Arginase I Attenuates Inflammatory Cytokine Secretion
Induced by Lipopolysaccharide in Vascular Smooth Muscle Cells

Xu-ping Wang, Yu-guo Chen, Wei-dong Qin, Wei Zhang, Shu-jian Wei, Juan Wang, Fu Qiang Liu, Lei Gong, Feng Shuang An, Yun Zhang, Zhe-Yu Chen, Ming-Xiang Zhang

Objective—Inflammation plays an important role in atherosclerosis. Arginase I (Arg I) promotes the proliferation of vascular smooth muscle cells; however, the effect of Arg I on inflammation remains unknown. The present study investigated the role of Arg I in inflammation in vitro and in vivo.

Methods and Results—Quantitative reverse transcription–polymerase chain reaction and Western blot analysis demonstrated that Arg I inhibited tumor necrosis factor-α production induced by lipopolysaccharide in human aortic smooth muscle cells. Inducible nitric oxide synthase substrate competition and nuclear factor-κB activation were main contributors to lipopolysaccharide-mediated inflammatory cytokine generation. However, Arg I could attenuate the function of inducible nitric oxide synthase and inhibit the subsequent nuclear factor-κB activation, leading to inhibition of tumor necrosis factor-α generation. Furthermore, upregulation of Arg I significantly decreased macrophage infiltration and inflammation in atherosclerotic plaque of rabbits, whereas downregulation of Arg I aggravated these adverse effects.

Conclusion—The results indicate the antiinflammatory effects of Arg I and suggest an unexpected beneficial role of Arg I in inflammatory disease. (Arterioscler Thromb Vasc Biol. 2011;31:1853-1860.)

Key Words: atherosclerosis ■ vascular biology ■ vascular muscle
deactivation and is involved in inflammatory responses to LPS and pathogens. Human aortic SMCs (hASMCs) contain constitutive Arg I, which plays an important role in SMC proliferation. However, the function of Arg I in inflammatory disease remains unknown. Arginase and NOS have reciprocal activities that may shift arginine metabolism to polyamine homeostasis or cytotoxic NO production, respectively. One potential mechanism for Arg I involvement in inflammation is through competition with NOS for the common substrate l-arginine.

To understand the role of Arg I in inflammatory disease, we aimed to determine the effect of Arg I on release of tumor necrosis factor-α (TNF-α) during LPS incubation in vitro and the function of Arg I in atherosclerotic plaques in vivo. Elevation of arginase activity should decrease the catalyzing substrate and inhibit the function of NOS.

Materials and Methods

Reagents and Antibodies

SMC medium was from ScienCell (Carlsbad, CA). LPS, dihydroethidium, aminoguanidine (AG), 1400W dihydrochloride (1400W), BAY-11-7082, L-arginine, a-isonitrosopropiophenone, and MnCl2 were from Sigma-Aldrich (St. Louis, MO). S-(2-Boronoethyl)-l-cysteine (BEC) was from Alexis Corp (San Diego, CA).

Cell Culture

hASMCs were from the American Type Cell Collection. Cells at up to passage 4 were used and cultured in SMC medium containing 2% fetal bovine serum (FBS) and 1% smooth muscle cell growth supplement (ScienCell) at 37°C for 24 to 96 hours. At 12 hours before each experiment, the complete medium was replaced by fresh SMC medium containing 0.5% FBS. BEC was a positive control of Arg I interference. The iNOS inhibitor 1400W, AG, and nuclear factor-κB (NF-κB) inhibitor BAY-11-7082 were applied 2 hours before LPS incubation.

Animal Model and Experimental Protocol

Sixty New Zealand White rabbits (1.5 to 2.0 kg) were randomly divided into 6 groups (n=10). After acclimatization to an atherogenic diet (containing 1% cholesterol) for 2 weeks, rabbits underwent balloon injury of the abdominal aorta and were fed 1% cholesterol. At the end of 12 weeks, physiological saline, Arg I lentivirus, pGC FU-green fluorescent protein-lentivirus (LV), rabbit ARGI-RNAi lentivirus (sequences: forward, 5′-GGUGGAUGCUCAUACUGAUTT-3′, reverse, 5′-AUCAGUAUACACAACAUAUCCCT-3′; Gene Pharma) and siRNA-NC (forward, 5′-UUCUCCGAACGUUGACUAGUTT-3′, reverse, 5′-ACGUACGACUGUGGAGAATT-3′; Gene Pharma), as well as hARGI-siRNA paired with pCDNA3.1(+)–hARGI, were transfected in hASMCs with double-strand DNA in OptiMEM medium (Gibco) mixed with Lipofectamine 2000 (Invitrogen) according to the manufacturers’ instructions. At 12 hours after transfection or interference, hASMCs were incubated in SMC medium containing 2% FBS for 4 hours. Arg I expression was measured by Western blot analysis.

Measurement of Arginase Activity

Arginase activity was measured in hASMC lysates as previously described. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol urea/minute.

Monocyte Chemotaxis and Migration

THP1 cells and hASMCs were cocultured in Transwell (0.4 and 8 μm pore size; Corning), with the former cells cultured in the upper well and the latter cells in the bottom well. After LPS treatment for 48 hours, coculture was performed (6 hours for 0.4-μm-pore Transwells, 12 hours for 8-μm-pore Transwells). Chemotaxis and migration of THP1 cells were determined by counting THP1 cells adhering to the bottom surface and to the external surface of the Transwell bottom, respectively.

Measurement of NO Activity

NO activity was determined by NO assay kit according to the manufacturer’s instructions (BioVision). All operations were performed at room temperature. Absorbance was measured at 542 nm by use of a Varioskan Flash multifunction plate reader.

ROS and O₂⁻ Assay

Cellular ROS levels were determined using the ROS Assay Kit (Beyotime, Haimen, China) according to the manufacturer’s instructions. For cellular O₂⁻ assay, cells were incubated with 2 μmol/L dihydroethidium for 30 minutes at 37°C and then underwent Hoechst 33342 staining for 5 minutes. Images were acquired with a Zeiss LSM 710 confocal microscope with a ×20 objective (number aperture 0.5) and an LD condenser (number aperture 0.55) (Zeiss, Germany).

Real-Time Polymerase Chain Reaction

The primers for iNOS were as follows: forward, 5′-CTCAGGCTCTGCCTATGCCCATC-3′, reverse, 5′-CTCAAGGGTGCCTAAGCTGC-3′, and for Arg I, forward, 5′-GGGCCGAAGCAGAATGATG-3′, reverse, 5′-TCCATGCTCAGCACTTACAAAC-3′. SYBR Green real-time polymerase chain reaction and quantitative assays involved the use of a sequence detector system (Bio-Rad). The relative expression of genes was obtained by the 2-ΔΔCt calculation method.

Western Blot Analysis

Protein expression was assayed with cell or tissue lysates of the same protein content (BCA method, Bio-Rad). Proteins were separated by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and then incubated with antibodies for mouse anti-Arg I (1:4000, BD, BD Biosciences), rabbit anti-iNOS (1:1000, Santa Cruz Biotechnology), mouse anti-TNF-α (1:2000, Abcam), and mouse or rabbit anti-β-actin (1:2000, Abcam), followed by horseradish peroxidase–conjugated goat anti-rabbit (1:5000) or goat anti-mouse IgG (1:5000, both from Santa Cruz Biotechnology), mouse anti-TNF-α, rabbit anti-iNOS, and mouse or rabbit anti-β-actin. Images were acquired with Alpha View software (Alpha).

Immunocytochemistry and Immunohistochemistry

Cells were incubated with antibodies for mouse anti-Arg I (1:100, BD) and rabbit anti-iNOS (1:100, Santa Cruz Biotechnology) or pCDNA3.1(+)–hARGI plasmid, pCDNA3.1(+)–LacZ plasmid and hARGI-small interfering RNA (siRNA) (sequences: forward, 5′-GAUUGUAUUGUAAUUGCUTT-3′, reverse, 5′-AGCGAUAGAUACACAUAUCCCT-3′; Gene Pharma) and siRNA-NC (forward, 5′-UUCUCCGAACGUUGACUAGUTT-3′, reverse, 5′-ACGUACGACUGUGGAGAATT-3′; Gene Pharma).
rabbit monoclonal anti-NF-κB p65 (1:50, Cell Signaling) overnight at 4°C, and then subsequent secondary antibodies (all 1:2000, Invitrogen, CA) were added. Before image acquisition, a drop of Prolong Gold antifade reagent with 4’,6-diamidino-2-phenylindole (Invitrogen) was needed.

For tissue sections, mouse anti-α-actin (1:100, Chemicon), mouse anti-iNOS (1:100, Abcam), mouse anti-Ram11 (1:100, Dako), and mouse anti-iNOS (1:100, Santa Cruz Biotechnology) antibodies were incubated overnight at 4°C. Addition of secondary antibody and color development were followed by manufacturer’s instructions (Jingmei, Shenzhen, China). Data were analyzed by use of ImagePro Plus 5.0 (Media Cybernetics).

Flow Cytometry

ONOO⁻ generation was assayed by flow cytometry. Briefly, cells were permeabilized with FACS Permeabilizing Solution 2 (BD), blocked for 30 minutes, and then incubated with mouse monoclonal nitrotyrosine antibody for 1 hour at 37°C. Alexa 488–conjugated goat anti-mouse IgG (1:1000, Invitrogen) was added. Before image acquisition, a drop of Prolong Gold antifade reagent with 4’,6-diamidino-2-phenylindole (Invitrogen) was needed.

For tissue sections, mouse anti-α-actin (1:100, Chemicon), mouse anti-iNOS (1:100, Abcam), mouse anti-Ram11 (1:100, Dako), and mouse anti-iNOS (1:100, Santa Cruz Biotechnology) antibodies were incubated overnight at 4°C. Addition of secondary antibody and color development were followed by manufacturer’s instructions (Jingmei, Shenzhen, China). Data were analyzed by use of ImagePro Plus 5.0 (Media Cybernetics).

Statistical Analysis

Data analysis involved use of SPSS v16.0 (SPSS Inc., Chicago, IL) and was the result of at least 3 independent experiments. Time- and dose-dependent data were assessed by Pearson bivariate correlation (2-tailed). Other data were assessed by 1-way ANOVA, followed by the Tukey t test (2-tailed). A value of \( P < 0.05 \) was considered statistically significant.

Results

LPS Increases TNF-α Production via iNOS Activity in hASMCs

To confirm the cells we used were hASMCs, cells were immunostained with anti-α-SM-actin antibodies. Results showed that all the cells were hASMCs (Supplemental Figure I, available online at http://atvb.ahajournals.org).

To test whether LPS could stimulate the generation of inflammatory cytokines, the level of TNF-α in the cell culture medium was measured. High concentrations of LPS caused significant TNF-α production (Figure 1A). Furthermore, a rapid increase in TNF-α release was detected with a short period of LPS incubation (>2 hours) (Figure 1B). To illustrate the role of iNOS in TNF-α release, we pretreated cells with 2 iNOS inhibitors, AG and 1400W, before LPS incubation. The release of TNF-α was abolished by ~70% to 80% in the presence of the inhibitors (\( P < 0.01 \)) (Figure 1C).

Effect of LPS on Coregulation of Arg I and iNOS

To analyze which isoform of Arg is involved in the LPS stimulation in hASMCs, we determined the expression of both Args. Only Arg I was constitutively expressed in hASMCs, and no Arg II was detected with or without LPS incubation (data not shown). To illustrate the effects of LPS on Arg I and iNOS, the mRNA level, protein content, and catalytic activity of both enzymes were determined. The induction of mRNA and protein levels of Arg I lagged behind that of iNOS (Figure 1D and 1E). Induced Arg I activity declined after 48 hours, whereas NO release continued to increase slowly. Moreover, we observed a slight increase in NO release after Arg I activity decreased. To validate the competitive relationship between iNOS and Arg I, we pretreated cells with l-arginine during LPS incubation. Both Arg I activity and NO release were increased after 12 hours and continued to increase even at 72 hours (Figure 1F).

Arg I Suppresses TNF-α Release and Inhibits Monocyte Chemotaxis and Migration

hASMCs were transiently transfected with human Arg I–expressed plasmid and bacterial β-galactosidase (LacZ)–expressed plasmid, human Arg I siRNA, negative siRNA, and human Arg I siRNA paired with Arg I–expressed plasmid. LacZ–expressed plasmid represented unrelated cytosolic protein and negative siRNA an irrelevant interference, respectively. Transfection efficiencies were >80% in our experiments (Supplemental Figure II). hASMCs transfected with human Arg I cDNA contained a high quantity of Arg I protein.
Arg I Inhibits NO Release Without Decreasing iNOS Expression

The data presented above suggest that Arg I inhibits LPS-induced TNF-α release and that iNOS participates in the process. We speculated that Arg I inhibited inflammation through t-arginine competition, which leads to iNOS dysfunction. However, many enzymatic pathways are compartmentalized in the cell to avoid direct competition for common substrates. To show the localization of these 2 enzymes, we performed immunocytofluorescence analysis. The 2 enzymes were largely colocalized in hASMCs with LPS incubation for 48 hours (Figure 3A). Subsequently, the influence of Arg I on iNOS expression and NO release was determined. In the presence of LPS, Arg I did not significantly reduce the expression of iNOS in hASMCs (all $P>0.05$ versus LPS alone) (Figure 3B and 3C). However, NO release was decreased by 22.5 ± 5.64% with Arg I elevation but increased to 130.03 ± 10.57% with Arg I inhibition and knockout, respectively (both $P<0.01$ versus LPS alone) (Figure 3D).

Arg I Decreases ROS, O$_2^\cdot$-, and ONOO$^-$ Generation in hASMCs

One possible mechanism of NO produced by iNOS provoke inflammation is its reaction with O$_2^\cdot$- to produce ONOO$^-$, O$_2^\cdot$- may generate from iNOS uncoupling or cellular ROS. To determine the source of O$_2^\cdot$-, ROS and O$_2^\cdot$- production were both determined. LPS-induced increase in ROS was attenuated to 54.77 ± 14.48% by Arg I elevation but was augmented to 133.98 ± 10.50% and 132.97 ± 13.52% by Arg I inhibition and knockdown, respectively (all $P<0.01$ versus LPS alone) (Figure 4A and 4B). Arg I elevation attenuated O$_2^\cdot$- production induced by LPS by 41.74 ± 12.23% ($P<0.01$ versus LPS alone), whereas Arg I inhibition or knockdown augmented O$_2^\cdot$- generation to 127.70 ± 16.03% and 124.81 ± 14.05%, respectively (both $P<0.05$ versus LPS alone) (Figure 4C and 4D). Reduced
ONOO\textsuperscript{−} generation was also observed with Arg I overexpression (27.74±3.87\%, \( P<0.01 \) versus LPS alone), whereas converse results were seen with Arg I inhibition or knockdown. (159.49±28.59\% and 157.24±36.99\%, respectively; \( P<0.01 \) versus LPS alone) (Figure 4E and 4F).

**Arg I Inhibits TNF-\alpha Release via NF-\kappaB Activation**

The localization of NF-\kappaB subunit P65 was determined by immunocytofluorescence analysis. LPS triggered the NF-\kappaB P65 subunit translocation from the cytoplasm to nucleus. However, iNOS inhibitors (AG and 1400W) and BAY-11-7082 suppressed P65 nuclear translocation. In addition, reduction of nuclear P65 translocation was seen with Arg I elevation, opposite to si-ARGI or BEC treatment (Figure 5A).

To determine a possible association of NF-\kappaB activation and magnitude of TNF-\alpha response to LPS, we pretreated cells with the selective iNOS inhibitor 1400W and the NF-\kappaB inhibitor BAY-11-7082. In the presence of the 2 inhibitors, LPS-induced
TNF-α release was attenuated by 24.68±4.92% and 49.50±3.14%, respectively (both P<0.01 versus LPS alone) (Figure 5B).

**Arg I Attenuated Atherosclerotic Plaque Inflammation**

Arg I regulation in vivo was accomplished through locally delivering ARGI-LV or si-ARG-LV. Arg I was upregulated to 282.17±53.85% by delivering ARGI-LV, whereas it was downregulated to 49.67±12.24% and 57.33±16.24% by si-ARGI-1-LV and si-ARGI-2-LV, respectively. (Supplemental Figure VI).

Immunohistochemistry results in aortaventralis of rabbit demonstrated that treatment with Arg I produced less TNF-α, which was also confirmed by Western blot analysis (Figure 6A and 6B). The opposite results from si-ARG groups were also interconfirmed by the 2 experimental approaches. Moreover, the results obtained in vivo agreed with the observations in cultured hASMCs (Figure 6A to 6C). To explore the contribution of SMCs to the release of TNF-α under Arg I regulation, we calculated the area ratio between TNF-α and SMCs. The area ratio was attenuated by Arg I upregulation to 20.17±7.81% but augmented by Arg I downregulation to 354.5±86.44% and 373.67±107.22%, respectively (all P<0.01 versus control group) (Figure 6D).

Arg I upregulation showed less macrophage content (31.67±9.05%, P<0.01, versus control group) and less iNOS expression (59.4±12.6%, P<0.01, versus control) in plaque (Figure 6E and 6F). Strikingly, si-ARGI increased macrophage content ~80% to 100% (both P<0.01 versus control) and iNOS expression ~35% (both P<0.05 versus control) (Figure 6E and 6F). However, the groups did not differ in blood serum TNF-α content (data not shown), which suggests that locally derived Arg I did not have a systemic anti-inflammation effect.

**Discussion**

Coronary artery disease and atherosclerosis are considered inflammatory processes. Although Arg I could be a new candidate gene of atherosclerosis resistance, no reports exist of the effects of Arg I in these diseases. As well, the fundamental mechanism still remains unexplored. We demonstrated that hASMCs constitutively express Arg I, and Arg I inhibited the inflammation both in LPS-induced hASMCs and in atherosclerotic plaque in rabbits. This knowledge may help in the control of VSMC inflammation or pathophysiological developments, such as atherosclerosis.

VSMCs exist in a extremely diverse range of phenotypes. Under physiological conditions, the predominant phenotype...
is contractile VSMCs, which have a major function in vasodilation and blood flow regulation. However, the synthetic phenotype is present when VSMCs are subjected to injury. The synthetic phenotype of VSMC increases the capacity to generate extracellular matrix protein or inflammatory cytokines and contributes to vascular remodeling. SMC-derived Arg I enhances polyamine generation and promotes VSMC proliferation but has no effect on cell migration. In the synthetic phenotype, migration and proliferation of VSMCs are key elements in atherosclerotic lesion development. However, VSMC proliferation appears to have paradoxical effects: it both promotes atherosclerotic lesions and stabilizes atherosclerotic plaque. The role of Arg I in atherosclerosis still remains unclear. To address this question, we systematically explored the effect of Arg I on inflammation in vitro and in vivo.

In vitro experiments, Arg I reduced inflammatory pathways by competing for the common substrate l-arginine with iNOS. Excess NO produced by iNOS reacts with O$_2^-$, leading to the production of ONOO$^-$. And ONOO$^-$ can cause oxidative damage and have a potent effect on NO$^-$ mediated inflammation. Both NO and O$_2^-$ generation, as well as ONOO$^-$ generation, were decreased with Arg I elevation during LPS incubation. However, these findings do not address whether O$_2^-$ generation was due only to iNOS uncoupling or to other sources, and then ROS generation was determined. The increase in ROS generation induced by LPS was also abolished by Arg I, the reason for which remains unknown. Subsequent mechanism research demonstrated that Arg I weakened LPS-induced TNF-$\alpha$ level by preventing NF-$\kappa$B translocation. The effect of correlation of degradation of I-$\kappa$B with NF-$\kappa$B translocation suggests that Arg I inhibits LPS-induced inflammation by preventing I-$\kappa$B degradation and then preventing NF-$\kappa$B activation and subsequent TNF-$\alpha$ release.

From our data, we developed a model describing the role of Arg I in inflammatory cytokine secretion in hASMCs.

This model, proinflammatory iNOS was induced by LPS, thus resulting in increasing NO and O$_2^-$ generation, which favors ONOO$^-$ generation. In addition to originating from iNOS uncoupling, other sources of O$_2^-$ may also contribute to the generation of O$_2^-$ . Excess ONOO$^-$ may contribute to NF-$\kappa$B activation and then inflammatory cytokine secretion. Elevated Arg I may suppress the inflammatory pathway though competitively inhibiting iNOS.

Atherosclerosis development is a complex process. Arg I was reported to enhance polyamine generation and VSMC proliferation and therefore was considered to have a proatherosclerotic effect. Our subsequent in vivo research in an atherosclerotic New Zealand rabbit challenged this viewpoint. To determine the role of Arg I in atherosclerosis, we used a local delivery viral vector method. This gene-targeting approach has been used successfully by our laboratory and elicits effective gene regulation. Arg I elevation in vivo decreased TNF-$\alpha$ expression and macrophage content in plaques, both of which aggravated pathological changes of atherosclerosis. The findings are in agreement with the observations in hASMCs under LPS incubation. A decrease in the hASMC content of TNF-$\alpha$ with Arg I elevation was also observed. This result is in agreement with a previous study in primary VSMCs, suggesting that Arg I has different roles under physical and pathological conditions. As well, Arg I overexpression attenuated the expression of iNOS in vivo, which was different from the observation in vitro. The reduction in macrophages, which also expresses iNOS in atherosclerotic conditions, was the main contributor to the decrease.

In the present study, we illustrated the role of Arg I in inflammatory cytokine secretion in hASMCs and in atherosclerotic plaques in rabbits. Further research is needed to consider Arg I a possible target gene in treating and preventing inflammatory diseases.

**Acknowledgments**

We thank Dr Yongxin Zou (Genetic Institutes, Shandong University) for providing THP1 cells.
Sources of Funding
This work was supported by the National 973 Basic Research Program of China (No. 2009CB521900), the State Program of National Natural Science Foundation of China for Innovative Research Group (Grant 81021001), and the National Natural Science Foundation of China for Innovative Program of China (No. 2009CB521900), the State Program of

Disclosures
None.

References
Arginase I Attenuates Inflammatory Cytokine Secretion Induced by Lipopolysaccharide in Vascular Smooth Muscle Cells
Xu-ping Wang, Yu-guo Chen, Wei-dong Qin, Wei Zhang, Shu-jian Wei, Juan Wang, Fu Qiang Liu, Lei Gong, Feng Shuang An, Yun Zhang, Zhe-Yu Chen and Ming-Xiang Zhang

Arterioscler Thromb Vasc Biol, 2011;31:1853-1860; originally published online May 26, 2011; doi: 10.1161/ATVBAHA.111.229302
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 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/31/8/1853

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/