Editorial Review

Antimicrobial effects of arginine and nitrogen oxides and their potential role in sepsis
Ines Hardy, Raid Alany, Bruce Russell and Gil Hardy

School of Pharmacy, Faculty of Medical and Health Sciences, University of Auckland, Auckland, New Zealand

Correspondence to Ines J Hardy, BPharm MRPharmS, School of Pharmacy, Faculty of Medical and Health Sciences, University of Auckland, Private Bag 92019, Auckland, New Zealand
Tel: +649 373 7599, ext. 88331; fax: +649 367 7192; e-mail: i.hardy@auckland.ac.nz


Abbreviations
ARG arginine
CIT citrulline
eNOS endothelial NOS
GLN glutamine
IFN-γ interferon gamma
iNOS inducible NOS
NOS nitric oxide synthase
RNI reactive nitrogen intermediates

Introduction
The amino acid arginine (ARG) (L-amino-5-guanidinovaleric acid) has traditionally been classified as non-essential for adults and children [1] because the human body can synthesize it. ARG, however, is essential for growth and development in many mammals and can be considered non-essential only in fully grown adult animals [2]. More recent data in humans now indicate that ARG can be considered a ‘conditionally essential’ amino acid because, under certain clinical conditions and disease states associated with metabolic stress, endogenous synthesis cannot keep up with increased demand. This seems to be the case particularly in critical illness and sepsis [3,4]. Sources of ARG other than de novo synthesis are protein catabolism and dietary intake. In healthy individuals, normal dietary ARG intake is approximately 5 g per day [2]. Fish and nuts are particularly ARG-rich foods [5].

Arginine metabolism
De novo ARG synthesis mainly takes place in the kidney, where ARG is formed from citrulline (CIT) in a two-step process via argininosuccinate [6]. The main source of CIT, in turn, is intestinal glutamine (GLN) metabolism. ARG also has an essential function in the immune system as a precursor for nitric oxide, which will be the main focus of this review. Apart from being incorporated into body proteins and serving as a precursor for other important amino acids, namely proline, glutamate and GLN, ARG is an important intermediate in the urea cycle. Under normal, healthy conditions, approximately 1.2% of total plasma ARG is used for nitric oxide production, whereas about 15% goes into the urea cycle [7]. These percentages may be quite different in severe infection and inflammation. No published data are available, however, on this particular aspect of ARG metabolism.

Nitric oxide synthase
In the presence of oxygen, the enzyme nitric oxide synthase (NOS) converts ARG to CIT and releases the nitric oxide radical from the guanidino group in this process. Several essential co-factors and co-enzymes also need to be present: nicotinamide adenine dinucleotide phosphate, tetrahydrobiopterine, flavin adenine dinucleotide, flavin adenine mononucleotide and protoporphyrin IX.
Three genetically distinct isoforms of NOS have been described [8]: NOS-1 neuronal NOS (nNOS) which is predominantly located in neuronal tissue in which nitric oxide acts as a neurotransmitter; NOS-3 endothelial NOS (eNOS) was first discovered in vascular endothelial cells, where nitric oxide has an important vasodilator function. Both the above-mentioned NOS isoforms are traditionally thought to be expressed constitutively, which means that they continuously produce low levels of nitric oxide. NOS-2 or inducible NOS (iNOS) is located mainly in immune cells, such as macrophages and neutrophils; however, iNOS activity has also been shown in a wide variety of other tissues. iNOS is induced by inflammatory mediators, such as interferon gamma (IFN-γ), tumour necrosis factor alpha (TNF-α) and the proinflammatory interleukins (IL-1, IL-2, IL-12). Other factors that induce nitric oxide production are bacterial toxins such as lipopolysaccharide (LPS) and hypoxia [9]. It is thought that a combination of several factors is required to induce the enzyme. iNOS can produce nitric oxide in much larger quantities (micromolar) than the two constitutively expressed isoforms (nanomolar).

**Nitric oxide antimicrobial effects**

Nitric oxide generated in significant amounts by iNOS is an important part of the body’s non-specific host defence. In-vitro studies of phagocytic immune cells and a variety of microbial targets have demonstrated that ARG-dependent cytokine-inducible microbistatic and microbicidal effects can be inhibited by competitive NOS inhibitors [10]. This demonstrates that the ARG–nitric oxide conversion is the principle behind the antimicrobial effect. The effect of nitric oxide on various microbes has been described comprehensively [11]. Nitric oxide has been shown to be effective against intra- and extracellular parasites [12–18], such as malaria [19] and trypanosoma [20], as well as a wide variety of bacteria, such as *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli* and *Pseudomonas aeruginosa* [21–23], *salmonella* [24], *mycobacteria* [9], fungi [23, 25] and viruses [26–28]. Nitric oxide has also been implicated in the defence against malignant cells [29].

Although a wide range of microbes appear to be sensitive to nitric oxide, it has been associated with host defence against intracellular pathogens, such as Leishmania [20], *mycobacteria* and *salmonella* [9]. This is thought to be related to the activity of nitric oxide scavengers outside the intracellular compartment. Nitric oxide scavenging by haemoglobin has been shown to counteract antimicrobial effects in in-vitro experimental systems [30], which could be clinically relevant, for example, in sepsis. The reaction between nitric oxide and oxyhaemoglobin, however, appears to be rather complex and there are also data suggesting that nitric oxide is not inactivated but remains bioactive by reacting with thiols, metals and superoxide [31].

Despite this growing evidence of nitric oxide as an antimicrobial agent, its role in bacterial infection has not yet been clearly defined and the exact mechanisms by which these antibacterial effects are achieved remain to be clarified. In addition to nitric oxide, other reactive nitrogen intermediates (RNI), such as nitrogen dioxide (NO₂), dinitrogen trioxide (N₂O₃) and peroxynitrite (ONOO⁻), are also involved in the antimicrobial defence. Further investigation is required, however, to elucidate the roles of individual RNI.

RNI other than nitric oxide itself may have more potent antimicrobial effects. Nitric oxide can react with superoxide (O₂⁻), which is generated in macrophages by the enzyme NADPH oxidase to form ONOO⁻. This compound – a very strong oxidant – is associated with a more effective antimicrobial defence; however, unfortunately, it can also inflict significant damage on the host’s own cells, causing inflammation [32].

It has been suggested that a direct microbicidal effect is exerted via the reaction of nitric oxide with iron or thiol groups in proteins forming iron nitrosyl complexes that inactivate enzymes essential for mitochondrial respiration and DNA replication [12].

In macrophages stimulated with IFN-γ, nitric oxide or other RNI produced in an ARG dependent mechanism inhibited the growth of *Francisella tularensis* – a bacterial pathogen that causes an acute febrile illness – whereas in a cell-free system, RNI were shown to be directly cytotoxic to *F. tularensis* [17]. The authors of this study suggest that RNI might act by inhibiting respiration of *F. tularensis* by promoting iron loss from the bacteria and that this RNI-related iron loss also occurs in activated macrophages, which would make them inhospitable for *F. tularensis*. A more recent study in murine macrophages [33] confirms that nitric oxide production from iNOS plays a major role in the host defence against *F. tularensis*. The data from this study also suggest that ONOO⁻ is the RNI most strongly associated with the destruction of *F. tularensis*.

The role of nitric oxide in antibacterial host defence against Gram-negative infection was investigated in a murine model with *Klebsiella pneumoniae* [12]. Administration of L-NAME (N⁶-nitro-L-arginine methyl ester) – an ARG analogue that non-selectively inhibits all three isoforms of NOS – impaired phagocytosis and rate of bacterial death. This resulted in a significant reduction in survival of the animals. The number of neutrophils and macrophages in the lungs of L-NAME-treated animals, however, was not significantly different from that in
controls, despite the fact that *K. pneumoniae* infection is known to cause a significant influx of these immune cells into the lungs of both humans and animals. It appears that nitric oxide does not have any significant effect on the recruitment of inflammatory cells to the site of infection; however, it seems to activate the resident alveolar macrophages. Thus, nitric oxide could be an important mediator of macrophage phagocytosis and *K. pneumoniae* destruction. The authors hypothesized that with the increasing problems of resistance to antibiotics, augmentation of nitric oxide synthesis or local administration of nitric oxide may improve the clinical management of patients with severe bacterial pneumonias. On that basis, supplementation with the nitric oxide precursor ARG could also have some beneficial effects in these patients.

Even though there is now a growing number of animal and in-vitro studies investigating nitric oxide-related antimicrobial activity, nitric oxide production in human mononuclear phagocytes has been called ‘one of the most controversial issues in nitric oxide biology’ [9]. It is now well established that nitric oxide production is elevated in infected patients, but the exact site(s) of origin remain(s) somewhat unclear. In several in-vitro studies, human macrophages produced only negligible quantities of nitric oxide when stimulated [9]. Other data, however, show low levels of ARG-dependent nitric oxide production in response to IL-4 or HIV infection [34–36]. Human neutrophils have also been shown to produce nitric oxide [37,38]. Furthermore, strong evidence of inducible nitric oxide generation by human macrophages has been observed in patients with inflammatory conditions, such as malaria [14], active pulmonary tuberculosis [39], acute respiratory distress syndrome and rheumatoid arthritis [40]. Interestingly, in children with malaria, increased nitric oxide production has been associated with better clinical outcome [14].

**Arginine and inflammation**

ARG is involved in the systemic inflammatory response through two different metabolic pathways. One is the production of nitric oxide via the iNOS pathway, which occurs mainly in macrophages [41,42]. During severe infection and sepsis, the pro-inflammatory T-helper 1 cytokines, such as IL-1, TNF-α and IFN-γ [41–45], which induce iNOS, are produced. Another aspect is the utilization of ARG for normal T-lymphocyte function and proliferation [46,47]. Under conditions in which the T-helper 2 (anti-inflammatory) cytokines (IL-4, IL-10 and IL-13) predominate, however, the enzyme arginase is induced. The Th-2 cytokines are associated with a down-regulation of the inflammatory response and favour antibody generation (humoral response). The arginase pathway converts ARG to ornithine (ORN) – a precursor of proline which is essential for collagen synthesis, cellular regeneration, wound healing and repair. Thus, in disease processes in which a pro-inflammatory response predominates iNOS expression and nitric oxide production are elevated, while during the phase of the anti-inflammatory response, arginase activity and the production of ORN-related metabolites predominate. Although arginase and iNOS compete for ARG availability and also inhibit each other through different mechanisms [48], the induction of iNOS or arginase is not a mutually exclusive phenomenon. No published studies on arginase expression in septic patients exist but data from murine models of endotoxemia show that arginase expression peaks at around 24 h after the onset of sepsis – much later than iNOS, at about 6 h. This would suggest a self-regulation of the two ARG pathways [49] over the clinical course of sepsis from an early pro-inflammatory phase to the anti-inflammatory response in the later stages of the disease.

**Arginine metabolism in sepsis**

Although it is clear that ARG plays an important metabolic role, there is still a lack of understanding about ARG metabolism and its interrelationship with nitric oxide metabolism in septic patients. Low ARG levels seem to be an indicator of poor prognosis in critically ill patients [50] and levels of nitrate – the stable end-product of nitric oxide metabolism – tend to be higher in survivors from septic shock than in those who succumbed to it [51]. However, use of ARG in sepsis is, at the present time, highly controversial. With respect to nitric oxide modulation, two diametrically opposed approaches have been used: enhancement of nitric oxide production to boost the host’s defence by dietary ARG supplementation [52,53] and that by administration of pharmacologic doses [54,55] have been proposed. On the other hand, major concerns about ARG supplementation have been raised regarding toxicity of increased nitric oxide and hemodynamic parameters [56–58].

Sepsis and septic shock are diseases characterized by increased nitric oxide production from iNOS. Excessive amounts of nitric oxide appear to be correlated with decreased systemic vascular resistance and hemodynamic instability in septic patients [59]. It has been suggested that decreasing plasma ARG concentration by administering arginase to limit excessive nitric oxide synthesis [60] may be beneficial in some patients. Another approach was to block the ARG-nitric oxide pathway by administering non-selective NOS inhibitors [61]. The theory behind this was that prohibiting nitric oxide generation in these patients would reduce the excessive proinflammatory response and prevent systemic vasodilatation. In several clinical studies [62,63], however, the investigators did not observe any change in proinflammatory cytokines and the modest hemodynamic benefits of the treatment were outweighed by an increase in mortality. This lead to the premature termination of a
phase III multicentre trial [64]. There was an excess of cardiovascular deaths, which was probably due to an impairment of microcapillary blood flow.

It appears that nitric oxide is actually very important to maintain adequate tissue perfusion in the heart and other organs. Inhibition of nitric oxide production seems to increase tissue damage rather than reduce it by avoiding cytotoxic injury mediated by nitric oxide and other RNI, as was thought previously. Attempting to inhibit excessive nitric oxide production by using non-selective NOS inhibitors during sepsis simultaneously shuts down the basal nitric oxide production that is essential for normal organ function. Selective NOS inhibitors that only affect the iNOS isoform should, in theory, avoid this particular problem. To date, this has only been investigated in animal models [65–67] but, again, an impairment of microcirculation and increased late mortality were observed [65,67]. Furthermore, apart from the adverse effects on microcirculation, there are other potential problems with the use of selective iNOS inhibitors in disease states such as sepsis [68], diabetes [69] or graft-versus-host disease [70]. As iNOS expression is essential for successful clearance of infecting organisms, it seems obvious that the inhibition of this enzyme would impair the antibacterial host defence and put these patients at an increased risk of overwhelming infections.

Regarding the other main concern about ARG supplementation in sepsis, namely nitric oxide cytotoxicity, the picture is equally unclear. It appears to make sense that the cytotoxic effects of nitric oxide exerts on microbial pathogens is not specific and also affects the host cells, although it has been suggested that nitric oxide can selectively damage microbial DNA without inflicting damage to mammalian DNA [9]. When considering nitric oxide toxicity, however, it is important to remember that in conditions of ARG depletion, the NOS enzyme starts to produce O$_2^-$ as well as nitric oxide, which leads to the sustained formation of ONOO$^-$ [71,72]. It has been shown that critically ill patients have very low ARG levels and that this correlates with higher mortality. Adequate ARG supplementation therefore seems to be warranted, not only to optimize the host’s immune defence, but also to minimize ONOO$^-$-mediated tissue damage.

Metabolic changes during catabolic stress such as severe infection and sepsis indicate that ARG could be considered an essential amino acid in these conditions. Some authors have proposed that sepsis might be an ARG-deficient state [73]. In both animals and humans with sepsis, plasma ARG concentrations were found to be significantly decreased [50,74–76], suggesting that endogenous synthesis and supply from dietary intake are unable to keep up with the increased demand. In infection body protein breakdown is increased [74] to make more ARG available for nitric oxide synthesis. Whereas de-novo ARG synthesis in the kidney is increased in moderate inflammation, and in the early phase of endotoxemia in a murine model [77], in severe inflammation, such as in sepsis/sepsis inflammatory response syndrome (SIRS), it appears to be reduced [78]. At the same time, ARG consumption is increased due to the up-regulation of the arginase and NOS pathways [49,74,79]. In addition to these alterations in ARG metabolism, dietary ARG intake is usually reduced, which further diminishes ARG supply, ultimately resulting in low plasma ARG levels.

A recent study [3] investigated plasma arginine kinetics in critically ill septic children in negative nitrogen balance. The main observation in this study was that ARG oxidation was increased, whereas de-novo ARG synthesis remained unchanged. There was also an increase in whole-body nitric oxide synthesis. The investigators concluded that as the rate of de-novo ARG synthesis appears not to be induced, it is reasonable to propose that ARG is a conditionally indispensable amino acid in septic children. Furthermore, it can be deduced that these patients are not only in negative nitrogen balance, but also in negative net ARG balance, eventually leading to ARG depletion.

The effects of ARG administration in sepsis were investigated [44] in a pig model with continuous 24-h lipopolysaccharide infusion. This model of sepsis has no mortality. The animals then received a continuous ARG infusion, which prevented an increase in pulmonary arterial blood pressure, improved muscle and liver protein metabolism, restored the intestinal motility pattern and improved tissue perfusion. ARG given to the septic animals was well tolerated and no adverse effects were observed. When a selective iNOS inhibitor was administered, however, it induced late mortality in the previously zero-mortality model.

**Clinical use of arginine in sepsis/infection**

A lack of published data about ARG administered as a single agent in patients with sepsis and other infections exists. ARG is usually given as a component of so-called ‘immunonutrition diets’ that may also include fish oil (omega-3 fatty acids), nucleotides, antioxidant vitamins and, occasionally, GLN. These diets, delivered enterally, have been shown to reduce infectious complications in surgical patients [80]. In critically ill septic patients, however, the use of immunonutrition is controversial. For the time being, the two important questions remain unanswered: Does stimulating the immune system in septic patients do more harm than good? Does ARG administration worsen septic shock and increase RNI-mediated tissue damage?
A few published studies on enteral ARG ‘monotherapy’ in other patient groups, such as head and neck, as well as gastrointestinal cancer patients, HIV and elderly patients with pressure ulcers, exist. Unfortunately, none of these investigations has shown any clear clinical or immunological benefits [81]. Only one published study of intravenous ARG administration (200 mg/kg bolus) in patients with septic shock exists [82]. This was a small study in only 15 patients, eight of which received a NOS inhibitor followed by the ARG bolus and seven patients received ARG alone. As expected, the group of patients receiving the NOS inhibitor experienced hypertension, a decrease in cardiac index and an increase in systemic vascular resistance. These effects were reversed by the subsequent ARG administration. In the group receiving ARG only, there was hypotension, cardiac index increased and systemic and pulmonary vascular resistance decreased; however, these hemodynamic effects were only transient and no other adverse events were observed.

It has been shown in animals [83] and human duodenal biopsies of healthy volunteers [84\textsuperscript{*}] that ARG administration can enhance nitric oxide production. In a pig model, ARG supplementation increased nitric oxide synthesis during both hyperdynamic endotoxemia and the recovery phase, irrespective of the route of administration [83]. Unfortunately, there are no published clinical data on either enteral or parenteral ARG monotherapy with regards to nitric oxide-mediated effects on infection.

On the basis of the data reviewed in this paper, however, one could speculate that the administration of ARG in an appropriate ‘nutraceutical’ dose via a novel dosage form to produce controlled release of nitric oxide might have some use as an antimicrobial agent to combat intensive care-acquired infections, such as sepsis, ventilator-associated pneumonia and wound infections.

**Measuring nitric oxide production**

As nitric oxide is a short-lived free radical with a half-life of 1–2 s, measuring nitric oxide biosynthesis can be a challenge, especially in vivo. Often, nitrate and nitrite – the stable end-products of oxidative nitric oxide degradation – are measured in plasma and urine [85,86]. This can be performed with the Griess diazotization reaction, which is a simple and inexpensive method [87\textsuperscript{*}]. The reaction is specific for nitrite; nitrate in samples, therefore, needs to be reduced to nitrite first. This is usually performed by enzymatic or chemical methods. The Griess method is a good option for in-vitro experiments in which there are no other sources of nitrite/nitrate such as various cell cultures. For in-vivo measurements, however, the situation is somewhat more complicated. As dietary intake of nitrate and nitrite affect nitrate excretion, the assay should preferably be used in the fasted state, or after elimination of dietary nitrite and nitrate sources such as green vegetables, particularly spinach, lettuce and kale, or any smoked and canned foods [88]. Plasma and urinary nitrate determinations with unrestricted dietary intake or in patients with impaired kidney function may not accurately reflect whole-body nitric oxide status. It has been suggested that renal patients’ nitric oxide measurements by the Griess method should be expressed as a nitrate/creatinine ratio [87\textsuperscript{*}]. Unfortunately, the Griess assay is not sensitive enough to detect nitric oxide production in the nano or picomolar range. It is therefore only suitable for measuring iNOS, but not eNOS and nNOS, activity. Another disadvantage of measuring nitrate/nitrite is that ongoing nitric oxide production would not be detected [89]. To accurately measure in-vivo nitric oxide metabolism, radio-labelled tracer studies measuring the conversion of ARG to CIT and nitric oxide have been used very successfully [74,83]. With this method, it is possible to measure nitric oxide in the small quantities (nano and picomolar ranges) generated from the eNOS and nNOS isoforms. The determination of NOS activity by radio-labelled tracers is also possible in cultured tissues and cells, such as gastric/intestinal biopsies [90–92] and leukocytes [89]. In viable biological samples and exhaled air, nitric oxide can also be measured directly by chemoluminescence [93]: nitric oxide reacts with ozone to form NO\textsubscript{2} and light. This assay is relatively simple to carry out, but it requires expensive equipment. Furthermore, nitric oxide reaction kinetics in biological samples can be determined quantitatively with a Clark-type electrode [94].

**Arginine dosage**

Despite the concerns about nitric oxide toxicity, there are arguments to support the case that ARG may be conditionally essential and supplementation may be beneficial for patients with sepsis and other infections. Nutritional requirements, as well as adequate ‘nutraceutical’ doses in sepsis and critical illness, however, remain to be established. In the context of enteral immunonutrition diets, the dose of ARG administered typically lies in the region of 12 g per 1000 kcal. In a recent multicentre trial [95] in nearly 600 ICU patients, the protocol prescribed a step-wise increase in the administration of the enteral diet corresponding to 4.5 g ARG on the first day, 9 g ARG on the second day, 13.5 g ARG on the third day and 18 g ARG from day 4 onwards. The formula also contained GLN, antioxidants and fish oil. The actual intake of the diet was between 60 and 70% of the prescribed amount. In the few publications that describe ARG monotherapy [81,82], the dose administered was between 17 and 25 g per day. It needs to be pointed out that no dose-ranging studies have been carried out and there are no data on what dose of ARG would be required to maintain adequate nitric oxide production to optimize the non-specific host defence.
Conclusion
Nitrergic oxide and other RNI have been shown to exert antimicrobial effects on a variety of different pathogens, such as bacteria, fungi, viruses and protozoan parasites. The mechanisms of nitric oxide-mediated antimicrobial actions, though not yet exhaustively investigated, may involve nitrosylation of enzymes essential for microbial respiration and DNA replication. The metabolic interactions between the nitric oxide-precursor arginine and other components of immunonutrition diets are not fully understood and there is a lack of clinical data on both enteral and parenteral ARG monotherapy.

There has been a lot of controversy about ARG supplementation in critically ill septic patients and general concerns about nitric oxide toxicity. Now, however, there is growing evidence that ARG may be conditionally essential in these patients. Low ARG levels seem to be an indicator of poor prognosis in critically ill patients and survivors from septic shock have been shown to have higher nitrate levels than non-survivors. Some evidence of the benefit of ARG in animal models of sepsis exists. Both selective and non-selective NOS inhibition was shown to produce more harm than benefit. Basal nitric oxide concentrations are essential to maintain cell and organ function as well as host response to infection. All of these points would appear to support the case for supplementation of ARG-depleted septic patients. Moreover, ARG may have an important role in the prevention and treatment of infection.

References and recommended reading
Papers of particular interest, published within the annual period of review, have been highlighted as:
• of special interest
•• of outstanding interest

5 A very good review that puts forward the argument that arginine is an essential amino acid in sepsis and infection.
30 Alspaugh JA, Granger DL. Inhibition of Cryptococcus neoformans by nitric oxide supports the role of these molecules as effectors of macrophage-mediated cytostasis. Infect Immun 1991; 59:2291–2296.
Effects of arginine and nitrogen oxides: editorial review


An interesting recent publication explaining the advantages and limitations of one of the most commonly used methods for measuring nitric oxide.


