PCS rats. So, a specific effect of PCS itself seems responsible for the changes observed. Increased turnover of protein and/or fat has been observed in cirrhosis (27,35), after trauma (6,28) and in sepsis (6,33) and has very recently also been reported in cancer cachexia (19). This suggests that stress-hormones may be concerned in the expression of the changes observed. Hyperinsulinism may be an integral part of a stressed state.

This study provides no definitive evidence that in PSS in vivo an increased catabolic rate is induced by increased breakdown of BCAA. Our data are in agreement with the hypothesis that increased in vitro catabolism of BCAA in PCS rats may be a consequence of increased α-KICA-DH activity in adipose tissue, induced by longlasting hyperinsulinism. Further studies to determine this α-KICA-DH activity in muscle and adipose tissue of PCS rats and their controls are described in chapter V. The increased in vitro catabolism of leucine in adipose tissue after PCS, may contribute to the decreased plasma leucine levels in PCS rats.

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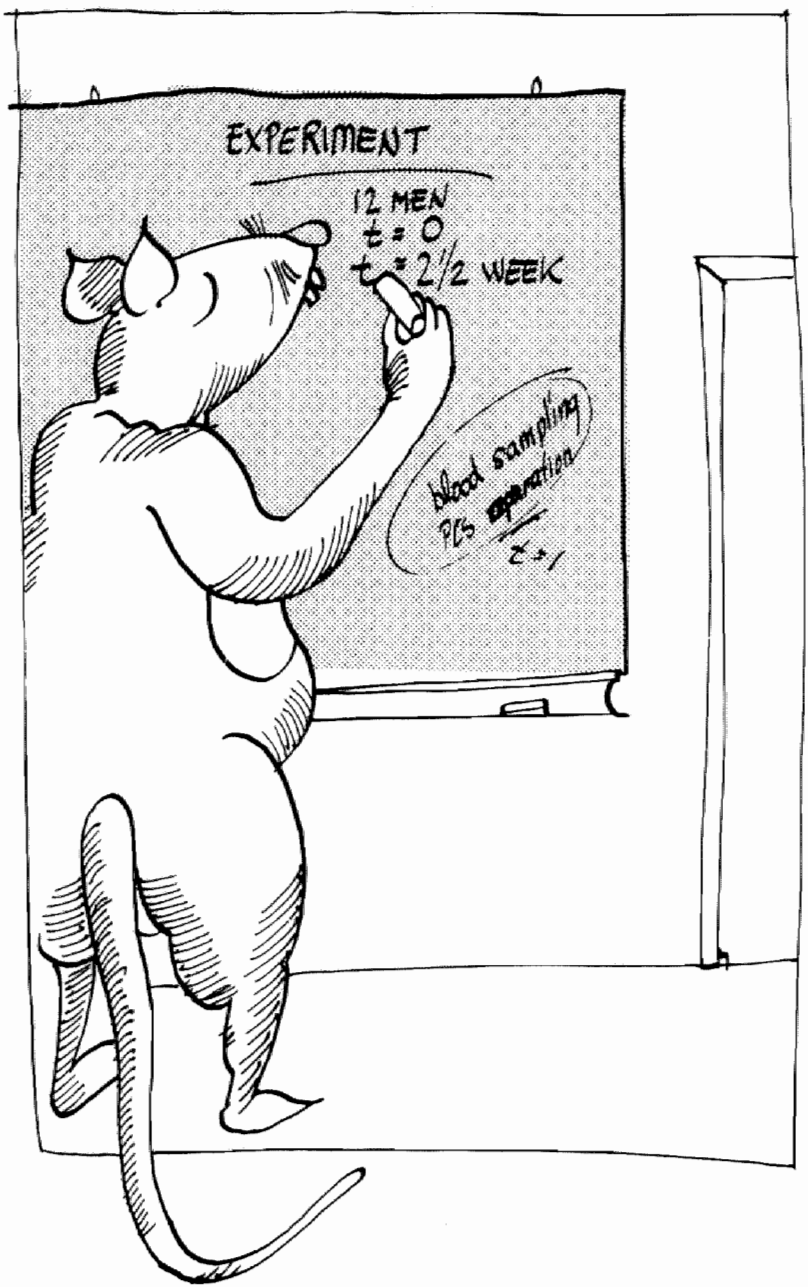

EXPERIMENT
12 MEN
t = 0
t = 2½ WEEK
blood sampling
PL's separation
Z = 1

CHAPTER IV

INFLUENCE OF PORTAL-SYSTEMIC SHUNTING ON ISOLEUCINE AND VALINE METABOLISM IN MUSCLE AND ADIPOSE TISSUE OF RATS

Summary

Decreased levels of branched chain amino acids (BCAA) in plasma exist in patients and experimental animals with portal-systemic shunting and hepatic insufficiency. The underlying mechanism is poorly understood. In a foregoing study, we reported an increased catabolism of leucine in vitro in peripheral tissues of porta-caval shunted (PCS) rats and the important role of adipose tissue in this catabolism. In this study, 2½ weeks after the PCS-operation, we investigate the in vitro metabolism of isoleucine and valine in diaphragm and adipose tissue from PCS-operated rats. The distribution of the incorporation of leucine-, isoleucine- and valine-derived carbon into different fat-fractions in adipose tissue is traced and the influence of addition of leucine on isoleucine- and valine catabolism studied. The results indicate that:

1. After PCS, in vitro isoleucine- and valine catabolism are increased in adipose tissue derived from fed rats and resembles the alterations measured with leucine under the same conditions: The increased decarboxylation rate of valine is augmented (isoleucine could not be measured) and a pronounced increase of incorporation of BCAA-derived carbon into fat in adipose tissue is found. In addition, increased catabolism of ^{14}C-(U)-valine-derived carbon to CO_2 is observed.
2. In muscle, the rate of decarboxylation of valine is decreased after PCS. The incorporation of isoleucine and valine into muscle protein is significantly increased after PCS.
3. The highest levels of incorporation of BCAA-derived carbon into fat in adipose tissue from both control- and PCS-operated rats are measured after incubation with leucine, whereas the lowest levels are found after incubation with valine.
4. The increased incorporation of BCAA-derived carbon into fat in adipose tissue after PCS is largely into the triglyceride fraction.
5. Incorporation of isoleucine-derived carbon into fat in adipose tissue (i.e. triglyceride synthesis) from both normal and PCS rats is enhanced in the presence of leucine in a physiological concentration.

The increased catabolism of all three BCAA in adipose tissue after PCS may contribute to the decrease of plasma BCAA levels after PCS. Because

increased protein synthesis and fatty acid synthesis in the PCS rats is accompanied by decreased muscle mass and fat mass, the data suggest that PCS induces increased muscle protein turnover and fatty acid turnover. In vivo, this may result in diminished growth or, in severe conditions, in net catabolism.

Introduction

Plasma branched chain amino acid (BCAA) levels are decreased in patients and experimental animals with impaired liver function and/or portal-systemic shunting (PSS) (3). BCAA are almost completely metabolized peripherally in muscle and adipose tissue. Kidney and brain play a minor role due to the correspondingly smaller amount of tissue (chapter I). In in vitro incubation studies, we investigated the metabolism of leucine in diaphragm and epididymal adipose tissue derived from normal and porta-caval shunt (PCS) operated rats which have been shown to exhibit decreased plasma BCAA levels (chapter II and III). It was demonstrated that, expressed per µg DNA, PCS increased in vitro in adipose tissue the catabolism of leucine by increasing irreversible decarboxylation of the C-1 atom of leucine in adipose tissue and subsequently by increasing the incorporation of leucine-derived carbon into fat (chapter III). These in vitro results suggested that the low plasma leucine levels are due to increased peripheral leucine catabolism in adipose tissue after PCS.

The rate-limiting enzyme in the degradation of leucine in most tissues, the α-keto isocaproicacid dehydrogenase (α-KICA DH), also catalyses the irreversible decarboxylation of the other BCAA, isoleucine and valine. Low plasma levels of these amino acids after PCS, might also be due to increased peripheral catabolism. To gain support for this hypothesis, we measured the influence of PCS on the in vitro catabolism of isoleucine and valine in muscle and adipose tissue in the presence of physiological concentrations of ^{14}C-(U)-isoleucine, ^{14}C-(1)-valine or ^{14}C-(U)-valine (0.1 mM); ^{14}C-(1)-isoleucine was not commercially available.

In the studies with leucine, the greatly increased incorporation of leucine-derived carbon into fat in adipose tissue after PCS, was an unexpected finding. We therefore determined, in this study, in which fraction(s) the leucine-derived carbon, and also isoleucine- and valine-

derived carbon, could be detected. In vivo, all three BCAA are present together and the metabolism of the BCAA may be influenced by each other. Special regulatory properties are ascribed to leucine (1,5,6). Therefore, the influence of leucine on the irreversible C-1 decarboxylation was measured. Furthermore, the distribution of isoleucine- and valine-derived carbon in the different fat-fractions was traced after incubation of the tissues with these amino acids in the presence of a physiological concentration of unlabeled leucine. In all these experiments, tissues from PCS operated and sham-operated rats were compared.

Materials and methods

PCS operations were performed in male Sprague-Dawley rats. Rats used in experiments were selected according to the criteria described before (chapter II). All these rats had proven hyperinsulinism, decreased plasma BCAA levels and positive nitrogen balance as evidenced by weight gain. Diaphragm and epididymal adipose tissue of these rats were used for the incubation experiments 2½ weeks postoperatively. The incubation procedure and the techniques to measure $^{14}CO_2$-release, incorporation into muscle protein and into fat in adipose tissue, from BCAA-derived carbon, have been described before (chapter III).

For determination of the distribution of BCAA-derived carbon in fat in adipose tissue, the following procedure was used. Immediately after incubation, the adipose tissue fragments (30-40 mg) were removed and frozen in liquid nitrogen. To minimize the action of lipolytic enzymes, the frozen tissue pieces were pulverized in an aluminium mortar that had been cooled in liquid nitrogen. Fat was extracted with a chloroform-methanol mixture (2:1) and dried under nitrogen atmosphere. For separation of the fat-fractions by thin-layer chromatography (TLC), the sample was dissolved in chloroform-methanol (1:1). One third of this sample was added to a TLC-plate (pre-coated TLC plates; silica gel 60 F-254, 0.5 mm, Merck, Darmstadt, Germany). The mobile phase existed of petroleum ether (boiling-range 60-80°C; 120 ml), diethyl ether (25 ml) and glacial acetic acid (1.5 ml) and contained butylated hydroxy toluene (o-cresol butylether; 50 mg/l), which was also present in the extraction fluid. Radioactivity in some

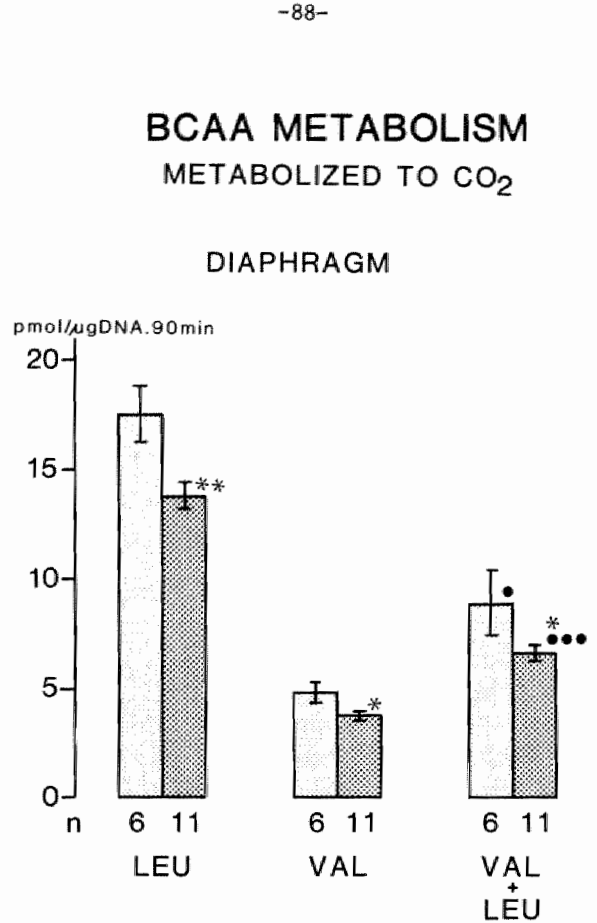


Figure 1a

Figure 1
Valine metabolism to CO_2 in vitro in diaphragm (1a) and epididymal adipose tissue (1b) derived from fed sham-operated and PCS-operated rats ($2\frac{1}{2}$ weeks p.o.), measured in the presence of 0.1 mM ^{14}C-(1)-L-valine, ^{14}C-(U)-L-valine (s.a. 0.25 mCi/mmol) or with extra unlabeled leucine (0.1 mM).

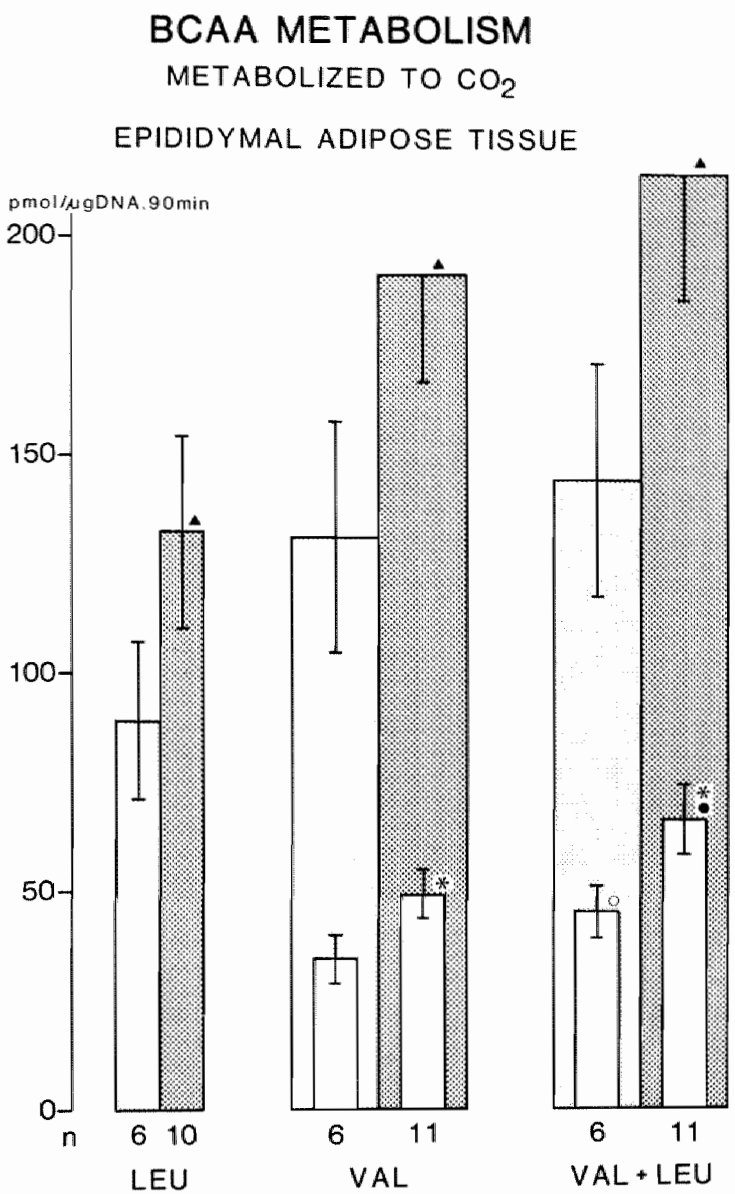


Figure 1b

Measurements with ^{14}C-(1)-L-leucine were included as control, allowing a valid comparison with experiments described in chapter III. Results are expressed in pmol/µg DNA.90 min. (mean ± sem). The narrow or inner bars represent results obtained with ^{14}C-1 labeled components; the wide bars represent results obtained with ^{14}C-(U) labeled components.

fractions was too low to allow detection by scanning the thin layer plates directly. Therefore, the fat fractions were identified by iodine vapour, scraped off and the radioactivity was measured by liquid scintillation counting. Identification of the fractions was facilitated by the use of purified standards (phospholipids, diglycerides, cholesterol, free fatty acids, triglycerides and cholesterol-esters) and/or by addition of tritium labeled purified components.

^{14}C-(U)-L-Isoleucine (s.a. 335 mCi/mmol), ^{14}C-(1)-L-valine (s.a. 48 mCi/mmol) and ^{14}C-(U)-L-valine (s.a. 295 mCi/mmol) were purchased from New England Nuclear Corp. (Boston, Mass.). Counting solution Pico-fluor 15 and the tissue solubilizer Soluene^{R}-350 were obtained from Packard-Becker B.V., (Chemical Operations, Groningen, The Netherlands). All other reagents were analytical grade. Glucose (5 mM) was present in the Krebs-Ringer incubation medium. The final concentration of isoleucine and valine was 0.1 mM and the s.a. 0.25 mCi/mmol. In the experiments in which BCAA-derived ^{14}C was traced in the different fat-fractions, the specific activity of the BCAA used was 2.5 mCi/mmol. Tissue DNA content was determined in muscle according to Burton (2) and in adipose tissue according to Rafael and Vsiansky (7).

Results

In accordance with data from earlier experiments, plasma insulin was increased by 35% two-and-a-half weeks after the PCS operation and plasma glucagon, by 100% (both measured after an overnight-fast), while plasma BCAA levels were decreased by approximately 30% and phenylalanine and tyrosine were increased (92 and 23%, respectively; amino acids measured in fed state). All results from the tissue-incubation experiments are derived with tissues from fed rats, and expressed in pmol/µg DNA.90 min. to correct for different numbers of cells.

Incubations with ^{14}C-(U)-L-isoleucine, ^{14}C-(1)-L-valine and with ^{14}C-(U)--L-valine.

Diaphragm.

Irreversible decarboxylation of ^{14}C-(1)-valine in muscle was significantly decreased after PCS ($p < 0.05$; fig.1a). The amount of isoleucine-

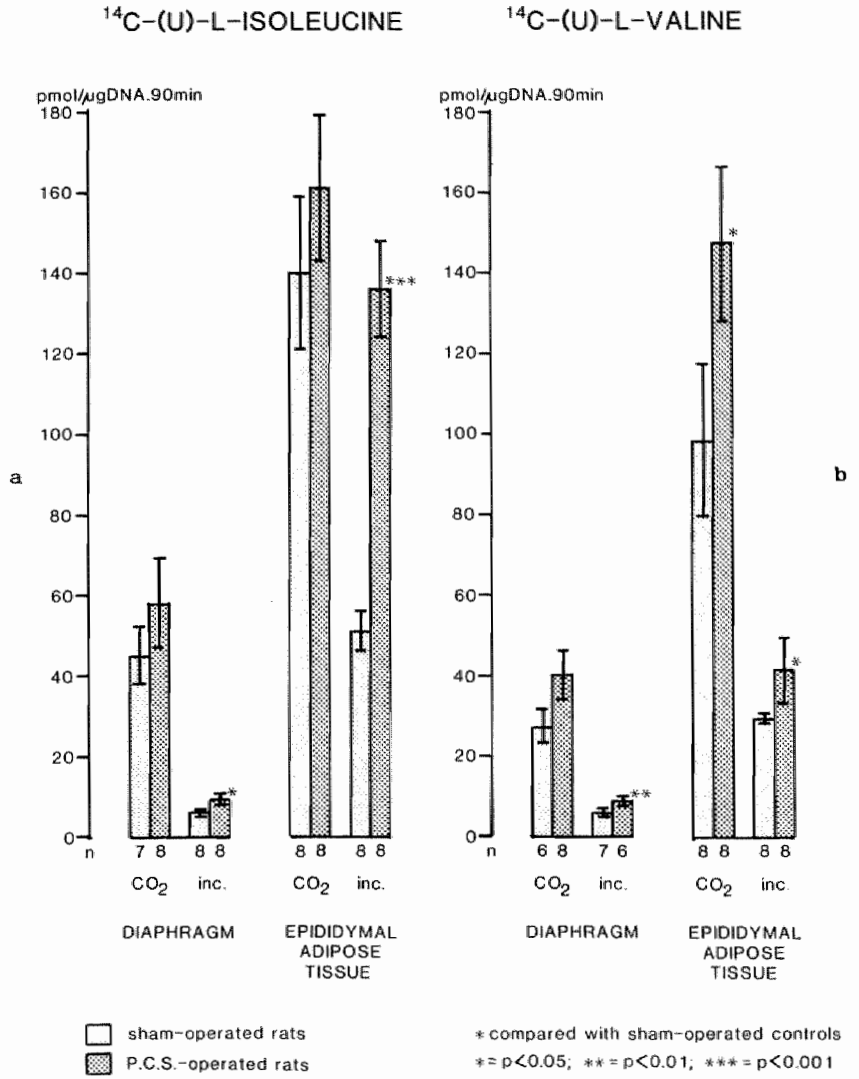


Figure 2
Isoleucine- and valine metabolism in vitro in diaphragm and epididymal adipose tissue derived from fed sham-operated and PCS-operated rats (2½ weeks p.o.) measured in the presence of 0.1 mM ^{14}C-(U)-L-isoleucine (2a) and 0.1 mM ^{14}C-(U)-L-valine (2b). Tissue pieces (30-40 mg) were, immediately after removal from the rats, incubated in Krebs-Ringer bicarbonate buffer pH 7.4, including 5 mM glucose, in a shakerbath at 38.5°C for 90 min. The reaction was started after a 10 min. preincubation by addition of the labeled substrates. Amino acid-derived carbon released as CO_2 by diaphragm, incorporation of amino acid into muscle protein, amino acid-derived carbon released as CO_2 by epididymal adipose tissue and incorporation of amino acid-derived carbon into fat in adipose tissue are given separately, from left to right. The procedure for these measurements has previously been described (chapter III). Results are expressed in pmol/ µg DNA.90 min. (mean ± sem).

carbon and valine-carbon derived CO_2, produced in vitro by diaphragm from PCS rats, was not significantly altered compared to the sham-operated controls in the presence of 0.1 mM ^{14}C-(U)-L-isoleucine or 0.1 mM ^{14}C-(U)-valine in the incubation medium but had a tendency to be increased after PCS, when 0.1 mM ^{14}C-(U)-L-valine was present ($p < 0.1$; fig. 2a and 2b). Incorporation of isoleucine and valine into muscle protein in vitro was increased after PCS ($p < 0.05$ and $p < 0.005$, respectively).

Adipose tissue.

Irreversible decarboxylation of ^{14}C-(1)-valine in adipose tissues was significantly increased after PCS ($p < 0.05$; fig.1b). CO_2-production from isoleucine-derived carbon by epididymal adipose tissue in vitro, was not significantly altered by PCS, while in the presence of valine a significant increase in CO_2-production was recorded ($p < 0.05$). The incorporation of both isoleucine- and valine-derived carbon into fat in adipose tissue was enhanced ($p < 0.001$ and $p < 0.05$ respectively; fig. 2a and 2b).

Levels that were recorded for valine-derived carbon were lower than for isoleucine-derived carbon, except in the incorporation of these amino acids into muscle protein, which was approximately equal in control rats.

Distribution of ^{14}C-(U)-L-BCAA derived carbon in fat from PCS-operated and sham-operated rats.

The differences in the in vitro rates of metabolism of leucine, isoleucine and valine, present at a 0.1 mM concentration, in muscle and adipose tissue from PCS- and sham-operated rats, were similar. The increased incorporation of BCAA-derived carbon into fat in adipose tissue of PCS-operated rats was most pronounced. The measured levels for both control- and PCS-rats decreased in the order leucine > isoleucine > valine, which was least incorporated into fat. In another group of control and PCS rats, selected according to the same criteria, the distribution of the BCAA-derived carbon incorporated in vitro into different fractions of fat, was investigated. Fat was extracted and separated as described in Materials and methods. The results are expressed in pmol/μg DNA. 90 min. and plotted in fig. 3a, 3b and 3c for leucine-, isoleucine- and valine-derived carbon, respectively. Fig. 3 demonstrates that the in vitro additionally incorporated BCAA-derived carbon into adipose tissue after PCS is largely

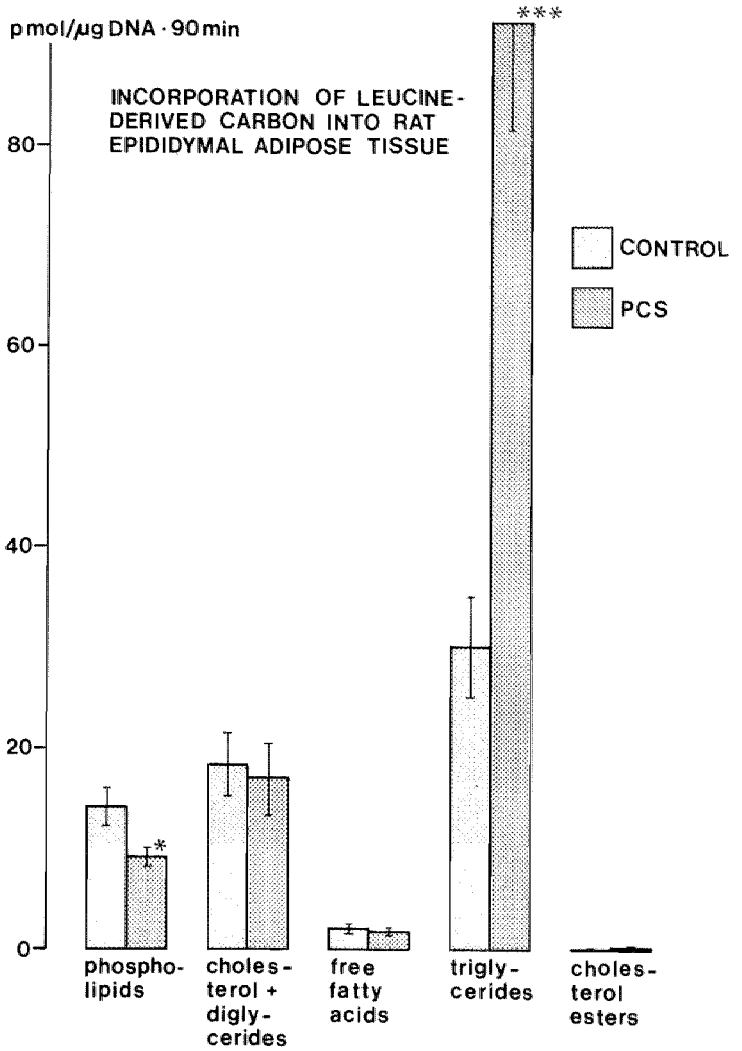
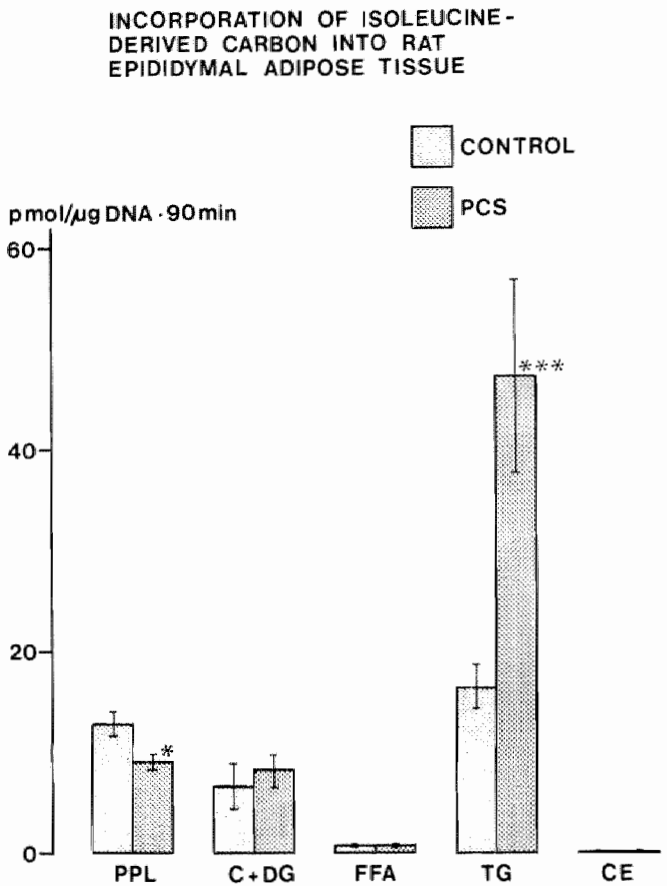


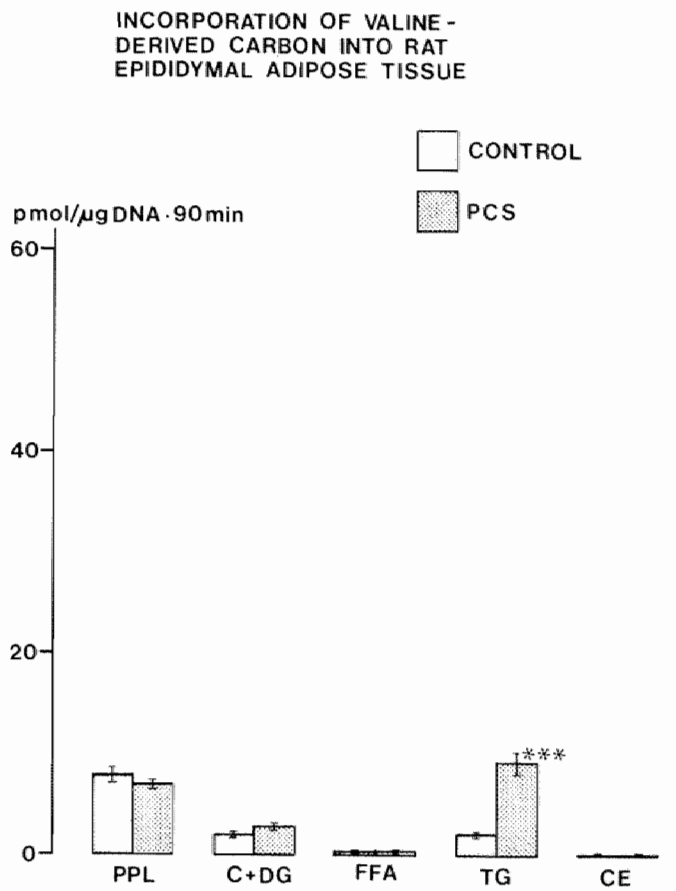
Figure 3a

Figure 3
Distribution of ^{14}C-(U)-L-BCAA derived carbon in fat in tissues from PCS-operated and sham-operated rats. For details see Materials and methods. The results are expressed in pmol/µg DNA.90 min. and given in fig. 3a, 3b and 3c for leucine-, isoleucine- and valine-derived carbon respectively (mean ± sem; n = 10, except in PCS group with leucine (n = 12) and sham-operated group with valine (n = 13)).



* compared with sham-operated controls
* = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$

Figure 3b
(see legend p. 93)



* compared with sham-operated controls
* = p<0.05; ** = p<0.01; *** = p<0.001

Figure 3c
(see legend p. 93)

accounted for by fatty acid synthesis which is mainly used for the formation of triglycerides. Diglycerides and cholesterol were poorly separated in these studies and are therefore shown as one fraction. The incorporation of leucine- and isoleucine-derived carbon into phospholipids was decreased in the PCS group ($p < 0.05$). As expected, all measured levels decreased in the order leucine > isoleucine > valine.

Influence of leucine on isoleucine and valine metabolism

A similar experiment was carried out with adipose tissue from a third group of PCS-rats and their sham-operated controls. In this experiment, the incorporation of amino acid-derived carbon into fat and the distribution over the fat-fractions were measured in the presence of 0.1 mM ^{14}C-(U)-L-isoleucine or 0.1 mM ^{14}C-(U)-L-valine in the incubation medium, and in the presence of these labeled amino acids (0.1 mM) in combination with 0.1 mM unlabeled L-leucine. The results are listed in table I. Comparison of the results derived with tissues from PCS rats and control rats are in good agreement with the experiments described above. Significant increases were measured in the incorporation in vitro of isoleucine- and valine-derived carbon into triglycerides after PCS ($p < 0.05$ and $p < 0.01$, respectively). In the presence of an additional 0.1 mM unlabeled leucine in the incubation medium, similar increases in incorporation into triglycerides after PCS were recorded with equal significances (table I; fig. 4). The presence of additional (unlabeled) leucine in the incubation medium (0.1 mM) increased the incorporation of ^{14}C-(U)-isoleucine-derived carbon into triglycerides in adipose tissue from PCS rats ($p < 0.05$) and tended to increase the incorporation in adipose tissue from control rats, however no significance was reached in this case. Incorporation of ^{14}C-(U)-valine-derived carbon into triglycerides in adipose tissue was not altered by the presence of additional leucine (fig. 4).

No significant increases were noted in incorporation into other fat fractions between PCS- and control rats. A decrease was noted in incorporation of valine-derived carbon into cholesterol in the PCS-group (table I; $p < 0.05$), when no unlabeled leucine was present.

	0.1 mM ^{14}C-(U)-isoleucine		0.1 mM ^{14}C-(U)-isoleucine + 0.1 mM leucine	
Fraction	Sham-op.	PCS-op.	Sham-op.	PCS-op.
Pl	15.2 ± 0.9	12.4 ± 1.6	17.1 ± 1.1	15.8 ± 1.6
DG	13.0 ± 3.0	9.0 ± 1.7	19.0 ± 4.0	12.4 ± 1.7*
Chol	1.9 ± 0.4	1.8 ± 0.5	2.4 ± 0.4	2.2 ± 0.5
FFA	0.7 ± 0.1	0.8 ± 0.2	0.7 ± 0.1	1.1 ± 0.2
TG	40.0 ± 9.0	69.0 ± 10*	48.0 ± 7.0	102.0 ± 18.0*○
CE	0.1 ± 0.1	0.1 ± 0.05	0.1 ± 0.1	0.1 ± 0.1

	0.1 mM ^{14}C-(U)-valine		0.1 mM ^{14}C-(U)-valine + 0.1 mM leucine	
Fraction	Sham-op.	PCS-op.	Sham-op.	PCS-op.
Pl	8.5 ± 0.5	8.0 ± 0.7	7.3 ± 0.8	7.2 ± 0.5
DG	3.3 ± 0.7	2.6 ± 0.6	3.3 ± 0.8	2.5 ± 0.5
Chol	0.8 ± 0.1	0.4 ± 0.1*	0.7 ± 0.1	0.6 ± 0.1
FFA	0.4 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	0.3 ± 0.1
TG	8.6 ± 1.2	20.0 ± 3.0**	9.0 ± 2.0	21.0 ± 3.0**
CE	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0

Table I.
Distribution of ^{14}C-(U)-L-isoleucine and ^{14}C-(U)-L-valine-derived carbon in fat in tissues from PCS-operated (n=8) and sham-operated rats (n=5), without and in the presence of unlabeled leucine (0.1 mM). Results are expressed in pmol/µg DNA.90 min. (mean ± sem). Significances between sham- and PCS operated rats are indicated by asterisks (* $p < 0.05$; ** $p < 0.01$); significances due to the presence of unlabeled leucine by open circles (○ $p < 0.05$).
PL: phospholipids; FFA: free fatty acids;
DG: diglycerides; TG: triglycerides;
Chol: cholesterol; CE: cholesterol esters.

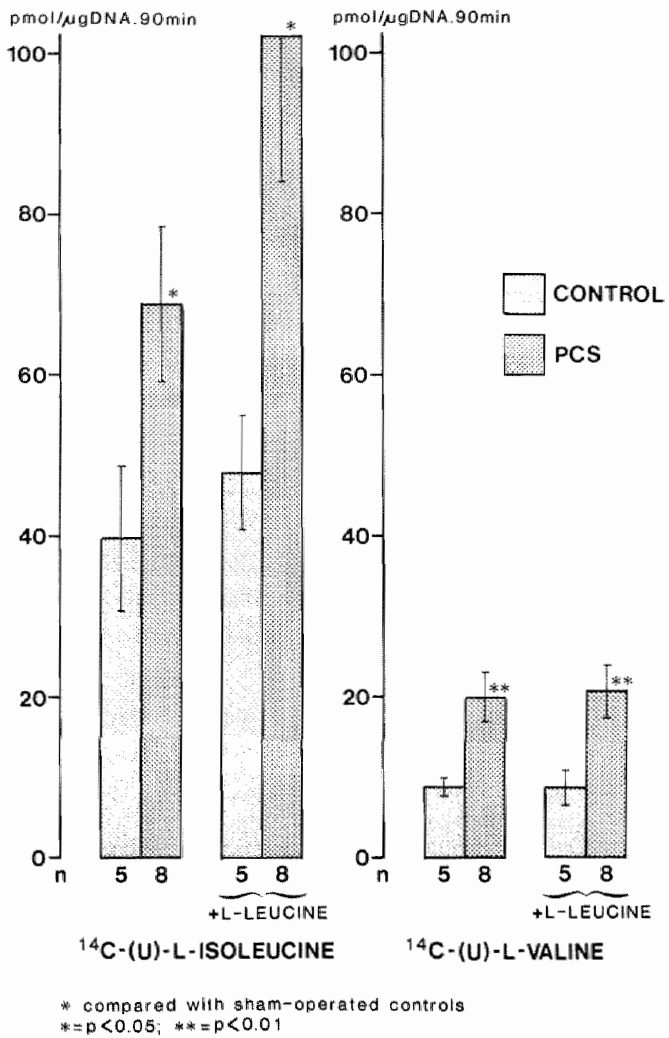


Figure 4
Incorporation of ^{14}C-(U)-L-isoleucine and ^{14}C-(U)-L-valine-derived carbon into triglycerides in adipose tissues from PCS-operated and sham-operated rats, without and in the presence of unlabeled leucine (0.1 mM). Results are expressed in pmol/ µg DNA.90 min. (mean ± sem). Significances are indicated as in table I.

Discussion

The metabolism of isoleucine and valine in muscle and adipose tissue from PCS rats and sham-operated controls, as measured in vitro in incubation experiments in the presence of 0.1 mM ^{14}C-(U)-isoleucine, ^{14}C-(1)-valine or ^{14}C-(U)-valine, greatly resembled the metabolism of leucine, measured under identical conditions: an increased BCAA catabolism in adipose tissue after PCS, and no change or a small decrease in BCAA catabolism, in muscle. The recorded levels, however, were generally lower with isoleucine and valine than with leucine (chapter III). The important role of adipose tissue in BCAA-catabolism, especially after PCS, was once more demonstrated. The enhanced incorporation of BCAA-derived carbon into the storage component triglyceride after PCS and the increased incorporation of BCAA into muscle protein, must imply that the turnover of fatty acids in adipose tissue and protein in muscle are increased, because of the generally smaller fat and muscle mass in PCS rats. The increased catabolism of BCAA in vitro in adipose tissue after PCS may contribute to the decreased BCAA-levels in plasma in vivo in case of portal-systemic shunting. The decarboxylation rates of leucine and valine are increased after PCS to approximately the same degree. Although the rate of decarboxylation of isoleucine could not be measured, it may be expected to be increased after PCS, judging from the results with universally labeled isoleucine. So, the rates of decarboxylation of all three BCAA appear to be increased to the same extent. This may explain the approximately equal decrease of BCAA levels in the plasma after PCS.

Metabolism from ^{14}C-(U)-isoleucine and ^{14}C-(U)-valine derived carbon to CO_2 by both muscle and adipose tissue (fig. 2a and 2b) were not significantly altered by PCS except in adipose tissue with valine, although a tendency to be increased after PCS was noted. Incorporation of isoleucine and valine into muscle protein was significantly increased in the PCS-operated group, while, after PCS, the increase of isoleucine- and valine derived carbon into fat in adipose tissue was most pronounced when isoleucine served as substrate. So, of all three BCAA, the metabolism of ^{14}C-(U)-valine to CO_2 in adipose tissue after PCS is proportionally more increased than fatty acid synthesis, while the increase in irreversible

decarboxylation from ^{14}C-(1)-valine after PCS is comparable to leucine (fig. 1b and fig. 2). Consequently, the valine carbon skeleton is, compared with leucine and isoleucine, more completely oxidized to CO_2 in adipose tissue after PCS.

Furthermore, it can be calculated from fig. 1 and fig. 2, by comparison of the data obtained with ^{14}C-(1)-valine and ^{14}C-(U)-valine, that the carbon atoms of all valine that is irreversibly decarboxylated in adipose tissue, are metabolized to CO_2 or used for fatty acid synthesis, as has been shown for leucine in chapter III. In contrast to the complete degradation of valine in adipose tissue, a significant part of the irreversibly decarboxylated valine in muscle cannot be accounted for by metabolism to CO_2, indicating an incomplete degradation in muscle. This was also shown for leucine (chapter III).

Differences in the recorded levels between leucine-, isoleucine- and valine-oxidation to CO_2 in adipose tissue from normal rats are in agreement with data reported by Rosenthal et al, Tischler et al and Frick et al (4,8,9). These differences may be explained by different rates of decarboxylation of the three BCAA determined by different affinities of the BCAA for the rate-limiting enzyme in the degradation in muscle and adipose tissue, and were also seen in vitro with tissues from PCS rats (fig. 1). Differences in transamination rates of leucine, isoleucine and valine may also occur in peripheral tissues, but are less relevant for the degradation rates of BCAA than the activity of the rate-limiting branched chain oxoacid dehydrogenase (BCOA-DH). The presence of leucine may have an additional effect on the activity of the BCOA-DH (1,4,5).

The increased incorporation of BCAA-derived carbon into fat in adipose tissue after PCS was very interesting. The increased incorporation of BCAA-derived carbon into triglycerides was completely responsible for this (fig. 3). Different levels of incorporation of leucine-, isoleucine- and valine-derived carbon into triglycerides were measured in tissues from both normal and PCS-rats. Apart from the differences in Km of BCOA-DH for the three BCAA, different levels of incorporation of BCAA-derived carbon into triglycerides will undoubtedly be influenced by the different degradation routes of the BCAA (for degradation routes of BCAA see addendum), distal of the oxidative decarboxylation and the subsequent partial β-oxidation step.

Leucine degradation results in the immediate formation of acetyl-CoA, the direct precursor in fatty acid synthesis, and in acetoacetate. The latter is readily converted to acetyl-CoA, and thus will also participate in fatty acid synthesis. Degradation of isoleucine, which is ketogenic and glycogenic, results in acetyl-CoA, ready for use in fatty acid synthesis, and, via propionyl-CoA, in succinyl-CoA, which can enter the tricarboxylic acid cycle to be converted to oxaloacetate. Oxaloacetate is part of the most important anaplerotic reaction of the tricarboxylic acid cycle, catalyzed by the mitochondrial enzyme, pyruvate carboxylase. In animal tissues, pyruvate may be carboxylated to produce more oxaloacetate whenever the tricarboxylic acid cycle is deficient in oxaloacetate or its precursors. Conversely, when oxaloacetate is in excess, it is disposed of by reversal of this reaction. The pyruvate so formed, can then be oxidized to completion via the cycle (via acetyl-CoA) but may also be used for fatty acid synthesis after being transported to the cytoplasm. Fatty acid synthesis from acetyl-CoA, derived from degradation of the glycogenic valine (via succinyl-CoA), is dependent on the same cumbersome pathway. Succinyl-CoA and oxaloacetate which originate from isoleucine and valine, may stimulate the tricarboxylic acid cycle, be oxidized to CO_2 in the cycle or used for fatty acid synthesis. Acetyl-CoA, formed from labeled BCAA, mixes with the total acetyl-CoA pool. This labeling will be lower with isoleucine and valine, than with leucine. Therefore, a lower level of incorporation of isoleucine-derived carbon and still lower levels for valine-derived carbon into triglycerides, compared with leucine-derived carbon, may be expected.

The decrease in incorporation of leucine- and isoleucine-derived carbon into phospholipids after PCS (fig. 3a and 3b; no significance was reached for valine) may be explained by the diminished amount of membrane per fat-cell after PCS (smaller fat cells). This explanation assumes, however, that phospholipid turnover is not increased after PCS, which is not proven.

In vivo, the catabolism of a single amino acid will be influenced by several regulatory substances. Leucine has been indicated as one of these regulators (1,4,5,6). The increased in vitro CO_2-production from ^{14}C-(1)-valine and the increased incorporation in vitro of ^{14}C-(U)-isoleucine-derived carbon into triglycerides in the presence of unlabeled leucine

(fig. 1 and fig. 4) support such a regulatory function of leucine in adipose tissue from both normal and PCS rats. The maintained level of incorporation of ^{14}C-(U)-valine derived carbon into triglyceride in the presence of unlabeled leucine, may be interpreted as an increased incorporation. Competition between valine and leucine for the rate-limiting BCOA-DH would have resulted in a decreased incorporation of degradation products of the labeled substrate (^{14}C-(U)-valine) in the presence of leucine. This stimulatory effect may be explained by an increased availability of substrate (acetyl-CoA) as a consequence of increased decarboxylation of valine in the presence of leucine (fig. 1), probably by activation of the BCOA-DH by leucine. Our findings are in agreement with the increased oxidation of ^{14}C-(1)-valine and ^{14}C-(U)-isoleucine in the presence of leucine in adipose tissue segments from normal rats, as reported by Frick and Goodman (4). Conversely, leucine oxidation was not stimulated in the presence of valine, a result that was also obtained in our laboratory (data not shown).

Irreversible decarboxylation of leucine and valine in adipose tissue, both increased after PCS, occurs at different rates, albeit of the same order of magnitude. Even after stimulation of the irreversible decarboxylation of valine in the presence of leucine approximately equal rates are not reached. CO_2-Production in adipose tissue from the three universally labeled BCAA are also in the same order in both PCS- and sham-operated rats. The greatest differences between the BCAA in recorded parameters were seen in the incorporation of BCAA-derived carbon into fat. These differences were maintained after stimulation in the presence of leucine. All these smaller or greater differences make, however, that equal rates of catabolism of all three BCAA, could not be demonstrated. This raises the question if the in vitro recorded elevations in catabolism of the BCAA in adipose tissue of PCS rats may in vivo be responsible for the observed decreases in plasma BCAA levels, each BCAA being decreased with about the same percentage. However, the approximately equal increase in the ability to decarboxylate BCAA in adipose tissue, may be expected to be of greater importance for the decrease of BCAA in plasma after PCS, than the absolute rates of BCAA degradation. Differences in absolute rates of BCAA degradation are also seen in normal rats with normal BCAA levels. Different

K_m-values of the BCOA-DH for the BCAA may, at least partially, be responsible for this. The observed differences with especially valine may, in addition, partially be caused by the fact that we used a relatively low concentration (0.1 mM) compared with normal valine plasma levels (ca. 0.15 mM). Besides the influence of leucine, other regulatory mechanisms in BCAA-catabolism will exert their influences simultaneously, especially in vivo. The role that adipose tissue may play in the catabolism of BCAA under non-pathological conditions is still often neglected. After PCS, adipose tissue seems to play an even more accentuated role evidenced by increased irreversible decarboxylation of BCAA, as measured for leucine and valine.

This study supports the view that the increased catabolism of BCAA after PCS may contribute to the decreased plasma BCAA levels in PCS animals.

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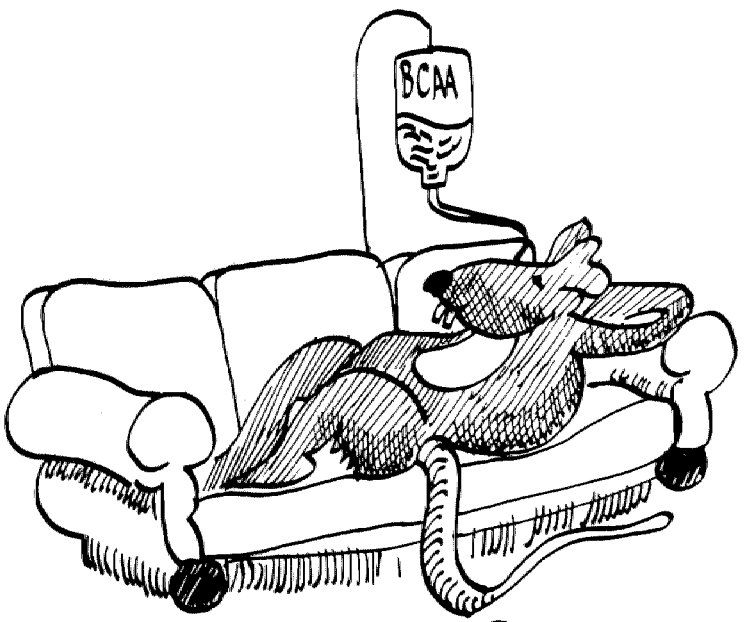

Sense or Nonsense

CHAPTER V

ACTIVITY OF BRANCHED CHAIN OXO ACID DEHYDROGENASE (BCOA-DH) IN ADIPOSE TISSUE AND DIAPHRAGM OF RATS: THE INFLUENCE OF PORTA-CAVAL SHUNT (PCS)

Summary

Porta-caval shunting (PCS) in man and experimental animals is accompanied by mildly elevated insulin levels and decreased branched chain amino acid (BCAA) levels in plasma from both overnight-fasted and fed subjects. In earlier experiments, we reported an increased catabolism of all three branched chain amino acids in adipose tissue of PCS-operated rats. In muscle, the catabolism of BCAA was not altered by PCS or was even decreased. Degradation of BCAA in muscle and adipose tissue from normal rats is limited by the activity of the branched chain oxo acid dehydrogenase (BCOA-DH; EC 1.2.4.4.). From these observations, an increased activity of BCOA-DH in adipose tissue from PCS-operated rats was suggested. In order to test this hypothesis, we determined in vitro the activity of BCOA-DH in muscle (diaphragm) and epididymal adipose tissue from fed PCS-operated rats and unoperated controls using α-ketoisocaproic acid (α-KICA) as substrate. In adipose tissue of PCS-operated rats, the BCOA-DH activity was increased two-fold when expressed in pmol α-KICA/μg DNA.min. In contrast, diaphragm of PCS rats exhibited a small decrease in BCOA-DH activity when expressed per μg DNA. These results confirm an important role of adipose tissue in BCAA-catabolism after PCS and suggest that adipose tissue causally contributes to the decreased plasma levels of BCAA after PCS. Short-term stimulation of BCOA-DH activity by insulin, as reported in the literature, was confirmed in adipose tissue from both normal and PCS-operated rats and seems to be superimposed on the stimulation of the BCOA-DH activity due to PCS, most probably caused by a long-term influence of insulin.

Introduction

Porta-caval shunting (PCS) in patients and experimental animals (2,6,20,22) leads to hyperinsulinism and decreased plasma levels of

branched chain amino acids (BCAA). Increased catabolism of BCAA in vitro in adipose tissue from overnight-fasted and fed rats with PCS was reported in chapter III and IV. In vitro BCAA catabolism was approximately equal in muscle from PCS-operated and from sham-operated rats. Therefore, adipose tissue seemed primarily responsible for the increased BCAA-catabolism in vivo after PCS.

Theoretically, there are at least three possible explanations for an increased processing of BCAA in adipose tissue:

1. An increased transport of BCAA and/or BCOA into the cell might be a simple explanation but assumes that this transport is rate-limiting. Transport of BCAA and BCOA across cell membranes does not occur by passive diffusion in contrast to e.g. that of pyruvate, but is mediated by transport systems as evidenced by specific inhibitors of the latter (9,10,16,32,34). However, no support could be found in the literature for a rate limiting transport of BCAA and/or BCOA, influencing BCAA-metabolism in peripheral tissues (12).
2. A second possibility would be a regulation at the receptor or post-receptor level. Using clamp techniques, insulin insensitivity has been reported in cirrhosis (23). There are some reports that suggest that the number of functional receptors for insulin is diminished in liver cirrhosis (1). However, such alterations would cause decreased metabolism of BCAA (23) and favour increased plasma BCAA levels.
3. Increased BCAA-catabolism in adipose tissue may be due to an increased enzymatic activity, possibly under hormonal influence or induced by increased substrate flow in coupled reactions (e.g. increased removal of glutamate by ammonia).

The first step in the degradative pathway of BCAA is the transamination of the amino group to α-ketoglutarate. Products of this reaction are glutamate and the corresponding branched chain α-keto acids: α-ketoisocaproic acid (ketoleucine), α-keto β-methylvaleric acid (keto-isoleucine) and α-keto-isovaleric acid (ketovaline). The second step in the degradation of BCAA is the oxidative decarboxylation of the BCOA, coupled to the reduction of NAD^+ and the binding of coenzyme A to form the corresponding branched chain thio-esters. While the first step, the transamination, is rate limiting in liver (37), the second step has been proven to be rate limiting in muscle and adipose tissue (5,11,29).

This study was undertaken to investigate the increased BCAA-catabolism in vitro in peripheral tissues, i.e. adipose tissue, from PCS-rats. Because changes in transport of BCAA and/or BCOA and altered tissue sensitivity (changes at the receptor level) are unlikely to be the cause of the increased BCAA-catabolism, we explored, in vitro, the influence of PCS on the activity of the rate-limiting enzyme in BCAA catabolism in muscle and adipose tissue, the branched chain aminoacid dehydrogenase (BCOA-DH; EC 1.2.4.4.). α-Ketoisocaproic acid was used as substrate. BCOA-DH activity in adipose tissue from normal rats can be influenced in vitro by insulin (7,12). We therefore also determined the activity of this enzyme, in tissues from both PCS-operated and sham-operated rats after preincubation with insulin. Any differences observed should be ascribed to short-term influence of insulin and may be additive to long-term effects of insulin on the BCOA-DH activity in tissues from PCS rats. On the other hand, it is possible that the short-term influence of insulin on the enzymatic activity in tissues from PCS-rats appears less or absent, when the same stimulus is responsible for both long-term and short-term effects and stimulation is maximal after long-term stimulation.

Materials and methods

PCS- and sham-operations were performed in male Sprague-Dawley rats weighing about 250 grams. Rats used in the experiment were selected according to criteria described earlier (chapter II). All PCS-operated rats exhibited, 2½ weeks post-operatively, hyperinsulinism, decreased plasma BCAA levels and had gained weight for at least one week. Two-and-a-half weeks after the operation, the diaphragm and epididymal adipose tissue were quickly removed from the rats (in fed state) and used immediately for the determination of the activity of the branched chain oxo acid dehydrogenase (BCOA-DH; EC 1.2.4.4.).

Determination of BCOA-DH activity

BCOA-DH activity was measured in tissue segments of diaphragm (approximately 50 mg) and epididymal adipose tissue (approximately 100 mg),

that were incubated in a shakerbath at 60 cycles per minute at 38.5°C. The incubation medium consisted of Tris-HCl buffer (Tris (hydroxymethyl) amino methane; 75 mM) pH 7.4 at 38.5°C and contained 3.3 mM $CaCl_2.2H_2O$, 5 mM $MgSO_4.7H_2O$, 5 mM $NaHCO_3$, 5 mM glucose and 75 mM sucrose. The tissues segments were preincubated in 1 ml of this mixture. After 30 minutes, the tissues were transferred to 1 ml fresh incubation medium containing 3.18 mM NAD^+ and 1.15 mM coenzyme A in addition to the consistuents mentioned above. The reaction was started by addition of 50 µl 6.42 mM α-keto (1-^{14}C) isocaproic acid (α-KICA; s.a. 65 µCi/mmol); consequently the concentration in the reaction vessel was 0.31 mM. The α-KICA-sodium salt was purchased from Amersham International, Amersham, Buckinghamshire, England, with a s.a. 54 mCi/mmol. Nonspecific radioactivity was lowered by flushing the labeled α-KICA solution with nitrogen to remove labeled CO_2. After 15, 30, 45 or 60 minutes incubation at 38.5°C in a shakerbath, the reaction was stopped by injection of 3 ml 0.66 M citric acid into the closed reaction vial. Subsequently, 0.33 ml Soluene^R-100 (0.5 N quaternary ammonium hydroxide in toluene; Packard Becker BV, Chemical Operations, Groningen, The Netherlands) was injected through the rubber stopper into the hanging center well (fixed to the stopper) to bind all the CO_2 released by the reaction medium at low pH. To be sure that all released CO_2 was collected in the center well, the reaction vial was placed back into the shakerbath for one hour. Thereafter, the stopper plus fixed center well was carefully removed, the outer surface of the center well was dried and the center well transferred to a counting vial containing 1 ml ethanol. After addition of 10 ml counting solution (Atomlight^R, New England Nuclear), the radioactivity was measured in a liquid scintillation counter for 5 minutes. Reaction velocities of BCOA-DH were derived from time-curves of at least three time points and corrected for blank values, derived from reaction vials that contained all constituents except tissue. All measurements were done in triplicate. Results were plotted in cpm/50 mg wet tissue weight for diaphragm and in cpm/100 mg for adipose tissue, to check linearity with time. The slopes of these curves represent the reaction velocities and were corrected for tissue DNA content. Final results were expressed in pmol α-KICA/µg DNA.min.

Tissue DNA content was determined in adipose tissue according to Rafael et al (35) and in diaphragm according to Burton (3). When **insulin** was

present during the (30 minute) preincubation of adipose tissue before recording the BCOA-DH activity, the insulin concentration was 1 mU/ml. Insulin was obtained from NOVO Industrie BV Amsterdam (Actrapid^{R}), 40 I.U./ml.

Results

Influence of PCS on BCOA-DH activity

BCOA-DH activity was measured in diaphragm and epididymal adipose tissue from 13 PCS-operated rats and 12 sham-operated controls. Tissues were taken from fed rats. The curves from which the reaction velocities were calculated after correction for tissue DNA content, are given in fig. 1 (diaphragm) and fig. 2 (adipose tissue). Fig. 1 demonstrates that,

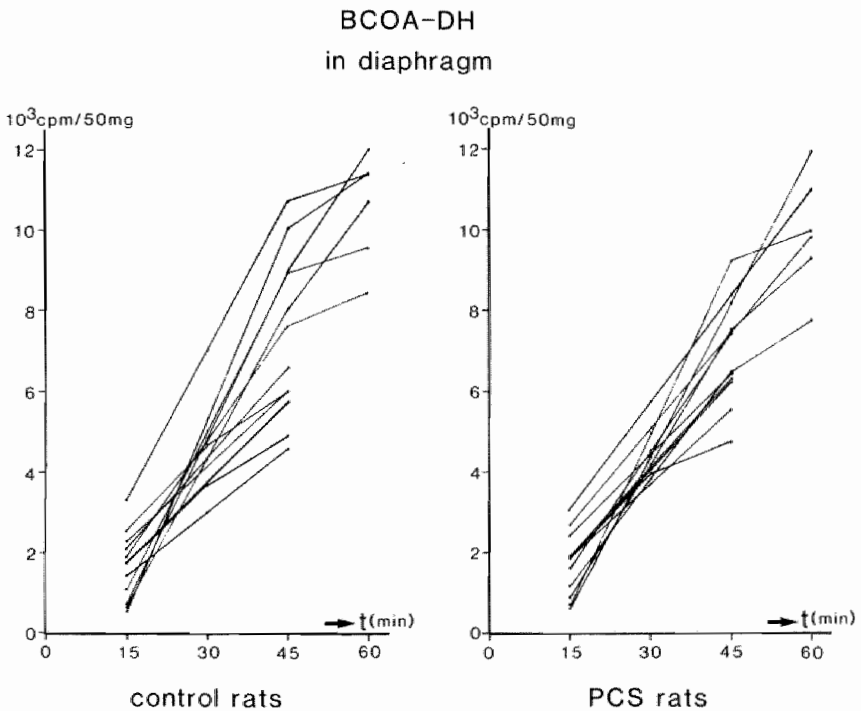

Figure 1
BCOA-DH activity in diaphragm from PCS-operated rats (n = 13) and sham-operated controls (n = 12) in the absorptive state. Each time-point was determined in triplicate. Data are expressed as cpm/50 mg wet tissue weight. For conditions used, see 'Materials and methods'.

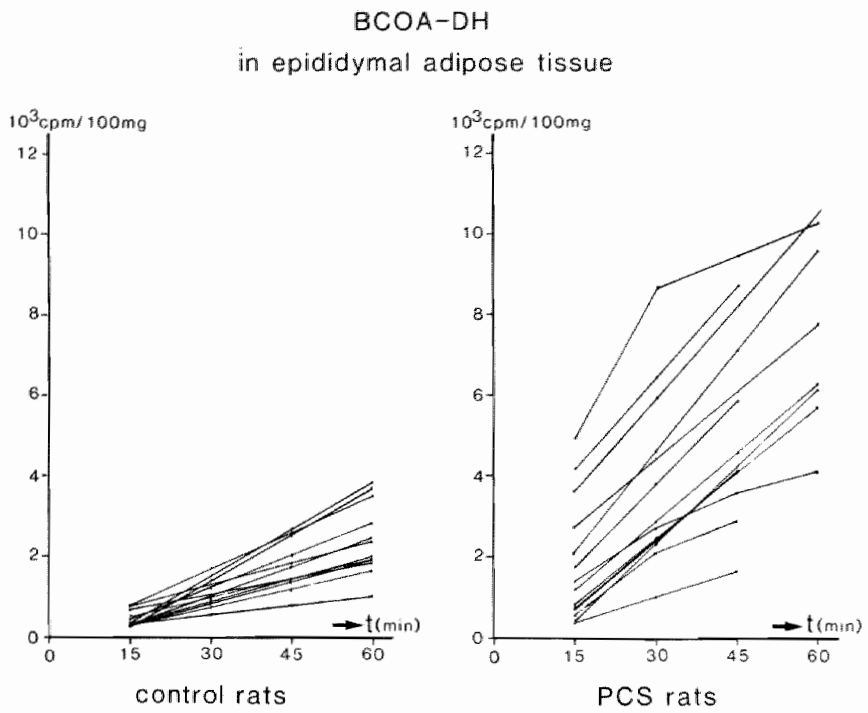

Figure 2
BCOA-DH activity in epididymal adipose tissue from PCS-operated rats (n = 13) and sham-operated controls (n = 12) in the absorptive state. Each time-point was determined in triplicate. Data are expressed as cpm/100 mg wet tissue weight. Conditions used are described in 'Materials and methods'.

under the described conditions, BCOA-DH activity in diaphragm is linear up to 45 min. When linearity was not maintained, the slope of the initial part of the curve was taken for further calculations. The same procedure was used for adipose tissue (fig. 2), where BCOA-DH activity was linear up to 60 min in tissues from all rats used, except in three PCS-operated rats. Figs. 1 and 2 indicate that BCOA-DH activity, as measured under the conditions described, was linear between 15 and 45 minutes after initiating the reaction with α-KICA in almost all rats used. It is further demonstrated that BCOA-DH activity in adipose tissue from PCS-rats is increased compared with sham-operated controls, while BCOA-DH activity in diaphragm is approximately similar in both groups. The data from figs. 1

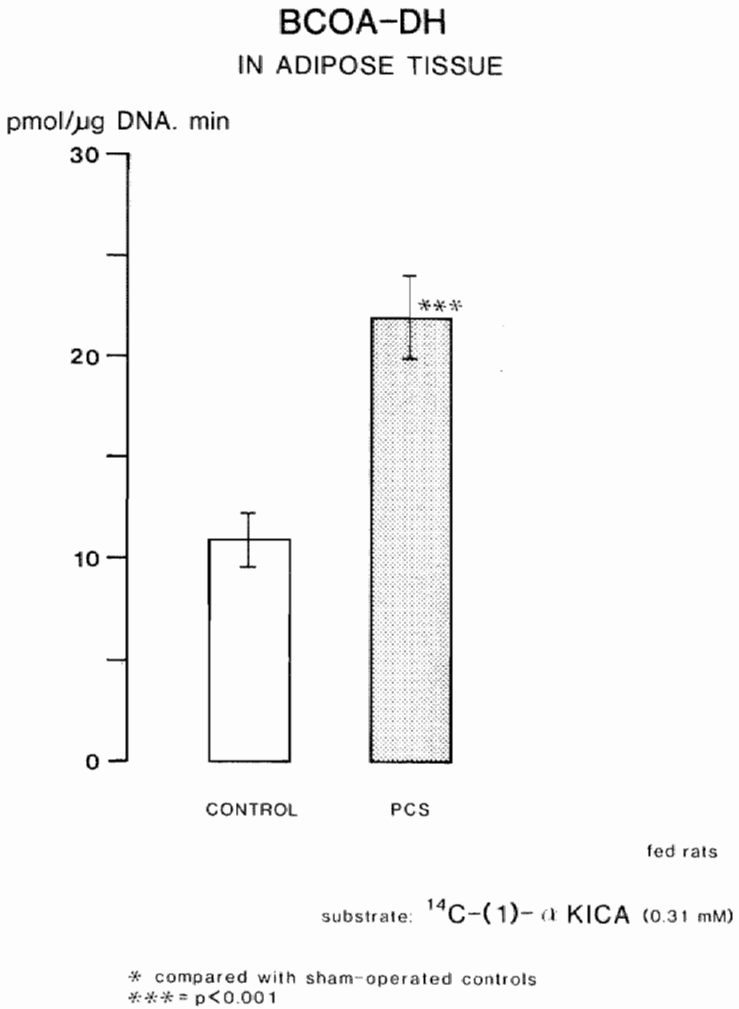

Figure 3
BCOA-DH activity in epididymal adipose tissue from PCS-operated rats (n = 13) and sham-operated controls (n = 12). Results are expressed in pmol α-KICA/µg DNA.min (mean ± sem). Data were derived from fig. 2.

and 2 were corrected for tissue DNA content of the individual rats and expressed in pmol α-KICA/µg DNA.min (figs. 3 and 4). The results shown in fig. 3, demonstrate that, also after correction for tissue DNA content, the activity of BCOA-DH in adipose tissue is still significantly increased

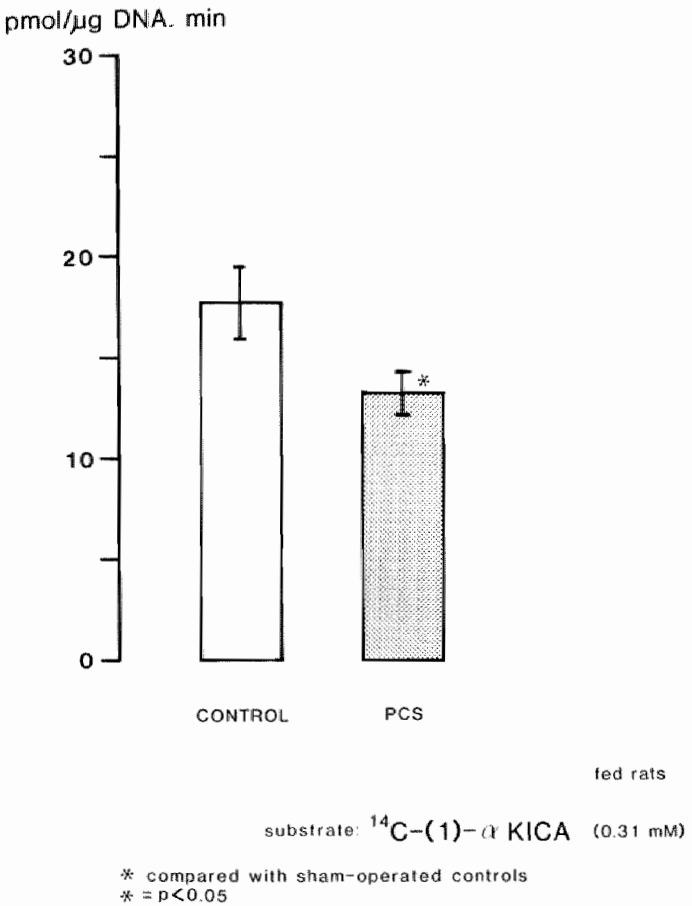

Figure 4
BCOA-DH activity in diaphragm from PCS-operated rats (n = 13) and sham-operated controls (n = 12). Data were derived from fig. 2. Results are expressed in pmol α-KICA / µg DNA.min (mean ± sem).

after PCS ($p < 0.0005$). In contrast, the BCOA-DH activity in diaphragm (fig. 4) was decreased in the PCS group ($p < 0.05$) when DNA correction was carried out.

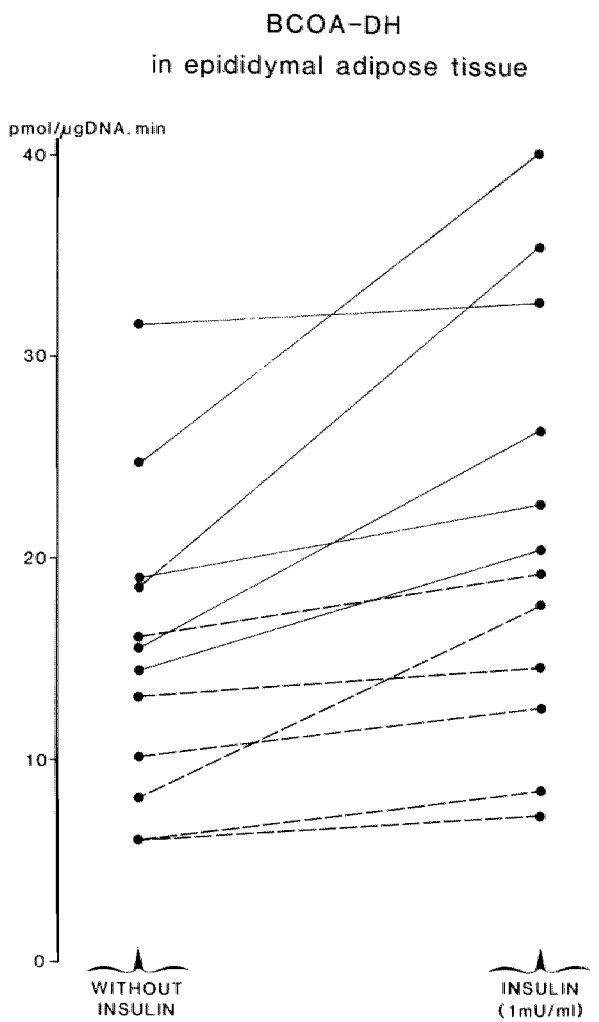

Figure 5
Short-term influence of insulin on the BCOA-DH activity in epididymal adipose tissue from PCS operated rats (n = 6; solid lines) and sham-operated controls (n = 6; broken lines) after 30' preincubation with insulin (1 mU/ml). Results are expressed as pmol α-KICA / µg DNA.min. All these rats were part of the study in which long-term influence of PCS was measured.

Short-term influence of insulin

In 6 PCS-operated rats and 6 sham-operated rats, used in the experiment described above, the in vitro BCOA-DH activity in adipose tissue was also recorded after a preincubation of 30 minutes in the presence of insulin (1

mU/ml). Data were processed in the same way as described above. The final results are presented in fig. 5. Preincubation with insulin stimulates in vitro BCOA-DH activity in adipose tissue from both PCS-operated and control rats. Stimulation varies greatly but seems to be more pronounced in tissues from PCS rats. Short-term influence of insulin on BCOA-DH activity in diaphragm (data not shown) could not be demonstrated.

Discussion

In chapters III and IV, we reported an increased in vitro catabolism of BCAA in adipose tissue from rats with a PCS. The demonstration of an increased activity of the rate-limiting enzyme in BCAA-degradation, BCOA-DH, in adipose tissue of PCS-operated rats is in accordance with these findings. After correction for tissue DNA content, the activity of BCOA-DH in adipose tissue after PCS was increased by twofold. In contrast, diaphragm exhibited in vitro a significant decrease in BCOA-DH activity after PCS, when results were expressed per µg DNA. These results emphasize the important role of adipose tissue in BCAA catabolism, especially after PCS, and suggest a role for adipose tissue in the decrease of BCAA levels in patients and experimental animals with liver insufficiency and/or portal-systemic shunting.

BCOA-DH is a membrane-bound enzyme located at the inner surface of the mitochondrial membrane (24). When measuring the enzymatic activity in tissue segments, one must keep in mind that the recorded activity may be submaximal due to the influence of membrane barriers, mediation by receptors, limitations in diffusion of substrate and the presence or absence of endogenous cofactors, activators, inhibitors, suppressors, etc. Despite all these possible influences, we decided to perform the activity measurements in whole tissue segments because this may resemble the physiological situation better than experiments with e.g. isolated or sonicated mitochondria. Furthermore, the obtained data are more comparable with the results from the BCAA-catabolism studies, performed in in vitro incubation experiments with tissues from normal and PCS-operated rats (chapters III and IV). We wanted the tissues from PCS rats to maintain, as

much as possible, the properties induced by the PCS-operation.

Other findings that, in addition, led us to our decision to use whole tissue instead of tissue homogenates or partly purified enzyme fractions, are now discussed. First of all, homogenizing had different effects on the BCOA-DH activity in different tissues. While the activity in liver greatly increased during homogenization, which is in accordance with data from May and associates (25), activity in muscle decreased with a variable percentage, and in adipose tissue, hardly any activity was maintained. Different conditions were used (e.g. different types of homogenizers, time of homogenizing, ultrasonic treatment, temperature, buffers, presence of glucose, sucrose, detergents, etc.) but no reproducible results could be obtained. Especially, no fixed ratio could be obtained between the activity present in the preparations and the activity measured in fresh whole tissue. Furthermore, the activity of the enzyme was often not high enough to guarantee reliable measurements. Detergents (Triton X-100 (0.02-1%) and CHAPS (3-((3 cholamidopropyl) dimethylammonio)-1-propanesulfonate; 0.02-1%) decreased the activity in liver homogenates, had no effect or decreased the activity even further in muscle homogenates (1% detergent) and had no effect at all in adipose tissue homogenates. Conflicting data with respect to BCOA-DH activity after homogenization and the use of different buffers and detergents have also been reported in the literature for different tissues (39). From adipose tissue we also prepared fat-poor homogenates (26), but, besides loss of activity due to homogenization, variable percentages of activity were lost by being trapped in the floating fat-mass which, moreover, is extremely difficult to handle.

Freezing of all tissues or tissue homogenates decreased the BCOA-DH activity further, if it had been detectable at all, as often occurred in adipose tissue homogenates. Freezing of whole tissue had, like the homogenizing of muscle and adipose tissue, a deleterious effect on the activity in these tissues. Loss of activity after freezing is also reported with variable results by others (25,39). By freezing, the local concentration of endogenous cofactors and/or facilitators may have become deficient by disruption of the integrity of the tissue. Repletion of the tissue preparations with these factors, absent in the reaction medium described, did not restore the enzymatic activity to levels recorded in fresh tissue segments. Mostly there was no measurable effect at all.

Calcium and bicarbonate tended to increase any remaining activity in frozen and homogenized tissue preparations, but had a similar effect in fresh tissue segments, probably by influencing membrane permeability. Addition of carnitine, ADP or thiamine pyrophosphate, singly or in combinations, to the reaction mixture, did not influence the activity of BCOA-DH in fresh tissue segments of muscle and adipose tissue. Omitting NAD^+ or CoASH from the reaction mixture, supposing that endogenous amounts would suffice (25), decreased the activity that could be measured after 30 minutes, creating non-linear curves between 15 and 45 minutes. This observation seems paradoxical because the reaction takes place on the inner surface of the mitochondrial membrane, where the enzyme complex is located (24). This membrane is impermeable to both NAD^+ and CoASH. Addition of NAD^+ may facilitate the reaction by removing reducing equivalents generated in the mitochondria and transferred to the cytosol via the malate-aspartate shuttle. Calcium, present in the reaction vial, may alter the membrane permeability for NAD^+ (15,19) and possibly also for CoASH.

Before the enzymatic reaction was started by addition of α-KICA to the reaction vial containing buffer, tissue and co-enzymes, the tissues were preincubated in buffer without co-enzymes for 30 minutes. This procedure was chosen after it was observed that preincubation stimulated the activity in adipose tissue. The same procedure was used for diaphragm. In both tissues, the lag-time before reaching linearity with time, was shortened by this preincubation. On the other hand, linearity in time was shortened from 60 and 90 minutes for muscle and adipose tissue, respectively, to 45 and 60 min.

In segments of diaphragm from 200-350 g rats, activity increased linearly with tissue weight from 25 to 100 mg. In adipose tissue, linearity with respect to weight of the tissue segments ranged from about 50 to 200 mg in 200-400 g rats, but was clearly dependent on the size of the fat cells, exhibiting more activity in leaner and younger rats.

The concentration of α-KICA (0.31 mM), used in the BCOA-DH assay, is nearly optimal with respect to Vmax and is about 3-10 times the Km as reported in preparations from rat liver, kidney, muscle and adipose tissue (7,25,39).

The increased BCOA-DH activity in vitro in adipose tissue after PCS is in agreement with the increased BCAA-catabolism in vitro in adipose tissue of PCS rats, as reported before (chapters III and IV) and may contribute to the existing lowered plasma levels of the BCAA in vivo after PCS.

The results from BCOA-DH activity measurements in diaphragm from normal and PCS-rats, are also in agreement with the rates of BCAA-catabolism in the tissues of these rats, namely decreased BCAA catabolism and decreased BCOA-DH activity after PCS when expressed per µg DNA. In muscle and muscle preparations, BCOA-DH activity is regulated by the ATP/ADP ratio influencing the ratio of inactive : active enzyme (18,27,28,30,31). The activity is further reported to be influenced by carnitine (stimulation; ref. 38), branched chain fatty acids and octanoate (feed back inhibition via CoA esters), as well as by ingested protein and leucine. Most of these factors may influence the rate of entry of BCAA into cells and thus intracellular concentrations of BCAA. After transamination of the BCAA in rat muscle, a significant proportion of the newly formed BCOA is released into the extracellular space (21), i.e. in the incubation medium, thereby maintaining low intracellular BCOA concentrations insufficient to saturate the BCOA-DH. After PCS, entry of BCAA into muscle may be lowered by decreased circulating plasma BCAA-levels, thereby, in addition, diminishing the stimulatory effect of leucine on BCOA-DH (4).

The mechanism by which the increased BCOA-DH activity in adipose tissue after PCS is induced, is difficult to assess. In the short term, the activity is reported to be influenced in vitro in adipose tissue or adipose tissue preparations by glucose (only in tissues from fasted rats; immediate effect), and by leucine and insulin within 30 minutes (7,8,9,11,12). In the long term, BCOA-DH activity in adipose tissue is also influenced by fasting, starvation, malnutrition, protein content of diets and unsupplied streptozotocin-induced diabetes (25,39). All these observations represent or simulate conditions that, in vivo, coincide with concomitant changes in insulin levels and plasma BCAA levels. This makes insulin a likely candidate to be involved in the increased BCOA-DH activity observed in adipose tissue from PCS rats, exhibiting hyperinsulinism and decreased plasma BCAA levels in both the fasted and fed state. In addition to the increased BCOA-DH activity in adipose tissue after PCS, a small and

variable further increase was measured after short-term preincubation with high dose insulin (1 mU/ml) which is suggested from fig. 5 to be more pronounced in adipose tissue from PCS rats than in sham-operated controls. If we assume that this further stimulation has an equal effect in both groups, the results suggest that the increased activity following PCS (fig. 3), is due to an increased quantity of enzyme, rather than a difference in the amount of activated enzyme. Some uncertainty about a causal relationship between insulin and BCOA-DH in adipose tissue remains. If insulin is instrumental, the greatest insulin effect, as after PCS, is under long-term regulation. The mechanism of action of insulin on BCOA-DH is completely unknown. Studies in which short-term influence by insulin on adipose tissue homogenates and whole tissue segments was explored (8,12), suggest that insulin lowers the apparent Km without affecting Vmax (which is suggested to be influenced by leucine). Long-term effects of insulin are probably mediated via enzyme synthesis, rather than via activation, and is possibly brought about by interference with suppressors. Wohlhueter and Harper were unable to draw conclusions from their studies in liver preparations from normal rats with inhibitors of protein synthesis, because one of these inhibitors, cycloheximide, had different effects on BCOA-DH activity after stimulation with leucine and with protein (39). However, different tissues may have different mechanisms of regulation of BCOA-DH activity. While BCAA, especially leucine, and protein seem to be common inducing agents in liver, kidney, muscle and adipose tissue, insulin does not have a stimulatory effect in all these tissues, neither do fasting or low protein diet (17,39,40). Insulin increases BCOA-DH activity in adipose tissue (7,11,12), but does not change or even decreases activity in muscle. Lack of insulin in unsupplied diabetes promotes BCOA-DH activity in liver (25) and decreases activity in adipose tissue (12; unpublished observation of our own group). In liver preparations, many other agents and conditions are reported to influence BCOA-DH activity. It has been further reported that muscle may play a special regulatory role in the activity of BCOA-DH in other tissues by mediation of a still unrecognized factor, probably a peptide or protein, released by muscle (33). Cytosolic α-KICA-dehydrogenase activity as present in liver tissue (13,14,25,36) (α-KICA specific, NAD^+ and CoA independent and therefore called α-KICA decarboxylase) is reported not to be present in muscle or adipose tissue and is not relevant for the

altered BCAA-catabolism in adipose tissue after PCS. In view of the lack of effect of insulin on the BCOA-DH activity in muscle, it is unlikely that decreased BCOA-DH activity in diaphragm of PCS rats is caused by hyperinsulinism but may be related to lower food intake and less favourable growing curves in PCS rats.

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CHAPTER VI

GENERAL DISCUSSION

In the preceeding chapters some aspects have been described of branched chain aminoacid (BCAA) metabolism in vitro in both muscle and adipose tissue from porta-caval shunt (PCS)-operated rats. Muscle and adipose tissue are quantitatively the most important sites of BCAA catabolism in the body. It has been shown that PCS itself can result in decreased levels of BCAA in plasma, also in the absence of accompanying superimposed disorders such as starvation, malnutrition, sepsis or serious liver failure, that may otherwise exhibit additional influences on plasma BCAA levels. Therefore, observed alterations in BCAA metabolism must be due to the PCS. The animal model described, served in these studies as one in which there are decreased plasma BCAA levels in combination with hyperinsulinism, in the absence of the above-mentioned superimposed disorders. Consequently, we dealt with relatively healthy, growing animals. This does not mean that other abnormalities, encountered in liver insufficiency, were completely absent. Liver enzyme activities in plasma deviated slightly; moderate elevations of ALT, AST, AP and GLDH were observed. Generally, however, these abnormalities were mild, both after the shunt operation and at the time of the experiments, when compared with reports in the literature and with results from rats that had a PCS operation in our laboratory according to the classical "suture" technique. Furthermore, increased plasma glucagon, ammonia and AAA levels occurred in these PCS rats (chapter II). It needs to be emphasized once more that, in spite of the observed mild disturbances in liver function, our "non-suture" PCS model, used according to the criteria described in chapter II, may not be considered as an animal model for chronic liver failure or hepatic encepha-

lopathy. Chronic liver insufficiency develops during many years and this cannot be simulated in rats in two weeks or a month. The PCS model, even when standardized as much as possible, is a variable model. Using only PCS rats fulfilling our criteria, implies that many rats that had a PCS-operation, could not be included in the studies. Only in this way could we be sure that the phenomena studied, resulted from the PCS itself rather than from secondary phenomena. The PCS rat model described, was used to obtain muscle and adipose tissue which may be expected to exhibit metabolic characteristics, that are the consequence of PCS itself.

In vitro incubation studies, in which muscle and adipose tissue from PCS- and sham-operated rats were used, demonstrated that BCAA catabolism in adipose tissue was increased after PCS. This was evidenced by increased irreversible decarboxylation of leucine and valine. ^{14}C-(1)-L-Isoleucine was not available for our experiments, and thus irreversible decarboxylation of isoleucine could not be studied. The increased decarboxylation of BCAA in adipose tissue, due to PCS, may contribute causally to the decreased plasma BCAA levels observed in PCS rats.

Increased decarboxylation of BCAA in adipose tissue after PCS, was accompanied by increased fatty acid synthesis and storage of BCAA-derived carbon into triglycerides. The levels of incorporation of BCAA-derived carbon into triglycerides, decreased in the order leucine to isoleucine to valine (100%, 50%, and 10% resp.), in tissues from both PCS-operated rats and sham-operated controls. In addition, PCS resulted in increased combustion of valine-derived carbon to CO_2. This increase was not found with leucine and isoleucine. This CO_2-production from universally labeled BCAA also decreased in the order leucine to isoleucine to valine in adipose tissue from both PCS-operated rats (100%, 94%, and 87% resp.) and sham-operated controls (100%, 77%, and 52% resp.). Furthermore, it was demonstrated that in adipose tissue from both PCS- and control rats, all leucine and valine that was decarboxylated, was used for either fatty acid synthesis or was degraded to CO_2. This indicates that other degradation pathways of BCAA in adipose tissue are negligible.

The differences in the levels of irreversible degradation of BCAA, of fatty acid synthesis and of combustion to CO_2 of the rest of the carbon skeletons of BCAA, can be explained by different rates of transamination (different Km's), of oxidative decarboxylation (different Km's; stimulating

effect of leucine on the activity of BCOA-DH) and by differences in the degradation routes of the BCAA (chapter IV; see addendum for degradation pathways of BCAA). This results in different amounts of acetyl-CoA, the direct precursor for fatty acid synthesis, while isoleucine and valine degradation also yield tricarboxylic acid cycle intermediates. The different rates of decarboxylation of leucine, isoleucine and valine in adipose tissues from both PCS operated and control rats, are not in conflict with a causal relationship in vivo between increased BCAA catabolism and decreased plasma BCAA levels after PCS. Different levels of degradation of BCAA in tissues from normal rats coincide with normal plasma BCAA levels. After PCS, the rates of decarboxylation of the BCAA appear to be increased to the same extent. This may explain the approximately equal decrease of BCAA levels in plasma after PCS.

Although adipose tissue was, in vitro, responsible for the increase in BCAA degradation, in vitro metabolism in muscle was not completely unaffected. Irreversible degradation of BCAA in vitro in muscle was not affected or was even decreased after PCS, but protein synthesis proved to be increased after PCS. Of course, increased protein synthesis results in an increased removal of BCAA from the incubation medium in vitro and from plasma in vivo, but, as pointed out before, the amount of BCAA required for protein synthesis is quantitatively less important with respect to the total amount of BCAA that is catabolized. Furthermore, protein degradation occurs at the same time and replenishes intracellular free BCAA pools. Moreover, BCAA used for protein synthesis are not degraded. In contrast to the complete degradation of BCAA in adipose tissue,it appears that after decarboxylation in muscle, a significant part of the carbon skeleton of BCAA is not degraded to CO_2, indicating an incomplete combustion in muscle. Most probably these carbon atoms are used for synthesis of glutamine via α-ketoglutarate and glutamate.

In health and after PCS, about half of the BCAA are catabolized in muscle, due to the total muscle mass and the activity of the rate limiting BCOA-DH in muscle. Decarboxylation rates did not consistently change after PCS. This indicates that in vivo, muscle is unlikely to contribute to the decrease of plasma BCAA levels after PCS. Our results strongly suggest however, that adipose tissue, despite its smaller total mass in the body as compared with muscle, is concerned in the regulation of plasma BCAA levels.

Futhermore, it should be realized that decarboxylation rates of BCAA are highest in adipose tissue and diaphragm and are much lover in skeletal muscle, when expressed per unit weight (chapter I). Generally, though, adipose tissue is still considered to be rather inactive and quantitatively unimportant in AA-metabolism.

As has already been mentioned, BCAA-catabolism in extrahepatic tissues is predominantly regulated by the activity of the BCOA-DH. This is supported by our findings that, after PCS, there is an increased activity of the enzyme in adipose tissue and a slightly decreased activity in muscle. These results were expected on the basis of the alterations in vitro in the degradation of BCAA after PCS.

In conclusion, adipose tissue has been indicated as an organ contributing to the lowering of plasma BCAA levels after PCS, and evidence is obtained that increased activity of BCOA-DH in adipose tissue might be responsible for the increased BCAA catabolism. The next question to be answered is: what mechanism regulates BCOA-DH activity in adipose tissue after PCS? And further, is that mechanism limited to PCS or is it applicable to other disease states with altered plasma BCAA levels as well? PCS-Operated animals and patients with surgical or spontaneous portal-systemic shunting exhibit, besides low plasma BCAA levels, hyperinsulinism and hyperglucagonism. A relationship between glucagon and BCAA has never been described, in contrast to one with insulin as discussed earlier (chapters I, III and V). In our view, insulin is the most likely candidate responsible for increased BCAA catabolism in adipose tissue after PCS. Many arguments support this view and many data are in agreement with this concept:

* Hyperinsulinism is present in our PCS animal model and has been documented in experimental, surgical and spontaneous shunting (chapters I, II and III; 2,4).
* The increased activity of BCOA-DH in adipose tissue after PCS is in accordance with the in vitro demonstrated stimulation of this enzymatic activity by insulin (5,6, and chapter V).
* The unaltered or slightly decreased activity of BCOA-DH in muscle is in agreement with the fact that insulin does not stimulate this activity in muscle in vitro (chapter V).

* The role of insulin is further supported by findings in other rat models with altered plasma insulin and BCAA levels (unpublished observations).

Unsupplied diabetes is accompanied by low insulin levels, high BCAA levels, decreased catabolism of leucine in adipose tissue in vitro and decreased BCOA-DH activity in adipose tissue. Diabetic rats with a PCS exhibit plasma insulin levels that are lower than from PCS rats and even lower than from untreated controls, but higher than from unsupplied streptozotocin-induced diabetic rats. The plasma BCAA levels in these rats are elevated but lower than from diabetics.

Rats fed with high protein diets for one week, have elevated insulin levels and exhibit increased BCOA-DH activity in adipose tissue. In contrast, long-term starvation is accompanied by low insulin levels, low BCAA levels and low BCOA-DH activity in adipose tissue.

Non-obese Zucker rats have slightly lower insulin levels than normal Sprague-Dawley rats and exhibit slightly higher plasma BCAA levels and moderately decreased leucine catabolism in adipose tissue, suggesting decreased BCOA-DH activity in adipose tissue.

Obese Zucker rats have, after feeding, highly elevated insulin levels, but exhibit insulin-resistance. These rats have, in the absorptive state, high BCAA levels and exhibit a decreased leucine catabolism in adipose tissue. Further support was derived from the extremely low BCOA-DH activities that we recorded in adipose tissue of obese patients (unpublished results).

In PCS rats, BCAA catabolism is increased even after an overnight fast (chapter III).

These findings suggest that insulin may also play a role in long-term regulation of BCOA-DH activity in adipose tissue. This supports an inverse correlation between plasma insulin levels and BCAA levels mediated by BCOA-DH activity in adipose tissue. These indicated relationships cannot be demonstrated in muscle of rats in a variety of experimental conditions exhibiting chronic changes in the nutritional or hormonal state.

* Increased protein synthesis observed in our rat model, may be the consequence of hyperinsulinism (chapters III and IV).
* Increased fatty acid synthesis in adipose tissue may also result from increased insulin levels (chapters III and IV).

All these arguments support a role for insulin in the regulation of plasma BCAA levels mediated by the activity of BCOA-DH in adipose tissue.

Objections have been raised against the role of insulin in the decrease of BCAA in liver disease because it has been shown that BCAA intolerance and glucose intolerance often exist in patients with liver disease (13,14), due to insulin insensitivity. BCAA Intolerance occurs, in particular, after protein loading. No changes in insulin receptor characteristics were observed in adipose tissue in liver cirrhosis (7). Therefore, BCAA intolerance can only be caused by a diminished capacity to degrade BCAA in muscle. This is consistent with the demonstration that BCOA-DH was decreased in muscle (chapter V). If we hypothesize, total capacity to clear BCAA is apparently reduced in liver cirrhosis, but in the fasting condition, faulty programming of adipose tissue leads to ongoing BCAA degradation and low plasma BCAA levels.

The mechanism of regulation of BCOA-DH activity in adipose tissue by insulin is still largely unknown. Recently, it has been demonstrated in vitro that BCOA-DH activity in liver can be influenced by a substance released by muscle, most probably a peptide (18). It is not yet excluded that insulin influences the synthesis and/or release of this substance by muscle which subsequently may exert its influence on the activity of BCOA-DH in adipose tissue. However, this seems unlikely because of the demonstration that BCOA-DH activity in kidney, heart and brain is not influenced by this muscle released factor.

The next unanswered question concerns the cause for hyperinsulinism after PCS and in liver insufficiency. This question is particularly interesting because, if insulin is instrumental in lowering the BCAA levels, the elevated insulin levels are inappropriate in relation to the quantity of food consumed, especially with respect to AA-metabolism because increased decarboxylation of BCAA and storage of BCAA-derived carbon into fat is induced. The increased protein synthesis in muscle also suggests the existence of an extremely well fed state in PCS rats compared to control rats, despite an equal or slightly decreased food intake by PCS rats. Even after an overnight fast, fatty acid synthesis from leucine derived carbon in PCS rats is higher than in fed control rats (chapter III, fig. 3). Compared with control rats, PCS rats used in the studies described in this thesis, were leaner, often consumed less food, had less muscle mass

(evidenced by tissue DNA content and the weights of the diaphragms excised) and had less fat (evidenced by tissue DNA content and the weight of the epididymal fat pads excised). PCS rats that did not gain weight and were excluded from the studies described for reasons mentioned (chapters I and II), were more ill, still leaner, had higher insulin levels (and glucagon levels) and exhibited the highest BCAA degradation rates and the highest rates of incorporation of BCAA-derived carbon into fat, that were recorded. These findings are not at all likely to be caused by malnutrition because the opposite would be expected to occur, but they suggest that the severity of the disease or the amount of stress may be a determining factor in the level of insulin and in the rate of BCAA catabolism. In this respect, it may be relevant that plasma levels of "stress hormones" are generally reported to be increased in liver cirrhosis (15,21,22,24,25,26). As already mentioned, glucagon levels are increased in PCS rats, but we are not aware of data concerning catecholamines and cortisol in PCS rats. Elevations of all three "stress hormones" are generally accompanied by increases in insulin levels. It is therefore possible that a "stressed state", although clinically not always apparent in cirrhotic patients and incompletely documented in PCS rats, is instrumental in the observed alterations, and that hyperinsulinism may be an integral part of the stressed state.

Recently, Owen (17) reported a study in patients with biopsy-proven alcoholic cirrhosis and healthy volunteers, in which the nature and quantity of fuels oxidized was measured after an overnight fast and after a longer period of starvation (only in volunteers). He demonstrated that, after an overnight fast, the caloric requirements of patients with (alcoholic) cirrhosis were normal, but the nature of fuels oxidized (fat - carbohydrate - protein) in these patients were similar to normal humans undergoing 2-3 days of total starvation (more fat, less carbohydrate). He concluded that these cirrhotic patients develop the catabolic state of starvation more rapidly than normal humans do. As mentioned before, our in vitro results in PCS rats appear to reflect a well-nourished state and suggest an inappropriate programming of adipose tissue with regard to BCAA metabolism. The normal fine tuning of net fatty acid synthesis and net fatty acid degradation is disturbed.

This well-nourished state is, of course, not present. Where increased protein synthesis in muscle coincides with muscle hypotrophy, increased

protein degradation must occur, possibly related to the increased catecholamine levels in plasma. In our PCS rats, this was supported by increased urinary 3-methylhistidine excretion. Even an equal 3-methylhistidine excretion might be interpreted as an increased protein degradation because of the smaller muscle mass after PCS and in cirrhosis. These findings indicate increased protein turnover which is also reported in patients with compromised liver function (16,27). In addition, cirrhotic patients have increased protein requirements to maintain nitrogen balance, compared with healthy individuals (27). This might be partially explained by compensation for increased BCAA catabolism in adipose tissue. These findings support the idea that cirrhotics fail to adapt to starvation (17,27).

The increased fatty acid synthesis in PCS rats is, as with the increased protein synthesis in muscle, accompanied by a smaller fat mass, indicating increased fatty acid turnover. Lipolysis might have been stimulated by elevated levels of glucagon, catecholamines and/or cortisol and is in accordance with the elevated plasma levels of free fatty acids (FFA) in liver disease (9). These increased plasma FFA levels may facilitate fatty acid utilization which is very much dependent on circulating FFA levels, when entrance into cells is not impaired. Such an increased fatty acid utilization, occurring in liver disease, leads to a diminished glucose utilization (19) and a diminished capacity to utilize glucose, resulting in glucose intolerance (13,14,21). In cirrhosis, a strong correlation between glucose intolerance and FFA-levels has been reported (21). The decreased capacity to utilize glucose has been mentioned as a possible cause for hyperinsulinism in cirrhosis.

Increased turnover of protein and fat is also observed after trauma and in sepsis (1,23) and was recently reported also in cancer cachexia (11). In both diseases, stress hormones and insulin seem to be involved. Therefore, the mechanism involved in regulating protein and fat turnover and in changing BCAA levels, appears to be more general. In malnutrition and long-term starvation in man, a decreased turnover of protein and fat is observed. Although in rats, the extent of decrease may not be as pronounced as in humans, the fact that, after PCS, protein turnover is increased, suggests that malnutrition does not play a role.

Another mechanism in lowering plasma BCAA levels in PSS and/or compromised liver function has been proposed by Holm and Leweling. They suggested that hyperammonemia intensifies BCAA utilization via stimulation of glutamine synthesis, thereby reducing the glutamate pool which is then, at least in part, restored by BCAA degradation in a coupled reaction. This hypothesis is in agreement with the demonstration by Imler (10) that, in liver patients, more glutamine and less alanine was released from muscle compared with healthy controls, and would explain the increased glutamine : alanine ratio. Support for this hypothesis was reported (12). It was demonstrated that continuous infusion of an ammonium acetate-ammonium bicarbonate mixture into rats decreased the plasma BCAA levels after two hours, and decreased intramuscular concentrations of BCAA six hours after initiating the infusion. The decrease in extracellular BCAA values was preceded by a decrease of glutamate and alanine as well as by an increase of glutamine in both plasma and muscle. These alterations were observed in the absence of significant hyperinsulinism, compared with untreated controls. Therefore, Holm and Leweling claim that this mechanism is independent of insulin. Scrutiny of the data reveals that there was a significant increase in insulin compared with the sodium acetate-sodium bicarbonate infused controls, due to a decrease of insulin in the sodium-infused group. Further support for this "ammonia-BCAA" hypothesis originates from our own group. In a pilot study we determined in vitro the irreversible decarboxylation of leucine in muscle in the presence of ammonium acetate (0, 0.25, 0.50 and 1.0 mM) and confirmed an increase in leucine decarboxylation dependent on the ammonia concentration present in the incubation medium (fig. 1). In addition, we recorded a much more pronounced increase in the irreversible decarboxylation of leucine in adipose tissue in the presence of ammonia (up to at least 0.5 mM) and a proportional increase in fatty acid synthesis from leucine-derived carbon (fig. 2). It is conceivable that the activity of the rate-limiting BCOA-DH may also play a decisive role in the ammonia-induced BCAA degradation.

The proposed mechanisms mediated by insulin and ammonia may operate simultaneously in vivo, insulin via adipose tissue and ammonia via both muscle and adipose tissue, possibly in part insulin independent. From our data, it is difficult to draw conclusions about the quantitative effects of these mechanisms on the decrease of plasma BCAA levels. Obviously, we

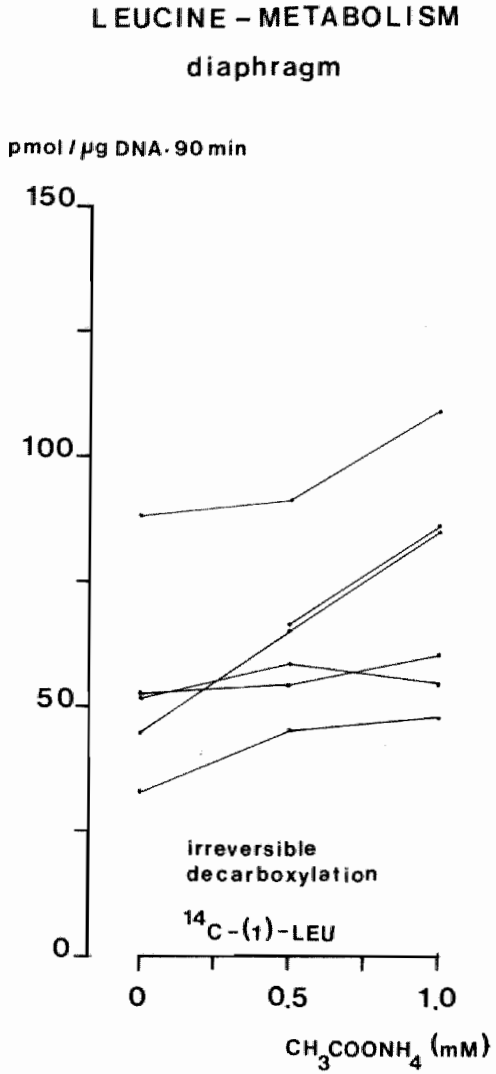

Figure 1
Influence of ammonia on the irreversible decarboxylation of ^{14}C-(1)-leucine (0.1 mM; s.a. 0.25 mCi/mmol) by diaphragm from six normal rats weighing approximately 280 g. In vitro measurements were carried out in triplicate according to the method described in chapter III.

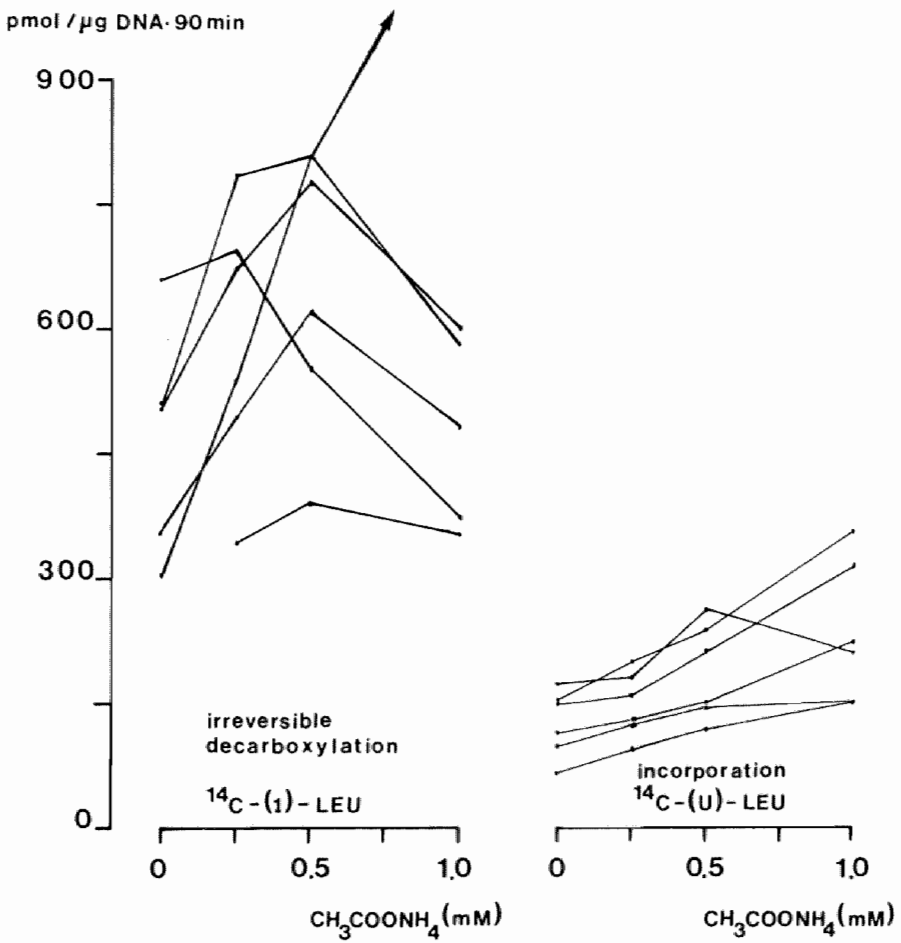

Figure 2
Influence of ammonia on the irreversible decarboxylation of ^{14}C-(1)-leucine (0.1 mM; s.a. 0.25 mCi/mmol) and on the incorporation of ^{14}C-(U)-leucine (0.1 mM; s.a. 0.25 mCi/mmol) -derived carbon into fat in epididymal adipose tissue from six normal rats of approximately 280 g. Measurements were performed in triplicate and carried out according to the method described in chapter III.

clearly showed that adipose tissue has a key role in these mechanisms.

A last point to be discussed is the rationale for BCAA enrichment in nutrional regimens. Do our data support BCAA therapy in patients with compromised liver function? It is still an unanswered question whether the distorted amino acid pattern in these patients, and in experimental animals with compromised liver function, is an imbalanced AA pattern. Do the diminished amounts of BCAA in plasma lead to diminished muscle protein synthesis and/or muscle wasting resulting in inhibition of growth or weight loss? Increased protein synthesis after PCS in rats and in cirrhotic patients (16,27), and the fact that tissue levels of free BCAA appear to be normal or even increased in liver patients (8), argue against the possibility that BCAA are rate-limiting for protein synthesis. If increased protein degradation occurs in an attempt to correct the distorted BCAA levels and to supply substrate for adipose tissue that seems to be programmed for increased BCAA degradation, resulting in net protein loss and less growth, then, supply of BCAA might be useful. In addition, if transamination of BCAA furnishes enough glutamate to decrease plasma ammonia levels by formation of glutamine, administration of BCAA might be useful in patients with liver failure. It may also be important that, in muscle, BCAA-derived carbon is used for α-ketoglutarate synthesis which is an extremely effective ammonia acceptor. Furthermore, it may be questioned whether increased AAA levels in liver disease are high enough to be toxic when BCAA, which compete with AAA for transport across cell membranes and through the blood-brain barrier, are low. Caution should be exerted in administration of imbalanced amino acid mixtures like excess valine (20) or excess leucine (3). Until now, BCAA therapy in liver patients has not been proven to be beneficial. It is beyond a doubt that clinical improvement must play the decisive role in the regimen followed.

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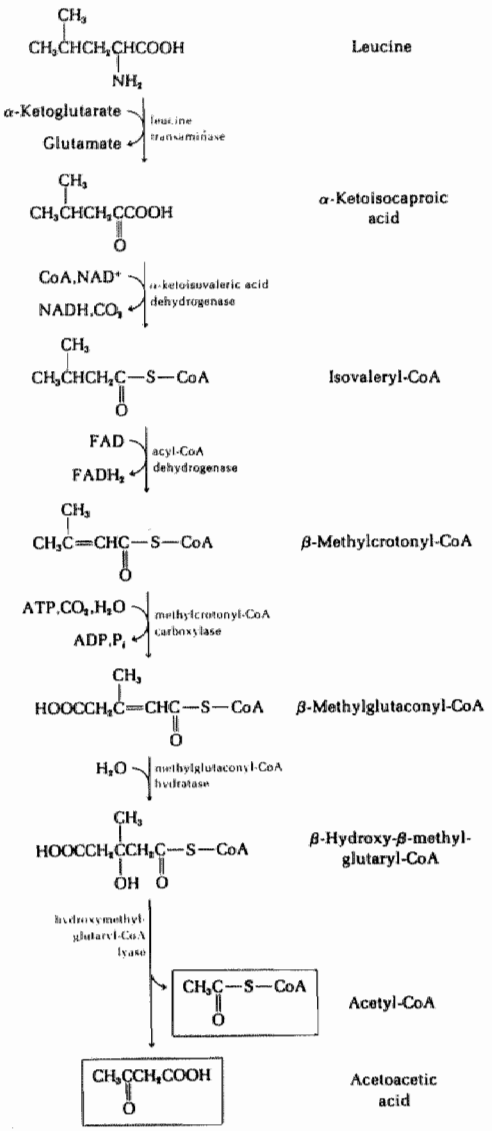
ADDENDUM

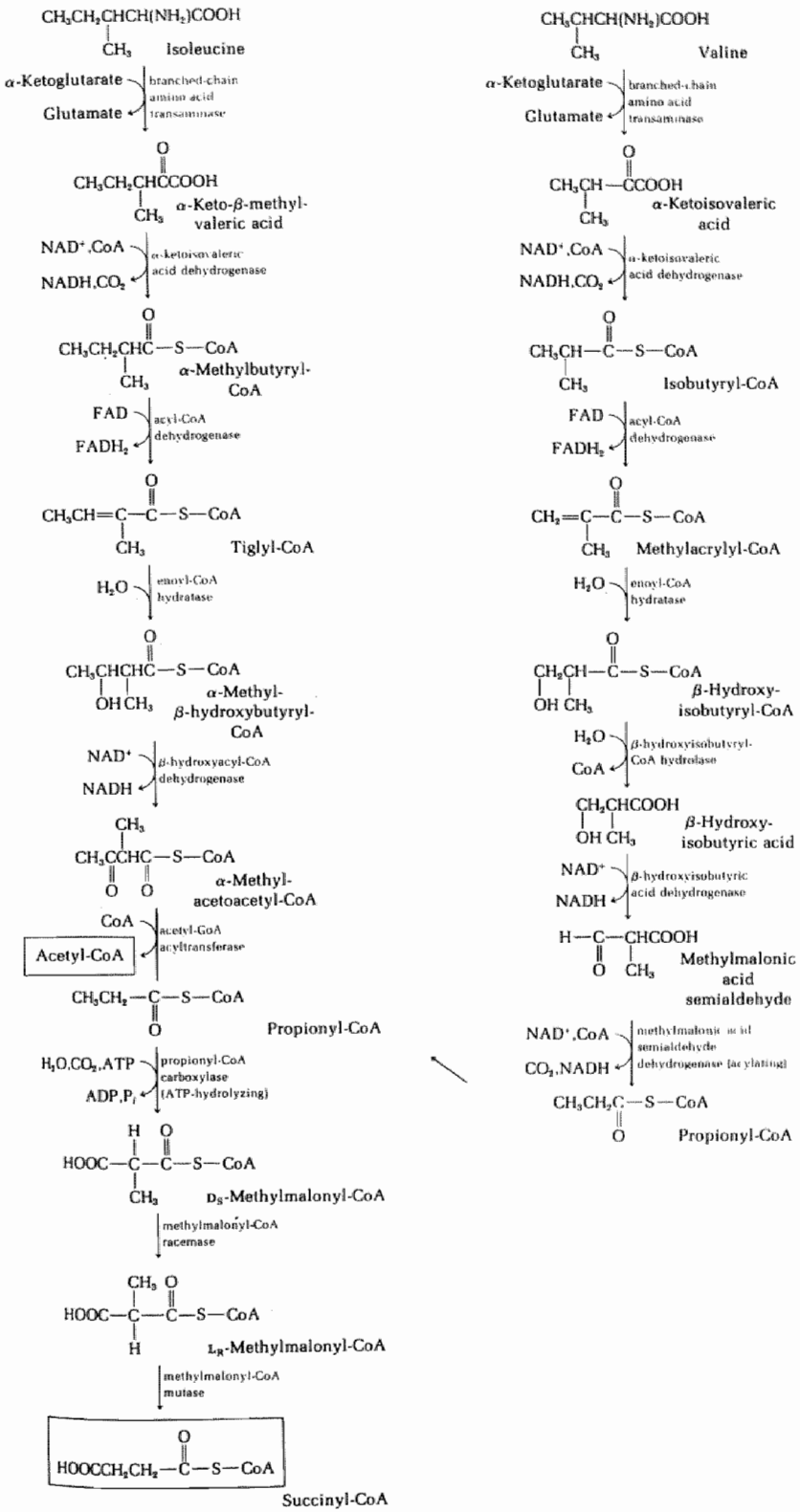
The degradation pathways of leucine, isoleucine and valine to acetyl-CoA, acetoacetic acid and succinyl-CoA are depicted below.

To be consistent with the way in which the data were presented in the experimental chapters, table I from chapter I, in which leucine degradation rates in several tissues were expressed per mg wet tissue weight, is included in this addendum in "DNA-corrected" form.

Most data presented in this thesis were expressed per µg DNA. Interpretation of the uncorrected data (data per mg wet tissue weight) would have been slightly different. Generally, the differences between controls and PCS-operated rat were greater, especially in adipose tissue. In muscle, correction for tissue DNA content sometimes abolished the observed differences, and sometimes even changed the direction of the differences. Therefore, some relevant data presented in this thesis are included in this addendum, expressed per mg wet weight.



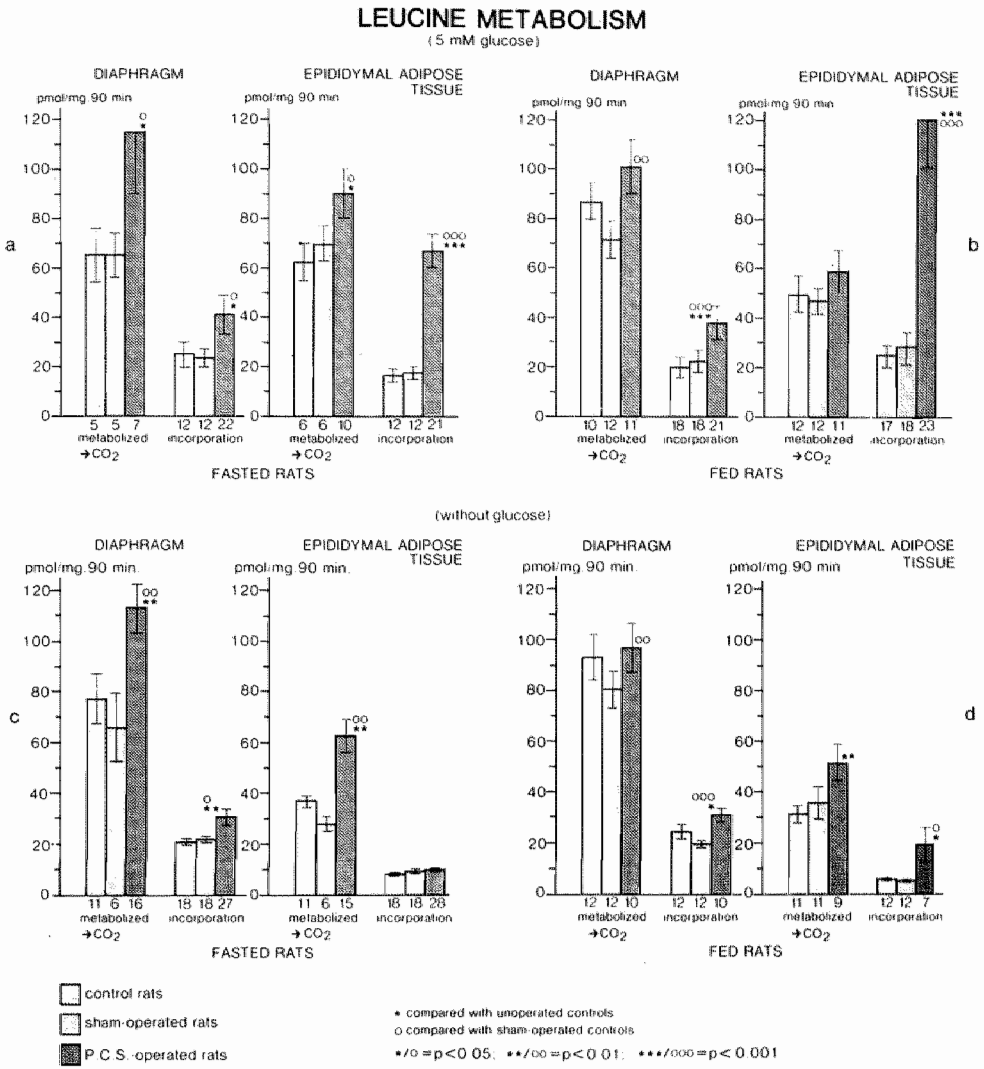
Catabolic pathway of leucine



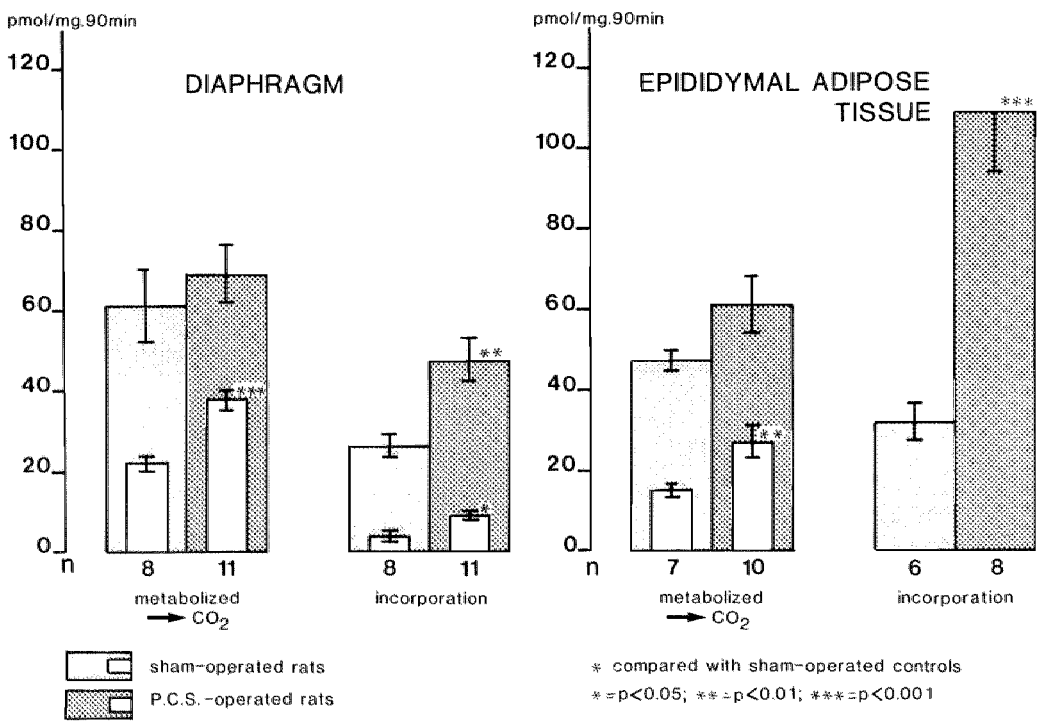
Catabolic pathway of isoleucine and valine

Tissue	Tissue DNA-content		irreversible leucine degradtion	
	µg DNA wet weight	activity pmol/µg DNA.90 min	% of total body activity based on skeletal muscle	based on diaphragm
adipose tissue	0.41	371	80.0	61.0
diaphragm	2.16	82		36.5
skeletal muscle	1.01	31	17.0	
heart muscle	2.28	24	0.1	0.1
small intestine	7.64	2	0.0	0.0
brain	1.15	132	1.2	0.9
liver	2.91	14	1.2	0.8
kidney	4.07	33	0.5	0.4

See table I, chapter I
Irreversible degradation of ^{14}C-(1)-L-leucine (s.a. 0.25 mCi/mmol; 0.1 mM) by different organs of normal male Sprague Dawley rats (n=4), determined in triplicate and expressed in pmol/ µg DNA.90 min. Average body weight was 290 g. To be able to calculate the activity per mg wet weight of the tissue, tissue DNA content is listed. A rough calculation of the percentual contribution of each organ is given, based on organ weights and on the assumption that adipose tissue is 15% of total body weight, and muscle 40% of total body weight. Because of the different activites that were recorded in diaphragm and skeletal muscle, two calculations are given (see text).



See figure 3 (chapter III)

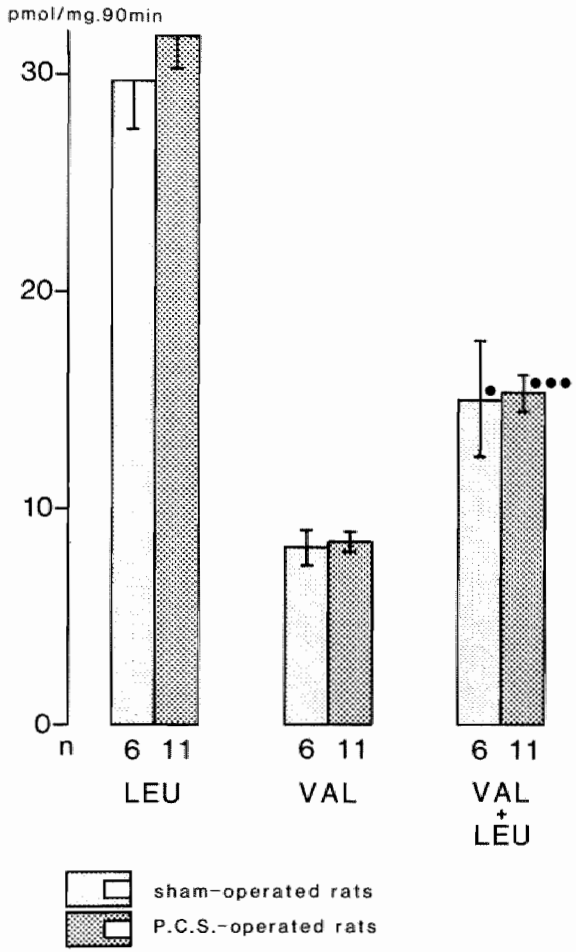


See figure 4 (chapter III)

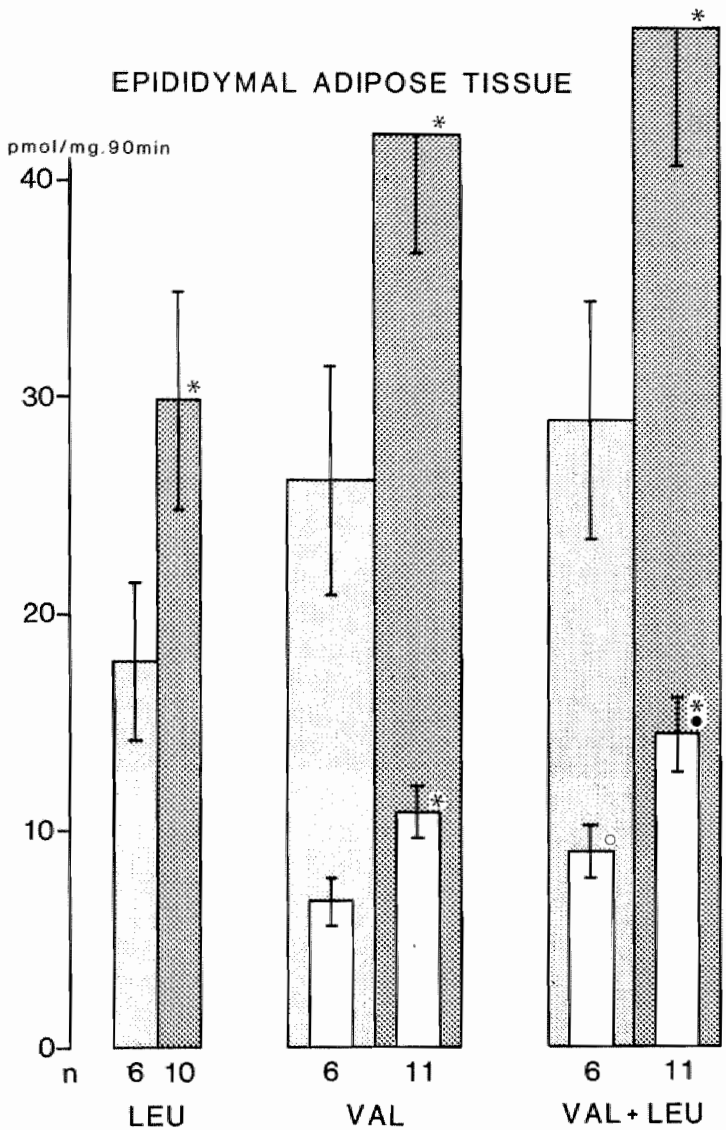
BCAA METABOLISM

METABOLIZED TO CO_2

DIAPHRAGM



See figure 1a (chapter IV)



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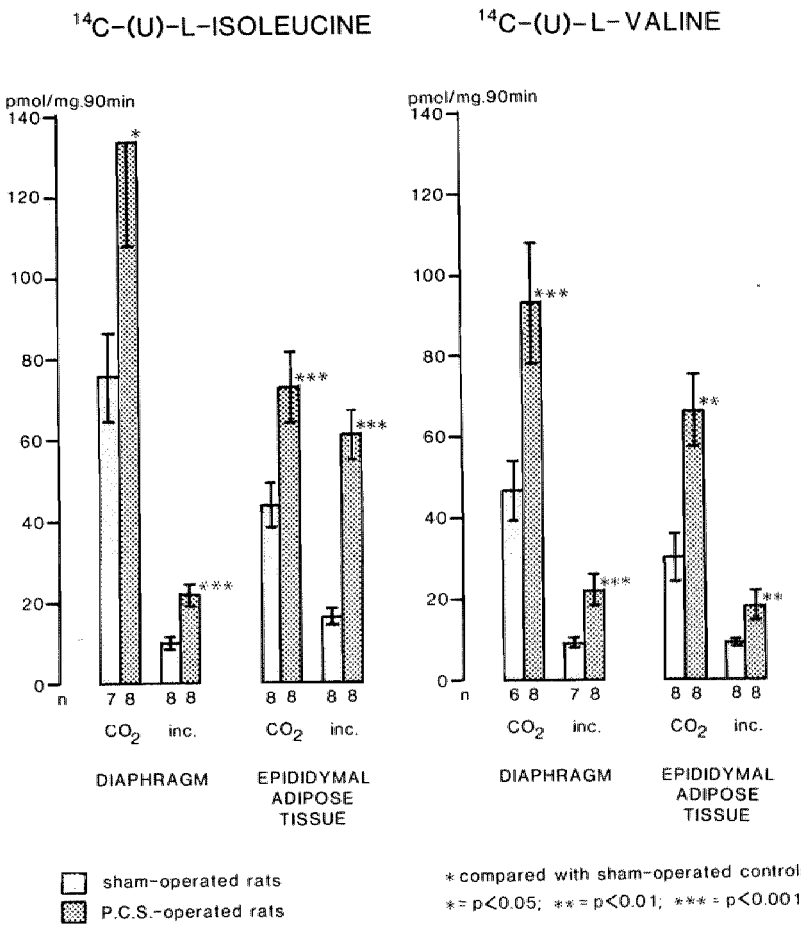
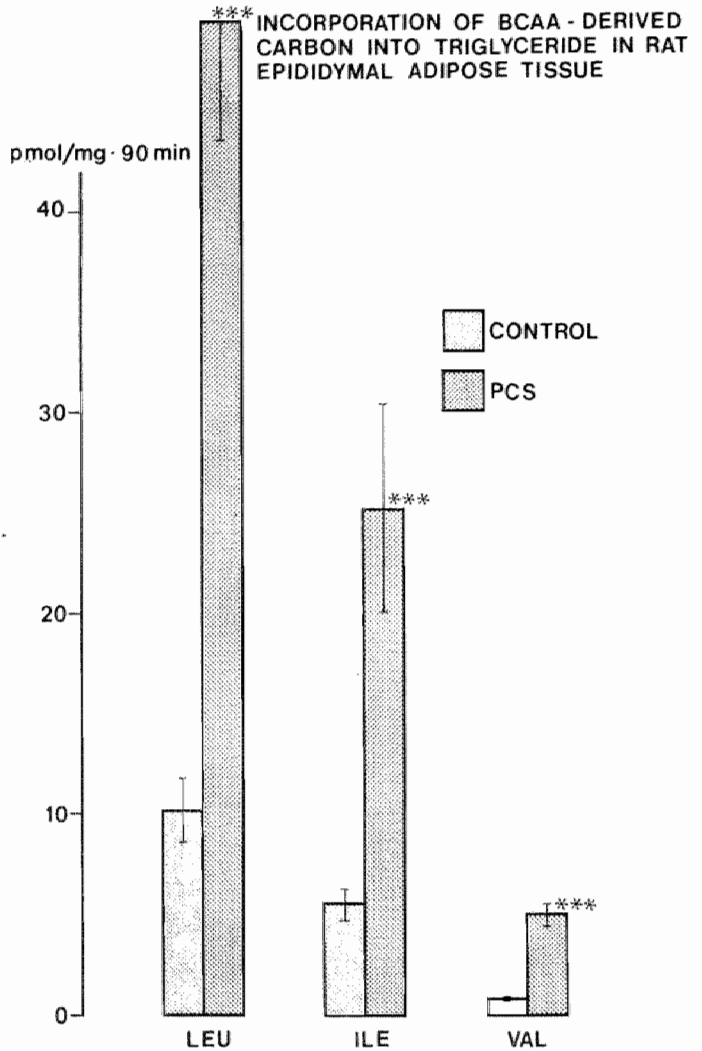
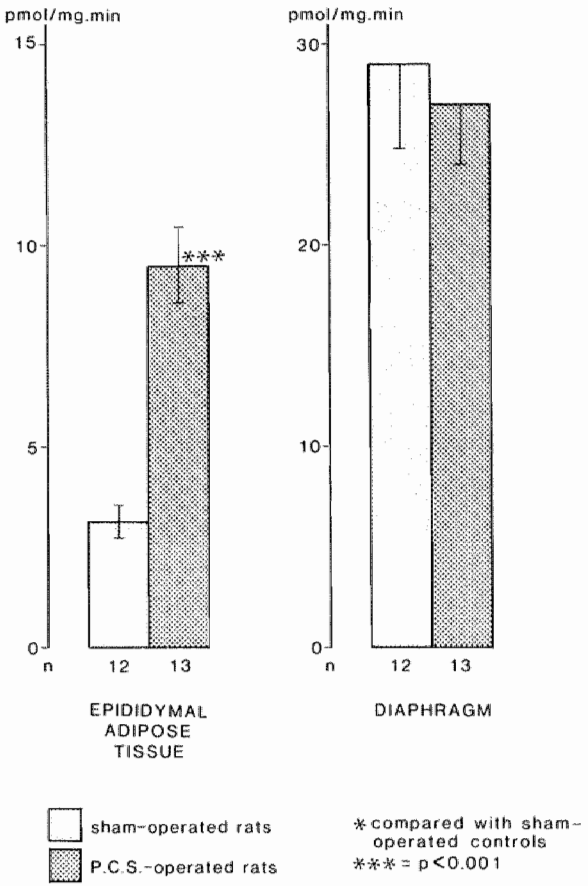


See figure 2 (chapter IV)



See figure 3a,b,c (chapter IV)

BCOA-DH



See figures 3 and 4 (chapter V)

SUMMARY

The subject of this thesis concerns the mechanism(s) involved in the genesis of decreased levels of branched chain amino acids (BCAA) in plasma from patients and experimental animals with liver failure and portal systemic shunting (PSS). Hyperinsulinism, encountered in liver patients and experimental animals with PSS, has been proposed to be a cause of an increased peripheral uptake of BCAA. This peripheral uptake, however, has never been well defined. Insight into the mechanism may be relevant to the understanding of the pathogenesis of hepatic encephalopathy (HE) and of the high catabolic rate prevailing in hepatic failure. In addition, a better understanding of the regulation of the plasma BCAA levels in pathologic conditions may lead to a rational application of BCAA-enriched infusion solutions in severely catabolic patients with low plasma BCAA levels.

In chapter I, a short review is given of the involvement of BCAA in various aspects of clinical interest. The role that BCAA play in several hypotheses, is briefly described. BCAA might serve as alternative energy substrate when carbohydrates and fat are not available or cannot be used in sufficient amounts. The validity of this hypothesis is questionable, however, because the contribution of BCAA degradation to overall energy requirement is very small. A second hypothesis claims that leucine, one of the BCAA, has special anabolic properties, and functions as a regulator of protein turnover. Furthermore, BCAA are implicated in a hypothesis that links encephalopathy to the altered levels of BCAA and aromatic amino acids (AAA) in the plasma of patients and experimental animals with liver failure and portal-systemic shunting. These hypotheses furnish the rationale for the administration of BCAA-enriched solutions.

Several disease states and altered nutritional states can influence plasma BCAA levels. Some of these conditions may accompany liver disease and may therefore exert an additional influence on the plasma BCAA levels.

The possible role of several organs and tissues in the in vivo regulation of plasma BCAA levels is discussed. Muscle and adipose tissue must play a predominant role in BCAA degradation judging from the ability to degrade BCAA and from the relative size of these tissues in the body. Some influences that may affect the BCAA degrading ability in vitro, may also be of relevance for the in vivo situation. These include transport of BCAA across cell membranes, sensitivity of the tissues for insulin and activities of enzymes involved in the degradation of BCAA.

To study mechanisms underlying the decrease of plasma BCAA levels in patients or animals with compromised liver function, an animal model is needed that exhibits both hyperinsulinism and low plasma BCAA levels. In addition, this animal model should not suffer from superimposed disorders that might influence plasma BCAA levels such as, e.g. malnutrition, starvations, diabetes or sepsis. Such an animal model (rats with a porta-caval shunt (PCS)) is described in chapter II. The model was carefully characterized with respect to relevant parameters in relation to the time after the operation. It was found that PCS rats exhibit hyperinsulinism and low plasma BCAA levels until 3 weeks after operation. Generally, PCS rats in our institute were in good health compared with those described in the literature.

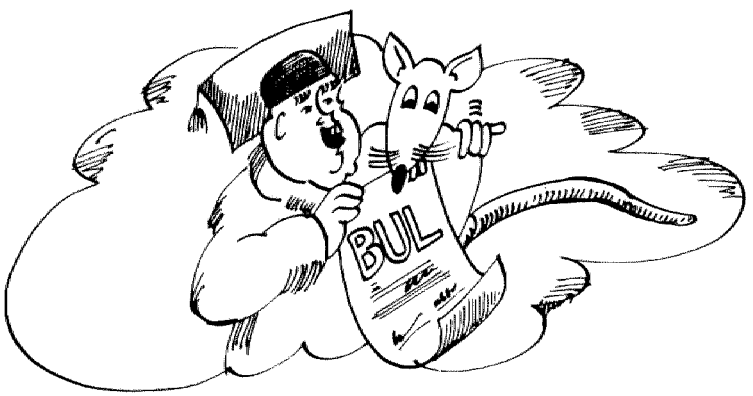
Muscle (diaphragm) and epididymal adipose tissue segments from PCS-operated and sham-operated rats were excised 2½ weeks post-operatively and used for in vitro studies of BCAA metabolism (chapters III and IV). Porta-caval shunting did not affect or even decreased the rate of irreversible degradation of BCAA in muscle but greatly increased this rate in adipose tissue. After PCS, the rates of decarboxylation of the BCAA in adipose tissue appeared to be increased to the same extent. The part of the BCAA carbon skeleton that remains after irreversible decarboxylation in adipose tissue, was used for incorporation into fat (mainly triglycerides) or degraded to CO_2, indicating that other pathways of BCAA catabolism in adipose tissue are negligible. In contrast, degradation of BCAA in muscle was incomplete. In adipose tissue from PCS rats were more BCAA degraded, even in the postabsorptive state, than in adipose tissue from fed control rats, indicating that this process is under long-term regulation. It was concluded that adipose tissue is extremely important in the degradation of BCAA. After PCS, this role is even more accentuated and it appears that

adipose tissue, and not muscle, contributes to the decrease in plasma BCAA levels after PCS. Protein synthesis in muscle and fatty acid synthesis in adipose tissue were increased after PCS. Because of the smaller muscle mass and fat mass in PCS rats compared with controls, these increases indicate that increased protein and fatty acid turnover must occur. Increased protein turnover has also been observed in cirrhotic patients.

The ability to degrade BCAA in muscle and adipose tissue is mainly determined by the activity of the branched chain oxoacid dehydrogenase (BCOA-DH), which is the rate-limiting enzyme in BCAA degradation in these tissues. The activity of this enzyme can be influenced by insulin in adipose tissue, but not in muscle. Based on the BCAA degradation rates, it was postulated that, after PCS, adipose tissue would exhibit an increased BCOA-DH activity, while this activity in muscle was expected to be unaffected or to be decreased. These expectations were confirmed in experiments described in chapter V. This may indicate that elevated insulin levels after PCS induce an increased BCOA-DH activity in adipose tissue. The relevance of these findings is discussed in chapter VI.

The results furnish support for the hypothesis that hyperinsulinism is instrumental in the decrease of the BCAA levels in plasma after PCS. Increased ammonia levels may exert an additional influence on plasma BCAA levels. The view is expressed that hyperinsulinism is a consequence of simultaneous increases of catecholamines, glucagon and cortisol levels encountered in liver disease and reflecting a stressed state. Findings in rats with diabetes or obesity, exhibiting altered plasma insulin and BCAA levels, suggest that the mechanism involved in changing plasma BCAA levels may be more general.

Increased protein and fatty acid synthesis and increased degradation of BCAA in adipose tissue all appear to reflect a well-fed state signalled by increased insulin levels. PCS rats, however, are generally less well-fed than control rats. Increased catabolism occurring in PCS rats, may therefore be induced by insulin, erroneously signalling the well-fed state. The BCAA carbon skeletons remaining after decarboxylation were less completely degraded in muscle after PCS which argues against a muscle energy deficit.


BUL

SAMENVATTING

Het onderwerp van dit proefschrift betreft het mechanisme dat verantwoordelijk is voor de verlaging van de spiegels van de vertakte keten aminozuren (VKAZ) in het plasma van patienten en proefdieren met leverlijden en shunting van portaal bloed buiten de lever om naar de vena cava (portaal systemische shunting (PSS)). Van oudsher zijn de verhoogde insulinespiegels die bij leverpatienten en bij proefdieren met PSS gevonden worden, genoemd als de mogelijke oorzaak van verhoogde perifere opname van VKAZ, zonder dat precies gespecificeerd is waar en hoe dit gebeurt. Inzicht in dit mechanisme is van belang voor de pathogenese van hepatische encephalopathie (HE) en voor de verhoogde netto afbraak van lichaamseiwit die vaak wordt waargenomen bij leverlijden. Bovendien is een beter begrip van de regulering van plasma VKAZ spiegels onder pathologische condities van belang voor een rationeel gebruik van infusievloeistoffen die verrijkt zijn met VKAZ, in ernstig katabole patienten met lage plasma VKAZ spiegels.

Hoofdstuk I geeft een kort overzicht van die aspecten van de VKAZ, die van belang zijn voor de kliniek. In het kort wordt de rol beschreven die aan de VKAZ wordt toegedacht in verschillende hypothesen. VKAZ zouden kunnen dienen als alternatieve energiebron in situaties waarin koolhydraten en vet niet voorhanden zijn of niet in voldoende mate gebruikt kunnen worden. Het is echter de vraag of deze hypothese juist is. De bijdrage die afbraak van VKAZ kan leveren aan de totale energiebehoefte, is namelijk maar heel gering. Volgens een tweede hypothese bezit leucine, één van de drie VKAZ, speciale anabole eigenschappen en vervult leucine een regulerende functie bij de opbouw en afbraak van eiwitten. Verder spelen VKAZ een rol in een hypothese die een verband legt tussen encephalopathie en veranderde plasma spiegels van VKAZ en aromatische aminozuren (AAZ) in het plasma van patienten en proefdieren met leverlijden en portaal-systemische shunting van bloed. Deze hypothesen verschaffen de argumenten om met VKAZ verrijkte voedingsoplossingen toe te dienen.

Diverse ziekten en veranderde voedingstoestanden kunnen de plasma VKAZ spiegels beinvloeden. Enkele van deze invloeden kunnen ook optreden bij leverlijden.

De mogelijke rol die verschillende organen in vivo hebben in de regulering van de plasma VKAZ spiegels, wordt besproken. Spierweefsel en vetweefsel zijn het belangrijkst voor de afbraak van VKAZ, geoordeeld naar het vermogen om VKAZ of te breken en naar de omvang van deze weefsels in het lichaam. Het vermogen van weefsels om VKAZ af te breken kan, zowel in vitro als in vivo, beinvloed worden door een aantal factoren. De belangrijkste zijn veranderingen in het transport van de VKAZ over de celmembraan, in de gevoeligheid van de weefsels voor insuline, en in de aktiviteit van de enzymen die betrokken zijn bij de afbraak van de VKAZ.

Om het mechanisme te kunnen bestuderen dat ten grondslag ligt aan de verlaging van de plasma VKAZ spiegels in patienten of proefdieren met een verminderde leverfunktie, dient beschikt te worden over een proefdier model dat zowel verhoogde plasma insuline spiegels heeft als verlaagde plasma VKAZ spiegels. Bovendien mogen zich in dat proefdier model niet gelijktijdig andere condities manifesteren die de plasma VKAZ spiegels zouden kunnen beinvloeden, zoals b.v. ondervoeding, hongeren, diabetes of sepsis. Zo'n diermodel (ratten met een porta-cavale shunt (PCS)) wordt beschreven in hoofdstuk II. Het model werd nauwkeurig gedefinieerd met betrekking tot relevante parameters in relatie tot de tijdsduur na de operatie. Tot 3 weken na de operatie vertoonden de PCS ratten hyperinsulinisme en lage plasma VKAZ spiegels. Over het algemeen waren deze ratten in ons instituut gezond, vergeleken met literatuurgegevens.

Twee en een halve week na de operatie werden spierweefsel (diaphragma) en epididymaal vetweefsel van PCS-geopereerde ratten en van controle ratten (sham operatie) gebruikt voor de bestudering in vitro van de metabolisering van VKAZ (hoofdstuk III en IV). De PCS had geen invloed op de mate van irreversibele afbraak van VKAZ in spierweefsel of verlaagde deze zelfs. In vetweefsel bleek de hoeveelheid VKAZ die irreversibel werd afgebroken sterk verhoogd als gevolg van de PCS. Alle drie de VKAZ werden in gelijke mate verhoogd afgebroken in vetweefsel van PCS ratten. Het gedeelte van het koolstofskelet dat overblijft na irreversibele decarboxylering in vetweefsel, wordt via acetyl-CoA ingebouwd in vet (voornamelijk in de triglyceriden) of wordt verbrand tot CO_2. Dit betekent dat andere afbraak routes voor

VKAZ in vetweefsel verwaarloosbaar zijn. Daarentegen is de afbraak van de VKAZ in spierweefsel onvolledig. In vetweefsel van PCS ratten worden, zelfs na een dag en een nacht vasten, nog steeds meer VKAZ afgebroken dan in vetweefsel van gevoede controles. Dit geeft aan dat dit proces aan lange termijn regulatie onderhevig is. Derhalve moet vetweefsel uiterst belangrijk zijn voor de afbraak van VKAZ. Na PCS is deze rol nog veel belangrijker en het lijkt er sterk op dat vetweefsel, en dus niet spierweefsel, bijdraagt aan de verlaging van de plasma VKAZ spiegels na PCS. De eiwitsynthese in spierweefsel en de vetzuursynthese in vetweefsel waren verhoogd na PCS. Omdat zowel de spiermassa als de vetmassa in PCS ratten kleiner is dan in controle ratten, betekenen deze verhogingen dat zowel de eiwitturnover als de vetturnover verhoogd moeten zijn. Verhoogde eiwitturnover is ook vastgesteld bij patienten met lever cirrhose.

Het vermogen van spierweefsel en vetweefsel om VKAZ af te breken, wordt voornamelijk bepaald door de aktiviteit van het vertakte keten oxozuur dehydrogenase (VKOZ-DH), dat snelheidsbeperkend is in de afbraak van VKAZ in deze weefsels. De aktiviteit van dit enzym kan in vetweefsel beinvloed worden door insuline. In spierweefsel is dit niet het geval. Gebaseerd op de hoeveelheden VKAZ die konden worden afgebroken, mocht na PCS een verhoogde VKOZ-DH aktiviteit verwacht worden in vetweefsel, en een onveranderde of verminderde aktiviteit van dit enzym in spierweefsel. Deze verwachtingen werden bevestigd in experimenten die beschreven zijn in hoofdstuk V. Dit betekent vrijwel zeker dat de verhoogde insuline spiegels die bestaan na PCS, effectief zijn in de inductie van VKOZ-DH aktiviteit in vetweefsel. De relevantie van deze bevindingen wordt besproken in hoofdstuk VI.

De resultaten steunen de hypothese dat hyperinsulinisme bijdraagt tot de verlaging van plasma VKAZ spiegels na een porta-cavale shunt. Verhoogde ammoniak spiegels zouden een additionele invloed op de VKAZ spiegels kunnen uitoefenen. Hyperinsulinisme zou het gevolg kunnen zijn van gelijktijdige verhogingen van catecholamines, glucagon en cortisol in het plasma. Deze verhogingen zijn in de literatuur beschreven zijn bij leverpatienten en weerspiegelen een stress-toestand. De resultaten van in vitro studies van de afbraak van VKAZ in vetweefsel van ratten met diabetes of obesitas, die eveneens veranderde plasma insuline- en VKAZ spiegels vertonen, suggereren dat het mechanisme dat zorgdraagt voor de verandering van de plasma VKAZ spiegels, meer algemene geldigheid heeft.

Een toename van de eiwit- en vetzuursynthese en verhoogde afbraak van VKAZ in vetweefsel na PCS lijken alle te wijzen op een goed gevoed proefdier, zoals aangegeven door hoge insuline spiegels. PCS ratten zijn over het algemeen echter minder goed gevoed dan controle ratten. De toename van eiwitafbraak of een verminderde groeisnelheid optredend bij PCS ratten zou daarom langs indirecte weg veroorzaakt kunnen worden door hoge insuline spiegels die een goede voedingstoestand suggereren die in werkelijkheid niet bestaat. Het koolstofskelet dat overblijft na decarboxylering van de VKAZ, werd na PCS minder volledig afgebroken in diaphragma. Dit pleit tegen de hypothese dat een toename van de afbraak van VKAZ in skeletspier het gevolg zou zijn van een energie tekort in spierweefsel.

ACKNOWLEDGEMENTS

Many people supported me during the preparation of this thesis. I cannot thank all these people in person, but I want to specifically acknowledge the contributions of those, without whom these investigations would not have been realized.

In the first place, I am very grateful to **Peter Soeters**, not only for the enormous amount of work that he has done to allow these investigations to be carried out, but also and especially for his warm personality, something which is not always encountered in the academic world. He initiated this work and guided as far as it could be combined with his clinical work. He introduced me into the field of liver disease, which is so immense, that in the beginning it was difficult to get an overall picture, and which has become so fascinating to me that I hope to somehow stay involved in it. The clinical relevance of the work especially attracted me. Peter and I spent many days, evenings and week-ends together, sometimes to the exasperation of both families. Publication of this thesis proves his valuable engagement in basic clinically-relevant research. He deserved another role at this promotion.

Prof.Dr.F. ten Hoor supported me greatly during the last years of the preparation of the thesis, for which I feel indebted to him.

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Johan van Dongen provided with his surgical skill the model which is so difficult to control. He was always joyful or tried to be, even though when it came time to do the experiments, sometimes half of the PCS rats could not be used because they failed to grow.

Mieke Janssen was a great help, especially in the difficult early period and in the first phase of the actual experiments.

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Hans van Eijk determined the parameters to check the model and helped out whenever necessary.

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CURRICULUM VITAE

Jurjen de Boer was born on May 10th 1948 in Amsterdam. After graduation from high school (Ir. Lely Lyceum, HBS^B, Amsterdam) in 1967, he studied to become a technician. From 1968 to 1969, he served Her Majesty in the military service. Thereafter, he worked as a technician at the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service in Amsterdam (1969-1975) in the Department of Blood Cell Chemistry (Dr. H.K. Prins, Dr. J.A. Loos, Dr. D. Roos and Dr. Ir. P. Diepenhorst) and in the laboratory for diagnosis and research on serum-hepatitis B virus (Dr. H.W. Reesink). There he was involved in the following fields: red blood cell enzyme deficiencies; lymphocyte-carbohydrate metabolism; preparation of leucocyte- and platelet-poor erythrocyte suspensions to be used into nephron dialysis patients with chance of transplantation or with antibodies against leucocytes and/or platelets; development of sensitive diagnostic tests for serum hepatitis B surface antigen; and development of heat-inactivated serum hepatitis B vaccine for active immunization. In his free time, he studied to become a laboratory assistant (HBO^B-biochemistry; graduation 1971) and for a teachers degree, specializing in chemistry and physics (MO^A; graduation 1975). He then studied at the University of Amsterdam and in 1976, did his candidates examination in chemistry and physics. He continued his study in the Department of Biochemistry (BCP Jansen Institute, University of Amsterdam; Prof. Dr. J.M. Täger, Dr. R. Wever, Dr. A.R.J. Bakkenist), where he was concerned with myeloperoxidase-ligand binding. His secondary field was clinical chemistry which he practised in the Haemostasis Laboratory of the Wilhelmina Gasthuis, University of Amsterdam (Dr. J.W. ten Cate); there he was concerned with characterization of clotting factor XII activity in the urine of a patient with nephrotic syndrome. Furthermore, he qualified in virology. He graduated as a biochemist in 1978.

From 1979 to 1984, he worked as a research scientist at the University of Limburg, Maastricht, in the Departments of Biochemistry and Surgery, where this dissertation was prepared.