The association effect of insulin and clonazepam on oxidative stress in liver of an experimental animal model of diabetes and depression

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Abstract

Context: It is known that oxidative stress occurs in peripheral blood in an experimental animal model of diabetes and depression, and acute treatment with insulin and clonazepam (CNZ) has a protective effect on oxidative stress in this model.

Objective: This study evaluated the effect of insulin plus CNZ on oxidative stress parameters in the liver of diabetic male rats induced with streptozotocin (STZ) and subjected to forced swimming test (FST).

Materials and methods: Diabetes was induced by a single intraperitoneal (i.p.) dose of STZ 60 mg/kg in male Wistar rats. Insulin (4 IU/kg) plus CNZ acute i.p. treatment (0.25 mg/kg) was administered 24, 5 and 1 h before the FST. Nondiabetic control rats received i.p. injections of saline (1 mL/kg). Protein oxidative damage was evaluated by carbonyl formation and the antioxidant redox parameters were analyzed by the measurements of enzymatic activities of the superoxide dismutase (SOD), catalase and glyoxalase I (GLO). Glycemia levels also were determined.

Results: Our present study has shown an increase in carbonyl content from diabetic rats subjected to FST (2.04 ± 0.55), while the activity of catalase (51.83 ± 19.02) and SOD (2.30 ± 1.23) were significantly decreased in liver from these animals, which were reverted by the treatment. Also, the activity of GLO (0.15 ± 0.02) in the liver of the animals was decreased.

Discussion and conclusion: Our findings showed that insulin plus CNZ acute treatment ameliorate the antioxidant redox parameters and protect against protein oxidative damage in the liver of diabetic rats subjected to FST.

Introduction

Diabetes mellitus (DM) is one of the most common endocrine metabolic disorders with increasing incidence and clinical relevance, contributing to high morbidity and mortality rates (Zimet et al., 2001). This disease is characterized by hyperglycemia resulting from defects on insulin secretion, insulin action or both (Robles & Sigh-Franc, 2009). The vast majority of diabetic patients develop serious chronic complications over time in target organspecific, such as eyes, kidneys, nerves, heart, liver and blood vessels (Baynes, 2003).

A large body of evidence suggests oxidative stress, an imbalance of oxidants/antioxidants in favor of oxidants, as a mechanism underlying insulin resistance, type 1 diabetes and diabetic complications (Ceriello et al., 2000). Hyperglycemia causes oxidative stress due to increased mitochondrial production of superoxide anion (Brownlee, 2001), nonglycemic glycation of proteins (Baynes, 2003) and glucose autoxidation (Bonnefont-Rousselot et al., 2004).

There is a well-recognized association between depression and diabetes; the prevalence of depression in diabetic patients is higher than in general population (Bouwman et al., 2010). Diabetics and depressive patients, compared to those with only diabetes, have been linked with poor self-care and adherence to medical treatment (Gonzalez et al., 2008), poorer glycemic control (Lustman et al., 2000), and more diabetes complications. Moreover, depression in diabetes patients is associated with a higher risk of morbidity (Katon et al., 2007). In this context, it is important to emphasize that clonazepam (CNZ), a positive GABA receptor modulator that was first used for seizure disorders, is now increasingly used to treat affective disorders, such as depression (Morishita, 2009).

Keywords

Carbonyl, catalase, free radicals, glyoxalase I, superoxide dismutase
Previous animal studies have demonstrated these depression-like behavioral changes, since the duration of immobility time in the forced swimming test (FST) is longer in diabetic animals when compared to nondiabetic animals (Gomez & Barros, 2000). Insulin (Hilakivi-Clarke et al., 1990) and CNZ treatment (da Silva Haeser et al., 2007) reversed the prolonged immobility in the FST of diabetic rats. Also, it was verified that the association of insulin plus CNZ in acute treatment was able to partially reverse this effect (Wayhs et al., 2010a). Furthermore, insulin, in association with CNZ, was capable of protecting against protein and lipid oxidative damage in plasma (Wayhs et al., 2010a), and against DNA oxidative damage in whole blood of streptozotocin (STZ)-induced diabetic rats subjected to FST (Wayhs et al., 2010b).

Diabetes was the first disease in which evidence emerged for increased formation of methylglyoxal (MG). Metabolism of MG by the glyoxalase system has been linked to the development of vascular complications of diabetes – nephropathy, retinopathy, neuropathy and cardiovascular disease. Increased formation of MG in hyperglycemia associated with diabetes and down-regulation of glyoxalase I (GLO) by inflammatory signalling in vascular cells leads to a marked increased modification of proteins by MG to form advanced glycation endproducts (AGEs) at the sites of vascular complications (Rabbani & Thornalley, 2011).

Liver is the main organ of oxidative and detoxification processes, as well as free radical reactions. It is a crucially important organ and, in a chronic hyperglycemic state, the oxidative stress of the liver is considered a relevant process. Studies have demonstrated alterations in the activity of antioxidant enzymes associated with liver oxidative injury (Ren et al., 2008). Besides, in many diseases, biomarkers of oxidative stress are elevated in the liver at an early stage (Di Naso et al., 2011). Since liver is subjected to ROS-mediated injury in diabetes, the present work aimed to investigate the effect of insulin plus CNZ on oxidative stress parameters in the liver of diabetic male rats induced with STZ and subjected to FST. Therefore, oxidative stress parameters such as carbonyl content, as well as the activities of the antioxidants enzymes catalase, superoxide dismutase (SOD) and GLO were determined.

Materials and methods

Animals

Male Wistar rats (250 ± 50 g) were obtained from the Animal House of Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA). The animals were housed in groups of four per polypropylene cage. Food and water were available ad libitum, except when otherwise stated, and the animals were maintained in a temperature-controlled room (22 ± 2°C) under a light–dark cycle (7:00 a.m. to 7:00 p.m.). The animals were divided into three groups: controls, diabetics (STZ), diabetics treated with insulin plus CNZ (STZ-INS + CNZ). All groups were subjected to FST plus STZ, except the control group that was not subjected to STZ. All in vivo experiments followed the guidelines of the International Council for Laboratory Animal Science (ICLAS) and were approved by the Ethical Committee for Animal Experimentation of UFCSPA (08404). All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

Reagents

All chemicals were of PA purity and purchased from Sigma-Aldrich (St. Louis, MO).

Drugs

Insulin (4 IU/mL; Humulin®, Lilly, Indianapolis, IN) was prepared in saline, CNZ (0.25 mg/mL; Rivotril®, Roche, São Paulo, Brazil) was prepared in saline with Tween 0.05% (v.v) and STZ (Sigma, St. Louis, MO) – 60 mg/mL was prepared in citrate buffer (pH 4.3). All solutions were prepared immediately before intraperitoneal (i.p.) administration.

Diabetes induction

Diabetes was induced by a single i.p. dose of STZ 60 mg/kg as already described (Gomez & Barros, 2000). Increased blood glucose levels (≥250 mg/dL) of the STZ group rats were confirmed with a glucometer (Accu Chek Advantage®, Roche, Grenzach-Wyhlen, Germany) after 72 h to confirm the hyperglycemic status. Nondiabetic control rats received i.p. injections of saline (1 mL/kg) and were also subjected to blood glucose measurement.

Forced swimming test

After 21 days of diabetes induction, animals were subjected to the FST (Figure 1). On the first day of the experiment (train session), 24 h before the FST, the animals were placed in the aquarium for 15 min (22 cm × 22 cm × 35 cm) with water of height 27 cm (temperature, 24–26°C). Soon after, the rats were dried with towels and the first drug dose was administered. Five and one hour before being subjected to the FST, the animals were again dosed with the indicated treatment. The FST session was recorded on videotape for subsequent analysis, by an observer blind to the drug treatment group (Porsolt et al., 1977).

Insulin and CNZ administration

All treatments were administered intraperitoneally. Insulin (4 IU/kg), CNZ (0.25 mg/kg) or saline (1 mL/kg) were administered according the indicated group, 24, 5 and 1 h before the FST.

Liver sample collection

Thirty minutes after the FST, animals were sacrificed by decapitation and liver samples were removed, dried (air-dried incubator, 45°C, 24 h), weighted, homogenized by buffer techniques and stored at −80°C until determinations. Excess of blood was removed from the liver of rats in order to reduce interference in the determination of oxidative stress parameters.

Carbonyl content

Protein carbonyl formation was quantified as an index of protein oxidative damage (Levine et al., 1990). This method is
based on the reaction of dinitrophenylhydrazine with protein carbonyl groups. The results were expressed as nmol carbonyl/mg protein.

**Catalase assay**

Catalase activity was assayed as previously described (Aebi, 1984) by measuring the absorbance decrease at 240 nm in a reaction medium containing 20 mM H₂O₂, 0.1% Triton X-100, 10 mM potassium phosphate buffer, pH 7.0, and 50 μg protein. One unit (U) of the enzyme is defined as 1 μmol of H₂O₂ consumed per minute and the specific activity was reported as U/mg protein.

**Superoxide dismutase**

SOD activity was quantified by the inhibition of superoxide-dependent adrenaline auto-oxidation to adrenochrome using spectrophotometric measurements at 480 nm, as previously described (Misra & Fridovich, 1972). The results were expressed as absorbance/time (s). The area under the curve of the graph was used for statistical analysis and compared against the control values. Five units of CuZnSOD were used to determine assay specificity.

**Glyoxalase I**

GLO activity was measured by the rate of formation of S-D-lactoylglutathione at 240 nm. The assay was carried out in 96-well microplates using a microplate spectrophotometer (Spectra Max 190, Molecular Devices, Sunnyvale, CA). Briefly, 10 μL of 1 mM glutathione (GSH) and 2 mM MG, pre-incubated for 30 min at room temperature, in 50 mM sodium phosphate buffer (pH 7.0) were added to each vial containing 190 μL samples (10 μg protein). The enzyme activity was calculated utilizing the molar extinction coefficient of 3300 M⁻¹ cm⁻¹ and expressed as units/mg protein, one unit being the amount of enzyme needed to produce 1 mmol/min of S-D-lactoylglutathione at 25°C (Mannervik et al., 1981).

**Protein quantification**

Protein content was measured as previously described (Lowry et al., 1951). All the results were standardized by protein content.

**Statistical analyses**

Data were expressed as mean ± standard deviation and were analyzed by one-way analysis of variance (ANOVA) followed by the Duncan multiple range test when F value was significant. A p value of less than 0.05 was considered to be significant. The Pearson correlation test was used to evaluate the correlations between the variables. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software (Chicago, IL) in a PC-compatible computer.

**Results**

Glycemia of the animals from different groups studied after FST and before decapitation is showed in Figure 2. It can be observed that insulin (INS) plus CNZ in acute treatment significantly decreased glycemia when compared to non-treated STZ rats \[F(2,28) = 17.319, p < 0.05\].

Figure 3 shows carbonyl content in liver from STZ-induced diabetic rats subjected to FST treated or untreated with INS plus CNZ. The level of protein oxidation expressed as carbonyl content indicates the extent of oxidative damage to proteins in liver and this value was significantly higher in the STZ group when compared to STZ-treated and control groups \[F(2,34) = 5.972, p < 0.05\].

The activity of the antioxidant enzymes catalase assay (CAT) and SOD are presented in Figures 4 and 5, respectively. It can be verified that CAT and SOD in liver were significantly decreased in the STZ group when compared to the control group. The INS plus CNZ in acute treatment reverted CAT and SOD to controls \[F(2,34) = 8.495, p < 0.05\] and \[F(2,34) = 21.329, p < 0.05\], respectively. Furthermore, a significant negative correlation between glycemia and CAT \(r = -0.4386\).
and between glycemia and SOD ($r = -0.4665$, $p < 0.05$) were observed in treated and untreated STZ-rats submitted to the FST.

The activity of the antioxidant enzyme GLO is presented in Figure 6. It was verified that STZ and STZ – INS + CNZ groups presented lower levels of the enzyme GLO when compared to the control group [$F_{(2,34)} = 80.687$, $p < 0.05$].

**Discussion**

Experimental animal models of diabetes and depression-like behavior, such as STZ-induced diabetic rats and FST, respectively, can be useful for the study of the underlying pathophysiology of diabetes-related depression and its complications. Considering that there is oxidative stress in peripheral blood of STZ-induced diabetic rats subjected to FST, and that acute treatment with insulin and CNZ has a protective effect on oxidative stress in this model...
CAT activities.

The role of oxidative protein damage in the pathogenesis of the diabetic state is being investigated extensively (Telci et al., 2000). Considerable evidence indicates that the maintenance of protein redox status is of fundamental importance for cell function, whereas structural changes in proteins are considered to be among the molecular mechanisms leading to diabetic complications (Altomare et al., 1997). In this study, protein oxidation expressed as carbonyl content was evaluated in the liver of the animals. A significantly higher carbonyl content in the STZ group was verified when compared to control rats. Treatment with INS plus CNZ significantly reduced the carbonyl content to similar levels of controls. Nonenzymatic modification of proteins in hyperglycemia is a major mechanism causing diabetic complications. These modifications can have pathogenic consequences when they target active site residues, thus affecting protein function (Cakatay, 2005; Pan et al., 2008). Accordingly, our results suggest that protein oxidative damage occurs in the liver of STZ diabetic rats subjected to FST, and that INS plus CNZ has a protecting action upon these processes.

Catalase is a major primary antioxidant defense component that catalyzes the decomposition of H$_2$O$_2$ to water and oxygen, sharing this function with GSH peroxidase (GPx). Therefore, both of these enzymes detoxify H$_2$O$_2$, which is derived from SOD activity, the enzyme that combats the damaging effect of superoxide radicals. There is no consensus about changes in the activities of antioxidant enzymes of different organs in diabetic rats. Although some studies measuring the activities of SOD and catalase in DM showed reductions in the levels of these enzymes (Coskun et al., 2005), other researchers reported increased activities in STZ-induced diabetic rats (Yılmaz et al., 2004). These apparently contradictory results could be due to tissue specificity, variation in severity and duration of the disease or other experimental conditions (Essani et al., 1996) like the age of the animals (Sadi & Guray, 2009).

In the present study, it was verified that the activity of CAT and SOD in liver of rats were significantly decreased in the STZ group when compared to the control group, and INS plus CNZ in acute treatment reverted CAT and SOD levels to controls. Moreover, a significant negative correlation between glycemia and CAT, as well as between glycemia and SOD, were observed in treated and untreated STZ-rats submitted to the FST. This decrease in the enzyme activity of CAT and SOD may thus be a response to the increase of reactive species in the liver tissue, and one of the reasons for this may be that reduced SOD activity decreases H$_2$O$_2$ production, leading to a reduction in CAT activity. Furthermore, Kono and Fridovich (1982) demonstrated that a decrease in SOD activity might lead to build up high concentration of superoxide radicals which might inhibit CAT activities.

GLO plays an important role in the detoxification of oxoaldehydes, mainly glyoxal and MG (Lorenzi et al., 2010). This enzyme catalyzes the formation of S-D-lactoylglutathione from MG and GSH, leading to reduced concentrations of its substrate. Moreover, decreased GLO activity can be implicated in decreased clearance of MG which can accumulate and lead to the formation of AGEs, which have been associated to diabetic complications, including diabetic nephropathy (Matsumoto et al., 2010) and retinopathy (Wu et al., 2011). In this study it was verified that diabetic rats presented lower liver levels of the enzyme GLO when compared to the control group. Our results are in agreement with the literature, since GLO plays a role in high glucose-mediated signaling by reducing MG accumulation and oxidative stress in DM (Kim et al., 2011). It is important to emphasize that there is a tendency to increase GLO in the STZ—INS + CNZ group when compared to the STZ-group, suggesting that insulin + CNZ treatment could increase the GLO levels, decreasing AGEs formation.

Our findings showed that protein is damaged and antioxidant status is decreased in the liver of this experimental animal model of diabetes and depression, and that INS + CNZ in acute treatment was able to revert or partially revert these alterations. The effect of insulin treatment can be explained by controlling the glycemia, avoiding the increase of free radicals and the reduction of the antioxidant potential (Wiersperger, 2003). Moreover, insulin may improve the total antioxidant capacity (Kocic et al., 2007), acting as an antioxidant scavenger, as well as CNZ, which also exerts an antioxidant effect in STZ-induced diabetic rats subjected to FST (da Silva Haeser et al., 2007; Gomez & Barros, 2000; Wayhs et al., 2010a,b). These findings support antioxidant therapy as important in the treatment of diabetes and its complications.

Declaration of interest

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The authors report no declarations of interest.

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