Arginase Promotes Neointima Formation in Rat Injured Carotid Arteries

Kelly J. Peyton, Diana Ensenat, Mohammed A. Azam, Amit N. Keswani, Sankaranarayanan Kannan, Xiao-ming Liu, Hong Wang, David A. Tulis, William Durante

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 after 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 N⁴-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 G₁ 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 after arterial injury. Arginase I represents a potentially new therapeutic target for the treatment of vasculoproliferative disorders. (Arterioscler Thromb Vasc Biol. 2009;29:488-494.)

Key Words: arginase ▶ vascular smooth muscle cell proliferation ▶ neointima

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 after arterial injury. However, the exact mechanism and mediators responsible for VSMC activation and neointima formation after 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 (see⁴⁻⁵). Two distinct isoforms of arginase exist, arginase I and II, that are encoded by distinct genes and exhibit different enzymatic properties. Arginase II is a mitochondrial protein that is concentrated in the kidney. Arginase I, whereas endothelial cells predominantly express arginase II.¹⁰⁻¹³ Interestingly, VSMCs and endothelial cells possess arginase activity yet preferentially express different arginase isoforms: VSMCs exclusively express arginase I, whereas 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 after ischemia-reperfusion (see⁵⁵). Besides limiting nitric oxide synthesis, arginase generates biologically significant molecules. In particular, the arginase...
product L-ornithine is further metabolized by ornithine de-carboxylase (ODC) to the polyamine putrescine which forms the successive polyamines, spermine and spermidine. Polyamines play an integral role in the mitogenic response of cells. VSMC proliferation is preceded by increases in polyamine synthesis, whereas inhibition of polyamine formation blocks cell growth.\(^\text{18–20}\) Interestingly, we previously reported that VSMCs possess arginase activity and postulated a physiological role for arginase in augmenting cell growth by optimizing polyamine synthesis in these cells.\(^\text{21}\) Indeed, subsequent studies found that overexpression of arginase I enhances polyamine generation and VSMC proliferation in culture while arginase inhibition suppresses VSMC growth.\(^\text{20,22}\) 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 to 450g; Charles River Laboratories, Wilmington, Mass) were anesthetized (ketamine, xylazine, and acepromazine; 0.7 mL/kg, intramuscularly; VetMed Drugs) and the left carotid artery injured with a Fogarty 2F embolectomy catheter (Baxter Healthcare Corporation), as we previously described.\(^\text{23,24}\) 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 200 mL of a 25% copolymer gel (PLF127; BASF Corporation) containing BEC (1 mg) or L-OHNA (1 mg) 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.\(^\text{25}\) PLF127 gels act as a rate-controlling barrier and serve 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.\(^\text{26}\) Based on this finding, we estimated a delivery dose of 55 \(\mu\)g 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 (5 \(\mu\)m) stained with Verhoff–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) to a Zeiss Axioskop 50 light microscope (Carl Zeiss).\(^\text{23–25}\)

### Arginase I Immunohistochemistry

Carotid arteries were perfusion-fixed and embedded in paraffin. Sections (5 \(\mu\)m) 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 TSA (5%) at room temperature for 30 minutes, and then incubated with biotinylated antirabbit arginase I antibody (1:50) at 4°C overnight. Slides were rinsed in PBS, incubated in biotinylated antirabbit IgG (1:100) in BSA (1%) for 1 hour, developed with peroxidase-labeled streptavidin and NovaRed, 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.\(^\text{24}\) Paraffin-embedded tissues were incubated with an anti-PCNA monoclonal antibody (1:25) followed by a biotinylated antirabbit 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 transerase (TdT)-measured dUTP nick end-labeling (TUNEL) method and TUNEL-positive cells confirmed microscopically, as we previously described.\(^\text{24}\)

### Endothelial Regrowth

Endothelial regrowth after arterial injury was examined using Evans blue dye, as we previously described.\(^\text{27}\) Animals were injected intravenously with Evans blue dye (5%) 10 minutes before 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. Denuded endothelialized 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.\(^\text{20,21}\) Cell cycle progression was determined by flow cytometry and the expression of arginase I, cyclins D1, cyclin A, cyclin E, p27, p21, and p53 monitored by Western or Northern blotting.\(^\text{23}\) Complete details of these experiments are available in the supplemental materials (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 2 days after injury, peaked after 1 week, and remained elevated 2 weeks after balloon injury. The peak increase in arginase activity observed 1 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, 1 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 nonimmune IgG abolished arginase I staining in all sections (data not shown).

Figure 2A shows representative cross-sections of perfusion-fixed, Verhoff–Von Gieson–stained tissues obtained from injured animals 2 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 3 groups of animals for circumference of the left carotid internal elastic laminae (gel control, 2.555 ± 0.041 mm; BEC-treated, 2.531 ± 0.083 mm; L-OHNA-treated, 2.541 ± 0.066 mm) or external elastic lamina (gel control, 2.883 ± 0.048 mm; BEC-treated, 2.757 ± 0.086 mm; L-OHNA–treated, 2.852 ± 0.0582 mm).

Representative cross-sections of vessels treated with an empty gel demonstrate substantial PCNA staining 2 days after 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 after 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 after arterial injury (Figure 3F).

Cultured VSMCs expressed high levels of arginase I protein and substantial arginase activity (Figure 4A and 4B). Treatment of VSMCs with the arginase inhibitors BEC or 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). Because polyamines play a fundamental role in cell cycle entry, succeeding experiments examined whether arginase influenced cell cycle progression in cultured VSMCs. Quiescent VSMCs accumulated in the G0/G1 phase of the cell cycle but the administration of serum for 24 hours induced cell cycle progression and the population of cells in G0/G1 decreased whereas the number of cells in S and G2/M increased (Figure 5 A). However, administration of the arginase inhibitors, BEC or L-OHNA, for 24 hours increased the fraction of cells in the G0/G1 phase of the cell cycle and this was accompanied by a decrease in the percentage 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-G0/G1 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<sub>0</sub>/G<sub>1</sub> and significantly decreased the percentage of cells in S and G<sub>2</sub>/M (Figure 5C). In contrast, nontargeting siRNA had no effect on 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 nontargeting siRNA failed to modify arginase I expression, confirming the efficacy and selectivity of the arginase I knockdown approach (Figure 5C).

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<sub>1</sub> 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), whereas nontargeting siRNA failed to modulate p21 expression (data not shown).

**Discussion**

The present study demonstrates that arginase plays a fundamental role in vascular growth after 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<sub>0</sub>/G<sub>1</sub>, 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 after arterial injury. Balloon injury of rat carotid arteries resulted in a sustained increase in arginase activity 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<sub>0</sub>/G<sub>1</sub>, 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.
The highest mitotic activity, 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. Mechanisms responsible for the induction of arginase activity after arterial injury are not known; however, the generation of growth factors and inflammatory mediators after vascular injury may be involved because a number of mitogens and inflammatory cytokines have been demonstrated to stimulate arginase I gene expression.

To determine the role of arginase in neointima formation, we applied the potent and selective arginase inhibitors BEC or L-OHNA 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 nonspecific effects associated with the systemic administration of drugs. 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. Because arginase may also contribute to endothelial cell growth, the effect of arginase inhibition on the reendothelialization of injured vessels was examined. Neither BEC nor L-OHNA affected endothelial regrowth after balloon injury.

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 (2 mmol/L) or L-OHNA (2 mmol/L). 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 (2 mmol/L) or L-OHNA (2 mmol/L). 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 (100 nmol/L) 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 (2 mmol/L) or BEC (2 mmol/L) for 24 hours. In some cases, VSMCs were pretreated with ArgI siRNA (100 nmol/L) 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±SEM of 3 to 4 experiments. *Statistically significant effect of serum. †Statistically significant effect of BEC, L-OHNA, or ArgI siRNA.
injury. Thus, arginase inhibitors appear to specifically reduce VSMC growth after 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, 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 but contrasts with a recent study that failed to detect arginase activity in a fetal VSMC line, 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. We also found that treatment of VSMCs with arginase inhibitors or knockdown of arginase I protein arrests cells in the G0/G1 phase of the cell cycle. Notably, 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 after arginase inhibition was not associated with changes in the expression of the G1 cyclins, cyclin D1, E, or A, but was linked with an increase in the expression of p21, a known mediator of G1 arrest. Similarly, knockdown of arginase I protein induced p21 expression in VSMCs. Arginase inhibition also stimulated the expression of the transcription factor, p53, a well-established inducer of p21, 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, because polyamine depletion secondary to ODC inhibition also leads to the induction of p21 and G1 arrest. Moreover, polyamines have been demonstrated to repress p21 gene transcription. 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 after 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 G0/G1 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.

Sources of Funding
This work was supported by National Institutes of Health Grants HL59976, HL74966, HL82774, and HL81720 and American Heart Association Midwest Affiliate Grant-in-Aid 095571SG.

Disclosures
None.

References


Arginase Promotes Neointima Formation in Rat Injured Carotid Arteries
Kelly J. Peyton, Diana Ensenat, Mohammed A. Azam, Amit N. Keswani, Sankaranarayanan Kannan, Xiao-ming Liu, Hong Wang, David A. Tulis and William Durante

Arterioscler Thromb Vasc Biol. 2009;29:488-494; originally published online January 22, 2009; doi: 10.1161/ATVBAHA.108.183392

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/29/4/488

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/