Arginine de novo and nitric oxide production in disease states

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Luiking YC, Ten Have GA, Wolfe RR, Deutz NE. Arginine de novo and nitric oxide production in disease states. Am J Physiol Endocrinol Metab 303: E1177–E1189, 2012. First published September 25, 2012; doi:10.1152/ajpendo.00284.2012.—Arginine is derived from dietary protein intake, body protein breakdown, or endogenous de novo arginine production. The latter may be linked to the availability of citrulline, which is the immediate precursor of arginine and limiting factor for de novo arginine production. Arginine metabolism is highly compartmentalized due to the expression of the enzymes involved in arginine metabolism in various organs. A small fraction of arginine enters the NO synthase (NOS) pathway. Tetrahydrobiopterin (BH4) is an essential and rate-limiting cofactor for the production of NO. Depletion of BH4 in oxidative-stressed endothelial cells can result in so-called NOS3 “uncoupling,” resulting in production of superoxide instead of NO. Moreover, distribution of arginine between intracellular transporters and arginine-converting enzymes, as well as between the arginine-converting and arginine-synthesizing enzymes, determines the metabolic fate of arginine. Alternatively, NO can be derived from conversion of nitrate. Reduced arginine availability stemming from reduced de novo production and elevated arginase activity have been reported in various conditions of acute and chronic stress, which are often characterized by increased NOS2 and reduced NOS3 activity. Cardiovascular and pulmonary disorders such as atherosclerosis, diabetes, hypercholesterolemia, ischemic heart disease, and hypertension are characterized by NOS3 uncoupling. Therapeutic applications to influence (de novo) arginine and NO metabolism aim at increasing substrate availability or at influencing the metabolic fate of specific pathways related to NO bioavailability and prevention of NOS3 uncoupling. These include supplementation of arginine or citrulline, provision of NO donors including inhaled NO and nitrite (sources), NOS3 modulating agents, or the targeting of endogenous NOS inhibitors like asymmetric dimethylarginine.

Arginine metabolism; nitric oxide; therapy; citrulline; sepsis

THE PURPOSE OF THIS REVIEW is to give an overview of and highlight recent developments on arginine metabolism and nitric oxide (NO) production in health and disease. The endogenous de novo production of arginine will be a special focus, and alterations in this pathway in disease and its relevance will be discussed. Finally, therapeutic applications to influence (de novo) arginine and NO metabolism will be summarized.

Physiology of Arginine and Arginine De Novo and NO Metabolism

Arginine is a conditionally essential amino acid in healthy adults, and therefore there is no specific nutritional requirement (11). However, dietary arginine is required in neonates, infants, and in certain conditions and diseases (171). In the 1980s, L-arginine was identified as the precursor for NO (127). Although first discovered in endothelial cells (116), NO appeared to be a ubiquitous molecule present in a variety of cells, including cells from the cardiovascular and nervous systems and also inflammatory cells. As such, NO has many physiological functions, and the relationship between arginine availability and NO production emphasizes the functional relevance of arginine.

Metabolic Pathways

Arginine is derived from dietary intake, body protein breakdown, or endogenous de novo arginine production (Fig. 1). In the postabsorptive state, whole body arginine flux in healthy adults is ~70–90 μmol·kg⁻¹·h⁻¹, which equals 15–20 g/day (31, 35; see Ref. 101 for review), while daily dietary arginine intake is about 4–6 g (76, 165). De novo arginine production, which contributes ~10–15% to whole body arginine production under normal conditions (31, 53), involves the conversion of citrulline to arginine and is catalyzed by the enzymes argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL) (54, 57). This conversion is part of the so-called intestinal-renal axis, with intestinal production of citrulline and renal synthesis of arginine (96, 160, 161, 170, 176). Citrulline availability is a limiting factor in this conversion (54).
Arginine is a constituent for body protein synthesis, 80% of which is derived from recycling amino acids originating from protein breakdown. Moreover, arginine plays a key role in several other metabolic pathways catalyzed by various enzyme systems (Fig. 2). Arginine metabolism is highly compartmentalized within the body, since the enzymes involved in arginine metabolism are expressed in various organs, although to different extents. The only exceptional organ is the liver, which contains the complete urea cycle and its related enzymes. However, in healthy conditions, arginine produced in the liver urea cycle is not released to plasma (173). Due to compartmentalization, arginine metabolism and recycling are only partly in balance with plasma arginine concentration. This so-called “arginine paradox” explains that acute exogenous arginine provison can still increase NO production even though the intracellular arginine concentration far exceeds the $K_m$ of endothelial NO synthase (eNOS) (60). It is also likely that, once transported into the cell, arginine can no longer gain access to the membrane-bound eNOS. This makes intracellular arginine less useful as a reference point (147).

Arginine flux as measured with an intravenously infused stable isotope and subsequent dilution of this isotope in the plasma compartment reflects the whole body appearance of arginine in plasma. This plasma arginine flux does not account for hidden compartments (such as liver cells) in which arginine is produced without first being released into plasma. Of the plasma arginine flux, 15% enters the (extrahepatic) arginase pathway (31) that degrades arginine to ornithine and urea.

There are two isoforms of the enzyme arginase. Type I (cytosolic) arginase is predominantly expressed in the liver, as part of the urea cycle, but was also demonstrated at lower levels in various extrahepatic organs in rodents with a main role in production of ornithine for polyamine biosynthesis (175). Type II (mitochondrial) arginase is expressed in low levels in extrahepatic tissues and cells (such as brain, kidney, small intestine, red blood cells, and immune cells) and is mainly involved in the syntheses of ornithine, proline, and glutamate (81, 119). Based on the variability of arginase I and II among organs in rodents, organ-specific roles of arginase isoforms have been suggested (42). Under normal conditions, ~40% of dietary arginine is extracted in the splanchnic area (33), which is likely due to the relatively high arginase activity in the intestinal mucosa. Arginine is a substrate for citrulline synthesis in the intestine through conversion by arginase II and ornithine transcarbamylase (OTC) metabolic pathways with interorgan exchange of ornithine (105, 107).

Arginine is substrate for creatine synthesis, which also requires glycine and methionine. Creatine synthesis consumes some 20–30% of arginine’s amidino groups, whether provided in the diet or synthesized within the body, and therefore imposes an appreciable burden on the metabolism of arginine. Creatine is excreted from the body as urinary creatinine. This is a nonenzymatic and unregulated breakdown process that occurs at a rate of ~1.7% of total body creatine and creatine phosphate per day (see Ref. 27 for review).

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**Fig. 1.** Extrahepatic systemic arginine availability in healthy humans. ARG, arginine; CIT, citrulline; GLN, glutamine; GLU, glutamate; ORN, ornithine; ADMA, asymmetric dimethylarginine; NO, nitric oxide.

**Fig. 2.** Compartments of arginine metabolism in healthy humans. ARG, arginine; ORN, ornithine; PRO, proline; GLU, glutamate; CIT, citrulline; NO, nitric oxide; NOS, nitric oxide synthase; Ca, calcium; OTC, ornithine transcarbamylase.
Role of Arginine and NO in Normal Physiology

Arginine is a constituent of body proteins and is an intermediate in the urea cycle in the liver. The urea cycle is a series of five reactions, in which urea synthesis is the final step in the detoxification of ammonia. Moreover, the urea cycle has been considered a major pathway for the removal of metabolically generated bicarbonate, and as such a role for the liver in pH homeostasis (72). Other roles of arginine are related to arginine-derived metabolites. These include, among others that we will not further specify here, ornithine and derived polyamines (putrescine, spermine, and spermidine), which are important for cell growth and differentiation. Proline, which is hydroxylated to hydroxyproline posttranslationally, can also be derived from arginine and has a role in collagen formation, tissue repair, and wound healing. Creatine is also derived from arginine and plays a role in energy metabolism in muscle and neurons (see Ref. 173 for review). Apart from actions via its metabolites, arginine directly activates p70 S6 kinase and phosphorylation of 4E-BP1 through the mTOR signaling pathway (9) with stimulation of protein synthesis in a NO-independent way (14). NO has various roles in normal physiology, but we will not review all roles in full detail. NO derived from NOS1 and NOS3 acts as a neurotransmitter and a vasodilator, respectively (114). NO in the brain regulates many physiological processes affecting behavior and cognitive function, including synaptic plasticity. In addition, NO controls brain blood flow, promotes angiogenesis, and maintains cellular redox state, cell immunity, and neuronal survival. However, despite the many diverse roles of NO, regulation of the amount produced is important, as overproduction of NO may lead to neurodegeneration (115). NO is synthesized at high levels by NOS2 when activated during inflammatory processes by elevated circulating cytokine concentrations (mainly TNF-α and IL-1, IL-6, and IL-8) and/or microbial products like LPS (68, 85, 114, 120). This NO has immune regulatory functions, such

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as control or killing of infectious pathogens, modulation of cytokine production, and T-helper cell development. Moreover, NO can act as a free radical scavenger (157). Local NO responses are concentration and exposure time dependent (156). In general, at low concentrations NO promotes cell survival and proliferation and at high concentrations promotes cell cycle arrest, apoptosis, and senescence. As such, arginine has an indirect role in NO-mediated functions, including immune modulation (52, 139), or acts as immune response enhancing during immunological challenge (95).

Factors That Mediate Arginine and NO Metabolism and Availability

The level of dietary arginine intake and endogenous production on the one hand, and the extent of utilization or clearance on the other hand influence arginine metabolism and availability (Fig. 4).

Systemic and intracellular arginine availability. Transporters for arginine uptake in the cell are often colocalized with arginine-converting enzymes and as such can modulate cellular arginine metabolism (173). For example, cationic amino acid transporter (CAT)-1 and NOS3 are colocalized in plasma membrane caveolae (109). This facilitates specific channeling of arginine to endothelial NO production without mixing with the total intracellular pool. This is in line with observations in vitro of extracellular rather than intracellular arginine being the major determinant for NO production in endothelial cells (147). Another example of the relation between arginine metabolism and its transporter is the upregulation by inflammatory cytokines of CAT-2 and downregulation of CAT-1 arginine transporters (137, 143) that result in increased availability of arginine to NOS2 and decreased availability to NOS3. Competition with lysine, ornithine, glutamine, and certain endogenous NOS inhibitors that use the same transporter as arginine may compromise intracellular arginine transport in conditions of low arginine (100). In addition to the link between arginine transport and intracellular arginine availability, the coupling between arginine-synthesizing and -converting enzymes or the competition between enzymes for arginine as a substrate determine its metabolic fate. For example, coupling between de novo arginine synthesis and NO production is supported by colocalization in endothelial cells of NOS3 and ASS/ASL (60). On the other hand, substrate competition between arginase and NOS reciprocally regulates NO levels in endothelial cells (10, 94, 173).

De novo arginine production from citrulline can be impaired by renal failure (24, 25, 39), but citrulline delivery to the kidney is the rate-limiting determinant of renal arginine production (39, 54). Impaired intestinal function is a major underlying reason for reduced citrulline availability (45; see Ref. 46 for review), supported by the observations that 80–90% of the citrulline is derived from conversion of glutamine to citrulline in the intestine (19, 20, 96, 97, 159).

Dietary arginine availability influences its own catabolism and that of other amino acids by controlling ureagenesis. Arginine is not only a substrate for ureagenesis, but also an activator of N-acetylglutamate synthetase, which is a key ureagenic enzyme (49). On a low-arginine diet, arginine catabolism (i.e., arginine hydroxylation with conversion to ornithine) is reduced with maintained de novo arginine production and reduced plasma arginine (32, 34, 155). This also applies when a low-protein (nitrogen) diet is fed and amino acids are more efficiently used for other processes than oxidation, and subsequently, less ammonia is present for detoxification in the liver urea cycle. However, a concept presented by the Cynober group states that, under the condition of low dietary protein...
intake, the intestinal conversion of arginine into citrulline by intestinal arginase and OTC is activated (47). The newly formed citrulline then bypasses liver metabolism and is converted back to arginine in the kidney (de novo arginine production), while at the same time ureogenesis is limited and arginine and other amino acids are spared for protein synthesis (50, 112).

**Endogenous NOS inhibitors and enzymatic cofactors.** Methylnormines, such as asymmetric dimethylarginine (ADMA) and N^\text{\textsubscript{O}}-methyl-L-arginine (L-NMMA), are the most powerful endogenous and competitive nonspecific NOS inhibitors that compete with l-arginine for the active site of NOS and for intracellular transport (30; see Ref. 92 for review). These methylarginines are derived from the catabolism of posttranslational modified proteins that contain methylated arginine residues. Methylnormines are eliminated from the body by a combination of renal excretion and metabolism through enzymatic degradation by dimethylaminohydrolase (DDAH) to citrulline and methylamines (126). Increased protein catabolism and impaired renal function can thus contribute to elevated levels of methylarginine. High hepatic expression of DDAH and uptake of ADMA make the liver important in the metabolism of ADMA, and hepatic dysfunction a prominent determinant of ADMA concentration (121, 122). Reference values (2.5th-97.5th %ile) for the l-arginine/ADMA ratio are between 74.3 and 225 (104).

Enzymatic cofactors for NO production, such as tetrahydrobiopterin (BH4), can be affected by several factors and can subsequently influence NO production and endothelial function. Factors that regulate BH4 activity are nutritional, therapeutical, and endothelium-derived factors. Vitamin C, folate, and other antioxidants enhance endothelial BH4 bioavailability through chemical stabilization or scavenging of reactive oxygen species (146). Depletion of BH4 by oxidation into 7,8-dihydrobiopterin (BH2) in oxidative-stressed endothelial cells can result in so-called NOS3 “uncoupling,” with production of superoxide instead of NO. It is now believed that the intracellular BH4-to-BH2 ratio, rather than absolute concentrations of BH4, is the key determinant of this NOS3 uncoupling (43).

**Metabolic Alterations Related to Age, Sex, Animal Species and Strain Differences**

In neonates, de novo arginine production via the interorgan intestinal-renal axis is not yet developed (171), but conversion of citrulline to arginine occurs in the intestine. The precursor of intestinal citrulline in this condition is proline. In addition, the absence of arginase in the placenta of the mother and in the neonatal intestines suggests a metabolic strategy to maximize the availability of arginine in the systemic circulation from mother to fetus and from maternal milk to neonate. In addition, polyamines and NO are essential for growth and angiogenesis. Therefore, limited de novo arginine production capacity makes arginine an essential amino acid in early life. In neonatal piglets, dietary arginine is conserved in times of deficiency by decreasing arginine hydroxylation and increasing recycling (158). Also, in a wide range of livestock reproduction processes, nonoptimum availability of arginine results in suppression or inhibition of arginine metabolism (171). NO bioavailability diminishes with aging and as such adds to the pathogenesis of disturbances in endothelium-dependent vasodilatation related to aging (153). This diminished NO bioavailability has been related to a decrease in BH4 and uncoupling of NOS3 with increased oxidative stress in aging (78, 149), but also to increased arginase activity with subsequently reduced arginine availability for NOS (see Ref. 142 for review). In the brain, the excitatory glutamate-NO-cGMP neurotransmission is normally in balance with the inhibitory GABA neurotransmission. In rats, an age-related regional imbalance of the glutamate/GABA balance was observed, caused by decreased glatamate levels. This is correlated with changes in levels of l-arginine and its metabolites in many brain regions (99).

In mice, differences in arginine metabolism are described between sexes and strains, such as between C57BL/6J and FVB mice, as well as between B6 and ICR mice (102, 106). In female mice, plasma arginine was reported to be higher, and strain differences with regard to whole body de novo arginine production and portal-drained visceral (PDV) arginine metabolism were found (102).

**Alterations of Arginine and NO Metabolism in Disease**

Arginine metabolism is altered in disease states with regard to both its synthesis and its catabolism. This can result in a disruption of the normal homeostasis between metabolic pathways and the fasted blood arginine level. Tang et al. (154) proposed the global arginine bioavailability ratio (GABR), defined as plasma arginine divided by the sum of ornithine and citrulline, to account for arginine catabolic metabolites. This ratio was derived as a better index of reduced NO synthetic capacity than systemic arginine levels alone. Impaired intestinal absorption (64), impaired organ function such as intestinal dysfunction (47) or renal dysfunction (36), enzyme competition, and impaired cellular uptake may further compromise (de novo) arginine metabolism and (cellular) arginine availability. Subsequent functional consequences of altered arginine metabolism, such as altered endothelial function with hemodynamic changes on systemic (especially hypertension) and (micro)circulatory level, as well as immune alterations, are well known.

**Metabolic Alterations of Arginine and Arginine De Novo and NO Under Stress Conditions**

Normal fasted plasma arginine ranges between 80 and 100 μmol/l (162), whereas recent reference values (2.5th-97.5th %iles) from relatively healthy subjects from the Framingham Offspring Cohort were reported to be lower between 41 and 114 μmol/l (104). This could well be related to the fact that in that study the special treatment of plasma samples for arginine (immediately cooling and cooled spinning down within 30 min) was not done (162). When care is taken of proper treatment of blood samples, under stressed conditions plasma arginine is decreased (164) to levels as low as 50 μmol/l in patients with sepsis (62, 103, 110). The intestinal-renal pathway resulting in de novo arginine synthesis from citrulline is impaired in sepsis (103), which may be the results of limited citrulline availability due to intestinal failure (103, 129) and impaired glutamate-to-citrulline conversion (83), or limited arginine production due to renal failure (54, 134). Higher-level proteolysis can mask the decline in de novo synthesis of arginine, and as a consequence total arginine availability may be maintained (6, 103).
Increased utilization of arginine for the synthesis of proteins associated with the stress response, such as the acute-phase proteins, will reduce arginine availability in these conditions and may result in alterations in (isoform-specific) NOS enzyme activity. Increased NOS2 activity during sepsis coincides with the downregulation of the activity of other NOS isoforms (15, 71, 144). This reduces NO production enzyme specifically in conditions of overall NO production being either lowered or not different from healthy controls (84, 103). Increased plasma arginine clearance (84) due to enhanced arginase activity also reduces arginine availability for other catabolic pathways. Moreover, increased arginine oxidation is observed during sepsis in pediatric patients (6). Nutritional status also affects the metabolic response to endotoxia, as demonstrated in a pig model of sepsis. A well-nourished condition before prolonged endotoxia in this model resulted in a better ability to adapt to endotoxin-induced metabolic deterioration of arginine-NO metabolism compared with reduced caloric intake before endotoxia (131).

Acute Conditions, Such as Trauma, Sepsis, and Acute Liver Failure

In sepsis, a reduced plasma arginine concentration was related to worse survival (62). Increased mortality in critically ill patients has also been related to elevated ADMA levels, which by substrate competition may be a causative factor in the development of multiple organ dysfunction (123). Sepsis, in particular septic shock, is characterized by elevated cardiac output and hypotension caused by vasodilatation that are associated with misdistribution of blood flow and low peripheral vascular resistance. These characteristics of sepsis have been attributed to increased NO production by NOS2 (4, 120). However, simultaneously decreased NOS3 expression may be related to microcirculatory shutdown and shunting, contributing to the reduced microvascular blood flow and impaired tissue oxygenation (79, 90, 128, 133, 140). Others have suggested that elevated NO production in critically ill patients impairs substrate and oxygen utilization by enhanced protein nitrosylation and inhibition of mitochondrial respiration (59, 132).

Increased NO production is likely also responsible for the hyperdynamic circulation found in patients with liver cirrhosis and may be an important mediator of the exaggerated circulatory abnormalities during acute systemic inflammation in so-called acute-on-chronic liver failure with increased cerebral blood flow (98). Upregulation of the NOS2 pathway in the endothelium with simultaneous downregulation of NOS3-mediated NO production was observed in critically ill cirrhotic patients after transjugular intrahepatic stent-shunt (TIPPS) placement through exacerbation of existing endotoxia (80). Recently, in a pig model with early-phase acute liver failure, arginine deficiency, and increased ADMA did not limit whole body NO production. Arginine deficiency was caused by arginine-related arginine clearance. The stimulated intestinal-renal axis was insufficient to compensate the arginine deficiency (145).

Chronic Conditions, Such as Obesity, Diabetes, and Cardiovascular Diseases

Abnormalities in NO production and transport in vascular systems result in various cardiovascular pathologies, including hypertension, atherosclerosis, and angiogenesis-associated disorders (for recent review on the role of NO in the vasculature see Ref. 40). Reduced basal, NOS3-mediated NO synthesis or action leads to vasoconstriction, elevated blood pressure, or thrombus formation. On the other hand, NO overproduction by NOS2 leads to vasodilation, hypotension, vascular leakage, disruption of cell metabolism, and atherosclerosis, either directly or indirectly via the formation of NO adducts such as peroxynitrite (115). Cardiovascular and pulmonary disorders such as atherosclerosis, diabetes, hypercholesterolaemia, ischemic heart disease, and hypertension are characterized by NOS3 uncoupling with formation of superoxide instead of NO (see Refs. 43 and 66 for recent reviews). The lower NO production in these conditions due to a deficiency of BH4 also underlies the impaired action of insulin in the vasculature of obese and diabetic subjects (172). Diet-induced oxidative scavenging of NO and reduced NO bioavailability were also shown to accompany early diet-induced insulin resistance (18). Oxidative stress causing S-glutathionylation of NOS3 in endothelial cells with loss of NO and gain of superoxide is increased in hypertensive vessels (38). By inhibiting NO production, elevated cellular levels of endogenous methylarginines can impair vascular relaxation and are mediators of vascular dysfunction in disease. Elevated ADMA levels have been reported in hypercholesterolemia, atherosclerosis, hypertension, chronic heart failure, diabetes mellitus, and chronic renal failure (30, 148). The L-arginine:ADMA ratio was positively associated with the estimated glomerular filtration rate and diastolic blood pressure in a large cohort study (104) and may act as a clinical diagnostic tool for improved cardiovascular risk assessment (148). ADMA was also identified as an independent risk marker for mortality in ambulatory patients with peripheral arterial disease (23).

Neurological Diseases

In the brain, arginine as a precursor for NO is necessary for cerebrovascular homeostasis (NOS3) and is involved in learning and memory capacities via the glutamate-NO-cGMP pathway (NOS1). In neurological diseases, one or both of these routes are impaired. Also, high amounts of induced NO production by inflammatory factors (NOS2) contribute to oxygen stress and therefore can play a role in the severity of the diseases. In the onset of brain stroke, cerebrovascular disease, and Alzheimer’s disease, hypoperfusion as an underlying cause of oxygen stress is one of the present avenues to understanding the initiation of these diseases (see Ref. 2 for review). A chronic imbalance of NOS in the brain is believed to be a key element.

In hepatic encephalopathy (HE), a neurocognitive disorder in which brain function is impaired and is associated with both acute and chronic liver dysfunction, hyperammonemia plays an important role in the pathophysiology. Alterations in the glutamate-NO-cGMP pathway are described, especially in acute HE and in relation with excessive glutamine production (ammonia detoxification by conversion of glutamate into glutamine) in the brain (1). Besides a direct effect on the glutamate neurotransmission cycle, glutamine can also limit the transport of arginine into neurons and astrocytes, because it competes with the glutamine transport (178). The implications are not clear yet and may differ in different stages of the disease.
Cancer

Humans with cancer have decreased systemic availability of arginine independent of the type of cancer, age, sex, or cachectic state (166). In mice, cancer affects de novo arginine production probably through diminished intestinal citrulline production (167). In addition, high arginase activity is observed via the myeloid suppressor cells in the microenvironment of tumors (177). Other research is focused on tumor growth and the arginine dependence of certain tumors that do not express ASS. Such tumors, such as melanoma and hepatocellular carcinoma, are sensitive to arginine depletion by arginine-degrading enzymes such as arginine deiminase (see Ref. 58 for review) or a recombinant form of human arginase I (88). A disturbed arginine metabolism could be a factor that is causing relative poor clinical outcome. NO is not only required for an adequate immune reaction during a surgical trauma after a tumor extraction but also contributes to cytotoxic-induced antitumor processes (77, 174).

Therapeutic Approaches to Influence Arginine and NO Metabolism

Reduced arginine intake in disease or malnutrition as well as increased metabolic needs can result in arginine deficiency or its increased requirement. Therapeutic approaches can aim at increasing substrate availability by supplementation or at influencing the metabolic fate of specific pathways related to NO bioavailability and prevention of NOS3 uncoupling.

Arginine Supplementation

Arginine supplementation varying between 3 and over 100 g/day has been used in clinical studies. Single doses of 3–8 g appear to be safe and rarely provoke adverse events (21), but single doses exceeding 9 g and especially when part of a dosing regimen of over 30 g/day have been associated with gastrointestinal discomfort, nausea, and (osmotic) diarrhea (67). Arginine has been used in supplemental nutrition for surgical patients, burn patients, and patients with sepsis and cancer to benefit regulation of blood pressure, wound healing, and immunomodulation or to serve as an anabolic stimulus. However, the benefits of arginine in these conditions are not uniformly proven and accepted.

Arginine supplementation in sepsis patients has been combined with a mixture of amino acids and other nutrients, referred to as immunonutrition (7, 17, 26, 63). Several reviews and opinion papers on its use have been published (65, 73–75, 86, 108, 152, 169), but conclusions regarding the benefits and potential use in sepsis are not uniform. Arginine treatment starting before endotoxemia in a pig model appeared beneficial by improved hepatopancreatic perfusion and oxygenation during prolonged endotoxemia without causing deleterious systemic side effects. This is probably mediated through enhanced NO synthesis (130). Arginine supplementation has inconsistent effects on intestinal ischemia-reperfusion injury, and a recent publication with long-term intraduodenal arginine supplementation in a rat model of intestinal ischemia and reperfusion injury did not show observable benefits on intestinal morphology or on the inflammatory response (89). Remarkably, serum NOx was even decreased with arginine supplementation in this study, which suggests that arginine was probably not available for NO production due to substrate competition for arginine. Supplementing arginine in partial enteral feeding in neonatal pigs modestly increased intestinal mucosal growth through an NO-independent mechanism (135).

Beneficial effects of arginine supplementation were observed in patients with sickle cell disease suffering from pulmonary hypertension, as well as in the prevention of age-related glomerular injury, in reversing impaired vasodilatation in clinically asymptomatic hypercholesterolemic adults, and improving wound healing (12, 22, 70, 117, 138). A growing body of evidence indicates that arginine supplementation is beneficial in growth, health, and disease and may provide novel and effective therapies for obesity, diabetes, and metabolic syndrome (171). Malnourished head and neck cancer patients who received arginine-enriched nutrition perioperatively demonstrated better 10-year survival (29). Moreover, arginine was proposed as a therapy in hypertension, to interrupt the vicious cycle that initiates and maintains low NO (136). A recent meta-analysis concluded that short-term oral l-arginine supplementation is effective in improving the fasting vascular endothelial function, i.e., flow-mediated vasodilation (FMD), when the baseline FMD is low, and thus endothelial dysfunction can be restored (8). FMD is an early pathophysiological feature of cardiovascular disease and reflects local bioavailability of NO under physiological stimulation. Whether long-term arginine supplementation is beneficial is debated, since exogenous arginine also increases arginase with subsequent diversion of arginine from NOS and subsequent NO production (55).

Citrulline Supplementation

Citrulline supplementation as a single oral dose of 2, 5, 10, or 15 g is safe and well tolerated in healthy adults, with no effect on plasma levels of insulin and growth hormone and with urinary excretion of citrulline remaining low (<5%) even at high doses. Citrulline supplementation has proved to be an effective precursor for arginine and ornithine, but saturation of the renal conversion of citrulline into arginine probably occurred at the highest citrulline dose (15 g) (113). Citrulline-malate is an alternative citrulline source that is also applied as antiasthenia treatment and quickly lowers ammonia levels in hyperammonemia (47).

Citrulline supplementation likely restores the optimal balance between arginine production and metabolism as well as improving NO production and related functions. In an arginine-deprived in vitro model of macrophages, addition of citrulline restored NO production, while glutamine interfered with citrulline-mediated NO production (28). Therefore, in conditions of acute or chronic inflammation with arginine deficiency, citrulline supplementation is a potentially powerful approach to restoring NO production (50). In sickle cell disease, oral citrulline supplementation maintained elevated arginine levels and maintained nearly normal total leukocyte and neutrophil counts and has therefore been suggested as a useful palliative therapy in this condition (168). Citrulline supplementation ameliorated the development of pulmonary hypertension and increased NO production in piglets exposed to chronic hypoxia (3); this suggests that neonates exposed to prolonged periods of hypoxia from cardiac or pulmonary causes may potentially benefit from citrulline supplementation. In middle-aged men, citrulline supplementation improved arterial stiff-
ness, which is considered a powerful predictor of cardiovascular disease (124). Citrulline supplementation restored nitrogen balance and generated large amounts of arginine in rats with short bowel syndrome (125).

**NO Donors and NOS3 Modulating Agents**

NO can also be derived from so-called NO donors, inhaled NO, and nitrite (sources). NO donors such as nitroglycerine are well known and used as vasodilators to treat heart conditions such as angina and chronic heart failure. In septic patients, nitroglycerine increased sublingual microvascular flow, even though arterial and central venous pressure dropped temporarily (151). The use of inhaled NO in the perioperative setting for the treatment of pulmonary hypertension in children is recommended (13). Short-term nitrite therapy reversed age-associated vascular endothelial dysfunction, large elastic artery stiffness, oxidative stress, and inflammation in old mice by restoring NO bioavailability through a NOS-independent conversion. Sandler et al. (150) therefore suggested that sodium nitrite may be a novel therapy for treating arterial aging in humans. Nitrite is also currently undergoing or being planned for clinical trials as a vasodilator drug in patients with cardiovascular diseases such as ischemic stress, sickle cell disease, coronary artery disease, and pulmonary hypertension (179). Nitrate-rich vegetable juice acutely increased nitrite (within 2.5 h) and reduced blood pressure as well as oxygen costs of moderate-intensity exercise in normotensive subjects. These effects were sustained during continuous juice intake over 15 days (163).

Other novel pharmacological approaches under development to increase NO bioavailability are targeted at preventing NOS3 uncoupling or enhancing NOS3 expression (see Refs. 43, 56, 61, 66 for recent reviews). Regarding the latter, BH4 or its synthetic versions may be a new therapeutic strategy to tackle myocardial and endothelial dysfunction (111). Other agents or therapies, e.g., statins, intravenous ascorbic acid administration, or exercise, act on preventing BH4 loss and on improving BH4 availability or on BH4 stability by scavenging superoxide; this functions by improved endothelial NOS coupling and vascular NO bioavailability (5, 61, 149). Local ascorbic acid infusion was demonstrated to improve NO-mediated muscle blood flow during exercise in elderly (44). While BH4 repletion only partly restored NOS activity and NO-dependent vasodilation, reversion of another redox-regulated mechanism controlling NOS function by thiol-specific reducing agents can restore vasodilation when NOS3 S-glutathionylation is increased (38). A pharmacological NOS3 enhancer (AVE3085) ameliorated endothelial dysfunction in db/db mice through increased NO bioavailability, which makes targeting NOS3 and NO a promising approach to combat diabetic vasculopathy (37).

**Targeting Endogenous NO Inhibitors**

An alternative approach to increase NO bioavailability is via targeting endogenous inhibitors of NO synthesis such as ADMA or arginase. Pharmacological modification of dimethylarginine dimethylaminohydrolase (DDAH) enzymes that metabolize ADMA (91) or treatment with the arginase inhibitor N(ω)-hydroxy-1-arginine (nor-NOHA) are options (69, 82). Nor-NOHA restored microvascular coronary artery function in type 2 diabetic rats and caused cardioprotection against myocardial ischemia-reperfusion injury in rats by a mechanism with increased utilization of arginine by NOS and increased NO availability (69, 82). Recently published reference levels for the L-arginine:ADMA ratio may be helpful for evaluation of the effects of L-arginine supplementation in participants with an impaired L-arginine/NO pathway (104).

**Therapies That Influence NO-Mediated Effects**

Patients receiving IL-2 cytokine treatment for advanced malignancy demonstrate increased endogenous nitrate synthesis (77), whereas NOS3 knockout mice were resistant to IL-2-induced hypotension and vascular leak. Methylene blue, by inhibiting guanylate cyclase and cGMP, could inhibit this NOS3-mediated vascular leak (141). Selective inhibition of p38 mitogen-activated protein kinase (MAPK), a mediator of vascular inflammation and activated by oxidized low-density lipoproteins, improved NO-mediated vasodilation in patients with hypercholesterolemia (41). Those authors suggested that p38 MAPK could therefore be a novel target for patients with cardiovascular disease.

**Summary, Conclusion, and Future Research**

While NO production is dependent on arginine availability as its precursor, the odd circumstance is that only a small percentage of arginine is used for NO synthesis and that either too much or too little NO is detrimental. This suggests that the relation between arginine availability and NO production is not simply a case of precursor availability; rather, it is more likely the combination of the availability of arginine along with cofactors and rate-limiting enzymes that determine the rate of production of NO. The compartmentalization of arginine metabolism plays a role here in what is also referred to as the “arginine paradox.”

During the past few years, it has become recognized that endothelial NO uncoupling and NOS3-dependent superoxide generation, induced by stress, are key mediators in the pathogenesis of cardiovascular and pulmonary diseases. Local arginine deficiency, which can be the result of arginine catabolism via arginase or competition with methylarginines, results in endothelial NO uncoupling. Modulating NO uncoupling and targeting NOS3-dependent ROS formation are recent developments that require further clinical testing. Arginine seems to have a critical and dual role here, both as a substrate for NO and as a radical scavenger. Since arginine released from local protein breakdown may not be available for NOS, coupling of the enzymes for de novo arginine and NOS3 could make citrulline a good and maybe even better source for NO. The antioxidant action of citrulline could further contribute to preventing NO uncoupling, but this is not yet known. Specific drugs that act on increasing local arginine availability for NO production, or those that mediate or provide cofactors for NO production, are also considered useful.

In conclusion, the complex regulation of NO synthesis and intracellular availability of arginine as its precursor probably require an approach beyond the primary provision of extra arginine. A multi-target approach addressing substrate competition, precursor availability, and cofactor availability may be useful, and future research could focus on developing such strategies that can optimize NO bioavailability. This can be applied to conditions of compromised or unbalanced NO pro-
production, such as those of endothelial dysfunction in various acute and chronic diseases.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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