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Evaluation of extracellular lipid peroxidation in brain cortex of anaesthetized rats by microdialysis perfusion and high-performance liquid chromatography with fluorimetric detection

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Abstract

A method for in vivo evaluation of lipid peroxidation in the extracellular space of anaesthetized rat brain cortex was developed. This method involved the use of microdialysis perfusion and high-performance liquid chromatography. The microdialysates, eluted from implanted probes, were reacted with thiobarbituric acid (TBA) prior to analysis by an HPLC system equipped with a fluorescence detector (excitation and emission wavelengths were 515 and 550 nm, respectively). Lipid peroxidation in the extracellular space was evaluated as the concentration of malondialdehyde, a lipid peroxidation end product which reacts with TBA to form a fluorescent conjugate. Significantly increased production of malondialdehyde following hydrogen peroxide perfusion (0.03%, 0.3% at a flow-rate of 1 μl/min) was observed in the brain cortex of anaesthetized rats.

Keywords: Lipids; Malondialdehyde; Thiobarbituric acid; Microdialysis

1. Introduction

Formation of oxygen-derived free radicals has been implicated in the pathophysiological progression of various disorders such as cerebral ischemia and Parkinson's disease [1–3]. One of the major mechanisms of oxygen-derived free radical-induced damages is the stimulation of lipid peroxidation [4–6]. Lipid peroxidation levels are commonly used to evaluate oxidative stress, as free radicals are difficult to directly measure due to their short half-lives.

Various methods can be used to assess biological lipid peroxidation [7–10]. Direct and indirect measurements of malondialdehyde (MDA), a side product of enzymic polyunsaturated fatty acid oxygenation and a secondary end product of autooxidative peroxide formation and decomposition [11,12], are commonly used. Direct measurement is usually by a high-performance liquid chromatography (HPLC) system equipped with a UV detector [13]. For indirect measurement, MDA can be derivatized with thiobarbituric acid (TBA) prior to analysis by an HPLC system equipped with a fluorescence detector [14–16].

Microdialysis perfusion, which provides a mini-

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mally invasive method for in vivo sampling of organ interstitial fluids from awake or anaesthetized animals, can be used in combination with high-performance liquid chromatography to evaluate in vivo oxidative stress. Recently, this combination has been used to determine the concentrations of major antioxidants such as glutathione [17,18] and ascorbic acid [19,20], and to determine hydroxyl radical production [21,22], in anaesthetized rats. Since lipid peroxidation is closely related to the production of oxygen-derived free radicals, we examined the possibility of measuring MDA content by microdialysis perfusion and an HPLC system equipped with a fluorescence detector. Additionally, this method was used to investigate the effect of hydrogen peroxide, a strong pro-oxidant that is commonly used to induce lipid peroxidation in biological matrices [23], on MDA production in the brain cortex of anaesthetized rats.

2. Experimental

2.1. Chemicals

The following chemicals were purchased from Sigma (St. Louis, MO, USA). Tetraethoxypropane (TEP), urethane, 2′-thiobarbituric acid, sodium phosphate, butylated hydroxytoluene and sodium biphosphate.

Monochloroacetic acid and tetrahydrofuran (THF) 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 deionized distilled water was used.

2.2. General procedure for microdialysis

The microdialysis system was obtained from Carnegie Medicine Associates (Stockholm, Sweden). Microdialysis probes (CMA/20) were purchased from CMA. The probe length was 24 mm. The membrane of the probes was made of polycarbonate, with 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 (1 μl/min) by a CMA-100 perfusion pump. The microdialysates were collected every 20 min period with a CMA-140 fraction collector.

2.3. Animal preparations

Male Sprague–Dawley rats (280–330 g) were used. The animals were anaesthetized with pentobarbital (50 mg/kg, i.p.). Body temperature was maintained at 37°C with a heating pad. Polyethylene catheters were inserted into the femoral artery for monitoring of systemic arterial blood pressure (SAP) via a Gould pressure processor. The rat's head was mounted on a stereotaxic apparatus (Davis 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 on the skull for insertion of a dialysis probe. A hollow needle was tied parallel to the microdialysis probe in such a way that the ends of the probe and needle were placed together. The needle, made from a stripped microdialysis probe was perfused with hydrogen peroxide solution at the same rate as the probe. This device was then implanted into the striatum (0 mm anterior and 6.0 mm lateral to the bregma, and 4 mm from the brain surface).

2.4. Analysis of MDA

Two TEP stock solutions were prepared. The original TEP stock consisted of the addition of 0.050 ml of standard TEP to 24.95 ml of 40% ethanol. The intermediate stock solution was prepared by the addition of 0.25 ml original standard TEP solution to 49.75 ml 40% ethanol solution. Final concentration for the intermediate standard TEP stock solution was 41.8 μ*M*. The stock solution was diluted with 0.01 *M* HCl to yield experimental concentrations. TEP standard solutions or microdialysates were mixed with 2′-thiobarbituric acid. (0.4% in 0.2 M HCl) and butylated hydroxytoluene (0.2% in 95% ethanol) at a ratio of 1:2:0.3. The mixture was heated at 90°C for 45 min. Once cooled, the mixture was subjected to HPLC analysis. Injection volume was 30 μl. Blank solutions were prepared using the same procedure for TEP solution except for the absence of TEP.

The HPLC system consisted of a type 1050 series quaternary pump, a 1050 series autosampler, a 1050

series on-line degasser, and a 1046A HPLC fluorescence detector, all obtained from Hewlett-Packard (Hewlett-Packard Taiwan Branch, manufactured in Waldbronn, Germany). Optimum responses were observed when excitation and emission wavelengths were set at 515 nm and 550 nm, respectively. Peak areas and concentrations were determined using Hewlett-Packard Chem Station Chromatographic Management System.

Separations were achieved using a Merck, LiChrospher 100, LiChroCART (5 μm) 4 mm×125 mm RP-18 cartridge column. Binary gradient elution was used. Mobile phase A consisted of 35% methanol and 65% 50 m*M* sodium phosphate buffer (final pH 7). Mobile phase B consisted of 90% methanol and 10% 50 m*M* sodium phosphate buffer. In all cases, the mobile phases were filtered (filter pore size: 0.45 μm). Binary gradient elution was performed at a flow-rate of 1.0 ml/min. The elution profile was: 0 to 7 min isocratic with mobile phase A, followed by a column wash with 90% mobile phase B for 5 min. Then, the column was re-equilibrated with mobile phase A for 5 min before the next injection.

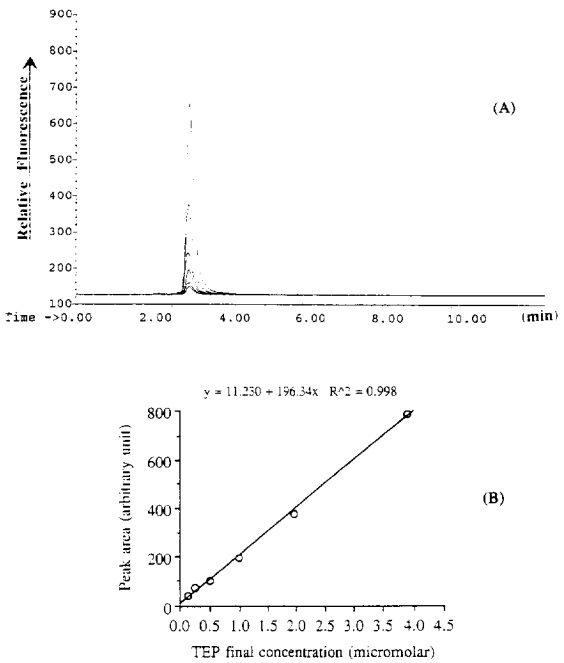

Fig. 1. Typical chromatograms (A) obtained from injections of reaction mixtures of standard TEP with TBA. The final concentrations for MDA–TBA conjugates are 0.16 μ*M*, 0.31 μ*M*, 0.62 μ*M*, 1.25 μ*M*, 2.50 μ*M* and 5.00 μ*M*, respectively. Typical calibration curve is shown in (B).

3. Results and discussion

Tetraethoxypropane (TEP), which yields MDA following hydrolysis, has been commonly used to prepare the standard solution in HPLC analysis. Fig. 1A shows the chromatograms obtained from injections of reaction mixtures of standard TEP with TBA. A good linearity (Fig. 1B) was usually observed in these chromatograms, which indicates the validity of TEP as a standard, as well as the appropriateness of derivatization and other sample preparations.

TEP was used as the standard solution for in vitro recovery in microdialysis perfusion. Ringer solution was continuously perfused through microdialysis probes placed in TEP solutions of four different concentrations (1.9 μ*M*, 3.9 μ*M*, 7.8 μ*M*, 15.6 μ*M*). The original standard TEP solutions and the their corresponding microdialysates were hydrolyzed and reacted with TBA, the MDA levels were analyzed and compared. A linear relation was observed between the standard TEP solutions before and after the microdialysis perfusion (Fig. 2). The linear response demonstrated that the operational system for MDA–TBA was adequate. Additionally, the slopes in Fig. 2 can be used to represent the in vitro recovery of TEP.

Shown in Fig. 3A is a typical chromatogram obtained from injection of MDA–TBA formed from the reaction of TBA with microdialysate collected from the perfusion of TEP solution. Fig. 3B is the injection of blank solution which was processed exactly the same as the TEP solution except the microdialysis probe was placed in the Ringer's solution. A very distinct MDA–TBA peak was observed in Fig. 3A, while no peak appeared in the same region in Fig. 3B. Additionally, the peak identification was also performed by the observation of corresponding increase in the peak areas from injections of TEP–TBA mixtures with various TEP concentrations.

Shown in Fig. 4C is a typical chromatogram

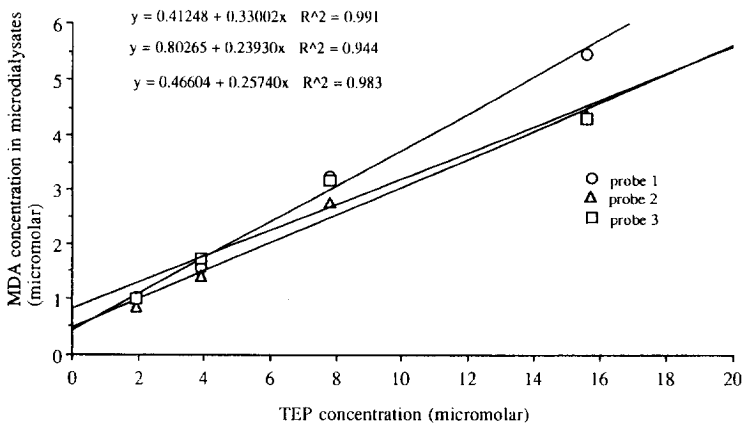

Fig. 2. Effect of various tetraethoxypropane (TEP) concentrations on the microdialysis probe recovery. TEP concentrations, represented as MDA–TBA conjugate concentrations, in microdialysates (*y*-axis) perfused through microdialysis probes are on the *y*-axis. Original TEP concentrations are on the *x*-axis. Perfusion time for each concentration was 60 min at 1 μl/min. The MDA–TBA concentrations shown were the average values from microdialysates collected from 20 min to 40 min and from 40 min to 60 min, respectively.

obtained from injection of microdialysates collected at 100 min after the probe implantation into anaesthetized rat brain cortex. MDA levels in the microdialysates were very low (from 0.02 μ*M* to 0.16 μ*M*, mean±S.D. 0.07±0.05 μ*M* from the average of 10 rats). Perfusion of 0.03% and 0.3% hydrogen peroxide significantly increased MDA levels (typical chromatograms for 0.03% and 0.3% hydrogen peroxide perfusions are shown in Fig. 4B and A, respectively). The profiles for MDA levels during the entire period of hydrogen peroxide perfusion are shown in Fig. 5. The increase in MDA was more prominent following 0.3% hydrogen peroxide perfusion than following 0.03% hydrogen peroxide perfusion. Sham-operated rats showed no variation in basal extracellular MDA levels over the entire experimental period of 4 h after probe implantation (data not shown).

Brain tissues are rich in phospholipids [24] and vulnerable to attack by oxygen-derived free radicals to initiate lipid peroxidation. In vivo data of organ extracellular MDA levels from anaesthetized or awake animals might thus provide valuable information for evaluation of oxygen radical-induced oxidative stress. However, the data is usually difficult to obtain due to obstacles in the in vivo sampling, that might be overcome by the microdialysis perfusion. Waterfall et al. used microdialysis perfusion and an HPLC system equipped with a UV detector to directly monitor MDA content in anaesthetized rat brain [25]. Althaugh HPLC–UV detection can be used for direct measurement of MDA, certain limitations exist [26,27]. For example, the UV detector may not have adequate sensitivity when used to monitor the low basal levels of MDA in brain extracellular space. Furthermore, MDA is not a very stable substance, immediate injection is needed to avoid possible variations. In the present study, we used a fluorescence detector in the HPLC system to analyze MDA–TBA conjugate in microdialysates. Fluorimetric detection provides good sensitivity, which is important since MDA levels are usually low in the extracellular space. Based on a signal to noise ratio of 3, the limit of detection in our assay was 1 pmol of MDA. The limit of detection for UV detection is 20 pmol, according to published results [25].

Additionally, the MDA–TBA conjugate compound is more stable than MDA itself, and immediate conjugation of MDA with TBA, following microdialysate collection might reduce variations in MDA levels.

We selected hydrogen peroxide perfusion for the present study due to hydrogen peroxide's ability to induce lipid peroxidation in both brain tissues and brain cell cultures [23,28,29]. Hydrogen peroxide

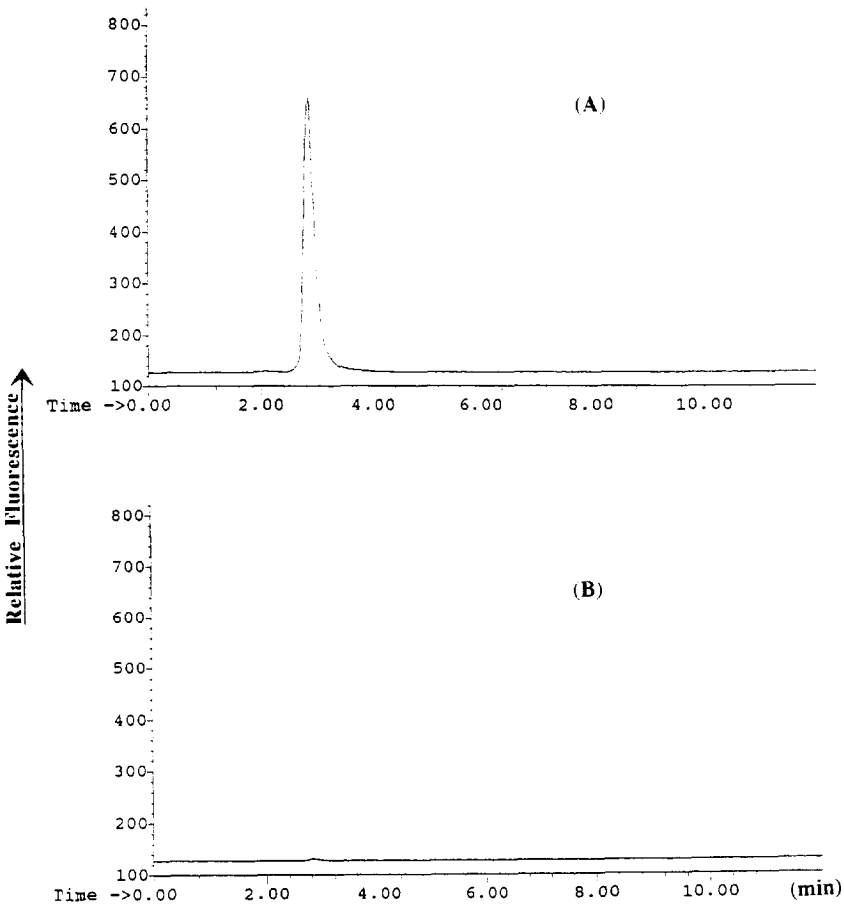

Fig. 3. Typical chromatograms obtained from injections of MDA–TBA conjugate resulting from reaction of TBA with microdialysate perfused through standard 20 μ*M* TEP solution (A), and Ringer's solutions (B). The procedure for preparing blank solution was described in Section 2.

perfusion-induced increase in MDA content in microdialysates demonstrates that the microdialysis–HPLC assay was adequate for monitoring extracellular lipid peroxidation. Although the concentration of hydrogen peroxide was high (0.03% and 0.3%), however, perfusion was conducted at a low flow-rate (1 μl/min). Production of oxygen-derived free radicals has been shown to be involved in various cerebral disorders such as cerebral ischemia [30–32], Parkinson's disease [33,34], etc. MDA measurements are commonly used to evaluate organ extracellular oxidative stress in isolated animal hearts following myocardial ischemia/reperfusion [35,36] and hepatic ischemia reperfusion [37,38]. Therefore, the assay reported in this paper might be of potential application to the investigations on lipid peroxidation in cerebral disorders, as well as many other physiological disorders that involve oxygen radicals.

Microdialysis is a minimally invasive method for in vivo sampling of organ extracellular fluids. It is especially useful for analysis of in vivo oxygen radical status due to simplified sample preparation and shortened analysis time by on-line and off-line HPLC. The production of oxygen-derived free radicals can be reflected in the concentration variations of three different groups of metabolites: major antioxidants, oxygen radicals themselves, cellular adducts from oxygen radical attack. In earlier studies, we used microdialysis coupled with an HPLC system to monitor major extracellular anti-

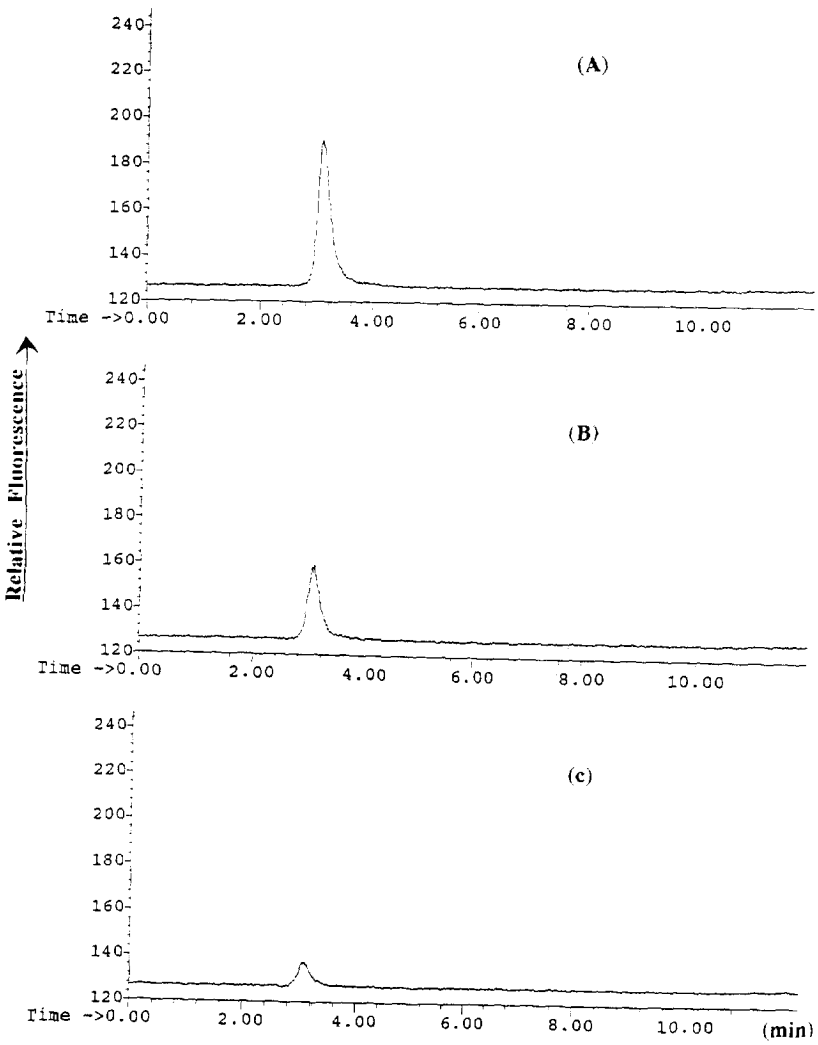

Fig. 4. Typical chromatograms obtained from injections of the reaction mixtures of TBA solution with microdialysates. Chromatogram (C) represents microdialysate before hydrogen peroxide infusion, and chromatograms (B) and (A) represent microdialysates after hydrogen peroxide infusion (0.03% and 0.3% respectively, 1 μl/min).

oxidants such as glutathione [17,18] and ascorbic acid [19,20], as well as hydroxyl radical production [21,22] in anaesthetized animals. Recently, we used microdialysis perfusion and an on-line HPLC system to monitor 8-hydroxydeoxyguanosine, a possible DNA oxidative damage marker following myocardial ischemia and reperfusion in anaesthetized rats [39]. Results from the present study show that microdialysis perfusion and HPLC analysis can be used to monitor in vivo lipid peroxidation, providing another marker for oxidative stress in anaesthetized animals.

In conclusion, we developed an assay, involving microdialysis perfusion and an HPLC system equipped with a fluorescence detector, to evaluate extracellular MDA content in anaesthetized rats. This assay has been applied to the dose-dependent increase in MDA production following hydrogen peroxide perfusion in the brain cortex of anaesthetized rats.

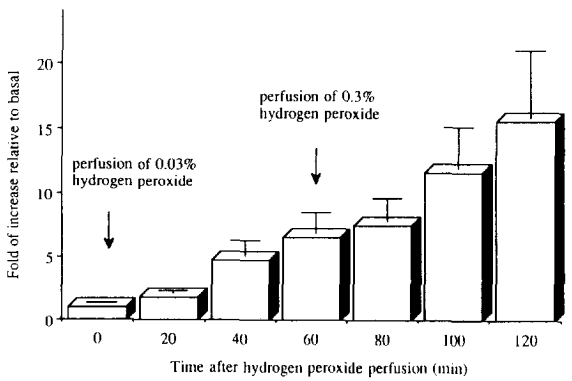

Fig. 5. Effect of hydrogen peroxide perfusion (0.03% and 0.3%) on brain cortex extracellular MDA contents in anaesthetized rats. Data are represented as fold of increase relative to basal status.

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