Insulin reduces cerebral ischemia/reperfusion injury in the hippocampus of diabetic rats: a role for glycogen synthase kinase-3β

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ABSTRACT

Objective. There is evidence that insulin reduces brain injury evoked by ischemia/reperfusion (I/R). However, the molecular mechanisms underlying the protective effects of insulin remain unknown. Insulin is a well-known inhibitor of glycogen synthase kinase-3β (GSK-3β). Here we investigate the role of GSK-3β inhibition on I/R-induced cerebral injury in a rat model of insulinopenic diabetes.

Research Design and Methods. Streptozotocin-induced diabetic rats were subjected to 30 min occlusion of common carotid arteries followed by 1 h or 24 h reperfusion. Insulin (2-12 IU/kg, i.v.) or the selective GSK-3β inhibitor, TDZD-8 (0.1-3 mg/kg, i.v.), were administered during reperfusion.

Results. Insulin or TDZD-8 dramatically reduced infarct volume and levels of S100B protein, a marker of cerebral injury. Both drugs induced phosphorylation of the Ser9-residue, thereby, inactivating GSK-3β in the rat hippocampus. Insulin, but not TDZD-8, lowered blood glucose. The hippocampi of the drug-treated animals displayed reduced oxidative stress at 1 h reperfusion as shown by the decreased generation of reactive oxygen species and lipid peroxidation. I/R-induced activation of nuclear factor-κB was attenuated by both drug treatments. At 24 h reperfusion, TDZD-8 and insulin significantly reduced plasma levels of tumor-necrosis-factor-α, neutrophil infiltration, measured as myeloperoxidase activity and intercellular-adhesion-molecule-1 expression, and cyclooxygenase-2 and inducible-NO-synthase expression.

Conclusions. Acute administration of insulin or TDZD-8 reduced cerebral I/R injury in diabetic rats. We propose that the inhibitory effect on the activity of GSK-3β contributes to the protective effect of insulin, independently of any effects on blood glucose.
Epidemiologic studies have shown that diabetes is a leading risk factor for ischemic cerebrovascular diseases (1). Animal and human studies demonstrate that insulin reduces brain damage evoked by ischemia/reperfusion (I/R) injury (2; 3). Glycemic control by insulin may be involved in this protective effect, but the molecular mechanisms underlying the protective effects of insulin are debated and still poorly understood (4). One important pharmacological effect of insulin is its ability to inhibit the activity of the glycogen synthase kinase (GSK)-3, a serine/threonine kinase that was originally identified for its key role in glucose metabolism (5). More recently, GSK-3 has emerged as a key regulatory switch in the modulation of neurodegeneration and inflammation (6; 7). There are two mammalian isoforms of GSK-3: GSK-3α and GSK-3β. GSK-3β is highly expressed in the central nervous system (8). Unlike most kinases, GSK-3β is constitutively active in cells and can be inactivated by phosphorylation at Ser9 (9). Binding of insulin to its receptor activates phoshatidylinositol 3-kinase leading to the subsequent activation of protein kinase B/Akt, and the inactivation of GSK-3β by phosphorylation on the regulatory Ser-9. This contributes to the insulin-induced stimulation of glycogen synthesis. We and others have recently reported that various inhibitors of GSK-3β attenuated brain injury in rat models of cerebral I/R injury, with a marked reduction in infarct size (10-12). However, the potential protective effects of GSK-3β inhibition against cerebral I/R injury have never been tested in animal models of insulinopenic diabetes, where the lack of insulin may drastically influence GSK-3β basal activity. Hence, this study was undertaken to investigate (i) the effects of insulin administration on the organ injury associated with cerebral I/R in a rat model of insulinopenic diabetes and (ii) the role of GSK-3β inhibition in mediating insulin effects. To weigh the role of GSK-3β inhibition in the observed effects of insulin, TDZD-8, a potent and selective inhibitor of GSK-3β, was used as a comparative pharmacological tool.

RESEARCH DESIGN AND METHODS

Animals and surgery. Male Wistar rats (Harlan-Italy; Italy) were provided with a Piccioni pellet diet (n.48, Gessate Milanese, Italy) and water ad libitum. Insulinopenic diabetes was induced in 8-week old rats by a single intravenous tail vein injection of streptozotocin (STZ) (50 mg/kg). A blood sample was collected 4 days after the STZ injection, and plasma glucose was determined using a glucose analyzer (Accu-Chek Compact System, Roche Diagnostics, Switzerland). Diabetes was defined by a blood glucose >300 mg/dl. Animals were used 6 weeks later without insulin supplements.

Animal care was in compliance with Italian regulations on the protection of animals used for experimental and other scientific purposes (D.M.116/92). The experimental protocol was performed as described elsewhere (13). Briefly, rats were anesthetized through i.p. injection (30 mg/kg) of Zoletil 100 (a mixture of tiletamine and zolazepam, Laboratoires Virbac, France). The anesthetized rats were placed onto a thermostatically-controlled heating pad, a rectal temperature probe was inserted and body temperature was monitored and maintained at 37 °C. Ischemia was achieved by clamping the bilateral common carotid arteries for 30 min using non-traumatic artery clamps. Recirculation of blood flow was established by releasing the clips and restoration of blood flow in the carotid
arteries was confirmed by careful observation. Reperfusion was allowed for 1 or 24 h. At the end of the reperfusion, the anaesthetized rats were killed by decapitation after aortic exsanguination. After decapitation, the forebrain was rapidly dissected at 0 °C and the hippocampus from both hemispheres was quickly removed and transferred to a ice-chilled homogenizing medium for biochemical assays.

**Drugs and treatments.** Animals were randomly assigned to the following experimental groups:

- Sham and STZ: non-diabetic and diabetic rats were subjected to the surgical procedure alone, without causing ischemia (n = 8 per group).
- I/R and STZ I/R: rats were subjected to 30 min ischemia followed by 1 or 24 h reperfusion (n = 10 per group).
- STZ I/R + TDZD-8: diabetic rats, that underwent I/R, were treated with TDZD-8 (0.2-3 mg/kg, tail vein injection) at the beginning of reperfusion and again after 6 h reperfusion (n = 10 per group).
- STZ I/R + INSULIN: diabetic rats that underwent I/R, were treated with insulin (2 – 12 IU/kg, tail vein injection) at the beginning of reperfusion and again after 6 h reperfusion (n = 10 per group).

Two additional groups of diabetic rats received TDZD-8 (3 mg/kg, i.v.) or insulin (12 IU/kg, i.v.) before the sham operation (n = 4 per group).

**Determination of infarct volume.** At 1 day of reperfusion, the rats were killed with an overdose of anesthetic and decapitated. The brains were removed and placed in a brain matrix and coronal sections were cut into 2-mm slices. Brain slices were immersed in 0.5% 2,3,5-triphenyltetrazolium chloride monohydrate (TTC) solution at 37 °C for 30 min, followed by 4% paraformaldehyde solution. The infarct area and hemisphere area of each section were traced, quantified by an image analysis system (Inquiry; Loats, Westminster, U.S.A.) and expressed as percentage of infarct area in the whole brain.

**Tissue extracts.** Cytosolic and nuclear extracts were prepared by the Meldrum method (14). Briefly, rat hippocampi were homogenized and centrifuged at 4,000 g for 5 min at 4°C. Supernatants were removed and centrifuged at 15,000 g at 4°C for 40 min to obtain the cytosolic fraction. The pelleted nuclei were resuspended in extraction buffer. The suspensions were centrifuged at 15,000 g for 20 min at 4°C. The resulting supernatants containing nuclear proteins were carefully removed and protein content was determined using a BCA protein assay following the manufacturers’ directions.

**Determination of reactive oxygen species and GSH.** Reactive oxygen species (ROS) were measured fluorimetrically in cytosolic fractions using 2',7'-dichlorofluorescein diacetate (DCFH-DA) and the results were expressed as units of fluorescence (UF)/mg protein. Antioxidant levels in the cytosolic fractions were evaluated in terms of reduced GSH content by using Ellman’s method (15).

**End products of lipid peroxidation.** Lipid peroxidation was investigated by measurement of the end-product of peroxidation, hydroxynonenal (HNE), in the cytosol fractions. HNE concentration was determined on cytosol fractions by Esterbauer’s method (16).

**Myeloperoxidase (MPO) activity.** MPO activity, which was used as an indicator of polymorphonuclear leukocytes (PMNs) infiltration into the hippocampus, was determined as previously described (17).

**Serum concentration of tumor necrosis factor (TNF)-α by enzyme-linked immunosorbent assay (ELISA).** Four milliliters of blood were obtained from a peripheral artery and centrifuged at 1500 rpm for 15 min at 4°C. The content of TNF-α was
determined by using a rat TNF-α ELISA kit (Diaclone, France).

**Western blot analysis.** Western blots were carried out as previously described (13). Proteins were separated by 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were incubated with primary antibody (rabbit anti-total GSK-3β, goat anti-pGSK-3β Ser9, rabbit anti-iNOS, rabbit anti-COX-2, mouse anti-NF-κB p65, goat anti-S100B, goat anti-ICAM-1). Blots were then incubated with secondary antibodies and developed with the ECL detection system. The immunoreactive bands were visualized by autoradiography and the density of the bands was evaluated densitometrically using Gel Pro® Analyser 4.5, 2000 software (Media Cybernetics, Silver Spring, MD, USA).

**Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis.** Total RNA was extracted from the rat hippocampi using the RNA fast kit (Molecular Systems, San Diego, USA). Total DNA was amplified using sense and antisense primers specific for the GSK3β (sense 5’-CGTGACCAGTTGCTGAGT-3’ and antisense 5’-CGGGACCCAAATGTCAAACA-3’) and for GAPDH (sense 5’-AGATCCACAACGGATACATT-3’ and antisense 5’-TCCCTCAAGATTTGTCAGCAA-3’). Experiments were performed on at least three independent cDNA preparations. PCR products were electrophoresed on 2% agarose gels and amplification products were stained with GelStar nucleic acid gel stain (FMC BioProducts, Rockland, USA). Gels were photographed and analyzed with Kodak 1D Image Analysis software. The net intensity of bands in each experiment was normalized for the intensity of the corresponding GAPDH band.

**Electrophoretic Mobility Shift Assay (EMSA).** EMSA was performed by the method of Pahl et al. (18). NF-κB consensus oligonucleotides (5’-AGTTGAGGGACCTTTCCAGG-3’) were labelled with 32PγATP using T4 polynucleotide kinase and purified on QIAquick Nucleotide Removal Kit. For the EMSA, 50 μg of nuclear proteins were used. Specificity of binding was ascertained by competition with a 25-fold molar excess of unlabelled oligonucleotides. Recombinant human NF-κB incubating with the radiolabelled NF-κB-probe served as positive control.

**Materials.** Unless stated otherwise, all compounds were purchased from the Sigma-Aldrich Company Ltd. (St. Louis, USA). TDZD-8 was from Alexis Biochemicals (San Diego, USA). The BCA Protein Assay kit and blocking buffer were from Pierce Biotechnology Inc. (Rockford, USA). Antibodies were from Santa Cruz Biotechnology (Santa Cruz, California, USA). Luminol ECL was from Amersham (Buckinghamshire, UK).

**Statistical analysis.** All values in both the text and figures are expressed as mean ± standard error of the mean (S.E.M.) for n observations. One-way analysis of variance with Dunnett’s post test was performed using GraphPad Prism 4.02 (GraphPad Software, San Diego, California, USA) and p values below 0.05 were considered to be significant.

**RESULTS**

**Body weight and blood glucose levels.** Mean weight ± S.E.M. of the non-diabetic rats was 245±8 g (n = 18) and STZ-induced diabetes caused a significant decrease in body weight (214±18 g; n = 96; p<0.05). Diabetic rats had significantly higher non-fasting blood glucose (423±32 mg/dl; p<0.05) compared to normal controls (108 ± 8 mg/dl). The dose-response curve of glycemic control by insulin was
measured after drug administration at 1 h reperfusion, with the maximal reduction after 12 IU/kg insulin (115±12 mg/dl; p<0.05). 2 IU/kg insulin resulted in a significant, but lower, decrease of blood glucose (192±11 mg/dl; p<0.05). The acute injection of TDZD-8 (3 mg/kg) did not significantly decrease blood glucose levels at the time of I/R injury (403±28 mg/dl).

**Effect of insulin and TDZD-8 on GSK-3β expression and phosphorylation.** As shown by RT-PCR, diabetic rats exhibited a two-fold increase in GSK-3β total expression compared with non-diabetic rats (Fig. 1A). Neither I/R nor administration of insulin or TDZD-8 to diabetic rats further modified the total GSK-3β mRNA levels (Fig. 1A). When GSK-3β inhibition was evaluated in terms of levels of Ser9 phosphorylation (Fig. 1B), diabetic rats showed a stronger basal activation of the enzyme as the ratio of Ser9 phosphorylated GSK-3β/total GSK-3β was lower in the diabetic group than in the control group (p<0.05). Densitometric analysis of the autoradiograms (Fig. 1B) showed that, in the hippocampi of sham-operated animals, almost 60% of total GSK-3β was phosphorylated on Ser9, while Ser9 phosphorylation was less than 30% of total GSK-3β in sham-operated diabetic rats. I/R had no effect on the levels of Ser9 phosphorylation, while both insulin and TDZD-8 increased Ser9 phosphorylation when administered to sham-operated diabetic rats (data not shown). As shown in Fig. 1C, administration of TDZD-8 and insulin to diabetic rats that had undergone I/R promoted GSK-3β phosphorylation at both 1 h and 24 h reperfusion, in a dose-dependent fashion. TDZD-8 administration induced Ser9 phosphorylation in the dose-range of 0.2–3 mg/kg, with maximum effect at 3 mg/kg, and this dose was used for all subsequent experiments. Insulin significantly increased Ser9 phosphorylation at the doses of 2, 6 and 12 IU/kg. The lowest dose of insulin, 2 IU/kg, evoked a significant level of Ser9 phosphorylation, quantitatively similar to that measured with 3 mg/kg TDZD-8 and not statistically different from that recorded in the presence of 12 IU/kg insulin. Therefore, based on these data and the following results on infarct size (see below), we chose 2 IU/kg as the reference dose for subsequent experiments.

**Insulin and TDZD-8 reduce the severity of cerebral infarction and neutrophil infiltration.** Rats which underwent cerebral ischemia followed by 24 h reperfusion showed an infarct volume of 23.4±3.9% of the total brain volume (I/R group; Fig. 2A). Infarct size was larger in diabetic animal exposed to I/R (STZ I/R group; 34.9±4.8%; p<0.05). Administration of insulin halved the I/R-induced infarct volume in diabetic animals, but no differences in efficacy were observed for any of the doses tested (2-12 IU/kg). A similar reduction in infarct volume was measured when TDZD-8 (3 mg/kg) was administered during reperfusion.

S100B, a calcium binding protein, which has been recognized as a marker of neuronal damage, was scantily detectable in the hippocampi of sham-operated non-diabetic and diabetic animals (Sham and STZ groups, respectively). Non-diabetic and diabetic rats subjected to I/R exhibited a two-fold and three-fold increase, respectively, when measured at 24 h reperfusion (Fig. 2B). Treatment with insulin and TDZD-8 almost completely abolished the increase in the hippocampal content of S100B, so that values of S100B measured in animals treated with insulin or TDZD-8 were similar to those measured in diabetic, sham-operated animals. The improvement in the outcome of I/R injury was associated with a reduced neutrophil infiltration measured in reperfused hippocampi at 24 h (Fig. 3). MPO activity was significantly elevated in diabetic rats subjected to I/R (70.17±4.12 µU MPO/tissue...
g) in comparison with diabetic sham-operated rats (14.27±5.14 µU MPO/tissue g) (Fig. 3A). In both insulin- and TDZD-8-treated diabetic animals, the MPO activity was significantly attenuated (47.28±2.22 and 41.93±2.87 µU MPO/tissue g, respectively, p < 0.05). The adhesion molecule ICAM-1, which is the endothelial ligand for the neutrophil receptor CD11b/CD18, was scarcely detected in the hippocampus from sham-operated diabetic animals and its expression was strongly induced by 24 h reperfusion (Fig. 3B). Insulin and TDZD-8 prevented the I/R-induced up-regulation of ICAM-1 (p < 0.05), without any significant differences between the two drugs.

**Effects of insulin and TDZD-8 on oxidative stress.** In order to gain a better understanding of the degree of oxidative stress associated with diabetes and cerebral I/R, we determined ROS formation and concentrations of GSH and HNE (a toxic end product of lipid peroxidation) in hippocampal homogenates obtained after cerebral ischemia followed by 1 h of reperfusion (Table 1). Diabetic rats showed an increase in oxidative stress when compared to their wild-type littermates. I/R evoked a 70% increase in ROS production, which was associated with a dramatic increase in HNE. The I/R-induced lipid peroxidation was strongly decreased in hippocampal homogenates obtained from animals subjected to cerebral I/R that were treated with either insulin or TDZD-8. Administration of TDZD-8 caused a significant decrease in ROS overproduction. Insulin also reduced ROS production, but this effect did not achieve statistical significance. GSH levels showed no statistical differences among any of the groups studied and were, hence, not significantly altered by either I/R or by drug treatment.

**Effects of insulin and TDZD-8 on NF-κB nuclear activity.** The activation of NF-κB was evaluated by both Western blot analysis and EMSA. Measurement of the nuclear translocation of the p65 subunit NF-κB from the cytosolic to the nuclear fraction of tissue extracts showed higher levels of p65 subunit in the nucleus than non-diabetic animals, thus suggesting a basal NF-κB activation secondary to diabetes (data not shown). A further increase in NF-κB translocation from cytosol to the nucleus was recorded in diabetic rats subjected to cerebral ischemia followed by 1 h of reperfusion (Fig. 4A), but not by 24 h of reperfusion (data not shown). Interestingly, both insulin and TDZD-8 produced a marked inhibition of the I/R-induced NF-κB activation. EMSA was performed to assess the effects of I/R and drug treatment on NF-κB DNA-binding activity (Fig. 4B). In the hippocampus of diabetic rats that underwent I/R, nuclear NF-κB signal was strongly activated. The administration of insulin or TDZD-8 significantly attenuated (at the same level) NF-κB activation.

**Effects of insulin and TDZD-8 on inflammatory markers.** Western blot analysis showed that the hippocampal expression of both COX-2 and iNOS proteins was higher in diabetic animals than in their wild-type littermates (data not shown). In diabetic rats, I/R was associated with an increase in COX-2 and iNOS protein expression at 24 h reperfusion (Fig. 5A and 5B), and this effect was attenuated to a similar degree by either insulin or TDZD-8. Similarly, TNF-α levels detected in the serum of diabetic animals were higher than those recorded in non-diabetic rats (Sham group: 19.8±4.8 pg/ml; STZ group: 65.8±8.8 pg/ml), and reached three-fold basal levels at 24 h of reperfusion (STZ I/R group: 195±10.7 pg/ml) (Fig. 5C). Treatment with insulin or TDZD-8 prevented the I/R-induced rise in the serum concentration of this cytokine (94.8±11 and 108±8.8 pg/ml, respectively, p < 0.05).
DISCUSSION

Our findings support previous studies that demonstrate that the acute administration of insulin reduces the brain injury associated with I/R (2; 3). Recent clinical trials have shown that insulin can be safely administered in ischemic stroke to both diabetic and non-diabetic patients (19-21). However, to date, the mechanisms by which insulin protects the brain remain unclear, and involvement of glycemic control as the sole underlying mechanism of the observed protection remains dubious (4). As insulin is a well-characterized inhibitor of the activation of GSK-3β (9), this study was designed to compare the effects of insulin with those of TDZD-8, a selective inhibitor of the activation of GSK-3β, on the degree of injury caused by cerebral I/R in the hippocampus, as this region of the brain (i) is most sensitive to I/R injury (22), and (ii) has the most pronounced expression of GSK-3β (8). A reduction in GSK-3β activity has been previously demonstrated to improve brain tolerance to I/R in non-diabetic animals (10; 11; 12), but its effects against I/R injury in the presence of diabetes has never been tested. The present study provides lines of evidence suggesting a potential association between diabetes and enhanced susceptibility to a cerebral ischemic insult and the protective effect of GSK-3β inhibition. Specifically, the I/R-induced infarct volume was increased by 40% in diabetic rats when compared with their non-diabetic littermates (control). Insulin administration significantly reduced infarct volume and a similar effect was recorded when rats were treated with the GSK-3β inhibitor TDZD-8. Inhibition of the activity of GSK-3β with either TDZD-8 or insulin also abolished the I/R-induced rise in the levels of S100B, a member of the S100 family of calcium-binding proteins, mainly expressed in the brain (23). Clinical studies indicate that the increase in the levels of S100B correlate with an impairment of hippocampal function as well as cerebral infarct size (24). In our study, diabetes caused a 50% increase in the I/R-induced expression of S100B protein, while TDZD-8 and insulin reduced this effect of I/R in diabetic animals. It has to be stressed, however, that the lack of behavioral testing related to the observed brain damage limits the interpretation of the clinical transferability of our findings. Thus, a further rigorous evaluation of effects of the tested compounds on any behavioral impairment caused by cerebral I/R is needed.

To demonstrate the involvement of the GSK-3β pathway in mediating the protective effects exerted by insulin and TDZD-8, we analyzed the effects of both drugs on the phosphorylation of the Ser9 residue on GSK-3β, which is the key site determining the activity of this kinase (with an increase in phosphorylation indicating the inhibition of this kinase). Both TDZD-8 and insulin markedly enhanced the hippocampal Ser9 phosphorylation and hence inhibition of GSK-3β activity, at both 1 and 24 h of reperfusion. Previous studies in non-diabetic animal models have shown that insulin and TDZD-8 increase GSK3β phosphorylation in the rodent brain (10; 25), but this is the first study demonstrating their effects on cerebral GSK-3β in the presence of diabetes. Another major observation made in the present study was that both basal GSK-3β expression and activity were up-regulated in the hippocampus of diabetic animals compared to those in age-matched normal animals. As insulin is reported to negatively regulate GSK3β activity, we propose that STZ-induced lack of insulin contributes to the resulting over-expression and activation of GSK3β in the early stages of experimental diabetes. Therefore, the insulinopenic rat can represent a suitable experimental model for testing the potential protective effects evoked by pharmacological inhibition of the GSK3β
signaling pathway. However, regulation of GSK3β in diabetic conditions differs dramatically among tissues and animal species and models. Other authors (26) showed that central administration of a very low STZ dose (1 mg/kg), that does not produce diabetes, increased GSK3β phosphorylation in the rat hippocampus, whereas the level of total GSK3β expression were unchanged. In diabetic animals, GSK3β activity has been reported to be increased in epididymal fat, slightly decreased in the liver, and unchanged in skeletal muscle (27-29). Others found that GSK3β activity was elevated approximately twofold in skeletal muscle samples from human patients with type 2 diabetes (30). Recent studies suggest that serotoninergic activity may regulate the inhibitory Ser9-phosphorylation of GSK3β in the rodent hippocampus (31), thus raising the question whether ligands of serotoninergic receptors may enhance the effects evoked by insulin. Similarly, further studies are needed to better elucidate whether TDZD-8 can enhance the protective action of insulin.

Oxidative stress and inflammation are known to be implicated in the pathogenesis of cerebral I/R injury (32-33). We recently observed (13) that 30 min ischemia followed by 1 h reperfusion causes significant oxidative stress, whereas the inflammatory response is delayed (by 6-24 h). Here we demonstrated that diabetic animals show a higher level of oxidative stress and inflammation when compared to non-diabetic animals and, most notably, insulin and TDZD-8 partially affect oxidative stress and cause a substantial decrease of the inflammatory response. TNF-α has been identified as one of the pivotal pro-inflammatory cytokines that exacerbate I/R injury (33) and, recently, the attenuation of insulin signaling cascade evoked by TNF-α, has been suggested to involve Ser9 phosphorylation of GSK3β (34). In our experimental model, the ability of both insulin and TDZD-8 to abolish the increase in serum levels of TNF-α and, at the same time, to reduce the expression of iNOS and COX-2 confirms the role of GSK3β in contributing to protection against I/R injury. An important marker of an inflamed and dysfunctional endothelium is the increased leukocyte adhesion. Experimental evidence indicates that leukocyte adhesion in response to I/R is increased in diabetic animals (35) and, thus, represents a common link between I/R injury and diabetes. Here we show that neutrophil infiltration of previously ischemic sections of the brain was reduced by GSK3β inhibition, as both insulin and TDZD-8 abolished the expression of the adhesion molecule ICAM-1 and attenuated (to a similar degree) the increase in tissue MPO activity.

NF-κB plays a fundamental role in the development of both I/R injury and diabetes (36;37) and GSK3β has been shown to affect NF-κB transcriptional activity in a promotor-specific manner, demonstrating that GSK3β selectively supports the expression of a subset of genes activated by NF-κB (38-40). As observed in the present study, GSK-3β inhibition with either insulin or TDZD-8 was associated with a significant reduction of the nuclear NF-κB activity, which may account for the observed reduction in the expression of COX-2, iNOS and ICAM-1, all of which are NF-κB-dependent proteins. As GSK-3β has been linked to the regulation of other transcription factors, including AP-1, NF-AT and CREB (40), further investigation are needed to gain a better insight into the role of these transcription factors in the protective effects caused by GSK-3β inhibition.

One particularly interesting finding was the qualitative difference of the effects of insulin and TDZD-8 on blood glucose levels. Specifically, acute administration of insulin rapidly lowered STZ-induced hyperglycemia, while the GSK-3β inhibitor TDZD-8 did not
affect blood glucose levels. As TDZD-8 treatment differed from insulin in the modulation of blood glucose levels, while the effects on infarct size and markers of oxidative stress and inflammation were similar, we would like to propose that the beneficial effects of insulin observed in our model of cerebral I/R are, at least in part, due to the inhibition of GSK3β activity, but not directly due to the lowering of blood glucose. This hypothesis warrants further investigation.

In conclusion, our results point to a role for GSK3β signaling in the protective effects exerted by insulin in a rat model of cerebral I/R injury. Both expression and activity of GSK3β were higher in the rat hippocampus of insulinopenic diabetic animals when compared with their non-diabetic littermates. We provide evidence that treatment of STZ-induced diabetic rats with insulin or TDZD-8 decreases experimental cerebral I/R injury, possibly by attenuating the signaling of GSK3β. However, we are aware that further studies evaluating insulin and/or TDZD-8 effects on the alterations in animal behaviour caused by cerebral I/R are warranted to clarify the potential clinical relevance of our findings.

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REFERENCES


TABLE 1
ROS, GSH and HNE content in hippocampi homogenates from non-diabetic or diabetic rats, exposed to 30 min ischemia and 1 h reperfusion without or with drug treatment.

<table>
<thead>
<tr>
<th></th>
<th>ROS (U.F./mg protein)</th>
<th>GSH (µg/mg protein)</th>
<th>HNE (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>89.5 ± 10.49*</td>
<td>57.08 ± 0.97</td>
<td>0.24 ± 0.16*</td>
</tr>
<tr>
<td>STZ</td>
<td>150.3 ± 9.50</td>
<td>37.07 ± 5.68</td>
<td>3.90 ± 0.21*</td>
</tr>
<tr>
<td>I/R</td>
<td>141.7 ± 8.52</td>
<td>39.67 ± 6.03</td>
<td>4.8 ± 0.3</td>
</tr>
<tr>
<td>STZ I/R</td>
<td>198.7 ± 16.65</td>
<td>32.67 ± 6.03</td>
<td>5.65 ± 0.26</td>
</tr>
<tr>
<td>STZ I/R + INS (2 IU/kg)</td>
<td>147.3 ± 18.79</td>
<td>44.89 ± 8.54</td>
<td>3.20 ± 0.34*</td>
</tr>
<tr>
<td>STZ I/R + TDZD-8 (3 mg/kg)</td>
<td>133.8 ± 25.11*</td>
<td>47.10 ± 10.68</td>
<td>3.29 ± 0.46*</td>
</tr>
</tbody>
</table>

Data are means ± S.E.M. of five animals/group
★ p< 0.05 vs STZ I/R
Figure 1. GSK-3β expression and Ser9 phosphorylation in the rat hippocampus. GSK-3β mRNA expression was analyzed by RT-PCR in the hippocampus of non-diabetic (Sham) and diabetic (STZ) rats and was compared with GSK-3β mRNA expression in diabetic rats that underwent 30 min cerebral ischemia followed by 24 h reperfusion (STZ I/R). Two groups of diabetic rats received TDZD-8 (3 mg/kg) or insulin (12 IU/kg) during reperfusion (STZ I/R + TDZD-8 and STZ I/R + INSULIN, respectively). (Panel A). Western blot analysis of total GSK-3β protein expression and Ser9 phosphorylation in both non-diabetic (Sham) and diabetic (STZ) animals is shown in Panel B. The reduced ratio Ser9 phosphorylated GSK-3β/total GSK-3β in the STZ group indicates a stronger basal activation of the enzyme in the presence of diabetes. The dose-response of TDZD-8 and insulin administration on GSK-3β phosphorylation detected at both 1 h and 24 h reperfusion is reported in Panel C. Densitometric analysis of the bands is expressed as relative optical density (O.D.) of GSK-3β phosphorylation at Ser9, corrected for the corresponding total GSK-3β content and normalized using the related sham-operated band. The data are means ± S.E.M. of three separate experiments.

● p < 0.05 versus Sham; ★ p < 0.05 versus STZ I/R.
Figure 2. Total infarct volume (Panel A) and hippocampal S100B expression (Panel B)

Diabetic rats that underwent 30 min ischemia followed by 24 h reperfusion (STZ I/R) showed higher levels of infarct volume and S100B expression than non-diabetic rats exposed to I/R (I/R). Additional groups of diabetic rats received TDZD-8 (STZ I/R + TDZD-8 3 mg/kg) or insulin (2 – 12 IU/kg) (STZ I/R + INSULIN) during reperfusion. The infarct volume data are means ± S.E.M. of four rats per group. The immunoblot of S100B protein expression and the corresponding β-actin are representative of three separate experiments. ● p < 0.05 versus I/R; ★ p < 0.05 versus STZ I/R.
Figure 3. Effects of insulin and TDZD-8 on I/R-induced neutrophil infiltration in the hippocampus of diabetic rats. Myeloperoxidase (MPO) activity (Panels A) and ICAM-1 expression (Panel B) were measured in hippocampi homogenates of sham-operated diabetic rats (STZ) and diabetic rats that underwent 30 min ischemia and 24 h reperfusion (STZ I/R). Insulin (2 IU/kg) and TDZD-8 (3 mg/kg) were administered during reperfusion (STZ I/R + INSULIN and STZ I/R + TDZD-8). Each immunoblot is from a single experiment and is representative of three separate experiments. Densitometric analysis of the bands is expressed as relative optical density (O.D.), corrected for the corresponding β-actin contents and normalized using the related sham-operated band. Data are means ± S.E.M. of three separate experiments for Western Blot and four animals/group for MPO. * p < 0.05 versus STZ I/R.
**Figure 4. Effect of insulin and TDZD-8 treatment on NF-κB activation evoked by cerebral I/R injury in diabetic rats.** NF-κB translocation from the cytosol to the nucleus was evaluated by Western blot analysis (Panel A) in sham-operated diabetic rats (STZ) and diabetic rats at 1 h reperfusion (STZ I/R), measuring NF-κB p65 subunit levels in both cytosol and nuclear fractions and expressing the results as nucleus/cytosol ratio. Insulin (2 IU/kg) or TDZD-8 (3 mg/kg) were administered at the beginning of reperfusion (STZ I/R + INSULIN and STZ I/R + TDZD-8, respectively). The data from bands densitometric analysis are means ± S.E.M. of three separate experiments. ★ p < 0.05 versus STZ I/R. DNA binding activity of NF-κB was evaluated by EMSA (Panel B) on hippocampus nuclear extracts isolated from sham-operated diabetic rats (STZ) and diabetic rats at 1 h reperfusion (STZ I/R) with or without insulin (2 IU/kg) or TDZD-8 (3 mg/kg) treatment. Specificity of binding was ascertained by competition with a 25-fold molar excess of unlabelled oligonucleotides (Cf). Recombinant human NF-κB incubated with the radiolabelled NF-κB probe served as a positive control (Cp); middle panel, DNA binding activity of NF-κB was quantified by densitometry in the different groups. The values are means ±S.D. of 6-7 rats per group and expressed as percent variation compared to DNA-binding activity of NF-κB of control.
Figure 5. Alteration in hippocampal expression of COX-2 and iNOS and serum levels of TNF-α induced by insulin or TDZD-8 treatment. Diabetic rats (STZ) were subjected to 30 min ischemia and 24 h reperfusion (STZ I/R). Insulin (2 IU/kg) or TDZD-8 (3 mg/kg) was administered during reperfusion (STZ I/R + INSULIN and STZ I/R + TDZD-8, respectively). COX-2 and iNOS protein expression was evaluated by Western Blot analysis (Panels A and B, respectively). Each immunoblot is from a single experiment and is representative of three separate experiments. Serum levels of TNF-α were analyzed by enzyme-linked immunosorbent assay (ELISA) (Panel C). Densitometric analysis of the bands is expressed as relative optical density (O.D.), corrected for the corresponding β-actin contents and normalized using the related sham-operated band. Data are means ± S.E.M. of three separate experiments for Western Blot and six animals/group for ELISA. * p < 0.05 versus STZ I/R.