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Analysis of interstitial concentrations of ATP from rat soleus muscle using microdialysis combined with ion-pairing high performance liquid chromatography (HPLC)

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***Abstract*—** In the present study, a method is described for the determination of ATP in small samples of interstitial fluid (< 100 μl) using microdialysis combined with ion-pairing high performance liquid chromatography (HPLC) in a gradient elution system which gives a clear separation of ATP from other interstitial constituents. Lactic acid infusion was performed to stimulate ATP to exit the cells to appear in the interstitial space. Microdialysis probe is used to in-vivo sample the molecules in the interstitial fluid. Interstitial ATP could be reliably detected at concentrations higher than 1×10^{-9} M. The present study provides a simple, sensitive and selective assay for endogenous extracellular concentrations of ATP from rat soleus muscle.

Keywords—ATP; Microdialysis; Reversed-phase high-performance liquid chromatography; pH depression

I. Introduction

It is known that the interstitial adenosine triphosphate (ATP) concentration increases during muscle contractions[1], and that this ATP is converted extracellularly to adenosine[2, 3]. However, it is not known how muscle contractions could give a rise to the increase in interstitial ATP; previous studies have found a strong correlation between the depression of muscle pH and the appearance of adenosine in the venous blood[4]. The present experiments were conducted to test directly whether a localized decrease in muscle pH would stimulate the efflux of ATP.

Tissue or cellular ATP contents have been measured by luciferin-luciferase assay[5]. However, the activity of the luciferase would be limited by the variance in sample pH. In present study, an experimental procedure was modified for studying the effects of lactic acid infusion on the ATP release, with an in-vivo microdialysis method combined with ion-pairing HPLC. We measured interstitial concentrations of ATP from rat soleus muscle before, during and in the recovery from acidosis.

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II. Materials and Methods

A. Muscle pH depression by infusion of graded doses of lactic acid

Sprague-Dawley male rats were under deep surgical anaesthesia with pentobarbitone sodium (Segatal, May and Baker Ltd, 60 mg/kg). The abdominal aorta was cannulated in order to perfuse the arterial supply of the rat hindlimb muscles with buffer. Hindlimb muscles were perfused at a flow rate of 1.5 ml/min for 90 minutes prior to the experiments using a modified Krebs-Henseleit bicarbonate buffer containing 2 g/L glucose, 59 g/L Dextran, 1 g/L albumin and 100 microliter/L antifoam A (Sigma, St Louis, USA) equilibrated against 95% O_2/5% CO_2 to give a pH of 7.4. Fig. 1 shows the schematic diagram of the perfusion circuit. Localized decreases in the muscle pH were induced by infusion of graded doses of lactic acid (0, 2.5, 5, or 10 mM) into the arterial supply of the muscle.

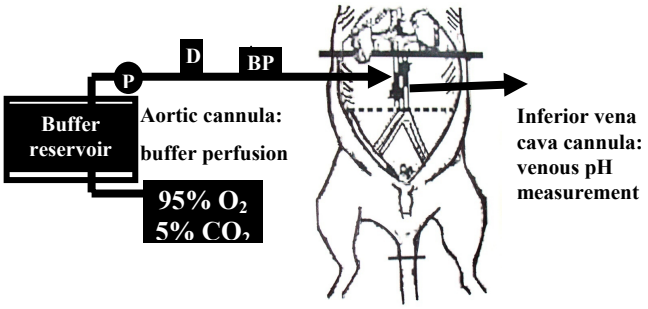

Fig. 1: Schematic diagram of the preparation and perfusion circuit for perfusion of rat hindlimb muscles. P = peristaltic pump (Cole Parmer, Model 7518-10); D = pulse damping chamber; BP = blood pressure.

B. Microdialysis study in rats

One of the hindlimbs was skinned down to the ankle; the distal tendon of the lateral gastrocnemius muscle was cut so that the soleus muscle was exposed. A microdialysis probe (LM10, Bioanalytical Systems, West Lafayette IN, USA) was inserted longitudinally into the soleus muscle of rats using a fine surgical needle, and was perfused at 2 microliter/min with a fluid of similar composition to the interstitial fluid (NaCl 142 mM, KCl 4 mM, $CaCl_2$ 2.4 mM, $MgSO_4$ 0.5 mM, NaH_2PO_4 1.2 mM, Glucose 5.6 mM). Probes were perfused for 90 mins prior to sample collection. The dialysate was collected in an ice-cooled vial for HPLC analysis.

C. Interstitial ATP concentrations analysis by HPLC

ATP in the samples was analyzed by ion-pair reverse-phase HPLC as previously described[4]. Samples of dialysate were analysed by HPLC, using 15-30 microliter dialysate mixed with 105-90 microliter ultrapure water. Samples were chromatographed on a 25 cm × 4.6 mm column, packed with 3 micrometer particles of LC-18T (Supelco, Bellefonte, PA USA). The mobile phase consisted of solution A (0.1 M KH_2PO_4, 0.004 M tetrabutyl ammonium chloride, pH 6.0 and solution B (20% methanol in solution A, pH 5.5) Adenine nucleotides were eluted with a solvent B gradien from 7 to 65 %, and the peak of ATP appeared in the phase of 65 % solvent B, run from 32 to 45 min. The solvent flow rate was 1 ml/min. The peak of ATP was identified by comparison of its retention times of the standard and the 'spiked' sample, in which, a certain ratio of the standard and the dialysate sample were mixed, and also by comparison of its absorption spectrum to that of the standard. ATP concentration was quantified using the area under the peak. The areas under peaks were measured either by an integrator (Empower, Waters, USA) or occasionally by cutting out peaks and weighing. The areas of the ATP peaks in chromatogram were adjusted to take account of dilution factors and standard ATP peak areas, using **the equation** $\boldsymbol{C_{ATP} = C_s \times [(A_{ATP}/A_s)/D]}$ where C = concentration, A = integrated peak area, s = standard ATP and D = dilution factor. Fig. 2 shows a schematic diagram of the instrument of HPLC.

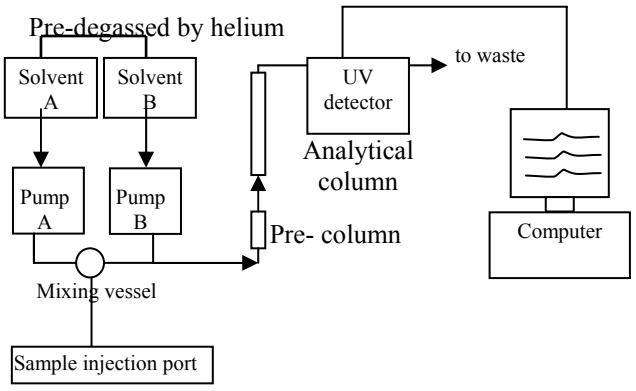

Fig. 2: A schematic diagram of instrument of HPLC set-up. Pre-column = guard column; Analytical column = main column.

D. Statistical analysis

All data are expressed as means ± SEM, and n represents the number of animals used. One-way ANOVA/Dunnett's *post hoc* test was performed for comparing multiple comparisons. In all cases significance was established at $P < 0.05$.

III. Results

1. Detection of ATP peak

Fig. 3 shows a typical HPLC chromatogram of adenosine, AMP, ADP and ATP standard sample.

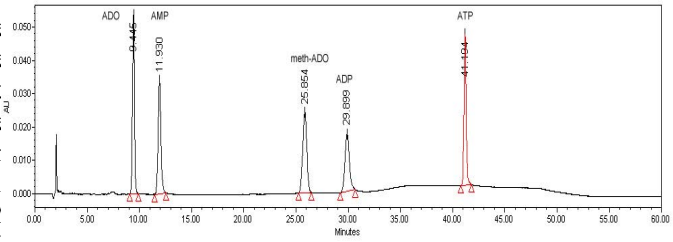

Fig. 3: A typical HPLC chromatogram of adenosine, AMP, ADP and ATP standard sample. ATP appeared at 41st min, separated by the gradient elution system.

2. pH depression by infusion of graded doses of lactic acid

Rat soleus muscles were perfused for 20 minutes with K-H buffer at pH 7.40, during which, the venous pH was stable at 7.22 ± 0.04. This was followed by the infusion of 2.5, 5 and then 10 mM lactic acid for 20 mins each: Fig. 4 shows that 2.5 mM lactic acid slightly reduced buffer pH but did not significantly reduce venous pH; 5 or 10 mM lactic acid produced significant dose-dependent decreases in both the buffer pH and the venous pH. When the lactic acid infusion was stopped and the muscle was again perfused with K-H buffer, the venous pH had returned to a value not significantly different from the precontrol level.

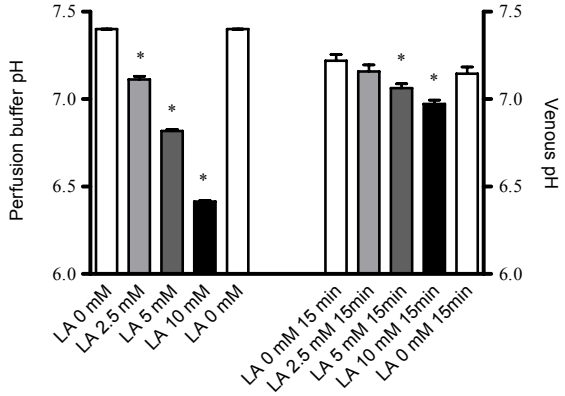

Fig. 4: Relationship between perfusion buffer pH and venous pH before, during or after addition of 2.5, 5 or 10 mM lactic acid to the perfusion medium. Data are expressed as mean ± SEM, n=11-13. *, $P < 0.05$ *vs* control group.

3. Effects of pH depression on the interstitial ATP of soleus muscle

The effects of lactic acid infusion on the interstitial ATP concentration of rat soleus muscle are shown in Fig. 5: interstitial ATP was significantly increased by 20 mins infusion of lactic acid at 5 mM or 10 mM. Interstitial ATP returned to a value not significantly different from the precontrol 20 mins after lactic acid infusion was stopped.

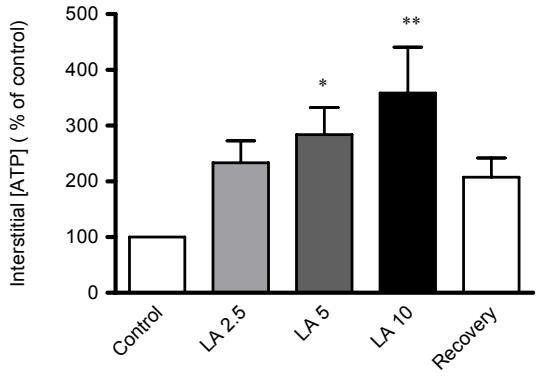

Fig. 5: Effects of graded doses of lactic acid infusion on the interstitial [ATP] of rat soleus muscle. Data are normalized to control interstitial ATP concentration. n=11-15, *, P <0.05, **, P <0.01, compared with control.

IV. DISCUSSION

Extracellular ATP and other adenine compounds, at concentrations ranging from nanomolar to millimolar, produce a large variety of pharmacological actions. In present study, *in vivo* microdialysis combined with HPLC was performed to examine the dynamic profile of extracellular ATP during acidosis challenge. The main features of ATP analysis by the methods outlined in this paper are: (1) a high sensitivity of detection in interstitial down to 1×10^{-9} mol/L, which is of physiological significance; (2) only 15-30 microliter of interstitial fluid is required; (3) Concentrations of ATP in interstitial fluid were calculated from chromatograms by integrating peak height with respect to time (s), and were adjusted to take account of dilution factors and standard ATP peak areas.

HPLC is a useful method to separate the compounds in a mixture, based on chemical and size differences between them, using a column. The reversed-phase column is composed of hydrocarbone chains attached to a porous silica base (stationary phase). The eluent (mobile phase) is water based. The hydrocarbon chains have a strong attraction by *Van der Waals* forces to the hydrophobic molecules, and therefore, hydrophobic molecules are strongly bound to the column. Addition of methanol to the mobile phase attracts the hydrophobic molecules away from the stationary phase and allows them to elute. In our experiment, the ion-pairing chromatography (IPC) was applied since the mixture contained nucleotides. The IPC is an extension of reversed-phase HPLC. In ion-pairing chromatography, the separation is based upon both electrostatic and lipophilic forces. The organic eluent is supplemented with a specific ion-pairing reagent. The IPC reagent in our experiment is tetrabutylammonium bisulfate.

There are few reports of the use of HPLC for muscle interstitial ATP analysis. As we mentioned in the introduction section, luciferin-luciferase assay was the most often used method to measure tissue or cellular ATP contents. The luciferase assay is based upon the bioluminescent measurement of firefly luciferase. In luminescent reactions, light is produced by the oxidation of a luciferin (a pigment), sometimes involving ATP. The rates of this reaction between luciferin and oxygen are extremely slow until they are catalyzed by luciferase, often mediated by the presence of calcium ions. However, under our experimental procedures, the variance in the fluid pH would cause a notable perturbation to this bioluminescent reaction. HPLC analysis described here provides more credible and quantitative data in assessing the effects of some extreme stimulus (such as acidosis) on the interstitial concentrations of ATP and other adenine compounds of rat skeletal muscles. As we shown in Fig. 4, there was a well correlation between a decrease in buffer pH with lactic acid and the depression of venous pH, and this acidosis induced by infusion of lactic acid caused a dose-dependent increase in soleus muscle interstitial ATP (Fig. 5).

Microdialysis is currently one of the most important *in vivo* sampling methods in physiology and pharmacology. It is used to determine the chemical components of the fluid in the extracellular space of tissue. As demonstrated by the present study, the small probe dimension caused minimal injury to skeletal muscle so the dialysate represented normal physiological conditions. Since the exchange of molecules through the dialysis membrane occurs in both directions, a microdialysis probe placed in a target tissue can be used to continuously deliver compounds into a localized area of tissue, as well as to sample endogenously-produced compounds.

As illustrated by the experimental results showed above, the microdialysis technique combined with HPLC can afford more credible and quantitative data in accessing and screening the effect of acid challenge on the extracellular ATP concentrations of rat soleus muscle. In conclusion, the present study affords a more precise, simple, and selective assay for submicromolar concentrations of ATP.

V. REFERENCE

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