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In vivo, continuous and automatic monitoring of extracellular ascorbic acid by microdialysis and on-line liquid chromatography

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Abstract

A system for in vivo, automatic, continuous monitoring of organ extracellular ascorbic acid in anesthetized rat is described. This system involves microdialysis perfusion and a LC system equipped with an electrochemical detector. Microdialysate, eluted from a microdialysis probe implanted in the brain cortex or in the left ventricular myocardium of anesthetized rats was collected in the sample loop of an on-line injector for direct injection onto the LC system. This automated method provides a shortened sample processing time. This system was utilized to investigate the effect of cerebral ischemia on cortex extracellular ascorbic acid and the effect of myocardial ischemia on left ventricular myocardium extracellular ascorbic acid in anesthetized rats. Basal ascorbic acid concentrations in the cortex and left ventricular myocardium ranged from 9.7 to 15.4 μM (mean$\pm$S.D., 12.7$\pm$2.5 μM from the results of eight rats) and from 9.3 to 36.0 μM (mean$\pm$S.D., 24.3$\pm$8.9 μM from the results of twelve rats), respectively. Cerebral ischemia significantly elevated ascorbic acid levels in the cortex extracellular space, while myocardial ischemia did not significantly alter ascorbic acid levels in the left ventricular myocardium extracellular space.

Keywords: Microdialysis; Vitamins; Ascorbic acid

1. Introduction

Ascorbic acid (vitamin C) is a physiologically important aqueous antioxidant [1,2]. Ascorbic acid is capable of scavenging oxygen-derived free radicals, which are thought to contribute to the development or exacerbation of many common diseases including cancer, heart attack, stroke, arthritis, etc. [3–6]. Ascorbic acid also plays an important protective role in the elimination of oxygen-derived free radicals associated with ischemia and reperfusion of organs such as the brain, heart and liver [7–10]. Therefore, the concentration of ascorbic acid can be used as an index for the evaluation of biological oxidative stress. Determination of this antioxidant in biological samples can be achieved via a high-performance liquid chromatography system equipped with either an absorbance detector [11] or an electrochemical detector [12].

Microdialysis perfusion provides a minimally invasive method for the sampling of low molecular mass metabolites in the extracellular fluids of anesthetized animals [13]. LC analysis of microdialysate is a popular method for determining extracellular metabolites in the organs of both awake and anesthetized animals. Microdialysates can be injected onto an LC system using either an off-line or an on-line injection mode. On-line injection has several

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advantages such as minimal sample preparation and automated injection. Additionally, a shortened sample preparation process is important when the metabolites to be analyzed are unstable or sensitive to air. On-line injection methods have been used to determine extracellular major antioxidants such as glutathione in the liver [14] and hydroxyl radical production [15] in brain of anesthetized rats.

Therefore, in the present study, we used microdialysis perfusion with an on-line LC system to measure the extracellular ascorbic acid levels in brain cortex and in left ventricular myocardium of anesthetized rats. Additionally, this system was used to investigate the effect of cerebral ischemia on brain cortex extracellular ascorbic acid levels and the effect of myocardial ischemia on left ventricular myocardium extracellular ascorbic acid levels in anesthetized rats.

2. Experimental

Ascorbic acid, tetrabutyl ammonium hydroxide and urethane were purchased from Sigma (St. Louis, MO, USA). Sodium acetate and Na_2EDTA were obtained from Merck (Darmstadt, Germany). Reagent-grade acetonitrile and methanol were obtained from J.T. Baker (Phillipsburg, NJ, USA). All other chemicals were of reagent grade and distilled deionized water was used.

2.1. General procedure for microdialysis

The microdialysis system and microdialysis probes (CMA/20) were obtained from Carnegie Medicine Associates (Stockholm, Sweden). Probe length was 24 mm. The membrane of the probe was made of polycarbonate and had a length and diameter of 4 mm and 0.5 mm, respectively. Molecular mass cut-off for the membrane was 20 000. The probe was perfused (2 μl/min) with a CMA-100 perfusion pump in corresponding outer medium with Ringer's solution for 30–60 min before measurement were taken, to avoid changes in relative recovery over time. The microdialysates were collected over 10-min intervals with a 19.6-μl loading loop of a CMA 160 on-line injector. The time taken for injection of the collected microdialysates into the LC system was 8 s.

2.2. Animal preparation for cerebral ischemia

Male Sprague–Dawley rats (280–330 g) were used. The animals were anesthetized with pentobarbital (50 mg/kg, i.p.). Body temperature was maintained at 37°C with a heating pad. A polyethylene catheter was inserted into the femoral artery in order to monitor systemic arterial blood pressure (SAP) with a Gould pressure processor. Prior to cerebral ischemia, the rat's head was mounted on a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) with the nose bar positioned 3.3 mm below the horizontal. Following a midline incision, the skull was exposed and one burr hole was drilled in the skull for insertion of a dialysis probe. The microdialysis probe was implanted into the striatum (0 mm anterior and 5.5 mm lateral to the bregma, and 4 mm from the brain surface). Ischemia was accomplished by clamping the bilateral common carotid arteries and the unilateral middle cerebral artery.

2.3. Animal preparation for myocardial ischemia

Female Sprague–Dawley rats (250–300 g) were used. The animals were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). A catheter was inserted into the femoral artery for direct measurement of blood pressure and heart rate via a Statham pressure transducer and a Gould Biotachnometer, respectively. An endotrachial tube was positioned and each animal was ventilated at a respiration rate similar to that of rats (60–80 strokes/min, 1 ml/100 g). Electrocardiograms were recorded from lead II limb leads, via an ECK Monitor (Cardiac Recorders, London, UK). A left thoractomy was performed in the fifth intercostal space and the head was exposed. A microdialysis probe (CMA-20) was implanted in the left ventricular myocardium. A ligature (6-0 silk suture) was placed around the left coronary artery. The rat was then allowed to equilibrate for 60 min prior to myocardial ischemia, which was induced by ligation of the coronary artery. The ischemic period lasted for 60 min. Blood pressure, heart rate and electrocardiogram results were monitored.

2.4. *LC instrumentation for ascorbic acid determination*

The LC system consisted of a BAS PM-80 isocratic pump (Bioanalytical System, Lafayete, IN, USA), a CMA on-line degasser (CMA 260) and a BAS LC-4C electrochemical detector with dual glassy carbon electrodes. Separation was achieved by using a Merck (Darmstadt, Germany) Lichrospher 100 and LichroCART (5 μm) 250×4 mm RP-18 cartridge column. The mobile phase consisted of 40 m*M* sodium acetate, 0.54 m*M* Na_2EDTA, 1.5 m*M* tetrabutylammonium hydroxide, 7.5% methanol (final pH 4.75). Elution was isocratic with a flow-rate of 0.7 ml/min. The settings for the electrochemical detector (working potential: +0.6 V vs. Ag/AgCl) were described previously [16]. Data collection and analysis were performed with a Chem Station Chromatographic Management System (Hewlett Packard, Taiwan Branch, Taipei, Taiwan).

3. Results and discussion

In this study, an on-line injection device was used for automatic injection of the microdialysates, which were continuously sampled from the brain cortex and left ventricular myocardium of anesthetized rats. This combination provided several advantages such as shortened sample preparation time and minimal contact of ascorbic acid with air. Since ascorbic acid is a major antioxidant, prolonged sample preparation and exposure to air might induce ascorbic acid oxidation and subsequently affect the accuracy of ascorbic acid determinations. Additionally, the simple and direct collection–injection device automated sample collection, injection and analysis.

3.1. *Determination of ascorbic acid from on-line injection onto an LC system*

Microdialysates perfused through probes placed in standard ascorbic acid solutions (200 μ*M*) were collected and automatically injected onto the HPLC system. The results are shown in Fig. 1. The ascorbic acid reached a plateau 30 min after probe implantation, indicating that the system had reached equilibrium.

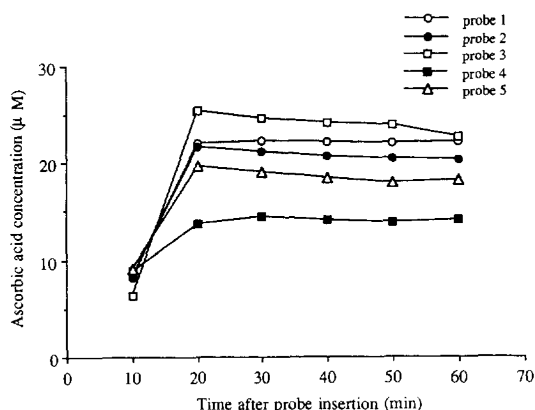

Fig. 1. Ascorbic acid concentrations in microdialysates collected every 10 min after probe placement in standard ascorbic acid solution (200 μ*M*).

3.2. *Effect of ascorbic acid concentration on microdialysis probe recovery*

Ringer solution was continuously perfused through microdialysis probes placed in ascorbic acid solutions of five different concentrations (25, 50, 100, 200 and 400 μ*M*). The perfusing time for each concentration was 1 h. The eluted microdialysates were analyzed by this on-line LC system for ascorbic acid concentrations. A linear relationship was observed between the ascorbic acid concentrations in microdialysates and the standard ascorbic acid concentrations (Fig. 2). This linear response demon-

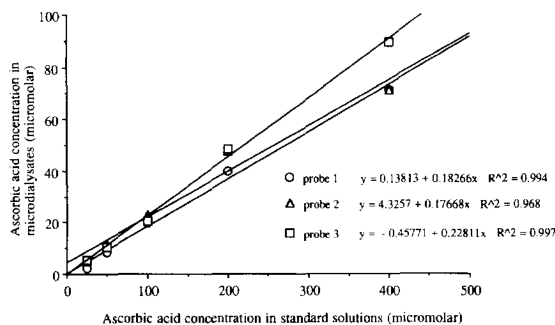

Fig. 2. Effect of various ascorbic acid concentrations on the microdialysis probe recovery. Ascorbic acid concentrations in microdialysates (*Y*-axis) eluted from microdialysis probes placed in standard ascorbic acid solutions (*X*-axis) are plotted. Perfusing time for each concentration was 1 h. The ascorbic acid concentrations in microdialysates were the average of three values obtained at 40, 50 and 60 min, respectively.

strated that the on-line system is working properly for determining ascorbic acid concentrations. Furthermore, the linear response also implied that the in vitro recovery of ascorbic acid from the microdialysis probe did not vary significantly between the different ascorbic acid concentrations. The in vitro recovery for three probes was examined on three consecutive days and the results are shown in Fig. 3. The in vitro recovery did not show significant variation among the experiments performed on each of the three days.

3.3. Determination of extracellular ascorbic acid levels in the brain cortex and left ventricular myocardium of anesthetized rats

Due to the intracellular abundance of ascorbic acid and the tissue damage resulting from probe insertion, ascorbic acid concentrations in the microdialysates from initial collections after probe implantation into brain cortex were very high, but decreased sharply over time (Fig. 4). The extracellular ascorbic acid reached an equilibrium at 180 min following probe implantation. Basal levels for ascorbic acid in extracellular fluids of rat brain cortex ranged from 9.7 to 15.4 μ*M* (average: mean±S.D., 12.7±2.5 μ*M*, from the results of twelve rats).

In contrast to brain cortex observations, probe insertion did not cause a significant variation in ascorbic acid levels in extracellular fluids from the left ventricular myocardium (Fig. 5) and an equilibrium was reached 60 min after probe implantation. Basal ascorbic acid levels in the extracellular fluids of the left ventricular myocardium ranged from 9.3 to 36.0 μ*M* (mean±S.D., 24.3±8.9 μ*M*). These observations might be due to levels of ascorbic acid in rat heart tissue (approximately 0.2 μmol/g wet weight) that are ten-fold lower than those found in the brain (approximately 2 μmol/g wet weight).

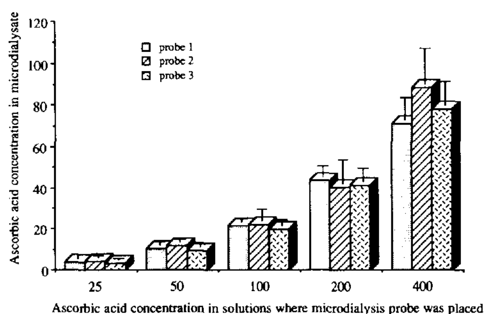

Fig. 3. Comparison of microdialysis probe recovery for ascorbic acid on three consecutive days. Experimental protocol was the same as described in the legend of Fig. 2.

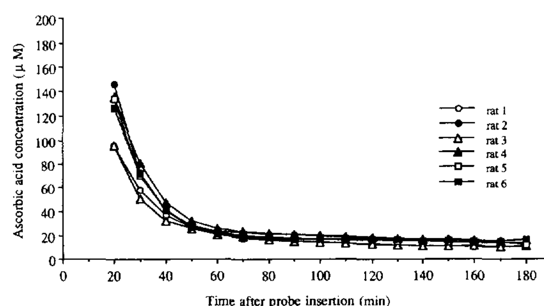

Fig. 4. Extracellular ascorbic acid concentrations after probe implantation into the brain cortex. The ascorbic acid concnetrations were very high for the first 10 min and thus are not shown in the figure.

3.4. Effect of cerebral ischemia on brain cortex extracellular ascorbic acid levels

A typical chromatogram obtained following injection of microdialysate collected during the first 10 min of the microdialysis period after cerebral ischemia is shown in Fig. 6A. Fig. 6B shows the chromatogram obtained from an injection of mi-

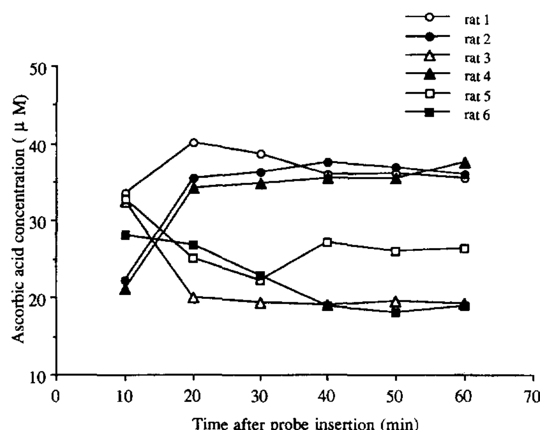

Fig. 5. Extracellular ascorbic acid concentrations after probe implantation into the left ventricular myocardium.

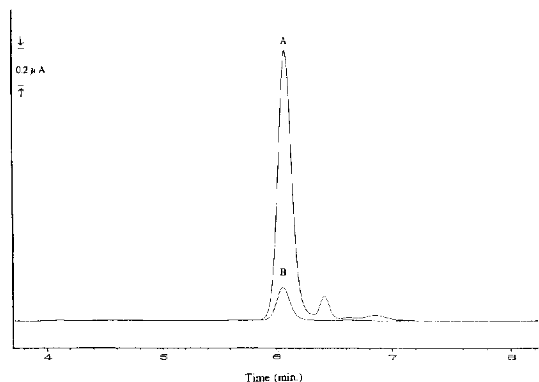

Fig. 6. Chromatograms of ascorbic acid obtained from the injection of microdialysates before (B) and after (A) the onset of cerebral ischemia in anesthetized rats.

crodialysate from basal extracellular fluids previously obtained from the same animal. From comparisons of Fig. 6A and Fig. 6B, it is evident that the ascorbic acid peak is significantly higher in Fig. 6A. The entire time profile obtained for eight rats for the extracellular ascorbic acid in the brain cortex before and after ischemia, which lasted for 120 min, is shown in Fig. 7. It is evident that cerebral ischemia immediately and significantly elevated cortex extracellular ascorbic acid. There are several possible reasons for the increase in extracellular ascorbic acid following cerebral ischemia. One possible cause is that ischemia damages the integrity of the cellular membrane of brain cells, and thus, intracellular ascorbic acid, which is present in much higher concentrations than in the extracellular space, is rapidly released into the latter compartment [16]. Another possible cause is that a large amount of ascorbic acid was released to scavenge the reactive oxygen species, which are known to be generated in cerebral ischemia [17,18].

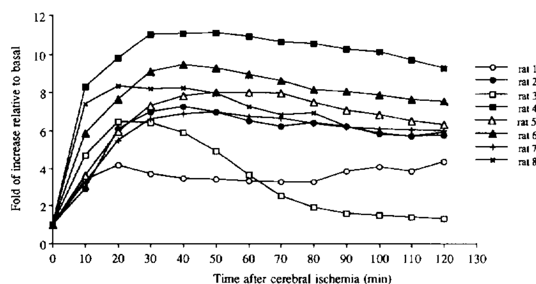

Fig. 7. Time profile for extracellular ascorbic acid in rat brain cortex following cerebral ischemia.

3.5. Effect of myocardial ischemia on the left ventricular myocardium extracellular ascorbic acid level

In contrast to observations of the brain, acute myocardial ischemia in anesthetized rats did not cause significant variation in left ventricular extracellular ascorbic acid (Fig. 8). This observation corresponds to the results of isolated working hearts [19]. One possible reason for this observation is that the ascorbic acid concentration in rat brain tissue (usually 2 μmol/g wet weight) is usually one order of magnitude higher than the ascorbic acid concentration in rat heart (usually 0.2 μmol/g wet weight), and thus cerebral ischemia-induced ascorbic acid release might be more prominent than the myocardial ischemia-induced ascorbic acid release.

In conclusion, an analytical method involving microdialysis perfusion, automatic on-line injection and LC for the continuous monitoring of extracellular ascorbic acid in the brain cortex and left ventricular myocardium of anesthetized rats has been developed. This method has been used for cerebral and myocardial ischemia/reperfusion investigations, which have provided evidence for significantly elevated extracellular ascorbic acid in the brain cortex of anesthetized rats.

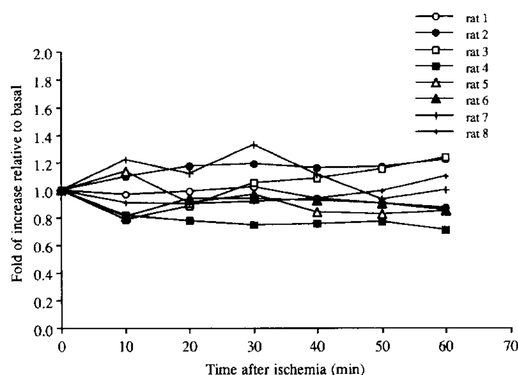

Fig. 8. Time profile for extracellular ascorbic acid in rat left ventricular myocardium following myocardial ischemia.

Acknowledgments

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