NADPH oxidase contributes to coronary endothelial dysfunction in the failing heart

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NADPH oxidase contributes to coronary endothelial dysfunction in the failing heart. Am J Physiol Heart Circ Physiol 296: H840–H846, 2009. First published January 23, 2009; doi:10.1152/ajpheart.00519.2008.—Increased reactive oxygen species (ROS) produced by the failing heart can react with nitric oxide (NO), thereby decreasing NO bioavailability. This study tested the hypothesis that increased ROS generation contributes to coronary endothelial dysfunction in the failing heart. Congestive heart failure (CHF) was produced in six dogs by ventricular pacing at 240 beats/min for 4 wk. Studies were performed at rest and during treadmill exercise under control conditions and after treatment with the NADPH oxidase inhibitor and antioxidant apocynin (4 mg/kg iv). Apocynin caused no significant changes in heart rate, aortic pressure, left ventricular (LV) systolic pressure, LV end-diastolic pressure, or maximum rate of LV pressure increase at rest or during exercise in normal or CHF dogs. Apocynin caused no change in coronary blood flow (CBF) in normal dogs but increased CBF at rest and during exercise in animals with CHF (P < 0.05). Intracoronary ACh caused dose-dependent increases of CBF that were blunted in CHF. Apocynin had no effect on the response to ACh in normal dogs but augmented the response to ACh in CHF dogs (P < 0.05). The oxidative stress markers nitrotyrosine and 4-hydroxy-2-nonenal were significantly greater in failing than in normal myocardium. Furthermore, coelenterazine chemiluminescence for O$_2^*$ was more than twice normal in failing myocardium, and this difference was abolished by apocynin. Western blot analysis of myocardial lysates demonstrated that the p47phox and p22phox subunits of NADPH oxidase were significantly increased in the failing hearts, while real-time PCR demonstrated that Nox2 mRNA was significantly increased. The data indicate that increased ROS generation in the failing heart is associated with coronary endothelial dysfunction and suggest that NADPH oxidase may contribute to this abnormality.

heart failure; superoxide anion

WE PREVIOUSLY OBSERVED THAT the increase in coronary blood flow in response to exercise was impaired in dogs with pacing-induced congestive heart failure (CHF) (35). Furthermore, the increase in coronary flow in response to endothelium-dependent vasodilators such as acetylcholine is also impaired in the failing heart (7), indicative of endothelial dysfunction. There is evidence that increased production of reactive oxygen species (ROS), which react with nitric oxide (NO), contributes to endothelial dysfunction in the failing heart (28). Several biological systems can contribute to ROS formation, including enzymes of the respiratory chain, cytochrome P-450 monoxygenases, xanthine oxidase, uncoupled NO synthases, and NADPH oxidase (3). Although each of these can contribute to the oxidative burden, evidence is accumulating that the predominant superoxide (O$_2^*$)-producing enzyme in vascular tissue is NADPH oxidase (3). Components of the NADPH oxidase enzyme system have been found in endothelial cells, vascular smooth muscle cells, and cardiomyocytes of several species (3, 4, 19). Furthermore, NADPH oxidase can be activated by cytokines, hormones, and mechanical forces that are known to be increased in CHF (4, 6).

Apocynin, a methoxy-substituted catechol, has been widely used as an NADPH oxidase inhibitor. Although apocynin is known to inhibit NADPH oxidase in leukocytes, Heumann et al. (18) recently reported that apocynin failed to inhibit NADPH activity in endothelial or vascular smooth muscle cells in vitro but did exert antioxidant effects in these cell types. They proposed that previous findings that apocynin abolished the increase in O$_2^*$ detected in response to TNF-α or ANG II in endothelial cells (9, 11) could be attributed to the antioxidant effects of the compound. In any case, apocynin has been demonstrated to exert significant in vivo biological effects on the cardiovascular system. Thus apocynin prevented the endothelial dysfunction that resulted from acute coronary hypertension in awake dogs (21) and ameliorated left ventricular (LV) remodeling and dysfunction in rabbits with myocardial infarction (29). However, no previous studies of the effect of apocynin on coronary blood flow or endothelial function in CHF are available. Here we show that the impaired coronary vasodilator response to acetylcholine in failing hearts was reversed by apocynin, implying that increased oxidative stress contributed to the endothelial dysfunction. Furthermore, Western blot analysis demonstrated increased expression of the p47phox and p22phox subunits of NADPH oxidase, and real-time PCR demonstrated significantly increased Nox2 mRNA in the failing hearts, suggesting that increased NADPH oxidase contributed to the oxidative stress and endothelial dysfunction.

METHODS

Studies were performed in adult mongrel dogs (25–30 kg body wt) of either sex in accordance with the “Position of the American Heart Association on Research Animal Use” and with prior approval of the Animal Care Committee of the University of Minnesota.

Surgical preparation. Animals were anesthetized with pentobarbita-

Address for reprint requests and other correspondence: R. J. Bache, Cardi- nal sodium (30–35 mg/kg iv), intubated, and ventilated with 1–2% isoflurane using oxygen-enriched room air. A left thoracotomy was intro-

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artery. An epicardial pacing lead was screwed into the right ventricle. A programmable pacing generator (model 5385, Medtronic, Minneapolis, MN) modified to allow rapid pacing was placed in a subcutaneous pocket and connected to the pacing lead. Effect of apocynin on the normal heart. The effect of apocynin was examined in normal dogs 10–14 days after surgical preparation. With the dogs standing quietly on the treadmill, resting hemodynamics and coronary blood flow were recorded. Subsequently, a two-stage treadmill exercise protocol [3.2 km/h at 0% grade (stage 1) and 6.4 km/h at 0% grade (stage 2)] was begun. Each exercise stage was 3 min in duration. Pressures and coronary flow were measured during the last 30 s of each exercise stage. After a 20-min rest period, apocynin (4 mg/kg iv) was infused. At 60 min after apocynin administration, all measurements were repeated at rest and during exercise as described above. Production of heart failure. After completion of the above-described protocol, rapid ventricular pacing was begun so that the same animals could be studied again after the development of CHF. The pacemaker was activated at 240 beats/min, and pacing was continued for 4 wk (7–35). Weekly assessments of hemodynamics and coronary blood flow were obtained with the dogs standing quietly in a sling in sinus rhythm 1 h after the pacemaker had been deactivated. CHF was demonstrated by an increase in the resting LV end-diastolic pressure (LVEDP) to >20 mmHg. Effect of apocynin on development of CHF. On the day of study, the pacemaker was deactivated and the dog was placed on the treadmill. At 1 h after pacemaker deactivation, resting hemodynamics and coronary blood flow were recorded. Subsequently, the two-stage treadmill exercise protocol was begun, and all measurements were obtained as previously described. After a 20-min rest period, apocynin (4 mg/kg iv) was infused, and all measurements were repeated at rest and during exercise. Endothelium-dependent and independent vasodilation. The effect of apocynin on endothelium-dependent and independent coronary vasodilation was examined in five of the dogs under control conditions and in all six dogs after the development of CHF. The increases in coronary flow produced by intracoronary infusion of the endothelium-independent vasodilator sodium nitroprusside (3.75–75 µg/min) and the endothelium-independent vasodilator acetylcholine (3.75–75 µg/min) were observed. After these measurements were completed, apocynin (4 mg/kg iv) was administered. At 60 min after drug administration, responses to acetylcholine and sodium nitroprusside were recorded as described above. Hemodynamic measurements. LV pressure was measured with the implanted micromanometer; maximum rate of LV pressure increase (dP/dt max) was obtained by electrical differentiation. Coronary blood velocity was measured with a Doppler-flowmeter system (Craig Hartley, Houston, TX). Data were recorded on an eight-channel recorder (Coulbourn Instruments, Lehigh Valley, PA). Measurement of O2 production. O2 production was determined by chemiluminescence using coelenterazine (Molecular Probes) as a pre- treatment (14) was added to the tissue suspension during the linear phase of the reaction, and cycle numbers obtained at this point were plotted against a standard curve prepared from serially diluted control samples. Results were normalized to GAPDH expression levels. The primers for Nox1, Nox2, Nox3, Nox4, Nox5, and GAPDH were designed from the canine cDNA database using D-LUX Designer (Invitrogen). The primers were as follows: 5′-ggggtgaggctggacggcggcggcgacccccg3′ (forward) and 5′-cggatatcaccatctggctacactccg3′ (reverse) for Nox1, 5′-gtcggtgctatccttttacccatgtgccg3′ (forward) and 5′-gaggtatttaaaagttctcctg3′ (reverse) for Nox2, 5′-cagtcgatctcgatggaagtttcccg3′ (forward) and 5′-gggccctaacattcctggaacc3′ (reverse) for Nox3, 5′-eg- gatgcttgtgcttttacccg3′ (forward) and 5′-ctctgcttggcttttgaggc3′ (reverse) for Nox4, 5′-gagatatcgctgctgcacggggac3′ (forward) and 5′-gggacctggctttgagcttgc3′ (reverse) for Nox5, and 5′-ctcagaggac3′ (forward) and 5′-gggaggactggagagggc3′ (reverse) for GAPDH. Data analysis. Heart rate, pressures, and coronary velocity were measured with the implanted micromanometer. Data were compared using a two-way ANOVA for repeated measures (exercise level and treatment). When a significant result was found, individual comparisons were performed using Scheffé’s method. Comparisons between normal and heart failure assays were made using Student’s t-test with Bonferroni’s correction. P < 0.05 was required for statistical significance. Values are means ± SE. RESULTS Effect of apocynin on the normal heart. Hemodynamic data from the six dogs during normal control conditions are shown in Table 1. Exercise resulted in significant progressive increases in heart rate, aortic pressure, coronary blood flow, LV systolic pressure, and LV dP/dt max. Apocynin caused no significant hemodynamic changes at rest or during exercise and did not alter coronary blood flow compared with control conditions (all P > 0.05). Effect of apocynin on CHF. Hemodynamic data from the same six dogs after the development of CHF are shown in Table 2. Exercise resulted in significant progressive increases in heart rate, aortic pressure, LV systolic pressure, and LV dP/dt max. Western blot analysis. LV myocardium from the six dogs with heart failure, as well as six normal control dogs, was homogenized in lysis buffer containing 20 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 µg/ml leupeptin, 2 µg/ml aprotinin, and 1 mM PMSF. The homogenates were clarified by centrifugation at 12,000 g at 4°C for 10 min, and equal amounts of protein (80 µg) were subjected to 10% SDS-PAGE, transferred to HyBond nitrocellulose membrane, incubated with primary antibody followed by horseradish peroxidase-labeled secondary antibody, and detected by enhanced chemiluminescent substrate (Amersham) as previously described (25, 37). Primary antibodies included 4-hydroxy-2-nonenal (4-HNE), obtained from Alexis Biochemicals (rabbit 210-767-R100); nitrotyrosine, obtained from Upstate Biotechnology (mouse 05-233); and p47phox (rabbit sc-14015), p22phox (rabbit sc-20781), and GAPDH (sc-32233), obtained from Santa Cruz Biotechnology. Protein levels were normalized to GAPDH levels. Real-time quantitative RT-PCR. Total RNA was isolated from myocardium using TRIzol reagent (Invitrogen) and treated with DNase (Turbo DNA-free, Ambion) and then reverse transcribed to cDNA (Advantage RT-for-PCR kit, Clontech). Quantitative real-time RT-PCR was prepared using DNA Master SYBR Green I (Roche) and analyzed using the Light Cycler Thermocycler (Roche Diagnostics) as previously described (8). Quantification was performed in the log-linear phase of the reaction, and cycle numbers obtained at this point were plotted against a standard curve prepared from serially diluted control samples. Results were normalized to GAPDH expression levels. The primers for Nox1, Nox2, Nox3, Nox4, Nox5, and GAPDH were designed from the canine cDNA database using D-LUX Designer (Invitrogen). The primers were as follows: 5′-ggggaagtcagcagcagaggcgcggcgacccccg3′ (forward) and 5′-cggatatcaccatctggctacactccg3′ (reverse) for Nox1, 5′-cagtcgatctcgatggaagtttcccg3′ (forward) and 5′-gaggtatttaaaagttctcctg3′ (reverse) for Nox2, 5′-cagtcgatctcgatggaagtttcccg3′ (forward) and 5′-gggccctaacattcctggaacc3′ (reverse) for Nox3, 5′-eg- gatgcttgtgcttttacccg3′ (forward) and 5′-ctctgcttggcttttgaggc3′ (reverse) for Nox4, 5′-gagatatcgctgctgcacggggac3′ (forward) and 5′-gggacctggctttgagcttgc3′ (reverse) for Nox5, and 5′-ctcagaggac3′ (forward) and 5′-gggaggactggagagggc3′ (reverse) for GAPDH.
Table 1. Effects of apocynin on rest and exercise hemodynamics in normal dogs

<table>
<thead>
<tr>
<th></th>
<th>Rest</th>
<th>Stage 1 (3.2 km/h)</th>
<th>Stage 2 (6.4 km/h)</th>
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<tr>
<td>Heart rate, beats/min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>108±3.7</td>
<td>158±3.7*</td>
<td>184±4.4*</td>
</tr>
<tr>
<td>Apocynin</td>
<td>118±4.7</td>
<td>158±4.2*</td>
<td>187±5.4*</td>
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<tr>
<td>Mean aortic pressure, mmHg</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>118±5.6</td>
<td>128±6.4*</td>
<td>131±5.9*</td>
</tr>
<tr>
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<td>107±3.1</td>
<td>122±5.9*</td>
<td>124±6.6*</td>
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<tr>
<td>LV systolic pressure, mmHg</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>136±5.6</td>
<td>150±6.8*</td>
<td>154±6.5*</td>
</tr>
<tr>
<td>Apocynin</td>
<td>126±3.1</td>
<td>142±6.3*</td>
<td>147±6.9*</td>
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<tr>
<td>LVEDP, mmHg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6±1.7</td>
<td>9.6±1.7*</td>
<td>11.8±1.8*</td>
</tr>
<tr>
<td>Apocynin</td>
<td>3.6±0.8</td>
<td>6.8±0.6*</td>
<td>10.6±0.6*</td>
</tr>
<tr>
<td>LV dP/dtmax, mmHg/s</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3,290±259</td>
<td>4,630±268*</td>
<td>5,665±392*</td>
</tr>
<tr>
<td>Apocynin</td>
<td>3,370±162</td>
<td>4,630±280*</td>
<td>5,780±323*</td>
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<tr>
<td>CBF, ml/min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>43±11</td>
<td>64±9.6*</td>
<td>75±9.1*</td>
</tr>
<tr>
<td>Apocynin</td>
<td>43±11</td>
<td>64±11*</td>
<td>75±11*</td>
</tr>
<tr>
<td>Rate-pressure product, mmHg·beat−min⁻¹×10⁻³</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>15±0.7</td>
<td>24±1.7*</td>
<td>28±1.7*</td>
</tr>
<tr>
<td>Apocynin</td>
<td>15±0.7</td>
<td>23±1.5*</td>
<td>28±2.0*</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 6). LV, left ventricle; LVEDP, LV end-diastolic pressure; LV dP/dtmax, maximal rate of LV pressure increase; CBF, coronary blood flow. *P < 0.05 vs. rest.

Apocynin caused no significant changes in heart rate, aortic pressure, or LV systolic pressure at rest or during exercise. Apocynin tended to decrease LVEDP at rest and during both exercise stages compared with control conditions, but this did not achieve statistical significance. Apocynin resulted in significant increases in coronary flow at rest and during exercise compared with control conditions (each P < 0.05).

Effect of apocynin on responses to endothelium-dependent and -independent vasodilators. The development of heart failure was associated with a significant reduction of the vasodilator response to intracoronary acetylcholine (Fig. 1). Responses to intracoronary infusion of acetylcholine are shown in Fig. 2. Intracoronary acetylcholine had no significant effect on mean aortic pressure, LV pressure, or heart rate. Acetylcholine caused dose-related increases in coronary blood flow in dogs under normal conditions and after the development of CHF, but the response was significantly blunted after the development of CHF. Apocynin caused no change in the response of coronary flow to acetylcholine under normal control conditions (Fig. 2A) but significantly augmented the acetylcholine-induced increase in coronary flow after the development of CHF (P < 0.05; Fig. 2B). Apocynin did not alter the response of coronary blood flow to sodium nitroprusside during normal conditions or after the development of CHF (data not shown).

Evidence of oxidative stress, O₂⁻ generation, and NADPH oxidase subunit expression. The oxidative stress markers nitrtyrosine and 4-HNE were significantly greater in failing than in normal myocardium (Fig. 3). Coelenterazine chemiluminescence was used to detect O₂⁻ production in myocardial lysates. As shown in Fig. 4A, the signal in myocardium from the failing hearts was more than twice as great as that from normal hearts. Apocynin significantly reduced O₂⁻ detected in normal and CHF myocardium; this effect was more prominent in the failing hearts so that after treatment with apocynin O₂⁻ production was not significantly different between normal and failing hearts. The specificity of the coelenterazine chemiluminescence O₂⁻ measurement was confirmed by addition of the SOD mimic Mn(III)-TBAP (10 µM) to the CHF tissue lysate before addition of coelenterazine (Fig. 4B).

Western blot analysis for p47phox and p22phox demonstrated significantly increased expression of both of these subunits of...
NADPH oxidase in myocardium from the failing hearts compared with normal hearts (Fig. 5). The mRNA levels of Nox1, Nox2, Nox3, Nox4, and Nox5 were measured by real-time PCR (Fig. 6). The highest RNA level in the heart tissue was expressed by Nox1, followed by Nox4 and Nox2. The Nox3 and Nox5 RNA levels were lowest, at less than one-third of the Nox1 level. The Nox2 RNA level was significantly increased in myocardium from the failing hearts (64.5 ± 21%, *P* < 0.05), while the others were not significantly different from normal heart tissue.

**DISCUSSION**

In the present study, apocynin caused significant increases in coronary blood flow in dogs with CHF at rest and during exercise but had no effect when the same animals were studied under normal control conditions. Apocynin also enhanced endothelium-dependent vasodilation in animals with heart failure, augmenting the response of coronary flow to intracoronary acetylcholine, but had no effect in the dogs under normal control conditions (Fig. 2). These findings were associated with increased coelenterazine enhanced chemiluminescence in the failing myocardium, implying that increased O$_2$ production contributed to endothelial dysfunction in the failing hearts. The findings of increased NADPH oxidase subunits, as well as increased Nox2 expression, support the view that O$_2$ generated by NADPH oxidase contributed to coronary endothelial dysfunction in the failing hearts.

We previously demonstrated that inhibition of NO synthesis with N-nitro-l-arginine attenuates the coronary vasodilator response to acetylcholine in normal dogs (13) and in dogs with pacing-induced CHF (34), implying that in the dog the response to acetylcholine is at least in part mediated by NO. In the present study, treatment with apocynin augmented the coronary vasodilator response to acetylcholine in animals with CHF.
heart failure and increased the coronary flow response to exercise. Although no previous data are available examining the effect of apocynin on coronary flow responses in vivo, Takayama et al. (31) reported that increased O₂/H₂O₂ generation contributed to endothelial dysfunction in the femoral vascular bed of dogs with tachycardia-induced CHF. The investigators found that the increased O₂/H₂O₂ produced by isolated femoral artery segments from animals with CHF was inhibited by apocynin. In in vitro buffer-perfused hearts from Tg q*44 mice in which cardiomyopathy develops secondary to constitutive activation of Gq signaling, Drelicharz et al. (12) reported impaired coronary vasodilation in response to NO-dependent and prostaglandin I₂-dependent vasodilators that was associated with increased myocardial O₂ production, which was inhibited by apocynin. In both of these studies, as in the present study, the investigators used apocynin to block NADPH activity, so their results are subject to the limitations of this agent (see below).

The finding that apocynin augmented coronary flow responses to exercise and acetylcholine in the present study supports the concept that increased ROS generation in the failing heart impaired NO-mediated coronary vasodilation and that apocynin improved the response by scavenging or decreasing the production of O₂⁻, thereby increasing NO bioavailability. Alternatively, NO and O₂⁻ are known to exert effects on cardiomyocyte metabolism/performance that might result in alterations of coronary flow regulation. Thus NO can inhibit mitochondrial respiration by competing with oxygen at cytochrome oxidase to decrease oxygen consumption (5). However, such a decrease in myocardial oxygen consumption would be expected to cause a decrease in coronary blood flow, rather than the increase observed in the present study. Over-production of O₂⁻, H₂O₂, and hydroxyl radical caused decreases in ADP-stimulated respiration in rat myocardial mitochondria by acting on specific mitochondrial targets (40). Again, this effect would have been expected to cause a decrease in coronary blood flow.

Cardiovascular O₂ production can arise from several sources, including xanthine oxidase (38), the mitochondria, uncoupled NO synthase (39), and nonphagocytic NADPH oxidase (3, 4, 19). Coronary vessels have been found to express components of NADPH oxidase, including the flavocytochrome b₅₅₈ subunits gp91phyox and p22phyox, as well as the cytosolic factors p47phyox and p67phyox (17). Vascular cells normally produce low amounts of O₂⁻, which can stimulate transcription factors and signaling cascades (3, 6, 17), but both experimental and clinical studies have provided evidence that NADPH oxidase can be a significant source of increased vascular O₂⁻ production in disease states such as hypertension, where increased ROS production contributes to smooth muscle hypertrophy and endothelial dysfunction (36). The activity of vascular NADPH oxidase has been found to increase in response to stimulation with ANG II, aldosterone, endothelin-1, and cytokines such as TNF-α, all of which are increased in CHF (3, 6).

In the present study, we found that p22phyox and p47phyox protein levels, as well as Nox2 mRNA, were increased in the failing hearts. p22phyox and p47phyox are regulatory proteins for Nox activation. p22phyox is membrane bound and acts to stabilize the Nox proteins and dock cytosolic factors of the complex, which leads to activation of the Nox enzyme (3). Previous studies have demonstrated that exposure of vascular smooth muscle or endothelial cells to transforming growth factor-β, TNF-α, or ANG II (which are increased in CHF) caused elevation of p22phyox mRNA (10, 15). The cytosolic components of NADPH oxidase (including p47phyox, p40phyox, p67phyox, and Rac1) have been detected at mRNA and protein levels in most cardiovascular cells (3, 6), and p47phyox was increased in thrombin-stimulated vascular smooth muscle cells (2, 3). ROS production in response to platelet-derived growth factor and ANG II was impaired in vascular smooth muscle cells and endothelial cells from p47phyox−/− mice (23, 24), demonstrating

![Fig. 5. Western blot analysis for NADPH oxidase subunits p22phyox and p47phyox in normal (n = 5) and CHF (n = 5 or 6) dogs. *P < 0.05 vs. normal.](http://ajpheart.physiology.org/)

![Fig. 6. Real-time PCR detection of RNA levels of Nox1, Nox2, Nox3, Nox4, and Nox5 in heart tissue from normal and CHF dogs (n = 6 for each). *P < 0.05 vs. normal.](http://ajpheart.physiology.org/)
that p47phox is an essential component of the vascular NADPH oxidase system.

Nox2 is one of the catalytic subunits of NADPH oxidase that is expressed in phagocytes as well as in nonphagocytic cells, including cardiomyocytes and all layers of coronary artery (6). Nox2 gene expression is induced in cardiomyocytes after acute myocardial infarction (22) and in aorta, heart, and resistance artery vascular smooth muscle cells in response to ANG II (3). The increase in p47phox, p22phox, and Nox2 in the failing hearts in the present study supports the concept that NADPH oxidase contributed to increased O2\textsuperscript{-} generation in the failing hearts. A limitation of the study is that we were not able to identify the cell(s) of origin of the increased NADPH oxidase subunits, which could be any of the coronary vessels, cardiomyocytes, or leukocytes. Regulation of coronary blood flow, which was examined in the present study, occurs at the level of the coronary resistance vessels; preparation and study of these microvessels were beyond the scope of this study.

Apocynin (4’-hydroxy-3’-methoxy-acetophenone) is a methoxy-substituted catechol that was first reported to inhibit ROS production by activated neutrophils (30). Apocynin has subsequently been used as an NADPH inhibitor in vascular preparations and various cell types for nearly two decades (3, 16, 21, 29). In these studies, apocynin was found to decrease ROS generation measured by various techniques, including lucigenin chemiluminescence, p47phox phosphorylation, or p47phox/p67phox translocation (33), and the results were taken as evidence of NADPH inhibition. Recently, however, Heumüller et al. (18) questioned the role of apocynin as an NADPH inhibitor in nonphagocytic cells. The investigators reported that, in HEK-293 cells overexpressing Nox1, Nox2, or Nox4, as well as vascular smooth muscle cells, apocynin did not inhibit NADPH oxidase but, rather, acted as an antioxidant. To act as an NADPH inhibitor, apocynin must first be metabolized by peroxidase to generate apocynin dimers, which then inhibit the membrane translocation of cytoplasmic subunits for assembly into the active enzyme complex (3). Heumüller et al. reported that the inhibitory action of apocynin for NADPH oxidase is restricted to myeloperoxidase (MPO)-expressing cells, and that the compound does not inhibit NADPH oxidase in MPO-free vascular cells. However, other evidence suggests that apocynin can form dimers and inhibit NADPH oxidase activity through peroxidases other than MPO (20). Furthermore, in vivo studies suggest that apocynin in vascular cells can be activated by MPO secreted by neutrophils (1). Astern et al. (1) demonstrated that MPO released into the vessel as a result of intravascular neutrophil degradation can be internalized by endothelial cells and impair endothelial signaling. This phenomenon would not be observed in isolated cell systems but could occur in vivo. Despite this controversy, the present study supports the concept that increased O2\textsuperscript{-} generation contributed to coronary endothelial dysfunction in the failing hearts and that apocynin modulated O2\textsuperscript{-}, by inhibiting NADPH oxidase activity or through radical scavenging (or both), to improve endothelium-mediated vasodilation and to increase coronary blood flow in the failing heart at rest and during exercise.

The effects of increased ROS production in the failing heart may be compounded by impaired ROS-scavenging mechanisms. We recently found that extracellular SOD (ecSOD) was decreased in hearts from dogs with pacing-induced CHF (7) and that ecSOD gene deficiency exacerbated the myocardial oxidative stress and LV remodeling that occurred in mice exposed to chronic pressure overload or myocardial infarct (25, 37). A decrease of ecSOD would facilitate the interaction of O2\textsuperscript{-} with NO, thereby removing the dilator effect of NO. There is evidence that H2O2 can act as a hyperpolarizing factor that can contribute to endothelium-dependent vasodilation (27). A decrease of ecSOD would decrease the generation of H2O2, which might also contribute to endothelial dysfunction.

In summary, the present data indicate that increased O2\textsuperscript{-} production may contribute to increased O2\textsuperscript{-} generation in the failing hearts. The finding of increased expression of NADPH oxidase subunits and Nox2 mRNA suggests that increased expression of NADPH oxidase may contribute to this endothelial dysfunction.

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