Human Coronary Arteriolar Dilation to Bradykinin Depends on Membrane Hyperpolarization
Contribution of Nitric Oxide and Ca\textsuperscript{2+}-Activated K\textsuperscript{+} Channels

Hiroto Miura, MD, PhD; Yanping Liu, MD, PhD; David D. Gutterman, MD

**Background**—K\textsuperscript{+} channel activation in vascular smooth muscle cells (VSMCs) plays a key role in regulating vascular tone. It has been proposed that endothelium-derived hyperpolarizing factor (EDHF) contributes to microvascular dilation more than nitric oxide (NO) does. Whether hyperpolarization is important for coronary arteriolar dilation in humans is not known. Bradykinin (BK), an endogenous vasoactive substance, is released from ischemic myocardium and regulates coronary resistance. Therefore, we tested the effects of inhibiting NO synthase, cyclooxygenase, and K\textsuperscript{+} channels on the changes in diameter and membrane potential (Em) in response to BK in isolated human coronary microvessels.

**Methods and Results**—Arterioles (97 ± 4 μm; n = 120) dissected from human right atrial appendages (n = 78) were cannulated at a distending pressure of 60 mm Hg and zero flow. Changes in vessel diameter (video microscopy) and VSMC Em (glass microelectrodes) were measured simultaneously. In vessels constricted and depolarized (Em; −50±3 to −28±2 mV) with endothelin-1 (ET), dilation to BK was associated with greater membrane hyperpolarization (−48±3 mV at 10\textsuperscript{−6} mol/L) than dilation to sodium nitroprusside (SNP) (−34±2 mV at 10\textsuperscript{−4} mol/L) for similar degrees of dilation. Treatment with N\textsuperscript{ω}-nitro-L-arginine methyl ester (L-NAME; 10\textsuperscript{−4} mol/L), an NO synthase inhibitor, partially decreased dilation to BK (maximum dilation 61±10% versus control 92±4%; P < 0.05). Charybdotoxin (CTX; 10\textsuperscript{−8} mol/L), a small-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel blocker, or apamin (10\textsuperscript{−6} mol/L), a small-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel blocker, inhibited both dilation (CTX 22±6% and apamin 45±10% versus control 69±6%; P < 0.05) and membrane hyperpolarization (CTX −31±2 mV and apamin −37±2 mV versus control −44±2 mV; P < 0.05) to BK, whereas glibenclamide (10\textsuperscript{−6} mol/L), an ATP-sensitive K\textsuperscript{+} channel blocker, was without effect.

**Conclusions**—Vasodilation of human coronary arterioles to BK is largely dependent on membrane hyperpolarization by Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel activation, with apparently less of a role for endothelium-derived NO. This suggests a role for K\textsuperscript{+} channel activation in regulating human coronary arteriolar tone. (Circulation. 1999;99:3132-3138.)

**Key Words:** bradykinin □ nitric oxide □ endothelin □ vasodilation □ potassium

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Bradykinin (BK) is a potent endogenous vasodilator that stimulates endothelial B\textsubscript{2} receptors, leading to release of a variety of vasoactive substances.\textsuperscript{1,2} Studies in vivo in humans suggest that BK contributes to basal and flow-mediated vaso-motor responses in the coronary circulation.\textsuperscript{3} BK may also mediate vasodilation of resistance arteries to ischemia in the canine heart.\textsuperscript{4} Thus, BK may play an important role in regulation of the coronary microcirculation in both physiological and diseased states. However, the mechanism of vasodilation to BK in humans is not understood.

Endothelial cells contribute to regulation of vascular tone by releasing ≥3 vasoactive compounds: nitric oxide (NO), prostacyclin (PGI\textsubscript{1}), and endothelium-derived hyperpolarizing factor (EDHF).\textsuperscript{5-7} Although NO is often responsible for conduit artery dilation in some species and vascular beds, EDHF predominates in smaller arterioles.\textsuperscript{8} Hyperpolarization and BK-induced vasorelaxation have been shown in human conduit arteries.\textsuperscript{9} However, the relative role of NO and EDHF in dilation of human coronary resistance vessels is not known.

The objectives of the present study were to determine whether human coronary arteriolar vasodilation to BK is dependent on membrane hyperpolarization of vascular smooth muscle cells (VSMCs) and to elucidate the contributions of NO and K\textsuperscript{+} channels.

**Methods**

**General Preparation**
All protocols were approved by The University of Iowa and the Medical College of Wisconsin committees on the use of human subjects in research. Human coronary arterioles were dissected from human right
atrial appendage tissue obtained from 78 patients (56±17 years of age; 57 men) undergoing valve replacement (aortic, n=6; mitral, n=1) and/or coronary bypass surgery (n=67). Baseline demographic data are summarized in Table 1. After surgical removal, tissue was placed in Krebs buffer solution (4°C) with the following composition (in mmol/L): NaCl 118, KCl 4.7, CaCl2 2.5, KH2PO4 1.2, MgSO4 1.2, NaHCO3 20, Na2EDTA 0.026, and dextrose 11, pH 7.4. Coronary arterioles were followed by a Bonferroni corrected t test when significant differences were noted. To compare sensitivities of the agents used, ED90 values (negative logarithm of the molar concentration of vasodilator that produced 90% of the maximal dilation to the agonist) were calculated. Percent maximal dilations and ED90 values were compared by ANCOVA. This approach optimizes our ability to infer whether the models can be generalized to nondiseased human arterioles. Although the design incorporated blinded assignment of vessels to specific treatment protocols, we used stepwise multiple regression analysis to assess whether coronary artery disease (CAD) or risk factors for CAD, age, or sex influenced the vasodilator response. If such a factor was found, our plan was to detect any independent influence using ANCOVA. This approach optimizes our ability to infer whether the models can be generalized to nondiseased human arterioles.

Results

Human right atrial appendages were obtained from 78 patients. One hundred twenty atrial vessels (mean internal diameter of 97±4 μm) were studied at a pressure of 60 mm Hg in Krebs buffer. Patient demographics, including diagnoses, are summarized in Table 1.

Figure 1 shows vasodilator responses to BK and SNP in human coronary arterioles. Both agents produced potent concentration-dependent dilations in vessels constricted with endothelin-1 (maximum dilation: BK 91±2% and SNP 100±0% by definition).

Measurement of Membrane Potential

In separate experiments, we simultaneously examined steady-state changes in vessel diameter and VSMC membrane potential (Em) in response to BK and sodium nitroprusside (SNP), as described previously. Em was measured with glass microelectrodes (impedance of 50 to 100 MΩ; tip potential ≤5 mV) filled with 3 mol/L KCl and connected to a high-impedance amplifier (Axo-clamp). The microelectrode was advanced through the adventitial side of the vessel (manual microdrive) in 0.5-μm increments while tip potential was monitored.

Criteria for successful impalements included an abrupt drop in potential to a new steady-state value for ≥10 seconds and a sudden return to the original baseline when the electrode was pulled from the VSMC. Multiple successful impalements of ≥3 distinct VSMCs were averaged to obtain each reported Em measurement.

Changes in vascular diameter and Em were measured simultaneously to elucidate the relationship between vasodilation and membrane hyperpolarization. At the end of each experiment, maximal dilatation was determined by SNP (10−6 mol/L).

Materials

Endothelin-1 was obtained from Peninsula Laboratories, Inc and was prepared in saline with 1% BSA. All other reagents were obtained from Sigma Chemical Co. Glibenclamide was prepared in 100% DMSO and diluted in saline. INDO was dissolved in saline with 1.0N NaOH, and pH was adjusted with 0.1N HCl to 7.4. Other agents were prepared in distilled water. Final molar concentrations in the organ chambers are reported. The addition of pharmacological agents produced ≤1% change in the volume of the circulating bath.

Statistical Analysis

Results are expressed as percent dilation, with 100% representing the change from constricted diameter with endothelin-1 or KCl to the diameter in the presence of SNP (10−6 mol/L). The diameter in the presence of this dose of SNP was similar to the maximal diameter achieved during the experiment (pressurized at 60 mm Hg, at a temperature of 20°C, early during incubation). Comparisons of percent vasodilation under different treatments were performed with 2-factor repeated-measures ANOVA with proc mixed modules in SAS version 6.2 with autoregressive covariance assumptions. Em values were compared with a 1-factor repeated-measures ANOVA. Both computations were followed by a Bonferroni corrected t test when significant differences were noted. To compare sensitivities of the agents used, ED90 values (negative logarithm of the molar concentration of vasodilator that produced 90% of the maximal dilation to the agonist) were calculated. Percent maximal dilations and ED90 values were compared by Bonferroni’s paired t test. Simple linear regression analysis was used to evaluate the relationship between vasodilation and hyperpolarization. Statistical significance was defined as P<0.05. All data are presented as mean±SEM; n indicates the number of patients.

Experimental Protocols

After a 60-minute stabilization period, vessels were constricted to 30% to 60% of resting diameter with endothelin-1. Vascular responses to cumulative logarthmic increases in the concentration of BK (10−14 to 10−6 mol/L) in the external bathing media were examined in the presence and absence of N-nitro-l-arginine methyl ester (L-NAME; 10−4 mol/L), a NO synthase inhibitor, alone or together with indomethacin (INDO; 10−3 mol/L), a cyclooxygenase inhibitor.

To test for the role of potassium channels, some vessels were constricted with KCl (45 mmol/L) instead of endothelin-1 or treated with tetraethylammonium chloride (TEA; 10−3 mol/L), a relatively selective blocker of large-conductance Ca2+-activated K+ channels (BKCa); tetrabutylammonium chloride (TBA; 10−3 mol/L), a distinct and less-selective K+ channel blocker; glibenclamide (10−6 mol/L), a selective blocker of ATP-sensitive K+ channels (Kir6.2); charybdotoxin (CTX; 10−4 mol/L), a selective blocker of BKCa; apamin (10−5 mol/L), a selective blocker of small-conductance Ca2+-activated K+ channels (SKCa); and 30 minutes before application of BK. All studies in this group of experiments, except those in which KCl was substituted for endothelin, were performed in the presence of L-NAME and INDO.
Because hyperpolarization may contribute to vasodilation, we examined the change in membrane potential (Em) to both BK and SNP. A typical tracing of Em in response to BK is shown in Figure 2A. In the absence of preconstriction, BK (10^{-6} mol/L) produced a transient small depolarization (−36 to −30 mV) followed by a larger and more sustained membrane hyperpolarization (−30 to −46 mV). In a separate example, after constriction and depolarization with endothelin-1, BK (10^{-6} mol/L) evoked a potent vasodilation (81%) with an associated decrease in Em (−20 to −45 mV) that was greater in magnitude than that observed without constriction (Figure 2B). This type of recording was possible in only a few cases because of vessel motion with change in diameter. Figure 2C shows a series of VSMC impalements in the presence of increasing doses of BK in a single arteriole. BK hyperpolarized and dilated this vessel in a concentration-dependent manner. The delay from application of BK to the onset of hyperpolarization approximates the delay of fluid pumped from the buffer reservoir, where drugs are added, to the vessel chamber.

Figure 3 summarizes responses to BK and SNP. Both agents simultaneously dilated and hyperpolarized vessels, although the magnitude of hyperpolarization was less with SNP (versus BK, P<0.05 at highest dose). Vasodilator responses correlated closely with changes in Em; however, the relationship was steeper for BK (Figure 4) than for SNP (BK versus SNP, P=0.0024). These findings indicate that in contrast to SNP, dilation to BK depends largely on membrane hyperpolarization, which implies that EDHF plays an important role in human coronary microvascular responses to BK.

In the presence of L-NAME (10^{-4} mol/L), vasodilation to BK was modestly but significantly decreased (maximum dilation 61±10% versus control 92±4%, P<0.05; ED50 9.7±0.8 versus control 9.8±0.4, P=NS; n=6). Addition of INDO to L-NAME produced no further reduction in dilation to BK (maximum dilation 77±7% versus control 95±1%; P<0.05 versus no inhibitors) (Figure 5).

Figure 6 summarizes changes in Em and diameter to BK in the presence and absence of L-NAME plus INDO. Although vasodilation was reduced after L-NAME plus INDO (60±5% versus control 82±7%; P<0.05), hyperpolarization was not. These results indicate that a small portion of the dilation to

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**Figure 1.** Human coronary arteriolar dilation to BK and SNP. BK and SNP produced potent dilation in a concentration-dependent manner in human coronary arterioles constricted with endothelin-1. ED50 was 10.0±0.3 in BK (n=25) and 7.1±0.2 in SNP (n=20). Values in this and subsequent figures represent mean±SEM.

**Figure 2.** Effect of BK and SNP on membrane potential (Em) and vascular diameter. A, Application of BK (10^{-6} mol/L) to superfusate reservoir induced deep and prolonged membrane hyperpolarization after modest and transient membrane depolarization in absence of vasoconstriction. B, After membrane depolarization and vasoconstriction with endothelin-1, BK induced rapid membrane hyperpolarization and vasodilation. C, Membrane hyperpolarizations to BK were concentration-dependent.

**Figure 3.** Summary of simultaneous changes in membrane potential and vascular diameter in response to vasodilators. A, BK induced membrane hyperpolarization and vasodilation in a concentration-dependent manner in vessels constricted with endothelin-1 (n=5). B, SNP produced potent dilation but with only modest membrane hyperpolarization (n=6). #P<0.05 vs endothelin-1.
BK is mediated primarily by NO, although NO does not contribute to the associated membrane hyperpolarization.

EDHF-induced dilation typically is mediated by activation of K<sup>1</sup> channels in VSMCs. Extraluminal KCl (45 mmol/L), which prevents membrane hyperpolarization via flux through K<sup>1</sup> channels, diminished vasodilation to BK (Figure 7; maximum dilation 33±15% versus control 92±4%; P<0.05). In this protocol, L-NAME and INDO were not included in the bathing solution. This result is consistent with an important role for K<sup>1</sup> channels in vasodilation and hyperpolarization to BK.

We tested the effect of several K<sup>1</sup> channel-blocking agents on dilation to BK (Table 2). In the presence of L-NAME plus INDO, TEA, a relatively selective blocker of BK<sub>Ca</sub>, attenuated coronary arteriolar dilation to BK, whereas TBA, a blocker of both BK<sub>Ca</sub> and SK<sub>Ca</sub>, reduced dilation even more. Glibenclamide, a selective blocker of K<sub>ATP</sub>, did not alter dilation to BK. CTX, a chemically distinct and more selective blocker of BK<sub>Ca</sub>, markedly reduced dilation to BK. Apamin, a selective blocker of SK<sub>Ca</sub>, decreased the ED<sub>50</sub> value. In addition to inhibiting the maximal dilation to BK, combined treatment with CTX and apamin or use of apamin alone reduced ED<sub>50</sub> (Table 2). In vessels from 6 patients, CTX was administered in the absence of L-NAME and INDO. As seen in Figure 8, CTX alone markedly attenuated dilation to BK (P<0.05).

To link the effects of K<sup>1</sup> channel blockers on diameter with changes in Em, we performed separate studies in which both were recorded simultaneously (Figure 9). Treatment with L-NAME plus INDO had no effect on resting Em or vascular tone. BK produced dilation (69±6%) and membrane hyperpolarization. The inhibitory effect of CTX (22±6% versus control; P<0.05) or apamin (45±10% versus control; P<0.05) on vasodilation to BK was associated with a parallel reduction in membrane hyperpolarization, whereas glibenclamide (66±7% vasodilation) did not affect either parameter. Therefore, Ca<sup>2+</sup>-activated K<sup>1</sup> channels play a key role in dilation to BK, inducing membrane hyperpolarization in VSMCs in human coronary resistance vessels.

Presence of disease and age and sex of the patients posed no significant influence on vasodilation in these experiments. However, all but 10 patients had CAD, and only 6 had no preexisting conditions. This limits the degree to which external validity regarding the influence of CAD can be imputed from the results.

**Discussion**

This study is the first to determine the role of vascular smooth muscle hyperpolarization in dilation of human coronary arterioles to BK. The major new findings are 3-fold. First, vasodilation of human coronary arterioles to BK is dependent on membrane hyperpolarization than was dilation to SNP. #P=0.0024 vs SNP.

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membrane hyperpolarization of VSMCs, largely through mechanisms other than activation of NO synthase and cyclooxygenase. Second, activation of both BK<sub>Ca</sub> and SK<sub>Ca</sub> channels contributes to membrane hyperpolarization and relaxation. Third, NO-induced dilation of human coronary arterioles is associated with much less membrane hyperpolarization than is dilation to BK. Taken together, these findings suggest that EDHF plays an important role in regulating human coronary arteriolar tone. These conclusions are confined to atrial arterioles; ventricular vessels may respond by different mechanisms.

**Role of NO and Cyclooxygenase in Vasodilation to BK**

In animal models, activation of endothelial B<sub>2</sub> receptors stimulates NO synthase and phospholipase A<sub>2</sub> to release NO and vasoactive metabolites of arachidonic acid, respectively. NO plays a major role in endothelium-dependent vasodilation in conduit vessels, whereas factors other than NO contribute more to the microvascular response in perfused rat hearts, intact canine hearts, and isolated coronary arterioles. This is consistent with reports that endothelial NO synthase activity is lower in resistance arteries than in conduit arteries.

NO synthase inhibitors only modestly reduce vasodilation to endothelium-dependent agents in human coronary arteries. Acetylcholine-induced increases in coronary flow in patients are also independent of NO. These findings are consistent with those of the present study suggesting that factors other than NO play a prominent role in endothelium-dependent vasodilation to BK in human coronary arterioles. However, this conclusion is made indirectly, because it is not possible to measure NO release in individual microvessels. Furthermore, the conclusion is based on the efficacy of L-NAME in inhibiting NO synthase. Although higher doses may be necessary in some preparations, in our laboratory this same dose inhibits human coronary arteriolar dilation to adrenomedullin by 50% (unpublished observations).

In the present study, addition of INDO, a cyclooxygenase inhibitor, to L-NAME did not further reduce vasodilation to BK (data not shown; n = 3). This is consistent with reports that cyclooxygenase-derived vasoactive substances such as PGI<sub>2</sub> do not contribute to endothelium-dependent vasodilation in coronary arteries from animals and humans.

**Role of K<sup>+</sup> Channel Activity**

The portion of the endothelium-dependent vasodilation resistant to inhibition of NO synthase and cyclooxygenase is...
thought to be mediated by an endothelium-derived substance that activates K⁺ channels, hyperpolarizes VSMCs, and has been termed EDHF. Kemp and Cockshott and Ohlmann et al reported a prominent contribution of K⁺ channel activation to BK-induced vasorelaxation in human coronary arteries. In the present study, inhibition of changes in membrane potential with exogenous KCl markedly diminished vasodilation to BK in human coronary resistance arteries, implying that K⁺ channels play a critical role.

We demonstrated that TBA, a blocker of BKCa and SKCa, reduces vasodilation to BK more than does TEA, which is primarily a blocker of BKCa channels. Furthermore, CTX or apamin decreased sensitivity, whereas their combination reduced both maximal response and sensitivity. However, glibenclamide, a selective blocker of KATP channels, had no effect. These results are consistent with those of Dong et al, who demonstrated that vasorelaxation of rabbit carotid artery to acetylcholine is completely inhibited by CTX and is partially inhibited by apamin. A similar role for CTX and apamin-sensitive potassium channels in vasorelaxation to BK and acetylcholine has been demonstrated in a variety of other models. These findings are consistent with our observations that both BKCa and SKCa K⁺ channels contribute to BK-induced vasodilation in human coronary arterioles.

Membrane Hyperpolarization and Vasodilation
EDHF has been defined pharmacologically as endothelium-dependent vasorelaxant resistant to inhibition of NO synthase and cyclooxygenase. A limitation of this definition is the failure to include frank hyperpolarization in association with vasodilation. In the present study, we demonstrated that BK-induced changes in membrane potential correlate with endothelium-dependent vasodilation in human coronary arterioles, consistent with a role for EDHF.

NO can activate a variety of K⁺ channels in VSMCs from conduit arteries of several species. In contrast, no role was seen for NO in the Em change to endothelium-dependent vasodilators in canine and rat mesenteric arteries or in rabbit cerebral arteries. We demonstrated directly that exogenous NO (SNP) induces minor membrane hyperpolarization despite significant dilation. This is consistent with other reports that high extracellular concentrations of KCl do not change vasodilation to exogenous NO in human coronary conduit arteries. In the present study, a portion of the dilation to BK was blocked by L-NAME, although no effect on membrane potential was observed. This suggests minimal contribution of membrane hyperpolarization to the vasodilation evoked by NO, although it should be considered that the dose of L-NAME may not have been sufficient to alter membrane potential.

Potential Limitations
Potassium channel blockers may act on the endothelium as well as vascular smooth muscle, potentially blocking the release or synthesis of relaxing factors. Ishizaka and Kuo showed that KATP channels in porcine coronary arteriolar endothelial cells are important in dilation to hyperosmolarity. However, there are no investigations of the effect of K⁺ channel blockers on the endothelial synthesis or release of EDHF. Increases in endothelial K⁺ efflux and [Ca²⁺], to shear stress are not altered by K⁺ channel-blocking agents in endothelial cells of bovine aorta and calf pulmonary arteries. Consistent with these results, KCl does not alter the release of PGI₂ or NO to BK in endothelial cells from bovine aorta. This finding suggests that endothelial metabolism of arachidonic acid or EDHF-mediated vasodilation to BK is not affected by blocking endothelial K⁺ channels. Nevertheless, we have not excluded a potential role for endothelial K⁺ channels in the dilation to BK.

This study found a limited contribution of NO to BK-induced dilation in human coronary arteries. Because all but 6 patients had CAD or coronary risk factors, we cannot exclude the possibility that the presence of conduit coronary atherosclerosis reduced the contribution by NO to coronary vasomotor response. However, inhibition of NO synthase in patients with angiographically normal coronary arteries does not affect basal or stimulated coronary blood flow responses to acetylcholine, suggesting an important role for other factors such as EDHF in the normal human coronary microcirculation. Analysis of risk factors for CAD showed no influence of hypertension, hypercholesterolemia, diabetes mellitus, sex, or age on vasodilator or membrane potential responses.

A limitation of the present study is the lack of a true control group, because healthy individuals do not undergo cardiopulmonary bypass. We attempt to deal with this limitation in a statistical fashion, identifying the influence of individual risk factors; however, conclusions are limited to patients with heart disease, and postulations about normal human coronary physiology are inferential.

Clinical Implications
Studies in both animals and humans demonstrate that diabetes mellitus, hypertension, hypercholesterolemia, and atherosclerosis reduce endothelium-dependent vasodilation by impairment of NO-mediated mechanisms. This could be explained by the demonstration that in disease states, hyperpolarizing mechanisms may be preserved or even play an enhanced role in regulating vascular tone. Thus, the observation of a prominent role for EDHF in human coronary arteriolar dilation may be a reflection of normal physiology or a compensatory response to loss of NO.

In summary, vasodilation of human coronary arterioles to BK is largely dependent on membrane hyperpolarization, whereas vasodilation to exogenous NO is less dependent. The vasodilator response to BK is mediated largely by activation of large- and small-conductance Ca²⁺-activated K⁺ channels, with a lesser contribution by NO. These findings indicate a prominent role for EDHF in regulating human coronary arteriolar tone.

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