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Clonazepam increases in vivo striatal extracellular glucose in diabetic rats after glucose overload

Rosane Gomez , Helena M.T. Barros*

Pharmacology Division, Fundação Faculdade Federal de Ciências Médicas de Porto Alegre, R. Sarmento Leite, 245, 90050-170 Porto Alegre, Brazil

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Abstract

Hyperglycemia modulates brain function, including neuronal excitability, neurotransmitter release and behavioral changes. There may be connections between the GABAergic system, glucose sensing neurons and glucose in the neuronal environment that shed light on the mechanism by which $GABA_A$ agents influence depressive behavior in diabetic rats submitted to the forced swimming test. We aimed to investigate whether clonazepam (CNZ), a $GABA_A$ receptor positive modulator, modifies in vivo striatal extracellular glucose levels in diabetic rats under fasting condition or after oral glucose overload. Streptozotocin diabetic and nondiabetic rats were submitted to in vivo striatal microdialysis. Perfusate samples were collected at baseline, during fasting and following administration of CNZ (0.25 mg/kg) and oral glucose overload. Blood glucose and striatal extracellular glucose were measured simultaneously at several time points. Fasting striatal glucose levels were higher in diabetic than in nondiabetic rats and the differences between these animals were maintained after glucose overload. The increases in extracellular striatal glucose after glucose overload were around 40% and blood to brain transference was decreased in diabetics. CNZ treatment paradoxically increased striatal glucose after glucose overload in diabetic rats, which may mark the dysfunction in brain glucose homeostasis.

Keywords: Microdialysis; Depression; Glucose ratio; Benzodiazepines; Metabolism

1. Introduction

Diabetes is a hyperglycemic chronic state that may modify central nervous system functions (Bellush et al., 1991; Lustman et al., 1992). The impact of diabetes on the central nervous system has been closely studied during the last decade (McCall, 1992) and a condition that may be referred to as diabetic encephalopathy is now accepted (Biessels et al., 2002). Besides the known consequences of the increased risk of strokes, diabetes mellitus is associated with moderate cognitive deficits and neurophysiological and structural changes in the brain (Biessels et al., 2002). Psychiatric manifestations seem to accompany this encephalopathy, since the prevalence of depression is as high as 39% in diabetic patients, much higher than in the general population (Gavard et al., 1993; Lustman et al., 1992; Téllez-Zentero and Cardiel, 2002).

The diabetic encephalopathy may be replicated in the experimental animal setting. The relationship between hyperglycemia and neuronal damage in the cerebral cortex has already been demonstrated in streptozotocin (STZ) diabetic rats and insulin treatment prevents this neuronal damage (Guyot et al., 2001). It has been shown that diabetic mice and rats also present depressive-like behavior in the forced swimming test (Gomez and Barros, 2000; Hilakivi-Clarke et al., 1990). Insulin treatment prevents the behavioral variation of diabetic rats (Hilakivi-Clarke et al., 1990). Clonazepam (CNZ), a $GABA_A$ receptor positive modulator, also counteracts the depressive behaviors in diabetic animals (Gomez and Barros, 2000).

Very few studies have examined the relationship between the GABAergic system and diabetes and the functioning of the GABAergic system in diabetic individuals' brains is controversial. Hyperglycemia is seen to increase (During et al., 1995; Levin, 2000; Levin and Dunn-Meynell, 1998; Othani et al., 1997), to decrease (Guyot et al., 2001; Honda et al., 1998) or not change GABA levels (Guyot et al., 2001), depending on the brain areas and if the animals are diabetic. Diabetic rats present brain GABA concentrations

* Corresponding author. Tel.: +55-51-3224-8822x151; fax: +55-51-3224-8822x121.
E-mail addresses: rgomez@terra.com.br (R. Gomez), helenbar@fffcmpa.tche.br (H.M.T. Barros).

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similar to nondiabetic rats when the animals are kept under basal laboratory conditions (Duarte et al., 2000; Gomez et al., 2003). Cerebral GABA uptake of diabetic Goto–Kakizeki rats also does not differ from that of Wistar rats (Duarte et al., 2000). However, differences in brain GABA concentrations seem to occur when the individuals are submitted to behavioral challenges, because diabetic rats present a delayed and slightly decreased extracellular striatal GABA response when submitted to the forced swimming test (Gomez et al., 2003).

The interaction between glucose homeostasis and GABA is highly complex due to the multiple points of interplay between the GABAergic system and the biochemistry of glucose in mammalian cells. Insulin reduces astrocytic uptake of GABA, increasing extracellular GABA and enhancing the number of functional $GABA_A$ receptors in postsynaptic neurons (as reviewed in Figlewicz, 1999; Guyot et al., 2001). In a previous study in our laboratory, we showed that diazepam increased fasting glycemia of STZ diabetic rats while it decreased hyperglycemia induced by glucose overload and increased plasma insulin concentrations in these diabetic animals (Gomez et al., 1999). These effects did not occur in nondiabetic animals and were not seen with THIP, a direct $GABA_A$ agonist. Low doses of CNZ, another $GABA_A$ receptor positive modulator, is known to reduce brain glucose utilization by 20% without changing blood glucose in free feeding nondiabetic mice (Ishizuka et al., 1989).

In the other hand, CNZ has been used in the clinical setting as a therapeutic adjunct to treat moderate depression (Morishita et al., 1998). In the preclinical setting, CNZ has shown to reduce depressive-like behaviors in diabetic rats submitted to an animal model of depression (Gomez and Barros, 2000). To better understand the effects of CNZ on brain glucose levels of diabetic rats, we studied both fasting and overload glucose levels modeling different alimentary conditions. Therefore, it was our objective to investigate whether this GABAergic agent modifies in vivo striatal extracellular glucose levels in nondiabetic and diabetic rats, in both fasting and glucose overload conditions.

2. Materials and methods

2.1. Animals

Male Wistar rats (250–350 g) were obtained from the Animal House of Fundação Faculdade Federal de Ciências Médicas de Porto Alegre (FFFCMPA). The animals were housed in groups of three per polypropylene cage, with wood shavings as bedding before the surgery. 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.–7:00 p.m.). 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 FFFCMPA. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

2.2. Drugs

CNZ (0.25 mg/ml; Rivotril, Roche, Brazil) was prepared in distilled water with Tween 0.05% (v.v.) immediately before intraperitoneal administration. This dose was chosen because it has antidepressant effects in diabetic rats (Gomez and Barros, 2000). The correspondent vehicle was prepared with saline and Tween 0.05%.

Glucose [D(+)-glucose, monohydrate (Merck, Rio de Janeiro, Brazil)] was dissolved in distilled water (50 g/100 ml) and administered by gavage.

STZ (Sigma, St. Louis, MO, USA) 60 mg/ml was prepared in phosphate buffer (pH 4.5).

2.3. Diabetes induction

Diabetes was induced by a single intraperitoneal dose of STZ 60 mg/kg. Increased blood glucose levels (≥ 200 mg/dl) of the STZ group rats were confirmed with a glucometer (Glucotrend, Boehringer Institute, Mannheim, Germany) after 24 h. Nondiabetic control rats received intraperitoneal injections of saline (1 ml/kg) and were also submitted to blood glucose measurement.

2.4. In vivo microdialysis and glucose analysis

One week after diabetes induction, the rats were anesthetized with intraperitoneal injections of a mixture of 10 mg/kg ketamine and 50 mg/kg xylazine, and were placed into the stereotaxic frame (David Kopf, Tujunga, CA). Unilateral guide-cannulae for CMA 12 probes were implanted in the left dorsal striatum (bregma: anteroposterior $+0.2$ mm, lateral $+3$ mm and vertical -5.0 mm), according to Paxinos and Watson (1986). The guide-cannulae assembly was then fixed to the skull with three anchorage screws and dental cement. After recovering from anesthesia, the animals were maintained in isolated acrylic cages ($35 \times 25 \times 40$ cm) with wood shaving beds and were observed closely for 1 week.

After 14 days of diabetes induction and 1 day before the microdialysis, rats from both groups [diabetes (STZ) and nondiabetic (CTR)] were individually habituated for 5 h to a containment system for freely moving animals, in which the animal is connected to the counterbalanced arm and swivel through a plastic collar (CMA/120, Acton, MA). During this habituation period, only water was offered to the animals and they remained fasted until the end of the experiment. Glucose recovery from the probes (0.5 mm diameter with 4 mm dialyzing membrane, CMA/12, Acton, MA) was eval-

uated in a 2.4-mM glucose solution at 1 μl/min. The in vitro recovery was 28 ± 3%.

On the microdialysis experiment day, the probes were inserted into the guide-cannulae and the fasting animals were introduced into the same containment system for freely moving animals described above. Probes were perfused with artificial cerebrospinal fluid (NaCl 147 mM; $CaCl_2$ 2.3 mM; KCl 4.0 mM; $MgCl_2$ 0.9 mM with unadjusted pH 7.1–7.3) at a constant flux rate of 1.0 μl/min with a microperfusion pump (CMA/102, Acton, MA). The microdialysis membrane was stabilized for 1 h and three samples were then collected at 20-min intervals to determine striatal baseline glucose levels.

At 120 min after the introduction of the probes, subgroups of STZ or CTR rats received 0.25 mg/kg of CNZ or vehicle. Three subsequent perfusate samples were collected at 20-min intervals for 1 h. At 180 min after the introduction of the probe, all rats received 1 ml of glucose solution by gavage and the perfusate samples were collected at 20-min intervals for 2 h more (see Fig. 1). All samples were frozen at −80 °C for glucose determination by an enzymatic method (Glucose oxidase GOD/PAP, Labtest Diagnóstica, Brazil), using a spectrophotometer at 505 nm (Ultrospec 2000, England).

Glucose was measured in blood taken from a superficial puncture in the distal end of the rat's tail. The blood drop was applied to the test zone of the strip for measurement of fasting-food glycemia in a Glucotrend device (Boehringer Mannheim, Germany). These measures were repeated every 40 min (20 and 60 min after CNZ and 40, 80 and 120 min after glucose overload) to reduce the frequency of manipulation of the animals (Fig. 1). All experiments were undertaken between 9:00 a.m. and 3:00 p.m.

2.5. Histology

After completion of the microdialysis experiments, the rats were euthanized with an overdose of pentobarbital (50 mg/kg) and perfused intracardially with around 20 ml 0.9% saline, followed by perfusion with 10% formalin. The brains were carefully removed and stored in a 10% formalin solution until they were sectioned into coronal 40 μm slices, collected consecutively through the extension of the area of implantation of guide cannulae.

2.6. Statistical analysis

Striatal baseline glucose levels were calculated by averaging levels from the three samples collected immediately before CNZ administration. Striatal glucose concentration changes after CNZ and after glucose overload were expressed as percent from baseline level for each individual. These absolute and percent values were analyzed using a three-way ANOVA test, considering as factors: diabetic/nondiabetic condition, CNZ/saline treatment and time. Blood glucose levels were also analyzed through a three-way ANOVA test. Pearson's correlation was used to measure the strength of the association between blood glucose and striatum glucose levels. Striatal glucose was divided by blood glucose to obtain an extracellular striatal/blood glucose ratio and these values were compared through a two-way ANOVA test. The Student–Newman–Keuls test was used for post hoc comparisons when appropriate. All data are expressed as mean ± standard error of the mean. A P value of less than .05 was considered significant.

3. Results

In Fig. 2, there is a representation of absolute values of glucose concentration in the striatum of rats. These values are estimates from data and reflect the probe recovery rate of 28% for glucose. Diabetic rats showed higher fasting extracellular striatal glucose baseline levels than nondiabetic rats [$F(1,135) = 58.721$, $P < .001$]. CNZ administration did not change absolute values of striatal glucose concentration during the fasting condition. Oral glucose overload induced a significant increase in extracellular glucose levels in all groups [$F(9,135) = 3.156$, $P = .002$]. The nondiabetic rats treated or not with CNZ and the diabetic rats that did not receive benzodiazepine treatment had significantly higher extracellular glucose levels 40 and 60 min after oral glucose overload. Diabetic rats treated with CNZ showed a significant interaction between treatment and glucose overload

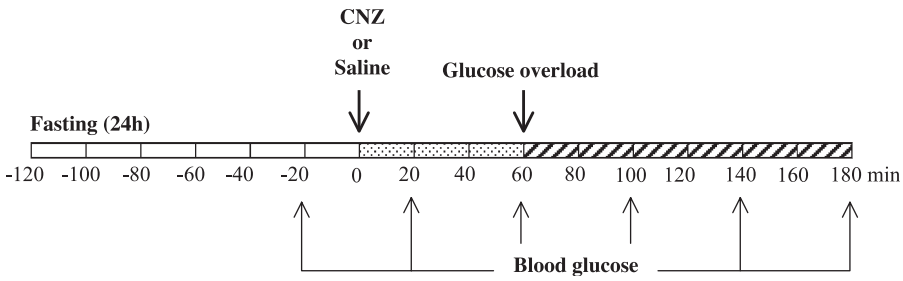

Fig. 1. Time line of microdialysis striatal sampling during experiments with fasted diabetic or nondiabetic rats treated or not with CNZ at time 0 and submitted to glucose overload after 1 h. The three 20-min samples before time 0 were used to calculate extracellular striatal baseline glucose levels. Blood glucose was determined every 40 min.

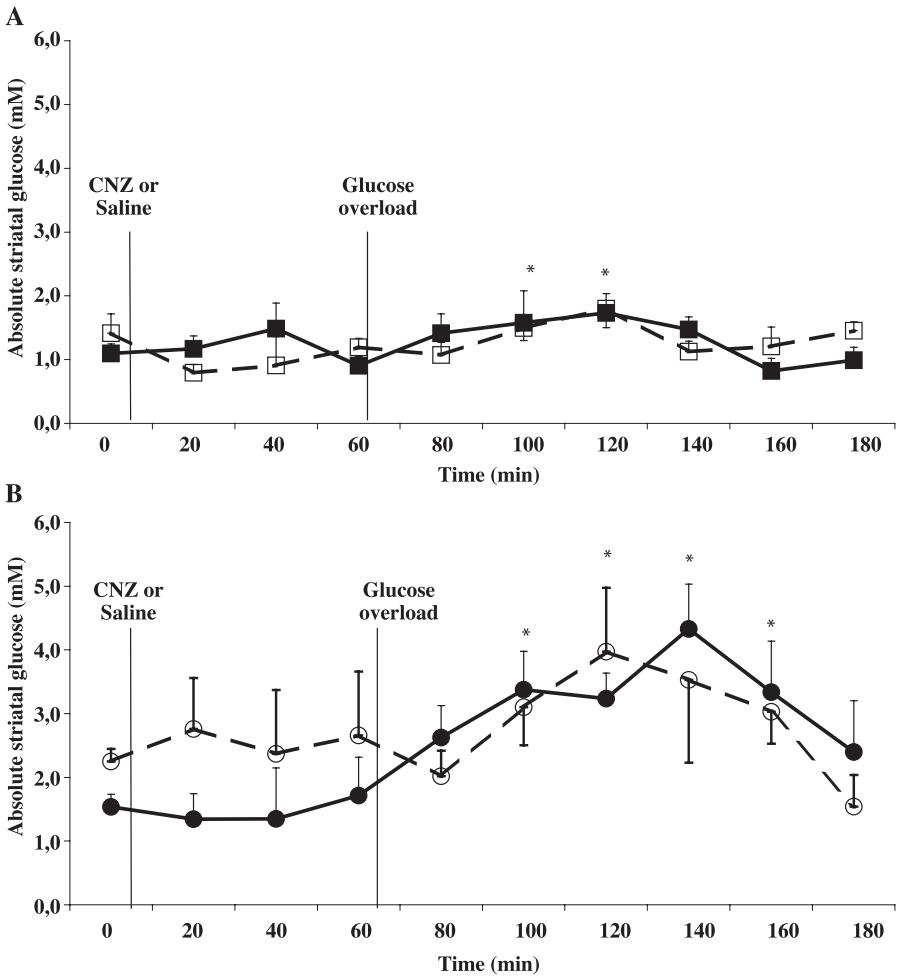

Fig. 2. Absolute concentrations of extracellular striatal glucose levels after 24 h fasting (time 0), after CNZ (0.25 mg/kg) and after oral glucose overload. (A) Nondiabetic rats: Open squares represent the rats treated with saline and full squares represent the rats treated with CNZ. (B) Diabetic rats: Open circles represent the diabetic rats treated with saline and full circles represent the rats treated with CNZ. Values are mean ± S.E.M. of four to five animals per group. * Different from baseline.

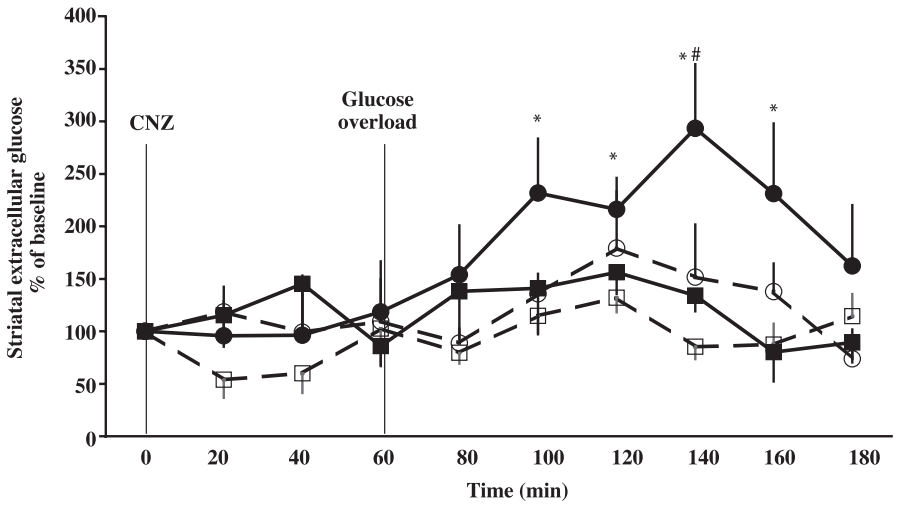

Fig. 3. Striatal glucose levels change after oral glucose overload in diabetic and CNZ-treated rats. Squares represent nondiabetic rats and circles represent diabetic rats. Open figures and lines represent vehicle treatment and full figures and lines represent CNZ (0.25 mg/kg) treatment. Values are means ± S.E.M.; * Differences from baseline for all groups; # Differences from CNZ of both groups of nondiabetic rats and nontreated diabetic rats.

[$F(9,135) = 2.069$, $P = .036$]. These animals showed a significantly higher increment in extracellular glucose levels compared with the animals of the other three groups from 40 to 100 min after glucose overload.

As represented in Fig. 3, diabetic rats present higher extracellular glucose percent changes from baseline when submitted to glucose overload [$F(1,84) = 15.268$, $P < .001$]. Higher extracellular glucose levels were achieved between 60 and 100 min after glucose overload [$F(6,84) = 2.85$, $P = .014$]. The main effect of CNZ was a significant increase in the striatal glucose concentration [$F(1,84) = 12.935$, $P < .001$]. Additionally, the benzodiazepine treatment and diabetic condition interacted significantly [$F(1,84) = 4.843$, $P = .031$] because CNZ increased striatal glucose concentrations to levels even higher in diabetic rats.

STZ diabetic rats presented higher blood glucose than nondiabetic rats [$F(1,78) = 280.777$, $P < .001$] as expected (Fig. 4). In diabetic rats, blood glucose peaked 80 min after glucose overload and in nondiabetic rats the peak was reached in 40 min after glucose overload [$F(5,78) = 5.370$, $P < .001$] and an interaction between diabetic condition and time was seen [$F(5,78) = 3.068$, $P = .014$]. This may have occurred because CNZ-treated diabetic rats tended to show a slightly higher fasting blood glucose level, which lead to a lower increase in blood glucose levels after glucose overload. CNZ did not significantly change blood glucose levels at any time point measured.

Higher blood glucose in diabetic rats was correlated with higher striatal glucose ($r = .525$, $P < .05$). As an exercise we decided to estimate glucose transfer from blood to brain by dividing the estimated striatal glucose levels by blood glucose levels. Diabetic rats showed a lower ratio than nondiabetic rats [$F(1,72) = 12.902$, $P < .001$]. CNZ treatment or glucose overload did not significantly change the estimated blood/brain ratio.

4. Discussion

In this experiment, we showed that the in vivo basal extracellular striatal glucose levels are around 50% higher in freely moving diabetic than in nondiabetic rats. Basal extracellular glucose levels of nondiabetic animals in this experiment were an intermediate value in respect to the levels showed by other laboratories. In vivo brain extracellular glucose levels in rats are described to be from around 0.4 to 2.4 mM (Fellows et al., 1992; Fray et al., 1996, 1997; McNay et al., 2001; Silver and Erecinska, 1994). In previous studies, extracellular glucose concentrations were estimated considering glucose plasma levels, glucose utilization by brain cells and blood–brain barrier transport kinetics as 2 to 4 mM (Lund-Anderson, 1979). Similar results of brain extracellular glucose values were estimated in vivo in anesthetized rats using glucose microelectrodes (Silver and Erecinska, 1994). Direct measurement of extracellular fluid glucose levels by microdialysis is a better method for studies using freely moving animals (Fellows et al., 1992), especially when the objective is to monitor concomitant changes in behavior. Brain glucose measurement through microdialysis is not as susceptible to overestimation due to contamination from plasma or due to brain metabolism compartmentalization (Gruetter, 2002; McNay et al., 2001). Use of the microdialysis technique also surpasses the need to keep animals under anesthesia, which is known to interfere with brain metabolism and to increase glucose efflux (Fellows et al., 1992). On the other hand, there are also differences in results of glucose extracellular levels between experiments using microdialysis. These differences depend on both the recovery of the substance relative to the membrane used and the flow of perfusion (Khan and Shuaib, 2001). They may also be due to the presence of glucose in the perfused artificial cerebrospinal fluid that

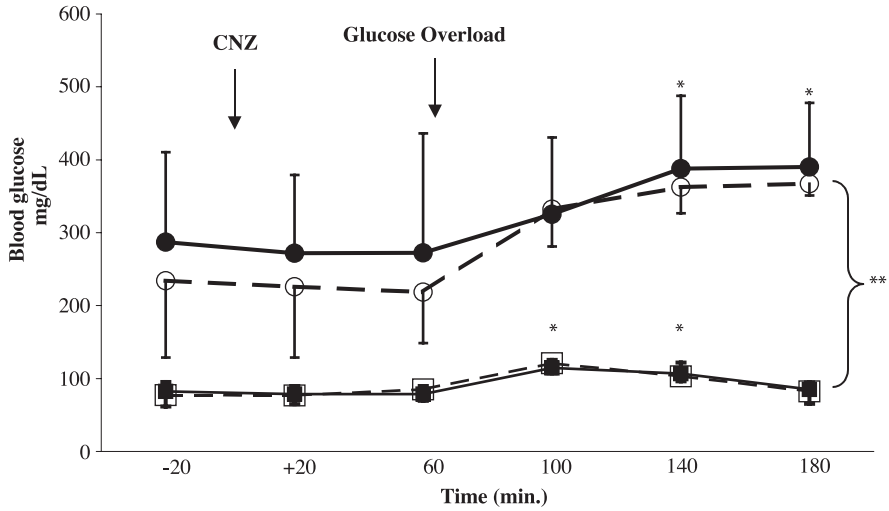

Fig. 4. Blood glucose levels of diabetic and nondiabetic fasting rats, after treatment with CNZ (0.25 mg/kg) and after oral glucose overload. Open squares represent the nondiabetic rats treated with saline and bold squares, the nondiabetic rats treated with CNZ. Open circles represent the diabetic rats treated with saline and bold circles the animals treated with CNZ. * $P < .05$, differs from other times; ** $P < .05$, differs from nondiabetic rats.

would correct the gradient and decrease the flow of glucose from brain to the perfusate (Fellows et al., 1992; Fray et al., 1996). In our case, the noncorrected glucose concentration was 0.35 mM, which represents only around one-third of the actual estimate (1.17 ± 0.13 mM), since the recovery of the probes used was 30%. Because we measured this recovery in vitro, it is possible that our values are underestimated, since in vivo recovery of glucose has been reported to be lower than in vitro recovery (Lönrut et al., 1987).

It has been described that the estimates of extracellular glucose concentration represent the steady state between blood delivery of glucose to the brain and the metabolic needs of neurons and glial cells (Fray et al., 1996). This explains the positive correlation between brain and blood glucose levels seen in this study. Therefore, the higher blood glucose levels of diabetic animals correlates to the higher extracellular glucose levels in the striatum during fasting (Figs. 2 and 4). However, brain glucose levels seen in diabetic animals is 45% higher than those of nondiabetic rats while blood glucose levels were much greater, around 250% of levels seen in nondiabetic rats. As foreseen, the estimated brain extracellular/blood glucose ratio is significantly lower in diabetic than in nondiabetic rats. The decreased ratio in chronic hyperglycemic diabetics may be justified by decreased glucose extraction by brain cells when plasma glucose is too high, saturating the blood–brain glucose transport mechanism (Lund-Anderson, 1979; Mooradian and Morin, 1991) or by changes in neural glucose transporters GLUT3 (Reagan et al., 1999), GLUT4 (Vannucci et al., 1998) and GLUT8 (Reagan et al., 2001). Any one of these mechanisms may explain the changes in glucose transfer rate from blood to the extracellular fluid and from fluid to neurons. We also tried to estimate the correlation between blood and striatal glucose levels after glucose overload of diabetic and nondiabetic freely moving rats. Nondiabetic rats had a transitory 30% increase in striatal extracellular glucose levels 40 to 60 min after an oral glucose overload while their blood glucose levels increase 40 to 60% at the same time point. In diabetic rats, the extracellular glucose levels increased almost 100% after glucose overload, while blood glucose increased around 50%. This illustrates that the increment of blood glucose is not accompanied by an increment in brain glucose levels at the same proportion in nondiabetic animals, reproducing the fine brain glucose homeostasis regulation, as stated in the glucostatic hypothesis (Levin, 2002; Silver and Erecinska, 1994). It also shows that this homeostasis regulation is deficient in diabetic rats.

The mechanisms for glucose homeostasis may involve glucosensing neurons that monitor the metabolic status of the body (Levin, 2002, Pénicaud et al., 2002). In glucose-excited neurons, glucose levels reproducing those seen in hyperglycemia inactivate the K_{ATP} channel, increasing neuronal firing rate and inducing release of GABA and other transmitters (During et al., 1995; Levin, 2000). STZ-induced diabetes up-regulates high-affinity sulfonylurea binding sites at GABA neurons and therefore one would expect that increased glucose levels lead to higher GABA release (Levin and Dunn-Meynell, 1998). In fact, increased GABA levels were detected in the hypothalamus of diabetic rats (Othani et al., 1997). However, other authors describe no change or decreased GABA levels in the cortex (Guyot et al., 2001) and in the superior colliculus of diabetic animals (Honda et al., 1998). Decreased GABA levels were also seen in the cortex of animals with acute hyperglycemia (Phillis et al., 1999). In a recent observation, we have demonstrated that diabetic rats present unchanged basal GABA levels, but a lower and delayed increase of extracellular GABA in the striatum during the forced swimming procedure (Gomez et al., 2003). The $GABA_A$ receptors participate in a very complex way in glucose homeostasis, depending on the brain area being studied (Levin, 2000). Acute administration of CNZ is known to reduce brain glucose utilization by 20%. This effect occurs with doses as low as 0.01 mg/kg and there is a nonlinear relationship between benzodiazepine receptor occupancy and the brain metabolic response (Ishizuka et al., 1989). We are not aware of studies that describe changes in the $GABA_A$ receptors during diabetes and chronic hyperglycemia. Surprisingly, in this study, the benzodiazepine CNZ induced an even higher glucose striatal level, compared with the baseline, only in the diabetic animals during glucose overload (Fig. 3). One possible explanation could involve CNZ pharmacokinetics changes induced by diabetes; however, there is no literature data to support such fact. We discarded the possibility of CNZ having a retarded effect, only after 1 h, on striatal glucose levels because it is very rapidly absorbed, with concentration peaks within minutes and shows an extended half-life, around 20 to 80 h, assuring us that the levels may be maintained in the 3-h period of our study. We also considered the possibility of tissue sequestration inducing a differential effect as time progresses, however, as described by Greenblatt et al. (1987), this does not happen with CNZ, which shows a constant brain–plasma concentration ratio and benzodiazepine receptor occupation by CNZ directly and predictably related to the drug's concentration in brain tissue. No change by CNZ was seen in nondiabetic rats reproducing previous studies with diazepam (Gomez et al., 1999). However, it was hypothesized that CNZ would decrease striatal extracellular glucose levels, because it reverses behavior that is influenced by high brain glucose levels such as those seen in diabetes (Gomez et al., 2003). This perplexing result could be explained by dysfunctional metabolic homeostasis in diabetic rats (Lund-Anderson, 1979). In fact, further studies are needed to prove this hypothesis and disregard other changes that might also occur, such as modification of $GABA_A$ receptor number or binding by diabetes, since diabetic animals showed supersensitive behavioral motor effects to CNZ (Bellush et al., 1991; Gomez, 1997).

Considerable evidence, including preclinical and clinical data, has accumulated, suggesting that GABA may play a

role in the pathophysiology of mood disorders such as depression (Gomez and Barros, 2000; Parent et al., 2002; Petty, 1995; Smith et al., 2002). How do we reconcile these observations of increased striatal glucose with the observations of decreased GABA in diabetes and the increased 'depressive mood' in diabetic rats as part of the diabetic encephalopathy? In humans, depression has been attributed to a failure of negative feedback from the frontal cortex and caudate to the amygdala (Drevets, 2000; Drevets et al., 2002). As demonstrated in an animal model of depression in diabetic rats (Gomez et al., 2003), decreased striatal GABA function in diabetic patients may be the cause of the increased amygdala activity. The decrease of GABA concentration could be a consequence of GAD activity inhibition due to an increase in intracellular ATP (Martin and Tobin, 2000), which accompanies high extracellular glucose. CNZ will overcome this deficiency by acting directly upon $GABA_A$ receptors, reversing the depressive-like behavioral changes, as seen in rats submitted to the forced swimming test. It remains to be tested if diabetic animals display different amounts of immobility in the forced swimming test after oral glucose overload and if CNZ still holds the same intensity of antidepressant effects in the glucose overload state. Therefore, determination of brain extracellular glucose levels during fasting and after glucose overload is important to investigate the mechanism by which glucose acts to modulate brain function, including modulation of neuronal excitability, neurotransmitter release and behavior in diabetic individuals. Both clinical and basic research needs to focus on the mechanisms involved in abnormal physiology of the brain in diabetes and the best ways to prevent chronic brain damage in patients.

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