Effects of hypoxia reoxygenation in brain slices from rats with type 1-like diabetes mellitus

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Abstract

Background  The aim of this study was to determine whether the brain tissue of type 1 diabetic animals is more susceptible to damage by hypoxia reoxygenation than healthy animals.

Methods  This study used rats with diabetes of 1, 2 and 3 months (N = 15 rats/group). Brain slices were subjected to hypoxia and reoxygenation for 180 min in vitro. We measured oxidative stress (lipid peroxidation, glutathione concentration and enzyme activities related to glutathione), concentration of prostaglandin E2 (PGE2) and nitric oxide (NO) pathway (nitrite + nitrate, activities of constitutive (cNOS) and inducible (iNOS) nitric oxide synthase). As a parameter of cell death we measured the efflux of lactate dehydrogenase (LDH).

Results  After reoxygenation LDH activity increased in comparison to nondiabetic animals by 40, 40.6 and 68.9% in animals with diabetes of 1, 2 and 3 months duration, respectively. These changes were accompanied by greater increases in lipid peroxides (25.4, 93.7 and 92.8%). PGE2 accumulated in significantly larger amounts in diabetic animals (62.5, 85.5 and 114%), and nitrite + nitrate accumulation was significantly greater in rats with diabetes of 2 (40.2%) and 3 months duration (24.0%). iNOS activity increased significantly in all the groups of diabetic animals, with the largest increases in rats with diabetes of 2 (18.6%) and 3 months duration (21.1%).

Conclusions  The biochemical pathways involved in oxidative stress and neuronal death are more sensitive to hypoxia reoxygenation in type 1-like diabetic, as compared to normal, rats. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords  diabetes; brain hypoxia; oxidative stress; nitric oxide

Introduction

In diabetes the formation of reactive oxygen species is a direct consequence of hyperglycemia [1]. It was shown that the accumulation of free radicals described in diabetes can give rise to nonenzymatic protein glycation, activation of the diacylglycerol-protein kinase C pathway, accumulation of sorbitol and activation of the expression of nuclear transcription factor κ-B in endothelial cells [2]. Reactive species of oxygen oxidize and damage DNA, proteins and lipids, playing an important role in the pathogenesis of late complications of diabetes [2,3]. In addition, diabetes has been characterized by a decline in almost all endogenous antioxidant activities [4,5].
One of the main mechanisms of brain damage during ischemia involves the formation of free radicals and the impairment of enzymatic mechanisms, which result in tissue oxidative stress [6]. Oxidative stress has been directly related with increased nitric oxide (NO) production mainly via the inducible pathway, and with altered intracellular prostaglandin production [7].

Diabetes mellitus is a risk factor for stroke [8]. Type 2 diabetes mellitus is usually related with this high incidence of stroke, but a similar incidence of these events both in type 1 and type 2 diabetes mellitus has recently been described [9]. The abnormalities described in type 1 diabetes mellitus include diminished prostacyclin synthesis and function, reduced constitutive NO synthesis (constitutive nitric oxide synthase, cNOS) and increased nitric oxide synthase (iNOS) activity via the inducible nitric oxide synthase (iNOS) pathway [10,11]. In addition, free radical production and platelet aggregation are increased, oxide synthase (iNOS) pathway [10,11]. In addition, free radical production and platelet aggregation are increased, giving rise to a vascular state that favors reduced blood flow to tissues and a tendency toward the formation of intravascular thromboses [10–13].

The aim of the present study was to determine the changes in ischemic damage pathways between type 1- like diabetes mellitus and normoglycemic rats in a model of hypoxia reoxygenation in brain slices.

Materials and methods

Material

Lactate dehydrogenase (LDH) reagent kits were obtained from Biosystem SA (Barcelona, Spain). L-(3H)Arginine and prostaglandin E2 (PGE2) enzyme immunoassay kits were from Amersham International plc (Little Chalfont, Buckinghamshire, UK). Nitrite + nitrate enzyme immunoassay kits were from Cayman Chemical Co. (Ann Arbor, Michigan, USA). Insulin NPH was from Novo Nordisk, Denmark). Glucopat reactive tips were obtained from Menarini Diagnóstica (Barcelona, Spain). All other reagents were from Sigma Chemical Corp. (St Louis, MO, USA).

Study design

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. All rats were used in accordance with current Spanish legislation for animal care, use and housing (RD 223/1998, based on European Directive 86/609/CEE). All efforts were made to minimize the number of animals used and their suffering. The recommendations in Principles of Laboratory Animal Care (NIH publication no. 86-23, revised 1985) were followed, as well as the Spanish Law on the Protection of Animals, where applicable. The study protocol was approved by the University of Malaga Ethics Committee for the Use of Animals.

After different periods of follow-up (1, 2 and 3 months), the animals were anesthetized with ether inhalation and 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

Experimental diabetes was induced with a single femoral intravenous injection of 50 mg/kg streptozotocin under ether anesthesia. Blood glucose concentration was measured after rats fasted overnight 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 two consecutive days. Rats in nondiabetic 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.

In vitro model of rat brain hypoxia reoxygenation

We used a previously described method of in vitro model of rat brain hypoxia reoxygenation [14]. After the cerebellum and brainstem were discarded, the brain (cortex and median brain including hippocampus) was coronally cut into 1-mm slices with a vibrating microtome (Capdem Instruments, San Francisco, CA, USA). The slices were placed in buffer (composition in molar: 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 × 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 3 × 10⁻³ M, that of MgSO₄ was 1 × 10⁻⁶ M, and glucose was not included. This solution was perfused with a mixture of 95% N₂ and 5% O₂ for 20 min (hypoxia). This hypoxic period was chosen on the basis of previous experiments (data not shown) in which this time was sufficient to alter the reoxygenation biochemical pathways without high significant changes in the biochemical ischemic pathways. This period simulates the ischemic process in humans in which neither oxygen nor glucose arrives to the brain. 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).

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 and (3) after 180 min of 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 within 7 days of freezing.
Analytical techniques

Lipid peroxidation
To quantify lipid peroxidation we measured thiobarbituric acid reactive substances (TBARS) under basal conditions [15]. Cell membrane-enriched fractions of the tissue samples were obtained as described by Bossman and Hemsworth [16]. Absorbance was determined spectrophotometrically at 532 nm (Perkin Elmer C-532001 spectrophotometer, USA). Blank samples were prepared in an identical manner except that they were incubated at 4°C to avoid TBARS production. The results were expressed as micromoles TBARS per milligram protein; the latter was determined by the method of Bradford [17].

Glutathione peroxidase. GSHpx activity was measured according to the method of Flohé and Gunzler [19]. Briefly, a volume equivalent to 25 µg protein was taken from each supernatant and 0.1 M phosphate-buffered saline was added to a volume of 880 µL, together with 53 µL glutathione reductase, 133 µL GSH, 100 µL nicotinamide adenine dinucleotide phosphate (NADPH) and 100 µL terbutylhydroperoxide. The preparation was shaken by inversion and incubated as described above for GSHpx. Then 6.1 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 transferase. GSHt activity was determined according to the method of Warholm et al. [20]. One hundred microliters of GSH was added to the volumes of sample and buffer as indicated above.

Lactate dehydrogenase (LDH) assay
Tissue damage was measured by examining the LDH efflux to the incubation solution [21]. Samples of this solution were obtained every 30 min and the enzyme activity was measured spectrophotometrically at 340 nm by following oxidation of NADH (decrease in absorbance) in the presence of pyruvate using an ELX-800 microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA).

Nitrite + nitrate concentration
As an indirect indicator of overall NO production, we determined nitrite + nitrate levels in the incubating buffer. One milliliter of buffer was filtered through Ultrafree MC microcentrifuge filters to remove high molecular weight substances released by cell lysis. The nitrite + nitrate level was measured with a commercial kit based on the Griess reaction, after the nitrates were converted to nitrates with nitrate reductase. Levels of nitrite/nitrate were determined spectrophotometrically at 540 nm and compared with a standard curve obtained with sodium nitrite.

Nitric oxide synthase activity
Brain tissue was snap-frozen in liquid nitrogen until the assay for NOS activity. Samples were homogenized (1:5 wt/vol) in buffer containing 10 mM HEPES, 320 mM sucrose, 1 mM EDTA, 1 mM dithiothreitol (DTT), 10 µg/mL leupeptin and 2 µg/mL aprotinin 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. First we determined protein concentration with the method of Bradford [17]. Enzymatic reactions were tested at room temperature for 30 min with a mixture of 40 µL of supernatant and 100 µL 40 mM potassium phosphate buffer (pH 7.0) consisting of 4.8 mM DL-valine, 1 mM NADPH, 1 mM MgCl₂, 2 mM CaCl₂, 20 µM L-arginine and 1.25 µL/mL L-[³H]arginine (59 Ci/mol, Amersham Life Science Inc.). For each assay three parallel samples were run: (A) a sample prepared as described above, (B) a sample that included 1 mM N⁵-methyl-L-arginine (nonspecific activity) and (C) a sample without calcium salts but with 1 mM EDTA and 1 mM EGTA. In all cases
the reaction was stopped by adding cold buffer that consisted of 0.2 mM EDTA. The samples were assayed with 50W-XA Dowex resin columns (Na+ form). One hundred microliters of supernatant was added to 5 mL scintillation fluid, and counts per minute (cpm) were recorded with a β counter. Net NOS activity was calculated as the difference between sample A counts and sample B counts (calcium-dependent activity, equivalent to constitutive NOS and cNOS), or the difference between sample A counts and sample C counts (calcium-independent activity, equivalent to inducible NOS, iNOS). The results were expressed as the production of [3H]citrulline per minute and per milligram of protein.

**Quantification of prostaglandin E₂**

Brain tissues were snap-frozen in liquid nitrogen and stored until analysis. The samples were homogenized (1:10 wt/vol) in 15% methanol with 0.1 N phosphate-buffered saline (pH 7.5), then centrifuged at 37,000 g for 15 min at 4°C. The supernatant was run through a C18 column (Bio-Rad Laboratories, Hercules, CA, USA) that had been activated with absolute methanol, followed by washing with distilled water. After the sample had been assayed, the column was washed with 15% methanol in distilled water followed by petroleum ether. Prostaglandins were eluted with methanoforine. The samples were then dried at room temperature under a nitrogen current and reconstituted with phosphate-buffered saline. The concentration of PGE₂ was measured with a commercial enzyme immunoassay (Amersham International plc).

**Statistical methods**

The data in the text, tables and figures are expressed as the mean ± standard error of 15 experiments. All statistical analyses were done with the Statistical Program for Social Sciences v 12.0 (SPSS Co., Chicago, IL, USA). One-way analysis of variance (ANOVA) followed by Bonferroni transformation was used, and differences were considered significant when p < 0.05.

**Results**

**Effect of experimental diabetes in oxygenated brain tissue**

In slices of oxygenated brain tissue the production of TBARS was greater in 2 months diabetic animals (Table 1). The content of reduced glutathione in brains from diabetic rats was not statistically different. However, after 3 months of diabetes the proportion of GSSG was 94.5% higher in the brain of diabetic animals than in normoglycemic animals. Enzyme activities responsible for the maintenance and functioning of the glutathione system showed no statistically significant alterations in diabetic animals except a lower GSHpx activity in rats.

Table 1. Mean values (mean ± SEM) of the parameters measured after 30 min of oxygenation in normoglycemic and diabetic rats (N = 15 rats per group)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Animals</th>
<th>1 month</th>
<th>2 months</th>
<th>3 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>Normoglycemic</td>
<td>237 ± 8.93</td>
<td>318 ± 6.31</td>
<td>370 ± 8.29</td>
</tr>
<tr>
<td></td>
<td>Diabetic</td>
<td>229 ± 14.29</td>
<td>256 ± 15.80*</td>
<td>305 ± 11.92*</td>
</tr>
<tr>
<td>Blood glucose (mg/dL)</td>
<td>Normoglycemic</td>
<td>106 ± 7.01</td>
<td>106 ± 6.13</td>
<td>102 ± 7.51</td>
</tr>
<tr>
<td></td>
<td>Diabetic</td>
<td>382 ± 12.06*</td>
<td>368 ± 10.94*</td>
<td>335 ± 9.54*</td>
</tr>
<tr>
<td>TBARS (nmol/mg protein)</td>
<td>Normoglycemic</td>
<td>0.56 ± 0.06</td>
<td>0.46 ± 0.03</td>
<td>0.64 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>Diabetic</td>
<td>0.69 ± 0.05</td>
<td>0.81 ± 0.08*</td>
<td>0.63 ± 0.06</td>
</tr>
<tr>
<td>GSH (µmol/g tissue)</td>
<td>Normoglycemic</td>
<td>3.89 ± 0.46</td>
<td>2.12 ± 0.13</td>
<td>2.73 ± 0.19</td>
</tr>
<tr>
<td></td>
<td>Diabetic</td>
<td>3.51 ± 0.29</td>
<td>2.02 ± 0.15</td>
<td>2.40 ± 0.37</td>
</tr>
<tr>
<td>% GSSG compared to GSH + GSSG</td>
<td>Normoglycemic</td>
<td>10.49 ± 1.04</td>
<td>7.33 ± 0.62</td>
<td>7.27 ± 0.69</td>
</tr>
<tr>
<td></td>
<td>Diabetic</td>
<td>10.07 ± 0.68</td>
<td>7.26 ± 0.58</td>
<td>14.14 ± 1.39*</td>
</tr>
<tr>
<td>GSHpx (µmol/min)</td>
<td>Normoglycemic</td>
<td>64.67 ± 3.63</td>
<td>58.45 ± 1.61</td>
<td>63.75 ± 0.95</td>
</tr>
<tr>
<td></td>
<td>Diabetic</td>
<td>62.00 ± 6.52</td>
<td>55.21 ± 2.14</td>
<td>53.40 ± 1.48*</td>
</tr>
<tr>
<td>GSSGrd (µmol/min)</td>
<td>Normoglycemic</td>
<td>15.44 ± 0.90</td>
<td>13.36 ± 1.30</td>
<td>8.25 ± 0.25</td>
</tr>
<tr>
<td></td>
<td>Diabetic</td>
<td>13.43 ± 0.92</td>
<td>10.00 ± 0.46</td>
<td>9.40 ± 0.76</td>
</tr>
<tr>
<td>GSHft (µmol/min)</td>
<td>Normoglycemic</td>
<td>25.67 ± 0.98</td>
<td>23.18 ± 1.36</td>
<td>17.50 ± 1.77</td>
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<tr>
<td></td>
<td>Diabetic</td>
<td>17.60 ± 0.69*</td>
<td>23.64 ± 1.11</td>
<td>13.00 ± 0.87</td>
</tr>
<tr>
<td>PGE₂ (pg/mg tissue)</td>
<td>Normoglycemic</td>
<td>7.26 ± 0.73</td>
<td>8.50 ± 0.92</td>
<td>14.73 ± 0.71</td>
</tr>
<tr>
<td></td>
<td>Diabetic</td>
<td>11.70 ± 0.81*</td>
<td>14.73 ± 0.17*</td>
<td>14.45 ± 1.22</td>
</tr>
<tr>
<td>NO₂⁻ + NO₃⁻ (mmol/mg protein)</td>
<td>Normoglycemic</td>
<td>1.49 ± 0.16</td>
<td>1.70 ± 0.18</td>
<td>1.08 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>Diabetic</td>
<td>1.35 ± 0.19</td>
<td>1.63 ± 0.18</td>
<td>1.02 ± 0.10</td>
</tr>
<tr>
<td>cNOS (pmol/mg/min)</td>
<td>Normoglycemic</td>
<td>165 ± 15</td>
<td>141 ± 7.88</td>
<td>130 ± 31</td>
</tr>
<tr>
<td></td>
<td>Diabetic</td>
<td>110 ± 15*</td>
<td>149 ± 24</td>
<td>120 ± 26</td>
</tr>
<tr>
<td>iNOS (pmol/mg/min)</td>
<td>Normoglycemic</td>
<td>154 ± 13</td>
<td>135 ± 17</td>
<td>128 ± 18</td>
</tr>
<tr>
<td></td>
<td>Diabetic</td>
<td>137 ± 21</td>
<td>135 ± 11</td>
<td>139 ± 12*</td>
</tr>
<tr>
<td>LDH (U/min/mg tissue)</td>
<td>Normoglycemic</td>
<td>1.226 ± 0.136</td>
<td>1.359 ± 0.087</td>
<td>1.262 ± 0.090</td>
</tr>
<tr>
<td></td>
<td>Diabetic</td>
<td>1.522 ± 0.142</td>
<td>1.429 ± 0.066</td>
<td>1.423 ± 0.158</td>
</tr>
</tbody>
</table>

TBARS, thiobarbituric acid reactive substances; GSH, reduced glutathione; GSSG, oxidized glutathione; GSHpx, glutathione peroxidase activity; GSSGrd, glutathione reductase activity; GSHft, glutathione transferase activity; PGE₂, prostaglandin E₂; cNOS, constitutive nitric oxide synthase; iNOS, inducible nitric oxide synthase; LDH, lactate dehydrogenase activity.

*p < 0.05 in comparison to normoglycemic group.
with 3 months of diabetes and GSHtf in rats with 1 month of diabetes.

Brain concentrations of PGE₂ were higher in diabetic rats after 1 month (61.15%) and after 2 months (73.29%), but after 3 months the difference between diabetic and normoglycemic animals was not statistically significant.

Total nitrite and nitrate levels did not differ significantly between the two groups. After 1 month of diabetes, cNOS activity was reduced by 33% in diabetic animals, and after 3 months, iNOS activity was 28.70% higher.

**Effect of the experimental model of hypoxia reoxygenation**

After 20 min hypoxia, changes in all of the parameters were not statistically different in comparison with prehypoxic values.

After 180 min of posthypoxia reoxygenation, LDH activity was 40, 40.67 and 68.90% higher in animals with diabetes of 1, 2 and 3 months duration, respectively, in comparison to nondiabetic animals (Figure 1). When we measured oxidative stress, we found that TBARS content increased after reoxygenation significantly more in diabetic than in normoglycemic animals (25.48, 93.75 and 92.81% after 1, 2 and 3 months of diabetes, respectively, Figure 2). The results for glutathione levels showed that GSH and GSSG concentrations decreased after reoxygenation, and that the decreases were greater in diabetic animals (Figure 3). These decreases were accompanied by a decrease in the activity of GSHpx and GSSGrd in comparison to normoglycemic rats (Figure 4).

After reoxygenation, PGE₂ accumulated in the brain slices, and the increase was significantly greater in diabetic animals (62.50, 85.51 and 114% greater after 1, 2 and 3 months of diabetes, respectively, in comparison to normoglycemic rats) (Figure 5).

The findings for the NO pathway showed an overall increase in nitrite + nitrate production after reoxygenation, with larger increases in animals with diabetes of 2 (40.21%) or 3 months duration (78.05%). cNOS activity was significantly reduced in all groups, although the largest reductions in comparison to nondiabetic animals were seen in animals with diabetes of 2 (16.71%) and 3 months duration (22.35%). iNOS activity was significantly increased in all groups, with the greatest increases in rats with diabetes of 2 (18.64%) and 3 months duration (27.14%) in comparison to nondiabetic animals (Figure 6).

**Discussion**

The biochemical changes that take place in the model of brain hypoxia reoxygenation used in the present study were described previously in nondiabetic animals [14,22,23] and are consistent with the observations reported here. This study therefore shows that the same biochemical pathways are altered in diabetic animals.

The oxidative stress data are compatible with other studies in streptozotocin-diabetic animals [24,25] and in humans with type 1 [26] and type 2 diabetes mellitus [4]. These studies reflect the increase in free radicals during diabetes because of the accumulation of glycation endproducts or activation of the sorbitol metabolic pathway. There were, however, no substantial changes in glutathione levels [27]. In humans with type 2 diabetes there were no significant changes in plasma or erythrocyte concentrations of glutathione [4], or in GSHpx activity, which was found to be reduced only in animals with prolonged diabetes [28].

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**Figure 1.** Lactate dehydrogenase efflux (LDH) in rat brain slices after 180 min of reoxygenation. The percentages of change with respect to prehypoxic values are shown below X axis. *p < 0.05, **p < 0.01 in comparison to the control nondiabetic group (each bar represents the mean ± SEM of 15 rats)
Figure 2. Thiobarbituric acid reactive substances (TBARS) in rat brain slices after 180 min of reoxygenation. The percentages of change with respect to prehypoxic values are shown below X axis. *p < 0.05, **p < 0.001 in comparison to the control nondiabetic group (each bar represents the mean ± SEM of 15 rats).

Figure 3. Reduced glutathione (GSH) and percentage of oxidized glutathione (GSSG) in comparison to total glutathione (GSH + GSSG) in rat brain slices after 180 min of reoxygenation. The percentages of change with respect to prehypoxic values are shown below X axis. *p < 0.05, **p < 0.001 in comparison to the control nondiabetic group (each bar represents the mean ± SEM of 15 rats).
Our results also show a higher concentration of prostaglandins in the brain of diabetic animals. With respect to the eicosanoid synthesis in diabetes, others have reported an imbalance in several tissues, particularly in prostanoids related with the cardiovascular system. The changes reported till date include decreased prostacyclin...
production in the endothelium [29,30] and in leukocytes [11], and increased platelet thromboxane synthesis [29–31].

The results for the NO pathway show that cNOS activity is reduced in the earlier phases of experimental diabetes, whereas iNOS activity is increased in more advanced phases. The buildup of NO in diabetes and the increase in cerebral ischemia have been explained as consequences of iNOS hyperactivity, because the enzyme is potently activated by the release of inflammatory mediators such as interleukin 1β and tumor necrosis factor alpha (TNFα), among others. This appears to occur in experimental diabetes when the disease is in more advanced stages [32,33].

Some parameters such as TBARS, GSHtf and cNOS activities and PGE2 concentration, showed modifications at 1 and/or 2 months evolution of diabetes but there are no differences at 3 months evolution; this could probably be due to a high biochemical brain damage in diabetic animals in which an active enzymatic activity should be affected more than in previous periods of evolution of diabetes.

When we analyzed the degree of cerebral involvement in the hypoxia reoxygenation model, the rates of cell death were higher after 180 min of reoxygenation in diabetic animals. In this connection, we have found earlier reports that neurones and brain tissues are more susceptible to ischemic damage in diabetes than in normoglycemic. Tekkök et al. [34] showed that in slices of hippocampus from the brain of streptozotocin-diabetic rats, synaptic transmission was slower when the animals were subjected to glucose deficiency than in the equivalent synapses of nondiabetic animals. Studies that used in vivo models of ischemia have mostly compared sensitivity in diabetic rat brains after transitory ischemia caused by ligating the common carotid artery [35] or the middle cerebral artery [36], or after permanent ischemia [37]. All studies concluded that neuronal damage was greater in diabetic animals, with higher grades of neuronal death and increases in the parameters that define and indicate the degree of neuronal apoptosis [36]. Our findings with regard to cell death are consistent with earlier results, and illustrate the changes in the main mechanisms of cell damage resulting from hypoxia reoxygenation. According to the literature, in this model, neurones are the main cell type affected after reoxygenation [38], and for that reason we suppose this is the most affected type in diabetic brain slices.

The parameters of oxidative stress in brain slices showed greater imbalances in diabetic than in normoglycemic animals after 180 min of reoxygenation. This stress increased TBARS production and decreased glutathione content in the brain to a greater degree in diabetic than in normoglycemic animals. In the former, the potential antioxidant defense mechanisms were less effective, as shown by the greater decrease in GSHpx activity. In this connection, Santos et al. [39] showed that brain and liver mitochondria from diabetic rats were more sensitive to the accumulation of lipid peroxides in response to oxidative attack, and that these changes were accompanied by a decrease in antioxidant substances such as vitamin E and coenzyme Q. Other antioxidant enzymes are also affected in diabetes, such as catalase or superoxide dismutase [4]. We measured the glutathione system because it is a more stable antioxidant mechanism than others and its antioxidant role has been demonstrated in several diseases.

Another pathway of cell damage – the accumulation of prostaglandins – is also exacerbated in diabetic animals. The determination of this prostaglandin was due to its relationship with oxidative stress, because in
Figure 6. Nitrites + nitrates (NO$_2^-$ + NO$_3^-$), constitutive (cNOS) and inducible (iNOS) nitric oxide synthase activities in rat brain slices after 180 min of reoxygenation. The percentages of change with respect to prehypoxic values are shown below X axis. *$p < 0.05$, **$p < 0.0001$ in comparison to the control nondiabetic group (each bar represents the mean ± SEM of 15 rats).

The prostaglandin synthesis, free radicals are produced and these radicals upregulate the prostaglandin synthesis. In a model of experimental diabetes, the results showed a significant decrease in prostacyclin synthesis along with a tendency toward increased thromboxane A$_2$ and PGE$_2$ in rat brain tissue [40]. In other words,
prostaglandins related with vasoconstriction and cytotoxicity predominated over those related with vasodilatation [11,29,30]. Moreover the increase in brain prostaglandin synthesis demonstrated in our study could support a proinflammatory status in rat diabetic brain before and after the hypoxia reoxygenation model.

NO production in brain tissue after reoxygenation is greater in diabetic animals than in nondiabetic animals. Both brain ischemia and diabetes activate inducible pathway enzymes such as iNOS, possibly because of the release of inflammatory mediators such as interleukin 1β, TNFα and others. In this connection, the brain of diabetic animals shows clear evidence of oxidative stress and increased NO, which give rise to ideal conditions for the production of potentially cytotoxic peroxynitrites and NO-derived radicals (nitrative stress) [32,33]. In this connection we could suppose that the increase in nitrite + nitrates may be due mainly to the great iNOS activity in diabetic animals.

We conclude that the increase in tissue oxidative stress, the greater production of prostaglandins and stimulation of the inducible pathway of NO production set the stage for increased cell damage in the brain of type 1-like diabetic animals, and account for the worse cell damage seen after hypoxia reoxygenation in comparison to nondiabetic animals. This conclusion is supported on the biochemical pathways analyzed in the present study and on the results obtained previously with drugs that inhibit these pathways, such as antioxidants [14], anti-inflammatory drugs [22] or inhibitors of iNOS [41]. Our findings thus strongly suggest that the greater incidence of ischemic stroke in the population of people with type 1 diabetes mellitus is not probably due to the result of higher incidence of arteriosclerosis and thrombosis (more frequently in type 2 diabetes mellitus). The high incidence of stroke in people with diabetes could be due to the increased risk factors for stroke, but according to our results, they could also reflect a possible greater susceptibility of the brain to ischemic injury. However, it is necessary to demonstrate this hypothesis in clinical trials.

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