Elevated arginase I expression in rat aortic smooth muscle cells increases cell proliferation

Liu Hua Wei*, Guoyao Wu†, Sidney M. Morris, Jr.‡, and Louis J. Ignarro*§

*Department of Molecular and Medical Pharmacology, Center for the Health Sciences, School of Medicine, University of California, Los Angeles, CA 90095; †Cardiovascular Research Institute and Department of Medical Physiology, Texas A&M University System Health Science Center, College Station, TX 77843; and ‡Department of Biochemistry and Molecular Genetics, University of Pittsburgh, Pittsburgh, PA 15261

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Arginase, which exists as the isoforms arginase I and II, catalyzes the hydrolysis of arginine to ornithine and urea. Ornithine is the principal precursor for production of polyamines, which are required for cell proliferation. Rat aortic smooth muscle cells (RASMC) contain constitutive arginase I, and arginase inhibitors cause inhibition of cell proliferation. The objective of this study was to determine whether the elevated expression of arginase I in RASMC causes increased cell proliferation. RASMC were stably transfected with either rat arginase I cDNA or a β-galactosidase control expression plasmid. Western blots and arginase enzymatic assays revealed high-level expression of cytosolic arginase I in arginase I-transfected RASMC. Moreover, this observation was associated with the increased production of urea and polyamines and higher rates of RASMC proliferation. The two selective inhibitors of arginase, N^G-hydroxy-L-arginine and *S*-(2-boronoethyl)-L-cysteine, inhibited arginase and decreased the production of urea and polyamines in arginase I-transfected RASMC, all of which were associated with the inhibition of cell proliferation. This study demonstrates that elevated arginase I expression increases RASMC proliferation by mechanisms involving increased production of polyamines. These observations suggest that arginase I plays a potentially important role in controlling RASMC proliferation.

nitric oxide | vascular smooth muscle growth | polyamines | atherosclerosis

Atherosclerosis is the underlying cause of coronary artery disease, which, in turn, is the most common cause of morbidity and untimely death in Western countries. An acute attack is typically precipitated by thrombosis occurring at the site of atherosclerotic plaque disruption. Atherosclerotic plaques consist of a fibrous cap overlaying a lipid-rich cone. Many cell types are involved in their formation, including platelets, endothelial cells, smooth muscle cells (SMC), macrophages, and leukocytes (1). A key event in the pathogenesis of the disease is the migration and proliferation of SMC from the medial layer to the intimal layer in arteries. Excessive intimal thickening contributes to failures in vascular graft and angioplasty procedures by causing luminal restenosis as a result of excessive SMC growth. The precise mechanisms that control arterial SMC proliferation have not been resolved. In an attempt to better understand such mechanisms, we have found that rat aortic SMC (RASMC) contain constitutive as well as IL-4- and IL-13-inducible arginase I, and that both IL-4 and IL-13 stimulate RASMC proliferation (2). Therefore, there seems to be a close association between arginase activity and SMC proliferation.

Arginase (EC 3.5.3.1), which exists as two distinct isoforms (arginase I and arginase II), catalyzes the conversion of arginine to ornithine plus urea, and ornithine decarboxylase (ODC) catalyzes the subsequent conversion of ornithine to putrescine (3). Putrescine and decarboxylated *S*-adenosylmethionine serve as substrates for the subsequent synthesis of spermidine, which is a precursor of spermine. Polyamines (putrescine, spermidine, and spermine) are required components for the progression of the cell cycle and, as such, play an important role in cell proliferation (4, 5). We hypothesized that at least one of the mechanisms by which IL-4 and IL-13 stimulate RASMC proliferation is by up-regulation of arginase I, which subsequently makes more ornithine available for polyamine synthesis (2). Our objective in the present study was to test this hypothesis by determining whether elevated arginase I expression in RASMC stimulates cell proliferation, and whether this effect is mediated by mechanisms involving increased polyamine production. The present study uses gene transfection specifically to manipulate the expression of arginase I to study its impact on cell proliferation in RASMC.

Materials and Methods

Chemicals and Solutions. Cell-culture medium, supplements, and reagents for urea determination, arginase assay, and protein determination were described (6). N^G-hydroxy-L-arginine (NOHA) was obtained from Cayman Chemicals (Ann Arbor, MI). *S*-nitroso-*N*-acetylpenicillamine (SNAP) was synthesized as described (7). *S*-(2-boronoethyl)-L-cysteine (BEC) was a generous gift from D. W. Christianson (Univ. of Pennsylvania, Philadelphia).

Vector Construction and Transfection of RASMC. pEF1-rARGI, a mammalian expression plasmid for rat arginase I, was constructed by inserting the *Eco*RI–*Bsa*AI 1194-bp coding region fragment of pARGr-2 (8) into the *Eco*RI/*Pme*I sites of the plasmid pEF1/Myc-His C. No additional epitope sequences were fused to the arginase I coding sequence in this construct. For control transfection, the β-galactosidase expression plasmid (pEF1/Myc-His/lacZ) alone, which contains the *Escherichia coli lacZ* gene under control of the human elongation factor (EF)-1α promoter (Invitrogen), was used to represent the expression of an unrelated exogenous protein. RASMC were transfected with pEF1/rARGI or pEF1/Myc-His/lacZ by using Lipofectamine (Life Technologies, Rockville, MD) according to the manufacturer's instructions. Stably transfected cells were selected with the antibiotic G418 (500 μg/ml) in complete DMEM. Cells were maintained at 37°C in complete DMEM containing 10% (vol/vol) FBS and 500 μg/ml G418. After ≈3 weeks, G418-resistant clones were isolated and analyzed individually for expression of arginase I. The individual clones were grown in DMEM containing 10% (vol/vol) FBS and 250 μg/ml G418. The G418 was omitted from the cell-culture medium beginning 2 days before initiating any experiments. Stably transfected RASMC were examined for expression of arginase I by Western blot analysis as described (2).

Abbreviations: SMC, smooth muscle cells; RASMC, rat aortic smooth muscle cells; ODC, ornithine decarboxylase; NOHA, N^G-hydroxy-L-arginine; SNAP, *S*-nitroso-*N*-acetylpenicillamine; BEC, *S*-(2-boronoethyl)-L-cysteine; LacZ, bacterial β-galactosidase.

§To whom reprint requests should be addressed. E-mail: lignarro@mednet.ucla.edu.

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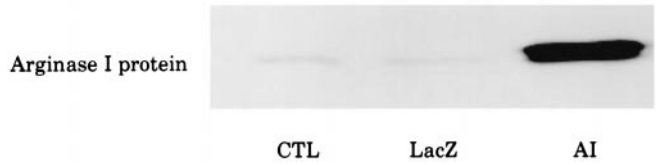

Fig. 1. Arginase I expression in RASMC stably transfected with rat arginase I cDNA. Control (CTL) represents untransfected RASMC; LacZ represents RASMC transfected with a β-galactosidase expression plasmid; AI represents RASMC transfected with a rat arginase I expression plasmid. Twenty micrograms of protein was loaded in each lane, and immunoreactive arginase I was detected by Western blot analysis. RASMC (5×10^6 cells per dish) were incubated at 37°C in cell-culture medium for 24 hr and then harvested, washed, and lysed. Cell lysates were used for Western blot analysis. Data illustrated are from a single experiment and are representative of a total of five separate experiments.

Cell Culture of RASMC and Measurement of Cell Proliferation. RASMC was a generous gift from S. Gross (Cornell Medical College, New York). Cells were plated, grown, subcultured, and cultured as described (2). In determining the rates of DNA synthesis, a modification of the [*methyl*-^{3}H]thymidine incorporation procedure described (9) was used, and cell proliferation data are expressed as a percent of control (9, 10).

Arginase Assay. Arginase activity was determined by methods that we have described (6). Briefly, RASMC (5×10^6 cells per sample) were washed twice with ice-cold PBS, harvested, pelleted by centrifugation, and then lysed. Supernatant fractions were assayed for arginase activity under optimal conditions of pH (9.6) and L-arginine concentration (20 mM) by monitoring the conversion of L-[*guanido*-^{14}C]arginine to [^{14}C]urea during a 10-min incubation.

Determination of Urea Concentrations in Cell-Culture Media. RASMC were analyzed for urea production by determination of urea released into the cell-culture medium. Cell-culture media were collected and analyzed spectrophotometrically for urea exactly as described (6).

Determination of Polyamine Concentrations in Cells. The concentrations of putrescine, spermidine, and spermine in RASMC were determined by a sensitive HPLC procedure (11). Briefly, RASMC were plated at a density of 10^6 cells per 100-mm dish and grown to 80% confluence before the start of experiments. Transfected cells (6×10^6 cells) were rinsed with PBS and then incubated at 37°C for 24 hr in complete DMEM containing 0.5% FBS and 0.4 mM L-arginine. After 24 hr, the cells were rapidly washed twice with ice-cold PBS and then lysed in 0.5 ml of 1.5 M $HClO_4$, and the solution was neutralized by the addition of 0.25 ml of 2 M K_2CO_3. The neutralized extracts were used for the determination of polyamines.

Statistical Analyses. Where indicated, data were analyzed statistically by using the Bonferroni *t* test for unpaired values. Values of $P < 0.05$ were taken to indicate statistical significance.

Results

Elevated Expression of Arginase I in RASMC. RASMC were stably transfected to express either rat arginase I or bacterial β-galactosidase (LacZ), the latter representing the control for expression of an unrelated cytosolic protein. Western blots demonstrated that RASMC transfected with rat arginase I cDNA expressed high levels of arginase I protein (Fig. 1). Control RASMC contained a much smaller quantity of arginase I present constitutively, which was not altered quantitatively by cell transfection with LacZ. Increased catalytic activity of arginase I in

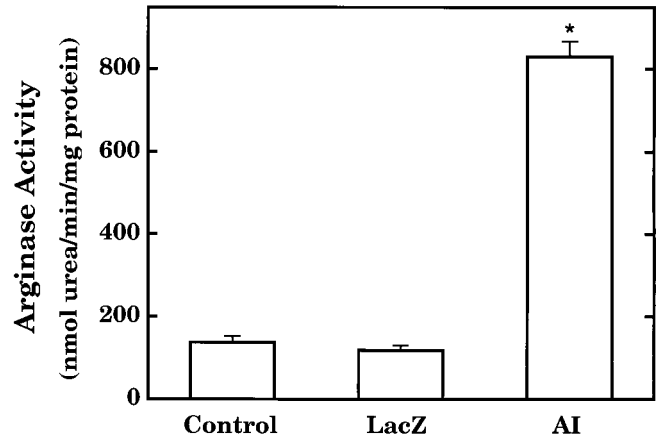

Fig. 2. Arginase I activity in RASMC stably transfected with rat arginase I cDNA. Control represents untransfected RASMC; LacZ represents RASMC transfected with a β-galactosidase expression plasmid; AI represents RASMC transfected with a rat arginase I expression plasmid. RASMC (5×10^6 cells per dish) were incubated at 37°C in cell-culture medium for 24 hr and then harvested, washed, and lysed; cell lysates were used for the determination of arginase activity. Cell lysates were the same cell lysates used for Western blot analysis (Fig. 1). Arginase activity was determined by monitoring the conversion of L-[*guanido*-^{14}C]arginine to [^{14}C]urea. Data represent means ± SE of duplicate determinations from five separate experiments. *, $P < 0.05$, significantly different from Control.

arginase I-transfected RASMC was confirmed by analysis of cell extracts for arginase activity. The specific activity of arginase in extracts of arginase I-transfected cells was 8- to 10-fold higher than that in either control RASMC or LacZ-transfected cells (Fig. 2). Consistent with the data on Western blot analysis and arginase activity, the production of urea by arginase I-transfected cells was 4-fold greater than that by control or LacZ-transfected cells (Fig. 3).

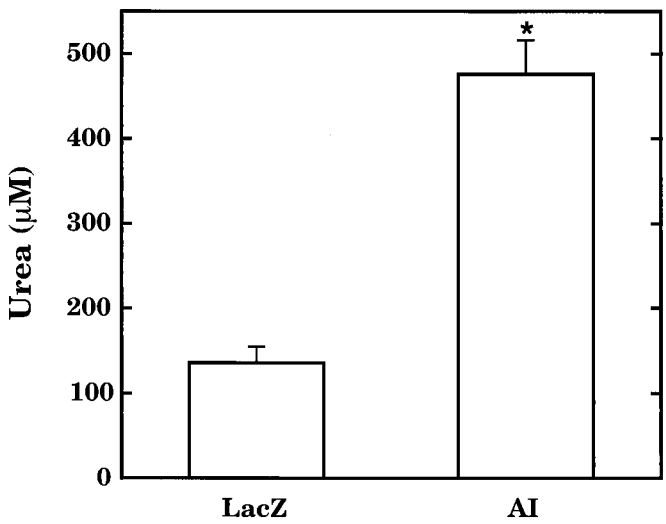

Fig. 3. Urea production as a measure of arginase activity in RASMC stably transfected with rat arginase I cDNA. LacZ represents RASMC transfected with a β-galactosidase expression plasmid; AI represents RASMC transfected with a rat arginase I expression plasmid. RASMC (5×10^6 cells per dish) were incubated at 37°C in cell-culture medium for 24 hr and then harvested, washed, and lysed; cell lysates were used for urea determinations. Data represent means ± SE of duplicate determinations from five separate experiments. *, $P < 0.05$, significantly different from Control.

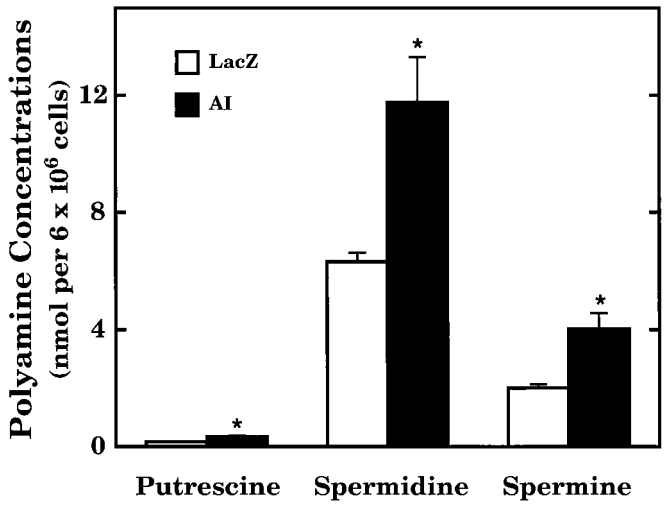

Fig. 4. Polyamine production in RASMC stably transfected with rat arginase I cDNA. LacZ (white bars) represents RASMC transfected with a β-galactosidase expression plasmid; AI (black bars) represents RASMC transfected with a rat arginase I expression plasmid. RASMC (6×10^6 cells per dish) were incubated at 37°C for 24 hr in complete DMEM containing 0.4 mM L-arginine and 0.5% FBS. Cells were then harvested, washed, and extracted as described in the text. Data represent means ± SE of duplicate determinations from three separate experiments. *, $P < 0.05$, significantly different from corresponding LacZ controls.

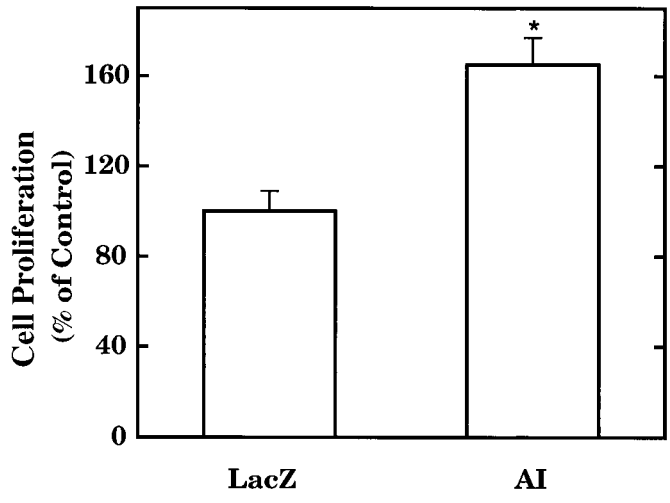

Fig. 5. Influence of increased arginase I expression on RASMC proliferation. LacZ represents RASMC transfected with a β-galactosidase expression plasmid; AI represents RASMC transfected with a rat arginase I expression plasmid. Cell proliferation was assessed by thymidine incorporation into DNA during the second day (24 hr) of a 2-day growth period as described in the text. Data were calculated as dpm per 10^5 cells per well and expressed as a percent of LacZ control (assigned 100%). Data represent means ± SE of duplicate determinations from four separate experiments. *, $P < 0.05$, significantly different from LacZ control.

Influence of Elevated Arginase I Expression on Polyamine Production and Proliferation in RASMC. Arginase I-transfected cells showed significantly higher levels of polyamines than LacZ-transfected cells (Fig. 4). Putrescine increased by 208%, spermidine increased by 186%, and spermine increased by 202% compared with LacZ-transfected cells. The concentration of putrescine in cells is generally about one to two orders of magnitude lower than that of either spermidine or spermine (5, 10, 11). The actual values for putrescine concentrations ranged from 0.20 ± 0.03 to 0.41 ± 0.06 nmol per 6×10^6 cells for LacZ-transfected RASMC and arginase I-transfected RASMC, respectively. The increase in polyamine production correlated with the increase in cell proliferation observed in arginase I-transfected RASMC (Fig. 5). Arginase I-transfected RASMC showed over a 60% increase in the rate of cell proliferation when compared with LacZ-transfected RASMC as determined by thymidine incorporation into DNA.

Influence of Arginase Inhibitors and NO on Polyamine Production and Proliferation in Arginase I-Transfected RASMC. The data presented above suggest that increased arginase I expression in RASMC leads to the increased proliferation of RASMC, and that increased growth may be attributed to increased polyamine production. Accordingly, the argument follows that inhibitors of arginase should cause both a decrease in polyamine production as well as a decrease in cell proliferation. Reports from this laboratory have indicated that arginase inhibitors and NO interfere with the proliferation of tumor cells and vascular SMC (9, 10). In contrast to the arginase inhibitors, NO interferes with cell growth by inhibiting ODC which, in turn, should lead to decreased polyamine production. In the present series of experiments, two arginase inhibitors and NO were compared with respect to their effects in arginase I-transfected RASMC. Fig. 6 illustrates that two arginase inhibitors, NOHA and BEC, and the NO donor agent, SNAP, each inhibited the proliferation of arginase I-transfected RASMC in a concentration-dependent manner.

Each of the two arginase inhibitors, but not SNAP, caused inhibition of arginase activity after incubation with arginase I-transfected RASMC (Fig. 7). Consistent with these findings, NOHA and BEC inhibited urea production by these cells (Fig. 8), whereas SNAP did not alter urea production (data not shown). To be certain that neither the arginase inhibitors nor SNAP was interfering with arginase I expression during cell culture, Western blot analysis was performed, and the data (Fig. 9) indicate clearly that none of the test agents interfered with arginase I expression in arginase I-transfected RASMC. The

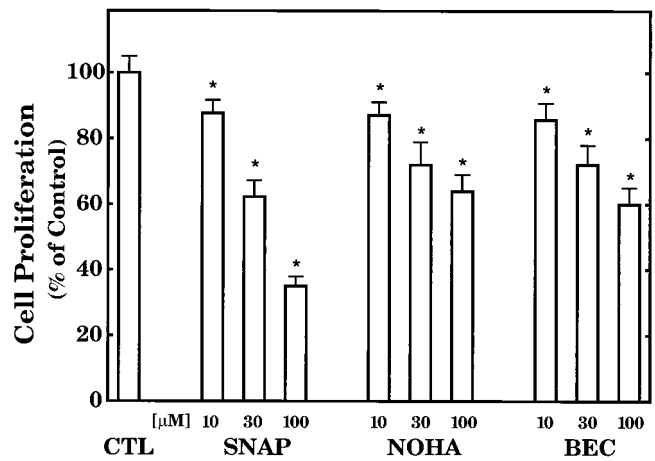

Fig. 6. Influence of arginase inhibitors and NO on the proliferation of RASMC stably transfected with rat arginase I cDNA. RASMC were transfected with a rat arginase I expression plasmid. Cell proliferation was assessed by thymidine incorporation into DNA during the second day (24 hr) of a 2-day growth period as described in the text. Test agents were added to the cells at the time of thymidine addition. Data were calculated as dpm per 10^5 cells per well and expressed as a percent of control (CTL; absence of added test agents; assigned 100%). CTL, arginase I-transfected RASMC. Data represent means ± SE of duplicate determinations from four separate experiments. *, $P < 0.05$, significantly different from CTL.

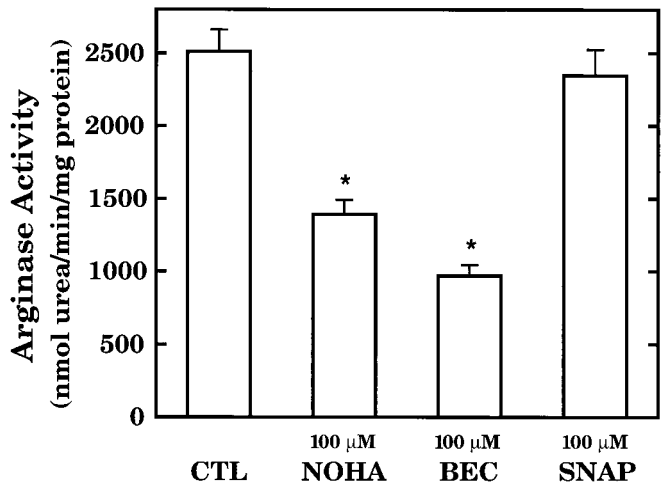

Fig. 7. Influence of arginase inhibitors and NO on arginase activity in RASMC stably transfected with rat arginase I cDNA. RASMC were transfected with a rat arginase I expression plasmid. RASMC (5×10^6 cells per dish) were incubated in the absence or presence of test agents at 37°C in cell-culture medium for 24 hr and then harvested, washed, and lysed; cell lysates were used for determination of arginase activity. Arginase activity was determined by monitoring the conversion of L-[*guanido*-^{14}C]arginine to [^{14}C]urea. Control (CTL), arginase I-transfected RASMC. Data represent means ± SE of duplicate determinations from three separate experiments. *, $P < 0.05$, significantly different from CTL.

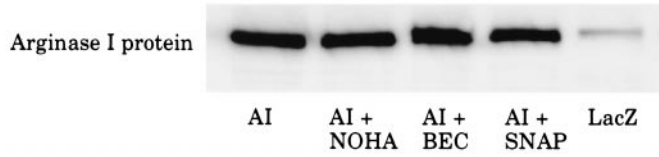

Fig. 9. Influence of arginase inhibitors and NO on arginase I expression in RASMC stably transfected with rat arginase I cDNA. AI represents RASMC transfected with a rat arginase I expression plasmid; LacZ represents RASMC transfected with a β-galactosidase expression plasmid. Twenty micrograms of protein was loaded in each lane, and immunoreactive arginase I was detected by Western blot analysis. RASMC (5×10^6 cells per dish) were incubated at 37°C in cell-culture medium for 24 hr in the absence or presence of 100 μM NOHA, 100 μM BEC, or 100 μM SNAP as indicated. Cells were then harvested, washed, and lysed; cell lysates were used for Western blot analysis. Data illustrated are from a single experiment and are representative of a total of four separate experiments.

probable mechanism by which NOHA, BEC, and SNAP interfere with proliferation of arginase I-transfected RASMC is inhibition of polyamine production (Fig. 10). NOHA, BEC, and SNAP, each at 100 μM, significantly inhibited the production of putrescine, spermidine, and spermine. The percentage inhibition of polyamine production ranged from 35% to 65%.

Discussion

There are two distinct isoforms of mammalian arginase (arginase I and arginase II) that are encoded by different genes and differ in molecular and immunological properties, tissue distribution, subcellular location, and regulation of expression (12, 13). Arginase I is highly expressed in the liver of higher animals to detoxify ammonia. Arginase I is also expressed to a much lesser extent in other cell types. Because extrahepatic tissues do not possess a complete urea cycle, the physiological effects of arginase I in those cells are unclear. The present study shows that elevated expression of arginase I markedly increases cellular DNA synthesis by RASMC, suggesting that arginase is important not only in the urea cycle but also in biochemical pathways essential to controlling cell proliferation. The importance of arginase in cell growth is supported by the knowledge that arginase is the principal enzyme that generates ornithine, the substrate for ODC. In turn, ODC catalyzes the conversion of ornithine to putrescine, a polyamine that serves as the precursor for two additional polyamines, spermidine and spermine. These polyamines are required for cell proliferation and seem to function by binding to DNA and modulating DNA–protein interactions (4, 5). The present findings that cellular polyamine levels increase about 2-fold in arginase I-transfected RASMC

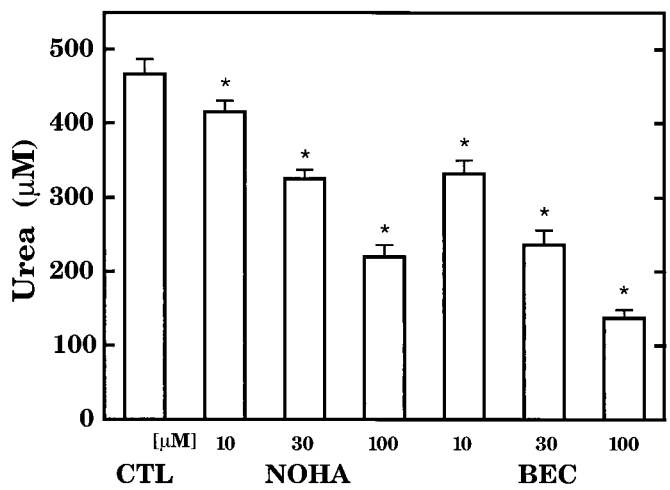

Fig. 8. Influence of arginase inhibitors on urea production in RASMC stably transfected with rat arginase I cDNA. RASMC were transfected with a rat arginase I expression plasmid. RASMC (5×10^6 cells per dish) were incubated in the absence or presence of test agents at 37°C in cell-culture medium for 24 hr and then harvested, washed, and lysed; cell lysates were used for urea determinations. Control (CTL), arginase I-transfected RASMC. Data represent means ± SE of duplicate determinations from three separate experiments. *, $P < 0.05$, significantly different from CTL.

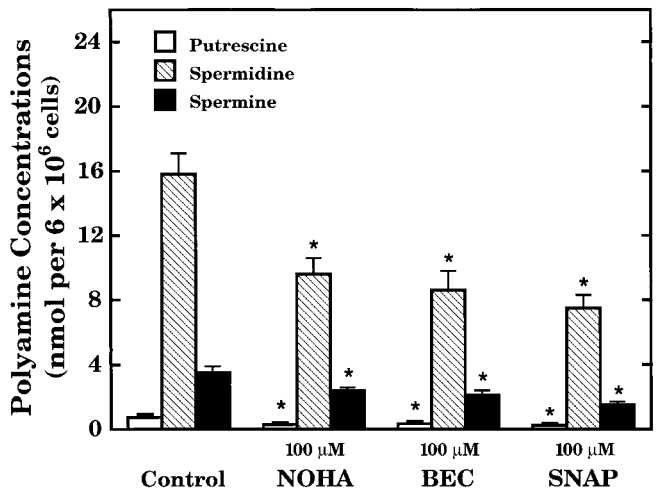

Fig. 10. Influence of arginase inhibitors and NO on polyamine production in RASMC stably transfected with rat arginase I cDNA. RASMC (6×10^6 cells per dish) were incubated at 37°C for 24 hr in complete DMEM containing 0.4 mM L-arginine, 0.5% FBS, and test agents as indicated. Cells were then harvested, washed, and extracted as described in the text. NOHA, BEC, and SNAP, each at 100 μM, significantly inhibited the production of putrescine (white bars), spermidine (hatched bars), and spermine (black bars). Data represent means ± SE of duplicate determinations from three separate experiments. *, $P < 0.05$, significantly different from corresponding Control values.

compared with LacZ-transfected cells support this view. These results are consistent with the view that arginase I-transfected cells produce more ornithine that is available for ODC, which subsequently produces more polyamines to support cell proliferation.

We have shown that NOHA, the major intermediate in the NO synthase-catalyzed conversion of arginine to NO and citrulline, is a potent inhibitor of both arginase and tumor-cell proliferation (9). Arginase is a binuclear Mn^{2+} metalloenzyme with a high binding affinity for boronic acid-based arginine analogs (14). BEC is one such analog and has been reported to be more potent than NOHA as an arginase inhibitor (14). Both NOHA and BEC are competitive inhibitors of arginase (15). In the present study, we found that arginase catalytic activity and urea production, but not arginase I protein expression, were markedly inhibited by NOHA and BEC in arginase I-transfected RASMC. These observations indicate that both arginase inhibitors permeate cells and decrease intracellular arginase activity. Inhibition of arginase activity and urea production was associated with a concomitant inhibition of polyamine production and cell proliferation in arginase I-transfected RASMC. These observations support the hypothesis that arginase activity is important in maintaining cell proliferation and that interference with the production of ornithine can result in impaired polyamine production and decreased cell proliferation.

NO also inhibits cell proliferation, and the mechanism seems to be cGMP-independent and attributed to the inhibition of ODC (9, 10, 16). In this regard, the action of NO resembles the action of the well known ODC inhibitor α-difluoromethylornithine, which has been shown to inhibit polyamine production in and proliferation of RASMC (10). The mechanism of inhibition of ODC by NO donor agents such as SNAP seems to be *S*-nitrosation of the active site Cys-360 residue by transnitrosation, thereby inactivating the enzyme because the Cys-360 sulfhydryl is essential for the expression of ODC catalytic activity (17, 18). The present data indicate that SNAP inhibits proliferation and polyamine production in arginase I-transfected RASMC by mechanisms that do not involve the inhibition of arginase activity. The mechanism by which SNAP interferes with the proliferation of arginase I-transfected RASMC is likely to be the same as that in control RASMC, namely, by inhibition of ODC. The present study demonstrates that both arginase I and ODC are important enzymes in controlling polyamine synthesis and proliferation in RASMC. The findings that these two sequential enzymes in the polyamine pathway are inhibited by two products of NO synthase (NOHA and NO) argue for a modulatory role for the arginine-NO pathway in vascular SMC proliferation.

ODC often has been considered to be the first and rate-limiting enzyme in polyamine synthesis from ornithine (19). However, this view was expressed before the availability of potent arginase inhibitors to test this hypothesis more vigorously. The data provided in this study using RASMC show clearly that overexpression with arginase I results in enhanced polyamine production and increased rates of cell proliferation. Moreover, two different arginase inhibitors cause reductions in polyamine production as well as cell proliferation. We do find, however, that the NO donor agent, SNAP, is more effective than NOHA or BEC in slowing cell proliferation despite the fact that their potencies as inhibitors of arginase or ODC are quite similar (9, 10, 14–16). Therefore, the ODC enzymatic reaction may be somewhat slower and more rate-limiting than the arginase reaction under certain conditions but, nevertheless, inhibition of either reaction can lower polyamine production and decrease cell proliferation.

Coronary artery disease and atherosclerosis in general are considered to be chronic inflammatory processes characterized by the invasion of vascular tissue by circulating blood cells such as monocytes, which then proliferate and elaborate factors that cause the underlying vascular SMC to proliferate, eventually resulting in plaque formation, lumen occlusion, and plaque rupture (20). Cytokines such as IL-4 and IL-13 produced in humans by activated $CD4^+$ cells have been found to be the predominant T cells in atherosclerotic lesions (21, 22). We have demonstrated that both IL-4 and IL-13 can up-regulate arginase I expression in RASMC and stimulate cell proliferation (2). Based on these observations and those of the present study, we forward the hypothesis that during atherogenesis, cytokines produced by local cells may up-regulate arginase I expression in SMC and thereby lead to increased rates of cell proliferation. In conclusion, the present study suggests that arginase I acts as a primary step in the pathway to polyamine formation and cell proliferation. This knowledge may aid in the understanding of the mechanisms that control vascular SMC proliferation under both physiological and pathophysiological conditions such as atherosclerosis, restenosis, and angiogenesis.

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