Effect of Exposure to Cigarette Smoke on Carotid Artery Intimal Thickening
The Role of Inducible NO Synthase

Takeo Anazawa, Paul C. Dimayuga, Hongyan Li, Shigemasa Tani, Jason Bradfield, Kuang-Yuh Chyu, Sanjay Kaul, Prediman K. Shah, Bojan Cercek

Objective—We investigated the role of inducible NO synthase (iNOS) in intimal thickening with exposure to cigarette smoke (CS).

Methods and Results—Intimal thickening in wild-type (WT) and iNOS-deficient (iNOS−/−) mice subjected to CS exposure was induced by placement of a cuff around the carotid artery. CS exposure in WT mice was associated with increased arterial iNOS expression, superoxide production, activator protein-1 (AP-1) activation, and serum NO. Intimal thickening 21 days after cuff placement was significantly greater in mice exposed to CS compared with air (0.023±0.013 mm2 versus 0.009±0.008 mm2; P<0.05). iNOS inhibitor mercaptoethylguanidine-treated WT mice exposed to CS had reduced iNOS activity and intimal thickening (0.006±0.005 mm2; P<0.05). Intimal thickening was significantly less in iNOS−/− mice compared with WT mice (0.006±0.005 mm2; P<0.01) and was not augmented with CS (0.002±0.002 mm2). The aryl hydrocarbon receptor (AhR) was detected in arteries in vivo and in smooth muscle cells (SMCs) in vitro. CS condensate treatment of SMCs increased AhR binding to the core xenobiotic-responsive element of the iNOS promoter and increased iNOS expression.

Conclusions—Increased arterial expression of iNOS, mediated at least in part by AhR signaling, may be an important mechanism by which CS increases carotid intimal thickening. CS exposure in mice was associated with increased arterial iNOS expression, superoxide production, AP-1 activation, serum NO expression, and intimal thickening. Inhibition or deletion of iNOS abrogated the effects of CS. (Arterioscler Thromb Vasc Biol. 2004;24:1-7.)

Key Words: smoking ■ iNOS ■ oxidative stress ■ intimal thickening

Cigarette smoking is an important risk factor for stroke,1–3 mediated in part by accelerated intimal thickening and plaque development.4,5 In the Atherosclerosis Risk in Communities Study, active smoking and exposure to environmental tobacco smoke were associated with accelerated progression of intimal/medial thickness of the carotid artery.6 The underlying mechanism(s) of these deleterious effects remain unknown.7,8 Increased transcription factor activation, expression of adhesion molecules, and redox gene inducible NO synthase (iNOS) have been demonstrated with exposure to cigarette smoke (CS)9–11 and may contribute to increased intimal thickening.

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor mediating enzyme induction in response to CS and may have a potentiating effect on CS carcinogenicity.12 The mouse iNOS promoter13 contains a site that corresponds to the core xenobiotic-responsive element (XRE) to which the AhR binds. Therefore, the AhR may mediate the negative effects of CS on the arterial intima.

We hypothesized that exposure to CS increases iNOS gene expression and activity in the arterial wall leading to augmented intimal thickening after injury. We used a model of intimal thickening induced by placement of a periadventitial cuff around the right carotid artery of mice exposed to CS. Cultured aortic smooth muscle cells (SMCs) were used to investigate the AhR pathway for increased iNOS expression after exposure to CS condensate (CSC).

Materials and Methods

Animals
Male wild-type (WT) and iNOS-deficient (iNOS−/−) mice with the C57BL/6J background strain (The Jackson Laboratory) were fed normal chow throughout the study period. At the age of 25 weeks, mice were anesthetized with Avertin (0.016 mL/g of 2.5% solution IP), and the right carotid artery was carefully isolated under a dissecting microscope. A nonocclusive plastic cuff was placed around the right carotid artery, and the skin incision was closed, as described previously.14 Mice were euthanized 3, 7, or 21 days after cuff placement. The carotid arteries were perfused with 0.9% saline.
for 10 minutes, embedded in OCT compound (Tissue-Tek; Alle-
giance), and frozen at −80°C.

WT mice were randomized into the following groups: (1) cuff
with exposure to air, (2) cuff with exposure to CS, (3) cuff
with exposure to air and treatment with mercaptoethylguanidine (MEG),
and (4) cuff with exposure to CS and treatment with MEG. Treatment
with MEG (30 mg/kg in 0.9% NaCl IP twice daily) was
started on the day of the injury. Other groups of mice received
injections with 0.9% NaCl. MEG was kindly provided by Inotek
Company (courtesy of Csaba Szabo, MD, PhD). iNOS+/− mice
were exposed to the same protocol of cuff placement and exposure
to CS at air and air as above.

Exposure to CS was performed in an apparatus obtained from
the Department of Physiology, Washington University School of
Medicine.15 Standardized reference research cigarettes (R-1F4 cigarette)
were purchased from University of Kentucky Tobacco and Health
Research Institute. Mice were exposed gradually to CS, starting at 20
weeks of age to the dose of 1 cigarette per day. Mice were allowed
≥60 minutes for a break between each one-half cigarette. Control
mice were exposed to filtered air in an identical apparatus. Blinded
operators performed the analysis. The Institutional Animal Care and
Use Committee approved the protocol for the study.

Blood Carboxy-Hemoglobin Level
Blood carboxy hemoglobin (CO-Hb) level was measured by CO-
Oximeter on heparinized blood taken from the right ventricle under
anesthesia immediately after CS exposure.

Immunohistochemistry
Antibodies were purchased from Santa Cruz Biotechnology. Sec-
tions of arteries harvested 3 days after cuff placement were incubated
with rabbit polyclonal iNOS antibody. Arterial sections 21 days after
cuff placement were incubated with rabbit polyclonal antiproliferat-
ing cell nuclear antigen (PCNA) or goat polyclonal antibody against
the AhR. Detection was performed using biotinylated secondary
antibodies with 3-amin-9-ethylcarbazole chromogen (DAKO) for
iNOS and AhR or nickel-enhanced diaminobenzidine stain for
PCNA, which produces a distinct black nuclear stain. Measurement
of the iNOS-stained area was taken with computer-assisted analysis
as described previously.14 PCNA-positive nuclei within the vessel
wall were counted under high-power magnification (×400) as
described previously.16

Western Blot
Carotid arteries were harvested 7 days after cuff placement, homog-
enized in cold lysis buffer (10 mmol/L HEPES, 1 mmol/L EDTA, 60
mmol/L KCl, 1 mmol/L dithiothreitol, proteinase inhibitor cocktail
[Roche], and 0.4% Nonidet P-40), and incubated for 30 minutes on
ice. After spinning at 12 000×g for 10 minutes, the supernatant
was collected as cytosolic extract. The pellet was
incubated for 2 minutes at 9000

Equal amounts of cytosolic protein were run on a reducing
cytoplasmic gel and transferred to nitrocellulose membrane. Mem-
branes were incubated with rabbit polyclonal iNOS antibody and
detected by the horseradish peroxidase–enhanced chemilumines-
cence method (Amersham).

Oxidative Fluorescent Microtopography
To assess the effect of exposure to CS on vessel wall 02
production, carotid arterial sections were incubated with hydroethidine. The
fluorescent dye hydroethidine has been used to detect reactive
oxygen species and specifically indicates 02

Unfixed frozen sections of noncuffed carotid arteries and carotid
arteries harvested 7 days after cuff placement were incubated with
0.25×10-6 mol/L of hydroethidine for 30 minutes at 37°C.

Serum NO Metabolite Measurement
Serum was collected from mice at the time of euthanasia before, 3,
and 7 days after cuff placement. Total serum NO metabolite (NOx;
nitrate and nitrite) levels were measured using a commercially
available kit (NO-540; OxisResearch).

Activator Protein-1 Electrophoretic Mobility
Shift Assay
Electrophoretic mobility shift assay (EMSA) was performed as
described previously.17 Briefly, activator protein-1 (AP-1) consensus
oligonucleotides (Promega) were labeled with [γ-32P] ATP and T4
kinase. The pellet obtained during cytosolic protein extraction from
carotid arteries harvested 7 days after cuff placement was washed in
lysis buffer and spun down, and the pellet was resuspended in cold
nuclear lysis buffer (250 mmol/L Tris-HCl, 1 mmol/L EDTA, 60
mmol/L KCl, 1 mmol/L dichlorodiphenyltrichloroethane, and
proteinase inhibitor cocktail [Roche]). Nuclear proteins were ex-
tacted by 3 freeze and thaw cycles on dry ice. Extracts were centrifuged at 12 000×g for 20 minutes and the supernatant containing
the nuclear protein extract collected.

A total of 2 μg of carotid artery nuclear extracts were used for the binding assay. DNA–protein complexes were separated on low ionic
4% polyacrylamide gel and the dried gel exposed to film.

Morphometric Measurement
Intimal thickening was assessed 21 days after cuff placement. Frozen
sections 6-μm thick from the cuffed segment of the carotid arteries
were analyzed as described previously.14 Computer-assisted mor-
phometric analysis with image analysis software was used.14 Mea-
surements from all the slides of each mouse were averaged for
statistical calculations.

In Vitro Studies
Mouse aortic vascular SMCs were cultured in 20% FBS-DMEM/
F12 media until confluence. Media was replaced with 1% FBS–
DMEM/F12 for 48 hours, and the cells were treated with CSC
(University of Kentucky Tobacco and Health Research Institute) in
100% ethanol for a final concentration of 25 μg/mL. Controls were
treated with an equal volume of 100% ethanol.

Cytosolic and nuclear proteins were extracted from the harvested
cells. Briefly, cells were washed, scraped in PBS, and centrifuged
for 2 minutes at 9000×g. Cells were resuspended in cold lysis buffer,
icubated for 20 minutes on ice, centrifuged for 5 minutes at 9000×g,
and the supernatant collected as cytosolic extract. The pellet was
washed once in nuclear lysis buffer, subjected to 3 freeze and thaw
cycles, and centrifuged at 14 000×g for 10 minutes. The supernatant
was collected as nuclear extract.

EMSA for the iNOS core XRE binding was performed using a
double-stranded oligonucleotide corresponding to the −401 to −381
site of the published mouse iNOS promoter18 labeled with 32P. Equal
amounts of nuclear extracts were used for the assay. Supershift was
performed using a polyclonal antibody against the AhR.

Western blots for the AhR and iNOS were performed using
cytoplasmic extracts with the same protocol used for the in vivo studies.
Loading was controlled for by Ponceau S stain.

Statistical Analysis
Numeric data are expressed as mean±SD. Group differences were
determined by 1-way ANOVA and Neuman–Keuls test for multiple
comparison. Student t test was used for comparison between 2
groups. P<0.05 was considered statistically significant.

Results
Carboxy-Hemoglobin
To document CS exposure, CO-Hb levels were measured in a
separate group of mice immediately after exposure to CS or
air in the smoking apparatus.15 CO-Hb levels were signifi-
cantly higher in mice exposed to CS compared with mice
exposed to air (15.9±4.4, n=5 versus 6.4±2.2, n=4, respectively; P<0.001). Hemoglobin levels were similar in
mice exposed to CS compared with mice exposed to air
(10.2±0.6 g/dL, n=9 versus 10.2±2.9 g/dL, n=7, respectively).
iNOS Expression

Three days after cuff placement, iNOS expression was detected by staining in the media of the injured arteries (n=5 in each group). Percentage of iNOS-positive stain area of the media was significantly increased in mice exposed to CS compared with mice exposed to air only (22% ± 10% versus 10% ± 2%; P<0.05). Treatment with MEG did not significantly affect the extent of staining in mice exposed to CS (21% ± 7%; Figure 1A through 1C). iNOS expression was not detectable in uninjured carotid arteries of mice exposed to air or CS (data not shown). As expected, there was no iNOS expression in iNOS−/− mice (Figure 1D).

iNOS expression 7 days after cuff placement was determined by Western blotting (Figure 1E; n=3 in each group). iNOS expression was higher in carotid arteries of mice exposed to CS compared with mice exposed to air (1.01 ± 0.13 versus 0.38 ± 0.09 densitometric units, respectively; Figure 1G; P<0.01). MEG treatment did not affect iNOS protein expression in mice exposed to CS or air (0.92 ± 0.66 and 0.54 ± 0.54 densitometric units, respectively). Again, there was no iNOS protein detected in extracts from carotid arteries of iNOS−/− mice (Figure 1F).

iNOS Activity Assessed by Serum NOx Levels and Ethidium Bromide Fluorescence

Serum NOx levels before cuff placement were similar in mice exposed to CS and mice exposed to air (25.2 ± 20.2 and 20.3 ± 23.5 µmol/L, respectively). Three days after cuff placement, NOx levels significantly increased in mice exposed to CS compared with air (39.1 ± 12.8 µmol/L, n=5 versus 22.3 ± 7.3 µmol/L, n=6; P<0.05; Figure 2A). MEG treatment did not affect NOx levels in mice exposed to CS 3 days after cuff placement (42.1 ± 26.2 µmol/L; n=5). Seven days after carring, serum NOx was increased in mice exposed...
to air and was similar to the CS-exposed mice (47.7±28.5 μmol/L, n=7 versus 50.9±41.8 μmol/L, n=9).

NOx levels 7 days after cuff placement was significantly reduced in CS-exposed mice treated with MEG compared with CS mice without MEG treatment (14.5±9.0 μmol/L; n=7; P<0.05).

NOx levels in iNOS−/− mice with or without exposure to CS were low at 3 days (12.5±8.6 versus 14.3±6.8 μmol/L, respectively; n=6 each) and 7 days after cuff placement (18.4±13.1 versus 12.3±7.7 μmol/L, respectively; n=6 each).

To test whether increased iNOS expression in CS mice was associated with O2− generation, arterial sections were stained with hydroethidine. Marginal ethidium bromide (EtdBr) fluorescence was detected in noncuffed arteries. Cuff placement for 7 days resulted in marked increase in EtdBr fluorescence. The fluorescence was further increased in arteries of mice exposed to CS compared with cuffed arteries of mice exposed to air, reflecting increased vascular O2− with cuff placement and exposure to CS. EtdBr fluorescence was minimal in iNOS−/− mice, even with exposure to CS (Figure 2B through 2E).

**Activator Protein-1 EMSA**

Exposure of mice to CS increased AP-1 nuclear binding compared with mice exposed to air (Figure 3). AP-1 binding in CS mice treated with MEG was similar in mice exposed to CS. There was minimal nuclear AP-1 binding in the carotid arteries of iNOS−/− mice, even with exposure to CS (Figure 3).

**PCNA Expression**

Cell proliferation assessed by PCNA staining 21 days after cuff placement was significantly increased in WT mice exposed to CS compared with mice exposed to air (22.6±5.4% versus 11.3±4.1%, n=5 each; P<0.05). In WT mice exposed to CS, treatment with MEG reduced cell proliferation (11.0±6.3%; n=5; P<0.05) compared with untreated mice.

Cell proliferation was significantly reduced in iNOS−/− mice exposed to CS (10.5±4.8%; n=5) compared with WT mice exposed to CS (P<0.01).

**Intimal Thickening**

Intimal thickening area measured at 21 days after cuff placement was significantly increased in WT mice exposed to CS compared with mice exposed to air (Table; P<0.05; Figure 4A and 4B). The intima/media ratio (I/M ratio) was also significantly increased in mice exposed to CS compared with mice exposed to air. In WT mice exposed to CS and treated with MEG, intimal thickening was reduced (Table; P<0.05), comparable to thickening in mice not exposed to CS (Figure 4C). There was no difference in medial area and total vessel area among the 3 groups (Table).

Intimal thickening area 21 days after cuff placement was significantly less in iNOS−/− mice exposed to CS compared to air and was similar to the CS-exposed mice (47.7±28.5 μmol/L, n=7 versus 50.9±41.8 μmol/L, n=9).

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with WT mice exposed to CS (Table; \( P < 0.01 \); Figure 4D). The I/M ratio was also less in iNOS\(-/-\) mice (Table).

### AhR Expression and Activation
AhR expression was detected in 21-day cuffed arteries in mice exposed to air (Figure 5A) and in mice exposed to CS (Figure 5B). Cultured SMCs also expressed the AhR protein as assessed by Western blot (Figure 5C). DNA binding activity on the core XRE sequence of the iNOS promoter was increased 2-fold after 30 minutes of CSC treatment (Figure 5D, left). Specificity to AhR was confirmed by supershift analysis using an AhR antibody (Figure 5D, right). iNOS protein expression was increased 2-fold in SMCs after a 1-hour pulse treatment with CSC and 24-hour incubation in 1% FBS media (0.15 ± 0.04 versus 0.35 ± 0.09 densitometric units; \( P < 0.05 \); \( n = 3 \) each; Figure 5E).

### Discussion
Although it has been reported that exposure to CS increased intimal thickening,1 the mechanism(s) involved has not been defined. Previous work has suggested that iNOS is involved in intimal thickening after injury.14 Furthermore, exposure to CS has resulted in increased iNOS expression in the lungs.11 Therefore, we postulated that exposure to CS augments iNOS expression in the injured arterial wall and is likely involved in the increased intimal thickening caused by CS. The current study shows that exposure to CS resulted in increased iNOS expression in the vascular tissues. Increased iNOS expression in the vessel wall was paralleled by increased nitrate radical production, increased SMC proliferation, and 2-fold increased intimal thickening in mice exposed to CS compared with mice exposed to room air. Effects of CS exposure on intimal thickening were markedly attenuated in mice treated with specific iNOS inhibitor MEG and in mice lacking the iNOS gene. These data suggest that the augmenting effects of exposure to CS on arterial wall thickening are mediated in part through increased iNOS expression.

The gas phase of CS contains abundant free radicals18 that may mediate the damaging effects of exposure to smoke on arteries19,20 through oxidative stress and inflammation.21 One of the redox genes implicated in the inflammatory response is iNOS.22 Whereas constitutively expressed endothelial NOS–produced NO is considered to have protective effects on the vessel wall, iNOS may have an important role in mediating oxidative stress.23 In addition, iNOS is also involved in the process of healing after injury.24 Mechanical injury to the vessel wall is associated with increased iNOS expression.25 Conversely, intimal thickening was reduced in iNOS\(-/-\) mice.14,24 Increased iNOS expression in the vessel wall after cuff placement in our study was further increased by \( \approx 2.5 \)-fold with exposure to CS compared with mice with exposure to air, strongly suggesting that it is implicated in the detrimental effects of CS on intimal thickening.

Tissue iNOS expression and serum NOx levels were significantly higher in mice exposed to CS compared with air 3 days after cufing, suggesting that CS exposure potentiated iNOS expression and activity. MEG inhibition of NOS is selective for the inducible isoform.26 In our study, MEG treatment did not inhibit iNOS expression but decreased serum NOx. Similar findings were reported by Szabo et al in a model of lung injury.26 Reduction of serum NOx by MEG treatment was evident 7 days after injury and not at 3 days, suggesting that several days of treatment are needed for its

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**Table: Carotid Arteries 21 Days After Cuff Placement**

<table>
<thead>
<tr>
<th></th>
<th>Weight (g)</th>
<th>EEL (mm²)</th>
<th>Media (mm²)</th>
<th>Intima (mm²)</th>
<th>I/M</th>
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<td>WT Mice</td>
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<td>Air (n=6)</td>
<td>30.3±1.8</td>
<td>0.072±0.029</td>
<td>0.026±0.011</td>
<td>0.009±0.008</td>
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<td>Air+MEG (n=5)</td>
<td>32.7±3.0</td>
<td>0.094±0.048</td>
<td>0.033±0.015</td>
<td>0.005±0.009†</td>
<td>0.09±0.14†</td>
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<tr>
<td>CS (n=5)</td>
<td>31.8±6.1</td>
<td>0.088±0.047</td>
<td>0.026±0.005</td>
<td>0.023±0.013*</td>
<td>0.88±0.51*</td>
</tr>
<tr>
<td>CS+MEG (n=6)</td>
<td>29.6±1.6</td>
<td>0.082±0.015</td>
<td>0.030±0.008</td>
<td>0.006±0.005†</td>
<td>0.21±0.17†</td>
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<tr>
<td>iNOS(-/-) mice</td>
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<tr>
<td>Air (n=5)</td>
<td>33.8±6.7</td>
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<td>0.031±0.008</td>
<td>0.006±0.005†</td>
<td>0.18±0.15†</td>
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<td>CS (n=5)</td>
<td>29.3±3.2</td>
<td>0.084±0.023</td>
<td>0.027±0.009</td>
<td>0.002±0.002†</td>
<td>0.08±0.07†</td>
</tr>
</tbody>
</table>

Air indicates exposure to room air; Air+MEG, exposure to room air, treatment with MEG; CS, exposure to CS; CS+MEG, exposure to CS, treatment with MEG; EEL, XXXX.

*\( P < 0.01 \) vs WT Air; †\( P < 0.01 \) vs WT CS.

**Figure 4.** Representative sections of carotid arteries 21 days after cuff placement. Intimal thickening in WT mice exposed to air (A), WT mice exposed to CS (B), WT mice exposed to CS and treated with MEG (C), and iNOS\(-/-\) mice exposed to CS (D). Arrows indicate intimal thickening. Bar=10 \( \mu \)m.
Inhibition of iNOS activity with MEG treatment was associated with inhibition of intimal thickening, suggesting that increased iNOS activity likely plays a role in augmented vessel wall thickening after exposure to CS.

Seven days after cufing, mice exposed to air also had increased serum NOx, indicating increased iNOS activity. Although tissue iNOS expression was significantly increased in CS mice compared with air, NO production was similar. It is possible that serum NOx 7 days after cufing reflects uncoupling of the NOx-producing function of iNOS from its protein expression. Increased EtdBr staining observed in CS mice compared with air at the 7-day time point suggests that increased iNOS protein expression was associated with increased O$_2$ generation. The minimal serum NOx levels and EtdBr presence in the carotid arteries of iNOS$^{-/-}$ mice were not affected by CS, suggesting that the O$_2$ generation potentiated by CS is mediated by iNOS. Results from the iNOS$^{-/-}$ mice also suggest that the increase in serum NOx after cufing and CS exposure is likely attributable to iNOS expression and activity. The possibility of other sources of NOx in addition to the cuffed artery cannot be discounted. The rodent model has been reported to produce notoriously high levels of NO compared with larger animals.

It is currently unclear how uncoupling may occur in CS-exposed mice. However, it is notable that L-arginine treatment prevented endothelial dysfunction in rabbits exposed to tobacco smoke and normalized coronary vasomotion in long-term smokers. Previously, we and others showed that mechanical injury to the vessel wall is associated with severalfold increase in nuclear factor κB (NF-κB) and AP-1 nuclear translocation and binding. Although exposure to CS smoke in this study did not influence NF-κB binding (data not shown), it did increase AP-1 activation and binding in the injured tissues. AP-1, activation has been observed in response to growth factors, cytokines, and stress signals and was demonstrated to be critical for cell proliferation in c-Jun$^{-/-}$ fibroblasts. In vivo inhibition of AP-1 binding with octreotide was associated with decreased intimal thickening. Decreased AP-1 activity in iNOS$^{-/-}$ mice suggests a regulatory role for iNOS in AP-1 activation. Reduced PCNA staining in iNOS$^{-/-}$ mice exposed to CS supports this notion.

The mechanism for increased iNOS expression after CS exposure is not clearly defined. Although CS contains multiple compounds, aryl hydrocarbons are known to activate signaling pathways. Carotid arteries and cultured SMCs express the AhR. The AhR was initially described in mice as a factor mediating the response to dioxin. It is a ligand-activated transcription factor that binds to the XRE of target genes regulating expression of a number of xenobiotic-metabolizing enzymes. The mouse iNOS promoter contains the core XRE sequence at positions −401 to −381 that could be involved in CS-mediated signaling. An oligonucleotide probe designed to investigate the potential binding activity of the AhR demonstrated that treatment of SMCs with CSC for 1 hour increased DNA-binding activity specific to the AhR. Furthermore, pulse treatment of SMCs with CSC for 1 hour also resulted in a significant increase in iNOS protein expression after 24 hours.

The results of the current study suggest that AhR signaling is a pathway for CS-induced increase in vascular iNOS expression. It supports the notion that CS exposure may have effects on the arterial wall, mediated by SMCs. This finding is in agreement with a previous report indicating that AhR signaling occurs in benzo(a)pyrene-treated SMCs, which is
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abrogated in AhR-null cells. However, systemic effects of CS in the current study cannot be excluded.

In conclusion, our study suggests that increased arterial expression of iNOS and SMC proliferation are important mechanisms by which exposure to CS augments intimal thickening. Inhibition of iNOS expression or activity may offer an opportunity to ameliorate the deleterious effects on the vasculature by exposure to CS.

Acknowledgments

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References


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