Agonist-dependent variability of contributions of nitric oxide and prostaglandins in human skeletal muscle

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Schrage, William G., Niki M. Dietz, John H. Eisenach, and Michael J. Joyner. Agonist-dependent variability of contributions of nitric oxide and prostaglandins in human skeletal muscle. J Appl Physiol 98: 1251–1257, 2005. First published November 24, 2004; doi:10.1152/japplphysiol.00966.2004.—The relative contributions of endothelium-dependent dilators [nitric oxide (NO), prostaglandins (PGs), and endothelium-derived hyperpolarizing factor (EDHF)] in human limbs are poorly understood. We tested the hypothesis that relative contributions of NO and PGs differ between endothelial agonists acetylcholine (ACh; 1, 2, and 4 μg·dl⁻¹·min⁻¹) and bradykinin (BK; 6.25, 25, and 50 ng·dl⁻¹·min⁻¹). We measured forearm blood flow (FBF) using venous occlusion plethysmography in 50 healthy volunteers (27 ± 1 yr) in response to brachial artery infusion of ACh or BK in the absence and presence of inhibitors of NO synthase [NOS; with N⁶-monomethyl-L-arginine (L-NMMA)] and cyclooxygenase (COX; with ketorolac). Furthermore, we tested the idea that the NOS + COX-independent dilation (in the presence of L-NMMA + ketorolac, presumably EDHF) could be inhibited by exogenous NO administration, as reported in animal studies. FBF increased ~10-fold in the ACh control; L-NMMA reduced baseline FBF and ACh dilation, whereas addition of ketorolac had no further effect. Ketorolac alone did not alter ACh dilation, but addition of L-NMMA reduced ACh dilation significantly. For BK infusion, FBF increased ~10-fold in the control condition; L-NMMA tended to reduce BK dilation (P < 0.1), and addition of ketorolac significantly reduced BK dilation. Similar to ACh, ketorolac alone did not alter BK dilation, but addition of L-NMMA reduced BK dilation. To test the idea that NO can inhibit the NOS + COX-independent portion of dilation, we infused a dose of sodium nitroprusside (NO-clamp technique) during ACh or BK that restored the reduction in baseline blood flow due to L-NMMA. Regardless of treatment order, the NO clamp restored baseline FBF but did not reduce the NOS + COX-independent dilation to ACh or BK. We conclude that the contribution of NO and PGs differs between ACh and BK, with ACh being more dependent on NO and BK being mostly dependent on a NOS + COX-independent mechanism (EDHF) in healthy young adults. The NOS + COX-independent dilation does not appear sensitive to feedback inhibition from NO in the human forearm.

brachial artery; intra-arterial infusion; human endothelium; cyclooxygenase

STUDIES IN ISOLATED BLOOD vessels from various species have long shown that stimulation of the endothelium may release several dilating substances from the vascular endothelium, including vasodilating prostaglandins (PGs) and endothelial-derived hyperpolarizing factor (EDHF), as well as nitric oxide (NO). The contributions of NO, PGs, and EDHF mediating endothelium-dependent dilation are difficult to define, as their importance may vary by vessel type and size, by disease state, and by the agonist chosen to stimulate the endothelium (6, 22, 30–32). For example, exposure to a single endothelial agonist may suggest “normal endothelial function,” and a second may not (11). Alternatively, normal function may be mediated by lesser or greater relative contributions of specific vasodilator mechanisms. Specifically, hypertensive rats exhibit normal dilation to acetylcholine (ACh) but mediate the dilation primarily through EDHF instead of NO (29). Thus agonist and inhibitor selection and study tissue are important points to consider for appropriate physiological testing and interpretation of endothelial function.

Taken together, the relative contributions of NO, PGs, and EDHF in human limbs are uncertain, due to considerable variability that seems to depend in large part on the pharmacological approach to the question (7, 16, 17, 20, 34). Another important consideration is that these dilator systems may interact in a redundant fashion.

A redundancy in endothelial dilator mechanisms, or interaction, is demonstrated in cultured endothelial cells, where inhibition of either NO synthase (NOS) or cyclooxygenase (COX) enzymes causes increased production of the nonblocked substance (26). In complex tissues like human limbs, one might overestimate the contribution of NO or PGs due to the redundancy of the other mechanisms in endothelium-dependent dilation. Alternatively, the relative importance of EDHF in mediating dilation may be overestimated during combined NOS and COX inhibition due to the normal suppression of the EDHF effects when NOS activity is intact (2, 24).

We are not the first to indirectly assess a role for EDHF in human skeletal muscle circulation. To date, reports in humans attempt to inhibit EDHF with inhibition of either a cytochrome P-450 or calcium-sensitive potassium (KCa) channels (16–18, 28). These approaches are based on evidence that the primary human EDHF is an arachidonic acid metabolite (epoxyeicosatrienoic acid) that hyperpolarized via KCa channels (6). Some reports support a role for this EDHF, but a definitive role is lacking and seems to depend, in part, on the agonist tested (8, 16–18, 28). Additionally, EDHF may be a class of substances, including K+ (H2O2, and even NO (6). Thus even “specific” EDHF inhibitors may be limited in defining a role for EDHF in human endothelial control of muscle blood flow. Until the identities of EDHFs are well defined in human muscle, we used the common approach to assume that the NOS + COX-resistant portion of agonist-mediated dilation was due to EDHF.

We, therefore, sought to compare the relative contributions of NOS, COX, and EDHF (by process of elimination) pathways in the forearm of healthy young adults. We tested the
primary hypothesis that the relative contributions of these three systems are specific to each endothelial agonist [ACH vs. bradykinin (BK)], and the secondary hypothesis that any agonist-mediated dilatation remaining after NOS + COX inhibition would be suppressed further by addition of exogenous NO, providing indirect evidence that the remaining NOS + COX-independent dilatation was due to “NO-suppressible” release of EDHF.

MATERIALS AND METHODS

All protocols and procedures were approved by the Institutional Review Board at the Mayo Clinic. Each subject provided his or her written, informed consent before participation in this study.

Subjects

Fifty healthy volunteers (32 women and 18 men), ranging in age from 19 to 39 yr, with average levels of physical fitness, were studied. Subjects were excluded for hypertension, hyperlipidemia, smoking, heart disease, and diabetes. All female subjects were not pregnant and were examined during the early follicular phase of their menstrual cycle (or placebo phase of oral contraceptives) to minimize effects of gender-specific hormones. Subjects reported to the laboratory after an overnight fast. Those studied in the afternoon ingested a light meal ≥3 h before the study. All subjects refrained from exercise, alcohol, caffeine, and any prescription or nonprescription drugs for 24 h before the study.

Brachial Artery Catheterization

Catheterization was done under aseptic technique after infiltration of the area with 1–2 ml of 1% lidocaine. A standard 5-cm 20-gauge Teflon catheter was inserted into the nondominant arm and continuously flushed with heparinized saline (2 U/ml, 3 ml/h). The brachial artery was catheterized for ~4 h in all of the subjects.

Measurement of Forearm Blood Flow

Forearm blood flow (FBF) was measured by venous occlusion plethysmography, using a standard mercury-in-Silastic strain gauge (15). Blood flow is expressed in milliliters per 100 milliliters forearm volume per minute (ml·100 ml⁻¹·min⁻¹).

Drug Administration

Drugs were administered via the brachial artery catheter using a three-port connector system that permitted simultaneous measurements of arterial pressure during drug infusions. All drugs were diluted in saline immediately before use. ACh (Michol-E, Novartis) and BK (Aerbio/Clinalfa) were dissolved in standard concentrations and infused at rates normalized to forearm volume as determined by water displacement. Pump infusion rates varied from 1.5 to 3.38 ml/min, depending on forearm volume, and saline infusion at these rates did not alter basal blood flow. Nω-nitro-L-arginine (L-NMMA; Aerbio/Clinalfa) was infused at 5 mg/min for 10 min (50-mg loading dose), followed by an infusion of 1 mg/min for the remainder of the experiment (maintenance dose). In three subjects infused with ACh, we infused Nω-nitro-L-arginine methyl ester (LNAME; Aerbio/Clinalfa) instead of L-NMMA to qualitatively compare efficacy of NOS inhibition. Ketorolac (Abbott) was infused (150 µg/min for 20 min, for a total dose of 300 µg/dl forearm) (36). Ketorolac was used because a recent production limit of indomethacin caused our institution to restrict the use of parenteral indomethacin to therapeutic interventions. In clinical and animal studies, ketorolac is several hundredfold more effective than aspirin and up to fivefold more effective than indomethacin in blocking the COX pathway (5, 14).

Restoration of Baseline Blood Flow With the “NO Clamp” After L-NMMA Treatment

Before the subjects received L-NMMA, FBF was measured during infusion of very small doses of NTP to increase basal FBF by 30–50%. The NTP level ranged from 2 to 15 ng·dl⁻¹·min⁻¹ between subjects. The goal was to determine a dose of NTP for infusion after L-NMMA to provide a “basal” level of NO to restore baseline blood flow to pre-L-NMMA values. This technique was previously reported as the NO-clamp technique (17).

Experimental Design

The protocols of this study were designed to systematically test whether NOS, COX, and EDHF pathways interact to cause endothelium-dependent vasodilation and whether NO exerts feedback inhibition of EDHF (assumed to be the portion of vasodilation resistant to combined NOS and COX inhibition). FBF was measured in response to four consecutive dose-response curves of ACh (1, 2, and 4 µg·100 ml⁻¹·min⁻¹) or BK (6.25, 25, and 50 ng·100 ml⁻¹·min⁻¹). In all studies, before and after the repeated exposures to ACh or BK, FBF responses to sodium nitroprusside (NTP, 0.25, 0.5, 1, and 2 µg·100 ml⁻¹·min⁻¹) were assessed to ensure that smooth muscle responsiveness to NO was not altered by repeated exposure to ACh, BK, or treatment with inhibitors of NOS and COX.

Control protocols. For control protocols, n = 22 subjects, 10 men and 12 women. Because our main protocols used repeated FBF

Fig. 1. Forearm blood flow (FBF) responses to brachial artery infusion of acetylcholine (ACH; 1–8 µg·dl⁻¹·min⁻¹). Infusion of ACh caused a dose-dependent dilatation, and this was blunted by Nω-nitro-L-arginine (L-NMMA) (A) but not ketorolac (B). Addition of exogenous nitric oxide (NO) [sodium nitroprusside (NTP)] restored baseline blood flow but did not blunt the ACh dilator response in the presence of L-NMMA + ketorolac. Values are means ± SE. Braces indicate significant differences between inhibitor treatments on the FBF response to ACh (P ≤ 0.05).
ACh was followed by ACh in the presence of either L-NMMA or ketorolac. Values are means ± SE. Braces indicate significant differences between inhibitor treatments on the FBF response to BK (P < 0.05). Addition of ketorolac did not alter the FBF response to ACh further (P > 0.5). When the order of inhibitors was reversed, addition of ketorolac alone did not alter the mean ACh-mediated dilation (P > 0.6).

Control Experiments

Four consecutive brachial artery infusions of ACh demonstrated that FBF responses were similar (interaction effect P > 0.7). For BK, four infusions produced similar FBF responses (P > 0.4). The NO clamp did not alter the dilator response to ACh, nor did it alter the dilator response of ACh in the presence of L-NMMA (P > 0.3). To keep the number of subjects studied manageable, we assumed that low-dose NTP has similar effects on BK-induced dilation, because a previous study showed that the NO clamp in the presence of L-NMMA had no effect on BK-mediated dilation (17).

Protocol I: Effects of Combined NOS and COX Inhibition on Dilator Responses to ACh

FBF increased in a dose-dependent manner to ACh (P < 0.0001). Addition of L-NMMA alone reduced the ACh response by ~50% (P < 0.01, Fig. 2A). Addition of ketorolac did not alter the FBF response to ACh further (P > 0.5). When the order of inhibitors was reversed, addition of ketorolac alone did not alter the mean ACh-mediated dilation (P > 0.6).

RESULTS

Subject Characteristics

Subject characteristics are summarized in Table 1. Subjects from each protocol exhibited similar characteristics. Because no discernable difference between genders was apparent, all subjects’ data were pooled.

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Addition of L-NMMA caused a significant reduction in the FBF response ($P < 0.02$; Fig. 2B).

**Does the NO Clamp After L-NMMA + Ketorolac Blunt the Remaining ACh-induced Dilation?**

Addition of the NO clamp restored baseline blood flow (Table 2) and surprisingly restored the ACh response to control conditions ($P > 0.7$) after double blockade with L-NMMA + ketorolac when L-NMMA was the first inhibitor infused (Fig. 2A). However, when L-NMMA was replaced by L-NAME ($n = 3$, not shown), or when ketorolac was infused first (Fig. 2B), the NO clamp did not alter the FBF response to ACh in the presence of NOS and COX inhibition ($P > 0.3$). Thus, in contrast to our hypothesis, the NO clamp did not suppress the ACh-mediated dilation that was measured after combined administration of L-NMMA plus ketorolac.

**Protocol II: Effects of Combined NOS and COX Inhibition on Dilator Responses to BK**

FBF increased in a dose-dependent manner to BK ($P < 0.0001$). Addition of L-NMMA tended to reduce the BK response ($P < 0.1$), and addition of ketorolac significantly blunted this response compared with control ($P < 0.03$, Fig. 3A). When inhibitor order was reversed, addition of ketorolac did not change the FBF response to BK ($P > 0.5$, Fig. 3B), whereas addition of L-NMMA reduced the FBF response to BK ($P < 0.02$).

**Does the NO Clamp After L-NMMA + Ketorolac Blunt the Remaining BK-induced Dilation?**

Addition of the NO clamp restored baseline blood flow (Table 2). The NO clamp did not alter the FBF response to BK in the presence of L-NMMA + ketorolac when L-NMMA was the first inhibitor infused ($P > 0.5$). When ketorolac was infused first, the NO clamp shifted the FBF response to BK ($P < 0.01$, Fig. 3), which was similar to control responses ($P > 0.3$). Thus, in contrast to our hypothesis, the NO clamp did not suppress the BK-mediated dilation that was measured after combined administration of L-NMMA plus ketorolac.

**Table 2. Baseline forearm blood flow in each protocol**

<table>
<thead>
<tr>
<th>Drug Order</th>
<th>Control</th>
<th>L-NMMA</th>
<th>Ketorolac</th>
<th>L-NMMA + Ketorolac</th>
<th>NO Clamp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine</td>
<td>2.5±0.5</td>
<td>1.5±0.2*</td>
<td>1.7±0.3*</td>
<td>1.8±0.2*</td>
<td>2.7±0.2</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>2.2±0.3</td>
<td>1.9±0.4*</td>
<td>1.4±0.2*</td>
<td>1.4±0.2*</td>
<td>2.3±0.4</td>
</tr>
</tbody>
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<tr>
<th>Drug Order</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine</td>
<td>2.4±0.3</td>
<td>2.4±0.3</td>
<td>1.8±0.2*</td>
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<td>2.5±0.4</td>
</tr>
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<td>2.7±0.3</td>
</tr>
</tbody>
</table>

Values are means ± SE in ml/100 ml⁻¹·min⁻¹. *Top shows drug order with N⁴-monomethyl-l-arginine (l-NAME) first; *bottom shows drug order with ketorolac first. Baseline forearm blood flow was similar between control trials. Nitric oxide (NO) synthase inhibition reduced forearm blood flow. The NO clamp restored baseline forearm blood flow to levels similar to control.

Does the NO Clamp After L-NMMA + Ketorolac Blunt the Remaining BK-induced Dilation? (Table 2) and surprisingly restored the ACh response to control conditions ($P > 0.7$). However, when L-NMMA was replaced by L-NAME ($n = 3$, not shown), or when ketorolac was infused first (Fig. 2B), the NO clamp did not alter the FBF response to ACh in the presence of NOS and COX inhibition ($P > 0.3$). Thus, in contrast to our hypothesis, the NO clamp did not suppress the ACh-mediated dilation that was measured after combined administration of L-NMMA plus ketorolac.

**Endothelium-independent Vasodilation**

All subjects dilated in a dose-dependent manner to both trials of NTP. The NTP response was similar ($P > 0.4–0.99$) before and after exposure to ACh or BK, regardless of the order of inhibitor treatment. These results suggest that the changes in FBF measured after administration of inhibitors and agonists were likely due to changes in endothelial vasodilator signals and not vascular smooth muscle responsiveness to dilator substances (Fig. 3).

**DISCUSSION**

The primary findings in these studies are that 1) ACh- and BK-induced dilation of the forearm are not significantly mediated by PGs; 2) NO mediates a large portion of dilation to ACh but not BK; 3) a small but significant interaction between NOS and COX is evident with BK; 4) combined inhibition of NOS and COX blocks up to 50% of the dilation to ACh and up to 30% of dilation to BK, suggesting that EDHF can account for at least 50% of dilation to ACh and BK; and 5) the NO clamp does not reduce NOS + COX-independent dilation to ACh or BK, suggesting that, if the remaining vasodilation is due to EDHF, the production or action of EDHF is not suppressed by NO in human forearm.

The FBF responses to ACh observed in this study are in line with previous studies in young healthy humans (10, 11, 23, 32,
Our results confirm that vasodilation to ACh is mediated in part by NO, as l-NMMA reduced FBF by about one-half (4, 12, 32). The lack of an effect of ketorolac on the FBF response to ACh is consistent with results using indomethacin (19, 33). Because ketorolac is at least as efficacious in inhibiting COX as indomethacin (14), the present results add to the growing body of evidence that normotensive young subjects do not produce significant amounts of vasoactive prostanoids, in contrast to hypertensive or older humans (33–35). An alternative explanation is that a balance exists between vasodilator and vasoconstrictor PGs, and COX inhibition does not alter this balance.

The fact that double blockade with l-NMMA + ketorolac produced similar relative reductions in FBF (Fig. 2) suggests that a third dilator pathway consistently mediates the remainder of ACh-induced dilation. We presumed, based on previous reports, that this dilation is mediated by EDHF (2, 7, 17, 24). Determination of the “normal” contribution of EDHF is complicated since EDHF may normally serve as a backup dilator mechanism when NO or PGs are compromised (2, 24, 29). In this context, use of a cytochrome P-450 inhibitor alone (micronazole or sulfaphenazole) to inhibit EDHF in human forearm showed no effect on ACh-induced dilation (13, 16, 18). Unfortunately, two of these studies (13, 16) did not test the effects of cytochrome P-450 inhibition in the presence of combined NOS and COX inhibition. Thus a reasonable hypothesis is that, during ACh stimulation, EDHF is “silent” in human forearm until NOS and COX are blocked simultaneously. This interpretation is consistent with animal data (2, 24). The other study, in Japanese subjects, showed no effect on ACh-mediated FBF with tetraethylammonium (TEA) alone or in combination with NOS + COX inhibition (18). An alternative explanation is that the EDHF substance produced by ACh is not the same as that produced by BK.

The present results do not support our secondary hypothesis, since restoration of basal blood flow with exogenous NO did not blunt the NOS + COX-independent increase in FBF to ACh or BK. These data contrast with results from animal studies (2, 24) and suggest that this interaction is not present in human forearm microcirculation. Our results confirm a lack of effect of the NO clamp on BK-induced dilation under double-blockade conditions (17) and extend the finding to endothelium-dependent dilation mediated via ACh. The NO clamp unexpectedly increased the FBF response to control levels while in the presence of NOS + COX inhibition (Fig. 2A). Even more unexpectedly, this was the case only when l-NMMA was administered first but not when ketorolac was first (Fig. 2B). The explanation for this is unclear, but we can conclude that exogenous NO infusion does not suppress the NOS + COX-independent dilation to ACh.

The control (no inhibitors) FBF responses to BK were similar to previous reports (4, 11, 16, 17, 27). Results with l-NMMA are in line with three previous reports (4, 17, 25), which show that NO plays no role or a minor role in BK-mediated dilation in human forearm (Fig. 2A), as l-NMMA displayed only borderline significant effects on BK dilation. However, one other study reported a significant reduction in BK-mediated vasodilation with l-NMMA (9). One main difference between our methods and those of Cockcroft et al. (9) is that they infused l-NMMA once they reached steady-state FBF with BK infusion, whereas we infused l-NMMA in a resting forearm. Take together, our findings are consistent with the majority of reports that point to a smaller portion of NO-mediated dilation to BK.

Dilator responses to BK during COX inhibition are consistent with results, using ACh, that PGs alone play little role in BK-mediated vasodilation. To our knowledge, prior studies of BK infusions have always used combined NOS and COX inhibition but not COX inhibition alone. However, one report of chronic (3 days) oral indomethacin (4) suggested that COX inhibition actually increased dilation to BK (and ACh). One possible explanation is that chronic COX inhibition causes a shift from balanced PG production to one primarily of vasoconstrictor PGs, like thromboxane A2.

Despite the differences in results with NOS inhibitors, responses to BK combined NOS + COX inhibition are consistent with the idea that some other substance (like EDHF) is responsible for the majority of the BK-induced vasodilation (16–18). Our data contrast with a previous report (17) that showed normal BK-mediated increases in FBF after infusion of l-NMMA and oral ingestion of carbasalate (to inhibit COX). One consideration is that intra-arterial ketorolac is a better inhibitor of COX than oral carbasalate. A second possible explanation for an apparent NOS-COX interaction in our results is that production of one signal compensates for the loss of the other (6, 26). Our results agree with a significant reduction in BK-mediated dilation after ibuprofen and l-NMMA (18). In previous work and the present study, it remains possible that EDHF is silent until NOS and COX are inhibited (13, 16), since these studies were not designed to directly inhibit the cytochrome P-450 pathway with and without combined NOS + COX inhibition. Reports using cytochrome P-450 inhibitors show no effect or modest reductions in BK dilation (16, 28). More consistent results have been reported using TEA to inhibit KCa channels, suggesting that TEA can attenuate the FBF response to BK (17, 18). Despite attempts to directly inhibit EDHF in human forearm, the exact identities and contributions of EDHF(s) in humans need further exploration.

To address our secondary hypothesis, we used the NO-clamp technique (17) to see if the NOS + COX-independent dilation to ACh or BK could be suppressed. We are limited in our interpretation because we did not use an inhibitor specific to EDHF. Inhibition of EDHF is a difficult problem to address, as the identity of EDHF may be different between species or type of arteries studied. Based on previous work in humans (3, 8, 16, 17, 21), whereas EDHF may be one or several factors mediating the NOS + COX-independent dilation to ACh and BK, our results suggest that exogenous NO at levels great enough to restore baseline blood flow do not reduce the ability of the forearm vessels to dilate.

Comparison of the present ACh and BK results supports our hypothesis that the contributions and interactions among NO, PGs, and EDHF are agonist specific in healthy humans. Primarily, it appears that ACh-mediated increases in FBF are more dependent on NO than BK, as l-NMMA blunts the ACh response more than the BK response. However, both ACh and BK appear resistant to COX inhibition, suggesting that PGs play a minor role in endothelium-dependent vasodilation in the forearm.
**Experimental Limitations**

Our findings should be considered in light of our main assumption that the portion of agonist-mediated increases in FBF after NOS and COX inhibition is due to EDHF. Our results may be significantly strengthened had we tested a role for EDHF with one of several reported EDHF inhibitors. Because EDHF is thought to be a cytochrome P-450 product, the most common approach is to use a sulfenazole or miconazole to inhibit cytochrome P-450 or to use TEA to inhibit KCa channels. If we assume that this is the only EDHF in human forearm, then we missed a potentially informative experiment in our approach. However, reports using this approach have reported no changes in FBF (16) or modest reductions in FBF (16, 17). Furthermore, a recent report using “triple blockade” with ibuprofen, L-NMMA, and sulfenazole had no effect on BK-induced dilation (28).

The methods and results from these studies highlight several important experimental considerations. First, an EDHF inhibitor alone may have no effect (16) due to the idea of redundancy between endothelial dilator systems. Second, miconazole may have direct effects on KCa channels (1), or perhaps a redundancy between endothelial dilator systems. Second, miconazole alone may have no effect (16) due to the idea of redundancy between endothelial dilator systems. If we assume that this is the only EDHF in human forearm, then we missed a potentially informative experiment in our approach. However, reports using this approach have reported no changes in FBF (16) or modest reductions in FBF (16, 17). Furthermore, a recent report using “triple blockade” with ibuprofen, L-NMMA, and sulfenazole had no effect on BK-induced dilation (28).

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