REVIEW ARTICLE

Arginine metabolism: nitric oxide and beyond

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Arginine is one of the most versatile amino acids in animal cells, serving as a precursor for the synthesis not only of proteins but also of nitric oxide, urea, polyamines, proline, glutamate, creatine and agmatine. Of the enzymes that catalyse rate-controlling steps in arginine synthesis and catabolism, argininosuccinate synthase, the two arginase isoenzymes, the three nitric oxide synthase isoenzymes and arginine decarboxylase have been recognized in recent years as key factors in regulating newly identified aspects of arginine metabolism. In particular, changes in the activities of argininosuccinate synthase, the arginases, the inducible isoenzyme of nitric oxide synthase and also cationic amino acid transporters play major roles in determining the metabolic fates of arginine in health and disease, and recent studies have identified complex patterns of interaction among these enzymes. There is growing interest in the potential roles of the arginase isoenzymes as regulators of the synthesis of nitric oxide, polyamines, proline and glutamate. Physiological roles and relationships between the pathways of arginine synthesis and catabolism *in vivo* are complex and difficult to analyse, owing to compartmentalized expression of various enzymes at both organ (e.g. liver, small intestine and kidney) and subcellular (cytosol and mitochondria) levels, as well as to changes in expression during development and in response to diet, hormones and cytokines. The ongoing development of new cell lines and animal models using cDNA clones and genes for key arginine metabolic enzymes will provide new approaches more clearly elucidating the physiological roles of these enzymes.

INTRODUCTION

There is a rich history of studies on arginine (2-amino-5-guanidinovaleric acid) and its metabolism over the past 100 years. This interesting amino acid was first isolated from lupin seedlings in 1886 [1], and soon afterwards (1895) was identified as a component of animal proteins [2]. The structure of arginine was established by alkaline hydrolysis to yield ornithine and urea in 1897 [3] and by synthesis from benzoylornithine in 1910 [4]. Subsequently, arginine was found in 1924 to be a major amino acid in the basic proteins of fish sperm [5], and its synthesis by mammals was deduced in the classic nutrition studies of W. C. Rose and his colleagues in 1930 [6]. Although high activities of arginase, the enzyme that hydrolyses arginine to ornithine and urea, had been identified in the liver in 1904 [7], it was the discovery of the ornithine cycle (urea cycle) by Krebs and Henseleit in 1932 [8] that led to the elucidation of prominent roles of arginine in physiology and metabolic pathways.

Physiological and nutritional studies in the late 1930s and 1940s started a new era of arginine research. Foster et al. [9] reported that arginine was required for the synthesis of creatine, the precursor of creatinine, which was known to scientists of the 19th century and had been proposed in 1926 as a clinical indicator of renal function [10]. Meanwhile, dietary arginine was shown to be required for growth of the chick [11] and for optimal growth of the young rat, but not for the healthy adult rat [12,13]. These findings led to extensive studies in the 1950s, 1960s and 1970s that resulted in the initial classification of arginine as a dispensable (non-essential) amino acid for healthy adult humans [14], but as an essential amino acid for young, growing mammals [15–17] and for carnivores [18,19].

With the discovery of novel pathways for arginine synthesis and catabolism in animals, the 1980s witnessed the beginning of another exciting era in arginine research. Windmueller and Spaeth [20] reported in 1981 that the small intestine is the major source of circulating citrulline for endogenous synthesis of arginine in the adult rat. This classical finding led to the elucidation of pathways for the intestinal synthesis of citrulline from glutamine/glutamate via L-Δ^1-pyrroline-5-carboxylate (P5C) synthetase in 1983 [21,22]. There was growing recognition during this decade that nitrogen-balance studies are not sufficiently sensitive to fully evaluate dietary requirements for arginine, and that arginine should be regarded as a conditionally essential amino acid in adult humans and other animals, particularly in cases of disease or trauma [23]. Meanwhile, much effort was directed towards identifying the endothelial cell-derived factor that was reported in 1980 by Furchgott and Zawadski [24] to play an obligatory role in the relaxation of arterial smooth muscle. Also, extensive research was conducted (e.g. [25]) to elucidate the metabolic basis for endogenous nitrate synthesis that had been discovered in humans and rats [26–28]. Key discoveries in 1987 included reports that arginine is the precursor for mammalian nitrite/nitrate synthesis [29] and that nitric oxide (NO) is the endothelium-derived relaxing factor [30,31]. In 1988, NO was identified as the biologically active intermediate of the arginine → nitrite + nitrate pathway in macrophages [32,33] and endothelial cells [34]. It is now known that many cell types utilize arginine to generate NO, which plays

Abbreviations used: ASL, argininosuccinate lyase; ASS, argininosuccinate synthase; CPS I, carbamoyl-phosphate synthase I; LPS, lipopolysaccharide; NOS, NO synthase; eNOS, endothelial NOS; iNOS, inducible NOS; nNOS, neuronal NOS; OAT, ornithine aminotransferase; OCT, ornithine carbamoyltransferase; ODC, ornithine decarboxylase; P5C, Δ^1-pyrroline-5-carboxylate.

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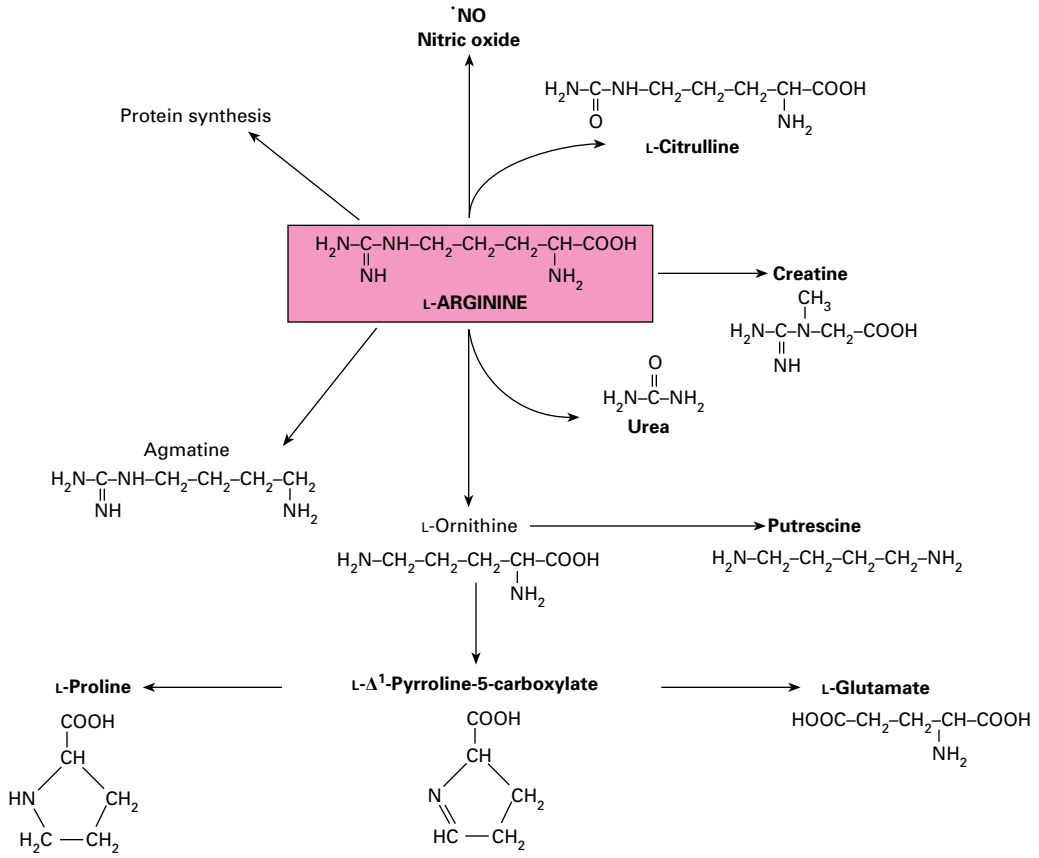

Figure 1 Metabolic fates of arginine in mammalian cells

The five enzymes on which the central limbs of the pathways are based include (clockwise from the top): nitric oxide synthase (NOS), arginine:glycine amidinotransferase, arginase, arginine decarboxylase and arginyl-tRNA synthetase.

important roles in many diverse processes, including vasodilation, immune responses, neurotransmission and adhesion of platelets and leucocytes [35,36]. The discovery of the novel arginine-dependent NO pathway has stimulated renewed interest in the biochemistry, physiology and nutrition of arginine in animals and humans.

Although arginine synthesis and transport are clearly key elements in the overall scheme of arginine metabolism, it is the processes of arginine catabolism (Figure 1) that have attracted the most interest in recent years. Three of the end-point products of arginine in Figure 1 are cell-signalling molecules: NO, glutamate and agmatine. Glutamate, which is also synthesized from glutamine, proline and branched-chain amino acids and via transamination, can give rise to yet another cell-signalling molecule, γ-aminobutyric acid ('GABA'). Although not commonly thought of as cell-signalling molecules, polyamines also can regulate key cellular processes, such as ion channel function [37]. The recognition that arginine is a precursor for these distinct types of cell-signalling molecules represents a dramatic revision of the traditional textbook view of arginine as primarily a precursor for the synthesis of proteins, urea and creatine.

Arginine itself plays other roles in physiology and metabolism. Arginyl-tRNA not only is an immediate precursor for protein synthesis, but is also involved in the post-translational conjugation of arginine with the N-termini of proteins bearing N-terminal aspartate or glutamate, thereby allowing these proteins to be targeted for degradation by the ubiquitin-dependent proteolytic pathway [38]. Arginine also acts as an allosteric activator of *N*-acetylglutamate synthase, which synthesizes *N*-acetylglutamate from glutamate and acetyl-CoA [39]. As *N*-acetylglutamate is an essential cofactor for carbamoyl-phosphate synthase I (CPS I) (Figure 2), a key enzyme in arginine and urea synthesis, arginine may play a regulatory role in its own metabolism. Furthermore, arginine can stimulate secretion of hormones, such as insulin, growth hormone, glucagon and prolactin [23,40]. Thus regulation of arginine homoeostasis, which depends on dietary arginine intake, whole-body protein turnover, arginine synthesis and catabolism, is of considerable nutritional and physiological significance. In this review, we will examine current views of arginine metabolism in mammals.

ARGININE SYNTHESIS

The metabolic pathway for arginine synthesis in mammals via P5C synthetase and proline oxidase is illustrated in Figure 2 [41,42]. Some of the enzymes in this pathway are present in a variety of cell types, while expression of other enzymes is highly restricted. Phosphate-dependent glutaminase, ornithine aminotransferase (OAT), argininosuccinate synthase (ASS), argininosuccinate lyase (ASL) and aspartate aminotransferase are widely distributed in animal tissues [42–45], whereas CPS I, ornithine carbamoyltransferase (OCT) and *N*-acetylglutamate

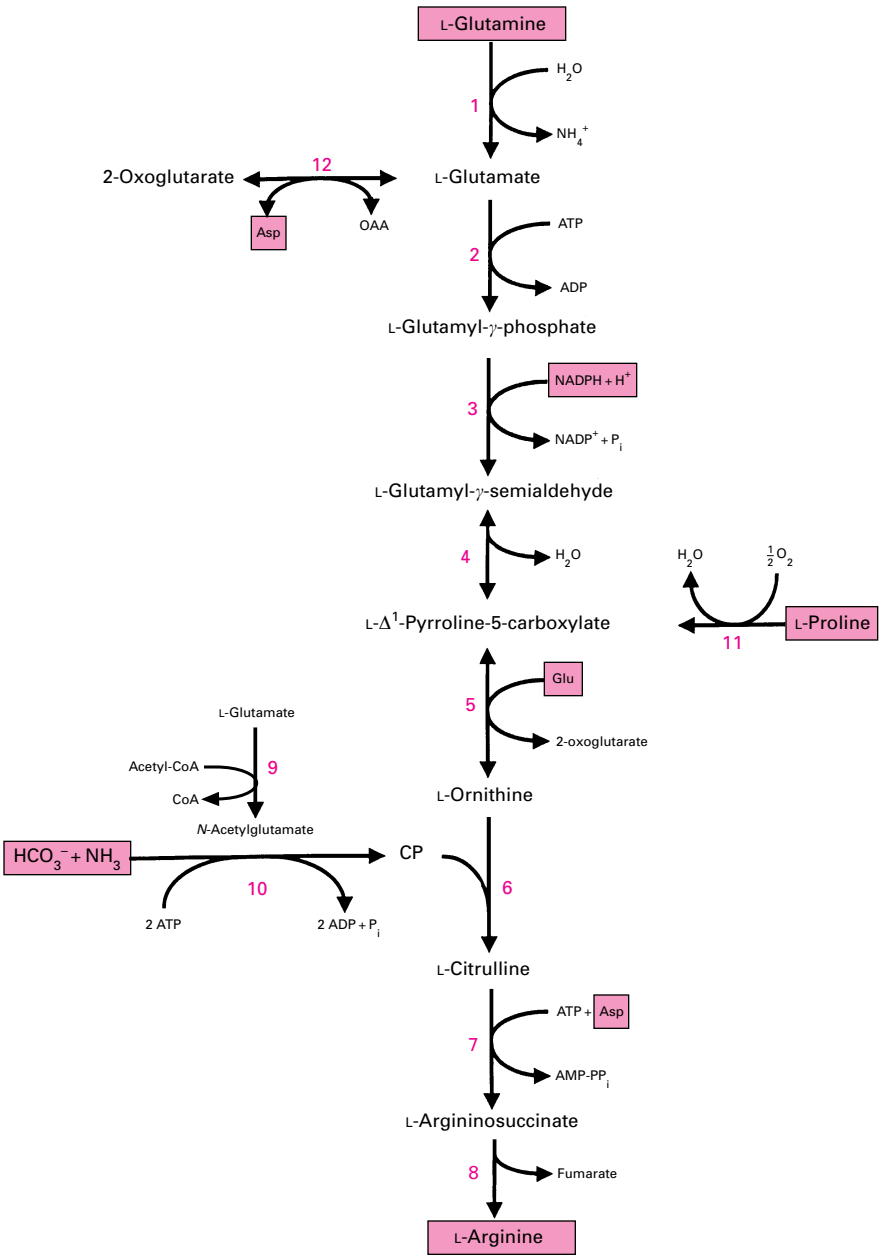

Figure 2 Pathways of arginine synthesis

Enzymes that catalyse the indicated reactions are: 1, phosphate-dependent glutaminase (EC 3.5.1.2); 2 and 3, P5C synthetase (EC number not assigned); 5, ornithine aminotransferase (OAT; EC 2.6.1.13); 6, ornithine carbamoyltransferase (OCT; EC 2.1.3.3); 7, argininosuccinate synthase (ASS; EC 6.3.4.5); 8, argininosuccinate lyase (ASL; EC 4.3.2.1); 9, *N*-acetylglutamate synthase (EC 2.3.1.1); 10, carbamoyl-phosphate synthase I (ammonia) (CPS I; EC 6.3.4.16); 11, proline oxidase (EC number not assigned); 12, aspartate aminotransferase (EC 2.6.1.1). Step 4 is a spontaneous, non-enzymic reaction. Glutamyl-γ-semialdehyde is in chemical equilibrium with P5C. The chemical equilibrium favours P5C formation [21,22]. P5C synthetase is a bifunctional polypeptide that exhibits both γ-glutamyl kinase (glutamate 5-kinase; EC 2.7.2.11) and γ-glutamylphosphate reductase (glutamate-5-semialdehyde dehydrogenase; EC 1.2.1.41) activities (reactions 2 and 3 respectively) [41]. Reactions 1–6 and 9–11 occur in mitochondria, reactions 7 and 8 take place in the cytosol, and reaction 12 can occur in both mitochondria and the cytosol. Abbreviations: OAA, oxaloacetate; CP, carbamoyl phosphate.

synthase are restricted to the liver and intestinal mucosa [46,47]. Proline oxidase is present mainly in the small intestine, liver and kidneys [42,48], but P5C synthetase is located almost exclusively in the intestinal mucosa, with only trace amounts in other tissues [42,47]. Proline can also be converted into P5C by L-pipecolic acid oxidase and sarcosine oxidase [49], but it is not known whether these enzymes play any significant role in endogenous arginine synthesis. The differential expression of the various enzymes has resulted in highly compartmentalized metabolic functions of different organs. Thus in most animals the complete pathway for net arginine synthesis outlined in Figure 2 is found only in the small intestine of neonates; in pigs, however, the complete pathway is also present in weaned animals. In adults, the majority of endogenous arginine synthesis involves an inter-organ pathway (also known as the intestinal–renal axis), in which the small intestine releases citrulline into the blood circulation which is then extracted primarily by the kidney for conversion into arginine [50,51].

Table 1 Plasma concentrations of citrulline and arginine and synthesis of arginine from citrulline in animals and humans receiving total parenteral nutrition (TPN) or enteral feeding

[1]Data are from Castillo et al. [52] for infants recovering from persistent pulmonary hypertension. [2]Data are from Beaumier [53] for healthy infants. [3]Data are from Castillo et al. [54]. [4]Data on plasma concentrations of citrulline and arginine are from Flynn and Wu [61]. Endogenous flux of arginine was calculated as the difference between the flux of plasma arginine and the exogenous supply of arginine [55]. Data on endogenous synthesis of arginine from citrulline were estimated as the difference on the basis of arginine requirement for protein deposition (68 μmol/h per kg body wt.), arginine catabolism (40 μmol/h per kg body wt.) and exogenous supply of arginine (12 μmol/h per kg body wt.) in the piglet fed an arginine-deficient diet [55]. The value for arginine requirement for protein deposition was estimated on the basis of daily weight gain of the piglet (75 g/day) [55], protein concentration in the body (12 g/100 g body wt.) [55] and arginine concentration in the pig (69 mg/g of protein) [57]. [5]Data on plasma concentrations of citrulline and arginine are from Wu et al. [42] for 28 kg pigs. The value for endogenous flux of arginine was calculated on the basis of estimated endogenous arginine synthesis [42], whole-body protein degradation (477 mg/h per kg body wt.) in 30 kg pigs [56] and arginine concentration in the pig (69 mg/g of protein) [57]. [6]Data are from Dhanakoti et al. [50] for plasma concentrations of citrulline and arginine. Data on endogenous synthesis of arginine from citrulline are from Windmueller and Spaeth [20]. Data for exogenous supply of arginine to the circulation and endogenous synthesis of arginine from citrulline were calculated on the basis of arginine intake (91 μmol/h per kg body wt.) [59] and on the fact that only 60% of intestinal luminal arginine is absorbed intact into the portal circulation in the adult rat [191]. Endogenous flux of arginine was calculated on the basis of whole-body protein degradation (8.1 g/day) in the adult rat (394 g) [58] and arginine concentration in the rat (73 mg/g of protein) [57]. [7]Data are from Yu et al. [60]. Abbreviation: Cit, citrulline. The symbol ?? means that data are not available.

Animals or humans	Nutritional state	Plasma concn. (μM)		Flux/supply/synthesis (μmol/h per kg body wt.)			
		Citrulline	Arginine	Flux of plasma Arg	Exogenous supply of Arg to circulation	Endogenous flux of Arg	Endogenous synthesis of Arg from Cit
Human infant[1]	TPN	11	115	125	29	96	??
Human infant[2]	TPN	??	??	209	69	140	15
Adult human[3]	Enteral	27	210	73	22	51	5.2
Neonatal pig[4]	Enteral	89	163	408	93	315	96
Growing pig[5]	Enteral	64	159	319	65	254	65
Adult rat[6]	Enteral	62	175	479	55	424	65
Adult dog[7]	Post-absorptive	31	178	225	0	204	9.4

Arginine synthesis *in vivo*

Table 1 summarizes representative data on plasma concentrations of citrulline and arginine, and *in vivo* flux and synthesis of arginine, in humans and other animals [20,50,52–60]. The sources of arginine entering the plasma (flux of plasma arginine) in the fed state are exogenous (diet) and endogenous (whole-body protein degradation plus endogenous synthesis from citrulline). Endogenous synthesis of arginine varies with species, nutritional status and developmental stage (Table 1). As *de novo* arginine synthesis accounts for only 5–15% of endogenous arginine flux in adult animals and humans (Table 1), the major contributor to endogenous arginine flux is whole-body protein turnover. In neonatal pigs, however, *de novo* arginine synthesis accounts for about 30% of the endogenous arginine flux. This difference between adult and neonatal animals probably reflects the great differences in net protein synthesis relative to protein turnover. A high requirement for endogenous arginine synthesis during the neonatal period is indicated by the fact that the relative abundance of arginine in milk protein is significantly less than that in total body protein [57]. Endogenous synthesis of arginine plays an important role in regulating arginine homoeostasis in neonatal and growing pigs [42,61], but does not play a major role in regulating arginine homoeostasis in healthy adult humans [54].

Virtually all flux of plasma citrulline is associated with endogenous synthesis of arginine in adult humans [62] and rats [20], but apparently not in adult dogs [60]. Endogenous synthesis of arginine from citrulline is not responsive to reduced dietary intake of arginine in adult rats [59] or humans [54].

Intestinal arginine synthesis

Enteral glutamine and glutamate, and plasma glutamine, are extensively catabolized by the small intestine [63–66] and serve as major precursors for intestinal synthesis of arginine or citrulline [66,67]. Windmueller and Spaeth [68] were the first to demonstrate uptake of arterial and luminal glutamine by the rat small intestine and release of citrulline as a product of glutamine. They later established that the small intestine is the major source of circulating citrulline in the adult rat [20]. The small intestine of the dog [69] and pig [70], as well as the portal-drained viscera of the human [71] and sheep [72], also release relatively large amounts of citrulline. The enterocyte is the cell type responsible for intestinal synthesis of citrulline or arginine from glutamine/glutamate [73–75]. Of all weaned or adult mammals studied, the pig is the only species whose small intestine releases endogenously synthesized arginine into the venous circulation [64,70], which results from the balance between arginine synthesis and catabolism by the intestinal mucosa. A small amount of citrulline is released by the canine large intestine [69], consistent with the recent finding that synthesis of citrulline from glutamine occurs also in rat colonocytes [76].

There are developmental changes in the intestinal synthesis of arginine from glutamine. At birth, the small intestine is the major site of net arginine synthesis [75,77], but gradually becomes the major site of net citrulline production as intestinal arginase expression increases [77–80]. This transition is compensated for by the gradually increasing capacity of the kidney to synthesize arginine from citrulline [73,79,81]. In pigs, net arginine synthesis from glutamine by enterocytes was greater than net citrulline production at birth (Figure 3) [82]. By day 14, net production of both arginine and citrulline had greatly declined, but net arginine synthesis still occurred in the oldest animals studied. Citrulline synthesis had recovered to approximately half its original rate by day 29 (8 days post-weaning) (Figure 3). The enzymic basis for the marked increase in citrulline synthesis from glutamine by enterocytes of post-weaning pigs has not been determined, but may result from the large increase in P5C synthetase activity between days 21 and 29 [74].

In addition to glutamine and glutamate, proline is also an important precursor for the intestinal synthesis of citrulline and arginine [82]. The developmental profiles of arginine and citrulline production from proline in pig enterocytes are generally similar to those for production from glutamine (Figure 3). Because there

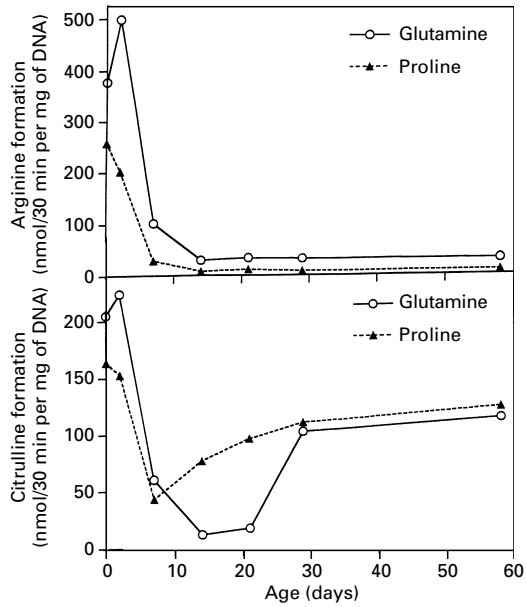

Figure 3 Developmental changes in synthesis of arginine and citrulline by pig enterocytes

Enterocytes were isolated from the jejenum of pigs at various times after birth (day 0) and incubated in Krebs bicarbonate buffer containing 2 mM L-glutamine or 2 mM [^{14}C]proline plus 2 mM L-glutamine. The incubation medium also contained 5 mM D-glucose. Pigs were weaned at day 21. Data are from Table 2 (L-glutamine) and Table 4 (L-proline) of Wu [82].

is no significant uptake of arterial proline by the small intestine [70], the enteral diet must be the major source of proline for citrulline synthesis in enterocytes, consistent with the recent finding that citrulline is a major product of enterally delivered proline in the pig [83]. Although it is often stated that proline oxidase is present mainly in the liver, kidney and brain [48,84] and that proline is not catabolized in the gut [47,85], relatively high activities of proline oxidase have been found in the small intestine and enterocytes of pigs [82,86] and rats [82]. In fact, the activity of proline oxidase in the pig small intestine is several-fold greater than that in the liver and kidney [42,86]. The failure to detect intestinal proline oxidase activity in earlier studies may have been due to the presence of a proline oxidase inhibitor in tissue homogenates or to a lack of proteinase inhibitors in the buffers used for tissue homogenization.

The essential role of the small intestine in arginine synthesis is graphically demonstrated by the arginine deficiencies that result when intestinal citrulline synthesis is blocked by inhibitors of OCT [87] or OAT [61] or by massive resection of the small bowel [88,89]. Similarly, arginine deficiencies occur in individuals with inherited defects in OCT [90], OAT [91,92] or P5C synthetase [93]. An analogous situation exists in strict carnivores, such as cats, which synthesize very little arginine and thus must rely on the diet to meet their needs for this amino acid [94]. Although feline kidneys contain the enzymes required for arginine synthesis [95], cats synthesize little citrulline because their intestines have relatively low activities of P5C synthetase and OAT [94,96]. The requirement for dietary arginine by cats and other carnivores (such as ferrets) is so stringent that ingestion of an arginine-free meal rapidly leads to hyperammonaemia, encephalopathy and, in the case of cats, even death [94,97].

Relatively little is known about how the intestinal synthesis of citrulline and arginine is regulated. Glucocorticoids precociously induce activities of several of the enzymes in the intestines of immature animals [98–101], indicating that these hormones probably play a role in the developmental maturation of this pathway, as is the case for many other developmentally regulated pathways in this organ [102]. This may explain why dexamethasone increases plasma concentrations of ornithine, citrulline and arginine in pre-term infants [103]. In contrast with the liver, activities of intestinal OCT [104,105] and OAT [106] in adult rats were modestly decreased by increasing the dietary protein intake, and expression of intestinal OCT and CPS I was unaffected by starvation [107]. The latter observation is consistent with the finding that citrulline production by the small intestine does not increase in rats and pigs fed an arginine-deficient diet, even when an additional substrate in the form of glutamate is included in the diet [108,109].

Renal arginine synthesis

Analyses of the arginine biosynthetic enzymes in kidney in the 1940s [110,111] paved the way for physiological studies which established the kidney as the major organ involved in endogenous arginine synthesis [50,51]. Approximately 60 % of net arginine synthesis in adult mammals occurs in the kidney [50,60], where citrulline is extracted from the blood and converted stoichiometrically into arginine by the action of ASS and ASL (Figure 2), which are localized within the proximal convoluted tubules [81,112–115]. A tight correlation between renal citrulline uptake and renal arginine output has been elegantly demonstrated for both humans and rats [50,116]. Furthermore, *in vivo* rates of arginine synthesis in adult rats are limited primarily by the amount of citrulline produced by other organs, such as the small intestine, rather than by the renal arginine biosynthetic capacity [50]. As the renal arginine biosynthetic capacity appears to be several-fold greater than the intestinal capacity for citrulline production, it is unclear why renal activities of, or mRNA levels for, ASS and ASL are increased by a high-protein diet [117,118]. Renal mRNA levels for ASS and ASL also increase during starvation [118], probably as an adaptive response to maintain plasma arginine levels in the absence of dietary protein. As noted in the preceding section, the renal capacity for arginine synthesis develops in late fetal stages and continues to increase after birth [81], complementing the developmental shift from release of arginine to release of citrulline by the small intestine. The kidney also expresses arginase, but expression of arginase and the arginine biosynthetic enzymes is highly segregated within different parts of the nephron, so that there is little or no co-expression of these opposing enzymic pathways within the same cell [113,119].

As expected, individuals with chronic renal insufficiency have elevated plasma levels of citrulline [116,120,121]. Surprisingly, however, there is little or no decrease in plasma arginine in these patients. The basis for the maintenance of plasma arginine at normal or near-normal levels is unknown, but probably involves a combination of factors [40,122], including increased release of arginine by protein catabolism in skeletal muscle, increased arginine synthesis at extrarenal sites, hypertrophy of proximal tubules, hyperfiltration (which increases the amount of citrulline filtered per nephron), and an increased rate of arginine synthesis due to elevated plasma levels of citrulline. The last point follows from the finding that rates of renal arginine synthesis are essentially a function of plasma citrulline levels [50]. In addition, the proposal that arginine degradation is more important than arginine synthesis in maintaining arginine homoeostasis in adult humans [54] raises the possibility of some compensatory decrease in arginine degradation.

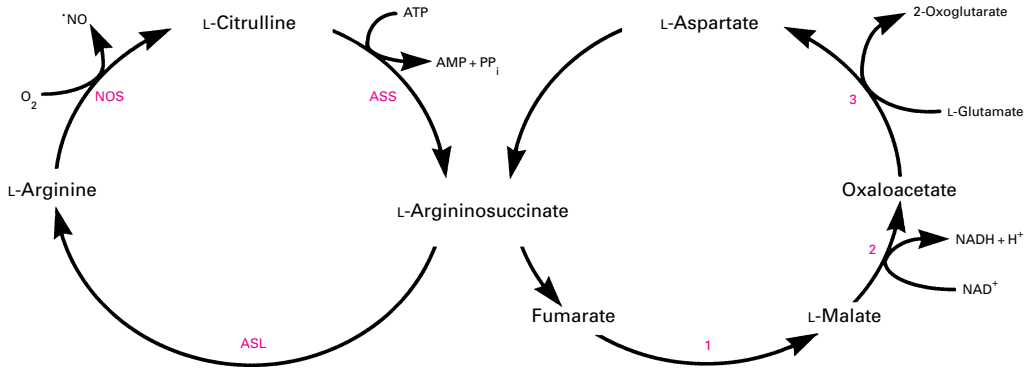

Figure 4 Citrulline/NO cycle

This cytosolic cycle can be coupled to the citric acid cycle, as shown on the right. Fumarate produced in the cytosol enters the citric acid cycle in the mitochondrion, where it is converted into oxaloacetate. Transamination converts oxaloacetate into aspartate, which is transported into the cytosol. Enzymes catalysing reactions 1–3 are: 1, fumarase (EC 4.2.1.2); 2, malate dehydrogenase (EC 1.1.1.37); 3, aspartate aminotransferase. Although reactions 1–3 are reversible, the diagram depicts the net unidirectional flow in NO-producing cells. (Modified from Figure 5 of Nussler et al. [143] and reprinted with permission of the American Society for Biochemistry and Molecular Biology.)

Hepatic arginine synthesis

The highest rates of arginine synthesis occur within the hepatic urea cycle, which is localized within periportal hepatocytes [39]. In healthy adult humans, for example, rates of urea production (239 μmol/h per kg in the fed state and 184 μmol/h per kg in the fasted state) are vastly greater than rates of NO synthesis (0.91 μmol/h per kg in the fed state and 1.00 μmol/h per kg in the fasted state) [62], and also much higher than rates of creatine synthesis (7.9 μmol/h per kg) [123]. Net arginine synthesis by the liver is only possible if the urea cycle is replenished by necessary intermediates, such as ornithine. The urea cycle enzymes are also organized in a metabolon [124], whereby the product of each enzymic reaction is efficiently channelled to the next enzyme in the pathway [124,125]. Thus the tight channelling of metabolites and the very high level of arginase in hepatocytes result in little or no net production of arginine by the liver. This is dramatically illustrated by the fact that individuals who cannot make arginine because of inherited defects in the urea cycle continue to require arginine in their diet after receiving liver transplants [90].

Expression of hepatic urea-cycle enzymes begins during fetal development and continues to increase after birth [126–128]. Subsequently, levels of the urea-cycle enzymes are co-ordinately induced by conditions involving increased protein and amino acid catabolism, such as increased dietary protein intake, starvation and increases in glucocorticoid levels or the glucagon/insulin ratio [46]. One exception is the cat, a strict carnivore in which levels of the urea-cycle enzymes are unaffected by changes in dietary protein intake [129]. These long-term adaptive increases largely reflect increased transcription rates of the genes encoding these enzymes. Rapid, short-term changes in urea-cycle activity occur primarily via changes in CPS I catalytic efficiency, which in turn is regulated by changes in the mitochondrial concentration of *N*-acetylglutamate [39]. As the mechanisms that regulate urea cycle activity have been extensively discussed in previous reviews [39,46,130,131], they will not be considered here.

In response to inflammatory conditions such as sepsis, hepatocytes can be induced to produce NO in addition to urea [132]. This fact raised the question as to whether the urea cycle also provides arginine for NO synthesis. To address this matter, several groups examined the metabolic consequences of perfusing various nitrogenous substrates into livers isolated from rats in which high-level hepatic NO synthesis had been induced [133–135]. In one such study, perfusion of isolated livers with glutamine or NH_4Cl resulted in large increases in urea synthesis without any change in NO synthesis, indicating that arginine made within the urea cycle was not available for NO synthesis [134]. In contrast, a similar study by another group did find an increase in NO production when livers were perfused with NH_4Cl [133]. These apparently disparate outcomes might have reflected differences in, for example, the integrity of the hepatocytes resulting from the different stimuli used to induce hepatic NO synthesis. In any event, both studies are consistent with the view that, if the urea cycle does provide any arginine for hepatic NO synthesis, it must represent only a tiny fraction of the total arginine synthesized within the urea cycle. Although competition for arginine between the urea cycle and hepatic inducible NO synthase (iNOS) was claimed also by a third group [135], their study only showed that exogenously supplied arginine could be utilized by both arginase and iNOS; it did not determine whether arginine synthesized within the urea cycle could be used for hepatic NO synthesis. Finally, the hepatic capacity for arginine synthesis within the urea cycle, as indicated by mRNA levels for ASS and ASL, is not affected by inflammatory stimuli such as lipopolysaccharide (LPS) [136,137].

Arginine synthesis in NO-producing cells

The arginine biosynthetic pathway represents a regulated and highly localized source of substrate for NO synthesis in a wide variety of non-hepatic cells [138,139]. Citrulline, which is co-produced with NO, can be recycled to arginine via a pathway that has been termed the citrulline/NO cycle [139] or the arginine/citrulline cycle [140] (Figure 4). This recycling is accomplished by the combined action of ASS and ASL, which are expressed to some degree in nearly all cell types. The existence of the citrulline/NO cycle is supported by the fact that total citrulline production is lower than total NO production for some cell types [141], and also by the demonstration that citrulline can replace arginine, at least in part, in supporting NO synthesis by intact cells [142–145]. Although the data clearly demonstrate the conversion of citrulline into arginine, there is no direct experimental evidence that the aspartate used for argininosuccinate formation is produced from fumarate as depicted in Figure 4, or by a possible alternative route [142] whereby malate is instead converted into pyruvate by malic enzyme, and pyruvate is then

converted into oxaloacetate by pyruvate carboxylase. The fact that citrulline accumulates to a considerable extent in the medium of NO-producing cells demonstrates that the citrulline/NO cycle is much less efficient than the hepatic urea cycle, indicating that the activity of ASS is appreciably less than the activity of iNOS and/or that there is little or no channelling of substrates and products between iNOS and ASS.

Following initial reports that NO synthesis and arginine biosynthetic capacity were co-induced in macrophages [146], several laboratories established that induction of iNOS in all non-hepatic mammalian cells examined to date is accompanied by induction of ASS, a rate-controlling enzyme in arginine biosynthesis [136,142–144,147–150]. In rat tissues, ASL is also co-induced with iNOS [136,137]. Because basal expression of ASS differs greatly among different cell types, the magnitude of ASS induction by inflammatory stimuli is highly variable. The observation that ASS and iNOS were co-induced led to the hypothesis [143,151] that regulation of the arginine recycling pathway could itself represent a potential mechanism for regulating inducible NO synthesis. This hypothesis was confirmed by the finding that vascular smooth muscle cells that had been transfected to overexpress ASS had higher levels of induced NO production at limiting extracellular arginine concentrations than did untransfected cells [152]. Importantly, this result also demonstrated that rates of arginine uptake were not sufficient to support maximal rates of NO synthesis, further supporting the general proposition that any mechanism that regulates the availability of arginine represents a potential control point for NO synthesis [151]. Despite the provocative results obtained with cultured cells, it should be emphasized that the contribution of the arginine recycling pathway to NO synthesis *in vivo* is completely unknown.

L-Glutamine and hypoxia are physiological regulators of arginine synthesis in NO-producing cells. Inhibition of arginine synthesis by glutamine has been reported for NO-producing endothelial cells [153–155], cerebral perivascular nerve tissues [156] and rat peritoneal macrophages [146], but not for a murine macrophage cell line [153]. Glutamine-dependent inhibition of endothelial arginine synthesis appeared to occur via (1) competitive inhibition of citrulline uptake [154] and (2) a decrease in ASS activity [155]. Several other amino acids (L-alanine, L-glutamate and L-lysine) were also tested, but did not mimic the effects of glutamine [154]. Inhibition of arginine synthesis by glutamine in rat peritoneal macrophages has not been characterized. Hypoxia was reported to inhibit arginine synthesis in endothelial cells by reducing ASS activity rather than citrulline uptake [155]; the basis for ASS inhibition is unknown.

ARGININE CATABOLISM

Arginine can be catabolized via multiple pathways (Figure 5), many of which are co-expressed within the same cell. For example, iNOS, the arginases and arginine decarboxylase can be co-expressed in murine macrophages, as described in the following sections. This can result in complex interactions, whereby the product of one enzyme may inhibit the activity of another enzyme, e.g. as in the inhibition of arginase by N^G-hydroxyarginine. The cellular distribution of enzyme expression varies widely. For example, iNOS can be expressed in almost any cell type which is exposed to the appropriate stimuli [157], whereas expression of arginine:glycine amidinotransferase is much more restricted, being limited principally to kidney, pancreas and, to a lesser extent, liver [158,159]. The type II isoenzymes of arginase and OAT are expressed in many cell types [42,47,160–162], indicating a widespread capacity for synthesis of proline and/or glutamate from arginine. As OAT and ornithine decarboxylase (ODC) are located in different subcellular compartments (mitochondria and cytosol respectively), the ornithine produced by mitochondrial or cytosolic arginases probably has differing metabolic fates. In short, arginine catabolism in mammals involves multiple organs and complex compartmentation at the cellular and systemic levels.

Arginine transport

As arginine transport systems may regulate substrate availability for arginine-requiring enzymes, a brief survey of this topic is appropriate. Because arginine transport is the subject of several reviews over the past few years [163–168], we will summarize points most relevant to the present review. In most mammalian cells, arginine requirements are met primarily by uptake of extracellular arginine via specific transporters, such as systems y^+, $b^{o,+}$, $B^{o,+}$ or y^+L [163,164,168]. Not all transporters are found in every cell type, and activities of specific transporters can be dynamically regulated in response to specific stimuli, such as bacterial endotoxin and inflammatory cytokines [166]. The most important mechanism for arginine uptake in most cell types is system y^+, a high-affinity, Na^+-independent transporter of arginine, lysine and ornithine. Recent studies have identified cDNAs encoding two transmembrane proteins, CAT-1 and CAT-2(B), which have amino acid transport properties consistent with system y^+ [165,167]. A cDNA encoding rCAT3, a brain-specific protein which also exhibits system-y^+ activity, has recently been isolated from rats [169]. Still unresolved is the question of whether system y^+ consists solely of these proteins or involves other proteins as yet unidentified. Because of its major role in arginine transport, regulation of system-y^+ expression or activity represents a potential target for modulating cellular arginine metabolism.

Other cationic amino acids and positively charged analogues are effective inhibitors of arginine uptake by system y^+. For example, arginine uptake can be competitively inhibited by lysine, ornithine, canavanine and certain NOS inhibitors, including N^G-monomethyl-L-arginine and N^G-iminoethyl-L-ornithine, but not by other NOS inhibitors such as aminoguanidine, N^G-nitro-L-arginine and N^G-nitro-L-arginine methyl ester [170–175]. Thus use of NOS inhibitors that are taken up via system y^+ may limit the availability of arginine for other enzymes that utilize this amino acid. It is important to note that N^G-monomethyl-L-arginine, N^G-nitro-L-arginine, N^G-nitro-L-arginine methyl ester and aminoguanidine have no significant activity as direct inhibitors of arginase [176,177].

The expression of system y^+ not only varies among different cell types, but can also be dynamically regulated at the pretranslational level. System y^+ is present in a variety of cell types, but is virtually absent from hepatocytes. Thus $> 85\%$ of the arginine delivered to the livers of rats [178] or dogs [60] is not taken up by the liver. However, system y^+ can be induced by inflammatory cytokines in hepatocytes and other cells [166,167]. In fact, system-y^+ expression is co-induced with iNOS in a wide variety of cell types [150,179–184], indicating that arginine transport capacity increases to support the elevated rates of NO synthesis. In rat astrocytes, induced NO synthesis is strictly dependent on co-induction of system y^+ [185]. It has not been determined whether induction of system y^+ is a general response in conditions where arginine consumption is elevated, e.g. when arginase is induced in the absence of iNOS induction.

Recent studies have indicated that the precise cellular localization of arginine transporters may be responsible for the 'arginine paradox' [186], the observation that endothelial NO synthesis

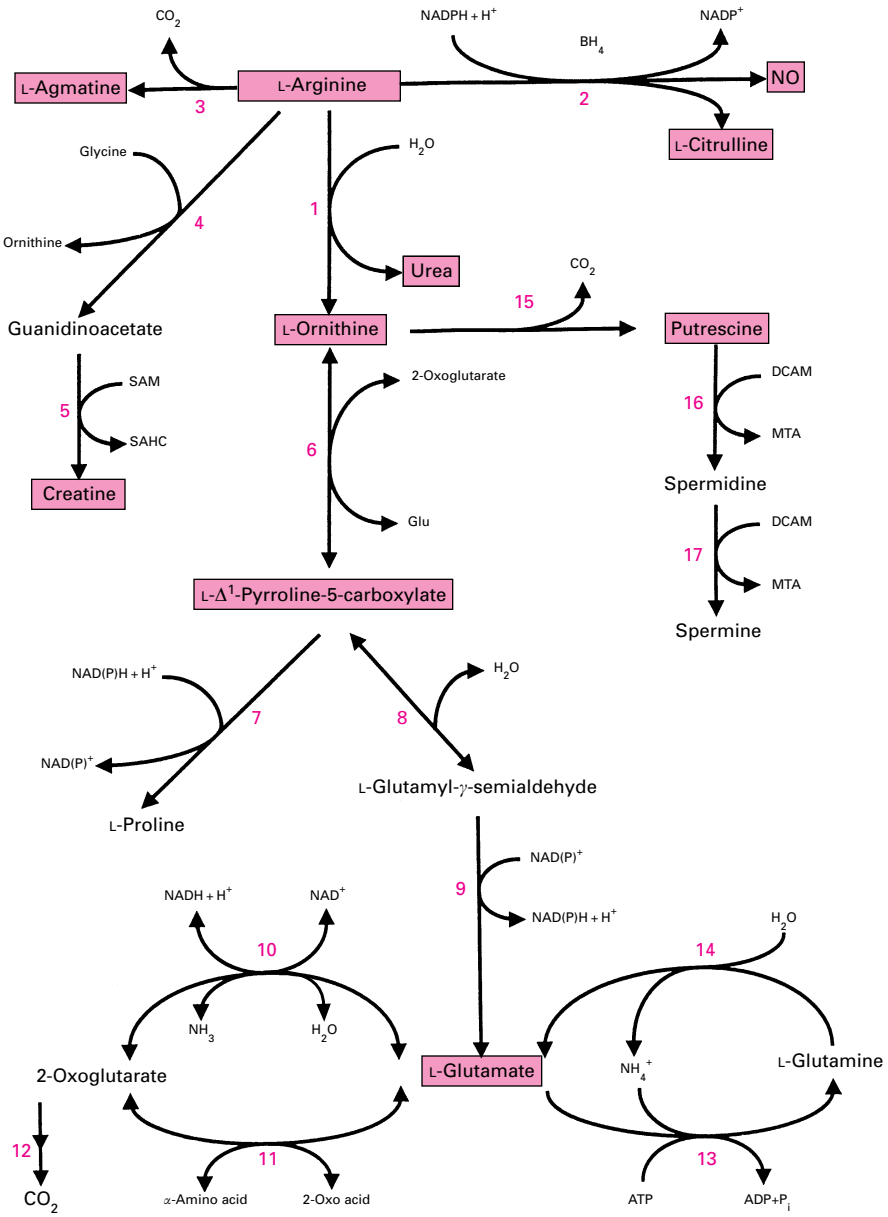

Figure 5 Pathways of arginine catabolism

Enzymes that catalyse the indicated reactions are: 1, arginase (EC 3.5.3.1); 2, NOS (EC 1.14.13.39); 3, arginine decarboxylase (EC 4.1.1.19); 4, arginine:glycine amidinotransferase (EC 2.1.4.1); 5, guanidinoacetate *N*-methyltransferase (EC 2.1.1.2); 6, OAT; 7, P5C reductase (EC 1.5.1.2); 9, P5C dehydrogenase; 10, glutamate dehydrogenase (EC 1.4.1.2); 11, alanine aminotransferase (EC 2.6.1.12), aspartate aminotransferase or branched-chain amino acid aminotransferase (EC 2.6.1.42); 13, glutamine synthetase (EC 6.3.1.2); 14, glutaminase (EC 3.5.1.2); 15, ornithine decarboxylase (ODC; EC 4.1.1.17); 16, spermidine synthase (EC 2.5.1.16); 17, spermine synthase (EC 2.5.1.22). Complete oxidation of arginine-derived 2-oxoglutarate occurs via the citric acid cycle (step 12). Step 8 is a spontaneous, non-enzymic reaction. See the legend to Figure 2. Abbreviations: DCAM, decarboxylated *S*-adenosylmethionine; MTA, methylthioadenosine; SAM, *S*-adenosylmethionine; SAHC, *S*-adenosylhomocysteine; BH_4, (6*R*)-5,6,7,8-tetrahydro-L-biopterin.

can be regulated by varying the extracellular arginine concentration, despite the fact that the reported intracellular arginine concentrations (0.1–1 mM) greatly exceed the K_m of endothelial NOS (eNOS) for arginine (2.9 μM). The apparent K_m of NO synthesis by intact cells for extracellular arginine is approx. 73–150 μM [187,188], which is within the range of the K_m values of the arginine transport systems (100–150 μM) [165] and of plasma arginine concentrations (Table 1). Immunohistochemical studies [189] demonstrated that CAT-1, eNOS and caveolin are co-localized in plasma-membrane caveolae, suggesting a preferential channelling or directed delivery of extracellular arginine to eNOS, as proposed previously [174]. It is not known whether preferential channelling of extracellular arginine also occurs for other arginine-requiring enzymes.

Arginine catabolism *in vivo*

Our knowledge of arginine catabolism *in vivo* is limited, due in large part to the complex compartmentalization of arginine metabolism at both the organ and subcellular levels. However,

our understanding of this subject is being expanded by recent tracer studies using stable isotopes. Only 5 % of urea production is derived from plasma arginine [62], reflecting very low uptake of arginine by the liver and the strict segregation of hepatic and plasma arginine pools. Relative rates of NO synthesis from plasma arginine are low. For example, in infants [52] and adult humans [62], NO synthesis represent only 0.48 % and 1.2 % respectively of the flux of plasma arginine. This accounts for about half of total NO production, because plasma arginine provides only 54 % of the arginine used in NO synthesis [62]; the remainder is presumably derived from endogenous sources, such as protein degradation and endogenous arginine synthesis at sites of NO synthesis. The fractions of plasma arginine flux associated with the synthesis of citrulline and NO are virtually identical in adult humans [62], strongly indicating that production of plasma citrulline from arginine *in vivo* is due entirely to NOS activity. In adult humans, an arginine-free diet reduced the flux of plasma arginine and endogenous NO synthesis [190]. In the neonatal pig, however, an arginine-deficient diet did not alter the flux of plasma arginine or its conversion into metabolic products [55], suggesting species or developmental differences in arginine metabolism *in vivo*.

Owing to a relatively high activity of arginase in the intestinal mucosa of adults, approx. 40 % of the arginine absorbed from the intestinal lumen is degraded in the first pass in rats [191] and humans [192], and the remainder of the absorbed arginine is released into the venous blood. About one-third of the ornithine produced from exogenous arginine is released by the rat small intestine [191] or isolated pig enterocytes [193]; the remaining two-thirds of the ornithine is further catabolized to various metabolites, as indicated in Figure 5. Thus changes in intestinal arginase expression can have a major impact on the metabolic fates of arginine and on the availability of dietary arginine to extra-intestinal tissues [66].

Arginase

Interest in the arginases as possible regulatory enzymes is growing because of their potential for regulating the availability of arginine for the synthesis of NO, polyamines, agmatine, proline and glutamate. Although much of the evidence for the role of the arginases in providing or depleting substrate for other biosynthetic pathways is circumstantial, the general conclusions drawn in many of the studies cited below are likely to be correct. As cloning of the arginase cDNAs [138,148,160,162] and development of potent arginase inhibitors [194–197] have now provided the means to test directly the validity of these conclusions, we anticipate that many of these studies will be revisited with new experimental tools.

It is important to recognize that there are two distinct isoenzymes of mammalian arginase, which are encoded by separate genes. They are quite similar with regard to enzymic properties and requirement for manganese, but differ with regard to subcellular localization, tissue distribution, regulation of expression and immunological reactivity [198,199]. Type I arginase, a cytosolic enzyme, is highly expressed in liver as a component of the urea cycle, and to a limited extent in a few other tissues. In contrast, type II arginase, a mitochondrial enzyme, is expressed at lower levels in kidney, brain, small intestine, mammary gland and macrophages, but there is little or no expression in liver [138,162,198,199]. Rat aortic endothelial cells and murine macrophages express both type I and type II arginases [200–202], and it is likely that other cell types also express both isoenzymes. The different subcellular localization of the arginase isoenzymes may provide a mechanism for regulating the metabolic fate of arginine, as postulated for enterocytes [203]. For example, differential expression of the arginase isoenzymes could provide a means to preferentially direct ornithine either to proline or glutamate synthesis via OAT or to polyamine synthesis via ODC (Figure 2). If so, this would imply that ornithine does not rapidly equilibrate between cytosolic and mitochondrial compartments, despite the existence of transporters via which ornithine can traverse the mitochondrial membrane [204–208]. So far as we are aware, no experiments to determine the effect of arginase localization on the metabolic fates of ornithine have been performed.

Arginase and ureagenesis

As noted above, the high levels of type I arginase in liver, together with the channelling of metabolites within the urea cycle, serve to ensure that this pathway for detoxifying waste nitrogen operates at high efficiency. Arginase is unique among the urea-cycle enzymes in that two distinct isoenzymes exist. Thus inherited defects in the hepatic (type I) arginase are partially compensated for by elevated expression of type II arginase in kidney [209,210], resulting in a less severe clinical disorder [130]. As Mn^{2+} can allosterically activate hepatic arginase in a pH-sensitive fashion, it has been suggested that pH-dependent regulation of arginase activity may contribute to the pH-dependence of hepatic urea production [211]. However, changes in hepatic amino acid transport [212,213] and possibly also in activities of key enzymes involved in amino acid catabolism, rather than changes in activities of the urea-cycle enzymes *per se*, are probably much more important in regulating pH-dependent alterations in hepatic urea synthesis.

Because all urea-cycle enzymes are present to some extent in the small intestine [214–216], several investigators have hypothesized that a metabolically significant urea cycle may function in this organ [79,216]. Wu [217] has demonstrated urea synthesis from both extracellular and intramitochondrially generated ammonia in enterocytes from post-weaning pigs, although the rate of ureagenesis is considerably less than in hepatocytes. This result not only shows that more than one organ is capable of synthesizing urea from ammonia in mammals, but also may help in understanding the complex kinetics of urea metabolism *in vivo* [218]. Although some urea may be formed, it is nonetheless clear that a major product of these enzymes in the small intestine is citrulline. Ureagenesis in the small intestine of weaned animals may constitute a first line of defence against the toxicity of ammonia which is generated by intestinal glutamine catabolism and by the microbial flora of the gut (reviewed in [219]).

Arginase and NO synthesis

At first glance, it might appear that arginase would not compete well with NO synthesis for arginine. The K_m for arginine is in the 2–20 mM range for mammalian arginases [198], but it is in the 2–20 μM range for the various NOS isoenzymes [220]. On the other hand, the V_{max} of arginase at physiological pH (approx. 1400 μmol/min per mg; calculated for rat liver arginase from [221]) is more than 1000 times that of the NOS enzymes (approx. 1 μmol/min per mg [220]), indicating similar rates of substrate usage for NO synthesis at low arginine concentrations. Sufficient quantities of arginase can limit the availability of arginine for NO synthesis by intact cells. For example, in wounds [222–224] and macrophage cultures [225,226], the extracellular fluid becomes almost completely depleted of arginine, whereas ornithine increases, indicative of high arginase activity. More recently, inhibition of arginase in LPS-treated rodent macrophages was shown to result in enhanced conversion of arginine into citrulline,

indicating that arginase and iNOS can compete for arginine [227,228]. One aspect of the cell culture studies that needs further clarification is the extent to which arginine depletion was due to extracellular compared with intracellular arginase, i.e. were arginase and iNOS in direct competition for an intracellular pool of arginine? This is an important point, because the rate of cellular NO synthesis may be limited by the rate of arginine uptake. Thus a decrease in the extracellular arginine concentration may have a more marked impact on the rate of NO synthesis than would a corresponding decrease in the intracellular arginine concentration.

In reality, the basis for interplay between arginase and NOS is more complex than the fact that they use a common substrate. For example, iNOS-expressing macrophages [228,229] and endothelial cells [200] can produce sufficient N^G-hydroxyarginine to inhibit arginase activity. Because endothelial cells in intact animals are constantly perfused, whereas cultured cells are not, it is not clear whether the former would be exposed to sufficient N^G-hydroxyarginine *in vivo* to inhibit cellular arginase activity. In evaluating these results it also must be borne in mind that arginine concentrations in standard tissue culture media are up to 10 times higher than plasma arginine concentrations, so that rates of N^G-hydroxyarginine production by cultured cells are probably much greater than *in vivo*. Nonetheless, limited perfusion at localized anatomical sites such as wounds may allow accumulation of inhibitory concentrations of N^G-hydroxyarginine. This possibility is supported by the finding that the plasma level of N^G-hydroxyarginine is about 9 μM, even in healthy adult humans [229], and becomes elevated in the serum of LPS-treated rats [230]. Further complicating the picture is the fact that N^G-hydroxyarginine can be oxidized to citrulline and NO by a variety of haem proteins, such as peroxidases, cytochromes *P*-450, haemoglobin and catalase [231,232], as well as by superoxide anions [233], suggesting that the half-life of N^G-hydroxyarginine, and thus its accumulation, may vary in a tissue-specific manner. Because of its potential importance, the role of N^G-hydroxyarginine in arginine metabolism bears further study.

Arginase and polyamine synthesis

Polyamines are essential for cell proliferation and differentiation [234]. Support for the hypothesis that arginase may regulate the availability of ornithine for polyamine synthesis comes from observations that arginase activity is often co-induced with ODC and that cells that are deficient in arginase cannot proliferate in serum-free medium unless ornithine or polyamines are provided [235]. In addition, arginase activity is greatly elevated in tumour cell lines that have become resistant to difluoromethylornithine, a potent inhibitor of ODC [234,236]; the high arginase activity apparently generates sufficient ornithine to compete intracellularly with the ODC inhibitor.

Correlations between changes in arginase activity and polyamine synthesis have been found in studies of the kidney. For example, androgens induce activities of both arginase [237–239] and ODC [239–241] in kidneys of female or castrated male mice. Hypertrophy generally occurs under these conditions, although there is no direct correlation between the increase in kidney size and renal arginase activity [236]. Androgens not only induce arginase and ODC in kidneys of female mice, but also lower OAT activity [237]. Taken together, these observations suggest that renal arginase activity increases to support enhanced polyamine synthesis, whereas the decline in OAT activity indicates reduced utilization of ornithine for proline or glutamate synthesis. These conclusions may be somewhat oversimplified, because no-

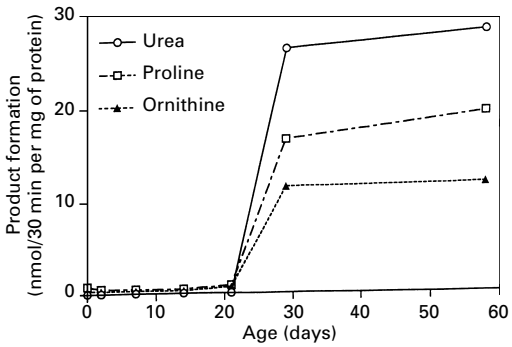

Figure 6 Developmental changes in arginine catabolism by pig enterocytes

Enterocytes were isolated from the jejenum of pigs at various times after birth (day 0) and incubated in Krebs Bicarbonate buffer containing 5 mM D-glucose and 2 mM arginine. Pigs were weaned at day 21. Data are from Table 3 of Wu [217].

one has determined whether the changes in expression of these enzymes are co-localized within the kidney.

Arginase [148,202,242] and ODC [243,244] are also induced in murine macrophage lines by LPS or cAMP. Moreover, induction of these enzymes is synergistic when LPS and cAMP are combined [202,244]. Although not proven, these observations suggest that arginase induction serves to enhance macrophage polyamine synthesis. There is also evidence for a link between arginase activity and polyamine synthesis in the small intestine. The synthesis of polyamines from arginine is negligible in enterocytes of newborn and suckling animals [245,246], due, at least in part, to low arginase activity. Polyamine synthesis increases in enterocytes of post-weaning animals, concurrent with the induction of both arginase and ODC [193]. This induction appears to be glucocorticoid-dependent [101,247], and may play an important role in intestinal remodelling.

Arginase and proline synthesis

Much of the ornithine generated by arginase outside of the urea cycle is metabolized by OAT to generate P5C (Figure 5) [91]. This assertion is supported by the occurrence of hyperornithinaemia in adult humans [91] and mice [92] when OAT is deficient or absent. As shown in Figure 5, P5C can subsequently be utilized for synthesis of proline or glutamate. OAT, P5C reductase and P5C dehydrogenase activities are present in a wide range of tissues [248], indicating the potential for synthesis of both proline and glutamate, but little is known regarding the relative usage of P5C by these two pathways in different cells, with the exception of the small intestine [193] and the mammary gland [249].

Intestinal synthesis of proline from arginine varies with species, developmental stage and nutritional state. For example, arginine-dependent proline synthesis is not detectable in enterocytes of newborn or suckling pigs, but does occur in cells from post-weaning pigs (Figure 6). The increase in arginine catabolism at day 29 corresponds to the sharp increase in arginase activity in enterocytes between days 21 and 29 [217]. Proline is the major product of arginine catabolism in enterocytes of post-weaning pigs (Figure 6), accounting for about 55% of the metabolized arginine carbon atoms [193]. This result may explain, in part, why proline is an essential dietary amino acid for neonatal pigs, but not for post-weaning pigs [66]. During the post-absorptive period, the proline released into the circulation by the small

intestine of the rat [68], dog [69] and pig [70] is probably synthesized from arterial glutamine, due to the fact that uptake of arterial arginine and glutamate by the small intestine is not significant [68,70]. Thus the route of delivery (enteral versus parenteral) plays a major role in determining whether arginine and glutamine/glutamate are used as precursors for intestinal proline synthesis.

Given the high activity of OCT in the small intestine, it is perhaps surprising that the major product of the metabolism of extracellular arginine or ornithine in this organ is proline rather than citrulline [193]. This may be explained as follows. First, enterocytes have an exceedingly high activity of OAT, but a low activity of CPS I [74]. Thus, in the mitochondrion, ornithine is preferentially metabolized via OAT to form P5C instead of citrulline. Secondly, enterocytes have a virtually negligible activity of P5C dehydrogenase (a mitochondrial enzyme), but a high activity of P5C reductase (a cytosolic enzyme) [193]; therefore P5C produced by OAT is not metabolized to glutamate in the mitochondria, but enters the cytosol for conversion into proline. Thus dietary or arterial ornithine is a poor precursor for the intestinal synthesis of citrulline and does not contribute significantly to maintaining arginine homoeostasis in humans [190], pigs [250], rats [251] or cats [252].

Earlier work from several laboratories [222–225,253,254] led to the notion that macrophage-derived arginase activity at the site of wounds plays a role in the recovery of host tissues from inflammation and infection, not only by removing arginine as substrate for NO synthesis but also by generating ornithine for the synthesis of proline, which is required for collagen synthesis. Supporting this proposition is the fact that there is an early burst of NO synthesis at the wound site, followed by depletion of arginine and a concomitant rise in the concentrations of ornithine and proline [224,253]. Arginine metabolism in wounds is complex, because the wound site contains arginase both in intact cells and in the wound fluid, the latter as a consequence of macrophage autolysis. Further complicating our understanding of arginine metabolism in wounds is the fact that murine macrophages express both isoenzymes of arginase [201,202], whereas rat macrophages express only type I arginase [201].

Arginase plays an important role in proline synthesis by the lactating mammary gland. The output of proline in the milk of goats [255], sheep [256], cows [257] and pigs [258] greatly exceeds the uptake of proline by the lactating mammary gland, whereas the uptake of plasma arginine by lactating mammary glands greatly exceeds the output of arginine in the milk [255–259]. Studies with lactating mammary tissues have demonstrated arginine-dependent production of proline [249,257], but there was little or no synthesis of proline from glutamate [249] because of the absence of P5C synthetase [47,249]. Uptake of ornithine and citrulline (potential precursors for proline synthesis) by the lactating mammary gland is relatively low compared with that of arginine [256,257]. There is virtually no proline catabolism by the mammary gland because it lacks proline oxidase [249]. Consequently there is a relative enrichment of proline and a relative deficiency of arginine in milk protein [57,260]. The enzymes required for the synthesis of proline from arginine (arginase, OAT and P5C reductase) are present in the mammary gland [249,261,262], and activities of these enzymes are co-ordinately induced during development of the lactating mammary gland [249,261]. The major isoenzyme of arginase in the mammary gland is type II [262,263], which is co-localized with OAT in the mitochondrion. Localization of these enzymes within the same subcellular compartment enhances the conversion of arginine-derived ornithine into P5C, which is subsequently converted into proline by P5C reductase in the cytosol.

Arginase and glutamate synthesis

Glutamate and glutamine are the most abundant amino acids in milk [260,264]. As the high content of these amino acids in milk greatly exceeds their accumulation via uptake by the lactating mammary gland [258,265], they must be synthesized within this organ. As in the case of proline synthesis during lactation, arginase also plays an important role in providing substrate for glutamate synthesis in the mammary gland via type II arginase, OAT and P5C dehydrogenase [249,257]. Although ornithine is used for the synthesis of both proline and glutamate in all stages of lactation, it is preferentially used for synthesis of glutamate plus glutamine in the later stages of lactation [249].

Although the liver contains all the enzymes needed to convert arginine into glutamate, there have been few studies to determine whether such conversion occurs. Perfusion of the liver with ^{14}C-labelled arginine or ornithine resulted in production of $^{14}CO_2$ [178], reflecting conversion of arginine or ornithine into glutamate via P5C (Figure 5) and the subsequent oxidation of glutamate via the citric acid cycle. Because similar results were obtained when the liver was perfused in the antegrade or retrograde direction, O'Sullivan et al. [178] inferred that arginase must be co-expressed with hepatic OAT, which is expressed in perivenous, but not periportal, hepatocytes [266]. Although the isoenzyme of arginase expressed in perivenous hepatocytes has not been identified, it is likely to be type II arginase, which would be co-localized in the mitochondrion with OAT. Thus arginine-dependent glutamate synthesis is highly restricted within the liver. As glutamine synthetase is also selectively expressed in perivenous hepatocytes [267,268], the co-expression of all of these enzymes would support the perivenous synthesis of glutamine in the intercellular glutamine cycle proposed by Haüssinger [269].

Arginine decarboxylase

Arginine decarboxylase, which produces CO_2 and agmatine [4-(aminobutyl)guanidine] from L-arginine, had long been known to be present in plants and bacteria, but was thought to be absent from mammalian cells [270]. However, arginine decarboxylase activity and agmatine synthesis have now been identified in brain, liver, kidney, adrenal gland, macrophages and small intestine [271–274]. This enzyme is localized within the mitochondrial fraction of cell homogenates [272,275]. Arginine decarboxylase activity is absent from pig enterocytes [193], suggesting either that there are species or developmental differences in intestinal expression of this enzyme or that the identity of the intestinal cell types that express arginine decarboxylase remains to be determined.

Although the physiological roles of agmatine are still under investigation [273,276,277], three lines of investigation have suggested possible functions of this arginine metabolite. Agmatine binds to α_2-adrenergic and imidazoline receptors [271], suggesting a role in cell signalling. Agmatine can also inhibit ODC activity by inducing synthesis of antizyme, thus suppressing cell proliferation by reducing cellular polyamine concentrations [278]. Finally, agmatine is a weak competitive inhibitor of the NOS isoenzymes [279], suggesting that it may be an endogenous regulator of NO synthesis if local agmatine concentrations are sufficiently high. It should be emphasized that concentrations of agmatine sufficient to inhibit synthesis of NO or polyamines may be difficult to achieve *in vivo* because agmatine is also a feedback inhibitor of arginine decarboxylase [271]. So far as we are aware, it has not been determined whether endogenously produced agmatine has a significant impact on NO or polyamine synthesis from arginine.

Both arginine decarboxylase and agmatinase, the enzyme that degrades agmatine, are constitutively expressed in the RAW 264.7 murine macrophage line [274]. Stimulation with LPS, which strongly induced iNOS, decreased arginine decarboxylase activity, but modestly increased agmatinase activity, so that the net effect was to decrease the agmatine concentration [274]. The simultaneous presence of both agmatine synthetic and degradative enzymes within the same cell underscores the need to evaluate the roles of endogenous agmatine. As arginase activities are much higher than those of arginine decarboxylase or agmatinase in macrophages, it is likely that changes in arginase activities will have a much more significant impact on NO synthesis and other arginine metabolic pathways.

NOS

Owing to the incredible diversity and often dramatic nature of the effects of NO, as well as to the virtually ubiquitous expression of NOS activity in animal tissues [157], the family of NOS isoenzymes has become the best-known group of arginine-metabolizing enzymes within the past several years. As the structure and function of these enzymes have been reviewed extensively in the past few years (e.g. [36,280–282]), only selected features of these enzymes will be noted here. Briefly, there are three NOS isoenzymes, encoded by distinct genes: iNOS (Type II NOS), neuronal NOS (nNOS; Type I NOS) and eNOS (Type III NOS). For the most part, nNOS and eNOS are constitutively expressed at low levels in a variety of cell types, whereas iNOS, which normally is not expressed in most cell types, is highly inducible by bacterial endotoxin and inflammatory cytokines. Activities of the constitutive NOS isoenzymes are dynamically regulated by Ca^{2+}/calmodulin, whereas iNOS, once expressed, is constitutively active. Thus the cellular capacity for NO synthesis is determined by the levels of NOS expression and by regulation of the catalytic efficiency of NOS via Ca^{2+}/calmodulin or the availability of essential cofactors such as tetrahydrobiopterin [151,157]. In addition to serving as a substrate for NOS, arginine plays a structural role by promoting the dimerization of NOS [282].

The NOS isoenzymes have distinct patterns of subcellular localization that are probably involved in the regulation of NOS activity, particularly in the cases of eNOS and nNOS. Such regulation probably involves dynamic changes in direct protein–protein interactions or placement near ion channels and transporters. For example, eNOS is associated with caveolae at localized regions of the plasma membrane [283]. This may allow more efficient modulation of eNOS activity via local changes in flow-induced shear stress and in calcium flux through the plasma membrane, and may also affect rates of NO production by placing eNOS in close apposition to arginine transporters in the plasma membrane [189]. Recent studies have shown that nNOS is primarily associated with the rough endoplasmic reticulum and postsynaptic membranes in brain and with the sarcolemma of skeletal muscle [284,285]. As in the case of eNOS, it is thought that the subcellular localization of nNOS near calcium channels permits highly precise regulation of its activity. Unlike eNOS and nNOS, iNOS is primarily cytosolic [157], although there is one report of its association with membrane vesicles in macrophages [286]. Our understanding of the subcellular localization of the NOS isoenzymes, as well as its regulation and physiological significance, is still incomplete and remains an area of active investigation (e.g. [287–289]).

The relatively low-level production of NO, compared with overall arginine catabolism, in the intact animal undoubtedly reflects its great potency as a cell signalling or cytotoxic agent. Within specific cell types, such as endothelial cells and macrophages, NO production can represent a much greater proportion of arginine degradation, although the proportion varies according to animal species and exposure of the cells to inflammatory stimuli. Ornithine/urea production is the predominant route of arginine catabolism in unactivated rat endothelial cells [200,290], but NO production predominates when endothelial cells are stimulated with the appropriate combination of cytokines [200]. NO/citrulline synthesis represents the vast majority of arginine metabolism in rat macrophages [223], but ornithine/urea production dominates in murine macrophages [187,254,291]. These observations reflect the fact that arginase activity is greater in murine macrophages than in rat macrophages [201,292]. In marked contrast with rodent macrophages, human macrophages normally express little arginase or iNOS [293], although instances of NO production in human macrophages have been reported (reviewed in [201,294]). These findings indicate either that there are species differences in the intrinsic capacity for expression of these enzymes in macrophages or that conditions for reproducibly eliciting arginase or iNOS expression in human macrophages have not been identified.

One theme that has emerged in this review is the complexity of inter- and intra-cellular interactions between various arginine metabolic pathways. Cellular NO synthesis rates can be regulated via a variety of mechanisms that control the availability of arginine and cofactors. Thus NO synthesis by intact cells can exhibit features that do not precisely match the properties of NOS as studied in the test tube. A typical example is the 'arginine paradox' for NO synthesis, as discussed in the section on arginine transport. Moreover, glutamine does not affect eNOS activity, but inhibits NO production by endothelial cells, and this inhibition can be antagonized by arginine [295,296]. This regulatory property of glutamine may contribute to the 'arginine paradox' in endothelial cells, and is likely to be of physiological importance. However, the mechanism responsible for the inhibition of endothelial NO synthesis by glutamine and its antagonism by arginine is not known.

Arginine:glycine amidinotransferase

Another well known pathway of arginine catabolism is creatine synthesis, which is initiated by arginine:glycine amidinotransferase, a mitochondrial enzyme [158]. This enzyme, which transfers the guanidino group from arginine to glycine to form guanidinoacetate (glycocyamine) and ornithine (Figure 5), is present predominantly in the renal tubules and pancreas, and to a much lesser extent in the liver and other organs [159,297]. The kidney is considered to be the principal site of guanidinoacetate production, although studies of the pancreas suggest that this organ may provide physiologically significant amounts of guanidinoacetate to the liver [298]. Although activities of arginine:glycine amidinotransferase in liver have been difficult to measure because of the very high levels of arginase, it is detectable in hepatocytes by immunohistochemistry [159]. However, it is unlikely that the liver accounts for a significant fraction of whole-body guanidinoacetate synthesis, because uptake of arginine by the liver is low [60,178] and arginine synthesized within the urea cycle is rapidly hydrolysed to urea [46].

Guanidinoacetate is methylated by guanidinoacetate *N*-methyltransferase (a cytosolic enzyme located primarily in liver, pancreas and, to a much lesser extent, kidney [158]) to form creatine, which is released into the circulation. Circulating creatine is actively taken up by skeletal muscle and nerves, where it is phosphorylated and eventually undergoes non-enzymic and irreversible dehydration to yield creatinine. Creatinine, which is

not utilized by muscle, is distributed in the total body water and filtered by the kidneys; its urinary excretion is the most widely used clinical marker of renal function [299]. Thus creatine homoeostasis primarily involves three major organs: kidney, liver and muscle. The importance of creatine synthesis is illustrated by a recently identified deficiency of guanidinoacetate *N*-methyltransferase in humans which causes a severe creatine deficiency and developmental abnormalities in muscle and brain during early infancy [300].

Creatine synthesis represents a sizeable fraction of total-body arginine usage. For example, an adult 70 kg man excretes about 1.5 g of creatinine per day [123]. To maintain creatine homoeostasis, this must be matched by equimolar synthesis of creatine, which requires about 2.3 g (13.3 mmol) of arginine per day, equivalent to approx. 10% of the total plasma arginine flux (Table 1). This is nearly 10 times the flux of plasma arginine represented by NO synthesis in a healthy adult [62].

Regulation of creatine synthesis occurs largely via changes in the levels of renal arginine:glycine amidinotransferase, the rate-controlling enzyme in creatine synthesis. Activities of this enzyme in kidney are regulated primarily by creatine and growth hormone [301]. Thus activities and mRNA levels for arginine:glycine amidinotransferase in rat kidney were greatly reduced by hypophysectomy or by feeding a diet containing creatine. Administration of growth hormone to hypophysectomized rats induced expression of arginine:glycine amidinotransferase, but this induction was greatly blunted when the rats were simultaneously fed a creatine-supplemented diet. The mechanism of the effects of growth hormone and creatine on the expression of arginine:glycine amidinotransferase remains to be elucidated.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

While considerable advances in our understanding of mammalian arginine metabolism have been made in recent years, much remains to be learned about arginine synthesis and catabolism in animals and humans. This statement applies to our knowledge at all levels of biological organization (whole body, organ and cellular), in health and disease, during adulthood as well as at other stages of development. At the whole-body level, for example, little is known regarding the relative contributions of proline and glutamine/glutamate to the synthesis of citrulline and arginine. Moreover, complete oxidation of arginine from dietary and endogenous sources is likely to involve interorgan co-operation, but there are few data on this topic. Activities of some metabolic pathways in the intact animal are difficult to analyse because of the compartmentalization of pathways owing to, for example, tissue-specific differences in the transport of metabolites or rapid consumption of intracellular metabolites via tight coupling among enzymes. For example, the high rates of arginine flux within the urea cycle in liver are virtually invisible in most studies of plasma arginine flux, and it may well be that the magnitude of arginine fluxes within other tissues has also been underestimated. As such problems present considerable technical challenges for the design of experiments as well as conceptual challenges for the interpretation of data, it may be necessary to develop new strategies for studying arginine metabolism *in vivo*.

The metabolic fates of arginine, and consequently the physiological functions of some arginine metabolic enzymes, are largely unknown for many cell types. In part, this is due to the fact that most studies focus on only one or two metabolites of arginine, so that the extent to which arginine may be utilized for other pathways goes unrecognized. The best example of an arginine metabolic enzyme whose physiological function is poorly understood is type II arginase. This enzyme is widely expressed throughout the body, but its precise role in most cell types (with the possible exception of its likely role in proline and glutamate synthesis in the lactating mammary gland) has not been established. The role of type I arginase in non-hepatic cells is similarly unclear. Definition of the multiple, possibly tissue-specific, roles of the arginine metabolic enzymes *in vivo* will probably require animal models in which enzyme activity has been selectively ablated by tissue-specific enzyme inhibitors (e.g. [87]) or disruption of gene expression. Although tissue-specific gene disruption has not been accomplished for any of the arginine metabolic enzymes, the approach is illustrated by the selective disruption of DNA polymerase β gene expression in T cells by using the *Cre–loxP* recombination system [302]. Once specific routes of arginine metabolism have been identified in specific cell types and analysed in cell culture, experiments should be conducted to determine whether the metabolic consequences observed in cultured cells occur *in vivo*.

At the molecular level, there is a great deal to learn about the signal transduction pathways and transcriptional/post-transcriptional mechanisms that regulate the expression of arginine metabolic enzymes in key cell types such as hepatocytes, enterocytes, endothelial cells, macrophages and neural cells. In particular, it will be of interest to determine how a signal that induces a response in one cell type can inhibit the response in a different cell type. One example of such dichotomy is the induction of iNOS expression by cAMP in rat smooth muscle and mesangial cells [303,304], whereas cAMP inhibits induction of iNOS in rat hepatocytes [305,306] and a murine macrophage line [202]. As the cDNAs for essentially all the enzymes identified in Figures 2 and 5 have now been isolated, the tools now exist for elucidating the molecular bases for the regulation of arginine metabolism during development and in response to hormones in health and disease.

Finally, it is instructive to recall that mammalian NOS enzymes were discovered only 10 years ago and mammalian arginine decarboxylase only 4 years ago. We therefore are open to the exciting possibility that additional enzymes involved in arginine metabolism remain to be discovered.

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