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Accessibility of endothelial and inducible nitric oxide synthase to the intracellular citrulline–arginine regeneration pathway

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Abstract

This study investigates our hypothesis that argininosuccinate synthase (AS), the rate-limiting enzyme for arginine (L-arg) regeneration from citrulline (L-cit), plays a pivotal role in supplying L-arg to endothelial (eNOS), but not inducible (iNOS) nitric oxide synthase, for nitric oxide (NO) production. Transgenic rat blood–brain barrier (TR-BBB) endothelial cells were used as a model to elucidate the accessibility of the L-arg compartments for NOS isozymes. NO production via eNOS or iNOS, with or without α-methyl-DL-aspartic acid (MDLA), an AS inhibitor, was measured by a fluorometric method. NO production via eNOS was activated by the calcium ionophore A23187, while via iNOS was induced by cytokines. AS activity was assayed by the amount of argininosuccinate regenerated from radioactive aspartic acid from cell extracts. Upon increased AS activity (5.9-fold) in cells grown in L-arg-free/L-cit-supplemented medium, A23187-activated NO production also significantly increased, however cytokine-induced NO production was not detected. A23187-activated NO production was observed not only in L-arg containing medium, but also L-arg-free and L-arg-free/L-cit-supplemented medium, and was abolished by MDLA regardless of medium type. Cytokine-induced NO production was only observed in L-arg containing medium, not in L-arg-free or L-arg-free/L-cit-supplemented medium, and it was not inhibited by MDLA in the L-arg containing medium. Our results indicate that extracellular L-arg was the only L-arg pool for cytokine-induced NO production and intracellular L-arg regenerated from L-cit via AS pathway was the major L-arg pool for A23187-activated NO production in TR-BBB endothelial cells. Therefore, modulation of AS activity could be a promising strategy to selectively alter NO production via eNOS, but not iNOS.

Keywords: Argininosuccinate synthase; Nitric oxide synthases; Endothelial; Arginine; Nitric oxide; MDLA

1. Introduction

The importance of the nitric oxide synthases (NOSs) and nitric oxide (NO) is evident in physiological and pathophysiological processes. It has been suggested and much has been written about disturbances in regulation regarding inducible nitric oxide synthase (iNOS) as the likely 'damaging' producer of NO and endothelial nitric oxide synthase (eNOS) as 'protective' [1,2]. Nitric oxide, produced by eNOS, is a key mediator for maintaining the function and integrity of endothelium, such as the regulation of vascular tone, the prevention of leukocyte filtration and thrombus formation, and angiogenesis, etc. [2]. However, down-regulation of eNOS activity is associated with a number of diseases, such as atherosclerosis [3], hypertension [4] and diabetes [5]. Up-regulation of eNOS activity is correlated with tumor growth and angiogenesis in vitro and in vivo [6]. Therefore, the manipulation of eNOS activity might be a promising therapeutic strategy for a variety of diseases.

eNOS activity can be regulated by factors such as protein expression, post-translational modification, concentration of substrate and cofactor, and cellular localization [7]. The unique modification of eNOS, as compared to

Abbreviations: CAT, cationic amino acid transporter; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; HPLC, high performance liquid chromatography; NO, nitric oxide; ADI, arginine deiminase; TR-BBB, transgenic rat-blood–brain barrier; AS, argininosuccinate synthase; L-arg, L-arginine; L-cit, L-citrulline; MDLA, α-methyl-DL-aspartic acid

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the other NOS isoforms, by myristoylation/palmitoylation of residues near the N terminus cause the particular cellular localization of eNOS in the caveolae of plasmalemma [8]. It has been shown that the mutant of the myristoyl or palmitoyl moiety of eNOS reduced NO generation, even though purified wild-type and mutant eNOS from cell extracts had identical catalytic activity [9,10]. Therefore, the association of eNOS and caveolae may render eNOS in close proximity to its substrate and cofactor for its proper functioning. Although arginine (L-arg) is the sole substrate for the NOSs, we reported previously that in vitro NO production via eNOS in transgenic rat blood–brain barrier (TR-BBB) endothelial cells is independent of extracellular L-arg, whereas iNOS solely depends on extracellular L-arg for its substrate [11]. This finding suggests that the intracellular source of L-arg, including the regenerated L-arg from argininosuccinate synthase (AS) and argininosuccinate lyase (AL), can be the substrate for eNOS, but not iNOS, in TR-BBB endothelial cells under extracellular L-arg deprivation. AS, the rate-limiting enzyme for the endogenous regenerated L-arg has been found to be co-localized with eNOS in caveolae in endothelial cells [12]. Therefore, we hypothesize that the regulation of AS activity can manipulate the NO production via eNOS.

Even though AS is recognized as a ubiquitous enzyme in mammalian tissue, the regulation of AS activity varies due to factors such as hormones, nutrients and cytokines [13]. AS has been reported to be involved in urea production in hepatocytes [14], L-arg production in enterocytes and kidney cells [15,16], NO production via eNOS in bovine aortic endothelial cells [17] and NO production via iNOS in macrophages and vascular smooth muscle cells [18,19]. However, this is the first report to elucidate the relationship between AS and NO production via eNOS and iNOS simultaneously in endothelial cells. It is crucial to understand the differential regulation of AS on NO production via eNOS and iNOS in endothelial cells. TR-BBB cells, cultured blood–brain barrier endothelial cells derived from a transgenic rat, are immortalized endothelial cells [20] and exhibit specific endothelial markers, e.g. spindle fiber-shaped morphology, von Willebrand factor and acetylated low-density lipoprotein uptake [20–22]. TR-BBB cells express eNOS, iNOS, and AS [11], and are therefore a useful model to investigate the role of AS in NO production. Here we present data to examine our hypothesis that AS, the rate-limiting enzyme for L-arg regeneration from L-cit, plays a pivotal role in the supplying L-arg to eNOS, but not iNOS, for NO production.

2. Materials and methods

2.1. Materials

Rat recombinant IFN-γ and TNF-α, nitrite standard, and Griess Reagent were purchased from Calbiochem. [^{14}C]Aspartic acid (200 mCi/mmol) was obtained from Moravek Biochemicals. Dowex 1-X8-200-400 resin was from Supelco (Bellefonte, PA). Micro BCA protein assay reagent kit was obtained from Pierce (Rockford, IL). Calcium ionophore A23187, 2,3-diaminonaphthalene (DAN), AS inhibitor α-methyl-DL-aspartic acid (MDLA), as well as all other chemicals and reagents were products from Sigma Chemical Company.

2.2. Cell culture

TR-BBB endothelial cells were cultured as described previously [11]. Briefly, the cells were maintained in Dulbecco's minimal essential medium/F12 from Cellgro and supplemented with 15 μg/mL endothelial cell growth factor (ECGF), 10% heat-inactivated fetal bovine serum (FBS) and 2.5 mM L-glutamine and antibiotics, penicillin/streptomycin. The cells were cultured in culture-ware coated with type I collagen and grown in a humidified incubator at 33 °C [20]. All cell culture reagents and L-arg/L-cit-free MEMα + medium were products of GIBCO-BRL, except ECGF, which was a product of Roche Diagnostics Corporation. For experiments in this report, the L-arg-free/L-cit-free MEMα + medium is designated as 'L-arg-free medium'. L-arg-free medium supplemented with 1 mM L-arg is designated as 'control medium' and when supplemented with 1 mM L-cit/1 mM ammonium chloride, designation is 'L-arg-free/L-cit-supplemented medium'. The FBS was thoroughly dialyzed using a dialysis tubing with a molecular weight cut-off of 2 kDa, therefore the concentration of L-arg or L-cit in the serum was negligible.

2.3. AS activity assay

Cells were cultured in control medium and L-arg-free/L-cit-supplemented medium, respectively in T75 flasks for 4 days. For cytokine-treatment, cells in control medium were treated with 50 U/mL IFN-γ and 5 ng/mL TNF-α on day 3 for 24 h. AS activity in cell homogenates was determined as described in our previous paper [23] and AS enzymatic activity expressed as pmol of argininosuccinate formed from L-citr and [^{14}C]aspartic acid per min per mg protein.

2.4. Nitrite production assay

2.4.1. Effect of AS induction on NO production

To investigate the effect of up-regulation of AS activity on NO production via eNOS and iNOS, TR-BBB cells were grown under L-arg-starvation condition as a method to induce AS activity [11,24]. Successful induction of AS activity occurred under the L-arg starvation condition; therefore this experimental model was used to investigate the effect of up-regulation of AS activity on eNOS and iNOS activity.

2.4.1.1. Effect of AS induction on NO production via eNOS

Cells were seeded (1×10^5 cells/well) into 6-well culture plates in control medium and L-arg-free/L-cit-supplemented medium. On day 4, the cells were washed with PBS and media replaced with control medium and L-arg-free/L-cit-supplemented medium, respectively. After 30-min incubation, 10 μM of the calcium ionophore, A23187, was added and the cells incubated for an additional 30 min. The nitrite concentration in the supernatant was measured by a fluorometric method as previously described [11]. Briefly, freshly prepared DAN was added to the cell culture supernatant, mixed immediately, and incubated for 15 min. The reaction was terminated with 2.8 M NaOH, and the samples were measured using the excitation wavelength of 375 nm and emission wavelength of 415 nm with a Hitachi F-2000 Fluorescence Spectrophotometer.

2.4.1.2. Effect of AS induction on NO production via iNOS

Cells were seeded (2×10^4 cells/well) into 24-well culture plates in control medium and L-arg-free/L-cit-supplemented medium. At 4 h post-seeding, the cells were treated with cytokines and incubated for 4 days. The nitrite concentration in the supernatant was measured by the Griess method as previously described [11]. The protein content in each well was determined by Pierce Protein Kit and results presented as nmol of nitrite in the cell culture supernatant per mg protein.

2.4.2. Effect of AS inhibition on NO production

To elucidate the effect of the down-regulation of AS activity on NO production via eNOS and iNOS, MDLA, an analogue of aspartate, was used as an AS inhibitor [25].

2.4.2.1. Effect of AS inhibition on NO production via eNOS

Cells were seeded (2×10^6 cells/well) into 6-well culture plates in control medium. On day 3 post-seeding, the cells were washed with PBS and media replaced with control, L-arg-free and L-arg-free/L-cit-supplemented medium, respectively. The cells were treated with 10 mM MDLA for 30 min. After incubation, 10 μM A23187 was added and the cells incubated for an additional 30 min. The nitrite concentration in the supernatant was measured by a fluorometric method.

2.4.2.2. Effect of AS inhibition on NO production via iNOS

Cells were seeded (2×10^4 cells/well) into 24-well culture plates in control medium for 48 h. On day 3 post-seeding, 50 U/mL IFN-γ and 5 ng/mL TNF-α were added to the cultures to induce iNOS activity. After 24 h incubation with cytokines, the media were replaced with control, L-arg-free and L-arg-free/L-cit-supplemented medium, respectively. The cells were then treated with 10 mM MDLA and incubated for 2 h, and then the nitrite concentration in the supernatant was measured by a fluorometric method.

2.5. Intracellular L-arg analysis by high performance liquid chromatography (HPLC)

Cells were cultured in control medium, L-arg-free/L-cit-supplemented medium, and L-arg-free/L-cit-supplemented medium with 10 mM MDLA, respectively in 6-well plates for 24 h. Cells were washed with PBS and lysed with methanol/0.5 M boric acid, 9:1 (v/v), pH 7.7 [26]. The supernatant after 15 min centrifugation at 13,000 rpm was used for pre-column derivatization with phenylisothiocyanate (PITC). Amino acid derivatives were analyzed by PICO.TAG amino acid analysis system (Waters chromatography) [27]. The amount of intracellular L-arg was normalized by the cell number.

2.6. Statistical analysis

All values are mean ± standard deviation, except where otherwise noted. The significant difference was evaluated by ANOVA followed by the modified *t*-test according to Tukey, when appropriate. Values of $p < 0.05$ were accepted as significant.

3. Results

3.1. Induction of AS activity

To investigate the effect of L-arg deprivation on AS activity in TR-BBB cells, the cells were grown in L-arg-free/L-cit-supplemented medium for 4 days. The data in Fig. 1 demonstrate that AS activity was increased both by L-arg deprivation and cytokine treatment. The intrinsic AS activity of TR-BBB cells in control medium is 29.1 ± 0.5 pmol/(min mg), and upon L-arg deprivation,

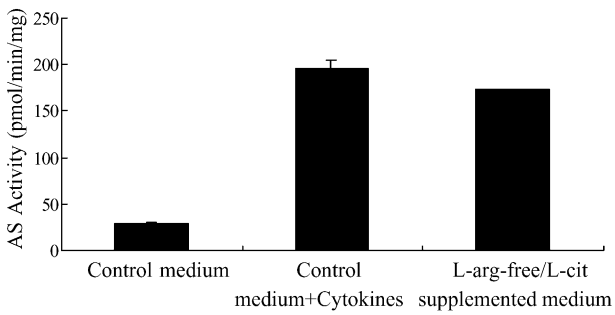

Fig. 1. Induction of AS activity by cytokines and L-arg-free/L-cit-supplemented medium. Cells were seeded into T75 collagen-coated culture flasks in control medium or L-arg-free/L-cit-supplemented medium and incubated at 33 °C, 95% air/5% CO_2. Cytokines (50 U/mL IFN-γ and 5 ng/mL TNF-α) were added on day 3. AS activity was assayed as described in Section 2.3 and error bars represent differences between duplicates.

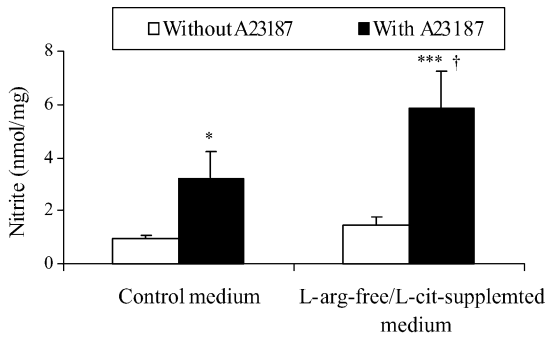

Fig. 2. Effect of AS induction on A23187-activated NO production. Cells were seeded into 6-well collagen-coated culture plates in control medium or L-arg-free/L-cit-supplemented medium and incubated at 33 °C, 95% air/5% CO_2. On day 4, calcium ionophore A23187 was added. NO concentration was determined by measuring nitrite concentration in the culture medium by the fluorometric assay described in Section 2.4. Error bars represent standard deviations ($n = 3$). $^{*}p < 0.05$, $^{***}p < 0.001$ arginine control group vs. each respective treatment. $^{\dagger}p < 0.05$ with A23187 in respective medium.

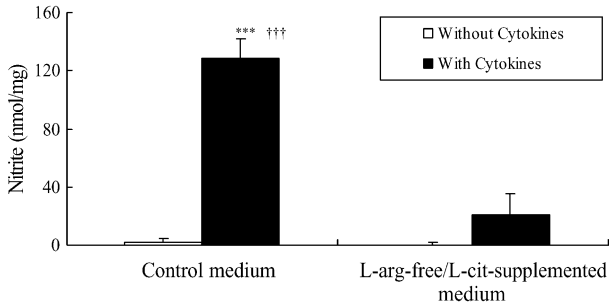

Fig. 3. Effect of AS induction on cytokine-induced NO production. Cells were seeded into 24-well collagen-coated culture plates in control medium or L-arg-free/L-cit-supplemented medium and treated with cytokines (50 U/mL IFN-γ and 5 ng/mL TNF-α), incubated at 33 °C, 95% air/5% CO_2. On day 4, nitrite concentration was measured using a commercially available Griess Assay kit. Error bars represent standard deviations ($n = 3$). $^{***}p < 0.001$ arginine control group vs. each respective treatment. $^{\dagger\dagger\dagger}p < 0.001$ with A23187 in respective medium.

increased to 173.0 ± 0.7 pmol/(min mg). Increase in AS activity also occurred upon 24 h of cytokine treatment, 195.3 ± 9.4 pmol/(min mg). These results demonstrated that successful induction of AS activity occurred.

3.2. Effect of AS induction on NO production

3.2.1. Effect of AS induction on A23187-activated NO production

By using A23187 to increase the permeability of the cell membrane to Ca^{2+}, eNOS, which is Ca^{2+}/calmodulin-dependent, is activated but not iNOS. As shown in Fig. 2, A23187 treatment enhanced the nitrite production from 0.93 ± 0.13 to 3.2 ± 0.6 nmol/mg in control medium and 1.42 ± 0.37 to 5.87 ± 1.38 nmol/mg ($p < 0.05$) in L-arg-free/L-cit-supplemented medium. The observed increased AS activity in L-arg-free/L-cit-supplemented medium parallels also with the observation of increased A23187-activated nitrite production (5.9 ± 1.4 nmol/mg versus control, 3.2 ± 0.6 nmol/mg, $p < 0.05$).

3.2.2. Effect of AS induction on cytokine-induced NO production

As shown in Fig. 1, an increase in AS activity occurred with the addition of cytokines or under L-arg deprivation. However, even though there was increased AS activity, the cytokine-induced NO production was 5-fold less in L-arg-free/L-cit-supplemented medium as compared to the NO induction in control medium (20.5 ± 15.2 nmol/mg versus control, 128.1 ± 13.8 nmol/mg, $p < 0.001$, Fig. 3).

3.3. Effect of AS inhibition on NO production

3.3.1. Effect of AS inhibition on A23187-activated NO production

A23187-activated nitrite production was not only observed in L-arg-free/L-cit-supplemented and control medium, but also detected in L-arg-free medium. As shown in Fig. 4, when treated with A23187 a 3.2-fold increase in control medium occurred, a 2.8-fold increase in L-arg-free and a 2.3-fold increase in L-arg-free/L-cit-supplemented as compared to no. A23187-treatment. Upon addition of 10 mM MDLA, nitrite production in all media was effectively abrogated ($p < 0.001$). The addition of MDLA had no effect on cell growth.

3.3.2. Effect of AS inhibition on cytokine-induced NO production

Twenty-four hours before reaching confluence (day 2) the cells were treated with cytokines to induce cytokine-activated NO production. On day 3, medium was replaced and 10 mM MDLA was added, and the nitrite concentration was

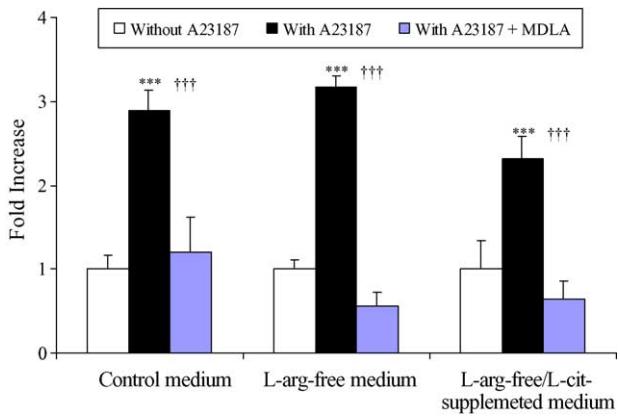

Fig. 4. Effect of AS inhibition on A23187-activated NO production. Cells were seeded into 6-well collagen-coated culture plates in control medium and incubated at 33 °C, 95% air/5% CO_2 for 3 days confluent. On day 3, cells were incubated in control medium, L-arg-free medium, and L-arg-free/L-cit-supplemented medium, respectively, and pre-treated with 10 mM MDLA for 1 h and then treated with A23187 for 0.5 h. Nitrite concentration was measured by fluorometric assay. Error bars represent standard deviations ($n = 3$). $^{***}p < 0.001$ without A23187 vs. with A23187 in respective medium. $^{\dagger\dagger\dagger}p < 0.001$ with A23187 vs. with A23187 and MDLA in respective medium.

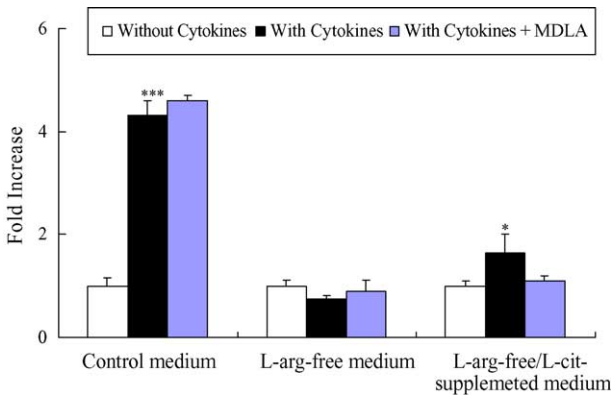

Fig. 5. Effect of AS inhibition on cytokine-induced NO production. Cells were seeded in to 24-well collagen-coated culture plates in control medium and incubated at 33 °C, 95% air/5% CO_2 for 3 days confluent. On day 2, cells were treated with cytokines (50 U/mL IFN-γ and 5 ng/mL TNF-α). After 24 h cytokine treatment, cells were incubated in fresh control, L-arg-free, and L-arg-free/L-cit-supplemented medium and treated with 10 mM MDLA for 2 h. Nitrite concentration was measured by fluorometric assay. Error bars represent standard deviations ($n = 3$). $^{*}p < 0.05$, $^{***}p < 0.001$ without cytokines vs. with cytokines in respective medium.

measured after 2 h. A significant 4.3-fold increase in cytokine-induced nitrite concentration was detected only in TR-BBB cells in control medium, not in L-arg-free or in L-arg-free/L-cit-supplemented medium, where the increase measured 0.7- and 1.6-fold, respectively (Fig. 5). There was no inhibitory effect by 10 mM MDLA on the cytokine-induced nitrite production. In control medium, the increase in nitrite concentration for cytokine-treatment alone and in combination with MDLA, as compared to no cytokine treatment, were comparable, 4.6-fold versus 4.3-fold.

3.4. Intracellular L-arg analysis

The effect of MDLA on the amount of intracellular L-arg was analyzed by a flurorescent HPLC method. The amount of intracellular L-arg was 1.41 ± 0.09 and 1.37 ± 0.08 pmol per 1000 cells in TR-BBB cells in control and L-arg-free/L-cit-containing medium, respectively (Table 1). However, a significant decrease in the amount of intracellular L-arg, from 1.41 ± 0.09 to 1.22 ± 0.03 pmol per 1000 cells, was observed in cells that were treated with 10 mM MDLA in L-arg-free/L-cit-containing medium (a 14% decrease, $p < 0.05$).

Table 1
Intracellular L-arg analysis in TR-BBB cells treated with MDLA

Treatment medium	Intracellular L-arg amount[a]	
	pmol/1000 cells	%
Control	1.41 ± 0.09	100 ± 6.4
L-Arg-free/L-cit-supplemented	1.37 ± 0.08	96.6 ± 5.9
L-Arg-free/L-cit-supplemented plus 10 mM MDLA	$1.22 \pm 0.03^{*}$	86.3 ± 2.2

[a] Mean ± S.D., $n = 3$.
* $p < 0.05$ L-arg control vs. each respective treatment.

4. Discussion

New studies of the separate L-arg pools, as well as the possible role of AS in eNOS regulation, are emerging due to the discovery of the L-cit-NO cycle [17,19]. However, details of the regulation and the mechanisms involved in L-arg compartmentalization are still largely unclear. Our results indicate that intracellular L-arg regenerated from L-cit via the AS pathway is the major L-arg pool for A23187-activated NO production in TR-BBB endothelial cells and that extracellular L-arg is the only arginine pool for cytokine-induced NO production. This is the first report to describe the relationship between AS and NO production via both eNOS and iNOS in endothelial cells. Results in this paper indicate that, AS does have an effect on NO production via eNOS, but not iNOS, suggesting that AS activity may play an important role in regulating NO production via different NOS isoforms.

As shown in Fig. 1, a model of the up-regulation of AS activity in TR-BBB cells under L-arg-deprivation conditions was successfully established and subsequently was used to investigate the effect of AS activity on eNOS and iNOS. Results from this study indicate that the eNOS activity in TR-BBB endothelial cells highly depends on AS activity. When AS activity was up regulated under the L-arg-depletion condition, A23187-activated nitrite concentration increased (Fig. 2), and this increased eNOS activity was abolished when AS activity was inhibited by MDLA (Fig. 4). This trend was even observed without the activation by A23187; however, basal levels of NO production via eNOS are too low for reliable detection and statistical analysis (data not shown). In contrast, iNOS activity was independent of AS activity in TR-BBB endothelial cells. Cytokine-activated NO production was not regulated by the induction or the inhibition of AS activity (Figs. 3 and 5). This finding, that the regulation of AS activity alters NO production via eNOS, but not iNOS, offers the possibility of exploiting AS as a differential modulator of the NOS isoforms. Previous publications have only linked the relationship between the regenerated-L-arg from L-cit and each of the three NOS isoforms separately and in different cell types [19,28,29].

Interestingly, our observation that A23187-activated NO production occurred not only in control and L-arg-free/L-cit-supplemented medium, but also in L-arg-free medium (Fig. 4), suggesting an important role for AS in eNOS regulation. This finding is in agreement with those of Arnal et al. [30] and Closs et al. [26], who showed that A23187-activated NO production could proceed in the absence of extracellular L-arg in bovine aortic and human endothelial cells, respectively. Also Goodwin et al., recently reported that the reduction of AS expression by RNAi knock-down decreased NO production via eNOS in bovine aortic endothelial cells [17], which is consistent with our result. However, the study focused on NO production via eNOS, and only speculated that AS would play the same role in

NO production via iNOS in endothelial cells without experimental support. In our study, data from experiments in endothelial cells indicate that a relationship not only between AS and eNOS, but also iNOS exists. These results demonstrate that AS may play a different role in NO production via eNOS and iNOS in endothelial cells. Further, both down- and up-regulation of AS activity were performed to confirm these results.

Taken together, the possible source of cellular L-arg to maintain eNOS activity is intracellular L-arg, e.g. de novo biosynthesis from L-cit and intracellular protein degradation, but not extracellular L-arg. The conclusion is further supported by the finding that MDLA, an AS inhibitor that inhibits the regeneration of L-arg from L-cit, abolished A23187-activated NO production (Fig. 4). The intracellular L-arg amount was decreased 14% in cells with MDLA treatment compared to control (Table 1). This indicates that even though the amount of intracellular L-arg was greater than 80% with MDLA treatment, the decrease caused by AS inhibition is sufficient to have an effect on the A23187-activated NO production. On the other hand, cytokine-induced NO production was observed only in L-arg-containing medium, not L-arg-free or L-arg-free/L-cit-supplemented medium (Fig. 5). This indicates that extracellular L-arg is the only substrate for iNOS, and not intracellular L-arg as shown in Table 1. In addition, cytokine-induced nitrite production was not altered whether or not AS function was up regulated or inhibited (Figs. 3 and 5). Therefore, it can be assumed that regenerated L-arg is not the substrate for iNOS, at least in some cell types. Compartmentalization of L-arg and the concept of the L-arg paradox are topics of much investigation and discussion, yet are not fully understood. Our findings in this endothelial cell model give further support to the assertion that even though L-arg concentrations found within the cell exceed the K_m value of the NOS enzymes, other mechanisms seem to be responsible for activation or inhibition [31]. It is possible that AS has different roles in NO production via iNOS in different types of cells. Xie and Gross observed that in vascular smooth muscle cells, AS was the rate-limiting enzyme to NO synthesis via iNOS using an AS-over-expression cell method [19]: this is in contrast to our finding. Additionally, this varied role could be explained by factors such as L-arg compartmentalization or transport, as well as NOS isoform expression in the various cell types. Primary cells, such as human umbilical vein endothelial cells (HUVEC) and bovine aortic endothelial cells (BAEC), are more biologically relevant than immortalized cells; however, the heterogeneity of functions and characteristics in different primary endothelial cells usually make it difficult to have consistent results from experiments with different cell passages or preparations [32,33]. TR-BBB endothelial cells, an immortalized cells, and have been shown to display stable growth and differentiated characteristics after more than 60 passages following cloning [20,22] and exhibit the biomarkers characteristic of normal endothelial cells [20,21]. Further, from our previously published data, TR-BBB cells express iNOS, eNOS and AS activity, and HUVEC cells do not have detectable endogenous, nor induced, AS activity [23]. Therefore, TR-BBB cells are a useful endothelial cell model to elucidate the relationship between AS activity and the two NOS isozymes.

The likely rationale for the differential effect of AS on eNOS and iNOS activity in TR-BBB cells is that the substrate for eNOS and iNOS are from different L-arg compartments. Feron, et al., have suggested that there are two compartments of intracellular L-arg pools [9]. Compartment 1 is the pool of regenerated-L-arg from L-cit by AS and AL which can be accessed by eNOS. Compartment 2 depends on extracellular L-arg uptake, which can be accessed by iNOS. Results in this report, as shown in Figs. 4 and 5, and Table 1, suggest that there may be a third compartment of intracellular L-arg which is accessible to neither eNOS nor iNOS. It is plausible that this third compartment represents L-arg generated from an intracellular source, however further investigation is needed.

The localization of eNOS in caveolae occurs because of the unique myristoylated/palmitoylated modification [9]. Liu et al. further demonstrated that the localization of eNOS in caveolae is necessary for optimal NO production even though palmitoylation-deficient mutant and wild type eNOS have similar kinetic properties [10]. Additionally, the L-arg regeneration enzymes, AS and AL, were also found to be co-localized in caveolae with eNOS [12]. This may contribute to the proximity between L-arg-regeneration and eNOS. Regenerated-L-arg has been shown to be channeled directly from AS to AL and, subsequently, from AL to arginase in the urea cycle in hepatocytes [34]. Therefore, it is plausible that such a channeling event may also occur between AS, AL, and eNOS in the caveolae of endothelial cells. The transport of the substrate, L-arg, into the cell may also have an effect on the role of AS in NO production. Since the cationic amino acid transporter 1 (CAT-1) has been reported to be co-localized with eNOS in the caveolar complex [35], it may be responsible in providing the transported L-arg from the extracellular space directly, as a substrate for eNOS. Some studies in vascular endothelial cells have shown that NO production was indeed increased by extracellular L-arg [36,37]. However, others reported that eNOS-mediated NO production could be measured in the absence of extracellular L-arg in bovine aortic endothelial cells and in human EA.hy926 endothelial cells [26,30], NO production was not increased in patients with L-arg supplementation [38,39], and eNOS and AS, but not CAT-1, were shown to be co-induced in the aorta of diabetic rats [5]. These reports would indicate that the AS induction has more direct of an effect toward eNOS activity than on the arginine transporters. The premise that extracellular L-arg might regulate eNOS activity, and not provide L-arg as eNOS substrate continues to be a controversial issue [40,41]. The mechanism of extracellular L-

arg and its role in an increased NO production needs further investigation. On the other hand, it is the consensus that the cationic amino acid transporter 2 (CAT-2) is responsible for the extracellular L-arg uptake as substrate for iNOS in macrophages [26]. Co-induction of CAT-2 and iNOS by bacterial lipopolysaccharide and IFN-γ, and subsequent increased iNOS-related NO production was also observed in brain astrocytes [42]. This is also consistent with our finding that the regulation of AS does not affect iNOS activity because the substrate for iNOS is the L-arg transported from the extracellular compartment by CAT-2, not the regenerated-L-arg by AS and AL.

AS, eNOS and iNOS play pivotal functional and regulatory roles under normal and disease states. Down-regulation of eNOS activity can cause various pathophysiological problems [3,4]. To activate or restore eNOS activity would maintain many important vascular physiological functions. However, if up-regulation of iNOS activity occurs, this would cause tissue damage as observed in inflammatory conditions [43,44]. The present study demonstrates that AS activity is strongly connected with eNOS, but not iNOS, activity. Therefore, modulation of AS activity could be a promising strategy to alter NO production via eNOS, with little alteration in iNOS activity. In vitro kinetic studies have shown fumonisin B1 to be an inhibitor of AS by altering sphingolipid metabolism [45]. On the other hand, recombinant arginine deiminase (rADI), the enzyme that catalyses the conversion of L-arg to L-cit, has been show to up-regulate AS, increase eNOS and inhibit iNOS activity in endothelial cells [11]. These and other AS modulators can serve as useful tools to elucidate the underlying mechanisms of the interrelation among AS, eNOS and iNOS, and consequently, may offer unique strategies for the treatment of NOS-related diseases.

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