Oxidative Stress Affects Synaptosomal γ-Aminobutyric Acid and Glutamate Transport in Diabetic Rats

The Role of Insulin

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Evidence suggests that oxidative stress is involved in the pathophysiology of diabetic complications and that insulin has a neuroprotective role in oxidative stress conditions. In this study, we evaluated the in vitro effect of insulin in the susceptibility to oxidative stress and in the transport of the amino acid neurotransmitters γ-aminobutyric acid (GABA) and glutamate in a synaptosomal fraction isolated from male type 2 diabetic Goto-Kakizaki (GK) rat brain cortex. The ascorbate/Fe^{2+}-induced increase in thiobarbituric acid reactive substances (TBARSs) was similar in Wistar and GK rats and was not reverted by insulin (1 μmol/l), suggesting that other mechanisms, rather than a direct effect in membrane lipid peroxidation, may mediate insulin neuroprotection. Diabetes did not affect GABA and glutamate transport, despite the significant decrease in membrane potential and ATP/ADP ratio, and insulin increased the uptake of both GABA and glutamate in GK rats. Upon oxidation, there was a decrease in the uptake of both neurotransmitters and an increase in extrasynaptosomal glutamate levels and in ATP/ADP ratio in GK rats. Insulin treatment reverted the ascorbate/Fe^{2+}-induced decrease in GABA accumulation, with a decrease in extrasynaptosomal GABA. These results suggest that insulin modulates synaptosomal GABA and/or glutamate transport, thus having a neuroprotective role under oxidizing and/or diabetic conditions. *Diabetes* **53:2110–2116, 2004**

In humans and experimentally diabetic rats, oxidative stress–mediated damage seems to be involved in the cause of diabetic complications (1). This hypothesis is based on reports of increased generation of reactive oxygen species (ROS), decreased antioxidant levels, and/or impaired generation of the reduced forms of the antioxidants (2–4), leading to lipid, protein, and DNA oxidation (5). Brain is one of the most important targets for ROS, because of its high levels of polyunsaturated fatty acids, high oxygen consumption, high content in transition metals (e.g., Fe^{2+}), and poor antioxidant defenses (6).

Several authors have shown that diabetes modifies the levels of free amino acids (7,8) and the accumulation of amino acid neurotransmitters. Vilchis and Salceda (8) reported an increase in taurine and γ-aminobutyric acid (GABA) uptake, whereas glycine and glutamate uptake remained unaffected in diabetic rat retina and retinal pigment epithelium. In a previous study performed in 6-month-old diabetic Goto-Kakizaki (GK) rat synaptosomes, we demonstrated a diabetes-induced decrease in glutamate accumulation, leading to the hypothesis that diabetes modifies glutamate transport and increases neuronal injury occurring under pathological conditions (3).

It has been widely accepted that peripherally synthesized insulin can be transported into the brain via the cerebrospinal fluid. However, recent molecular biological evidence suggests that it can also be synthesized de novo by neurons, because the presence of preproinsulin I and II mRNA or insulin receptor mRNA was observed in cultured neurons. Moreover, insulin immunoreactivity occurs in the endoplasmic reticulum and Golgi apparatus in vivo (9,10). In the central nervous system, insulin seems to play an important role, particularly in the complications caused by diabetes, involving the regulation of brain metabolism (11,12), neuronal growth and differentiation (9,13), or neuromodulation (13,14). Insulin may also protect against brain damage induced by stress conditions, such as oxidative stress or ischemia (12,15). Brain glucose utilization and metabolism are essential to cognitive functions, and a disturbance in both or in the desensitization of brain insulin receptors may be involved in the intellectual decline in Alzheimer's disease and related neurodegenerative disorders (16,17), in which excitotoxicity and oxidative stress have been shown to occur (18).

GK rats are a nonobese animal model of type 2 diabetes, produced by repeated selective breeding of Wistar nondiabetic rats, using glucose intolerance as a selective index (19). In the present study, we evaluated the interactions among abnormal glucose metabolism, preincubation with insulin, and the accumulation of the amino acid neurotransmitters GABA and glutamate in synaptosomes isolated from the cerebral cortex of GK diabetic rats under oxidative stress conditions. The oxidant pair ascorbate/

Fe^{2+} was used to induce oxidative injury, and the extent of lipid peroxidation was followed by measuring the thiobarbituric acid reactive substances (TBARSs) production.

RESEARCH DESIGN AND METHODS

Male spontaneously diabetic GK rats (31.9 ± 0.7 weeks of age) were obtained from our local breeding colony (Animal Research Center Laboratory, University Hospitals, Coimbra, Portugal), established in 1995 with breeding couples from the colony at the Tohoku University School of Medicine (Sendai, Japan; courtesy of Dr. K.I. Susuki). Control animals were nondiabetic male Wistar rats of similar age (28.2 ± 1.4 weeks of age) obtained from the same colony. Animals were kept under controlled light and humidity conditions, with free access to powdered rodent diet (diet C.R.F. 20; Charles River, L'Arbilesle, France) and water. Glucose tolerance tests were used as a selection index.

All chemicals used were of the highest grade of purity commercially available. [^{3}H]GABA (65 Ci/mmol) and L-[G-^{3}H]glutamate (49 Ci/mmol) were from Amersham International (Little Chalfont, U.K.).

Blood glucose level determination. Blood glucose levels were determined immediately after the animals were killed, through the glucose oxidation reaction, using a glucometer (Glucometer Elite; Bayer, Carnaxide, Portugal) and compatible reactive tests.

Preparation of synaptosomes. Crude synaptosomes were prepared from brain of male Wistar and GK rats, according to a preestablished method (20), with some modifications. After animal decapitation, the whole cerebral cortices were rapidly removed and homogenized in 10 vol of homogenization medium (0.32 mol/l sucrose, 10 mmol/l HEPES, and 0.5 mmol/l EGTA-K$^+$, buffered with Tris at pH 7.4). The homogenate was centrifuged at $1,000g$ for 5 min, and the synaptosomal fraction was isolated from the supernatant by centrifugation at $12,000g$ for 10 min and then centrifuged again at $12,000g$ for 10 min in 10 ml of washing medium (0.32 mol/l sucrose, 10 mmol/l HEPES, buffered at pH 7.4 with Tris). The white and fluffy synaptosome layer without contaminant mitochondria (mitochondria free), was then resuspended, respun, and resuspended in the sucrose medium at a protein concentration of 15–20 mg/ml, as determined by the biuret method (21). Experiments were carried out within 3 h after synaptosomal fraction preparation.

Induction of oxidative stress. The oxidizing agents ascorbic acid and iron (Fe^{2+}, ferrous sulfate) were used to induce oxidative stress (22). Synaptosomes (1 mg/ml) were peroxidized by incubation for 15 min at 30°C in a standard medium that contained (in mmol/l) 132 NaCl, 3 KCl, 1 MgCl$_2$, 1 NaH$_2$PO$_4$, 1.2 CaCl$_2$, 10 glucose, and 20 mmol/l HEPES adjusted to pH 7.4 with Tris, supplemented with 0.8 mmol/l ascorbic acid and 2.5 μmol/l Fe^{2+}. Ascorbic acid and Fe^{2+} solutions were prepared immediately before use and protected from light. Similar experiments were carried out in the presence of 1 μmol/l insulin, with synaptosomes preincubated for 15 min before the addition of ascorbate/Fe^{2+}.

Evaluation of lipid peroxidation. The extent of lipid peroxidation was determined by measuring TBARSs, using the thiobarbituric acid assay (23). The amount of TBARSs formed was calculated using a molar extinction coefficient of 1.56×10^5 (mol/l)$^{-1} \times$ cm^{-1} and expressed as nanomoles of TBARSs per milligram of protein.

Determination of [^{3}H]GABA and [^{3}H]glutamate uptake. The uptake of [^{3}H]GABA and [^{3}H]glutamate was determined as previously described (24,25). Synaptosomes (0.5 mg of protein/ml) were equilibrated at 30°C in a Na$^+$ medium that contained (in mmol/l) 125 NaCl, 3 KCl, 1.2 MgSO$_4$, 1.2 CaCl$_2$, 3 glucose, and 10 HEPES-Tris at pH 7.4 in the absence or presence of ascorbic acid (0.8 mmol/l) plus Fe^{2+} (2.5 μmol/l). The same experimental procedure was carried out in the presence or absence of 1 μmol/l insulin, with synaptosomes preincubated for 15 min before the addition of ascorbate/Fe^{2+}. Amino-oxyacetic acid (10 μmol/l) was included in all media when studying the uptake of [^{3}H]GABA to prevent GABA metabolism. After 15 min of incubation, the uptake reaction was started by adding [^{3}H]GABA (final concentration 0.5 μmol/l, 0.25 μCi/ml) or [^{3}H]glutamate (final concentration 10 μmol/l, 49 Ci/mmol). Uptake was stopped at 10 min by rapid filtration of the samples (0.2 ml) under vacuum, through glass fiber filters (Whatman GF/B), followed by a wash with 10 ml of the Na$^+$-containing glucose-free medium (washing medium). The filters were placed in scintillation vials with 3 ml of scintillation UniverSol cocktail (ICN, Irvine, CA), and radioactivity was counted in a liquid scintillation analyzer Packard Tri-Carb 2500 TR. The quenching of radioactivity in the samples was corrected automatically by using an efficiency correlation curve obtained for ^{3}H-quenched standards by the external standardization method. The results were presented as percentage of the control, considering the mean of Wistar rat synaptosomes not treated with ascorbate/Fe^{2+} as 100%.

Fluorimetric determination of extrasynaptosomal levels of amino acids after high-performance liquid chromatography separation. The extrasynaptosomal levels of the amino acids GABA and glutamate were analyzed fluorometrically after o-phthaldialdehyde/2-mercaptoethanol derivatization and high-performance liquid chromatography (HPLC) separation, as described by Sitges et al. (26), with some modifications. The amino acids were separated by reverse-phase Gilson-ASTED HPLC system, composed of a Spherisorb ODS column (particle size, 5 μm; 150-mm long; 4.6 mm i.d.) at 25°C and a Gilson model 121 fluorescence detector set at 340 nm (excitation wavelength) and at 410 nm (emission wavelength). A linear gradient elution program carried out over 30 min was applied for amino acid elution: eluent A (30 mmol/l sodium acetate buffer [pH 6.8]) from 100 to 50%, and eluent B (methanol) from 0 to 50%, with a flow rate of 2.5 ml/min. Amino acids were detected as fluorescence derivatives after precolumn derivatization with o-phthaldialdehyde/2-mercaptoethanol. The integration of the amino acid peak area and further calculations were carried out by Gilson system software, and quantification was allowed by running standard amino acids solutions under the same conditions.

Synaptosomal membrane potential measurements. The synaptosomal transmembrane potential ($\Delta\Psi_m$) was monitored by evaluating the transmembrane distribution of tetraphenylphosphonium (TPP$^+$) with a TPP$^+$-sensitive electrode prepared according to Kamo et al. (27), using a calomel electrode as reference. Reactions were carried out in a chamber with magnetic stirring in 1 ml of Na$^+$ medium that contained (in mmol/l) 125 NaCl, 3 KCl, 1.2 MgSO$_4$, 1.2 CaCl$_2$, 3 glucose, and 10 HEPES-Tris at pH 7.4. Synaptosomes (0.5 mg/ml) were equilibrated in Na$^+$ medium for 15 min at 30°C in the absence or presence of 1 μmol/l insulin and then incubated for the same time with or without ascorbate/Fe^{2+}. The experiments were started by adding 3 μmol/l TPP$^+$, and the membrane potential was estimated from the decrease in TPP$^+$ concentration in the reaction medium.

Determination of adenine nucleotides (ATP and ADP). At the end of the $\Delta\psi_m$ measurements, each synaptosomal suspension was rapidly centrifuged at 14,000 rpm for 2 min with 0.3 mol/l perchloric acid. The supernatants were neutralized with 10 mol/l KOH in 5 mol/l Tris and centrifuged at 14,000 rpm for 2 min. The resulting supernatants were assayed for adenine nucleotides by separation in a reverse-phase HPLC, as described by Stocchi et al. (28). The chromatographic apparatus used was a Beckman-System Gold, consisting of a 126 Binary Pump Model and 166 Variable UV detector, controlled by a computer. The detection wavelength was 254 nm, and the column used was a Lichrospher 100 RP-18 (5 μm) from Merck (Darmstadt, Germany). An isocratic elution with 100 mmol/l phosphate buffer (KH$_2$PO$_4$; pH 6.5) and 1.0% methanol was performed with a flow rate of 1 ml/min. The required time for each analysis was 6 min. Peak identity was determined by the retention time compared with standards. The amounts of ATP and ADP were determined by a concentration standard curve, and the results were presented as percentage of the control, considering the mean of Wistar rat synaptosomes not treated with ascorbate/Fe^{2+} as 100%.

Data analysis and statistics. Results are presented as mean $\pm$ SE of the indicated number of experiments. Statistical significance was determined using the t test or the one-way ANOVA test for multiple comparisons. $P < 0.05$ was considered significant.

RESULTS

General characterization of GK rats. When the rats were killed, blood glucose levels were significantly higher in the diabetic GK group (241.8 ± 26.5 mg/dl) as compared with control Wistar rats (93.5 ± 5.1 mg/dl).

Synaptosomal susceptibility to oxidative stress induced by ascorbate/Fe^{2+}. Oxidative stress was induced after 15 min of synaptosomal incubation with 0.8 mmol/l ascorbate and 2.5 μmol/l Fe^{2+}. Under these conditions, an increase in lipid peroxidation, as determined by the formation of TBARS, has been reported (3,4,15). In the absence of ascorbate/Fe^{2+}, synaptosomal levels of TBARS were similar in both Wistar and GK rats (2.6 ± 0.3 and 2.7 ± 0.2 nmol/mg protein, respectively; Fig. 1). In GK rat synaptosomes, insulin did not significantly change TBARS levels (2.7 ± 0.2 and 2.6 ± 0.3 nmol/mg protein in the absence and in the presence of insulin, respectively). Moreover, an 8.7-fold increase in TBARS formation induced by ascorbate/Fe^{2+} was observed and was not
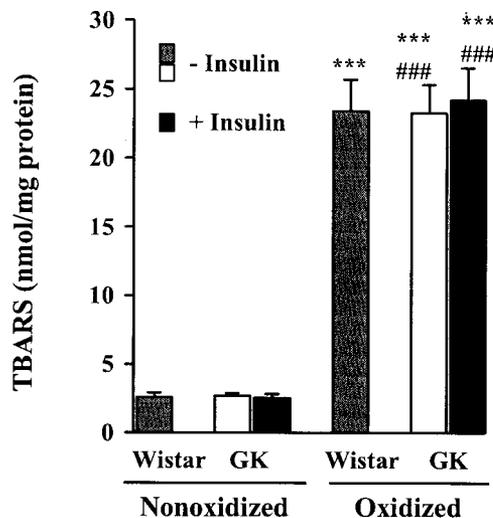
FIG. 1. Effect of oxidative stress induced by ascorbate/Fe^{2+} on the extent of lipid peroxidation on synaptosomes isolated from GK rat brain. Synaptosomes were preincubated in Na$^+$ medium for 15 min at 30°C in the absence or in the presence of 1 μmol/l insulin and then incubated in the absence (nonoxidized) or in the presence (oxidized) of 0.8 mmol/l ascorbate and 2.5 μmol/l Fe^{2+} for 15 min. The extent of lipid peroxidation was evaluated by determining the production of TBARSs and expressed as nmol/mg protein. Results obtained for Wistar rat synaptosomes submitted or not to oxidative stress are also represented. Data are the means ± SE of five to seven independent experiments. ***P < 0.001 vs. the respective control (Wistar or GK rat synaptosomes) not treated with ascorbate/Fe^{2+}; ###P < 0.001 vs. GK rat synaptosomes treated with 1 μmol/l insulin in the absence of oxidizing conditions.

reverted by preincubation with insulin (24.2 ± 2.3 nmol/mg protein).

Effect of insulin on the uptake of [^{3}H]GABA and [^{3}H]glutamate under oxidative stress conditions. Under nonoxidant conditions, [^{3}H]GABA uptake was similar in Wistar and GK rats, and preincubation with insulin increased [^{3}H]GABA uptake in GK rat synaptosomes, to 136 ± 13.7% of Wistar controls under nonoxidant conditions (Fig. 2A). The presence of ascorbate/Fe^{2+} induced a

90% decrease in [^{3}H]GABA uptake in GK rat synaptosomes, which was almost completely prevented by preincubation with insulin.

Under nonoxidizing conditions, there were no significant differences in synaptosomal [^{3}H]glutamate accumulation between Wistar and GK rats. Insulin incubation increased [^{3}H]glutamate uptake in diabetic rats by ~23% (Fig. 2B). Ascorbate/Fe^{2+} treatment decreased [^{3}H]glutamate uptake to 48.0 ± 9.8% of Wistar control rat synaptosomes. However, the protective effect of insulin was lower compared with that seen in [^{3}H]GABA. Under nonoxidizing conditions, the synaptosomal accumulation of [^{3}H]GABA and [^{3}H]glutamate in Wistar rat synaptosomes was 118.7 pmol/mg protein and 0.8 nmol/mg protein, respectively.

Effect of oxidative stress and/or insulin on the extrasynaptosomal levels of GABA and glutamate. In the absence of ascorbate/Fe^{2+}, extrasynaptosomal GABA levels were similar in both normal and diabetic rats (9.2 ± 0.6 and 8.9 ± 1.2 nmol GABA/mg protein, respectively; Fig. 3A), and insulin (1 μmol/l) treatment did not affect its levels in GK rat synaptosomes. In these animals, ascorbate/Fe^{2+}-induced oxidation increased 1.2-fold the extrasynaptosomal levels of GABA, which was prevented by insulin treatment (5.3 ± 0.6 nmol GABA/mg protein).

Under control conditions, extrasynaptosomal levels of glutamate were similar in Wistar and GK rats (17.8 ± 0.4 and 16.4 ± 1.2 nmol glutamate/mg protein, respectively), and incubation with insulin did not affect the extrasynaptosomal content of glutamate in diabetic rats (Fig. 3B). Upon oxidizing conditions, a 1.8-fold increase in extracellular glutamate levels was observed, which was not prevented by insulin.

Effect of oxidative stress and/or insulin on synaptosomal $\Delta\Psi_m$. Synaptosomal $\Delta\Psi_m$ was significantly lower in diabetic rats relative to normal Wistar rat synaptosomes ($-101.8 ± 4.7$ and $-80.4 ± 7.7$ mV in Wistar and GK rat synaptosomal membranes, respectively). Insulin (1 μmol/l) incubation did not significantly affect GK rat
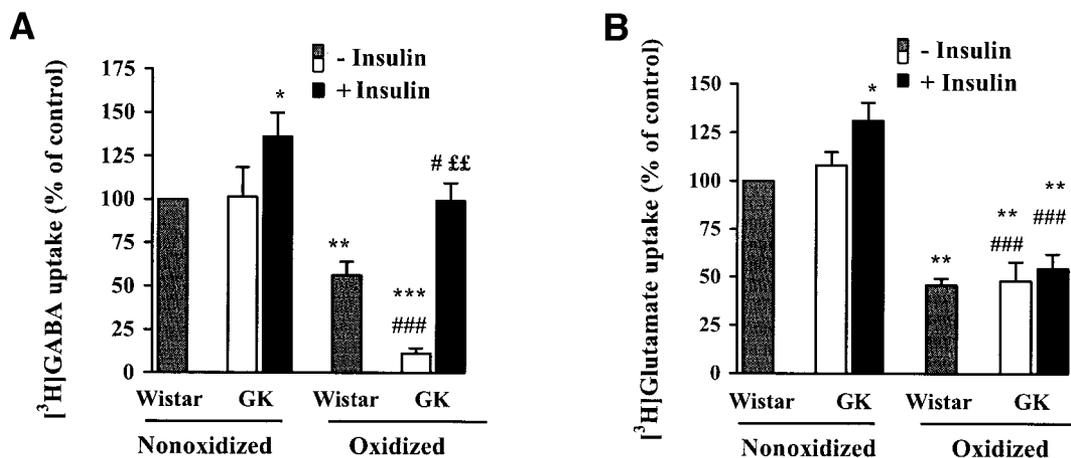

FIG. 2. Effect of insulin on the uptake of [^{3}H]GABA (A) and [^{3}H]glutamate (B) by GK rat synaptosomes submitted (oxidized) or not (nonoxidized) to oxidative stress induced by ascorbate/Fe^{2+}. Synaptosomes were equilibrated in Na$^+$ medium for 15 min at 30°C in the absence or in the presence of 1 μmol/l insulin and then incubated for 15 min with or without ascorbate/Fe^{2+}. Results obtained for Wistar rat synaptosomes submitted or not to oxidative stress are also presented. Data, expressed as a percentage of [^{3}H]GABA uptake or [^{3}H]glutamate uptake in Wistar control rat synaptosomes (considering 137.9 pmol [^{3}H]GABA/mg protein and 0.7 nmol [^{3}H]glutamate/mg protein as 100%), are the means ± SE of three to six and of three to seven different experiments, respectively. *P < 0.05; **P < 0.01; ***P < 0.001 vs. the respective control (normal or diabetic rat synaptosomes) not treated with ascorbate/Fe^{2+}; #P < 0.05; ###P < 0.001 vs. GK rat synaptosomes treated with 1 μmol/l insulin in the absence of ascorbate/Fe^{2+}; §§P < 0.01 vs. synaptosomes from GK rats submitted to oxidizing conditions.
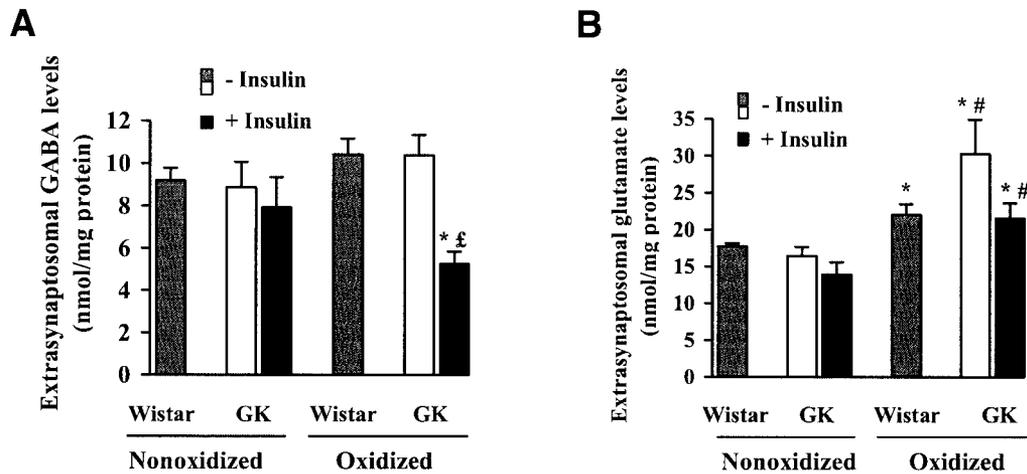
A

B

FIG. 3. Effect of insulin on the release of the endogenous amino acids GABA (*A*) and glutamate (*B*) by GK rat synaptosomes submitted (oxidized) or not (nonoxidized) to oxidative stress induced by ascorbate/Fe^{2+}. Results obtained for Wistar rat synaptosomes submitted or not to oxidative stress are also presented. The values, expressed in nmol/mg protein, are the mean ± SE of three to five independent experiments. *$P < 0.05$ vs. the respective control (Wistar or GK rat synaptosomes) not treated with ascorbate/Fe2+; #$P < 0.05$ vs. GK rat synaptosomes preincubated with insulin. £$P < 0.05$ vs. GK rat synaptosomes upon oxidative stress conditions.

synaptosomal $\Delta\Psi_m$. In these rats, neither ascorbate/Fe^{2+} nor insulin treatment under oxidizing conditions significantly altered $\Delta\Psi_m$ values (Fig. 4).

Effect of oxidative stress and/or insulin on synaptosomal ATP/ADP ratio. The effects of insulin and/or ascorbate/Fe^{2+} treatment on the synaptosomal ATP/ADP ratio are represented in Fig. 5. In the absence of oxidizing conditions, the ATP/ADP ratio was significantly lower in diabetic rats relative to normal Wistar rats, and synaptosomal incubation with 1 μmol/l insulin did not significantly change this value. Under oxidizing conditions, diabetic rats

showed a 1.7-fold increase in the ATP/ADP ratio, which was not altered upon insulin incubation. The ATP/ADP ratio in Wistar rat synaptosomes under nonoxidizing conditions was 2.9 ± 0.9.

DISCUSSION

Nonenzymatic glycation and irreversible protein cross-linking that occur under hyperglycemia may result in the formation of advanced glycation end products and ROS (29,30), supporting the idea that the increase in blood
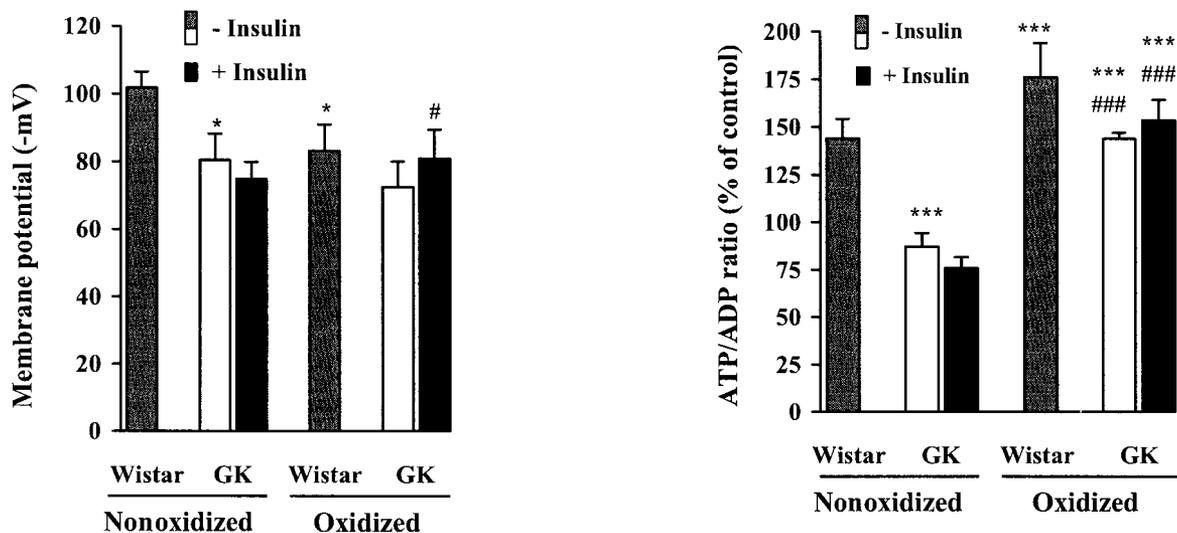

FIG. 4. Effect of insulin on the $\Delta\Psi_m$ in GK rat synaptosomes submitted (oxidized) or not (nonoxidized) to oxidative stress induced by ascorbate/Fe^{2+}. Synaptosomes were equilibrated in Na$^+$ medium for 15 min at 30°C in the absence or in the presence of insulin and then incubated for 15 min with or without ascorbate/Fe^{2+}. Membrane potential was determined by following the decrease of TPP$^+$ in the medium. Results obtained for Wistar rat synaptosomes submitted or not to oxidative stress are also presented. Data, expressed as −mV, are the means ± SE of 9–13 different experiments. *$P < 0.05$ vs. the respective control (Wistar or GK rat synaptosomes) not treated with ascorbate/Fe^{2+}; #$P < 0.05$ vs. the GK rat synaptosomes preincubated with 1 μmol/l insulin in the absence of oxidizing conditions.

FIG. 5. Effect of insulin in the ATP/ADP ratio in GK rat synaptosomes submitted (oxidized) or not (nonoxidized) to oxidative stress induced by ascorbate/Fe^{2+}. After the $\Delta\psi_m$ measurements, each synaptosomal suspension was treated with 0.3 mol/l perchloric acid and the supernatants were neutralized with 10 mol/l KOH in 5 mol/l Tris. Results obtained for Wistar rat synaptosomes submitted or not to oxidative stress are also presented. Data, expressed as a percentage of the ATP/ADP ratio in Wistar control rat synaptosomes (considering 2.9 ± 0.9 as 100%), are the means ± SE of 9–13 different experiments. ***$P < 0.001$ vs. the respective control (Wistar or GK rat synaptosomes) not treated with ascorbate/Fe^{2+}; ###$P < 0.001$ vs. the GK rat synaptosomes preincubated with 1 μmol/l insulin in the absence of oxidizing conditions.

glucose levels (3,31) may render diabetic rats exposed to oxidative stress (5,29).

Aberrant synaptic signaling has been implicated in neurodegenerative disorders, such as Alzheimer's disease (32). Synaptosomes isolated from mammalian brain constitute a useful in vitro model to study several nerve functions, because they are metabolically active and retain many properties of nerve endings, namely neurotransmitter uptake (33,34).

In previous studies (3,4), we observed that GK rat brain synaptosomes were less susceptible to ascorbate/Fe^{2+}-induced oxidative stress than Wistar rats, suggesting a protective mechanism against ROS-induced damage. In the present study, we found that basal levels of TBARS were similar in both animal models, as previously reported (35). Our recent observation that insulin did not reverse the ascorbate/Fe^{2+}-induced increase in TBARS levels in Wistar rats suggests that other mechanisms, rather than a direct action of insulin on lipid peroxidation, may mediate its neuroprotective role under stress conditions (15).

The controversial observations reported by some authors on alterations in brain amino acid transport using diabetic rats may be related to the diabetic model or to the experimental approach used. Morrison et al. (36) reported that streptozotocin-induced diabetes (a type 1 diabetes model) does not change the transport mechanism or the receptor sensitivity to GABA. Li et al. (37) reported an increased accumulation of extracellular glutamate in the neocortex submitted to hyperglycemic ischemia, which was correlated with the cell damage observed. In this study, we reported that diabetes had no effect on both synaptosomal GABA and glutamate transport, which is partially in accordance with the observations of Vilchis and Salceda (8). These authors demonstrated an increment in GABA uptake, without changes in glutamate uptake in diabetic rat retina and retinal pigment epithelium. These observations suggest that the modification of transmembrane electrical gradient may affect the accumulation of amino acid neurotransmitters. Our observation of a diabetes-related decrease in both synaptosomal transmembrane gradient ($\Delta\Psi_m$) and ATP/ADP ratio, resulting from a decrease in ATP and/or an increase in ADP levels (data not shown), further supports this hypothesis. The decrease in ATP production is a hallmark of a disturbance in glucose metabolism, which could be partially balanced by the use of endogenous brain substrates, such as glutamate and fatty acids (17). Synaptosomal uptake of neurotransmitters is an energy-dependent process that requires the integrity of membrane systems (33) and functional mitochondria. Thus, our observation of a diabetes-mediated decrease in synaptosomal membrane potential and ATP/ADP ratio suggests a glucose-dependent protective mechanism in GK rat brain, with higher glucose levels leading to the loss of the GABA-dependent protection against glutamate neurotoxicity.

Our observation of an oxidative stress–induced decrease in synaptosomal GABA uptake, accompanied by an increase in its extrasynaptosomal accumulation, suggested that GK rats may have developed a protective mechanism against oxidative stress–related neuronal death (38,39). The decrease in glutamate uptake and increased extrasynaptosomal levels induced by ascorbate/

Fe^{2+} in GK rats further support this hypothesis, because oxidative stress–induced damage to neuronal cell membrane leads to the release and subsequent extracellular accumulation of glutamate, contributing to excitotoxicity (38) and further promoting ROS generation (40). Sah and Schwartz-Bloom (41) reported that ROS-decreased inhibitory neurotransmission also promotes neuronal damage in oxidative stress–related diseases and aging. These effects of oxidative stress on amino acid accumulation may be due to membrane lipoperoxidation, which may impair the GABA transporter, leading to a decrease of GABA accumulation (42), and/or it may increase the release and extracellular accumulation of aspartate and glutamate (38,40). Other mechanisms that could be involved in the inhibition of glutamate uptake by ROS may be the direct oxidation of the transporter sulfhydryl groups (43,44) or the impairment of Na^+,K^+ ATPase activity (45).

The modulatory effect of insulin on amino acid accumulation in brain remains controversial (14,46). In some studies, 3 μmol/l insulin stimulated glutamate and GABA accumulation in rat brain synaptosomes (14), whereas in others, the same concentration was shown to inhibit GABA uptake in embryonic chick retina and newborn mouse cerebral cortex astroglial cells (11,47). According to Rhoads et al. (14), insulin dose-dependently stimulates synaptosomal amino acid uptake, and 1 μmol/l was the lowest insulin concentration that increased glutamate uptake by cortical synaptosomes, by an effect maintained during 1–20 min of in vitro incubation. In the present study, we used an insulin concentration of 1 μmol/l, incubated for 15 min with the synaptosomal fraction, in the absence or presence of the oxidizing pair ascorbate/Fe^{2+}. In a recent article, we reported that, in Wistar rat synaptosomes, insulin reverted both the oxidative stress–related inhibition in the uptake and the increase in GABA and glutamate extrasynaptosomal accumulation (15).

In this study, we observed that 1 μmol/l insulin per se stimulated intrasynaptosomal GABA accumulation in diabetic rat brain synaptosomes, suggesting that the neuroprotective role of insulin may be exerted directly on membrane GABA transporters. Some authors (11,14) hypothesized that insulin-induced changes in GABA transport can be exerted indirectly through the stimulation of Na^+,K^+ ATPase activity, thus increasing the transmembrane Na^+ gradient and the $\Delta\Psi_m$. However, Guyot et al. (48) postulated that these changes could be related to stimulation of glycolysis and pyruvate dehydrogenase, increasing lactate and ATP levels, which stimulate ion pumps and, ultimately, the synaptosomal amino acid transport. Our results did not show insulin-induced changes in $\Delta\Psi_m$ or ATP/ADP ratio, suggesting that the increase in the uptake of both GABA and glutamate might be related to a direct effect of insulin in both amino acid transporters. However, the activation of insulin receptor signaling cascade via its tyrosine kinase activity, which activates the uptake of amino acids (49), cannot be ruled out. We also observed that insulin reverted the effect of ascorbate/Fe^{2+}, increasing GABA uptake and decreasing its extrasynaptosomal levels, supporting the idea of a neuroprotective role for insulin. This hypothesis is also supported by a nonsignificant decrease in extrasynaptosomal glutamate levels observed under these conditions.

Recent evidence suggests that a dysfunction of the neuronal insulin receptor signaling cascade, with the subsequent abnormalities in glucose/energy metabolism, may affect amyloid precursor protein metabolism and induce τ hyperphosphorylation. These results suggest that insulin dysfunction could be involved in the cause of Alzheimer's disease (12,17) and supports the hypothesis of a neuroprotective role of insulin under stress conditions. The data obtained in the present study support the idea that a direct effect on amino acid neurotransmitter transport system (mainly GABA) could be one of the mechanisms responsible for the neuroprotective role of insulin in oxidative stress conditions, suggested to occur in diabetes and neurodegenerative diseases.

ACKNOWLEDGMENTS

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