Arginase inhibition increases nitric oxide production in bovine pulmonary arterial endothelial cells

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1Vascular Physiology Group and Department of Pediatrics, University of New Mexico Health Sciences Center, Albuquerque, New Mexico 87131; and 2Center for Developmental Pharmacology and Toxicology, Columbus Children’s Research Institute, Department of Pediatrics, The Ohio State University, Columbus, Ohio 43205

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Chicoine, Louis G., Michael L. Paffett, Tamara L. Young, and Leif D. Nelin. Arginase inhibition increases nitric oxide production in bovine pulmonary arterial endothelial cells. Am J Physiol Lung Cell Mol Physiol 287: L60–L68, 2004. First published February 20, 2004; 10.1152/ajplung.00194.2003.—Nitric oxide (NO) is produced by NO synthase (NOS) from l-arginine (l-Arg). Alternatively, l-Arg can be metabolized by arginase to produce l-ornithine and urea. Arginase (AR) exists in two isoforms, ARI and ARII. We hypothesized that inhibiting AR with l-valine (l-Val) would increase NO production in bovine pulmonary arterial endothelial cells (bPAEC), bPAEC were grown to confluence in either regular medium (EGM; control) or EGM with lipopolysaccharide and tumor necrosis factor-α (L/T) added. Treatment of bPAEC with L/T resulted in greater ARI protein expression and ARII mRNA expression than in control bPAEC. Treatment of bPAEC with L/T increased urea production and NO production, in both control and L/T-treated bPAEC. In a second set of experiments, control and L/T bPAEC were grown in EGM, EGM with 30 mM l-Val, EGM with 20 mM l-Arg, or EGM with both 10 mM l-Arg and 30 mM l-Val. In both control and L/T bPAEC, treatment with l-Val decreased urea production and increased NO production. Treatment with l-Arg increased both urea and NO production. The addition of the combination l-Arg and l-Val decreased urea production compared with the addition of l-Arg alone and increased NO production compared with l-Val alone. These data suggest that competition for intracellular l-Arg by AR may be involved in the regulation of NOS activity in control bPAEC and in response to L/T treatment.

l-Arginine (l-Arg) is the substrate for both nitric oxide synthase (NOS) and arginase (AR). Metabolism of l-Arg by NOS produces l-citrulline and nitric oxide (NO), whereas metabolism of l-Arg by AR produces l-ornithine and urea. There are three described isoforms of NOS, neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS), of which iNOS and eNOS are abundantly expressed in endothelial cells (1, 19). We have previously found that treatment of bovine pulmonary arterial endothelial cells (bPAEC) with l-Val alone and increased NO production compared with l-Val alone. These data suggest that competition for intracellular l-Arg by AR may be involved in the regulation of NOS activity in control bPAEC and in response to L/T treatment.

l-Arginine; l-ornithine; l-citrulline; l-arginine; l-valine

METHODS

Pulmonary Arterial Endothelial Cell Culture

bPAEC were cultured as previously described (19). Briefly, bPAEC were obtained from Clonetics (San Diego, CA). Upon arrival bPAEC were placed in T-25 flasks with 5 ml of endothelial growth media (EGM, Clonetics), which contains ~250 μM l-Arg. When the bPAEC were 80–90% confluent, the bPAEC were passaged with trypsin-EDTA followed by trypsin-neutralizing solution. The bPAEC

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were centrifuged at 1,200 g for 5 min, and the bPAEC pellet was resuspended in EGM. Nine milliliters of EGM were placed in a T-75 flask, and then 1 ml of the resuspended bPAEC pellet was added, and the T-75 flask was returned to the incubator at 37°C in 5% CO2-balance air. bPAEC between passages 3 and 8 were used for these studies.

On the day of study, the bPAEC were washed three times with 4 ml of HEPES balanced salt solution (HBSS, Clonetics). Then 5 ml of EGM were placed on the bPAEC (control), and the bPAEC were returned to the incubator at 37°C in 5% CO2-balance air for 24 h. In the LT-treated bPAEC, 0.5 µg/ml LPS (Sigma Chemical, St. Louis, MO) and 0.5 ng/ml TNF-α (Sigma Chemical) were included in the EGM as previously described (18). After 24 h, the medium was removed, stored in 1-ml aliquots, and frozen at −70°C. The bPAEC were washed three times with 4 ml of HBSS and treated with either lysis buffer for protein extraction or TRIzol (Life Technologies) for RNA isolation.

**bPAEC Protein Isolation**

Protein was isolated from the bPAEC as previously described (19). Briefly, bPAEC were washed with HBSS, and 750 µl of lysis buffer (0.2 M NaOH, 0.2% SDS) were added to each flask. Thirty minutes before use, the following protease inhibitors were added to each milliliter of lysis buffer: 0.2 µl aprotinin [10 mg/ml double distilled (dd) H2O], 0.5 µl leupeptin (10 mg/ml ddH2O), 0.14 µl pepstatin A (5 mg/ml methanol), and 5 µl of phenylmethylsulfonyl fluoride (34.8 mg/ml methanol). This was sterile filtered in a syringe and added to each T-75 flask of bPAEC. The bPAEC were scraped and placed in sterile centrifuge tubes on ice. The supernatant was stored in 1-ml aliquots, and frozen at −70°C. The bPAEC were washed three times with 4 ml of HBSS and treated with either lysis buffer for protein extraction or TRIzol (Life Technologies) for RNA isolation.

**bPAEC RNA Isolation**

RNA was isolated from the bPAEC as previously described (19). Briefly, 1 ml of TRIzol (Life Technologies) was added to the flask containing the bPAEC and incubated for 5 min at room temperature. Chloroform (0.2 ml) was added, and the tubes were shaken for 15 s and then incubated at room temperature for 3 min. The mixture was centrifuged at 12,000 g for 15 min at 4°C. The supernatant was transferred to a fresh 15-ml tube. Isopropanol alcohol (0.5 ml) was added, and the mixture was incubated at room temperature for 10 min and then centrifuged at 12,000 g for 15 min at 4°C. The supernatant was discarded, and the pellet was washed with 75% ethanol and centrifuged at 7,500 g for 5 min at 4°C. The supernatant was discarded, and the pellet was partially dried, dissolved in RNAse-free water, and stored at −70°C.

**Nitrite Assay**

The EGM samples were assayed in duplicate for nitrite (NO2−) with a chemiluminescence NO analyzer (model 270B; Sievers Instruments, Boulder, CO) as previously described (19, 23). Briefly, 100 µl of sample were placed in a reaction chamber containing a mixture of NaCl in glacial acetic acid to reduce NO2− to NO. The NO gas was carried into the NO analyzer by a constant flow of He gas. The analyzer was calibrated with an NaNO2 standard curve.

**Nitrite/Nitrate Assay**

EGM samples were assayed in duplicate for nitrite/nitrate (NO3−) using a chemiluminescence NO analyzer (model 280, Sievers Instruments) as previously described (18). Briefly, 100 µl of sample were injected into a reaction chamber containing a mixture of vanadium (III) chloride in 2 M HCl heated to 90°C to reduce NO3− to NO gas. The NO gas was carried into the analyzer by a constant flow of He gas. The analyzer was calibrated with an NaNO3 standard curve.

**Urea Assay**

The EGM samples were assayed in duplicate for urea colorimetrically as previously described (18, 19, 23). Briefly, 100 µl of sample were added to 3 ml of chromogenic reagent [5 mg thiosemicarbazide, 250 mg diacetyl monoxime, and 37.5 mg FeCl3 in 150 ml 25% (vol/vol) H2SO4 and 20% (vol/vol) H3PO4] or the same reagents with 0.5 units of urease added. After 1 h at 37°C, the mixtures were vortexed and then boiled at 100°C for 5 min. The mixtures were cooled to room temperature, and the difference in absorbance (530 nm) with and without urease was determined and compared with a urea standard curve.

**[3H]-Arg Uptake**

After a 24-h incubation in either EGM or LT, the bPAEC were washed three times with HBSS, and [3H]-Arg uptake was measured as previously described (19). Briefly, 4 ml of HBSS with 1 µCi/ml of [3H]-Arg were plated on the bPAEC in the T-75 flask. Two 100-µl samples of the [3H]-Arg/HBSS were placed in scintillation counting cocktail and placed in a scintillation counter. After 10 min, the [3H]-Arg was removed, and the bPAEC were washed three times with ice-cold HBSS. Lysis buffer (300 µl) was added to the bPAEC in the T-75 flask and incubated at room temperature overnight. Then 100-µl samples of lysed bPAEC sample were placed in the scintillation counting cocktail and placed in a scintillation counter.

**Western Blotting**

The lysed bPAEC were assayed for ARI protein by Western blot analysis as previously described (18, 19). Aliquots of cell lysate were diluted 1:1 with SDS sample buffer, heated to 80°C for 15 min, and then centrifuged at 10,000 g at room temperature for 2 min. Aliquots of the supernatant were used for SDS-polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene difluoride membranes and blocked overnight in phosphate-buffered saline with 0.1% Tween (PBS-T) containing 5% nonfat dried milk and 3% albumin. The membranes were then incubated with the primary antibody ARI (1:1,000; Transduction Laboratories) for 4 h and then washed with PBS-T with 1% nonfat dried milk. The membranes were then incubated with the biotinylated IgG secondary antibody (1:5,000; Vector Laboratories) for 1 h, washed, and then incubated with streptavidin-horseradish peroxidase conjugate (1:1,500; Bio-Rad) for 30 min. The bands for ARI were visualized by chemiluminescence (Amersham ECL) and quantified by densitometry (Sigma Gel, Jandel Scientific, Corte Madera, CA). The Authentic ARI (Transduction Laboratories) was used as a positive control. To control for protein loading, the blots were then stripped with a stripping buffer (each 100 ml contained 6.25 ml 1 M Tris-HCl, pH 6.8, 20 ml 10% SDS, 0.7 ml 2-mercaptoethanol, and 73 ml ddH2O). The blots were reprobed for β-actin (1:10,000; Abcam) as described above.

**RT-PCR**

RT-PCR was performed as previously described (18, 19). Briefly, 2 µg of total RNA were reversed transcribed in 2.5 µM dT16 (Applied Biosystems), 20 units AMV-RT, 1 mM dNTP, 1× buffer (Promega), and balance RNAse-free water, with a total volume of 40 µl. The samples were incubated in a PCR-iCycler (Bio-Rad) at 42°C for 60 min, 95°C for 5 min, and stored at −20°C. Multiplex PCR for the expression of the ARII gene was internally standardized by direct comparison to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression in the same reaction. PCR reactions (total volume of 50 µl) contained 5 µl of RT product, 1 mM MgCl2, 1.25 units AmpliTaqGold (Applied Biosystems), 0.2 mM dNTP (Promega), and 15 mM forward (5′-TTGTTGTTAGCTGGTTGATGC-3′) and reverse (5′-TGCTTTCTCGATAAGTCAGTC3′) primers for ARII. The mixed samples were heated to 94°C for 4 min and then cycled as follows: 94°C for 1 min, 53°C for 1 min, and 72°C for 2 min for 17
cycles, and then 15 μM of forward (5’-GAAGACTGTTGATGCCCCTC-3’) and reverse (5’-GTTGAGGGCAATGGCACAGCCCTC-3’) primers for GAPDH were added to each sample and allowed to run for an additional 18 cycles. The PCR products were visualized and sized by 2.0% agarose gel electrophoresis and poststained with Syber Gold (Molecular Probes) for 30 min. The gels were scanned with a MultiGenticus Bio Imaging System (Syngene), and band density analysis was performed on a personal computer with SigmaGel (Jandel Scientific) software. The PCR product sizes were the expected 422 and 356 bp for ARII and GAPDH, respectively. Preliminary PCR reactions run at various total cycle numbers between 20 and 45 demonstrated that 35 total cycles was well within the linear range for each reaction product.

**Experimental Protocols**

**Time-dependent NO and urea production.** We have previously shown that L/T treatment of bPAEC increased both NO and urea production (19). Therefore in the first set of experiments, we determined the 24-h time course of NO₂⁻ and urea production in bPAEC. Immediately after placing 5 ml of EGM or EGM containing L/T on bPAEC, we removed a 0.5-ml sample of the medium and placed the control (n = 6) and L/T (n = 6) bPAEC in an incubator. At 2, 4, 6, 8, and 24 h, 0.5-ml samples of medium were removed; after the 24-h sample was removed, protein was isolated as described above. The samples were assayed for NO₂⁻ and urea production.

NO₂⁻ vs. NOX in medium. To determine the concentrations of NO₂⁻ and NOX in the medium from bPAEC we did the following study. Immediately after placing 5 ml of EGM containing L/T on bPAEC (n = 3), we obtained a 0.5-ml sample of medium and placed the bPAEC in an incubator. At 6 and 24 h, 0.5-ml samples of medium were removed. The samples were assayed for NO₂⁻ and NOX concentrations as described above.

**Effect of L/T on ARI and ARII expression.** In these experiments, the effect of L/T treatment on ARI protein expression (n = 3 in each group) and ARII mRNA expression (n = 8 in each group) was determined. Control and L/T bPAEC were incubated for 24 h and washed, and either the protein was extracted or the RNA was extracted as described above. We used a commercially available antibody against ARI (Transduction Laboratories). There is no commercially available antibody directed against ARII at this time; therefore, we determined changes in ARII mRNA expression.

**Concentration-dependent effect of l-Val on NO production by bPAEC.** In these experiments the effect of increasing concentrations of l-Val on NO and urea production was determined in control (n = 4 for each concentration) and L/T-treated bPAEC (n = 4 for each concentration). Control and L/T bPAEC were incubated for 24 h in EGM containing 1 mM l-Arg and 3, 10, 30, or 100 mM l-Val added to the medium. We chose a concentration of 1 mM l-Arg to approximate plasma l-Arg concentration (6, 13). The l-Val concentrations were chosen given that l-Val is a competitive inhibitor of AR. The concentration of l-Val on NO and urea production was determined in control (n = 10 for each treatment) and L/T (n = 10) bPAEC. BPAEC were incubated for 24 h as described above with vehicle, 30 mM l-Val, 10 mM l-Arg, or both 30 mM l-Val and 10 mM l-Arg added to the medium. The medium was collected and assayed for NO₂⁻ and urea production. These experiments were repeated (n = 5 for each treatment and group) with another competitive inhibitor of AR, l-nornorvaline.

**Effect of l-Val on l-Arg uptake by bPAEC.** We have previously shown that L/T increases l-Arg uptake in bPAEC and that both urea and NO production depend on l-Arg uptake (19). Thus to determine whether l-Val had any effect on l-Arg uptake, we did the following studies. First, the effect of inhibiting l-Arg uptake on urea production was determined. Two sets of L/T bPAEC (n = 4 for each l-Arg concentration) were incubated for 24 h with 0.3, 1, 3, or 10 mM l-Arg added to the medium. In one set, 30 mM l-leucine (a competitive inhibitor of transporter-dependent l-Arg uptake) was included in the medium. In the second set, the vehicle for l-leucine was included in the medium. The medium was collected and assayed for urea production. In a separate set of experiments after a 24-h incubation with L/T, the uptake of [³H]-l-Arg was measured as described above, in the presence of vehicle, 30 mM l-Val, 10 mM l-Arg, or both 30 mM l-Val and 10 mM l-Arg (n = 3 for each group) added to the medium.

**Statistical Analysis**

Values are means ± SE. One-way analysis of variance was used to compare the densitometry data between control and L/T bPAEC and to compare the effect of the additives on either NO₂⁻ or urea production. Significant differences were identified by a Newman-Keuls post hoc test. Differences were considered significant when P < 0.05.

**RESULTS**

**Time-dependent NO and Urea Production**

There was a time-dependent increase in NO₂⁻ production in both control and L/T-treated bPAEC (Fig. 1A). There was no difference in NO₂⁻ production in control or L/T-treated bPAEC at 2 h of incubation; however, by 4 h of incubation with L/T, there was greater NO₂⁻ production than in control bPAEC. Although, the 24-h time course of NO₂⁻ production was not exactly linear, there was continued NO₂⁻ production throughout the 24-h incubation, such that a straight line could be fit to the data (Fig. 1A, r = 0.991 for control and r = 0.989 for L/T bPAEC). The slope of the regression line approximated the NO₂⁻ production rate in these bPAEC, and the NO₂⁻ production rate was ~0.19 nmol·mg protein⁻¹·h⁻¹ in control bPAEC and ~0.32 nmol·mg protein⁻¹·h⁻¹ in L/T (different from control, P < 0.05). There were no significant differences in the y-intercepts (0.27 for control bPAEC and 0.44 for L/T bPAEC).

There was also a time-dependent increase in urea production in both control and L/T-treated bPAEC (Fig. 1B). There was no difference in urea production in control or L/T-treated bPAEC at 2 or 4 h of incubation; however, by 6 h of incubation with L/T there was greater urea production than in control bPAEC. Although the 24-h time course of urea production may not be exactly linear, there was continued urea production throughout the 24-h incubation, such that a straight line could be fit to the data (Fig. 1B, r = 0.99 for control and r = 0.99 for L/T bPAEC). The slope of the regression line approximated the urea production rate in these bPAEC, and the urea production rate was ~21 nmol·mg protein⁻¹·h⁻¹ in control bPAEC and ~46 nmol·mg protein⁻¹·h⁻¹ in L/T (different from control, P < 0.02). There were no significant differences in the y-intercepts (7 for control bPAEC and ~5 for L/T bPAEC). Because the increase in NO₂⁻ production occurred at ~4 h and was maintained for 24 h, and the increase in urea production occurred at ~6 h and was maintained for 24 h, therefore, 24-h
The urea production vs. time curve was fitted for the incubation period. The NO₂ increased with time (Fig. 2). The increase in NO₂ line was a measure of NO production. Therefore, to improve the sensitivity of our measurements for NO production, we measured NO₂ production at 24 h. In these bPAEC NO₂ concentrations were employed in the remainder of the experiments.

Effect of L/T on ARI and ARII Expression

Consistent with our previous study (19), the treatment of bPAEC with L/T resulted in significantly greater NO₂ production than in control bPAEC (2.05 ± 0.13 nmol/24-h vs. 1.60 ± 0.09 nmol/24-h control, n = 10, P < 0.01) and a significantly greater urea production than in control bPAEC (161 ± 30 nmol/24-h L/T vs. 43 ± 20 nmol/24-h control, n = 10, P < 0.01). The greater urea production in the L/T bPAEC was associated with a significantly greater ARI protein expression in L/T-treated bPAEC than in control bPAEC (Fig. 3). The expression of ARII mRNA was also significantly greater in L/T-treated bPAEC than in control bPAEC (Fig. 4). Thus L/T increased the expression of both ARI and ARII in these bPAEC.

Concentration-dependent Effect of l-Val on NO Production by bPAEC

As expected, the addition of l-Val to the incubation medium of control bPAEC resulted in inhibition of urea production.
with 10, 30, and 100 mM l-Val resulting in undetectable urea production (Fig. 5A). The inhibition of urea production in the control bPAEC was associated with a concentration-dependent increase in NO\(_2\)\(_\text{\textsuperscript{-}}\) production (Fig. 5A). The increase in NO\(_2\)\(_\text{\textsuperscript{-}}\) production was seen even though urea production was undetectable; this is probably due to the chemiluminescence NO\(_2\) assay being more sensitive than the colorimetric urea assay. In L/T-treated bPAEC, the urea production with 3 mM l-Val added to the incubation medium was significantly greater than in control bPAEC (142 ± 31 nmol/24-h L/T vs. 9.5 ± 8.0 nmol/24-h control, \textit{P} < 0.05). In the L/T-treated bPAEC, l-Val caused a concentration-dependent inhibition of urea production with 100 mM l-Val resulting in undetectable urea production (Fig. 5B). This inhibition of urea production in L/T-treated bPAEC was associated with a concentration-dependent increase in NO\(_2\)\(_\text{\textsuperscript{-}}\) production (Fig. 5B).

To exclude an effect of l-Val on the L/T-induced expression of NOS, we studied control bPAEC and L/T bPAEC after a 2-h incubation, a time point chosen to eliminate L/T-induced increases in NO\(_2\) or urea production. NO\(_2\)\(_\text{\textsuperscript{-}}\) production was increased only in the L/T + l-Val-treated bPAEC incubated for 2 h (NO\(_2\)\(_\text{\textsuperscript{-}}\) production: 1.68 ± 0.13 nmol/mg protein EGM, 2.12 ± 0.15 nmol/mg protein L/T, and 3.49 ± 0.13 nmol/mg protein L/T + l-Val; L/T + l-Val different from other two groups, \textit{P} < 0.005). Urea production was decreased to undetectable levels in the L/T + l-Val bPAEC incubated for 2 h (urea production: 116 ± 11 nmol/mg protein EGM, 151 ± 20 nmol/mg protein L/T, and undetectable in L/T + l-Val; L/T + l-Val different from other two groups, \textit{P} < 0.005).

**Effect of l-Val, l-Arg, or both l-Arg and l-Val on NO Production by bPAEC**

In control bPAEC, as expected, the addition of 30 mM l-Val to the incubation medium resulted in significantly less urea production than in vehicle-treated bPAEC (Fig. 6A). The decrease in urea production was associated with a significantly greater NO\(_2\)\(_\text{\textsuperscript{-}}\) production (Fig. 6B). The addition of 10 mM l-Arg to the incubation medium resulted in significantly greater urea and NO\(_2\)\(_\text{\textsuperscript{-}}\) production than in vehicle-treated bPAEC (Fig. 6, A and B). The addition of both 30 mM l-Val and 10 mM l-Arg resulted in significantly less urea production than the addition of 10 mM l-Arg alone (Fig. 6A), whereas the NO\(_2\)\(_\text{\textsuperscript{-}}\) production was significantly more than with the addition of 30 mM l-Val alone (Fig. 6B). In the L/T-treated bPAEC a similar pattern of change in urea (Fig. 7A) and NO\(_2\)\(_\text{\textsuperscript{-}}\) production (Fig. 7B) was seen, although the NO\(_2\)\(_\text{\textsuperscript{-}}\) and urea production were greater than in control bPAEC. Substituting l-norvaline for l-Val in these experiments led to a similar pattern of response as described above for both control and L/T-treated bPAEC (data not shown).

**Effect of l-Val on l-Arg Uptake by bPAEC**

Figure 8 demonstrates the effect of inhibiting l-Arg uptake on urea production in L/T-treated bPAEC. In L/T-treated bPAEC, the addition of l-Arg led to a concentration-dependent increase in urea production. Including 30 mM l-leucine in the medium resulted in a lower urea production at each l-Arg concentration than in L/T bPAEC without l-leucine included in the medium (Fig. 8). The effect of l-Val on NO\(_2\)\(_\text{\textsuperscript{-}}\) and urea production in L/T-treated bPAEC was not due to inhibition of l-Arg uptake as demonstrated in Fig. 9. There was no significant difference in \([\text{^3H}]\text{l-Arg}\) uptake in L/T bPAEC with either vehicle or 30 mM l-Val added to the medium. However, as expected, addition of 10 mM nonlabeled l-Arg nearly completely inhibited \([\text{^3H}]\text{l-Arg}\) uptake, and this was true when 10 mM unlabeled l-Arg and 30 mM l-Val were added together (Fig. 9).
DISCUSSION

The main findings of this study were that 1) L/T treatment increased ARI protein expression and increased ARII mRNA expression, 2) the addition of L-Val to the media resulted in a concentration-dependent decrease in urea production and a concentration-dependent increase in NO production in both control and L/T-treated bPAEC, 3) the addition of both L-Arg and L-Val to the medium had a greater effect on NO production than did L-Val alone, and 4) L-Val did not affect L-Arg uptake by bPAEC. This suggests that L/T increased urea production, at least in part, by increased expression of ARI and ARIII and that inhibition of AR results in an increase in NO production via an increase in bioavailability of L-Arg to NOS. Together these data support our hypothesis and suggest that NOS and AR compete for a common pool of intracellular L-Arg.

Treatment with L/T resulted in an increase in NO\textsubscript{2} production starting at ~4 h and an increase in urea production starting at ~6 h. Therefore, it appears that both NOS and AR were upregulated in these bPAEC relatively rapidly. Furthermore, the bPAEC continued to produce both NO and urea throughout the 24-h incubation period, as shown by the reasonable fit of the NO\textsubscript{2} and urea production vs. time data by linear regression. This suggests that the necessary cofactors and substrates were available to NOS throughout the 24-h incubation period in both control and L/T-treated bPAEC. Therefore, the changes in NO\textsubscript{2} and urea production found with inhibition of AR (L-Val), addition of substrate (L-Arg), or inhibition of L-Arg uptake (L-leucine) most likely reflect enzymatic interactions rather than nonspecific effects.

Inhibiting L-Arg uptake with L-leucine decreased urea production. L/T-treated bPAEC were incubated with increasing concentrations of L-Arg with either vehicle (solid bars) or 30 mM L-leucine (open bars) added to the medium. Increasing extracellular L-Arg increased urea production (*different from 0.3 mM L-Arg, P < 0.05). The addition of 30 mM L-leucine significantly inhibited the L-Arg-induced increased urea production (#30 mM L-leucine different from vehicle at same L-Arg concentration, P < 0.01).

Fig. 6. A: effect of the addition of vehicle, L-Val, L-Arg, or both L-Val and L-Arg on urea production in control bPAEC. The addition of 30 mM L-Val tended to decrease urea production, the addition of 10 mM L-Arg increased urea production, and the addition of both decreased urea production, although urea production was greater than with vehicle (*different from vehicle, P < 0.05; #different from L-Val, P < 0.05). B: effect of the addition of vehicle, L-Val, L-Arg, or both L-Val and L-Arg on NO production in control bPAEC. The addition of 30 mM L-Val or 10 mM L-Arg increased NO production compared with vehicle treatment. The addition of both increased NO production compared with vehicle and L-Val (*different from vehicle, P < 0.05; #different from L-Val, P < 0.05).

Fig. 7. A: effect of the addition of vehicle, L-Val, L-Arg, or both L-Val and L-Arg on urea production in L/T-treated bPAEC. The addition of 30 mM L-Val decreased urea production, the addition of 10 mM L-Arg increased urea production, and the addition of both decreased urea production, such that urea production with both was no different from urea production with vehicle (*different from vehicle, P < 0.05; #different from L-Val, P < 0.05). B: effect of the addition of vehicle, L-Val, L-Arg, or both L-Val and L-Arg on NO production in L/T-treated bPAEC. The addition of 30 mM L-Val or 10 mM L-Arg increased NO production compared with vehicle treatment. The addition of both increased NO production compared with vehicle and L-Val (*different from vehicle, P < 0.05; #different from L-Val, P < 0.05).

Fig. 8. Inhibiting L-Arg uptake with L-leucine decreased urea production. L/T-treated bPAEC were incubated with increasing concentrations of L-Arg with either vehicle (solid bars) or 30 mM L-leucine (open bars) added to the medium. Increasing extracellular L-Arg increased urea production (*different from 0.3 mM L-Arg, P < 0.05). The addition of 30 mM L-leucine significantly inhibited the L-Arg-induced increased urea production (#30 mM L-leucine different from vehicle at same L-Arg concentration, P < 0.01).
NO can be oxidized to NO$\textsuperscript{2-}$, and NO$\textsuperscript{2-}$ can be further oxidized to NO$\textsuperscript{3-}$. It is of interest to note that the NO$\textsuperscript{2-}$ concentration was \(\sim 25\%\) of the total NOX concentration in the medium after 24 h. This finding is consistent with a report by Ignarro et al. (11), wherein they found that, although in an oxygenated buffer solution the principal oxidation product of NO was NO$\textsuperscript{2-}$, in the unpurified cytosolic fractions from rat cerebellum the molar ratio of NO$\textsuperscript{2-}$/NO$\textsuperscript{3-}$ was \(\sim 0.25\). Furthermore, in the bPAEC in this study, there was a sizable NOX concentration in the medium at time 0, \(\sim 48\%\) of the NOX concentration at 24 h, whereas the concentration of NO$\textsuperscript{2-}$ in the medium from the bPAEC at time 0 was negligible, only \(\sim 12\%\) of the 24-h medium NO$\textsuperscript{3-}$ concentration. Thus to improve sensitivity we employed measurement of the NO$\textsuperscript{2-}$ concentration as a marker of NO production rather than measurement of the NOX concentration.

Treatment with L/T increased urea production, ARI protein expression, and ARII mRNA expression in bPAEC in our study. These findings suggest that L/T treatment increased urea production by increasing the expression of AR and are in agreement with previous studies. For example in rat aortic endothelial cells, LPS and IFN-\(\gamma\) treatment increased urea production, had no effect on ARI mRNA expression, and increased ARII activity (1). Similarly, hyperoxic exposure increased AR activities and expression in the lungs of rats (21). In macrophages, treatment with LPS resulted in increased ARI and ARII protein expression (5, 13, 17). LPS treatment of mice increased AR activities, protein, and mRNA expression in the lungs (22). The role of increased urea production in the bPAEC is unclear. If bPAEC in culture are representative of in vivo conditions, then the increased AR expression and activity may be involved in the formation of polyamines and/or L-proline from L-ornithine, which are important in tissue healing after injury (10, 12a, 12b). Consistent with this concept is the finding in murine macrophages that T helper (Th) 2 cytokines (IL-4 and IL-10) are potent inducers of AR, whereas a Th1 cytokine (IFN-\(\gamma\)) is a potent inducer of iNOS (16). Similarly, in mouse peritoneal exudate cells, the growth factor transforming growth factor-\(\beta\) attenuated IFN-\(\gamma\)-induced increased NOS activity and resulted in increased AR activity (24). Thus together these studies suggest that AR induction leading to increased urea production may be associated with tissue repair in some conditions.

We found that L/T treatment increased both urea and NO production. It has been suggested that the co-induction of AR with iNOS may limit l-Arg bioavailability to iNOS and thereby serve to decrease NO production (3, 4, 25, 27, 28). On the other hand, Buga et al. (1) found in rat aortic endothelial cells that when NO production was increased \(\sim 20\)-fold by LPS/IFN-\(\gamma\) treatment, AR activity was inhibited. The authors found that this effect was due to the intermediate in NO production from l-Arg, \(N^a\)-hydroxy-l-Arg. The \(K_i\) for \(N^a\)-hydroxy-l-Arg inhibition of AR was \(\sim 10\) \(\mu\)M. Similarly, Waddington et al. (26) found in macrophages that \(N^a\)-hydroxy-l-Arg inhibited AR activity at concentrations of 20 and 200 \(\mu\)M but not at a concentration of 2 \(\mu\)M. Because the levels of NO produced in our culture media were \(\sim 1–6\) \(\mu\)M and because treatment with l-Val decreased urea production and increased NO production even in the L/T-treated bPAEC, it is unlikely that the levels of \(N^a\)-hydroxy-l-Arg produced would be sufficient to inhibit urea production. Therefore, in situations where both AR and iNOS are induced, the degree of iNOS induction may determine whether AR activity will limit l-Arg bioavailability to iNOS or whether enough \(N^a\)-hydroxy-l-Arg will be produced to inhibit AR and thereby increase l-Arg bioavailability to iNOS. Further studies are needed to examine the cellular mechanisms involved in iNOS and AR induction and the interrelationship between iNOS and AR activities during inflammation.

In this study we found that inhibiting AR increased NO production. This finding is consistent with a recent study in coronary microvascular endothelial cells, wherein NO production in response to serotonin or adenosine was significantly enhanced by inhibition of AR (28). In LPS-activated macrophages, inhibition of AR with l-norvaline decreased urea production and increased NO production, and l-norvaline resulted in a concentration-dependent increase in NO production (3), which is similar to what we found in bPAEC in this study. In macrophages, treatment with LPS and IL-13 induced both ARI and iNOS protein expression and increased urea and NO production; furthermore, treatment of these macrophages with l-norvaline increased NO production (4). Together these results demonstrate that inhibiting AR activity in a wide variety of cell types increases NO production.

There is no evidence that a product of AR activity inhibits NOS; therefore, the effect of AR inhibition on NO production suggests that AR and NOS compete for intracellular l-Arg. Considering that AR is the major l-Arg metabolic pathway in bPAEC, a decrease in AR activity might be expected to result in an increase in the intracellular concentration of l-Arg. However, when one considers that the l-Arg \(K_m\) for the various NOS isoforms is \(\sim 3–10\) \(\mu\)M and that the intracellular concentration of l-Arg in cultured endothelial cells is \(\sim 100–800\) \(\mu\)M (6, 13), it seems unlikely that the changes in l-Arg concentration from inhibition of AR would contribute significantly to l-Arg bioavailability to NOS. However, we (19, 23) and others (1, 3, 7) have previously shown that, despite an l-Arg \(K_m\) of \(\sim 3–10\) \(\mu\)M for the various NOS isoforms, increasing the extracellular concentration of l-Arg increases NO production. This phenomenon has been termed the l-Arg paradox (15). Thus a reasonable interpretation of our findings in light of these studies is that despite the l-Arg \(K_m\) for isolated NOS isoforms,
AR and NOS do indeed compete for their common substrate L-Arg. Further support for the concept that AR and NOS compete for intracellular L-Arg comes from experiments wherein AR is overexpressed in cells. For example in HEK-293 cells with stable overexpression of nNOS, transfection with ARI decreased NO production, and the addition of 1 mM L-Arg to the culture medium restored NO production (20). Furthermore, transfection of coronary venular endothelial cells with either ARI or ARII increased urea production and decreased NO production (12a). Similarly, transfection of J774A.1 cells with ARI increased LPS-induced urea formation and decreased LPS-induced NO production compared with nontransfected cells, and NO production increased when the transfected ARI was inhibited with L-norvaline (2). Together these studies suggest that AR and NOS compete for intracellular L-Arg, such that increasing AR activity leads to a decrease in NO production, whereas decreasing AR activity leads to an increase in NO production. Thus changes in AR activities may be involved in the cellular regulation of NO production.

Extracellular L-Arg is involved in both NO and urea production, since increasing the extracellular L-Arg concentration increased both NO and urea production, and inhibition of L-Arg uptake decreased urea production. An increase in urea production when the extracellular L-Arg concentration is increased from 0.3 to 10 mM is consistent with the L-Arg Km for AR of ~1–3 mM (14). Although the increase in bPAEC NO production with the addition of 10 mM L-Arg to the medium is consistent with our previous findings (19) and has been described by others (1, 7) in endothelial cells, it is more difficult to explain on a purely biochemical basis, since as discussed above the L-Arg Km for the NOS isoforms is ~3–10 μM. It has been suggested that the L-Arg paradox may involve the association of the cationic amino acid transporter-1 (CAT-1) with eNOS in the cell membrane, such that eNOS preferentially utilizes extracellular L-Arg (15). However, in this case, one would expect that inhibiting intracellular ARI would have little effect on NO production. Thus although association of eNOS and CAT-1 is involved in the L-Arg paradox, a significant portion of NO production in these endothelial cells also utilizes the same L-Arg pool as AR. Both ARI and iNOS are found in the cytosol, and they may occupy a common intracellular compartment (12a). Therefore, the effect of L-Val on NO production may represent an effect on this common intracellular compartment. However, further studies will need to be done to determine exactly which isoforms and which intracellular compartments utilize a common pool of L-Arg.

L-Arg uptake by endothelial cells has been found to be primarily by the CAT family of amino acid transporters (6, 14). In cultured endothelial cells ~70% of uptake occurs via CAT, whereas ~30% occurs via the Na⁺-dependent system b₀+/H⁺ amino acid transporters, also known as the broad-scope amino acid transport (BAT) proteins (6, 14). BAT proteins transport not only cationic amino acids like L-Arg but also neutral amino acids like L-Val (6). Thus to determine whether some of the effect of the addition of 30 mM L-Val to the medium was due to attenuation of L-Arg transport, we measured the uptake of [³H]L-Arg. L-Val had no significant effect on the uptake of [³H]L-Arg, suggesting that L-Val was taken up by bPAEC predominantly through mechanisms different from those responsible for L-Arg uptake. As expected, the addition of 10 mM unlabeled L-Arg nearly completely inhibited the uptake of [³H]L-Arg, and the small amount of [³H]L-Arg uptake in the presence of 10 mM unlabeled L-Arg represents nontransporter-dependent uptake (19), demonstrating that the majority of L-Arg is transported into bPAEC via a saturable process most likely representing CAT and/or BAT activities (6, 14, 19).

In conclusion, we found that treatment of bPAEC with L/T resulted in increased expression of ARI protein and ARII mRNA, as well as increased urea and NO production. Inhibition of AR with L-Val or L-norvaline led to a concentration-dependent increase in NO production. Both urea and NO production were increased by extracellular L-Arg, and the addition of both L-Arg and L-Val led to the greatest increase in NO production. L-Val is transported into bPAEC by a mechanism different from that which accounts for L-Arg uptake. Together these data demonstrate that AR and NOS compete for their common substrate, L-Arg, in bPAEC even under control conditions. We speculate that AR may represent a therapeutic target for the manipulation of NO production, particularly in cases of NO overproduction such as seen in inflammatory lung diseases.

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