Upregulation of Arginase by H₂O₂ Impairs Endothelium-Dependent Nitric Oxide–Mediated Dilation of Coronary Arterioles

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Objective—Overproduction of reactive oxygen species such as hydrogen peroxide (H₂O₂) has been implicated in various cardiovascular diseases. However, mechanism(s) underlying coronary vascular dysfunction induced by H₂O₂ is unclear. We studied the effect of H₂O₂ on dilation of coronary arterioles to endothelium-dependent and endothelium-independent agonists.

Methods and Results—Porcine coronary arterioles were isolated and pressurized without flow for in vitro study. All vessels developed basal tone and diluted dose-dependently to activators of nitric oxide (NO) synthase (adenosine and ionomycin), cyclooxygenase (arachidonic acid), and cytochrome P450 monoxygenase (bradykinin). Intraluminal incubation of vessels with H₂O₂ (100 μmol/L, 60 minutes) did not alter basal tone but inhibited vasodilations to adenosine and ionomycin in a manner similar as that by NO synthase inhibitor L-NAME. H₂O₂ affected neither endothelium-dependent responses to arachidonic acid and bradykinin nor endothelium-independent dilation to sodium nitroprusside. The inhibited adenosine response was not reversed by removal of H₂O₂ but was restored by excess L-arginine. Inhibition of L-arginine consuming enzyme arginase by α-difluoromethylornithine or N⁴-hydroxy-N-L-arginine also restored vasodilation. Administering deferoxamine, an inhibitor of hydroxyl radical production, prevented the H₂O₂-induced impairment of vasodilation to adenosine. Western blot and reverse-transcription polymerase chain reaction results indicated that arginase I was upregulated after treating vessels with H₂O₂.

Conclusions—H₂O₂ specifically impairs endothelium-dependent NO-mediated dilation of coronary microvessels by reducing L-arginine availability through upregulation of arginase. The formation of hydroxyl radicals from H₂O₂ may contribute to this process. (Arterioscler Thromb Vasc Biol. 2006;26:2035-2042.)

Key Words: endothelium ■ free radicals ■ hydrogen peroxide ■ nitric oxide

Reactive oxygen species (ROS) from mitochondria and other subcellular sources have been regarded as toxic byproducts of metabolism, especially when excessive production of ROS outstrips endogenous antioxidant defense mechanisms. However, ROS are also known to influence the expression of a number of genes and signal transduction pathways and are thought to act as subcellular messengers for certain growth factors. Interestingly, several cardiovascular diseases with diverse etiologies, such as atherosclerosis, hypertension, vascular complications in diabetes, and after ischemia/reperfusion injury, are associated with the common hallmarks of increased oxidative stress and endothelial cell dysfunction. Although the molecular basis of endothelial dysfunction is not completely understood, numerous studies point to the reduction of nitric oxide (NO) biosynthesis and/or bioactivity as a major mechanism. However, the underlying cellular mechanisms contributing to the reduction of NO-mediated effects remain unclear.
pressed in the endothelium and plays a counteracting role in the stimulated NO production and in NO-mediated vasodilatory function in coronary microcirculation. In addition, there is substantial evidence that the expression of arginase is elevated in a variety of cells and tissues under the conditions with inflammation and oxidative stress. It is plausible that the upregulation of arginase and its competition with NOS for their common substrate L-arginine may be involved in the microvascular dysfunction induced by H2O2. Because NO released from the endothelium plays a major role in the development of coronary vasomotor activity, it is important to understand the direct effect of H2O2 on coronary arteriolar function and to elucidate the underlying mechanism responsible for the impairment of NO-mediated dilation in these microvessels. Herein, we tested the hypothesis that H2O2 specifically inhibits endothelium-dependent NO-mediated dilation of coronary arterioles by reducing L-arginine availability through upregulated arginase. Using an isolated vessel preparation, we examined the effect of H2O2 on vasodilatory function of coronary arterioles in response to various endothelium-dependent and endothelium-independent agonists. The role of arginase in influencing vasomotor function was addressed using pharmacological, molecular, and immunohistochemical tools.

Methods

Effect of H2O2 on Vasodilatory Function of Isolated Coronary Arterioles

The procedures followed were in accordance with guidelines set by the Laboratory Animal Care Committee at Texas A&M University. See the online-only data supplement for detailed description of methods (http://atvb.ahajournals.org). Pigs (Milberger Farms, Kurten, Tex) were anesthetized with pentobarbital (20 mg/kg) and the heart was quickly excised. Individual coronary arterioles (60 to 120 µm in internal diameter in situ) were dissected from the subepicardium of left ventricle for in vitro study as previously described. Vessels were cannulated and pressurized to 60 cmH2O intraluminal pressure. After development of stable basal tone, the effects of H2O2 on coronary arteriolar dilations mediated by different signaling mechanisms were examined before and after intraluminal incubation with H2O2 (100 µmol/L) for 60 minutes. Preliminary studies indicated that 60 minutes but not 30 minutes of exposure to 100 µmol/L H2O2 was sufficient to inhibit adenosine-induced vasodilation. First, to assess the signaling mechanisms, we used adenosine and ionomycin as activators for NO-mediated vasodilation through receptor-dependent and receptor-independent mechanisms, respectively. Second, endothelium-dependent agonists bradykinin and arachidonic acid were used to stimulate vasodilation mediated by the cytochrome P450 monoxygenase and the cyclooxygenase pathway in vasodilation. Third, pinacidil and sodium nitroprusside were used as the endothelium-independent agonists to assess the vasodilatory function in response to ATP-sensitive potassium (KATP) channel and guanylyl cyclase activation, respectively. Fourth, the involvement of NOS and cyclooxygenase pathways in vasodilation was examined before and after extraluminal incubation of the vessels with the specific inhibitors N-nitro-L-arginine methyl ester (L-NAME) (10 µmol/L, 30 minutes) and indomethacin (10 µmol/L, 30 minutes), respectively. Fifth, the role of endothelial cytochrome P450 monoxygenase pathway in vasodilation was examined by intraluminal incubation of the vessels with its inhibitor miconazole (30 µmol/L, 30 minutes). Finally, to rule out time-dependent and nonspecific effects of H2O2, the vasodilatory responses were also examined in a separate series of experiments after a 60-minute intraluminal incubation of the vessels with vehicle (PSS).

Specificity of H2O2 in Impairing Vascular Function

The specificity of the effect of H2O2 on vasodilator responses was examined by intraluminal administration of H2O2 solution (100 µmol/L) containing catalase (1000 U/mL). To determine whether the impaired vascular function can be restored after H2O2 removal, in another group of vessels the agonist-induced vasodilations were initially studied in the presence of intraluminal H2O2 (60-minute incubation) and then re-examined at 30 minutes after replacing the intraluminal H2O2 with PSS. To evaluate whether superoxide anions or hydroxyl radicals contribute to the vascular dysfunction elicited by H2O2, coronary arteriolar vasodilation to agonists was examined before and after intraluminal administration of H2O2 solution containing cell permeable superoxide anion scavenger, polyethylene glycol (PEG)-superoxide dismutase (PEG-SOD) (100 U/mL), or hydroxyl radical production inhibitor, deferoxamine (100 µmol/L).26

Role of L-Arginine and Arginase in Vascular Dysfunction

To determine whether the deficiency of L-arginine contributes to the impaired NO-mediated response, the adenosine-induced and ionomycin-induced vasodilations in the presence of H2O2 were further examined after extraluminal incubation of the vessels with L-arginine (3 mmol/L) for 30 minutes. In addition, to determine whether arginase plays a role in vascular dysfunction, the vessels were initially treated with H2O2 and then the vasodilations to adenosine and ionomycin were examined after intraluminal incubation of the vessels with arginase inhibitors α-difluoromethylornithine (DFMO) (0.4 mmol/L) or N-hydroxy-nor-L-arginine (nor-NOHA) (0.1 mmol/L) for 30 minutes.

RNA Isolation and Reverse-Transcription Polymerase Chain Reaction Analysis

Total RNA was isolated from porcine subepicardial coronary arterioles (3 to 4 vessels, ∼100 µm diameter, 2 to 3 mm length) after incubation with H2O2 (100 µmol/L) or vehicle for 60 minutes at 37°C, based on the protocols described previously. RNA isolated from liver tissue and kidney tissue were used as positive control for arginase I and arginase II, respectively. Using primers specific for arginase I, arginase II, endothelial NOS (eNOS), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes, reverse-transcription polymerase chain reaction was conducted as delineated previously.

Western Blot Analysis

Isolated coronary arterioles (4 to 5 vessels per sample, 60 to 120 µm diameter, 3 to 4 mm length) were incubated with H2O2 (100 µmol/L) or vehicle for 60 minutes at 37°C. The vessels were then homogenized and prepared for Western blot analysis, as described previously with slight modification. Five micrograms of protein per lane were separated by 10% SDS-PAGE under reducing conditions, transferred onto a nitrocellulose membrane, and then allowed to react with a primary antibody for arginase I (1:1000; BD Transduction Laboratories, Lexington, Ky) or β-actin (1:1000; Ambion, Austin, Tex). The antigen–antibody complexes were revealed with horseradish peroxidase-labeled goat anti-rabbit IgG secondary antibody (Alpha Diagnostic International, San Antonio, Tex) by an enhanced chemiluminescence assay (Amersham Pharmacia, Piscataway, NJ).

Immunohistochemical Analysis

Isolated coronary arterioles (∼100 to 150 µm in diameter) were pressurized and incubated with intraluminal H2O2 (100 µmol/L) or vehicle for 60 minutes. The vessels were removed from the cannulating pipettes and prepared for immunohistochemical analysis, as described previously. Sections (12-µm-thick) were immunolabeled with anti-arginase I antibody or anti-eNOS antibody (1:100, BD Transduction Laboratories) and observed by means of confocal microscopy, as described previously.
Effect of Cycloheximide on Vascular Dysfunction
To determine the regulatory level of arginase activation by H$_2$O$_2$, the vessels were incubated with cycloheximide (CHX) (20 μg/mL, intraluminal incubation), a protein synthesis inhibitor, for 60 minutes and followed by the treatment of H$_2$O$_2$ (100 μmol/L) containing CHX for 60 minutes. The vasodilatory function was then evaluated by adenosine. Finally, the same vessels were prepared for immunohistochemical analysis of arginase I expression as described.

Data Analysis
Diameter changes in response to vasodilator agonists were normalized to the maximum diameter changes in response to 100 μmol/L sodium nitroprusside in an ethylenediaminetetraacetic acid (1 mmol/L) calcium-free PSS and expressed as a percentage of maximal dilation. Statistical comparisons were performed by means of 2-way ANOVA or Student t test. P<0.05 was considered significant. Data are presented as mean±SEM (n=number of vessels).

Results
Effect of H$_2$O$_2$ on Endothelium-Dependent and Endothelium-Independent Vasodilations
All isolated coronary arterioles developed a similar level of basal tone (eg, constricted to 62±1% of their maximal diameter) and dilated to adenosine (Figure 1A) and ionomycin (Figure 1B) in a concentration-dependent manner. In the presence of NOS inhibitor L-NAME, the basal vascular tone was slightly increased but did not reach statistical significance (before L-NAME: 62±3% of maximal diameter; after L-NAME: 60±4% of maximal diameter); however, the dilation of these vessels to adenosine and ionomycin was significantly inhibited (Figure 1A and 1B).

In another set of experiments, the vasodilations to adenosine and ionomycin were examined before and after treating the vessels with intraluminal H$_2$O$_2$. The resting vascular tone was not altered by H$_2$O$_2$ (before H$_2$O$_2$: 62±1%; after H$_2$O$_2$: 63±1%), but the dilation to adenosine and ionomycin was significantly inhibited in the same manner as by L-NAME (Figure 1A and 1B). Subsequent administration of L-NAME to the H$_2$O$_2$-treated vessels did not further reduce the vasodilator responses (data not shown). Contrarily, H$_2$O$_2$ did not affect the endothelium-dependent vasodilation to a cytochrome P450 monoxygenase activator bradykinin (supplemental Figure I, available online at http://atvb.ahajournals.org). Activation of cyclooxygenase pathway by arachidonic acid (10 μmol/L) caused a 77±5% dilation of coronary arterioles; and this dilation was not altered by H$_2$O$_2$ (ie, 79±4% dilation, n=5; data not shown). Furthermore, H$_2$O$_2$ also had no effect on the vasodilation elicited by a smooth muscle K$_{ATP}$ channel opener pinacidil (supplemental Figure I) or a guanylyl cyclase activator sodium nitroprusside (supplemental Figure I). To rule out the possible nonspecific endothelial deterioration during the experimental procedure, we examined the effect of luminal incubation of arterioles with a vehicle solution or a low concentration of H$_2$O$_2$ (10 μmol/L) for 60 minutes. As shown in supplemental Table I, dose-dependent dilations of adenosine and sodium nitroprusside were not altered by these treatments.

Effect of H$_2$O$_2$ Removal and ROS Scavengers on Vascular Dysfunction
The impaired vasodilations to adenosine were not restored after removing H$_2$O$_2$ from the lumen for 30 minutes (Figure 2A). In contrast, co-administration of H$_2$O$_2$ with catalase, but not superoxide scavenger PEG-SOD, prevented the inhibitory effect of H$_2$O$_2$ (Figure 2A). A similar result was observed in the vessels challenged with a receptor-independent NO-mediated vasodilator ionomycin (Figure 2B).

Role of L-Arginine and Arginase in Vascular Dysfunction
As shown in Figure 3, administration of L-arginine completely restored the H$_2$O$_2$-impaired vasodilatation to adenosine (Figure 3A) and ionomycin (Figure 3B). Restoration of vasodilations to these agonists was also observed in the vessels treated with an arginase inhibitor DFMO (Figure 3A and 3B). Administration of another specific arginase inhibitor nor-NOHA also restored the impaired vasodilation to adenosine and ionomycin (n=3, data not shown). It should be noted that L-arginine did not enhance NO-mediated dilations to adenosine and ionomycin in control arterioles (n=4, data not shown) as demonstrated in our previous studies. In contrast, catalase partially restored the impaired vasodilations to adenosine and ionomycin in H$_2$O$_2$-treated arterioles (n=4, data not shown) as demonstrated in our previous studies.21,23,24
an inhibitor of hydroxyl radical formation, on vascular dysfunction. Deferoxamine prevented the H$_2$O$_2$-induced inhibitory effect on adenosine-induced vasodilation (Figure 4) but did not alter the adenosine-induced response of control vessels (Figure 4) or the sodium nitroprusside-induced vasodilation in the presence of H$_2$O$_2$ (supplemental Figure I).

Effect of H$_2$O$_2$ on Arginase and eNOS Expression

Reverse-transcription polymerase chain reaction studies showed that coronary arterioles express arginase I (liver tissue was used as a positive control) but not arginase II (kidney tissue was used as a positive control) (Figure 5A). Treating coronary arterioles with H$_2$O$_2$ for 60 minutes increased arginase I mRNA by ≈2-fold without altering eNOS expression (Figure 5B). At the protein level, immunoblotting showed that H$_2$O$_2$ treatment also stimulated a 2-fold increase in arginase I protein in arterioles (Figure 6A). For cellular localization of arginase, immunohistochemical analyses indicated that arginase I protein was expressed in the vascular wall with relatively low levels. Treating the vessels with H$_2$O$_2$ significantly increased arginase I expression mainly in endothelial cells (Figure 6B). This upregulation was not observed in the vessels pretreated with a protein synthesis inhibitor CHX (Figure 6C). However, the eNOS protein expression was not altered by H$_2$O$_2$ (Figure 6C). CHX also protected the adenosine-induced and ionomycin-induced vasodilation from the inhibitory effect of H$_2$O$_2$ (supplemental Figure II).

Discussion

Previous studies have shown that H$_2$O$_2$ can cause vasodilation of small porcine$^{25}$ and human$^{30}$ coronary arterioles when it is administered extraluminally. In de-endothelialized pig coronary artery rings, H$_2$O$_2$ caused transient contraction and a subsequent relaxation.$^{31,32}$ However, there are few studies examining the intraluminal effect of H$_2$O$_2$ on arteriolar function, despite the evidence showing that a substantial increase in H$_2$O$_2$ was detected in the luminal surface of the vessels subjected to oxidative stress.$^{33,34}$ To the best of our knowledge, there is limited information on the endogenous level of H$_2$O$_2$ in the intact vascular wall. However, a level from 2.5 μmol/L to 50 μmol/L has been reported in human
In general, it is regarded that H\textsubscript{2}O\textsubscript{2} at the concentration \(\leq 50\) \(\mu\text{mol/L}\) exhibits limited cytotoxicity in many cell types.\textsuperscript{40} It appears that endothelial cells are less susceptible to H\textsubscript{2}O\textsubscript{2} because a relatively high concentration of H\textsubscript{2}O\textsubscript{2} (ie, \(\geq 200\) \(\mu\text{mol/L}\)) is generally required to produce irreversible endothelial barrier dysfunction\textsuperscript{41,42} and induce apoptosis.\textsuperscript{43,44} In the context of neutrophil-endothelial interaction, the H\textsubscript{2}O\textsubscript{2} released from activated neutrophils are capable of destroying endothelial cells,\textsuperscript{45,46} suggesting a high level of H\textsubscript{2}O\textsubscript{2} can be reached at the local circulation during inflammation. However, the direct effect of intraluminal H\textsubscript{2}O\textsubscript{2} on vasomotor function has not been systematically examined.

A recent study on KCl precontracted pig coronary arteries indicated that NO-mediated relaxation was attenuated after luminal perfusion with 500 \(\mu\text{mol/L}\) H\textsubscript{2}O\textsubscript{2},\textsuperscript{10} however, the underlying mechanism has not been fully investigated. At the microvascular levels, our present findings indicate that the intraluminal exposure of coronary arterioles to a sublethal level of H\textsubscript{2}O\textsubscript{2} (100 \(\mu\text{mol/L}\)) leads to a selective impairment of NO-mediated vasodilation independent of endothelial receptors. There are several lines of evidence to support this contention. First, endothelium-dependent vasodilation to NO-mediated agonists adenosine (receptor-dependent)\textsuperscript{29} and ionomycin (receptor-independent)\textsuperscript{23} were inhibited by intraluminal H\textsubscript{2}O\textsubscript{2} and L-NAME in an identical fashion. We have previously shown that adenosine-induced dilation in coronary arterioles is mediated by the activation of endothelial NO pathway and smooth muscle K\textsubscript{ATP} channels.\textsuperscript{22} Because vasodilation in response to the activation of K\textsubscript{ATP} channel by pinacidil was not altered, the impaired adenosine response appears to be caused by the selective action of H\textsubscript{2}O\textsubscript{2} on endothelial NO pathways. Second, the dilations induced by bradykinin (cytochrome P450 pathway\textsuperscript{24}) and arachidonic acid (cylooxygenase pathway\textsuperscript{24}) were unaltered. Third, the H\textsubscript{2}O\textsubscript{2}-treated vessels exhibited normal dilation to sodium nitroprusside, an NO donor, which activates smooth muscle guanylyl cyclase. Furthermore, H\textsubscript{2}O\textsubscript{2}-induced impairment is not caused by the time-dependent deterioration of NO-mediated function, because a 60-minute incubation of the vessels with either vehicle solution or a low concentration of H\textsubscript{2}O\textsubscript{2} (10 \(\mu\text{mol/L}\)) did not affect the vasodilatory response to NOS activators and to the NO donor sodium nitroprusside. It should be noted that to avoid the potential confounding influences imposed on these microvessels caused by the prolonged experimental protocol, we chose a 60-minute incubation as the cutoff point. Therefore, it is not known whether 10 \(\mu\text{mol/L}\) H\textsubscript{2}O\textsubscript{2} is sufficient to elicit endothelial dysfunction if a prolonged incubation (ie, \(>60\) minutes) were allowed. Nevertheless, the adverse effect caused by 100 \(\mu\text{mol/L}\) H\textsubscript{2}O\textsubscript{2} was not extended to the smooth muscle cells because there was no significant change in vascular tone by luminal H\textsubscript{2}O\textsubscript{2} and the vessels exhibited normal response to endothelium-independent vasodilators sodium nitroprusside and pinacidil. It appears that vascular smooth muscle function...
was preserved and the endothelium plays an important role in protecting smooth muscle cells against luminal H$_2$O$_2$. This result is in agreement with the finding in large arteries that H$_2$O$_2$ does not readily get across the endothelium to exert its cytotoxicity. It is possible that a high level of catalase in the endothelium allows protection of the underlying smooth muscle cells.

Our findings on the improvement of NO-mediated dilation of H$_2$O$_2$-treated vessels by L-arginine suggested that a reduction in the availability of NOS precursor was involved in the vascular dysfunction. In terms of L-arginine metabolism, beside NOS isoforms, arginase is another major L-arginine consuming enzyme that converts L-arginine to L-ornithine and urea. To date, 2 arginase isoforms have been identified. Arginase I isoform is expressed most abundantly, but not exclusively, in the liver, whereas arginase II is expressed in the kidney and many other extrahepatic tissues. The main function of the hepatic arginase is for ammonia detoxification via the urea cycle. However, the biological role of the extrahepatic arginase remains obscure. Nonetheless, our previous studies have shown that arginase I can modulate coronary arteriolar function by reducing NO production from NOS. It is plausible that upregulation of arginase in the H$_2$O$_2$-treated vessels causes a reduction of L-arginine availability to NOS and thus compromises NO-mediated vasodilation.

Indeed, we found that administration of arginase inhibitors, DFMO or nor-NOHA, effectively restored vasomotor function impaired by H$_2$O$_2$, suggesting the involvement of arginase in vascular dysfunction. It is worth noting that we and other laboratories have previously shown that these inhibitors effectively reduce arginase activity without affecting NOS function. Interestingly, H$_2$O$_2$ appears to upregulate the gene and protein expression of arginase I in coronary arteriolar wall, especially in endothelial cells. At the present time, the mechanism underlying the upregulation of arginase remains unclear. However, the induction of arginase protein synthesis appears to be involved in the vascular dysfunction because administration of the protein synthesis inhibitor CHX before H$_2$O$_2$ exposure not only inhibited the increased arginase expression but also preserved the NOS-dependent vasodilation. Although it is somewhat surprising that a significant arginase induction in coronary microvessels can be achieved within such a short period (ie, 60 minutes) of exposure to H$_2$O$_2$, previous studies have shown that pharmacological and pathophysiological stimulations can alter the expression of mRNA and/or protein within 60 minutes. Interestingly, our recent studies demonstrated that vascular arginase I was upregulated leading to impaired NO-mediated dilation in the porcine heart subjected to either chronic hypertension (8 weeks) or an acute episode of ischemia-reperfusion. Because ROS, including H$_2$O$_2$, play an important role in the vascular dysfunction in hypertension and ischemia-reperfusion injury, it is speculated that H$_2$O$_2$ may be the molecule that triggers the overexpression of vascular arginase and consequently leads to the impairment of NOS-mediated vascular function under these pathophysiological conditions.

In addition to the involvement of arginase, other potential mechanisms such as NOS expression and the production of ROS that could potentially influence NO-mediated vasodilation by H$_2$O$_2$ should be considered. Interestingly,
H$_2$O$_2$ has been shown to increase, rather than decrease, NOS expression in both mRNA and protein levels in cultured endothelial cells. However, these phenomena were not observed in our study in intact coronary arterioles since both mRNA and protein expressions in these vessels did not appear to be affected by H$_2$O$_2$. This discrepancy may be related to the differences in experimental model (cultured endothelium versus intact tissue) and/or incubation time (minutes versus hours) for H$_2$O$_2$ treatment. Nevertheless, in the present study it is unlikely that the reduced NO-mediated vasodilation by H$_2$O$_2$ is mediated by the alteration of NOS expression. Another possible route for reducing NO-mediated vasodilation is through the production of superoxide. It has been shown that H$_2$O$_2$ may lead to an increase in other ROS such as superoxide, which can directly inactivate NO to form peroxynitrite leading to an increased cellular redox stress. However, treating the vessels with catalase, but not PEG-SOD (a cell-permeable superoxide scavenger), preserved the NO-mediated vascular function (Figure 2). It should be noted that the concentration of PEG-SOD used in the present study is sufficient to eliminate superoxide effect on coronary arterial function. Furthermore, immunohistochemical studies with superoxide-sensitive dye (dihydroethidium) did not detect an increase in superoxide in the coronary arterioles after H$_2$O$_2$ treatment (100 μmol/L, n = 3, data not shown). Thus, it is unlikely that superoxide plays a role in arteriolar dysfunction associated with H$_2$O$_2$. However, we found that treatment of the vessels with deferoxamine, an inhibitor of hydroxyl radical formation, prevented the H$_2$O$_2$-induced impairment of vasodilation to adenosine. The effect appeared to be specific because deferoxamine did not alter the vasodilator response of control vessels to adenosine. Because H$_2$O$_2$ can be rapidly converted to hydroxyl radical, these results suggest that formation of this ROS may contribute to the reduction of endothelium-dependent NO-mediated vasodilation. Interestingly, H$_2$O$_2$ and hydroxyl radicals have been shown to activate the p38 MAP kinase and cAMP pathways and activation of both p38 and cAMP can cause arginase induction in some cells. It is speculated that these ROS-induced signaling cascades may be involved in the upregulation of arginase expression.

In summary, we demonstrate that H$_2$O$_2$ inhibits endothelium-dependent NO-mediated dilation of coronary arterioles by upregulating arginase expression. Administration of L-arginine or inhibition of arginase activity restores the impaired vascular function. These results may suggest potential therapeutic interventions targeting L-arginine administration and/or inhibition of arginase induction/activity to improve compromised coronary arteriolar function during oxidative stress.

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Disclosures
None.

References


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Supplementary Methods

Animal Preparation. The procedures followed were in accordance with guidelines set by the Laboratory Animal Care Committee at Texas A&M University. Pigs (8 to 12 weeks old, either sex, Milberger Farms, Kurten, TX) were sedated with an intramuscular injection of Telazol (tiletamine and zolazepam, 1:1, 4.4 mg/kg) and xylazine (2.2 mg/kg) and then anesthetized and anticoagulated with an intravenous administration of pentobarbital sodium (20 mg/kg) and heparin (1,000 U/kg), respectively, via the marginal ear vein. Pigs were intubated and ventilated with room air. After a left thoracotomy was performed, the heart was electrically fibrillated, excised, and immediately placed in cold (5°C) saline solution.

Isolation and Cannulation of Coronary Microvessels. To eliminate confounding influences from hemodynamic, neurohumoral and metabolic factors on vasomotor function, individual coronary arterioles (60–120 µm, in internal diameter in situ) were dissected from the subepicardium of left ventricle for in vitro study as previously described.1 Vessels were cannulated with glass micropipettes and pressurized to 60 cmH₂O intraluminal pressure. The cannulated vessel was bathed in physiological salt solution (PSS) (mmol/L: NaCl 145.0, KCl 4.7, CaCl₂ 2.0, MgSO₄ 1.17, NaH₂PO₄ 1.2, glucose 5.0, pyruvate 2.0, EDTA 0.02, and MOPS 3.0) containing bovine serum albumin (BSA) (1%, USB Corporation, Cleveland, OH) at 37°C.

Effect of H₂O₂ on Vasodilatory Function of Coronary Arterioles. After developing a stable basal tone (~60 minutes), the effects of H₂O₂ on coronary arteriolar dilations mediated by different signaling mechanisms were examined before and after intraluminal incubation with H₂O₂ (100 µmol/L) for 60 minutes. Preliminary studies indicated that 60 minutes but not 30 minutes of exposure to 100 µmol/L H₂O₂ was sufficient to inhibit adenosine-induced
vasodilation. First, to assess the signaling mechanisms, we used adenosine (0.1 nmol/L to 10 µmol/L)\(^3,4\) and ionomycin (0.1 nmol/L to 0.3 µmol/L)\(^4,5\) as activators for NO-mediated vasodilation through receptor-dependent and receptor-independent mechanisms, respectively. Second, endothelium-dependent agonists bradykinin (0.1 pmol/L to 1 nmol/L) and arachidonic acid (10 µmol/L) were employed to stimulate vasodilation mediated by the cytochrome P450 monooxygenase\(^6\) and the cyclooxygenase-derived prostanoid pathways.\(^6\) Third, pinacidil (30 nmol/L to 3 µmol/L)\(^3\) and sodium nitroprusside (1 nmol/L to 10 µmol/L)\(^7\) were used as the endothelium-independent agonists to assess the vasodilatory function in response to ATP-sensitive potassium (K\(_{\text{ATP}}\)) channel and guanylyl cyclase activation, respectively. Fourth, the involvement of NOS and cyclooxygenase pathways in vasodilation was examined before and after extraluminal incubation of the vessels with the specific inhibitors \(N^\text{G}\)-nitro-L-arginine methyl ester (L-NAME, 10 µmol/L, 30 minutes)\(^6\) and indomethacin (10 µmol/L, 30 minutes)\(^6\), respectively. Fifth, the role of endothelial cytochrome P450 monooxygenase pathway in vasodilation was examined by intraluminal incubation of the vessels with its inhibitor miconazole (30 µmol/L, 30 minutes)\(^8\). Finally, to rule out time-dependent and nonspecific effects of H\(_2\)O\(_2\), the vasodilatory responses were also examined in a separate series of experiments after a 60-minute intraluminal incubation of the vessels with vehicle (PSS).

**Specificity of H\(_2\)O\(_2\) in Impairing Vascular Function.** The specificity of the effect of H\(_2\)O\(_2\) on vasodilations induced by adenosine and ionomycin was examined by intraluminal administration of H\(_2\)O\(_2\) solution (100 µmol/L) containing catalase (1,000 U/mL). To determine whether the impaired vascular function can be restored after H\(_2\)O\(_2\) removal, in another group of vessels the agonist-induced vasodilations were initially studied in the presence of intraluminal H\(_2\)O\(_2\) (60-minute incubation) and then re-examined at 30 minutes after replacing the intraluminal
H₂O₂ with PSS. To evaluate whether superoxide anions or hydroxyl radicals contribute to the vascular dysfunction elicited by H₂O₂, coronary arteriolar dilations to adenosine and ionomycin were examined before and after intraluminal administration of H₂O₂ solution containing cell permeable superoxide anion scavenger, polyethylene glycol-superoxide dismutase (PEG-SOD, 100 U/mL), or hydroxyl radical production inhibitor, deferoxamine (100 µmol/L).⁹,¹⁰

**Role of L-arginine and Arginase in Vascular Dysfunction.** To determine whether the deficiency of L-arginine contributes to the impaired NO-mediated response, the adenosine- and ionomycin-induced vasodilations in the presence of H₂O₂ were further examined after extraluminal incubation of the vessels with L-arginine (3 mmol/L) for 30 minutes. In addition, to determine whether arginase plays a role in vascular dysfunction, the vessels were initially treated with H₂O₂ and then the vasodilations to adenosine and ionomycin were examined after intraluminal incubation of the vessels with arginase inhibitors α-difluoromethylornithine (DFMO, 0.4 mmol/L)⁵,¹¹ or Nω-hydroxy-nor-L-arginine (nor-NOHA, 0.1 mmol/L)¹² for 30 minutes.

**RNA Isolation and RT-PCR Analysis.** To study the expression of arginase and endothelial NOS (eNOS) messages, total RNA was isolated from porcine subepicardial coronary arterioles (3-4 vessels, ~100 μm diameter, 2-3 mm length) after incubation with H₂O₂ (100 µmol/L) or vehicle for 60 minutes at 37°C, based on the protocols described previously.⁴,⁵ RNA isolated from liver tissue and kidney tissue were used as positive control for arginase I and arginase II, respectively.⁵ Sets of primers specific for arginase I (sense: 5’-ACC CAT CTT TCA CAC CAG CTA CTG-3’, antisense: 5’-TGG GTT CAC TTC CAT TAT ATC TA-3’)¹³ and arginase II (sense: 5’-GAT CTG CTG ATT GGC AAG AGA CAA-3’, antisense: 5’-CTA AAT TCT CAC ACG TGC TTG ATT-3’)¹⁴ genes and glyceraldehyde-3-phosphate dehydrogenase
(GAPDH) (gene accession no. U48832, sense: 5’-CCA CCC ACG GCA AGT TCC ACG GCA-3’, antisense: 5’-GGT GGT GCA GGA GGC ATT GCT GAC-3’) gene and eNOS (gene accession no. AF146041, sense: 5’-GTG TTT GGC CGA GTC CTC ACC-3’, antisense: 5’-CTC CTG CAA GGA AAA GCT CTG-3’) were engineered (Sigma-Genosys, The Woodlands, TX). RT-PCR was conducted as delineated previously. Briefly, equal amounts of total RNA (0.5 µg) for each sample were annealed to the 3’-specific primers and RT reaction was performed using Thermoscript RT (Invitrogen, Carlsbad, CA) according to the manufacturer’s procedure. To determine whether the PCR reaction was amplifying genomic DNA, a first-strand cDNA synthesis reaction was performed with and without RT. Two microliters of RT cDNA sample were used to perform a PCR reaction with Expanding High Fidelity PCR enzyme (Roche, Indianapolis, IN). The PCR reaction was optimized and its products were within the linear assay range. The PCR amplified products were electrophoresed on a 1.8% agarose gel and visualized with ethidium bromide staining. Images of stained products were acquired with the Gel Doc 2000 system (Bio-Rad Laboratories, Hercules, CA) and quantified using volume integration (Multi-Analyst software, Macintosh). The level of expression of interested transcripts was normalized to that of GAPDH transcripts.

**Western Blot Analysis.** Isolated coronary arterioles (4-5 vessels per sample, 60-120 µm diameter, 3-4 mm length) were incubated with H$_2$O$_2$ (100 µmol/L) or vehicle for 60 minutes at 37°C and then homogenized in complete proteinase inhibitor lysis buffer (Roche). The protein content of each tissue lysate was determined using BCA protein assay kit (Pierce, Rockford, IL). Five micrograms of protein per lane were separated by 10% SDS-PAGE under reducing conditions. After electrophoresis, proteins were transferred onto a nitrocellulose membrane (Bio-Rad Laboratories), followed by blocking for overnight at 4°C with 5% dry milk in PBS.
Afterward, the membrane was allowed to react for 1 hour at room temperature with a primary antibody for arginase I (1:1000, BD Transduction Laboratories, Lexington, KY) or β-actin (1:1000, Ambion, Austin, TX). After washing for 30 minutes with PBS, the secondary antibody labeled with horseradish peroxidase was added for 60 minutes at room temperature. The blots were washed again for 30 minutes with PBS and antigen-antibody complexes were revealed with horseradish peroxidase-labeled goat anti-rabbit IgG secondary antibody (Alpha Diagnostic International, San Antonio, TX) by an enhanced chemiluminescence assay (Amersham Pharmacia, Piscataway, NJ). The blot bands were quantified with a densitometer (Bio-Rad Laboratories). The level of expression of arginase I protein was normalized to that of β-actin protein.

**Immunohistochemical Analysis.** Isolated coronary arterioles (~100-150 µm in diameter) were pressurized and incubated with intraluminal H₂O₂ (100 µmol/L) or vehicle for 60 minutes. The vessels were removed from the cannulating pipettes and embedded in Optimal Cutting Temperature (OCT) compound 4583 (Tissue-Tek). The embedded vessels were cut (Leica CM1850, Meyer Instruments, Houston, TX) into sections (12 µm thick) and placed on glass slide. The sectioned slides were dried at room temperature for 20 minutes, fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 1 hour, and then rinsed with PBS. The tissue slides were incubated with blocking diluent (5% normal goat serum, and 5% BSA in PBS) at room temperature for 1 hour, and then with anti-arginase I monoclonal antibody or anti-eNOS monoclonal antibody, at 4°C overnight (1:100, BD Transduction Laboratories). The slides were rinsed 3 times (5 minutes each) in PBS and then were incubated with goat anti-mouse antibody tagged with Oregon Green 448 (1:500, Invitrogen-Molecular Probes, Carlsbad, CA) at room temperature for 60 minutes. Subsequently, coverslips were applied and the slides were dried at
room temperature overnight. Control tissues were exposed for the same duration to non-immune mouse serum (1:100, Jackson ImmunoResearch Laboratories) in place of primary antibodies. Fluorescence images from the immunoreactive proteins were obtained and analyzed using the Ultima-Z 312 laser scanning confocal microscope (Meridian Instruments, Okemos, MI). Control and experimental tissues were placed on the same slide and processed under the same conditions. Laser settings for image acquisition were identical for both control and experimental tissues.

**Effect of Cycloheximide on Vascular Dysfunction.** To determine the regulatory level of arginase activation by H$_2$O$_2$, the vessels were incubated with cycloheximide (CHX, 20 µg/mL, intraluminal incubation), a protein synthesis inhibitor, for 60 minutes and followed by the treatment of H$_2$O$_2$ (100 µmol/L) containing CHX (20 µg/mL) for 60 minutes. The vasodilatory function was then evaluated by adenosine. Finally, the same vessels were prepared for immunohistochemical analysis of arginase I expression as described above.

**Chemicals.** Drugs were obtained from Sigma, except as otherwise stated. Adenosine, bradykinin, catalase, H$_2$O$_2$, iberiotoxin, L-arginine, L-NAME, nor-NOHA, sodium nitroprusside and PEG-SOD were dissolved in PSS. Deferoxamine was dissolved in water. Arachidonic acid, indomethacin and pinacidil were dissolved in ethanol, and subsequent concentrations were diluted in PSS. The final concentration of ethanol in the vessel bath was 0.1%. CHX, ionomycin (Calbiochem) and miconazole were dissolved in DMSO as a stock solution (10 mmol/L), and subsequent concentrations were diluted in PSS. The final concentration of DMSO in the vessel bath was 0.03%. Vehicle control studies indicated that the final concentration of ethanol and DMSO had no effect on arteriolar function.

**Data analysis.** At the end of each experiment, the vessel was dilated with sodium nitroprusside (100 µmol/L) in an ethylenediaminetetraacetic acid (1 mmol/L)-calcium-free PSS
to obtain its maximal diameter at 60 cmH₂O intraluminal pressure. All diameter changes in response to agonists were normalized to the vasodilation induced by sodium nitroprusside and expressed as a percentage of maximal dilation. All data are presented as mean±SEM. n = number of vessels. Statistical comparisons of vasomotor responses under different treatments were performed with two-way ANOVA and tested with Bonferroni multiple-range test. Differences in resting diameter before and after pharmacological interventions, as well as protein and mRNA expression, were compared by paired Student’s t-tests and P values <0.05 were considered to be significant.

References


Table I. Effect of lumenal vehicle solution or a low concentration of H$_2$O$_2$ on vasodilations to adenosine and sodium nitroprusside

<table>
<thead>
<tr>
<th>Dose (log mol/L)</th>
<th>% of Maximal Dilation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-9</td>
</tr>
<tr>
<td>Adenosine</td>
<td></td>
</tr>
<tr>
<td>Control (n=8)</td>
<td>6±1</td>
</tr>
<tr>
<td>Vehicle (n=4)</td>
<td>5±1</td>
</tr>
<tr>
<td>H$_2$O$_2$ (n=4)</td>
<td>5±1</td>
</tr>
<tr>
<td>Sodium Nitroprusside</td>
<td></td>
</tr>
<tr>
<td>Control (n=8)</td>
<td>0</td>
</tr>
<tr>
<td>Vehicle (n=4)</td>
<td>0</td>
</tr>
<tr>
<td>H$_2$O$_2$ (n=4)</td>
<td>0</td>
</tr>
</tbody>
</table>

All values are mean±SEM. Dose-dependent dilations of isolated coronary arterioles to adenosine and sodium nitroprusside were examined before (control) and after intraluminal incubation of the vessels with vehicle or a low concentration of H$_2$O$_2$ (10 µmol/L) for 60 minutes. n = number of vessels.
Figure I. Effect of H$_2$O$_2$ on arteriolar dilation to bradykinin, pinacidil, and sodium nitroprusside. Intraluminal H$_2$O$_2$ did not alter dilation of vessels to bradykinin (A, n=6), pinacidil (B, n=5), and SNP (C, n=12). Deferoxamine also had no affect on vasodilation to SNP (C, n=4).
Figure II. Effect of cycloheximide (CHX) on H$_2$O$_2$-induced vascular dysfunction. Arterioles were intraluminally incubated CHX for 60 minutes prior to the addition of H$_2$O$_2$ (60 minutes). The vasodilatory responses to adenosine (A, n=4, resting diameter = 56±4 µm, maximal diameter = 87±7 µm) and ionomycin (B, n=4, resting diameter = 76±5 µm, maximal diameter = 121±6 µm) were not altered by H$_2$O$_2$ in the vessels pretreated with CHX.