Arginine biosynthesis in organ development and function

Citation for published version (APA):

Marion, M. V. V. (2009). *Arginine biosynthesis in organ development and function*. [Doctoral Thesis, Maastricht University]. Maastricht University. <https://doi.org/10.26481/dis.20090326mm>

Document status and date:

Published: 01/01/2009

DOI:

[10.26481/dis.20090326mm](https://doi.org/10.26481/dis.20090326mm)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
- The final author version and the galley proof are versions of the publication after peer review.
- The final published version features the final layout of the paper including the volume, issue and page numbers.

[Link to publication](#)

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal.

If the publication is distributed under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license above, please follow below link for the End User Agreement:

www.umlib.nl/taverne-license

Take down policy

If you believe that this document breaches copyright please contact us at:

repository@maastrichtuniversity.nl

providing details and we will investigate your claim.

**ARGININE BIOSYNTHESIS
IN ORGAN DEVELOPMENT
AND FUNCTION**

Vincent Marion

To my little Dutchman Evan

and my little Frenchman Noah

Arginine biosynthesis in organ development and function

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Maastricht, op gezag van de
Rector Magnificus Prof. mr. G.P.M.F. Mols, volgens het besluit van het College van
Decanen in het openbaar te verdedigen op 26 donderdag maart 2009 om 12:00 uur

door

M.V.Vincent Marion

Geboren te Mauritius

Promotor

Prof.Dr W.H. Lamers

Co-promotor

Dr. S. E. Köhler

Beoordelingscommissie

Prof.Dr. A. Schols, voorzitter

Prof.Dr. L. Cynober, Paris

Prof.Dr. M. Hofker, RUG

Prof.Dr. J. de Mey

The studies presented in this thesis were performed at the School for Nutrition, Toxicology and Metabolism (NUTRIM), which participates in the Graduate School VLAG (Food Technology, Agrobiotechnology, Nutrition) accredited by the Royal Netherlands Academy of Art and Sciences (KNAW).

The work presented in this thesis was carried out at the Department of Anatomy & Embryology of Maastricht University and was supported by a grant of NUTRIM

TABLE OF CONTENTS

Chapter 1	Fates and functions of arginine Scope of the thesis
Chapter 2	Chronic arginine deprivation represses the GH-IGF-1 axis via the GCN2-mediated stress response
Chapter 3	Experiences from the construction of three conditionally knock out mice
Chapter 4	Physiological adaptations associated to intestinal inactivation of arginine biosynthesis in suckling mice
Chapter 5	The function of L-arginine in vascular physiology
Chapter 6	Concluding discussion
Nederlandse samenvatting	
Acknowledgements	
Curriculum vitae	

Chapter 1

Introduction

Fates and functions of arginine

I- Arginine requirement

II- Arginine synthesis

- a) Hepatic arginine metabolism
- b) Intestinal arginine synthesis
- c) Renal arginine synthesis
- d) Endothelial arginine synthesis
- e) Neuronal arginine synthesis
- f) Lymphocytic arginine synthesis

III- Arginine metabolism

- a) Ornithine
 - 1) *Ornithine cycle localization*
 - 2) *Ornithine cycle disorders (OCDs)*
- b) Other arginine metabolites
 - 1) *Proline & glutamate*
 - 2) *Creatine*
 - 3) *Agmatine*
 - 4) *Nitric oxide*
- c) Arginine in the immune system
- d) Arginine, a building block for protein synthesis
- e) Arginine as metabolic regulator
 - 1) *Arginyl tRNA*
 - 2) *N-terminal argininylation*

3) *Arginine, a potent secretagogue*

IV- Arginine transporters and their regulation

- a) Nomenclature of arginine transporters
- b) Arginine transporters

V- Arginine-related proteins and their transgenic models

VI- Scope of thesis

I- Arginine requirement

The sources of free arginine within the body are dietary protein and endogenous synthesis. Dietary sources of arginine account for about 5-6 grams per day (1, 2) which corresponds to ~25% of the daily whole-body arginine flux (3-5). In adult rodents and humans, ~40% of dietary arginine is catabolized by the intestine before entering the circulation. Most of the remaining arginine required is provided by endogenous synthesis of arginine from citrulline, with the kidneys being the major net producer (6). Arginine is classified as a conditionally essential amino acid, meaning that under normal conditions, the endogenous biosynthesis of arginine suffices to meet daily requirements in healthy adults. In rapidly growing infants and children, and in adults under catabolic stress or with intestinal or renal dysfunction, arginine becomes an essential amino acid (7-9).

Arginine catabolism is the major regulator of arginine homeostasis (10) as is indicated by the finding that the endogenous biosynthesis rate of arginine from citrulline is essentially unaffected by circulating arginine concentrations (5). This was demonstrated in homozygous arginase II- knockout mice which show elevated plasma arginine levels thereby indicating that arginase II participates in regulating arginine concentration. Furthermore, healthy human volunteers fed an arginine-free diet show a decrease in serum arginine but unaltered rates of whole body synthesis (5).

II- Arginine synthesis

All mammals are capable of covering their arginine requirements under normal physiological conditions through endogenous arginine biosynthesis (6, 11-13), except the Felidae, which have very low levels of pyrroline-5-carboxylate synthase and ornithine aminotransferase in the intestinal mucosa and can, therefore, not produce sufficient ornithine for arginine biosynthesis (14-17). Figure 1 depicts the enzymes that are involved in *de novo* arginine synthesis from proline and glutamine. Proline and glutamine metabolism to citrulline occurs in the mitochondria, whereas the conversion of citrulline to arginine takes place in the cytoplasm (18). Citrulline derives from three sources *in vivo*. The major source of citrulline is its synthesis in the small intestine enterocytes from proline in the perinatal period and from glutamate/glutamine in adulthood (19-21) (Figure 1). Furthermore, small quantities of citrulline are produced by

nitric oxide synthase (NOS) and from the degradation of asymmetric dimethylarginine (ADMA), a metabolic by-product of protein modification in the cytoplasm.

Figure 1. Enzymes involved in the synthesis of arginine. Abbreviations: 1, phosphate-dependent glutaminase; 2 and 3, P5C synthase; 5, ornithine aminotransferase; 6, ornithine carbamoyltransferase; 7, argininosuccinate synthetase; 8, argininosuccinate lyase; 9, N-acetylglutamate synthase; 10, carbamoylphosphate synthetase I; 11, proline oxidase; 12, aspartate aminotransferase. OAA, oxaloacetate; CP, carbamoylphosphate.

a) Hepatic arginine metabolism

The liver is the only organ, which expresses all the enzymes of the ornithine cycle. Here the highest rate of arginine synthesis is found (22), but this does not result in net arginine production due to the abundant expression of arginase 1. The ornithine cycle functions efficiently due to the organization of the five major ornithine cycle enzymes in a so-called metabolon (23) carbamoylphosphate synthetase I (CPS) and ornithine transcarbamoylase in the mitochondrial matrix, and argininosuccinate synthetase, argininosuccinate lyase and arginase 1 in the cytosol. The three soluble cytoplasmic enzymes of the ornithine cycle are grouped in such a way around the mitochondria that direct product transfer between consecutive enzymes occurs without substrate diffusion (24) Expression of the ornithine cycle enzymes in the liver lobule is restricted to the periportal compartment (25) and is regulated by glucagon, insulin and glucocorticoids.

There are controversial data on the hepatic absorption of arginine. Some recent tracer studies using stable isotopes showed that only 5% of the urea produced is derived from plasma arginine (4) implying a very low uptake of arginine by the liver and the strict segregation of hepatic and plasma arginine pools. Other authors showed rapid absorption of arginine in the liver specially after burn injury in rats (26) probably via a Na^+ -independent transport system γ^+ (27).

b) Intestinal arginine synthesis

In rapidly growing mammals like piglets and rats, the arginine extracted from milk is not enough for optimized growth and metabolic function (28-31). This is reflected by the fact that the percentage of arginine in milk is $\sim 3.3\%$ of the total protein whereas the percentage of arginine in total carcass protein is 7% in rats (29). This apparent deficiency accounts for the presence of an important amount of arginine being synthesized by the neonatal intestine to compensate for the low levels of arginine present in the milk. Significant intestinal arginine production has been demonstrated in neonatal rodents and pigs (32-34). The two cell types of the intestine in which arginine biosynthesis can take place are the enterocytes and the neurons of the enteric nervous system (35). The difference between these two sites of arginine production is that the enterocytes produce arginine only in the perinatal period (6), whereas neurons retain the capacity to produce arginine throughout life. Enterocytes differ from hepatocytes in that they have the capacity to produce ornithine (19). This capacity is due to the co-expression of 2-

pyrroline-5-carboxylate synthase (2-P5C-S) and ornithine aminotransferase (OAT) in the small intestine (36). OAT catalyzes the reversible reaction between ornithine and glutamate as shown in figure 1. The direction of the reaction evolves through development in the sense that during the neonatal period, OAT favors production of ornithine whereas in the post-weaning period OAT favors the synthesis of the P5C (37-39). Complementary to this, there seems to be no significant uptake of arterial proline by the enterocytes in young pigs, indicating that the enteral diet is the major perinatal source of proline for citrulline synthesis in the gut (40). Interestingly, parentally fed neonates develop hyperammonemia (37, 41) which implies that the absence of intestinal proline catabolism to citrulline prevents proper functioning of the ornithine cycle. The expression of the enzymes that are necessary for local arginine biosynthesis in the perinatal gut is accompanied by little, if any, arginase (42). After weaning, the enterocytes of rodents cease to express ASS and ASL (43), while the expression of ASS in human and porcine enterocytes becomes markedly reduced (42). It has also been shown that in postweaned pig and rat (35) enterocytes, intestinal type II arginase is expressed in a glucocorticoid-dependent fashion (42). After weaning, the intestinal contribution to arginine biosynthesis becomes limited to the production of citrulline, which is secreted to the blood and used for arginine synthesis in the kidneys and elsewhere (44, 45).

The essential role of the small intestine in arginine or arginine precursor synthesis in postnatal mammals is demonstrated by the arginine deficiencies that result when intestinal citrulline synthesis is blocked. Inhibitors of OTC or OAT or an arginine-free diet after near-complete resection of the small bowel (39, 46) resulted in weight loss, a negative nitrogen balance, a decrease in circulating arginine concentrations, sparse fur, elevated blood pressure and low organ arginine concentration (46-48) in these animals. The OAT knockout mice exhibited neonatal hypornithinaemia and lethality, and could be rescued by arginine administration (39). One of the questions that we have addressed in this thesis is the role of perinatal intestinal arginine synthesis. It has been speculated that neonatal intestinal arginine synthesis compensates for the low arginine concentration in milk and insufficient renal arginine synthesis. It was assumed that around weaning, the kidneys have reached their full biosynthetic capacity, making arginine synthesis in the small intestine superfluous (31, 49, 50). We and others (43) have, however, recently shown that the kidneys express substantial amounts of ASS

already in 14-day-old mice, roughly a week before natural weaning (see chapter IV) a finding that reduces the plausibility of this argument.

c) Renal arginine synthesis

Although the arginine synthesizing enzymes are already present in the kidneys of rodents in the perinatal period (51) their expression in suckling rodents is considered to be low (43) With the decline of arginine production in the gut in the post-weaning period, renal arginine production is increased to ensure sufficient arginine availability for whole-body requirements (43) Intestinal citrulline is transported via the blood to the kidneys for arginine production (52) the two organs, therefore, form the arginine “intestinal-renal” axis (6) . In the kidneys, ASS and ASL are localized in the proximal convoluted tubules (50, 53-55) Renal arginine synthesis in humans amounts to ~2g of arginine per day (56) which accounts for approximately 60% of net endogenous whole-body arginine biosynthesis, and for 10% of the total daily arginine requirements of a healthy adult (1, 2, 4) The rate of arginine synthesis depends on citrulline delivery and does not appear to be regulated by dietary arginine availability (57, 58) This mechanism fails in individuals suffering from chronic renal insufficiency, who have elevated plasma levels of citrulline (59, 60) However, there is little or no decrease in plasma arginine. The basis for the maintenance of plasma arginine probably involves a combination of increased arginine synthesis at extrarenal sites and hypertrophy of unaffected proximal tubules (61, 62) Another important regulator of circulating arginine levels is arginine catabolism to ornithine (57, 63)

d) Endothelial arginine biosynthesis

Arginine is the sole precursor for nitric oxide, the endothelium-derived relaxing factor which is crucial in regulating vascular tone and hemodynamics (64-67) In order to sustain nitric oxide production in the endothelium lining the arterial walls, a constant arginine supply for endothelial NOS (NOS3) needs to be maintained. The availability of arginine to NOS3 is governed by the import of arginine from circulating plasma, the recycling of citrulline, the other product of NOS, to arginine by ASS and ASL or its degradation via arginase (68) The enzymes ASS and ASL are ubiquitously expressed (69) including the endothelium (18) Endothelial recycling of citrulline to arginine and NO production take place in the caveolae, a subcompartment of the plasma membrane

16

(see Figure 2). During activation of NO production, recycling is co-activated to the extent that more than 80% of the citrulline produced is recycled to arginine (70)

Figure2. Endothelial arginine metabolism. Arginine is transported into the cell by CAT-1. NOS3 converts intracellular arginine into NO and citrulline. Citrulline is recycled to arginine via ASS. Intracellular arginine can be catabolized by arginase. At low intracellular arginine concentrations, NOS3 uncouples and produces superoxide (red) rather than NO. Note that NOS3, ASS and CAT-1 all co-localize at the caveolar membrane and that a cell contains numerous caveolae (invaginations of the plasma membrane) (diameter: ~50 nm).

The importance of arginine resynthesis in substrate delivery to NOS3, has been demonstrated by knocking down ASS expression in aortic endothelial cells. ASS-specific small interfering RNA diminished their capacity to produce NO, despite the presence of saturating levels of arginine in the medium (71) Resynthesis of arginine from citrulline is also the major route for NO production in rat blood-brain barrier endothelial cells (72) and porcine endothelial cells of the cerebral arteries (73).

e) Neuronal arginine synthesis

ASS expression has been detected in the neurons of the myenteric plexus of mice (35) and dogs (74) We have recently also demonstrated the presence of ASS in ganglia of the human small intestine in samples of all ages (ranging from fetal to elderly). These ganglia that are important in intestinal relaxation for food propulsion, co-express NOS1, suggestive of similar arginine recycling from citrulline as demonstrated in endothelial cells (see Figure 2). Brain neurons, on the other hand, seem to have a spatially different expression pattern for arginine- and NO-synthesizing neurons. ASS and ASL are expressed constitutively in neurons, but contrary to the intestinal ganglia, ASS and ASL

hardly co-localize with each other or with NOS1 in the same neurons (75) Therefore, trafficking of citrulline and arginine between neurons necessitates transport capacities in these cells, which are fulfilled by the cationic and neutral amino acids transporters (75).

f) Lymphocytic arginine synthesis

Macrophages and microglia (76) are able to use citrulline as a substrate for arginine synthesis, which is then metabolized either by arginase or nitric oxide synthase (to be discussed in section II of this introduction). Although ASS and ASL have been detected in these cells, extracellular arginine seems to be the major source for cytokine-induced NO production by NOS2 (73).

III- Arginine metabolism

Quantitatively, the availability of arginine is primarily regulated by arginase, which metabolizes arginine into ornithine and urea, and, to a lesser extent, by nitric oxide synthase, which metabolizes arginine into citrulline and nitric oxide. In addition, arginine is a building block for protein synthesis, and a precursor for creatine, and agmatine. The knowledge of arginine catabolism *in vivo* is, nevertheless, limited. This is largely due to the complex compartmentalization of arginine metabolism at tissue and cellular levels.

Figure 3. Arginine metabolism

a) Ornithine

1) *Ornithine cycle localization*

In the liver, the ornithine cycle (Figure 2) ensures that excess ammonia released by protein breakdown is detoxified by conversion of arginine into urea. The ornithine cycle is active in the periportal hepatocytes of the liver. The capacity of the liver for urea production is much higher than the normal basal rates of ammonia production. Furthermore, the K_M of the ornithine cycle for ammonia is high, namely, about 0.6 mM in human fetal liver and 0.45 mM in adult liver (77). Both features ensure that the cycle can immediately respond to acute increases in circulating ammonia concentration. All ornithine-cycle enzymes have also been detected in the post-weaning small intestine (78, 79) and several investigators have hypothesized that a metabolically significant ornithine cycle may function in this organ (49, 79). Indeed, it has been shown in young pigs, that the enterocytes were able to generate urea from glutamine, ammonium, ornithine and aspartate (80) but the responsible arginase isoform, i.e. arginase 1 or 2 has not been identified. In rodents, neither Arg1 nor Arg2 are found in the small intestine before weaning. Our group has been able to show (manuscript submitted (81)) that in healthy human small intestine, only arginase 2, a mitochondrial enzyme but not arginase 1, the cytosolic enzyme is expressed. This could explain why intestinal urea production functions at a considerably lower rate than in hepatocytes (49, 79, 82) since it requires transport of arginine across the mitochondrial membrane.

2) *Ornithine Cycle Disorders (OCDs)*

Whenever one or more of the enzymes involved in the ornithine cycle is inactivated, the transfer of nitrogen from ammonia to urea is stopped. Five autosomal recessive disorders involving different defects of the enzymes of the ornithine cycle have been described: Ornithine transcarbamylase (OTC) (83) deficiency, carbamoylphosphate synthetase deficiency, argininosuccinate synthetase (ASS) deficiency, or citrullinemia, argininosuccinate lyase (84, 85) deficiency, and arginase deficiency (86). For instance, citrullinemia is characterized by increased levels of blood citrulline and ammonia (87). Two major types of citrullinemia can be distinguished. In classical citrullinemia mutations in the ASS gene lead to either abnormal kinetics (20-40,000-fold higher K_M

than normal for citrulline (0.05 mM) (88)) or to undetectable or very low enzyme levels (88, 89) This form of citrullinemia is classified as a neonatal disease with an approximately 100-fold elevated plasma concentration of citrulline in young children (90) The other type of citrullinemia is an adult-onset disease caused by a dysfunction of the citrin protein. This mitochondrial carrier protein is encoded by the SLC25A13 gene localized at chromosome 7q21.3 (91, 92) and is predominantly expressed in liver and kidneys (93). It is an aspartate/glutamate carrier (AGC) which is differentially expressed in different organs during development (94) to be afterwards predominantly expressed in the liver. It functions to supply aspartate to the cytosol for urea, protein and nucleotide synthesis by exchanging mitochondrial aspartate for cytosolic glutamate and proton, and transporting cytosolic NADH reducing equivalent to mitochondria as a member of malate aspartate shuttle essential for aerobic glycolysis (95) Although the function of the citrin transporter have been elucidated, it is still not known how its deficiency leads to the type II citrullinemia. These ornithine cycle disorders (classically known as urea cycle disorders or UCDs) are accompanied by the accumulation of metabolites upstream of the reaction catalyzed by the inactive enzyme. OCDs are characterized by the triad of hyperammonemia, encephalopathy and respiratory alkalosis due to accumulation of bicarbonate not neutralized in urea.

b) Other arginine metabolites

1) *Proline & Glutamate*

Much of the ornithine generated by arginase outside the liver is metabolized by OAT to generate P5C. P5C can subsequently be utilized for the synthesis of proline and glutamate. In enterocytes of post-weaning pigs, proline is the major product of arginine catabolism, accounting for about 55% of the metabolized arginine carbon atoms (96) OAT deficiency accordingly leads to the occurrence of hyperornithinaemia in adult humans and mice (39).

2) *Creatine*

The kidney is the main organ, in which the first step of creatine synthesis, namely the conversion of arginine to guanidoacetate by L-Arginine:Glycine Amidinotransferase

(GATM) takes place. Guanidoacetate is then methylated, a reaction that primarily takes place in the liver (*S*-adenosyl-L-methionine:*N*-guanidinoacetate methyltransferase (GAMT)). The resulting creatine is then phosphorylated by creatine kinase using ATP as phosphate donor. Creatine phosphate serves as a storage form of high-energy phosphate. The latter reaction is reversible such that when energy demand is high (e.g. during muscle contraction), creatine phosphate donates its phosphate to ADP to yield ATP. Both creatine and creatine phosphate are found in muscle, brain and blood (97, 98).

3) *Agmatine*

Agmatine is formed by the decarboxylation of L-arginine through arginine decarboxylase (ADC) in bacteria and plants. Although a putative cDNA encoding arginine decarboxylase has been reported (99-101), other groups have not been able to confirm that it actually encodes arginine decarboxylase (99, 100). One group actually suggested that the predicted arginine decarboxylase is an ornithine decarboxylase antizyme (100). It is therefore not yet clear whether the known agmatine functions such as activation of imidazoline receptors and the α_2 adrenoceptor (102-105) are also important as a signaling pathway of endogenous agmatine or are relevant only in a pharmacologic context. Recently, it was reported that agmatine also acts as a blocker of the NMDA glutamate receptor (106) and an inhibitor of nitric oxide synthase in neonatal rats (107). Agmatine seems also to play a role in macrophage metabolism since both the putative ADC and agmatinase are modulated by both LPS and cytokines (108).

4) *Nitric Oxide*

NO, also known as endothelium-derived relaxing factor, is a naturally occurring gas that in the body serves as a short-lived signaling molecule with diverse functions. NO is important in the maintenance of vascular tone, has neurotransmitter functions in both the central and peripheral nervous system, and mediates cellular defense. In addition, NO interacts with mitochondrial systems to regulate cell respiration and to augment the generation of reactive oxygen species, thus triggering mechanisms of cell survival or death. To date, no nucleated mammalian cell has been described that does not express at least one of the three major NOS isoforms (109).

i) NO synthesis

L-arginine is the only endogenous substrate for the nitric oxide synthases (NOS).

Relative rates of NO synthesis from plasma arginine are low. In healthy adults, NO synthesis from plasma arginine represents only 1.2% of the flux of plasma arginine (4)

Figure 4. NO production catalyzed by NOS. In the presence of oxygen and co-factors like nicotinamide adenine dinucleotide phosphate (NADPH), and (6R)-5, 6, 7, 8-tetrahydrobiopterin (BH4), arginine is used for NO production

This accounts for about half of the total NO production. The arginine necessary for the remaining NO production is presumably derived from intracellular sources, such as local arginine synthesis at sites of NO synthesis as discussed above.

ii) NOS isoforms

Three isoforms of nitric oxide synthase exist, namely, neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2) and endothelial NOS (eNOS or NOS3). They all three have the same substrate, arginine, and reaction mechanism but differ in their dependence on Ca²⁺ ions, as well as in their expression pattern and activities. NOS3 and NOS1 are constitutively expressed and activated by an elevation in intracellular Ca²⁺. In contrast, NOS2 is not activated by Ca²⁺ influx but is only expressed upon external stimulation. All NOS enzymes function as homo-dimers. The dimerization process of the two monomers for all three isoforms is dependent on the presence of the co-factors and its substrate, since both BH4 (110-112) and arginine can promote and stabilize the active dimeric form of all three isoforms (110, 113, 114). In addition, arginine availability has a regulatory function for the three NO synthases. Both arginase and NOS activity deplete the local arginine pool. This decrease in arginine concentration causes the GCN2-mediated phosphorylation of the eukaryotic initiation factor (eIF2α), which in turn, downregulates translation of NOS mRNA. Substrate availability, sensed by GCN2, a kinase sensitive to amino-acid deprivation in cells, therefore, represents an important step in the regulation of NOS activity (115). Table 1 below shows the distribution of the three different isoforms of NOS in different organs.

NOS1

Neuronal NOS (NOS1) is a cytosolic enzyme present in central and peripheral neural tissues but also in skeletal muscle, epithelial cells and cells of the immune system (117). In neurons, constitutively expressed NOS1 is activated by agonists that increase intracellular Ca^{2+} ion levels and enhance calmodulin binding. The NO synthesized by NOS1 acts as a neurotransmitter. Bound to caveolin, NOS1 is also found in skeletal muscle (118, 119). It is now considered as the most complex human gene yet described in terms of promoter diversity and alternative splicing variants (120-123). Indeed, nine unique exon 1 variants expressed from a unique 5'-flanking region have been observed, combined with elaborate splicing patterns of different exons (122).

NOS2

NOS2 expression can be induced in most tissues (Table 1), but due to its well-described role in innate immune defense in macrophages, it is also known as macNOS. Its expression is induced by cytokines, such as IFN- γ , TNF- α and IL-1 and microbial products such as LPS (124, 125). The major difference compared to the two other isoforms is its Ca^{2+} -independent activation (126). It shares 51% amino acid homology with NOS1 with the greatest degree of divergence in the calmodulin binding domain. NO produced by NOS2 has been described to have microbiocidal, antiviral, antiparasital and antitumoral effects (109, 126). However, aberrant NOS2 induction may be involved in the pathology of many chronic inflammatory diseases (asthma, arthritis, multiple sclerosis, colitis, psoriasis), neurodegenerative diseases, tumor development, transplant rejection, and septic shock (109, 127). The level of expression of NOS2 is tightly controlled both on the transcriptional and the translational level. Moreover, protein stability is influenced by TGF- β at least in some cells, where enzyme degradation through the proteasome is stimulated. Posttranslational modification and substrate availability are additional mechanisms to control enzyme activity.

NOS3

NOS3 is localized in the small invaginations of the plasma membrane called caveolae. It is responsible for most of the vascular NO production and generates NO primarily in response to shear stress (66). The NO generated by NOS3 plays an important role in vascular smooth-muscle proliferation, platelet aggregation and extravasation of

leukocytes (67, 128-131). Two sources of arginine are available for NOS3 to sustain NO production: the recycling of citrulline via ASS and ASL within the cell and the uptake from the blood (depicted in Figure2). The relative contribution of these respective arginine sources has so far remained unknown

Organ	NOS1	NOS2	NOS3
Brain	Cytoplasm, mitochondria, RER, postsynaptic membrane	Primarily cytoplasm	Cytoplasm, plasma membran caveolae
Brown adipose	Weakly expressed in cytoplasm	Cytoplasm, nucleus	Cytoplasm, nucleus, plasma membrane caveolae
White adipose	Weakly expoesred in cytoplasm	Cytoplasm, plasma membrane	Cytoplasm, plasma membrane caveolae
Heart	Sarcoplasmic reticulum, mitochondria	Primarily cytoplasm	plasmalemmal & T-tubular caveolae, sarcoplasm
Kidneys	mitochondria	Primarily cytoplasm	Cytoplasm, plasma membrane caveolae
Liver	mitochondria	Primarily cytoplasm in periportal hepatocytes	Cytoplasm, plasma membrane caveolae
Skeletal muscle	mitochondria, sarcoplasm, sarcolemmal membrane	sarcoplasm, sarcolemmal membrane	Sarcoplasm, SR, sarcolemmal membrane, caveolae

Table 1. NOS isoform expression profile in animal tissues (adapted from (116)).

NO and its functions

NO functions are numerous as it can influence the activity of transcription factors and signaling cascades, mRNA stability and translation (109) These functions are mediated through cGMP, nitrosylation of proteins and peroxynitrite formation and will be precised in the following subchapters.

1 Cyclic guanosine monophosphate (cGMP)

Many of the physiological actions of NO, including the regulation of vascular tone and blood pressure, prevention of platelet aggregation and inhibition of vascular smooth muscle proliferation, are mediated via the activation of soluble guanylyl cyclase and the subsequent production of cGMP (66). There are three known targets for cGMP: cGMP-dependent protein kinase (132), cGMP-regulated phosphodiesterase and cGMP-gated ion channels (133). In smooth muscle cells, cGMP-dependent protein kinase phosphorylates the inositol 1,4,5-triphosphate receptor, leading to a decrease in Ca^{2+} ion concentration and, ultimately, smooth muscle relaxation. The third target of cGMP, cyclic nucleotide-gated ion channels are non-specific cation channels found in many tissues.

2 S-nitrosylation

S-nitrosylation is a process by which the nitric oxide group is reversibly transferred to cysteine sulfhydryls on proteins thereby transforming them into S-nitrothiols (134-136). S-nitrosylation represents a form of redox modulation with mostly an inhibitory effect on the function of the S-nitrosylated protein. It was discovered in red-blood cells where the S-nitrothiol form of hemoglobin inhibits platelet aggregation (137, 138). Interestingly, the enzymatic activity of ASS is also suppressed by S-nitrosylation (139) and this may represent a feedback mechanism to control the amount of NO produced in cells through substrate availability. In humans, of the five cysteine residues present in ASS, only cysteine132 is S-nitrosylated, resulting in enzyme inhibition. The same kind of feedback regulation via S-nitrosylation was also shown both *in vitro* and *in vivo* for NOS3 (140). The transcription factor NF- κ B that regulates the expression of numerous genes active in the inflammatory response, including NOS2 is also inhibited by S-nitrosylation (141). Moreover, nitric oxide inhibits apoptosis and inflammation by S-nitrosylation of the active site cysteine of caspases (142). This represents a feedback mechanism to prevent excessive inflammatory processes. In addition, NO has been described to regulate the phosphorylation of creatine to creatine phosphate by S-nitrosylation of the creatine kinase.

3 Peroxynitrite

Under sub-saturating arginine or BH4 concentrations or in the presence of NOS inhibitors, all three NOS isoforms are able to produce detectable quantities of superoxide

next to NO (143, 144). The very rapid reaction of superoxide with NO which is under the control of diffusion rates, results in peroxynitrite formation (145). Peroxynitrite is an oxidant that can react with DNA, proteins, and lipids under physiological conditions, leading to cellular damage and cytotoxicity. In proteins, peroxynitrite attacks tyrosine residues, forming nitrotyrosines. In inflammatory cells, peroxynitrite activates cyclooxygenase and increases prostaglandin synthesis, which is associated with a variety of vascular diseases, diabetes, chronic inflammatory diseases, cancer, and neurodegenerative disorders (146). With a short half-life and a low steady-state concentration direct measurement of peroxynitrite in vivo is very difficult (147).

c) Arginine in the immune system

In order to generate an efficient immune response, T cells require the assistance of myeloid-derived accessory cells that make antigen available to the clonotypic T-cell receptor (TCR) expressed by T cells. This immune response is controlled by myeloid suppressor cells (MSCs), a subset of immunosuppressive myeloid cells that enter the lymphoid organs and peripheral tissues during immune stress. They use two enzymes involved in arginine metabolism to control T-cell responses: NOS2 and arginase 1 (ARG1). The MSCs exploit the metabolism of arginine to render lymphocytes unresponsive to antigen stimulation. NOS2 blocks T-cell function by interfering with the IL-2 pathway whereas arginase 1 depletes L-Arg from the local environment, leading to the GCN2-mediated stress response in T-cells, resulting in a repression of the pro-inflammatory response (148). MSCs also use another enzyme, namely indoleamine dioxygenase, to deplete the amino acid tryptophan with similar consequences (148). Classically and alternatively activated macrophages use the same arginine metabolizing enzymes to fulfill their different functions, defense and healing, respectively (149). The T_H1 cytokine IFN- γ , which drives the classical pathway of macrophage activation increases NO release through NOS2 activity and inhibits IL-4 or IL-10-driven ARG1 activity. Alternative activation is regulated by T_H2 cytokines, such as IL-4 and IL-13, and IL-10 and TGF β , all of which inhibit NOS2 activity and induce ARG1 (see Figure 6). Classically activated macrophages release pro-inflammatory cytokines leading to microbiocidal activity and cellular immunity. In contrast, alternatively activated macrophages are essential for humoral immunity and are also involved in tissue repair and fibrosis via the production of polyamines and proline by ARG1 (150). They play a

role in tolerance in pregnancy and in the gut-associated lymphoid tissue. These T_H2 -driven macrophages are also involved in allergic and antiparasitic responses (151).

d) Arginine, a building block for proteins

Like most other amino acids, arginine is a building block for proteins. Initiation of protein synthesis in eukaryotic cells begins with the formation of the 43S “preinitiation complex” that is composed of a ternary complex of eukaryotic initiation factor 2 (eIF2) bound to GTP and Met-tRNA^{Met} associated with a small (40S) ribosomal subunit. This 43S complex binds mRNA near its capped 5'-end, migrates downstream along the mRNA and, upon reaching the first AUG codon, joins with the 60S subunit to form the 80S “initiation complex” (for a review of this “scanning mechanism”, see (153)). Following AUG recognition, the GTP bound to eIF2 is hydrolyzed and eIF2 is released as an inactive eIF2-GDP binary complex. Exchange of the bound GDP with GTP is catalyzed by eIF2B. Once correctly positioned, the 80S ribosomal complex stepwise adds amino acids by in-frame translation of the mRNA in a process termed elongation.

Figure 5. Arginine metabolism in macrophages. Activated macrophages can use arginine in response to either inflammatory signals leading to TH1 activation or inflammatory signals leading to TH2 activation. NO generated in activated macrophages is an important cytotoxic molecule in the defense against pathogens and tumor cells (152) in a TH1 response. The immune effects of arginine-depletion in a TH2 response remain to be defined.

The key steps in the elongation process are the entry of each succeeding aminoacyl-tRNA, the formation of a peptide bond and the translocation of the ribosome with respect to the mRNA. Arginyl-tRNA synthetase is the enzyme catalyzing the ligation of arginine to its cognate tRNA, arginyl-tRNA. Arginine is incorporated whenever one of the following 6 codons is encountered: AGG, AGA, CGA, CGC, CGG or CGU. Intriguingly, there are only three amino acids that are encoded by 6 different codons, namely leucine, serine and arginine, whereas all other amino acids have to do with less. Once a stop codon is detected, specific proteins are recruited which cause the release and dismantling of the ribosomal complex.

e) Arginine as metabolic regulator

To date, three major pathways, in which arginine functions as signaling molecule, have been identified, namely, through its absence resulting in uncharged arginyl-tRNA, stimulation of protein turnover via their N-terminal arginylation, and induction of secretion of hormones. In addition, arginine can inhibit the activity of its own synthesizing enzymes ASS and ASL in some non-hepatic cell-lines (154) but this effect is probably caused by S-nitrosylation (section II-b-4-2).

1) *Arginyl-tRNA*

Arginine binds to its cognate tRNA (arginyl-tRNA) to allow protein synthesis. When the intracellular pool of arginine decreases, the concentration of uncharged arginyl-tRNA increases. Uncharged tRNAs bind to general control non-derepressible 2 kinase (GCN2), which causes a conformational change activating the kinase domain (155, 156). This kinase phosphorylates the α -subunit of eukaryotic initiation factor 2 (eIF2) on serine52 (115) (Figure 6), which, in turn, prevents the recycling of eIF2 by eIF2B and blocks the formation of the preinitiation complex (157) (see chapter protein synthesis) and, thereby, general protein synthesis. Parallel to this blockade of general protein translation, translation of specific mRNAs is upregulated, including those for the transcription factor ATF4 and the cationic amino acid transporter CAT-1 (153). In this respect, arginine itself acts as a signaling molecule, since its decline causes a dose-dependent and reversible inhibition of general protein synthesis (153). Any enzyme or transporter that decreases local arginine concentrations can therefore induce this rather general control

mechanism. For example, the myeloid suppressor cells (MSCs), described above, use this of arginine depletion to control cell function (148).

2) *N-terminal arginylation*

Protein arginylation, although discovered more than 40 years ago, remains poorly understood at the molecular level (159). It is mediated by Arg-transfer RNA protein transferase which transfers arginyl from tRNA to the N-terminus of proteins (160, 161). This post-translational arginylation is believed to be critical in mouse embryogenesis, cardiovascular development and angiogenesis (162). More recently, it was shown that β -actin is a target for N-arginylation that then inhibits actin filament clustering thereby regulating the actin cytoskeleton and cell motility (162). Regeneration of the central nervous system (CNS) following injury requires degradation of certain proteins, which is triggered by transfer RNA-mediated posttranslational addition of amino acids to the N-terminus of acceptor proteins (163). Arginine is one of the most frequently added amino acids. This so-called N-terminal arginylation is part of the ubiquitin-dependent N-end rule pathway of protein degradation. In mammals, aspartate, glutamate and cysteine are substrates for arginylation by Arg-tRNA-protein transferases (164).

3) *Arginine, a potent secretagogue*

Arginine is a secretagogue for growth hormone, insulin, adrenal hormones and prolactin (165-167). In humans, arginine administration results in a two-fold increase in plasma growth hormone levels (168). Growth hormone secretion from the anterior pituitary gland is primarily governed by growth-hormone-releasing-hormone (GHRH). GHRH secretion is mediated by ghrelin (169) and somatostatin (170-172). These two hormones have antagonist actions in the hypothalamus, ghrelin promoting the release of growth hormone and somatostatin blocking its secretion. Arginine may either block the release of somatostatin (168, 173) or induce the release of GH directly by the same mechanism that promotes insulin release from the islands of Langerhans (174, 175), that is, depolarization of the plasma membrane. There, absorption of the positively charged extracellular arginine into the pancreatic cells causes depolarization of the plasma membrane which subsequently causes an insulin burst via a Ca^{2+} -mediated pathway (176).

Figure 6. GCN2-mediated stress response. In absence of arginine binding to its cognate arginyl-tRNA, the growing population of uncharged tRNA causes the activation of GCN2 kinase. The subsequent phosphorylation of the α -subunit of the eIF2 causes an arrest in protein translation by inhibiting the formation of the ternary preinitiation complex. mRNAs with special 3'-UTRs such as the transcription factor ATF4 and the cationic amino acid transporter CAT-1 will be preferentially translated under these conditions. ATF4 then induces the transcription of other transcription factors and genes involved in amino acid synthesis and transport. ,GCN2-activation leading to a decrease in NOS translation (115, 158). Interestingly, this GCN2-mediated inhibition of translation is not specific for arginine deprivation but rather general as indicated by its name and can be activated by a lack in any amino acid.

IV- Arginine transporters and their regulation

1) *Nomenclature of arginine transporters*

The supply of arginine to cells may become rate-limiting for enzymatic reactions that use arginine as a substrate. In the past decades, arginine carrier proteins were defined as belonging to systems y^+ , $b^{0,+}$, $B^{0,+}$ or y^+L (177-179) based on functional properties such as ion-dependence, kinetics, substrate specificity, and regulation of activity (177) Table 2 links these transporter groups to the genes of the solute carrier (SLC) family.

2) *Arginine transporters*

Among the different transporters that are part of the y^+ group, there are the high affinity, Na^+ -independent transporters of arginine, lysine and ornithine, which are responsible for a major part in the arginine transport. These transporters, also known as the cationic amino acid transporters (CAT) are CAT-1, -2B and -3 (Table 2). These CAT transporters (SLC7A) have 14 putative transmembrane segments and are glycosylated. Among them, CAT-1 and CAT-3 are most closely related (~ 60 % identity) (182).

The first member of the SLC7 family (mCAT-1, for mouse cationic amino acid transporter) was originally identified as the receptor for the ecotropic murine retroviruses. CAT-1 seems to be the major system y^+ transporter and is expressed almost ubiquitously with the exception of adult liver where it is virtually absent. Expression levels in other tissues vary considerably. CAT-1 expression is modulated by cell proliferation, growth factors, cytokines and hormones (188).

In contrast to normal cap-dependent protein synthesis that is down-regulated during nutrient limitation, translation of CAT-1 mRNA is increased under these conditions (189) and its mRNA (190) is stabilized.

A remarkable property of CAT-1 is its ability to bind and form complexes with other proteins, such as NOS3 in aortic endothelial cells, which enhances the production of NO (191). Moreover, it was shown that CAT-1 is complexed with caveolin in endothelial cells (192), that is, in the same compartment where NOS3, ASS and ASL are present, suggesting that arginine transport and recycling are essential for NOS3 function (see Figure 2 above). In renal epithelial cells, CAT-1 is restricted to the basolateral membrane where it regulates the transport of arginine from and to these cells (193). The

importance of CAT-1 is reflected in the finding that homozygous CAT-1 knockout mice are 25% smaller than their littermates at birth, die on postnatal day 1, and suffer from severe anemia, whereas heterozygous littermates exhibit no apparent phenotype (194). CAT-2A and -2B are splice variants of the SLC7A2 gene and differ in a stretch of 42 amino acids. CAT-2A is expressed in liver, muscle and skin and is a low affinity arginine transporter (177, 195), whereas CAT-2B is a high-affinity transporter, which is expressed in brain, lung, testis and activated T-cells (177, 196). CAT-2B expression is co-induced with NOS2 in a wide variety of cell types (197-201) indicating that arginine transport capacity needs to be increased to support the elevated rates of NO synthesis . It was also demonstrated in vivo by generating a CAT-2 knockout mouse, that, indeed, CAT-2B is required to allow sustained NO production by macrophages after inflammatory stimuli (202).

Plasma membrane transporters accepting arginine as substrate					
Protein	Gene	Transport system	Na ⁺ -dependent	Apparent K _M for arginine (mmol/L)	Ref.
CAT-1	SLC7A1	y ⁺	no	0.10-0.16	(180)
CAT-2A	SLC7A2	ND	no	3.40-3.90	(180)
CAT-2B	SLC7A2	y ⁺	no	0.25-0.70	(180)
CAT-3	SLC7A3	y ⁺	no	0.20-0.50	(181)
CAT-4	SLC7A4	-	-	-	(182)
y ⁺ LAT2 ⁺	SLC7A6	y ⁺ L	yes	0.12±0.035	(182, 183)
y ⁺ LAT1 ⁺	SLC7A7	y ⁺ L	no/yes	0.091/0.34	(182, 184)
B ^{0,+} AT ⁺	SLC7A9	b ^{0,+}	no	-	(182)
rBAT	SLC3A1	b ^{0,+}	no	0.08-0.20	(185, 186)
4F2hc	SLC3A2	y ⁺ L	no	0.12-0.34	(183, 184)
ATB ^{0,+}	SLC6A14	B ^{0,+}	yes ⁺ Cl ⁻	0.10-0.15	(187)

V- Arginine-related proteins and their transgenic models

In order to elucidate the metabolic fates of arginine, several transgenic animal models were developed in the last 3 decades. A list of some of the transgenic models related to arginine metabolism is given in Table 3.

gene	phenotype	ref
Ass	1-2 days postnatally lethal; hyperammonemia, hypercitrullinemia	(203)
Asl	~ 48 hours postnatally lethal; hyperammonemia, hyperargininosuccinemia, hypoargininemia	(204)
Arginase-I	~10 days postnatally lethal; hyperargininemia, hyperammonemia	(10)
Arginase-II	Viable, only hyperargininemia	(10)
Cat-1	1 day postnatally lethal; severe anemia & 25% smaller than littermates	(194)
Cat-2	Healthy, fertile; >90% decreased NO production & 95% decreased arginine uptake by macrophages	(202)
Nos1	Sphincter dysfunction and hypertrophy	(205)
Nos3	Hypertension	(206)
Nos2	Less resistant to infections	(207)
Citrin	No phenotype	(208)

Table 3. List of some constitutive KO mice for arginine-associated enzymes or transporters and their observed phenotypes.

These animal models were often used to study human arginine-related pathologies.

Although humans and mice share homologous genes, knocking out a gene in the mouse does not always result in the same phenotype as observed in humans. The mitochondrial carrier, citrin is an example. Citrin deficiency in humans causes type II citrullinemia (93) but the citrin knockout mouse has no phenotype. On the other hand, inactivation of ASS or arginase 1 yield the same phenotype in humans and in mice.

VI- Synopsis and scope of thesis

Arginine is a precursor for proteins, creatine and the signaling molecules agmatine and nitric oxide. Arginine is also a secretagogue for growth hormone, insulin, glucagon, and prolactin. Arginine deficiency acts as a signal, controlling general protein synthesis via activation of the stress kinase GCN2. Furthermore, arginine is an intermediate of the ornithine cycle. This thesis intends to identify how the availability of arginine controls organ development and function by using genetically modified mice.

The suckling small intestine of the mouse was identified as a producer of arginine from proline. This capacity is lost after weaning, when ASS and ASL are no longer expressed. To investigate the relevance of this transient biosynthetic capacity, a murine model of chronic arginine deficiency was developed by overexpression of arginase I in the enterocytes of the small intestine (the “F/A” mice). The deficiency of arginine caused severe retardation in general growth in these mice. In Chapter II we characterized this mouse model in vivo in combination with several in vitro models. We show that arginine deficiency inhibits the release of the growth hormone from the pituitary gland and ubiquitously activates the stress kinase GCN2 leading to a general shut-down of protein synthesis. Our findings demonstrate the signaling properties of arginine on the growth hormone axis and on the protein synthesizing machinery.

Overexpression of arginase may, however, suffer from a lack of specificity. Since arginase is a very active enzyme, overexpression of arginase in the gut may transform this organ into an arginine sink, meaning that not only newly synthesized arginine is degraded but also dietary arginine and plasma arginine are broken down excessively. To specifically investigate intestinal arginine synthesis but also synthesis in any other tissue where arginine metabolism is important, we created a conditional knock out mouse for argininosuccinate synthetase (ASS). The lethal effect of total inactivation of ASS but above all the ability to specifically inactivate the gene in a given tissue led us to opt for conditional inactivation of the gene. To date, 50 different mutations in the human ASS gene have been identified, some of which result in total inactivation of the enzyme (209). The absence of exon 7 or 13 in ASS mRNA is a consequence of abnormal splicing. In the case of exon 13 this is caused by a single base change in the intron region IVS-13+5 (a transition of G to A at the 5th nucleotide position within the 5' splice cleavage site). Exon 13 deletion leads to a reduction of ASS activity to <0.5 % of controls (210). As the ASS enzymatic activity is completely eliminated by the absence of exon 13, deletion of

exon 13 represents the perfect choice to generate a conditional knock-out mouse with specific excision of this particular exon.

To specifically inactivate a gene *in vivo* one needs to target the gene in mouse embryonic stem (ES) cells (211). Tissue-specific gene inactivation is achieved by excision of the critical region of the gene in the tissue of interest. In order to obtain the specific excision, the critical region is flanked on either side with a small recognition sequence for a certain recombinase enzyme. This recombinase enzyme identifies the 2 recognition sequences and eliminates the flanked region. Tissue-specificity of the recombinase is achieved by using a tissue-specific promoter to drive expression of the recombinase. Nowadays, the two mostly widely used recombinases are FLP recombinase from the yeast 2-micron episome and Cre recombinase from the coliphage P1 (212). A comparative biochemical analysis showed that Cre and FLP have temperature optima concordant with their original hosts –the temperature optimum of Cre is at 37°C and the temperature optimum of FLP is at 30°C (212), making Cre the recombinase of choice in most applications in mice. Chapter III describes how we succeeded in generating conditional knock-out mice for proteins regulating intracellular arginine concentration, viz. ASS, arginase 1 and the major arginine transporter CAT-1. This chapter is a troubleshooting guide for the generation of a conditional KO mouse.

It is believed that perinatal intestinal arginine synthesis compensates for insufficient arginine concentrations in maternal milk to ensure appropriate development. To test this hypothesis, we inactivated arginine synthesis in the gut by specifically inactivating ASS expression in the enterocytes of the small intestine. The characterization of these mice is reported in Chapter IV. ASS protein was totally absent in the mouse enterocytes but was normally present in all other tissues and cell types. In contrast to mice overexpressing arginase 1 in the enterocytes, mice deficient of ASS in their enterocytes showed no major phenotype. Absence of intestinal arginine biosynthesis leads to an increased production of citrulline by the gut and an increased hepatic absorption of arginine. Furthermore, arginine synthesis in the kidneys was increased. In the absence of arginine synthesis in the perinatal small intestine, other organs can apparently compensate for the resulting arginine shortage. By comparing the two arginine-deficient mice described in Chapter II and IV, we were able to demonstrate that intestinal arginase overexpression indeed renders the gut an active arginine sink. This change in function of the gut causes a

global reduction in circulating arginine, which in turn causes the phenotypes described in Chapter II.

Arginine is not only important for development and growth but it also plays a role in vascular function as the precursor for nitric oxide. Endothelial cells are the sites of nitric oxide production. ASS together with argininosuccinate lyase (ASL) co-localizes with NOS3 in these cells. In mice in which ASS, arginase 1 or CAT-1 were specifically inactivated in the endothelium, we were able to preliminarily characterize the importance of local arginine recycling, arginine import, and arginine catabolism for endothelial function (Chapter V).

References

1. Heys SD, Gardner E. Nutrients and the surgical patient: current and potential therapeutic applications to clinical practice. *J R Coll Surg Edinb* 1999;44:283-293.
2. Visek WJ. Arginine needs, physiological state and usual diets. A reevaluation. *J Nutr* 1986;116:36-46.
3. Cabella C, Gardini G, Corpillo D, Testore G, Bedino S, Solinas SP, Cravanzola C, et al. Transport and metabolism of agmatine in rat hepatocyte cultures. *Eur J Biochem* 2001;268:940-947.
4. Castillo L, Beaumier L, Ajami AM, Young VR. Whole body nitric oxide synthesis in healthy men determined from [15N] arginine-to-[15N]citrulline labeling. *Proc Natl Acad Sci U S A* 1996;93:11460-11465.
5. Castillo L, Sanchez M, Vogt J, Chapman TE, DeRojas-Walker TC, Tannenbaum SR, Ajami AM, et al. Plasma arginine, citrulline, and ornithine kinetics in adults, with observations on nitric oxide synthesis. *Am J Physiol* 1995;268:E360-367.
6. Wu G, Morris SM, Jr. Arginine metabolism: nitric oxide and beyond. *Biochem J* 1998;336 (Pt 1):1-17.
7. Ardawi MS, Newsholme EA. Glutamine metabolism in lymphocytes of the rat. *Biochem J* 1983;212:835-842.
8. Flynn NE, Meininger CJ, Haynes TE, Wu G. The metabolic basis of arginine nutrition and pharmacotherapy. *Biomed Pharmacother* 2002;56:427-438.
9. Windmueller HG, Spaeth AE. Source and fate of circulating citrulline. *Am J Physiol* 1981;241:E473-480.
10. Shi O, Morris SM, Jr., Zoghbi H, Porter CW, O'Brien WE. Generation of a mouse model for arginase II deficiency by targeted disruption of the arginase II gene. *Mol Cell Biol* 2001;21:811-813.
11. Heird WC, Nicholson JF, Driscoll JM, Jr., Schullinger JN, Winters RW. Hyperammonemia resulting from intravenous alimentation using a mixture of synthetic l-amino acids: a preliminary report. *J Pediatr* 1972;81:162-165.

12. Mertz ET, Beeson WM, Jackson HD. Classification of essential amino acids for the weanling pig. *Arch Biochem Biophys* 1952;38:121-128.
13. Rose WC, Haines WJ, Warner DT. The amino acid requirements of man. V. The role of lysine, arginine, and tryptophan. *J Biol Chem* 1954;206:421-430.
14. Anderson LC, Lewis AJ, Peo ER, Jr., Crenshaw JD. Effects of excess arginine with and without supplemental lysine on performance, plasma amino acid concentrations and nitrogen balance of young swine. *J Anim Sci* 1984;58:369-377.
15. Anderson PA, Baker DH, Corbin JE. Lysine and arginine requirements of the domestic cat. *J Nutr* 1979;109:1368-1372.
16. Czarnecki GL, Baker DH. Urea cycle function in the dog with emphasis on the role of arginine. *J Nutr* 1984;114:581-590.
17. Morris JG, Rogers QR. Arginine: an essential amino acid for the cat. *J Nutr* 1978;108:1944-1953.
18. Pendleton LC, Goodwin BL, Solomonson LP, Eichler DC. Regulation of endothelial argininosuccinate synthase expression and NO production by an upstream open reading frame. *J Biol Chem* 2005;280:24252-24260.
19. Brunton JA, Bertolo RF, Pencharz PB, Ball RO. Proline ameliorates arginine deficiency during enteral but not parenteral feeding in neonatal piglets. *Am J Physiol* 1999;277:E223-231.
20. Murphy JM, Murch SJ, Ball RO. Proline is synthesized from glutamate during intragastric infusion but not during intravenous infusion in neonatal piglets. *J Nutr* 1996;126:878-886.
21. Wilkinson DL, Bertolo RF, Brunton JA, Shoveller AK, Pencharz PB, Ball RO. Arginine synthesis is regulated by dietary arginine intake in the enterally fed neonatal piglet. *Am J Physiol Endocrinol Metab* 2004;287:E454-462.
22. Meijer AJ, Lamers WH, Chamuleau RA. Nitrogen metabolism and ornithine cycle function. *Physiol Rev* 1990;70:701-748.
23. Watford M. The urea cycle: a two-compartment system. *Essays Biochem* 1991;26:49-58.
24. Cheung CW, Cohen NS, Rajman L. Channeling of urea cycle intermediates in situ in permeabilized hepatocytes. *J Biol Chem* 1989;264:4038-4044.
25. Gaasbeek Janzen JW, Lamers WH, Moorman AF, de Graaf A, Los JA, Charles R. Immunohistochemical localization of carbamoyl-phosphate synthetase (ammonia) in adult rat liver; evidence for a heterogeneous distribution. *J Histochem Cytochem* 1984;32:557-564.
26. Chen CL, Fei Z, Carter EA, Lu XM, Hu RH, Young VR, Tompkins RG, et al. Metabolic fate of extrahepatic arginine in liver after burn injury. *Metabolism* 2003;52:1232-1239.
27. Inoue Y, Bode BP, Beck DJ, Li AP, Bland KI, Souba WW. Arginine transport in human liver. Characterization and effects of nitric oxide synthase inhibitors. *Ann Surg* 1993;218:350-362; discussion 362-353.
28. Davis TA, Nguyen HV, Garcia-Bravo R, Fiorotto ML, Jackson EM, Lewis DS, Lee DR, et al. Amino acid composition of human milk is not unique. *J Nutr* 1994;124:1126-1132.
29. Davis TA, Nguyen HV, Garcia-Bravo R, Fiorotto ML, Jackson EM, Reeds PJ. Amino acid composition of the milk of some mammalian species changes with stage of lactation. *Br J Nutr* 1994;72:845-853.

30. Wu G, Knabe DA. Free and protein-bound amino acids in sow's colostrum and milk. *J Nutr* 1994;124:415-424.
31. Wu G, Knabe DA. Arginine synthesis in enterocytes of neonatal pigs. *Am J Physiol* 1995;269:R621-629.
32. Blachier F, M'Rabet-Touil H, Posho L, Darcy-Vrillon B, Duce PH. Intestinal arginine metabolism during development. Evidence for de novo synthesis of L-arginine in newborn pig enterocytes. *Eur J Biochem* 1993;216:109-117.
33. Wu G. Synthesis of citrulline and arginine from proline in enterocytes of postnatal pigs. *Am J Physiol* 1997;272:G1382-1390.
34. Wu G, Knabe DA, Yan W, Flynn NE. Glutamine and glucose metabolism in enterocytes of the neonatal pig. *Am J Physiol* 1995;268:R334-342.
35. De Jonge WJ, Dingemans MA, de Boer PA, Lamers WH, Moorman AF. Arginine-metabolizing enzymes in the developing rat small intestine. *Pediatr Res* 1998;43:442-451.
36. Flynn NE, Wu G. An important role for endogenous synthesis of arginine in maintaining arginine homeostasis in neonatal pigs. *Am J Physiol* 1996;271:R1149-1155.
37. Bertolo RF, Brunton JA, Pencharz PB, Ball RO. Arginine, ornithine, and proline interconversion is dependent on small intestinal metabolism in neonatal pigs. *Am J Physiol Endocrinol Metab* 2003;284:E915-922.
38. Cleary MA, Dorland L, de Koning TJ, Poll-The BT, Duran M, Mandell R, Shih VE, et al. Ornithine aminotransferase deficiency: diagnostic difficulties in neonatal presentation. *J Inher Metab Dis* 2005;28:673-679.
39. Wang T, Lawler AM, Steel G, Sipila I, Milam AH, Valle D. Mice lacking ornithine-delta-aminotransferase have paradoxical neonatal hypornithinaemia and retinal degeneration. *Nat Genet* 1995;11:185-190.
40. Wu G, Borbolla AG, Knabe DA. The uptake of glutamine and release of arginine, citrulline and proline by the small intestine of developing pigs. *J Nutr* 1994;124:2437-2444.
41. Batshaw ML, Wachtel RC, Thomas GH, Starrett A, Brusilow SW. Arginine-responsive asymptomatic hyperammonemia in the premature infant. *J Pediatr* 1984;105:86-91.
42. Flynn NE, Meininger CJ, Kelly K, Ing NH, Morris SM, Jr., Wu G. Glucocorticoids mediate the enhanced expression of intestinal type II arginase and argininosuccinate lyase in postweaning pigs. *J Nutr* 1999;129:799-803.
43. Husson A, Brasse-Lagnel C, Fairand A, Renouf S, Lavoinne A. Argininosuccinate synthetase from the urea cycle to the citrulline-NO cycle. *Eur J Biochem* 2003;270:1887-1899.
44. Berthold HK, Reeds PJ, Klein PD. Isotopic evidence for the differential regulation of arginine and proline synthesis in man. *Metabolism* 1995;44:466-473.
45. Wu G. Intestinal mucosal amino acid catabolism. *J Nutr* 1998;128:1249-1252.
46. Wakabayashi Y, Yamada E, Yoshida T, Takahashi H. Arginine becomes an essential amino acid after massive resection of rat small intestine. *J Biol Chem* 1994;269:32667-32671.

47. Wakabayashi Y, Yamada E, Yoshida T, Takahashi H. Deficiency of endogenous arginine synthesis provokes hypertension by exhausting substrate arginine for nitric oxide synthesis. *Biochem Biophys Res Commun* 1994;205:1391-1398.
48. Wakabayashi Y, Yamada E, Yoshida T, Takahashi N. Effect of intestinal resection and arginine-free diet on rat physiology. *Am J Physiol* 1995;269:G313-318.
49. Hurwitz R, Kretchmer N. Development of arginine-synthesizing enzymes in mouse intestine. *Am J Physiol* 1986;251:G103-110.
50. Morris SM, Jr., Sweeney WE, Jr., Kepka DM, O'Brien WE, Avner ED. Localization of arginine biosynthetic enzymes in renal proximal tubules and abundance of mRNA during development. *Pediatr Res* 1991;29:151-154.
51. Goutal I, Fairand A, Husson A. Expression of the genes of arginine-synthesizing enzymes in the rat kidney during development. *Biol Neonate* 1999;76:253-260.
52. van de Poll MC, Soeters PB, Deutz NE, Fearon KC, Dejong CH. Renal metabolism of amino acids: its role in interorgan amino acid exchange. *Am J Clin Nutr* 2004;79:185-197.
53. Levillain O, Hus-Citharel A, Morel F, Bankir L. Localization of arginine synthesis along rat nephron. *Am J Physiol* 1990;259:F916-923.
54. Levillain O, Hus-Citharel A, Morel F, Bankir L. Arginine synthesis in mouse and rabbit nephron: localization and functional significance. *Am J Physiol* 1993;264:F1038-1045.
55. Wakui H, Komatsuda A, Itoh H, Kobayashi R, Nakamoto Y, Miura AB. Renal argininosuccinate synthetase: purification, immunohistochemical localization, and elastin-binding property. *Ren Physiol Biochem* 1992;15:1-9.
56. Brosnan ME, Brosnan JT. Renal arginine metabolism. *J Nutr* 2004;134:2791S-2795S; discussion 2796S-2797S.
57. Castillo L, Sanchez M, Chapman TE, Ajami A, Burke JF, Young VR. The plasma flux and oxidation rate of ornithine adaptively decline with restricted arginine intake. *Proc Natl Acad Sci U S A* 1994;91:6393-6397.
58. Dhanakoti SN, Brosnan JT, Brosnan ME, Herzberg GR. Net renal arginine flux in rats is not affected by dietary arginine or dietary protein intake. *J Nutr* 1992;122:1127-1134.
59. Tizianello A, De Ferrari G, Garibotto G, Gurreri G, Robaudo C. Renal metabolism of amino acids and ammonia in subjects with normal renal function and in patients with chronic renal insufficiency. *J Clin Invest* 1980;65:1162-1173.
60. Tizianello A, De Ferrari G, Garibotto G, Robaudo C. Amino acid metabolism and the liver in renal failure. *Am J Clin Nutr* 1980;33:1354-1362.
61. Bouby N, Hassler C, Parvy P, Bankir L. Renal synthesis of arginine in chronic renal failure: in vivo and in vitro studies in rats with 5/6 nephrectomy. *Kidney Int* 1993;44:676-683.
62. Reyes AA, Karl IE, Klahr S. Role of arginine in health and in renal disease. *Am J Physiol* 1994;267:F331-346.
63. O'Sullivan D, Brosnan JT, Brosnan ME. Catabolism of arginine and ornithine in the perfused rat liver: effect of dietary protein and of glucagon. *Am J Physiol Endocrinol Metab* 2000;278:E516-521.

64. Lamas S, Perez-Sala D, Moncada S. Nitric oxide: from discovery to the clinic. *Trends Pharmacol Sci* 1998;19:436-438.
65. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. *N Engl J Med* 1993;329:2002-2012.
66. Moncada S, Higgs EA. The discovery of nitric oxide and its role in vascular biology. *Br J Pharmacol* 2006;147 Suppl 1:S193-201.
67. Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 1991;43:109-142.
68. Huynh NN, Chin-Dusting J. Amino acids, arginase and nitric oxide in vascular health. *Clin Exp Pharmacol Physiol* 2006;33:1-8.
69. Pendleton LC, Goodwin BL, Flam BR, Solomonson LP, Eichler DC. Endothelial argininosuccinate synthase mRNA 5'-untranslated region diversity. Infrastructure for tissue-specific expression. *J Biol Chem* 2002;277:25363-25369.
70. Solomonson LP, Flam BR, Pendleton LC, Goodwin BL, Eichler DC. The caveolar nitric oxide synthase/arginine regeneration system for NO production in endothelial cells. *J Exp Biol* 2003;206:2083-2087.
71. Goodwin BL, Solomonson LP, Eichler DC. Argininosuccinate synthase expression is required to maintain nitric oxide production and cell viability in aortic endothelial cells. *J Biol Chem* 2004;279:18353-18360.
72. Shen LJ, Beloussow K, Shen WC. Accessibility of endothelial and inducible nitric oxide synthase to the intracellular citrulline-arginine regeneration pathway. *Biochem Pharmacol* 2005;69:97-104.
73. Lee TJ, Yu JG. L-Citrulline recycle for synthesis of NO in cerebral perivascular nerves and endothelial cells. *Ann N Y Acad Sci* 2002;962:73-80.
74. Daniel EE, Wang YF, Salapatek AM, Mao YK, Mori M. Argininosuccinate synthetase, argininosuccinate lyase and NOS in canine gastrointestinal tract: immunocytochemical studies. *Neurogastroenterol Motil* 2000;12:317-334.
75. Wiesinger H. Arginine metabolism and the synthesis of nitric oxide in the nervous system. *Prog Neurobiol* 2001;64:365-391.
76. Mori M, Gotoh T. Arginine metabolic enzymes, nitric oxide and infection. *J Nutr* 2004;134:2820S-2825S; discussion 2853S.
77. Karsai T, Elodi P. Urea cycle enzymes in human liver: ontogenesis and interaction with the synthesis of pyrimidines and polyamines. *Mol Cell Biochem* 1982;43:105-110.
78. Hall LM, Johnson RC, Cohen PP. The presence of carbamyl phosphate synthetase in intestinal mucosa. *Biochim Biophys Acta* 1960;37:144-145.
79. Rajzman L. Citrulline synthesis in rat tissues and liver content of carbamoyl phosphate and ornithine. *Biochem J* 1974;138:225-232.
80. Wu G. Urea synthesis in enterocytes of developing pigs. *Biochem J* 1995;312 (Pt 3):717-723.
81. Köhler ES, Sankaranarayanan S, van Ginneken CJ, van Dijk P, Vermeulen JLM, Ruijter JM, Lamers WH, et al. The human neonatal small intestine has the potential for arginine synthesis. manuscript submitted 2008.

82. Morris SM, Jr. Regulation of enzymes of the urea cycle and arginine metabolism. *Annu Rev Nutr* 2002;22:87-105.
83. Brusilow SW, Danney M, Waber LJ, Batshaw M, Burton B, Levitsky L, Roth K, et al. Treatment of episodic hyperammonemia in children with inborn errors of urea synthesis. *N Engl J Med* 1984;310:1630-1634.
84. Simard L, O'Brien WE, McInnes RR. Argininosuccinate lyase deficiency: evidence for heterogeneous structural gene mutations by immunoblotting. *Am J Hum Genet* 1986;39:38-51.
85. Stadler S, Gempel K, Bieger I, Pontz BF, Gerbitz KD, Bauer MF, Hofmann S. Detection of neonatal argininosuccinate lyase deficiency by serum tandem mass spectrometry. *J Inher Metab Dis* 2001;24:370-378.
86. Scaglia F, Lee B. Clinical, biochemical, and molecular spectrum of hyperargininemia due to arginase I deficiency. *Am J Med Genet C Semin Med Genet* 2006;142:113-120.
87. Goldblum OM, Brusilow SW, Maldonado YA, Farmer ER. Neonatal citrullinemia associated with cutaneous manifestations and arginine deficiency. *J Am Acad Dermatol* 1986;14:321-326.
88. Kobayashi K, Shaheen N, Terazono H, Saheki T. Mutations in argininosuccinate synthetase mRNA of Japanese patients, causing classical citrullinemia. *Am J Hum Genet* 1994;55:1103-1112.
89. Saheki T, Kobayashi K, Ichiki H, Matuo S, Tatsuno M, Imamura Y, Inoue I, et al. Molecular basis of enzyme abnormalities in urea cycle disorders. With special reference to citrullinemia and argininosuccinic aciduria. *Enzyme* 1987;38:227-232.
90. Thoene J, Batshaw M, Spector E, Kulovich S, Brusilow S, Walser M, Nyhan W. Neonatal citrullinemia: treatment with keto-analogues of essential amino acids. *J Pediatr* 1977;90:218-224.
91. Kobayashi K, Sinasac DS, Iijima M, Bortright AP, Begum L, Lee JR, Yasuda T, et al. The gene mutated in adult-onset type II citrullinaemia encodes a putative mitochondrial carrier protein. *Nat Genet* 1999;22:159-163.
92. Ohura T, Kobayashi K, Tazawa Y, Nishi I, Abukawa D, Sakamoto O, Iinuma K, et al. Neonatal presentation of adult-onset type II citrullinemia. *Hum Genet* 2001;108:87-90.
93. Iijima M, Jalil A, Begum L, Yasuda T, Yamaguchi N, Xian Li M, Kawada N, et al. Pathogenesis of adult-onset type II citrullinemia caused by deficiency of citrin, a mitochondrial solute carrier protein: tissue and subcellular localization of citrin. *Adv Enzyme Regul* 2001;41:325-342.
94. Begum L, Jalil MA, Kobayashi K, Iijima M, Li MX, Yasuda T, Horiuchi M, et al. Expression of three mitochondrial solute carriers, citrin, aralar1 and ornithine transporter, in relation to urea cycle in mice. *Biochim Biophys Acta* 2002;1574:283-292.
95. Saheki T, Kobayashi K, Iijima M, Moriyama M, Yazaki M, Takei Y, Ikeda S. Metabolic derangements in deficiency of citrin, a liver-type mitochondrial aspartate-glutamate carrier. *Hepatol Res* 2005;33:181-184.
96. Wu G, Knabe DA, Flynn NE, Yan W, Flynn SP. Arginine degradation in developing porcine enterocytes. *Am J Physiol* 1996;271:G913-919.
97. Bessman SP. The creatine phosphate energy shuttle--the molecular asymmetry of a "pool". *Anal Biochem* 1987;161:519-523.

98. Walker JB. Creatine: biosynthesis, regulation, and function. *Adv Enzymol Relat Areas Mol Biol* 1979;50:177-242.
99. Coleman CS, Hu G, Pegg AE. Putrescine biosynthesis in mammalian tissues. *Biochem J* 2004;379:849-855.
100. Lopez-Contreras AJ, Lopez-Garcia C, Jimenez-Cervantes C, Cremades A, Penafiel R. Mouse ornithine decarboxylase-like gene encodes an antizyme inhibitor devoid of ornithine and arginine decarboxylating activity. *J Biol Chem* 2006;281:30896-30906.
101. Zhu MY, Iyo A, Piletz JE, Regunathan S. Expression of human arginine decarboxylase, the biosynthetic enzyme for agmatine. *Biochim Biophys Acta* 2004;1670:156-164.
102. Fairbanks CA, Schreiber KL, Brewer KL, Yu CG, Stone LS, Kitto KF, Nguyen HO, et al. Agmatine reverses pain induced by inflammation, neuropathy, and spinal cord injury. *Proc Natl Acad Sci U S A* 2000;97:10584-10589.
103. Raasch W, Schafer U, Chun J, Dominiak P. Biological significance of agmatine, an endogenous ligand at imidazoline binding sites. *Br J Pharmacol* 2001;133:755-780.
104. Regunathan S, Reis DJ. Characterization of arginine decarboxylase in rat brain and liver: distinction from ornithine decarboxylase. *J Neurochem* 2000;74:2201-2208.
105. Reis DJ, Regunathan S. Is agmatine a novel neurotransmitter in brain? *Trends Pharmacol Sci* 2000;21:187-193.
106. Askalany AR, Yamakura T, Petrenko AB, Kohno T, Sakimura K, Baba H. Effect of agmatine on heteromeric N-methyl-D-aspartate receptor channels. *Neurosci Res* 2005;52:387-392.
107. Feng Y, Piletz JE, Leblanc MH. Agmatine suppresses nitric oxide production and attenuates hypoxic-ischemic brain injury in neonatal rats. *Pediatr Res* 2002;52:606-611.
108. Sastre M, Galea E, Feinstein D, Reis DJ, Regunathan S. Metabolism of agmatine in macrophages: modulation by lipopolysaccharide and inhibitory cytokines. *Biochem J* 1998;330 (Pt 3):1405-1409.
109. Bogdan C. Nitric oxide and the regulation of gene expression. *Trends Cell Biol* 2001;11:66-75.
110. Klatt P, Schmidt K, Lehner D, Glatter O, Bachinger HP, Mayer B. Structural analysis of porcine brain nitric oxide synthase reveals a role for tetrahydrobiopterin and L-arginine in the formation of an SDS-resistant dimer. *Embo J* 1995;14:3687-3695.
111. Schmidt HH, Murad F. Purification and characterization of a human NO synthase. *Biochem Biophys Res Commun* 1991;181:1372-1377.
112. Stuehr DJ, Cho HJ, Kwon NS, Weise MF, Nathan CF. Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: an FAD- and FMN-containing flavoprotein. *Proc Natl Acad Sci U S A* 1991;88:7773-7777.
113. Alderton WK, Boyhan A, Lowe PN. Nitroarginine and tetrahydrobiopterin binding to the haem domain of neuronal nitric oxide synthase using a scintillation proximity assay. *Biochem J* 1998;332 (Pt 1):195-201.
114. Giovanelli J, Campos KL, Kaufman S. Tetrahydrobiopterin, a cofactor for rat cerebellar nitric oxide synthase, does not function as a reactant in the oxygenation of arginine. *Proc Natl Acad Sci U S A* 1991;88:7091-7095.

115. Lee J, Ryu H, Ferrante RJ, Morris SM, Jr., Ratan RR. Translational control of inducible nitric oxide synthase expression by arginine can explain the arginine paradox. *Proc Natl Acad Sci U S A* 2003;100:4843-4848.
116. Jobgen WS, Fried SK, Fu WJ, Meininger CJ, Wu G. Regulatory role for the arginine-nitric oxide pathway in metabolism of energy substrates. *J Nutr Biochem* 2006;17:571-588.
117. Forstermann U, Boissel JP, Kleinert H. Expressional control of the 'constitutive' isoforms of nitric oxide synthase (NOS I and NOS II). *Faseb J* 1998;12:773-790.
118. Brenman JE, Chao DS, Xia H, Aldape K, Brecht DS. Nitric oxide synthase complexed with dystrophin and absent from skeletal muscle sarcolemma in Duchenne muscular dystrophy. *Cell* 1995;82:743-752.
119. Sander M, Chavoshan B, Harris SA, Iannaccone ST, Stull JT, Thomas GD, Victor RG. Functional muscle ischemia in neuronal nitric oxide synthase-deficient skeletal muscle of children with Duchenne muscular dystrophy. *Proc Natl Acad Sci U S A* 2000;97:13818-13823.
120. El Dwairi Q, Guo Y, Comtois A, Zhu E, Greenwood MT, Brecht DS, Hussain SN. Ontogenesis of nitric oxide synthases in the ventilatory muscles. *Am J Respir Cell Mol Biol* 1998;18:844-852.
121. Hall AV, Antoniou H, Wang Y, Cheung AH, Arbus AM, Olson SL, Lu WC, et al. Structural organization of the human neuronal nitric oxide synthase gene (NOS1). *J Biol Chem* 1994;269:33082-33090.
122. Wang Y, Newton DC, Robb GB, Kau CL, Miller TL, Cheung AH, Hall AV, et al. RNA diversity has profound effects on the translation of neuronal nitric oxide synthase. *Proc Natl Acad Sci U S A* 1999;96:12150-12155.
123. Xie J, Roddy P, Rife TK, Murad F, Young AP. Two closely linked but separable promoters for human neuronal nitric oxide synthase gene transcription. *Proc Natl Acad Sci U S A* 1995;92:1242-1246.
124. Miles PR, Bowman L, Rao KM, Baatz JE, Huffman L. Pulmonary surfactant inhibits LPS-induced nitric oxide production by alveolar macrophages. *Am J Physiol* 1999;276:L186-196.
125. Xie QW, Cho HJ, Calaycay J, Mumford RA, Swiderek KM, Lee TD, Ding A, et al. Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science* 1992;256:225-228.
126. MacMicking J, Xie QW, Nathan C. Nitric oxide and macrophage function. *Annu Rev Immunol* 1997;15:323-350.
127. Kleinert H, Pautz A, Linker K, Schwarz PM. Regulation of the expression of inducible nitric oxide synthase. *Eur J Pharmacol* 2004;500:255-266.
128. Ahluwalia A, Hobbs AJ. Endothelium-derived C-type natriuretic peptide: more than just a hyperpolarizing factor. *Trends Pharmacol Sci* 2005;26:162-167.
129. Kubes P, Suzuki M, Granger DN. Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc Natl Acad Sci U S A* 1991;88:4651-4655.
130. Selemidis S, Cocks TM. Endothelium-dependent hyperpolarization as a remote anti-atherogenic mechanism. *Trends Pharmacol Sci* 2002;23:213-220.
131. Vane JR, Botting RM. Pharmacodynamic profile of prostacyclin. *Am J Cardiol* 1995;75:3A-10A.

132. Lohmann SM, Vaandrager AB, Smolenski A, Walter U, De Jonge HR. Distinct and specific functions of cGMP-dependent protein kinases. *Trends Biochem Sci* 1997;22:307-312.
133. Zagotta WN, Siegelbaum SA. Structure and function of cyclic nucleotide-gated channels. *Annu Rev Neurosci* 1996;19:235-263.
134. Foster MW, McMahon TJ, Stamler JS. S-nitrosylation in health and disease. *Trends Mol Med* 2003;9:160-168.
135. Liu L, Yan Y, Zeng M, Zhang J, Hanes MA, Ahearn G, McMahon TJ, et al. Essential roles of S-nitrosothiols in vascular homeostasis and endotoxic shock. *Cell* 2004;116:617-628.
136. Stamler JS, Lamas S, Fang FC. Nitrosylation. the prototypic redox-based signaling mechanism. *Cell* 2001;106:675-683.
137. Catani MV, Bernassola F, Rossi A, Melino G. Inhibition of clotting factor XIII activity by nitric oxide. *Biochem Biophys Res Commun* 1998;249:275-278.
138. Pawloski JR, Swaminathan RV, Stamler JS. Cell-free and erythrocytic S-nitrosohemoglobin inhibits human platelet aggregation. *Circulation* 1998;97:263-267.
139. Hao G, Xie L, Gross SS. Argininosuccinate synthetase is reversibly inactivated by S-nitrosylation in vitro and in vivo. *J Biol Chem* 2004;279:36192-36200.
140. Ravi K, Brennan LA, Levic S, Ross PA, Black SM. S-nitrosylation of endothelial nitric oxide synthase is associated with monomerization and decreased enzyme activity. *Proc Natl Acad Sci U S A* 2004;101:2619-2624.
141. Marshall HE, Stamler JS. Inhibition of NF-kappa B by S-nitrosylation. *Biochemistry* 2001;40:1688-1693.
142. Brune B, von Knethen A, Sandau KB. Nitric oxide and its role in apoptosis. *Eur J Pharmacol* 1998;351:261-272.
143. List BM, Klosch B, Volker C, Gorren AC, Sessa WC, Werner ER, Kukovetz WR, et al. Characterization of bovine endothelial nitric oxide synthase as a homodimer with down-regulated uncoupled NADPH oxidase activity: tetrahydrobiopterin binding kinetics and role of haem in dimerization. *Biochem J* 1997;323 (Pt 1):159-165.
144. Strocs E, Hijmering M, van Zandvoort M, Wever R, Rabelink TJ, van Faassen EE. Origin of superoxide production by endothelial nitric oxide synthase. *FEBS Lett* 1998;438:161-164.
145. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci U S A* 1990;87:1620-1624.
146. Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. *Physiol Rev* 2007;87:315-424.
147. Szabo C, Ischiropoulos H, Radi R. Peroxynitrite: biochemistry, pathophysiology and development of therapeutics. *Nat Rev Drug Discov* 2007;6:662-680.
148. Bronte V, Serafini P, Mazzoni A, Segal DM, Zanovello P. L-arginine metabolism in myeloid cells controls T-lymphocyte functions. *Trends Immunol* 2003;24:302-306.
149. Noel W, Raes G, Hassanzadeh Ghassabeh G, De Baetselier P, Beschin A. Alternatively activated macrophages during parasite infections. *Trends Parasitol* 2004;20:126-133.

150. Bronte V, Zanovello P. Regulation of immune responses by L-arginine metabolism. *Nat Rev Immunol* 2005;5:641-654.
151. Gordon S. Alternative activation of macrophages. *Nat Rev Immunol* 2003;3:23-35.
152. Bronte V, Kasic T, Gri G, Gallana K, Borsellino G, Marigo I, Battistini L, et al. Boosting antitumor responses of T lymphocytes infiltrating human prostate cancers. *J Exp Med* 2005;201:1257-1268.
153. Holcik M, Sonenberg N. Translational control in stress and apoptosis. *Nat Rev Mol Cell Biol* 2005;6:318-327.
154. Schimke RT. Repression of enzymes of arginine biosynthesis in mammalian tissue culture. *Biochim Biophys Acta* 1962;62:599-601.
155. Padyana AK, Qiu H, Roll-Mecak A, Hinnebusch AG, Burley SK. Structural basis for autoinhibition and mutational activation of eukaryotic initiation factor 2alpha protein kinase GCN2. *J Biol Chem* 2005;280:29289-29299.
156. Qiu H, Dong J, Hu C, Francklyn CS, Hinnebusch AG. The tRNA-binding moiety in GCN2 contains a dimerization domain that interacts with the kinase domain and is required for tRNA binding and kinase activation. *Embo J* 2001;20:1425-1438.
157. Zhang P, McGrath BC, Reinert J, Olsen DS, Lei L, Gill S, Wek SA, et al. The GCN2 eIF2alpha kinase is required for adaptation to amino acid deprivation in mice. *Mol Cell Biol* 2002;22:6681-6688.
158. El-Gayar S, Thuring-Nahler H, Pfeilschifter J, Rollinghoff M, Bogdan C. Translational control of inducible nitric oxide synthase by IL-13 and arginine availability in inflammatory macrophages. *J Immunol* 2003;171:4561-4568.
159. Kaji H, Novelli GD, Kaji A. A Soluble Amino Acid-Incorporating System from Rat Liver. *Biochim Biophys Acta* 1963;76:474-477.
160. Balzi E, Choder M, Chen WN, Varshavsky A, Goffeau A. Cloning and functional analysis of the arginyl-tRNA-protein transferase gene ATE1 of *Saccharomyces cerevisiae*. *J Biol Chem* 1990;265:7464-7471.
161. Kaji H. Further studies on the soluble amino acid incorporating system from rat liver. *Biochemistry* 1968;7:3844-3850.
162. Karakozova M, Kozak M, Wong CC, Bailey AO, Yates JR, 3rd, Mogilner A, Zebroski H, et al. Arginylation of beta-actin regulates actin cytoskeleton and cell motility. *Science* 2006;313:192-196.
163. Chakraborty G, Ingoglia NA. N-terminal arginylation and ubiquitin-mediated proteolysis in nerve regeneration. *Brain Res Bull* 1993;30:439-445.
164. Kwon YT, Kashina AS, Davydov IV, Hu RG, An JY, Seo JW, Du F, et al. An essential role of N-terminal arginylation in cardiovascular development. *Science* 2002;297:96-99.
165. Bode-Boger SM, Boger RH, Löffler M, Tsikas D, Brabant G, Frolich JC. L-arginine stimulates NO-dependent vasodilation in healthy humans--effect of somatostatin pretreatment. *J Investig Med* 1999;47:43-50.

166. Giugliano D, Marfella R, Verrazzo G, Acampora R, Coppola L, Cozzolino D, D'Onofrio F. The vascular effects of L-Arginine in humans. The role of endogenous insulin. *J Clin Invest* 1997;99:433-438.
167. MacAllister RJ, Calver AL, Collier J, Edwards CM, Herreros B, Nussey SS, Vallance P. Vascular and hormonal responses to arginine: provision of substrate for nitric oxide or non-specific effect? *Clin Sci (Lond)* 1995;89:183-190.
168. Alba-Roth J, Muller OA, Schopohl J, von Werder K. Arginine stimulates growth hormone secretion by suppressing endogenous somatostatin secretion. *J Clin Endocrinol Metab* 1988;67:1186-1189.
169. Kamegai J, Tamura H, Shimizu T, Ishii S, Tatsuguchi A, Sugihara H, Oikawa S, et al. The role of pituitary ghrelin in growth hormone (GH) secretion: GH-releasing hormone-dependent regulation of pituitary ghrelin gene expression and peptide content. *Endocrinology* 2004;145:3731-3738.
170. Hall TR, Harvey S, Scanes CG. Control of growth hormone secretion in the vertebrates: a comparative survey. *Comp Biochem Physiol A* 1986;84:231-253.
171. Kineman RD, Kamegai J, Frohman LA. Growth hormone (GH)-releasing hormone (GHRH) and the GH secretagogue (GHS), L692,585, differentially modulate rat pituitary GHS receptor and GHRH receptor messenger ribonucleic acid levels. *Endocrinology* 1999;140:3581-3586.
172. Wass JA. Growth hormone neuroregulation and the clinical relevance of somatostatin. *Clin Endocrinol Metab* 1983;12:695-724.
173. Ghigo E, Bellone J, Mazza E, Imperiale E, Procopio M, Valente F, Lala R, et al. Arginine potentiates the GHRH- but not the pyridostigmine-induced GH secretion in normal short children. Further evidence for a somatostatin suppressing effect of arginine. *Clin Endocrinol (Oxf)* 1990;32:763-767.
174. Adriaio M, Chrisman CJ, Bielavsky M, Olinto SC, Shiraishi EM, Nunes MT. Arginine increases growth hormone gene expression in rat pituitary and GH3 cells. *Neuroendocrinology* 2004;79:26-33.
175. Villalobos C, Nunez L, Garcia-Sancho J. Mechanisms for stimulation of rat anterior pituitary cells by arginine and other amino acids. *J Physiol* 1997;502 (Pt 2):421-431.
176. Newsholme P, Brennan L, Rubi B, Maechler P. New insights into amino acid metabolism, beta-cell function and diabetes. *Clin Sci (Lond)* 2005;108:185-194.
177. Closs EI, Simon A, Vekony N, Rotmann A. Plasma membrane transporters for arginine. *J Nutr* 2004;134:2752S-2759S; discussion 2765S-2767S.
178. Deves R, Boyd CA. Transporters for cationic amino acids in animal cells: discovery, structure, and function. *Physiol Rev* 1998;78:487-545.
179. Kilberg MS, Stevens BR, Novak DA. Recent advances in mammalian amino acid transport. *Annu Rev Nutr* 1993;13:137-165.
180. Closs EI, Graf P, Habermeyer A, Cunningham JM, Forstermann U. Human cationic amino acid transporters hCAT-1, hCAT-2A, and hCAT-2B: three related carriers with distinct transport properties. *Biochemistry* 1997;36:6462-6468.

181. Vekony N, Wolf S, Boissel JP, Gnauert K, Closs EI. Human cationic amino acid transporter hCAT-3 is preferentially expressed in peripheral tissues. *Biochemistry* 2001;40:12387-12394.
182. Verrey F, Closs EI, Wagner CA, Palacin M, Endou H, Kanai Y. CATs and HATs: the SLC7 family of amino acid transporters. *Pflugers Arch* 2004;447:532-542.
183. Broer A, Wagner CA, Lang F, Broer S. The heterodimeric amino acid transporter 4F2hc/y+LAT2 mediates arginine efflux in exchange with glutamine. *Biochem J* 2000;349 Pt 3:787-795.
184. Pfeiffer R, Rossier G, Spindler B, Meier C, Kuhn L, Verrey F. Amino acid transport of y+L-type by heterodimers of 4F2hc/CD98 and members of the glycoprotein-associated amino acid transporter family. *Embo J* 1999;18:49-57.
185. Kanai Y, Fukasawa Y, Cha SH, Segawa H, Chairoungdua A, Kim DK, Matsuo H, et al. Transport properties of a system y+L neutral and basic amino acid transporter. Insights into the mechanisms of substrate recognition. *J Biol Chem* 2000;275:20787-20793.
186. Rajan DP, Kekuda R, Huang W, Wang H, Devoe LD, Leibach FH, Prasad PD, et al. Cloning and expression of a b(0,+)-like amino acid transporter functioning as a heterodimer with 4F2hc instead of rBAT. A new candidate gene for cystinuria. *J Biol Chem* 1999;274:29005-29010.
187. Sloan JL, Mager S. Cloning and functional expression of a human Na(+) and Cl(-)-dependent neutral and cationic amino acid transporter B(0+). *J Biol Chem* 1999;274:23740-23745.
188. Malandro MS, Kilberg MS. Molecular biology of mammalian amino acid transporters. *Annu Rev Biochem* 1996;65:305-336.
189. Fernandez J, Bode B, Koromilas A, Diehl JA, Krukovets I, Snider MD, Hatzoglou M. Translation mediated by the internal ribosome entry site of the cat-1 mRNA is regulated by glucose availability in a PERK kinase-dependent manner. *J Biol Chem* 2002;277:11780-11787.
190. Yaman I, Fernandez J, Sarkar B, Schneider RJ, Snider MD, Nagy LE, Hatzoglou M. Nutritional control of mRNA stability is mediated by a conserved AU-rich element that binds the cytoplasmic shuttling protein HuR. *J Biol Chem* 2002;277:41539-41546.
191. Li C, Huang W, Harris MB, Goolsby JM, Venema RC. Interaction of the endothelial nitric oxide synthase with the CAT-1 arginine transporter enhances NO release by a mechanism not involving arginine transport. *Biochem J* 2005;386:567-574.
192. Mann GE, Yudilevich DL, Sobrevia L. Regulation of amino acid and glucose transporters in endothelial and smooth muscle cells. *Physiol Rev* 2003;83:183-252.
193. Kizhatil K, Albritton LM. System y+ localizes to different membrane subdomains in the basolateral plasma membrane of epithelial cells. *Am J Physiol Cell Physiol* 2002;283:C1784-1794.
194. Perkins CP, Mar V, Shutter JR, del Castillo J, Danilenko DM, Medlock ES, Ponting IL, et al. Anemia and perinatal death result from loss of the murine ecotropic retrovirus receptor mCAT-1. *Genes Dev* 1997;11:914-925.
195. Closs EI, Albritton LM, Kim JW, Cunningham JM. Identification of a low affinity, high capacity transporter of cationic amino acids in mouse liver. *J Biol Chem* 1993;268:7538-7544.
196. MacLeod CL. Regulation of cationic amino acid transporter (CAT) gene expression. *Biochem Soc Trans* 1996;24:846-852.

197. Bogle RG, Baydoun AR, Pearson JD, Moncada S, Mann GE. L-arginine transport is increased in macrophages generating nitric oxide. *Biochem J* 1992;284 (Pt 1):15-18.
198. Cendan JC, Topping DL, Pruitt J, Snowdy S, Copeland EM, 3rd, Lind DS. Inflammatory mediators stimulate arginine transport and arginine-derived nitric oxide production in a murine breast cancer cell line. *J Surg Res* 1996;60:284-288.
199. Gill DJ, Low BC, Grigor MR. Interleukin-1 beta and tumor necrosis factor-alpha stimulate the cat-2 gene of the L-arginine transporter in cultured vascular smooth muscle cells. *J Biol Chem* 1996;271:11280-11283.
200. Simmons WW, Closs EI, Cunningham JM, Smith TW, Kelly RA. Cytokines and insulin induce cationic amino acid transporter (CAT) expression in cardiac myocytes. Regulation of L-arginine transport and no production by CAT-1, CAT-2A, and CAT-2B. *J Biol Chem* 1996;271:11694-11702.
201. Stevens BR, Kakuda DK, Yu K, Waters M, Vo CB, Raizada MK. Induced nitric oxide synthesis is dependent on induced alternatively spliced CAT-2 encoding L-arginine transport in brain astrocytes. *J Biol Chem* 1996;271:24017-24022.
202. Nicholson B, Manner CK, Kleeman J, MacLeod CL. Sustained nitric oxide production in macrophages requires the arginine transporter CAT2. *J Biol Chem* 2001;276:15881-15885.
203. Patejunas G, Bradley A, Beaudet AL, O'Brien WE. Generation of a mouse model for citrullinemia by targeted disruption of the argininosuccinate synthetase gene. *Somat Cell Mol Genet* 1994;20:55-60.
204. Reid Sutton V, Pan Y, Davis EC, Craigen WJ. A mouse model of argininosuccinic aciduria: biochemical characterization. *Mol Genet Metab* 2003;78:11-16.
205. Huang PL, Dawson TM, Brecht DS, Snyder SH, Fishman MC. Targeted disruption of the neuronal nitric oxide synthase gene. *Cell* 1993;75:1273-1286.
206. Huang PL, Huang Z, Mashimo H, Bloch KD, Moskowitz MA, Bevan JA, Fishman MC. Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature* 1995;377:239-242.
207. MacMicking JD, Nathan C, Hom G, Chartrain N, Fletcher DS, Trumbauer M, Stevens K, et al. Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell* 1995;81:641-650.
208. Sinasac DS, Moriyama M, Jalil MA, Begum L, Li MX, Iijima M, Horiuchi M, et al. Slc25a13-knockout mice harbor metabolic deficits but fail to display hallmarks of adult-onset type II citrullinemia. *Mol Cell Biol* 2004;24:527-536.
209. Gao HZ, Kobayashi K, Tabata A, Tsuge H, Iijima M, Yasuda T, Kalkanoglu HS, et al. Identification of 16 novel mutations in the argininosuccinate synthetase gene and genotype-phenotype correlation in 38 classical citrullinemia patients. *Hum Mutat* 2003;22:24-34.
210. Kobayashi K, Kakinoki H, Fukushige T, Shaheen N, Terazono H, Saheki T. Nature and frequency of mutations in the argininosuccinate synthetase gene that cause classical citrullinemia. *Hum Genet* 1995;96:454-463.

211. Thomas KR, Capecchi MR. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* 1987;51:503-512.
212. Schaft J, Ashery-Padan R, van der Hoeven F, Gruss P, Stewart AF. Efficient FLP recombination in mouse ES cells and oocytes. *Genesis* 2001;31:6-10.

Chapter 2

Chronic arginine deprivation represses the GH-IGF-1 axis via the GCN2-mediated stress response

Vincent Marion¹, Selvakumari Sankaranarayanan¹, Paul van Dijk¹, Chiel de Theije¹, Patrick Lindsey², Henny M.W. van Straaten¹, Marinus C. Lamers³, Heather P. Harding⁴, David Ron⁴, Wouter H. Lamers^{1,5} and S. Eleonore Köhler¹

¹ Department of Anatomy & Embryology, and ² Department of Population Genetics, Genomics & Bioinformatics, Maastricht University, P.O. box 616, 6200 MD, Maastricht, The Netherlands; ³ Max Planck Institute for Immunobiology, Freiburg, Germany; ⁴ Skirball Institute, New York University School of Medicine, New York, NY 10016, USA; ⁵ AMC Liver Center, Academic Medical Center, University of Amsterdam, P.O. box 22660, 1100 DD, Amsterdam, The Netherlands.

Summary

Transgenic "F/A2" mice overexpress arginase-I in their enterocytes. Suckling F/A2 mice develop a syndrome (hypoargininemia, reduced hair and muscle growth, impaired B-cell maturation) that resembles IGF-1 deficiency. We hypothesized that the syndrome results from an interaction of the growth-hormone/IGF-1 axis and the amino acid-sensing GCN2 stress-kinase pathway. Cells were cultured in different arginine concentrations. F/A2 and Gcn2^{-/-} mice were studied. Stress-kinase activation was assayed as eIF2 α phosphorylation, and induction of ATF4 and CHOP expression. Arginine deficiency inhibited GH secretion in pituitary cells. In F/A2 mice, pituitary GH content was 2.5-fold elevated, whereas liver and muscle Igf-1 mRNA content was 8-10-fold decreased. GH supplementation increased tissue Igf-1 mRNA 3-fold in wild-type and F/A2 mice, but did not enhance growth. In vitro, the Gcn2 pathway was activated at <50 μ M arginine, whereas IGF-1 enhanced myoblast differentiation at $\geq$ 25 μ M arginine only. In 1-day-old F/A2 mice, circulating and muscle arginine levels were 4.5-fold decreased and the Gcn2 pathway was activated in all organs investigated. Gcn2-deficient F/A2 mice did not increase ATF4 and CHOP expression, and died shortly after birth, demonstrating that GCN2 mediated the stress-kinase response. Conclusion: The arginine-deficiency syndrome develops due to GCN2 activation, suppression of GH secretion, and interference with the growth-stimulatory effects of IGF-1.

Abbreviations:

F/A2	mice overexpressing arginase-I under control of Fabp _i promoter
GCN2	general control non-derepressible 2 (Eif2ak4) kinase
GH	growth hormone
IGF-1	insulin-like growth factor-1
ND	neonatal day
CI	confidence interval

Introduction

Arginine is a substrate for the synthesis of proteins, ornithine, creatine, agmatine and nitric oxide (1). In addition, it functions as a secretagogue for hormones, such as growth hormone (GH) and insulin (2-4). Under normal conditions, endogenous arginine synthesis in adult mammals suffices to sustain daily requirements (5). A dietary source of arginine may, however, become necessary when demand increases under anabolic or catabolic conditions (5). For this reason, arginine is considered a conditionally essential amino acid.

The enterocytes of the small intestine of suckling rodents express all enzymes necessary to synthesize arginine (6-9), but not the major arginine-catabolizing enzyme arginase (10). To examine the function of intestinal arginine synthesis, a transgenic mouse model that overexpresses arginase-I in the enterocytes of the small intestine was produced (11-13). During the suckling period, these “F/A2” mice suffer from a selective deficiency of circulating arginine levels (which decline to ~30% of controls), reduced growth of hair and skeletal muscle, and inhibition of B-cell maturation (11). Thus far, we have been able to rule out deficiency of creatine or polyamine production, and ADP-ribosylation of proteins as causes of the described symptoms (11-14). Furthermore, mice that are deficient in all three nitric-oxide synthases do not show the same phenotype as F/A2 mice in the suckling period (15) (and Tsutsui, personal communication). This leaves two functions of arginine as likely candidates to account for the highly characteristic phenotype of arginine deficiency in suckling mice, viz. its role as a building block in protein synthesis and as a secretagogue of hormones.

Aminoacyl-tRNA synthetases catalyze the charging of tRNAs with their corresponding amino acids. Whenever the intracellular concentration of arginine decreases below 10-20 μ M, the charging process of its corresponding tRNA slows down and the concentration of uncharged tRNA increases (16). Uncharged tRNAs bind to the “general control non-derepressible2” kinase (Gcn2/Eif2ak4), which then phosphorylates the α -subunit of the eukaryotic initiation factor 2 (eIF2 α) (17) and thus initiates the “integrated stress response” (18,19). This response causes the arrest of 5' cap-dependent protein synthesis and an increased expression or activation of transcription factors such as ATF2, ATF4 and CHOP (20), which induce the transcription of genes necessary for amino-acid synthesis and transport. GCN2 is expressed in almost all mouse tissues and is

required for the adaptation to amino-acid deprivation (21). In the present study, we addressed the question whether the integrated stress response is activated in arginine-deficient F/A2 mice.

The most remarkable phenotypic features of F/A2 mice, namely impaired muscle and hair growth and impaired B-cell maturation, are all affected by insulin-like growth factor-1 (IGF-1) (22-26). Because the growth hormone (GH)/IGF-1 axis plays a predominant role in growth and development (27) and because arginine is a potent GH secretagogue (28), we also investigated the function of the GH-IGF-1 axis in F/A2 mice. Our findings show that arginine deficiency in the neonatal and suckling period activates the integrated stress response and interferes with GH/IGF-1 signaling.

Methods

Cell cultures

Myoblasts of the murine skeletal muscle cell line C2C12 (ATCC # CRL 1772, Manassas, VA) were cultured on gelatinized Petri dishes (0.1% gelatin in PBS) in Iscove's Modified Dulbecco's Medium (IMDM; Gibco, The Netherlands), 50U/mL penicillin, 50µg/mL streptomycin (Gibco), 1x Glutamax (Gibco), 10% fetal bovine serum (Gibco). Cultures were grown to 80% confluence. To induce myogenesis, fetal bovine serum was replaced by 2% heat-inactivated horse serum ("differentiating medium"; Gibco). In some experiments, the arginine concentration in differentiating medium was varied and/or 10ng/mL insulin-like growth factor-1 (IGF-1) was added. The rat pituitary cell line GH3 (ATCC # CCL-82.1) was also grown in IMDM. For the primary B-cell culture, the IL-7 secreting mouse myeloma cell-line J558-IL7 was cultured on ST2 medium (DMEM; Gibco, 10% FBS, 100U/mL penicillin, 100µg/mL streptomycin, 2mM Glutamax). Throughout the culture period, the cells were continuously shaken and IL-7-conditioned medium was harvested after 4 days. The ST2 cells, used as feeder cells for the B cells, were irradiated with a Tandemtron irradiator (IONEX) with 20 gray. The cells were then seeded at a density of 3×10^5 cells/cm². Mice were sacrificed at 2 weeks after birth. The bone marrow from the femur and tibia of the hind legs was removed by flushing the marrow with 2mL 2% FBS in PBS via a 25-gauge needle. After centrifugation at 1,000 rpm (250g) for 5 min at 4°C, the pellet was washed with PBS and centrifuged. The pellet was re-suspended in ice-cold erythrocyte-

lysis buffer (1mM NH_4HCO_3 , 114mM NH_4Cl) and incubated on ice for 3 min. The cell solution was diluted 10 times with ice-cold PBS and centrifuged at 1,000 rpm for 5 min at 4°C. The pellet containing B-cells was re-suspended in B-cell medium (IMDM containing 2 mM Glutamax, 0.4mM arginine, 36mM NaHCO_3 , 50 μM β -mercaptoethanol, 100U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin, 100 $\mu\text{g/mL}$ gentamycin, 5% foetal bovine serum and 2% IL-7 (ST2) conditioned medium) and seeded on an irradiated feeder layer of ST2 cells at a density of 3×10^5 cells/cm². Cells were counted using a Neubauer cell counter.

Growth-hormone ELISA

GH3 culture media were harvested and stored at -80°C until used. GH3 cells were washed, trypsinized, washed again, sonicated on ice and kept at -80°C until used. The GH content was measured in 50 μL of 10x-diluted medium or in sonicated cell lysates, using a one-step sandwich-type immunoassay with the ACTIVE Mouse/Rat Growth-Hormone ELISA kit (Diagnostic Systems Laboratories, Inc., Texas, USA). The absorbance was measured at 450nm. The quantity of GH in each sample was based on a standard curve.

Myogenic differentiation in C2C12 cells

The myogenic index (M.I.) was determined on May-Grünwald-Giemsa-stained cells (Sigma, Saint Louis, MO). To determine muscle creatine kinase (CK) activity, cells were washed twice, lysed in 0.5% Triton X-100, and scraped off the dish with a rubber policeman. Lysates were centrifuged for 2 min at 16,000g and the supernatant was stored at -80°C. CK activity was determined using the CK (UV-RATE) Stanbio (Texas, USA) kit (# 0910-206). Protein was measured with the Bradford method (43).

Transgenic mouse lines and animal husbandry

Transgenic mice that overexpress arginase-I in their small-intestinal enterocytes (F/A2 line(11)) were bred hemizygotously. $\text{Gcn2}^{-/-}$ mice (38) (129SvEv background) originated from the colony of D. Ron (New York, NY). All mice were housed in humidity- and temperature-controlled rooms on a 12-hour light/12-hour dark cycle with food and water ad libitum. Control animals were littermates of the experimental animals. Animal studies were reviewed and approved by the committee for animal care and ethics of Maastricht University (DEC).

GH administration

Litters were limited to 5 animals on neonatal day 1 (ND1) and weighed daily. Starting on ND3, 3mg/kg body weight of human recombinant growth hormone (hrGH; Humatrope, Lilly, kindly provided by Dr. Gerver, Dept. of Endocrinology, Academic Hospital Maastricht) (44) was administered subcutaneously in 250µL of vehicle twice daily from day 3 to day 21 postnatal. Control animals were injected with vehicle alone.

Blood and tissue collection

Animals were sacrificed by decapitation. Blood was collected into heparin-containing tubes and centrifuged at 2,000g for 5 min at 4°C. 80µL of plasma was added to 6.4mg of lyophilized sulphosalicylic acid, vortexed and stored at -20°C. For amino acid measurements, the tissues were homogenized with silica mini-beads in 0.33M sulphosalicylic acid. Tissues were collected at ND0, ND10, ND17 and ND21. Amino-acid concentrations were determined by HPLC, as described (45). Tissues for protein and mRNA analysis were isolated, snap frozen in liquid nitrogen and stored at -80°C. For histological analysis, tissues were fixed in 4% buffered formalin immediately after isolation and embedded in paraffin.

RNA extraction and cDNA synthesis

The TRI Reagent (Sigma) was used for RNA isolation from cells and tissues. RNA integrity was assessed by denaturing gel electrophoresis and purity by the ratio of the optical density at 260 and 280nm. RNA concentration was determined with a NanoDrop-ND1000 spectrophotometer (Isogen Life Science BV, The Netherlands). 1µg of total RNA was used for cDNA synthesis with the iScript cDNA synthesis kit (Bio-Rad, CA, USA).

Real-time quantitative PCR

The primers and their characteristics are given in Table 1. PCR was performed using qPCR Mastermix Plus for SYBR Green I (Eurogentec, Belgium). cDNA samples for mRNA determination were diluted 60-fold before use and those for 18S rRNA 200-fold. The reaction volume was 25 µL. CHOP, ATF-4 and 18S RNA levels were measured using the RNA TaqMan® Gene Expression Assays (ABI Applied Biosystems, USA). Primary fluorescent data were exported and analyzed with the Lin-Reg Analysis program

(46). Between-session variations in replicate experiments were corrected using factor correction (47).

Western blots, immunostaining and immunofluorescence

For Western-blotting assays, 100µg of protein were loaded per lane. Antibodies used are described in Table 2. Antibody binding was visualized using the Super Signal West Femto Maximum Sensitivity Substrate (Pierce, USA). For detection of the phosphorylated and unphosphorylated forms of eIF2 α , the Odyssey Infrared Imaging system (Li-cor Bioscience, UK) with a two-color detection program was used. For immunostaining, 5µm sections of paraffin-embedded tissues were prepared. After deparaffination, the sections were pre-incubated with Teng-T (10mM Tris (pH=7.6), 5mM EDTA, 150mM NaCl, 0.25% gelatin, 0.05% Tween-20)/10% normal goat serum (NGS) for 30 min, followed by an overnight incubation with the primary antibody diluted 1:100 in Teng-T/10% NGS. Slides were then washed with PBS and incubated with Teng-T/10% NGS for 15 min followed by 90 min incubation with the secondary antibody (Table 2) in Teng-T/10% NGS. The slides were washed and incubated with alkaline phosphatase substrate (NBT/BCIP tablets; Roche) at 37°C. Staining was stopped by washing with Millipore water, followed by dehydration in graded ethanols and UltraClear (Mallinckrodt Baker BV, The Netherlands). Coverslips were mounted using UltraKitt Histograde (Mallinckrodt Baker). Digital images of the sections were captured with a Sony 3CCD camera mounted onto a Leica DM RXA microscope. The images were analyzed with the Leica Quantimet 500 Image Analysis System, v3.2, as described (48). For immunofluorescence studies, ATF4, eIF2 α , and [pS51] phospho-specific eIF2 α antibodies, in combination with fluorescently labeled secondary antibodies, were used (Table 2). For nuclear staining, Hoechst 33258 (Molecular Probes, The Netherlands), diluted 1: 10,000 in 90% glycerol, was used. Pictures were captured using a Leica CCD camera and the fluorescent staining intensities were quantified using the QWin program (Leica). All results shown are representative of at least 3 separate experiments and 3 individual animals.

Statistical analysis

The significant difference for one variable between two distinct groups was analyzed using an unpaired student t-test with a P-value given. For more complex analyses of

significance for variables with different controls, each gene was analyzed using Gaussian linear regression including 18S as housekeeping gene. When appropriate, additional explanatory variables such as tissue type, strain, and time were included in the model. The treatment group was then added to the model. Finally, interactions among the included variables were considered. The inference criterion used for comparing the models is their ability to predict the observed data, i.e. models are compared directly through their minimized minus log-likelihood. When the numbers of parameters in models differ, they are penalized by adding the number of estimated parameters, a form of the Akaike information criterion (AIC) (49). If the most likely model contains the treatment effect, the fold change (FC) representing the ratio between the variable of the treated group and the same variable for the control can be computed as well as its 95% confidence interval (CI).

Results

Arginine deprivation blocks growth-hormone release

The phenotype of F/A2 mice is compatible with a deficiency of GH and IGF-1. We, therefore, investigated the GH content in the somatotropes of the pituitary gland by quantitative immunohistochemistry (Figure 1A). The somatotropes of 3-week-old homozygous F/A2 mice contained 2.5-fold more GH than those of their wild-type counterparts (Figure 1B). The somatotropes from hemizygous mice had an intermediate GH content, indicating a correlation with the degree of arginine deficiency (11,13). The rat pituitary cell line GH3, which produces GH (29,30), was then used to assess the direct effects of arginine on GH production and secretion. GH content in the GH3 cells was assessed by quantitative immunohistochemistry (Figure 1C and D) and ELISA (Figure 1E). Both assays showed that, after 24 hours of culture in the presence of 0 - 200 μ M arginine, the cellular GH content decreased from ~50pg to ~25pg GH per cell at 0 μ M and 200 μ M arginine in the medium, respectively (Figure 1E). This 2-fold decrease in tissue GH content was accompanied by a 1.5-fold increase in concentration of GH in the medium, revealing a pronounced effect of arginine on GH secretion. After 72 hours, the arginine-dependent GH secretion profile was similar to that observed after 24 hours (not shown). These data show that below 50 μ M ambient arginine GH secretion is reduced.

Figure 1. Detection of growth hormone in somatotropes of 21-day-old-mice and in GH3 cells. **Panel A, left side:** Sections of pituitary glands of 21-day-old homozygous *F/A2* mice (*F/A2tg/tg*), hemizygous *F/A2* mice (*He*; *F/A2tg/-*), and wild-type (*WT*; *F/A2-/-*) littermates were stained for the presence of growth hormone (GH). Bar: 5 μ m. **Panel A, right side:** Optical density (OD) in GH-positive cells as measured with the *QWin* program (mean $\pm$ SEM; *n*=3 mice per genotype; significance: wild-type vs. hemizygotes: *P* = 0.009 (unpaired student's *t*-test); hemizygotes vs. homozygotes: *P* = 0.02). **Panel C:** The pituitary cell line GH3 was cultured in the indicated arginine concentrations for 24 hours. Left row: DAPI-stained nuclei; right, purple-stained row: the same GH3 cells after staining for the presence of GH. Bar: 10 μ m. **Panel D:** Quantification of GH-staining intensity with correction for the number of cells using the DAPI fluorescence. The intensity of immunostaining decreased with increasing arginine concentrations in the medium with a half maximal staining intensity at $\sim$ 35 μ M arginine. **Panel E:** GH content in GH3 cells and in the medium as quantified with ELISA (mean of three experiments, $\pm$ SEM). Intracellular GH content decreased with increasing arginine concentration in the medium, whereas GH concentration in the medium increased, indicating that arginine induces GH secretion.

Figure 2. The effect of GH treatment on growth, and on GH-receptor and Igf-1 expression in suckling F/A2 mice. **Panel A:** Change in body weight of F/A2 mice and their wild-type littermates during treatment with GH (twice daily 3mg hrGH/kg body weight subcutaneously) between postnatal day 3 and day 21 ($n=6$ animals per genotype). F/A2 mice show a lower growth rate compared to wild type mice. Injection of GH had no effect on the growth rate in either wild-type or F/A2 mice. **Panel B:** Expression of the GH receptor in liver. Total RNA from the liver of 0-, 10- and 21-day old wild-type and F/A2 mice was quantified by RT-PCR. NC: negative control. Both wild-type and F/A2 mice expressed comparable levels of GH-receptor mRNA. **Panel C:** Quantification of Igf-1 mRNA in liver and muscle of 17-day-old mice. Igf-1 mRNA levels in both tissues were ~11-fold higher in wild-type than in homozygous F/A2 mice (CI: 8.2-14.3). Growth-hormone injections induced a 2.5-fold increase in Igf-1 mRNA in wild-type tissues (CI: 1.9-3.3) and an 8-fold increase F/A2 tissues (CI: 6.2-10.2). Data represent the mean $\pm$ SEM of 3 experiments.

The GH/IGF-1 axis is functional but repressed in F/A2 mice

As early as ND3, F/A2 mice can be distinguished from their wild-type littermates by a lower body weight(11). To assess whether the typically hypomorphic tissues of F/A2 mice (myocytes, hairs, and B-cells (12)) respond to GH treatment, the mice were injected subcutaneously twice daily with 3mg/kg human recombinant (hr)GH between day 3 and day 21 after birth. Figure 2A shows that the growth rate of vehicle-treated F/A2 mice (control) was ~50% of that of wild-type mice. Treatment with rhGH did not

change the growth rate of either wild-type or F/A2 mice. GH action is mediated by GH receptors (GHR) and results in the synthesis and secretion of IGF-1 in target tissues. Figure 2B shows that GHR mRNA is equally expressed in ND1, ND10 and ND21 wild-type and F/A2 livers. To assess the functionality of the GHRs, Igf-1 mRNA levels were quantified in liver and muscle of GH-treated and control mice (Figure 2C).

In both tissues, Igf-1 mRNA levels were decreased 8-10-fold in F/A2 relative to control mice. rhGH injections increased Igf-1 mRNA 8-fold in F/A2 liver and muscle as compared to 2.5-fold in wild-type liver and muscle. These observations show that F/A2 mice suffer from low tissue IGF-1 levels, which respond strongly to GH stimulation, indicating the presence of fully functional GHRs. Nevertheless, absolute IGF-1 levels remain much lower in GH-supplemented F/A2 than in wild-type animals. The data shows that muscle is quantitatively more affected than liver.

Arginine is essential for muscle development

To investigate the role of arginine during muscle growth and development, C2C12 myoblasts were cultured in both growth and differentiation medium in the presence of different concentrations of arginine (Figure 3). Figure 3A and B shows representative pictures after 4 days of myogenic differentiation in the absence or presence of arginine (200 μ M), respectively. Both the myogenic index (the fraction of nuclei residing in cells containing three or more nuclei; Figure 3C) and the cellular concentration of the late differentiation marker creatine kinase-M (CK; Figure 3D) increased with increasing arginine concentration to plateau at $\sim$ 50 μ M arginine. Myogenic differentiation is, therefore, clearly dependent on arginine availability, with a half-maximal effect at $\sim$ 20 μ M arginine in the medium.

Arginine deficiency induces the integrated stress response in vitro

Amino-acid deficiency can activate the GCN2 stress-kinase pathway (31). The first step is the phosphorylation of translation factor eIF2 α (32). We, therefore, investigated whether eIF2 α becomes phosphorylated in arginine-deprived myocyte cultures. C2C12 cells were grown to 80% confluence in standard growth medium and were then deprived of arginine. Western blots with phospho-specific eIF2 α antibodies revealed that eIF2 α

was phosphorylated immediately after arginine deprivation and remained phosphorylated for at least 2 hours (Figure 4A).

Figure 3. The effects of arginine on myogenic differentiation. C2C12 myoblasts were cultured for 4 days in differentiation medium containing 0-200 μ M arginine. Panel A: Culture grown in the absence of arginine (left column) and in the presence of 200 μ M arginine (right column). Bar: 50 μ m. Cells were stained with May-Grünwald-Giemsa. Panel B: Myogenic index (number of nuclei in myotubes as a fraction of the total number of nuclei; mean $\pm$ SEM of 5 experiments) in cultures shown in panel A. The formation of myotubes is arginine-dependent. Panel C: Dependence of the late myogenic differentiation marker creatine kinase on ambient arginine concentration (mean $\pm$ SEM of 3 experiments).

Figure 4. Arginine deficiency activates the integrated stress response. Panel A: Western blot of C2C12 cell extracts cultured for the indicated times in 0 or 200 μ M arginine. Top two panels: total eIF2 α (36 kDa); middle two panels: p51-phosphorylated eIF2 α (no phosphorylated eIF2 α was detected in the presence of arginine (left column)); bottom panels: superimposition of top and middle panels (phosphorylated eIF2 α colocalizes with total eIF2 α (right column)). Data shown are representative of 3 Western blots. Panels B and C: Dependence of ATF4 (panel B) and CHOP mRNA expression (panel C) on ambient arginine concentration in C2C12 (left Y-axis), and GH3 cells and B-lymphocytes (right Y-axis). Values were corrected for the concentration of 18S rRNA. The ATF4 mRNA concentration was only increased in the total absence of arginine (panel B); ATF4 levels were ~3.5-fold higher in C2C12 than in GH3 cells (CI: 2.2-5.3). The CHOP mRNA was reduced to basal levels at 50 μ M, with CHOP levels being ~3-fold higher in C2C12 than in GH3 cells (CI: 1.5-6.3). Data represent the mean $\pm$ SEM of 3 experiments.

Phosphorylation of eIF2 α induces the accumulation of the transcription factors ATF4 and CHOP. C2C12, GH3 and mouse primary B lymphocytes were cultured for 24 hours in the presence of varying concentrations of arginine (Figure 4B and C) before ATF4 and

Figure 5. The stress-kinase pathway is activated in the F/A2 mice. Panel A: Sections of liver (L), muscle (M) and small intestine (G) of a 1-day-old F/A2 mouse and a wild-type littermate stained for the presence of total eIF2 α (green) and phosphorylated eIF2 α (red). Phosphorylated eIF2 α was only expressed in F/A2 mice (top three panels of the right column). NEG: incubation without primary antibody. Representative for $n=3$ animals per genotype. Panel B: ATF4 mRNA concentration in skeletal muscle (M), liver (L) and small intestine (G) of 1- and 21-day-old F/A2 and wild-type mice, after correction for 18S rRNA content. The ATF4 mRNA content in F/A2 muscle of 1-day-old mice was ~3.5-fold higher than in wild-type muscle (CI: 2.5-4.5). Panel C: CHOP mRNA concentration was ~6-fold increased in muscle, liver and gut of 1-day-old F/A2 compared to wild-type mice (CI: 4.3-8.8). At 21 days of age, CHOP mRNA concentration was still 4-fold increased (CI: 2.9-6.0). Data represent the mean $\pm$ SEM of 8-12 mice per genotype. Panel D: Arginine concentration in muscle, liver and small intestine of 1- and 21-day-old mice. At D1, arginine concentration was 3-fold lower in the muscle of the F/A2 mice compared to the controls ($P=0.01$; unpaired t -test). At D21, muscle arginine concentration in F/A2 mice had increased 4.5-fold over that at D1 ($P=0.05$).

CHOP mRNA levels were determined. In C2C12 and GH3 cells, ATF4 mRNA was >5-fold higher when the cells were kept in arginine-free medium than when $\geq 12.5\mu\text{M}$ arginine was present. The dependence of ATF4 mRNA on the concentration of arginine in the medium was identical for the 2 cell lines, but C2C12 cells expressed ~3.5-fold

more ATF4 mRNA than GH3 cells. The response of CHOP mRNA expression to arginine deprivation was identical for the three cell lines with C2C12 cells expressing 3-fold more CHOP mRNA than the GH3 and the B cells. The CHOP mRNA level gradually decreased with increasing ambient arginine and reached a baseline at 50 μ M arginine. These data reveal that the integrated stress response is induced at arginine concentrations in the medium below 50 μ M. The data also confirm that CHOP mRNA is a more sensitive marker for the activation of the stress response than ATF4 mRNA. The stress-kinase pathway is activated in F/A2 mice

Based on the findings in vitro, we determined the activation of the integrated stress response in F/A2 mice in vivo by staining sections of ND1 liver, muscle, and small intestine for the presence of total and phosphorylated eIF2 α (Figure 5A). All tissues tested expressed eIF2 α in both wild-type and F/A2 animals, but the phosphorylated form of eIF2 α was only demonstrable in F/A2 mice.

We then measured ATF4 and CHOP mRNA levels in liver, small intestine and muscle of ND1 and ND21 mice. A significant 3.5-fold upregulation of ATF4 mRNA was found in ND1 muscle (Figure 5B), but ATF4 mRNA levels were not significantly different from wild-type animals in the other two tissues. At 21 days, there was no difference in ATF4 expression in any of the tissues investigated. CHOP mRNA levels were significantly elevated in all F/A2 tissues investigated at ND1 and at ND21 (Figure 5C), but the difference at ND1 was 3-4-fold that at ND21.

We measured the tissue concentration of arginine in the three target tissues in 1- and 21-day-old mice (Figure 5D). Interestingly, a significant 4.5-fold lower arginine concentration was found in F/A2 than in control muscle (Figure 5D), the only tissue that also showed induction of ATF4 mRNA, while the local arginine concentrations in liver and gut were 2-fold lower in F/A2 than in wild-type mice. The low concentration of arginine in the liver relative to the intestine can be ascribed to the high concentration of the urea-cycle enzyme arginase-1 in the liver. At 21 days, the concentration of arginine in F/A2 muscle had increased 2-fold relative to that at 1 day after birth and was no longer different from that in controls (Figure 5D). However, the concentration of arginine in liver and gut remained 2-fold lower in F/A2 compared to wild-type mice. In these newly weaned mice, arginine levels in the liver declined 3-fold and that in the gut even 10-fold relative to that of in neonatal mice, probably because of further maturation of the urea cycle in the liver.

Phosphorylation of eIF2 α induces translation of ATF4 (33). We determined ATF4 protein levels in muscle of ND1 mice by Western blot analysis (Figure 6A). All muscles of F/A2 mice showed the expected 38kDa band (bold arrow in Figure 6A) and a much weaker band at ~50kDa (arrow), whereas these bands were absent in wild-type mice. A much weaker band of ~47kDa was present in both F/A2 and wild-type muscle. The ~47kDa and ~50kDa bands may correspond to the ubiquitinated forms of ATF4 (34). Because of the apparent specificity of the ATF4 antiserum, we stained whole-body sagittal sections of ND1 mice immunohistochemically for the presence of ATF4 protein (Figure 6B). Quantification of the optical density of the staining signal revealed ~4-fold higher levels of ATF4 protein in most organs of F/A2 mice when compared to wild-type animals (Figure 6C), but in the brain, only a 2-fold difference was found. Together, these data show that the stress-response pathway is strongly activated in neonatal F/A2 mice. This activation persists to ND21, albeit that basal expression in wild-type control mice did increase.

Activation of the stress-kinase pathway coincides with inhibition of IGF-1 signaling

To test whether the activation of the integrated stress response by arginine deficiency inhibits IGF-1-mediated signaling, C2C12 cells were cultured in the presence of different arginine concentrations with or without IGF-1 for 48 hours (Figure 7). Immunofluorescent staining of the cells (Figure 7A) revealed that ATF4 accumulated at arginine concentrations below 25 μ M, whereas the size of the myotubes and the cellular concentration of myosin heavy chain (MHC) protein increased with increasing arginine concentrations.

CHOP mRNA levels decreased at increasing ambient arginine concentration as shown before, irrespective of whether IGF-1 was present or not (Figure 7B). As expected from the arginine-dependent increase in the size of the myotubes and the accumulation of MHC protein, MCK mRNA accumulated at $\geq 25\mu$ M arginine. More importantly, IGF-

1

Figure 6. ATF4 protein is expressed ubiquitously in F/A2 mice. Panel A: Western blots of muscle extracts of 1-day-old mice stained for the presence of ATF4. Lanes 1-4: 1-day-old F/A2 mice; lanes 5-7: 1-day-old wild-type mice. The left panel is stained with Ponceau-S to demonstrate equal protein loading, while the right panel shows the blot after staining for ATF4. Native form of ATF4 corresponds with the prominent 38kDa band (bold arrow). A fainter band at ~50 kDa was also present in F/A2 extracts only (thin arrow) which corresponds to the ubiquitinated form. Panel B: Immunodetection of ATF4 protein in sagittal sections of 1-day-old F/A2 and wild-type mice. The 4 subpanels show magnifications of muscle (M), lung (Lu), liver (L), and small intestine (G). Representative of $n = 3$ per genotype. Panel C: Optical density (OD) in ATF4-positive cells as measured with the QWin program. Significant differences in OD were found between wild-type and F/A2 mice in muscle ($P = 0.001$), gut ($P = 0.021$), liver ($P = 0.000$), lung ($P = 0.001$), and brain ($P = 0.05$). Data represent mean $\pm$ SEM of 3 mice per genotype.

Figure 7. IGF-1-mediated muscular hypertrophy is inversely proportional to stress-response activation. *Panel A:* Expression of myosin heavy chain (MHC; green) and ATF4 (red) after 2 days of culture in differentiation medium plus 2 days of culture in the presence of the indicated concentration of arginine with or without 10ng/ μL IGF-1. Lower arginine concentrations resulted in higher ATF4 expression, smaller myotubes, and less MHC expression. *Panel B:* CHOP mRNA concentration declines with increasing arginine concentration in the culture medium, irrespective of the presence of IGF-1. *Panel C:* MCK mRNA expression increased at $\geq 25\mu\text{M}$ arginine in the culture medium. Addition of IGF-1 to the culture medium increased MCK mRNA concentration at $\geq 25\mu\text{M}$ arginine. Data represent the means of 3 experiments $\pm$ SEM.

also increased MCK expression only at $\geq 25\mu\text{M}$ arginine (Figure 7C). These data show that muscle differentiation and the effects of IGF-1 on muscle differentiation are inhibited at low ambient arginine concentration, probably as a result of the activation of the stress response. GCN2 mediates the activation of the stress response in arginine-deficient neonatal mice

To demonstrate that GCN2 is the stress kinase that is activated in neonatal F/A2 mice, we crossed $\text{Gcn2}^{-/-}$ mice with F/A2 mice. Figure 8 lists the genotypes of the offspring and compares the observed frequencies with the expected Mendelian ratios. Inspection of these data shows that all mice that were transgenic for arginase and deficient for GCN2 ($\text{F/A2}^{\text{tg}}/\text{Gcn2}^{-/-}$) died within 72 hours after birth. F/A2 neonates had a 3-fold lower plasma arginine concentration than control neonates irrespective of

Figure 8. GCN2 is responsible for the induction of the integrated stress response in arginine-deficient mice. **Panel A:** Distribution of genotypes in the offspring of crosses of hemizygous F/A2 (F/A2tg/-) mice and heterozygous Gcn2 (Gcn2+/-) mice. Six different genotypes could be identified by PCR (no difference could be made between transgenic F/A2tg/- and F/A2tg/tg mice; F/A2-/- mice denote wild-type animals). A total of 60 mice (dead and alive) were analyzed. 4 newborn mice disappeared from the nests in the first two days and could not be analyzed. A chi-square test revealed that the chance of survival of a F/A2tg/?/Gcn2-/- neonate was <0.0001. **Panel B:** Arginine concentration in trunk blood of 1-day-old mice with genotype F/A2-/-/Gcn2+/+ (wild type; group 1), F/A2tg/?/Gcn2+/+ (arginine-deficient; group 2), and F/A2tg/?/Gcn2-/- (both arginine- & Gcn2-deficient; group 3). Compared to the wild-type mice, the circulating arginine concentration was decreased 3.5-fold in F/A2 mice, irrespective of whether Gcn2 was functional or not (F/A2tg/?/Gcn2+/+ and F/A2tg/?/Gcn2-/-, respectively). **Panel C:** CHOP mRNA expression in the same three genotypes as shown in panel B. F/A2 mice lacking active Gcn2 (F/A2tg/?/Gcn2-/-) cannot mount the induction of CHOP seen in mice with an active Gcn2 gene (F/A2tg/?/Gcn2+/+). In muscle, there was a 12-fold increase in the group 2 animals compared to group 1 (CI: 8.3-18.5) and a 14-fold increase in group 2 compared to group 3 (CI: 8.3-25.0). In liver, there was a ~6-fold increase in group 2 compared to group 1 (CI: 3.3-9.7), ~2.5-fold in group 2 compared to group 3 (CI: 1.5-4.4), and ~2-fold in group 3 compared to group 1 (CI: 1.5-3.3). In gut, there was a ~12-fold increase in group 2 compared to group 1 (CI: 8.3-18.5), ~6-fold in group 2 compared to group 3 (CI: 3.5-9.0), and a ~2-fold in group 3 compared to group 1 (CI: 1.5-3.3).

whether GCN2 was or was not expressed (Figure 8B). In addition to GCN2, three other stress kinases can phosphorylate eIF2 α .

To investigate whether GCN2 was solely responsible for activating the stress response, we determined CHOP mRNA levels in neonatal wild-type mice (F/A2^{-/-}/Gcn2^{+/+}), F/A2 mice (F/A2^{tg/tg}/Gcn2^{+/+}) and F/A2 mice that were also GCN2-deficient (F/A2^{tg/tg}/Gcn2^{-/-}) (Figure 8C). As expected, CHOP mRNA levels were low in muscle, liver and gut of wild-type (F/A2^{-/-}/Gcn2^{+/+}) mice and elevated in the same organs of F/A2 (F/A2^{tg/tg}/Gcn2^{+/+}) mice. However, the elevation of CHOP mRNA levels was not found in muscle, liver and gut of the F/A2 neonates that were, in addition, deficient for Gcn2 (F/A2^{tg/tg}/Gcn2^{-/-}). These findings demonstrate that GCN2 activation is necessary for postnatal survival in F/A2 mice and that the induction of CHOP expression is dependent on GCN2 activation.

Discussion

Our study shows that arginine-deficient F/A2 mice suffer from an inhibition of GH secretion by the pituitary gland, from an absolute, but not a relative decline of IGF-1 production upon a GH challenge in target organs, such as liver and intestine, and from activation of the GCN2-dependent stress response. The activation of this latter response was shown to be necessary to cope with deficiency of arginine in the neonatal period.

The GH/IGF-1 axis is repressed during arginine shortage

We investigated the fate of GH in the somatotropes of the pituitary gland of 21-day-old mice, because F/A2 mice show the most pronounced phenotype at this age. The increase in GH content in the somatotropes was clearly related to the genotype of the mice and, hence, with the circulating concentration of arginine (11,13). Since the increased cellular content of GH could be due to a block in secretion or an increase in synthesis, we studied the effects of arginine on the secretion of GH in rat GH3 cells. These experiments revealed that ambient arginine concentrations below 50 μ M inhibited secretion (at 0 μ M arginine, GH content of the cells and the medium combined was ~90% of that at 100 μ M arginine). *In vivo*, the effects of arginine deficiency on growth become apparent when circulating arginine levels decline below 30-35% of the age-matched control animals (11). It is well established that GH does not affect growth until after the second postnatal week, whereas IGF-1 affects growth from the fetal stage of development onward

(35,36)). The growth characteristics of F/A2 mice, therefore, resemble those of a general deficiency of IGF-1 rather than GH. In agreement, Igf-1 mRNA levels in 2 target tissues of GH, liver and muscle, were 11-fold lower in F/A2 mice than in wild-type controls. Although GH injections elevated liver and muscle Igf-1 mRNA levels substantially, they remained 2-3-fold lower than in untreated wild-type controls. However, the absence of any effect of the GH treatment suggested that additional mechanisms inhibited growth.

Arginine deficiency induces the integrated stress response

The lack of a single amino acid is sufficient to activate the integrated stress response via GCN2 kinase (21,37). In agreement, arginine withdrawal caused phosphorylation of the translation factor eIF2 α in muscle cells *in vitro*. Of the more downstream targets of GCN2 kinase (38), CHOP was the more sensitive marker for arginine deprivation at the mRNA level, whereas ATF4 was, as expected (39), mostly regulated at the protein level. This response to arginine deprivation was seen in all tissues investigated. Based on the degree of phosphorylation of eIF2 α and the expression of CHOP mRNA, the GCN2 signaling pathway was already activated at postnatal day 1 in all tissues of F/A2 mice tested. ATF4 mRNA levels were upregulated in skeletal muscle of 1-day-old mice, but not in liver or small intestine, in agreement with the severe muscle phenotype of F/A2 mice. The arginine concentration in plasma and muscle of F/A2 mice was 3- and 4.5-fold lower, respectively, than that in control mice, with a 4-5-fold concentration gradient between plasma and muscle in both mouse lines. F/A2 hemizygotes and F/A1 homozygotes have a milder muscle phenotype, even though circulating arginine concentrations are only slightly higher than in F/A2 homozygotes (11). Furthermore, F/A2 homozygotes resume growth around weaning (11), in all likelihood due to the increase in cellular arginine concentration. In combination, these data, therefore, indicate that the GCN2 signaling pathway in muscle *in vivo* becomes activated when muscle arginine levels decrease below ~500 μ M arginine. The neonatal gut appears to have a similar sensitivity to arginine with respect to GCN2 activation, but we have no explanation why this sensitivity declines towards weaning. Furthermore, we do not know why the liver is less sensitive to activation of GCN2 by arginine. In any case, GCN2 becomes activated in muscle and intestine at intracellular levels of arginine that exceed the K_M of arginyl-tRNA synthetase >20-fold (16).

Expression of ATF4 upon activation of the stress response is primarily regulated at the translational level (40). We therefore used ATF4 immunohistochemistry in 1-day-old mice to evaluate whether the response to arginine deprivation was general or restricted to a few organs only. ATF4 protein was ~4-fold upregulated in all tissues of F/A2 mice except brain, indicating that nearly all tissues reacted similarly to arginine deficiency. This finding is in agreement with earlier data that at least one of the three known isoforms of GCN2 is expressed in any mouse organ (21). The 2-fold lower response of ATF4 protein expression in brain suggests that this organ may be relatively shielded against amino-acid deficiencies during its rapid postnatal growth phase. The cerebral expression of the protein “Impact”, which inhibits the effects of GCN2 activation by inhibiting eIF2 α phosphorylation (41), may account for this protection.

Gcn2 is necessary to survive arginine deficiency

Although our *in vitro* data implied that GCN2 mediated the effects of arginine deficiency, we used GCN2-deficient mice to demonstrate a causal relation. Since GCN2 deficiency indeed prevented eIF2 α phosphorylation and CHOP expression in F/A2 mice, the anticipated causal relation apparently exists. In addition, the experiment showed that GCN2 was essential for the survival of the arginine-deficient pups. Although it has been demonstrated previously that GCN2 is required to survive a deficiency of the essential amino acid leucine (21), our experiments with F/A2 mice show that the deficiency of a conditionally essential amino acid also activates GCN2 and that this activation is necessary for survival.

The integrated-stress response opposes the effects of GH-IGF-1 signaling

IGF-1 and the integrated-stress response signaling pathway that is activated by GCN2 have opposite effects on mRNA translation – IGF-1 promotes global protein synthesis and cell growth, whereas the integrated-stress response mediates the repression of global protein synthesis. As might perhaps be expected, the GCN2-mediated effects prevail over the IGF-1-dependent effects. In fact, we observed *in vitro* a reciprocal relation between the activation of the stress response as deduced from CHOP expression and the growth- and differentiation-promoting effects of IGF-1, with a transition between both effects at ~25 μ M arginine in the medium.

Figure 9. Proposed mechanism underlying the arginine-deficient phenotype in mice. Arginine is the precursor for NO, polyamines, and creatine synthesis, and regulates ADP-ribosylation. The developmental impairment of F/A2 mice is, however, not mediated by these molecules. Instead, the GH/IGF-1 axis did not function properly in the absence of arginine. This deficiency could not be rescued by supplementation due to activation of the Gcn2-mediated integrated stress response. The integrated stress response blocks the GH-IGF-1 axis and general protein synthesis.

Conclusion

The discussion of arginine-dependent signaling in tissues often focuses on arginine metabolites, such as NO, agmatine, polyamines, creatine, or arginine-mediated modifications such as ADP-ribosylation. This and previous studies of the F/A2 mouse (11-14) demonstrate, however, that the arginine-deficient phenotype does not involve arginine metabolism (Figure 9). Instead, arginine deficiency appears to be a signaling entity in its own right, activating the Gcn2 signaling pathway. We would like to propose that many of the effects of arginase-I overexpression are mediated by GCN2 activation (Figure 9). Similarly, growth of F/A2 neonates could be rescued by arginine-supplementation (11). Unfortunately, the premature death of F/A^{tg}/Gcn2^{-/-} mice

prevented us from definitively establishing that arginine deprivation suppresses IGF-1-mediated growth via the GCN2-mediated integrated stress response.

Our studies have further shown that the low circulating levels of arginine also suppress GH secretion. However, the low levels of tissue IGF-1 and the diminished response to exogenous GH administration probably reflect the dominant effects of GCN2 activation (Figure 9). Our study of F/A2 mice, therefore, strongly supports the conclusion that the phenotype of arginine deficiency is determined by activation of GCN2 kinase and the subsequent inhibition of the growth-promoting activity of IGF-1.

Acknowledgements

We thank Dr. O. Bakker (Endocrinology, AMC, Amsterdam) for making GH3 cells available for this study. We further thank Dr. W. Gerver (Pediatrics, azM, Maastricht) for providing us with recombinant human growth hormone.

Appendix:

Table 1. Primers and probe set specifications

primer name	sequence 5' - 3'	annealing T	manufacturer
Igf-1 Fow	CAAGCATGCGTGAGAGGATA	70	Genosys, Sigma
Igf-1 Rev	CAAACCTTTCCCTCCTTTCC	70	Genosys, Sigma
MCK Fow	AGGTTTTCCGCCGCTTCT	60	Genosys, Sigma
MCK Rev	CGGTGCCCAGGTTGGA	60	Genosys, Sigma
18S Fow	TCCGGAAGTGAAGCCATGAT	60	Genosys, Sigma
18S Rev	CGAACCTCCGACTTTCGTTCT	60	Genosys, Sigma
primer & probe set	code	annealing T	manufacturer
Chop	Mm 00492097_m1	60	ABI
Atf4	Mm 00515324_m1	60	ABI
18S	Hs 99999901_s1	60	ABI

Table 2. Antisera and incubation conditions

Antibody name	dilutions used	cat #	manufacturer
eIF2 α ; anti-mouse monoclonal	1:500	AHO0802	Biosource
eIF2 α [p51]-phospho-specific antibody; rabbit anti-mouse polyclonal	1:1000	44-728G	Biosource
ATF-4; rabbit polyclonal	1:1000	sc-200	Santa Cruz
Growth Hormone;rabbit monoclonal antibody	1:1000	4750-2809	Biogenesis
MHC; monoclonal, skeletal, fast	1;200	M4276	Sigma-Aldrich
Alexa fluor @555 goat anti-rabbit IgG	1:500	A21429	Invitrogen
Goat anti-mouse IgG FITC	1:500	M32101	Invitrogen
Goat anti-rabbit AP conjugate	1:50	A3687	Sigma-Aldrich
IRDye 800 CW Goat anti-mouse	1:50	926-32210	Licor
IRDye 680 Goat anti-rabbit	1:50	926-32221	Licor

References

1. Wu G, Morris SM, Jr. Arginine metabolism: nitric oxide and beyond. *Biochem J* 1998;336 (Pt 1):1-17.
2. Alba-Roth J, Muller OA, Schopohl J, von Werder K. Arginine stimulates growth hormone secretion by suppressing endogenous somatostatin secretion. *J Clin Endocrinol Metab* 1988;67:1186-1189.
3. Rosati B, Marchetti P, Crociani O, Lecchi M, Lupi R, Arcangeli A, Olivotto M, et al. Glucose- and arginine-induced insulin secretion by human pancreatic beta-cells: the role of HERG K(+) channels in firing and release. *Faseb J* 2000;14:2601-2610.
4. Van Haeften TW, Van Faassen I, Van der Veen EA. Repetitive stimulation of insulin secretion with arginine and glucose. *Diabetes Res* 1988;9:187-191.
5. Visek WJ. Arginine needs, physiological state and usual diets. A reevaluation. *J Nutr* 1986;116:36-46.
6. Hurwitz R, Kretchmer N. Development of arginine-synthesizing enzymes in mouse intestine. *Am J Physiol* 1986;251:G103-110.
7. Riby JE, Hurwitz RE, Kretchmer N. Development of ornithine metabolism in the mouse intestine. *Pediatr Res* 1990;28:261-265.
8. Shenoy V, Roig JC, Kubilis P, Neu J. Characterization of glutaminase in the developing rat small intestine. *J Nutr* 1996;126:1121S-1130S.
9. Windmueller HG. Glutamine utilization by the small intestine. *Adv Enzymol Relat Areas Mol Biol* 1982;53:201-237.
10. Wu G. Intestinal mucosal amino acid catabolism. *J Nutr* 1998;128:1249-1252.
11. de Jonge WJ, Hallemeesch MM, Kwikkers KL, Ruijter JM, de Gier-de Vries C, van Roon MA, Meijer AJ, et al. Overexpression of arginase I in enterocytes of transgenic mice elicits a selective arginine deficiency and affects skin, muscle, and lymphoid development. *Am J Clin Nutr* 2002;76:128-140.
12. de Jonge WJ, Kwikkers KL, te Velde AA, van Deventer SJ, Nolte MA, Mebius RE, Ruijter JM, et al. Arginine deficiency affects early B cell maturation and lymphoid organ development in transgenic mice. *J Clin Invest* 2002;110:1539-1548.
13. de Jonge WJ, Marescau B, D'Hooze R, De Deyn PP, Hallemeesch MM, Deutz NE, Ruijter JM, et al. Overexpression of arginase alters circulating and tissue amino acids and guanidino compounds and affects neuromotor behavior in mice. *J Nutr* 2001;131:2732-2740.
14. Kwikkers KL, Ruijter JM, Labruyere WT, McMahon KK, Lamers WH. Effect of arginine deficiency on arginine-dependent post-translational protein modifications in mice. *Br J Nutr* 2005;93:183-189.
15. Tsutsui M, Shimokawa H, Morishita T, Nakashima Y, Yanagihara N. Development of genetically engineered mice lacking all three nitric oxide synthases. *J Pharmacol Sci* 2006;102:147-154.

16. Vellekamp G, Sihag RK, Deutscher MP. Comparison of the complexed and free forms of rat liver arginyl-tRNA synthetase and origin of the free form. *J Biol Chem* 1985;260:9843-9847.
17. Hinnebusch AG. Translational regulation of yeast GCN4. A window on factors that control initiator-tRNA binding to the ribosome. *J Biol Chem* 1997;272:21661-21664.
18. Ron D. Translational control in the endoplasmic reticulum stress response. *J Clin Invest* 2002;110:1383-1388.
19. Wek RC, Jiang HY, Anthony TG. Coping with stress: eIF2 kinases and translational control. *Biochem Soc Trans* 2006;34:7-11.
20. Averous J, Bruhat A, Jousse C, Carraro V, Thiel G, Fafournoux P. Induction of CHOP expression by amino acid limitation requires both ATF4 expression and ATF2 phosphorylation. *J Biol Chem* 2004;279:5288-5297.
21. Zhang P, McGrath BC, Reinert J, Olsen DS, Lei L, Gill S, Wek SA, et al. The GCN2 eIF2alpha kinase is required for adaptation to amino acid deprivation in mice. *Mol Cell Biol* 2002;22:6681-6688.
22. Foulstone EJ, Huser C, Crown AL, Holly JM, Stewart CE. Differential signalling mechanisms predisposing primary human skeletal muscle cells to altered proliferation and differentiation: roles of IGF-I and TNFalpha. *Exp Cell Res* 2004;294:223-235.
23. Kim H, Barton E, Muja N, Yakar S, Pennisi P, Leroith D. Intact insulin and insulin-like growth factor-I receptor signaling is required for growth hormone effects on skeletal muscle growth and function in vivo. *Endocrinology* 2005;146:1772-1779.
24. Landreth KS, Narayanan R, Dorshkind K. Insulin-like growth factor-I regulates pro-B cell differentiation. *Blood* 1992;80:1207-1212.
25. Sumita K, Hattori N, Inagaki C. Effects of growth hormone on the differentiation of mouse B-lymphoid precursors. *J Pharmacol Sci* 2005;97:408-416.
26. Weger N, Schlake T. Igf-I signalling controls the hair growth cycle and the differentiation of hair shafts. *J Invest Dermatol* 2005;125:873-882.
27. Le Roith D, Bondy C, Yakar S, Liu JL, Butler A. The somatomedin hypothesis: 2001. *Endocr Rev* 2001;22:53-74.
28. Laron Z. Growth hormone secretagogues. Clinical experience and therapeutic potential. *Drugs* 1995;50:595-601.
29. Adriaio M, Chrisman CJ, Bielavsky M, Olinto SC, Shiraishi EM, Nunes MT. Arginine increases growth hormone gene expression in rat pituitary and GH3 cells. *Neuroendocrinology* 2004;79:26-33.
30. Liu YL, Zhong YQ, Chi SM, Zhu YL. [Effect of leptin on growth hormone secretion and apoptosis of GH3 cells.]. *Sheng Li Xue Bao* 2005;57:254-258.
31. Gebauer F, Hentze MW. Molecular mechanisms of translational control. *Nat Rev Mol Cell Biol* 2004;5:827-835.
32. Harding HP, Zhang Y, Zeng H, Novoa I, Lu PD, Calton M, Sadri N, et al. An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. *Mol Cell* 2003;11:619-633.

33. Holcik M, Sonenberg N. Translational control in stress and apoptosis. *Nat Rev Mol Cell Biol* 2005;6:318-327.
34. Lassot I, Segéral E, Berlioz-Torrent C, Durand H, Groussin L, Hai T, Benarous R, et al. ATF4 degradation relies on a phosphorylation-dependent interaction with the SCF(betaTrCP) ubiquitin ligase. *Mol Cell Biol* 2001;21:2192-2202.
35. Jones JJ, Clemmons DR. Insulin-like growth factors and their binding proteins: biological actions. *Endocr Rev* 1995;16:3-34.
36. Lupu F, Terwilliger JD, Lee K, Segre GV, Efstratiadis A. Roles of growth hormone and insulin-like growth factor I in mouse postnatal growth. *Dev Biol* 2001;229:141-162.
37. Hao S, Sharp JW, Ross-Inta CM, McDaniel BJ, Anthony TG, Wek RC, Cavener DR, et al. Uncharged tRNA and sensing of amino acid deficiency in mammalian piriform cortex. *Science* 2005;307:1776-1778.
38. Harding HP, Novoa I, Zhang Y, Zeng H, Wek R, Schapira M, Ron D. Regulated translation initiation controls stress-induced gene expression in mammalian cells. *Mol Cell* 2000;6:1099-1108.
39. Wek RC, Cavener DR. Translational control and the unfolded protein response. *Antioxid Redox Signal* 2007;9:2357-2371.
40. Yukawa K, Tanaka T, Tsuji S, Akira S. Regulation of transcription factor C/ATF by the cAMP signal activation in hippocampal neurons, and molecular interaction of C/ATF with signal integrator CBP/p300. *Brain Res Mol Brain Res* 1999;69:124-134.
41. Pereira CM, Sattlegger E, Jiang HY, Longo BM, Jaqueta CB, Hinnebusch AG, Wek RC, et al. IMPACT, a protein preferentially expressed in the mouse brain, binds GCN1 and inhibits GCN2 activation. *J Biol Chem* 2005;280:28316-28323.
42. Munn DH, Sharma MD, Baban B, Harding HP, Zhang Y, Ron D, Mellor AL. GCN2 kinase in T cells mediates proliferative arrest and anergy induction in response to indoleamine 2,3-dioxygenase. *Immunity* 2005;22:633-642.
43. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248-254.
44. Liu JL, Yakar S, LeRoith D. Mice deficient in liver production of insulin-like growth factor I display sexual dimorphism in growth hormone-stimulated postnatal growth. *Endocrinology* 2000;141:4436-4441.
45. van Eijk HM, Rooyakkers DR, Deutz NE. Rapid routine determination of amino acids in plasma by high-performance liquid chromatography with a 2-3 microns Spherisorb ODS II column. *J Chromatogr* 1993;620:143-148.
46. Ramakers C, Ruijter JM, Deprez RH, Moorman AF. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci Lett* 2003;339:62-66.
47. Ruijter JM, Thygesen HH, Schoneveld OJ, Das AT, Berkhout B, Lamers WH. Factor correction as a tool to eliminate between-session variation in replicate experiments: application to molecular biology and retrovirology. *Retrovirology* 2006;3:2.

48. van Straaten HW, He Y, van Duist MM, Labruyere WT, Vermeulen JL, van Dijk PJ, Ruijter JM, et al. Cellular concentrations of glutamine synthetase in murine organs. *Biochem Cell Biol* 2006;84:215-231.
49. Akaike H. Information theory and an extension of maximum likelihood principle. In: *Second International Symposium on Information Theory*; 1973; Akademia Kiado; 1973. p. pp. 267–281.

Chapter 3

Experiences with the construction of conditional knock out mice

Selvakumari Sankaranarayanan^{1,*}, Vincent Marion ^{1,*}, Chiel de Theije ^{1,*},
Wouter H. Lamers^{1,2} and S. Eleonore Koehler¹

¹Department of Anatomy & Embryology, Maastricht University, P.O. box 616, 6200
MD, Maastricht, The Netherlands, ² AMC Liver Center, Academic Medical Center,
University of Amsterdam, P.O. box 22660, 1100 DD, Amsterdam, The Netherlands.

*: These authors contributed equally

Table of contents

I- Introduction

II- Construction of targeting Plasmid

- a) Selection of target region to be excised
- b) Prerequisites for homologous recombination: length of homologous regions
- c) Recombinase systems
- d) Selection tools
- e) Cloning the target and flanking regions
- f) Electroporation of the targeting construct

III- Screening for homologously recombined clones

- a) Elimination of randomly integrated clones
- b) Selection of homologously recombined clones

IV- Removal of selection tools

- a) Clonality of the recombined clone (“deep split”)
- b) Removal of the neo-tk cassette

V- Injection of ES cells into blastocysts

VI- Breeding Schemes

- a) Chimeras
- b) Breeding tissue-specific knockout mice

VII- Conclusions

I- Introduction

The development of techniques that allow the modification of the DNA sequence of an allele of a gene in embryonic stem cells enables us, in combination with microinjection techniques, to deliberately modify the expression of genes in a living organism, usually the mouse. The deletion (knock-out) or replacement (knock-in) of specific genes has proven to be a powerful tool. In this chapter, we comment on our experience with the generation of conditional knockout mice of the genes that code for Argininosuccinate Synthetase (ASS; VM), Arginase 1 (Arg1; SS) and Cationic Amino-Acid Transporter 1 (CAT1; CdT).

Conventional knock-out mice (deletion of a specific gene in every cell of the organism) are often not viable. This problem can be overcome by making “conditional” knock-out mice that allow the deletion of a gene in a specific tissue or at a specific developmental stage. Deletion in specific tissues or at a specific time point can be achieved with just one genetic modification of the gene of interest, that is, the insertion of two recognition sites for an enzyme with recombinase activity. Mice harboring such a modified allele are crossed with mice expressing the recombinase under control of a promoter that determines the spatiotemporal expression of the recombinase and thereby the spatiotemporal deletion of the DNA fragment surrounded by the recombinase recognition sites. Two systems are most widely used and discussed in this chapter, viz, the cre/loxP system and the *flp*/fRT systems. LoxP and fRT refer to the recombinase recognition sites, and cre and flp to the respective recombinases (1-4). There are now more than 100 cre mice available (see <http://www.mshri.on.ca/nagy/>), permitting deletion of genes in specific cell types (macrophages, B-cells, enterocytes, hepatocytes, endothelial cells to name a few) or at specific times using a recombinase that is modified to contain the ligand-binding domain of a member of the steroid-hormone receptor family. Most often, this ligand-binding domain is modified itself to respond only to a ligand that is not endogenously produced. In practice, the combination of a modification of the estrogen-receptor ligand-binding domain and the ligand tamoxifen is most often used.

The first step in the generation of a conditional knock-out mouse is the design and construction of an appropriate vector that is then introduced into embryonic stem cells by transfection. This chapter describes a PCR-based method for vector construction and specifies screening protocols for the embryonic stem cells after transfection.

II- Construction of the targeting plasmid

A targeting plasmid consists of two regions with target homology (hereafter called flanking regions; Figure 2), the region of the gene to be deleted, and genes that are used as selection tool. The flanking regions are placed at either end of the sequence to be deleted and are identical to the sequences surrounding target sequence. The flanking regions are required for homologous recombination, that is, for the site-specific insertion of the construct into genomic DNA. In our strategy, the exon(s) or gene to be deleted is flanked by loxP sites, whereas the selection tools (neomycin phosphotransferase (“neo”) and viral thymidine kinase (“tk”)) are surrounded by frt sites.

Figure 1: Overview of the design and construction of the targeting plasmid, the selection and characterization of the targeted ES cells, and the procedure to modify the germline of mice.

Vector construction has been greatly facilitated by the availability of the complete mouse genome (<http://www.ncbi.nlm.nih.gov>, <http://www.onderzoekinformatie.nl>) and

additional information on SNPs and strain differences. This made it possible to use a PCR-based approach for vector construction, which also facilitates the introduction of additional restriction sites necessary for later cloning steps. The advantages and pitfalls of this approach will be discussed.

Figure 2: Structure of a targeting construct. The fragment to be deleted is surrounded by "loxP" sequences ("floxed"). Adjacent to the floxed sequence, a selection cassette is inserted, which itself is surrounded by "frt" sequences for later removal. The target sequence and the selection cassette are located in between the "flanking regions", which are identical to the sequences surrounding the part of the gene that is to be deleted. At both ends of the construct, fragments of the vector can be left behind to facilitate selection. See text for details.

a) Selection of target region to be excised

One can delete one or more crucial exons or the entire coding sequence to inactivate the gene of interest. Deletion of one or a few exons is often preferable, since the efficiency of the CRE recombinase seems to be at least partially dependent on the distance between the loxP sites (5).

The region chosen for deletion should either code for domains that are important for the function of the gene product (eg. the active site of an enzyme) or should result in a frame shift with premature stop codons. One should nevertheless be aware of alternative splicing which could result in a partially functional gene product excision (e.g. (6)). Literature data on lethal mutations in man and mouse should be consulted, because they can be very valuable for the development of the knockout strategy. Examples are: natural mutants (in the case of the arginosuccinate synthetase gene, we selected exon 13 for deletion, because the deletion results in a frame shift and natural mutations in this exon led to undetectable levels of enzyme activity (7) or results known from conventional knockout mice (in the case of the arginase-1 gene, we selected exon 4 for deletion, because the conventional arginase I knockout mouse was made by deleting this exon and died between 10-14 days after birth (8)).

b) Prerequisites for homologous recombination: length of the homologous regions

Between 2 and 10 kb, the relationship between the length of the homologous region and the frequency of targeting events is exponential and shows saturation at 14 kb of homology (9). Therefore, a minimum length of 2 kb is recommended for the flanking region to support optimal homologous recombination. However, 1.5 kb of homologous region at one end and 5 kb at the other end proved sufficient for efficient recombination of the Glutamine Synthetase gene (Y. HE, AMC Liver Center, Amsterdam). In our case, we used approximately 4.5 kb of homologous region on either side of the construct. Although this was thought to be advantageous for homologous recombination, long flanking regions as e.g. found in pROSA26 (10) make screening of clones by Southern or PCR more difficult. For this reason, many constructs contain a long and a short flanking region.

Comment:

An important parameter that is often neglected when selecting a target region for vector construction is the presence of repeats in the sequence to be amplified. Such repeats should be avoided since they are troublesome during amplification by PCR.

c) Recombinase systems

Cre-loxP system

The P1 bacteriophage cyclization recombination (*cre*) recombinase recognizes a pair of *loxP* sites flanking a DNA sequence. *LoxP* sites are 34 bp long and composed of an 8bp core, which determines directionality, flanked by 13 bp of palindromic sequence on either side (Figure 3A). The natural occurrence of this exact sequence is unlikely in any eukaryotic genome. Sequences flanked by *loxP* sites are said to be “floxed”. The *cre* enzyme brings the two *loxP* sites into juxtaposition. If the *loxP* sites flank a DNA segment in a cis arrangement and are oriented in the same direction, CRE recombinase mediates excision and circularization of the flanked segment (Figure 3B). If the *loxP* sites flank the DNA segment in a cis arrangement and are oriented in opposite directions, CRE recombinase mediates the inversion of the segment (Figure 3C).

*Flp-*frt* system*

The yeast *flp* recombinase (4, 11) recognizes target sites called *frt* (12, 13). A full-length *frt* site is 48 bps long and, similar to a *loxP* site, consists of an asymmetric central 8bp spacer sequence, flanked by 13bp inverted repeats (Figure 4). However, in contrast to *loxP* sites, it contains a third 13bp direct repeat at one side (14). Because the Flp/*frt* system is derived from *S. cerevisiae*, its optimal working temperature is 25-30°C. At 37°C, the activity of the enzyme is dramatically reduced (15, 16), so that Flp is less suitable and less frequently used to engineer genome modifications in mammals.

Figure 3: (A): The nucleotide sequence of loxP sites. Note the inverted repeat surrounding the asymmetric core sequence. Due to their asymmetric core, the orientation and position of the loxP sites determines the outcome of the recombination event. In conditional knockout strategies, the aim is excision of the fragment which is achieved by placing 2 loxP sites on each side of the sequence of interest in the same orientation (B). With an opposite orientation of the loxP sites, an inversion of the floxed region is obtained in presence of Cre (C).

Attempts to overcome this limitation have led to the development of an “enhanced” mutant FLP (FLPe) by cycling mutagenesis. FLPe is said to have a better thermostability and activity (16, 17) and a more efficient function in mammalian cells (16, 18).

d) Selection tools

A homologous recombination is a very rare event compared to random integration. Based on our experience, homologous recombination occurs in ~50% (ROSA locus; M. Konings) to 0.05% of all integration events (arginase I locus). Selection is therefore necessary to identify the clones in which homologous recombination has occurred

Figure 4: The nucleotide sequence of frt sites. Note inverted repeat surrounding asymmetric core sequence, which contains a convenient XbaI restriction site. Also note that the left-sided repeat needs to be present as 2 copies for higher efficiency.

and to distinguish these clones from the clones in which the modified sequence is randomly integrated into the genomic DNA. To achieve this, a positive-negative selection protocol is commonly used (19). The positive selection tool is used to identify ES cells with the targeting sequence integrated into their DNA. Neomycin phosphotransferase (neo) is often used as a positive selection marker to select for ES clones in which the construct is integrated. Only clones that have integrated the construct will be able to grow in G418-containing medium as neomycin phosphotransferase will detoxify the compound.

The negative marker can be used to select for homologous recombination of the targeting construct or to select for successful removal of the positive selection marker in the ES cells prior to blastocyst injection. As a tool to select for homologous recombination, the negative selection marker has to be placed at the outer end of one of the flanking regions, whereas the positive selection marker is placed in the middle of the construct.

Positioning the negative selection marker at one extremity of the targeting construct (Figure 6A) will lead to elimination of this marker during homologous recombination. Addition of the selection compound to the medium will then kill all cells that still express the negative selection marker due to random integration, whereas clones with homologous recombination will not be affected. Viral thymidine kinase (tk) is a commonly used negative selection marker, as it confers sensitivity to ganciclovir in the ES cells. Phosphorylated ganciclovir is a toxic substance, so that cells can only grow if thymidine kinase is not or no longer present.

Removal of selection markers

To select for successful removal of the positive selection marker, both positive and negative selection tools have to be present in between the two flanking regions (Figure 2). There are 2 strategies to remove the selection markers. One approach uses three loxP sites that surround the target sequence and the selection marker. In the other approach, the target sequence and the selection markers are surrounded by loxP and frt sites, respectively. After selection for the positive selection marker and identification of a correct clone by the appropriate screening protocols, the selection tools are removed by

Figure 5: Gene targeting using solely loxP sites can be used to inactivate a specific gene in a desired cell type. In the standard homologous recombination protocol, three loxP sites are introduced along with a selection tool “M” at the target locus “A”. Subsequent transient transfection of a cre recombinase-expression plasmid or breeding a chimeric mouse with a deleter-cre mouse results in recombination between the three loxP sites. In the desired Type I recombination, only the selection tool is removed. Both other recombinations generate conventional unconditional knockouts.

Figure 6: The two applications of a negative selection marker. In panel A, a negative selection marker is placed at either end of the construct to select against random integration. In panel B, the negative marker is placed in the middle of the construct and surrounded by recombinase recognition sites for removal after recombination.

transient transfection with either a *cre*- or *flpe*-expressing plasmid, depending on whether the selection tools are surrounded by loxP (Figure 5) or *frt* sites (Figure 2). *In vivo*, loxP sites are preferable over *frt* sites as recognition sites for a recombinase, because CRE is a more efficient recombinase than FLP and because there are many more mouse strains harboring tissue-specific expression of *cre* than *flp*.

Therefore, the gene fragment to be excised *in vivo* is usually surrounded by loxP sites. Figure 5 shows the use of 3 loxP sites for gene targeting. Transient transfection of ES cells with a *cre*-expressing plasmid will ideally yield three products: cell clones, in which only the selection tool is deleted, cell clones in which only the target sequence is deleted, and cell clones with excision of both the selection tools and the target sequence. The first type of clones is the desired outcome, whereas the second and third type of clones will generate conventional knockouts. It is unfortunately not yet possible to predict if the desired type of clones can be obtained. We, therefore, chose for an approach in which the targeted gene fragment is placed between loxP sites and both positive and negative selection tools are present in the middle of the construct in between *frt* sites (Figure 2). This allowed us to first select for integration of our constructs into the

genomic DNA and then to select for deletion of the positive-negative selection tool before the injection of ES cells into blastocysts.

Comments

1. An alternative to “select” against random integration by including a negative selection marker at the outer end of the targeting construct is the use of a high throughput screening protocol, e.g. a PCR-based screening for the presence of remaining vector sequence. This strategy will be discussed in section III A.
2. Multiple copies of loxP can lead to chromosomal instability during expression of *cre* (20).
3. An alternative to plasmid transient transfection with *cre* or *flp* to remove the selection tools is to cross mice carrying the properly recombined targeting construct that still contains the selection marker with a mouse expressing *cre* or *flp* in early development, i.e., before the germline is set apart. An example of a *cre*-expressing mouse is the *Il2a-cre* mouse (21), while the “flipper” mouse is an example of an often used *flpe*-expressing mouse (22). The availability of the efficient flipper mouse (~50% frt excision (22)) has made the use of a negative selection marker like thymidine kinase obsolete, because the positive selection marker can now be removed *in vivo*. The use of solely the *neo* selection tool and its removal *in vivo* has 3 additional advantages:
4. One round of *in vitro* selection can be avoided (see section IV B), which decreases the chance that pluripotency of the ES cell is lost; crossing the mice with a *flpe*-deleter mouse on a pure background spares one round of breeding towards a new background; a targeted allele containing *neo* is available. Such alleles are often cripple (19, 23-26), allowing one to obtain not only animals with 0, 50% and 100% gene expression of the gene of interest, but also animals with intermediate expression levels (0-50% and 50-100%).

Cloning the target and flanking regions

PCR

Homologous recombination requires a 1.5 kb - 5 kb stretch of DNA on either side of the construct that is identical to the sequence to the wild type DNA (27). The efficiency of homologous recombination depends on the degree of sequence similarity. When the

construct is generated by PCR, the use of a high fidelity of the DNA polymerase for the amplification of the flanking regions from genomic DNA is therefore a prerequisite.

The different commercially available thermostable DNA polymerases show a ~20-fold difference in error rates (28, 29). The DNA polymerases from thermophilic bacteria (e.g. *Thermus aquaticus*) miss proof-reading 3'-5'- exonuclease activity and lack the ability to synthesize products larger than 5 Kb. DNA polymerases from hyperthermophilic archaea (e.g. *Thermococcus fumiculans* ("Tfu") and *litoralis* ("Vent") and especially *Pyrococcus furiosus* ("Pfu") and *abyssi*) have low error rates, but the stronger the proofreading activity, the lower the product yield of long DNA fragments. Therefore, blends of the non-proofreading Taq and a proof-reading enzyme are often used: "LA" (long and accurate (30)) PCR. According to the manufacturer, the Accutag LA DNA Polymerase (Sigma), which we used for amplifying the homologous arms, has a 6.5-fold greater fidelity than Taq polymerase. However, in 3 different targetings, we observed 27 differences with published sequences in 6.9 kb of DNA (0.4%). Some of these differences may reflect strain differences (published sequences are often C57BL/6 or BALB/c, whereas the ES cell line came from 129P2/OlaHsd mice). In the case of arginase I construct, we found 5 mismatches in the exonic sequences which did not lead to a change in amino-acid coding. We did not correct sequences, if they did not affect a consensus splice site or a coding sequence. However, when a coding sequence was affected (e.g. (GCG (Ala) to GTG (Val) in exon 6 of the arginase-1 gene), we did correct the mismatch.

Another problem of the PCR approach that we encountered was the presence of highly repetitive sequences in the region to be amplified. In the 5'-flanking region of the ASS targeting construct, for instance, a 36 bp TA repeat was present in the middle of the 4.2 kb fragment. To amplify this sequence, we tried several high-fidelity Taq enzymes (Pfu "Ultra High" Fidelity and "Herculase" DNA Polymerases (Stratagene); AccuTaq Polymerase (Sigma), but none of them was able to amplify the sequence. Eventually, we had to break up this flanking region into 4 fragments of approx. 1 kb each.

Comment

Our choice to amplify the flanking regions using genomic rather than plasmid- (BAC-) derived DNA was based on the limited availability of BAC clones when we designed our approach. However, since BAC-derived DNA is ~30,000-fold purified per weight unit

for a particular 100 kb fragment relative to primary genomic DNA, the number of PCR cycles necessary to amplify the DNA is reduced by ~17, which strongly reduces the fidelity issue.

Electroporation of the targeting construct

Purification of the targeting construct

Prior to electroporation in the ES cells, the construct is either linearized by restriction enzymes cutting in the vector backbone or digested with two restriction enzymes to remove the vector backbone. Complete removal of the backbone of the vector has the advantage of working with a “clean” construct without additional sequences that might interfere with homologous recombination in the cell. On the other hand, linearization avoids extra steps of purification of the targeting construct. Furthermore, the additional vector sequences can be used for a PCR screening strategy to eliminate clones with random integration quickly. This approach is described in section *III.a (Elimination of randomly integrated clones)*.

Figure 7: *Panel A: Schematic representation of the electro-elution system used to purify the targeting vector prior to electroporation. The two cylindrical reservoirs communicate through a buffer compartment on top. DNA migrates in an electric field (open arrow) from the agarose slices (in large reservoir, left) onto the dialysis membrane (in small reservoir, right). Panel B: after electroelution, the DNA concentrates on the dialysis membrane. The left picture shows an efficient transfer of DNA to the dialysis membrane, whereas the right picture shows an inefficient transfer (a high proportion of DNA remained in the agarose).*

There are several methods to purify large DNA fragments by electrophoresis (purification with commercial kits, electrophoresis in low melting point agarose followed by acid-phenol extraction or electro-elution). We used electro-elution, using a bridging

system to concentrate the DNA on a dialysis membrane (Figure 7A). The purified DNA was then precipitated in ethanol for electroporation.

Comments

1 DNA that is to be used to generate transgenic animals should be protected as much as possible from UV light (i.e. day light, UV light box). Long-wave UV light or a Dark Reader® using visible light (Clarechemicals), is recommended to visualize DNA. We are aware of a case in which undue exposure to UV light caused mutations in the majority of otherwise successfully targeted ES cells.

2 Residual ethidium bromide will interfere with the efficiency of homologous recombination. Traces of ethidium bromide in a DNA preparation can be easily detected on an agarose gel from which ethidium bromide has been omitted. If DNA is detectable under UV light, the preparation should be re-extracted. For the same reason, working surfaces and equipment (UV tray; scalpels) have to be clean, when isolating fragments from agarose gels.

3 During electroelution of DNA, the agarose blocks should be stacked properly to ensure efficient transfer (Figure 7B, left picture). Floating pieces of agarose result in inefficient transfer of DNA (Figure 7B, right picture) and are a major source of DNA loss.

Choice of ES cell line

The choice of ES cell line is an important factor to achieve high success rates. The 129P2/OlaHsd-derived IB10 ES cells (a subclone from (male) E14 ES cells (31)) was used successfully by our group for the generation of 4 conditional knockout and 3 knockin mice.

Electroporation of IB10 ES cells

ES cells from an 80% confluent 25cm² culture flask were electroporated with 25 µg DNA in a final volume of 200 µL sterile PBS. At a setting of the capacitance at 1 µF, the time constant should not exceed 0.1. After electroporation, the ES cells were seeded in two 10 cm dishes, containing medium without selection compound.

Figure 8: The phenotype of ES cell pictures cultured in vitro. Picture A: a colony of undifferentiated ES cells cultured on mouse embryonic fibroblasts (MEFs). The cells are spherical and show no boundary at the circumference of the colony. Picture B: a colony of differentiated ES cell with part of the cells exhibiting a high degree of differentiation (arrow). The cells are no longer spherical. Picture C: a colony of ES cells grown in BRL-conditioned medium. Although still undifferentiated, this ES colony is very different in appearance from one grown on MEFs.

The exposure of the electroporated ES cells to the selection medium (200 $\mu\text{g/mL}$ G418) was started 24 hours after electroporation. Five days later, resistant colonies become visible, with individual colonies becoming visible after 6 to 8 days. The colonies should not be left to grow too big, since this induces differentiation of the ES cells. Figure 8A shows an undifferentiated ES colony with cells retaining their spherical shape. On the other hand, when the ES cells become differentiated, they acquire a rectangular shape (Figure 8B) and should be discarded. Series of 16 undifferentiated colonies were picked and transferred to 96-well plates containing neomycin-resistant mouse embryonic fibroblasts (neo-MEF). After 2-3 days, when the cultures are around 60% confluent, they are split onto two plates, one containing neo-MEFs and the other 150 μL of BRL (Buffalo Rat Liver) conditioned medium. The cultures on neo-MEFs were grown to semiconfluence (2-3 days) and frozen. The colonies on BRL medium were expanded for isolation of genomic DNA. Note that these ES cells look different from MEF-supported ES cells (Figure 8C).

Comment

Recombinant ES cells should be grown under selection pressure until the selection tool has been excised to suppress the growth of wild-type cells. The requirement for a “deep split” (see section IV.a.) shows that selection pressure should be a standard part of the procedure.

III- Screening for homologously recombined clones

Once neomycin-resistant clones originating from single cells have been picked, they need to be screened for proper integration of the construct into the targeted gene. The picked clones are seeded individually in 96-well plates. It was common to observe differences in the growth rate among the recombinant cells. Therefore, care was taken not to allow cells to grow too confluent, thereby avoiding differentiation. Because screening is a tedious and time-consuming procedure, it is recommended to temporarily freeze an aliquot of promising ES colonies, while going through the selection with the remaining portion of the clone. Two ways of freezing down the cells were used. A computer-assisted cooling device in which the cryotubes were placed in a temperature-controlled chamber with a reduction in the temperature of 1°C per minute to -80°C before transferring the tubes to liquid nitrogen. When this machine was not available, we cooled the cryotubes in a foam box containing liquid isopropanol based on the same principle as the Mr Frosty labware (Nalgene) in a -80°C freezer before transferring them to liquid nitrogen.

a) Elimination of randomly integrated clones

After electroporation of the targeting vector, the vector either integrates randomly in the genomic DNA (RI) or recombines homologously with the gene of interest (HR). An interesting difference between these two ways of insertion is that the extremities of the vector are kept during random integration, but lost during homologous recombination (Figure 9A). We used this difference for a preliminary test to remove clones with randomly integrated DNA. We left the multiple cloning region (MCR) on either end of the linearized fragment or only linearized targeting construct. A MCR- or vector-specific primer and a construct-specific primer were used. Since the outer ends of the construct are lost during homologous recombination, only randomly integrated clones still have the MCR- or vector-specific sequence necessary for primer binding (Figure 9B). This “short” PCR approach worked well for some targeting vectors, e.g. ASS, that is, ~70% of the clones that were randomly integrated could be eliminated. We therefore suggest linearizing the targeting construct near the shortest flanking sequence. When the construct was restricted at the outer side of the MCRs before electroporation, leaving no backbone, this short PCR did not work efficiently. Probably, the extremities of the construct are nibbled off by nuclease activity before integration. Clones yielding a PCR

product were discarded; clones negative for this PCR were treated as potential candidates for proper homologous integration and investigated further.

b) Selection of homologously recombined clones

Long PCR

Clones negative for the “short PCR” were subjected to a “long PCR”. To discriminate the recombined allele from the wild-type allele, the primers for the long PCR were chosen to be specific for the recombined allele. The target-specific primer sequences

Figure 9: Panel A: Configuration of a targeting construct after random integration or after homologous recombination. The vector-specific sequences at the ends of the targeting construct (black) remain only present if the construct is randomly integrated. Panel B: This structural feature allows a negative selection for properly integrated clones by “short” PCR. Primers are designed specifically for these sequences which will only give a band for randomly integrated clones. It is obvious that PCR at the right side (long vector sequence left) is less sensitive to “nuclease nibbling” than PCR at the left side. Panel C: Screening for homologous recombination by “long” PCR. By using primers in the endogenous DNA outside the targeting construct and in the selection marker or recombination sites, a PCR product that is unique for the recombined allele is produced. This PCR product can then be used to sequence crucial parts of the modified allele without interference from the wild-type allele.

were chosen in the selection markers, the lox P sites, or the frt sites in the center of the construct and in the genomic sequence outside that present in the targeting vector

sequence (Figure 9C). This PCR yields a specific PCR product with a known size, when homologous recombination has taken place. The PCR product is then restricted with appropriate enzymes and sequenced to confirm the exact insertion of the targeting vector. The long PCR yields very detailed information on the integration of the DNA. We, therefore, recommend performing this long PCR on both sides of the integrated vector, if this is possible. Finally, all crucial sequences are sequenced to check for any mutation.

Southern blot

Clones positive for the long PCR on both sides were further tested by Southern blot to check for clonality, that is, checked for the presence of an equal number of copies of the recombined and the unmodified endogenous allele to test for the presence of wild-type ES cells that were not removed during the positive selection procedure. This Southern-blot does also reveal the presence or absence of additional random integrations.

The DNA sequence used to probe such a Southern blot should be localized external to the flanking regions of the construct. The quality of the probe determines the quality of the result. We found that a relatively short probe that is specific and contains no repetitive elements is more efficient than longer probes containing such repetitive elements. Also, it should be close to the boundaries of the construct to avoid excessively long sequences. Exons are preferable to introns because introns more often contain repetitive sequences and, therefore, bind non-specifically to sequences elsewhere, resulting in smears as depicted in Figure 10A. However, sometimes the next exon is so far away that intronic sequences have to be used as probe. Any candidate probe should be checked for its specificity by blasting for the occurrence of relatively short sequences (>12 bp) that are shared with other regions (comparison with the mouse genomic database) and then tested on a Southern. There is no golden rule to select a sequence for a Southern probe, in the sense that intronic probes can be as good as exonic probes as shown in Figure 10B, where we used an intronic sequence of 300 bp for the Southern probe and obtained high specificity.

*Figure 10: Southern blotting as tool to identify homologous recombination. Panel A: ES-cell DNA was digested with *Xmn*-I, blotted, and hybridized with an external intronic ASS probe (1.17kb). The probe generated the same staining pattern as ethidium bromide, demonstrating non-specific hybridization. Panel B: ES-cell DNA was digested with *Bln*-I, blotted, and hybridized with a short 300bp intronic Arginase-1 probe. The wild-type allele is 6.1 kb long and the recombined allele 8.5 kb. Note the difference in intensity of the wild-type and recombined allele. The excess of wild-type DNA suggests that wild-type cells had escaped removal during neomycin selection. R: Recombinant DNA fragment; E: Endogenous DNA fragment.*

Comment

DNA isolated for genotyping may be difficult to dissolve. If the centrifugational force is limited, a more homogenous solution is usually obtained. Prolonged centrifugation during precipitation should, therefore, be avoided.

IV- Removal of selection tools

a) Clonality of the recombined clone (“deep split”)

The picked ES colonies are often contaminated with WT ES cells. These contaminant cells need to be removed before blastocyst injection. The clonality test, a Southern blot-based technique, allows us to visualize the intensity (that is, the prevalence in the population) of both the endogenous and recombinant alleles and to compare them. If the ES cells used on the blot originate from a pure recombinant clone, the intensities of the

two bands should be equal. On the other hand, if the intensity of the endogenous band is stronger than the recombinant one, there is most likely a contamination with wild-type ES cells (Figure 10B). We observed contamination with wild-type ES cells in many cases as long as we stopped the G418 selection after picking the clones. It is therefore highly recommended to continue the drug selection throughout the whole procedure. To remove the remaining wild-type cells, a deep split has to be done and the resulting “single-cell” ES cultures have to be subjected to a new round of G418 selection.

b) Removal of the neo-tk cassette

The presence of a selection cassette can interfere with the expression of a nearby gene (19, 23-26). Furthermore, ES cells that express tk are less likely to be incorporated into the male germ line (23, 32). It is therefore recommended to remove the neo-tk cassette or any other selection cassette in the selected ES-cell clones prior to blastocyst injection. The excision of the cassette can be achieved by transient expression of either *flpe* or *cre* recombinase through electroporation of either a *flpe*- or *cre*-expressing plasmid. In our constructs, we use the Cre-LoxP system to achieve specific gene inactivation, so that the Flp-Frt system remained to remove our selection cassette.

Comments

The *flpe*-expression plasmid (Figure 11A) is a fastidious plasmid. After electroporation into e.g. DH5- α bacteria according to the Biorad standard electroporation protocol, the incubation temperature of the bacteria should not exceed 30°C. At 37°C, the apparent size of the plasmid doubled (Figure 11B). Forty-eight hours after transformation, colonies were picked for inoculation of 5 mL cultures. When incubated at 30°C with a constant agitation of 180 rpm (instead of 270 rpm), these cultures yielded the proper-sized plasmids (Figure 11C). Large-scale cultures were grown under the same conditions. 25 μ g aliquots of the plasmid were stored in sterile PBS at -20°C until electroporation.

The thymidine kinase protein that is encoded in the neo-tk cassette is as stable as cellular bulk protein with an estimated long half-life of around 40 hours (33), meaning that the deletion of the gene does not make the cell insensitive to ganciclovir for several days. In order to make sure that no tk is left in the cells, they were grown in selection-free medium for 5 days before ganciclovir was added. Due to this long time period, the

cultures have to be split between transfection and selection. Care should therefore be taken that independent clones are generated. After the deep split, a pure ES cell clone was expanded to 25 cm², trypsinized, and electroporated with 25µg FLPe plasmid. One tenth of the cells were seeded onto the wells of a 6-well plate containing wild-type mouse embryonic fibroblasts (wtMEFs). Two-three days after electroporation, the cells of each 6-well were split onto two 6-well plates at a dilution of 1:30 and 1:100. After another 2-3 days, ganciclovir selection was started.

Ganciclovir is a nucleotide analogue that may cause mutations. We therefore resequenced crucial sections of the recombined alleles. In the ASS allele, we did indeed find a mutation in the targeted exon that resulted in a frameshift (ATC to TTT – Ala to Val). This mutation was not present before FLPe-mediated excision and ganciclovir selection, and would have inactivated the enzymatic activity (34).

Flpe-deleter (“flipper”) mice (22) are available and have already shown their usefulness in excising selection cassettes (see II.d.1, comment 3).

V- Injection of ES cells into blastocysts

A clone that passed all tests was karyotyped before injection into the blastocyst. Despite all quality checks, ES cells may incur damage during transfection, freezing, selection, and culture procedures that is not detected. Therefore, the karyotype of the ES cells of each ES line was routinely tested. At least 15 metaphase spreads were counted, of which at least 80% should have the proper karyotype (40 chromosomes).

To exclude artifacts due to DNA modifications in the ES cells, germ-line chimeras of at least two independent modified clones should be generated. To eliminate DNA mutations outside the locus of interest that have accumulated during many generations of *in vitro* culture of the ES cells, newly generated genetically modified mice should be bred into wild-type mice for several generations.

Figure 11: Picture A: Schematic representation of the pCAGGS- expression vector. *Flpe* expression is driven by the chimeric chicken actin/*CMV* promoter-enhancer (18). The ampicillin (*amp*) and puromycin resistance genes are present for selection in bacteria and eukaryotic cells, respectively. The *flpe* sequence harbours four amino-acid changes (P2S, L33S, Y108N, S294P) that collectively improve the *in vitro* recombination activity 4-fold at 37°C compared with wild-type *flpe* (16). Picture B: The plasmid was electroporated into *XL-1 Blue* bacteria, using a standard mini-preparation. The observed size of the supercoiled form of the 7.7 kb plasmid appeared to exceed 12 kb. Picture C: Under optimized bacterial culture conditions (180rpm; 30°C), the linearized plasmid was 7.7kb long and the uncut supercoiled form ~5.5kb.

Comment

Prior to blastocyst injection, the ES cells should pass the following checklist:

Correct DNA stoichiometry by Southern hybridization (1:1 ratio between wild-type and recombinant allele).

Morphology check (undifferentiated status, Figure 8B)

Karyotyping

VI- Breeding Schemes

a) Chimeras

A rule of the thumb is that the best – in the sense of germline transmission - chimeras should be moderately chimeric. However, we do not know in advance whether the genetically modified ES cells that we produced are still sufficiently pluripotent. In the case of arginase I, the best chimeras gave 5 floxed animals out of 20-25 offspring. Since the percentage of ES cells that populates the germline is always relatively small and since an ES cell can populate the germline without carrying the modified allele, it can be very reassuring to identify a germline transmission of a modified ES cell that, as a result of meiosis, does not carry the floxed allele. This is possible based on the coat colour of the offspring.

The 129P2/HsdOla line carries the coat colour alleles A/A and c^{ch}/c^{ch} , whereas the important colour alleles of the C57Bl/6 donors of the blastocysts are a/a and C/C. A stands for agouti and is the normal brown wild-type colour of mice, whereas “a” stands for non-agouti, making the mice black. In addition, however, the c or C alleles influence the development of the colour determined by the a/A alleles. C stands for albino, meaning a c/c mouse will be white, whereas C will support whatever colour is determined by A. The allele c^{ch} stands for chinchilla and will result in dilution of the colour determined by the A-allele, yielding beige or grey mice. An a/a, C/C mouse is a black mouse, such as the C57Bl6. An A/A, c^{ch}/c^{ch} mouse (donor of the stem cells) shows a beige coat (and red eyes). In the chimera, those cells originating from the donor blastocyst are a/a C/C, whereas those cells originating from the modified ES cells are A/A c^{ch}/c^{ch} . The fur coat therefore reveals whether offspring is chimeric.

When chimeric males are bred with C57Bl6 or 129P2/HsdOla females, the beige coat colour of the offspring will indicate that there was germline transmission of the ES cells injected into the blastocysts, independent of the demonstration of the recombinant gene. This is because the male chimera can donate either a/C (wild type) or A/ c^{ch} (derived from the ES cells) sperm cells, while a C57Bl6 mother has a/C eggs. After fertilization, the offspring will either be a/a C/C (pure C57Bl6), yielding a black and therefore wild-type animal. If the offspring is A/a C/ c^{ch} , resulting in a brown (agouti-coloured) animal, germ line transmission of the ES cell line has taken place.

Coat-colour can be used in a similar way to check for germline transmission in a crossing of male chimeras with 129P2/HsdOla mice. Again the male chimera provides

sperm cells that are either a/C or A/c^{ch}, while the female 129P2/HsdOla has A/c^{ch} egg cells. Two types of offspring colour can be expected: a brown (agouti) animal with the genotype A/a C/c^{ch}, showing that the chimera donated a wild-type allele, or a cream-coloured, red-eyed animal (like the mother), meaning that the chimera donated the colour alleles from the injected ES-cells. Therefore, cream-coloured animals are evidence for germline transmission.

Animals with a proper coat colour still need to be genotyped for genetic modification, because crossing over may segregate the floxed allele from those conferring coat colours. Nevertheless, an animal with a proper coat colour shows that the chimeric male that sired, is a promising candidate for offspring carrying the floxed allele. A chimeric mouse that only yields black offspring (>20 pups) should not be used for further breedings. Furthermore, even when brown or light-brown offspring are obtained, one might have to analyze several nests before ever getting a positive offspring showing germline transmission. For the ASS project, a chimeric male breeding with Ola females gave 10 nests with a total of 61 offspring. Although the offspring were predominantly brown, the first 52 offspring (first 9 nests) gave no positive animals for the ASS construct. We had to wait for the 10th nest to get one mouse positive for the ASS construct.

There are actually more alleles determining coat colour, that these alleles do not play a role here. Depending on the ES cell line used, it may be worthwhile to check the genetics beforehand and use this information to decide on crossings that yield a 2-fold higher rate of ES-cell derived offspring. A comprehensive review of coat colour alleles and their interaction can be found on www.jax.org by Willys K. Silvers (1979), *The coat Colors of Mice; a model for mammalian gene action and interaction*.

b) Breeding tissue-specific knockout mice

The ES cells injected into the blastocysts have one floxed allele and one wild-type allele. The chimeras obtained are bred to find offspring that carry the floxed genotype in their germline. This mouse is then bred to increase the number of floxed offspring. It is also crossed with a Cre mouse, in which *cre* is expressed under control of the EIIa promoter. This promoter is expressed at the zygotic stage, i.e. before the germline is set aside, so that we obtain a hemizygous knockout mouse (35). This hemizygous knockout will be crossed with hemizygous floxed mice.

Figure 12: Breeding scheme to generate tissue-specific knockout mice. Chimeras are bred with wild-type mice to obtain mice with 100% germline transmission and with “del-cre” mice to obtain mice which are heterozygous for the target gene. The resulting $X^{-/flox}$ mice (left arm of scheme) are interbred to obtain $X^{flox/flox}$ mice, which can be produced with a high efficiency. The heterozygous $X^{+/-}$ mice (right arm of scheme) are bred with cre mice to generate $cre^{+/-}/X^{+/-}$ mice. These mice are then crossed with the $X^{flox/flox}$ mice to obtain $cre^{+/-}/X^{-/flox}$ mice, which have gene *X* deleted in a tissue-specific manner. This scheme is advisable if *cre* is suspected to remove the floxed fragment inefficiently. However, all cells in the organism have the $X^{+/-}$ genotype. If *cre* efficiently removes floxed fragments, one can better use mice with a $cre^{+/-}/X^{flox/flox}$ genotype, because the targeted sequence is deleted in the tissue of interest only.

It takes at least three generations of mice from the chimera to the genetically modified animal (Figure 12). If we first work with a floxed allele and a tissue-specific

cre, we go for a safe breeding protocol, meaning that $X^{fl/fl}$ mice are crossed with $X^{+/-}/cre^{+/-}$ mice. The advantage of this approach is that *cre* has to inactivate only one allele in the tissue of interest, doubling the chance that the cells in the tissue of interest do not express the gene X. The disadvantage of this approach is that all cells in the body only contain 1 functional allele of gene X and, hence, usually only 50% of the normal protein content. If excision proves efficient, we prefer matings between $X^{fllox/fllox}$ and $X^{+/-fllox}/cre^{+/-}$ mice, because all cells in these mice contain 2 functional copies of gene X.

VII- Conclusions

The following are the important factors to be considered from our experience.

Constructing the targeting vector: If using the PCR-based approach to amplify the homologous arms from genomic DNA, BAC clones should be used to decrease the number of amplications. Furthermore, repeats in the regions to be amplified should be avoided as they are very difficult to amplify by PCR. Finally, sequence differences between strains should be taken into account when selecting the homologous sequences: they may interfere with the efficiency of the homologous recombination and should not be interpreted as mutations.

Selection of the recombinant ES clone: The clone to be injected into blastocysts should contain only homologously recombined cells. To achieve this, selected colonies should be continuously cultured in presence of the appropriate selection drug to avoid the growth of wild-type cells.

Screening against random integration: The “short” PCR method to eliminate the randomly integrated clones described in III.a. is an easy, fast and reliable method to eliminate randomly integrated clones, if the target DNA is not be located totally at the end of the constructs.

Screening for homologous recombination: The “long” PCR procedure to identify and characterize recombination events is faster and more informative than Southern blots. A draw back is that the primer pair cannot be tested and optimized before the recombinant clone is available.

Selection of probes for Southern blotting: Intronic probes have to be checked for the presence of repeats. A 300bp probe can suffice for sensitive and specific detection.

Selection with ganciclovir: Ganciclovir causes mutations. It is therefore necessary to resequence the clone after the excision of the selection cassette.

Coat colour as a marker for germline transmission: Coat colour as a marker doubles the chance to identify a male with proper germline transmission of the floxed allele.

Breeding set-up: The best genotypes to study physiological effects of gene knock out are flox/flox and flox/flox-Cre. In all our modified mice (ASS, Arg-1 and CAT-1), we obtained very good excision efficiency *in vivo* of both alleles with *cre*. It is therefore recommended to set up breeding schemes that yield these genotypes.

References:

1. Sauer B, Henderson N. Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proc Natl Acad Sci U S A* 1988;85:5166-5170.
2. Kuhn R, Schwenk F, Aguet M, Rajewsky K. Inducible gene targeting in mice. *Science* 1995;269:1427-1429.
3. Dymecki SM. FLP recombinase promotes site-specific DNA recombination in embryonic stem cells and transgenic mice. *Proc Natl Acad Sci U S A* 1996;93:6191-6196.
4. Sadowski PD. The FLP recombinase of the 2-microns plasmid of *Saccharomyces cerevisiae*. *Prog Nucleic Acid Res Mol Biol* 1995;51:53-91.
5. Zheng B, Sage M, Sheppard EA, Jurecic V, Bradley A. Engineering mouse chromosomes with Cre-loxP: range, efficiency, and somatic applications. *Mol Cell Biol* 2000;20:648-655.
6. McCright B, Lozier J, Gridley T. Generation of new Notch2 mutant alleles. *Genesis* 2006;44:29-33.
7. Kobayashi K, Kakinoki H, Fukushige T, Shaheen N, Terazono H, Saheki T. Nature and frequency of mutations in the argininosuccinate synthetase gene that cause classical citrullinemia. *Hum Genet* 1995;96:454-463.
8. Iyer RK, Yoo PK, Kern RM, Rozengurt N, Tsoa R, O'Brien WE, Yu H, et al. Mouse model for human arginase deficiency. *Mol Cell Biol* 2002;22:4491-4498.
9. Deng C, Capecchi MR. Reexamination of gene targeting frequency as a function of the extent of homology between the targeting vector and the target locus. *Mol Cell Biol* 1992;12:3365-3371.

10. Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet* 1999;21:70-71.
11. Sadowski PD. The Flp double cross system a simple efficient procedure for cloning DNA fragments. *BMC Biotechnol* 2003;3:9.
12. Kilby NJ, Snaith MR, Murray JA. Site-specific recombinases: tools for genome engineering. *Trends Genet* 1993;9:413-421.
13. Bode J, Schlake T, Iber M, Schubeler D, Seibler J, Snezhkov E, Nikolaev L. The transgeneticist's toolbox: novel methods for the targeted modification of eukaryotic genomes. *Biol Chem* 2000;381:801-813.
14. Andrews BJ, Proteau GA, Beatty LG, Sadowski PD. The FLP recombinase of the 2 micron circle DNA of yeast: interaction with its target sequences. *Cell* 1985;40:795-803.
15. Buchholz F, Ringrose L, Angrand PO, Rossi F, Stewart AF. Different thermostabilities of FLP and Cre recombinases: implications for applied site-specific recombination. *Nucleic Acids Res* 1996;24:4256-4262.
16. Buchholz F, Angrand PO, Stewart AF. Improved properties of FLP recombinase evolved by cycling mutagenesis. *Nat Biotechnol* 1998;16:657-662.
17. Rodriguez CI, Buchholz F, Galloway J, Sequerra R, Kasper J, Ayala R, Stewart AF, et al. High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. *Nat Genet* 2000;25:139-140.
18. Schaft J, Ashery-Padan R, van der Hoeven F, Gruss P, Stewart AF. Efficient FLP recombination in mouse ES cells and oocytes. *Genesis* 2001;31:6-10.
19. Jaenisch R. Transgenic animals. *Science* 1988;240:1468-1474.
20. Mansour SL, Thomas KR, Capecchi MR. Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. *Nature* 1988;336:348-352.
21. Holzenberger M, Lenzner C, Leneuve P, Zaoui R, Hamard G, Vaulont S, Bouc YL. Cre-mediated germline mosaicism: a method allowing rapid generation of several alleles of a target gene. *Nucleic Acids Res* 2000;28:E92.
22. Farley FW, Soriano P, Steffen LS, Dymecki SM. Widespread recombinase expression using FLPeR (flipper) mice. *Genesis* 2000;28:106-110.
23. Hug BA, Wesselschmidt RL, Fiering S, Bender MA, Epner E, Groudine M, Ley TJ. Analysis of mice containing a targeted deletion of beta-globin locus control region 5' hypersensitive site 3. *Mol Cell Biol* 1996;16:2906-2912.
24. Pham CT, MacIvor DM, Hug BA, Heusel JW, Ley TJ. Long-range disruption of gene expression by a selectable marker cassette. *Proc Natl Acad Sci U S A* 1996;93:13090-13095.
25. Clark AJ, Harold G, Yull FE. Mammalian cDNA and prokaryotic reporter sequences silence adjacent transgenes in transgenic mice. *Nucleic Acids Res* 1997;25:1009-1014.
26. Kolb AF. Genome engineering using site-specific recombinases. *Cloning Stem Cells* 2002;4:65-80.

27. Hasty P, Rivera-Perez J, Bradley A. The length of homology required for gene targeting in embryonic stem cells. *Mol Cell Biol* 1991;11:5586-5591.
28. Cline J, Braman JC, Hogrefe HH. PCR fidelity of pfu DNA polymerase and other thermostable DNA polymerases. *Nucleic Acids Res* 1996;24:3546-3551.
29. Dietrich J, Schmitt P, Zieger M, Preve B, Rolland JL, Chaabihi H, Gueguen Y. PCR performance of the highly thermostable proof-reading B-type DNA polymerase from *Pyrococcus abyssi*. *FEMS Microbiol Lett* 2002;217:89-94.
30. Barnes WM. PCR amplification of up to 35-kb DNA with high fidelity and high yield from lambda bacteriophage templates. *Proc Natl Acad Sci U S A* 1994;91:2216-2220.
31. Robanus-Maandag E, Dekker M, van der Valk M, Carrozza ML, Jeanny JC, Dannenberg JH, Berns A, et al. p107 is a suppressor of retinoblastoma development in pRb-deficient mice. *Genes Dev* 1998;12:1599-1609.
32. Hasty P, Rivera-Perez J, Chang C, Bradley A. Target frequency and integration pattern for insertion and replacement vectors in embryonic stem cells. *Mol Cell Biol* 1991;11:4509-4517.
33. Sherley JL, Kelly TJ. Regulation of human thymidine kinase during the cell cycle. *J Biol Chem* 1988;263:8350-8358.
34. Gao HZ, Kobayashi K, Tabata A, Tsuge H, Iijima M, Yasuda T, Kalkanoglu HS, et al. Identification of 16 novel mutations in the argininosuccinate synthetase gene and genotype-phenotype correlation in 38 classical citrullinemia patients. *Hum Mutat* 2003;22:24-34.
35. Lakso M, Pichel JG, Gorman JR, Sauer B, Okamoto Y, Lee E, Alt FW, et al. Efficient in vivo manipulation of mouse genomic sequences at the zygote stage. *Proc Natl Acad Sci U S A* 1996;93:5860-5865.

Chapter 4

Physiological adaptation to inactivation of intestinal arginine biosynthesis in suckling mice

Vincent Marion¹, Selvi Sankarayanan¹, Chiel de Theije¹, Paul van Dijk¹,
Theo B.M. Hakvoort², Wouter H. Lamers^{1,2} & S. Eleonore Köhler¹

1: Department of Anatomy & Embryology, Maastricht University, Postbus 616,
6200 MD, Maastricht, The Netherlands

2: AMC Liver Center, Academic Medical Center University of Amsterdam,
Postbus 22660, 1100 DD Amsterdam, The Netherlands

Summary

In mice and man, neonatal deficiency of ornithine transaminase and ornithine transcarbamoylase causes severe hypoargininemia, because endogenous citrulline synthesis must offset the deficient supply of arginine via the milk. Suckling mammals, including mice, differ from adults in the abundant expression of enzymes synthesizing arginine from citrulline in their enterocytes, but arginine can also be synthesized outside the gut. The importance of the small intestine for whole-body arginine synthesis was tested by crossing “Ass^{fl/fl}” mice (that have exon 13 of the argininosuccinate-synthetase gene surrounded by loxP sites) with “VilCre^{tg/+}” mice (that transgenically express Cre in their enterocytes). To reduce the extra-intestinal capacity to synthesize arginine, VilCre mice were also crossed with ASS^{-fl} mice. In suckling VilCre^{tg/+}/Ass^{fl/fl} and VilCre^{tg/+}/Ass^{-fl} mice, ASS was completely deleted in the enterocytes, but no signs of the arginine-deficiency syndrome (hypoargininemia, deficient muscle and hair growth, or underdevelopment of Peyer’s patches) developed. The systemic loss of one Ass allele, nevertheless, increased circulating citrulline with ~50μM and the loss of intestinal ASS expression with an additional ~80μM. Using microspheres, we showed that 80±3% of the transhepatic blood flow of neonatal day 14 mice originated in the portal vein and 19±3% in the hepatic artery. Kidney perfusion was only 20±3% of that through the splanchnic region. Relative to Ass^{+/+} mice, arginine production in the splanchnic region was abolished in all experimental genotypes, whereas citrulline production had increased. The elimination of ASS expression in the enterocytes with genetic tools demonstrates that arginine biosynthesis in the small intestine is dispensable for murine neonates.

Abbreviations:

ASS:	argininosuccinate synthetase [EC 6.3.4.5];
ND:	neonatal day;
VilCre ^{tg/-} mice:	mice that transgenically express Cre in their small-intestinal enterocytes;
VilCre ^{tg/-} /Ass ^{+/+} mice:	control animals;
VilCre ^{tg/-} /Ass ^{fl/fl} mice:	mice with absent ASS expression in the enterocytes only;
VilCre ^{tg/-} /Ass ^{+/-} mice:	mice with a single functional Ass allele only;
ASS-KO/I mice:	mice without ASS expression in the small-intestinal enterocytes (VilCre ^{tg/-} /Ass ^{-/fl} or VilCre ^{tg/-} /Ass ^{fl/fl}).

Introduction

Arginine is a substrate for the synthesis of nitric oxide, urea, ornithine, citrulline, creatine, agmatine, glutamate and proline (1, 2). The rate-determining enzyme of arginine synthesis from citrulline and aspartate is argininosuccinate synthetase (ASS) (EC 6.3.4.5) (3). The endogenous biosynthetic capacity for arginine suffices to meet the daily requirement under normal conditions, but a dietary source of arginine becomes necessary when demand increases under anabolic or catabolic conditions (4-6). For this reason, arginine is a conditionally essential amino acid.

These general conclusions apply to postweaning individuals that can adapt their sources of food. However, neonatal arginine metabolism differs considerably from that in the adult, probably because the rapidly growing suckling animals have no choice of food. Since the supply of arginine via the milk is not sufficient to support protein synthesis (7-9), suckling animals need a higher endogenous capacity for arginine synthesis. The abundant expression of the enzymes necessary for *de novo* arginine biosynthesis in the enterocytes of the small intestine of perinatal rodents (10-13), piglets (14-17) and humans (Koehler et al., submitted) and the absence of cytosolic arginase expression (18, 19) implicate the intestine as an important site for neonatal arginine production. In fact, it has been demonstrated that the small-intestinal mucosa accounts for ~50% of the endogenous production of arginine in normally fed suckling piglets and that hypoargininemia develops when this source of arginine production is bypassed as during parenteral feeding (18, 20-22). Finally, we have shown that suckling mice that transgenically overexpress arginase-I in the small-intestinal enterocytes suffer from a deficient hair and muscle growth and underdevelopment of Peyer's patches due to impaired B-cell maturation (23, 24). The capacity of the intestine to produce arginine becomes limited to citrulline synthesis in the weaning period, when the expression of ASS and ASL in the intestines declines in humans and pigs (Koehler et al, submitted) (10, 25-27), and stops in rodents (10, 25-27). It is, however, not known whether intestinal arginine biosynthesis is essential for suckling animals, as would be expected from the above mentioned arguments.

To specifically address the question to what extent small-intestinal arginine synthesis is essential for suckling mice, we have eliminated the expression of argininosuccinate synthetase (ASS) in the enterocytes, using the Cre-loxP recombinase

system. Since the homozygous deletion of *Ass* is lethal (28), exon 13 of the murine *Ass* gene was surrounded by loxP sites, using genetic techniques. Excision of the “floxed” exon was accomplished using the transgenic *VilCre* mouse (29), which expresses the recombinase Cre protein in the enterocytes of the small intestine only. Suckling *VilCre^{tg/-}/Ass^{-/-}* and *VilCre^{tg/-}/Ass^{fl/fl}* mice were completely devoid of ASS in their enterocytes, but unexpectedly did not suffer from growth retardation or, as far as we have been able to establish, any other functional impairment. Even though intestinal citrulline synthesis is essential for neonatal mice to maintain normal circulating arginine levels (30), the present model shows that intestinal arginine biosynthesis is not essential for normal growth and development and implicates that other tissues can compensate for the loss of arginine synthesis in the gut.

Methods

Plasmid construction and recombinant ES cell selection

The mouse *Ass* gene is located on chromosome 2. Protocols were obtained from (31, 32). The targeting vector (14.384 Kb) consisted of the 3' 4.2 Kb of intron 12, a Neo-TK selection cassette flanked by *frt* sites (33, 34), exon 13 flanked by loxP sites (35, 36) and the 5' 3.5 Kb of intron 13 (Figure 1). The targeting construct was sequence verified with respect to splice junctions, exon 13 and recombinase-recognition sites, digested with *AscI* and *PmeI* (introduced by PCR for cloning purposes), and purified by electrophoresis and electro-elution. Twenty-five µg of the targeting fragment was electroporated into the mouse ES cell line E14IB10 (129/Ola). Selection with G418 (200 µg/mL) was started 24 hours after electroporation. Short vector sequences that were left on either end of the targeting construct allowed a PCR-based screen against random integrations. Long-distance PCR, using AccuTaq (Sigma), an ASS-specific primer outside the targeting construct and a loxP-specific primer, was used to demonstrate homologous recombination at the 3' side in the remaining clones. Proper recombination of the 5' end was demonstrated with Southern blotting and probing with a Neo fragment. The Neo-TK cassette was removed by transient transfection with an FLPe recombinase expression vector (kindly provided by Dr. Francis Stewart, EMBL, Germany). Selection with ganciclovir (5µM) was started 5 days after electroporation. The recombined allele was again sequence verified (ganciclovir introduced a mutation in 1 of 4 clones tested). Two clones containing 40 chromosomes were selected for blastocyst injection.

Generation of transgenic mice and husbandry

ASS-recombinant ES cells were injected into C57Bl/6J blastocysts. Chimeric males were bred with females on an Ola background (The Jackson Laboratories, Bar Harbor, Maine) and with deleter-Cre females on a C57Bl/6 background (kindly provided by Dr. Ursula Lichtenberg, University of Cologne, Germany) to delete Ass exon 13 in the germ line (37). Mice were genotyped with primers ASS-F1 and ASS-R1 (supplementary Table 1, Figure 1), yielding a 360 bp wild-type allele and a 390 floxed allele. The Cre-excised allele was detected by PCR with the primers ASS-F2 and ASS-R1. To specifically delete the floxed ASS allele (Ass^{fl}) in the enterocytes of the small intestine, mice were crossed with VilCre mice (29) that were obtained from Jackson Laboratories. Animals were genotyped for the presence of VilCre by PCR with primers Vil-F and Vil-R (see supplementary Table 1), yielding a 1,100 bp band for VilCre-positive animals.

Animals were housed in humidity- and temperature-controlled rooms on a 12-hour light/ 12-hour dark cycle with food and water ad libitum. The animal studies were reviewed and approved by the institutional animal care and ethical committee (DEC) of Maastricht University.

Blood and tissue collection

Animals were sacrificed by decapitation on neonatal day (ND) 14, 18, 21, and 35. Blood samples from the hepatic vein, portal vein, renal vein and left ventricular cavity were taken from 14-day-old mice under 2% isoflurane (Abbott, # B506)/ 90% O₂ anesthesia, as described for adult mice (38). Tissues were fixed in 4% buffered formalin and embedded in paraplast.

Amino-acid concentration

Blood was collected into heparin-coated tubes and centrifuged at 2,000*g for 5 min at 4°C. 80 µL of plasma was added to 6.4 mg of lyophilized sulphosalicylic-acid, vortexed and stored at -20°C. Amino-acid concentrations were determined by HPLC as described (39). Amino-acid fluxes were calculated as described (38).

Blood flow distribution using radioactive microspheres

Radioactive microspheres (15 µm diameter), labeled with the radioisotope Ruthenium-103, were purchased from Perkin Elmer USA (NEM082A100UC). Fourteen-day old mice (~6 gram bodyweight) were anesthetized by intraperitoneal injection of 100 µg/g ketamine (Pfizer)/ 20 µg/g xylazine. After opening the thorax through the fourth left intercostal space, 50 µL of a 0.01% Tween-80/saline solution containing 10,000 microspheres (0.56 µCi) was injected into the left ventricle of the mouse. Organs were isolated, transferred to a plastic counting vial containing 2 mL of saline and measured with a gamma counter (Wallac 1480).

Western blotting and histology

Tissues were crushed in a liquid nitrogen-cooled mortar, sonicated in RIPA buffer (25 mM Tris•HCl (pH=7.6), 150 mM NaCl, 1% NP-40, 1% Na-deoxycholate, 0.1% Na-dodecylsulfate), containing a protease inhibitor cocktail (Complete; ROCHE, The Netherlands). Twenty-five µg of protein, as determined with the Bradford assay, were loaded per lane. ASS was visualized by incubation the blots with rabbit anti-mouse ASS antiserum (1:1,500; (23, 24)), followed by alkaline phosphatase-coupled goat anti-rabbit antibody and “Super Signal West Femto Maximum Sensitivity Substrate” (Pierce # 34095). Duodenum (2 cm distal to the pyloric sphincter) and ileum (2 cm proximal to the ileocecal junction) were sectioned at 7 µm. Goblet cells were visualized with 1% Alcian blue (BDH, The Netherlands) in 3% acetic acid (pH = 2.5) for 30 min. Nuclei were visualized with 1% Nuclear Fast Red (Chroma, Fluka) for 3 min. For immunostaining, sections were subjected to antigen retrieval (10 min at 100°C in 10 mM Na citrate (pH 6.0)), blocked with Teng-T (10 mM Tris (pH=7.6), 5 mM EDTA, 150 mM NaCl, 0.25% gelatin, 0.05% Tween-20) /10% normal goat serum (NGS, Gibco, The Netherlands) for 30 min, and incubated overnight in a 1:1,000 dilution of the ASS antibody (23, 24) in Teng-T/10 % NGS. After washing with PBS and another blocking step with Teng-T /10% NGS for 15 min, sections were incubated with goat anti-rabbit antiserum (Sigma A3687; diluted 1:200 in Teng-T /10% NGS) for 90 min. After washing, the slides were incubated with nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP; Roche 169471) at 37°C until staining reached an optical density not exceeding 0.8.

Visualization of Peyer's patches

The small intestine was immersed for 5 min in 10% acetic acid (v/v) before fixing them in 10% neutral-buffered formalin, as described previously (40).

Quantitative PCR

RNA was extracted from cells and tissues with guanidinium thiocyanate (TRI Reagent, Sigma). RNA integrity was assessed by denaturing gel electrophoresis and RNA concentration with a NanoDrop-ND1000 spectrophotometer (Isogen Life Sciences). One μ g of total RNA was reverse transcribed with the iScript cDNA synthesis kit (Bio-Rad). PCR was performed using iQTM SYBR® Green Supermix (Biorad 170-8882) in an iQ5 real-time PCR detection system (Biorad). The extension temperature was 60°C for all primer pairs. cDNA samples were diluted 60-fold before use for mRNAs and 600-fold for 18S. Primary fluorescent data were exported and analyzed using the Lin-RegPCR program (41). All primers (supplementary Table 1) were obtained from Genosys (Sigma).

Statistics

Between-session variation in replicate experiments was corrected using Factor Correction (42). All quantitative values are presented as means $\pm$ SEM. Statistical differences for the amino acids fluxes between genotypes were based on homogeneity of variances between the blood sources per genotype followed by one-way analysis of variances using known means and standard deviations. P-values were calculated through multiple comparisons (43).

Results

Characterization of Ass conditional knockout mouse

To study the function of ASS in the enterocytes of the small intestine, we generated a conditional ASS knockout, in which the critical exon 13 is flanked by loxP sites. Mice with $Ass^{-/n}$ and $Ass^{n/n}$ genotypes are healthy and fertile. Figure 2A shows that flanking exon 13 with loxP sites does not affect ASS protein expression. To test if deletion of exon 13 in the mouse is as effective (lethal) as deletion of exon 4 (28), we interbred $Ass^{+/-}$ mice (generated by crossing $Ass^{+/n}$ mice with deleter-Cre mice (37)). Figure 2B demonstrates that none of the $Ass^{-/-}$ neonates survived longer than 1 day after birth, as

Figure 1. Production and molecular analysis of conditional *Ass* knockout mice. Panel A: schematic representation of the *Ass*-targeting construct (*Ascl* - *PmeI*). The *Ascl* and *PmeI* sites flanking the construct (14.38 Kb) were introduced by PCR. The *XmnI* and *EcoRI* sites upstream from the recombination arms of the targeting construct were used for Southern-blot characterization. Panel B: Identification of successfully targeted ES cells. Top subpanel: PCR amplification of vector sequences downstream of *Ascl* (left) or upstream of *PmeI* (right) identify clones that carried random integrations of the targeting vector. Middle subpanel: Long PCR to demonstrate homologous recombination on 3' side with primer in *loxP* site and primer downstream of *PmeI*, yielding a band of 3.7 kb. Bottom subpanel: Southern blot of ES cell DNA with proper 3' homologous recombination after digestion with *XmnI* and *EcoRI*. Two fragments of the expected size (8.663 kb and 10.158 kb) were detected with the internal *Neo* probe. Panel C: Wild-type (*Ass*⁺) exon-13 allele, floxed (*Ass*^{fl}) allele, and *Ass*-deficient (*Ass*⁻) allele, together with remnant *frt* and *loxP* sites. *F1*, *F2*, and *R1* indicate the position of the primers used to identify these genotypes (for sequence, see supplementary Table 1). Panel D: Mouse genotyping. Top subpanel: The *F1* and *R1* primer pair yield a band of 360 bp for the *Ass*⁺ allele and 390 bp for the *Ass*^{fl} allele. Middle subpanel: The *F2* and *R1* primer pair yield a band of 340 bp for the *Ass*⁻ allele. Bottom subpanel: The presence of the *VilCre* construct was detected with primer pair *Vil-F* and *Vil-R*, giving a band of ~1,100 bp.

was found for the conventional knockout mouse (28). Together, these data show that mice harboring the *Ass*^{fl} allele are a good model to investigate the effects of tissue-specific *Ass* deletions.

VilCre efficiently and specifically eliminates Ass in the enterocytes

To evaluate the development of the small intestine in the absence of ASS expression in the enterocytes, intestines of ND14, ND18, and ND21 mice were sectioned and stained with haematoxylin and eosin or with Alcian blue and Nuclear Fast Red. No qualitative differences in the architecture of the duodenum and ileum were found between VilCre^{tg/-}/Ass^{fl/fl}, VilCre^{tg/-}/Ass^{-fl} and VilCre^{tg/-}/Ass^{+/+} mice at any of the ages investigated (not shown). Staining the duodenum and the ileum of these mice (Figure 3) for the presence of ASS reveals a complete elimination of ASS expression in the enterocytes, whereas ASS expression in the enteric ganglia remained unaffected. Although both ASS and villin are expressed in the proximal tubules of the kidney (44, 45), the VilCre transgene is not (29). Accordingly, we did not observe ablation of ASS expression in the kidney (Figures 2C and 3).

ASS expression in the skin was lower in VilCre^{tg/-}/Ass^{fl/fl} mice (Figure 2C). Although we did not anticipate ablation of Ass in the skin, both ASS and villin are coexpressed in the epidermis (46, 47). Development in the absence of ASS expression in the enterocytes F/A2 mice, which cannot synthesize arginine in their enterocytes due to the transgenic expression of arginase-1 in these cells, suffer from impaired hair and muscle growth and underdevelopment of Peyer's patches due to impaired B-cell development during the suckling period (23, 24).

Despite the complete elimination of ASS expression in their enterocytes, VilCre^{tg/-}/Ass^{-fl} and VilCre^{tg/-}/Ass^{fl/fl} mice show an unimpaired increase in body weight (Figure 3D), hair growth (Figure 3E), and development of Peyer's patches (Figure 3F).

Changes in the circulating concentration of amino acids in the absence of ASS expression in the intestine

Circulating amino acids in arterial, portal, hepatic and renal blood of Ass^{+/+}, Ass^{+/-}, Ass^{-fl}, and Ass^{fl/fl} mice expressing Cre in their enterocytes (VilCre^{tg/-}) were determined at ND14 (supplementary Table 2). In addition, the summed concentrations of all amino acids are presented. Compared to wild-type mice, no significant changes in the circulating concentration of arginine were observed in the arterial blood of the experimental groups, while its levels had decreased to ~70% of wild-type values in the portal blood of Ass^{-fl} mice and to ~70% of wild-type values in the hepatic blood of all

three experimental groups. In the renal vein, arginine concentrations had increased to 130% of control values in of $Ass^{+/-}$ mice.

Circulating levels of the arginine precursor citrulline changed more profoundly (Figures 4 and 5B), increasing 1.5-2-fold in the arterial and portal blood, and 2-3-fold in the renal-vein blood of all experimental animals. In the hepatic vein, citrulline levels had increased ~1.6- and ~2.5-fold in $Ass^{+/-}$ and $Ass^{-/-}$ mice, respectively. These changes could be attributed to a ~50 μ M increase for the systemic loss of one functional *Ass* allele, while the loss of the capacity to synthesize arginine in the enterocytes amounted to approximately 80 μ M (Figure 5B). We could not reliably determine the level of aspartate, the other substrate of arginine biosynthesis. Changes in the levels of further upstream substrates, such as ornithine, glutamate, and alanine (Figure 5A), did not change in a uniform manner (Figure 4). Lysine, which shares the transmembrane CAT transporters with arginine, had decreased markedly in the portal vein of $Ass^{-/-}$ mice only (Figure 4).

Changes in the fluxes of amino acids in the absence of ASS expression in the intestine

To be able to estimate the fluxes across the intestine, liver and kidneys of the suckling ND14 mice, we determined the distribution of radioactive microspheres injected into the left ventricular cavity (Figure 5C). At this age, $80 \pm 3\%$ of the transhepatic blood flow originates in the portal vein and $19 \pm 3\%$ in the hepatic artery.

We were not able to measure references samples in the abdominal aorta of these small mice, so that we could only use the data to calculate relative metabolic fluxes in the portal drained viscera and liver (therefore expressed as “units” in Figure 4). Assuming no large differences in the cardiac output between the genotypes (all genotypes grew equally well during the entire suckling period (Figure 3D), we calculated the flux of these amino acids across the portal-drained viscera, the liver, and the kidneys.

Figure 2. Biological effects of modification of exon 13 of the mouse *Ass* gene. Gray bars indicate VillCre^{tg}/Ass^{+/+} and black bars VillCre^{tg}/Ass^{fl/fl} mice. Panel A: Western blot demonstrating that the loxP sites surrounding exon 13 do not affect ASS expression at the protein level (25 μ g total protein was loaded per lane; quantification of band intensity on right-sided subpanel). Panel B: Cre-mediated homozygous elimination of exon 13 causes neonatal death, as was previously shown for elimination of exon 4 (58, 59). Panel C: ASS protein in ND14 VillCre^{tg}/Ass^{fl/fl} mice is eliminated in the enterocytes of the small intestine only. Note that the residual ASS expression in the small intestine reflects the presence of the ASS-positive enteric ganglia (see Figure 3). Panels D-F: VillCre^{tg}/Ass^{fl/fl} mice grow normally during the first 4 postnatal weeks (D), have normal hair growth (ND14; E) and normal development of Peyer's patches in the small intestine (ND14; F).

Figure 3. Expression of ASS in the small intestine, kidney and liver of ND14 mice. Gray bars indicate *VilCretg*^{-/-}*Ass*^{+/+} and black bars *VilCretg*^{-/-}*Ass*^{fl/fl} mice. ASS protein expression in the duodenum (left 2 columns) and ileum (right 2 columns) of control (gray bar) or *VilCretg*^{-/-}*Ass*^{fl/fl} animals (black bar) is visualized by semiquantitative immunohistochemistry. The second row of panels shows magnifications of the top row. Note that expression of ASS in controls is limited to the top portion of the villus. Further note that the elimination of ASS expression is complete in the enterocytes of *VilCretg*^{-/-}*Ass*^{fl/fl} mice, but unaffected in their enteric ganglia (arrows). The bottom row of panels shows expression of ASS in the S3 segment of the proximal tubule of the kidney and the periportal region of the liver of *VilCretg*^{-/-}*Ass*^{+/+} and *VilCretg*^{-/-}*Ass*^{fl/fl} mice. Bars: 8 μ m (overviews) and 1 μ m (magnifications).

Figure 4. Circulating levels and fluxes of amino acids in ND14 mice. Panel A: Circulating concentrations of arginine (Arg), citrulline (Cit), ornithine (Orn), glutamate (Glu), alanine (Ala), lysine (Lys) and the sum of all amino acids (Σ AA) in *VilCretg*^{-/-}/*Ass*^{+/+} (white), *VilCretg*^{-/-}/*Ass*^{fl/fl} (light gray), *VilCretg*^{-/-}/*Ass*^{+/-} (dark gray), and *VilCretg*^{-/-}/*Ass*^{-/-} (black) mice. The source of the blood is indicated. **Panel B:** Production and consumption of arginine, citrulline, ornithine, glutamate, alanine, lysine, and the sum of all amino acids across the splanchnic region, portal-drained viscera (PDV) and liver, assuming the blood flows as determined by microsphere injection (Figure 5C).

Relative to *Ass*^{+/+} mice, the production of arginine by the splanchnic region had become non-significant in all experimental genotypes, whereas citrulline production was increased, as expected, in *Ass*^{fl/fl} and *Ass*^{-/-} relative to *Ass*^{+/+} mice. The sum of all amino acids produced in the splanchnic region was similar in *Ass*^{+/+} and *Ass*^{-/-} mice, but ~1.5-fold lower in *Ass*^{+/-} and ~4-fold lower in *Ass*^{fl/fl} mice than in *Ass*^{+/+} mice (Figure 4). The differences between the respective genotypes in the splanchnic production of most amino

acids followed this trend (supplementary Table 3) only in $Ass^{-/n}$ mice, whereas citrulline production had increased in only $Ass^{n/n}$ mice. Ornithine production was highest in $Ass^{+/+}$ and $Ass^{n/n}$ and lowest in $Ass^{+/-}$ mice, while glutamate production was similar in all genotypes. Lysine production was highest in $Ass^{n/n}$ and lowest in $Ass^{-/n}$ mice. As noticed for the splanchnic region, the differences between the respective genotypes in the intestinal production of the remaining amino acids (supplementary Table 3) could be largely understood from the fact that the sum of all amino acids produced was similar in $Ass^{+/+}$ and $Ass^{n/n}$ mice, but ~2-fold lower in $Ass^{+/-}$ mice and ~4-fold lower in $Ass^{-/n}$ mice than in $Ass^{+/+}$ mice (Figure 5).

The liver of $Ass^{n/n}$ and $Ass^{+/-}$ mice consumed some arginine, while citrulline was released from the liver of $Ass^{-/n}$ mice and ornithine from that of $Ass^{+/+}$, $Ass^{+/-}$, and $Ass^{-/n}$ mice. The liver of $Ass^{n/n}$ mice was a net consumer of amino acids, whereas the sum of all amino acids produced in the liver of $Ass^{+/+}$ mice was similar to that of $Ass^{+/-}$ and $Ass^{-/n}$ mice, although the production in $Ass^{-/n}$ livers was almost 2-fold bigger than that in $Ass^{+/-}$ livers (Figure 5). The differences between the respective genotypes in the hepatic production of most amino acids followed this trend (supplementary Table 3).

Adaptive changes in metabolism of the kidney.

The main difference between the genotypes in the concentration of amino acids in the renal vein were those of citrulline, that was ~2-fold increased in $Ass^{+/-}$ and $Ass^{fl/fl}$ and ~3-fold in $Ass^{-/fl}$ mice relative to $Ass^{+/+}$ mice ($260 \pm 15 \mu M$; supplementary Table 2). However, arginine levels were not different in the experimental genotypes relative to the $Ass^{+/+}$ genotype. Furthermore, branched-chain amino acids, reflecting similar trends in valine, isoleucine and leucine, were ~1.5-fold increased in $Ass^{fl/fl}$ relative to $Ass^{+/+}$ mice. Due to the relative tiny rate of perfusion of the kidneys ($20 \pm 3\%$ of that through the splanchnic region), the contribution of the kidney to amino-acid metabolism is still minor at 14 days after birth (supplementary Table 3).

Discussion

The main findings of the present study are that the elimination of ASS expression from the enterocytes of the small intestine of suckling $VilCre^{tg/-}/Ass^{n/n}$ and $VilCre^{tg/-}/Ass^{-/n}$ mice was complete and specific for enterocytes (Figure 3), but unexpectedly did not affect the animal's growth and development. In agreement, no differences in the

do so in animals in which the expression of the “floxed” gene is eliminated in the targeted tissue only ($Ass^{fl/fl}$ genotype) or the expression of Ass in other ASS-positive cells is additionally reduced by 50% ($Ass^{-fl/fl}$ genotype). In the latter mice, the compensatory capacity of arginine-producing cells outside the gut would presumably be compromised. We chose to surround exon 13 with loxP sites, as deletion of this exon causes the complete loss of ASS expression in human neonates (48). Floxing exon 13 of the mouse Ass gene did not affect its expression (Figure 2A), whereas the Cre-mediated deletion of exon 13 in the germ line caused neonatal death, as was observed after homozygous deletion of exon 4 in the mouse (Figure 2B; (28)).

Elimination of exon 13 in the small-intestinal enterocytes by VilCre-mediated excision resulted in undetectable levels of the ASS protein in the targeted cells only (Figures 2 and 3). The observation that excision of 1 floxed Ass allele (as in $Ass^{-fl/fl}$ mice) was as complete as that of 2 alleles (as in $Ass^{fl/fl}$ mice) demonstrates the highly efficient excision obtained with the VilCre transgene and the easy accessibility of the floxed Ass alleles to the Cre enzyme. It was, therefore, to some extent unexpected that the VilCre-mediated complete elimination of Ass from the enterocytes of neonatal mice did not cause hypoargininemia or any other detectable arginine-dependent deficiency, irrespective of whether these mice carried the $Ass^{fl/fl}$ or $Ass^{-fl/fl}$ genotype. We, therefore, carefully evaluated the consequences of the genetic modification. Since the gut of ASS-KO/I mice still contained ~20% of ASS that was present in control mice (Figure 2C), we checked ASS expression throughout the small intestine and found it absent in the enterocytes only (Figure 3). ASS expression in other tissues, such as the enteric ganglia, kidney tubules, and hepatocytes was not affected (Figures 2 and 3).

The metabolic consequences of intestinal deletion of ASS

We had anticipated that we would be able to differentiate the effects of the Ass^{fl} and Ass^{-fl} alleles by pair-wise comparisons of the $VilCre^{tg/+}/Ass^{+/+}$, $VilCre^{tg/+}/Ass^{+/-}$, $VilCre^{tg/+}/Ass^{fl/fl}$, and $VilCre^{tg/+}/Ass^{-fl/fl}$ mice, but the metabolic consequences of deleting ASS in the enterocytes proved less distinct than those of overexpression arginase-1 in these cells (23, 24). The most remarkable difference was, of course, the maintenance of a normal circulating arginine level in the ASS-deficient mice and the selective decline of this level in arginase-overexpressing mice. The present finding that arterial arginine levels remain in the control range in animals without intestinal arginine synthesis

demonstrates that extra-intestinal tissues must have a considerable and adaptively adjustable capacity to synthesize arginine. Unfortunately, we have not yet been able to identify this tissue, even though its capacity still suffices in the $\text{VilCre}^{\text{tg}/-}/\text{Ass}^{-/\text{fl}}$ genotype, which misses both the intestinal contribution to arginine synthesis and 50% of the whole-body capacity due to the ubiquitous presence of the inactive Ass^{-} allele. The kidneys do contain a sizable concentration of ASS, but only have a marginal metabolic role in suckling mice, as their perfusion is still minor. Only for citrulline, we found a significant correlation between circulating concentration and genotype, the concentration increasing $\sim 50 \mu\text{M}$ for the systemic loss of one functional Ass allele and $\sim 80 \mu\text{M}$ for the loss of the capacity to synthesize arginine in the small intestines.

We have tried to assess the fluxes of amino acids across the portal drained viscera, liver and kidney. The most pronounced differences were found between the $\text{VilCre}^{\text{tg}/-}/\text{Ass}^{\text{fl}/\text{fl}}$ $\text{VilCre}^{\text{tg}/-}/\text{Ass}^{-/\text{fl}}$ genotypes and the $\text{VilCre}^{\text{tg}/-}/\text{Ass}^{+/+}$ and $\text{VilCre}^{\text{tg}/-}/\text{Ass}^{\text{fl}/\text{fl}}$ genotypes, although the latter did not differ significantly for any amino acid in the portal drained viscera. In contrast, the $\text{VilCre}^{\text{tg}/-}/\text{Ass}^{+/-}$ and $\text{VilCre}^{\text{tg}/-}/\text{Ass}^{-/\text{fl}}$, and in particular the $\text{VilCre}^{\text{tg}/-}/\text{Ass}^{+/+}$ and $\text{VilCre}^{\text{tg}/-}/\text{Ass}^{+/-}$ were remarkably similar. Furthermore, the much larger number of significant differences in the flux across the liver contrasted with the paucity of significant differences across the gut as target organ. At present, we have no explanation for these trends.

Because we could not measure the cardiac output, we have assessed to what extent this (lack of) information can affect our interpretations. A change in the relative contributions of the portal vein and hepatic artery to liver perfusion from 80:20 to 70:30 or 85:15 hardly affected the calculated fluxes. If the cardiac output would differ between the genotypes without effect on the distribution of the blood flow, the patterns would not change either. If the blood-flow distribution would also change, it would result in comparing in the extreme case a 70:30 distribution for one genotype with a 85:15 for another. Although such an unnoticed difference could (partially) undo the relatively low production of amino acids in the portal drained viscera of $\text{VilCre}^{\text{tg}/-}/\text{Ass}^{-/\text{fl}}$ mice, it would not change another unexplained observation, viz. the relatively high uptake of amino acids in $\text{VilCre}^{\text{tg}/-}/\text{Ass}^{\text{fl}/\text{fl}}$ livers. We, therefore, do not think that our lack of information of the cardiac output and distribution of the blood affects the outcome of our study in any major way.

Comparison with the transgenic F/A2 model

Even though ASS expression and, hence arginine biosynthesis, was completely eliminated from the enterocytes of ASS-KO/I mice, we did not observe deficient hair and muscle growth and underdevelopment of Peyer's patches (Figure 2D-F), features that characterize "F/A" mice, in which intestinal arginine biosynthesis is eliminated by transgenic overexpression of arginase-1 in the enterocytes (23, 24). The circulating concentration of arginine and the phenotype of F/A mice correspond with the degree of overexpression of arginase-1 in the enterocytes (23, 24). Except for the circulating concentration of arginine and glycine, no amino acid changes in concentration in F/A mice. The "healthy" phenotype of mice that lack ASS expression in their enterocytes as compared with mice that overexpress arginase in their enterocytes demonstrates that the phenotype of F/A mice is not only due to elimination of de novo arginine biosynthesis in the gut. Instead, the much lower circulating arginine concentration in F/A mice compared to that in ASS-KO/I mice shows that overexpression of arginase-1 causes the gut to function additionally as a sink for circulating arginine that passes the intestine. Apparently, the capacity of this sink exceeds the supply of arginine by its endogenous production and the absorption from the milk. In agreement, the half-life of a subcutaneous bolus of arginine was shortened at least 3-fold in F/A2 relative to wild-type mice (49). The comparison of the ASS-KO/I and F/A models therefore demonstrates that elimination of intestinal arginine biosynthesis does not produce an arginine-deficient phenotype in mice.

Comparison with other arginine-deficient mouse models

The intestine is the only net producer of ornithine, the precursor of citrulline and arginine (3). In the suckling intestine, ornithine is produced from proline rather than glutamate (50). A deficiency of ornithine aminotransferase (OAT), a common enzyme in the conversion of glutamate and proline into ornithine, and expressed at ~5-fold higher levels in the suckling than the weaned small intestine (30), results in a severe neonatal deficiency of ornithine, citrulline and arginine (30). Similarly, the Spf mutation in the enzyme ornithine transcarbamoylase (OTC), which severely decreases its capacity to convert ornithine into citrulline, causes a deficiency in circulating citrulline and arginine levels in suckling mice (51). These findings show that the suckling mouse needs to produce arginine endogenously to maintain normal circulating levels of this amino acid.

Since the present study demonstrated that intestinal arginine synthesis from citrulline is dispensable, the extra-intestinal production of arginine can apparently compensate completely for the elimination of arginine synthesis from the intestine in the suckling mouse.

Comparison of intestinal arginine synthesis in suckling mice and piglets

Hyperammonemia rapidly develops if piglets are parenterally fed with an arginine-deficient diet (18). Extensive metabolic studies in chronically instrumented piglets have subsequently shown that oral feeding and intestinal arginine production are necessary to sustain normal circulating arginine levels and growth in piglets (20-22). In enterally fed piglets, proline can replace arginine in the diet (18), because the enterocytes convert proline into ornithine and arginine (20). Proline is preferred over glutamate as precursor of ornithine synthesis, because enteral glutamate is extensively catabolized in the gut (52-54). The capacity to synthesize citrulline is determined by the intestinal mass (21) and limits the capacity of arginine production to ~50% of the daily requirement of arginine (20, 22). Citrulline is, therefore, a better exogenous precursor for arginine synthesis than proline or ornithine (55). In piglets, intestinal arginine production cannot be adaptively upregulated when arginine becomes deficient, but extra-intestinal tissues can upregulate arginine synthesis from proline ~3-fold (56). The liver does not contribute to arginine production (57). These data show that arginine metabolism in suckling piglets and mice are very similar.

Comparison with the human condition

The analyses of the experimental genetic mouse models have demonstrated that intestinal citrulline, but not arginine synthesis is necessary to maintain normal circulating arginine levels during the suckling period. The outcomes of the nutritional experiments in piglets largely concur with this conclusion. It is therefore of interest that the necessarily less complete human data also show that total parenteral nutrition in premature neonates is associated with hypoargininemia and hyperammonemia (58, 59). In addition, some polymorphisms of the carbamoylphosphate synthetase (CPS-1) gene, a rate-determining enzyme of citrulline synthesis, are associated with deficient NO production (60). The interest in endogenous arginine synthesis was further boosted by

the hypothesis that the fore-mentioned CPS-1 polymorphisms (61) and hypoargininemia predispose premature infants to the development of necrotizing enterocolitis (62, 63).

In the premature piglet, parenteral feeding has also been reported to sensitize the animal to a condition that closely resembles necrotizing enterocolitis (64), but, to the best of our knowledge, a condition closely resembling necrotizing enterocolitis has not been described in neonatal mice. In fact, the circulating arginine concentration in neonatal F/A2 mice is only ~30% of control values (23, 24, 49), but we never observed any sign of ischemic lesions in their intestine.

Conclusion

For suckling mice, endogenous citrulline and arginine synthesis is necessary (30) to cope with the insufficient supply of arginine in the milk (51). In this respect, mice resemble piglets (7-9) and probably humans (18, 20, 21). Under normal physiological conditions, the gut releases arginine that is either absorbed from the maternal milk or produced by the enterocytes from proline and, to a lesser extent, glutamine (Figure 6A). In mice overexpressing arginase-1 in their enterocytes, this and circulating arginine is degraded (Figure 6B). After enterocyte-specific ASS inactivation, dietary arginine is still normally absorbed and transported, but the de novo synthesized citrulline is no longer converted into arginine (Figure 6C). Instead, it is secreted into the portal vein (Figure 6C). Since the selective and complete elimination of ASS from the enterocytes does not produce an observable phenotype, our experiments have convincingly demonstrated that intestinal arginine synthesis from citrulline is not crucial to sustain normal development and that an as yet unidentified extra-intestinal tissue is able to upregulate its arginine production to maintain circulating arginine levels.

Figure 6. Adaptation in arginine synthesis in F/A2 and Ass-KO/I suckling mice. Panel A. In control mice, the gut absorbs arginine from the milk (yellow arrow) and produces it de novo from citrulline (red arrow). In addition, some citrulline that is secreted to the portal vein (narrow red arrow) can be converted to arginine elsewhere, e.g. in the kidney. Panel B: Suckling F/A2 transgenic mice suffer from a severe arginine deficiency due to overexpression of arginase-1 in their enterocytes (23, 24). Circulating citrulline levels in these animals are unchanged (our unpublished observations), but it is unknown whether extra-intestinal arginine biosynthesis is upregulated. Panel C: Ass-KO/I intestine cannot synthesize arginine de novo, leaving milk as the only source. Due to the absence of intestinal arginine synthesis, the AssS-KO/I intestine produces more citrulline, while as yet unidentified "peripheral" organs (not the kidney) increase the expression of ASS.

Appendix:

Supplementary Table 1: Primer sequences, annealing temperatures and expected PCR-product lengths

primer	sequence	°C	length
Ass-F1 (exon13)	5'gaggaagggacagtggggtg3'	60	Ass ⁺ : F1→←R1: 360bp Ass ⁿ : F1→loxP←R1: 390bp Ass ⁿ : F2→loxP-exon13-loxP←R1: 1,000bp Ass ⁻ : F2→loxP←R1: 340bp
Ass-R1 (intron13)	5'actgctcagggcacgcatgtg3'		
Ass-F2 (*)	5'tctagaactagtggtcacctcag3'		
Vil-F	5'tgcctggcctaaagctcac3'	62	1,100 bp
Vil-R	5'cgacagtatcggcctcagg3'		

*: new sequence between frt and loxP sites

Supplementary Table 2: Amino acid concentrations in plasma (μM ; mean $\pm$ SEM) of $VilCre^{tg}/Ass^{+/+}$, $VilCre^{tg}/Ass^{fl/m}$ and $VilCre^{tg}/Ass^{+/+}$ and $VilCre^{tg}/Ass^{fl/fl}$ mice. Statistical evaluation was performed by ANOVA.

		GLU	ASN	SER	GLN	HIS	GLY	THR	CIT	ARG	ALA	TAU	TYR	VAL	MET	ILE	PHE	TRP	LEU	ORN	LYS
aorta	+/+	86 $\pm$ 9	67 $\pm$ 5	256 $\pm$ 21	504 $\pm$ 12	76 $\pm$ 4	516 $\pm$ 28	258 $\pm$ 16	94 $\pm$ 8	232 $\pm$ 13	339 $\pm$ 42	541 $\pm$ 45	142 $\pm$ 9	215 $\pm$ 13	86 $\pm$ 6	101 $\pm$ 5	70 $\pm$ 4	87 $\pm$ 4	142 $\pm$ 6	131 $\pm$ 11	504 $\pm$ 25
	fl/fl	146 $\pm$ 20	79 $\pm$ 12	259 $\pm$ 33	556 $\pm$ 65	90 $\pm$ 9	569 $\pm$ 39	273 $\pm$ 36	140 $\pm$ 21	269 $\pm$ 38	593 $\pm$ 94	870 $\pm$ 41	135 $\pm$ 17	281 $\pm$ 57	104 $\pm$ 16	124 $\pm$ 20	73 $\pm$ 8	87 $\pm$ 5	173 $\pm$ 30	161 $\pm$ 38	536 $\pm$ 86
	+/+	79 $\pm$ 11	74 $\pm$ 4	256 $\pm$ 20	523 $\pm$ 23	90 $\pm$ 4	509 $\pm$ 54	259 $\pm$ 14	147 $\pm$ 6	235 $\pm$ 10	487 $\pm$ 44	610 $\pm$ 64	153 $\pm$ 13	266 $\pm$ 19	100 $\pm$ 5	122 $\pm$ 7	85 $\pm$ 4	91 $\pm$ 4	174 $\pm$ 9	205 $\pm$ 21	523 $\pm$ 20
	fl/-	75 $\pm$ 16	78 $\pm$ 4	272 $\pm$ 13	629 $\pm$ 31	82 $\pm$ 4	573 $\pm$ 27	273 $\pm$ 11	196 $\pm$ 13	204 $\pm$ 16	483 $\pm$ 36	797 $\pm$ 30	119 $\pm$ 12	234 $\pm$ 15	88 $\pm$ 4	117 $\pm$ 6	69 $\pm$ 3	83 $\pm$ 4	162 $\pm$ 9	129 $\pm$ 13	473 $\pm$ 39
vena portae	+/+	138 $\pm$ 13	112 $\pm$ 7	401 $\pm$ 16	408 $\pm$ 25	110 $\pm$ 5	678 $\pm$ 25	363 $\pm$ 17	114 $\pm$ 7	342 $\pm$ 16	668 $\pm$ 37	772 $\pm$ 49	164 $\pm$ 7	270 $\pm$ 14	111 $\pm$ 5	141 $\pm$ 4	94 $\pm$ 3	96 $\pm$ 3	187 $\pm$ 6	233 $\pm$ 17	601 $\pm$ 49
	fl/fl	169 $\pm$ 20	131 $\pm$ 14	478 $\pm$ 64	584 $\pm$ 90	134 $\pm$ 21	601 $\pm$ 47	440 $\pm$ 49	223 $\pm$ 24	316 $\pm$ 42	901 $\pm$ 125	880 $\pm$ 52	189 $\pm$ 16	403 $\pm$ 54	129 $\pm$ 13	176 $\pm$ 16	101 $\pm$ 9	103 $\pm$ 6	248 $\pm$ 27	231 $\pm$ 42	796 $\pm$ 76
	+/+	124 $\pm$ 11	119 $\pm$ 6	392 $\pm$ 20	442 $\pm$ 23	108 $\pm$ 5	596 $\pm$ 27	340 $\pm$ 12	169 $\pm$ 8	338 $\pm$ 17	679 $\pm$ 34	675 $\pm$ 41	186 $\pm$ 11	279 $\pm$ 14	127 $\pm$ 5	143 $\pm$ 6	95 $\pm$ 4	107 $\pm$ 4	197 $\pm$ 9	196 $\pm$ 12	627 $\pm$ 23
	fl/-	96 $\pm$ 9	97 $\pm$ 5	326 $\pm$ 19	451 $\pm$ 38	91 $\pm$ 8	611 $\pm$ 43	324 $\pm$ 10	222 $\pm$ 17	222 $\pm$ 20	643 $\pm$ 36	752 $\pm$ 64	120 $\pm$ 11	284 $\pm$ 12	100 $\pm$ 4	324 $\pm$ 49	101 $\pm$ 8	81 $\pm$ 4	147 $\pm$ 17	173 $\pm$ 10	366 $\pm$ 59
vena hepatica	+/+	189 $\pm$ 14	127 $\pm$ 14	478 $\pm$ 45	547 $\pm$ 48	138 $\pm$ 9	684 $\pm$ 51	400 $\pm$ 21	132 $\pm$ 12	355 $\pm$ 27	646 $\pm$ 41	666 $\pm$ 79	204 $\pm$ 16	337 $\pm$ 29	127 $\pm$ 6	175 $\pm$ 10	112 $\pm$ 9	114 $\pm$ 5	236 $\pm$ 17	341 $\pm$ 34	709 $\pm$ 42
	fl/fl	127 $\pm$ 12	122 $\pm$ 10	453 $\pm$ 36	482 $\pm$ 21	130 $\pm$ 19	585 $\pm$ 36	350 $\pm$ 28	213 $\pm$ 15	239 $\pm$ 18	629 $\pm$ 67	740 $\pm$ 130	220 $\pm$ 19	277 $\pm$ 20	113 $\pm$ 6	131 $\pm$ 10	103 $\pm$ 8	94 $\pm$ 4	182 $\pm$ 17	249 $\pm$ 31	661 $\pm$ 38
	+/+	110 $\pm$ 13	105 $\pm$ 5	361 $\pm$ 24	551 $\pm$ 16	120 $\pm$ 8	711 $\pm$ 47	354 $\pm$ 17	164 $\pm$ 14	260 $\pm$ 20	646 $\pm$ 45	888 $\pm$ 72	180 $\pm$ 10	302 $\pm$ 15	116 $\pm$ 6	168 $\pm$ 11	97 $\pm$ 5	106 $\pm$ 5	234 $\pm$ 17	285 $\pm$ 21	786 $\pm$ 37
	fl/-	140 $\pm$ 23	117 $\pm$ 8	412 $\pm$ 28	592 $\pm$ 33	118 $\pm$ 10	632 $\pm$ 60	373 $\pm$ 22	336 $\pm$ 33	242 $\pm$ 44	708 $\pm$ 65	855 $\pm$ 70	189 $\pm$ 20	356 $\pm$ 39	130 $\pm$ 6	244 $\pm$ 42	100 $\pm$ 8	108 $\pm$ 7	252 $\pm$ 19	327 $\pm$ 31	641 $\pm$ 28
vena renalis	+/+	149 $\pm$ 9	94 $\pm$ 4	331 $\pm$ 17	531 $\pm$ 26	105 $\pm$ 6	572 $\pm$ 39	301 $\pm$ 13	60 $\pm$ 3	263 $\pm$ 14	476 $\pm$ 29	560 $\pm$ 31	185 $\pm$ 10	229 $\pm$ 11	113 $\pm$ 9	97 $\pm$ 5	73 $\pm$ 3	94 $\pm$ 3	134 $\pm$ 8	152 $\pm$ 8	646 $\pm$ 29
	fl/fl	130 $\pm$ 12	102 $\pm$ 11	335 $\pm$ 49	614 $\pm$ 73	104 $\pm$ 9	519 $\pm$ 47	344 $\pm$ 48	140 $\pm$ 21	356 $\pm$ 43	577 $\pm$ 67	668 $\pm$ 47	162 $\pm$ 24	355 $\pm$ 64	90 $\pm$ 12	146 $\pm$ 20	78 $\pm$ 9	84 $\pm$ 5	201 $\pm$ 32	219 $\pm$ 41	691 $\pm$ 110
	+/+	118 $\pm$ 10	91 $\pm$ 5	326 $\pm$ 24	508 $\pm$ 21	90 $\pm$ 5	703 $\pm$ 57	323 $\pm$ 16	139 $\pm$ 8	283 $\pm$ 14	506 $\pm$ 44	583 $\pm$ 53	142 $\pm$ 11	284 $\pm$ 23	91 $\pm$ 8	127 $\pm$ 10	83 $\pm$ 4	86 $\pm$ 5	176 $\pm$ 14	198 $\pm$ 16	568 $\pm$ 33
	fl/-	140 $\pm$ 8	94 $\pm$ 3	350 $\pm$ 20	516 $\pm$ 19	106 $\pm$ 6	534 $\pm$ 45	326 $\pm$ 20	203 $\pm$ 14	303 $\pm$ 29	528 $\pm$ 37	636 $\pm$ 26	207 $\pm$ 26	246 $\pm$ 26	110 $\pm$ 10	106 $\pm$ 11	67 $\pm$ 4	86 $\pm$ 6	143 $\pm$ 12	187 $\pm$ 18	691 $\pm$ 31

Supplementary Table 3: Amino-acid fluxes (mean $\pm$ SEM) of VilCre^{fl/y}/Ass^{+/+}, VilCre^{fl/y}/Ass^{fl/fl} and VilCre^{fl/y}/Ass^{+/+} mice. Production is indicated in black and consumption in red numbers. Statistical evaluation was performed by ANOVA.

	GLU	ASN	SER	GLN	HIS	GLY	THR	CIT	ARG	ALA	TAU	TYR	VAL	MET	ILE	PHE	TRP	LEU	ORN	LYS
splanchnic	44* 103 $\pm$ 17	63 $\pm$ 15	272 $\pm$ 49	43 $\pm$ 49	50 $\pm$ 10	167 $\pm$ 58	143 $\pm$ 27	37 $\pm$ 13	124 $\pm$ 39	367 $\pm$ 59	325 $\pm$ 91	63 $\pm$ 18	122 $\pm$ 32	40 $\pm$ 8	74 $\pm$ 11	42 $\pm$ 9	27 $\pm$ 5	97 $\pm$ 18	210 $\pm$ 35	205 $\pm$ 49
muscle	61* 15 $\pm$ 23	43 $\pm$ 15	155 $\pm$ 48	74 $\pm$ 33	48 $\pm$ 21	15 $\pm$ 53	77 $\pm$ 46	74 $\pm$ 26	33 $\pm$ 40	35 $\pm$ 16	121 $\pm$ 10	85 $\pm$ 25	4 $\pm$ 31	9 $\pm$ 17	51 $\pm$ 22	30 $\pm$ 11	7 $\pm$ 5	9 $\pm$ 34	87 $\pm$ 49	145 $\pm$ 94
fat	32 $\pm$ 17	31 $\pm$ 7	125 $\pm$ 31	27 $\pm$ 26	30 $\pm$ 9	101 $\pm$ 71	51 $\pm$ 22	16 $\pm$ 15	25 $\pm$ 22	160 $\pm$ 53	278 $\pm$ 95	27 $\pm$ 16	35 $\pm$ 24	17 $\pm$ 8	45 $\pm$ 13	12 $\pm$ 7	15 $\pm$ 6	50 $\pm$ 19	90 $\pm$ 30	243 $\pm$ 41
RL	84 $\pm$ 28	39 $\pm$ 9	140 $\pm$ 31	35 $\pm$ 45	35 $\pm$ 11	53 $\pm$ 56	100 $\pm$ 25	142 $\pm$ 35	35 $\pm$ 48	225 $\pm$ 75	58 $\pm$ 76	71 $\pm$ 23	122 $\pm$ 42	41 $\pm$ 9	127 $\pm$ 42	32 $\pm$ 9	25 $\pm$ 8	50 $\pm$ 21	159 $\pm$ 33	167 $\pm$ 47
potentially drained	44* 42 $\pm$ 13	35 $\pm$ 7	116 $\pm$ 21	77 $\pm$ 22	27 $\pm$ 55	127 $\pm$ 30	84 $\pm$ 18	15 $\pm$ 7	59 $\pm$ 17	230 $\pm$ 45	165 $\pm$ 53	18 $\pm$ 9	44 $\pm$ 15	30 $\pm$ 6	33 $\pm$ 5	18 $\pm$ 4	7 $\pm$ 4	37 $\pm$ 7	82 $\pm$ 15	78 $\pm$ 44
viscera	61* 18 $\pm$ 23	42 $\pm$ 15	175 $\pm$ 57	22 $\pm$ 86	35 $\pm$ 18	25 $\pm$ 49	134 $\pm$ 49	57 $\pm$ 26	39 $\pm$ 45	247 $\pm$ 125	81 $\pm$ 53	43 $\pm$ 19	55 $\pm$ 63	20 $\pm$ 17	41 $\pm$ 20	23 $\pm$ 9	13 $\pm$ 6	53 $\pm$ 32	96 $\pm$ 45	208 $\pm$ 92
RL	35 $\pm$ 12	35 $\pm$ 5	105 $\pm$ 22	25 $\pm$ 25	16 $\pm$ 5	13 $\pm$ 45	35 $\pm$ 15	13 $\pm$ 8	33 $\pm$ 18	154 $\pm$ 45	52 $\pm$ 51	25 $\pm$ 13	11 $\pm$ 18	22 $\pm$ 6	17 $\pm$ 7	8 $\pm$ 5	13 $\pm$ 4	18 $\pm$ 10	53 $\pm$ 27	84 $\pm$ 24
RL	17 $\pm$ 14	16 $\pm$ 5	45 $\pm$ 18	11 $\pm$ 29	7 $\pm$ 7	30 $\pm$ 41	41 $\pm$ 12	21 $\pm$ 17	14 $\pm$ 21	123 $\pm$ 41	38 $\pm$ 50	1 $\pm$ 13	40 $\pm$ 15	9 $\pm$ 5	155 $\pm$ 99	25 $\pm$ 7	1 $\pm$ 5	12 $\pm$ 15	35 $\pm$ 13	51 $\pm$ 35
liver	44* 51 $\pm$ 18	24 $\pm$ 16	105 $\pm$ 48	120 $\pm$ 53	33 $\pm$ 10	40 $\pm$ 57	59 $\pm$ 27	22 $\pm$ 13	35 $\pm$ 31	27 $\pm$ 56	140 $\pm$ 92	45 $\pm$ 18	75 $\pm$ 32	20 $\pm$ 8	41 $\pm$ 11	23 $\pm$ 9	20 $\pm$ 6	50 $\pm$ 18	128 $\pm$ 38	127 $\pm$ 42
RL	37 $\pm$ 24	2 $\pm$ 17	19 $\pm$ 69	47 $\pm$ 58	4 $\pm$ 27	10 $\pm$ 68	57 $\pm$ 54	7 $\pm$ 28	4 $\pm$ 34	210 $\pm$ 139	135 $\pm$ 139	42 $\pm$ 25	10 $\pm$ 39	11 $\pm$ 15	35 $\pm$ 20	7 $\pm$ 12	4 $\pm$ 5	57 $\pm$ 22	5 $\pm$ 11	60 $\pm$ 37
fat	43 $\pm$ 17	32 $\pm$ 7	15 $\pm$ 31	81 $\pm$ 28	16 $\pm$ 9	112 $\pm$ 58	24 $\pm$ 21	3 $\pm$ 16	3 $\pm$ 25	71 $\pm$ 8	205 $\pm$ 86	11 $\pm$ 15	25 $\pm$ 21	5 $\pm$ 8	29 $\pm$ 13	5 $\pm$ 7	2 $\pm$ 6	42 $\pm$ 19	96 $\pm$ 25	155 $\pm$ 43
RL	45 $\pm$ 25	23 $\pm$ 9	57 $\pm$ 33	105 $\pm$ 49	29 $\pm$ 12	29 $\pm$ 73	59 $\pm$ 24	121 $\pm$ 37	24 $\pm$ 48	98 $\pm$ 74	94 $\pm$ 95	70 $\pm$ 22	52 $\pm$ 42	37 $\pm$ 9	33 $\pm$ 20	6 $\pm$ 11	27 $\pm$ 8	102 $\pm$ 25	152 $\pm$ 33	253 $\pm$ 62
kidney	44* 13 $\pm$ 2	5 $\pm$ 1	15 $\pm$ 15	5 $\pm$ 5	5 $\pm$ 2	11 $\pm$ 10	18 $\pm$ 4	7 $\pm$ 7	6 $\pm$ 4	25 $\pm$ 10	44 $\pm$ 11	8 $\pm$ 3	3 $\pm$ 3	5 $\pm$ 2	7 $\pm$ 4	0 $\pm$ 3	1 $\pm$ 1	2 $\pm$ 2	4 $\pm$ 3	25 $\pm$ 9
RL	3 $\pm$ 6	4 $\pm$ 3	15 $\pm$ 12	12 $\pm$ 15	3 $\pm$ 3	10 $\pm$ 12	14 $\pm$ 12	0 $\pm$ 6	17 $\pm$ 12	3 $\pm$ 71	4 $\pm$ 10	5 $\pm$ 6	15 $\pm$ 17	0 $\pm$ 4	4 $\pm$ 6	1 $\pm$ 2	1 $\pm$ 1	5 $\pm$ 9	12 $\pm$ 11	31 $\pm$ 23
fat	8 $\pm$ 3	3 $\pm$ 1	14 $\pm$ 6	5 $\pm$ 3	0 $\pm$ 1	19 $\pm$ 16	5 $\pm$ 4	2 $\pm$ 3	10 $\pm$ 3	4 $\pm$ 12	5 $\pm$ 17	2 $\pm$ 3	4 $\pm$ 6	2 $\pm$ 2	1 $\pm$ 3	0 $\pm$ 1	1 $\pm$ 1	1 $\pm$ 3	1 $\pm$ 5	9 $\pm$ 8
RL	13 $\pm$ 4	3 $\pm$ 2	15 $\pm$ 5	22 $\pm$ 7	5 $\pm$ 2	8 $\pm$ 11	11 $\pm$ 5	1 $\pm$ 4	20 $\pm$ 7	5 $\pm$ 11	22 $\pm$ 5	18 $\pm$ 5	2 $\pm$ 6	4 $\pm$ 2	2 $\pm$ 2	0 $\pm$ 1	1 $\pm$ 1	4 $\pm$ 3	12 $\pm$ 4	44 $\pm$ 10

References

1. Morris SM, Jr. Regulation of enzymes of the urea cycle and arginine metabolism. *Annu Rev Nutr* 2002;22:87-105.
2. Wu G, Morris SM, Jr. Arginine metabolism: nitric oxide and beyond. *Biochem J* 1998;336 (Pt 1):1-17.
3. Meijer AJ, Lamers WH, Chamuleau RA. Nitrogen metabolism and ornithine cycle function. *Physiol Rev* 1990;70:701-748.
4. Flynn NE, Meininger CJ, Haynes TE, Wu G. The metabolic basis of arginine nutrition and pharmacotherapy. *Biomed Pharmacother* 2002;56:427-438.
5. Southern LL, Baker DH. Arginine requirement of the young pig. *J Anim Sci* 1983;57:402-412.
6. Visek WJ. Arginine needs, physiological state and usual diets. A reevaluation. *J Nutr* 1986;116:36-46.
7. Davis TA, Fiorotto ML, Reeds PJ. Amino acid compositions of body and milk protein change during the suckling period in rats. *J Nutr* 1993;123:947-956.
8. Davis TA, Nguyen HV, Garcia-Bravo R, Fiorotto ML, Jackson EM, Lewis DS, Lee DR, et al. Amino acid composition of human milk is not unique. *J Nutr* 1994;124:1126-1132.
9. Wu G, Knabe DA. Free and protein-bound amino acids in sow's colostrum and milk. *J Nutr* 1994;124:415-424.
10. De Jonge WJ, Dingemans MA, de Boer PA, Lamers WH, Moorman AF. Arginine-metabolizing enzymes in the developing rat small intestine. *Pediatr Res* 1998;43:442-451.
11. Herzfeld A, Raper SM. Enzymes of ornithine metabolism in adult and developing rat intestine. *Biochim Biophys Acta* 1976;428:600-610.
12. Hurwitz R, Kretchmer N. Development of arginine-synthesizing enzymes in mouse intestine. *Am J Physiol* 1986;251:G103-110.
13. Nagy LE, Kretchmer N. Utilization of glutamine in the developing rat jejunum. *J Nutr* 1988;118:189-193.
14. Dekaney CM, Wu G, Jaeger LA. Ornithine aminotransferase messenger RNA expression and enzymatic activity in fetal porcine intestine. *Pediatr Res* 2001;50:104-109.
15. Dekaney CM, Wu G, Jaeger LA. Gene expression and activity of enzymes in the arginine biosynthetic pathway in porcine fetal small intestine. *Pediatr Res* 2003;53:274-280.
16. Wu G. Synthesis of citrulline and arginine from proline in enterocytes of postnatal pigs. *Am J Physiol* 1997;272:G1382-1390.
17. Wu G, Knabe DA. Arginine synthesis in enterocytes of neonatal pigs. *Am J Physiol* 1995;269:R621-629.
18. Brunton JA, Bertolo RF, Pencharz PB, Ball RO. Proline ameliorates arginine deficiency during enteral but not parenteral feeding in neonatal piglets. *Am J Physiol* 1999;277:E223-231.
19. Hoogenraad N, Totino N, Elmer H, Wraight C, Alewood P, Johns RB. Inhibition of intestinal citrulline synthesis causes severe growth retardation in rats. *Am J Physiol* 1985;249:G792-799.

20. Bertolo RF, Brunton JA, Pencharz PB, Ball RO. Arginine, ornithine, and proline interconversion is dependent on small intestinal metabolism in neonatal pigs. *Am J Physiol Endocrinol Metab* 2003;284:E915-922.
21. Urschel KL, Evans AR, Wilkinson CW, Pencharz PB, Ball RO. Parenterally fed neonatal piglets have a low rate of endogenous arginine synthesis from circulating proline. *J Nutr* 2007;137:601-606.
22. Urschel KL, Raffi M, Pencharz PB, Ball RO. A multitracer stable isotope quantification of the effects of arginine intake on whole body arginine metabolism in neonatal piglets. *Am J Physiol Endocrinol Metab* 2007;293:E811-818.
23. de Jonge WJ, Hallemesch MM, Kwikkers KL, Ruijter JM, de Gier-de Vries C, van Roon MA, Meijer AJ, et al. Overexpression of arginase I in enterocytes of transgenic mice elicits a selective arginine deficiency and affects skin, muscle, and lymphoid development. *Am J Clin Nutr* 2002;76:128-140.
24. de Jonge WJ, Kwikkers KL, te Velde AA, van Deventer SJ, Nolte MA, Mebius RE, Ruijter JM, et al. Arginine deficiency affects early B cell maturation and lymphoid organ development in transgenic mice. *J Clin Invest* 2002;110:1539-1548.
25. Blachier F, M'Rabet-Touil H, Posho L, Darcy-Vrillon B, Duee PH. Intestinal arginine metabolism during development. Evidence for de novo synthesis of L-arginine in newborn pig enterocytes. *Eur J Biochem* 1993;216:109-117.
26. Dugan ME, Knabe DA, Wu G. The induction of citrulline synthesis from glutamine in enterocytes of weaned pigs is not due primarily to age or change in diet. *J Nutr* 1995;125:2388-2393.
27. Wu G, Borbolla AG, Knabe DA. The uptake of glutamine and release of arginine, citrulline and proline by the small intestine of developing pigs. *J Nutr* 1994;124:2437-2444.
28. Patejunas G, Bradley A, Beaudet AL, O'Brien WE. Generation of a mouse model for citrullinemia by targeted disruption of the argininosuccinate synthetase gene. *Somat Cell Mol Genet* 1994;20:55-60.
29. Madison BB, Dunbar L, Qiao XT, Braunstein K, Braunstein E, Gumucio DL. Cis elements of the villin gene control expression in restricted domains of the vertical (crypt) and horizontal (duodenum, cecum) axes of the intestine. *J Biol Chem* 2002;277:33275-33283.
30. Wang T, Lawler AM, Steel G, Sipila I, Milam AH, Valle D. Mice lacking ornithine-delta-aminotransferase have paradoxical neonatal hypornithinaemia and retinal degeneration. *Nat Genet* 1995;11:185-190.
31. Holland P, Hogan B, Constantini F, Lacy E. Manipulating the mouse embryo: A laboratory manual. New York: Cold Spring Harbor Laboratory, 1986: 228-242.
32. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: A laboratory manual. New York: Cold Spring Harbor Laboratory, 1989.
33. Dymecki SM. FLP recombinase promotes site-specific DNA recombination in embryonic stem cells and transgenic mice. *Proc Natl Acad Sci U S A* 1996;93:6191-6196.
34. Sadowski PD. The FLP recombinase of the 2-microns plasmid of *Saccharomyces cerevisiae*. *Prog Nucleic Acid Res Mol Biol* 1995;51:53-91.

35. Kuhn R, Schwenk F, Aguet M, Rajewsky K. Inducible gene targeting in mice. *Science* 1995;269:1427-1429.
36. Sauer B, Henderson N. Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proc Natl Acad Sci U S A* 1988;85:5166-5170.
37. Schwenk F, Baron U, Rajewsky K. A cre-transgenic mouse strain for the ubiquitous deletion of loxP-flanked gene segments including deletion in germ cells. *Nucleic Acids Res* 1995;23:5080-5081.
38. Hallemeesch MM, Ten Have GA, Deutz NE. Metabolic flux measurements across portal drained viscera, liver, kidney and hindquarter in mice. *Lab Anim* 2001;35:101-110.
39. van Eijk HM, Rooyakkers DR, Deutz NE. Rapid routine determination of amino acids in plasma by high-performance liquid chromatography with a 2-3 microns Spherisorb ODS II column. *J Chromatogr* 1993;620:143-148.
40. Koni PA, Flavell RA. A role for tumor necrosis factor receptor type 1 in gut-associated lymphoid tissue development: genetic evidence of synergism with lymphotoxin beta. *J Exp Med* 1998;187:1977-1983.
41. Ramakers C, Ruijter JM, Deprez RH, Moorman AF. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci Lett* 2003;339:62-66.
42. Ruijter JM, Thygesen HH, Schoneveld OJ, Das AT, Berkhout B, Lamers WH. Factor correction as a tool to eliminate between-session variation in replicate experiments: application to molecular biology and retrovirology. *Retrovirology* 2006;3:2.
43. Altman DG. *Practical statistics for medical research*. London: Chapman & Hall, 1991.
44. Levillain O, Hus-Citharel A, Morel F, Bankir L. Arginine synthesis in mouse and rabbit nephron: localization and functional significance. *Am J Physiol* 1993;264:F1038-1045.
45. Maunoury R, Robine S, Pringault E, Leonard N, Gaillard JA, Louvard D. Developmental regulation of villin gene expression in the epithelial cell lineages of mouse digestive and urogenital tracts. *Development* 1992;115:717-728.
46. Goldblum OM, Brusilow SW, Maldonado YA, Farmer ER. Neonatal citrullinemia associated with cutaneous manifestations and arginine deficiency. *J Am Acad Dermatol* 1986;14:321-326.
47. Toyoshima K, Seta Y, Takeda S, Harada H. Identification of Merkel cells by an antibody to villin. *J Histochem Cytochem* 1998;46:1329-1334.
48. Kobayashi K, Kakinoki H, Fukushige T, Shaheen N, Terazono H, Saheki T. Nature and frequency of mutations in the argininosuccinate synthetase gene that cause classical citrullinemia. *Hum Genet* 1995;96:454-463.
49. de Jonge WJ, Marescau B, D'Hooge R, De Deyn PP, Hallemeesch MM, Deutz NE, Ruijter JM, et al. Overexpression of arginase alters circulating and tissue amino acids and guanidino compounds and affects neuromotor behavior in mice. *J Nutr* 2001;131:2732-2740.
50. Murphy JM, Murch SJ, Ball RO. Proline is synthesized from glutamate during intragastric infusion but not during intravenous infusion in neonatal piglets. *J Nutr* 1996;126:878-886.
51. Malo C. Free amino acid levels in serum and small intestine during the post-natal development of normal and sparse-fur mutant mice. *Comp Biochem Physiol A Physiol* 1994;109:1049-1057.

52. Janeczko MJ, Stoll B, Chang X, Guan X, Burrin DG. Extensive gut metabolism limits the intestinal absorption of excessive supplemental dietary glutamate loads in infant pigs. *J Nutr* 2007;137:2384-2390.
53. Lambert BD, Filip R, Stoll B, Junghans P, Derno M, Hennig U, Souffrant WB, et al. First-pass metabolism limits the intestinal absorption of enteral alpha-ketoglutarate in young pigs. *J Nutr* 2006;136:2779-2784.
54. Reeds PJ, Burrin DG, Stoll B, Jahoor F. Intestinal glutamate metabolism. *J Nutr* 2000;130:978S-982S.
55. Urschel KL, Shoveller AK, Uwiera RR, Pencharz PB, Ball RO. Citrulline is an effective arginine precursor in enterally fed neonatal piglets. *J Nutr* 2006;136:1806-1813.
56. Wilkinson DL, Bertolo RF, Brunton JA, Shoveller AK, Pencharz PB, Ball RO. Arginine synthesis is regulated by dietary arginine intake in the enterally fed neonatal piglet. *Am J Physiol Endocrinol Metab* 2004;287:E454-462.
57. Urschel KL, Shoveller AK, Pencharz PB, Ball RO. Arginine synthesis does not occur during first-pass hepatic metabolism in the neonatal piglet. *Am J Physiol Endocrinol Metab* 2005;288:E1244-1251.
58. Batshaw ML, Wachtel RC, Thomas GH, Starrett A, Brusilow SW. Arginine-responsive asymptomatic hyperammonemia in the premature infant. *J Pediatr* 1984;105:86-91.
59. Wu G, Jaeger LA, Bazer FW, Rhoads JM. Arginine deficiency in preterm infants: biochemical mechanisms and nutritional implications. *J Nutr Biochem* 2004;15:442-451.
60. Summar ML, Hall L, Christman B, Barr F, Smith H, Kallianpur A, Brown N, et al. Environmentally determined genetic expression: clinical correlates with molecular variants of carbamyl phosphate synthetase I. *Mol Genet Metab* 2004;81 Suppl 1:S12-19.
61. Moonen RM, Paulussen AD, Souren NY, Kessels AG, Rubio-Gozalbo ME, Villamor E. Carbamoyl phosphate synthetase polymorphisms as a risk factor for necrotizing enterocolitis. *Pediatr Res* 2007;62:188-190.
62. Becker RM, Wu G, Galanko JA, Chen W, Maynor AR, Bose CL, Rhoads JM. Reduced serum amino acid concentrations in infants with necrotizing enterocolitis. *J Pediatr* 2000;137:785-793.
63. Zamora SA, Amin HJ, McMillan DD, Kubes P, Fick GH, Butzner JD, Parsons HG, et al. Plasma L-arginine concentrations in premature infants with necrotizing enterocolitis. *J Pediatr* 1997;131:226-232.
64. Sangild PT, Siggers RH, Schmidt M, Elnif J, Bjornvad CR, Thymann T, Grondahl ML, et al. Diet- and colonization-dependent intestinal dysfunction predisposes to necrotizing enterocolitis in preterm pigs. *Gastroenterology* 2006;130:1776-1792.

Chapter 5

The role of arginine-metabolizing enzymes in vascular physiology

Vincent Marion¹, Merlijn Meens^{1,2}, Chiel de Theije¹, Paul van Dijk¹, Gregorio Fazzi², Jo de Mey², Wouter H. Lamers¹ and S. Eleonore Köhler¹

¹Depart of Anatomy & Embryology, and ²Depart of Pharmacology & Toxicology,
Maastricht University, Postbus 616, 6200MD, Maastricht, The Netherlands

Summary

An early feature of endothelial dysfunction is a decline in arginine-dependent nitric oxide (NO) production, but the relatively mild phenotype of mice lacking *all* three NO-synthesizing (NOS) enzymes suggests that NO bioavailability itself is not highly pathogenic. NOS produces NO and citrulline in the presence of arginine and tetrahydrobiopterin. If either substrate is deficient, NOS “uncouples” and produces the oxygen radical superoxide. These findings suggest that arginine deficiency is more detrimental for endothelial function than NO deficiency. We therefore investigated the effects of endothelial inactivation of argininosuccinate synthetase (Ass; arginine resynthesis from the NOS product citrulline) or Arginase-1 (Arg1; arginine breakdown to ornithine and urea) by crossing mice with genetically modified Ass (Assflox) or Arg1 (Arg1flox) alleles with Tie2-Cre mice that specifically express Cre in the endothelium. Ass and NOS3 are co-expressed in the endothelial cells of arteries of young wild-type animals, whereas no arginase1 was detected in these vessels. Arteries lacking either the expression of Ass or Arg1 did not differ from wild-type arteries in their contractile response to phenylephrine or their relaxation response to acetylcholine. Only after challenging the arteries with repeated contractions-relaxation cycles, we could demonstrate that the relaxation of Ass-deficient arteries was rescued by arginine, but not citrulline, whereas the relaxation of Arg1-deficient arteries had become more sensitive to both added arginine and citrulline than wild-type arteries. The exact mechanism by which arginine deprivation causes endothelial dysfunction will be further investigated.

Abbreviations

CRC:	Concentration Response Curve	NO:	Nitric Oxide
PGI ₂ :	Prostacyclin		
EDHF:	Endothelial- derived Hyperpolarizing Factor		
CNP:	C-type natriuretic peptide		
NOS:	Nitric Oxide Synthase		
COX:	Cyclooxygenase		
ED:	Endothelial Dysfunction		
ROS:	Reactive Oxygen Species		
ASS:	Argininosuccinate Synthetase		
ASL:	Argininosuccinate Lyase		
CAT-1:	Cationic Amino Acid Transporter 1		
oxLDL:	oxidized LDL		
ARG1:	Arginase1		
ARG2:	Arginase2		

Introduction

The endothelium is the metabolically active tissue that lines the lumen of all blood vessels. Its vasoprotective effects include the suppression of platelet aggregation and monocyte adhesion, and smooth-muscle cell growth and relaxation (1). Endothelium-dependent agonists such as acetylcholine and bradykinin, in addition to physical vascular stimuli such as shear stress, stimulate endothelial cells to release the vasodilators nitric oxide (NO), prostacyclin (PGI₂), and endothelium-derived hyperpolarizing factor (EDHF). The identity of EDHF is still unknown, although K⁺ ions, cyclic AMP, cytochrome P₄₅₀-2C products, H₂O₂, spread of electric current, and, most recently, C-type natriuretic peptide (CNP) have been suggested (2, 3). As long as EDHF remains unidentified, its involvement in regulating vascular tone is defined as the response that persists after blocking NO synthase (NOS) and cyclooxygenase (COX) (2). For this reason, NOS3^{-/-}/Cox1^{-/-} double-knockout mice are also known as EDHF mice (1). NO and PGI₂ are the predominant endothelium-derived vasodilators in conduit arteries, whereas the influence of EDHF is more prominent in resistance arteries (4), probably because basal NO production tonically inhibits the synthesis of prostaglandins and the responses to EDHF (1, 5). Male NOS3^{-/-}/Cox1^{-/-} knockout mice suffer from hypertension, whereas their female counterparts do not, suggesting that EDHF could be more functional in females than in males (1, 2, 5, 6).

Endothelial dysfunction (ED) is the impaired ability of vascular endothelium to stimulate vasodilatation (7, 8). An early and key feature of ED is a decline in the bioavailability of NO as a result of a reduced synthesis and/or an increased degradation by reactive oxygen species (ROS). ED is, therefore, also associated with abnormal platelet aggregation and monocyte adhesion, and predisposes to both small-(hypertension) and large-artery disease (atherosclerosis).

Endothelial NO is produced from L-arginine by the NOS isoform 3 (NOS3) (7). The functional link between arginine metabolism and NO synthesis is reflected in the common localization of NOS3, the L-arginine synthesizing enzymes argininosuccinate synthetase (ASS) and lyase (ASL), and the L-arginine transporter CAT-1 in the endothelial caveolae (Figure 1) (9-11). Factors that enhance the formation of caveolae, e.g. polymerization of the microtubular cytoskeleton, increase NO production (2), whereas factors that disrupt caveolae, such as oxidized LDL (oxLDL),

decrease NO production (12, 13). NOS compete with arginase for their common substrate L-arginine. Mitochondrial arginase (ARG2) appears to be the constitutively expressed arginase isoform in the endothelium, whereas cytosolic ARG1 expression is upregulated in ageing (14, 15), diabetes (16), ischemia (17), and hypertension (18), implying that it is the inducible isoform.

Figure 1: Arginine and its pivotal role in the endothelial signaling. Three sources of arginine are available for the endothelial cell: 1) the synthesis from citrulline imported from the extracellular environment via the citrulline transporter (SN2), followed by the action of ASS and AL; 2) from the recycling of the NOS3 metabolite citrulline back to arginine, using the same two enzymes; or 3) from the direct absorption of arginine from the blood via an arginine transporter such as CAT-1. Two enzymes in the endothelial cell compete for arginine as substrate: 1) NOS3, which produces NO and citrulline; and 2) arginase, which produces ornithine and urea. NOS3 is activated by an increase in intracellular Ca²⁺ ions following Ach binding to its receptor (AChR) and is localized in a specific compartment, called the caveolae. The NO produced readily diffuses to the neighboring smooth-muscle cell. At low intracellular arginine concentrations, NOS3 uncouples and produces the oxygen radical superoxide (red) rather than NO. NO activates soluble guanylate cyclase (sGC), which metabolizes GTP into cyclic GMP (cGMP) causing relaxation of the smooth-muscle cell (vasodilatation). Phenylephrine (Phe), on the other hand, by binding to its receptor on the smooth-muscle cell, causes contraction of this cell (vasoconstriction). In a similar way, K⁺ ions increase the concentration of the Ca²⁺ ions inside the smooth-muscle cell and cause a contraction.

The relatively mild phenotype of mice lacking all three NO-synthesizing (NOS) enzymes (19) indicates that NO bioavailability in itself is not the major determinant of ED. NOS produces NO and L-citrulline from L-arginine in the presence of the co-factor tetrahydrobiopterin (BH4). If either becomes deficient, NOS “uncouples” and oxidizes oxygen to superoxide (O₂⁻) (20, 21), which enhances oxidative stress and BH4 oxidation (21, 22). These findings suggest that deficiency of L-arginine or BH4 is more detrimental for endothelial function than deficiency of NO.

Many aspects of endothelial arginine metabolism, in particular the degree to which import of L-arginine from the plasma, intracellular recycling of L-citrulline to L-arginine, and L-arginine catabolism by arginase contribute to the availability of L-arginine as a substrate for NOS3 (Figure 1) remain incompletely understood. Obviously, mechanisms can be sorted out in cultured endothelial cells, but pathogenetic and therapeutic studies require animal models. In this preliminary study, we show that it is possible to generate mice which do not express Ass and Arg1 in the arterial endothelium by crossing mice with genetically modified (“floxed”) Ass or Arg1 alleles with Tie2-Cre mice, which express the Cre recombinase in their endothelium. These mice do already exhibit a functional phenylephrine phenotype as young adults upon endothelial L-arginine depletion with repeated contraction-dilation cycles of vessel segments. As a proof of principle, we could further show that exogenous L-arginine yielded a higher acetylcholine-dependent vasodilation in endothelial Arg1-deficient vessels than in endothelial Ass-deficient vessels, and that L-citrulline supported vasodilation in this condition in endothelial Arg1-deficient, but not in endothelial Ass-deficient mice.

Methods

Animal husbandry and genotyping

Animal studies were reviewed and approved by the institutional animal care and use committee of Maastricht University. Animals were housed in humidity- and temperature-controlled rooms on a 12-hour light/ 12-hour dark cycle with food and water ad libitum. The generation of conditional knockout mice, in which “flox” sequences surround exon 13 of argininosuccinate synthetase (Ass^{fl}) or exon 4 of arginase-1 (Arg1^{fl}) is described in chapters 2 and 3. The presence of Ass^{fl} or Arg1^{fl} alleles was determined by PCR using the primers (Sigma Genosys) given in Table 1. Specific excision of the Ass^{fl} or Arg1^{fl}

alleles in the endothelium was achieved by crossing floxed mice with Tie2-Cre mice (23).

Tissue collection

Mice were sacrificed by O₂/CO₂-inhalation. The thoracic aorta, mesenteric (first order side branches), femoral and saphenous arteries were dissected. During dissection, adipose and connective tissue were carefully removed from the arteries. After dissection, arteries were collected in Krebs-Ringer bicarbonate buffer (118.5 mM NaCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 1.25 mM NaHCO₃, 2.5 mM CaCl₂, and 5.5 mM glucose) and kept at 4°C until assayed. Other parts of the vessels were fixed in 4% buffered formalin and embedded in paraplast.

Histology

The aortic arch, the thoracic aorta, and the mesenteric and femoral arteries were sectioned at 7µm. Some sections were stained with haematoxylin and eosin. For immunostaining, paraffin-embedded tissues were sectioned at 5 µm. After deparaffination, the sections were pre-incubated with Teng-T (10 mM Tris (pH=7.4), 5 mM EDTA, 150 mM NaCl, 0.25% gelatin, 0.05% Tween-20) /10 % normal goat serum (NGS) for 30 min, followed by an overnight incubation with the ASS, ARG1 or NOS3 primary antibodies diluted in Teng-T/10 % NGS. After washing with PBS and another pre-incubation with Teng-T/10 % NGS for 15 min, sections were incubated with goat-anti-rabbit secondary antiserum (Sigma A3687; diluted in Teng-T/10 % NGS) for 90 min. After washing, the slides were incubated with Nitroblue tetrazolium/5-Bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) substrate (Roche 169471) at 37°C until staining reached an optical density not exceeding 0.8. After washing and dehydration, the slides were exposed to UltraClear and mounted with UltraKit Histograde (J.T.Baker).

Myograph measurements

Two-millimeter segments of the arteries were mounted on steel wires (diameter 40 µm) in a myograph organ bath (model 610, Danish Myotechnology, J.P. Trading, Denmark) for isometric force measurements. Organ baths were filled with Krebs-Ringer bicarbonate buffer, maintained at 37°C, and aerated with carbogen gas (95% O₂/5% CO₂). The arterial segments were stretched until a transmural pressure corresponding to

13.3 N/m² was reached. Arterial viability was assessed using a single dose of 10⁻⁵ M phenylephrine (Phe; Sigma P6126, The Netherlands) to stimulate contraction. Endothelium was judged to be intact, when subsequent addition of 3.10⁻⁵ M acetylcholine (Ach) induced an almost complete relaxation of the segments. Whenever this initial relaxation in response to acetylcholine was <50%, the endothelium was considered to be damaged and the vessel was discarded. Contraction-response curves (CRC) for Ach (10⁻⁸-3.10⁻⁵ M in half-log steps) were made following precontraction with Phe (3.10⁻⁵ M). In addition, Ach-dependent vasodilation after precontraction of the vessel rings with 4.10⁻² M potassium was tested. Furthermore, the effect of 10⁻⁴ M of the L-arginine analogue L-NAME, in the presence of either 10⁻³ M arginine or 10⁻³ M citrulline, was investigated. NO-dependent vasodilatation was also assessed by exposing the vessel rings to the NO-donor sodium nitroprusside (SNP; 10⁻⁸-3.10⁻⁵ M in half-log steps).

L-Arginine depletion experiments

Two-millimeter segments of femoral arteries were mounted and stretched in a myograph and subjected to 14 CRC cycles with Phe and Ach at 15 min intervals. Thereafter, the vessel segments were incubated in either 10⁻³ M arginine or 10⁻³ M citrulline in Krebs medium prior to a final Phe-Ach CRC.

Statistics

Results are shown as means ± standard deviation. Significance of the results was determined by paired *t* tests. A value of *P* < 0.05 was considered to denote statistical significance and was marked with an asterisk.

Results

ASS, NOS3, and ARG1 expression in the endothelium

To confirm the presence of ASS, NOS3, and ARG1 in the endothelial cells, segments of different arteries were processed histologically and stained with haematoxylin/eosin to demonstrate the presence of endothelium and with specific antisera to demonstrate the presence of ASS and NOS3. Representative pictures of the aorta and the mesenteric and femoral arteries are shown in Figure 2.

The Figure shows that the endothelium, internal elastic lamina, tunica media and tunica adventitia were all similar in control and experimental mice. ASS protein was found in the endothelial cells in all three types of arteries, indicating that ASS is expressed both in the endothelial cells of the large elastic arteries and of the medium-sized muscular arteries.

Figure 2: Cellular localization of ASS and NOS3. Aorta and femoral artery stained for the presence of Ass and NOS3. The endothelium of both vessels expresses Ass and NOS3 (middle and right columns, respectively). Adjacent sections of both arteries (left panel) were stained with H&E to verify the presence of endothelial cells. Original magnification: 40X. staining is also observed in the tunica media of the aorta and in the tunica adventitia of the mesenteric artery. These cells correspond with Ass-positive nerve cells. The NOS3 antiserum produced a similar staining pattern as the ASS antiserum (Figure 2, right column) in the endothelial cells. This indicates that the endothelial cells of these arteries can produce NO and resynthesize L-arginine from L-citrulline, the other NOS product. No staining for ARG1 could be detected in the arteries of these young healthy mice (data not shown).

Generation of mice with a deletion of ASS or ARG1 in the endothelium

We initiated breedings to obtain mice that were deficient in their endothelium for Ass, Arg1, or the cationic transporter Cat1. Breedings were set to obtain the control groups Ass^{+/+}/Tie2-Cre^{+/+}, Arg1^{+/+}/Tie2-Cre^{+/+} and Cat^{+/+}/Tie2-Cre^{+/+}, and the corresponding experimental groups Ass^{-/-}/Tie2-Cre^{+/+}, Arg1^{-/-}/Tie2-Cre^{+/+} and Cat^{-/-}/Tie2-Cre^{+/+}. We opted to delete one allele in all cells to make sure that Tie2-Cre would excise the single

remaining floxed allele in as many endothelial cells as possible. Unfortunately, $Cat^{fl}/Tie2-Cre^{+/+}$ animals died 2-3 days after birth with the same symptoms as $Cat^{-/-}$ mice, viz severe anemia (C. de Theije et al, unpublished observations). The viable offspring was genotyped using specific primer pairs (Table 1) to detect the wild-type, floxed and excised alleles, and the Tie2-Cre construct. Figure 3 shows the typical result of genotyping *Ass* animals, yielding 360bp and 390bp bands with the *AssF1* and *AssR1* primer pair for Ass^{+} and Ass^{fl} alleles, respectively (top panel). The middle panel shows that the *excised* Ass^{fl} allele yields a 340bp band with the *AssF2* and *AssR1* primer pair, while the bottom panel shows that the Tie2-Cre-specific PCR yields a band of 360bp. For *Arg1* mice, primer pair *ArgF1* and *ArgR1* yields bands of 351bp and 384bp for the $Arg1^{+}$ and $Arg1^{fl}$ alleles, respectively, while the excised $Arg1^{fl}$ allele yields a 298bp band with primer pair *AssF2* and *Arg1-R1*.

Figure 3: Genotyping the Tie2-Cre/*Ass*^{+/fl} mice. Panel A: Typical genotyping data to demonstrate the presence of the wild-type and floxed *Ass* alleles (top panel, obtained when using primer pair *AssF1* & *AssR1* described in Table 1) in arteries. The middle panel reveals the presence of the excised *Ass* allele (obtained with the primer pair *AssF2* and *AssR1*). In the bottom panel, the expression of *Cre* in arterial isolates (obtained with the *Tie2* primer pair). Left to right: positive control, H₂O (negative) controls, femoral 1, femoral 2, saphenous 1, saphenous 2, mesenteric 1 and mesenteric 2.

Endothelial-specific inactivation of ASS expression

To verify that specific inactivation of Ass by Cre-mediated recombination had taken place inside the endothelial cells, paraffin-embedded sections of arteries from experimental animals (Ass^{-fl}/Tie2-Cre^{+/+}) and the corresponding controls (Ass^{-fl}/Tie2-Cre^{+/+}) were stained for Ass. Figure 4 shows representative results for the femoral artery. There is clear staining for Ass in the endothelial cells in the control artery (top panel) compared to the experimental animals (middle panel). The negative control (bottom panel) shows no staining for Ass. The right column shows adjacent H&E- stained sections to confirm the presence of the endothelial cells. Similar results were obtained for the other arteries (data not shown).

Role of L-arginine in the regulation of the vascular tone

Vascular tone refers to the ability of vascular smooth muscle to maintain a permanent partial constriction based on equilibrium between the effects of vasodilator and vasoconstrictor antagonists. To establish whether L-arginine and endogenous NO production could play a role in contraction, the response of the aorta, femoral, saphenous and mesenteric arteries to cumulatively increasing concentrations of Phe was analyzed. The results of experiments conducted on the femoral artery are depicted in Figure 5A. These results were typical for all arteries analyzed, irrespective of genotype. The amplitude of the response in Krebs buffer was set at 100%. In Krebs buffer, the log IC₅₀ for contraction was -4.9 ± 0.1 . In the presence of L-arginine (1mM), the response to Phe did not change (log IC₅₀: -4.8 ± 0.5), but in the presence of 1mM L-citrulline, the log IC₅₀ declined to -4.2 ± 0.2 . Supplementing extracellular L-arginine or L-citrulline did not alter the contraction of arteries, irrespective of whether ASS was absent or not. Pre-contracted arteries were then assessed for their ability to dilate in response to Ach in the presence of either L-arginine or L-citrulline. In Krebs buffer, the log IC₅₀ of Ach for dilatation was -7.3 ± 0.4 . There was no significant change in the response to Ach in the presence of L-arginine (log IC₅₀ Ach: -7.5 ± 1.7 (Figure 5B). Surprisingly, the vascular response to Ach was significantly less sensitive in the presence of L-citrulline (log IC₅₀ Ach: -6.5 ± 0.6 ; Figure 5C). While L-arginine therefore tended to decrease the sensitivity of the arteries to Ach-mediated dilatation, L-citrulline decreased it by more than 10-fold, irrespective of genotype.

Figure 4: Specificity of Tie2-Cre-mediated endothelial inactivation of Ass. Expression of ASS in the femoral artery of Ass^{+/-}/Tie2-Cre^{+/-} control mice (top panel) and Ass^{-/-}/Tie2-Cre^{+/-} experimental animals (middle panel). The endothelial cells of the Ass^{+/-}/Tie2-Cre^{+/-} animals do show a positive signal for Ass, whereas the simultaneously stained artery of an Ass^{-/-}/Tie2-Cre^{+/-} mice did not. If no primary antiserum was added, the control artery did not stain either (bottom panel). To confirm the presence of endothelial cells in the arteries, adjacent sections were stained with H&E (right column).

Figure 5: Contractile and dilatory properties of experimental arteries The arterial contraction in the presence of phenylephrine (10^{-8} - $10^{-4.5}$ M) in absence or presence of 1mM arginine or citrulline was registered (panel A). The amplitude of the response in Krebs buffer was set at 100%. The contractile response was not dependent on the genotype. Furthermore, neither arginine nor citrulline affected the contraction. The log IC_{50} for contraction was -4.9 ± 0.1 in the control condition (Krebs buffer only), -4.8 ± 0.5 in the presence of 1mM arginine, and -3.2 ± 0.3 in the presence of 1mM citrulline. Citrulline decreased the sensitivity to phenylephrine significantly. The amplitude of the contraction did not change in any condition. In panels B and C, the arteries were precontracted with phenylephrine prior to exposure to 1mM arginine or citrulline (panel B and C, respectively). In Krebs buffer alone, the log IC_{50} for dilatation was -7.3 ± 0.4 . The addition of arginine to the incubation medium had no significant effect (log IC_{50} : -7.5 ± 1.7), whereas the vascular response to Ach declined in the presence of L-citrulline (log IC_{50} : -6.5 ± 0.6).

L-NAME inhibition of endothelial vasodilation

L-NAME is a nonselective competitive and, therefore, reversible NOS inhibitor that is often used to block NOS3-mediated NO production. The amplitude of the response to Phe was significantly higher in presence of L-NAME ($P < 0.05$) (Figure 6A). We then tried to reverse the effect of L-NAME by adding either L-arginine or L-citrulline (Figure 6B). However, neither L-arginine nor L-citrulline could, at the concentrations used (100 and 1000 μ M, respectively) revert the effect of L-NAME on Phe-mediated arterial contractions (log IC_{50} : -5.0 ± 0.1). We then investigated the effect of L-NAME on Ach-mediated relaxation for the arteries. For both control and experimental groups of arteries, L-NAME significantly prevented the arterial relaxation of the arteries treated with Ach (log IC_{50} : -6.7 ± 0.8 ; Figure 6C). Exogenous L-arginine or L-citrulline added at a concentration of 1mM to the experimental medium could also not rescue the vascular response to Ach in the presence of L-NAME (log IC_{50} : -7.1 ± 0.8 and -6.6 ± 0.5 respectively; Figure 6D).

The findings shown in Figure 6 suggest that the effects of the L-arginine analogue L-NAME on NOS3 activity in the mouse are not easily reversible. In the rat, however, L-NAME has been described as a reversible inhibitor with inhibitory

properties that are reverted by a 10-fold excess (100 μ M) L-arginine (24-26). To verify our findings in the mouse, we checked the reversibility of the effects of L-NAME in rat aortic rings that were mounted in the myograph exactly as the mouse arteries. After Phe-mediated precontraction, we induced arterial relaxation by addition of Ach in presence normal medium with or without supplementation with either 100 μ M L-arginine or 1000 μ M L-citrulline. In all three conditions, Ach induced a vasodilatory response (Figure 7A).

Figure 6: Effect of arginine and citrulline on inhibition of NOSs by L-NAME. Arterial segments were precontracted with phenylephrine. The logIC₅₀ for phenylephrine was -5.0 ± 0.1 , whether or not 0.1mM L-NAME was present (panel A). However, the amplitude of the response to phenylephrine increased significantly in the presence of L-NAME ($P < 0.05$). Addition of 100 μ M arginine or citrulline did not reverse the effect of L-NAME (panel B; log IC₅₀: -5.0 ± 0.2 and -4.9 ± 0.1 for arginine and citrulline, respectively). The amplitude of the response remained unchanged. In the presence of L-NAME (10-4M), vasodilation in response to acetylcholine was decreased significantly (log IC₅₀: -6.7 ± 0.8 ; panel C). The addition of 1mM arginine or citrulline (panel D) did not restore the vascular response to acetylcholine (logIC₅₀: -7.1 ± 0.8 and -6.6 ± 0.5 , respectively). In addition, no affect on the amplitude of the response was seen.

The effects of L-NAME are reversible in rat vessels

Pretreatment with L-NAME completely inhibited the Ach-induced vasodilation (Figure 7B; square plot). This inhibition could be partially restored by supplementing the medium with extra L-arginine or L-citrulline (upright and inverted triangles plots, respectively). This confirms the reversibility of the effects of L-NAME in rats and strongly suggests that the effects of L-NAME are not as readily reversible in the mouse.

The contribution of the intracellular arginine pool to vasodilatation

To test if NOS3 activity becomes more sensitive to external L-arginine or L-citrulline after depletion of the intracellular arginine pools, femoral artery segments were mounted in the myograph and subjected to repeated CRC cycles in L-arginine- and L-citrulline-free Iscove's culture medium. After the 14th CRC cycle, the vasodilatory response to Ach was only 10% of the original one (Figure 8A; right and left top panels, respectively). After the 13th CRC cycle, arterial segments were then incubated for 1 hour in either 100 μ M L-arginine or 1000 μ M L-citrulline, pre-contracted for the 14th time with Phe, and then subjected to Cre arteries and panel C with Ass^{- η} /Tie2-Cre arteries. In both cases, the incubation with L-arginine restored the vasodilation, with a higher degree of vasodilation in Arg1^{- η} /Tie2-Cre arteries (~50% dilation at 10⁻⁵ M Ach) than in Ass^{- η} /Tie2-Cre arteries (~30% dilation at 10⁻⁵ M Ach). Treatment with L-citrulline, on the other hand, only caused a increasing concentrations of Ach. Panel B shows the results obtained with Arg1^{- η} /Tie2- significant increase in dilation in Arg1^{- η} /Tie2-Cre arteries. These findings show that the arterial endothelium in mice is sensitive to extracellular L-arginine and can synthesize L-arginine from L-citrulline, in agreement with the presence of Ass protein (Figures 2 and 4).

Discussion

Performance of the animal models

The aim of our study was to delete the expression of Ass, Arg1 and Cat-1 in the arterial endothelium to interfere with intra-endothelial recycling of L-citrulline to L-arginine, to inhibit intra-endothelial breakdown of L-arginine and to decrease L-arginine uptake from the plasma. We could not use endothelial Cat-1-deficient mice, because Cat-1 ^{η/η} /Tie2-Cre mice died neonatally with much the same phenotype as Cat-1^{-/-} mice (C. de Theije et

Figure 7: Reversibility of NOS3 inhibition by L-NAME in the rat aorta. L-citrulline, but not L-arginine significantly sensitized the rat aorta to the vasodilatory effect of acetylcholine (panel A). In the presence of 10⁻⁴M L-NAME (panel B), acetylcholine-dependent vasodilation was totally abolished (square plot), but vasodilation was restored by supplementing the medium with either arginine or citrulline only at high Ach concentrations (upright and inverted triangles plot).

al, unpublished observations). We used mice with an *Ass*^{-fl}/Tie2-Cre or *Arg1*^{-fl}/Tie2-Cre genotype, that is, with one allele already inactivated systemically, to assure that the floxed allele would be deleted in as many endothelial cells as possible. We followed this strategy with the reasoning that if Cre is not 100% efficient due to e.g. low expression levels, deletion of a single allele is more probable than deletion of two alleles. However, thus far, alleles appear to be removed equally efficient from fl/fl genotypes as from -/fl genotypes (data not shown). We have succeeded in the first 2 aims, because endothelial segments that were depleted of L-arginine by repeated contraction-relaxation cycles (Ach-dependent relaxation became near extinct) could be rescued by L-arginine. Because *Ass*^{-fl}/Tie2-Cre artery segments could not be rescued by L-citrulline, whereas *Arg1*^{-fl}/Tie2-Cre artery segments were efficiently rescued, citrulline-to-arginine cycling must have been effectively abolished. Furthermore, *Arg1*^{-fl}/Tie2-Cre artery segments were much more effectively rescued by L-arginine than *Ass*^{-fl}/Tie2-Cre artery segments, which implies that deletion of Arg-1 made more exogenous L-arginine available to NOS3. Interestingly, Arg1 was not detectable in the endothelium of these femoral segments when they were taken from the body. We will have to explore if Arg1 did become expressed in the course of this experiment, which took several hours, or whether

Figure 8: *Ass*- and *Arg1*-deficient mouse arteries become sensitive to the effects of arginine and citrulline after repeated contraction-relaxation cycles. Femoral arteries from *Tie2-Cre^{+/+}/Ass^{-fl}* and *Tie2-Cre^{+/+}/Arg1^{-fl}* mice were mounted in a myograph. Directly after isolation, the $\log IC_{50}$ of acetylcholine in *Ass*- and *Arg1*-deficient arteries (-6.4 ± 0.4 and -6.5 ± 0.2 , respectively; panel A, left) did not differ from that of wild-type arteries ($\log IC_{50}$: 6.7 ± 0.8). After 14 cycles of 10^{-5} M phenylephrine-dependent precontractions and acetylcholine-dependent dilations (10^{-8} – $10^{-4.5}$ M), the $\log IC_{50}$ was reduced to -3.5 ± 0.25 and -5.1 ± 0.2 (panel A; right). The initial vasodilator response was 75% at 10^{-5} M acetylcholine, but after 14 cycles only ~10% remained. In panels B and C, *Ass*- (panel C) or arginase-deficient arteries (panel B) were (colored line) or were not (black line) incubated for 1 hour in the presence of either 100 μ M arginine or citrulline after contraction-dilation cycle #14. In the *Arg1*-deficient arterial segments, the vascular response to acetylcholine significantly increased in the presence of L-arginine or L-citrulline (panel B, right). In the *Ass*-deficient arterial segments, no significant change in dilatation was found in the presence of L-citrulline (panel C, right; 35% relaxation), whereas in the presence of arginine (panel C, left), a significant response to 10^{-5} M acetylcholine was seen (55% relaxation).

very low levels of Arg1 suffice to interfere with the intracellular availability of L-arginine to NOS3.

Response to extracellular L-arginine and arginine pools in the endothelium

Although *Ass^{-fl}/Tie2-Cre* and *Arg1^{-fl}/Tie-2-Cre* mice did respond as expected, they did so only after the intra-endothelial arginine pool had been depleted by repeated contraction-relaxation cycles. The problem with arginine pools in the endothelium is well established and often referred to as the “arginine paradox”. In fact, the problem seems to be that the endothelium maintains several intracellular arginine pools (27). One pool can be depleted by extracellular lysine, which acts via the L-arginine transporters Cat-1 and CD98, whereas the second pool is not freely exchangeable with the extracellular space, but accessible to NOS3. Recycling of L-citrulline to L-arginine occurs in the second pool and could be inhibited by activating transporter System N1 (SN1; Slc38A3), presumably by the removal of intracellular L-citrulline, one of its substrates. A third pool could not be depleted by any of the cationic or neutral amino acids tested and is fed by protein breakdown. This pool does not only generate L-arginine, but also the posttranslationally modified L-arginine products NG-monomethyl-L-arginine (L-NMMA) and asymmetric dimethylarginine (ADMA), which are inhibitors of NOS3. Since it is postulated that supplementation of L-arginine is primarily effective for conditions, in which endothelial ADMA production is enhanced (28), we should investigate whether the beneficial effects of L-arginine on the relaxation capacity after repeated contraction-relaxation cycles are due to enhanced proteolysis and ADMA release.

Limitations of this study

Vasodilation of arteries is primarily based on the additive action of NO, prostanoids and EDHF. The relative contribution of these factors depends on the location in the vascular tree (4, 29), the sex of the animals (1, 5, 6) and the adaptive upregulation of the remaining factors in case a factor is inhibited (1, 5). Most experiments that study endothelium-dependent relaxation, including those in mice (30, 31), analyze vessel rings with inhibitors for all these relaxants separately and in combination. We have not done so in the present study and should do so in future experiments.

Surprisingly, vascular dilatation in response to Ach did not respond to extracellular L-arginine or L-citrulline without prior L-arginine depletion. Rather, the addition of L-citrulline and, to a lesser extent, L-arginine inhibited the response to Ach in Phe-pre-contracted vessels. It has been reported that high concentrations of L-arginine (>100 μ M) exert an inhibitory effect on NOS3 activity by a process called “substrate inhibition” (29). We tested the optimal arginine concentrations in our settings and found it to be 100 μ M for arginine and 1000 μ M for citrulline (data not shown).

The absence of a more extensive relaxation in the presence of extra ambient L-arginine could be due to inaccessible intracellular arginine pools in the endothelial cell that feed NO production (see above) or to an Ach-mediated vasodilation that is independent of L-arginine and NO. Indeed, it was recently demonstrated that under oxidative stress or tetrahydrobiopterin (BH4) depletion, the endothelium can produce and release hydrogen peroxide (H_2O_2) after exposure to Ach (32). H_2O_2 , in turn, activates the downstream target of cyclic GMP (PKG1 α) independently of cyclic GMP (33). Under these conditions, NOS3 is probably uncoupled, because H_2O_2 -dependent regulation of the vessels wall is L-NAME-sensitive (32).

As expected, the vasodilatory responses to Ach declined significantly in the presence of the competitive NOS inhibitors L-NAME and S-methylisothiourrea (data not shown). However, supplementation with neither L-arginine nor L-citrulline restored the vasodilator response, even though these compounds should be able to compete with the L-arginine look-alike inhibitors. Therefore, L-NAME and S-methylisothiourrea appear to behave as irreversible inhibitors in mice, even though they do not behave in this way in other species (24, 25). We could, for example, show that in a similar experimental set-up, the effects of L-NAME in aortic-ring segments of the rat were reversible (Figure 7). In agreement with our findings, it has been shown that 1mM L-arginine in the medium cannot undo the effects of L-NAME in mice (34). However, 30mM L-arginine could effectively antagonize L-NAME in that study, showing that mice may differ quantitatively, but not qualitatively from rats.

Conclusions

In the present exploratory study, we did show that mice with floxed *Ass* or *Arg1* alleles are, after crossing with the deleter strain Tie2-Cre, useful models to study L-arginine metabolism in the arterial endothelium. Unfortunately, the Tie2-Cre deleter strain

generates Cat^{-/-} mice that die neonatally, so that we have to try other endothelium-specific Cre mice to delete Cat-1 mediated L-arginine import. The immunohistochemical assays showed that Ass is indeed expressed in all arterial endothelium we have studied so far, but that Arg1 was not detectable in the endothelium of young adult mice, in agreement with the notion that Arg1 only becomes induced in dysfunctional endothelium. Our myograph measurements yielded the expected genotype-dependent responses to L-arginine and L-citrulline, although thus far only after repeated contraction-dilatation cycles. Finally, recent experiments showed that NO production *in vivo* was reduced ~2-fold in the kidney of Ass^{-fl}/Tie2-Cre mice compared to Ass^{-fl} mice (van Faassen & Koehler, unpublished observations). In conclusion, our observations therefore show that Ass^{-fl}/Tie2-Cre and Arg1^{-fl}/Tie-2-Cre mice are promising mouse models to study the relation between endothelial arginine metabolism and endothelial dysfunction.

Appendix:

primer	sequence	primer pair	allele detected	band size (bp)
Ass F1	5'GAGGAAGGGACAGTGGGGTG 3'	AssF1; AssR1	Ass ⁺ & Ass ^{fl}	360 & 390
Ass R1	5'ACTGCTCAGGGCACGCATGTG 3'	AssF2; AssR1	Ass ⁻	340
Ass F2	5'TCTAGAACTAGTGGATCACCTC 3'			
Tie2F	5'CGCATAACCAGTGAAACAGCATTGC 3'	Tie2F; Tie2R	Cre ^{tg}	360
Tie2R	5'CCCTGTGATAAGACAGAAATGAGA 3'			
Arg F1	5'GGAGAGCCTTCCTGCACTTT 3'	ArgF1; ArgR1	Arg ⁺ & Arg ^{fl}	351 & 384
Arg R1	5'GTGCCTTGGTCTACATTGAACATAC 3'	AssF2; ArgR1	Arg ⁻	298

Supplementary Table 1: Primers used in the present study. Primer pairs AssF1 and AssR1 were used to detect the wild-type allele and floxed alleles as bands of 360bp (wild-type allele) or 390bp (floxed allele). The other primer pair Ass-F2 and Ass-R1 was used to detect the excised allele (exon 13) producing a band of 340bp. In a similar manner, primer pair Arg-F1 and Arg-R1 was used to detect the wild-type allele and floxed alleles as bands of 351bp (wild-type allele) or 384bp (floxed allele). The primer pair Ass-F2 and Arg-R1 was used to detect the excised allele (exon 4) yielding a band of 298bp. Finally, the primer pair Tie2F and Tie2R yields a band of 360bp in animals transgenic for the Tie2-Cre construct.

References

1. Villar IC, Francis S, Webb A, Hobbs AJ, Ahluwalia A. Novel aspects of endothelium-dependent regulation of vascular tone. *Kidney Int* 2006.
2. Su Y, Zharikov SI, Block ER. Microtubule-active agents modify nitric oxide production in pulmonary artery endothelial cells. *Am J Physiol Lung Cell Mol Physiol* 2002;282:L1183-1189.
3. Feletou M, Vanhoutte PM. Endothelium-dependent hyperpolarizations: past beliefs and present facts. *Ann Med* 2007;39:495-516.
4. Shimokawa H, Yasutake H, Fujii K, Owada MK, Nakaike R, Fukumoto Y, Takayanagi T, et al. The importance of the hyperpolarizing mechanism increases as the vessel size decreases in endothelium-dependent relaxations in rat mesenteric circulation. *J Cardiovasc Pharmacol* 1996;28:703-711.
5. Sun D, Liu H, Yan C, Jacobson A, Ojaimi C, Huang A, Kaley G. COX-2 contributes to the maintenance of flow-induced dilation in arterioles of eNOS-knockout mice. *Am J Physiol Heart Circ Physiol* 2006;291:H1429-1435.
6. Kuhlencordt PJ, Gyurko R, Han F, Scherrer-Crosbie M, Aretz TH, Hajjar R, Picard MH, et al. Accelerated atherosclerosis, aortic aneurysm formation, and ischemic heart disease in apolipoprotein E/endothelial nitric oxide synthase double-knockout mice. *Circulation* 2001;104:448-454.
7. Southan GJ, Szabo C. Selective pharmacological inhibition of distinct nitric oxide synthase isoforms. *Biochem Pharmacol* 1996;51:383-394.
8. Yang Z, Kaye DM. Endothelial dysfunction and impaired L-arginine transport in hypertension and genetically predisposed normotensive subjects. *Trends Cardiovasc Med* 2006;16:118-124.
9. Sbaa E, Frerart F, Feron O. The double regulation of endothelial nitric oxide synthase by caveolae and caveolin: a paradox solved through the study of angiogenesis. *Trends Cardiovasc Med* 2005;15:157-162.
10. Flam BR, Hartmann PJ, Harrell-Booth M, Solomonson LP, Eichler DC. Caveolar localization of arginine regeneration enzymes, argininosuccinate synthase, and lyase, with endothelial nitric oxide synthase. *Nitric Oxide* 2001;5:187-197.
11. McDonald KK, Zharikov S, Block ER, Kilberg MS. A caveolar complex between the cationic amino acid transporter 1 and endothelial nitric-oxide synthase may explain the “arginine paradox”. *J Biol Chem* 1997;272:31213-31216.
12. Blair A, Shaul PW, Yuhanna IS, Conrad PA, Smart EJ. Oxidized low density lipoprotein displaces endothelial nitric-oxide synthase (eNOS) from plasmalemmal caveolae and impairs eNOS activation. *J Biol Chem* 1999;274:32512-32519.
13. Zhang WZ, Venardos K, Finch S, Kaye DM. Detrimental effect of oxidized LDL on endothelial arginine metabolism and transportation. *Int J Biochem Cell Biol* 2007.
14. White AR, Ryoo S, Li D, Champion HC, Steppan J, Wang D, Nyhan D, et al. Knockdown of arginase I restores NO signaling in the vasculature of old rats. *Hypertension* 2006;47:245-251.

15. Berkowitz DE, White R, Li D, Minhas KM, Cernetich A, Kim S, Burke S, et al. Arginase reciprocally regulates nitric oxide synthase activity and contributes to endothelial dysfunction in aging blood vessels. *Circulation* 2003;108:2000-2006.
16. Romero MJ, Platt DH, Tawfik HE, Labazi M, El-Remessy AB, Bartoli M, Caldwell RB, et al. Diabetes-induced coronary vascular dysfunction involves increased arginase activity. *Circ Res* 2008;102:95-102.
17. Gao X, Xu X, Belmadani S, Park Y, Tang Z, Feldman AM, Chilian WM, et al. TNF-alpha contributes to endothelial dysfunction by upregulating arginase in ischemia/reperfusion injury. *Arterioscler Thromb Vasc Biol* 2007;27:1269-1275.
18. Demougeot C, Prigent-Tessier A, Bagnost T, Andre C, Guillaume Y, Bouhaddi M, Marie C, et al. Time course of vascular arginase expression and activity in spontaneously hypertensive rats. *Life Sci* 2007;80:1128-1134.
19. Tsutsui M, Shimokawa H, Morishita T, Nakashima Y, Yanagihara N. Development of genetically engineered mice lacking all three nitric oxide synthases. *J Pharmacol Sci* 2006;102:147-154.
20. Liu VW, Huang PL. Cardiovascular roles of nitric oxide: A review of insights from nitric oxide synthase gene disrupted mice. *Cardiovasc Res* 2008;77:19-29.
21. Rabelink TJ, van Zonneveld AJ. Coupling eNOS uncoupling to the innate immune response. *Arterioscler Thromb Vasc Biol* 2006;26:2585-2587.
22. Forstermann U, Munzel T. Endothelial nitric oxide synthase in vascular disease: from marvel to menace. *Circulation* 2006;113:1708-1714.
23. Kisanuki YY, Hammer RE, Miyazaki J, Williams SC, Richardson JA, Yanagisawa M. Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. *Dev Biol* 2001;230:230-242.
24. Rees DD, Palmer RM, Schulz R, Hodson HF, Moncada S. Characterization of three inhibitors of endothelial nitric oxide synthase in vitro and in vivo. *Br J Pharmacol* 1990;101:746-752.
25. Dalziel HH, Thornbury KD, Ward SM, Sanders KM. Involvement of nitric oxide synthetic pathway in inhibitory junction potentials in canine proximal colon. *Am J Physiol* 1991;260:G789-792.
26. Yan ZQ, Kramer K, Bast A, Timmerman H. The involvement of nitric oxide synthase in the effect of histamine on guinea-pig airway smooth muscle in vitro. *Agents Actions* 1994;41 Spec No:C111-112.
27. Simon A, Plies L, Habermeyer A, Martine U, Reining M, Closs EI. Role of neutral amino acid transport and protein breakdown for substrate supply of nitric oxide synthase in human endothelial cells. *Circ Res* 2003;93:813-820.
28. Boger RH. L-Arginine therapy in cardiovascular pathologies: beneficial or dangerous? *Curr Opin Clin Nutr Metab Care* 2008;11:55-61.
29. Su Y, Couch M, Block ER. Substrate inhibition of nitric oxide synthase in pulmonary artery endothelial cells in culture. *Nitric Oxide* 1997;1:469-475.

30. Fitzgerald SM, Kemp-Harper BK, Parkinson HC, Head GA, Evans RG. Endothelial dysfunction and arterial pressure regulation during early diabetes in mice: roles for nitric oxide and endothelium-derived hyperpolarizing factor. *Am J Physiol Regul Integr Comp Physiol* 2007;293:R707-713.
31. Fitzgerald SM, Bashari H, Cox JA, Parkinson HC, Evans RG. Contributions of endothelium-derived relaxing factors to control of hindlimb blood flow in the mouse in vivo. *Am J Physiol Heart Circ Physiol* 2007;293:H1072-1082.
32. Leung HS, Leung FP, Yao X, Ko WH, Chen ZY, Vanhoutte PM, Huang Y. Endothelial mediators of the acetylcholine-induced relaxation of the rat femoral artery. *Vascul Pharmacol* 2006;44:299-308.
33. Burgoyne JR, Madhani M, Cuello F, Charles RL, Brennan JP, Schroder E, Browning DD, et al. Cysteine redox sensor in PKGI α enables oxidant-induced activation. *Science* 2007;317:1393-1397.
34. Harrington LS, Carrier MJ, Gallagher N, Gilroy D, Garland CJ, Mitchell JA. Elucidation of the temporal relationship between endothelial-derived NO and EDHF in mesenteric vessels. *Am J Physiol Heart Circ Physiol* 2007;293:H1682-1688.

Chapter 6

Concluding discussion

In this thesis, I have described the experimental analysis of mice with a modified metabolism of arginine due to overexpression of arginase-1 or due to a tissue-selective elimination of argininosuccinate synthetase (ASS) in their small-intestinal enterocytes, or due to elimination of ASS and arginase-1 in the endothelium. The major findings are that overexpression of arginase-1 in the small-intestinal enterocytes causes the surprisingly specific “neonatal arginine deficiency syndrome” (hypoargininemia, deficient muscle and hair growth, or underdevelopment of Peyer’s patches) in the suckling period (Chapter 2), whereas elimination of ASS in the same tissue does not (Chapter 4). Preliminary studies in mice with an endothelial deletion of ASS or arginase-1 show that these manipulations do affect endothelial arginine availability, but only after stimulation of arginine turnover (Chapter 5).

Overexpression of a specific gene in a given tissue can be brought about by combining that gene with a tissue-selective promoter/enhancer and introducing this DNA construct either into the germ line (“classical” genetically modified or transgenic mice) or directly into the somatic cells of a host animal. For metabolic studies, the large majority of host cells should express the transgenic construct to maximize the effect of the intervention. For this reason, only classical transgenic mice or mice virally transduced with genes are used. The level of expression of the transgene in classical transgenic mice can vary depending on its site of integration in the DNA and the degree of mosaic expression (1, 2). Mosaic expression, that is, the heterogeneous expression of the transgene in a tissue, is the rule rather than the exception in a classical transgenic mouse line. In the case of our F/A mice, for instance, 6 founder lines were available. Four of these were strongly mosaic and without phenotype, even though the expression of the transgene in the F/A-3 and F/A-6 lines was similar to that in the F/A1 line, which did have a phenotype. Furthermore, the level of expression of arginase-1 in the 2 lines that expressed this transgene in all small-intestinal enterocytes, differed 2-fold (3), emphasizing the effect of the integration site of the transgene. The importance of the level of expression is further demonstrated by the fact that the phenotype of mice with the highest expression of the transgene (line F/A2) is more severe than that of mice with the lowest expression (line F/A1). Moreover, in both lines homozygotes had a more severe phenotype than hemizygotes.

Because a time-consuming extensive characterization of the pattern and level of expression of transgenes in classical transgenic mice is always necessary before the

line can be used in experiments, viral transduction can be attractive. In vivo, adenoviruses have a strong preference for the liver and can infect >90% of the hepatocytes (4). Although adenoviral transduction of genes is an attractive alternative to transgenesis for the liver, the situation is much less favorable for other tissues, including the endothelium (e.g. (5)).

Deletion of the arginine-metabolizing enzymes ASS and arginase-1, and of the arginine-transporter CAT-1 is not compatible with life (6-8). For this reason, we have produced conditional knockout mice, that is, mice in which the gene of interest can be selectively deleted in a specific tissue. Because the production of such mice is a long and tedious process, in which many steps can be optimized for a better efficiency, we have compiled our experience and recommendations in Chapter 3. In this “practical approach”-like chapter, we have also included the experiences that were simultaneously gathered in the generation of a conditional glutamine-synthetase knockout (He et al., submitted) and green-fluorescent protein “knockins” into the glutamine-synthetase and the phosphoenolpyruvate-carboxykinase loci at our Amsterdam location. The major modification we would probably apply if we were to make yet another conditional knockout mouse would be to surround the neomycin phosphotransferase selection gene by “frt” sites and eliminate it only after chimeras are produced. The selection cassette would then be removed by crossing the chimeric mice with “flipper” mice (9), that is, mice that transgenically express the Flpe recombination enzyme before the establishment of the germ line, so that the selection gene is also eliminated in the germ line (10). This approach was not possible with the modified embryonic stem cells we made, because we used viral thymidine kinase as selection gene. This is because all fertile chimeric mice are males (the embryonic stem cell line used is male) and viral thymidine kinase does not allow transmission through the germ line (11).

The modified approach would have the advantage that no negative selection of the embryonic stem cells with Ganciclovir is necessary. This would be preferable, because Ganciclovir can induce mutations, as we observed ourselves (Chapter 3). The other advantage would be that lines with the selection gene integrated inside the modified gene would become available. Because the expression of the modified gene is often hampered if another transcriptionally active gene is present within one of its introns, this approach would allow the production of mouse lines with attenuated expression from the allele still containing the selection marker (12-14). Especially in

cases where tissue-specific deletion of the gene of interest is lethal (as e.g. in the case of the Tie2-mediated deletion of the CAT-1 gene (Chapter 5), such intermediate levels of expression may be advantageous for an experimental analysis. Furthermore, the possibility to vary tissue-specific expression not only between 0 (complete knockout), ~50% (heterozygous knockout), and 100% (wild-type mouse), but also in between ($0\% < \text{expression/activity} < 50\%$ or $50\% < \text{expression/activity} < 100\%$) would allow one to firmly establish the metabolic control of a particular enzyme in a pathway.

The finding that F/A2 mice (and, to a lesser extent, F/A1 mice) suffer from the neonatal arginine-deficiency syndrome, indicates that a drain of arginine via overexpression of arginase-1 in the small-intestinal enterocytes is more effective in decreasing circulating arginine levels (Chapter 2) than eliminating arginine production in the intestine (Chapter 4). This finding is remarkable, since the mere size of the small intestine indicates that it should materially contribute to whole-body arginine production. In fact, it does so in the neonatal piglet, in which it accounts for ~50% of the daily endogenous synthesis (15, 16). The reason for this quantitative difference between suckling piglets and mice is not clear. Possibly, extra-intestinal arginine synthesis has a higher adaptive capacity in the mouse than in the piglet. Such a difference might also explain why the piglet appears to be more sensitive to the development of necrotizing enterocolitis (17), which may well be an arginine-deficiency disease (18-21).

The arginine that is available to suckling mice, originates from the food (milk), from endogenous synthesis, and from protein catabolism (Figure 1). Mammalian milk, including mouse milk, does not contain sufficient arginine for growth (22-24). The arginine that derives from protein catabolism cannot serve as a steady-state source unless catabolism of arginine-containing proteins is balanced by the synthesis of arginine-containing proteins. For these 2 reasons, the mouse neonate has to produce arginine endogenously. Arginine can only be synthesized from citrulline. Citrulline, in turn, is generated from ornithine and carbamoylphosphate. The only organ that can produce ornithine and carbamoylphosphate in sufficient quantities to provide the body with citrulline for arginine biosynthesis is the small intestine (25, 26). In fact, the circulating citrulline concentration is a sensitive marker for the amount of healthy gut tissue (27). Ornithine derives from glutamate or proline (28). Because of the very rapid metabolism of glutamate in the neonatal intestine (29-31), proline is quantitatively the most important ornithine precursor.

Figure 1. Sources of arginine

In mammals, including mice and man, a neonatal deficiency of ornithine aminotransferase (which produces ornithine from either glutamate or proline) or ornithine transcarbamoylase (which produces citrulline from ornithine and carbamoylphosphate) does induce a severe citrulline and arginine deficiency (32-35), underscoring the crucial role of the small intestine in citrulline production.

Human neonates often develop hyperammonemia and hypoargininemia, especially if premature and fed parenterally (19, 36). These observations mimic the finding in neonatal piglets that proline can only prevent hyperammonemia and hypoargininemia if administered enterally (16, 37) and demonstrate that intestinal feeding is necessary to allow endogenous citrulline production. By using intragastric and intraportal administration of label, it was then demonstrated that the small intestine does indeed contribute materially to whole-body arginine synthesis in the piglet (15, 38). In agreement with these findings, we and others have found that the enterocytes of the small intestine of all mammalian neonates investigated thus far, including those of human neonates, express the enzymes necessary to produce arginine from citrulline (39-43), but do not express the main cytosolic arginine catabolizing enzyme arginase-1 (39, 40, 44). In piglets at least, intestinal biosynthesis of arginine cannot be adaptively upregulated (45), suggesting extra-intestinal sites of arginine synthesis from citrulline. Our findings in mice indicate that, in this species, an as yet unidentified extraintestinal tissue can completely replace the intestine as far as synthesis of arginine from citrulline is concerned. Although the kidneys of suckling mice can produce arginine from

citrulline, their size is too minute to contribute materially. The skin as a very large organ would be another possibility, but its ASS expression declines in Ass-KO/I due to villin, and hence, villin-Cre expression (Chapter 4).

The provision of arginine for cellular functions can proceed by import from the blood or by endogenous synthesis from citrulline. Since the distribution of ASS and argininosuccinate lyase (ASL) is near ubiquitous, many tissues can synthesize arginine from citrulline. Since arginine is rapidly catabolized by arginase, whereas the only metabolic fate of citrulline in mammals is arginine synthesis, citrulline is also a much more efficient source of cellular arginine than arginine itself. This could be important if arginine from arginine import and arginine synthesized from citrulline occupy different intracellular compartments, as has been suggested (46). In the special case of the arginine-metabolizing enzyme nitric-oxide synthase (NOS), the products of the reaction are nitric oxide (NO) and citrulline, so that resynthesis of arginine in the cell producing NO is possible. Resynthesis of arginine from citrulline has been shown in neurons and endothelium (47-49). Since the upregulation of arginase-1 expression seems to be characteristic for endothelial dysfunction (50-55) increased arginase expression may be a cause of the decline in arginine levels in dysfunctional endothelium and may explain why arginine supplementation is not always effective (56, 57). The preliminary findings that we report in Chapter 5 indicate that repetitive stimulation with acetylcholine, that is, repetitive rounds of NO synthesis are necessary to decrease endothelial arginine levels in young adult endothelium, even if that lacks ASS expression. This finding resembles a similar observation in sphincters, in which relaxation becomes dependent on exogenous arginine only after several rounds of stimulation (58). The upregulation of arginase-1 may therefore be the precondition that makes dysfunctional endothelium arginine-deficient.

Among the many functions of arginine (Figure 2), signal transduction has drawn much attention lately. Our analysis of the arginine-deficiency syndrome in neonates heavily leaned on our hypothesis that this syndrome strongly resembled a deficiency of insulin-like growth factor-1 (IGF-1; Chapter 2). Even though it is well established that signaling via the growth-hormone (GH)-IGF-1 axis becomes more efficient after weaning (59, 60), we observed a significantly lower IGF-1 concentration in several organs of arginine-deficient compared to wild-type neonatal mice.

Figure 2. Functions of arginine

Injection of GH increased tissue IGF-1 levels, but to a lesser extent in arginine-deficient than in control mice, which indicates that arginine deficiency negatively affects GH-IGF-1 signaling. This finding led us to investigate the signaling pathways activated by amino-acid deficiencies.

A deficiency of amino acids leads to the inhibition of protein synthesis via 2 routes (Figure 3). A lack of activation of the mTOR (mammalian target of rapamycin) pathway (Figure 3) causes, among others, dephosphorylation of p70S6 kinase and eIF4E-binding protein (61). This dephosphorylation inactivates p70S6 kinase and activates eIF4E-binding protein. Active 4E-binding protein inactivates translation by binding and sequestration of the cap-binding protein eIF4E. Both leucine and arginine are very strong agonists of the mTOR pathway, whereas histidine, isoleucine, lysine, methionine, phenylalanine, threonine, tryptophan, cysteine, glutamine, and tyrosine are not (62). In some studies, glutamine even inhibits the mTOR-dependent phosphorylation of p70S6kinase (63). These data show that amino acids differentially affect the activation of the mTOR pathway. We still have to demonstrate whether or not arginine deficiency affects mTOR signaling in F/A2 mice.

Activation of GCN2 (general control non-derepressing) by amino-acid deficiency causes phosphorylation of eIF2 α and, hence, inhibition of the binding of the methionyl-tRNA/mRNA complex to the 40S ribosomal subunit(64, 65). Our analysis has primarily focused on the activation of the GCN2 pathway. Not all amino acids appear

Figure 3. The effects of amino-acid deficiency on protein translation. The figure illustrates the stages in translation initiation involving the binding of methionyl-tRNAⁱ (met) and mRNA to the 40S ribosomal subunit. The eukaryotic initiation factor (eIF) eIF4E is depicted as a hexagon with the label 4E in the center of the shape. In addition, the regulation of eIF2B activity by phosphorylation of the α -subunit of eIF2 and the sequestration of eIF4E by eIF4E binding protein-1 (4E-BP1) are illustrated. mTOR, mammalian target of rapamycin; AUG, the nucleoside triplet adenosine, uridine, guanosine; GCN2, general control nonderepressing eIF2 protein kinase 2. Green: Synthetic loop blocked by GCN2 activation; red: synthetic loop blocked by mTOR inactivation. Modified from (96).

equally potent in this respect. Thus, leucine appears to be more powerful than glycine, which in turn is more powerful than tryptophan (65). Our data, as well as similar data from the literature (66, 67) show that arginine deficiency is also a powerful stimulator of GCN2 (Chapter 2), but the hierarchy in the activation potential among amino acids remains to be established. Furthermore, our findings show that not all tissues are equally

sensitive: neonatal muscle is exquisitely sensitive, whereas liver, almost devoid of arginine due to the action of the urea cycle enzyme arginase-1, hardly responds (Chapter 2; cf. ref. (68) for a similar situation in piglets). Although circulating arginine levels do not increase after weaning in arginine-deficient F/A2 mice (3), muscle growth resumes, so that adult mice are only slightly smaller than their wild-type controls. Very recently, it was shown that C2C12 myoblasts do respond to amino-acid deficiency, whereas the more differentiated myotubes do not (69, 70). The exquisite sensitivity of neonatal skeletal muscles to arginine deficiency may, therefore, well be due to the fact that the 3rd phase of myoblast fusion, which generates the mature, multinuclear striated muscles, occurs perinatally in rodents (71). The rapid activation of protein synthesis by growth hormone requires signaling through mTOR (72). We hypothesize that activation of the GCN2 pathway, with or without inactivation of mTOR-dependent phosphorylation, blocks GH-IGF-1 signaling. To test the hypothesis that this hierarchical relation between amino-acid deficiency and GCN2 signaling exists, GH-IGF-1 signaling in C2C12 cells that constitutively express GCN2-specific RNAi is presently being investigated.

Perspectives

With the newly available conditional knockouts for ASS, arginase-1 and CAT-1 (Figure 4), we can, in conjunction with already available constitutive knockouts, manipulate intracellular arginine levels. The limitations to generate attractive combinations are the complex breeding schemes (it may take more than a year of breeding to generate a specific double knockout with an appropriate Cre expressor) and the fact that we cannot yet delete the heterodimeric arginine transporters, which all share the 4F2hc heavy chain (Slc3A2) (73). We aim to specifically address arginine metabolism in endothelial cells and in macrophages. As we stated earlier, endothelial cells resynthesize arginine from the citrulline that is one of the products of NO synthesis. NO is a major vasodilatory agent. Endothelial dysfunction (ED) is the impaired ability of vascular endothelium to stimulate vasodilation (74) and predisposes to both small- (hypertension) and large-artery disease (atherosclerosis). An early and key feature of ED is a decline in the bioavailability of NO as a result of its reduced synthesis and/or increased degradation by reactive oxygen species (ROS) (75). Quantitatively, NO synthesis is small compared to arginine synthesis and turnover in most tissues. This, and the fact that the K_M of NOS for arginine is in the low micromolar range (76) should confer little control of arginine

Figure 4. Genetically modified mice that are useful for the study of arginine metabolism *in vivo*. Arginine is represented by its one-letter code R. The rectangle depicts a schematic cell. Red dots indicate arginine and citrulline transporters. NO: nitric oxide, AS: argininosuccinate; CAT: Cationic transporter (Slc7A).

availability over NO synthesis. The relatively mild phenotype of mice lacking all three NO-synthesizing (NOS) enzymes (77, 78) suggested to us, however, that NO bioavailability in itself may not be the major determinant of ED. NOS produces NO and citrulline in the presence of arginine and the co-factor tetrahydrobiopterin (BH4). If either becomes deficient, NOS “uncouples” and releases free superoxide (O_2^-) radicals (79, 80), which enhance oxidative stress and oxidize the NOS co-factor BH4 (75, 81). These findings suggest that deficiency of arginine (or BH4) is more detrimental for endothelial function than deficiency of NO *per se*. We can test this hypothesis by using a mouse model which is sensitive to ED (e.g. the low-density lipoprotein (LDL)-receptor knockout (82, 83)) and is impaired in the synthesis of arginine (endothelial ASS knockout; this deletion should sensitize the animal to vascular pathology) and a mouse

model which is sensitive to ED (the same LDL-receptor knockout) and is impaired in the degradation of arginine (endothelial arginase-1 knockout; this deletion should protect the animal against vascular pathology). We have recently obtained a grant from the Dutch Heart Foundation to test this hypothesis.

Macrophages use arginine to synthesize NO or ornithine and urea. The macrophage population is heterogeneous, but two main phenotypes can be distinguished (84). The classical, IFN- γ -induced activation of (M1) macrophages is associated with cell-mediated immunity to intracellular pathogens and with TH1 lymphocyte responses. M1 macrophages express NOS2 and have a high microbicidal activity, immunostimulatory functions and cytotoxicity. The alternative, IL4 and IL-13-induced activation of (M2) macrophages is strongly associated with extracellular parasitic infections, allergy, humoral immunity, and fibrosis. M2 macrophages express arginase-1 and have a high scavenging ability, promote tissue repair and angiogenesis, tune the adaptive TH1 response and favour tumour progression (Figure 5). Since arginase-1 catabolizes arginine, M2 macrophages have little arginine available for the synthesis of NO. Furthermore, the arginine deficiency can activate GCN2 (Chapter 2; (85)) and suppress the expression of T-cell receptor ζ -chain in neighbouring effector T cells, which diminishes their activation and proliferation (86-88). The alveolar, M2-polarized macrophage plays a key role in the development and maintenance of asthma (89). For this reason, we presently investigate whether deletion of arginase-1 in macrophages affects the severity of ovalbumin-induced allergic asthma in mice. Tumor-associated macrophages (TAMs) also have an M2 phenotype and are thought to tolerize the host to tumors (90). Using the same approach as developed for asthmatic mice, we plan to study these macrophages.

In addition to these 2 tissues, i.e. endothelium and macrophages, there are several other tissues in which arginine metabolism is intriguing. Since the kidney is considered the single most important tissue for arginine synthesis in its proximal tubules (91, 92), it would be most interesting and important to assess the consequences of its deletion. Unfortunately, a mouse line which selectively expresses the recombinase Cre in the P1-3 segments of the kidney tubule is not yet available. The ASS-expressing thymocytes (Chapter 4) are another intriguing tissue for which the significance of arginine remains to be determined. The major limitation to the use of floxed conditional knockout mice is that these mice have their floxed gene deleted from the time the Cre

arginine metabolism in macrophages

Figure 5. Arginine metabolism in polarized macrophages. M1 and M2 macrophages are polarized by the cytokines secreted by the TH1 and TH2 lymphocytes, and express predominantly NOS2 and arginase-1, respectively. NOS: nitric oxide synthase; IL: interleukin; IFN: interferon; TGF: tumor-growth factor. Modified from (97).

enzyme becomes expressed. The approach would therefore become even more elegant, if not only spatial (tissue specificity), but also temporal control of the moment of gene inactivation would be possible. In principle, such temporal control can be achieved by using tamoxifen-dependent Cre variants (93, 94). Unfortunately, the amount of tamoxifen (and, more importantly, its vegetable-oil solvent) that is necessary to achieve gene inactivation is too high to allow metabolic assessment soon after this treatment (Y. He, T.B.M. Hakvoort & W.H. Lamers, unpublished). Possibly, the development of more sensitive doxycyclin-dependent Cre-expressing transgenes (95) will meet the requirements.

References

1. Dobie K, Mehtali M, McClenaghan M, Lathe R. Variegated gene expression in mice. *Trends Genet* 1997;13:127-130.
2. Weis J, Fine SM, Sanes JR. Integration site-dependent transgene expression used to mark subpopulations of cells in vivo: an example from the neuromuscular junction. *Brain Pathol* 1992;2:31-37.
3. de Jonge WJ, Hallemeesch MM, Kwikkers KL, Ruijter JM, de Gier-de Vries C, van Roon MA, Meijer AJ, et al. Overexpression of arginase I in enterocytes of transgenic mice elicits a selective arginine deficiency and affects skin, muscle, and lymphoid development. *Am J Clin Nutr* 2002;76:128-140.
4. Ferry N, Heard JM. Liver-directed gene transfer vectors. *Hum Gene Ther* 1998;9:1975-1981.
5. Beck C, Uramoto H, Boren J, Akyurek LM. Tissue-specific targeting for cardiovascular gene transfer. Potential vectors and future challenges. *Curr Gene Ther* 2004;4:457-467.
6. Patejunas G, Bradley A, Beaudet AL, O'Brien WE. Generation of a mouse model for citrullinemia by targeted disruption of the argininosuccinate synthetase gene. *Somat Cell Mol Genet* 1994;20:55-60.
7. Iyer RK, Yoo PK, Kern RM, Rozengurt N, Tsoa R, O'Brien WE, Yu H, et al. Mouse model for human arginase deficiency. *Mol Cell Biol* 2002;22:4491-4498.
8. Perkins CP, Mar V, Shutter JR, del Castillo J, Danilenko DM, Medlock ES, Ponting IL, et al. Anemia and perinatal death result from loss of the murine ecotropic retrovirus receptor mCAT-1. *Genes Dev* 1997;11:914-925.
9. Farley FW, Soriano P, Steffen LS, Dymecki SM. Widespread recombinase expression using FLP_eR (flipper) mice. *Genesis* 2000;28:106-110.
10. Matsui Y, Okamura D. Mechanisms of germ-cell specification in mouse embryos. *Bioessays* 2005;27:136-143.
11. Braun RE, Lo D, Pinkert CA, Wiedera G, Flavell RA, Palmiter RD, Brinster RL. Infertility in male transgenic mice: disruption of sperm development by HSV-tk expression in postmeiotic germ cells. *Biol Reprod* 1990;43:684-693.
12. Meyers EN, Lewandoski M, Martin GR. An Fgf8 mutant allelic series generated by Cre- and Flp-mediated recombination. *Nat Genet* 1998;18:136-141.
13. Nagy A, Moens C, Ivanyi E, Pawling J, Gertsenstein M, Hadjantonakis AK, Purity M, et al. Dissecting the role of N-myc in development using a single targeting vector to generate a series of alleles. *Curr Biol* 1998;8:661-664.
14. Olson EN, Arnold HH, Rigby PW, Wold BJ. Know your neighbors: three phenotypes in null mutants of the myogenic bHLH gene MRF4. *Cell* 1996;85:1-4.
15. Bertolo RF, Brunton JA, Pencharz PB, Ball RO. Arginine, ornithine, and proline interconversion is dependent on small intestinal metabolism in neonatal pigs. *Am J Physiol Endocrinol Metab* 2003;284:E915-922.

16. Urschel KL, Evans AR, Wilkinson CW, Pencharz PB, Ball RO. Parenterally fed neonatal piglets have a low rate of endogenous arginine synthesis from circulating proline. *J Nutr* 2007;137:601-606.
17. Sangild PT, Siggers RH, Schmidt M, Elnif J, Bjornvad CR, Thymann T, Grondahl ML, et al. Diet- and colonization-dependent intestinal dysfunction predisposes to necrotizing enterocolitis in preterm pigs. *Gastroenterology* 2006;130:1776-1792.
18. Becker RM, Wu G, Galanko JA, Chen W, Maynor AR, Bose CL, Rhoads JM. Reduced serum amino acid concentrations in infants with necrotizing enterocolitis. *J Pediatr* 2000;137:785-793.
19. Wu G, Jaeger LA, Bazer FW, Rhoads JM. Arginine deficiency in preterm infants: biochemical mechanisms and nutritional implications. *J Nutr Biochem* 2004;15:442-451.
20. Zamora SA, Amin HJ, McMillan DD, Kubes P, Fick GH, Butzner JD, Parsons HG, et al. Plasma L-arginine concentrations in premature infants with necrotizing enterocolitis. *J Pediatr* 1997;131:226-232.
21. Reber KM, Nankervis CA, Nowicki PT. Newborn intestinal circulation. Physiology and pathophysiology. *Clin Perinatol* 2002;29:23-39.
22. Davis TA, Nguyen HV, Garcia-Bravo R, Fiorotto ML, Jackson EM, Lewis DS, Lee DR, et al. Amino acid composition of human milk is not unique. *J Nutr* 1994;124:1126-1132.
23. Wu G, Knabe DA. Free and protein-bound amino acids in sow's colostrum and milk. *J Nutr* 1994;124:415-424.
24. Davis TA, Fiorotto ML, Reeds PJ. Amino acid compositions of body and milk protein change during the suckling period in rats. *J Nutr* 1993;123:947-956.
25. Meijer AJ, Lamers WH, Chamuleau RA. Nitrogen metabolism and ornithine cycle function. *Physiol Rev* 1990;70:701-748.
26. Wakabayashi Y. Tissue-selective expression of enzymes of arginine synthesis. *Curr Opin Clin Nutr Metab Care* 1998;1:335-339.
27. Crenn P, Messing B, Cynober L. Citrulline as a biomarker of intestinal failure due to enterocyte mass reduction. *Clin Nutr* 2008;27:328-339.
28. Wu G, Morris SM, Jr. Arginine metabolism: nitric oxide and beyond. *Biochem J* 1998;336 (Pt 1):1-17.
29. Janeczko MJ, Stoll B, Chang X, Guan X, Burrin DG. Extensive gut metabolism limits the intestinal absorption of excessive supplemental dietary glutamate loads in infant pigs. *J Nutr* 2007;137:2384-2390.
30. Reeds PJ, Burrin DG, Stoll B, Jahoor F. Intestinal glutamate metabolism. *J Nutr* 2000;130:978S-982S.
31. Lambert BD, Filip R, Stoll B, Junghans P, Derno M, Hennig U, Souffrant WB, et al. First-pass metabolism limits the intestinal absorption of enteral alpha-ketoglutarate in young pigs. *J Nutr* 2006;136:2779-2784.
32. Wang T, Lawler AM, Steel G, Sipila I, Milam AH, Valle D. Mice lacking ornithine-delta-aminotransferase have paradoxical neonatal hypoorithinaemia and retinal degeneration. *Nat Genet* 1995;11:185-190.

33. Malo C. Free amino acid levels in serum and small intestine during the post-natal development of normal and sparse-fur mutant mice. *Comp Biochem Physiol A Physiol* 1994;109:1049-1057.
34. Cleary MA, Dorland L, de Koning TJ, Poll-The BT, Duran M, Mandell R, Shih VE, et al. Ornithine aminotransferase deficiency: diagnostic difficulties in neonatal presentation. *J Inherit Metab Dis* 2005;28:673-679.
35. Wendel U, Wieland J, Bremer HJ, Bachmann C. Ornithine transcarbamylase deficiency in a male: strict correlation between metabolic control and plasma arginine concentration. *Eur J Pediatr* 1989;148:349-352.
36. Batshaw ML, Wachtel RC, Thomas GH, Starrett A, Brusilow SW. Arginine-responsive asymptomatic hyperammonemia in the premature infant. *J Pediatr* 1984;105:86-91.
37. Brunton JA, Bertolo RF, Pencharz PB, Ball RO. Proline ameliorates arginine deficiency during enteral but not parenteral feeding in neonatal piglets. *Am J Physiol* 1999;277:E223-231.
38. Urschel KL, Shoveller AK, Uwiera RR, Pencharz PB, Ball RO. Citrulline is an effective arginine precursor in enterally fed neonatal piglets. *J Nutr* 2006;136:1806-1813.
39. De Jonge WJ, Dingemans MA, de Boer PA, Lamers WH, Moorman AF. Arginine-metabolizing enzymes in the developing rat small intestine. *Pediatr Res* 1998;43:442-451.
40. Hurwitz R, Kretchmer N. Development of arginine-synthesizing enzymes in mouse intestine. *Am J Physiol* 1986;251:G103-110.
41. Wu G. Synthesis of citrulline and arginine from proline in enterocytes of postnatal pigs. *Am J Physiol* 1997;272:G1382-1390.
42. Wu G, Knabe DA. Arginine synthesis in enterocytes of neonatal pigs. *Am J Physiol* 1995;269:R621-629.
43. Wu G, Knabe DA, Flynn NE. Synthesis of citrulline from glutamine in pig enterocytes. *Biochem J* 1994;299 (Pt 1):115-121.
44. Wu G. Urea synthesis in enterocytes of developing pigs. *Biochem J* 1995;312 (Pt 3):717-723.
45. Wilkinson DL, Bertolo RF, Brunton JA, Shoveller AK, Pencharz PB, Ball RO. Arginine synthesis is regulated by dietary arginine intake in the enterally fed neonatal piglet. *Am J Physiol Endocrinol Metab* 2004;287:E454-462.
46. Closs EI, Simon A, Vekony N, Rotmann A. Plasma membrane transporters for arginine. *J Nutr* 2004;134:2752S-2759S; discussion 2765S-2767S.
47. Toda N, Herman AG. Gastrointestinal function regulation by nitrergic efferent nerves. *Pharmacol Rev* 2005;57:315-338.
48. Mori M. Regulation of nitric oxide synthesis and apoptosis by arginase and arginine recycling. *J Nutr* 2007;137:1616S-1620S.
49. Solomonson LP, Flam BR, Pendleton LC, Goodwin BL, Eichler DC. The caveolar nitric oxide synthase/arginine regeneration system for NO production in endothelial cells. *J Exp Biol* 2003;206:2083-2087.
50. Demougeot C, Prigent-Tessier A, Bagnost T, Andre C, Guillaume Y, Bouhaddi M, Marie C, et al. Time course of vascular arginase expression and activity in spontaneously hypertensive rats. *Life Sci* 2007;80:1128-1134.

51. Kuhlencordt PJ, Gyrko R, Han F, Scherrer-Crosbie M, Aretz TH, Hajjar R, Picard MH, et al. Accelerated atherosclerosis, aortic aneurysm formation, and ischemic heart disease in apolipoprotein E/endothelial nitric oxide synthase double-knockout mice. *Circulation* 2001;104:448-454.
52. Romero MJ, Platt DH, Tawfik HE, Labazi M, El-Remessy AB, Bartoli M, Caldwell RB, et al. Diabetes-induced coronary vascular dysfunction involves increased arginase activity. *Circ Res* 2008;102:95-102.
53. Scotland RS, Madhani M, Chauhan S, Moncada S, Andresen J, Nilsson H, Hobbs AJ, et al. Investigation of vascular responses in endothelial nitric oxide synthase/cyclooxygenase-1 double-knockout mice: key role for endothelium-derived hyperpolarizing factor in the regulation of blood pressure in vivo. *Circulation* 2005;111:796-803.
54. Sun D, Liu H, Yan C, Jacobson A, Ojaimi C, Huang A, Kaley G. COX-2 contributes to the maintenance of flow-induced dilation in arterioles of eNOS-knockout mice. *Am J Physiol Heart Circ Physiol* 2006;291:H1429-1435.
55. White AR, Ryoo S, Li D, Champion HC, Steppan J, Wang D, Nyhan D, et al. Knockdown of arginase I restores NO signaling in the vasculature of old rats. *Hypertension* 2006;47:245-251.
56. Boger RH. L-Arginine therapy in cardiovascular pathologies: beneficial or dangerous? *Curr Opin Clin Nutr Metab Care* 2008;11:55-61.
57. Wilson AM, Harada R, Nair N, Balasubramanian N, Cooke JP. L-arginine supplementation in peripheral arterial disease: no benefit and possible harm. *Circulation* 2007;116:188-195.
58. Chakder S, Rattan S. L-arginine deficiency causes suppression of nonadrenergic noncholinergic nerve-mediated smooth muscle relaxation: role of L-citrulline recycling. *J Pharmacol Exp Ther* 1997;282:378-384.
59. Jones JL, Clemmons DR. Insulin-like growth factors and their binding proteins: biological actions. *Endocr Rev* 1995;16:3-34.
60. Lupu F, Terwilliger JD, Lee K, Segre GV, Efstratiadis A. Roles of growth hormone and insulin-like growth factor 1 in mouse postnatal growth. *Dev Biol* 2001;229:141-162.
61. Averous J, Proud CG. When translation meets transformation: the mTOR story. *Oncogene* 2006;25:6423-6435.
62. Hara K, Yonezawa K, Weng QP, Kozlowski MT, Belham C, Avruch J. Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. *J Biol Chem* 1998;273:14484-14494.
63. Deldicque L, Sanchez Canedo C, Horman S, De Potter I, Bertrand L, Hue L, Francaux M. Antagonistic effects of leucine and glutamine on the mTOR pathway in myogenic C2C12 cells. *Amino Acids* 2008;35:147-155.
64. Hao S, Sharp JW, Ross-Inta CM, McDaniel BJ, Anthony TG, Wek RC, Cavener DR, et al. Uncharged tRNA and sensing of amino acid deficiency in mammalian piriform cortex. *Science* 2005;307:1776-1778.

65. Zhang P, McGrath BC, Reinert J, Olsen DS, Lei L, Gill S, Wek SA, et al. The GCN2 eIF2alpha kinase is required for adaptation to amino acid deprivation in mice. *Mol Cell Biol* 2002;22:6681-6688.
66. Lee J, Ryu H, Ferrante RJ, Morris SM, Jr., Ratan RR. Translational control of inducible nitric oxide synthase expression by arginine can explain the arginine paradox. *Proc Natl Acad Sci U S A* 2003;100:4843-4848.
67. Rodriguez PC, Quiceno DG, Ochoa AC. L-arginine availability regulates T-lymphocyte cell-cycle progression. *Blood* 2007;109:1568-1573.
68. Yao K, Yin YL, Chu W, Liu Z, Deng D, Li T, Huang R, et al. Dietary arginine supplementation increases mTOR signaling activity in skeletal muscle of neonatal pigs. *J Nutr* 2008;138:867-872.
69. Deval C, Talvas J, Chaveroux C, Maurin AC, Mordier S, Cherasse Y, Parry L, et al. Amino-acid limitation induces the GCN2 signaling pathway in myoblasts but not in myotubes. *Biochimie* 2008.
70. Talvas J, Obled A, Fafournoux P, Mordier S. Regulation of protein synthesis by leucine starvation involves distinct mechanisms in mouse C2C12 myoblasts and myotubes. *J Nutr* 2006;136:1466-1471.
71. Stockdale FE. Myogenic cell lineages. *Dev Biol* 1992;154:284-298.
72. Hayashi AA, Proud CG. The rapid activation of protein synthesis by growth hormone requires signaling through mTOR. *Am J Physiol Endocrinol Metab* 2007;292:E1647-1655.
73. Palacin M, Nunes V, Font-Llitjos M, Jimenez-Vidal M, Fort J, Gasol E, Pineda M, et al. The genetics of heteromeric amino acid transporters. *Physiology (Bethesda)* 2005;20:112-124.
74. Yang Z, Kaye DM. Endothelial dysfunction and impaired L-arginine transport in hypertension and genetically predisposed normotensive subjects. *Trends Cardiovasc Med* 2006;16:118-124.
75. Forstermann U, Munzel T. Endothelial nitric oxide synthase in vascular disease: from marvel to menace. *Circulation* 2006;113:1708-1714.
76. Bode-Boger SM, Scalera F, Ignarro LJ. The l-arginine paradox: Importance of the l-arginine/asymmetrical dimethylarginine ratio. *Pharmacol Ther* 2007;114:295-306.
77. Nakata S, Tsutsui M, Shimokawa H, Suda O, Morishita T, Shibata K, Yatera Y, et al. Spontaneous myocardial infarction in mice lacking all nitric oxide synthase isoforms. *Circulation* 2008;117:2211-2223.
78. Tsutsui M, Shimokawa H, Morishita T, Nakashima Y, Yanagihara N. Development of genetically engineered mice lacking all three nitric oxide synthases. *J Pharmacol Sci* 2006;102:147-154.
79. Liu VW, Huang PL. Cardiovascular roles of nitric oxide: a review of insights from nitric oxide synthase gene disrupted mice. *Cardiovasc Res* 2008;77:19-29.
80. Rabelink TJ, van Zonneveld AJ. Coupling eNOS uncoupling to the innate immune response. *Arterioscler Thromb Vasc Biol* 2006;26:2585-2587.
81. Katusic ZS. Mechanisms of endothelial dysfunction induced by aging: role of arginase I. *Circ Res* 2007;101:640-641.
82. Whitman SC. A practical approach to using mice in atherosclerosis research. *Clin Biochem Rev* 2004;25:81-93.

83. Zadelaar S, Kleemann R, Verschuren L, de Vries-Van der Weij J, van der Hoorn J, Princen HM, Kooistra T. Mouse models for atherosclerosis and pharmaceutical modifiers. *Arterioscler Thromb Vasc Biol* 2007;27:1706-1721.
84. Gordon S. Macrophage heterogeneity and tissue lipids. *J Clin Invest* 2007;117:89-93.
85. Marion V, Sankaranarayanan S, van Dijk P, de Theije C, Lindsey P, van Straaten H, Lamers M, et al. Chronic arginine deprivation represses the GH-IGF-1 axis via the Gcn2-mediated stress response. manuscript in revision.
86. Fallarino F, Grohmann U, You S, McGrath BC, Cavener DR, Vacca C, Orabona C, et al. The combined effects of tryptophan starvation and tryptophan catabolites down-regulate T cell receptor zeta-chain and induce a regulatory phenotype in naive T cells. *J Immunol* 2006;176:6752-6761.
87. Kusmartsev S, Nefedova Y, Yoder D, Gabrilovich DI. Antigen-specific inhibition of CD8+ T cell response by immature myeloid cells in cancer is mediated by reactive oxygen species. *J Immunol* 2004;172:989-999.
88. Rodriguez PC, Zea AH, Culotta KS, Zabaleta J, Ochoa JB, Ochoa AC. Regulation of T cell receptor CD3zeta chain expression by L-arginine. *J Biol Chem* 2002;277:21123-21129.
89. Peters-Golden M. The alveolar macrophage: the forgotten cell in asthma. *Am J Respir Cell Mol Biol* 2004;31:3-7.
90. Sica A, Schioppa T, Mantovani A, Allavena P. Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy. *Eur J Cancer* 2006;42:717-727.
91. Brosnan ME, Brosnan JT. Renal arginine metabolism. *J Nutr* 2004;134:2791S-2795S; discussion 2796S-2797S.
92. Levillain O, Hus-Citharel A, Morel F, Bankir L. Arginine synthesis in mouse and rabbit nephron: localization and functional significance. *Am J Physiol* 1993;264:F1038-1045.
93. Indra AK, Warot X, Brocard J, Bornert JM, Xiao JH, Chambon P, Metzger D. Temporally-controlled site-specific mutagenesis in the basal layer of the epidermis: comparison of the recombinase activity of the tamoxifen-inducible Cre-ER(T) and Cre-ER(T2) recombinases. *Nucleic Acids Res* 1999;27:4324-4327.
94. Sohal DS, Nghiem M, Crackower MA, Witt SA, Kimball TR, Tymitz KM, Penninger JM, et al. Temporally regulated and tissue-specific gene manipulations in the adult and embryonic heart using a tamoxifen-inducible Cre protein. *Circ Res* 2001;89:20-25.
95. Zhou X, Vink M, Klaver B, Berkhout B, Das AT. Optimization of the Tet-On system for regulated gene expression through viral evolution. *Gene Ther* 2006;13:1382-1390.
96. Kimball SR, Farrell PA, Jefferson LS. Invited Review: Role of insulin in translational control of protein synthesis in skeletal muscle by amino acids or exercise. *J Appl Physiol* 2002;93:1168-1180.
97. Mills CD. Macrophage arginine metabolism to ornithine/urea or nitric oxide/citrulline: a life or death issue. *Crit Rev Immunol* 2001;21:399-425.

Nederlandse samenvatting

In dit proefschrift staat het aminozuur arginine centraal. Arginine is een voorloper voor de synthese van eiwitten, stikstofoxide, creatine en agmatine, en is een intermediair in de ontgiftiging van ammoniak via de ornithine cyclus. Arginine behoort niet tot de essentiële aminozuren. In zoogdieren vindt de endogene aanmaak van arginine vooral in de proximale tubulus contortus van de nier plaats uit citrulline, dat op zijn beurt uitsluitend in de epitheelcellen van de dunne darm (enterocyten) gevormd wordt uit glutamine of proline. De capaciteit om zelf arginine te synthetiseren is laag in vleeseters zoals katten en fretten, intermediair in de mens, en relatief hoog in snel and blijvend groeiende dieren zoals varkens en knaagdieren. In de volwassen mens voorziet de endogene synthese van arginine onder normale omstandigheden in de dagelijkse behoefte. Onder sterk anabole of katabole omstandigheden wordt een externe bron van arginine noodzakelijk. Om deze reden wordt arginine tegenwoordig een “conditioneel” essentieel aminozuur genoemd. In snelgroeiende pasgeboren zoogdieren voorziet de relatief geringe bijdrage van arginine uit de melk niet in de behoefte voor eiwitsynthese ten behoeve van groei, zodat endogene synthese van arginine in deze periode noodzakelijk is. Verschillende onderzoeken, inclusief onderzoek uit onze groep, wijzen erop dat de darm in deze periode een belangrijker bron van arginine vormt dan de nier.

In hoofdstuk 1 wordt allereerst een overzicht gegeven van het evenwicht tussen de behoefte, de eigen aanmaak, en de stofwisseling van arginine in het lichaam.

Vervolgens wordt de regulering van het transport van arginine over de celmembraan besproken en tenslotte komen de tot nu toe beschreven muizen met een deletie van een van de genen uit de aanmaak, transport, en afbraak van arginine aan de orde.

Transgene "F/A2" muizen brengen arginase-1 in hun enterocyten tot expressie. Het gevolg daarvan is dat F/A2 zuigelingen lijden aan te lage bloedspiegels van arginine, verminderde spier- en haargroei, en een geremde rijping van B cellen. Het arginine deficiëntiesyndroom lijkt op een tekort aan Insuline GroeiFactor (IGF)-1. In hoofdstuk 2 hebben wij daarom de hypothese getoetst of het arginine deficiëntie syndroom het gevolg is van een interactie tussen de GroeiHormoon (GH)/IGF-1 as en de GCN2 stress kinase signaaltransductie route die geactiveerd wordt door een tekort aan een bepaald aminozuur. De activering van GCN2 kan gevolgd worden door de fosforylering van translatie initiatie factor eIF2 α en de concentratietoename van de transcriptiefactoren ATF4 en CHOP te bepalen. Deze bepalingen zijn uitgevoerd in cellen die in vitro gekweekt werden onder verschillende arginine concentraties en in F/A2, Gcn2^{-/-}, of

F/A2//Gcn2^{-/-} muizen. Een tekort aan arginine remt de secretie van GH in hypofyse cellen. In arginine deficiënte F/A2 muizen was de GH eiwitconcentratie in de hypofyse 2.5-voudig verhoogd en de Igf-1 mRNA concentratie in lever en spier 8-10-voudig verlaagd. Tweemaal daags toedienen van GH verhoogde de Igf-1 mRNA concentratie 3-voudig in zowel wildtype als F/A2 muizen, maar stimuleerde de groei niet. In celkweken werd de GCN2 signaaltransductie route geactiveerd zodra de arginine spiegel van het medium onder 50 µM daalde, terwijl IGF-1 myoblast differentiatie stimuleerde indien de arginine spiegel hoger dan 25 µM was. In 1-dag oude F/A2 muizen was de arginine concentratie 4.5-voudig verlaagd en was GCN2 in alle onderzochte organen geactiveerd. Muizen waarvan het Gcn2 gen geïnactiveerd was (Gcn2^{-/-}) vertoonden geen toegenomen ATF4 and CHOP expressie en stierven vlak na de geboorte. Deze waarneming laat zien dat alleen het stress kinase Gcn2 verantwoordelijk was voor de stress kinase response. Op grond van deze waarnemingen hebben wij geconcludeerd dat de symptomen van het arginine deficiëntiesyndroom ontstaan door GCN2 activering, onderdrukking van GH secretie, en remming van de groeistimulerende effecten van IGF-1.

Binnen de afdeling Anatomie & Embryologie zijn de afgelopen jaren "conditionele" knockout muizen gemaakt voor de arginine-gerelateerde eiwitten argininosuccinaat synthetase, arginase-1 en de cationic amino-acid transporter-1. In een conditionele muis wordt een essentieel deel van het structurele gen geflankeerd door een 32bp lang DNA fragment ("loxP") dat de functie van het gen niet beïnvloedt. Doordat dit fragment herkend wordt door een enzym met "recombinase" activiteit ("Cre"), kan het geflankeerde DNA selectief verwijderd worden door kruising met muizen die Cre transgeen tot expressie brengen. Indien de transgene muis Cre via een weefselspecifieke promotor/enhancer tot expressie brengt, wordt een weefselspecifieke knockout verkregen. In hoofdstuk 3 hebben we onze ervaringen met de vervaardiging van deze 3 conditionele knockout muizen samengevat. Belangrijke aspecten om rekening mee te houden blijken te zijn:

De gekozen strategie om de "targeting" vector (het DNA molecule dat modificatie van het DNA in de embryonale stamcel (ES) teweeg brengt) te construeren. Indien een op PCR gebaseerde methode wordt gebruikt om de homologe "armen" van de vector te kloneren, dient bij voorkeur een "bacterial artificial chromosome" (BAC) kloon als uitgangsmateriaal te worden genomen om het aantal amplificatiestappen te

minimaliseren. Ook dienen gebieden waarin veel "repeats" (herhaalde, aansluitend achter elkaar liggende sequenties van ≥ 2 nucleotiden) voorkomen vermeden te worden omdat ze moeilijk foutloos te amplificeren zijn.

De selectie van de recombinante ES kloon voor injectie in blastocysten. Om te bewerkstelligen dat de geselecteerde kloon daadwerkelijk alleen homoloog gerecombineerde ES cellen bevat dienen geselecteerde ES klonen voortdurend onder selectiedruk gekweekt te worden om de overleving van wildtype cellen te voorkomen. De eerste screening tegen "random integration" van een targeting vector in het DNA van een ES cel kan het best geschieden door aan de zijde van de "lange" homologe arm een stuk vector DNA te laten zitten. Deze vector sequentie komt niet voor in het DNA van de ES cel en verdwijnt bij homologe recombinatie. De aanwezigheid ervan bewijst random integratie.

Screening op homologe recombinatie met behulp van een lange PCR aan de zijde van de "korte" homologe arm van de targeting vector is sneller en informatiever dan de klassieke Southern blot. Een nadeel van deze aanpak is dat het te gebruiken paar PCR "primers" pas niet kan worden getest en geoptimaliseerd voordat de recombinante ES kloon beschikbaar is.

Sondes ("probes") om Southern blots te screenen dienen, indien ze uit intronen afkomstig zijn, te worden gecontroleerd op de aanwezigheid van repeats. Een probe van ~300bp blijkt voldoende lang te zijn voor een gevoelige en specifieke selectie. Na selectie met ganciclovir om de selectiecassette te verwijderen dienen ES klonen opnieuw gesequenced te worden omdat ganciclovir mutageen gebleken is.

Succesvol homoloog gerecombineerde ES cellen zijn slechts op één van beide allelen gemodificeerd. De kleur van de vacht is een goede marker voor de aanwezigheid van homoloog gerecombineerde cellen in de kiembaan van chimere muizen. Dit kan van groot belang zijn indien niet onmiddellijk muizen met een gefloxt gen worden verkregen. De zorg dat de activiteit van transgeen Cre recombinase onvoldoende is om twee gefloxt allelen in één cel te verwijderen is ongegrond gebleken. De beste genotypes om de fysiologische effecten van gen deletie te bestuderen blijken flox/flox and flox/flox-Cre te zijn, omdat het fenotype van dieren met één constitutief en een conditioneel verwijderd allel moeilijk te interpreteren is.

Afwezigheid van het enzym ornithine transaminase of ornithine transcarbamoylase veroorzaakt een ernstig tekort aan arginine in pasgeboren muizen en

mensen, omdat endogene synthese van citrulline nodig is om het voor groei te geringe gehalte aan arginine in de melk te compenseren. Pasgeborenen verschillen van volwassenen in de zin dat de enzymen die nodig zijn voor de synthese van arginine uit citrulline in sterke mate tot expressie komen in de epitheelcellen ("enterocyten") van de dunne darm. In hoofdstuk 4 hebben wij het belang van de arginine synthese in de dunne darm voor de arginine voorziening van de muizenzuigeling onderzocht door het enzym argininosuccinaat synthetase (ASS) selectief in de enterocyten van de dunne darm te elimineren. Eliminatie van ASS expressie werd bewerkstelligd door $Ass^{fl/fl}$ muizen (in deze muizen wordt exon 13 van het Ass gen geflankeerd door loxP sites) te kruisen met transgene "Vil" muizen (deze muizen brengen het recombinase Cre selectief in de enterocyten tot expressie). Om de bijdrage van ASS activiteit buiten de darm te beperken werden bovendien $Ass^{fl/fl}$ met Vil muizen gekruist. De gevolgde strategie bleek effectief om ASS expressie in de enterocyten geheel te elimineren. Desondanks ontstond geen tekort aan arginine in muizenzuigelingen met het Vil/ $Ass^{fl/fl}$ of Vil/ $Ass^{fl/fl}$ genotype (de verschijnselen van een tekort aan arginine in zuigelingen zijn in hoofdstuk 2 beschreven). De systemische eliminatie van één Ass allel deed de circulerende citrulline spiegel met $\sim 50\mu M$ toenemen, terwijl de eliminatie van een Ass allel in de enterocyten verantwoordelijk was voor een additionele stijging met $\sim 80\mu M$. Met behulp van microsferen konden we aantonen dat $\sim 80\%$ van het bloed dat door de lever van 14 dagen oude muizen stroomt van de vena portae afkomstig is en $\sim 20\%$ van de arteria hepatica. Ten opzichte van wildtype (Vil/ $Ass^{+/+}$) muizen bleek de arginine productie in alle experimentele genotypes effectief geëlimineerd, terwijl de citrulline productie, zoals verwacht, toegenomen was. In zuigelingen dragen de nieren nog nauwelijks bij aan de productie van arginine omdat de doorstroming van de nier slechts $\sim 20\%$ vertegenwoordigt van dat wat door het maagdarmkanaal stroomt. Deze resultaten tonen aan dat arginine synthese in de dunne darm niet essentieel is voor muizenzuigelingen.

Een van de eerste kenmerken van disfunctie van het endotheel (de cellen die bloedvaten bekleden) is een vermindering van de productie van het vaatverwijdende stikstofoxide (NO) uit arginine. Het relatief milde fenotype van muizen waarin alle 3 NO synthetiserende (NOS) enzymen zijn geëlimineerd impliceert dat een tekort aan NO op zich niet zeer pathogeen is. NOS enzymen produceren NO en citrulline in aanwezigheid van arginine en de co-factor tetrahydrobiopterine. Als een van beide in onvoldoende mate aanwezig is, "ontkoppelt" NOS en produceert het het zuurstofradicaal superoxide in

plaats van NO. Op grond hiervan hebben wij gehypothetiseerd dat een tekort aan arginine schadelijker is voor de functie van het endotheel dan een tekort aan NO. Om die reden hebben wij (hoofdstuk 5) de effecten van een selectieve eliminatie van ASS en arginase-1 in het endotheel nagegaan door $Ass^{fl/fl}$ of $Arg1^{fl/fl}$ muizen te kruisen met Tie2-Cre muizen (deze muizen brengen het Cre recombinase selectief in het endotheel tot expressie). ASS en het endotheel specifieke NOS3 komen samen tot expressie in het arteriële endotheel van jonge wildtype muizen, terwijl Arginase1 daarin (nog niet) tot expressie komt. Arteriën zonder ASS of Arginase1 expressie in het endotheel verschilden niet van wildtype arteriën in hun vernauwing na blootstelling aan phenylephrine of in hun verwijding na blootstelling aan acetylcholine. Alleen na herhaalde inductie van vaatvernauwing en -verwijding bleek dat toevoeging van arginine aan het orgaanbad de vaatverwijding in arteriën zonder ASS herstelde, terwijl citrulline (zoals verwacht) geen effect had. Na dezelfde voorbehandeling bleken arteriën zonder Arginase1 daarentegen gevoeliger voor toegevoegd arginine en citrulline dan wildtype controles. Deze voorlopige resultaten laten zien dat resynthese van arginine uit citrulline in het endotheel ook in vivo optreedt en belangrijk is voor een goede functie van het arteriële epitheel.

Acknowledgements

Finally, after all these months of writing and corrections, I am able to write this acknowledgment section. This is actually more difficult than anticipated and this is why I took all the thesis books from my once-was-an-A.I.O. fellows and tried to figure out how they did it. Unfortunately, this approach was not very fruitful since most of the acknowledgements sections are in Dutch. Damn I should really have worked more on my Taal Vital lessons. Anyway, I will try my best to thank each and everyone who helped me through all these years I spent working on my PhD.

To start, I am very grateful to Professor Toon Stegmann. As it was one said, Holland occupies less than one percent of the earth's surface... but like its airline (KLM for those who don't know), the Dutch scientists cover the rest. Indeed, you can always meet a Dutch scientist in every big western city and this is how I met Toon Stegman at the University Paul Sabatier in Toulouse. I was searching a PhD position and he was the one who suggested me the Netherlands and helped me got one. So *erg bedankt*, Toon.

Wout, it has been a pleasure being your PhD student. I really appreciated the scientific as well as the non-scientific discussions we shared. Moreover, you invested all your knowledge and experience in keeping the course of my PhD, especially through the doubtful moments we've been through and I'm really grateful for this.

Naturally, the next person I have to thank is Leo, my co-promotor and friend. She has always been very good in making the experiments go smoothly. Through the years we spent working in generating and studying the mice, she has tried her best to find the appropriate answers to the numerous questions I had. Her most frequent answer which I dislike was: Vincent, you have to do it again!!! Also a big thank for your *non-stop caring* in making our everyday life as enjoyable as possible.

Also big thanks to my 2.122-room-mates, Rinus and Selvi for all these years of turmoil and laughs we had together. My buddy Rinus, *de palm boom*, has always made sure that all our data were safely stored. I am still amazed how you could memorise all our technical stuff and use them as if you were also doing a PhD on arginine metabolism. To my other room-mate, Selvi, our Indian lady. I am sure everyone in the department remembers our fights just about anything. It's true that it took a while for us to become

friends and you to stop calling me a “*paradesi*” which means beggar for those who don’t understand Tamil. But once the turmoil was over, I really enjoyed the time I spent with you in the lab and outside during the dinners where I would be choking due to your spicy food. So thank you for your help in the lab and for your friendship.

I would also like to thank all the members of the Anatomy & Embryology Department for their help. A special thank to Paul, my histological mentor, who taught me all the tricks to obtain nice results from making sections, staining them and taking pictures. Thank you also for your extra attention like your home-made saté sauce. I would never have been able to cut all these sections without it. That was *lekker*. I also want to thank Chiel who made sure I would have everything available to perform my research. He was also able to find out my errors and made me correct them which in the end made me get my mouse. I am also very grateful to Johan in helping me with all the mouse experiments and tried, in vain, to teach me golf. To Greta, if I recall well you have a cup on which is written “the best secretary”. This is totally true; you always manage to have all paper work fixed while I would be bringing you a cup of coffee. Also a big thank-you for having been such a pleasant neighbour who took care of my plants when I was gone. I don’t forget the other members of the department, Els, Henny, Arno, Leon, Rogier, Hans, and Professor Drukker and all the others for their support during these years.

Big thanks to the members of the Population Genetics Department Franck, Hanneke, and Sofia and to all others who were always friendly when I came to perform my experiments. Also to Kim Salvekouls and Sherry Wheppler from the Maastricht Lab who gave me all the required technical support during my experiments.

Of course all the experiments would not have been possible without the work from the members of the animal facility (CPV) and in particular Richard. It’s been a pleasure working with you.

Last but not least, I would like to express all my gratitude to my ever-supporting wife Celine, my father Daniel, my mother Denise, my brothers François and Gérald, my sister Vanessa and to the rest of *my family and other animals*

Thanks! That's what makes it all worthwhile.

Curriculum vitae

Vincent Marion was born April 21st, 1976 in Mauritius. He received his secondary schooling at the catholic college of Saint Esprit, where he obtained his Higher School Certificated (H.S.C.) from Cambridge University (U.K.) in 1995. He then started studying Biological Sciences at the University of Paul Sabatier in Toulouse, France. He obtained his Master in Science in Structural and Functional Biochemistry in 2002, after performing an internship at the University Hospital Center of Purpan, Toulouse, France, where he took part in the characterization of an enzymatic activity required by the human cytomegalovirus for infecting human cells. In October 2002, he obtained a PhD position in the Department of Anatomy & Embryology at Maastricht University to work on a project aimed at finding the role of the amino acid arginine in the organ development and function. Currently, he has a post-doc position in the Laboratory of Medical Genetics at the University of Strasbourg, France, where he is focusing on how the primary cilium regulates the development of tissues in humans.