Estimation of Skeletal Muscle Interstitial Adenosine During Forearm Dynamic Exercise in Humans

Fernando Costa, John Heusinkveld, Robert Ballog, Stephen Davis, Italo Biaggioni

Abstract—It has been proposed that adenosine is a metabolic signal that triggers activation of muscle afferents involved in the exercise pressor reflex. Furthermore, exogenous adenosine induces sympathetic activation that mimics the exercise pressor reflex, and blockade of adenosine receptors inhibits sympathetic activation induced by exercise. Thus, we hypothesize that adenosine is released locally by the muscle during exercise. We used microdialysis probes, placed in the flexor digitorium superficialis muscle, to estimate muscle interstitial adenosine levels in humans. We estimated resting in vivo muscle interstitial adenosine concentrations (0.292±0.058 μmol/L, n=4) by perfusing increasing concentrations of adenosine to determine the gradient produced in the dialysate. Muscle interstitial adenosine concentrations increased from 0.23±0.04 to 0.82±0.14 μmol/L (n=14, P<0.001) during intermittent dynamic exercise at 50% of maximal voluntary contraction. Lactate increased from 0.8±0.1 to 2.3±0.3 mmol/L (P<0.001). Lower intensity (15% maximal voluntary contraction) intermittent dynamic exercise increased adenosine concentrations from 0.104±0.02 to 0.42±0.16 μmol/L (n=7). The addition of ischemia to this low level of exercise produced a greater increase in adenosine (from 0.095±0.02 to 0.48±0.2 μmol/L) compared with nonischemic exercise (0.095±0.02 to 0.25±0.12 μmol/L). These results indicate that microdialysis is useful in estimating adenosine concentrations and in reflecting changes in muscle interstitial adenosine during dynamic exercise in humans. (Hypertension. 2000;35:1124-1128.)

Key Words: microdialysis ■ adenosine ■ muscle, skeletal ■ exercise ■ lactates

Microdialysis is a relatively new and minimally invasive technique that allows continuous sampling of extracellular substances of small molecular weight that will diffuse through the dialysis membrane. It has been used to estimate interstitial concentrations of lactate, glucose, glutamate, norepinephrine, and adenosine, among others. Over the last few years, the microdialysis technique has been introduced in human research, mostly to study skeletal muscle and subcutaneous metabolites. We theorized that this technique would be particularly useful in the determination of extracellular concentrations of metabolites in which rapid cellular uptake and/or degradation constitutes a major limitation to their measurement by traditional techniques. Adenosine is one such metabolite.

We have previously presented evidence supporting a role of adenosine in the triggering of the exercise pressor reflex, the sympathetic activation resulting from isometric handgrip. We have found that exogenous adenosine induces sympathetic activation that mimics the exercise pressor reflex and that blockade of adenosine receptors attenuates sympathetic activation induced by isometric exercise. We postulate, therefore, that endogenous adenosine is released from skeletal muscle during ischemic exercise and activates muscle afferent fibers involved in the triggering of this reflex.

It is difficult to test this hypothesis directly because of the challenges involved in measuring adenosine levels. Previous studies have used tissue biopsies to measure adenosine levels during ischemia. Whole tissue levels, however, do not discriminate between increases produced in the intracellular compartment or the interstitial space, the probable site of action. Attempts have also been made to measure adenosine levels in venous effluent. However, once outside the cell, adenosine is rapidly metabolized by adenosine deaminase and is reuptaken into cells via a very effective transporter. These processes explain the extremely short half-life of adenosine in human blood, reportedly <1 second. Furthermore, the endothelium acts as an effective barrier to adenosine.

The microdialysis technique offers theoretical advantages in the estimation of interstitial adenosine concentrations from samples taken directly from the muscle interstitial fluid. Neither cells nor enzymes, such as adenosine deaminase, cross the microdialysis membrane, which only allows passage of low-molecular-weight molecules. Once adenosine crosses the dialysis membrane, it is protected from degradation. We, therefore, explored its use to estimate interstitial levels of adenosine in the human forearm and to determine how interstitial levels are affected by intermittent dynamic exercise. Interstitial lactate concentrations were also determined.
simultaneously. We hypothesize that, for endogenous adenosine to play a role in the activation of the exercise pressor reflex, interstitial levels of adenosine should increase during forearm exercise.

**Methods**

**Subjects**

We studied a total of 19 normal, healthy volunteers of both genders, age 20 to 43 years (mean, 32.5 ± 1.9 years). Subjects were nonsmokers and free of medications, and they were asked to abstain from methylxanthines for ≥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. All the procedures followed were in accordance with institutional guidelines.

**Transcutaneous Muscle Microdialysis**

Microdialysis probes were introduced into the flexor digitorium superficialis muscle of the nondominant forearm by the following procedures. Lidocaine 2% was administered subcutaneously and above the muscle fascia at the site of each probe insertion. A guide cannula was inserted in a 45° angle, parallel to the fiber direction, and 15 mm into the tissue from the point of penetration of the muscle fascia. Before insertion of the probe, the steel guide cannula was removed, leaving only a polytetrafluoroethylene (Teflon) guide tubing in the tissue, through which the microdialysis probe was gently introduced. The polytetrafluoroethylene part of the guide was removed by splicing on retraction. The point of penetration of the skin was 20 to 50 mm distal to the elbow flexure. When 2 probes were needed, they were placed parallel to each other at a distance of 20 to 30 mm.

The microdialysis probe, CMA/20 (CMA) has been previously described in detail. Briefly, a dialysis tubing (10×0.5 mm in size with a 20 000 molecular-weight cutoff) is attached to the end of a double-lumen cannula. The perfusion solvent 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 inlet tubing of the probe was connected to a microinjection pump (CMA/102 Microdialysis Pump) and was continuously perfused with isotonic saline at a perfusion rate of 2 μL/min (“perfusate”). The effluent (“dialysate”) was collected continuously to obtain 30-μL samples over 15-minute periods.

**Microdialysis at Rest and During Exercise**

In 14 subjects (10 men and 4 women) who were age 20 to 43 years, we performed additional in vivo recovery of adenosine from the skeletal muscle with a technique previously described. In 7 of these 14 subjects studied, we performed additional 15-minute dialysate samples were collected as described previously, and a 60-minute rest period was included between interventions.

**In Vitro Calibration for Adenosine and Lactate**

In 8 of the subjects studied, we performed an in vitro calibration of the microdialysis probes to estimate fractions of adenosine and lactate recovered from the interstitial fluid across the microdialysis membranes. At the end of each study, the microdialysis probes used for adenosine and lactate determinations were removed from the muscle and placed in solutions that contained 2.5 μmol/L adenosine or 5 mmol/L lactate, respectively. These solutions were homogeneously mixed and the in vitro calibrations were performed in steady-state conditions. The probes were continuously perfused with saline at 2 μL/min, and the dialysate was collected over 30 minutes in two 15-minute fractions. Two 30-μL samples were also collected directly from each solution (2.5 μmol/L adenosine or 5 mmol/L lactate). These sets of samples were processed and the percentage recoveries were calculated for adenosine and lactate. Dialysate concentrations were divided by adenosine concentration measured from the 2.5 μmol/L adenosine solution or by lactate concentration measured from the 5 mmol/L lactate solution, respectively.

**In Vivo Equilibrium Microdialysis for Adenosine**

In 4 subjects (3 men and 1 woman) who were age 23 to 43 years, the in vivo recovery of adenosine from the skeletal muscle with a technique previously described.10 One hour after insertion, the microdialysis probe was perfused at a constant rate (2 μL/min) with increasing concentrations of adenosine (0, 100, 200, 400, and 600 nmol/L) for 30 minutes each. Dialysate was collected during the last 15 minutes of each perfusion period for adenosine determinations. Samples from each adenosine perfusate were also collected and processed. A linear relationship can be established between the perfusate concentration and the net increase of dialysate adenosine. The differences between adenosine dialysate and adenosine perfusate concentrations measured are considered the net increase in dialysate adenosine for each perfusate. The concentration of perfusate adenosine that does not result in a net change in dialysate adenosine can be used as an estimate of the interstitial concentration of adenosine surrounding the dialysis membrane. In vivo recovery was calculated by dividing each dialysate concentration by its correspondent perfusate concentration, which was expressed as a percentage. At the end of each of these studies, we also performed an in vitro calibration of the microdialysis probes, as described above.

**Dialysate Samples**

Dialysate samples were collected in ice-cooled 200-μL polyethylene vials, with a fraction collector (CMA/142, CMA). Collection vials for adenosine determinations contained 15 μL of internal standard (2-methyladenosine, 20 ng/15 μL), and collection vials for lactate determinations contained 180 μL of saline. Samples for adenosine determinations were immediately taken to dryness by centrifugation under vacuum (Savant SpeedVac SC100, Savant Instruments Inc) and then stored at −20°C until analyzed. Samples for lactate determinations were stored at −20°C until analysis.

**Adenosine and Lactate Determinations**

Samples for adenosine determinations were analyzed with a microbore high-pressure liquid chromatography system (Isco microLC system, Isco Inc) in a method previously described. The mobile phase consisted of 85% phosphoric acid, pH 3.0, with 2% acetonitrile (vol/vol). The phosphoric acid buffer and acetonitrile were filtered separately through a 0.22-μm filter (Millipore Corp) and degassed under vacuum. After mixing, the mobile phase was degassed further by sonication. The mobile phase was run isocratically with an Isco L500 micropump at 20 μL/min through an in-line degasser (Valco) equipped with a 1-μL injection-valve rotor. Detection was done with a UV absorbance monitor set as follows: deuterium lamp; 260-nm wavelength; 3.2-second rise time;
and sensitivity, 0.005 absorbance unit full scale. The output from the detector was recorded on an Isco Model 615A recorder (paper speed=12 cm/h). Immediately before sample injection, dried samples were reconstituted in 4 μL of mobile phase and 1 μL was injected into the column. Retention times for adenosine and 2-methyladenosine were 9 and 14 minutes, respectively. A standard curve was constructed with each run of samples by injecting increasing amounts of adenosine containing a constant amount of internal standard. Standards were evaporated to dryness, stored, and reconstituted before injection to simulate the handling of the samples. The ratios of the peak heights of adenosine and internal standard were correlated to the amount of adenosine injected by linear regression. The mean correlation coefficient of 4 representative standard curves was 0.996±0.001. The amount of adenosine in the dialysate sample was calculated by measuring the peak height ratio of adenosine to the internal standard and applying this ratio in the regression equation. Lactate was measured by an automated fluorometric method described previously. The buffer used was glycine 0.5 mol/L (pH 9.6), containing 0.2 mol of hydrazine and 2 g of disodium ethylenediaminetetraacetate per liter.

Statistical Analysis
Data were analyzed in a microcomputer with the Number Cruncher Statistical System (NCSS). Statistical evaluation was performed by ANOVA with repeated measures within subjects for multiple comparisons. Single comparisons were evaluated by 2-tailed unpaired t-test by Prism (Graph Pad Software). Values of P<0.05 were considered significant. Results are expressed as mean±SEM. Baseline values from microdialysis experiments were taken as the average of two 15-minute or 5-minute measurements depending on the duration of exercise. Linear regression analysis was used for construction of standard curves and calculation of samples.

Results

Estimation of Interstitial Levels of Adenosine in Resting Skeletal Muscle and Recovery of Metabolites From the Interstitium
Figure 1 shows the results from the in vivo microdialysis calibration performed in 4 subjects. A linear relationship was observed between the different adenosine concentrations perfused into the probe and their corresponding change in adenosine levels in the dialysate. Interstitial concentrations of adenosine were estimated at 0.29±0.058 μmol/L from the perfuse adenosine concentrations, which did not result in a net change of dialysate adenosine concentrations. The average recovery across the dialysis membrane in these in vivo studies was 33±1%. Recovery of adenosine through the dialysate probe was also measured in these same probes in vitro and was estimated to be 34±6%. There was agreement, therefore, between these 2 methods in the in vitro recovery for the estimation of interstitial levels of adenosine.

The estimated in vitro recovery for lactate from 12 of the subjects studied averaged 29±7%. This represents the fraction of lactate recovered throughout the microdialysis procedure and sample processing.

Effect of Exercise on Interstitial Metabolites
Dialysate concentrations of adenosine and lactate increased significantly immediately after the insertion of the microdialysis probe, but both levels decreased to a stable baseline within 1 hour. For this reason, all measurements were made after 1 hour of probe insertion. Figure 2 shows a representative time course for adenosine and lactate dialysate concentrations from 1 of the subjects studied with high-intensity exercise. Each measurement represents a 15-minute sample collection that began immediately after the insertion of the microdialysis probes.

During high-intensity intermittent dynamic exercise (50% of MVC), adenosine and lactate dialysate concentrations increased 3.6- and 2.9-fold, respectively. The average dialysate concentrations for adenosine and lactate at rest (baseline measurements), during high-intensity intermittent dynamic exercise, and during a 60-minute recovery period are shown in Figure 3. This graph shows parallel changes in adenosine and lactate dialysate concentrations, and each value represents a 15-minute collection period. The increase in lactate concentration during intermittent dynamic handgrip at 50% of MVC reassures that nonaerobic metabolism was induced. Adenosine dialysate concentrations increased significantly during high-intensity intermittent dynamic exercise, from 0.23±0.04 to 0.82±0.14 μmol/L (P<0.001, n=14), and recovered after exercise. A similar trend was observed with dialysate concentrations of lactate obtained simultaneously, which increased significantly, from 0.8±0.1 to 2.3±0.3 mmol/L (P<0.001, n=14). No obvious gender differences in dialysate concentrations at rest or in response
to exercise were observed in this relatively small number of subjects.

During low-intensity intermittent dynamic exercise (15% of MVC), adenosine concentrations increased by 300%, from 0.095 ± 0.02 to 0.25 ± 0.12 μmol/L (n=7), compared with a 528% increase when the subjects were studied on a different day with a higher intensity of exercise (50% of MVC, from 0.297 ± 0.08 to 1.237 ± 0.42 μmol/L, Figure 4).

Low-intensity intermittent dynamic exercise (15% of MVC) of shorter duration (5 minutes) produced a nonsignificant increase in adenosine dialysate concentrations, from 0.095 ± 0.02 to 0.25 ± 0.12 μmol/L (P=0.2). This increase was greater when ischemia was superimposed to low-intensity exercise (0.095 ± 0.02 to 0.48 ± 0.2 μmol/L, P=0.05, n=6, Figure 5).

Discussion

There are 2 novel contributions derived from these in vivo human experiments. First, we were able to estimate interstitial levels of adenosine in skeletal muscle at rest with an in vivo calibration approach. Second, we have shown that adenosine concentrations increase in skeletal muscle during exercise. Our motivation in performing these studies was our incomplete knowledge about the metabolites involved in the activation of the exercise pressor reflex. Previous studies have found that adenosine injected intrabrachially triggers sympathetic activation that mimics the exercise pressor reflex.5,13 We have also found that the adenosine antagonist theophylline blunts the exercise pressor reflex induced by forearm exercise.5 These earlier studies would suggest that adenosine is a metabolic trigger of the exercise pressor reflex. Central to this postulate is the notion that interstitial levels of adenosine must increase in the forearm during exercise, and our present results support this concept.

Our reasoning for measuring lactate concentrations was to monitor the metabolic state of the muscle throughout the studies. In particular, lactate was used as an indicator that the exercise could not be sustained by aerobic metabolism. Adenosine release is thought to result from an increase in metabolic demands that exceed oxygen supply and would be expected to correlate with anaerobic metabolism. Our results indeed show a parallel increase in adenosine and lactate dialysate concentrations during high-intensity exercise, an observation not previously reported. We have recently reported that interstitial adenosine does not increase in the forearm muscle during ischemia alone, whereas it does increase in the intravascular compartment.14 Taken together, these results support the concept that adenosine is released during ischemia in metabolically active tissue. In the case of the resting skeletal muscle in humans, it is possible that ischemia alone is not a sufficient enough stimulus to evoke adenosine release, unless it is associated with the increased metabolic demands of exercise.

Interstitial myocardial concentrations of adenosine have been estimated in animals with microdialysis, ranging from 0.2 ± 0.05 to 1.1 ± 0.2 μmol/L.15–17 Subcutaneous adenosine concentrations have been estimated in vivo in humans (0.13 ± 0.03 μmol/L from periumbilical subcutaneous interstitial fluid).10 Hellsten et al,18 who also used the microdialysis technique, have recently reported interstitial concentrations of adenosine in the vastus lateralis muscle of the leg of 0.22 ± 0.1 μmol/L. They also found a substantial increase in interstitial adenosine during low intensities of leg exercise (10 watts) and lesser incremental increases with more intense exercise (from 10 to 50 watts).18 Our results show an adenosine dialysate concentration from resting skeletal muscle ranging from 0.095 ± 0.2 to 0.23 ± 0.04 μmol/L and estimated interstitial concentrations of 0.29 μmol/L (range 0.07 to 0.42 μmol/L) similar to what was reported by Hellsten et al. There are differences in the experimental design between these 2 studies and the conclusions reached. The design of their microdialysis probe differs from ours in that they use a continuous hollow tube that is introduced at
one end of the muscle and exits at a different site. No data were provided by Hellsten et al to determine if adenosine concentrations returned to baseline values in between exercise periods. In our study, we waited longer between exercise periods to allow dialysate levels to return to baseline. We were concerned with the possibility that the presence of the probe would provoke trauma in surrounding tissues during dynamic exercise and that this would spuriously increase adenosine levels. Hellsten et al disregarded trauma induced during muscle contraction as an explanation for the apparent increase in interstitial adenosine levels during low-intensity exercise. We believe, however, that this is a real concern, given that we observed increases in dialysate adenosine during very-low levels of intermittent dynamic exercise, although they were not statistically significant. Global ischemia superimposed to these mild levels of exercise did produce a greater and significant increase in adenosine levels, showing that this technique can detect greater imbalance between oxygen supply and metabolic demands. We cannot rule out, however, that the apparent increase in our study and that of Hellsten et al is due to contraction-induced trauma. Given the differences in probe design, it is also possible that one probe could produce greater contraction-induce “trauma” compared with the other. On the other hand, it is reassuring that Hellsten et al found no change in recovery of adenosine during the different levels of exercise.

It is important to note the limitations of the microdialysis technique. Because there is no gold standard to measure interstitial levels of adenosine, absolute values obtained by microdialysis should be considered only an estimate. Adenosine and lactate concentrations were particularly high immediately after probe insertion but rapidly fell to stable levels thereafter. This transient increase in metabolite concentrations is a universal finding with this technique and is attributed to insertion trauma. In the process of inserting the probe, there is obviously tissue damage with the subsequent release of intracellular components. Because levels of adenosine and lactate returned to a stable state within an hour, we do not believe that the initial muscle trauma could be affecting our results. Finally, there remains a concern about the occurrence of trauma during muscle contraction, as discussed above.

In conclusion, we found microdialysis to be particularly useful in estimating interstitial levels of adenosine in human skeletal muscle at rest. We believe it also reflects changes in muscle metabolism during high-intensity exercise when metabolic demands exceed oxygen supply. It is possible, however, that local trauma induced by muscle contraction overestimates the increase in interstitial adenosine observed during exercise. This overestimation is likely to be greater during low levels of exercise. Absolute levels of adenosine reached during exercise, calculated by this technique, should be interpreted with caution.

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References

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