Effects of S-adenosyl-L-methionine on lipid peroxidation and glutathione levels in rat brain slices exposed to reoxygenation after oxygen-glucose deprivation

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Received 6 July 2001; received in revised form 6 November 2001; accepted 6 November 2001

Abstract

We analyzed the effects of S-adenosyl-L-methionine (AdoMet) on tissue oxidative stress in rat brain slices exposed to reoxygenation after oxygen-glucose deprivation. The thiobarbituric acid reactive substances (TBARS), total and oxidized glutathione, and lactate-dehydrogenase efflux (LDH) from tissue to the incubation medium, were measured. Brain slices were incubated without glucose and with N2, then glucose was added and O2 was perfused. After the anoxic-reoxygenation period, increase in TBARS, oxidized glutathione and LDH efflux, and decrease in total glutathione levels, were observed. The incubation with AdoMet before the anoxic period reduced TBARS (31–1000 μmol/l), glutathione production was increased (31–1000 μmol/l), LDH efflux decreased 6.41% with 15 μmol/l and 61.5% with 500 μmol/l). In the ex vivo experiments, we administered 50 mg/kg per day p.o., AdoMet for 3 days, then brain slices were collected and the anoxiareoxygenation experiment was carried out. AdoMet led to the inhibition of brain lipid peroxidation and increased total glutathione production, after 3 h-reoxygenation. The increase of LDH efflux in non-treated rats was reduced by 77%. We conclude that AdoMet exerts citoprotective effects in an experimental model of brain slices reoxygenation after oxygen-glucose deprivation. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: S-adenosyl-L-methionine; Oxidative stress; Lipid peroxidation; Glutathione; Free radicals

Oxygen-derived free radicals play an important role in the pathophysiological processes of damage during brain ischemia and reperfusion, both during ischemia and when the tissue supply of oxygen is restored [2,6,15]. Changes caused by ischemia-reperfusion can include an increase in oxidant factors, impairment of endogenous antioxidant systems, or both. These effects have led to studies designed to identify drugs with antioxidant activity in models of ischemia-reperfusion, especially agents able to inhibit lipid peroxidation [1,7–9]. However, another possibility might be to analyze the role of drugs that increase glutathione production, such as S-adenosyl-L-methionine (AdoMet). AdoMet has shown antioxidant effects in kidney and liver tissue in several models of ischemic or toxic damage [10], and in some control rat and human organs [4]. In a study of the rat brain we recently showed that AdoMet modulates cellular oxidative status, mainly by inhibiting lipid peroxidation and enhancing the glutathione system [5]. This study was performed in control rats, the present study was designed to determine whether these pharmacological effects also occurred in an experimental model of 20 min oxygen-glucose deprivation, followed 180 min of reoxygenation, in which oxidative stress is enhanced [7].

The study was done with brain tissue from male Wistar rats (body weight 300–350 g). The study was performed in compliance with international guidelines for the care and handling of laboratory animals. The rats were killed by decapitation and the whole brain was removed immediately. The cerebellum and brain stem were discarded and the remaining tissue was cut transversally into 0.2 mm slices with a vibratome (Capdem Instruments, San Francisco, CA, USA) according to a previously described technique [17] with slight modification. The slices were placed in buffer (composition in M: 0.1 NaCl, 5 × 10^{-4} KCl, 2.4 × 10^{-2} NaHCO3, 5.5 × 10^{-4} KH2PO4, 5 × 10^{-6} CaCl2, 2 × 10^{-3}}
MgSO₄, $9.8 \times 10^{-3}$ glucose, pH 7.4) perfused with a mixture of 95% O₂ and 5% CO₂. After 30 min to reach equilibrium the slices were placed in fresh buffer of the same composition except that the concentration of CaCl₂ was $3 \times 10^{-3}$ M, that of MgSO₄ was $1 \times 10^{-6}$ M, and no glucose was included. This solution was perfused with a mixture of 95% N₂ and 5% CO₂ for 20 min (anoxia), then perfused again for 180 min with 95% O₂ and 5% CO₂ (reoxygenation). One brain slice was analyzed for each of the following conditions: (1) after 30 min of incubation and before N₂ perfusion; (2) after 20 min of perfusion with N₂; (3) 3 h after reoxygenation. For all studies the tissues were quickly frozen in liquid nitrogen and stored at $-80 \, ^{\circ}C$ until the day of the experiment, which was done within 7 days of freezing.

In the in vitro experiments, AdoMet was incubated (15–1000 μmol/l) in the buffer from the equilibration period to the end of the experiment ($n = 10$ animals per group). In the ex vivo experiments, intraperitoneal injections of normal saline (1 ml/kg) ($n = 10$ animals) or AdoMet (50 mg/kg) ($n = 10$ animals) were given every day (09:00 h) for 3 days. One hour after last dose was given, brain was collected and slices were obtained, then the experimental model of anoxia-reoxygenation was carried out. This dose and duration of AdoMet was chosen in order to plan an acute treatment against brain damage, and in these conditions, this was the lower dose that inhibited lipid peroxidation and increased glutathione after the experimental model of anoxia-reoxygenation was performed.

To quantify lipid peroxidation we measured thiobarbituric acid reactive substances (TBARS) under basal conditions [3] in cell membrane-enriched fractions of the tissue. Absorbance was determined spectrophotometrically at 532 nm (Perkin Elmer C-532001 spectrophotometer, USA). The results were expressed as μmol TBARS per mg protein.

Total glutathione was measured spectrofluorometrically according to the technique described by Hissin and Hill [13]. Brain tissue was homogenized in 0.1 M sodium phosphate buffer (pH 8.0) with 25% phosphoric acid at a proportion of 1:20, then centrifuged at 13,000 × g for 15 min at 4 °C to obtain the supernatant. Duplicate cuvettes were prepared for spectrofluorometry with sodium phosphate buffer, supernatant for each sample, and o-phthaldehyde. To determine the proportions of oxidised and reduced glutathione we incubated supernatant from each sample with 4vinylpyridine, then proceeded as for total glutathione.

Tissue damage was measured by examining the lactate-dehydrogenase (LDH) efflux to the incubation solution. Samples of this solution were taken every 30 min (an equivalent volume of the corresponding buffer was replaced) and the enzyme activity was measured spectrophotometrically at 340 nm by following oxidation of NADH (decrease in absorbance) in the presence of pyruvate using a microplate reader ELX-800 (Bio-Tek Instruments Inc., Winooski, VT, USA).

The data in the text, tables and figures are expressed as the mean ± standard error of the mean from 10 experiments in samples from different animals. All statistical analyses were done with the Social Program for Statistical Sciences (SPSS v. 6.0). One-way analysis of variance followed by Bonferroni transformation was used, and differences were considered significant when $P < 0.05$.

With the in vitro model of anoxia-reoxygenation used here we found changes in all components of oxidative status in rat brain slices. A 20-min period of anoxia led to no significant changes in any of the parameters. Reoxygenation after anoxia increased the production of TBARS (maximum increase $60.2 \pm 4.8\%$ after 3 h in comparison to the preanoxia value) (Fig. 1A). Incubation with AdoMet reduced, in a concentration-dependent way, TBARS content in brain slices, reaching statistical significance with 31 μmol/l. Glutathione production decreased 3 h after reoxygenation by $52.9 \pm 3.4\%$ in comparison with the preanoxic value (Fig. 2A, whereas the percentage of oxidised glutathione...
increased by a maximum of $102 \pm 7.4\%$ 3 h after reoxygenation) (Fig. 2B). Incubation with AdoMet diminished the glutathione consumption: restrained the reduction of total glutathione, due to the reoxygenation, and the increase of its oxidised form. In control samples (oxygenated slices), LDH efflux increased (with respect to preanoxic values) $30.8 \pm 2.1\%$ after 3 h incubation, whereas in samples under anoxia-reoxygenation conditions, LDH efflux increased 258% (Fig. 1B). After the incubation of samples with AdoMet (from the equilibration period), LDH efflux after 3 h reoxygenation, decreased with respect to oxygenated samples, $3.84\%$ (15.6 μmol/l), 42.30% (31.25 μmol/l), 64.74% (62.5 μmol/l), 89.42% (125 μmol/l) and 89.74% (500 μmol/l).

The administration of 50 mg/kg per day of AdoMet induced few changes in brain tissues under oxygenated conditions (Table 1): 16.6% in TBARS content; 9.4% increase in glutathione levels; and 7.3% decrease in the percentage of oxidised glutathione (GSSG). None of these differences were significant. In rats whose brain was induced to the in vitro experimental anoxia-reoxygenation model, the administration of AdoMet reduced 44.9% in TBARS content; in non-treated animals after 3 h of reoxygenation, glutathione levels increased 130% with respect to non-treated animals, reduced by 52.4% the amount of glutathione in oxidised form, and reduced by 55.4% the LDH release with respect to non-treated animals (Table 1).

In our model, tissue reoxygenation led to biochemical changes (increased TBARS production) and evidence that a tissular defence mechanism was triggered (increased percentage of GSSG). However, the concomitant increase in lipid peroxidation suggests that this defence mechanism was neither sufficient nor entirely effective, even more if we consider that these changes correlated with an increase in LDH efflux, as an indirect index of cellular death. Recently, Moro et al. [16] have demonstrated that the main proportion of the LDH efflux increase corresponds to neuronal death, measured as increases in neuronal specific enolase. For that reason, we suppose that the cellular death measured in our experimental model is due to neuronal death.

The presence of AdoMet in the incubation medium, and the intraperitoneal administration of AdoMet, brought about a partial re-equilibration of oxidative status, decreasing oxidizing factors and preventing the decrease in the antiox-

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>O2</th>
<th>N2/O2</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>AdoMet</td>
</tr>
<tr>
<td>TBARS (nmol/mg protein)</td>
<td>0.78 ± 0.08</td>
<td>0.65 ± 0.09</td>
</tr>
<tr>
<td>GSH + GSSG (μmol/g tissue)</td>
<td>4.97 ± 0.29</td>
<td>5.44 ± 0.31</td>
</tr>
<tr>
<td>% GSSG</td>
<td>10.08 ± 0.77</td>
<td>9.35 ± 0.91</td>
</tr>
<tr>
<td>LDH (unit/mg tissue/min)</td>
<td>1.10 ± 0.11</td>
<td>1.00 ± 0.09</td>
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</tbody>
</table>

*P < 0.05 respect to oxygenated control, **P < 0.05 respect to N2/O2 control.

Our results indirectly support the findings of some authors [14,18], who reported that AdoMet protects from the neuronal damage in rats after brain ischemia. However, in these three studies the dose of AdoMet was 100–120 mg/kg, twice as high as the dose we used. Our study demonstrates the importance of the ex vivo antioxidant effect of AdoMet in brain tissue under reoxygenated conditions.

The mechanisms by which AdoMet palliates the damage caused by ischemia-reperfusion in the brain have not yet to be explained, although several hypotheses have been put forward: (a) prevention of the damage to choline phospholipids in the cell membrane [19]; (b) an increased erythrocyte membrane fluidity [12]; (c) a normalization of the ATP levels that had been lowered in a model of brain ischemia [21]; (d) antioxidant action, as other antioxidant substances have been shown to have a neuroprotective effect [11]. In a recent study [20] we demonstrated that AdoMet reduced brain damage in an in vivo model of brain ischemia-reperfusion; in this study, the effect of the drug could be due to a possible effect on blood perfusion or to a direct effect to the brain tissue. Our findings support the latter possibility; restoration of brain oxidative status may be an important mechanism in the ability of AdoMet to protect tissues from oxidative damage in an in vitro model of brain slices exposed to reoxygenation after oxygen-glucose deprivation.

We thank Antonio Pino Blanes for his expert technical assistance, and Laboratorios Boehringer Ingelheim España, S.A. for the financial support of this study.

[15] Michowiz, S.D., Melamed, E., Pikarsky, E. and Rappaport,


