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Arginase Promotes Neointima Formation in Rat Injured Carotid Arteries

Kelly J. Peyton, MA[a,b], **Diana Ensenat, MS**[b], **Mohammed A. Azam, MS**[a], **Amit N. Keswani, BS**[b], **Sankaranarayanan Kannan, PhD**[a,b], **Xiao-ming Liu, MD**[a,b], **Hong Wang, MD, PhD**[c], **David A. Tulis, PhD**[d,e], and **William Durante, PhD**[a,b]

a*Department of Medical Pharmacology and Physiology, School of Medicine, University of Missouri, Columbia, MO, USA*

b*Department of Medicine, Baylor College of Medicine, Houston, TX, USA*

c*Department of Pharmacology, Temple University, Philadelphia, PA, USA*

d*J.L. Chambers Biomedical/Biotechnology Research Institute and Department of Biology, North Carolina Central University, Durham, NC, USA*

e*Department of Physiology, Brody School of Medicine, East Carolina University, Greenville, NC, USA*

Abstract

Objective—Arginase stimulates the proliferation of cultured vascular smooth muscle cells (VSMCs); however, the influence of arginase on VSMC growth *in vivo* is not known. This study investigated the impact of arginase on cell cycle progression and neointima formation following experimental arterial injury.

Methods and Results—Balloon injury of rat carotid arteries resulted in a sustained increase in arginase activity in the vessel wall and the induction of arginase I protein in both the media and neointima of injured vessels. Furthermore, local perivascular application of the potent and selective arginase inhibitors *S*-(2-boronoethyl)-L-cysteine (BEC) or N^G-hydroxy-nor-L-arginine (L-OHNA) immediately after injury markedly attenuated medial and neointimal DNA synthesis and neointima formation. Substantial arginase I protein and arginase activity was also detected in rat cultured aortic VSMCs. Moreover, treatment of VSMCs with BEC or L-OHNA, or knockdown of arginase I protein, arrested cells in the G_0/G_1 phase of the cell cycle and induced the expression of the cyclin-dependent protein kinase inhibitor, p21.

Conclusion—This study demonstrates that arginase is essential for VSMCs to enter the cell cycle and that arginase I contributes to the remodeling response following arterial injury. Arginase I represents a potentially new therapeutic target for the treatment of vasculoproliferative disorders.

Correspondence: William Durante, PhD, Department of Medical Pharmacology and Physiology, M409 Medical Sciences Building, University of Missouri-Columbia, One Hospital Drive, Columbia, MO 65212, USA, Tel: 573-882-3886, Fax: 573-884-4276, Email: durantew@health.missouri.edu.

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Introduction

The proliferation of vascular smooth muscle cells (VSMCs) plays a critical role in the formation of vascular lesions, and is the major pathophysiologic mechanism responsible for the failure of interventional therapeutic approaches to treat occlusive vascular disorders, including vein bypass failure, transplant arteriosclerosis, and restenosis after balloon angioplasty (1–3). With the recognition of the essential involvement of VSMC proliferation in occlusive vascular disorders, considerable effort has been directed at characterizing biochemical and molecular pathways that regulate the proliferative response of VSMC following arterial injury. However, the exact mechanism and mediators responsible for VSMC activation and neointima formation following injury of the vessel wall remain elusive.

Arginase is a binuclear manganese metalloenzyme that catalyzes the hydrolysis of L-arginine to L-ornithine and urea (see 4,5). Two distinct isoforms of arginase exist, arginase I and II, that are encoded by distinct genes and exhibit different tissue distribution and subcellular localization but possess similar enzymatic properties. Arginase I is a cytosolic enzyme that is abundantly expressed in the liver and plays a central role in the urea cycle while arginase II is a mitochondrial protein that is concentrated in the kidney. Recent studies have documented the presence of arginase I and II in blood vessels from numerous animal species, including humans (6–9). Interestingly, VSMCs and endothelial cells possess arginase activity yet preferentially express different arginase isoforms: VSMCs exclusively express arginase I while endothelial cells predominantly express arginase II (10–13).

Emerging studies indicate that arginase plays an important role in regulating vascular cell function. Arginase impairs endothelial function by competing with endothelial nitric oxide synthase for substrate L-arginine (6–8,12–15). In this respect, we recently reported that arginase contributes to arteriolar nitric oxide dysfunction in salt-sensitive hypertensive rats by depleting endothelial cells of L-arginine (16). Arginase-mediated endothelial dysfunction has also been documented in other models of hypertension as well as in aging, diabetes, and following ischemia-reperfusion (see 4,5). Besides limiting nitric oxide synthesis, arginase generates biologically significant molecules. In particular, the arginase product L-ornithine is further metabolized by ornithine decarboxylase (ODC) to the polyamine putrescine which forms the successive polyamines, spermine and spermidine (17). Polyamines play an integral role in the mitogenic response of cells. VSMC proliferation is preceded by increases in polyamine synthesis while inhibition of polyamine formation blocks cell growth (18–20). Interestingly, we previously reported that VSMCs possess arginase activity and postulated a physiologic role for arginase in augmenting cell growth by optimizing polyamine synthesis in these cells (21). Indeed, subsequent studies found that overexpression of arginase I enhances polyamine generation and VSMC proliferation in culture while arginase inhibition suppresses VSMC growth (20,22). However, the underlying mechanisms by which arginase promotes VSMC proliferation have not been fully characterized. Moreover, the involvement of arginase in governing vascular growth *in vivo* is not known. Accordingly, the present study investigated the role of arginase in regulating cell cycle progression and neointima formation in a rat carotid artery injury model.

Materials and Methods

Rat Carotid Artery Balloon Injury

Male Sprague Dawley rats (400–450g; Charles River Laboratories, Wilmington, MA) were anesthetized (ketamine, xylazine, and acepromazine; 0.7ml/kg, intramuscularly; VetMed Drugs, Houston, TX) and the left carotid artery injured with a Fogarty 2F embolectomy catheter (Baxter Healthcare Corporation, Houston, TX), as we previously described (23,24). Immediately after injury, a local polymer-based delivery system was used to administer arginase inhibitors to the injured vessel wall. The delivery system consisted of 200µl of a 25% copolymer gel (PLF127; BASF Corporation, Florham Park, NJ) containing BEC (1mg) or L-NOHA (1mg) that was applied in a circumferential manner to the exposed adventitia of the carotid artery. A separate group of animals received empty gel, which has previously been shown to have no effect on vascular remodeling (25). PLF127 gels act as a rate-controlling barrier and serves as a vehicle for the sustained release of drug releasing an accumulative 5.5% original dose of a drug over 3 hours when using a 25% PLF gel (26). Based on this finding, we estimated a delivery dose of 55µg after 3 hours.

Histology

Animals were euthanized and the vasculature perfusion-fixed with 10% buffered formalin. The common carotid artery was excised, paraffin-embedded, and sections (5µm) stained with Verhoff's-Von Gieson for measurement of vessel dimensions. Microscopic quantification of vessel dimensions was performed using Image-Pro Plus (Media Cybernetics) and Adobe Photoshop software linked through a digital camera (Leaf Microlumina; Leaf Systems, USA) to a Zeiss Axioskop 50 light microscope (Carl Zeiss, Germany) (23–25).

Arginase I Immunohistochemistry

Carotid arteries were perfusion-fixed and embedded in paraffin. Sections (5µm) were dried at room temperature for 20 minutes, fixed in acetone, and treated with 0.3% hydrogen peroxide in methanol to block endogenous peroxidase activity. After washing with PBS, tissues were incubated with BSA (5%) at room temperature for 30 minutes, and then incubated with anti-rabbit arginase I antibody (1:50) at 4°C overnight. Slides were rinsed in PBS, incubated in biotinylated anti-rabbit IgG (1:100) in BSA (1%) for 1 hour, developed with peroxidase-labeled streptavidin and NovaRed (Burlingham, CA), and counterstained with hematoxylin. Slides were viewed and analyzed using a Zeiss Axioskop 50 light microscope (Carl Zeiss, Germany).

DNA Synthesis and Apoptosis

DNA synthesis was determined by measuring the expression of proliferating cell nuclear antigen (PCNA) in the vessel wall by immunostaining (24). Paraffin-embedded tissues were incubated with an anti-PCNA monoclonal antibody (1:25) followed by a biotinylated anti-mouse secondary antibody (1:100). Slides were treated with avidin-biotin block and exposed to DAB black with nuclear fast red counterstain and analyzed by light microscopy. Data are represented as a PCNA-labeling index (LI), defined as the percentage of total cells within a given area positive for PCNA staining. Apoptotic cells were detected by the terminal deoxynucleotidyl transferase (TdT)-measured dUTP nick end-labeling (TUNEL) method and TUNEL-positive cells confirmed microscopically, as we previously described (24).

Endothelial Regrowth

Endothelial regrowth following arterial injury was examined using Evans blue dye, as we previously described (27). Animals were injected intravenously with Evans blue dye (5%) 10 minutes prior to sacrifice. Injured carotid arteries were perfusion-fixed with 10% neutral buffered formalin, removed, cut longitudinally, pinned on a silicon dissecting dish and photographed under a dissecting microscope. Deendothelialized areas were defined as those that stained blue and were determined by planimetry using the Image-Pro Plus program (Media Cybernetics).

Cultured VSMCs

VSMCs were isolated from rat thoracic aorta and arginase activity and polyamine synthesis monitored by measuring the metabolism of radiolabeled L-arginine (10,21). Cell cycle progression was determined by flow cytometry and the expression of arginase I, cyclin D1, cyclin A, cyclin E, p27, p21, and p53 monitored by western and/or northern blotting (23). Complete details of these experiments are available online at http://atvb.ahajournals.org/.

Results

Balloon injury of rat carotid arteries resulted in a time-dependent increase in arginase activity (Figure 1A). A significant increase in arterial arginase activity was detected two days after injury, peaked after one week, and remained elevated two weeks following balloon injury. The peak increase in arginase activity observed one week after arterial injury was associated with an approximate 2-fold increase in arginase I protein expression in the vessel wall (Figure 1B). Immunohistochemistry detected minimal arginase I expression (shown in brown) in control, uninjured carotid arteries (Figure 1C). However, one week after arterial injury there was arginase I staining throughout the vessel wall but intense arginase I expression was observed in the neointima of injured vessels. Omission of the primary antibody or replacement of the primary antibody with non-immune IgG abolished arginase I staining in all sections (data not shown).

Figure 2A shows representative cross-sections of perfusion-fixed, Verhoff-Van-Giesson-stained tissues obtained from injured animals two weeks after balloon injury. Animals treated with empty gel exhibited a significant and concentric neointima. In contrast, a markedly diminished neointima was observed in vessels exposed to gel containing the arginase inhibitors, BEC or L-OHNA. Morphometric measurements indicate that neointimal area, neointimal thickness, and neointimal/medial wall area ratio were all significantly reduced by BEC- or L-OHNA (Figure 2B). Despite the fact that the neointimal/medial wall ratio was reduced by approximately 65%, medial wall area was not significantly changed by either BEC- or L-OHNA. No significant differences were observed between the three groups of animals for circumference of the left carotid internal elastic laminae (gel control, 2.555±0.041mm; BEC-treated, 2.531±0.083mm; L-OHNA-treated, 2.541±0.066mm) or external elastic lamina (gel control, 2.883±0.048mm; BEC-treated, 2.757±0.0886mm; L-OHNA-treated, 2.852 ±0.0582mm).

Representative cross-sections of vessels treated with an empty gel demonstrate substantial PCNA staining 2 days following arterial injury whereas BEC-treated arteries are nearly devoid of staining (Figure 3A). Temporal analysis of medial wall DNA replication in empty gel-treated vessels revealed that arterial injury induced substantial PCNA-labeling 2 and 7 days following balloon injury (Figure 3B). However, this increase in medial wall DNA synthesis was significantly attenuated in vessels treated with BEC- or L-OHNA-containing gels. Similarly, the high rate of PCNA-labeling observed in the neointima 7 and 14 days after injury was blocked by BEC or L-OHNA (Figure 3C). Interestingly, treatment of injured arteries with L-OHNA resulted in a significant 2-fold increase in the expression of the cyclin-dependent kinase inhibitor, p21, compared to gel-treated vessels (Figure 3D). Control, uninjured carotid arteries exhibit minimal VSMC apoptosis (1.5±0.3%); however, balloon injury resulted in significant VSMC apoptosis two days after arterial injury (Figure 3E). Application of either BEC or L-OHNA failed to noticeably alter the rate of apoptosis. Finally, BEC or L-OHNA treatment had no effect on endothelial regrowth 14 days following arterial injury (Figure 3F).

Cultured VSMCs expressed high levels of arginase I protein and substantial arginase activity (Figure 4A and B). Treatment of VSMCs with the arginase inhibitors BEC and L-OHNA markedly reduced arginase activity, confirming the efficacy of these pharmacological inhibitors (Figure 4B). In addition, treatment of VSMCs with BEC or L-OHNA blocked the ability of VSMCs to generate the polyamine putrescine from L-arginine (Figure 4C). Since polyamines play a fundamental role in cell cycle entry (17), succeeding experiments examined whether arginase influenced cell cycle progression in cultured VSMCs. Quiescent VSMCs accumulated in the G_0/G_1 phase of the cell cycle but the administration of serum for 24 hours induced cell cycle progression and the population of cells in G_0/G_1 decreased while the number of cells in S and G_2/M increased (Figure 5A). However, administration of the arginase inhibitors, BEC or L-OHNA, for 24 hours increased the fraction of cells in the G_0/G_1 phase of the cell cycle and this was accompanied by a decrease in the percentage of cells in S and G_2/M (Figure 5A). BEC or L-OHNA decreased the number of cells in S phase by nearly 80% (Figure 5B). No apparent toxicity was noted with either inhibitor, as reflected by the lack of a sub-G_0/G_1 fraction (Figure 5A). In addition, trypan blue staining confirmed the lack of toxicity by either arginase inhibitor (data not shown). Treatment of VSMCs with arginase I siRNA also arrested cells in G_0/G_1 and significantly decreased the percentage of cells in S and G_2/M (Figure 5C). In contrast, non-targeting siRNA had no effect cell cycle progression (data not shown). In addition, transfection of VSMCs with arginase I siRNA suppressed arginase I protein expression by nearly 80% whereas the non-targeting siRNA failed to modify arginase I expression, confirming the efficacy and selectivity of the arginase I knockdown approach (Figure 5D).

To elucidate the mechanisms by which arginase inhibition disrupts cell cycle progression, we examined the effects of L-OHNA or BEC on cell cycle regulatory proteins. L-OHNA failed to inhibit basal or serum-stimulated protein expression of the G_1 cyclins D1, E or A, or the serum-mediated downregulation of the cyclin-dependent kinase inhibitor, p27 (Figure 6A). In contrast, L-OHNA increased the protein expression of the cyclin-dependent kinase inhibitor, p21, and this was associated with an elevation in p53 protein expression in serum-treated cells (Figure 6A). L-OHNA also stimulated p21 mRNA expression in serum-deprived VSMCs and enhanced the serum-mediated induction of p21 mRNA (Figure 6B). Similar findings were also observed with BEC (data not shown). Finally, transfection of VSMCs with arginase I siRNA likewise stimulated the expression of p21 protein (Figure 6C) while non-targeting siRNA failed to modulate p21 expression (data not shown).

Discussion

The present study demonstrates that arginase plays a fundamental role in vascular growth following injury. Arterial injury stimulates arginase activity and arginase I protein expression in the vessel wall, and local arginase inhibition leads to a significant decline in VSMC DNA synthesis and reduced intimal thickening. In addition, this study shows that arginase promotes the entry of VSMCs into the cell cycle. Arginase inhibition or arginase I knockdown arrests VSMCs in G_0/G_1 and this is associated with the induction of the cyclin-dependent protein kinase inhibitor, p21. These findings illustrate a critical role for arginase in cell cycle progression and identify arginase I as a novel therapeutic target for the treatment of occlusive vascular disorders.

In the present study, we are the first to demonstrate that arginase plays an integral role in the remodeling response following arterial injury. Balloon injury of rat carotid arteries resulted in a sustained increase in arginase activity in the vessel wall. A significant increase in arginase activity was observed 2 days after injury, peaked at 7 days, and remained elevated after 14 days. The induction of arterial arginase activity following injury was associated with an approximate 2-fold increase in arginase I protein. While arginase I protein expression was minimally expressed in control, uninjured arteries, substantial arginase I protein was detected in the neointima of injured vessels. Since these cells exhibit the highest mitotic activity (28), arginase I expression may parallel the proliferative status of cells. This may explain the absence of arginase I in the quiescent VSMCs resident in the media of control arteries and the strong expression of arginase I noted in our proliferating cultured VSMCs. Our finding that arginase is upregulated in proliferative vascular lesions is consistent with a recent clinical report demonstrating increased arginase activity in hyperplastic human uterine arteries (29).

Mechanisms responsible for the induction of arginase activity following arterial injury are not known; however, the generation of growth factors and inflammatory mediators following vascular injury may be involved since a number of mitogens and inflammatory cytokines have been demonstrated to stimulate arginase I gene expression (10,11,21).

To determine the role of arginase in neointima formation, we applied the potent and selective arginase inhibitors BEC or L-OHNA (30) topically to the adventitia of the blood vessel using a specific local delivery copolymer. This drug-targeting approach has been used successfully by our laboratory and others, and elicits a sustained release of agent over the course of several days while avoiding possible non-specific effects associated with the systemic administration of drugs (23–25,31). We found that local perivascular application of BEC or L-OHNA markedly diminished neointima formation after injury. Furthermore, vessel caliber was unaffected by arginase inhibition, indicating that positive remodeling did not occur. Moreover, the inhibition of neointima development by BEC or L-OHNA was independent of overt signs of toxicity or with enhanced apoptosis, but was associated with a significant decline in medial and neointimal VSMC DNA replication. Interestingly, the perivascular administration of L-OHNA stimulated the expression of p21 protein, an established inhibitor of arterial injury-induced intimal thickening (32). Since arginase may also contribute to endothelial cell growth (33), the effect of arginase inhibition on the reendothelialization of injured vessels was examined. Neither BEC nor L-OHNA affected endothelial regrowth following balloon injury. Thus, arginase inhibitors appear to specifically reduce VSMC growth following arterial injury. The ability of arginase inhibitors to selectively target VSMCs may confer a significant therapeutic advantage over currently used antiproliferative drugs that also repress endothelial cell growth (34), an undesired side-effect that enhances neointimal development.

Although arginase has been shown to stimulate VSMC growth, the biological basis for this remains largely unknown. Herein, we demonstrate that arginase promotes the entry of VSMCs into the cell cycle. We found that cultured VSMCs express substantial arginase activity. This result is in agreement with previous reports in primary VSMCs (10,11) but contrasts with a recent study that failed to detect arginase activity in a fetal VSMC line (35), suggesting that arginase activity may be differentially regulated in primary and transformed VSMCs. Variable expression of arginase has also been observed in different breast cancer cells, further emphasizing the cell-specific nature of arginase expression (36). We also found that treatment of VSMCs with arginase inhibitors or knockdown of arginase I protein arrests cells in the G_0/G_1 phase of the cell cycle. Noteably, arginase inhibition blocks serum-mediated cell cycle progression in the absence of cell death indicating that arginase inhibition acts via cytostatic rather than cytotoxic mechanisms. We further determined that cell cycle arrest following arginase inhibition was not associated with changes in the expression of the G_1 cyclins, cyclin D1, E or A, but was linked with an increase in the expression of p21, a known mediator of G_1 arrest (37). Similarly, knockdown of arginase I protein induced p21 expression in VSMC. Arginase inhibition also stimulated the expression of the transcription factor, p53, a well established inducer of p21 (38), raising the possibility that the induction of p21 and p53 are causally related in serum-treated VSMCs. Interestingly, our finding that arginase blockade inhibits polyamine biosynthesis may also contribute to the upregulation of p21 expression, since polyamine depletion secondary to ODC inhibition also leads to the induction of p21 and G_1 arrest (39,40). Moreover, polyamines have been demonstrated to repress p21 gene transcription (41). Collectively, these findings provide novel insight into the mechanisms behind the antiproliferative actions of arginase inhibitors and highlight a critical role for arginase in cell cycle progression.

In conclusion, the present study demonstrates that arginase plays a fundamental role in cell cycle progression and neointima formation following arterial injury. In particular, we found that balloon injury of rat carotid arteries induces arginase I expression and activity, and that the local application of arginase inhibitors blocks VSMC proliferation and intimal thickening. In addition, we show that arginase inhibition or arginase I knockdown arrests VSMCs in the G_0/G_1 phase of the cell cycle and this is associated with an increase in p21 expression. These results provide important new insights into the mechanism by which arginase promotes intimal thickening and identify arginase as an attractive therapeutic target in treating occlusive vascular disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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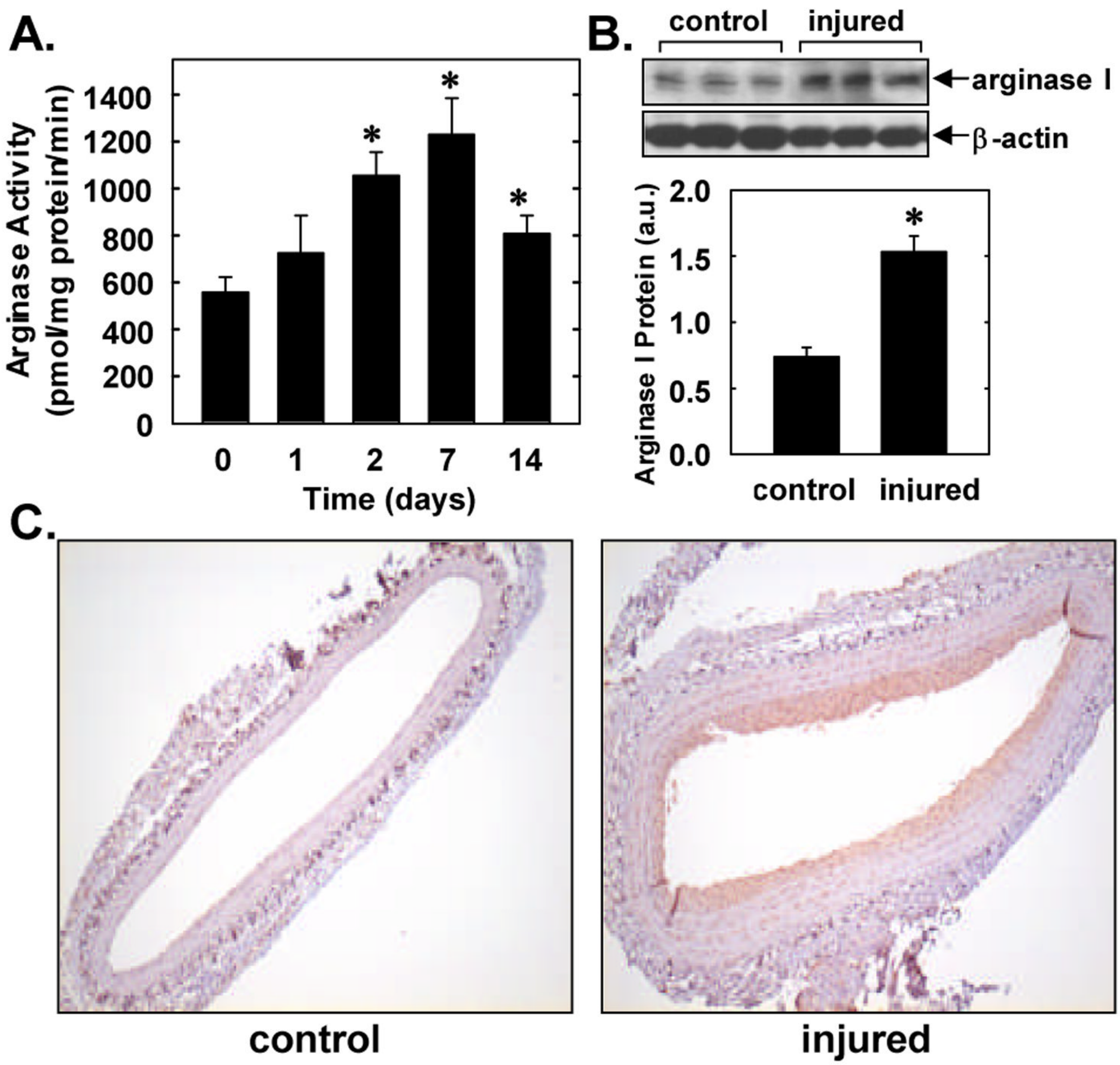

Figure 1. Effects of arterial injury on arginase activity and expression

A. Arginase activity in rat control or balloon injured carotid arteries. Results are mean ± SEM of 5 experiments. *Statistically significant effect of arterial injury. B. Western blot of arginase I protein in control carotid arteries or in vessels 7 days post-injury. Arginase I protein levels were quantified by scanning densitometry, normalized with respect to β-actin, and expressed in arbitrary units (a.u.). Results are mean ± SEM of 3. *Statistically significant effect of arterial injury. C. Immunohistochemistry demonstrating arginase I expression (shown in brown) in control carotid arteries or in vessels 7 days post-injury (magnification 100x). Data are representative of 5 separate experiments.

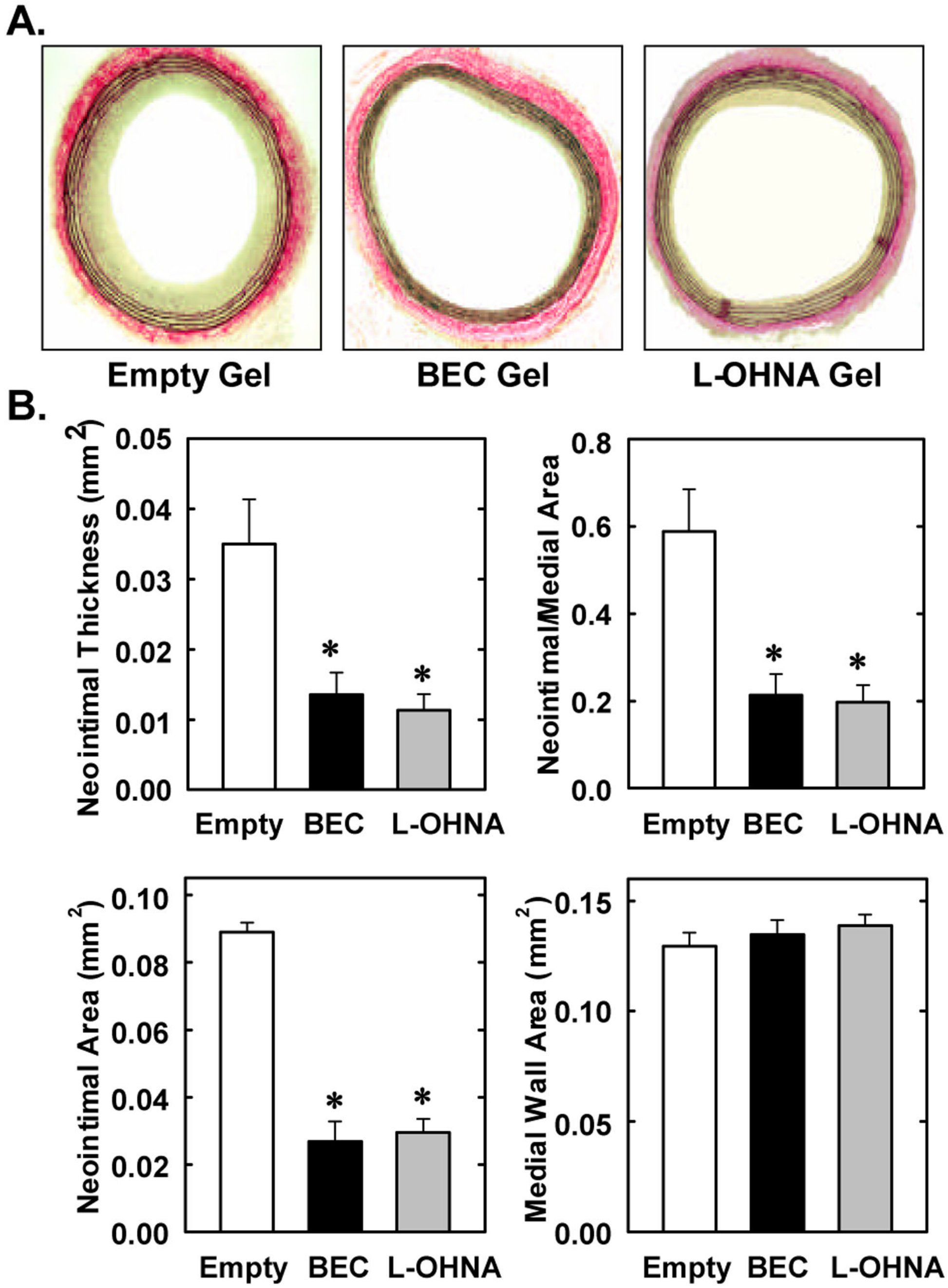

Figure 2. Effects of arginase inhibition on neointima formation after rat carotid artery balloon injury

A. Injured arteries were treated topically immediately after injury with empty gel or with gel containing BEC (1mg) or L-OHNA (1mg) and harvested two weeks after injury (magnification 100x). B. Morphometric analysis of neointima formation two weeks post-injury. Results are mean ± SEM of 8–10 experiments. *Statistically significant effect of BEC or L-OHNA.

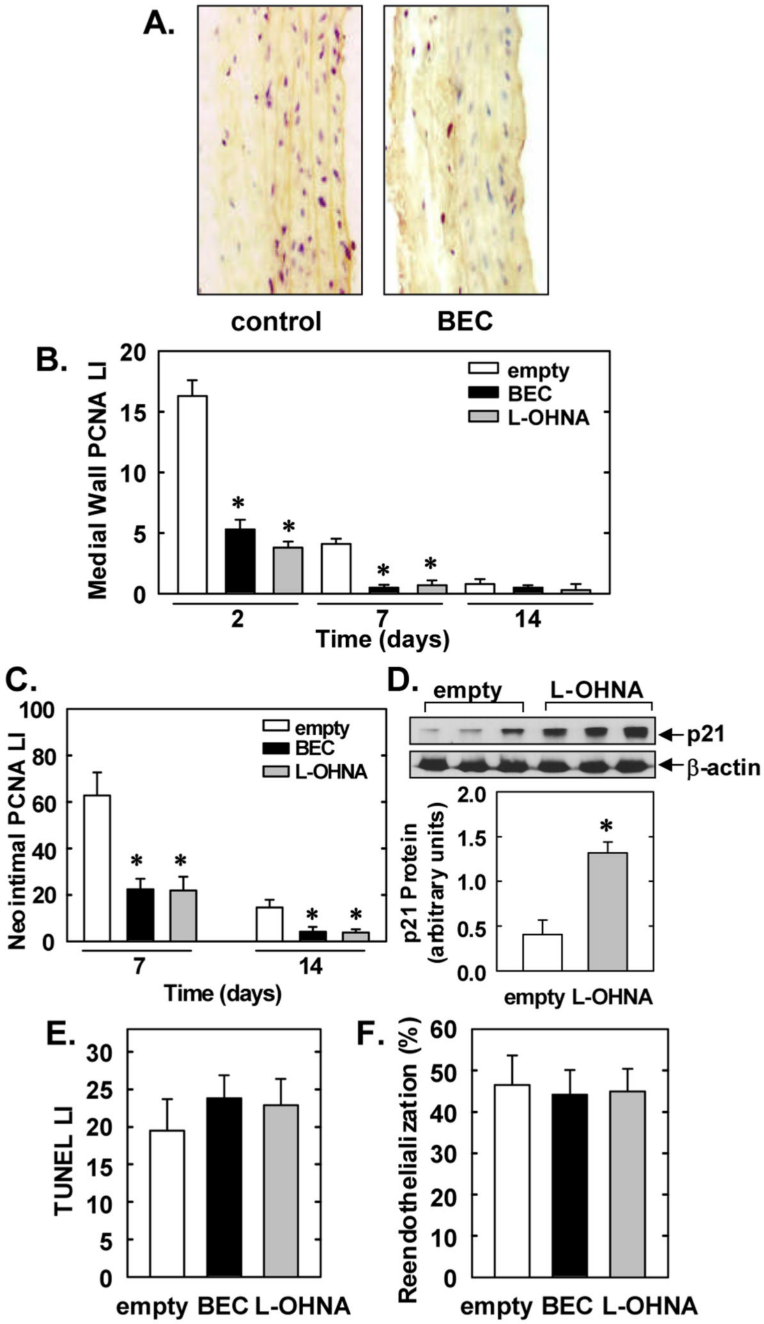

Figure 3. Effects of arginase inhibition on DNA synthesis, p21 protein expression, apoptosis and endothelial regrowth after rat carotid artery balloon injury

A. Representative immunostaining for PCNA (shown in red) in injured carotid arteries 2 days post-injury. B. PCNA labeling index (LI) in the media at 2, 7, and 14 days post-injury. C. PCNA LI in the neointima at 7 and 14 days post-injury. D. Western blot of p21 protein in injured carotid arteries 2 days post-injury. E. TUNEL LI two days post-injury. F. Reendothelialization of carotid arteries 14 days post-injury. Arteries were treated topically with an empty gel, or with gel containing BEC (1mg) or L-OHNA (1mg) immediately after injury. Results are mean ± SEM of 4–6 experiments. *Statistically significant effect of BEC or L-OHNA.

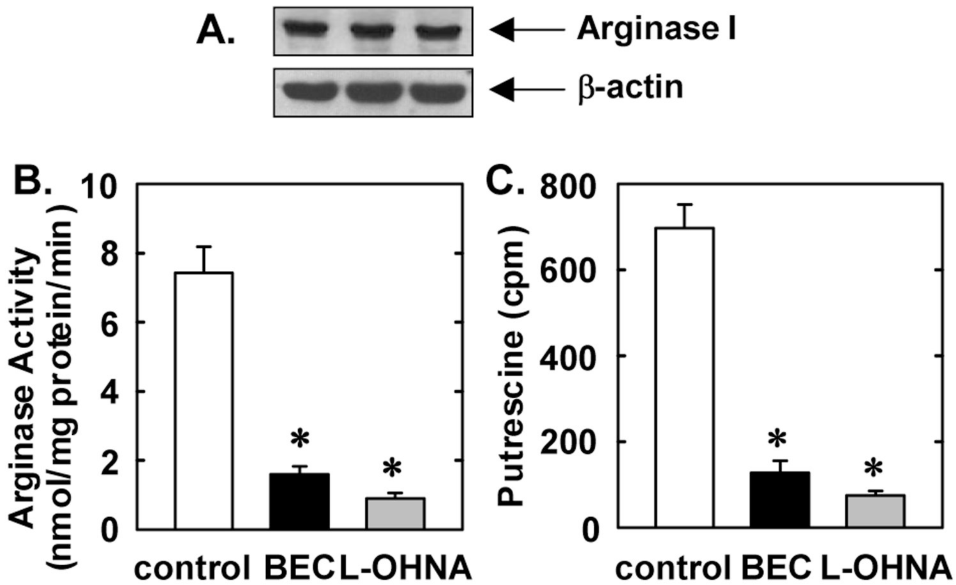

Figure 4. Arginase expression and activity and in rat cultured VSMCs

A. Expression of arginase I protein in VSMCs. Data are representative of 3 separate experiments. B. Inhibition of VSMC arginase activity by BEC (2mM) or L-OHNA (2mM). C. Inhibition of VSMC putrescine formation by BEC (2mM) or L-OHNA (2mM). Results are mean ± SEM of 3–5 experiments. *Statistically significant effect of BEC or L-OHNA.

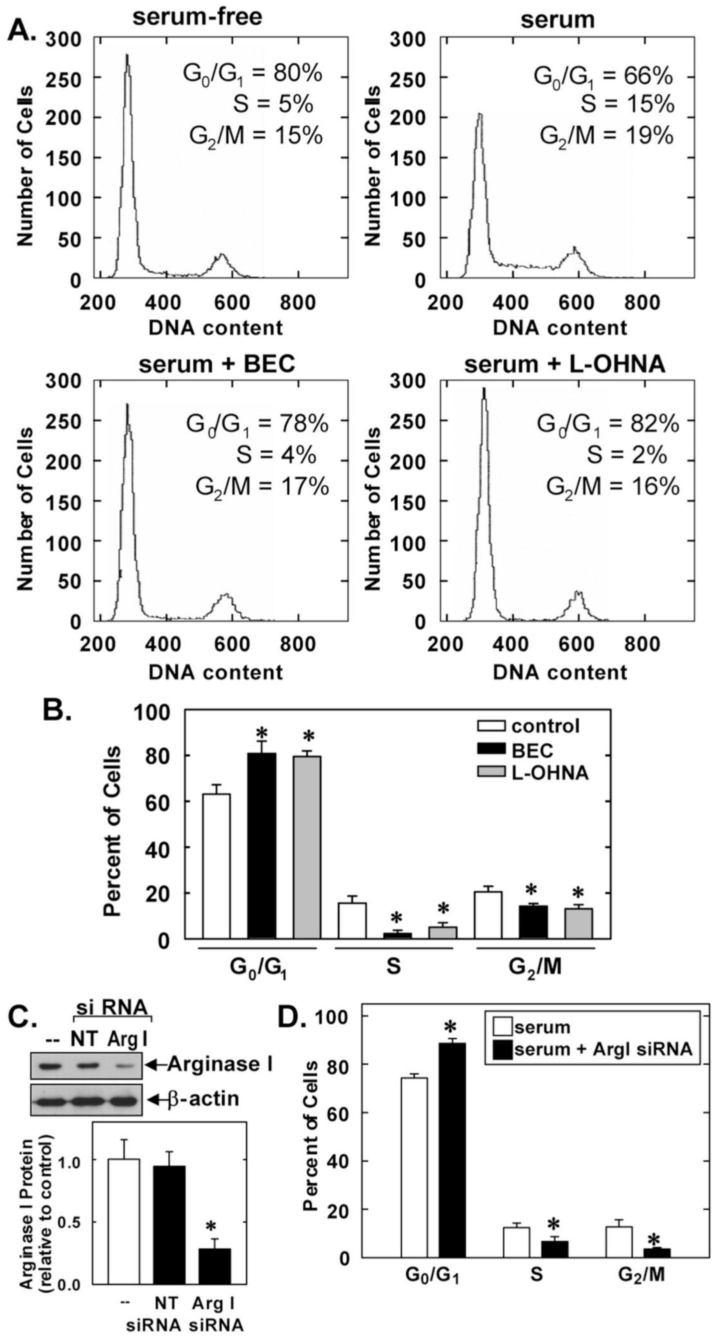

Figure 5. Role of arginase in cell cycle progression in rat cultured VSMCs

A. Representative histograms of VMSC DNA content in cells treated with serum (5%) for 24 hours in the presence or absence of BEC (2mM) or L-OHNA (2mM). Data are representative of 3 experiments. B. Effect of arginase inhibitors on the distribution of VSMCs in the cell cycle. VSMCs were treated with serum (5%) for 24 hours in the presence or absence BEC (2mM) or L-OHNA (2mM). Results are mean ± SEM of 5 experiments. C. Effect of arginase I siRNA (ArgI siRNA) on the distribution of VSMCs in the cell cycle. VSMCs were pretreated with ArgI siRNA (100nM) for 2 days and then exposed to serum (5%) for 24 hours. Results are mean ± SEM of 4 experiments. *Statistically significant effect of BEC, L-OHNA, or ArgI siRNA.

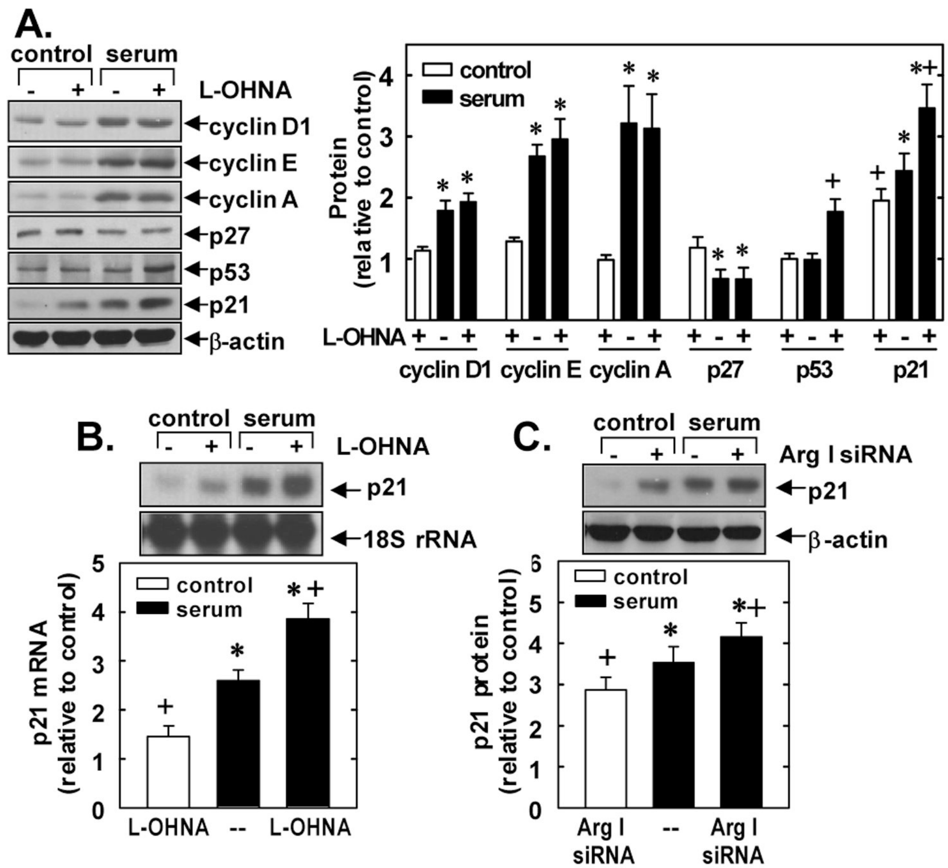

Figure 6. Effect of arginase inhibition on VSMC expression of cell cycle regulatory proteins

A. Effect of L-OHNA on the expression of cyclin D1, E, and A, p21, p27 and p53 protein. B. Effect of L-OHNA and BEC on p21 mRNA expression. C. Effect of arginase I siRNA (ArgI siRNA) on p21 protein expression. VSMCs were treated with serum (5%) in the presence or absence of L-OHNA (2mM) or BEC (2mM) for 24 hours. In some cases, VSMC were pretreated with ArgI siRNA (100nM) for 2 days and then exposed to serum (5%) for 24 hours. Quantification of protein or mRNA expression by scanning densitometry relative to serum-free controls. Results are mean ± of 3–4 experiments. *Statistically significant effect of serum. †Statistically significant effect of BEC, L-OHNA, or ArgI siRNA.