NAD(P)H Oxidase Inhibition Improves Endothelial Function in Rat and Human Blood Vessels

Carlene A. Hamilton, M. Julia Brosnan, Sammy Al-Benna, Geoffrey Berg, Anna F. Dominiczak

Abstract—The NO/superoxide (O_2^-) balance is a key regulator of endothelial function. O_2^- levels are elevated in many forms of cardiovascular disease; therefore, decreasing O_2^- should improve endothelial function. To explore this hypothesis, internal mammary arteries and saphenous veins, obtained from patients undergoing coronary artery revascularization, and aortic and carotid arteries from Wistar-Kyoto and spontaneously hypertensive stroke-prone rats were incubated with O_2^- dismutase or NAD(P)H oxidase inhibitors. O_2^- levels were measured using lucigenin chemiluminescence; NO bioavailability was assessed in organ chambers; and mRNA expression of NAD(P)H oxidase components was quantified by use of a Light Cycler. In rat arteries, phenylarsine oxide, 4-(2-aminoethyl)-benzenesulfanyl fluoride, and apocynin all decreased NADH-stimulated O_2^- production, but only apocynin increased NO bioavailability. In human internal mammary arteries and saphenous veins, apocynin decreased NAD(P)H-stimulated O_2^- generation and caused vasorelaxation that was endothelium dependent and reversed on addition of the NO synthase inhibitor N^6^-nitro-L-arginine methyl ester. In addition, it increased NO production from cultured human endothelial saphenous vein cells. Polyethylene-glycolated O_2^- dismutase also increased NO bioavailability in rat carotid arteries and human blood vessels, but the effects were smaller than those observed with apocynin. NADH-generated O_2^- and mRNA expression of p22^{phox}, gp91^{phox}, and nox-1 were comparable between the 2 strains of rat. This is the first study to demonstrate pharmacological effects of apocynin in human blood vessels. The increases in NO bioavailability shown here suggest that the NAD(P)H oxidase pathway may be a novel target for drug intervention in cardiovascular disease. (Hypertension. 2002;40:755-762.)

Key Words: apocynin ■ NAD(P)H oxidase ■ nitric oxide ■ superoxide ■ humans ■ blood vessels

Endothelial dysfunction and decreased NO bioavailability are associated with many forms of cardiovascular disease. In most cases, the reduction in NO bioavailability has been shown to be related not to decreased NO synthesis but to increased superoxide (O_2^-) production. Thus, decreasing O_2^- levels has potential therapeutics implications. O_2^- dismutase mimetics have been shown to improve vascular function in some, but by no means all, cases, whereas gene transfer of intracellular Cu/Zn O_2^- dismutase as a method of improving NO bioavailability has proven disappointing. An alternative strategy would be to limit O_2^- production. There are a number of sources of O_2^-, including uncoupled eNOS, xanthine oxidase, and NAD(P)H oxidase. In the human, however, the levels of xanthine oxidase are reported to be low, and NADPH oxides have been proposed to be the major source of O_2^- in vasculature. NAD(P)H oxidases generate O_2^- through the assembly of a multi-subunit protein complex. The complex consists of a membrane-integrated cytochrome b_{558}, which is itself composed of 2 subunits (gp91^{phox} and p22^{phox}) and at least 3 cytosolic proteins (p47^{phox}, p67^{phox}, and p21 rac). In some tissues, gp91^{phox} may be wholly or partially replaced by homologs such as nox-1. Levels of NAD(P)H-stimulated O_2^- production have been reported to be elevated in hyperinsulinemic rats and hypercholesterolemic rabbits and to be positively correlated with endothelial dysfunction and clinical risk factors for atherosclerosis in humans. Expression of p22^{phox} has been reported to be increased in aorta from hypertensive rats, to increase with age in both normotensive and hypertensive rats, and to be increased in atherosclerotic coronary arteries from humans. Angiotensin II has been shown to increase p67 and gp91^{phox} in aortas from angiotensin II-infused mice. A number of inhibitors of NAD(P)H oxidase exist. These include phenylarsine oxide (PAO), 4-(2-aminoethyl)-benzenesulfanyl fluoride (ABF), and apocynin. PAO fully and reversibly inhibits NAD(P)H oxidase in neutrophils. It is believed to act at a step distal to the translocation of the oxidase subunits and proximal to the starting point of O_2^- generation. ABF is an irreversible serine protease inhibitor. It inhibits NAD(P)H oxidase activity in phagocytes by interfering with the binding of p47^{phox} and/or p67^{phox} to cytochrome b_{558}, probably by a direct effect on cytochrome...
Apocynin is a reversible inhibitor of NAD(P)H oxidase activity that impedes assembly of the p47phox subunit with the membrane complex.18 It has recently been proposed as a potential therapeutic agent in the treatment of atherosclerotic disease by Meyer and Schmitt.19 The aim of the present work was (1) to investigate the ability of these inhibitors of NAD(P)H oxidase to increase NO bioavailability in vivo in arteries from rats with both normal and perturbed endothelial function, and (2) from these studies, to select the compound(s) with the greatest potential for further investigation in human blood vessels.

Methods

Tissues

Internal mammary arteries (IMAs) and saphenous vein (SV) surplus to requirement at the time of surgery were obtained from patients undergoing coronary artery revascularization. Informed consent was obtained from all subjects, and the study was approved by the local ethics committee. Clinical details of the patients donating vessels are shown in the data supplement in Table 1. Carotid arteries and aortae were obtained from Wistar Kyoto rats (WKY) and stroke-prone spontaneously hypertensive rats (SHRSP) from the colonies established in our department in Glasgow by brother-sister mating.20 Four groups were studied: 3- to 4-month-old WKY and SHRSP, and 9- to 12-month-old WKY and SHRSP.

O2•− Measurement

Lucigenin chemiluminescence was used for measurement of O2•− levels. To study NADH/NADPH-stimulated O2•− generation, homogenates of rat aortae, IMAs, and SVs were prepared in 50 mmol/L phosphate buffer (pH 7.8). Either NADH or NADPH (250 μmol/L) and lucigenin (5 μmol/L) were added before measuring O2•−. In rings of blood vessels, lucigenin (25 μmol/L) was used for O2•− measurement, and results were expressed as milligrams/wet weight.

Vascular Reactivity

Rings of rat carotid arteries, human IMAs, or SVs were set up in organ baths.21,22 Cumulative vasorelaxation curves to apocynin (0.01 to 1 mol/L) or polyethylene glycolated superoxide dismutase (PEG-SOD; 10 to 300 U/mL) were constructed in rings precontracted with phenylephrine. In some cases, the NO synthase inhibitor N‘-nitro-l-arginine methyl ester (L-NAME; 200 μmol/L) was added to reverse NO-mediated vasodilation. In other experiments, vasorelaxation to the calcium ionophore A23187 (0.1 to 10 μmol/L) was also studied. In studies on rat carotid arteries, cumulative concentration response curves to phenylephrine (0.01 to 10 μmol/L) were obtained first in the absence and again in the presence of 100 μmol/L L-NAME. The increase in tension in the presence of L-NAME was calculated over the full concentration response curve expressed as an area under the curve and provided a measure of the effect of NO on basal tone.

NO Measurement in Human SV Endothelial Cells

Cultured endothelial cells were prepared from human SVs. Cells (passage 3 to 4) were plated into 6-well plates and grown to confluence. NO production over a 4-hour period was measured in acidified potassium iodide by use of a Sievers 280A NO meter.

Expression of mRNA for p22phox

RNA was extracted using RNeasy (Qiagen) and quantified by use of the Ribogreen kit (Molecular Probes), and 100 ng was reverse transcribed by use of the Promega reverse transcription (RT) kit according to the manufacturer’s instructions. Real-time RT–polymerase chain reaction (PCR) was performed by use of a LightCycler (Roche), with primers designed specifically for each transcript (Table 2; online data supplement). Validation of equal material in the RT mix was confirmed by performing RT-PCR using primers for GAP. Standard curves were generated for rat p22phox using linearized pSport (a gift from Kathy Griendling, Emory University, Atlanta, Ga) and plasmids containing rat gp91phox and nox-1 (plasmids provided by Bernard Lassègue, Emory University).

Statistical Analysis

For the studies on O2•− levels in rat and human blood vessels and NO bioavailability in rat carotid arteries, vehicle and drug-treated samples from the same individual were studied in parallel and differences examined using paired t tests. Unpaired t tests with Bonferroni correction were used in comparisons between age groups of rats and for comparison of NADH- and NADPH-generated O2•− generation. General linear modeling was used to compare vasorelaxation to apocynin in IMAs and SVs, and ANOVA with Bonferroni correction was used in comparisons between age groups of rats and for comparison of NADH- and NADPH-generated O2•− generation.

Table 1. NADH- and NADPH-Stimulated O2•− Generation

<table>
<thead>
<tr>
<th>Vessel</th>
<th>n</th>
<th>NADH-Stimulated O2•−</th>
<th>n</th>
<th>NADPH-Stimulated O2•−</th>
<th>P</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY aorta</td>
<td>7</td>
<td>535±125</td>
<td>6</td>
<td>276±49</td>
<td>0.049</td>
<td>1–530</td>
</tr>
<tr>
<td>SHRSP aorta</td>
<td>12</td>
<td>633±92</td>
<td>7</td>
<td>298±68</td>
<td>0.003</td>
<td>156–591</td>
</tr>
<tr>
<td>IMAs</td>
<td>11</td>
<td>565±76</td>
<td>12</td>
<td>254±36</td>
<td>0.002</td>
<td>165–552</td>
</tr>
<tr>
<td>SVs</td>
<td>11</td>
<td>409±11</td>
<td>7</td>
<td>211±5</td>
<td>0.035</td>
<td>13–302</td>
</tr>
</tbody>
</table>

O2•− generation measured in vessel homogenates and shown as mean±SEM (nmol/min per milligram protein). NADH- and NADPH-stimulated O2•− generation compared by unpaired t tests.

Table 2. Inhibition of O2•− Generation by Apocynin in Human Blood Vessels

<table>
<thead>
<tr>
<th>Vessel</th>
<th>Apocynin (μmol/L)</th>
<th>n</th>
<th>Vehicle</th>
<th>Apocynin</th>
<th>P</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMA</td>
<td>0.1</td>
<td>6</td>
<td>1.11±0.27</td>
<td>0.78±0.16</td>
<td>0.049</td>
<td>0.002–0.283</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>7</td>
<td>0.78±0.15</td>
<td>0.48±0.09</td>
<td>0.020</td>
<td>0.095–0.718</td>
</tr>
<tr>
<td>SV</td>
<td>0.1</td>
<td>6</td>
<td>0.47±0.07</td>
<td>0.40±0.07</td>
<td>0.019</td>
<td>0.018–0.128</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>6</td>
<td>0.48±0.08</td>
<td>0.39±0.09</td>
<td>0.020</td>
<td>0.021–0.167</td>
</tr>
</tbody>
</table>

Results expressed as mean±SEM. Vehicle- and apocynin-treated vessel segments from the same individual were compared using paired t tests.
tions to compare levels of p22phox, nox-1, and gp91phox in different groups of rats. All results are expressed as mean±SEM.

An expanded Methods section can be found in an online supplement available at http://www.hypertensionaha.org.

**Results**

**O$_2^-$ Generation in Rat and Human Blood Vessels**

NADH-stimulated O$_2^-$ generation was greater than NADPH-stimulated levels in both SHRSP and WKY (Table 1). No significant differences were observed between WKY and SHRSP. ABF, PAO, and apocynin all concentration-dependently inhibited NADH-stimulated O$_2^-$ generation in rat aorta (Figure 1a).

Levels of NADH- and NADPH-stimulated O$_2^-$ generation in human blood vessels are shown in Table 1. Both NADH- and NADPH-stimulated O$_2^-$ generation were attenuated by apocynin, (Figures 1c and 1d). Apocynin also reduced total O$_2^-$ levels in rings of IMAs and SVs as shown in Table 2.

**Expression of mRNA for NAD(P)H Oxidase Components**

Levels of mRNA encoding nox-1 were 0.13±0.03, 0.49±0.30, 0.10±0.03, and 0.15±0.02 amol/100 ng RNA for 3- to 4-month-old WKY, 3- to 4-month-old SHRSP, 9- to 12-month-old WKY, and 9- to 12-month-old SHRSP, respectively (n=4 per group). No significant differences between the groups were observed. Levels of expression of gp91phox were higher: 0.66±0.14 and 1.82±0.22 fmole/100 ng RNA in the older WKY and SHRSP. Levels of expression of p22phox mRNA were also in the femtomole range but ~10-fold higher. In contrast to nox-1 and gp91phox, differences between the groups with respect to expression of p22phox were observed as shown in Figure 1b. Most notable was the increase in expression in the older animals (WKY: P=0.001, 95% confidence interval CI=0.052, 0.124; SHRSP: P=0.001, 95% CI=0.064, 0.138). There was also a trend toward higher levels in the young SHRSP compared with young WKY (P=0.099, 95% CI=−0.056, 0.007) and in the

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**Figure 1.** a, Effect of NAD(P)H oxidase inhibitors on NADH-stimulated O$_2^-$ generation in rat aorta. Hatched bars indicate SHRSP; dotted bars, WKY. Apo indicates apocynin. Results are mean±SEM; n=3 to 6. b, Expression of mRNA for p22phox in rat aorta. Results are mean±SEM; n=4 per group. c, Inhibition of NADH-stimulated O$_2^-$ generation by apocynin in human blood vessels. Solid bars indicate IMA; empty bars, SV. Results are mean±SEM; n=4 to 10. d, Inhibition of NADPH-stimulated O$_2^-$ generation by apocynin in human blood vessels. Solid bars indicate IMA; empty bars, SV. Results are mean±SEM; n=4 to 10.
9- to 12-month-old SHRSP compared with 9- to 12-month-old WKY (P = 0.065, 95% CI = -0.076, 0.003).

**NO Bioavailability**

Neither PAO nor ABF over the concentration range tested was able to improve endothelial function in carotid arteries from 3-month-old SHRSP, NO bioavailability being decreased rather than increased (Figures 2a and 2b). In contrast, PEGSOD and apocynin increased NO bioavailability in a concentration-dependent manner in carotid arteries from the 3-month-old SHRSP (Figures 2c and 3). Consistent with these findings in arteries preconstricted with phenylephrine, vasorelaxation to apocynin, but not to PAO or ABF, was rapidly reversed on addition of L-NAME (data not shown). Both ABF and PAO, but not apocynin, caused attenuation of contractile responses to phenylephrine in endothelium-denuded rings (ABF: 0.5 mmol/L, -48±12%; PAO: 0.01 mmol/L, -42±14%; apocynin: 0.1 mmol/L, -4±17%). Furthermore, at concentrations of 10 and 0.1 mmol/L, respectively, ABF and PAO caused cell death as measured by toluidine blue uptake by cultured vascular smooth muscle cells from SHRSP (20±1% and 31±2%, respectively) No toluidine blue uptake was observed with apocynin up to 100 mmol/L.

Further studies into the effects of apocynin on NO bioavailability were undertaken in 3-month-old WKY and in older (9- to 12-month-old) WKY and SHRSP. In carotid arteries from the 3-month-old WKY, apocynin was able to increase NO bioavailability, but only at higher concentrations (0.3 and 3.0 mmol/L). At 0.03 mmol/L, apocynin had no significant effect on NO bioavailability in arterial rings from these animals. In the older WKY, all 3 concentrations examined enhanced NO bioavailability, as shown in Figure 3. In the 9- to 12-month-old SHRSP, apocynin was less effective than it was in the younger SHRSP, NO bioavailability in the presence of 3 mmol/L apocynin being significantly lower in the older animals (P = 0.022, 95% CI = 0.420, 4.009), PEGSOD (50 U/mL), similar to 0.03 mmol/L apocynin, increased NO bioavailability in carotid arteries from the 3-month-old SHRSP but not in blood vessels from the 3-month-old WKY. In arteries from the older animals, a significant increase in NO bioavailability was achieved for the WKY and SHRSP, although in the case of the older SHRSP, significance was marginal (Figure 2c). A higher concentration of PEGSOD (200 U/mL) was also used in carotid arteries from 3-month-old SHRSP, but no increase in NO bioavailability above that observed with 50 U/mL was observed (data not shown).

Vasorelaxation to apocynin was also observed in IMAs and SVs preconstricted with 3 μmol/L phenylephrine, as shown in Figure 4a. Vasorelaxation was endothelium dependent, with no relaxation being observed in blood vessels, which failed to respond to the calcium ionophore A23187. Furthermore, vasorelaxation to apocynin could be reversed by...
SVs were more responsive to apocynin than were IMAs, with significant differences being observed at concentrations of 0.1 mmol/L. This difference between arteries and veins was not owing to greater endothelial integrity of veins. In a subset of vessels in which relaxation to both apocynin and A23187 was studied, 10 μmol/L A23187 caused 63±9% relaxation in IMAs (n=7) and 36±8% in SVs (n=7), whereas 0.3 mmol/L apocynin caused 40±10% and 52±16% relaxation in the same rings of IMAs and SVs. Statistical analysis showed a significant difference in responses between IMAs and SVs (P=0.006, 95% CI = −50.7, −13.3). Confirmation that apocynin increased NO bioavailability was obtained using an NO meter. In cultured human SV endothelial cells incubated with apocynin, A23187 or vehicle NO output into the medium was concentration-dependently increased in the cells exposed to A23187 and apocynin (Figure 4c).

In contrast to apocynin, PEGSOD (10 to 300 U/mL) produced only small decreases in vasorelaxation in human blood vessels preconstricted with phenylephrine (Figure 4a).

### Discussion

Increased levels of reactive oxygen species, in particular O$_2^-$, are a major cause of endothelial dysfunction in many forms of cardiovascular disease. One of the most important sources of O$_2^-$ is NAD(P)H oxidases. In the present study, we have shown that treatment of isolated rat arteries with apocynin decreased NADH-stimulated O$_2^-$ generation and increased NO bioavailability. In addition, in human arteries and veins, NADH- and NADPH oxidase–mediated O$_2^-$ generation was inhibited by apocynin; endothelium-dependent vasodilation was improved; and NO production from human SV endothelial cells was enhanced.

Apocynin interferes with p47$^{phox}$ and p67$^{phox}$ association with the cell membrane compounds of the NAD(P)H oxidase complex. It has been used by Peruvian Indians as an antiinflammatory agent and has been reported to attenuate O$_2^-$ levels in hyperinsulinemic rats and DOCA-salt hypertensive rats. However, this is the first investigation into the O$_2^-$ lowering capacity of apocynin in human blood vessels and the first to show improved NO bioavailability and endothelial function in either animal or human blood vessels.
In addition, the present study highlights the crucial role of the balance between NO and O$_2^\cdot$/H$_2$O$_2$ in determining NO bioavailability and regulating endothelial function. In the rat arteries, NO bioavailability was expressed as the increase in contractile responses to phenylephrine in the presence of the NO synthase inhibitor L-NAME. We have previously validated this method to calculate NO bioavailability in rat carotid arteries infused with genes encoding NO synthase and superoxide dismutase$^5,22$ and to investigate effects of antihypertensive drugs on NO bioavailability in vivo and in vitro. When expressed in this format, the apocynin-induced increases in NO bioavailability were greater than those observed in any of the previous studies and were greater than that achieved by scavenging of O$_2^\cdot$/H$_2$O$_2$ in the present study.

When arteries from the 3-month-old SHRSP and WKY were compared, no differences were observed in NAD(P)H-stimulated O$_2^\cdot$/H$_2$O$_2$ generation, apocynin inhibition of this O$_2^\cdot$/H$_2$O$_2$ generation, or levels of expression of mRNA for the membrane-integrated components of the NAD(P)H oxidase system. Despite this, arteries from young SHRSP showed a greater sensitivity to apocynin-mediated enhancement of NO bioavailability than these from age-matched WKY. O$_2^\cdot$/H$_2$O$_2$ levels are reported to be higher in SHRSP and SHR than in WKY.$^{34,12}$ The overproduction of O$_2^\cdot$/H$_2$O$_2$ in the young 3-month-old SHRSP is believed to originate from eNOS$^{24}$ and may be accompanied by increased NOS expression and activity but decreased NO bioavailability.$^{20}$ Thus, it appears that the relative proportions of O$_2^\cdot$/H$_2$O$_2$ and NO are critical in maintaining endothelial function, and that manipulation of O$_2^\cdot$/H$_2$O$_2$ levels has a greater effect on NO bioavailability when the O$_2^\cdot$/H$_2$O$_2$/NO balance is disturbed. PEGSOD also improved NO bioavailability in the 3-month-old SHRSP but not WKY. In the older WKY, which have O$_2^\cdot$/H$_2$O$_2$ levels similar to those observed in the young SHRSP, both apocynin and PEGSOD had effects on NO bioavailability comparable to those observed in 3-month-old SHRSP. Consistent with our hypothesis, carotid arteries from SHRSP infected with first generation Ad vector expressing eNOS from the cytomegalo-virus immediate early promoter (AdCMV eNOS) in vivo have been previously shown to exhibit enhanced NO bioavailability when harvested 24 hours later, whereas function in vessels from WKY was unchanged.$^{22}$

The same argument might be used to explain the increased vasorelaxation to apocynin in SVs compared with IMAs. As NO levels have been reported to be lower in veins than in arteries,$^{25,26}$ attenuating O$_2^\cdot$/H$_2$O$_2$ production might have a propor-
tionally greater effect on the $O_2^{-}$/NO balance and NO bioavailability in SVs compared with IMAs. Alternatively, apocynin may have additional vasorelaxant effects that are more prominent in SVs than IMAs. In addition, age, sex, duration of disease, and treatment might all affect the ability of blood vessels to relax in response to apocynin.

If the relative $O_2^{-}$ and NO levels are the primary factors regulating endothelial function, the most dramatic effects of apocynin and PEGSOD might be expected in the older SHRSP, but this was not the case. However, arteries from these animals are more rigid, have lost elasticity, and may be unable to respond as effectively to changes in the $O_2^{-}$/NO balance.

Zalba and colleagues reported an association among p22phox gene expression, NAD(P)H oxidase activity, and impaired endothelial function and media hypertrophy in aortic from spontaneously hypertensive rats. The possibility that differences in responses to apocynin observed in our studies were related to levels of expression of p22phox or other membrane components of the NAD(P)H oxidase were therefore investigated in WKY and SHRSP. However, although age, and possibly hypertension, appeared to regulate levels of mRNA encoding p22phox, no simple relationship emerged between levels of gene expression and either sensitivity to apocynin or NO bioavailability.

It has been proposed that reactive oxygen species and myeloperoxidase or other peroxidases are involved in activation of apocynin. Tissues from animals or humans in which levels of oxidative stress are elevated could prove more sensitive to apocynin thus providing a further explanation for some of the findings reported in the present study.

Although apocynin was a potent enhancer of NO bioavailability, the 2 other NAD(P)H oxidase inhibitors showed no beneficial effects on vascular responses decreasing NO bioavailability and modifying endothelium-independent responses. This is not surprising considering that neither compound is a specific inhibitor of NAD(P)H oxidase. PAO is an inhibitor of tyrosine phosphatases; it also affects glucose transport and inhibits receptor endocytosis and insulin-mediated activation of p21 ras. Apocynin, in contrast, showed no adverse effects in the present study. It has been given to rats in vivo to lower $O_2^{-}$ levels and decrease blood pressure in deoxycorticosterone acetate–salt hypertensive rats, but the present study is the first to demonstrate pharmacological effects of apocynin in human blood vessels.

Perspectives

Excess $O_2^{-}$ production occurs in many forms of cardiovascular disease. NAD(P)H oxidases have been proposed to be the major source of $O_2^{-}$ in the vasculature. In the present study, we undertook a systematic investigation of NAD(P)H oxidase inhibitors in the vasculature of rats with both normal and abnormal endothelial function. The effects of apocynin, the inhibitor that showed the most potential in rat tissue, were then examined in human blood vessels. The studies revealed apocynin as an NAD(P)H oxidase inhibitor that reduced $O_2^{-}$ production and improved endothelial function in both rat and human blood vessels. The present study suggests that the NAD(P)H oxidase signaling pathway is an important new target for drug discovery in cardiovascular disease.

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