Effects of aspirin plus alpha-tocopherol on brain slices damage after hypoxia-reoxygenation in rats with type 1-like diabetes mellitus


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Abstract

Diabetes mellitus is a risk factor for cerebrovascular ischemic disease. Aspirin (acetylsalicylic acid) is the most widely used drug for the secondary prevention of thrombotic phenomena. It has been also recently demonstrated that α-tocopherol influenced in vitro the antiplatelet effect of aspirin. The aim of the present study is to evaluate the effects aspirin plus α-tocopherol on cerebral oxidative stress, prostaglandin production and the nitric oxide pathway in a model of hypoxia-reoxygenation in rat brain slices. Our results show an imbalance in brain oxidative status (reflected mainly as the increase in lipid peroxides) as a result of diabetes itself rather than a failure of the glutathione-based antioxidant system. Moreover, our results also show a higher concentration of prostaglandins in the brain of diabetic animals and a higher nitric oxide concentration, mainly through a high iNOS activity. After 180 min of post-hypoxia reoxygenation, LDH activity was 40.6% higher in animals with diabetes, in comparison to non-diabetic animals. The increase of the LDH efflux observed in non-treated rats was reduced by 31.2% with aspirin, by 34.7% with α-tocopherol and by 69.8% with the association aspirin-α-tocopherol. The accumulation of prostaglandin E2 observed in diabetic non-treated rats was reduced statistically after the treatment with aspirin (34.2% inhibition), α-tocopherol (19.3% inhibition) or the association aspirin-α-tocopherol (54.4% inhibition). Nitric oxide production after 180 min reoxygenation was significantly reduced in aspirin (36.4%), α-tocopherol (22.7%) and aspirin-α-tocopherol (77.8%) treated rats with respect to diabetic non-treated animals; this was related mainly with a reduction in iNOS activity. The association between aspirin and alpha tocopherol could protects against brain ischemic-reperfusion damage with a better profile than aspirin alone.

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Keywords: Acetylsalicylic acid; Alpha-tocopherol; Brain hypoxia; Oxidative stress; Nitric oxide

Diabetes mellitus is a risk factor for cerebrovascular ischemic disease. Age, race, hypertension, diabetic neuropathy, coronary disease and peripheral vascular disease are all risk factors for cerebrovascular accidents in diabetes [2]. Type 2 diabetes mellitus is usually related with this high incidence of cerebrovascular ischemic disease, but it has it recently been described a high incidence of these events in type 1 diabetes mellitus [19].

One of the mechanisms of brain damage during ischemia involves the formation of free radicals and the impairment of enzymatic mechanisms, which result in tissue oxidative stress [17]. Oxidative stress has been directly related with increased nitric oxide (NO) production mainly via the inducible pathway, and with altered intracellular prostaglandin production [4], among others. These alterations increase cell death and apoptotic pathways [21].

Aspirin (acetylsalicylic acid) is the most widely used drug for the secondary prevention of thrombotic phenomena in the heart, brain and peripheral circulation [1]. In the brain, aspirin reduces the incidence of thrombotic events in patients who have had a prior stroke. This effect is due mainly to its antiplatelet action [14], which prevents the formation of arterial platelet thrombi. It has been recently demonstrated the ability of aspirin and its main metabolite salicylic acid, to prevent cell damage after a hypoxic model in rat brain slices by inhibiting oxidative stress, prostaglandin accumulation and inducible nitric oxide synthesis [6,15].

Alpha-tocopherol is an antioxidant protecting unsaturated fatty acids, protein and DNA from oxidation. The administration of α-tocopherol to rats reduces neuronal cell death caused...
by ischemia and reperfusion in rat models through its antioxidant effect [24,26]. It has also been recently demonstrated that α-tocopherol influenced in vitro the antiplatelet effect of aspirin, mainly by an increase in the prostacyclin and nitric oxide production [12]. For that reason, the aim of the present study is to evaluate the effects aspirin plus α-tocopherol on cerebral oxidative stress, prostaglandin production and the nitric oxide pathway in a model of hypoxia-reoxygenation in rat brain slices.

The experimental animals were adult male Wistar rats (body weight 200–250 g) that were 2 months old at the time of inclusion in the study. The study was performed in compliance with international guidelines for the care and handling of laboratory animals (European Community Directive 86/609 EEC). Rats were divided into five groups: (1) normoglycemic animals, (2) diabetic rats treated with saline, (3) diabetic rats treated with 2 mg/kg/day p.o. of aspirin, (4) diabetic rats treated with 25 mg/kg/day p.o. α-tocopherol, and (5) diabetic rats treated with aspirin plus α-tocopherol at the same dose referred above. Drugs were administered in a single daily dose between 9.00 and 10.00 a.m. After the period of follow-up (2 months), the animals were anaesthetised with ether inhalation, then killed by decapitation. The brain was removed to prepare slices which were exposed to hypoxia-reoxygenation in vitro. In all cases, 15 diabetic animals and 15 matched controls were used for each type of experiment. Experimental diabetes was induced with a single femoral injection of 50 mg/kg streptozotocin under ether anaesthesia. Blood glucose concentration was measured by placing a Glucocard Memory II glucosimeter (Menarini, SA, Barcelona, Spain) in contact with blood from a small incision in the tail. Animals were considered to have diabetes if blood glucose was >200 mg/dl for 2 consecutive days. Rats in non-diabetic control groups received a single intravenous injection of isotonic saline solution, and blood glucose was measured in the same way as in animals that were made diabetic.

We used a previously described method of in vitro brain slices hypoxia-reoxygenation [7]. Brains were isolated, the cerebellum and brain stem were discarded and the remaining tissue was cut transversally into 0.1-mm slices with a vibrating microtome (Capdem Instruments, San Francisco, CA, USA). 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^{-7} MgSO4, 9.8 × 10^{-3} glucose, pH 7.4) and 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 5 × 10^{-3} M, that of MgSO4 was 1 × 10^{-6} M, and no glucose was included. This solution was perfused with a mixture of 95% O2 and 5% O2 for 20 min (hypoxia). Then the slices were placed in fresh buffer containing glucose and the solution was perfused with a mixture of 95% oxygen and 5% CO2 (reoxygenation). For all studies the tissues were quickly frozen in liquid nitrogen and stored at −80 °C until the day of the experiment, which was done within 7 days of freezing.

To quantify lipid peroxidation we measured thiobarbituric acid reactive substances (TBARS) [5] in cell membrane-enriched fractions of the tissue at 532 nm (Perkin Elmer C-532001 spectrophotometer, USA). Total glutathione was measured spectrophotometrically [16]. Brain tissue was homogenized in 0.1 M sodium phosphate buffer (pH 8.0), then centrifuged at 13,000 × g for 15 min at 4 °C to obtain the supernatant. Duplicate cuvettes were prepared for spectrophotometry with sodium phosphate buffer, supernatant for each sample, and α-phthalaldehyde. To determine the proportions of oxidized and reduced glutathione we incubated supernatant from each sample with 4-vinylpyridine. Glutathione peroxidase (GSHpx), glutathione reductase (GSSGrd) and glutathione transference (GSHtr) were determined by spectrophotometric kinetics. Tissue samples were diluted in 0.1 M phosphate-buffered saline (pH 7.0) and 25% phosphoric acid. The mixture was homogenized and centrifuged at 13,000 × g for 15 min at 4 °C. The supernatant was used to determine protein concentration, after neutralization with 0.1N NaOH, and to determine the enzyme activities. For glutathione peroxidase (GSHpx) activity [11], 25 μg protein [3] was taken from each supernatant and 53 μl glutathione reductase, 133 μl GSH, 100 μl nicotinamide adenine dinucleotide phosphate (NADPH) and 100 μl terbutyl-hydroperoxide were added. The preparation was read at 340 nm. For glutathione reductase (GSSGrd) activity [11] 100 μl of NADPH (0.12 mg/ml) was added, then 6 μl GSSG was added, the cuvette was again shaken by inversion, and the preparation was read at 340 nm. The decrease in absorbance was recorded every 30 s for 5 min. Glutathione transference (GSHtr) activity was determined according to the method of Warholm et al. [28]. One hundred microliters of GSH was added to the volumes of sample and buffer indicated above.

Nitric oxide synthase activity was measured in brain tissue samples that were homogenized (1.5, w/v) in buffer containing 10 mM HEPES, 320 mM sucrose, 1 mM EDTA, 1 mM n,N-diethylthioleotrotil (DDT), 10 μg/ml leupeptin and 2 μg/ml aprotonin at 0 °C. The homogenates were centrifuged at 12,000 × g for 20 min at 4 °C, and the supernatant was used to measure NOS synthase, according to a previously described method [13] based in the transformation of L-^{1}

\[^{3}H\]arginine (59 Ci per mole, Amersham Life Science Inc.) into \[^{3}H\]citrulline; in each determination, a calcium-dependent test and a calcium-independent test were carried out in order to discriminate the constitutive and inducible isofoms of NOS.

Prostaglandin E2 was measured in brain tissues samples that were homogenized (1:10, w/v) in 15% methanol with 0.1N phosphate-buffered saline (pH 7.5), then centrifuged at 37,000 × g for 15 min at 4 °C, according to a previously described method [13]. The concentration of PGE2 was measured with a commercial enzyme immunoassay (Amersham International plc).

Tissue damage was measured by examining the lactate dehydrogenase (LDH) efflux to the incubation solution. 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 (BioTek Instruments Inc., Winooski, VT, USA).

The data in the text, tables and figures are expressed as the mean ± standard error of the mean from six experiments in sam-
to non-diabetic animals (Fig. 1). When we measured oxidative stress, we found that TBARS content increased after reoxygenation significantly more in diabetic than in normoglycemic animals (91.5%). The results for glutathione levels showed that tocopherol reduced with statistical significance the LDH activity in diabetic rats without treatment or treated with 2 mg/kg/day p.o. aspirin (ASA), 25 mg/kg/day p.o. α-tocopherol (AT) or its association. Treated animals showed brain concentrations of prostaglandin E2 lower than non-treated diabetic rats (Fig. 1). Percentages of inhibition, respect to control group, were as follows: 67.4% for aspirin, 75.5% for α-tocopherol and 65.0% for its association (data not shown). All of the three treatments increased constitutive nitric oxide synthase activity and mainly through a high iNOS activity (Figs. 1 and 2).

One-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test was used, and differences were done at P<0.05. Our results show an imbalance in brain oxidative status (reflected mainly as the increase in lipid peroxides) as a result of diabetes itself rather than a failure of the glutathione-based antioxidant system (Table 1). This may lead to the assumption that after oxidative damage the brains of diabetic animals are rendered more susceptible to membrane damage. Moreover, our results also show a higher concentration of prostaglandins in the brain of diabetic animals and a higher nitric oxide concentration, reflecting mainly as the increase in lipid peroxides) as a result of diabetes itself rather than a failure of the glutathione-based antioxidant system (Table 1). This may lead to the assumption that after oxidative damage the brains of diabetic animals are rendered more susceptible to membrane damage. Moreover, our results also show a higher concentration of prostaglandins in the brain of diabetic animals and a higher nitric oxide concentration, mainly through a high iNOS activity (Figs. 1 and 2).

Table 1

Mean values of the oxidative stress parameters measured before hypoxia and after reoxygenation in normoglycemic rats, and before hypoxia and after reoxygenation in diabetic rats without treatment or treated with 2 mg/kg/day p.o. aspirin (ASA), 25 mg/kg/day p.o. α-tocopherol (AT) or its association.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TBARS (nmol/mg prot)</th>
<th>GSH (mM/g tissue)</th>
<th>%GSSG with respect to GSH+GSSG</th>
<th>GSHpx (μmol/min)</th>
<th>GSGrd (μmol/min)</th>
<th>GSHtf (μmol/min)</th>
<th>GSSGrd (μmol/min)</th>
<th>GSH (mM/g tissue)</th>
<th>%GSSG with respect to GSH+GSSG</th>
<th>GSHpx (μmol/min)</th>
<th>GSGrd (μmol/min)</th>
<th>GSHtf (μmol/min)</th>
<th>GSSGrd (μmol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-hypoxia</td>
<td>0.46 ± 0.03</td>
<td>2.12 ± 0.13</td>
<td>7.33 ± 0.62</td>
<td>58.45 ± 1.61</td>
<td>13.36 ± 1.30</td>
<td>23.18 ± 1.36</td>
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<tr>
<td>Reoxygenation</td>
<td>0.83 ± 0.09*</td>
<td>1.35 ± 0.11*</td>
<td>11.33 ± 1.01*</td>
<td>71.09 ± 1.03</td>
<td>9.91 ± 0.81*</td>
<td>14.00 ± 1.31*</td>
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<td>Diabetic rats</td>
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<tr>
<td>Pre-hypoxia</td>
<td>0.81 ± 0.08</td>
<td>2.02 ± 0.15</td>
<td>7.36 ± 0.58</td>
<td>55.21 ± 2.14</td>
<td>10.00 ± 0.46</td>
<td>23.64 ± 1.11</td>
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<tr>
<td>ASA</td>
<td>0.01 ± 0.08</td>
<td>2.36 ± 0.05</td>
<td>7.49 ± 0.84</td>
<td>40.62 ± 1.27</td>
<td>12.08 ± 0.37</td>
<td>27.69 ± 1.59</td>
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<tr>
<td>ASA + AT</td>
<td>0.81 ± 0.04</td>
<td>2.27 ± 0.05</td>
<td>6.46 ± 0.24</td>
<td>55.86 ± 1.04</td>
<td>12.14 ± 0.69</td>
<td>27.71 ± 1.48</td>
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<tr>
<td>Reoxygenation</td>
<td>1.59 ± 0.11*</td>
<td>1.14 ± 0.07*</td>
<td>5.20 ± 0.59</td>
<td>55.57 ± 2.42</td>
<td>7.21 ± 0.41*</td>
<td>12.00 ± 1.50*</td>
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<tr>
<td>ASA</td>
<td>1.73 ± 0.04</td>
<td>1.04 ± 0.09*</td>
<td>11.09 ± 1.11</td>
<td>56.54 ± 1.40</td>
<td>8.77 ± 0.56*</td>
<td>16.46 ± 0.70*</td>
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<tr>
<td>ASA + AT</td>
<td>1.32 ± 0.20*</td>
<td>1.41 ± 0.07*</td>
<td>7.54 ± 0.81</td>
<td>44.86 ± 1.38</td>
<td>5.57 ± 0.30*</td>
<td>24.07 ± 1.01</td>
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<tr>
<td>ASA + AT</td>
<td>1.19 ± 0.12*</td>
<td>1.31 ± 0.07*</td>
<td>11.59 ± 0.41</td>
<td>46.10 ± 1.19</td>
<td>10.00 ± 0.55</td>
<td>27.40 ± 1.04</td>
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Table 1: Mean values of the oxidative stress parameters measured before hypoxia and after reoxygenation in normoglycemic rats, and before hypoxia and after reoxygenation in diabetic rats without treatment or treated with 2 mg/kg/day p.o. aspirin (ASA), 25 mg/kg/day p.o. α-tocopherol (AT) or its association.

- TBARS, thiobarbituric acid reactive substances; GSH, reduced glutathione; GSSG, oxidized glutathione; GSHpx, glutathione peroxidase activity; GSGrd, glutathione reductase activity; GSHtf, glutathione transferase activity.
- *P<0.05 with respect to healthy control group.
- †P<0.05 in comparison to aspirin group.

Respect to the oxidative stress parameters, in oxygenated brain slices (Table 1), only an increase in glutathione transferase activity in diabetic rats treated with aspirin plus α-tocopherol was observed. None of the others determinations were statistically modified after 2 months of treatment with aspirin, α-tocopherol or its association. Treated animals showed brain concentrations of prostaglandin E2 lower than non-treated diabetic rats (Fig. 1). Percentages of inhibition, respect to control group, were as follows: 67.4% for aspirin, 75.5% for α-tocopherol and 65.0% for its association (data not shown). All of the three treatments increased constitutive nitric oxide synthase activity and reduced the inducible isomform activity. Only the aspirin-treated group showed a higher effect that the other treatment in the increase of cNOS activity (Fig. 2). The association aspirin-α-tocopherol reduced with statistical significance the LDH activity in oxygenated brain slices (Fig. 1).

After 180 min of post-hypoxia reoxygenation, LDH activity was 40.6% higher in animals with diabetes, in comparison to non-diabetic animals (Fig. 1). When we measured oxidative stress, we found that TBARS content increased after reoxygenation significantly more in diabetic than in normoglycemic animals (91.5%). The results for glutathione levels showed that...
Fig. 2. Nitrites + nitrates (NO$_2^-$ + NO$_3^-$), constitutive (cNOS) and inducible (iNOS) nitric oxide synthase activities in rat brain slices before the period of hypoxia and after 180 min of reoxygenation.

P $< 0.05$ in comparison to the control group, † P $< 0.05$ with respect to healthy control slices (n = 14 rats per group).

GSH concentration decreased both in non-diabetic and in diabetic rats, while percentage of GSSG (respect to GSH + GSSG) decreased after reoxygenation in diabetic rats, and that the decreases were greater in diabetic animals. These decreases were accompanied by a minor activity of GSHpx and GSSGrd in comparison to normoglycemic rats (Table 1). After reoxygenation, prostaglandin E$_2$ accumulated in the brain slices, and the increase was significantly greater in diabetic animals (83.6% greater in comparison to normoglycemic rats) (Fig. 1).

The findings for the NO pathway showed an overall increase in nitrite + nitrate production after reoxygenation, with larger increases in animals with diabetes (33.8% with respect to non-diabetic animals). Constitutive nitric oxide synthase activity was significantly reduced both in normoglycemic and in diabetic rats, although the largest reductions in comparison to non-diabetic animals were seen in animals with diabetes (23.2%). Inducible nitric oxide synthase activity was significantly increased in both groups, with the greatest increases in rats with diabetes (18.64% after reoxygenation, with respect to non-diabetic animals; Fig. 2).

These differences between normoglycemic and diabetic animals are in agreement with some studies in which a high sensitivity in brain tissue has been demonstrated in diabetic animals after different ischemic models in rats [21,25,27]. For that reason it is important to consider a possible neuroprotective therapy that extends the prophylactic effect of the antithrombotic drug aspirin.

In this experimental model, in treated rats, after 180 min reoxygenation, the increase of the LDH efflux observed in non-treated rats was reduced by 31.2% with aspirin, by 34.7% with α-tocopherol and by 69.8% with the association aspirin-α-tocopherol (Fig. 1). The accumulation of lipid peroxides (TBARS) after reoxygenation in diabetic non-treated rats was only reduced with statistical significance after the treatment with α-tocopherol (23.2% reduction with respect to control group) and with the association aspirin-α-tocopherol (25.1% reduction) (Table 1). The concentration of reduced glutathione (GSH) was inhibited after reoxygenation in control group; this inhibition was not reached in diabetic rats treated with α-tocopherol alone or in combination with aspirin. Moreover, the percentage of oxidized glutathione (GSSG) was increased after the three types of treatments: 111% increase in aspirin group, 45% in α-tocopherol group and 123% in the aspirin-α-tocopherol group.

After 180 min reoxygenation, glutathione reductase activity was not modified, but glutathione peroxidase and glutathione transferase activities were increased significantly with respect to control group, in α-tocopherol and aspirin-α-tocopherol groups.

In aspirin-treated diabetic rats, the decrease in GSH levels was higher than in α-tocopherol group, but aspirin reached GSHpx activity in order to increase the proportion of glutathion in the oxidized form, for that reason we think the redox status was re-equilibrated in reoxigenated brains. Another observation with aspirin is the apparent reduction in the glutathione system in oxygenated slices and the increase in reoxygenated slices; this could be due to a preservation of this system when it is not necessary (oxygenated conditions) and a reaction when it is useful (reoxygenated conditions) induced with aspirin. The accumulation of prostaglandin E$_2$ observed in diabetic non-treated rats was reduced statistically after the treatment with aspirin (34.2% inhibition with respect to control group), α-tocopherol (19.3% inhibition) or the association aspirin-α-tocopherol (54.4% inhibition; Fig. 1). Nitric oxide production after 180 min reoxygenation was significantly reduced in aspirin (36.4% inhibition), α-tocopherol (22.7% inhibition) and aspirin-α-tocopherol (77.8% inhibition).
inhibition) treated rats with respect to diabetic non-treated animals. Nitric oxide synthase activities were also modified with the three treatments after 180 min reoxygenation: the constitutive isoform activity was increased with aspirin, α-tocopherol and its association (71.3%, 101% and 193%, respectively, with respect to control group) and the inducible isoform activity was inhibited (48.1%, 62.6% and 79.6%, respectively, with respect to control group; Fig. 2).

Despite the antioxidant effects of aspirin and α-tocopherol, this is clearly lower than that previously demonstrated in non-diabetic rats [6,15,26]. In our opinion, the inhibition in lipid peroxidation and the increase in glutathione levels and GSHtf activity are not enough to explain the neuroprotective effects of its association. However, the effects on prostaglandin accumulation and nitric oxide production after reoxygenation closely correlated with this neuroprotective effect. This could be explained through their effects on inflammatory pathway in damaged tissues, such as ischemic brain. Inflammation is one of the mechanisms of injury after ischemic insult and during reperfusion time; several transcription factors (NF-κB, interferon-regulatory factor, etc.) and inflammatory mediators (tumor necrosis factor alpha—TNFs, interleukins, etc.) are activated by hypoxia; these mediators induce the synthesis of some enzymes, such as cyclooxygenase-2 (COX-2) or inducible nitric oxide synthase (iNOS) [8,29]. Aspirin inhibits NF-κB expression [18]. TNFα gene expression in murine tissue macrophages [23], nitric oxide release from glial cells induced with bacterial polyinosinic-polycytidylic acid [20] or in cardiac fibroblasts induced with interferon alpha plus TNFα [10]. Alpha tocopherol inhibits COX-2 activity [22] and attenuates COX-2 transcription and synthesis in murine microglial cells [9]. For that reason, both compounds and mainly their combination could induce quiescence to pathways that are associated with acute or chronic inflammatory conditions in the central nervous system [9]. In this sense, the classical antioxidant effect of α-tocopherol does not seem to play an important role in its neuroprotective effect.

These mechanisms could explain the profile in prostaglandin, nitric oxide production and NOS activities in the experimental model used in our study. Moreover, this could be the explanation to the higher neuroprotective effect of the association of aspirin and α-tocopherol showed in our results. Prostaglandin E2 production and iNOS activity increased with aspirin and α-tocopherol [26], however, the neuroprotective effect in these animals was proportionally lower than in diabetic rats. For that reason the association between aspirin and alpha tocopherol could protects against brain ischemic-reperfusion damage with a better profile than aspirin alone. However, this asseveration needs further studies mainly in patients suffering from stroke.

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References