Mechanisms of ATP-mediated vasodilation in humans: modest role for nitric oxide and vasodilating prostaglandins

Anne R. Crecelius,1 Brett S. Kirby,1 Jennifer C. Richards,1 Leora J. Garcia,1 Wyatt F. Voyles,2 Dennis G. Larson,2 Gary J. Luckasen,2 and Frank A. Dinenno1

1Human Cardiovascular Physiology Laboratory, Department of Health and Exercise Science, Vascular Physiology Research Group, Department of Biomedical Sciences, Colorado State University, Fort Collins; and 2Medical Center of the Rockies Foundation, Poudre Valley Health System, Loveland, Colorado

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Crecelius AR, Kirby BS, Richards JC, Garcia LJ, Voyles WF, Larson DG, Luckasen GJ, Dinenno FA. Mechanisms of ATP-mediated vasodilation in humans: modest role for nitric oxide and vasodilating prostaglandins. Am J Physiol Heart Circ Physiol 301: H1302–H1310, 2011. First published July 22, 2011; doi:10.1152/ajpheart.00469.2011.—ATP is an endogenous messengers that bind to P2X and P2Y receptors on various cell types including vascular smooth muscle cells, neutrophils, platelets, and the nervous system. ATP is released following cell damage and as a cotransmitter with norepinephrine, and can act to blunt sympathetic vasoconstriction (4). In contrast, circulating ATP can bind to P2Y receptors on the vascular endothelium, which results in vasodilation, the magnitude of which can reach levels observed during exercise in the peripheral circulation (3, 16). Accumulating evidence indicates that circulating ATP may play an important role in the regulation of skeletal muscle vascular tone in humans, specifically during physiological stimuli such as exercise and hypoxia (13, 16) where it is thought to assist in the regulation of blood flow and oxygen delivery to meet the metabolic demands of the tissue. However, our current understanding of the signaling mechanisms involved in ATP-mediated vasodilation in humans remains poor.

In vitro studies in a variety of tissues have demonstrated that ATP-mediated vasodilation is endothelium dependent (11) and occurs through the activation of endothelial G-protein-coupled P2Y receptors (3). It is well established that endothelial-derived vasodilation occurs through the production of 1) nitric oxide (NO); 2) prostacyclin, the predominant vasodilator prostaglandin (PG); or 3) other endothelial-derived factors that result in smooth muscle cell hyperpolarization (15). Limited studies have addressed the role of these pathways in ATP-mediated vasodilation in humans, and the results are conflicting, some showing no significant role for NO or PGs (28, 32, 37) and others claiming these pathways are involved in ATP vasodilation (24).

There are several issues to consider when integrating the findings to date regarding the potential mechanisms underlying ATP-mediated vasodilation. First, in vitro evidence suggests that within the same vasculature, low-dose ATP-induced vasodilation is sensitive to NO and PG inhibition, whereas higher doses are not (30, 33). This latter finding is of interest when considering the dual properties of ATP in the human vasculature whereby it not only evokes direct vasodilation but also can limit sympathetic α-adrenergic vasoconstriction (20, 29). In this context, our laboratory has demonstrated 1) that NO and PGs are not obligatory to observe the normal blunting of sympathetic vasoconstriction in contracting muscle (10), and 2) that the unique ability of ATP to blunt sympathetic vasoconstriction is graded with ATP concentrations, where high levels are sympatholytic but low levels are not (20). Thus, when taken together, it seems plausible to speculate that low doses of ATP may be more dependent on NO and PGs to evoke vasodilation compared with high doses.

Second, the majority of studies in humans have not tested for interactions between NO and PGs in ATP-mediated vasodilation (28, 32, 37). Intracellular endothelial cell calcium release events and calcium concentrations are a point of crossover in the stimulus for the synthesis of NO, PGs, and hyperpolarizing

ADENOSINE TRIPHOSPHATE (ATP) is an important nucleotide with various functions including diverse effects on the cardiovascular system (3). With respect to the control of vascular tone, ATP can be released as a cotransmitter with norepinephrine from sympathetic nerve endings, bind to P2X receptors on smooth muscle cells, and evoke subsequent vasoconstriction...
factors (38), and in fact, a portion of PG-mediated vasodilation may be dependent on NO (27). During physiological stimuli such as exercise and hypoxia where ATP is thought to play a role in the control of vascular tone, we and others (1, 23, 25, 31) have observed a significant interaction of NO and PGs. A third consideration relates to the timing of inhibitor administration and subsequent quantification of the potential contribution of a specific pathway to the observed vasodilatory response. To date, all experiments have been designed such that NO or PGs are inhibited before ATP infusion. However, in certain circumstances such as exercise and hypoxia, inhibition of the synthesis of vasodilatory substances can have differential effects depending on whether it occurs before or during vasodilator infusion and this may be of physiological importance (12, 31). This approach to examining vasodilatory mechanisms of exogenous ATP has never been tested in humans.

With this information and these experimental considerations as a background, the purposes of the present investigation were to (1) determine the individual, combined, and potential interactive roles of NO and PGs in ATP-mediated vasodilation; and (2) determine whether a potential role for NO and PGs is affected by the dose of exogenous ATP infused or the timing of inhibition of NO and PG synthesis. We hypothesized (1) that individual inhibition of NO or PGs does not impact ATP-mediated vasodilation, whereas combined inhibition will significantly reduce the response; (2) that the potential role of NO and PGs in the dilatory response is greatest at low doses of exogenous ATP administration; and (3) that the impact of NO/PG blockade on the dilatory response will be more robust during (vs. before) ATP infusion.

METHODS

Subjects

With Colorado State University Institutional Review Board approval and after written informed consent, a total of 38 young healthy adults [protocol 1: 10 men, 8 women; age = 22 ± 1 yr; weight = 71.7 ± 4.1 kg; height = 172 ± 10 cm; body mass index = 24.2 ± 1.4 kg/m²; forearm volume (FAV) = 1.030 ± 0.90 ml; protocol 2: 15 men, 5 women; age = 22 ± 1 yr; weight = 78.4 ± 2.3 kg; height = 178 ± 2 cm; body mass index = 24.7 ± 0.6 kg/m²; means ± se] participated in the present study. All subjects were sedentary to moderately active, nonsmokers, nonobese, normotensive (resting blood pressure <140/90 mmHg), and not taking any medications. Studies were performed after a 4-h fast and 24-h abstention from caffeine and exercise with subjects in the supine position with the experimental arm abducted to 90° and slightly elevated above heart level on a tilt-adjustable table (5). 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

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 and blood sampling. The catheter was connected to a three-port connector as well as a pressure transducer for mean arterial pressure (MAP) measurement and continuously flushed at 3 ml/h with heparinized saline. The two side ports were used for drug infusions (5, 20).

Body Composition and FAV

Body composition was determined by dual-energy X-ray absorptiometry (DEXA; Hologic, Bedford, MA). Total FAV was calculated from regional analysis of the experimental forearm (from the proximal to distal radioulnar joint) from whole body DEXA scans with QDR software for normalization of the dose of ATP (19). Body mass index was calculated as body weight (kg) divided by height (meters) squared.

Forearm Blood Flow and Vascular Conductance

Protocol 1. Forearm blood flow (FBF) was measured via venous occlusion plethysmography using mercury-in-salistic strain gauges (17, 19). A pediatric blood pressure cuff was placed around the wrist of the experimental arm and inflated to suprasystolic pressure (~200 mmHg) to arrest the hand circulation. Additionally, a venous occlusion cuff was placed around the upper portion of the experimental arm and rapidly cycled between inflation at ~50 mmHg (7 s) and deflation (8 s) yielding one blood flow measurement every 15 s. FBF was expressed as milliliters per 100 milliliters of tissue per minute (ml-100 ml⁻¹·min⁻¹). 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-100 ml⁻¹·min⁻100 mmHg⁻¹. In an effort to minimize the contribution of cutaneous blood flow to FBF measurements, a fan was directed at the experimental arm throughout the experimental protocol.

Protocol 2. A 12-MHz linear-array ultrasound probe (Vivid 7; General Electric, Milwaukee, WI) was used to determine brachial artery mean blood velocity (MBV) and brachial artery diameter. The probe was securely fixed to the skin over the brachial artery proximal to the catheter insertion site as previously described (9). For blood velocity measurements, the probe insonation angle was maintained at <60° and the frequency used was 5 MHz. The Doppler shift frequency spectrum was analyzed via a Multigon 500V TCD (Multigon Industries, Mt. Vernon, NY) spectral analyzer from which mean velocity was determined as a weighted mean of the spectrum of Doppler shift frequencies (5). Brachial artery diameter measurements were made in duplex mode at end-diastole and during steady-state conditions in triplicate. FBF was calculated as:

\[ FBF = MBV \times \pi \times \left(\frac{brachial\ artery\ diameter}{2}\right)^2 \times 60, \]

where the FBF is in ml/min, the MBV is in cm/s, the brachial diameter is in cm, and 60 was used to convert from ml/s to ml/min. FVC was calculated as (FBF/MAP) × 100, and expressed as ml-100 ml⁻¹·min⁻¹00 mmHg⁻¹. In an effort to minimize the contribution of cutaneous and hand blood flow to FBF measurements, a fan was directed at the experimental arm throughout the experimental protocol and a pediatric blood pressure cuff was placed around the wrist of the experimental arm and inflated to suprasystolic pressure (~200 mmHg) to arrest the hand circulation.

We chose to use the Doppler ultrasound measure of FBF in protocol 2 and not plethysmography as we had in protocol 1 to be able to measure FBF on a beat-to-beat basis and observe any transient responses to vasoactive drug infusions, specifically when combined NO/PG inhibition is performed during the vasodilatory condition (31).

Vasoactive Drug Administration

In protocol 1, the P2Y-receptor agonist ATP (Sigma A7699) was infused at 1.25, 2.5, and 5 μg/dl MAP⁻¹·min⁻¹ for 4 min each (19, 20). ATP was confirmed sterile and free of fungus/endotoxin with a standard microbiology report (JCB-Analytical Research Labs, Wichita, KS) before use (19, 20).

To determine the role of NO in the vasodilatory response to ATP, the NO synthase (NOS) inhibitor NG⁻²-monomethyl-L-arginine (L-NMMA; Clinalfa/Bachem, Weil am Rhein, Germany) was admin-
istered intra-arterially to inhibit the production of NO. A loading dose totaling 25 mg (5 mg/min for 5 min) was given, and a maintenance dose (1.25 mg/min) was then infused for the duration of the study to ensure continuous blockade. This dose of L-NMMA has been previously shown to significantly reduce basal vascular tone and also the vasodilatory effects of acetylcholine (8, 9), consistent with effective NOS inhibition (36). Further, our dose of L-NMMA is within the range of incremental doses that were previously shown to lower venous plasma nitrite concentrations at rest and inhibit NO release in response to intrabrachial acetylcholine infusion, both of which were associated with corresponding reductions in FBF (22).

The contribution of PGs to the vasodilatory response to ATP was determined by performing regional cyclooxygenase (COX) inhibition. Ketorolac (trade name Toradol; Hospira, Lake Forest, IL), a nonselective COX inhibitor, was administered intra-arterially to inhibit the synthesis ofPGs. A loading dose totaling 6 mg (600 μg/min for 10 min) and a maintenance dose (300 μg/min) were infused for the duration of the study to ensure continuous blockade. This dose of ketorolac is twice that which was previously demonstrated to reduce circulating PGF_{1α} (a stable breakdown product of PGs) at rest and during handgrip exercise (10).

In protocol 2, two doses of ATP, 0.64 and 4.6 μg·dl·FAV^{-1}·min^{-1} were administered in separate subjects. These doses were used in a previous study in our laboratory where we found that the high dose (4.6) significantly reduces sympathetic vasoconstrictor responses whereas the low dose (0.64) does not (20), and thus it is possible that the signaling mechanisms differ for the two doses of ATP as has been shown in vitro (33). To determine the contribution of NO to ATP-mediated vasodilation, the same dose of L-NMMA administered in protocol 1 was used in this protocol. The same dose of ketorolac administered in protocol 1 was also used in this protocol; however, the loading dose (6 mg) occurred over a 5-min period, rather than 10 min to match the timing of the loading dose of L-NMMA. To address the combined contributions of NO and PGs to the steady-state vasodilation mediated by ATP, the loading doses of L-NMMA and ketorolac occurred during the infusion of ATP as opposed to before ATP infusion. We (5, 23, 31) have previously demonstrated the ability of these inhibitors to cause significant reductions in vasodilation when administered during a hyperemic stimulus.

**Experimental Protocols**

**Protocol 1.** The purpose of protocol 1 was to determine the individual and combined contributions of NO and PGs to the vasodilatory response to intra-arterial ATP. A timeline of the experimental study is depicted in Fig. 1A. Two minutes of resting data were acquired before the start of all ATP infusions. Trial 1 served as the control ATP response, with 4-min infusions of ATP at each of three progressive doses (1.25, 2.5, and 5 μg·dl·FAV^{-1}·min^{-1}). Following the control response, during the 15-min rest period, half of the subjects (n = 9) received L-NMMA to assess the individual role of NO to ATP-mediated vasodilation and the other half (n = 9) of subjects received ketorolac to assess the individual role of PGs to the response. A second ATP dose-response trial was then performed with this single vasodilatory pathway inhibition. Following the second ATP trial, whichever inhibitor was not previously given was administered during the 15-min rest period and a third and final ATP dose-response trial was performed with the combined inhibition of NO and PGs.

**Protocol 2.** The purpose of protocol 2 was to investigate whether the contribution of NO and PGs to ATP-mediated vasodilation may be dependent on 1) the dose of ATP administered and/or 2) whether these vasodilatory pathways are inhibited before ATP infusion vs. during vasodilatory infusion. A timeline of the experimental study is depicted in Fig. 1B. The same general experimental approach was performed on both subject groups; those who received low dose ATP (n = 8; 0.64 μg·dl·FAV^{-1}·min^{-1}) and those who received high dose ATP (n = 8; 4.6 μg·dl·FAV^{-1}·min^{-1}). Two minutes of resting data were acquired before the start of all ATP infusions. Trial 1 served as the time control response, where ATP infusion occurred for 10 min.
Fifteen minutes of rest followed before performing a second trial beginning with 2 min of resting data acquisition before the initiation of a 10-min ATP infusion. Five minutes into ATP infusion, combined inhibition of NO and PG synthesis (via L-NMMA and ketorolac, respectively) occurred for the remaining 5 min of the trial. After another 15 min of rest (and continued maintenance doses of the L-NMMA and ketorolac), a third and final trial (5 min) was performed. In all trials and protocols, saline was used as a control infusion at matched rates to the vasodilator and inhibitor infusions.

**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; DDATAQ Instruments, Akron, OH). MAP was determined from the arterial pressure waveform and heart rate (HR) was determined via the standard three-lead ECG. In protocol 1, FBF was determined from the derivative of the forearm plethysmogram signal. FBF, HR, and MAP represent an average of the last minute (protocol 1) or 30 s (protocol 2) of steady-state conditions (e.g.: baseline rest, steady-state vasodilator infusion, combined NO/PG inhibition, etc.). Although we designed our studies to capture any transient effects of the combined NO/PG inhibition as observed in our previous studies during exercise, we found this did not occur and that the peak responses always occurred at the end of the L-NMMA and ketorolac infusion (minute 10 of trial 2) and thus these last 30 s were used for data analysis.

Given the existence of individual differences in baseline vascular tone, individual differences in forearm vascular tone during vasodilator infusion, as well as the potential influence of the NOS and COX inhibition on baseline vascular tone, our primary interest was in the relative (%) change in FVC, as percent changes in FVC track changes in blood vessel radius independent of the initial level of vascular tone and therefore is the most appropriate index of changes in vasomotor tone. Thus, in both protocols 1 and 2, to quantify the vasodilatory response to ATP, the percent increase in FVC due to vasodilator infusion in each trial was calculated as:

\[
\text{(FVC vasodilator infusion – FVC baseline)/(FVC baseline)} \times 100.
\]

**Statistics**

Data are presented as means ± SE. For protocol 1, differences within and between trials and conditions for each group were determined via two-way [dose and condition (control, single inhibition, combined inhibition)] repeated-measures ANOVA. When significance was observed, the Fisher’s least significant difference method was used to make individual comparisons. In protocol 2, specific hypothesis testing related to differences between conditions within the same experimental group were made using paired two-tailed Student’s t-tests. Differences between experimental groups in both protocols were analyzed with unpaired two-tailed Student’s t-tests. Paired two-tailed Student’s t-tests were used to determine baseline forearm hemodynamic changes in both protocols. Significance was set a priori at \( P < 0.05 \).

**RESULTS**

**Protocol 1: Effects of Independent and Combined Inhibition of NO and PGs on ATP-Mediated Vasodilation (Assessed via Venous Occlusion Plethysmography)**

Absolute FBF, FVC, MAP, and HR for protocol 1 are presented in Table 1. The vasodilatory responses expressed as the percentage increase in FVC from baseline during ATP infusion in those subjects who received ketorolac first (independent PG inhibition) and those who received L-NMMA first (independent NO inhibition) are presented in Fig. 2, A and B.
There was no significant effect of independent PG inhibition (Fig. 2A) nor independent NO inhibition (Fig. 2B) on ATP-mediated vasodilation. Further, the vasodilatory response to ATP was similar under control and combined NO/PG blockade conditions in both groups of subjects (Fig. 2, A and B). Minimal changes in MAP (3–6 mmHg) and HR (2–5 beats/ min) did occur between trials, and these were more common in conditions that followed l-NMMA infusion, possibly indicating a minor systemic effect of our NOS inhibition.

Protocol 2: Time Control of ATP Infusion (Trial 1, Assessed via Doppler Ultrasound)

Absolute FBF, FVC, MAP and HR for protocol 2 are presented in Table 2. During trial 1, vasodilatory responses expressed as the percentage increase in FVC from baseline during ATP infusion were not different at minute 5 and minute 10 for both low dose (196 ± 31 vs. 204 ± 34%; P = 0.63) and high dose (728 ± 75 vs. 688 ± 75%; P = 0.26). Further, these vasodilatory responses were similar to those achieved within the first 5 min of ATP infusion during trial 2 (low dose: 198 ± 24%; high dose: 706 ± 79%; P = n.s. for both).

Protocol 2: Effect of Combined NO and PG Inhibition During ATP Infusion (Trial 2)

In trial 2, combined inhibition of NO and PGs was performed from minutes 5 to 10 of ATP infusion. Combined NO/PG inhibition during ATP infusion resulted in a decrease in absolute FVC for both low and high doses (P < 0.05; Fig. 3), and the magnitude was similar for low (~34.4 ml·min⁻¹·100 mmHg⁻¹) and high dose ATP (25.0 ml·min⁻¹·100 mmHg⁻¹; P = 0.27). The relative impact of NO/PG inhibition during steady-state vasodilation was significantly greater for the low dose (ΔFVC = −35 ± 3%) compared with that for the high dose of ATP (ΔFVC = −12 ± 2%; P < 0.05).

Protocol 2: Effect of Combined NO and PG Inhibition Before ATP Infusion (Trial 3)

To determine the effect of prior NO and PG inhibition on the vasodilatory response to ATP, we compared the percent increase in FVC from baseline during trial 2 and trial 3 (Fig. 4). Combined NO/PG inhibition before ATP infusion resulted in a decrease in the vasodilatory response (%FVC) for both the low

![Graph](image-url)

Fig. 2. Protocol 1: forearm vasodilatory responses to ATP. A: control, independent PG inhibition (ketorolac) and combined PG/NO inhibition (ketorolac + l-NMMA) vasodilatory responses to 3 doses of intrabrachial ATP infusion. B: control, independent NO inhibition (l-NMMA) and combined NO/PG inhibition (l-NMMA + ketorolac) vasodilatory responses to 3 doses of intrabrachial ATP infusion. No significant differences were observed across all conditions in either group.

respectively. As expected, infusion of ATP at all doses resulted in a significant vasodilatory response under all conditions in both groups (P < 0.05). Baseline FBF and FVC were significantly reduced from control in all blockade conditions (Table 1).

![Table 2](image-url)

Table 2. Protocol 2: forearm and systemic hemodynamics

<table>
<thead>
<tr>
<th></th>
<th>Trial 1: Time Control</th>
<th>Trial 2: Inhibition During SS</th>
<th>Trial 3: Prior Inhibition</th>
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<tr>
<td></td>
<td>Baseline</td>
<td>Minute 5</td>
<td>Minute 10</td>
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<tr>
<td>ATP dose of 0.64 μg·dl FAV⁻¹·min⁻¹</td>
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<tr>
<td>FBF</td>
<td>30 ± 3</td>
<td>81 ± 7*</td>
<td>84 ± 7*</td>
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<tr>
<td>FVC</td>
<td>32 ± 3</td>
<td>89 ± 8*</td>
<td>91 ± 7*</td>
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<tr>
<td>HR</td>
<td>56 ± 2</td>
<td>56 ± 2</td>
<td>55 ± 2</td>
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<tr>
<td>MAP</td>
<td>96 ± 4</td>
<td>92 ± 3</td>
<td>92 ± 3</td>
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<td>ATP dose of 4.6 μg·dl FAV⁻¹·min⁻¹</td>
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<tr>
<td>FBF</td>
<td>27 ± 4</td>
<td>220 ± 42*</td>
<td>205 ± 31*</td>
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<tr>
<td>FVC</td>
<td>30 ± 4</td>
<td>246 ± 39*</td>
<td>227 ± 28*</td>
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<tr>
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<td>51 ± 3</td>
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Data are means ± SE. SS, steady state. *P < 0.05 vs. baseline (within trial). †P < 0.05 vs. trial 1 (within time point). ‡P < 0.05 vs. trial 2 (within time point). §P < 0.05 vs. minute 5 (within trial).
and high dose of ATP (P < 0.05; Fig. 4). Here, the magnitude of the impact of NO/PG inhibition when occurring before ATP infusion was not different between the low (Δ = −31 ± 12%) and high dose (Δ = −25 ± 11%; P = n.s.).

DISCUSSION

The purposes of the present study were to directly determine 1) the individual, combined, and potential interactive roles of NO and PGs in ATP-mediated vasodilation in humans; and 2) whether a potential role for NO and PGs is affected by the dose of exogenous ATP infused or the timing of inhibition of NO and PG synthesis. The primary novel findings of this study are as follows. First, in protocol 1, we observed no significant contribution of NO nor PGs, alone or in combination, to the vasodilatory response to graded infusions of ATP in the forearm using venous occlusion plethysmography to determine FBF (Fig. 2). Second, in protocol 2, where Doppler ultrasound was used to determine FBF, combined NO/PG inhibition that occurred during ATP infusion revealed a modest contribution of NO and PGs for the low and high dose of ATP, and this relative contribution appeared to be greater than that for the high dose (Fig. 3). When combined NO/PG inhibition occurred before vasodilator infusion, there was again a modest contribution of NO and PGs, and here the relative modest contribution was similar for both the low and high dose of ATP (Fig. 4). Overall, the collective data from the present investigation indicate that depending on the technique used to assess forearm vasodilation, there is at most a modest contribution of NO and PGs to ATP-mediated dilation and the majority of vasodilation that occurs in response to exogenous ATP is independent of these putative endothelium-dependent pathways in humans.

Exogenous ATP-Mediated Vasodilation in the Human Vasculature

The findings from the present study add to the growing body of knowledge regarding the potential mechanisms of vasodilation to exogenous ATP infusions in humans (24, 28, 32, 37). The independent roles of NO and PGs in ATP-mediated vasodilation have been addressed in separate studies by Rongen and colleagues (28, 37) in an experimental forearm model similar to that used in the present study. In these previous studies, only single inhibition of NO or PGs were performed in separate subjects, and thus any potential interaction between these two endothelium-derived vasodilators in mediating ATP vasodilation was not determined. This is an important question to address based on previous observations in humans during exercise (31) and hypoxia (23), physiological stimuli in which ATP is thought to play a role in vascular regulation (14, 16), demonstrating a significant interaction between NO and PGs in the regulation of vascular tone. Thus we hypothesized that combined inhibition of NO and PGs would have a greater impact on ATP-mediated vasodilation than individual inhibition of these pathways. Our findings from protocol 1 indicate no significant independent or combined contribution of NO and PGs to the ATP-mediated vasodilatory response when FBF is measured via venous occlusion plethysmography.

In protocol 2 of the present investigation, we aimed to address whether or not the timing of NOS/COX inhibition could potentially reveal an important combined role of NO and PGs to ATP-mediated vasodilation in humans. In all previous studies on this topic to date (including protocol 1), administration of NOS and/or COX inhibitors occurred before the infusion of ATP. Again, drawing from previous findings during physiological stimuli such as exercise and hypoxia in which ATP may be involved, it was possible that a potential role of NO and PGs was not observed in our first protocol because other vasodilatory pathways were able to acutely compensate for the loss of NO and PG synthesis to achieve the same vasodilatory response as had occurred in the control condition (12, 23, 31). In this protocol, FBF was measured via Doppler ultrasound to allow us to capture any potential transient response of the inhibitors suggestive of compensatory mechanisms of vasodilation, as has occurred in previously (31). In these subjects, infusion of L-NMMA and ketorolac to inhibit NO and PGs during ATP significantly reduced steady-state vasodilator responses from baseline to steady-state vasodilation (minute 5) in control conditions (trial 2) and with prior NO/PG inhibition (trial 3) for low and high dose ATP. *P < 0.05 vs. control vasodilator response (trial 2).
state FVC to both low and high doses of ATP (Fig. 3). Interestingly, we also found in a third trial of ATP infusion that prior combined inhibition of NO and PGs significantly reduced the vasodilatory response to both doses of ATP (~25–30%), a finding in stark contrast with data obtained in protocol 1.

Recently, Mortensen et al. (24) demonstrated a modest role for both NO and PGs in ATP-mediated vasodilation in the leg circulation of humans. In this study, femoral blood flow was measured via Doppler ultrasound at rest and via the constant-infusion thermodilution technique during ATP infusions in young subjects with blockade of NO (via L-NMMA) and/or PGs (via indomethacin) on different days. These investigators attributed the divergent findings with regards to the roles of NO and PGs in mediating ATP vasodilation to potential differences in vascular control in the leg compared with the forearm circulation. In the present study, we found a significant contribution of both NO and PGs to ATP-mediated vasodilation when quantified via Doppler ultrasound, and this is consistent with Mortensen et al. It should be emphasized that all studies to date on this topic utilizing the forearm model have employed use of venous occlusion plethysmography to assess forearm hemodynamics. Thus perhaps the divergent findings regarding the possible involvement of NO and PGs in ATP-mediated vasodilation are not limb specific in humans but rather reflect differences in measurement techniques (see Experimental Considerations). Collectively, the available evidence indicates a potential modest role of NO and PGs to ATP-mediated vasodilation in humans. Importantly, however, the majority of the observed vasodilation appears independent of these endothelium-dependent pathways.

Another question addressed in protocol 2 of the present investigation was whether the signaling pathways may differ depending on the dose of ATP administered. In addition to support from isolated vessels (30, 33) and nonstatistical evaluation of the results of previous human studies (28, 37), we were interested in examining the effect of dose given the previous findings by our laboratory and others regarding the ability of ATP to limit sympathetic α-adrenergic vasoconstriction (20, 29). In this context, we (20) have recently demonstrated that this “sympatholytic” ability of ATP is graded with dose, where high levels of ATP are sympatholytic but low levels are not, much like the intensity-dependent nature of functional sympatholysis within contracting muscles (20, 35). Related to the contributions of NO and PGs, we (9, 10) have demonstrated that NO and PGs are not obligatory for sympatholysis in contracting muscle. Thus we hypothesized that the nonsympatholytic low dose of ATP we had previously used would be mediated predominantly through NO and PGs, whereas the high dose of ATP would be independent of these substances. In this protocol, when combined NOS and COX inhibition was performed during ATP infusion, the absolute reduction in FVC was similar for low and high doses; however, the relative (%) reduction in FVC was greater during low dose ATP (~30%) compared with high dose ATP (~10%). In trial 3 of this protocol, prior combined NOS and COX inhibition attenuated the vasodilator response to both doses of ATP similarly (~25–30%), and thus the collective data indicate no dose dependency of the combined contribution of NO and PGs to ATP-mediated vasodilation.

In a subgroup of subjects (n = 4), we attempted to address this apparent discrepancy regarding the dose dependency of NO and PGs in ATP-mediated vasodilation depending on whether quantifying the absolute vs. relative reduction in FVC during ATP infusion. Given the differences in the magnitude of vasodilation and subsequent elevation in FBF in the high vs. low ATP dose condition, we questioned whether any apparent smaller role of NO and PGs to the high ATP vasodilator response could be attributed to a lower concentration in the blood due to dilution and thus less effectiveness of the inhibitors. In this subgroup of subjects, we infused a high dose of acetylcholine, an endothelium-dependent agonist known to rely primarily on the NO and PG pathways to evoke vasodilation in the human forearm (6, 34), and essentially replicated the experimental trials used to test our ideas related to ATP. Our findings indicate that combined NOS/COX inhibition modestly reduces FVC during acetylcholine infusion (~30%), whereas combined inhibition before acetylcholine attenuated the vasodilation by nearly 80%. We interpret our collective data to indicate that the effectiveness of combined NOS/COX inhibition could be less during the high ATP dose due primarily to a dilution effect, and thus we have slightly underestimated the combined contributions of NO and PGs under this experimental condition. Given this proposition, we conclude that any modest reliance on the NO and PG pathways to evoke vasodilation is not different for low compared with high ATP doses. When integrating the current findings of a lack of dose-dependent contributions of NO and PGs with how ATP can modulate sympathetic vasoconstriction, we speculate that it is the net effect of the remaining (yet currently unknown) downstream P2Y-receptor signaling that must limit α-adrenergic receptor responsiveness in humans.

Experimental Limitations and Considerations

In many human studies employing the use of pharmacological inhibitors to probe vasodilatory mechanisms, it is difficult to assess the effectiveness of the drugs used. In protocol 1 of the present study, we did not directly test the efficacy of our inhibitors, L-NMMA and ketorolac. However, the significant (~80%) inhibition of the vasodilatory response to acetylcholine in a subgroup of subjects in protocol 2 in which the same doses of L-NMMA and ketorolac were used, the reductions in resting vascular tone observed in both protocols, as well as the established use of these doses of inhibitors in previous studies (see METHODS) leads us to believe that the lack of an effect in protocol 1 was not a result of insufficient blocking of the NOS and COX enzymes.

Given that there is some discrepancy in the findings from our two protocols regarding whether or not there is any significant contribution of NO and PGs to ATP-mediated vasodilation in humans, it is important to consider our experimental approach that utilized two different methods of determining FBF. Various methods of determining limb blood flow in vascular research studies exist and, given the appropriate expertise of the investigators utilizing these techniques, are well accepted as reliable measures limb blood flow (18). The contribution of NO and PGs differed in the present study depending on whether we used venous occlusion plethysmography or Doppler ultrasound to measure FBF. Within our own laboratory, and in the literature, we have observed divergent findings related to vasodilator responsiveness within a population based on which method is used to measure FBF (7, 19, 21, 26). Further
investigations into the potential differences of these established assessment techniques and how they may provide differing insight on similar experimental questions may be merited.

Based on our findings from protocol 1 of no significant interaction between NO and PGs in mediating the vasodilatory response to exogenous ATP, we utilized the approach of combined inhibition of NOS and COX for our studies in protocol 2, which somewhat limits the conclusions from this protocol regarding the relative contributions of NO and PGs to the response. The recent data from Mortensen et al. (24) would suggest that the contributions of NO and PGs to ATP-mediated vasodilation are approximately equal, although it is important to note that the dilatory responses in this study were quantified as an absolute change from rest (and thus does not take into account the reduction in baseline leg blood flow that occurred with independent or combined NOS/COX inhibition), and therefore the total contribution of NO and PGs to ATP-mediated vasodilation was overestimated. The collective findings from our study and that of Mortensen et al. (24) suggest that ATP infusions into resting muscle tissue fails to mimic the vascular control observed during physiological stimuli in which one primary goal is the matching of blood flow and oxygen delivery to tissue oxygen demand. This point may help explain why our observed lack of an effect of timing of NO/PG inhibition on ATP-mediated vasodilation is dissimilar from previous studies of exercise (31) and hypoxia (23), despite the fact that ATP may be playing a role in regulating vascular tone during these stimuli.

**Perspectives and Conclusions**

The impetus for the present study was to comprehensively address a current, highly relevant, and somewhat unclear issue regarding the mechanisms of a unique and increasingly important vasodilator (ATP) in the human vasculature. The results from the present investigation demonstrate that in combination NO and PGs have at most a modest role in mediating ATP vasodilation in the forearm circulation of healthy humans. However, the method used to assess FBF responses may affect whether any significant role of NO and PGs is observed. Based on our collective findings, the contribution of NO and PGs to the response is not dose dependent nor does it depend on the timing of inhibition of these endothelium-dependent vasodilatory pathways. We believe that these findings strongly indicate that other mechanisms beyond these traditional endothelium-dependent vasodilators are significantly involved in the vascular responses to circulating ATP and likely also contribute to its unique vasoactive properties in the human vasculature (direct vasodilation and sympatholytic) (20, 29). Thus future studies designed to understand the primary pathways involved in ATP-mediated vasodilation in humans could be important for understanding how blood flow and oxygen delivery may be impaired in patient populations whom demonstrate endothelial dysfunction and/or impaired circulating ATP, particularly during stressors that evoke increases in sympathetic nervous system activity (e.g., exercise, hypoxia).

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**REFERENCES**


