Perivascular Superoxide Anion Contributes to Impairment of Endothelium-Dependent Relaxation

Role of gp91phox

Federico E. Rey; Xiao-Chun Li, MD; Oscar A. Carretero, MD; Jeffrey L. Garvin, PhD; Patrick J. Pagano, PhD

Background—Like endothelial and smooth muscle cells, vascular adventitial fibroblasts contain a substantial NAD(P)H oxidase superoxide anion (O$_2^-$)-generating system activated by angiotensin II (Ang II). Based on the ability of nitric oxide (NO) to diffuse rapidly through tissue and the fast reaction rate of NO and O$_2^-$, we postulated that the interaction between NO and adventitial NAD(P)H oxidase–derived O$_2^-$ contributes to impairment of endothelium-dependent relaxation (EDR).

Methods and Results—C57Bl/6 mouse abdominal aortas were simultaneously perfused intraluminally and suffused adventitially with physiological buffer at 37°C. After constricting the vessels with phenylephrine, an acetylcholine dose-response curve was obtained while monitoring changes in diameter by videomicroscopy. Endogenous O$_2^-$ was increased by treating the adventitial side of the aortas with Ang II (10 pmol/L), leading to impairment of EDR. EDR impairment was reversed by adventitial suffusion of superoxide dismutase (SOD) of aortas from wild-type mice. Ang II–treated aortas from gp91phox null mice, which lack significant adventitial O$_2^-$, exhibited greater EDR and were not affected by SOD. Adventitially suffused SOD failed to penetrate the media, indicating that the effects of SOD were localized to the adventitia. Adventitial application of the O$_2^-$-generating system xanthine/xanthine oxidase or the potent NO scavenger oxyhemoglobin impaired EDR.

Conclusions—O$_2^-$ derived from adventitial gp91phox-based NAD(P)H oxidase contributes to impairment of the action of endothelium-derived NO.

Key Words: aorta ■ endothelium-derived factors ■ angiotensin ■ nitric oxide ■ free radicals

In many vascular studies, the adventitia is damaged or removed. The simple removal of the blood vessel from the animal is likely to injure the adventitia, and during dissection the adventitia is often reduced or excluded by attempts to remove adherent adipose tissue. Moreover, endothelial removal commonly involves rolling a vessel ring using forceps pressed on its luminal surface, which is likely to cause adventitial or medial damage. These and other manipulations are likely to skew assessment of changes in vascular tone under both normal and pathological conditions, and significant variation in vascular responsiveness is likely to result depending on the degree of damage.

Numerous studies have implicated superoxide anion (O$_2^-$) in hypertension. O$_2^-$ interferes with nitric oxide (NO)-dependent vasodilatation and participates in endothelium-dependent constriction. It also inhibits basal cGMP formation in vascular smooth muscle cells as well as the increase in cGMP produced by S-nitroso-N-acetylpenicillamine. Although NO has been considered highly reactive and ephemeral in nature, several theoretical and empirical reports suggest that it diffuses relatively unimpeded through tissue unless opposed by its two major scavengers, O$_2^-$ and oxyhemoglobin.

An inducible phagocyte-like NAD(P)H oxidase has been reported in the endothelium and smooth muscle and has been cited in the regulation of NO bioactivity. Several key studies described a functional oxidase in these cells and implicated their contribution to impaired endothelial function. Because these O$_2^-$ sources are near the site of eNOS-derived NO, it is broadly accepted that they interfere with the actions of endothelium-derived NO. We reported finding significant O$_2^-$ generation by an angiotensin II (Ang II)–inducible NAD(P)H oxidase in the adventitia, and Wang et al suggested involvement of this source in the development of passive tone. However, the ability of adventitial O$_2^-$ to reduce endothelium-dependent relaxation (EDR) remains in question. We examined the possibility that the adventitia contributes significantly to impairment of NO-dependent EDR through the generation of O$_2^-$. 

Received May 15, 2002; revision received August 13, 2002; accepted August 16, 2002.

From the Hypertension & Vascular Research Division, Henry Ford Hospital, Detroit, Mich.
Correspondence to Patrick J. Pagano, PhD, Room 7044, E&R Building, Henry Ford Hospital, 2799 W Grand Blvd, Detroit, MI 48202-2689. E-mail ppagano1@hfhs.org

© 2002 American Heart Association, Inc.

Circulation is available at http://www.circulationaha.org DOI: 10.1161/01.CIR.0000038108.71560.70

2497
37°C in the dark. After incubation, tubes were placed in a luminometer and luminescence integrated over 5 minutes (10 cycles). Tiron (10 μmol/L) was added, and 20 more cycles were read, averaging the final 3 values. Differences between the average values of the first 10 readings and the last 3 readings (with Tiron) were expressed as Δ chemiluminescence/min per mg tissue weight.

**Biotinylation of Superoxide Dismutase** SOD was biotinylated using an EZ-Link kit (Pierce). Sulfo-NHS-LC-biotin was added to Cu/Zn-SOD in 12-fold molar excess and incubated for 2 hours at 4°C, then separated from free biotin using a dextran desalting column.

**Histochemistry** Frozen aortas suffused with biotinylated or unmodified SOD were cut into 4- to 5-μm sections and dried for 1 hour at room temperature. Sections were fixed in acetone (10 minutes), dried at room temperature, washed with PBS, blocked with goat serum (Sigma) (1:100) for 30 minutes, and incubated with ABC reagent (Vector) for 30 minutes. Sections were incubated with peroxidase substrate (AEC) for 5 minutes to obtain the desired stain intensity. After washing with PBS, sections were photographed at ×10 and ×40 magnification.

**Statistics** Data are expressed as mean±SEM. Comparisons were made using ANOVA. If differences were noted between treatment groups, a t test was performed for point analyses and the results adjusted using Hochberg’s method. *P*<0.05 was considered significant.

**Results**

**EDR Response to Adventitial Application of Oxyhemoglobin** To examine whether selective addition of an NO scavenger to the perivascular space attenuates EDR, 10 μmol/L oxyhemoglobin was applied to the adventitial suffusate, with the knowledge that proteins with a lower molecular weight are unable to penetrate beyond the vascular adventitia. Oxyhemoglobin impaired EDR compared with vehicle (Figure 1).

**Effect of Adventitial Xanthine/Xanthine Oxidase Suffusion on EDR** To test whether exogenously applied adventitial O$_2$ impairs EDR, preconstricted aortas were adventitially suffused with vehicle, xanthine (X, 500 μmol/L) plus xanthine oxidase (XO, 0.01 U/mL), or X/XO plus SOD. Acetylcholine-induced relaxation was impaired in the presence of X/XO and was reversed by adventitial suffusion of SOD (300 U/mL), but SOD suffusion did not improve relaxation in the vehicle group (Figure 2).

**Effect of Adventitial Application of SOD on Ang II–Induced EDR Impairment** Endogenous adventitial O$_2$ was increased by suffusing the adventitia for 3 hours with 10 μmol/L Ang II, which increases O$_2$ in the mouse aorta without increasing diameter (5.4±0.2 versus 5.5±0.2 arbitrary units). Phenylephrine-induced contraction was the same in vehicle and Ang II groups; however, Ang II shifted acetylcholine-induced relaxation to the right. EDR impairment was reversed by adventitial suffusion of SOD (300 U/mL), but SOD suffusion did not improve relaxation in the vehicle group (Figure 3). To test whether adventitiously applied SOD could penetrate other vascular layers, we suffused the adventitia with biotinylated SOD. SOD was detected in the adventitial space.
surrounding the external elastic lamina (Figure 4); aortas not suffused with biotinylated SOD did not stain.

Comparison of EDR in Ang II–Treated Aortas From gp91phox−/− Versus Wild-Type Mice
Our previous data suggested that adventitial fibroblasts contain a gp91phox-based NAD(P)H oxidase that is primarily responsible for O2− generation in the adventitia. The present study revealed that in the absence of SOD, Ang II–treated aortas from gp91phox−/− mice showed greater relaxation than those from Ang II–treated wild-type mice. Moreover, SOD had no effect on EDR of Ang II–treated aortas from gp91phox−/− mice (Figure 5).

Effect of H2O2 on Phenylephrine-Constricted Mouse Aortas
To test whether the relaxant effect of SOD is partly attributable to generation of H2O2, we examined the ability of adventitiously applied H2O2 to relax phenylephrine-constricted aortas; however, contrary to our hypothesis, we observed vasoconstriction with H2O2 (Figure 6).

Lack of Effect of Oxygenation on O2− Production by Mouse Aortas
To examine whether in vitro buffer oxygenation with 95% O2 in this preparation enhances aortic O2− production, we compared nonoxygenated aortas with those oxygenated with 95% O2/5% CO2 but observed no difference in O2− detection (8.7 ± 4.2 versus 9.5 ± 4.7 mU/min per mg tissue, oxygenated versus nonoxygenated). This is likely related to a low Km for O2− of NAD(P)H oxidase.

Discussion
Our findings support the hypothesis that adventitial NAD(P)H oxidase–derived O2− impairs endothelium-derived NO-dependent relaxation. Adventitial delivery of the O2−-generating system X/XO caused significant impairment of EDR that was reversed by coadministration of SOD. Ang II–induced impairment of EDR could be ameliorated by adventitial delivery of SOD. Moreover, Ang II–treated aortas from gp91phox−/− deficient mice exhibited enhanced EDR (compared with wild-type controls), whereas adventitial SOD delivery had no effect. These data suggest that O2− derived from adventitial gp91phox–NAD(P)H oxidase can impair EDR.
Undoubtedly, endothelial and smooth muscle NAD(P)H oxidase–derived O$_2^−$ can interfere with NO. Numerous studies have implicated both oxidases in impairment of endothelium-dependent relaxation, and both are widely accepted as important regulators of vascular tone. G"orlach et al$^a$ clearly demonstrated the functional role of endothelial oxidase in O$_2^−$ production, showing that phorbol ester–induced aortic O$_2^−$ was blocked by removal of the endothelium or gp91$^{phox}$ deletion. They also showed that gp91$^{phox}$ deletion improves acetylcholine-induced relaxation of mouse aortas under basal conditions. Mollnau et al$^b$ associated Ang II–induced NAD(P)H oxidase expression with uncoupling of NOS and decreased NO–induced signaling via guanylate cyclase and cGMP-dependent protein kinase, suggesting that endothelial or smooth muscle NAD(P)H oxidase impairs endothelium-dependent relaxation. In the present study, we focused on the role of adventitial NAD(P)H oxidase in endothelium-dependent relaxation impairment, independent of the effect of endothelial and smooth muscle oxidase.

Although NO– scavengers are known to impair vascular relaxation, we questioned whether adding a scavenger to the perivascular space could attenuate EDR. Applying oxyhemoglobin to the adventitia impaired EDR, consistent with the notion that adventitial scavenging of NO reduces overall vascular NO– derived from the endothelium and thus its relaxant effect. These findings support remote NO– scavenging reducing EDR.

Our results suggest that in addition to sources adjacent to the endothelium, stimulation of adventitia-derived O$_2^−$ can contribute to impairment of EDR. Previous studies showed that the adventitia is a significant O$_2^−$ source and suggest that adventitial O$_2^−$ acts as a barrier to exogenously applied NO– and affects passive tone$^{22}$; however, its ability to interfere with EDR was not explored. Adventitial application of X/XO impaired EDR, which was reversed by adventitial SOD suffusion, consistent with adventitial O$_2^−$ impairing endothelium–derived NO– bioactivity. The apparent convergence of dose–response curves at high acetylcholine concentrations may be explained by the ability of endogenous NO– to overcome ambient O$_2^−$ levels or the possibility that peroxynitrite (formed by the reaction of these two free radicals) is contributing to vasodilatation.$^{23}$ The fact that in the presence of X/XO, SOD enhanced acetylcholine-induced relaxation more than in vehicle controls may partly reflect SOD conversion of O$_2^−$ to very high concentrations of H$_2$O$_2$, which cause relaxation.$^{24}$ Thus, H$_2$O$_2$ accumulation in this scenario could enhance the relaxation observed with X/XO plus SOD. Previous studies showed that endothelial NOS plays a significant role in relaxation of the mouse thoracic aorta, but this was not always true for smaller blood vessels.$^{25}$ The markedly reduced ability of acetylcholine to relax preconstricted abdominal aortas from eNOS$^{-/-}$ versus wild-type mice in our study (not shown) confirmed that eNOS–derived NO– plays a highly significant role in acetylcholine–induced relaxation of the abdominal aorta.

We previously described an Ang II–inducible NAD(P)H oxidase in the rabbit aortic adventitia,$^{16}$ which is also present in rats$^{26}$ and mice.$^{17}$ In the present study, Ang II–induced EDR impairment was reversed by adventitial SOD suffusion, suggesting that elevated adventitial O$_2^−$ lowers vascular steady–state NO– levels in the same way as oxyhemoglobin (the sink hypothesis).$^2$ In contrast, adventitial SOD suffusion did not improve relaxation of vehicle–treated aortas, consistent with low basal O$_2^−$ under normal conditions. Indeed, the highly impenetrable elastic lamina blocks proteins and adenosivl vectors$^{26}$ and would sequester large molecules such as SOD, XO, and oxyhemoglobin. Adventitial suffusion of biotinylated SOD (the smallest of the 3 proteins) resulted in SOD being detected only in the adventitial space. This confirmed that SOD was not penetrating the media and is consistent with adventitial O$_2^−$ impeding EDR.

Interestingly, relaxations of aortas (treated adventitially with Ang II) from gp91$^{phox}$–/– mice were greater than those of wild-type mice, suggesting that adventitial gp91$^{phox}$–/–

![Graph](https://example.com/graph.png)

**Figure 5.** EDR was greater in aortas from Ang II–treated gp91$^{phox}$–/– vs Ang II–treated wild-type mice. SOD had no effect on EDR in Ang II–treated gp9$^{phox}$–/– mouse aortas. (n=5 to 6). Ang II 10 pmol/L suffused from the adventitial side of mouse aortas for 3 hours did not induce active tone development. While continuing the Ang II infusion, aortas were preconstricted with 1 μmol/L phenylephrine, and a dose–response curve for acetylcholine was obtained. SOD (300 U/mL) was added to the adventitial suffusate 20 minutes before and during the dose–response curve to acetylcholine. ANOVA revealed a significant difference (P=0.003) comparing relaxation in wild-type and gp91$^{phox}$–/– mice; *P<0.05, −/− vs wild-type mice at 10$^{-7}$ mol/L acetylcholine (t test adjusted by Hochberg’s method; n=6) but no significant difference between aortas from gp91$^{phox}$–/− treated or not treated with SOD.

![Graph](https://example.com/graph2.png)

**Figure 6.** Adventitial suffusion of H$_2$O$_2$ causes dose–related contraction of mouse abdominal aortas. Arteries were preconstricted with 1 μmol/L phenylephrine, and a dose–response curve for adventitial suffusion of H$_2$O$_2$ was obtained (n=5). ANOVA revealed significant contraction produced by H$_2$O$_2$ (P<0.01); *P<0.05 at 10$^{-4}$ mol/L (t test adjusted by Hochberg’s method).
oxidase could interfere with EDR. Moreover, adventitial SOD suffusion had no effect on EDR of Ang II–treated aortas from gp91phox−/− mice, consistent with the reduced ability of the aortic adventitia in gp91phox−/− mice to produce Ang II–stimulated O2−. This suggests that the O2− source impairing EDR is adventitial gp91phox-based NAD(P)H oxidase. The data also corroborate that the effects of SOD we observed were specific to O2− scavenging. Although previous studies addressed the role of ubiquitous gp91phox NAD(P)H oxidase in normal acetylcholine-induced EDR, our present study tested whether adventitial gp91phox-containing NAD(P)H oxidase might contribute to impaired EDR. We are presently developing methods of assessing the relative contribution of the various cellular sources of NAD(P)H oxidases to impaired EDR.

We postulated that adventitial O2− scavenges endothelium-derived NO− and thus impairs EDR; yet O2− could be constricting the outer vascular medial layers or stimulate vasoconstrictor release from the adventitia. For instance, reactive oxygen species (ROS) activate cyclooxygenase and enhance the vasoconstrictor action of prostaglandin H2. 28 They also stimulate vascular smooth muscle cells to release heat shock protein-90 and cyclophilin A, 29 which can activate ERK1/2 in an autocrine fashion and mediate smooth muscle cell contraction.30 These studies strongly support a paracrine effect of oxidative stress in the vasculature; and although we are not aware of studies examining such a role, it is tempting to speculate that such mechanisms also exist in adventitial fibroblasts.

We initially postulated that the ability of SOD to improve relaxation was partly attributable to generation of H2O2. In fact, under physiological conditions, Ang II can activate endogenous extracellular SOD.31 Thus, when adventitial O2− is elevated and endogenous SOD is enhanced, the resulting product, H2O2, could become a paracrine signaling agent, diffusing from the adventitia into the media, because it is stable under biological conditions and can penetrate cell membranes.32 Attempts to determine the direct contribution of H2O2 to the effect of SOD on EDR were impeded by the ability of the peroxide scavenger catalase to decrease NO− as well.33 For this reason, we examined the ability of exogenous H2O2 to relax mouse aortas. Contrary to our hypothesis, we observed a constrictor response to adventitially applied H2O2 between 10−6 and 10−4 mol/L. A previous report by Fujimoto et al 24 demonstrated only a relaxant effect of H2O2 in mouse aortas at concentrations of 10−4 mol/L; however, they applied H2O2 to an organ chamber bath with access to the entire vessel ring, whereas we applied it directly to the adventitia. Thus, the difference we observed might be related to a constrictor effect of H2O2 on the outer media or secondary release of a vasoconstrictor from the adventitia, whereas the effect they observed could have been mediated by direct smooth muscle relaxation or release of a vasodilator from the endothelium. Nevertheless, because vascular O2− is reported to be in the nanomolar range,34 application of exogenous SOD would at most be expected to cause acute production of nanomolar concentrations of H2O2. Such concentrations did not relax the aorta and are therefore unlikely to have contributed to the ameliorative relaxant effect of SOD.

Hypertension, atherosclerosis, and vascular injury all activate perivascular cells and increase macrophage levels in the perivascular space,35–37 which, combined with characteristic fibroblast proliferation, will likely result in increased perivascular O2− production and hence EDR impairment. It is conceivable that generation of Ang II or cytokines by perivascular adipose tissue38 and interstitium39 as well as macrophages and mast cells present in the adventitia37 could potentiate endogenous O2− production. Moreover, adventitial NAD(P)H oxidase activity may affect nitrergic neurotransmission; eg, adventitial fibroblasts are juxtaposed to adventitial nerve endings that produce NO. Therefore, in vivo adventitial O2− may play an even more important role in the control of vascular tone.

In summary, we believe our data support the hypothesis that adventitial O2− plays a significant role in the impairment of EDR and perhaps a modulatory role in endothelial function. Thus, previous preparations of blood vessels that damaged the adventitia may have overlooked an important modulator of tone. These data also highlight the potential role of an activated adventitia in vascular disease and support the concept of cross-talk between vascular segments via ROS. Additional studies targeting adventitial ROS are needed to clarify their role in vascular physiology and pathophysiology.

Acknowledgments
This work was supported by National Institutes of Health grants HL55425 and HL28982 and by American Heart Association grants 95011900 and 9808086W. We thank Dr William C. Sessa for his assistance and insightful discussion.

References
12. Touyz RM, Chen X, Tabet F, et al. Expression of a functionally active gp91phox-containing neutrophil-type NADPH oxidase in smooth


Perivascular Superoxide Anion Contributes to Impairment of Endothelium-Dependent Relaxation: Role of gp91phox
Federico E. Rey, Xiao-Chun Li, Oscar A. Carretero, Jeffrey L. Garvin and Patrick J. Pagano

Circulation. 2002;106:2497-2502
doi: 10.1161/01.CIR.0000038108.71560.70
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/106/19/2497

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation 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 Circulation is online at:
http://circ.ahajournals.org//subscriptions/