# Insulin Reduces Cerebral Ischemia/Reperfusion Injury in the Hippocampus of Diabetic Rats

## A Role for Glycogen Synthase Kinase-3β

**Massimo Collino,[1] Manuela Aragno,[2] Sara Castiglia,[1] Chiara Tomasinelli,[2] Christoph Thiemermann,[3] Giuseppe Boccuzzi,[4] and Roberto Fantozzi[1]**

**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—**Rats with streptozotocin-induced diabetes were subjected to 30-min occlusion of common carotid arteries followed by 1 or 24 h of reperfusion. Insulin (2–12 IU/kg i.v.) or the selective GSK-3β inhibitor TDZD-8 (0.2–3 mg/kg i.v.) was 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 of 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 of 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. ***Diabetes*** **58:235–242, 2009**

From the [1]Department of Anatomy, Pharmacology, and Forensic Medicine, University of Turin, Turin, Italy; the [2]Department of Experimental Medicine and Oncology, University of Turin, Turin, Italy; the [3]Centre for Translational Medicine and Therapeutics, William Harvey Research Institute, Barts and the London School of Medicine and Dentistry, Queen Mary University of London, London, U.K.; and the [4]Department of Clinical Pathophysiology, University of Turin, Turin, Italy.

Corresponding author: Massimo Collino, massimo.collino@unito.it.

Received 22 May 2008 and accepted 26 September 2008.

Published ahead of print at http://diabetes.diabetesjournals.org on 7 October 2008. DOI: 10.2337/db08-0691.

M.C. and M.A. contributed equally to this work.



Epidemiological 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 phosphatidylinositol 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, in which the lack of insulin may drastically influence GSK-3β basal activity. Hence, this study was undertaken to investigate *1*) the effects of insulin administration on the organ injury associated with cerebral I/R in a rat model of insulinopenic diabetes and *2*) 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, San Pietro al Natisone, Italy) were provided with a Piccioni pellet diet (48; Piccioni, Gessate Milanese, Italy) and water ad libitum. Insulinopenic diabetes was induced in 8-week-old rats by a single intravenous tail vein injection of 50 mg/kg streptozotocin (STZ). 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, Basel, 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 previously (13). Briefly, rats were anesthetized through injection of 30 mg/kg Zoletil 100 i.p. (a mixture of tiletamine and zolazepam; Laboratoires Virbac, Carros, 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 nontraumatic 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 anesthetized 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. *1*) Sham and STZ: nondiabetic and diabetic rats were subjected to the surgical procedure alone, without causing ischemia ($n = 8$ per group). *2*) I/R and STZ I/R: rats were subjected to 30 min of ischemia followed by 1 or 24 h of reperfusion ($n = 10$ per group). *3*) STZ I/R + TDZD-8: diabetic rats that underwent I/R were treated with 0.2–3 mg/kg TDZD-8 (tail vein injection) at the beginning of reperfusion and again after 6 h of reperfusion ($n = 10$ per group). *4*) STZ I/R + insulin: diabetic rats that underwent I/R were treated with 2–12 IU/kg insulin (tail vein injection) at the beginning of reperfusion and again after 6 h of reperfusion ($n = 10$ per group). Two additional groups of diabetic rats received 3 mg/kg TDZD-8 i.v. or 12 IU/kg insulin 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 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, MD), 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 bicinchoninic acid (BCA) protein assay following the manufacturer's directions.

**Determination of reactive oxygen species and glutathione.** Reactive oxygen species (ROS) were measured fluorimetrically in cytosolic fractions using 2′,7′-dichlorofluorescein diacetate, and the results were expressed as units fluorescence per milligram protein. Antioxidant levels in the cytosolic fractions were evaluated in terms of reduced glutathione (GSH) content 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 activity.** Myeloperoxidase (MPO) activity, which was used as an indicator of polymorphonuclear leukocyte infiltration into the hippocampus, was determined as previously described (17).

**Serum concentration of tumor necrosis factor-α by enzyme-linked immunosorbent assay.** Four milliliters of blood were obtained from a peripheral artery and centrifuged at 1,500 rpm for 15 min at 4°C. The content of tumor necrosis factor-α (TNF-α) was determined using a rat TNF-α ELISA kit (Diaclone, Besançon, France).

**Western blot analysis.** Western blots were carried out as previously described (13). Proteins were separated by 8% SDS-PAGE and transferred to polyvinyldenedifluoride membranes. The membranes were incubated with primary antibody (rabbit anti–total GSK-3β, goat anti–pGSK-3β Ser9, rabbit anti–inducible nitric oxide synthase (iNOS), rabbit anti–cyclo-oxigenase (COX)-2, mouse anti–nuclear factor-κB [anti–NF-κB] p65, goat anti-S100B, and goat anti–intercellular adhesion molecule (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).

**RT-PCR analysis.** Total RNA was extracted from the rat hippocampi using the RNA fast kit (Molecular Systems, San Diego, CA). Total DNA was amplified using sense and antisense primers specific for the GSK-3β (sense, 5′-CGTGACCAGTGTTGCTGAGT-3′, and antisense, 5′-CGGGACCCAAATGTCAAACA-3′) and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense, 5′-AGATCCACAACGGATACATT-3′, and antisense, 5′-TCCCTCAAGATTGTCAGCAA-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, ME). 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.** Electrophoretic mobility shift assay (EMSA) was performed by the method of Pahl et al. (18). NF-κB consensus oligonucleotides (5′-AGTTGAGGGGACTTTCCCAGG-3′) were labeled with [$\gamma$-$^{32}$P]ATP using $T_4$ polynucleotide kinase and purified on QIAquick Nucleotide Removal kit. For the EMSA, 50 μg nuclear proteins was used. Specificity of binding was ascertained by competition with a 25-fold molar excess of unlabeled oligonucleotides. Recombinant human NF-κB incubating with the radiolabeled NF-κB probe served as positive control.

**Materials.** Unless stated otherwise, all compounds were purchased from Sigma-Aldrich (St. Louis, MO). TDZD-8 was from Alexis Biochemicals (San Diego, CA). The BCA Protein Assay kit and blocking buffer were from Pierce Biotechnology (Rockford, IL). Antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Luminol ECL was from Amersham (Buckinghamshire, U.K.).

**Statistical analysis.** All values in both the text and figures are expressed as means ± SE for $n$ observations. One-way ANOVA with Dunnett's post test was performed using GraphPad Prism 4.02 (GraphPad Software, San Diego, CA), and $P$ values $<0.05$ were considered to be significant.

## RESULTS

**Body weight and blood glucose levels.** Mean weight ± SE of the nondiabetic 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 nonfasting blood glucose (423 ± 32 mg/dl; $P < 0.05$) compared with normal controls (108 ± 8 mg/dl). The dose-response curve of glycemic control by insulin was measured after drug administration at 1 h of reperfusion, with the maximal reduction after 12 IU/kg insulin (115 ± 12 mg/dl; $P < 0.05$). A significant, but lower, decrease of blood glucose (192 ± 11 mg/dl; $P < 0.05$) resulted from 2 IU/kg insulin. The acute injection of 3 mg/kg TDZD-8 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 twofold increase in GSK-3β total expression compared with nondiabetic rats (Fig. 1*A*). Neither I/R nor administration of insulin or TDZD-8 to diabetic rats further modified the total GSK-3β mRNA levels (Fig. 1*A*). When GSK-3β inhibition was evaluated in terms of levels of Ser9 phosphorylation (Fig. 1*B*), diabetic rats showed a stronger basal activation of the enzyme because the ratio of Ser9 phosphorylated GSK-3β to total GSK-3β was lower in the diabetic group than in the control group ($P < 0.05$). Densitometric analysis of the autoradiograms (Fig. 1*B*) showed that in the hippocampi of sham-operated animals, ~60% of total GSK-3β was phosphorylated on Ser9, whereas Ser9 phosphorylation was <30% of total GSK-3β in sham-operated diabetic rats. I/R had no effect on the levels of Ser9 phosphorylation, whereas both insulin and TDZD-8 increased Ser9 phosphorylation when administered to sham-operated diabetic rats (data not shown). As shown in Fig. 1*C*, administration of TDZD-8 and insulin to diabetic rats that had undergone I/R promoted GSK-3β phosphorylation at both 1 and 24 h of 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

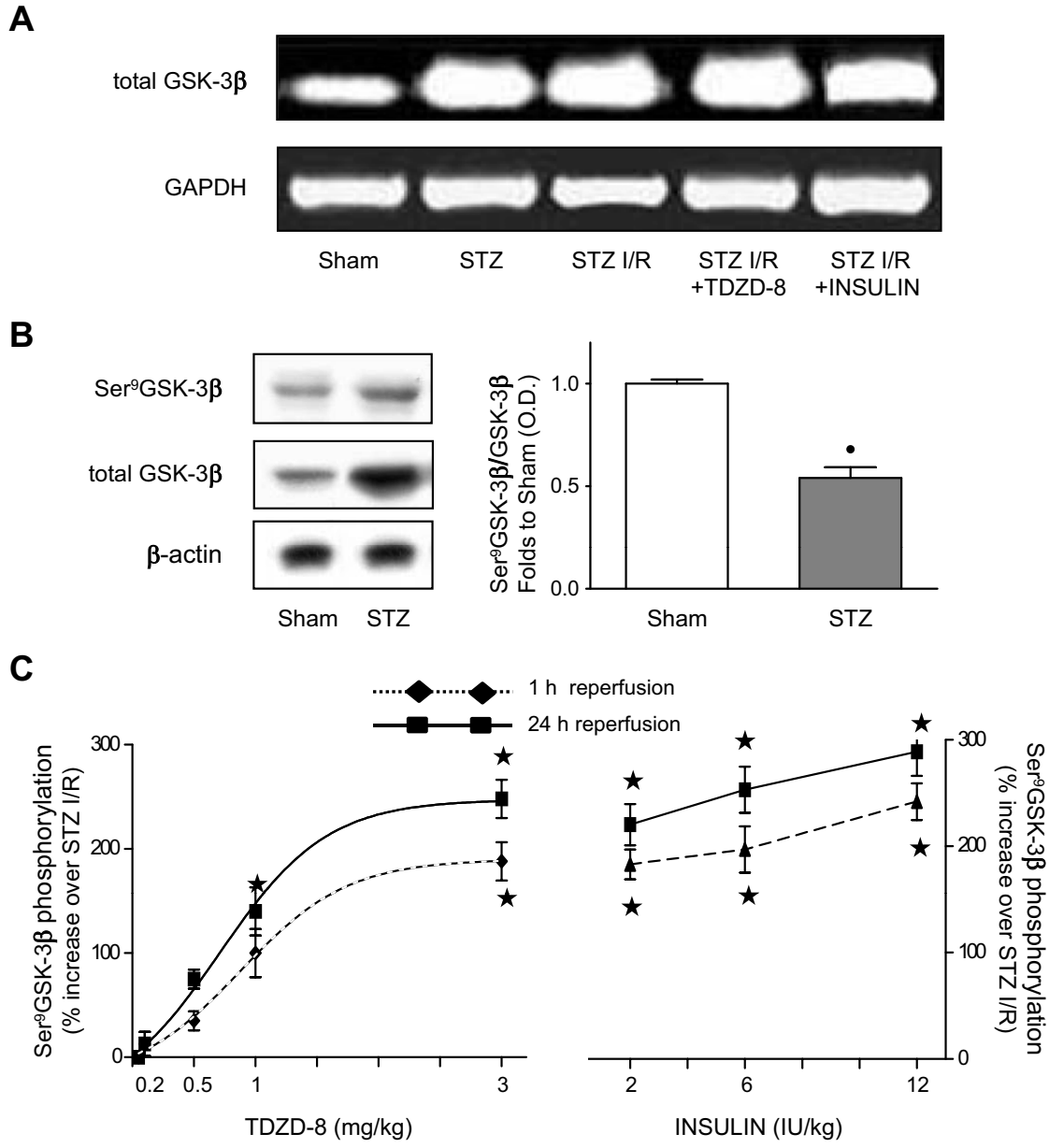


**FIG. 1. GSK-3β expression and Ser9 phosphorylation in the rat hippocampus. *A*: GSK-3β mRNA expression was analyzed by RT-PCR in the hippocampus of nondiabetic (Sham) and diabetic (STZ) rats and was compared with GSK-3β mRNA expression in diabetic rats that underwent 30-min cerebral ischemia followed by 24 h of reperfusion (STZ I/R). Two groups of diabetic rats received 3 mg/kg TDZD-8 or 12 IU/kg insulin during reperfusion (STZ I/R + TDZD-8 and STZ I/R + INSULIN, respectively). *B*: Western blot analysis of total GSK-3β protein expression and Ser9 phosphorylation in both nondiabetic (Sham) and diabetic (STZ) animals. 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. *C*: The dose response of TDZD-8 and insulin administration on GSK-3β phosphorylation detected at both 1 and 24 h of reperfusion. Densitometric analysis of the bands is expressed as relative optical density of GSK-3β phosphorylation at Ser9 corrected for the corresponding total GSK-3β content and normalized using the related sham-operated band. Data are means ± SE of three separate experiments. ●*P* < 0.05 vs. Sham; ⋆*P* < 0.05 vs. STZ I/R.**

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 that underwent cerebral ischemia followed by 24 h of reperfusion showed an infarct volume of 23.4 ± 3.9% of the total brain volume (I/R group; Fig. 2*A*). Infarct size was larger in diabetic animals 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 3 mg/kg TDZD-8 was administered during reperfusion.

S100B, a calcium binding protein that has been recognized as a marker of neuronal damage, was scantily detectable in the hippocampi of sham-operated nondiabetic and diabetic animals (sham and STZ groups, respectively). Nondiabetic and diabetic rats subjected to I/R exhibited a two- and threefold increase, respectively, when measured at 24 h of reperfusion (Fig. 2*B*). Treatment

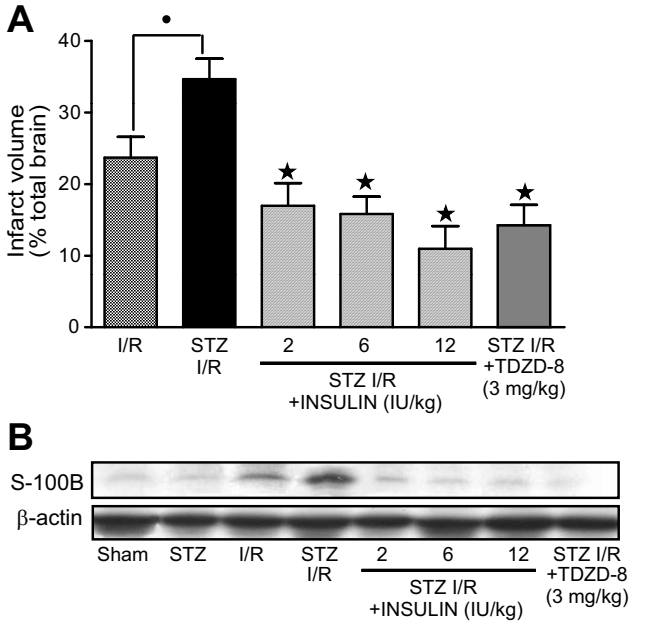


**FIG. 2. Total infarct volume (*A*) and hippocampal S100B expression (*B*). Diabetic rats that underwent 30 min of ischemia followed by 24 h of reperfusion (STZ I/R) showed higher levels of infarct volume and S100B expression than nondiabetic rats exposed to I/R. Additional groups of diabetic rats received 3 mg/kg TDZD-8 (STZ I/R + TDZD-8) or 2–12 IU/kg insulin (STZ I/R + INSULIN) during reperfusion. The infarct volume data are means ± SE 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 vs. I/R; ⋆*P* < 0.05 vs. STZ I/R.**

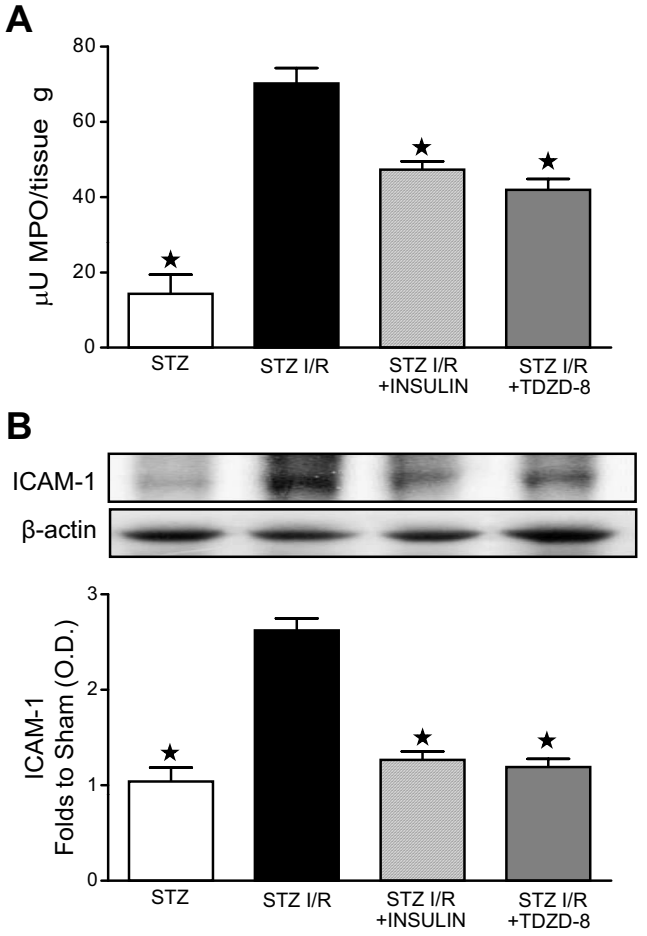


**FIG. 3. Effects of insulin and TDZD-8 on I/R-induced neutrophil infiltration in the hippocampus of diabetic rats. MPO activity (*A*) and ICAM-1 expression (*B*) were measured in hippocampi homogenates of sham-operated diabetic rats (STZ) and diabetic rats that underwent 30 min of ischemia and 24 h of 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 ± SE of three separate experiments for Western blot and four animals per group for MPO. ⋆*P* < 0.05 vs. STZ I/R.**

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/g tissue) (Fig. 3*A*). 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/g tissue, 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 of reperfusion (Fig. 3*B*). Insulin and TDZD-8 prevented the I/R-induced upregulation of ICAM-1 ($P < 0.05$), without any significant differences between the two drugs.

**Effects of insulin and TDZD-8 on oxidative stress.** 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 with 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 nondiabetic 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. 4*A*) but not by 24 h of reperfusion (data not shown). Interestingly, both insulin and TDZD-8 produced a marked inhibition of the I/R-

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

| | ROS (units fluorescence/mg protein) | GSH (μg/mg protein) | HNE (μmol/l) |
|---|---|---|---|
| Sham | 89.5 ± 10.49* | 57.08 ± 0.97 | 0.24 ± 0.16* |
| STZ | 150.3 ± 9.50 | 37.07 ± 5.68 | 3.90 ± 0.21* |
| I/R | 141.7 ± 8.52 | 39.67 ± 6.03 | 4.8 ± 0.3 |
| STZ I/R | 198.7 ± 16.65 | 32.67 ± 6.03 | 5.65 ± 0.26 |
| STZ I/R + INS (2 IU/kg) | 147.3 ± 18.79 | 44.89 ± 8.54 | 3.20 ± 0.34* |
| STZ I/R + TDZD-8 (3 mg/kg) | 133.8 ± 25.11* | 47.10 ± 10.68 | 3.29 ± 0.46* |

Data are means ± SE of five animals/group. *$P < 0.05$ vs. STZ 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. 4*B*). 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 of reperfusion (Fig. 5*A* and *B*), 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 nondiabetic rats (sham group, 19.8 ± 4.8 pg/ml; STZ group, 65.8 ± 8.8 pg/ml) and reached threefold basal levels at 24 h of reperfusion (STZ I/R group, 195 ± 10.7 pg/ml) (Fig. 5*C*). 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 nondiabetic 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). Because 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, because this region of the brain *1*) is most sensitive to I/R injury (22) and *2*) 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 nondiabetic animals (10–12), 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 nondiabetic 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 and cerebral infarct size (24). In our study, diabetes caused a 50% increase in the I/R-induced expression of S100B protein, whereas 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 nondiabetic animal models have shown that insulin and TDZD-8 increase GSK-3β 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 upregulated in the hippocampus of diabetic animals compared with those in age-matched normal animals. Because insulin is reported to negatively regulate GSK-3β activity, we propose that STZ-induced lack of insulin contributes to the resulting overexpression and activation of GSK-3β 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 GSK-3β signaling pathway. However, regulation of GSK-3β 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 GSK-3β phosphorylation in the rat hippocampus, whereas the level of total GSK-3β expression was unchanged. In diabetic animals, GSK-3β activity has been reported to be increased in epididymal fat, slightly decreased in the liver, and unchanged in skeletal muscle (27–29). Others found

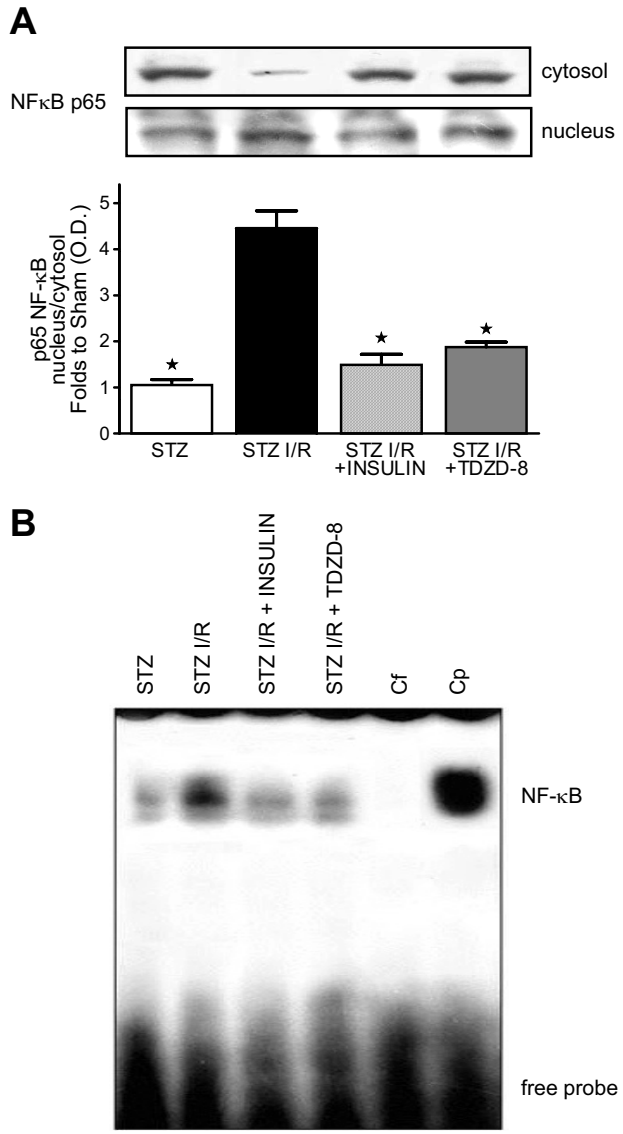


**FIG. 4. Effect of insulin and TDZD-8 treatment on NF-κB activation evoked by cerebral I/R injury in diabetic rats. *A*: NF-κB translocation from the cytosol to the nucleus was evaluated by Western blot analysis in sham-operated diabetic rats (STZ) and diabetic rats at 1 h of reperfusion (STZ I/R), measuring NF-κB p65 subunit levels in both cytosol and nuclear fractions and expressing the results as nucleus-to-cytosol ratio. Insulin (2 IU/kg) or TDZD-8 (3 mg/kg) was 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 ± SE of three separate experiments. ⋆*P* < 0.05 vs. STZ I/R. *B*: DNA binding activity of NF-κB was evaluated by EMSA on hippocampus nuclear extracts isolated from sham-operated diabetic rats (STZ) and diabetic rats at 1 h of reperfusion (STZ I/R) with or without 2 IU/kg insulin or 3 mg/kg TDZD-8 treatment. Specificity of binding was ascertained by competition with a 25-fold molar excess of unlabeled oligonucleotides (Cf). Recombinant human NF-κB incubated with the radiolabeled NF-κB probe served as a positive control (Cp).**

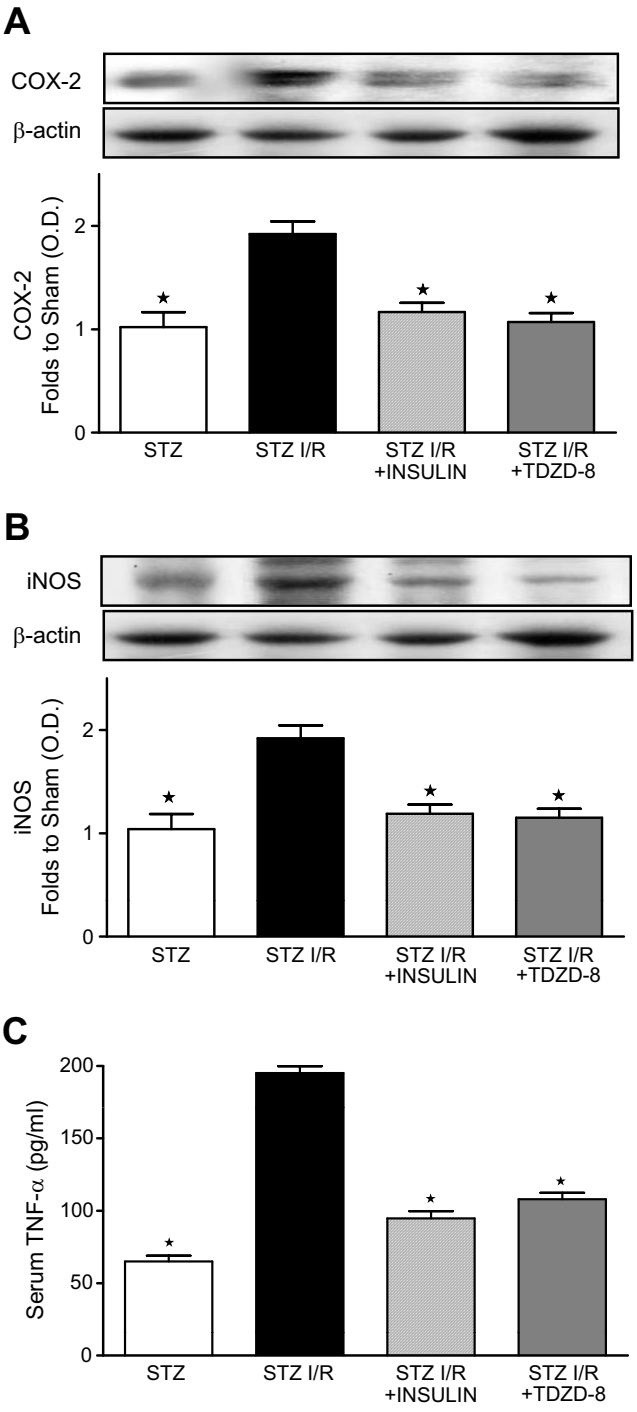


**FIG. 5. Alteration in hippocampal expression of COX-2 and iNOS and serum levels of TNF-α induced by insulin or TDZD-8 treatment. Diabetic rats (STZ) were subjected to 30 min of ischemia and 24 h of reperfusion (STZ I/R). Insulin (2 IU/kg) or TDZD-8 (3 mg/kg) was administered during reperfusion (STZ I/R + INSULIN and STZ I/R + TDZD-8, respectively). COX-2 and iNOS protein expression was evaluated by Western blot analysis (*A* and *B*, respectively). Each immunoblot is from a single experiment and is representative of three separate experiments. *C*: Serum levels of TNF-α were analyzed by enzyme-linked immunosorbent assay (ELISA). 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 ± SE of three separate experiments for Western blot and six animals per group for ELISA. ⋆*P* < 0.05 vs. STZ I/R.**

that GSK-3β 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 GSK-3β in the rodent hippocampus (31), thus raising the question of 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 of ischemia followed by 1 h of 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 with nondiabetic 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 proinflammatory 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 GSK-3β (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 GSK-3β 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 GSK-3β inhibition, because 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 GSK-3β has been shown to affect NF-κB transcriptional activity in a promoter-specific manner, demonstrating that GSK-3β 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. Because GSK-3β has been linked to the regulation of other transcription factors, including activated protein-1, nuclear factor of activated T-cells, and cAMP response element binding (40), further investigations 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, whereas the GSK-3β inhibitor TDZD-8 did not affect blood glucose levels. Because TDZD-8 treatment differed from insulin in the modulation of blood glucose levels, whereas 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 GSK-3β 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 GSK-3β signaling in the protective effects exerted by insulin in a rat model of cerebral I/R injury. Both expression and activity of GSK-3β were higher in the rat hippocampus of insulinopenic diabetic animals when compared with their nondiabetic 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 GSK-3β. However, we are aware that further studies evaluating insulin and/or TDZD-8 effects on the alterations in animal behavior caused by cerebral I/R are warranted to clarify the potential clinical relevance of our findings.

## ACKNOWLEDGMENTS

C.T. has received support from the William Harvey Research Foundation. This study was supported by Turin University funding, Regione Piemonte, Ministry of Education, University and Research (Progetti di Ricerca di Interesse Nazionale, 2007) and Alfieri Project (Cardiomiopatia Diabetica: Individuazione di Nuove Strategie Terapeutiche).

No potential conflicts of interest relevant to this article were reported.

## REFERENCES

1. Tanne D: Impaired glucose metabolism and cerebrovascular diseases. *Adv Cardiol* 45:107–113, 2008
2. Rizk NN, Rafols JA, Dunbar JC: Cerebral ischemia-induced apoptosis and necrosis in normal and diabetic rats: effects of insulin and C-peptide. *Brain Res* 1096:204–212, 2006
3. Hui L, Pei DS, Zhang QG, Guan QH, Zhang GY: The neuroprotection of insulin on ischemic brain injury in rat hippocampus through negative regulation of JNK signaling pathway by PI3K/Akt activation. *Brain Res* 1052:1–9, 2005
4. Donnan GA, Levi C: Glucose and the ischaemic brain: too much of a good thing? *Lancet Neurol* 6:380–381, 2007
5. Embi N, Rylatt DB, Cohen P: Glycogen synthase kinase-3 from rabbit skeletal muscle: separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase. *Eur J Biochem* 107:519–527, 1980
6. Martinez A, Castro A, Dorronsoro I, Alonso M: Glycogen synthase kinase 3 (GSK-3) inhibitors as new promising drugs for diabetes, neurodegeneration, cancer, and inflammation. *Med Res Rev* 22:373–384, 2002
7. Dugo L, Collin M, Thiemermann C: Glycogen synthase kinase 3beta as a target for the therapy of shock and inflammation. *Shock* 27:113–123, 2007
8. Leroy K, Brion JP: Developmental expression and localization of glycogen synthase kinase-3beta in rat brain. *J Chem Neuroanat* 16:279–293, 1999
9. Cohen P, Goedert M: GSK3 inhibitors: development and therapeutic potential. *Nat Rev Drug Discov* 3:479–487, 2004
10. Collino M, Thiemermann C, Mastrocola R, Gallicchio M, Benetti E, Miglio G, Castiglia S, Danni O, Murch O, Dianzani C, Aragno M, Fantozzi R: Treatment with the glycogen synthase kinase-3β inhibitor, TDZD-8, affects transient cerebral ischemia/reperfusion injury in the rat hippocampus. *Shock* 30:299–307, 2008
11. Nonaka S, Chuang DM: Neuroprotective effects of chronic lithium on focal cerebral ischemia in rats. *Neuroreport* 9:2081–2084, 1998
12. Kelly S, Zhao H, Hua Sun G, Cheng D, Qiao Y, Luo J, Martin K, Steinberg GK, Harrison SD, Yenari MA: Glycogen synthase kinase 3beta inhibitor Chir025 reduces neuronal death resulting from oxygen-glucose deprivation, glutamate excitotoxicity, and cerebral ischemia. *Exp Neurol* 188:378–386, 2004
13. Collino M, Aragno M, Mastrocola R, Benetti E, Gallicchio M, Dianzani C, Danni O, Thiemermann C, Fantozzi R: Oxidative stress and inflammatory response evoked by transient cerebral ischemia/reperfusion: effects of the PPAR-alpha agonist WY14643. *Free Radic Biol Med* 41:579–589, 2006
14. Meldrum DR, Shenkar R, Sheridan BC, Cain BS, Abraham E, Harken AH: Hemorrhage activates myocardial NFkappaB and increases TNF-alpha in the heart. *J Mol Cell Cardiol* 29:2849–2854, 1997
15. Ellman GL: Tissue sulphydryl groups. *Arch Biochem Biophys* 82:70–77, 1959
16. Esterbauer H, Koller E, Slee RG, Koster JF: Possible involvement of the lipid peroxidation product 4-hydroxynonenal in the formation of fluorescent chromolipids. *Biochem J* 239:405–409, 1986
17. Mullane KM, Kraemer R, Smith B: Myeloperoxidase activity as a quantitative assessment of neutrophil infiltration into ischemic myocardium. *J Pharmacol Methods* 14:157–167, 1985
18. Pahl HL, Bauerle PA: Expression of influenza virus hemagglutinin activates the transcription factor NF-kB. *J Virol* 69:1480–1484, 1995
19. Walters MR, Weir CJ, Lees KR: A randomised, controlled pilot study to investigate the potential benefit of intervention with insulin in hyperglycaemic acute ischaemic stroke patients. *Cerebrovasc Dis* 22:116–122, 2006
20. Gray CS, Hildreth AJ, Sandercock PA, O'Connell JE, Johnston DE, Cartlidge NE, Bamford JM, James OF, Alberti KG, GIST Trialists Collabo-

ration: Glucose-potassium-insulin infusions in the management of post-stroke hyperglycaemia: the U.K. Glucose Insulin in Stroke Trial (GIST-UK). *Lancet Neurol* 6:397–406, 2007
21. Bruno A, Kent TA, Coull BM, Shankar RR, Saha C, Becker KJ, Kissela BM, Williams LS: Treatment of hyperglycemia in ischemic stroke (THIS): a randomized pilot trial. *Stroke* 39:384–389, 2008
22. Sharma BK, Kumar K: Role of proinflammatory cytokines in cerebral ischemia: a review. *Metab Brain Dis* 13:1–8, 1998
23. Foerch C, Singer OC, Neumann-Haefelin T, du Mesnil de Rochemont R, Steinmetz H, Sitzer M: Evaluation of serum S100B as a surrogate marker for long-term outcome and infarct volume in acute middle cerebral artery infarction. *Arch Neurol* 62:1130–1134, 2005
24. Heizmann CW, Fritz G, Schafer BW: S100 proteins: structure, functions and pathology. *Front Biosci* 7:1356–1368, 2002
25. Planel E, Miyasaka T, Launey T, Chui DH, Tanemura K, Sato S, Murayama O, Ishiguro K, Tatebayashi Y, Takashima A: Alterations in glucose metabolism induce hypothermia leading to tau hyperphosphorylation through differential inhibition of kinase and phosphatase activities: implications for Alzheimer's disease. *J Neurosci* 24:2401–2411, 2004
26. Salkovic-Petrisic M, Tribl F, Schmidt M, Hoyer S, Riederer P: Alzheimer-like changes in protein kinase B and glycogen synthase kinase-3 in rat frontal cortex and hippocampus after damage to the insulin signalling pathway. *J Neurochem* 96:1005–1015, 2006
27. Eldar-Finkelman H, Schreyer S, Shinohara M, LeBoeuf R, Krebs E: Increased glycogen synthase kinase-3 activity in diabetes- and obesity-prone C57BL/6J mice. *Diabetes* 48:1662–1666, 1999
28. Semiz S, Orvig C, McNeill J: Effects of diabetes, vanadium, and insulin on glycogen synthase activation in Wistar rats. *Mol Cell Biochem* 231:23–35, 2002
29. Henriksen EJ, Kinnick TR, Teachey MK, O'Keefe MP, Ring D, Johnson KW: Modulation of muscle insulin resistance by selective inhibition of GSK-3 in Zucker diabetic fatty rats. *Am J Physiol Endocrinol Metab* 284:892–900, 2003
30. Nikoulina SE, Ciaraldi TP, Mudaliar S, Mohideen P, Carter L, Henry RR: Potential role of glycogen synthase kinase-3 in skeletal muscle insulin resistance of type 2 diabetes. *Diabetes* 49:263–271, 2000
31. Li X, Zhu W, Roh MS, Friedman AB, Rosborough K, Jope RS: In vivo regulation of glycogen synthase kinase-3beta (GSK3beta) by serotonergic activity in mouse brain. *Neuropsychopharmacology* 29:1426–1431, 2004
32. Schaller B, Graf R: Cerebral ischemia and reperfusion: the pathophysiologic concept as a basis for clinical therapy. *J Cereb Blood Flow Metab* 24:351–371, 2004
33. Del Zoppo GJ: Stroke and neurovascular protection. *N Engl J Med* 354:553–555, 2006
34. Gupta D, Varma S, Khandelwal RL: Long-term effects of tumor necrosis factor-alpha treatment on insulin signaling pathway in HepG2 cells and HepG2 cells overexpressing constitutively active Akt/PKB. *J Cell Biochem* 100:593–607, 2007
35. Granger DN: Ischemia-reperfusion: mechanisms of microvascular dysfunction and the influence of risk factors for cardiovascular disease. *Microcirculation* 6:167–178, 1999
36. Nichols TC: NF-kappaB and reperfusion injury. *Drug News Perspect* 17:99–104, 2004
37. Cameron NE, Cotter MA: Pro-inflammatory mechanisms in diabetic neuropathy: focus on the nuclear factor kappa B pathway. *Curr Drug Targets* 9:60–67, 2008
38. Steinbrecher KA, Wilson W III, Cogswell PC, Baldwin AS: Glycogen synthase kinase 3β functions to specify gene-specific, NF-κB-dependent transcription. *Mol Cell Biol* 25:8444–8455, 2005
39. Dugo L, Collin M, Allen DA, Murch O, Foster SJ, Yaqoob MM, Thiemermann C: Insulin reduces the multiple organ injury and dysfunction caused by coadministration of lipopolysaccharide and peptidoglycan independently of blood glucose: role of glycogen synthase kinase-3beta inhibition. *Crit Care Med* 34:1489–1496, 2006
40. Martin M, Rehani K, Jope RS, Michalek SM: Toll-like receptor-mediated cytokine production is differentially regulated by glycogen synthase kinase 3. *Nat Immunol* 6:777–784, 2005