Intravascular Source of Adenosine During Forearm Ischemia in Humans
Implications for Reactive Hyperemia

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Abstract—It is believed that adenosine is released in ischemic tissues and contributes to reactive hyperemia. We tested this hypothesis in the human forearm using microdialysis to estimate interstitial and intravascular levels of adenosine and caffeine withdrawal to potentiate endogenous adenosine and determine its effect on reactive hyperemia. Forearm blood flow response to ischemia was measured by air plethysmography before and 60 hours after the last dose of caffeine (250 mg TID for 7 days, n=6). Forearm blood flow increased by 274±66% and 467±97% after 3 minutes of forearm ischemia, before and during caffeine withdrawal, respectively (P<0.05). Thus, caffeine withdrawal enhances reactive hyperemia. To determine the source of adenosine, we measured interstitial adenosine with the use of a microdialysis probe inserted into the flexor digitorum superficialis muscle of the forearm, and we measured intravascular adenosine with the use of a microdialysis probe inserted retrogradely into the medial cubital vein. Dialysate samples were collected at 15-minute intervals during resting, forearm ischemia, and recovery periods. Forearm ischemia failed to increase muscle dialysate concentrations of adenosine but did increase intravascular dialysate adenosine 2.1-fold, from 0.61±0.12 to 1.28±0.39 μmol/L (P<0.01, n=8). Intravascular dialysate concentrations of thromboxane B₂ did not increase during ischemia, ruling out platelet aggregation as a source of adenosine. These results support the hypothesis that endogenous adenosine contributes to reactive hyperemia and indicate that the major source of adenosine in the human forearm is intravascular. We speculate that endothelial cells are the source of intravascular adenosine during ischemia. (Hypertension. 1999;33:1453-1457.)

Key Words: adenosine ■ ischemia ■ muscle ■ microdialysis

Local vasodilation is an important protective response to ischemia. This reactive hyperemia, present in all vascular beds with the exception of the kidneys, is largely due to metabolic factors produced by the mismatch between oxygen supply and metabolic demand. Adenosine has been identified as one of the metabolic products involved in this process. The contribution of adenosine to reactive hyperemia has been extensively studied in coronary circulation,¹ and adenosine has also been proposed to contribute to blood flow regulation in several other vascular beds, including skeletal muscle.²⁻⁴

Because the actions of adenosine are mediated by cell membrane receptors, its importance in modulating reactive hyperemia will be proportional to the extracellular concentrations it reaches during ischemia. Adenosine is released in tissues when metabolic demands exceed oxygen supply, but extracellular concentrations are limited by efficient mechanisms of cellular uptake and metabolism. Cellular uptake is particularly potent in humans and accounts for the extremely short half-life of adenosine in blood, estimated at <1 second.⁵ Previous attempts to assess how much of an increase in extracellular adenosine is produced by ischemia in humans have relied on sampling from the venous drainage of the organ of interest. This, however, has 2 serious limitations. First, the very short half-life of adenosine in human blood and the likelihood that adenosine is released during sampling by activated platelets or hemolized red blood cells introduces errors that underestimate or overestimate, respectively, blood levels of adenosine, even when blood is drawn directly into a cocktail of enzyme inhibitors. Second, it is clear that the endothelium functions as an impermeable metabolic barrier for adenosine.⁶ Therefore, it is possible that little if any of the adenosine released into the interstitium will reach the vascular compartment.

An alternative approach to estimating adenosine concentrations is based on the microdialysis technique: a microdialysis probe is inserted into the tissue of interest and interstitial components are dialyzed and sampled. This method has the advantage that, once inside the dialysis probe, adenosine is protected from cellular reuptake or degrading enzymes. In the present study we used this approach to test the hypothesis...
that adenosine is increased in skeletal muscle interstitium during ischemia in humans. We also used microdialysis to estimate intravascular concentrations of adenosine in humans. Finally, to confirm the contribution of adenosine to reactive hyperemia in our model, we determined the effect of caffeine withdrawal on reactive hyperemia of the forearm, taking advantage of our previous findings that the actions of adenosine are enhanced during withdrawal of long-term caffeine consumption in humans.7

Methods

Subjects
We studied a total of 28 healthy volunteers of both genders, 18 to 42 years of age. Subjects were asked to abstain from methylxanthines for at least 72 hours before the study day. The protocol was approved by the Vanderbilt University Institutional Review Board. Volunteers were informed of the characteristics of the study and gave written consent, in accordance with institutional guidelines.

Instrumentation
For each study session, subjects fasted and were placed in the supine position. FBF was determined by venous occlusion air plethysmography as previously described.8 For intramuscular microdialysis, we used a probe with a dialysis membrane (10×0.5 mm, 20 000 molecular weight cutoff) attached to the end of a double-lumen cannula (CMA/20, CMA). The perfusate enters the probe through the inner cannula, passes down to the tip of the probe, streams upward in the space between the inner cannula and the outer dialysis membrane, and leaves the probe through the outer cannula via a sidearm from which it is collected. The probe was introduced into the flexor digitorum superficialis muscle of the nondominant forearm using 2% lidocaine for local anesthesia. A steel guide cannula covered by a Teflon sheath was inserted at a 45° angle, 15 mm into the muscle. The guide cannula was then removed, leaving only the Teflon sheath in the tissue, through which the microdialysis probe was gently introduced. The Teflon sheath was removed by splitting it as it was retracted. The inlet tubing of the probe was connected to a microinjection pump (CMA) and continuously perfused with saline at 2 μL/min (perfusion). The effluent (dialysate) was collected continuously.

A larger probe with a greater dialysis surface (30×0.5 mm) was used for intravenous microdialysis (CMA/60). Lidocaine 2% was administered subcutaneously and a stainless steel introducer containing the microdialysis probe was inserted retrogradely into the medial cubital vein of the nondominant forearm. The introducer was then pulled out and the probe was secured with transparent surgical tape. The probe was perfused as described above.

Experimental Protocols

Protocol 1: FBF During Caffeine Withdrawal
Six subjects were given 250 mg TID caffeine for 7 days and studied before caffeine was started (day 0) and 60 hours after the last dose of caffeine (day 10). On each study day, subjects were instrumented for FBF measurements as described above and were then allowed to rest for 20 to 30 minutes. We measured the increase in FBF produced by 15, 30, 45, 60, 90, 120, and 180 seconds of forearm ischemia which was induced in the left arm by inflating the proximal pneumatic cuff to 50 mm Hg above the systolic blood pressure. Three of these subjects underwent a similar protocol but received placebo instead of caffeine with the use of a double-blind crossover design. Caffeine and placebo capsules were prepared by the Department of Pharmaceutical Services, Vanderbilt University Medical Center. The analysis of the FBF tracings was done by investigators blinded to the treatment received (placebo or caffeine) and to the study day (day 0 or day 10).

Protocol 2: Intramuscular Microdialysis and Forearm Ischemia
In 14 subjects, an intramuscular probe was inserted as described previously. After a 1-hour equilibration period, 2 consecutive 15-minute dialysate samples were collected to determine baseline adenosine levels. Circulatory arrest (ischemia) was induced by inflating a proximal pneumatic cuff to 50 mm Hg above the systolic blood pressure for 15 minutes and a dialysate sample was collected during this period. Immediately after the cuff was deflated, 2 15-minute recovery samples were collected. The dialysate collection period was shifted by 1 minute in relation to the ischemic period to account for the lag time produced by the length of the collecting tubing.

Protocol 3: Intravenous Microdialysis During Forearm Ischemia
In 8 subjects, an intravascular probe was inserted as described previously. After a 1-hour equilibration period, 2 consecutive 15-minute dialysate samples were collected to determine baseline adenosine levels. Circulatory arrest (ischemia) was induced by inflating a proximal pneumatic cuff to 50 mm Hg above the systolic blood pressure for 15 minutes and a dialysate sample was collected during this period. Immediately after the cuff was deflated, 2 15-minute recovery samples were collected. In 5 of these subjects, we repeated the intravascular microdialysis protocol on a different day to measure intravascular thromboxane B2 concentrations.

Protocol 4: In Vitro Calibration of Microdialysis Probes
In vitro calibration of the microdialysis probe was performed in 8 subjects participating in the intramuscular microdialysis protocol and in 5 subjects participating in the intravenous microdialysis protocol to estimate the fraction of adenosine recovered across the microdialysis membrane. The probe was removed from the muscle or vein at the end of the study and was placed in a solution containing 2.5 μmol/L adenosine. This probe was continuously perfused with saline at 2 μL/min. The dialysate was collected over 30 minutes in two 15-minute fractions. Two 30 μL samples were also collected directly from the 2.5 μmol/L adenosine solution. These 2 sets of samples were processed and the percentage recovery was calculated by dividing the dialysate concentration by the adenosine concentration measured from the 2.5 μmol/L adenosine solution.

Analytical and Statistical Methods
FBF was measured from the original tracings with a digitizer tablet coupled to Sigma Scan software (Jandel Scientific) and was expressed in units of ml/100 mL of forearm volume/min. We averaged the first 10 blood flow determinations immediately after each intervention. This average, an estimate of circulatory debt repayment, was used to assess the magnitude of reactive hyperemia. We also used the single largest blood flow during each intervention to analyze peak blood flow.

Samples for adenosine determinations were analyzed with the use of a microbore high pressure liquid chromatography system (Isco microLC system, Isco Inc) with the use of a method previously described.9 Dialysate samples were collected in ice-cooled 200 μL polyethylene vials containing 15 μL of internal standard (2-methyl-adenosine, 20 ng/15 μL), using a fraction collector (CMA) for the intramuscular protocol and manual collection for the intravenous protocol. Samples for adenosine determinations were immediately dried by centrifugation under vacuum (Savant SpeedVac, Savant Instruments Inc) and then stored at −20°C until analyzed. The dried samples were reconstituted in 4 μL of mobile phase immediately before 1 μL was injected into the column. A standard curve was constructed for each experiment with increasing amounts of authentic adenosine and processed in a manner identical to the corresponding samples, including evaporation to dryness.

Samples for thromboxane B2 were stored at −70°C until analysis, with a modification of a previously described method.10 Briefly, 2 ng of tetradeuterated thromboxane B2 was introduced into 100 μL of each sample as an internal standard. Samples were acidified with 0.5% formic acid and extracted with ethyl acetate. After concentra-
The increase in FBF produced by ischemia was significantly greater when subjects were studied during caffeine withdrawal ($P<0.01$ by ANOVA, $n=6$). FBF was measured at 12-second intervals for 2 minutes after each ischemic period, and the average of these 10 measurements was used as an indicator of circulatory debt repayment. The lower panel shows the time course of reactive hyperemia after 3 minutes of ischemia. The peak blood flow after ischemia was enhanced during caffeine withdrawal ($P<0.01$ by ANOVA for the differences between curves; asterisks denote significant difference in individual time points between groups by Duncan’s test).

Results are expressed as mean±SEM. Baseline values from microdialysis experiments were taken as the average of two 15-minute measurements. We used ANOVA with repeated measures within subjects for multiple comparisons. Linear regression analysis was used for construction of standard curves and calculation of samples. Data were analyzed with the use of the Number Cruncher Statistical System (NCSS). Values of $P<0.05$ were considered significant.

**Effect of Caffeine Withdrawal on Reactive Hyperemia**

The increase in FBF produced by ischemia was significantly greater when subjects were studied during caffeine withdrawal versus baseline ($P<0.01$, $n=6$, Figure 1). FBF increased in response to 3 minutes of ischemia from $2.4±0.5$ to $7.61±0.82$ mL/100 mL forearm volume/min (274±66%) at baseline (day 0), and from $2.25±0.3$ to $11.45±0.88$ mL/100 mL forearm volume/min (467±97%, $P<0.05$) during caffeine withdrawal (day 10). This potentiation was particularly apparent at peak blood flow (Figure 1). By comparison, no differences in reactive hyperemia were found in the control group of subjects receiving placebo instead of caffeine; FBF increased by $333±95\%$ in response to 3 minutes of ischemia at baseline (day 0), and by $325±128\%$ during placebo withdrawal (day 10, $n=3$).

**Effect of Ischemia on Intramuscular and Intravascular Adenosine**

Dialysate concentrations of adenosine were high immediately after the insertion of the intramuscular microdialysis probe ($0.97±0.23$ μmol/L), but levels decreased to a stable baseline within 1 hour. For this reason, baseline collections were started at least 1 hour after probe insertion. Adenosine dialysate concentrations were not significantly different during forearm ischemia ($0.28±0.08$ μmol/L) versus either baseline period ($0.22±0.03$ and $0.24±0.03$ μmol/L, Figure 2). A slight but not significant increase in adenosine dialysate concentrations occurred during the recovery periods after ischemia ($0.34±0.09$ and $0.29±0.05$ μmol/L). In vitro recovery for adenosine from the intramuscular microdialysis probe averaged $34±6\%$.

Dialysate adenosine concentrations were stable immediately after insertion of the intravascular probe, but collection for baseline intravenous adenosine concentrations was also started at least 1 hour after probe insertion, to reproduce the intramuscular protocol. Adenosine dialysate concentrations increased significantly during forearm ischemia, from $0.61±0.12$ to $1.28±0.39$ μmol/L ($120±43\%$, $P<0.01$, $n=8$, Figure 2) and returned to baseline thereafter. In vitro recovery for adenosine from the intravascular probe averaged $81±8\%$. The higher recovery of adenosine is explained by the 3-fold larger surface area of the intravascular probe compared with
the intramuscular probe. No significant increase in thromboxane dialysate concentrations was observed during forearm ischemia. Conversely, a decrease from 0.45±0.11 to 0.27±0.01 pg/L was observed (Figure 3).

**Discussion**

A new and unexpected finding from this study is that ischemia does not increase interstitial muscle adenosine in the human forearm. There is ample evidence that adenosine is released from virtually any metabolically active tissue during ischemic conditions to act as a protective metabolite. We do not believe that the lack of increase in interstitial adenosine reported here contradicts this premise. Our results need to be interpreted considering that forearm ischemia was imposed on resting, metabolically inactive skeletal muscle. It is likely that if ischemia was induced in exercising muscle an increase in interstitial muscle adenosine levels would be observed, as suggested by preliminary studies.

The lack of increase of interstitial adenosine during forearm ischemia could be interpreted as negating the hypothesis that adenosine contributes to reactive hyperemia. This seems unlikely given the substantial evidence from animal experiments supporting a role of adenosine in reactive hyperemia. Nonetheless, we thought it was important to use caffeine withdrawal to test this hypothesis in our model. Numerous animal studies have shown that long-term caffeine administration upregulates adenosine receptors, and we have previously shown potentiation of adenosine actions during caffeine withdrawal in humans. The potentiation of reactive hyperemia found in this study supports the role of adenosine in this process.

Taken together, therefore, our results suggest that, at least in the human forearm, the source of adenosine that contributes to reactive hyperemia is located within the intravascular compartment. This conclusion is based on the finding that ischemia selectively increases intravascular levels of adenosine. From our studies we cannot determine the origin of intravascular adenosine or the mechanism by which it mediates reactive hyperemia. Platelet aggregation occurs when a foreign body is placed in a vessel, as could be the case with venous microdialysis. This would result in the release of adenine nucleotides which can be a source of intravascular adenosine and that would explain our results. However, platelet aggregation occurs rapidly, within a few minutes or less. Therefore, we would expect an early increase in intravascular adenosine concentrations immediately after the insertion of the microdialysis probe or a gradual increase during the baseline period, neither of which was observed. Furthermore, we found that intravascular thromboxane B2 levels did not increase during ischemia, which indicates the lack of significant platelet activation when intravascular adenosine concentrations were highest.

Because very little if any adenosine injected into the vascular compartment reaches the underlying vascular smooth muscle, it has been suggested that adenosine mediates vasodilation by interacting with the endothelium. The nature of such interaction remains controversial. Adenosine-induced vasodilation appears to be mediated by endothelium-derived nitric oxide in some vascular beds, but whether this occurs in the human forearm is controversial, and it does not appear to occur in human coronary arteries. It has also been proposed that adenosine mediates vasodilation through an endothelium-derived hyperpolarizing factor. Finally, it is possible that endothelial cells release adenosine not only into the lumen but also into the underlying smooth muscle cells in sufficient quantity to induce local vasodilation but not enough to be detected by our assay.

We have previously proposed that adenosine contributes to the triggering of the exercise pressor reflex, a sympathetically-mediated pressor reflex elicited by isometric handgrip. It is well known that ischemia alone is not sufficient to trigger this reflex and the results obtained in this study may provide an explanation for this observation; ischemia alone was not a sufficient stimulus to increase adenosine levels in the interstitium, where afferent fibers involved in the exercise pressor reflex are located.

There are limitations to our study that need to be discussed. With the use of the microdialysis technique we had no difficulty measuring basal resting interstitial levels of adenosine in the skeletal muscle. Our methodology, therefore, was sensitive enough to measure an increase in interstitial adenosine during forearm ischemia. We cannot exclude, however, the possibility of a transient increase in interstitial adenosine during ischemia that, given our sampling period, we were unable to detect. However, it is uncertain if a transient increase would play a role in the subsequent reperfusion period, when reactive hyperemia is observed. Also, our conclusions are based on the assumption that changes in intravascular adenosine measured in an antecubital vein reflect changes at the level of resistance vessels responsible for reactive hyperemia. Currently, it is not possible to test the validity of this assumption because of the risks of introducing a microdialysis probe in the arterial circulation and the inaccessibility of resistance vessels to this or other techniques. It should be noted, however, that similar plasma levels are found in both the arterial and venous circulations.

Finally, because of the dynamics of our model, it is unlikely that a true equilibrium is reached between both sides of the dialysis membrane. It is important to emphasize, therefore, that the reported dialysate values represent estimates, rather than absolute interstitial adenosine concentrations. We are confident, however, in the direction of the changes observed, particularly when the same technique was applied in the intravascular and interstitial spaces.

Our results are in agreement with those of Kurz et al, who studied the cardioprotection elicited by GP531, an analog of...
acadesine and an adenosine regulating agent that alters adenosine metabolism and promotes its accumulation during ischemia. They found that GP531 improved posts ischemic myocardial functions in pigs in association with an increase in vascular adenosine concentrations, whereas interstitial adenosine concentrations were no different versus control animals.

In conclusion, our results indicate that adenosine plays a role in the increase in FBF in response to ischemia (reactive hyperemia) in humans. This protective action is mediated by an increase in intravascular adenosine because measurements of interstitial adenosine remained unchanged. We speculate that endothelial cells are an important source of adenosine during ischemia, as previously suggested by Gerlach et al.18

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References

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