

Review Article

Understanding the Metabolic Possibilities for L-Arginine Tolerance Development

*Srinidi Mohan and Charity Benson

Department of Pharmaceutical Sciences, College of Pharmacy, University of New England, Portland, USA

*Corresponding author: Srinidi Mohan, Department of Pharmaceutical Sciences, College of Pharmacy, University of New England, Portland, 716 Stevens Avenue, Portland, Maine 04103-2670, USA; T: 207-221-4058; F: 207-523-1926; Email: smohan@une.edu

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Abstract

L-arginine (ARG) has gained popularity as a dietary supplement in the last decade after its role as the endogenous substrate for endothelial nitric oxide synthase (eNOS) was identified. Of concern, the therapeutic benefits of ARG, often clearly observed during short-term dosing, are not evident after long-term use. Initial metabolic studies in cells show ARG tolerance in endothelial cells to be mediated by eNOS down-regulation, secondary to oxidative stress and glucose accumulation. Modulation in ARG transport mechanism (via cationic amino acid transporters), eNOS cofactor (tetrahydrobiopterin), arginase, as well as the metabolic intermediate (N^ω-hydroxyl ARG) formed during ARG utilization by eNOS have all shown promising potential towards the development of ARG tolerance. While asymmetric-dimethyl-L-arginine is a potent inhibitor of eNOS and competitive analog of ARG, it has been found not to be responsible in developing ARG tolerance under physiological or diseased conditions. AMP-activated protein kinase has been recently identified as the fundamental modulator of short-term and long-term ARG responses in cells. Translatability of these findings *in vivo* will prove beneficial and crucial for the design of safe and effective use of ARG as alternative and complementary medicine.

Keywords: Tolerance; ADMA; eNOS; AMPK; ARG

Introduction

L-arginine (ARG) is an important conditionally essential amino acid that plays an important role not only in the mere removal of ammonia, but is also involved in the regulation of numerous physiological processes [1]. Endogenously, ARG is synthesized as a by-product of glutamine, glutamate and proline catabolism [2]. L-Citrulline (CIT) reabsorption by the kidney is also known to be involved in the formation of ARG [3]. Since ARG is not an essential amino acid, and is usually generated in sufficient amounts endogenously, initial reports suggested the real necessity for ARG supplementation only during conditions of malnutrition or for elderly patients who lack sufficient production of ARG.

However, the use of ARG gained its popularity as a dietary supplement in the last decade after its role as the endogenous substrate for endothelial nitric oxide synthase (eNOS) was identified [4]. The use of ARG, in as many as 44 diseases and diagnoses, were discussed and categorized (in Medlineplus) according to the strength of scientific evidence. Examples of conditions that have shown improvement with ARG treatment include pituitary disorders, coronary artery disease, critical illness, heart failure, migraine headache, peripheral vascular disease/ Claudication, diabetes, erectile dysfunction, myocardial infarction, and pre-eclampsia, among others [5]. Benefits of supplementation were also seen in patients with stable angina pectoris [6,7], and those with congestive heart failure [8,9], whereby their exercise tolerance, and prolonged exercise duration, respectively were observed. In addition, ARG has been found to improve immunity [10-12], in patients under critical care [13,14] and in sickle cell disease [15,16]. Thus, the range of diseases that are benefited by ARG supplementation is quite wide, and is also commercially endorsed by Nobel Laureates.

Of concern, the therapeutic beneficial effects of ARG that are clearly observed during short-term studies are not evident after long-term use. Wilson et al. [17] showed, in the “Nitric Oxide Peripheral Arterial Insufficiency” (NO-PAIN) trial that ARG supplementation (3 g/d) for 6 months in 133 subjects, “did not increase NO synthesis or improve vascular reactivity, and the expected placebo effect observed in studies of functional capacity was attenuated in the ARG-treated group”. These authors characterized the findings as indications of “ARG tolerance”. A 2005 report by Bernarz et al., [9] showed that ARG oral supplementation at 9 g/day for 1 month in 792 patients with acute myocardial infarction resulted in marginal benefits in the reduction of major clinical events [9]. In the follow-up Vascular Interaction With Age (VINTAGE) trial [18] in myocardial infarction patients, a total of 153 patients after myocardial infarction were randomly assigned ARG (goal dose of 3 g) or matching placebos for 6 months. The results showed no improvement in vascular stiffness measurements or ejection fraction. Strikingly, 6 patients in the ARG group died during the study period versus none in the placebo group. The authors therefore concluded that ARG “may be associated with high post-infarct mortality”, and stated that ARG “should not be recommended following acute myocardial infarction”. Animal studies also revealed that chronic use of ARG may be harmful. Chen et al. [19] showed that ARG fed to apoE knockout mice (25g/L for 16-24 weeks) on a western diet did not affect lesion formation but “it negates the protective effect of inducible NOS gene deficiency”. However, commercial available form of ARG, such as N.O.XPLODE, NiteWorks and ARG extreme; are being used by body builders without medical supervision.

The development of this ARG tolerance (and possible toxicity)

upon chronic dosing represents a major hindrance for the use of this important amino acid to benefit patients. Because of the extensive involvement of NOS in many physiological systems, a wide variety of disease states are expected to benefit from increased NO bioavailability through increased ARG supply. Thus, if the metabolic events causing ARG tolerance and toxicity can be understood, and suitable alternative approaches can be devised to circumvent these effects, the beneficial effects of ARG supplementation can be extended to benefit patients in whom short-term therapeutic effects have already been demonstrated.

Analyses and Interpretation

ARG tolerance events

While studies have identified the potential development of tolerance during long-term or continuous ARG supplementation, better understanding of the biochemical and pharmacological consequences of long-term ARG supplementation will greatly help to inform the design and evaluation of future clinical trials regarding ARG. A recent study by Mohan et al., has shown ARG tolerance in endothelial cells to be mediated by eNOS down-regulation, secondary to oxidative stress and glucose accumulation [20]. This study is considered highly significant on several counts. First, the study has led to the development of a cellular model for the study of ARG tolerance that has allowed experimental manipulations for mechanistic studies to be carried out without the need for long-term *in vivo* studies, which are otherwise not possible in animal models and human trials. Second, the study demonstrated, for the first time, that continuous ARG exposure leads to eNOS down-regulation, thus providing a direct mechanism to explain ARG tolerance, and the endothelial dysfunction that accompanies this phenomenon. Third, the study also showed that ARG tolerance is accompanied by increased glucose accumulation and oxidative stress. These results, when applicable *in vivo*, would have tremendous impact in the consideration of ARG supplementation for the large number of patients with cardiovascular and metabolic diseases.

However, it is not known whether the major three effects observed during ARG tolerance, viz., superoxide ($O_2^{\cdot-}$) production, eNOS down-regulation and increased cellular glucose concentration, are connected with each other, and how interference with one factor may affect the others. If we find that these events are sequential to each other, identification of the initiating event would provide insights concerning how these events can be prevented. Additionally, to what extent eNOS uses ARG supplied exogenously versus those from the endogenous stores has not been determined conclusively. One possible mechanism that has been suggested in better understanding exogenous versus endogenous ARG utilization has linked it to ARG transport pathway.

The cellular transport of ARG by itself is complex [21,22] and involves four cationic amino acid transporters (CAT 1-4) of the y^+ carrier system. However, in endothelial cells, 70-95% of ARG uptake has been attributed to the CAT-1 [23]. Suppression of the CAT-1 mediated transport of extracellular ARG strongly depressed the endothelial cell NO response to a wide range of physiological stimuli. Furthermore, modulation in tetrahydrobiopterin (BH_4 , the cofactor for eNOS) [5], asymmetric dimethyl arginine (ADMA), N^w -hydroxyl-ARG (NOHA) and arginase have all been suggested as potential

factors involved in tolerance development during continuous ARG supplementation. Hence, an in-depth characterization of these metabolic pathways becomes crucial to gain sufficient insights of the molecular basis for such tolerance development.

Role of ADMA

ADMA is a naturally occurring analog of ARG and is usually derived during proteolysis [24-26]. Clinical evidences have suggested ADMA in the serum as a novel cardiovascular risk factor and ADMA has also been associated with impaired endothelial function in humans [27,28]. Patients of hypertension [29], hyperlipidemia [30], hyperhomocysteinemia [31], coronary artery disease [32], peripheral arterial occlusive disease [33], congestive heart failure [34], stroke [35], pulmonary hypertension [36], and end-stage renal disease [37] have all shown elevation in their plasma ADMA concentrations. Studies have demonstrated that ADMA induces oxidative stress in vascular tissues. Veresh et al., [38] suggested ADMA to increase $O_2^{\cdot-}$ production by angiotensin II-NADPH oxidase pathway, thereby impairing NO mediated arteriolar function. However, Antoniadou et al., [39] found no correlation between elevated serum ADMA and NADPH-stimulated vascular $O_2^{\cdot-}$. Thus the exact role of NADPH oxidase in mediating ADMA-induced vascular $O_2^{\cdot-}$ accumulation is still unclear.

While it is undisputable that ADMA is a potent inhibitor of eNOS ($K_i = 0.9 \mu M$), its circulating concentration of about $0.5 \mu M$ in healthy subjects is too low [23] to exert a significant inhibition of NOS. However, ARG has been reported to inhibit dimethylarginine dimethylaminohydrolase [40], thus cellular ADMA concentration could potentially increase during ARG supplementation. On the other hand, ARG trans-stimulates the efflux of ADMA from cells through CAT 1-4, potentially reducing intracellular ADMA load [41]. Recent studies have concluded that the modest changes in ADMA concentration observed as a result of ARG exposure, still couldn't explain the ARG tolerance phenomenon, unless substantial intracellular compartmentalization of ADMA takes place [41]. We are unaware of any literature report suggesting specific cellular compartments for ADMA. Thus, the general consensus is that this pathway is less-likely to be important in developing tolerance during continuous ARG exposure. However evidence for ADMA-induced eNOS uncoupling and involvement of BH_4 are presented [41].

Role of eNOS cofactor: BH_4

As a redox sensitive NOS cofactor, BH_4 is required for normal NO synthesis by all NOS isoforms. While fully reduced BH_4 supports NOS catalysis, the oxidized pterin species of BH_4 (for example; 7,8-dihydrobiopterin, BH_2) are catalytically non-functional [42-44]. Depletion in BH_4 level have resulted in eNOS uncoupling from ARG, as well as in causing endothelial dysfunction via product switching from NO to $O_2^{\cdot-}$ [5], which were restored during subsequent dosing with BH_4 in chronic smokers [45] and patients with diabetes [46], ischemia-reperfusion injury [47], or hypercholesterolemia [48]. Besides supplementing BH_4 to improve endothelial function, exposure to antioxidants such as Glutathione, vitamin C or E, that are capable of providing chemical stabilization to BH_4 (thereby preventing BH_4 oxidation) increased eNOS efficacy and NO synthesis [49,50]. These studies provide the initial evidences to suggest oxidation of BH_4 to be

the basis for eNOS uncoupling and vascular dysfunction during ARG supplementation. The metabolically more critical literature finding was the ability of BH₂ to bind to eNOS with greater avidity than BH₄ alone [51], as well as the importance in maintaining the ratio between BH₂ to BH₄ (rather than maintaining the level of BH₄ alone) for proper vascular function and NO generation during continuous ARG supplementation [5]. Thus, both the level of BH₄ and the ratio of BH₂ to BH₄ are to be considered equally important determinants during continuous ARG treatments.

Role of Arginase and NOHA

Although the arginase K_m for the utilization of ARG is ~ 1000 fold greater than those of NOSs, the V_{max} of the arginase is ~1000 fold higher than that of the NOS enzymes [52]. So, in principle, the arginase should be able to compete with the NOS enzymes for ARG, thereby limiting NO production. Arginase has been shown to contribute to endothelial oxidative stress [53] and its inhibition restores NOS coupling and reverses endothelial dysfunction [54]. So, the interaction among ARG, O₂⁻ and arginase needs to be further explored.

Studies of the competitive nature between NOS and arginase in response to inflammatory cytokines [55,56], showed a substantial increase in the amount of the intermediate NOHA. NOHA has been reported to accumulate in the culture medium up to 20-30% of the amount of NO generated [57,58], and is a potent inhibitor of Arginase (with a K_i in the range of 40-150 μM). Additionally NOHA has an NO independent mechanism leading to cytostasis and/or apoptosis by inhibiting synthesis of polyamines (by inhibiting ornithine decarboxylase) [59].

Since polyamine are formed from ARG and L-Ornithine via Arginase and ornithine decarboxylase (ODC) [60], an inhibition of both arginase activity and ODC by NOHA, can potentially affect L-Ornithine level. Fluctuation in L-Ornithine is known to inhibit NOS mediated NOHA generation (and thus, subsequent NO generation) [61]. Based on these literature evidences, it is logical to hypothesize that short-term beneficial effects of ARG supplementation is predominantly dominated by NOHA's influence on inhibiting arginase and polyamines, while a continuous supplementation of ARG can allow fluctuation in L-Ornithine, that in turn inhibits NOS mediated NOHA generation, thereby favoring tolerance and possible toxicity (via arginase activation). However additional validation is required at this instance to better delineate NOHA response towards ARG tolerance.

Metabolic switch for ARG tolerance : AMP-activated protein kinases (AMPK)

While it would be metabolically interesting to validate the potential factors involved in tolerance development during continuous ARG supplementation, the fundamental question of what drives the cascade of events needs to be better addressed. Not much progress has been reached until recently, where AMPK has been suggested as the potential modulator of the events associated with ARG tolerance (and potential toxicity) [62,63].

AMPK plays a significant role in energy-sensing/ signaling system utilized by cells to detect and respond to changes in energy levels [64]. The NO generated by eNOS via ARG utilization, is

known to be required for the initial activation of AMPK [65], possibly via calmodulin dependent protein kinase kinase [66,67] or other mechanistic pathways [68,69]. eNOS knockdown in mice, or shear stress in endothelial cells [70] suppressed AMPK activity, emphasizing the importance of endogenous NO in AMPK activation and subsequent metabolism of energy substrates.

When AMPK is activated, processes of ATP-consumption, such as lipogenesis or gluconeogenesis are switched off, whereas ATP-producing pathways like fatty acid and glucose oxidation are switched on. AMPK activation also increases NO synthesis by eNOS under various physiological and pathological conditions [71,72]. While NO controls AMPK function through activation of guanylyl cyclase; peroxynitrite that is formed by the reaction between NO and O₂⁻; can also impose its regulation on AMPK activation by impairing guanylyl cyclase [73-75]. The recent AMPK modulation study suggests that ARG mediated short-term therapeutic benefits to be initiated via the activation of AMPK, which stimulates downstream NO release by maintaining eNOS activity and allowing glucose to accumulate only via cellular transport [62]. The dysfunction in AMPK enzyme activity affected eNOS function, decreased glucose uptake from medium, increased cellular glucose synthesis and oxidative stress. All of these events seen during AMPK dysfunction are concomitant with those reported to occur during continuous ARG supplementation [20].

ARG versus CIT response

An alternative problem-solving approach to avoid ARG tolerance development would be to indirectly deliver ARG to eNOS for NO production. The CIT to ARG recycling pathway is well known [52], and CIT supplementation has been found to be beneficial to cardiovascular functions [23,40,76-80], although no long-term tolerance and toxicity studies about CIT supplementation have appeared. Further reduction in arginase toxicity with CIT has been recently reported, however, the exact mechanism involved is still unclear [81]. In essence, the use of CIT offers a safe and effective alternative to circumvent the ARG tolerance sparing effects, and to extend the beneficial effects of ARG supplementation among patients in whom short-term therapeutic effects have already been demonstrated.

Conclusion and future direction

In this review on ARG tolerance development, we have identified some of the potential metabolic factors that are crucial in better delineating this phenomenon. This review has shown evidence to support ADMA to be not a crucial factor in the development of ARG tolerance. AMPK has been identified as the modulating switch for determining ARG mediated beneficial versus deleterious events. Further detail metabolic studies are warranted to better understand the pharmacological events involving Arginase, NOHA, BH₄/BH₂ and CAT systems during AMPK regulation on continuous ARG supplementation, to better inform the design and evaluation of future ARG clinical trials. The mechanistic basis for the use of CIT as a safe and effective alternative to circumvent the ARG tolerance-sparing effects needs to be further explored.

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