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Review

The caveolar nitric oxide synthase/arginine regeneration system for NO production in endothelial cells

Larry P. Solomonson*, Brenda R. Flam, Laura C. Pendleton, Bonnie L. Goodwin and Duane C. Eichler

Department of Biochemistry and Molecular Biology, University of South Florida College of Medicine, Tampa, FL 33612, USA

*Author for correspondence (e-mail: lsolomon@hsc.usf.edu)

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Summary

The enzyme endothelial nitric oxide synthase (eNOS) catalyzes the conversion of arginine, oxygen and NADPH to NO and citrulline. Previous results suggest an efficient, compartmentalized system for recycling of citrulline to arginine utilized for NO production. In support of this hypothesis, the recycling enzymes, argininosuccinate synthase (AS) and argininosuccinate lyase (AL), have been shown to colocalize with eNOS in caveolae, a subcompartment of the plasma membrane. Under unstimulated conditions, the degree of recycling is minimal. Upon stimulation of NO production by bradykinin, however, recycling is co-stimulated to the extent that more than 80% of the citrulline produced is recycled to arginine. These results suggest an efficient caveolar recycling complex that supports the receptor-mediated stimulation of endothelial NO production. To investigate the molecular basis for the unique location and function of endothelial AS and AL, endothelial AS mRNA was compared with liver AS mRNA. No differences were found in the coding region of the mRNA species, but significant differences were found in the 5′-untranslated region (5′-UTR). The results of these studies suggest that sequence in the endothelial AS-encoding gene, represented by position –92 nt to –43 nt from the translation start site in the extended AS mRNA 5′-UTRs, plays an important role in differential and tissue-specific expression. Overall, a strong evidential case has been developed supporting the proposal that arginine availability, governed by a caveolar-localized arginine regeneration system, plays a key role in receptor-mediated endothelial NO production.

Key words: nitric oxide, eNOS, endothelial nitric oxide synthase, arginine, citrulline, arginine regeneration system, argininosuccinate synthase, argininosuccinate lyase, caveolae, nitric oxide production.

Introduction

Endothelial nitric oxide synthase (eNOS), the enzyme that catalyzes the production of NO from the amino acid arginine in endothelial cells, plays a key role in vasoregulation as well as in other important physiological processes such as angiogenesis. Impaired production of endothelial NO has been associated with hypertension, heart failure, hypercholesterolemia, atherosclerosis and diabetes (Govers and Rabelink, 2001; Vallance and Chan, 2001; Maxwell, 2002). Circulating effectors, such as bradykinin, bind to receptors on the luminal surface of endothelial cells, signaling the transient release of NO to the adjacent smooth muscle layer and resulting in relaxation of the vessel wall.

The signal for eNOS activation is a transient increase in intracellular calcium, which activates the enzyme through binding of a calcium–calmodulin complex (Ca–Cam). Endothelial NOS activation also occurs in response to shear stress (Govers and Rabelink, 2001; Maxwell, 2002). Consistent with the important physiological roles of eNOS, the enzyme appears to be subject to multiple modes of regulation, in addition to primary regulation through reversible Ca–Cam binding and activation. These include reversible phosphorylation and palmitoylation, substrate and cofactor availability, dimerization of enzyme subunits, intracellular translocation and protein–protein interactions (Govers and Rabelink, 2001). Several of these potential modes of regulation appear to be interrelated. As a component of caveolae, a subcompartment of the plasma membrane that serves to sequester proteins involved in cell signaling, eNOS may transiently interact with several different caveolar components. Previous work from several different laboratories has suggested that a diverse group of proteins, including calmodulin, caveolin-1, bradykinin B2 receptor, heat shock protein 90, argininosuccinate synthase (AS), argininosuccinate lyase (AL), Raf-1, Akt, extracellular signal-related kinase,

eNOS interacting protein, eNOS traffic inducer and unidentified tyrosine-phosphorylated proteins (Hellermann et al., 2000; Govers and Rabelink, 2001; Maxwell, 2002; Nedvetsky et al., 2002), may be transiently and functionally associated with eNOS.

A potential limiting factor for endothelial NO production is the availability of the substrate, arginine. Intracellular levels of arginine have been estimated to range from 100 μmol l^{-1} to 800 μmol l^{-1}, which is well above the K_m value of 5 μmol l^{-1} for eNOS (Harrison, 1997). Endothelial NO production can, nonetheless, be stimulated by exogenous arginine (Vallance and Chan, 2001). This phenomenon, termed the 'arginine paradox', suggests the existence of a separate pool of arginine directed to endothelial NO synthesis. As illustrated in Fig. 1, arginine has a number of metabolic roles in addition to NO production, including production of major metabolites such as urea, polyamines, creatine, ornithine and methylarginine derivatives. The observed stimulation of endothelial NO production by exogenous arginine suggests that the arginine directed to NO production may be segregated from bulk cellular arginine utilized for these other metabolic roles.

One possible site of control is at the level of arginine uptake. McDonald et al. (1997) showed that the CAT1 transporter, responsible for 60–80% of total carrier-mediated arginine transport into endothelial cells, colocalizes with eNOS in caveolae. They proposed that the arginine utilized by eNOS might, at least in part, be maintained by the CAT1 transporter. Another important mechanism for controlling the availability of arginine directed to NO production may be the regeneration of arginine from the other product of the eNOS-catalyzed reaction, citrulline. Hecker et al. (1990) initially demonstrated that citrulline, produced in the conversion of arginine to NO, can be recycled to arginine. A possible link between NO production and arginine regeneration from citrulline was subsequently established for other cell types (Nussler et al., 1994; Shuttleworth et al., 1995). This regeneration is catalyzed by the enzymes AS and AL, both of which also play an essential role in the urea cycle in liver. The potential importance of this regeneration system for endothelial NO production was supported by a report of two infants with a deficiency of AL who were shown to be hypertensive (Fakler et al., 1995). Upon infusion of arginine, the blood pressure of these infants decreased to near normal levels, suggesting a critical role for arginine regeneration in the regulation of systemic blood pressure. More recent evidence from DNA microarray analysis suggests an important role for the arginine regeneration system by clearly demonstrating significant and coordinate upregulation of AS-encoding gene expression in response to shear stress stimulation of endothelial NO production (McCormick et al., 2001). It was concluded that available arginine is a prerequisite for NO production and that in the absence of synthesis of additional eNOS, shear stress-induced increases in NO synthesis depend on an increase in synthesis of arginine from citrulline through increased AS expression. Although supplemental arginine can be beneficial in some cases (Wu and Meininger, 2002), in other cases it may lead to adverse effects owing to the multiple metabolic roles of arginine (Chen et al., 2003; Loscalzo, 2003).

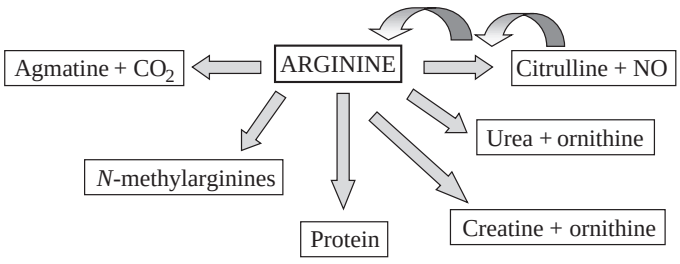

Fig. 1. Metabolic roles and fates of arginine. In addition to incorporation into protein, arginine serves as a metabolic precursor for several important metabolites, as indicated by the arrows. Also indicated is the two-step conversion of citrulline to arginine.

Recent work further supports the hypothesis that the arginine regeneration system, comprised of a caveolar complex that includes eNOS, AS and AL, plays an important, and most likely essential, role in the receptor-mediated production of NO by vascular endothelial cells.

Effects of exogenous arginine and citrulline on endothelial NO production

Endothelial NOS is localized in plasmalemmal caveolae. The localization of eNOS in this signaling subcompartment of the plasma membrane may have important implications with regard to the regulation and catalytic efficiency of eNOS (Everson and Smart, 2001; Shaul, 2002). We have recently found evidence for an efficient cycling of citrulline to arginine, raising the possibility of a channeling complex of eNOS and the enzymes of the citrulline–arginine cycle (AS and AL) localized in caveolae. Our initial research effort that led to this finding was designed to test the hypothesis that an intracellular pathway exists for the generation of methylarginines to regulate NO production in nitric oxide-producing tissues. The goal of this initial work was to determine the physiological significance of intracellular methylarginines as regulators of NOS activity. To examine the levels of endogenous methylarginines, we developed methods that allowed for the rapid and quantitative analysis (by HPLC) of arginine, citrulline and the methylarginines from endothelial cell extracts. There was no apparent change in levels of methylarginines following stimulation of endothelial cells with either bradykinin or the calcium ionophore A23187. In an attempt to raise intracellular methylarginine levels, and further test our hypothesis, we added citrulline, which we expected to inhibit dimethylarginine dimethylaminohydrolase, the enzyme that converts N^G-methylarginine or N^G,N^G-dimethylarginine to citrulline and monomethylamine or dimethylamine, respectively. The objective was to determine whether inhibition of the degradation of methylarginines would increase their intracellular concentrations and thereby inhibit NO production. To our surprise, stimulation of NO production by bradykinin was increased by the addition of citrulline, rather

than decreased, and there was no apparent change in methylarginine levels. To further examine the molecular basis for the stimulation of NO production by citrulline, we compared the effect of exogenous citrulline with the effect of exogenous arginine on NO production and levels of intracellular arginine following bradykinin activation. Surprisingly, added arginine did not cause as great an increase in endothelial NO production as did added citrulline. In addition, there was a much larger increase in intracellular arginine in response to exogenous arginine compared with exogenous citrulline. Added citrulline caused only a modest increase in intracellular arginine, while added arginine caused a substantial increase. Thus, there appeared to be no correlation between total intracellular arginine levels and endothelial NO production. To the best of our knowledge, this represents the first attempt to correlate NO production with the levels of intracellular arginine. Furthermore, the effects of arginine and citrulline on NO production appeared to be synergistic, since a combination of arginine and citrulline stimulated endothelial NO production more than did either arginine or citrulline alone (Flam et al., 2001). Since arginine has a number of potential metabolic fates, while citrulline has only one known metabolic fate (Fig. 1), the efficiency of NO production could be enhanced if a separate pool of arginine is maintained by endothelial cells. Recycling the product of the NOS-catalyzed reaction, citrulline, back to arginine *via* the enzymes of the arginine regeneration system, AS and AL, would maintain this separate pool. The pool of arginine used for NO synthesis would be essentially isolated from the bulk of intracellular arginine through the efficient operation of an arginine regeneration system. The apparent efficiency of the process suggests a channeling of intermediates and a compartmentalized complex of eNOS and enzymes of the arginine regeneration system. These results further support a model in which eNOS is localized together with this arginine regenerating system, and regulatory components, to ensure optimal efficiency of NO production and regulation without affecting other arginine-dependent cellular processes.

Caveolar localization of arginine regeneration enzymes with eNOS

Endothelial NOS is targeted by acylation to caveolae, where it interacts with caveolin-1 (Everson and Smart, 2001; Shaul, 2002). In liver cells, the arginine-generating enzymes AS and AL are associated with the outer mitochondrial membrane, reflecting the functional role of these enzymes in the production of urea (Cohen and Kuda, 1996). To test the model for a colocalization of AS and AL with eNOS, we used two different fractionation protocols for the purification of caveolae (Smart et al., 1995; Song et al., 1996). Both protocols generated a caveolar membrane fraction that was highly enriched in caveolin-1, eNOS, AS and AL (Flam et al., 2001). These results support the proposal that a separate pool of arginine, directed to NO synthesis, is effectively separated from the bulk of intracellular arginine through the functional localization of arginine regeneration enzymes and eNOS with plasmalemmal caveolae. A possible consequence of this functional association would be the channeling of intermediates through AS, AL and eNOS such that intermediates of the complex would not equilibrate with bulk intracellular arginine.

Degree of recycling

Cellular activity of eNOS has been estimated by measuring the rate of conversion of [^{3}H]arginine to [^{3}H]citrulline (Hardy and May, 2002). If recycling of citrulline to arginine is tightly coupled to NO production, this measurement would underestimate the cellular activity of eNOS. Estimating cellular activity of eNOS by measuring rate of production of NO (as the degradation product nitrite), on the other hand, should give a better estimate of cellular activity of eNOS. To test this hypothesis, and to estimate the degree of recycling of citrulline to arginine, we simultaneously measured the apparent rate of arginine-to-citrulline conversion and the rate of production of NO under both unstimulated and stimulated (addition of bradykinin) conditions. The ratio of these activities was close to one under unstimulated conditions. An increase in the ratio of NO produced to citrulline produced was approximately eight upon exposure of endothelial cells to agonist (B. R. Flam, D. C. Eichler and L. P. Solomonson, unpublished), indicating that recycling and NO production were costimulated. These preliminary results suggest an efficient caveolar complex for the regeneration of arginine directed to receptor-mediated production of NO in endothelial cells and an efficiency of greater than 80% for the recycling of citrulline to arginine under conditions of maximum stimulation of NO production. Although recycling of citrulline to arginine has been assumed to be important for conservation and efficient utilization of arginine, the degree of recycling relative to NO production has not, to the best of our knowledge, been quantified. Our results suggest that this recycling, especially under stimulated conditions, may play a more important role in endothelial NO production than previously recognized.

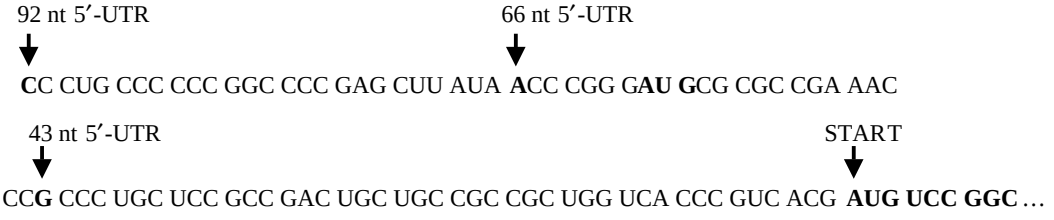

Fig. 2. Novel 5′ untranslated regions (UTRs) of endothelial argininosuccinate synthase mRNA.

Molecular basis for functional role and location of endothelial AS

In liver tissue, AS plays an essential role in urea synthesis and appears to be associated with the outer mitochondrial membrane (Cohen and Kuda, 1996). By contrast, endothelial AS appears to be the rate-limiting enzyme in the recycling of citrulline to arginine used for NO synthesis and is localized in caveolae (Flam et al., 2001). Immunoblotting experiments suggested small differences in subunit molecular masses and isoelectric points of endothelial AS compared with liver AS (B. R. Flam, D. C. Eichler and L. P. Solomonson, unpublished). We speculated that these differences could be due to a splice variant, but analysis of the coding sequence of AS mRNA indicated no differences between the mRNA from endothelial cells and liver (Pendleton et al., 2002). Because upstream and downstream untranslated regions (UTRs) of mRNA can influence regulation of gene expression, we carried out both 5′-RACE (rapid amplification of cDNA ends) and 3′-RACE analysis to investigate possible differences in the UTRs. We found AS mRNA species with three different length 5′-UTRs in endothelial cells (Fig. 2). Only one of these products, the shortest 5′-UTR of 43 nt, was quantitatively expressed in liver. No significant variation was found in the 3′-UTR. The 5′-RACE analysis identified endothelial AS mRNA species with extended 5′-UTRs of 66 nt and 92 nt, in addition to a major 43 nt 5′-UTR AS mRNA (Fig. 2). Compositional analysis revealed that all three AS mRNA 5′-UTRs were enriched in G+C content (approximately 76%) and were likely to form complex and stable secondary structures. An upstream open reading frame (uORF) that was out-of-frame with the AS mRNA AUG start codon was detected in the 66 nt and 92 nt 5′-UTRs. RNase protection analysis (RPA) and real-time reverse transcriptase-PCR (RT-PCR) verified and quantified the differential expression of the extended 5′-UTR species relative to the major 43 nt 5′-UTR AS mRNA. Estimates from RPA of the amount of the 92 nt and 66 nt species, relative to the 43 nt species, were approximately 15% and 13%, respectively.

Features of mRNA UTRs, specifically uORFs, are regarded as important determinants of translational efficiency and may have important biological implications for the regulation of translation. We therefore designed experiments to determine to what extent the various 5′-UTRs of AS mRNA influenced translation. Translational efficiencies for the 66 nt and 92 nt AS 5′-UTR constructs were 70% and 25%, respectively, of the translational efficiency for the 43 nt 5′-UTR AS mRNA. Sequential deletions, starting with the 5′-terminus of the 92 nt 5′-UTR construct, resulted in a corresponding increase in translational efficiency, but the most pronounced effect resulted from mutation of the uORF, which restored translational efficiency to that observed with the 43 nt species. When the different AS mRNA 5′-UTRs, cloned in front of a luciferase reporter gene, were transfected into endothelial cells, the pattern of luciferase expression was nearly identical to that observed for the different 5′-UTR AS mRNAs in endothelial cells. These results suggest that a complex transcriptional/translational infrastructure exists to coordinate AS expression and NO production (Pendleton et al., 2002).

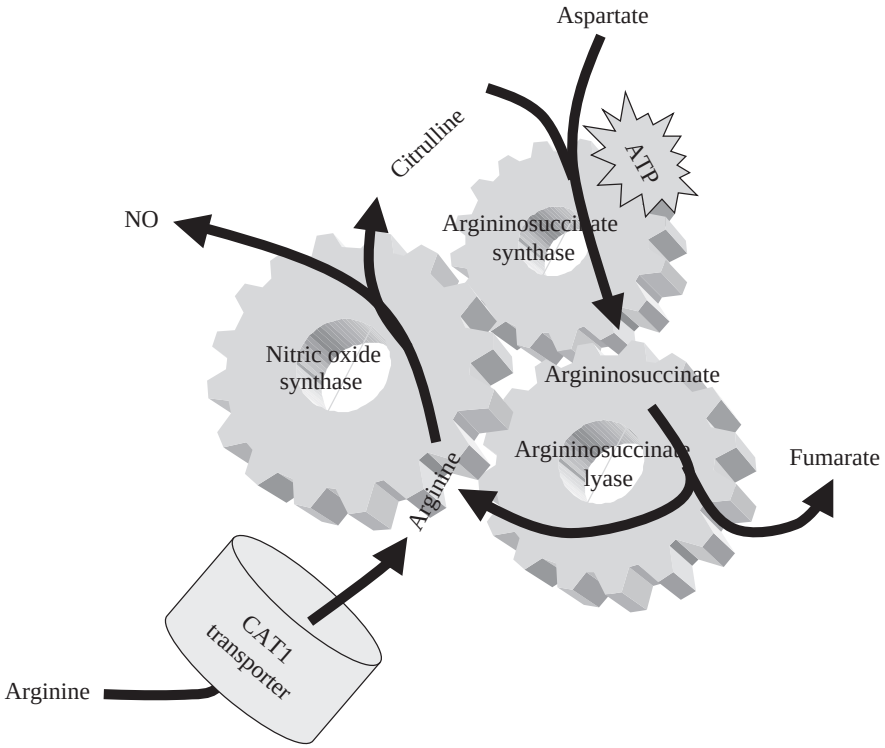

Fig. 3. Model for the coupling of endothelial NO production to the regeneration of the substrate, arginine, from the product, citrulline. Shown is the CAT1 transporter involved in arginine transport and the complex of argininosuccinate synthase and argininosuccinate lyase with endothelial nitric oxide synthase.

Model for coupling of arginine regeneration to endothelial NO production

A model depicting our view of the coupling of arginine regeneration to endothelial NO production through the compartmentalized complex of AS, AL and eNOS is shown in Fig. 3. This coupling may be largely ‘disengaged’ under unstimulated conditions but is ‘engaged’ and tightly coupled in response to agonists such as bradykinin. The molecular determinants and mechanisms involved in this coupling are not fully understood at this time. Based on our studies, and evidence from other labs, we believe the coupling of arginine regeneration to endothelial NO production is important for the overall regulation of endothelial NO production and may be essential for agonist-stimulated endothelium-dependent vasorelaxation.

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