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Reactive Hyperemia Occurs via Activation of Inwardly-Rectifying Potassium Channels and Na⁺/K⁺-ATPase in Humans

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Abstract

Rationale—Reactive hyperemia (RH) in the forearm circulation is an important marker of cardiovascular health yet the underlying vasodilator signaling pathways are controversial and thus remain unclear.

Objective—We hypothesized RH occurs via activation of inwardly-rectifying potassium (K_{IR}) channels and Na⁺/K⁺-ATPase and is largely independent of the combined production of the endothelial autocooidsnitric oxide (NO) and prostaglandins (PGs) in young healthy humans.

Methods and Results—In 24 (23±1 years) subjects, we performed RH trials by measuring forearm blood flow (FBF; venous occlusion plethysmography) following 5 minutes of arterial occlusion. In Protocol 1, we studied 2 groups of 8 subjects and assessed RH in the following conditions; Group 1: control (saline), K_{IR} channel inhibition (barium chloride; BaCl₂), combined inhibition of K_{IR} channels and Na⁺/K⁺-ATPase (BaCl₂+ouabain, respectively), and combined inhibition of K_{IR} channels, Na⁺/K⁺-ATPase, NO and PGs (BaCl₂+ouabain+L-NMMA+ketorolac, respectively). Group 2 received ouabain rather than BaCl₂ in the 2nd trial. In Protocol 2 (n=8), 3 RH trials were performed: control, L-NMMA+ketorolac, and L-NMMA+ketorolac +BaCl₂+ouabain. All infusions were intra-arterial (brachial). Compared to control, BaCl₂ significantly reduced peak FBF (-50±6%; *P*<0.05) whereas ouabain and L-NMMA+ketorolac did not. Total FBF (area under curve) was attenuated by BaCl₂ (-61±3%) and ouabain (-44±12%) alone and this effect was enhanced when combined (-87±4%), nearly abolishing RH. L-NMMA+ketorolac did not impact total RH FBF prior to or after administration of BaCl₂+ouabain.

Conclusions—Activation of K_{IR} channels is the primary determinant of peak RH, whereas activation of both K_{IR} channels and Na⁺/K⁺-ATPase explains nearly all of total RH in humans.

Keywords

Blood flow regulation; vasodilation; ischemia; hyperpolarization

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Introduction

Following ischemia caused by temporary arterial occlusion, there is significant vasodilation and a rapid marked increase in blood flow in most tissues, including the human forearm¹. This phenomenon of reactive hyperemia (RH) is thought to occur as a result of myogenic and local metabolic or endothelial factors within the resistance vasculature and thus can be used as a test of microvascular function³. Attenuated RH responses have been documented in populations demonstrating a variety of risk factors that increase cardiovascular morbidity and mortality including hypertension⁴⁻⁷, atherosclerosis⁸, peripheral artery disease⁹, congestive heart failure¹⁰, and advanced age¹¹. Recently, peak RH flow was determined to be predictive of future cardiovascular events in a healthy population¹² as well as in at-risk patient populations¹³, and this predictive value may be greater than that of commonly assessed macrovascular function via flow-mediated brachial artery vasodilation^{12, 14}. Despite the utility of the RH test as a measure of vascular health, the underlying mechanisms of local vasodilation that contribute to this response in humans are largely unknown.

Given the strong associations between impaired RH, cardiovascular disease risk, and attenuated endothelial-dependent and metabolic vasodilation^{15, 16}, a variety of previous investigations in humans have attempted to determine the role of numerous endothelial-derived and metabolically-dependent substances or pathways involved in the response including nitric oxide (NO)^{8, 17-20}, prostaglandins (PGs)^{18, 21-23}, ATP-dependent potassium (K_{ATP}) channels^{17, 24} and adenosine^{22, 25, 26}. The results of these studies are largely equivocal and to date, even when the production or action of these substances are inhibited in combination^{17, 18, 22}, a significant portion of both the peak and total RH remains unexplained. There is growing interest in vasodilation that occurs via non-NO and -PG mechanisms due to hyperpolarization of endothelial and vascular smooth muscle cells^{27, 28}. Endothelial-derived hyperpolarization (EDH) can be broadly categorized into two groups: “classical” EDH associated with activation of calcium-activated potassium channels (K_{Ca}) and subsequent direct electrical communication or activation of inwardly-rectifying potassium (K_{IR}) channels and Na^+/K^+ -ATPase resulting in hyperpolarization of vascular smooth muscle cells and the second category of EDH, diffusible factors such as hydrogen peroxide (H_2O_2) and the cytochrome-P450 metabolites [e.g. epoxyeicosatrienoic acid (EET)]²⁹. In this context, we recently demonstrated the ability to block the local vasodilation to intra-arterial infusions of potassium chloride (KCl) via inhibition of K_{IR} channels and Na^+/K^+ -ATPase in the human forearm, and further have shown that these pathways can contribute to vasodilator responses to pharmacological stimulation of the endothelium³⁰ as well as increased metabolic demand³¹. Whether or not vascular hyperpolarization via K_{IR} channel and Na^+/K^+ -ATPase activation contributes to RH in humans has never been tested.

With this information as background, we directly tested the hypothesis that vascular hyperpolarization via K_{IR} channels and Na^+/K^+ -ATPase contributes to forearm RH following temporary arterial occlusion in healthy humans. Further, we sought to determine whether combined inhibition of NO and PGs attenuates reactive hyperemia, as several studies have demonstrated that individual inhibition of these endothelial pathways may not impact hyperemic responses to a variety of physiological stimuli, whereas combined inhibition can reveal a significant role in vascular control^{32, 33}.

Methods

A detailed and expanded Methods section is available in the online-only Supplemental Materials.

Subjects

With Institutional Review Board approval and after written informed consent, a total of 24 young healthy adults [18 men, 6 women; age=23±1 years (range:18-34 years); weight=73.1±1.5 kg; height=175±1 cm; body mass index=23.9±0.5 kg/m²; forearm volume (FAV)=945±39 ml; means±s.e.m.] participated in the present study. All studies were performed according to the *Declaration of Helsinki*.

Arterial catheterization, arterial blood pressure and heart rate

A 20 gauge, 7.6 cm catheter was placed in the brachial artery of the non-dominant arm under aseptic conditions after local anesthesia (2% lidocaine) for local administration of study drugs, blood sampling, and mean arterial pressure (MAP) measurement. Heart rate (HR) was determined using a 3-lead electrocardiogram (Cardiopap/5, Datex-Ohmeda Louisville, CO).

Forearm blood flow and vascular conductance

Forearm blood flow (FBF) was measured via venous occlusion plethysmography using mercury-in-silastic strain gauges and techniques as previously described³⁰. FBF was expressed as milliliters per deciliter of tissue per minute (ml/dl FAV/min). As an index of forearm vasodilation and to account for individual differences in baseline vascular tone, forearm vascular conductance (FVC) was calculated as (FBF/MAP) × 100 expressed as ml/dl FAV/min/100mmHg. Immediately following the release of the occlusion cuff for the RH (see below), the same cuffcycled between inflation at ~50 mmHg (4 seconds) and deflation (3 seconds) to cause venous occlusion and this yielded one blood flow measurement every 7 seconds for the first 56 seconds (8 flow measures). After 8 flow measures, the inflation:deflation cycle was changed back to 7:8 seconds, as was used at rest.

RH protocol

After measurement of baseline FBF, the cuff on the upper arm was rapidly inflated to 200 mmHg for 5 minutes of ischemia. This location and duration of ischemia was chosen to mimic the RH protocol utilized in investigations of the contributions of various endothelial-derived vasodilator pathways to the RH response^{18, 19, 22, 23} and importantly, has recently demonstrated peak RH flow to be more strongly associated with cardiovascular disease risk than measures of flow-mediated vasodilation¹². After 5 minutes, the cuff was rapidly deflated and flow measures commenced for 2.5 minutes (150 seconds). To determine the effect of repeated bouts of RH, 8 additional young subjects were instrumented non-invasively and underwent four successive bouts of RH separated by 20 minutes of rest (see Online Supplemental Materials for details).

Vasoactive drug infusion

All drug infusions were through the brachial artery catheter to create a local effect in the forearm, we recompleted during baseline measures prior to the arterial occlusion, and saline was utilized as a control infusate. Specific timing and duration of infusions is provided below in the *Experimental Protocols* section.

To inhibit K_{IR} channels, barium chloride (BaCl₂; K_{IR} channel inhibitor; 10% w/v BDH3238, EMD Chemicals, Gibbstown, NJ) was infused at 0.9 μmol/dl FAV/min within an

absolute range of 8 $\mu\text{mol}/\text{min}$ to 10 $\mu\text{mol}/\text{min}$ for five minutes prior to each arterial occlusion. To inhibit Na^+/K^+ -ATPase, ouabain octahydrate (Na^+/K^+ -ATPase inhibitor; Sigma 03125, St. Louis, MO) was infused at 2.7 nmol/min for 15 minutes prior to arterial occlusion^{30, 34}. On subsequent RH trials, ouabain was reinfused for 5 minutes prior to arterial occlusion to provide continuous inhibition. This approach of using BaCl_2 and ouabain to inhibit K_{IR} channels and Na^+/K^+ -ATPase, respectively, has been used previously by our group^{30, 35} and others^{36, 37}. We administered N^G -monomethyl-L-arginine (L-NMMA; NO synthase inhibitor; Clinalfa/Bachem, Weil am Rhein, Germany) to inhibit the production of NO in combination with ketorolac (non-selective cyclooxygenase inhibitor; Hospira, Lake Forest, IL) to inhibit the synthesis of PGs. The doses of L-NMMA and ketorolac were 5 mg/min and 1200 $\mu\text{g}/\text{min}$ respectively and given for 5 minutes³⁰ prior to arterial occlusion.

Experimental protocols

In all experimental protocols, subjects rested quietly for 30 minutes after insertion of the catheter before the first experimental trial and for 20 minutes between each RH trial.

Protocol 1: Independent and combined effects of K_{IR} channel and Na^+/K^+ -ATPase inhibition—This protocol was designed to primarily address the role of K_{IR} channels and Na^+/K^+ -ATPase in the RH response. In total, 16 subjects participated in this protocol. Eight of these subjects (Group 1) underwent RH trials in the following conditions: (1) control (saline), (2) independent K_{IR} channel inhibition (BaCl_2), (3) combined K_{IR} channel and Na^+/K^+ -ATPase inhibition (BaCl_2 +ouabain), and (4) inhibition of K_{IR} channels, Na^+/K^+ -ATPase, as well as NO and PGs (BaCl_2 +ouabain+L-NMMA+ketorolac). In the other eight subjects (Group 2), the protocol was the same except that the second trial consisted of independent inhibition of Na^+/K^+ -ATPase via ouabain versus BaCl_2 infusion.

Protocol 2: Effects of combined inhibition of NO and PGs—To further address the combined role of NO and PGs in RH and assess the role of K_{IR} channel and Na^+/K^+ -ATPase activation, we performed a second protocol (n=8) that consisted of RH trials in the following conditions: (1) control (saline), (2) combined NO and PG inhibition (L-NMMA+ketorolac), and (3) inhibition of the production of NO and PGs as well as K_{IR} channels and Na^+/K^+ -ATPase (L-NMMA+ketorolac+ BaCl_2 +ouabain).

Protocol 3: Control vasodilator stimulus—In a subset of subjects (n=6), sodium nitroprusside (SNP; Nitropress, Hospira Inc., Lake Forest, IL) was infused at 2 $\mu\text{g}/\text{dl}$ FAV/min for 5 minutes³⁸ in control (saline) conditions and after prior administration of all four antagonists (BaCl_2 +ouabain+L-NMMA+ketorolac) as a negative control to confirm intact capacity of the forearm resistance vasculature to vasodilate.

Data acquisition and analysis

Data were collected and stored on a computer at 250 Hz and were analyzed off-line with signal-processing software (WinDaq, DATAQ Instruments, Akron, OH). MAP was determined from the arterial pressure waveform. FBF was determined from the derivative of the forearm plethysmography signal. For resting hemodynamic measures, the average of the last minute of baseline was used. To quantify the RH response, we averaged and plotted values from each subject at all FBF time points (7, 14, 21, 28, 35, 42, 49, 56, 60, 75, 90, 105, 120, 135, 150 seconds post-cessation of arterial occlusion) and the total reactive hyperemic FBF [area under the curve (AUC)] was determined as the sum of FBF above baseline at each time point. The peak RH FBF and vasodilation (FVC) was determined for each subject individually and these values were also averaged. In all subjects, these individual peaks occurred at either the first, second, or third flow measurements. When FBF/FVC measurements for all subjects were averaged at each time point, the peak nearly always

occurred at the first flow measurement (see Results). To quantify the impact of the vasoactive inhibitors, the magnitude of inhibition ($\% \Delta$) was calculated as: $(\text{FBF}_{\text{peak/total inhibition}} - \text{FBF}_{\text{peak/total control}}) / (\text{FBF}_{\text{peak/total control}}) \times 100$ and always quantified from the control condition. For the SNP control trials, FBF was averaged across the last minute of baseline and SNP infusion.

Statistics

Data are presented as mean \pm s.e.m. Dynamic post-occlusion FBF values were analyzed via two-way repeated measures ANOVA (time \times condition). To make comparisons of peak and total RH FBF and baseline hemodynamics between each of the experimental conditions within a given protocol, we used one-way repeated measures ANOVA. For comparisons between protocols, a one-way ANOVA was utilized. In all cases, Student-Newman-Keuls *post hoc* pairwise comparisons were made when a significant *F* was observed. Significance was set *a priori* at $P < 0.05$.

Results

No significant differences in subject characteristics were detected between the 3 experimental groups. Baseline systemic hemodynamics (HR, MAP) and FBF for all experimental protocols are presented in Table 1 and baseline FVC values are presented in Table 2. For all protocols, there were no significant changes in HR or MAP during or following the 5 minutes of arterial occlusion (data not shown).

Protocol 1: Independent and combined effects of K_{IR} channel and Na^+/K^+ -ATPase inhibition

In Group 1 of Protocol 1, subjects received BaCl_2 alone following the control trial in order to assess the independent role of K_{IR} channels in RH. A representative tracing of one subject who participated in this protocol is provided in Figure 1 in control conditions (Panel A) and following BaCl_2 infusion (Panel B). Baseline FBF and FVC are presented in Tables 1 and 2. During RH, BaCl_2 significantly reduced the peak response ($-50 \pm 6\%$; Figure 2A and B) and impaired FBF for the first 75 seconds (Figure 2A). Taken together, the total RH FBF was also significantly reduced from control levels ($-62 \pm 3\%$; Figure 2C). The addition of ouabain did not further impact peak RH FBF ($-60 \pm 7\%$; BaCl_2 vs $\text{BaCl}_2 + \text{ouabain}$; $P = 0.25$) but there was a strong trend towards an additional effect on total RH FBF ($-82 \pm 4\%$; $P = 0.07$). The addition of L-NMMA+ketorolac did not have a further impact (Peak: $-68 \pm 7\%$; Total: $-88 \pm 3\%$). Changes in peak vasodilation (FVC) paralleled those of FBF (Table 2).

In Group 2 of Protocol 1, subjects received ouabain alone following the control trial in order to assess the independent role of Na^+/K^+ -ATPase in RH (Figure 3). Ouabain had no effect on peak RH FBF ($2 \pm 6\%$; Figure 3A and B) but did significantly reduce FBF during 14-90 seconds of hyperemia, resulting in a significant attenuation of the total RH FBF ($-44 \pm 12\%$; Figure 3C). The addition of BaCl_2 significantly reduced peak RH FBF ($-62 \pm 8\%$) as well as further reduced total RH FBF ($-92 \pm 8\%$) whereas there was no additional effect of L-NMMA+ketorolac on either peak ($-63 \pm 7\%$) or total RH FBF ($-94 \pm 8\%$). Changes in peak vasodilation (FVC) paralleled those for FBF (Table 2).

Protocol 2: Effects of combined inhibition of NO and PGs

In Protocol 2, we assessed the combined contribution of NO and PGs to RH and subsequently inhibited K_{IR} channels and Na^+/K^+ -ATPase (Figure 4). As would be expected with effective inhibition, L-NMMA+ketorolac significantly reduced baseline FBF and FVC (Tables 1-3). The mean of the first FBF measures was augmented with L-NMMA+ketorolac (Figure 4A); however, when each individual subjects' peak response was averaged, this

comparison only approached being significant ($+18\pm 8\%$; $P=0.07$; Figure 4B). FBF was attenuated with L-NMMA+ketorolac 30-60 seconds following the end of arterial occlusion (Figure 4A), yet the total RH FBF remained similar to control ($-10\pm 12\%$; $P=0.24$; Figure 4C). The additional inhibition of K_{IR} channels and Na^+/K^+ -ATPase via $BaCl_2$ and ouabain, respectively, significantly attenuated both peak ($-61\pm 8\%$; Figures 4A and B) and total ($-69\pm 6\%$; Figure 4C) RH FBF.

Comparison of RH protocols

A summary of the relative ($\% \Delta$) effects of independent and combined roles of K_{IR} channels and Na^+/K^+ -ATPase, as well as combined NO and PGs as compared to control conditions is presented in Figure 5. In these pooled comparisons ($BaCl_2$: $n=8$; ouabain: $n=8$; $BaCl_2$ +ouabain: $n=16$; L-NMMA+ketorolac: $n=8$; $BaCl_2$ +ouabain+L-NMMA+ketorolac: $n=24$) the reduction of peak FBF from $BaCl_2$ alone ($-50\pm 6\%$) to combined $BaCl_2$ +ouabain ($-61\pm 6\%$) was similar ($P=0.15$). Total RH FBF was attenuated by $BaCl_2$ alone ($-62\pm 3\%$) and ouabain alone ($-44\pm 12\%$) and this effect was enhanced when these inhibitors were combined ($-87\pm 4\%$), nearly eliminating the hyperemic response to ischemia. L-NMMA+ketorolac had no independent effects on peak ($P=0.68$) or total RH FBF ($P=0.69$) nor did they enhance any inhibition beyond that which occurred with $BaCl_2$ +ouabain infusion (Peak: $-64\pm 4\%$; $P=0.60$; AUC: $-84\pm 4\%$; $P=0.62$).

Protocol 3: Control vasodilator stimulus

Given the profound effects of our inhibitors on total RH, we wanted to confirm preserved vasodilator capacity after administration of $BaCl_2$ +ouabain+L-NMMA+ketorolac. To do so, SNP was administered in control (saline) conditions and at the end of the experimental protocol in a subgroup of 6 subjects. Baseline FBF and FVC were reduced following infusion of $BaCl_2$ +ouabain+L-NMMA+ketorolac, however there was no significant reduction in the absolute level, absolute change, or relative change in FBF and FVC during SNP infusion (Table 3).

Discussion

The primary novel finding from the current study is that activation of K_{IR} channels and Na^+/K^+ -ATPase and presumed subsequent vascular hyperpolarization explains nearly 90% of the total RH response to temporary ischemia, whereas NO and PGs have no significant combined role in this response (Figure 5). K_{IR} channels appear to be involved in both the peak and total FBF response; however, Na^+/K^+ -ATPase only contributes to the total RH FBF and not the peak FBF. The present findings lend novel and significant mechanistic insight into this basic microvascular response that has been shown to have clinical relevance in a variety of conditions that increase cardiovascular disease morbidity and mortality.

Historical overview of RH in humans

Beginning with the initial observation nearly 70 years ago of a rapid and profound hyperemia in response to a period of ischemia, there was interest in determining the underlying signals for this response^{1, 2, 7, 20, 23}. Early experiments determined that an intact nervous system was not requisite to observe this response^{2, 10} and subsequent studies pursued investigating local mechanisms of vascular control that might be involved^{8, 17-20, 22, 24}. Alongside these studies aimed to determine the physiological basis of RH, the test itself began to be used as a measure of vascular health in a variety of at-risk populations^{4-7, 9, 10}. Different groups of subjects that demonstrated “endothelial dysfunction” as commonly assessed by intra-arterial infusion of endothelium-dependent vasodilators (e.g. acetylcholine) or flow-mediated vasodilation of the brachial artery were shown to have attenuated RH responses^{4-7, 9, 10}. These associations among cardiovascular

disease, endothelial health, and impaired RH have further stimulated an interest in the potential underlying mechanisms of this response. Importantly, recent evidence indicates that peak RH flow in response to five minutes of ischemia (via upper arm cuff inflation) may in fact be a better predictor of cardiovascular events than the more commonly-assessed brachial flow-mediated vasodilation¹². Thus, elucidating the mechanisms underlying this physiological response has clear significant clinical implications.

Several potential mediators and downstream targets underlying RH have been postulated and experimentally tested in both humans and experimental animals. Due to the ischemic nature of the stimulus, classic metabolic candidates for regulating RH include adenosine or K_{ATP} channel activation. Augmenting adenosine signaling through caffeine (adenosine receptor antagonist) withdrawal or dipyridamole (inhibitor of cellular uptake of adenosine) does improve RH; however, direct inhibition of adenosine receptors (via theophylline or caffeine) does not impair peak RH FBF^{22, 26} and has a minimal effect on total FBF²². Similarly, results from inhibition of K_{ATP} channels have been equally as unsuccessful in explaining RH. Inhibition of K_{ATP} channels via sulfonylureas such as tolbutamide or glibenclamide modestly reduces total RH FBF but has no impact on the peak response in some studies^{17, 24}, whereas other investigators have demonstrated no effect of K_{ATP} channel inhibition on either peak or total RH³⁹.

Endothelium-derived NO and PGs contribute little to RH in humans

It is well known that endothelial-derived NO contributes to cardiovascular health in humans due to its multifaceted cardioprotective properties²⁷. Whether NO mediates RH was a logical proposition and has been investigated in a variety of existing studies^{8, 17-20}. There is discrepancy within the literature, and our present finding that NO (in combination with PGs) does not contribute to peak RH FBF fits with the results of most^{5, 20, 36, 40} but not all^{8, 41} of these studies. Some of the previous work has shown a modest role for NO in the total hyperemic response^{17, 18, 20}. Additionally, previous studies demonstrated only a minimal contribution of endothelial-derived PGs to peak and/or total RH^{18, 21, 22}. However, it is important to note that significant cross-talk occurs between these two endothelial pathways, such that inhibition of one pathway often does not impact vascular responses to a variety of stimuli, whereas combined inhibition reveals a significant role^{32, 33}. Only one study to date inhibited NO and PGs in combination, and this was done with intra-arterial L-NMMA and oral ibuprofen¹⁸. In this prior study, there was no impact on the peak change in FBF in response to ischemia, whereas there was some reduction (~35%) in the prolonged hyperemic response¹⁸. Our current findings agree with these observations that even in combination, NO and PGs do not contribute to peak RH FBF, and while we observed a reduction in absolute FBF in the latter portion of RH, this was not of sufficient magnitude to impair the total RH FBF (Figure 4). An interesting observation in the present study was that peak RH was somewhat augmented after combined NO and PG inhibition, and this could also reflect a critical role for vascular hyperpolarization in the response as NO is capable of suppressing EDH-mediated vasodilation⁴². Although presently unclear, another explanation for this augmented response is our inhibition of cyclooxygenase may have shifted arachadonic acid to the cytochrome p450 pathway, thus increasing the production of EETs to cause additional vasodilation²⁷.

Critical role for K_{IR} channel and Na^+/K^+ -ATPase activation in RH in humans

Based on our assessment in the present study, there is a prominent role for K_{IR} channels and Na^+/K^+ -ATPase in RH in humans (Figure 5). Interestingly, only K_{IR} channel activation, but not Na^+/K^+ -ATPase contributed to the peak RH FBF. Selective inhibition of K_{IR} channels reduced the peak hyperemic response ~50%, and a total of ~60% was observed when inhibition of Na^+/K^+ -ATPase was performed simultaneously. Inhibition of K_{IR} channels and

Na⁺/K⁺-ATPase independently reduced the total RH FBF by ~60% and ~40% respectively, and this effect is enhanced when these are inhibited in combination. In this context, there is a remarkable reduction in the total response (-87±4%) from control, nearly abolishing RH. Collectively, the magnitude of the observed attenuation due to K_{IR} channel inhibition on peak hyperemia, and combined K_{IR} and Na⁺/K⁺-ATPase inhibition on the total hyperemic response, is by far the greatest in the known studies to date on this topic.

Endothelium-dependent vasodilation that occurs beyond NO and PGs causes hyperpolarization of endothelial cells and vascular smooth muscle cells²⁷. ‘Classic’ EDH is sensitive to inhibition of K_{Ca} channels that when activated cause hyperpolarization of endothelial and smooth muscle cells through direct electrical communication or stimulation of K_{IR} channels and Na⁺/K⁺-ATPase²⁹. Our findings of a significant role for K_{IR} channel and Na⁺/K⁺-ATPase activation in RH in humans is consistent with the classic proposed mechanism of EDH. However, we must also recognize that the RH response may be endothelium-independent, yet still occur through vascular smooth muscle cell hyperpolarization²⁸. Recently, K_{IR} channels have been shown to be particularly important for the amplification of hyperpolarizing stimuli as they are directly responsive to changes in membrane potential⁴³ and thus, this unique property may explain the profound impact of BaCl₂ administration we observe on both peak and total reactive hyperemia. We are not able to address cell-specific issues related to the K_{IR} and Na⁺/K⁺-ATPase activation that we observe in the present study due to the limitations of our human *in vivo* model.

Experimental considerations

All of the inhibitors utilized were administered prior to arterial occlusion and RH. This may lead one to question the efficacy of our inhibitors after the 5 minutes of occlusion and subsequent large increases in blood flow. While not directly assessed, we used doses previously established in our laboratory³⁰ and given the large magnitude of the effects on peak and total RH FBF we observed, do not think this consideration affects our primary conclusions. If anything, we maybe potentially *underestimating* a role for K_{IR} channels and Na⁺/K⁺-ATPase in RH. Further, we show that RH responses are largely repeatable over time, with only a slight decline (-9±5%) in peak RH in the 4th trial as compared to the 1st trial (see Supplemental Figure I) and no change in AUC. Thus, the marked impact of BaCl₂ and ouabain we observe cannot be attributed to reduced responses with repeated trials.

BaCl₂ has been demonstrated to be primarily selective for K_{IR} channels up to a concentration of 100 μmol/L⁴⁴. Dawes and colleagues demonstrated that a dose at half of what we used increased antecubital venous plasma concentrations in the infused forearm to 50 μmol/L³⁶ and thus, it can be assumed that our dose would result in concentrations within the selective range for K_{IR} channels. A direct assessment of the selectivity of BaCl₂ for K_{IR} channels is difficult in humans as it is not possible to make membrane potential measurements or isolate selective stimulation of this channel. Along these lines, at greater concentrations, BaCl₂ has been shown to inhibit other potassium channels, most prominently K_{ATP} channels. While we believe that BaCl₂ in the dose we administered is selective for K_{IR} channels, if we are in fact inhibiting K_{ATP} channels, this likely does not provide an alternate explanation for our findings as the majority of existing data shows little-to-no impact of inhibiting K_{ATP} channels on peak and/or total RH^{17, 24, 39}. Further, our group^{30, 31, 35} and others^{36, 37} have demonstrated that each of the pharmacological inhibitors we use in this investigation do not impact overall vascular responsiveness nor have systemic effects in the doses utilized.

Presently, the exact stimulus for vascular hyperpolarization in response to local ischemia is unknown. Potential candidates include substances that have been shown to cause vasodilation through K_{IR} channels and/or Na⁺/K⁺-ATPase such as K⁺^{30, 45}, ATP³⁰,

bradykinin³⁷, H₂O₂⁴⁶, and EETs⁴⁷ and it is possible that concentrations of these substances may rise during ischemia as has been observed in animal models, particularly in the coronary circulation⁴⁸. However, to the best of our knowledge, limited studies in humans have made interstitial measures of the candidate substances during ischemia in skeletal muscle, and to date, no significant increases have been observed⁴⁹. Alternatively, evidence suggests that mechanosensitive mechanisms such as the myogenic response and stretch of endothelial cells contribute to the earliest portion of RH⁴⁰. In this context, recent data indicate that low intravascular pressure can stimulate transient receptor potential channels that elicit changes in endothelial cell calcium which can stimulate vasodilation via hyperpolarization⁵⁰ and this might also serve as a stimulus for K_{IR} channel and/or Na⁺/K⁺-ATPase activation. Identifying the stimulus for K_{IR} channel and Na⁺/K⁺-ATPase activation that occurs during RH represents an intriguing future area of research and potentially would provide valuable insight into explaining impaired RH responses in clinical populations.

Conclusions

Following temporary arterial occlusion, there is a significant increase in blood flow in the forearm vasculature of humans, the magnitude of which reflects microvascular function and is an important marker of overall vascular health and future cardiovascular disease risk. Here, we show that the majority of this response, in terms of both the initial peak hyperemia as well as the total hyperemia above baseline that occurs throughout the duration of the response depends on activation of K_{IR} channels and Na⁺/K⁺-ATPase. Additionally, our findings support the previous investigations that showed little-to- no role for NO and PGs in RH in humans, despite associations between RH and endothelial function⁴⁻¹³. Given the strong relation between attenuated RH responses and cardiovascular disease morbidity and mortality¹², and as a result of this study, RH and vascular hyperpolarization via K_{IR} channels and Na⁺/K⁺-ATPase, these vasodilator pathways present an exciting future direction for studies in patient populations and suggest that “vascular health” may extend beyond the commonly-assessed NO bioavailability. Moreover, these findings could be particularly important for populations that exhibit microvascular dysfunction and may serve as a target for specific therapies to improve microvascular blood flow control in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Nonstandard Abbreviations and Acronyms

AUC	area under curve
BaCl₂	barium chloride
EDH	endothelial-derived hyperpolarization
EET	epoxyeicosatrienoic acid
FAV	forearm volume
FBF	forearm blood flow
FVC	forearm vascular conductance
HR	heart rate
H₂O₂	hydrogen peroxide
K_{ATP}	ATP-dependent potassium channel
K_{Ca}	calcium-activated potassium channel
K_{IR}	inwardly-rectifying potassium channel
I-NMMA	N ^G -monomethyl-L-arginine
MAP	mean arterial pressure
NO	nitric oxide
PGs	prostaglandins

RH reactive hyperemia
SNP sodium nitroprusside

Novelty and Significance

What Is Known?

- Reactive hyperemia (RH) describes the rapid, large increase in blood flow that occurs in response to a brief circulatory occlusion.
- Impaired reactive hyperemic responses are associated with increased cardiovascular disease risk, yet the underlying mechanisms of RH in humans are not clear.

What New Information Does This Article Contribute?

- In young healthy humans, activation of inwardly-rectifying potassium (K_{IR}) channels contributes substantially to both peak and total (area under the curve) RH measured by changes in forearm blood flow.
- Activation of Na^+/K^+ -ATPase contributes to total RH but not peak RH in the forearm.
- There is no combined role of nitric oxide (NO) and prostaglandins (PGs) to either peak or total RH in the forearm.

Despite the use of RH as a test of vascular function and as a marker of cardiovascular disease risk, the underlying signaling mechanisms that contribute to this response remain unclear. To date, inhibition of vasodilator substances such as NO and PGs, have not been able to explain RH. Activation of K_{IR} channels and Na^+/K^+ -ATPase can lead to vascular hyperpolarization and vasodilation, however; these signaling pathways have not been studied with respect to RH. In young healthy humans, we demonstrate that intra-arterial inhibition of K_{IR} channels reduces both peak (~50%) and total (~60%) RH in the human forearm. Activation of both K_{IR} channels and Na^+/K^+ -ATPase explains nearly all (~90%) of the total RH response. Our findings now provide important connections among vascular hyperpolarization, RH and cardiovascular disease risk and may have significant implications for patient populations that demonstrate impaired microvascular function.

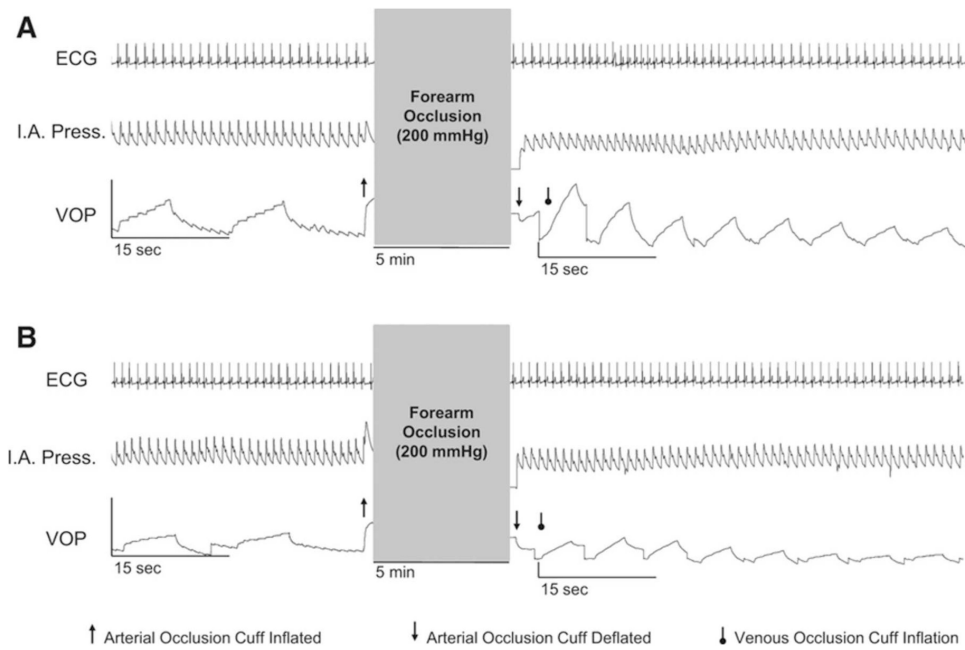


Figure 1. Representative Tracing of Baseline and Reactive Hyperemia

Representative tracing (n=1) of the last 30 seconds of rest and the first minute of the reactive hyperemia response in control (saline; A) conditions and with inhibition of K_{IR} channels via $BaCl_2$ (B). Tracings are shown for the electrocardiogram (ECG), intra-arterial pressure (I.A. Press.), and venous occlusion plethysmography (VOP) output from which heart rate, mean arterial pressure, and forearm blood flow, respectively, are calculated and/or derived. Notes: The vertical scale for VOP is 4 times greater during rest (pre-occlusion) than during reactive hyperemia (post-occlusion). Vertical deflections indicate balancing of the plethysmography signal to maintain a consistent baseline.

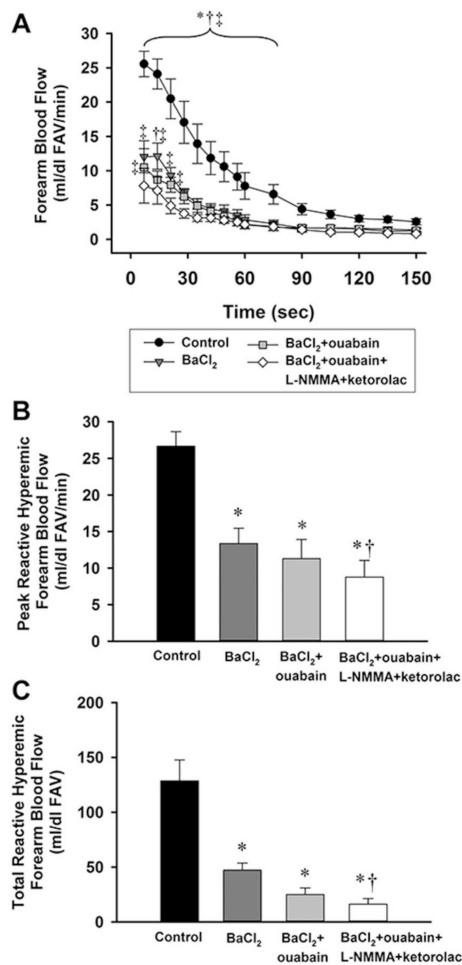


Figure 2. Protocol 1: Independent effects of K_{IR} channel inhibition (Group 1)

A. Forearm blood flow (FBF) response following 5 minutes of arterial occlusion in the following conditions: control (black circles), independent K_{IR} channel inhibition ($BaCl_2$; dark grey triangles), combined K_{IR} channel and Na^+/K^+ -ATPase inhibition ($BaCl_2$ +ouabain; light grey squares), and combined inhibition of K_{IR} channels, Na^+/K^+ -ATPase, NO and PGs ($BaCl_2$ +ouabain+L-NMMA+ketorolac; white diamonds) conditions. $BaCl_2$ significantly inhibited the response for 75 seconds and there was little additional effect of ouabain, or L-NMMA+ketorolac. * $P < 0.05$ vs $BaCl_2$; † $P < 0.05$ vs $BaCl_2$ +ouabain; ‡ $P < 0.05$ vs $BaCl_2$ +ouabain+L-NMMA+ketorolac. B. Peak reactive hyperemic FBF was significantly attenuated from control by $BaCl_2$, and ouabain had no additional effect whereas there was a slightly greater reduction with the addition of L-NMMA+ketorolac. * $P < 0.05$ vs Control; † $P < 0.05$ vs $BaCl_2$. C. Similarly, total reactive hyperemic FBF (area under curve) was significantly reduced from control by $BaCl_2$, and ouabain had no additional effect whereas L-NMMA+ketorolac further reduced this response. * $P < 0.05$ vs Control; † $P < 0.05$ vs $BaCl_2$.

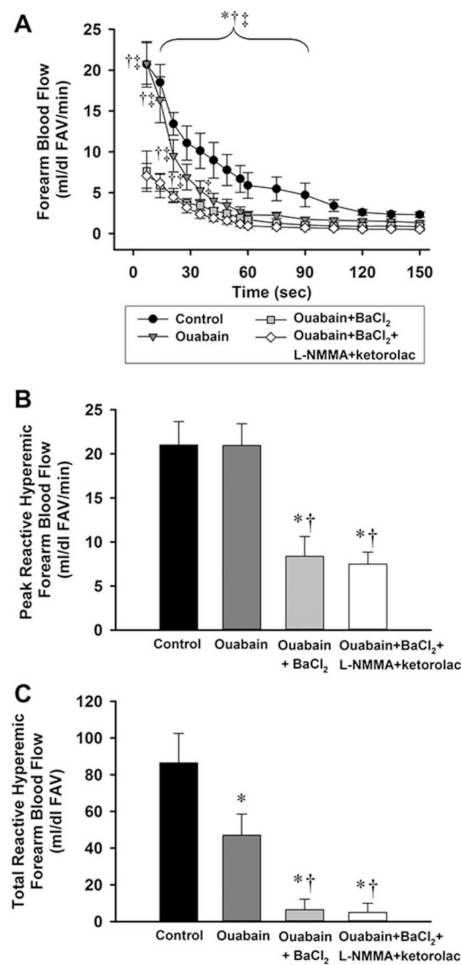


Figure 3. Protocol 1: Independent effects of Na⁺/K⁺-ATPase inhibition (Group 2)

A. Forearm blood flow (FBF) response following 5 minutes of arterial occlusion in control (black circles), independent Na⁺/K⁺-ATPase inhibition (Ouabain; dark grey triangles), combined Na⁺/K⁺-ATPase and K_{IR} channel inhibition (Ouabain+BaCl₂; light grey squares), and combined inhibition of Na⁺/K⁺-ATPase, K_{IR} channels, NO and PGs (Ouabain +BaCl₂+L-NMMA+ketorolac; white diamonds) conditions. Ouabain did not affect initial FBF, but thereafter reduced FBF from control until 90 seconds post-cuff deflation. The addition of BaCl₂ further attenuated FBF for 30 seconds, whereas addition of L-NMMA+ketorolac had no further effect. **P*<0.05 vs Ouabain; †*P*<0.05 vs Ouabain+BaCl₂; ‡*P*<0.05 vs Ouabain+BaCl₂+L-NMMA+ketorolac. B. Peak reactive hyperemic FBF was not affected by ouabain. Infusion of BaCl₂ significantly reduced peak FBF from control, and L-NMMA+ketorolac had no further impact. **P*<0.05 vs Control; †*P*<0.05 vs Ouabain. C. Total reactive hyperemic FBF (area under curve) was significantly reduced from control by ouabain, and BaCl₂ had an additional effect whereas L-NMMA+ketorolac did not. **P*<0.05 vs Control; †*P*<0.05 vs Ouabain.

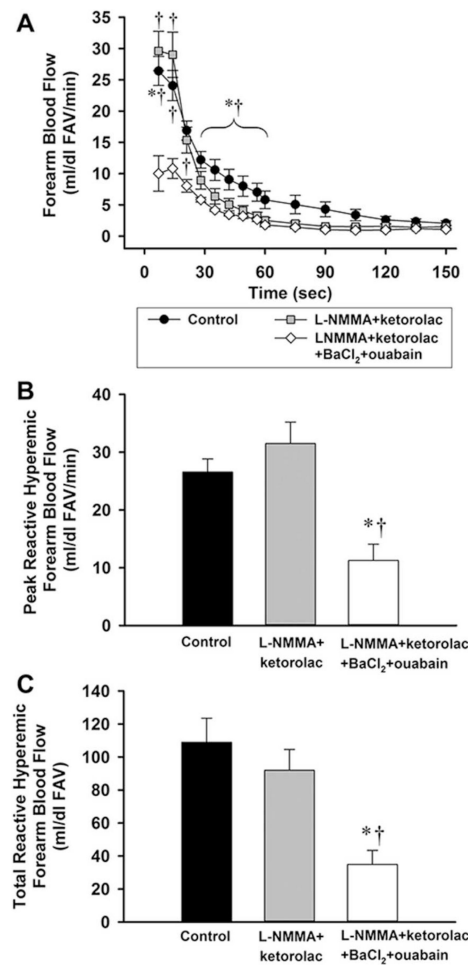


Figure 4. Protocol 2: Effects of combined inhibition of nitric oxide and prostaglandins

A. Forearm blood flow (FBF) response following 5 minutes of arterial occlusion in control (black circles), combined inhibition of NO and PG synthesis (L-NMMA+ketorolac; light grey squares), and combined inhibition of NO, PGs, K_{IR} channels and Na^+/K^+ -ATPase (L-NMMA+ketorolac +BaCl₂+ouabain; white diamonds) conditions. L-NMMA+ketorolac attenuated the response from control only from 30-60 seconds post-cuff deflation. The addition of BaCl₂+ouabain significantly reduced FBF for 30 seconds and thereafter had no further effect. * $P < 0.05$ vs L-NMMA+ketorolac; † $P < 0.05$ vs L-NMMA+ketorolac +BaCl₂+ouabain. B. Peak reactive hyperemic FBF was not affected by L-NMMA+ketorolac and was significantly attenuated by L-NMMA+ketorolac +BaCl₂+ouabain. * $P < 0.05$ vs Control; † $P < 0.05$ vs L-NMMA+ketorolac. C. Similar to peak, total reactive hyperemic FBF (area under curve) was not affected by L-NMMA+ketorolac and was significantly attenuated by L-NMMA+ketorolac +BaCl₂+ouabain. * $P < 0.05$ vs Control; † $P < 0.05$ vs L-NMMA+ketorolac.

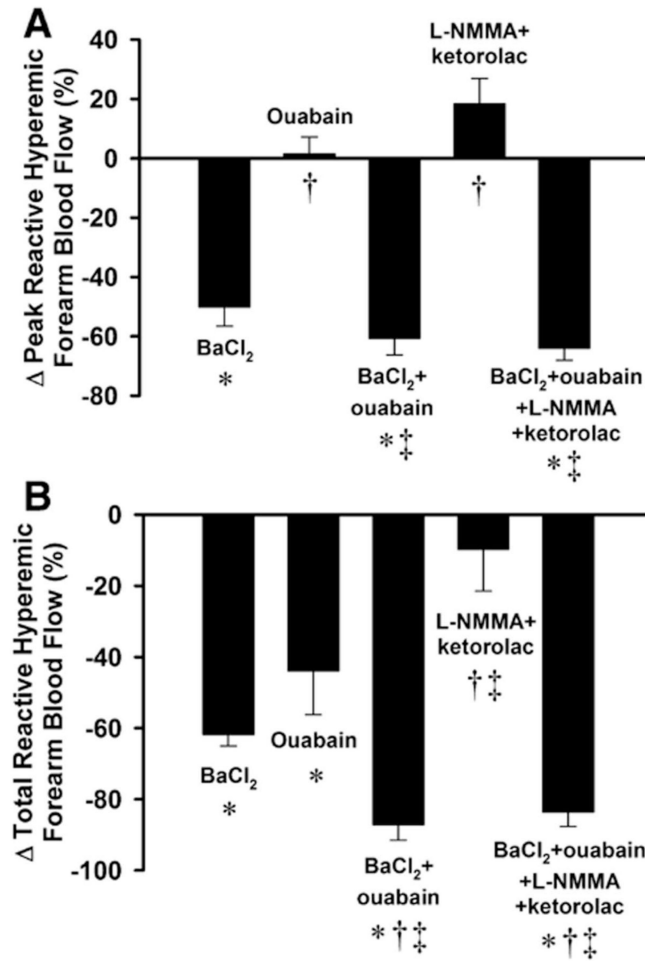


Figure 5. Summary: Effects of inhibition of K_{IR} channels, Na^+/K^+ -ATPase, nitric oxide and prostaglandins on peak and total reactive hyperemia

Combined results from the three experimental protocols are presented for relative impact (% Δ) on both peak (A) and total (B) reactive hyperemic forearm blood flow (FBF) in each experimental condition (BaCl₂: n=8; Ouabain: n=8; BaCl₂+ouabain: n=16; L-NMMA+ketorolac: n=8; BaCl₂+ouabain+L-NMMA+ketorolac: n=24). BaCl₂ reduced peak FBF and this attenuation was unchanged with the addition of ouabain or L-NMMA+ketorolac. Neither ouabain alone nor L-NMMA+ketorolac attenuated peak FBF. BaCl₂ and ouabain both independently reduced total FBF and in combination (BaCl₂+ouabain), the reduction was enhanced. There was no additional reduction by L-NMMA+ketorolac, nor did L-NMMA+ketorolac independently reduce total FBF. * P <0.05 vs zero; † P <0.05 vs BaCl₂; ‡ P <0.05 vs Ouabain

Table 1

Baseline forearm and systemic hemodynamics for all protocols

Protocol 1 – Group 1	Control	BaCl ₂	BaCl ₂ +ouabain	BaCl ₂ +ouabain+L-NMMA+ketorolac
HR	56±3	58±3	58±4	56±3
MAP	86±3	87±3	87±2	91±4
FBF	2.3±0.5	1.5±0.2*	2.3±0.3	1.9±0.2
Protocol 1 – Group 2	Control	Ouabain	Ouabain+BaCl ₂	Ouabain+BaCl ₂ +L-NMMA+ketorolac
HR	57±4	57±4	58±4	58±5
MAP	83±1	84±2	89±2* [†]	91±3* [†]
FBF	2.5±0.3	2.3±0.4	2.2±0.2	1.8±0.1*
Protocol 2	Control	L-NMMA+ketorolac	L-NMMA+ketorolac+BaCl ₂ +ouabain	
HR	58±4	54±3	55±4	
MAP	90±3	89±4	92±4	
FBF	2.1±0.3	1.5±0.2*	1.5±0.1* [†]	

n=8 in all groups;

* $P < 0.05$ vs 1st Trial (i.e. control);

[†] $P < 0.05$ vs 2nd Trial (i.e. ouabain); HR=heart rate (beats/min); MAP=mean arterial pressure (mmHg); FBF=forearm blood flow (ml/dl forearm volume/min)

Table 2

Resting and peak reactive vasodilation in all protocols

		Forearm Vascular Conductance (ml/dl FA V/min/100 mmHg)			
Protocol 1 – Group 1		Control	BaCl ₂	BaCl ₂ +ouabain	BaCl ₂ +ouabain+L-NMMA+ketorolac
Rest		2.8±0.6	1.8±0.3*	2.6±0.4	2.1±0.3
Peak		36.3±3.4	18.0±3.2*	13.8±3.0*	10.4±2.8* [†]
Protocol 1 – Group 2		Control	Ouabain	Ouabain+BaCl ₂	Ouabain+BaCl ₂ + L-NMMA+ketorolac
Rest		3.0±0.4	2.7±0.4	2.5±0.2	2.0±0.3*
Peak		28.5±3.5	26.5±3.1	9.8±2.7* [†]	8.8±1.7* [†]
Protocol 2		Control	L-NMMA+ketorolac	L-NMMA+ketorolac+BaCl ₂ +ouabain	
Rest		2.3±0.3	1.6±0.2*	1.6±0.1*	
Peak		34.5±4.1	39.0±5.3	14.5±3.9* [†]	

n=8 in all groups;

* $P < 0.05$ vs 1st Trial (i.e. control);[†] $P < 0.05$ vs 2nd Trial (i.e. BaCl₂)

Table 3

Protocol 3: Control vasodilator stimulus

Control (saline)	Baseline	SNP 2 $\mu\text{g}/\text{dl}$ FAV/min	Absolute Δ	% Δ
FBF	2.2 \pm 0.3	12.4 \pm 1.5	10.2 \pm 1.4	499 \pm 66
FVC	2.5 \pm 0.4	15.3 \pm 2.1	12.7 \pm 1.8	546 \pm 72
BaCl ₂ +ouabain +L-NMMA+ketorolac	Baseline	SNP 2 $\mu\text{g}/\text{dl}$ FAV/min	Absolute Δ	% Δ
FBF	1.5 \pm 0.3*	10.1 \pm 2.0	8.6 \pm 1.7	611 \pm 107
FVC	1.6 \pm 0.2*	12.1 \pm 2.6	10.6 \pm 2.4	672 \pm 100

n=6;

* $P < 0.05$ vs control; FAV=forearm volume; FBF=forearm blood flow (ml/dl FAV/min); FVC=forearm vascular conductance (ml/dl FAV/min/100 mmHg); SNP=sodium nitroprusside