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

Anne R. Crecelius, Jennifer C. Richards, Gary J. Luckasen, Dennis G. Larson, Frank A. Dinенно

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 that 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 autacoids nitric oxide (NO) and prostaglandins 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) after 5 minutes of arterial occlusion. In protocol 1, we studied 2 groups of 8 subjects and assessed RH in the following conditions. For group 1, we studied control (saline), \(K_{IR}\) channel inhibition (\(BaCl_2\)), combined inhibition of \(K_{IR}\) channels and Na+/K+ -ATPase (\(BaCl_2\) and ouabain, respectively), and combined inhibition of \(K_{IR}\) channels, Na+/K+ -ATPase, NO, and prostaglandins (\(BaCl_2\), ouabain, 1-NMMA [\(N^\omega\)-monomethyl-l-arginine] and ketorolac, respectively). Group 2 received ouabain rather than \(BaCl_2\) in the second trial. In protocol 2 (n=8), the following 3 RH trials were performed: control; 1-NMMA plus ketorolac; and 1-NMMA plus ketorolac plus \(BaCl_2\) plus ouabain. All infusions were intra-arterial (brachial). Compared with control, \(BaCl_2\) significantly reduced peak FBF (−50±6%; \(P<0.05\)), whereas ouabain and 1-NMMA plus ketorolac did not. Total FBF (area under the curve) was attenuated by \(BaCl_2\) (−61±3%) and ouabain (−44±12%) alone, and this effect was enhanced when combined (−87±4%), nearly abolishing RH. 1-NMMA plus ketorolac did not impact total RH FBF before or after administration of \(BaCl_2\) and 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 the total (AUC) RH in humans. (Circ Res. 2013;113:1023-1032.)

Key Words: blood flow regulation ■ ischemia ■ hyperpolarization ■ vasodilation

After ischemia caused by temporary arterial occlusion, there is significant vasodilation and a rapid marked increase in blood flow in most tissues, including the human forearm.\(^1\) 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 function.\(^2\) Attenuated RH responses have been documented in populations demonstrating a variety of risk factors that increase cardiovascular morbidity and mortality, including hypertension,\(^3,4,7\) atherosclerosis,\(^8\) peripheral artery disease,\(^9\) congestive heart failure,\(^10\) and advanced age.\(^11\) Recently, peak RH flow was determined to be predictive of future cardiovascular events in a healthy population\(^12\) as well as in at-risk patient populations,\(^13\) and this predictive value may be greater than that of commonly assessed macrovascular function via flow-mediated brachial artery vasodilation.\(^12,14\) Despite the use 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 is
inhibited in combination, a significant portion of both the peak and total RH remains unexplained. There is growing interest in vasodilation that occurs via non-NO and non-PG mechanisms due to hyperpolarization of endothelial and vascular smooth muscle cells. Endothelial-derived hyperpolarization (EDH) can be broadly categorized into 2 groups: classical EDH associated with activation of calcium-activated potassium channels (KCa) and subsequent direct electric communication or activation of inwardly rectifying potassium (KIR) channels and Na+/K+-ATPase, resulting in hyperpolarization of vascular smooth muscle cells; and the second category of EDH involving diffusible factors such as H2O2 and NO2. A 20-gauge, 7.6-cm catheter was placed in the brachial artery of the nondominant 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 ECG (Cardiograph; Datex-Ohmeda, Louisville, CO).

Methods

A detailed and expanded Methods section is available in the Online Data Supplement.

Subjects

With Colorado State University 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±SEM) participated in the present study. All studies were performed according to the Declaration of Helsinki.

Arterial Catheterization, Arterial Blood Pressure, and Heart Rate

Forearm Blood Flow and Vascular Conductance

Forearm blood flow (FFB) was measured using venous occlusion plethysmography using mercury-in-silastic strain gauges and techniques as previously described. FFB was expressed as milliliters per deciliter of tissue per minute (mL/dL FA V per minute). As an index of forearm vasodilation and to account for individual differences in baseline vascular tone, forearm vascular conductance (FVC) was calculated as (FFB/MAP)×100, expressed as mL/dL FA V per minute per 100 mm Hg. Immediately after the release of the occlusion cuff for the RH, the same cuff cycled between inflation at ±50 mm Hg (4 seconds) and deflation (3 seconds) to cause venous occlusion, and this yielded 1 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 FFB, the cuff on the upper arm was rapidly inflated to 200 mm Hg for 5 minutes of ischemia. This location and duration of ischemia were chosen to mimic the RH protocol used in investigations of the contributions of various endothelial-derived vasodilator pathways to the RH response and, importantly, peak RH flow has recently been demonstrated to be more strongly associated with cardiovascular disease risk than measures of flow-mediated vasodilation. 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 noninvasively and underwent 4 successive bouts of RH separated by 20 minutes of rest (see online only Data Supplement for details).

Vasoactive Drug Infusion

All drug infusions were through the brachial arterial catheter to create a local effect in the forearm and were completed during baseline measures before the arterial occlusion, and saline was used as a control infused. Specific timing and duration of infusions is provided in the Experimental Protocols section.

To inhibit KIR channels, BaCl2 (KIR channel inhibitor; 10% wt/vol BDH3238; EMD Chemicals, Gibbstown, NJ) was infused at 0.9 µmol/dL FA V per minute within an absolute range of 8 to 10 µmol/min for 5 minutes before each arterial occlusion. To inhibit Na+/K+-ATPase, ouabain octahydrate (Na+/K+-ATPase inhibitor; Sigma, St Louis, MO) was infused at 2.7 nmol/min for 15 minutes before arterial occlusion. In subsequent RH trials, ouabain was reinfused for 5 minutes before arterial occlusion to provide continuous inhibition. This approach of using BaCl2 and ouabain to inhibit KIR channels and Na+/K+-ATPase, respectively, has been used previously by our group and others. We administered Nω-monomethyl-l-arginine (l-NMMA; NO synthase inhibitor; Chinalfa/Bachem, Weil am Rhein, Germany) to inhibit the production of NO in combination with ketorolac (nonselective 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 µg/min, respectively, and were administered for 5 minutes before 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 KIR Channel and Na+/K+-ATPase Inhibition
This protocol was designed to primarily address the role of KIR 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 during the following conditions: control (saline); independent KIR channel inhibition (BaCl2); combined KIR channel and Na+/K+-ATPase inhibition (BaCl2 plus ouabain); and inhibition of KIR channels, Na+/K+-ATPase, as well as NO and PGs (BaCl2 plus ouabain plus l-NMMA plus ketorolac). In the other 8 subjects (group 2), the protocol was the same except that the second trial consisted of independent inhibition of Na+/K+-ATPase via ouabain versus BaCl2 infusion.

Protocol 2: Effects of Combined Inhibition of NO and PGs
To further address the combined role of NO and PGs in RH and to assess the role of KIR channel and Na+/K+-ATPase activation, we performed a second protocol (n=8) that consisted of RH trials during the following conditions: control (saline); combined NO and PG inhibition (l-NMMA plus ketorolac); and inhibition of the production of NO and PGs as well as KIR channels and Na+/K+-ATPase (l-NMMA plus ketorolac plus BaCl2 plus ouabain).

Protocol 3: Control Vasodilator Stimulus
In a subset of subjects (n=6), sodium nitroprusside (SNP; Nitropress; Hospira, Lake Forest, IL) was infused at 2 μg/dL FAV per minute for 5 minutes during control (saline) conditions and after previous administration of all 4 antagonists (BaCl2 plus ouabain plus l-NMMA plus 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 offline 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, and 150 seconds postocclusion 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 the first, second, or third flow measurement. 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 (%Δ) was calculated as (FBFpeak/total control–FBFpeak/total inhibition–FBFpeak/peak control–FBFpeak/peak control)×100 and was 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 means±SEM. Dynamic postocclusion FBF values were analyzed via 2-way repeated measures ANOVA (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 1-way repeated measures ANOVA. For comparisons between protocols, a 1-way ANOVA was used. 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 after the 5 minutes of arterial occlusion (data not shown).

Protocol 1: Independent and Combined Effects of KIR Channel and Na+/K+-ATPase Inhibition
In group 1 of protocol 1, subjects received BaCl2 alone after insertion of the catheter before the first experimental trial and for the second trial (ie, ouabain). To further address the combined role of NO and PGs in RH and to assess the role of KIR channel and Na+/K+-ATPase activation, we performed a second protocol (n=8) that consisted of RH trials during the following conditions: control (saline); combined NO and PG inhibition (l-NMMA plus ketorolac); and inhibition of the production of NO and PGs as well as KIR channels and Na+/K+-ATPase (l-NMMA plus ketorolac plus BaCl2 plus ouabain).

Table 1. Baseline Forearm and Systemic Hemodynamics for All Protocols

<table>
<thead>
<tr>
<th>Protocol 1, Group 1</th>
<th>Control</th>
<th>BaCl2</th>
<th>BaCl2+ouabain</th>
<th>BaCl2+ouabain+ l-NMMA+ketorolac</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR</td>
<td>56±3</td>
<td>58±3</td>
<td>58±4</td>
<td>56±3</td>
</tr>
<tr>
<td>MAP</td>
<td>86±3</td>
<td>87±3</td>
<td>87±2</td>
<td>91±4</td>
</tr>
<tr>
<td>FBF</td>
<td>2.3±0.5</td>
<td>1.5±0.2*</td>
<td>2.3±0.3</td>
<td>1.9±0.2</td>
</tr>
<tr>
<td>Protocol 1, Group 2</td>
<td>Control</td>
<td>Ouabain</td>
<td>Ouabain+BaCl2</td>
<td>Ouabain+BaCl2+ l-NMMA+ketorolac</td>
</tr>
<tr>
<td>HR</td>
<td>57±4</td>
<td>57±4</td>
<td>58±4</td>
<td>58±5</td>
</tr>
<tr>
<td>MAP</td>
<td>83±1</td>
<td>84±2</td>
<td>89±2*†</td>
<td>91±3*†</td>
</tr>
<tr>
<td>FBF</td>
<td>2.5±0.3</td>
<td>2.3±0.4</td>
<td>2.2±0.2</td>
<td>1.8±0.1*†</td>
</tr>
<tr>
<td>Protocol 2</td>
<td>Control</td>
<td>l-NMMA+ketorolac</td>
<td>l-NMMA+ketorolac +BaCl2+ouabain</td>
<td></td>
</tr>
<tr>
<td>HR</td>
<td>58±4</td>
<td>54±3</td>
<td>55±4</td>
<td></td>
</tr>
<tr>
<td>MAP</td>
<td>90±3</td>
<td>89±4</td>
<td>92±4</td>
<td></td>
</tr>
<tr>
<td>FBF</td>
<td>2.1±0.3</td>
<td>1.5±0.2*</td>
<td>1.5±0.1†</td>
<td></td>
</tr>
</tbody>
</table>

FBF indicates forearm blood flow (mL/dL forearm volume per min); HR, heart rate (beats per min); l-NMMA, Nω-monomethyl-l-arginine; and MAP, mean arterial pressure (mm Hg). n=8 in all groups. *P<0.05 vs first trial (ie, control). †P<0.05 vs second trial (ie, ouabain).
in this protocol is provided in control conditions (Figure 1A) and after BaCl₂ infusion (Figure 1B). Baseline FBF and FVC are presented in Tables 1 and 2. During RH, BaCl₂ significantly reduced the peak response (−50±6%; Figure 2A and 2B) and impaired FBF for the first 75 seconds (Figure 2A). Taken together, the total RH FBF was also significantly reduced from control levels (−62±3%; Figure 2C). The addition of ouabain did not further impact peak RH FBF (−60±7%; BaCl₂ versus BaCl₂ plus ouabain; P=0.25), but there was a strong trend toward an additional effect on total RH FBF (−82±3%; P=0.07). The addition of l-NMMA plus ketorolac did not have a further impact (peak: −68±7%; total: −88±3%). Changes in peak vasodilation (FVC) paralleled those of 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 subsequent inhibition of K IR channels and Na+/K+-ATPase (Figure 4). As would be expected with effective inhibition, l-NMMA plus ketorolac significantly reduced baseline FBF and FVC (Tables 1–3). The mean of the first FBF measures was augmented with l-NMMA plus ketorolac (Figure 4A); however, when each individual subject’s peak response was averaged, this comparison only approached being significant (+18±8%; P=0.07; Figure 4B).

<table>
<thead>
<tr>
<th>Table 2. Baseline and Peak Reactive Vasodilation in All Protocols</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forearm Vascular Conductance (mL/dL forearm volume per min per 100 mmHg)</td>
</tr>
<tr>
<td>Protocol 1, Group 1</td>
</tr>
<tr>
<td>Baseline</td>
</tr>
<tr>
<td>Peak</td>
</tr>
<tr>
<td>Protocol 1, Group 2</td>
</tr>
<tr>
<td>Baseline</td>
</tr>
<tr>
<td>Peak</td>
</tr>
<tr>
<td>Protocol 2</td>
</tr>
<tr>
<td>Baseline</td>
</tr>
<tr>
<td>Peak</td>
</tr>
</tbody>
</table>

l-NMMA indicates NG-monomethyl-l-arginine.
n=8 in all groups.
*P<0.05 vs first trial (ie, control).
†P<0.05 vs second trial (ie, BaCl₂).
FBF was attenuated with \( l \)-NMMA plus ketorolac 30 to 60 seconds after the end of arterial occlusion (Figure 4A), yet the total RH FBF remained similar to control (−10±12%; \( P = 0.24 \); Figure 4C). The additional inhibition of \( K_r \) channels and Na\(^+/K^+\)-ATPase via BaCl\(_2\) and ouabain, respectively, significantly attenuated both peak (−61±8%; Figure 4A and 4B) and total (−69±6%; Figure 4C) RH FBF.

**Comparison of RH Protocols**

A summary of the relative (\( \% \Delta \)) effects of independent and combined roles of \( K_r \) channels and Na\(^+/K^+\)-ATPase and combined NO and PGs as compared with control conditions are presented in Figure 5. In these pooled comparisons (BaCl\(_2\), \( n = 8 \); ouabain, \( n = 8 \); BaCl\(_2\) plus ouabain, \( n = 16 \); \( l \)-NMMA plus ketorolac, \( n = 8 \); BaCl\(_2\) plus ouabain plus \( l \)-NMMA plus ketorolac, \( n = 24 \)), the reduction of peak FBF from BaCl\(_2\) alone (−50±6%) to combined BaCl\(_2\) plus ouabain (−61±6%) was similar (\( P = 0.15 \)). Total RH FBF was attenuated by BaCl\(_2\).
alone (−62±3%) and ouabain alone (−44±12%), and this effect was enhanced when these inhibitors were combined (−87±4%), nearly eliminating the hyperemic response to ischemia. L-NMMA plus 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 BaCl₂ plus ouabain infusion (peak: −64±4%; P=0.60; AUC: −84±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 BaCl₂ plus ouabain plus L-NMMA plus 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 after infusion of BaCl₂ plus ouabain plus L-NMMA plus 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 KIR 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). KIR 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 ≈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 a 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 (eg, acetylcholine) or flow-mediated vasodilation of the brachial artery were shown to have attenuated
KATP channels have been equally as unsuccessful in explaining the modest effect on total FBF. Similarly, results from inhibition of KATP channels via sulfonylureas, such as tobutamide or glibenclamide, modestly reduces total RH FBF but has no impact on the peak response in some studies, whereas other investigators have demonstrated no effect of KATP channel inhibition on either peak or total RH.

**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 because of its multifaceted cardioprotective properties. Whether NO mediates RH was a logical proposition and has been investigated in a variety of existing studies. 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, but not all, of these studies. Some of the previous work has shown a modest role for NO in the total hyperemic response. Additionally, previous studies demonstrated only a minimal contribution of endothelial-derived PGs to peak or total RH. However, it is important to note that significant cross-talk occurs between these 2 endothelial pathways, such that inhibition of 1 pathway often does not impact vascular responses to a variety of stimuli, whereas combined inhibition reveals a significant role. Only 1 study to date showed inhibition of NO and PGs in combination, and this was performed with intra-arterial l-NMMA and oral ibuprofen. In this previous study, there was no impact on the peak change in FBF in response to ischemia, whereas there was some reduction in the prolonged hyperemic response. Our findings agree with these observations that even in combination NO and PGs do not contribute to peak RH FBF, and although 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 because NO is capable of suppressing EDH-mediated vasodilation. Although presently unclear, another explanation for this augmented response is our inhibition of cyclooxygenase may have shifted arachidonic acid to the cytochrome p450 pathway, thus increasing the production of epoxyeicosatrienoic acids to cause additional vasodilation.

**Critical Role for KIR Channel and Na+/K+-ATPase Activation in RH in Humans**

Based on our assessment in the present study, there is a prominent role for KIR channels and Na+/K+-ATPase in RH in humans (Figure 5). Interestingly, only KIR channel activation, but not Na+/K+-ATPase, contributed to the peak RH FBF. Selective inhibition of KIR 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 KIR channels and Na+/K+-ATPase independently reduced the total RH FBF by ≈60% and ≈40%, respectively, and this effect was enhanced when these were inhibited in combination. In this context, there was a remarkable reduction in the total response (≈87±4%) from control, nearly abolishing RH. Collectively, the magnitude of the observed attenuation attributable to KIR channel inhibition on peak hyperemia

**Figure 5. Summary: Effects of inhibition of inwardly rectifying potassium channels, Na+/K+-ATPase, nitric oxide (NO) and prostaglandins on peak and total reactive hyperemia.**

Combined results from the 3 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 [N°-monomethyl-l-arginine]+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.

RH responses. 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 5 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 vasodilation. 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 humans and in experimental animals. Because of the ischemic nature of the stimulus, classic metabolic candidates for regulating RH include adenosine or KATP channel activation. Augmenting adenosine signaling through caffeine (adenosine receptor antagonist) withdrawal or dipryridamole (inhibitor of cellular uptake of adenosine) does improve RH; however, direct inhibition of adenosine receptors (via theophylline or theophylline) does not impair peak RH FBF and has a minimal effect on total FBF. Similarly, results from inhibition of KATP channels have been equally as unsuccessful in explaining RH. Inhibition of KATP channels via sulfonylureas, such as
and combined KIR and Na+/K+-ATPase inhibition on the total hyperemic response are 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 cells.23 Classic EDH is sensitive to inhibition of KCa channels when which when activated, cause hyperpolarization of endothelial and smooth muscle cells through direct electric communication or stimulation of KIR channels and Na+/K+-ATPase.29 Our finding of a significant role for KIR 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 but still occurs through vascular smooth muscle cell hyperpolarization.24 Recently, KIR channels have been shown to be particularly important for the amplification of hyperpolarizing stimuli because they are directly responsive to changes in membrane potential43 and, thus, this unique property may explain the profound impact of BaCl2 administration we observed on both peak and total RH. We are not able to address cell-specific issues related to KIR and Na+/K+-ATPase administration we observed on both peak and total RH. We used doses previously established in our laboratory30 and, given the large magnitude of the effects on peak and total RH FBF we observed, we do not think this consideration affects our primary conclusions. If anything, we may be potentially underestimating a role for KIR channels up to a concentration of 100 μmol/L.44 Dawes et al36 demonstrated that a dose at half of what we used in this study on RH and vascular hyperpolarization via KIR channels and Na+/K+-ATPase, these vasodilator pathways present a direct assessment of the selectivity of BaCl2 and ouabain we observed cannot be attributed to reduced responses with repeated trials.

BaCl2 has been demonstrated to be primarily selective for KIR channels up to a concentration of 100 μmol/L.44 Dawes et al36 demonstrated that a dose at half of what we used increased antecubital venous plasma concentrations in the infused forearm to 50 μmol/L and, thus, it can be assumed that our dose would result in concentrations within the selective range for KIR channels. A direct assessment of the selectivity of BaCl2 for KIR channels is difficult in humans because 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 KATP channels. Although we believe that BaCl2 in the dose we administered is selective for KIR channels, if we are in fact inhibiting KATP channels, this likely does not provide an alternative explanation for our findings because the majority of existing data show little to no impact of inhibiting KATP channels on peak or total RH.17,24,39 Further, our group30,31,35 and others6,37 have demonstrated that each of the pharmacological inhibitors we used in this investigation does not impact overall vascular responsiveness and does not have systemic effects in the doses used.

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 KIR channels or Na+/K+-ATPase, such as K+, ATP,30 bradykinin,37 H2O2,46 and epoxyeicosatrienoic acids,47 and it is possible that concentrations of these substances may increase during ischemia, as has been observed in animal models, particularly in the coronary circulation.48 However, to the best of our knowledge, limited studies in humans have made intermediary measures of the candidate substances during ischemia in skeletal muscle and, to date, no significant increases have been observed.49 Alternatively, evidence suggests that mechanosensitive mechanisms such as the myogenic response and stretch of endothelial cells contribute to the earliest portion of RH.40 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 hyperpolarization,50 and this might also serve as a stimulus for KIR channel or Na+/K+-ATPase activation. Identifying the stimulus for KIR 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**

After 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 KIR 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 function.4–13 Given the strong relation between attenuated RH responses and cardiovascular disease morbidity and mortality,12 and as a result of this study on RH and vascular hyperpolarization via KIR 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.

**Acknowledgments**

We thank the subjects who volunteered to participate, and Leora J. Garcia and Devin V. Dinenno for their assistance.

**Sources of Funding**

National Institutes of Health awards HL102720 and HL095573 (to F.A.D.).
Disclosures

None.

References


44. Pomposiello S, Rhalbe NB, Alva M, Carretero OA. Reactive oxygen species: role in the relaxation induced by bradykinin or arachidonic acid via...


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 (KIR) 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) in 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 has not been able to explain RH. Activation of KIR 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 KIR channels reduces both peak (≈50%) and total (≈60%) RH in the human forearm. Activation of both KIR 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.
Reactive Hyperemia Occurs Via Activation of Inwardly Rectifying Potassium Channels and Na+/K+-ATPase in Humans
Anne R. Crecelius, Jennifer C. Richards, Gary J. Luckasen, Dennis G. Larson and Frank A. Dineno

Circ Res. 2013;113:1023-1032; originally published online August 12, 2013;
doi: 10.1161/CIRCRESAHA.113.301675

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/113/8/1023

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2013/08/12/CIRCRESAHA.113.301675.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
SUPPLEMENTAL MATERIAL

Reactive hyperemia occurs via activation of inwardly-rectifying potassium channels and Na⁺/K⁺-ATPase in humans

Anne R. Crecelius¹, Ph.D., Jennifer C. Richards¹, M.S., Gary J. Luckasen³, M.D., Dennis G. Larson³, M.D., Frank A. Dinenno¹², Ph.D.

¹Human Cardiovascular Physiology Laboratory
Department of Health and Exercise Science
²Vascular Physiology Research Group
Department of Biomedical Sciences
Colorado State University
Fort Collins, CO 80523-1582

³Medical Center of the Rockies Foundation
University of Colorado Health
Loveland, CO 80538
SUPPLEMENTAL METHODS

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 subjects were sedentary to moderately active, non-smokers, non-obese, normotensive (resting blood pressure <140/90 mmHg), and not taking any medications. Studies were performed after an overnight fast and 24 hour abstention from caffeine and exercise with subjects in the supine position with the experimental arm abducted to 90° and slightly elevated above heart level upon a tilt-adjustable table in a cool environment (20-22°C). Female subjects were studied during the early follicular phase of their menstrual cycle or placebo phase of oral contraceptive use to minimize any potential cardiovascular effects of sex-specific hormones. 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 and blood sampling. The catheter was connected to a 3-port connector as well as a pressure transducer for mean arterial pressure (MAP) measurement and continuously flushed at 3 ml/hr with heparinized saline. The two side ports were used for drug infusions. Heart rate (HR) was determined using a 3-lead electrocardiogram (Cardiocap/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 (Hokanson, Bellevue, WA) and techniques as previously described. Briefly, a pediatric blood pressure cuff (TMC 7, Hokanson, Bellevue, WA) was placed around the wrist of the experimental arm and inflated to suprasystolic pressure (~200 mmHg via AG101 Cuff Inflator Air Source and E20 Rapid Cuff Inflator, Hokanson, Bellevue, WA) to arrest the hand circulation and isolate the forearm tissue. Additionally, a venous occlusion cuff (SC12D, Hokanson, Bellevue, WA) was placed around the upper portion of the experimental arm and rapidly cycled between inflation at a pressure of 50 mmHg (7 seconds) and deflation (8 seconds) yielding one blood flow measurement every 15 seconds. 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 reactive hyperemia (RH; see below), the venous occlusion cuff cycled between inflation (4 seconds) and deflation (3 seconds) yielding 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 sec, as was used at rest.

RH protocol
The same cuff that was placed around the upper portion of the arm and used for venous occlusion for the measurement of FBF was used to cause arterial occlusion for each RH trial. After measurement of baseline FBF, the occlusion cuff was rapidly inflated to 200 mmHg for 5 minutes of ischemia. In order to provide the most relevant insight, 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 and importantly, that has recently demonstrated peak RH flow to be more strongly associated with cardiovascular disease risk than measures of flow-mediated vasodilation. After 5 minutes, the cuff was rapidly deflated and flow measures commenced for 2.5 minutes (150 sec). Our interest in RH was not to attempt to understand mechanisms of maximal vasodilation as is more appropriately assessed with longer duration ischemia, ischemic exercise, or pharmacological infusion.
Rather, we were interested in testing the underlying vasodilator pathways of RH, given the relationship with impaired responses to a 5-minute ischemic stimulus and increased cardiovascular disease risk\(^6,9\).

The protocol we utilized for our RH trials is repeatable over time as demonstrated in a previous study\(^3\), as well as in 8 additional subjects (5M:3F) in the present investigation. These subjects were studied in the same conditions and instrumented similarly to the experimental subjects except MAP was obtained with non-invasive beat-to-beat photoplethysmography (Finometer, Finapres Medical Systems, Amsterdam, The Netherlands). We performed 4 consecutive RH trials consisting of 3 minutes of baseline forearm blood flow measurement, 5 minutes of arterial occlusion, and 2.5 minutes of RH flow measurements. Each trial was separated by 20 minutes. Baseline forearm and systemic hemodynamics are presented in Supplemental Table I. As shown in Supplemental Figure IB, there was a slight attenuation in peak FBF in Trial 4 versus Trial 1; however, this was not significant for peak dilation (FVC; Supplemental Table II). Importantly, any small changes observed in Trial 4 do not impact the primary findings from the present investigation (see Discussion of manuscript).

**Vasoactive drug infusion**

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

To inhibit K\(_{IR}\) channels, barium chloride (BaCl\(_2\); K\(_{IR}\) channel inhibitor; 10% w/v BDH3238, EMD Chemicals, Gibbstown, NJ) was infused at 0.9 \(\mu\)mol/dl FAV/min with a range of a minimum dose of 8 \(\mu\)mol/min to a maximum dose of 10 \(\mu\)mol/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\(^1\). On subsequent RH trials, ouabain was reinfused for 5 minutes prior to arterial occlusion to provide continuous inhibition. We administered \(NG\)-monomethyl-L-arginine [L-NMMA; nitric oxide (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 prostaglandins (PGs). The doses of L-NMMA and ketorolac were 5 mg/min and 1200 \(\mu\)g/min respectively and given for 5 minutes\(^1\) prior to arterial occlusion. IND numbers were obtained for the investigational use of BaCl\(_2\) (110141), ouabain (110203), and L-NMMA (101256). BaCl\(_2\) and ouabain were prepared in saline and confirmed sterile and free of fungus/endotoxin and particulate matter with a standard microbiology report (JCB-Analytical Research Labs, Wichita, KS) prior to use. Forearm volume used for normalization of specific vasoactive drugs was determined from regional analysis of whole-body dual-energy X-ray absorptiometry scans (QDR series software, Hologic, Inc., Bedford, MA). A subregion was manually defined by a trained user from the head of the radius to the intersection of the radius and ulna with the carpus, encompassing the entire forearm tissue. Forearm fat mass and fat-free mass was then used to calculate FAV, assuming densities of 0.9 g/ml and 1.1 g/ml, respectively\(^10\).

**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 KIR 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 KIR channels and Na+/K+-ATPase (L-NMMA+ketorolac+BaCl2+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 µg/dl FAV/min for 5 minutes in control (saline) conditions and after prior administration of all four antagonists (BaCl2, ouabain, L-NMMA and 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 plethysmogram 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 sec post-cession 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 (Δ%) was calculated as: (FBFpeak/total inhibition − FBFpeak/total control)/(FBFpeak/total control)×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±S.E.M. Dynamic post-occlusion FBF values were analyzed via two-way repeated measures ANOVA (time × 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.
SUPPLEMENTAL RESULTS

Supplemental Table I. Baseline forearm and systemic hemodynamics for time control protocol

<table>
<thead>
<tr>
<th></th>
<th>Trial 1</th>
<th>Trial 2</th>
<th>Trial 3</th>
<th>Trial 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR</td>
<td>58±1</td>
<td>58±2</td>
<td>58±1</td>
<td>58±1</td>
</tr>
<tr>
<td>MAP</td>
<td>86±2</td>
<td>86±2</td>
<td>84±3</td>
<td>88±1</td>
</tr>
<tr>
<td>FBF</td>
<td>1.8±0.3</td>
<td>1.8±0.3</td>
<td>1.5±0.3</td>
<td>1.6±0.3</td>
</tr>
</tbody>
</table>

n=8; HR=heart rate (beats/min); MAP=mean arterial pressure (mmHg); FBF=forearm blood flow (ml/dl forearm volume/min)
Table II. Resting and peak reactive vasodilation for time control protocol

<table>
<thead>
<tr>
<th></th>
<th>Trial 1</th>
<th>Trial 2</th>
<th>Trial 3</th>
<th>Trial 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rest</td>
<td>2.0±0.3</td>
<td>1.9±0.3</td>
<td>1.9±0.4</td>
<td>1.9±0.3</td>
</tr>
<tr>
<td>Peak</td>
<td>36.6±6.3</td>
<td>37.9±5.5</td>
<td>39.4±7.4</td>
<td>34.6±5.6</td>
</tr>
</tbody>
</table>

n=8; FAV=forearm volume
Supplemental Figure I. Repeated Trials of Reactive Hyperemia

A. Forearm blood flow (FBF) response following 5 min of arterial occlusion, in Trial 1 (black circles), Trial 2 (dark grey triangles), Trial 3 (light grey squares), and Trial 4 (white diamonds) conditions. Twenty minutes separated each trial. There were minimal effects of time or repeated reactive hyperemia (RH) bouts. *P<0.05 Trial 1 vs Trials 3 and 4; †P<0.05 Trial 4 vs Trials 1 and 2. B. Peak reactive hyperemic FBF was slightly attenuated from the first RH (Trial 1) in the 4th trial. *P<0.05 vs Trial 1. C. There were no significant differences in the total reactive hyperemic response between any of the trials.
REFERENCES


