Cytokine treatment increases arginine metabolism and uptake in bovine pulmonary arterial endothelial cells

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Nelin, Leif D., Heather E. Nash, and Louis G. Chicoine. Cytokine treatment increases arginine metabolism and uptake in bovine pulmonary arterial endothelial cells. Am J Physiol Lung Cell Mol Physiol 281: L1232–L1239, 2001.—L-Arginine (L-Arg) is metabolized to nitric oxide (NO) by NO synthase (NOS) or to urea by arginase (AR). L-Arg is transported into bovine pulmonary arterial endothelial cells (BPAECs) by cationic amino acid transporter-2 (CAT-2). We hypothesized that cytokine treatment would increase L-Arg metabolism and increase CAT-2 mRNA expression. BPAECs were incubated for 24 h in medium (control) or medium with lipopolysaccharide and tumor necrosis factor-α (L-T). L-T increased nitrite production (3.1 ± 0.4 nmol/24 h vs. 1.8 ± 0.1 nmol/24 h for control; \( P < 0.01 \) ) and urea production (83.5 ± 29.5 nmol/24 h vs. 17.8 ± 8.6 nmol/24 h for control; \( P < 0.05 \)). L-T-treated BPAECs had greater endothelial and inducible NOS mRNA expression compared with control cells. Increasing the medium L-Arg concentration resulted in increased nitrite and urea production in both the control and the L-T-treated BPAECs. L-T treatment increased L-Arg metabolism to both NO and urea in BPAECs and resulted in increased levels of CAT-2 mRNA. This suggests that induction of NOS and/or AR is linked to induction of CAT-2 in BPAECs and may represent a mechanism for maintaining L-Arg availability to NOS and/or AR.

urea; cationic amino acid transport; tumor necrosis factor; lipopolysaccharide; nitric oxide synthase; arginase

ENDOTHELIAL CELL-DERIVED NITRIC OXIDE (NO) has many important functions in the lungs including vasodilation and involvement in oxidative injury and as an inflammatory mediator (15). NO is synthesized from L-arginine (L-Arg) via NO synthase (NOS). There are three isoforms of NOS: type I or neuronal NOS (nNOS), type II or inducible NOS (iNOS), and type III or endothelial NOS (eNOS). The nNOS and eNOS isoforms are constitutively expressed and are calcium dependent (15). The iNOS isoform is induced in response to cytokines, is calcium independent, and produces greater quantities of NO than either nNOS or eNOS (15). The enzyme arginase (AR) also metabolizes L-Arg to produce urea and L-ornithine (L-Orn). In the lung, endothelial cell-derived L-Orn can be utilized in the synthesis of proline and polyamines, which are important in recovery from lung injury (8). There are two isoforms of AR: AR1, which is constitutively expressed in liver and many other organs, and AR2, which is inducible by cytokines (9). Because isoforms of both NOS and AR are inducible by cytokines and the production of NO (2, 15, 21) and urea (2, 9) by endothelial cells can be increased by cytokines, we used cytokine treatment as a model of increased L-Arg metabolism in the present study. This model mimics the inflammatory processes seen during pulmonary diseases such as acute asthma, acute respiratory distress syndrome (ARDS), and pneumonia.

Because cytokine treatment increases the metabolism of L-Arg by NO to NO and by AR to urea, an increase in L-Arg metabolism might deplete intracellular L-Arg unless L-Arg uptake is also increased by cytokines. L-Arg is transported into endothelial cells mainly via the system y+ amino acid transporters (1, 6). The genes encoding the system y+ transporters have been cloned and designated as cationic amino acid transporter (CAT)-1 and CAT-2 (7, 24, 29). The availability of L-Arg to NOS and AR may be a critical factor in NO and L-Orn production by cells. For example, we have previously found that both NO and urea production in rat alveolar macrophages can be increased by increasing the extracellular concentration of L-Arg (22). Thus the purpose of this study was to examine the hypothesis that cytokine treatment of bovine pulmonary arterial endothelial cells (BPAECs) would result in an increase in both L-Arg metabolism and L-Arg uptake. To test this hypothesis, we measured nitrite \((\text{NO}_2^-)\) and urea production as well as eNOS and iNOS protein levels in BPAECs that were incubated in normal medium or medium with lipopolysaccharide (LPS) and tumour necrosis factor-α (TNF-α) added. We also measured NO\(_2^-\) and urea in medium with increasing concentrations of L-Arg added. Finally, we measured the expression of CAT-2 mRNA and the uptake of L-Arg into BPAECs that were incubated in normal medium or medium with LPS and TNF-α added.

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METHODS

BPAEC culture. BPAECs were obtained from Clonetics (San Diego, CA). On arrival, BPAECs were placed in T-25 flasks with 5 ml of endothelial growth medium (EGM; Clonetics) that contained ~250 μM L-Arg. When the BPAECs were 80–90% confluent, the cells were passaged with trypsin-EDTA followed by a trypsin-neutralizing solution. The BPAECs were then centrifuged at 1,200 g for 5 min, and the pellet was resuspended in EGM. We placed 9 ml of EGM in a T-75 flask and added 1 ml of the resuspended BPAEC pellet. The resuspended BPAEC pellet was resuspended in EGM. We placed 9 ml of EGM in a T-75 flask and added 1 ml of the resuspended BPAEC pellet before returning the T-75 flask to the incubator at 37°C in 5% CO₂-95% air. We used BPAECs between passages 3 and 8 for these studies.

On the day of study, the BPAECs were washed three times with 4 ml of HEPES balanced salt solution (HBSS; Clonetics). EGM (5 ml) was then placed on the BPAECs (control), and the cells were returned to the incubator at 37°C in 5% CO₂-95% air for 24 h. In the cytokine-treated group, 0.5 μg/ml LPS (Sigma, St. Louis, MO) and 0.5 ng/ml TNF-α (L-T) were included in the EGM. In preliminary studies, we found that adding LPS alone at doses between 0.1 and 10 μg/ml had little effect on either NO₂⁻ or urea production; therefore, we used the combination of TNF-α and LPS. After 24 h, the medium was removed and stored in 1-ml aliquots frozen at −70°C. The BPAECs were then washed three times with 4 ml of HBSS. The cells were treated with either lysis buffer for protein extraction or TRIzol reagent (GIBCO BRL, Life Technologies) for RNA isolation.

BPAEC protein isolation. After the cells were washed three times with HBSS as described, 300 μl of lysis buffer (containing 0.4 ml of 10% SDS, 0.6 ml of 1 M Tris-HCl (pH 6.8), and 9.0 ml of distilled water) were added. The flask was shaken by hand for 3 min, during which time a thick mucous film developed. The flask was then scraped with a cell scraper, and 100-μl aliquots were frozen at −70°C for later Western blotting. One aliquot from each flask was reserved for total protein determination using a commercially available assay (Bio-Rad).

BPAEC RNA isolation. TRIzol reagent (1 ml) was added to the flask containing the BPAECs, which was then incubated for 5 min at 30°C. Chloroform (0.2 ml) was then added, and the tubes were shaken for 15 s and incubated at 30°C for 3 min. The mixture was centrifuged at 12,000 g for 15 min at 2°C before the supernatant was transferred to a fresh 15-ml tube. Isopropyl alcohol (0.5 ml) was added, and the mixture was incubated at 30°C for 10 min and then centrifuged at 12,000 g for 15 min at 2°C. The supernatant was then discarded, and the pellet was washed with 75% ethanol and centrifuged at 7,500 g for 5 min at 2°C. The supernatant was again discarded, and the pellet was partially dried, dissolved in RNase-free water, and stored at −70°C.

NO₂⁻ assay. In oxygenated medium, NO is rapidly oxidized to NO₃⁻. Therefore, EGM samples were assayed in duplicate for NO₂⁻ using a chemiluminescence NO analyzer (model 270B, Sievers Instruments, Boulder, CO) as previously described (5, 16). Briefly, 100 μl of sample were placed in a reaction chamber containing a mixture of NaI in glacial acetic acid to reduce NO₂⁻ to NO and allow the liberation of NO gas from the aqueous mixture. The NO gas was carried into the NO analyzer using a constant flow of nitrogen gas. The analyzer was calibrated using a NaNO₂ standard curve (5, 16).

Urea assay. The EGM samples were colorimetrically assayed in duplicate for urea as previously described (22). Each sample (100 μl) was added to 3 ml of chromogenic reagent (which contained 5 mg of thiosemicarbazide, 250 mg of diacetyl monoxime, 37.5 mg of FeCl₃ in 150 ml of 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 differences in absorbance (530 nm) with and without urease were determined and compared with a urea standard curve (22).

L-[³H]Arg uptake. After a 24-h incubation in either EGM or L-T, the BPAECs were washed three times with HBSS. To determine total L-[³H]Arg uptake, 4 ml of HBSS with 1 μCi/ml L-[³H]Arg were placed on the BPAECs in the T-75 flask. To determine the nonspecific uptake of L-[³H]Arg, additional BPAEC flasks had 4 ml of HBSS with 1 μCi/ml L-[³H]Arg and 10 mM nonlabeled L-Arg placed on the BPAECs in the T-75 flask. Two 100-μl samples of L-[³H]Arg-HBSS were placed in scintillation counting cocktail and inserted into a scintillation counter. After 15 min, the L-[³H]Arg was removed and the BPAECs were washed three times with ice-cold HBSS. Lysis buffer (300 μl) was added to the BPAECs in the T-75 flask and incubated at room temperature overnight. The lysis buffer and BPAECs were then placed in scintillation counting cocktail and inserted into a scintillation counter. Specific L-[³H]Arg uptake was determined by subtracting total L-[³H]Arg uptake from the nonspecific L-[³H]Arg uptake.

Western blotting. The lysed BPAECs were assayed for either eNOS or iNOS protein using Western blot analysis as previously described (5). Aliquots of cell lysate were diluted in a 1:1 ratio 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-PAGE. The proteins were transferred to polyvinylidene difluoride membranes and blocked overnight in phosphate-buffered saline with 0.1% Tween (PBS-T) containing 5 g of nonfat dried milk and 3 g of albumin. The membranes were incubated with the primary antibody, iNOS (1:500 dilution; Transduction Laboratories) or eNOS (1:1,500 dilution; Transduction Laboratories), for 4 h and then washed with PBS-T with 1% nonfat dried milk. The membranes were incubated with the biotinylated IgG secondary antibody (1:5,000 dilution; Vector Laboratories) for 1 h, washed, and incubated with streptavidin-horseradish peroxidase conjugate (1:1,500 dilution) for 30 min. The bands for iNOS and eNOS were visualized using chemiluminescence (ECL, Amersham) and quantified using densitometry (Sigma Gel, Jandel Scientific). Authentic iNOS and eNOS were used as positive controls.

RT-PCR. RT-PCR was performed as previously described (20). RT reactions (20 μl) contained 1.0 μg of total cellular RNA, 200 U of Moloney murine leukemia virus reverse transcriptase (PerkinElmer), 5 μM oligo(dT)₁₆, 1 mM deoxyribonucleotide triphosphates, and 3 mM MgCl₂. Reactions were incubated at room temperature for 10 min, at 42°C for 1 h, and then at 94°C for 5 min. PCRs contained 1.0 μM specific oligonucleotide primers for either eNOS or CAT-2. The eNOS primers were the previously described (20) forward 5'-TACGGAGGACCAATCCAC-3' and reverse 5'-CAGGTCGTACGCTCTTGTGAT-3'. The CAT-2 primers were the previously described (7) forward 5'-AAGGCTGTTATTTGACCCTTGTT-3' and reverse 5'-GGTGACCTGGACTGCTGACTCT-3'. Additional components of the PCR included 5.0 μl of RT product, 3 mM MgCl₂, PCR Buffer II (PerkinElmer), 0.2 mM deoxyribonucleotide triphosphates, and 2.5 U of AmpliTaq polymerase (PerkinElmer). PCRs were denatured at 94°C for 4 min and then cycled at 94°C for 1 min, 53°C for 1 min, and 72°C for 2 min for a total of 30 cycles. Final extension was 5 min at 72°C. PCR products were visualized and sized by 1%
agarose gel (0.5 mg/ml ethidium bromide) electrophoresis. Gels were photographed using Polaroid 667 film and digitized using an Epson 636 scanner. PCR product sizes were the expected 819 bp for eNOS and 613 bp for CAT-2.

Experimental protocols. The following experiment was performed to determine whether L-T treatment increased NOS and AR activities. Confluent control and L-T-treated BPAECs were incubated for 24 h, and the medium was sampled for NO₂⁻ and urea concentration. The BPAEC protein was harvested for Western blot analysis of eNOS and iNOS as well as for protein concentration. Because we found an increase in eNOS protein expression, we then performed RT-PCR for eNOS on mRNA isolated from control or L-T-treated BPAECs.

In separate experiments, the contribution of iNOS activity to total NO production was determined. Confluent control and L-T-treated BPAECs were incubated for 24 h with either 1) nothing additional added to the EGM, 2) 10 μM L-N⁶-(1-iminoethyl)lysine (L-NIL, a more selective iNOS inhibitor) added to the EGM, or 3) 10 μM L-NAME, a nonselective NOS inhibitor) added to the EGM. The medium was then sampled for NO₂⁻ concentration.

The contribution of extracellular L-Arg concentration to NOS and AR activities was determined in separate experiments. Confluent BPAECs were incubated for 24 h in control or L-T medium containing either 1, 3, 10, or 30 mM L-Arg. The medium was then sampled for NO₂⁻ and urea concentrations.

The expression of CAT-2 mRNA was determined in separate experiments. Confluent control and L-T-treated BPAECs were incubated for 24 h. mRNA was harvested from the BPAECs as described above, and RT-PCR was performed for CAT-2.

In separate experiments, the effect of L-T treatment on L-Arg uptake was determined. After a 24-h incubation, control and L-T BPAECs were washed with HBSS, and the 15-min uptake of L-[³H]Arg was measured as described (see L-[³H]Arg uptake).

Statistical analysis. Values are means ± SE. The production of NO₂⁻ and urea are expressed as nanomoles per 24 h. One-way ANOVA was used to compare control and L-T-treated groups, and significant differences were identified using a Newman-Keuls post hoc test. Differences were considered significant when P < 0.05.

RESULTS

The effect of L-T treatment on NO₂⁻ and urea production is shown in Fig. 1. L-T treatment resulted in a doubling of NO production (Fig. 1A) and a fourfold increase in urea production (Fig. 1B). There was no measurable production of either NO₂⁻ or urea when medium was incubated for 24 h without BPAECs. The protein concentrations did not differ between control BPAECs (0.67 ± 0.11 mg/ml) and L-T-treated BPAECs (0.69 ± 0.18 mg/ml), which suggests that the total number of BPAECs in each flask was similar. The number of BPAECs in each flask was also counted, and there was no difference between control BPAECs (3.75 × 10⁶ ± 0.20 × 10⁶ cells) and L-T-treated BPAECs (3.28 × 10⁶ ± 0.35 × 10⁶ cells). Thus NO and urea production were increased by L-T treatment in BPAECs.

The effect of L-T on eNOS protein levels is shown in Fig. 2. L-T treatment resulted in an increase in eNOS protein. This somewhat unexpected result led us to confirm the Western blot findings by performing RT-PCR for eNOS on RNA isolated from the BPAECs, and the results are shown in Fig. 3. L-T treatment resulted in the appearance of detectable eNOS mRNA bands. Control BPAECs probably have a steady-state eNOS mRNA level that is below the level of detection of our RT-PCR analysis. L-T treatment under the culture conditions of these experiments led to an increase in both eNOS protein and mRNA. The effect of L-T treatment on iNOS protein is shown in Fig. 4. L-T treatment resulted in an increase in iNOS protein. Thus the increase in NO production caused by L-T treatment was due to an increase in both eNOS and iNOS proteins.

Although L-T treatment increased both eNOS and iNOS proteins, the production of NO due to iNOS was a significant portion of the total NO production in the L-T-treated cells (as demonstrated in Fig. 5). Figure 5 represents the inhibition of NO production by L-NIL and L-NAME as a percent of the basal NO production. Both L-NIL and L-NAME had a greater effect in the L-T-treated cells, which suggests an increase in NO production by both eNOS and iNOS. However, the greater effect of L-NIL in the L-T-treated BPAECs suggests that NO produced by iNOS was a significant portion of the increased NO production caused by L-T treatment.

The production of NO could be influenced by the extracellular L-Arg concentration (as illustrated in Fig.
6). In both control and L-T-treated cells, increasing the extracellular L-Arg concentration resulted in a dose-dependent increase in NO production, and the effect was greater in L-T-treated BPAECs compared with control BPAECs. The production of urea could also be influenced by the extracellular L-Arg concentration, which is shown in Fig. 7. In both control and L-T-treated cells, increasing the extracellular L-Arg concentration resulted in an increase in urea production, and the effect was greater at 10 mM L-Arg in L-T-treated BPAECs compared with controls.

To determine whether the increase in L-Arg metabolism by the BPAECs was accompanied by an increase in mRNA expression for CAT-2, RT-PCR was performed for CAT-2 mRNA in control and L-T-treated BPAECs (Fig. 8). In control BPAECs, there was no detectable CAT-2 mRNA in any of the four experiments; however, in L-T-treated BPAECs, there was detectable CAT-2 mRNA in five of the six experiments performed.

To determine whether the increase in CAT-2 mRNA expression was associated with an increase in L-Arg uptake, the uptake of L-[3H]Arg was measured after a 24-h incubation in control and L-T-treated BPAECs. There was no difference in the nonspecific uptake of L-[3H]Arg between control and L-T-treated BPAECs (0.39 ± 0.01 vs. 0.36 ± 0.01 pmol). Figure 9 demonstrates that specific L-[3H]Arg uptake was significantly (P < 0.05) greater in L-T-treated BPAECs compared with control BPAECs. Taken together, the mRNA and L-[3H]Arg data suggest that the increase in L-Arg uptake was due, at least in part, to an increase in transporter number.

**DISCUSSION**

The main findings of this study were that 1) cytokine treatment increased L-Arg metabolism by both NOS and AR, 2) the increase in NO production was due to an increase in both eNOS and iNOS, 3) NO and urea production could be increased by increasing the extracellular concentration of L-Arg, 4) cytokine treatment increased CAT-2 mRNA, and 5) cytokine treatment increased L-Arg uptake in BPAECs. These findings support our hypothesis that cytokine treatment results in increased L-Arg metabolism by both NOS and AR as well as increased uptake of extracellular L-Arg in BPAECs. The mRNA results suggest that the increase in L-Arg uptake was due, at least in part, to an increase in CAT-2 transporter numbers.

We demonstrate for the first time that the treatment of BPAECs with LPS and TNF-α increased the mRNA for CAT-2. Furthermore, this finding suggests that L-T...
induced an increase in CAT-2 transporter numbers in BPAECs. This is consistent with findings in cardiac microvascular endothelial cells where treatment with interleukin-1β (IL-1β) and interferon-γ led to increased CAT-2 mRNA expression (25). CAT-2 has been shown to consist of two subgroups: CAT-2A and CAT-2B. CAT-2B clones are consistent with the system y⁺ transporter (7, 24, 25), whereas CAT-2A clones represent a phenotype consistent with a low-affinity CAT that is insensitive to transstimulation (7, 24, 25). Thus it is likely that the CAT-2B transporter numbers in the BPAECs were increased by cytokine treatment in our study. This concept is consistent with a previous study in rats, which found that treatment with LPS led to an increase in CAT-2B mRNA but not CAT-2A mRNA in the lung (7).

In our BPAEC culture system, we found that the increase in CAT-2 mRNA caused by L-T treatment was also associated with an increase in the uptake of extracellular L-Arg. This suggests that the L-T-induced increase in CAT-2 mRNA led to increased CAT-2 transporter expression. Previous studies have demonstrated an increase in pulmonary arterial endothelial cell L-Arg uptake by cytokine treatment (10, 17). Those studies reported an increase in the kinetic maximal velocity parameter ($V_{max}$) for L-Arg uptake (10, 17), a finding that is consistent with an increase in transporter number. Furthermore, the kinetic data from pulmonary arterial endothelial cells is consistent with the premise that the majority of L-Arg uptake is occurring via the system y⁺ transporter (6, 18). Thus, taken together, these results suggest that cytokine treatment increases expression of system y⁺ transporters in BPAECs, and that CAT-2 transporters are likely to be responsible for a portion of the increase.

Treatment with LPS and TNF-α increased eNOS protein and mRNA expression in BPAECs in our study. Previous studies have suggested that induction of iNOS may lead to a decrease in eNOS protein expression (4, 12, 21). For example, de Frutos and colleagues (4) found that when BPAECs were grown in coculture with bovine vascular smooth muscle cells (BVSMCs), treatment with IL-1β resulted in iNOS induction in BVSMCs but decreased eNOS protein expression in BPAECs. This effect was not due to NO overproduction because incubation with NO donors had no effect on eNOS protein expression. However, TNF-α levels were increased in the medium, and incubation with anti-TNF-α antibody prevented the decrease in eNOS protein. This led the authors to conclude that IL-1β treatment increased the production of TNF-α by the BVSMCs, which resulted in decreased eNOS protein expression in BPAECs. In contrast, Liu and coworkers (11) found that in cirrhotic rats, plasma TNF-α levels and nitrates were elevated compared with controls and that the expression of eNOS protein in the aorta was greater than in controls. This finding suggested that increased TNF-α levels were associated with increased eNOS protein expression. In the study by de Frutos and colleagues (4), the reported TNF-α levels in the media were 20–50 pg/ml, and in the study by Liu and coworkers (11), the plasma TNF-α levels were ~17 pg/ml for controls and ~48 pg/ml for cirrhotic rats. The present study utilized a TNF-α concentration of 500 pg/ml. Thus it may be that the effect of TNF-α on eNOS protein expression is dose dependent. Interestingly, the TNF-α concentrations employed in these two studies were similar, and yet the effect on eNOS protein was opposite (4, 11). Therefore, the cell type and culture conditions may also determine whether eNOS is upregulated or downregulated by iNOS induction. Our results, however, demonstrate that eNOS protein and mRNA expression were induced in BPAECs under the conditions employed in this study, which suggests that eNOS may contribute to the increase in NO production in L-T-treated BPAECs.

Treatment with LPS and TNF-α increased iNOS protein expression in BPAECs in this study. The increase in NO production and protein expression with L-T treatment is consistent with the well-described phenomenon of iNOS induction in a wide variety of cell types (2–4, 7, 11, 12, 24). It is of interest to note that under our culture conditions, iNOS protein was present in the control BPAECs. This may be due to the presence of fetal bovine serum in the medium, which would result in activation of the control BPAECs. In terms of data analysis, this “activation” would be expected to decrease the differences between control and L-T-treated BPAECs. However, in the conditions employed in this study, L-T treatment increased iNOS
protein expression and NO production in our BPAECs. Furthermore, the l-NIL data suggest that iNOS activity in these BPAECs was also increased by L-T treatment.

Treatment with LPS and TNF-α increased urea production in BPAECs in our study. It has previously been shown that LPS induces AR protein expression in macrophages (3, 23, 26, 28). Similarly, hyperoxic exposure has been shown to induce AR protein and activity in the lungs of rats (19). Interestingly, we found that urea production in the BPAECs was 10-fold greater than NO production during control conditions and ~25-fold greater than NO production after L-T treatment. This suggests that AR is the major l-Arg metabolic pathway in these BPAECs and is consistent with previous studies measuring AR and NOS activities in rat aortic endothelial cells (2) and in lungs from rats (19). The role of increased urea production in the BPAECs is unclear. If BPAECs in culture are representative of in vivo conditions, then the increased urea production may be involved in the formation of polyamines and proline from l-Orn. These processes are important in tissue healing after injury (8, 23).

A previous study on rat lungs demonstrated that hyperoxic exposure induced AR activity but did not induce iNOS activity. Therefore, the induction of AR was felt to be associated with lung repair (8). In murine macrophages, the T helper type 2 (Th2) cytokines IL-4 and IL-10 appear to be potent inducers of AR, whereas the T helper type 1 (Th1) cytokine interferon-γ appears to be a potent inducer of iNOS (14). In general, Th2 cytokines are considered to be anti-inflammatory, and Th1 cytokines are considered to be proinflammatory (14). Thus it may be that AR induction leading to increased urea production in certain conditions is associated with tissue repair, whereas iNOS induction leading to increased NO production is associated with the inflammatory response. However, we found that both urea and NO production were increased by the combined treatment of LPS and TNF-α. This finding is consistent with previous studies in rat aortic endothelial cells (2).

In macrophages, it has been suggested that the coinduction of AR with iNOS may limit l-Arg availability to iNOS and thereby decrease NO production during the inflammatory response (28). On the other hand, Buga and colleagues (2) found that when NO production was increased ~20-fold by LPS-interferon-γ treatment, AR activity was inhibited. The authors found that this effect was due to the intermediate in NO production from l-Arg, Nω-hydroxy-L-arginine. The inhibition constant (K_i) for Nω-hydroxy-L-arginine inhibition of AR was ~10 μM. The levels of NO produced in our culture medium were ~1 μM, and, therefore, it is unlikely that the levels of Nω-hydroxy-L-arginine produced would be sufficient to inhibit urea production by AR. Although it is unclear why both urea and NO production were increased by L-T treatment, it does appear that some stimuli will lead to induction of both AR and iNOS, whereas other stimuli will only induce one or the other enzyme. Therefore, in situations where both AR and iNOS are induced, the degree of iNOS induction may determine whether AR activity will limit l-Arg availability to iNOS or whether enough Nω-hydroxy-L-arginine will be produced to inhibit AR and thereby increase the availability of l-Arg 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.

The importance of l-Arg uptake to NO and urea production was demonstrated by the finding that increasing the extracellular concentration of l-Arg led to increased NO and urea production in both control and
L-T-treated BPAECs. The Michaelis constant ($K_m$) for both eNOS and iNOS for l-Arg is $\sim 10$ μM. Thus the cause of the increase in NO production with increasing extracellular l-Arg is difficult to explain in terms of enzyme kinetics alone. However, this finding is consistent with the study by Buga and colleagues (2) on control and cytokine-treated rat aortic endothelial cells. The effect of extracellular l-Arg concentration on NO synthesis has been termed the l-Arg paradox (13). In BPAECs, it has been shown that system y$^+$ transporters exist in close proximity to eNOS in the cell membrane (13), which suggests that eNOS may preferentially utilize extracellular l-Arg. The complex of arginine transporter and eNOS may explain in part the increase in NO production with increasing l-Arg concentration in our study. In terms of AR, the $K_m$ for l-Arg is $\sim 1$ mM; thus the increased urea production with increasing l-Arg concentrations from 1 to 30 mM may in large part be explained by AR kinetics. Because both NOS and AR utilize l-Arg as a substrate, it may be that the intracellular partitioning of the enzymes influences the amount of l-Arg available to the respective enzymes. Further studies will be needed to determine the exact mechanism responsible for the increase in NO and urea production with increasing extracellular l-Arg concentration.

In summary, we found that treatment with LPS and TNF-α led to an increase in NO production via induction of both eNOS and iNOS in BPAECs and to an increase in urea production via AR. We demonstrated for the first time that treating BPAECs with LPS and TNF-α leads to an increase in CAT-2 mRNA expression. The LPS-TNF-α-induced increase in CAT-2 mRNA expression was associated with an increase in the uptake of l-$[^{3}$H]Arg by BPAECs. These results suggest that inflammatory stimuli that increase NO and urea production in BPAECs also induce CAT-2 expression. This may represent a mechanism for the endothelial cell to maintain adequate intracellular l-Arg concentrations in the face of increased l-Arg metabolism. Furthermore, interruption of l-Arg uptake or expression of l-Arg transporters may represent future therapeutic targets in diseases characterized by NO overproduction such as septic shock and ARDS.

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