Activities of arginase I and II are limiting for endothelial cell proliferation

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1Department of Animal Science and Faculty of Nutrition, Texas A&M University; 2Cardiovascular Research Institute and Department of Medical Physiology, Texas A&M University System Health Science Center, College Station, Texas, 77843; and 3Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

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Li, Hui, Cynthia J. Meinninger, Katherine A. Kelly, James R. Hawker, Jr., Sidney M. Morris, Jr., and Guoyao Wu. Activities of arginase I and II are limiting for endothelial cell proliferation. Am J Physiol Regulatory Integrative Comp Physiol 282: R64–R69, 2002.—Polyamines are essential for cell proliferation; therefore, we hypothesized that arginase I or arginase II activities, via production of ornithine for polyamine synthesis, may be limiting for proliferation of endothelial cells (EC). Bovine coronary venular EC stably transfected with a lacZ gene (lacZ-EC, control), rat arginase I cDNA (AI-EC), or mouse arginase II cDNA (AII-EC) were utilized to test this hypothesis. Cell-proliferation assays showed that EC proliferation was markedly increased in AI-EC and AII-EC compared with lacZ-EC. Expression of proliferating cell nuclear antigen was also enhanced in AI-EC and AII-EC. DL-o-difluoromethylornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase, was used to establish that increased polyamine synthesis was involved in mediating the enhanced growth of AI-EC and AII-EC. Addition of 5 mM DFMO to the culture medium completely abolished the differences in cellular putrescine concentrations and reduced the differences in spermidine concentrations among AI-EC, AII-EC, and lacZ-EC. The DFMO treatment also prevented an increase in AI-EC and AII-EC proliferation compared with lacZ-EC. Addition of 10 and 50 μM putrescine dose-dependently increased AI-EC, AII-EC, and lacZ-EC growth to the same extent. These results demonstrate that either arginase isofrom can potentially play a role in modulating EC proliferation by regulating polyamine synthesis.

ornithine; polyamines; cell transfection

ARGINASE IS PHYSIOLOGICALLY the first enzyme for synthesis of polyamines (putrescine, spermidine, and spermine) in mammalian cells (20, 30). Arginase hydrolyzes L-arginine into urea and L-ornithine, which is decarboxylated by ornithine decarboxylase (ODC) to form putrescine. The latter is converted into spermidine and spermine by spermidine synthase and spermine synthase, respectively (Fig. 1). As polycationic compounds, polyamines interact with nucleic acids, proteins, and other negatively charged molecules and modulate their biosynthesis (8). Thus polyamines are essential for cell growth and function (6, 16, 27). When cells are stimulated with growth factors, one of the first important events in cell proliferation is the induction of polyamine synthesis, which precedes increases in DNA replication as well as increases in RNA and protein syntheses (6, 16). In contrast, depletion of cellular polyamines by inhibition of arginase or ODC arrests cell growth (e.g., see Refs. 3, 9, 22, 24, 26, 28).

There are two distinct isofroms of arginase in mammalian cells (11, 32). Arginase I, which is colocalized with ODC and other polyamine synthetic enzymes in the cytosol, is highly expressed in the liver and to a much less extent in a few other tissues and cell types. Arginase II, a mitochondrial enzyme, is widely distributed in extrahepatic cells and tissues. These two isofroms are encoded by two different genes and differ in molecular and immunological properties, tissue distribution, subcellular location, and regulation of expression (11, 23). Recent studies have shown that altering expression of either arginase I (12, 14) or arginase II (14) can alter polyamine synthesis in macrophages and EC. These findings suggest the potential for an important role of the arginases in cell proliferation.

Proliferation of EC, an initial and necessary step in angiogenesis, is regulated by cellular polyamine levels (22, 26). Angiogenesis plays a key role both in physiological events such as wound healing and placental growth and in pathological conditions such as myocardial infarction, tumor growth, and diabetic retinopathy (2). Thus understanding and manipulating the growth of EC have important clinical implications. The objective of this study was to test the hypothesis that activities of arginase I or arginase II, via production of ornithine for polyamine synthesis, may be limiting for EC proliferation.

MATERIALS AND METHODS

Materials. Putrescine, spermidine, spermine, bovine serum albumin (BSA, essentially fatty-acid free), and o-phthal-
and 0 or 5 mM DFMO or 5 mM DFMO plus 10 or 50 μM putrescine. The concentrations of DFMO and putrescine were chosen on the basis of previous studies with EC (22, 26). Culture medium was changed every other day in all experiments.

Western blot analysis of PCNA and actin. Western blot analysis of PCNA was performed as described by Meiningher and Wu (17). Briefly, transfected EC were plated at 5,000 cells/cm² in complete DMEM containing 10% FBS and cultured for 4 days. On day 4 of the culture, cells were lysed with a buffer consisting of 10 mM Tris-HCl (pH 8.0), 0.1% SDS, 1% sodium deoxycholate, 1% NP-40, 0.14 M NaCl, and a protease inhibitor cocktail. Protein concentration was determined using the bicinchoninic acid protocol (Pierce, Rockford, IL) with BSA as a standard. Total cell protein (10 μg) was loaded on a 9.5–16% gradient gel. To determine PCNA, the primary antibody (mouse monoclonal anti-PCNA) and the secondary antibody (peroxidase-labeled donkey anti-mouse IgG) were used at 1:1,000 and 1:30,000 dilutions, respectively. Peroxidase activity was visualized using a SuperSignal West Dura Extended Duration Substrate (Pierce) according to the manufacturer’s instructions and by exposing blots to Kodak Biomax ML film (Kodak, Rochester, NY) for 1 min. For Western blot analysis of actin, the same blots used for PCNA analysis were washed with Restore Western Blot Stripping Buffer (Pierce). The primary antibody (mouse monoclonal anti-actin) and the secondary antibody (peroxidase-labeled donkey anti-mouse IgG) were used at 1:1,000 and 1:75,000 dilutions, respectively. Bands were visualized as described.

HPLC analysis of polyamines. Cellular concentrations of putrescine, spermidine, and spermine were determined by HPLC as previously described (29, 33). Briefly, 1 × 10⁶ cells were lysed in 100 μl of 1.5 M HClO₄ and then neutralized with 50 μl of 2 M K₂CO₃. The neutralized extracts were used for polyamine analysis by an ion-pairing HPLC method that involved precolumn derivatization with o-phthaldialdehyde. The assay mixture contained 10 μl of sample, 140 μl of HPLC water, and 10 μl of 1.2% benzoic acid (in 40 mM sodium borate, pH 9.5). An aliquot (25 μl) of the assay mixture was derivatized in an autosampler (model 712 WISP, Waters, Milford, MA) with 25 μl of 30 mM o-phthaldialdehyde (in 3.1% Brij-35, 50 mM 2-mercaptoethanol, and 40 mM sodium borate, pH 9.5), and 25 μl of the derivatized mixture was injected into a 3-μm reversed-phase C18 column (150 × 4.6 mm inside diameter) (Supelco, Bellefonte, PA). Polyamines were separated using a solvent gradient consisting of solution A (0.1 mM sodium acetate, 4 mM SDS, 0.5% tetrahydrofuran, and 9% methanol, pH 7.2) and solution B (methanol and 4 mM SDS). Putrescine, spermidine, and spermine quantities in samples were measured by comparison with known amounts of authentic standards.

Statistical analysis. Data were analyzed by one-way ANOVA with Student-Newman-Keuls test for identifying differences among means, or by unpaired Student’s t-test (25). P < 0.05 was taken to indicate statistical significance.

RESULTS

Effect of elevated arginase expression on cell proliferation. AI-EC and AII-EC expressed high levels of arginase compared with lacZ-EC (14). Elevated expression of arginase I or arginase II markedly increased (P < 0.05) EC proliferation (Fig. 2). For example, on day 4 of the cell culture in complete DMEM containing 10% FBS, cell numbers of AI-EC and AII-EC were increased (P < 0.05) by 69% and 75%, respectively, compared

Fig. 1. Polyamine synthesis from arginine in mammalian cells. Cit, L-citrulline; DCAM, decarboxylated 5-adenosylmethionine; Glu, L-glutamate; α-KG, α-ketoglutarate; MTA, methylthioadenosine; OAT, ornithine aminotransferase; PSC, L-α-pyrroline-5-carboxylate.

diaidehyde were obtained from Sigma-Aldrich (St. Louis, MO). Dulbecco’s modified Eagle’s medium (DMEM), Dulbecco’s phosphate-buffered saline (DPBS), penicillin, streptomycin, and amphotericin B were obtained from GIBCO (Grand Island, NY) and fetal bovine serum (FBS) from Summit (Greeley, CO). HPLC-grade methanol and water were purchased from Fisher Scientific (Houston, TX). DL-α-difluoromethylornithine (DFMO) and G-418 sulfate were obtained from Calbiochem (San Diego, CA) and mouse monoclonal anti-actin antibody was obtained from Santa Cruz (Santa Cruz, CA) and mouse monoclonal anti-actin from Sigma-Aldrich. Peroxidase-conjugated donkey anti-mouse IgG was obtained from Jackson (West Grove, PA).

Preparation and culture of stably transfected EC. Bovine coronary venular EC stably transfected with a lacZ gene (lacZ-EC, control), rat arginase I cDNA (AI-EC), or mouse arginase II cDNA (AII-EC) were produced and characterized as previously described (14). Transfected cells were cultured at 37°C in complete DMEM (DMEM with 20 mM d-glucose, 2 mM l-glutamate, 0.4 mM l-arginine, 100 U/ml penicillin, 100 μg/ml streptomyacin, 0.25 μg/ml amphotericin B, and 600 μg/ml G-418) containing 10% FBS. Cells were passaged by trypsinization in Ca²⁺/Mg²⁺-free DPBS containing 0.25% trypsin and 0.02% EDTA.

Cell proliferation assays. Cell-proliferation assays were performed as described by Meiningher and Wu (17). Briefly, transfected EC were seeded at 5,000 cells/cm² in 24-well trays (day 0), and cells were cultured in complete DMEM containing 10% FBS for 4 days. On days 2, 4, and 6, three wells of cells for each treatment group were trypsinized, and cell numbers were determined using a hemacytometer. In experiments to determine the effect of DFMO on cell proliferation, EC were first cultured for 24 h in serum-free DMEM to minimize cellular polyamines and then cultured for 4 days in complete DMEM containing 10% FBS

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with lacZ-EC. There were no significant differences (\(P > 0.05\)) in cell proliferation between AI-EC and AII-EC.

Enhanced EC proliferation was also confirmed by Western blot analysis of PCNA (Fig. 3), which is an index of cell proliferation (5). After proliferation in complete DMEM containing 10% fetal bovine serum (FBS) for 4 days, expression of PCNA was greater in AI-EC and AII-EC compared with lacZ-EC. The level of cellular actin, which reflects expression of a growth-unrelated gene, was not affected by elevated expression of arginase I or arginase II in EC.

**Effect of DFMO on cell proliferation and cellular polyamine content.** To investigate whether the enhanced proliferation of AI-EC and AII-EC was mediated by increased polyamine synthesis, the effects of DFMO (an irreversible inhibitor of ODC) on EC proliferation and cellular polyamine content were examined. After a 24-h period of culture in serum-free medium, cellular polyamine concentrations reached basal values and did not differ among AI-EC, AII-EC, and lacZ-EC (data not shown). Cell-proliferation assays were then performed in complete DMEM containing 10% FBS and one of the following: 1) 0 or 5 mM DFMO, 2) 5 mM DFMO plus 10 \(\mu\)M putrescine (Put), or 3) 5 mM DFMO plus 50 \(\mu\)M putrescine. On day 2, the medium was replaced with fresh medium. On day 4, cell numbers were determined. Data are means \(\pm\) SE of 5 independent experiments, each performed in triplicate; \(*P < 0.05\), different from control lacZ-EC.

![Fig. 2. Growth curves of stably transfected endothelial cells (EC). Cells were transfected with lacZ gene (lacZ-EC), arginase I cDNA (AI-EC), or arginase II cDNA (AII-EC). Transfected EC were plated at 5,000 cells/cm\(^2\) in 24-well trays on day 0 and cultured in complete DMEM containing 10% fetal bovine serum (FBS). Cell numbers were determined every other day using a hemacytometer. Data are means \(\pm\) SE of 5 independent experiments, each performed in triplicate; \(*P < 0.05\), different from control lacZ-EC.](image)

![Fig. 3. Western blot analysis of proliferating cell nuclear antigen (PCNA) and actin in stably transfected EC. Cells were transfected with lacZ gene, arginase I cDNA, or arginase II cDNA. Transfected EC were plated at 5,000 cells/cm\(^2\) in medium containing 10% FBS and were cultured for 4 days. On day 4 of the culture, cells were lysed and subjected to Western blot analysis of PCNA and actin. Lane 1, lacZ-EC (10 \(\mu\)g of cell protein); lane 2, AI-EC (10 \(\mu\)g of cell protein); and lane 3, AII-EC (10 \(\mu\)g of cell protein).](image)

![Fig. 4. Effect of DL-\(\alpha\)-difluoromethylornithine (DFMO) on growth of stably transfected EC. Cells were plated at 5,000 cells/cm\(^2\) in serum-free DMEM for 24 h before initiation of experiments. On day 0, medium was changed to DMEM containing 10% FBS and one of the following: 1) 0 or 5 mM DFMO, 2) 5 mM DFMO plus 10 \(\mu\)M putrescine (Put), or 3) 5 mM DFMO plus 50 \(\mu\)M putrescine. On day 2, the medium was replaced with fresh medium. On day 4, cell numbers were determined. Data are means \(\pm\) SE of 5 independent experiments, each performed in triplicate; \(*P < 0.05\), different from lacZ-EC under the same treatment; \(a, b, c P < 0.05\), means with different letters are different within each EC group.](image)
lacZ-EC (Fig. 5), but there were no significant differences ($P > 0.05$) in intracellular spermine concentrations as we previously reported (14). The addition of 5 mM DFMO completely abolished the difference in cellular putrescine concentration among AI-EC, AII-EC, and lacZ-EC (Fig. 5), but spermidine concentration remained greater ($P < 0.05$) in AI-EC and AII-EC compared with lacZ-EC (Fig. 5). Spermine concentrations were unaffected by DFMO treatment (Fig. 5).

**DISCUSSION**

Besides playing an essential role in hepatic and intestinal urea synthesis from ammonia, arginase also may play a role in mammalian polyamine synthesis by producing L-ornithine as substrate for ODC (32). Our recent study has shown that overexpression of either arginase I or arginase II can enhance polyamine synthesis in EC (14). Likewise, overexpression of arginase I increases polyamine production in macrophages (12) and vascular smooth muscle cells (9, 28). In addition, induction of arginase II correlates with enhanced polyamine generation in enterocytes during weaning (30) or in the lactating mammary gland (19). Collectively, these findings suggest that intracellular L-ornithine availability is a limiting factor for polyamine synthesis in a wide variety of cell types and that both arginase isoforms have the ability to modulate polyamine synthesis. Although ODC is located in the cytosol, mitochondrially generated L-ornithine is available as substrate for this enzyme for polyamine synthesis in cells such as EC (this study) and enterocytes (29, 30), which is likely due to the presence of an L-ornithine transporter on the mitochondrial membrane (4, 10).

The essential role of polyamine synthesis in cell proliferation has been known for several decades (6). Elevated polyamine synthesis is an initial and necessary event in the cell cycle, and thus inhibition of polyamine synthesis arrests cell growth (16). A novel and important finding of this study is that expression of arginase I or arginase II can modulate EC proliferation on the basis of increases in both cell numbers (see Fig. 2) and PCNA expression (see Fig. 3) after overexpression of either arginase isoform. In agreement with these findings, arginase I expression was recently shown to be a limiting factor for the proliferation of vascular smooth muscle cells (9, 28). Although the present results are strong evidence for the role of arginase expression in cultured EC, we cannot state with certainty that arginase expression is limiting for EC proliferation in vivo. This matter will be addressed in future studies using mice with targeted knockout of arginase expression in EC.

To establish that elevated polyamine synthesis was involved in mediating the enhanced proliferation of AI-EC and AII-EC, the ODC inhibitor DFMO was employed. The addition of 5 mM DFMO to the culture medium markedly decreased intracellular putrescine and spermidine concentrations in EC, markedly reduced EC proliferation, and completely abolished any differences in the proliferation of AI-EC, AII-EC, and lacZ-EC. Furthermore, the addition of 10 or 50 μM putrescine to the culture medium dose-dependently restored the proliferation rates of DFMO-treated AI-EC, AII-EC, and lacZ-EC to the same extent (see Fig. 4), which indicates that there were no intrinsic differences in capability for proliferation among these cell lines. These data support our earlier conclusion that arginase can be a limiting factor for endothelial polyamine synthesis (14). Our findings also suggest that the increase in EC proliferation brought about by elevated arginase I or arginase II expression was mediated by an increase in polyamine synthesis as was recently shown for vascular smooth muscle cells (9, 28). A role for arginase in cell proliferation is also consistent with previous findings that 1) arginase-deficient Chinese hamster ovary cells could not grow in medium that lacked L-ornithine or polyamines (7), and 2) inhibition of arginase decreased the proliferation of the Caco-2 human colon carcinoma cell line (3) and the MDA-MB-468 human breast cancer cell line (24). Of note, under the present experimental conditions, spermine concentrations did not differ among AI-EC, AII-EC, and lacZ-EC, which indicates that spermine levels are not a determinant of EC proliferation rates.

Our findings may have important implications for EC proliferation under physiological and pathological conditions. Angiogenesis, the formation of new capillary blood vessels from preexisting microvessels, involves the migration and proliferation of EC, breakdown and reassembly of extracellular matrix, and construction of an endothelial tube (2). The essential role of polyamines in EC proliferation and angiogenesis has been well documented (1, 22, 26). Because we showed previously that elevated arginase expression in EC increased L-arginine-dependent synthesis of L-proline and polyamines (14), we also tested whether proline availability was limiting for EC proliferation rates. Addition of 0.1–2.0 mM L-proline to the tissue culture

![Graph](http://ajpregu.physiology.org/)

**Fig. 5.** Polyamine contents in stably transfected EC. Experiments were performed as described for Fig. 4. On day 4, cellular levels of putrescine were measured by HPLC. Data are means ± SE, $n = 4$; $^*P < 0.05$, different from control (0 mM DFMO treatment) within each cell group; $^+P < 0.05$, different from corresponding value for lacZ-EC within each treatment. DFMO, 5 mM.

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medium did not result in an increase in the growth of lacZ-EC, AI-EC, or AII-EC (31), which indicates that proliferation rates of AI-EC and AII-EC are not dependent on rates of proline synthesis.

It is noteworthy that arginase activity is severely deficient in EC of the spontaneously diabetic BB rat [an animal model of human type 1 diabetes mellitus (31)]. Interestingly, these cells also exhibit a marked impairment in proliferation (15). Arginase activity is also low in platelets of diabetic rats and humans compared with nondiabetic subjects (18). Because arginase can modulate EC proliferation through an increase in polyamine synthesis, elevating the expression of arginase I or arginase II in EC may provide a novel gene-therapy approach for improving angiogenesis and wound healing in diabetes mellitus.

**Perspectives**

Arginase is a major enzyme for providing intracellular ornithine for polyamine synthesis in many mammalian cells (32). Results of this study demonstrate that arginase I or II expression may be a limiting factor for EC proliferation. An increase in arginase activity results in enhanced synthesis of ornithine, polyamines, and proline (the major component of collagen) by EC (Ref. 14 as well as this study). As such, arginase may be a novel and attractive target for modulation of EC proliferation, angiogenesis, and vascular remodeling. An important application of this concept would be to enhance wound healing, improve microcirculatory function, and treat neovascular diseases. It therefore would be of great interest to investigate expression of endothelial arginase I and II in response to physiological and pathological factors that are known to promote or inhibit angiogenesis. Collectively, these studies may help establish an important but hitherto unrecognized role for arginase in the regulation of cardiovascular function.

In conclusion, results of this study demonstrate for the first time that arginase is normally a limiting factor for endothelial cell proliferation. Consequently, elevated expression of either arginase I or arginase II results in enhanced EC proliferation via an increase in polyamine synthesis from L-arginine. As EC proliferation is an initial and necessary step in angiogenesis, regulation of arginase expression may have important implications for wound healing and cardiovascular function.

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