Bradykinin-Induced Dilation of Human Coronary Arterioles Requires NADPH Oxidase–Derived Reactive Oxygen Species

Brandon T. Larsen, Aaron H. Bubolz, Suelhem A. Mendoza, Kirkwood A. Pritchard Jr, David D. Gutterman

Objective—Hydrogen peroxide (H₂O₂) is an endothelium-derived hyperpolarizing factor in human coronary arterioles (HCAs). H₂O₂ mediates bradykinin (BK)-induced vasodilation and reduces bioavailability of epoxyeicosatrienoic acids (EETs); however, the cellular and enzymatic source of H₂O₂ is unknown.

Methods and Results—NADPH oxidase expression was determined by immunohistochemistry. Superoxide and H₂O₂ production was assayed in HCAs and human coronary artery endothelial cells (HCAECs) using dihydroethidium and dichlorodihydrofluorescein histofluorescence, respectively. Superoxide was quantified by HPLC separation of dihydroethidium products. Diameter changes of HCAs were measured by videomicroscopy. NADPH oxidase subunits Nox1, Nox2, Nox4, p22, p47, and p67 were each expressed in HCA endothelium. In HCAs or HCAECs incubated with dihydroethidium and dichlorodihydrofluorescein, BK induced superoxide and H₂O₂ formation, which was inhibited by gp91ds-tat or apocynin but not by gp91scram-tat or rotenone. HPLC analysis confirmed that BK specifically induced superoxide production. Gp91ds-tat reduced vasodilation to BK but not to papaverine. 14,15-EEZE (an EET antagonist) further reduced the residual dilation to BK in the presence of gp91ds-tat, but had no effect in the presence of gp91scram-tat, suggesting that NADPH oxidase–derived ROS modulate EET bioavailability.

Conclusion—We conclude that endothelial NADPH oxidase is a functionally relevant source of H₂O₂ that mediates agonist-induced dilation in the human heart. (Arterioscler Thromb Vasc Biol. 2009;29:739-745.)

Key Words: oxidant stress • endothelium • coronary circulation • neurohumoral control of circulation • hypertension–basic studies

H₂O₂ is a reactive oxygen species (ROS) that functions as an endothelium-derived hyperpolarizing factor (EDHF) in some circulatory beds, including HCAs.1–4 EDHF is particularly important in the microcirculation, where vascular resistance is regulated. As an EDHF, H₂O₂ may compensate to maintain adequate perfusion in states of heightened oxidative stress such as cardiovascular disease, where nitric oxide (NO)-mediated vasodilation is impaired. In addition to its direct vasodilator properties, H₂O₂ can modulate bioavailability of EETs, cytochrome P450 (CYP)-derived metabolites of arachidonic acid that also function as EDHFs.5

H₂O₂ arises by enzymatic or spontaneous dismutation of the superoxide anion. Superoxide is generated from numerous intracellular sources, including mitochondria,4 CYPs,6 and NADPH oxidasés.7 The canonical Nox2-containing NADPH oxidase was originally identified in phagocytes, where it mediates the microbicidal respiratory burst; however, Nox2 as well as alternative NADPH oxidases containing Nox1 or Nox4 are now recognized as an important source of ROS in vascular tissue as well.8 Although the pathological role of NADPH oxidase is well defined by its heightened participation in a variety of vascular diseases,7,9 its possible contribution to physiological vascular stimuli is less clear, especially in the human heart.

This study was conducted to examine the putative role of NADPH oxidase in mediating dilation to BK in HCAs. Published data indicate that both flow-induced3 and BK-induced5 dilation of HCAs are sensitive to catalase, implicating a role for H₂O₂ in these responses. Evidence points to the mitochondrial electron transport chain as a critical source of ROS in response to shear stress,4 but the enzymatic source of H₂O₂ in response to BK is not known. The present study provides the first evidence that NADPH oxidase produces ROS in the human coronary microcirculation and that this enzyme is critical to the mechanism of dilation to BK, a physiological vasomotor agonist. Specifically, we examined whether NADPH oxidase is (1) expressed in HCAs, (2) responsible for BK-induced ROS production, (3) required for BK-induced dilation, and (4) responsible for oxidative inhibition of the EET-mediated component of BK-induced dilation.

Methods

For complete details of immunohistochemistry, fluorescence microscopy, HPLC, and videomicroscopy protocols, as well as a listing of
materials, please refer to the online supplement at http://atvb.ahajournals.org.

**Tissue Acquisition**

Fresh right atrial appendages were obtained as discarded surgical specimens from patients undergoing cardiopulmonary bypass procedures, as reported previously. All tissue acquisition procedures and experimental protocols were approved by the appropriate Institutional Review Boards. Demographic data and diagnoses were obtained at the time of surgery.

**Immunohistochemistry**

Immunohistochemistry was performed to visualize NADPH oxidase subunit expression as reported previously.

**Superoxide and H₂O₂ Detection by Fluorescence Microscopy**

Fluorescence detection of superoxide and H₂O₂ in HCAECs or HCAs was performed using dihydroethidium (DHE) and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH), respectively. Briefly, HCAECs or HCAs were incubated with gp91ds-tat (5×10⁻⁵ mol/L), a specific peptide inhibitor of NADPH oxidase, gp91scram-tat (5×10⁻⁵ mol/L), a scrambled control peptide, apocynin (3×10⁻³ mol/L, an NADPH oxidase inhibitor), catalase (1000 U/mL), rotenone (10⁻⁶ mol/L, a mitochondrial Complex I inhibitor), or vehicle. HCAECs or HCAs were then loaded with DCFH and DHE and exposed to BK (10⁻⁶ mol/L). Fluorescence detection of H₂O₂, and superoxide was performed by krypton-argon laser excitation at 488 and 585 nm while recording emission at 523 nm and 610 nm, respectively.

**HPLC Measurement of Superoxide**

HPLC separation and quantification of oxidized DHE products were performed as described previously. Briefly, HCAECs or HCAs were incubated with the NADPH oxidase inhibitor apocynin (3×10⁻³ mol/L) or vehicle. DHE was then added and HCAs were incubated with BK (10⁻⁶ mol/L) or vehicle. DHE metabolites were then extracted, separated by HPLC, and quantified.

**Measurement of HCA Dilation by Videomicroscopy**

Dilation of HCAs was observed by videomicroscopy as described previously. Briefly, isolated HCAs were cannulated, pressurized, equilibrated, and constricted with KCl (50 mmol/L) to assess viability. Only vessels that contracted >30% were used for subsequent experiments. After washing, vessels were constricted 30% to 50% with endothelin-1 (ET-1, 5×10⁻¹⁰ to 10⁻⁹ mol/L). Although published data indicate little contribution of NO or prostacyclin to BK-induced dilation of HCAs, all experiments were performed in the presence of Nω-nitro-L-arginine methyl ester (10⁻⁴ mol/L, an NO synthase inhibitor) and indomethacin (10⁻⁵ mol/L, a cyclooxygenase inhibitor) to eliminate any residual confounding effects of these vasodilators. Cumulative concentrations of BK (10⁻¹⁰ to 10⁻⁶ mol/L) were then added and steady state diameters were measured. After washing, gp91ds-tat (5×10⁻⁵ mol/L) or gp91scram-tat peptide (5×10⁻⁵ mol/L) was introduced both in the superfusate and intraluminally under a brief period of flow. In other vessels, 14,15-epoxyeicosa-5(Z)-enoic acid (EEZE, 10⁻⁵ mol/L, an EET antagonist) was coadministered with gp91ds-tat or gp91scram-tat. After equilibration, HCAs were again constricted with ET-1, and cumulative concentrations of BK were again applied. At the end of each experiment, papaverine (10⁻⁴ mol/L, an endothelium-independent vasodilator) was added to determine the maximum diameter.

**Statistical Analyses**

HPLC data, maximum percent vasodilation (%MD), EC₅₀, and fluorescence intensities were evaluated using paired or unpaired Student t tests, whenever applicable. To compare concentration–response relationships, a 2-factor repeated measures ANOVA was used. When a significant difference was observed between curves (P<0.05), responses at individual concentrations were compared using a Holm-Sidak multiple comparison test. Multiple stepwise regression analyses were used to detect the influence of underlying diseases, age, and gender on vasodilation at various concentrations. All analyses were performed using SigmaStat, version 3.1. Statistical significance was defined as P<0.05. All data are described as mean±SEM; n=number of patients or experiments.

**Results**

Atrial appendages were obtained from 55 patients, yielding 135 HCAs with a mean internal diameter of 164±7 μm. Patient demographic information is summarized in the Table.

**Expression of NADPH Oxidase Subunits**

To investigate whether NADPH oxidase may be a source of ROS in HCAs, immunohistochemistry was used to evaluate expression of NADPH oxidase subunits. As shown in Figure 1, the catalytic subunits Nox1, Nox2, and Nox4 as well as the regulatory subunits p22, p47, and p67 are each expressed in the endothelium. Nonspecific staining was not observed in the absence of primary antibodies or in the presence of preimmune isotype control antibodies. Consistent with these findings, Western blot analysis of HCAEC protein demonstrated expression of Nox2, Nox4, and p22 (supplemental Figure II). These findings provide indirect evidence that NADPH oxidase may be a source of ROS in HCAs.

**Bradykinin-Induced Reactive Oxygen Species Production in HCAECs**

It was recently reported that the endothelium is required for BK-induced ROS production in intact HCAs; however, it is not known whether ROS actually originate from endothelial cells in this context, nor has the enzymatic source of ROS been determined. To assess whether endothelial NADPH oxidase is required for ROS production in response to BK, superoxide and H₂O₂ formation was assayed by DHE and DCFH histofluorescence, respectively. In HCAECs, superoxi-
ide and H₂O₂ were increased by BK (fluorescence ratio 1.80 ± 0.18 and 1.87 ± 0.17, n = 14 for both, respectively, P < 0.05), as shown in Figure 2. BK-induced ROS production was inhibited by apocynin (1.04 ± 0.03 and 0.97 ± 0.04, n = 6, respectively, P < 0.05 versus BK) but not by rotenone (1.68 ± 0.12 and 1.93 ± 0.16, n = 4, respectively, P = not significant [ns] versus BK), suggesting that ROS originate from NADPH oxidase and not the mitochondrial proximal electron transport chain. Importantly, catalase completely blocked the increase in DCFH but not DHE fluorescence, indicating that DCFH fluorescence was specific for H₂O₂. Taken together, these results suggest that BK induces endothelial ROS production through activation of NADPH oxidase.

**Bradykinin-Induced Reactive Oxygen Species Production in HCAs**

To further assess whether NADPH oxidase is required for BK-induced ROS production, DHE and DCFH histofluorescence was performed on intact HCAs. As shown in Figure 3, BK increased superoxide and H₂O₂ formation (fluorescence ratio 1.52 ± 0.10 and 2.24 ± 0.31, n = 9 for both, respectively, P < 0.05). Importantly, BK-induced ROS production was completely inhibited by gp91ds-tat (0.89 ± 0.05 and 0.98 ± 0.06, n = 4, respectively, P < 0.05 versus BK) or apocynin (0.90 ± 0.18 and 0.84 ± 0.24, n = 5, respectively, P < 0.05 versus BK) but not by gp91scram-tat (1.40 ± 0.15 and 1.96 ± 0.29, n = 4, respectively, P = ns versus BK). Importantly, gp91ds-tat and apocynin similarly affected baseline and BK-induced fluorescence, indicating that nonspecific effects of apocynin, if any, did not significantly influence ROS production. Taken together, these results suggest that NADPH oxidase is an important source of microvascular ROS.

**Quantification of Bradykinin-Induced Superoxide Production**

Although DHE histofluorescence suggests that BK induces superoxide formation, this method has an important limita-
tion. When oxidized, DHE forms 2 fluorescent products (2-hydroxyethidium, 2-OH-E\(^+\); and ethidium, E\(^+\)) with overlapping emission spectra; however, only 2-OH-E\(^+\) is specific for superoxide. To specifically assess and quantify superoxide, we separated oxidized products of DHE from isolated HCAs using HPLC. As seen in Figure 4, 2-OH-E\(^+\) formation increased in the presence of BK (36.2±14.3 versus 24.6±4.6 pmol/µg protein, ratio 1.52±0.19 versus control, n=6, P<0.05). In contrast, BK did not increase E\(^+\) production. Apocynin had little effect on baseline production of 2-OH-E\(^+\) (or E\(^+\), data not shown) but attenuated the increase in 2-OH-E\(^+\) in response to BK (24.1±6.8 pmol/µg protein, ratio 1.05±0.12 versus BK alone, n=6, P<0.05). This suggests that BK does indeed stimulate superoxide production in a NADPH oxidase–dependent manner.

**Contribution of NADPH Oxidase to Bradykinin-Induced Dilation**

To determine whether NADPH oxidase is a functionally relevant source of H\(_2\)O\(_2\), HCA vasodilation was measured using videomicroscopy. As shown in Figure 5, BK elicited a concentration-dependent dilation (% maximum dilation [MD] 93±3 and -logEC\(_{50}\) 7.8±0.3, n=10) that was inhibited by gp91ds-tat (%MD 49±5 and -logEC\(_{50}\) 6.7±0.1, n=5, P<0.05 versus BK alone) but not by gp91scram-tat (%MD 82±3 and -logEC\(_{50}\) 7.7±0.3, n=5, P=ns versus BK alone). Interestingly, BK-induced dilation was not influenced by sex, age, surgical procedure, or underlying disease. Dilation to papaverine was not altered in the presence of either gp91ds-tat or gp91scram-tat, indicating that these peptides do not directly impair vascular smooth muscle cell (VSMC) vasodilator capacity. Taken together, these results suggest that NADPH oxidase is required for BK-induced dilation.

**NADPH Oxidase–Derived H\(_2\)O\(_2\) Reduces the EET-Mediated Component of BK-Induced Dilation**

Previous studies indicate that BK-induced dilation is predominantly mediated by H\(_2\)O\(_2\); however, a prominent residual dilation occurs in the presence of catalase that is mediated by EETs. This secondary mechanism is masked by an inhibitory effect of H\(_2\)O\(_2\) on CYP enzymatic activity, which reduces EET production; however, the source of ROS in this context is unknown. To determine whether NADPH oxidase–derived H\(_2\)O\(_2\) reduces the EET-mediated component of BK-induced dilation, EEZE was coadministered with gp91ds-tat or gp91scram-tat. Interestingly, the residual dilation to BK in the presence of gp91ds-tat was markedly inhibited by EEZE (%MD 9±3 versus 45±6 and -logEC\(_{50}\) 6.5±0.1 versus 6.7±0.1 with gp91ds-tat alone, n=5, P<0.05, Figure 5).
However, there was no effect of EEZE in the presence of gp91scram-tat. These results suggest that EETs mediate dilation to BK when NADPH oxidase is inhibited, and that NADPH oxidase–derived H2O2 modulates the bioavailability or action of EETs.

Discussion
This study is the first to directly investigate a role for NADPH oxidase in the human coronary microcirculation. The novel findings of the present study are 4-fold. First, NADPH oxidase is expressed in HCAs. Second, BK induces endothelial ROS production in an NADPH oxidase–dependent manner. Third, NADPH oxidase is required for BK-induced dilation. Fourth, an EET-mediated component of BK-induced dilation is unmasked when NADPH oxidase is inhibited. Taken together, these data suggest that NADPH oxidase is a functionally relevant source of ROS that may modulate vasomotor tone not only by producing H2O2, but also by modulating EET bioavailability.

NADPH Oxidase and Agonist-Induced ROS Production
Vascular NADPH oxidase activity is induced by several physiological stimuli. The contribution of NADPH oxidase to angiotensin II (AngII)-induced ROS production in VSMCs is well established, where it plays a role in AngII-dependent hypertension and VSMC hypertrophy.18–20 NADPH oxidase is also activated by mechanical stimulation,21 and its activation may play a role in atherogenesis in areas of oscillatory shear stress.22

Despite evidence that BK induces H2O2 production in the coronary circulation,2,5 little is known about the source of ROS in response to this agonist. BK induces H2O2 release from porcine coronary microvessels in a manner that requires the endothelium; however, the enzymatic source of H2O2 in this model has not yet been characterized.7 The present study suggests that NADPH oxidase in endothelial cells may be a source of ROS in response to BK. Inhibition of NADPH oxidase reduces BK-induced dilation, indicating that this enzyme complex is a functionally relevant mediator of vasodilation. Although gp91ds-tat does not completely abolish this response, the magnitude of inhibition is similar to the published inhibitory effect of catalase on BK-induced dilation in these vessels.5

It is a somewhat unexpected finding that NADPH oxidase mediates a vasodilator response to BK in light of substantial evidence implicating this enzyme as an effector of AngII signaling and impaired NO-dependent vasodilation.19,20 Animal studies indicate that AngII-induced NADPH oxidase activity acutely impairs endothelium-dependent vasodilation23 and chronically promotes VSMC hypertrophy18 and hypertension.12,23 It is not known how AngII and BK elicit opposing vascular effects through activation of the same enzyme; however, the present study suggests that critical agonist-dependent or cell-dependent differences in NADPH oxidase activation occur. Whereas NADPH oxidase in VSMCs serves as an effector of AngII to promote vasoconstriction and hypertension, NADPH oxidase in endothelial cells may serve as an effector of BK to modulate acute vasodilatory function. It is also possible that NADPH oxidase may have differential roles in normal versus diseased vessels. In nondiseased vessels where NO-mediated dilation is significant,10 activation of NADPH oxidase may have a net constricting effect by reducing NO bioavailability. In contrast, diseased HCAs mediate dilation via EDHFs and not via NO10; therefore, activation of NADPH oxidase to generate H2O2 may have a net dilating effect. If so, the ratio of NADPH oxidase activation in endothelial cells versus
VSMCs may be an important factor in the regulation of cardiovascular parameters such as blood pressure and tissue perfusion. It is widely recognized that the ratio of BK to AngII plays an important role in blood pressure regulation. BK receptor B₂ knockout mice exhibit elevated basal blood pressure as well as an exaggerated blood pressure response to AngII, suggesting that BK dampens the vasoconstrictive effects of AngII. Angiotensin I converting enzyme (ACE) inhibitors exploit this relationship between BK and AngII. ACE catalyzes not only AngII formation from its inactive precursor, angiotensin I, but also inactivates BK. Importantly, the beneficial cardiovascular effects of ACE inhibitors have been attributed not only to reduced AngII bioavailability, but also in part to enhanced BK bioavailability. The present study suggests an intriguing mechanism that may underlie the antihypertensive effect of ACE inhibitors; namely, that they decrease blood pressure not only by suppressing AngII-induced NADPH oxidase activity in VSMCs, but also by promoting NAPDH oxidase activation in endothelial cells through enhanced BK bioavailability. Although ACE inhibitors inhibit NADPH oxidase activity in human mononuclear leukocytes and an AngII receptor blocker reduces AngII-induced activation of NADPH oxidase in rats, additional studies will be necessary to define the effect of ACE inhibitors on endothelial and smooth muscle NADPH oxidase in humans.

Potential Study Limitations
An important limitation of the present study is the lack of quantification of H₂O₂. Although HPLC of DHE metabolites may be used to quantify microvascular superoxide, similar methods for quantification of H₂O₂ have not yet been developed. We previously detected flow-induced H₂O₂ production from HCAs by electron spin resonance; however, accurate quantification of H₂O₂ is not possible by this method because of the limited number of arterioles that can be isolated from a patient sample. Fortunately, animal models are not fraught with the limitation of tissue availability. Indeed, BK (10⁻⁶ mol/L) induces endothelial release of H₂O₂ from porcine coronary microvessels at an approximate concentration of 1.5×10⁻⁶ mol/L. Although the concentration of H₂O₂ released from HCAs is unknown, the present study suggests that it may be similar, if not higher. Catalase and gp91ds-tat each inhibit HCA dilation to 10⁻⁶ mol/L BK by ≈40%, a response that requires exogenous addition of H₂O₂, at a concentration of ≈10⁻⁵ mol/L, indirectly suggesting that BK induces H₂O₂ production in HCAs in the micromolar range.

An additional limitation of this study is a lack of direct EET measurements. Although numerous attempts were made to quantify EETs released from HCAs using HPLC or liquid chromatography electrospray-ionization mass spectrometry, the sensitivity of currently available methods is insufficient to consistently and reproducibly measure EETs from such small samples. We were also unable to demonstrate EET production from HCAECs, a finding consistent with the observation that endothelial cells rapidly lose CYP expression and activity in culture. An alternative explanation is that HCAs may not, in fact, produce EETs. Although this possibility cannot be ruled out, we believe it is less likely, as CYPs and the EET-metabolizing soluble epoxide hydrolase enzyme are robustly expressed in HCA endothelium, suggesting that HCAs are capable of producing and metabolizing EETs. Indeed, vasodilation in the presence of gp91ds-tat is blocked by EEZE, indirectly suggesting that EETs are produced when NADPH oxidase is inhibited. This finding is consistent with published data indicating that EET production is sensitive to H₂O₂.

The present study indicates that Nox1, Nox2, and Nox4 are each expressed in HCAs; however, their relative physiological importance in this model is unknown. BK-induced dilation is reduced by gp91ds-tat, suggesting that Nox2 mediates this response; however, other Nox isoforms may also contribute to this response, as gp91ds-tat cross-reactivity is theoretically possible. Nox4 in particular may contribute to H₂O₂-mediated vasodilation, as Nox4 produces much more H₂O₂ than superoxide. Highly specific inhibitors will therefore be necessary to determine whether Nox1 or Nox4 are also functional in HCAs.

An additional limitation of this study is the lack of heart tissue from healthy subjects, as this is rarely obtainable. However, this limitation is offset by the unique advantage of studying NADPH oxidase in the clinically-relevant context of chronic cardiovascular disease and its risk factors, conditions that cannot be adequately mimicked in animal models. Recent evidence indicates that NADPH oxidase activity may be elevated in cardiovascular disease, rendering an evaluation of NADPH oxidase critical in the diseased human heart.

Clinical Implications
With the expanding body of evidence implicating NADPH oxidase in the pathogenesis of atherosclerosis, hypertension, and inflammation, pharmacological inhibition of this enzyme has emerged as a potential therapeutic approach for these diseases. This study suggests a potential limitation of this approach, as NADPH oxidase inhibitors may acutely reduce agonist-induced vasodilation. On the other hand, the acutely detrimental effects of NADPH oxidase inhibition, if any, may be partially offset by enhanced EET bioavailability. H₂O₂ is a proinflammatory and proatherosclerotic molecule, but EETs have antiinflammatory and vasculoprotective effects, similar to NO. Although NADPH oxidase inhibition affects human coronary microvascular function in vitro, additional studies will be necessary to understand the global effects of NADPH oxidase inhibitors in vivo.

Conclusions
The present study supports a role for NADPH oxidase as a modulator of vasomotor tone in the human coronary microcirculation. NADPH oxidase is a functionally relevant source of ROS that not only mediates agonist-induced vasodilation, but may also reduce EET bioavailability. NADPH oxidase activity may therefore influence coronary vascular resistance and myocardial perfusion, but its physiological importance in vivo remains to be determined.
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Disclosures

None.

References


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Expanded Materials and Methods

**Immunohistochemistry:** Immunohistochemistry was performed to visualize NADPH oxidase subunit expression in human atrial tissue as reported previously.\(^1\) Briefly, right atrial appendages were fixed for 24 hours in zinc formalin buffer, embedded in paraffin, and sliced into 4 µm sections. Sections were immunolabeled with a goat anti-human polyclonal antibody against Nox1 (Santa Cruz), goat anti-human polyclonal antibody against Nox2 (Santa Cruz), goat anti-human polyclonal antibody against Nox4 (Santa Cruz), a rabbit anti-human polyclonal antibody against p22 (Santa Cruz), a mouse anti-human monoclonal IgG\(_1\) antibody against p47 (BD Biosciences), or a mouse anti-human monoclonal IgG\(_{2b}\) antibody against p67 (BD Biosciences) (1:25, 1:75, 1:25, 1:75, 1:50, and 1:50 dilutions, respectively). Immunostains were visualized using an avidin-biotin horseradish peroxidase system (Vectastain Universal Quick Kit, Vector Laboratories). Additional experiments were performed on sections where the primary antibody was omitted\(^2\) or replaced with a preimmune isotype control antibody to assess for nonspecific binding.

**Western blotting:** Western blotting was performed on protein isolated from cultured HCAECs by standard methods using the same Nox2, Nox4, or p22 antibodies that were used for immunohistochemical studies. Briefly, HCAECs were homogenized in ice-cold lysis buffer (50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1% deoxycholic acid, 0.1% SDS, 0.5% NP40) supplemented with a protease inhibitor cocktail (Roche) and centrifuged at 12,000 g for 10 min at 4 °C. Protein samples (10 µg) were subjected to
10% SDS-PAGE, transferred to PVDF membranes, blotted for Nox2 (1:200 dilution), Nox4 (1:200 dilution), or p22 (1:200 dilution), and visualized by a horseradish peroxidase-based detection system (ECL-Plus western blotting kit, VWR International).

*Superoxide and H₂O₂ detection by fluorescence microscopy:* Human coronary artery endothelial cell (HCAEC) cultures were seeded and grown in an endothelial cell-specific growth medium according to the manufacturer’s protocol (EGM-2-MV BulletKit, Cambrex). Dishes containing HCAECs or human coronary arterioles (HCAs) were filled with HEPES buffer containing Nω-nitro-L-arginine methyl ester (L-NAME, 10⁻⁴ mol/L, a nitric oxide [NO] synthase inhibitor) and indomethacin (10⁻⁵ mol/L, a cyclooxygenase inhibitor) (pH 7.4 at 37°C). After equilibration at 37°C for 60 min, gp91ds-tat (5x10⁻⁵ mol/L, a specific peptide inhibitor of NADPH oxidase complex formation), gp91scram-tat (5x10⁻⁵ mol/L, a corresponding scrambled control peptide that does not inhibit NADPH oxidase), apocynin (3x10⁻³ mol/L, an NADPH oxidase inhibitor), catalase (1000 U/ml), rotenone (10⁻⁶ mol/L, an inhibitor of Complex I of the mitochondrial electron transport chain), or vehicle was added to the dishes, and HCAECs or HCAs were loaded for 30 min in the dark with the fluorescence probes 2',7'-dichlorodihydrofluorescein diacetate (DCFH, 5x10⁻⁶ mol/L, Molecular Probes) and dihydroethidium (DHE, 5x10⁻⁶ mol/L, Molecular Probes). After loading the probes, HCAECs or HCAs were exposed to BK (10⁻⁶ mol/L) or vehicle for 10 min, rinsed in fresh HEPES, and mounted on glass slides in Fluorescence Mounting Medium (Dako Corp.). Fluorescence detection of H₂O₂ and superoxide was performed by krypton-argon laser excitation at 488 and 585 nm while recording emission at 523 nm and 610 nm,
respectively. All fluorescence studies on vessels from a single patient were performed on the same day with identical microscope settings for all experiments. Images were obtained with a laser scanning imaging system mounted on an inverted microscope using a 20x objective lens. Images were obtained on a computer with the software program MetaMorph (Universal Imaging Corp.) and analyzed with ImageJ (NIH). Fluorescence intensity was normalized as the relative change in intensity from baseline (100%).

**HPLC measurement of superoxide:** Separation and quantification of oxidized DHE products were performed by adapting an established high-performance liquid chromatography (HPLC) cell culture-based method for use in intact microvascular tissue. DHE was prepared in DMSO under argon gas and stored at -20°C until use. Single isolated HCAs were incubated for 60 min at 37°C with the NADPH oxidase inhibitor apocynin (3x10⁻³ mol/L) or vehicle in HEPES buffer consisting of (in mol/L) 138 NaCl, 4 KCl, 1.6 CaCl₂, 1.2 MgSO₄, 0.026 Na₂ETDA, 1.2 KH₂PO₄, 10 HEPES, and 6 glucose). DHE (10⁻⁵ mol/L) was then added and HCAs were incubated 30 min with BK (10⁻⁶ mol/L) or vehicle. HCAs were washed with fresh buffer and homogenized on ice in a 1:1 v/v solution of HPLC-grade methanol and 10⁻³ mol/L HCl. Homogenates were centrifuged at 15,000 g for 20 min at 4°C. The pellet was reserved for protein determination, and the supernatant was diluted 3:10 in 1 mol/L HCl and subjected to HPLC. DHE metabolites were separated on a C₁₈ reverse-phase column (Partisil ODS-3 250 X 4.5 mm, Alltech Associates, Deerfield, IL) by a linear solvent gradient from 10% to 70% CH₃CN in H₂O over 46 min and monitored by electrochemical detection at 280 mV. A representative HPLC chromatogram is shown in Supplementary Figure S1, below.
Microvascular content of DHE products was then calculated from HPLC chromatograms using the area under the respective peaks and a standard curve derived from authentic standards at known concentrations. Finally, DHE product content was normalized for protein content.

Measurement of HCA dilation by videomicroscopy: Internal diameter measurements were performed on isolated, pressurized HCAs by videomicroscopy as reported previously.\textsuperscript{2, 6, 8-12} Briefly, isolated HCAs were cannulated on glass micropipettes and secured in an organ chamber containing a physiological saline solution (PSS) consisting of (in mol/L) 123 NaCl, 4.7 KCl, 2.5 CaCl\textsubscript{2}, 1.2 MgSO\textsubscript{4}, 16 NaHCO\textsubscript{3}, 0.026 Na\textsubscript{2}ETDA, 1.2 KH\textsubscript{2}PO\textsubscript{4}, and 11 glucose. The preparation was transferred to the stage of an inverted microscope (magnification 200X, Olympus CK2) coupled to a CCD video camera (WV-BL200, Panasonic), video monitor (Panasonic), and calibrated videomicrometer (VIA-100K, Boeckeler Instruments Inc., resolution = 0.4 µm). The vessels were slowly pressurized to 60 mmHg and incubated without intraluminal flow for 60 min. Bath temperature was maintained at 37°C and was bubbled continuously with 21% O\textsubscript{2}, 5% CO\textsubscript{2}, 74% N\textsubscript{2} gas to maintain pH = 7.40 ± 0.05, P\textsubscript{O\textsubscript{2}} = 140 ± 10 mmHg.

After the initial equilibration period, vessels were constricted with KCl (50 mmol/L final concentration) to assess viability. Vessels that constricted >30% were used for subsequent experiments.\textsuperscript{2, 6, 8-10, 12} After washing with fresh buffer to restore the concentration of K\textsuperscript{+} in the bath to baseline (4.7 mol/L), endothelin-1 (ET-1, 5x10\textsuperscript{-10} ~ 10\textsuperscript{-9} mol/L) was added to constrict the vessel by 30-50% of its diameter observed at the time of initial pressurization to 60 mmHg. While published data indicates little contribution of
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Supplement Material

NO or prostacyclin to BK-induced dilation of HCAs,\(^9\) all experiments were performed in the presence of L-NAME (10\(^{-4}\) mol/L) and indomethacin (10\(^{-5}\) mol/L) to eliminate any residual confounding effects of these vasodilators. Cumulative concentrations of BK (10\(^{-10}\) \(-\) 10\(^{-6}\) mol/L) were then added to the bath and steady state diameter measurements were taken 3 min. after each application. After washing the vessels with PSS, gp91ds-tat (5x10\(^{-5}\) mol/L) or gp91scram-tat peptide (5x10\(^{-5}\) mol/L) was introduced both in the superfusate and intraluminally under a brief period of flow, flow was stopped, and the vessels were incubated for 30 min. HCAs were again constricted with ET-1 in the same manner, followed by a second application of the same BK concentrations. At the end of each experiment, papaverine (10\(^{-4}\) mol/L, an endothelium-independent vasodilator) was added to determine the maximum internal diameter for normalization of dilator responses. Agonist-induced dilations are expressed as a percent, with 100% dilation representing the change from the constricted diameter to the maximal diameter obtained by addition of papaverine. All drugs were added directly to the bath, and the change in bath volume over the course of experiments was less than 1%.

Materials: Gp91ds-tat and gp91scram-tat peptides were synthesized according to their respective amino acid sequences as originally described\(^3\) using an Applied Biosystems 432A solid-phase peptide synthesizer that employs FMOC chemistry coupled with HBTU activation. Peptide identity and purity was monitored by mass spectrometry. Following their synthesis, each peptide was dissolved in PSS containing 5 mmol/L acetic acid as described previously.\(^3\) EEZE was a kind gift from Dr. John R. Falck (University of Texas Southwestern, Dallas, Texas). Catalase isolated from bovine liver was obtained.
from Calbiochem. HCAECs were obtained from Cambrex, DCFH and DHE were obtained from Molecular Probes, and all other chemicals were obtained from Sigma Chemical Co. DCFH and DHE were dissolved in DMSO. Apocynin and EEZE were prepared in ethanol. The final concentration of ethanol present in the bath during vasodilator studies was 0.05%. Vehicle control studies indicated that the final concentration of ethanol had no effect on basal tone or function of arterioles. Indomethacin was dissolved in 20 mmol/L Na₂CO₃. All other chemicals were dissolved in distilled water. All concentrations represent final molar concentrations (mol/L) in the organ chamber.
Supplementary Results

Supplementary Figure S1. Representative electrochemical signal at 280 mV of oxidized DHE products from single isolated HCAs. As indicated by the blue squares and red circles, two distinct peaks with elution times of 17 and 19 min comigrated with authentic 2-OH-E⁺ and E⁺ standards, respectively.
Supplementary Figure S2. Expression of NADPH oxidase subunits in HCAECs. A-C, Representative Western blots of protein isolated from HCAECs. While immunohistochemical staining of atrial appendages suggests that NADPH oxidase is expressed in the endothelium of the human coronary circulation, Western blots were performed on protein isolated from cultured HCAECs to determine whether NADPH oxidase proteins are indeed present in these cells. As seen above, Nox2, Nox4, and p22 are each expressed in HCAECs.
Reference List


