ARGINASE IS RESPONSIBLE FOR the hydrolysis of arginine into ornithine and urea, thus playing an essential role in hepatic and intestinal urea synthesis from ammonia as well as providing ornithine for the synthesis of polyamines, proline, and glutamate (13, 29, 36). There are two distinct isoforms of mammalian arginase (arginase I and II), which are encoded by different genes and differ in molecular and immunological properties, tissue distribution, subcellular location, and regulation of expression (9, 19). Arginase I (a cytosolic enzyme) is highly expressed in the liver and to a much lesser extent in a few other cell types, whereas expression of arginase II (a mitochondrial enzyme) is widespread (20, 25). Intracellular compartmentation of arginases may have important implications for arginine metabolism in mammalian cells. For example, because of its colocalization with ornithine decarboxylase (ODC) in the cytosol, arginase I may preferentially direct ornithine to polyamine synthesis, whereas arginase II may preferentially direct ornithine to proline and glutamate production due to the colocalization of arginase II and ornithine aminotransferase (OAT) in mitochondria (Fig. 1).

Almost all mammalian cells contain one of the isoforms of nitric oxide (NO) synthase (NOS) that synthesizes NO and L-citrulline from L-arginine (11, 16). Interestingly, both arginase and NOS use arginine as a common substrate, and thus arginase may play a role in regulating NO synthesis by modulating intracellular arginase availability (19, 26, 36). This idea is supported by reports that inhibition of arginase activity results in enhanced NO production by inducible NOS (iNOS) in activated macrophages (6, 8, 28), although conflicting findings have also been reported (4). Although relatively little is known about the role of arginase in NO synthesis by constitutive NOS (cNOS), recent studies using arginase inhibitors suggest that endogenous arginase activity partially inhibits NO synthesis by cNOS isoforms (1, 3).

Endothelial cells (EC) represent a good model for studying the metabolic roles of the arginases because of the following reasons: they express endothelial NOS...
(eNOS), a cNOS that is essential for the regulation of vascular tone and permeability (18); they express OAT (34) and therefore are potentially capable of synthesizing both proline and glutamate from arginine; and they also synthesize polyamines from ornithine (23). Both arginase isoforms can be expressed by EC (2), but the role(s) of arginase expression in endothelial arginine metabolism is unknown. Thus the objective of this study was to test the hypothesis that arginase activity may be a limiting or regulatory factor in endothelial syntheses of NO, proline, glutamate, or polyamines by analyzing the impact of elevated expression of arginase I or arginase II on arginine metabolism in bovine microvascular venular EC.

MATERIALS AND METHODS

Materials. All L-amino acids, putrescine, spermidine, spermine, BSA (essentially fatty-acid free), HEPES, DL-dithiothreitol, sucrose, EDTA, phenylmethylsulfonyl fluoride, aprotinin, chymostatin, pepstatin A, o-phthaldialdehyde, and 2,3-diaminonaphthalene were obtained from Sigma-Aldrich (St. Louis, MO). Nitrate reductase, NADPH, and leupeptin were obtained from Fisher Scientific (Houston, TX). DMEM, Dulbecco’s PBS (DPBS), dialyzed FBS, penicillin, streptomycin, and amphotericin B were obtained from GIBCO (Grand Island, NY).

Cell culture. Bovine coronary venular EC were isolated from coronary venules (15 μm in diameter) using a bead perfusion technique, as previously described (24, 33). EC were cultured at 37°C in complete DMEM (DMEM with 1 mM sodium pyruvate, 2 mM L-glucose, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 10 U/ml heparin) containing 10% dialyzed FBS. Cell lines were passaged by trypsinization in Ca²⁺ - and Mg²⁺-free DPBS containing 0.25% trypsin and 0.02% EDTA.

Vector construction. pEF1-rARGI, a mammalian expression plasmid for rat arginase I, was constructed by inserting the EcoRI-BsaI 1194-bp coding region fragment of pARGr-2 (10) into the EcoRI/Ipme I sites of the plasmid pEF1/Myc-His C (Invitrogen, San Diego, CA). pEF1-mARGII, a mammalian expression vector for mouse arginase II, was constructed by inserting the EcoRI-BstEII 1249-bp coding region fragment of mouse arginase II cDNA (25) into the EcoRI/Ipme I sites of pEF1/Myc-His C expression vector. In these two constructs, no epitope tags were fused to the arginases. For control transfection, pEF1/Myc-His/lacZ (In-
vitro) was used to represent the expression of an unrelated exogenous protein.

Transfection of EC. Bovine coronary venular EC at passages 6–9 were transfected with pEF1-rARGI, pEF1mARGII, or pEF1/Myc-His/lacZ using TransIT-LT2 Polyamine Transfection Reagent (PanVera, Madison, WI) according to the manufacturer’s instructions. Stably transfected cells were selected with antibiotic G418 (600 μg/ml) in complete DMEM and then were examined for expression of arginases I and II by Western blot analysis, as described below. Transfected cells were maintained in complete DMEM containing G418 (600 μg/ml) and were used at passages 10–20.

Western blot analysis of arginases I and II. Stably transfected cells were lysed in 0.1% Triton X-100 containing 2 mM Pefabloc, 2 μg/ml pepstatin A, and 10 μg/ml leupeptin. Western blot analysis for arginase I and II proteins was performed as described previously (20, 21). Before use for immunoblots, arginase I or arginase II antibody was diluted 1:50,000 or 1:10,000, respectively, in a solution containing 1% nonfat dry milk, 1% BSA, 20 mM Tris–HCl (pH 7.6), 137 mM NaCl, 5 mM MgCl₂, and 0.1% Tween 20. The secondary antibody was peroxidase-conjugated rabbit anti-chicken IgY (Jackson ImmunoResearch Laboratories, West Grove, PA), which was diluted 1:10,000, respectively, in a solution containing 1% nonfat dry milk, 1% BSA, and 20 mM Tris–HCl (pH 7.6). Net production of nitrite plus nitrate was measured by this HPLC method after its conversion to nitrite by nitrate reductase (31). The detection limit for nitrite and nitrate by the HPLC method was 10 nM. Production of nitrate plus nitrite was calculated after subtracting blank values for culture media without cells.

For determining medium urea and amino acids, an aliquot (200 μl) of the culture medium was acidified with 100 μl of 1.5 M HClO₄, and the solution was neutralized with 50 μl of 2 M K₂CO₃. Neutralized extracts were used for (1) urea analysis and (2) an enzymatic procedure involving urease and glutamate dehydrogenase (29) and (2) amino acid analysis by HPLC methods involving precolumn derivatization with o-phthalaldehyde (33). [¹⁴C]Citrulline, [¹⁴C]glutamate, [¹⁴C]proline, and [¹⁴C]ornithine fractions were collected from the HPLC column, and their radioactivities were measured by a Packard 1900 liquid scintillation counter, as previously described (30). Culture media without cells were used as blanks. Net production of [¹⁴C]citrulline, [¹⁴C]glutamate, [¹⁴C]proline, and [¹⁴C]ornithine from [¹⁴C]arginine was calculated after subtracting blank values on the basis of the specific radioactivity of intracellular [¹⁴C]arginine as previously described (30). Net production of urea was calculated after subtracting blank values for culture media without cells. Arginine consumption was calculated on the basis of arginine disappearance from culture media after correction of blank values for culture media without cells.

For determining cellular amino acids and polyamines, EC seeded in culture dishes were rapidly washed three times with 10 ml ice-cold DPBS (within a total of 20 s) to remove extracellular metabolites. Cells (5 × 10⁶) were then lysed in 200 μl of 1.5 M HClO₄, and the solution was neutralized with 100 μl of 2 M K₂CO₃. The neutralized extracts were used for determining amino acids and polyamines (putrescine, spermidine, and spermine) by HPLC methods, as previously described by Wu et al. (37). In preliminary studies to determine the efficiency of the cell-washing procedure in removing medium amino acids and polyamines, [¹⁴C]sucrose was added to cell cultures immediately before removal of the conditioned medium. We found that at the end of the third washing, there were no [¹⁴C]sucrose, arginine, or polyamines in collected DPBS and no [¹⁴C]sucrose in cells, indicating little contamination of cell pellets by the medium. To determine whether intracellular arginine might exit from cells during the washing procedure, EC were preloaded with [guanido-¹⁴C]arginine by incubation in DMEM at 37°C for 2 h and then were used for determining the release of [¹⁴C]arginine after the medium was removed, and cells were washed three times with ice-cold DPBS. We found that the cell-washing procedure resulted in the release of 4.3% of intracellular free arginine from EC, indicating that our analysis of arginine in cell lysates only slightly underestimated cellular arginine content.

Uptake of L-arginine by EC. The uptake of L-arginine by transfected EC was measured at 37°C in oxygenated (95% O₂,5% CO₂) Krebs-Henseleit bicarbonate (KHB) buffer containing 20 mM HEPES, 5 mM glucose, 1% BSA, and 0.4 mM
l-[U-14C]arginine (0.05 μCi/ml), as previously described (34). Arginine transport was initiated by addition of cells and terminated in 3 min by addition of 0.2 ml of ice-cold 10 mM L-arginine containing 0.05 μCi [2-3H]mannitol as an extracellular marker. The solution was mixed immediately and was transferred to a 1.6-ml microcentrifuge tube that contained 0.7 ml of an oil mixture (bromododecane/dodecane, 20:1, vol/vol) overlaid on 0.2 ml of 1.5 M HClO₄ (acid layer). Cells were separated from the medium through the oil layer into the acid layer by centrifugation (10,000 g, 1 min). The oil layer was washed three times with fresh KHB buffer, and the acid layer was used for the measurement of 3H and 14C using a dual-channel counting program in a Packard 1900 liquid scintillation counter. A small amount of [3H]mannitol was present in the acid layer, the value of which was used to correct for contamination of the cellular extracts by the incubation medium. The specific radioactivity of [14C]arginine uptake by EC. Preliminary studies showed that the amount of [14C]ornithine and [14C]proline (the major products of arginine catabolism) released from EC to the incubation medium during a 3-min incubation period represented only 3.8 and 2.0% of total intracellular 14C, respectively, indicating that intracellular accumulation of 14C was a valid indicator of [14C]arginine uptake by EC.

Measurement of protein. Protein content of whole cells, cytosol, and mitochondria was determined by a modified Lowry method, using BSA as a standard (32).

Statistical analysis. Data were analyzed by one-way ANOVA with the Student-Newman-Keuls test for identifying differences among means (27). P < 0.05 was taken to indicate statistical significance.

RESULTS

Elevated expression of arginase I and II in EC. To obtain EC with elevated expression of arginase I or arginase II, cells were stably transfected with expression plasmids containing rat arginase I cDNA or mouse arginase II cDNA. EC stably transfected with the bacterial β-galactosidase gene (lacZ) represented the control for expression of an exogenous protein from the elongation factor-1 plasmid. Western blots demonstrated that bovine venular EC transfected with rat arginase I cDNA (AI-EC) or mouse arginase II cDNA (AII-EC) expressed high levels of arginase I or arginase II protein, whereas the low endogenous levels of both arginase isoforms were not detectable by this method in control EC transfected with lacZ (lacZ-EC; Fig. 2). Consistent with Western blots, arginase activity was low but measurable in lacZ-EC, averaging 0.43 and 0.21 nmol·mg protein⁻¹·min⁻¹, respectively, in the cytosol and mitochondria and was markedly increased (P < 0.01) in the cytosol of AI-EC and mitochondria of AII-EC (Fig. 3). These results reflected the known subcellular localization of arginases I and II in mammalian cells (9, 20, 25). Arginase specific activity was 258% greater (P < 0.01) in AI-EC than in AII-EC.

Urea production was elevated (P < 0.01) by 616% in AI-EC and by 157% in AII-EC compared with lacZ-EC (Fig. 4). Net production of urea was similar to arginine consumption by EC (P > 0.05). Net production of ornithine differed markedly (P < 0.01) among lacZ-EC, AI-EC, and AII-EC and was greatest in AI-EC. Net production of ornithine from arginine was 5, 36, or 12% of net urea production for lacZ-EC, AI-EC, and AII-EC, respectively, reflecting further metabolism of ornithine in these cells.

Effect of elevated arginase expression on NO synthesis. The synthesis of NO (indicated by production of nitrite plus nitrate) was 60 and 47% lower (P < 0.05) in AI-EC and AII-EC, respectively, compared with lacZ-EC (Fig. 5). Net production of nitrite from [14C]arginine (an alternative indicator of NO synthesis) was also markedly reduced (P < 0.05) in AI-EC and in AII-EC compared with lacZ-EC (Fig. 5). Because citrulline can be recycled to arginine in EC (7, 33), the measurement of net [14C]citrulline formation from

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**Fig. 2.** Western blot analysis of arginases I and II in transfected endothelial cells (EC). *Lane I,* recombinant rat arginase I (15 ng, as positive control for arginase I and negative control for cross-reactivity with arginase II); *lane II,* recombinant human arginase II (1 ng, as positive control for arginase II and negative control for cross-reactivity with arginase I); *lanes 1 and 2,* EC transfected with rat arginase I cDNA (AI-EC; 20 μg cell protein); *lanes 3 and 4,* EC transfected with mouse arginase II cDNA (AII-EC; 20 μg cell protein); *lanes 5 and 6,* control lacZ-EC (20 μg cell protein).

**Fig. 3.** Arginase activity in transfected EC. Data are means ± SE; n = 4. Bovine venular EC stably transfected with the Escherichia coli lacZ gene (lacZ-EC, control), AI-EC, or AII-EC were used for arginase assay, as described in text. Means with different letters (a-c) are different (P < 0.01).
Effect of elevated arginase expression on arginine transport. Arginine transport by AI-EC and AII-EC was 31–33% greater ($P < 0.05$) than that by lacZ-EC. The values were $1.26 \pm 0.03$, $1.24 \pm 0.09$, and $0.95 \pm 0.02$ nmol mg$^{-1}$ protein$^{-1}$ min$^{-1}$ (means $\pm$ SE; $n = 6$), respectively, in AI-EC, AII-EC, and lacZ-EC, respectively. There was no difference ($P > 0.05$) in arginine uptake between AI-EC and AII-EC.

DISCUSSION

Arginase expression and arginine consumption. High-level expression of arginase I or arginase II in bovine venular EC was confirmed by Western blot analysis, increased arginase activity, and the appropriate distribution of arginase activity in subcellular fractions of the transfected cells (Figs. 2 and 3). As a result, arginine consumption was markedly enhanced in EC with elevated expression of arginase I or arginase II (Fig. 4) and was associated with a modest increase in arginine transport via a yet unknown mechanism. As reported previously for rat coronary microvascular EC (34), net production of urea in bovine EC closely matched arginine consumption, indicating that utilization of arginine by arginase was far greater than its utilization for synthesis of NO or proteins.

Ornithine is a coproduct of arginase. However, the ornithine accumulated at the end of a 24-h culture period does not represent total production of ornithine from arginine, as ornithine can be further metabolized to proline and glutamate (Fig. 6) as well as to CO$_2$ (34). Interestingly, net production of ornithine, expressed as a percentage of net urea formation, increased in direct proportion to cellular arginase activity, suggesting that OAT is a limiting factor for endothelial ornithine metabolism when arginase activity is enhanced.

Effect of elevated arginase expression on synthesis of proline and glutamate. These data are summarized in Fig. 6. Net production of proline from arginine was increased ($P < 0.01$) by 928% in AI-EC and by 295% in AII-EC compared with lacZ-EC. Net production of glutamate from arginine was enhanced ($P < 0.01$) by 164% in AI-EC and by 79% in AII-EC compared with lacZ-EC. Proline and glutamate syntheses from arginine were greater ($P < 0.01$) in AI-EC than in AII-EC. In all EC studied, proline was the major product of arginine catabolism.

Effect of elevated arginase expression on cellular content of arginine, ornithine, and polyamines. Figure 7 shows the cellular content of ornithine and polyamines. Elevated expression of arginase I, but not arginase II, increased ($P < 0.05$) cellular content of ornithine by 108% compared with lacZ-EC. Total cellular contents of arginine were 25 and 11% lower ($P < 0.05$) in AI-EC and AII-EC, respectively, compared with lacZ-EC. Proline and spermidine contents among the three groups of EC were increased ($P < 0.01$) by 158 and 43% in AII-EC, respectively, compared with lacZ-EC. There were no differences ($P > 0.05$) in cellular spermine contents among the three groups of EC. There was no detectable accumulation of polyamines in media after a 24-h culture period.
Arginase expression and NO synthesis. Both arginase and NOS utilize arginine as substrate, thus raising the question of whether these two enzymes directly compete for intracellular arginine (36). Such an interplay between arginase and NOS is particularly complex in the case of EC, because these cells contain both cytosolic arginase I and mitochondrial arginase II (2). Further complicating this picture is the fact that eNOS is located in both the membrane-bound caveolae and the cytoplasm (11, 16), and thus it is not known whether arginase I and II may differentially modulate NO production by EC. The cloning of arginase I and II cDNAs (10, 20, 25) has provided a useful tool to study the potential roles of arginase isoforms in regulating endothelial NO synthesis.

Results of this study demonstrate that increased expression of arginase I or arginase II markedly reduced basal NO synthesis in EC (Fig. 5). The reductions in NO synthesis suggested a possible depletion of intracellular arginine in AO-EC and A-II-EC, and this was consistent with modest but significant decreases in intracellular arginine content in these cells (Fig. 5). On the basis of cell volume (0.462 ± 0.04 ml/10^6 cells) and protein content (268 ± 6 mg/10^6 cells) in bovine coronary venular EC (14) and HPLC analysis of cellular arginine (expressed as nmol/mg protein; Fig. 5), intracellular arginine concentrations in lacZ-EC, AI-EC, and AII-EC were calculated to be 2.16 ± 0.04, 1.61 ± 0.08, and 1.92 ± 0.05 mM (means ± SE; n = 6), respectively. These values are similar to those (1–2 mM) previously reported for freshly isolated EC or EC cultured in the presence of 0.4 mM arginine (36) and are substantially higher than the Michaelis-Menten value (2.9 μM) of purified eNOS for L-arginine (11). Consequently, the declines in NO synthesis could not be accounted for by the declines in intracellular arginine concentration. These results suggest that a distinct intracellular pool of arginine available for NO synthesis exists that was more depleted than indicated by the decrease in total intracellular arginine content. Thus the apparent arginine paradox in endothelial NO synthesis remains to be fully explained (35).

Arginase I may be colocalized or closely associated with eNOS such that high-level expression of arginase I may reduce arginine concentrations at or near the site of NO production. However, it is not clear how arginase II (a mitochondrial enzyme) might compete with eNOS for intracellular arginine. A possible explanation for this interplay is that an increase in mitochondrial arginine degradation by arginase II may result in enhanced transport of arginine from the cytosol into mitochondria, thereby reducing the availability of cytosolic arginine for NO synthesis. Interestingly, Gotoh and Mori (5) also found that elevated expression of arginase II reduced NO production by iNOS in activated RAW 264.7 murine macrophages.

Bovine and human EC can synthesize arginine from citrulline (7, 33), as macrophages and smooth muscle cells (19, 31). On the basis of the production of nitrite plus nitrate vs. the net accumulation of citrulline, the efficiency of citrulline-to-arginine recycling was calculated to be 60, 80, and 74% in lacZ-EC, AI-EC, and AII-EC, respectively. Thus activities of the arginine-synthetic enzymes in bovine venular EC were not sufficient to completely recycle citrulline to arginine, even at the low basal rate of NO synthesis. Results from the
bovine venular EC contrast with the apparent lack of such recycling in rat coronary microvascular EC (34), indicating species or anatomical differences in the ability of EC for this recycling.

Arginase expression and production of proline, glutamate, and polyamines. Ornithine is the common substrate for the synthesis of proline, glutamate, and polyamines in mammalian cells (Fig. 1). OAT (a mitochondrial enzyme) converts ornithine into pyroline-5-carboxylate (P5C), which is subsequently converted to glutamate by P5C dehydrogenase (a mitochondrial enzyme) or to proline by P5C reductase (a cytosolic enzyme; see Ref. 36). Elevated expression of arginase I or arginase II increased the production of both proline and glutamate from arginine by EC, indicating that these synthetic pathways in EC normally are limited by availability of the substrate ornithine. Thus ornithine generated in either the cytosol or mitochondria is available for conversion to P5C by mitochondrial OAT. Net production of proline was greater than that of glutamate in all EC studied. This may be explained by our finding that P5C reductase activity was much greater than P5C dehydrogenase activity in EC (Wu and Meininger, unpublished data) and by the fact that glutamate can readily participate in transamination reactions. Proline is a major amino acid for the synthesis of collagen and the generation of extracellular matrix, and thus an increase in proline synthesis from arginine may play an important role in vascular remodeling.

ODC is the first and rate-controlling enzyme in polyamine synthesis from ornithine (36). Because ODC and arginase I are colocalized in the cytosol, the ornithine generated by arginase I is readily utilized for polyamine synthesis (10a, 22). However, the role of arginase II in polyamine synthesis is unknown. On the basis of our recent finding that mitochondrially generated ornithine can serve as a substrate for cytosolic polyamine synthesis in enterocytes (32), we anticipated that an increase in ornithine production by arginase II would stimulate polyamine synthesis in EC. Results of the current study demonstrate that elevated expression of arginase I or arginase II did enhance the cellular content of putrescine and spermidine in EC (Fig. 7), indicating an increase in their synthesis from arginine. Similarly, we also found that elevated expression of arginase I stimulated the synthesis of putrescine and spermidine in activated macrophages (10a, 22). Because polyamines are essential to EC proliferation (23), enhanced synthesis of polyamines from arginine may play an important role in angiogenesis and wound healing. In this regard, it is noteworthy that arginase activity is severely deficient in EC of the diabetic BB rat (an animal model of human type I diabetes mellitus; see Ref. 34), and these cells exhibit a marked impairment in proliferation (15). Elevating expression of arginase I or arginase II in diabetic EC may provide a novel gene therapy for improving angiogenesis and wound healing in diabetes mellitus.

In conclusion, our results demonstrate for the first time that increased expression of arginase I or arginase II reduced NO synthesis, promoted the production of glutamate and proline, and enhanced cellular polyamine content in EC. Because of the significant metabolic changes that occur when expression of either arginase is enhanced, we conclude that arginase normally is a limiting or regulatory factor in venular EC arginine metabolism. Because EC play an essential role in regulating vascular tone and angiogenesis, physiological or pathophysiological variations in expression of the arginase isoforms in EC may have important implications for cardiovascular function in health and disease.

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REFERENCES


