



BRAZILIAN JOURNAL
OF MEDICAL AND BIOLOGICAL RESEARCH

www.bjournal.com.br

ISSN 0100-879X
Volume 42 (11) 993-1118 November 2009

BIOMEDICAL SCIENCES
AND
CLINICAL INVESTIGATION

Braz J Med Biol Res, November 2009, Volume 42(11)1044-1049

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The Brazilian Journal of Medical and Biological Research is partially financed by



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Stem cell factor protects against neuronal apoptosis by activating AKT/ERK in diabetic mice

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Abstract

Neuronal apoptosis occurs in the diabetic brain due to insulin deficiency or insulin resistance, both of which reduce the expression of stem cell factor (SCF). We investigated the possible involvement of the activation of the MAPK/ERK and/or AKT pathways in neuroprotection by SCF in diabetes. Male C57/B6 mice (20-25 g) were randomly divided into four groups of 10 animals each. The morphology of the diabetic brain in mice treated or not with insulin or SCF was evaluated by H&E staining and TUNEL. SCF, ERK1/2 and AKT were measured by Western blotting. In diabetic mice treated with insulin or SCF, there was fewer structural change and apoptosis in the cortex compared to untreated mice. The apoptosis rate of the normal group, the diabetic group receiving vehicle, the diabetic group treated with insulin, and the diabetic group treated with SCF was $0.54 \pm 0.077\%$, $2.83 \pm 0.156\%$, $1.86 \pm 0.094\%$, and $1.78 \pm 0.095\%$ (mean \pm SEM), respectively. SCF expression was lower in the diabetic cortex than in the normal cortex; however, insulin increased the expression of SCF in the diabetic cortex. Furthermore, expression of phosphorylated ERK1/2 and AKT was decreased in the diabetic cortex compared to the normal cortex. However, insulin or SCF could activate the phosphorylation of ERK1/2 and AKT in the diabetic cortex. The results suggest that SCF may protect the brain from apoptosis in diabetes and that the mechanism of this protection may, at least in part, involve activation of the ERK1/2 and AKT pathways. These results provide insight into the mechanisms by which SCF and insulin exert their neuroprotective effects in the diabetic brain.

Key words: Stem cell factor; Diabetes; MAPK/ERK; AKT

Introduction

Although structural abnormalities in neural tissue such as synaptic and neuronal degeneration and neuronal apoptosis have been demonstrated in type 1 and type 2 diabetic rodents (1-3), very little is known about the mechanisms leading to these defects. Many researchers have suggested that neuronal apoptosis in diabetes is related to hyperglycemia (4), and insulin, proinsulin C peptide and insulin-like growth factor (IGF) have anti-apoptotic effects (5,6). Furthermore, insulin or insulin-like growth factor-1 (IGF-1) induced the expression of stem cell factor (SCF)

and a lower expression of SCF was observed in the mouse gut during diabetic gastroparesis (7,8). SCF, also termed Kit ligand, steel factor, mast cell growth factor, or c-kit ligand, is a receptor of tyrosine kinase. Both SCF and Kit support neuronal survival and function and influence cell differentiation, migration, and proliferation possibly by mutual interaction (9,10). Knockdown of SCF results in abnormalities in learning and memory in mice (11,12). SCF is a cytokine highly expressed in the central nervous system and protects neurons from apoptosis (13,14). SCF-mediated signal

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Research supported by the National Nature Science Foundation (#30470612, #30670739) of China, the Doctoral Program Foundation of the Ministry of Education of China (#20060284044), and the International Cooperation Program and Outstanding Researcher Program (#BZ2006045, #06-B-002, #RC2007006) of Jiangsu Province of China.

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Received December 27, 2008. Accepted August 24, 2009. Available online October 5, 2009.

transduction may involve many signaling pathways. The cytoprotection induced by SCF is associated with activation of the AKT and/or MAPK signaling pathways (14,15). In the present study, we tested the hypothesis that SCF protects neurons from apoptosis in diabetes via a mechanism that partially involves the AKT and ERK pathways.

Material and Methods

Animal model

The animal study was conducted in accordance with National Regulations of Experimental Animal Administration. All experimental animal protocols were approved by the Committee of Experimental Animal Administration of Nanjing Medical University. Diabetes was induced in male C57/B6 mice (20-25 g) with a single intraperitoneal injection of streptozotocin (100 mg/kg in 0.1 M citrate buffer, pH 4.4; Sigma, USA); 72 h later the plasma glucose level of the mice (diabetes type I model) was above 300 mg/dL (OneTouch® Ultra Blood Glucose Monitoring System, Johnson, USA) (16). Plasma glucose was evaluated weekly for 4 weeks (see plasma glucose level in Figure 1). Control mice received an equal volume of citrate buffer. Mice were randomly divided into four groups of 10 animals each, i.e., normal control (CON), diabetic receiving vehicle (DM), diabetic treated with insulin (DM + INS: 40 U·kg⁻¹·day⁻¹; Wanbang Bio-pharm, China), and diabetic treated with SCF (DM + SCF: 0.1 mg·kg⁻¹·48 h⁻¹; BD, USA). Four weeks later seven brains from each group were rapidly removed and the cerebral cortices and hippocampi were isolated. Three brains from each group were transcidentally perfused with cold 0.1 M phosphate buffer, pH 7.4, followed by 4.0% paraformaldehyde. The samples were stored at -80°C for later experiments.

HE staining and TUNEL assay

Brain sections were stained with hematoxylin and eosin

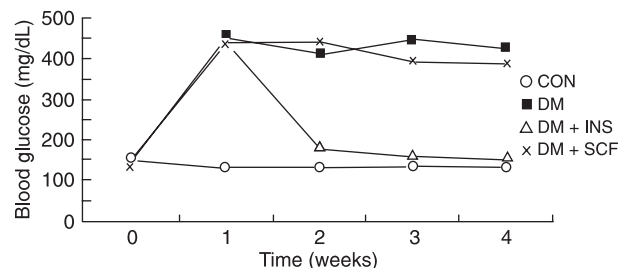


Figure 1. Plasma glucose curve. Type 1 diabetes was induced in mice by a single intraperitoneal injection of streptozotocin (100 mg/kg), with plasma glucose levels exceeding 250 mg/dL 72 h later. Plasma glucose was then measured weekly. CON = normal mice injected with citrate buffer; DM = diabetic mice injected with vehicle; DM + INS = diabetic mice treated with insulin (40 U·kg⁻¹·day⁻¹); DM + SCF = diabetic mice treated with stem cell factor (0.1 mg·kg⁻¹·48 h⁻¹).

for histological assay. Cell damage was also assessed by TUNEL (Boster, Wuhan, China), which was performed as previously described (17). Briefly, 20- μ m brain sections were incubated with TdT labeling buffer containing dUTP mix, Mn²⁺ and TdT enzyme for 60 min at 37°C to incorporate biotinylated nucleotides at the sites of DNA breaks. Sections were also incubated with diluted extraavidin-peroxidase link for 30 min at 37°C, followed by the addition of DAB (diaminobenzidine tetrahydrochloride) at room temperature to develop a brown color. They were counterstained with modified Harris' hematoxylin, changing to blue with TBS and observed microscopically. The number of cells exhibiting a positive TUNEL signal was quantified with the Soft Imaging System (Image Pro Plus 5.02, Media Cybernetics, Inc., USA).

Western blotting

Total protein and protein expression by the cortex was assayed as described previously (17). Membranes were probed with primary antibodies against SCF (1:500, BD), c-kit (1:500, Santa Cruz Biotechnology, USA), phosphorylated ERK1/2, AKT (cell signaling, 1:1000), total ERK1/2, AKT or GAPDH (cell signaling, 1:1000). Proteins were detected using horseradish peroxidase-conjugated anti-goat or anti-rabbit secondary antibodies and visualized by chemiluminescence using an ECL kit (Amersham Pharmacia Biotech, USA) and exposure to film. The intensity of the blots was quantified by densitometry (AlphaEaseFC, Alpha Innotech Corporation, USA). GAPDH, total ERK or total AKT were used as loading controls.

Statistical analysis

Data are reported as means \pm SEM. One-way ANOVA followed by multiple comparisons was used for statistical comparisons. In all statistical procedures, a probability value of $P < 0.05$ was used as a cutoff for statistical significance.

Results and Discussion

Cortical apoptosis appeared in the diabetic mice

Model type 1 diabetic mice (blood glucose level 300-450 mg/dL) were prepared using streptozotocin. To confirm previous studies, pathology and cell apoptosis were determined in the diabetic brains. Histological results confirmed strong staining of the nuclei, an intensely eosinophilic cytoplasm and widening of Virchow-Robin spaces. Along with these changes, neuronal apoptosis occurred in the same areas (Figure 2B,C). CON, DM + SCF or INS animals showed few pathological changes of this kind (Figure 2A). The pathological injuries in the cortex of diabetic mice, including structural changes and apoptosis, were fewer in animals pretreated with insulin or SCF. However, in contrast to previous studies, the hippocampus did not show structural changes or apoptosis (Figure 3A,B). In other words, there

is a diabetes-related programmed neuronal cell death in the hippocampus, which results in neuronal loss in type 1 diabetes (18, 19). Different results might be obtained using different models or at different times after experimental hyperglycemia. To test this model, we used a lower dose of streptozotocin and performed the experiment by the 4th week. The observed difference in structure changes may have been related to milder diabetic damage.

SCF protects against apoptosis in the diabetic brain

There is evidence that SCF expression levels can influence neuronal apoptosis in the diabetic brain. It is well known that diabetes results from insulin deficiency or variable degrees of insulin resistance. However, insulin or IGF-1 is a critical factor for inducing SCF expression and maintaining SCF signaling (7,8). Reduced SCF resulted

in smooth muscle myopathy and loss of interstitial cells of Cajal in murine diabetic gastroparesis (8). SCF and its receptor (c-kit) are expressed throughout the adult nervous system. SCF includes both membrane-bound SCF (M-SCF) and soluble SCF (S-SCF), the latter consisting of cleaved soluble forms of SCF (9,10). The present study showed that the levels of both M-SCF and S-SCF in the diabetic cortex were lower than that in the normal cortex (Figure 4) and that insulin can increase both M-SCF and S-SCF in the diabetic brain. Further study revealed that cortical apoptosis appeared in the diabetic mice and that SCF could attenuate it (Figure 2).

In an increasing number of studies, it has been shown that neuronal expression of SCF is induced in various forms of brain injury and that SCF expression after brain injury is correlated with neurogenesis and the mechanism of brain

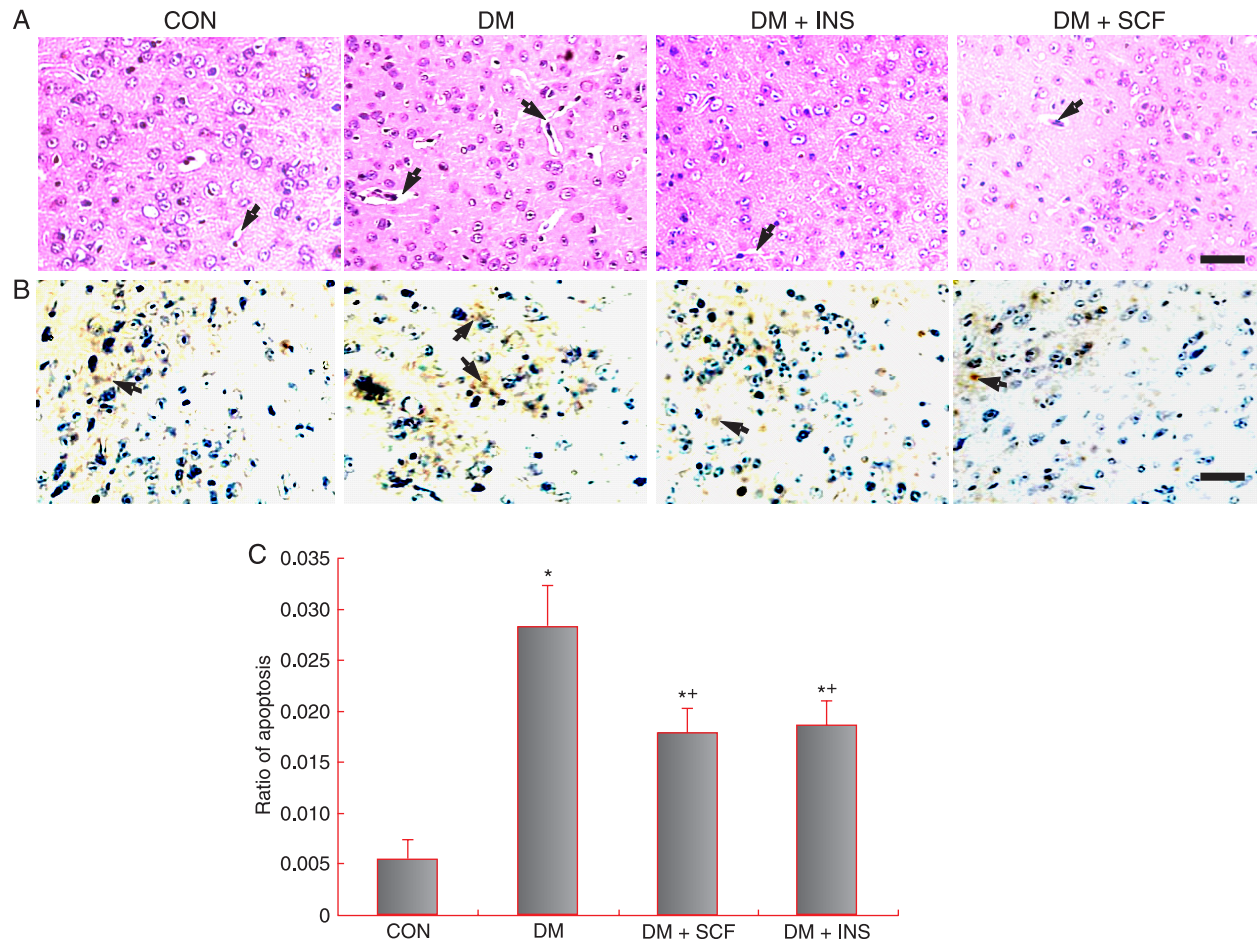


Figure 2. Morphology of the cortex. *A*, Hematoxylin and eosin staining. The DM group exhibited intensely eosinophilic cytoplasm, condensed nuclei, and widening of Virchow-Robin spaces (arrows). CON, DM + SCF, or DM + INS showed few of these pathological changes. Bar = 25 μ m. *B*, TUNEL assay. Many apoptotic cells (arrows, brown color) appeared in the DM group, and less apoptosis occurred in DM + SCF, and DM + INS groups. Bar = 40 μ m. *C*, Quantitation of TUNEL assay. The apoptosis rates of CON, DM, DM + INS, and DM + SCF groups were $0.54 \pm 0.077\%$, $2.83 \pm 0.156\%$, $1.86 \pm 0.094\%$, and $1.78 \pm 0.095\%$, respectively. * $P < 0.05$ compared to CON group, \dagger compared to DM group (one-way ANOVA). For abbreviations, see legend to Figure 1.

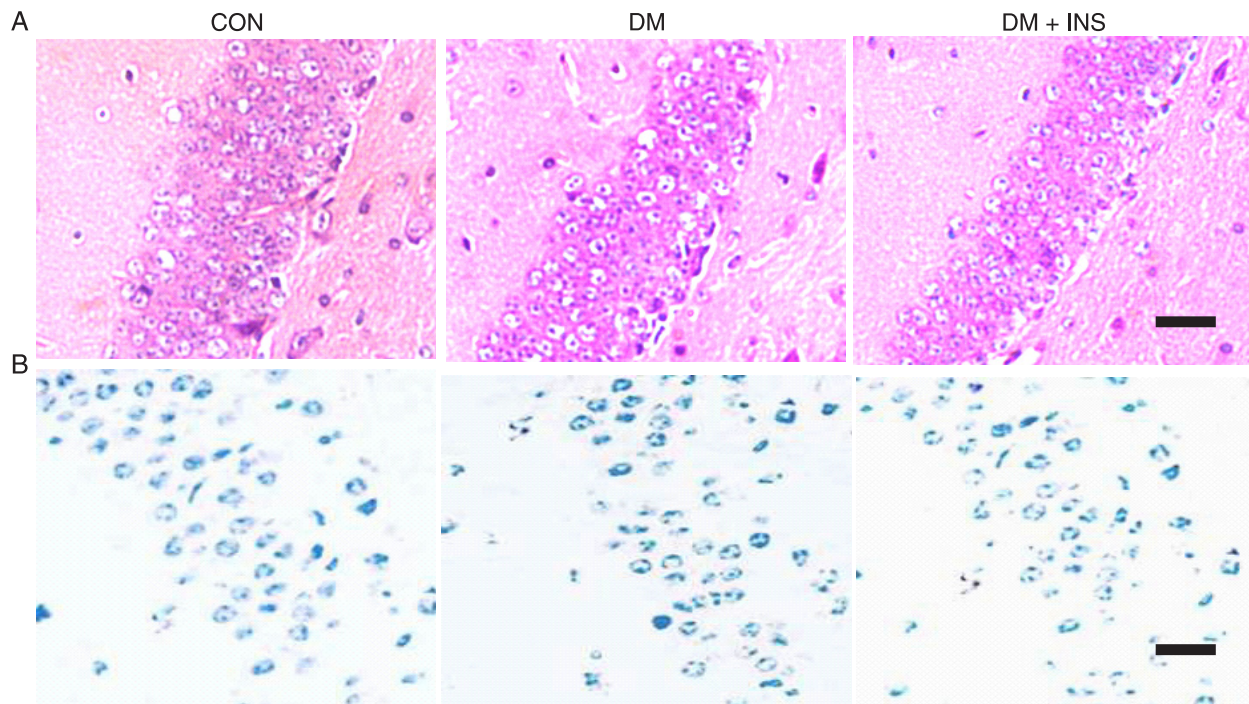


Figure 3. Morphology of the hippocampus. *A*, Hematoxylin and eosin staining. No significant structure change among the three groups was found in the hippocampus. Bar = 40 μ m. *B*, TUNEL assay. No apoptosis was observed in the hippocampus among the three groups. Bar = 25 μ m. For abbreviations, see legend to Figure 1.

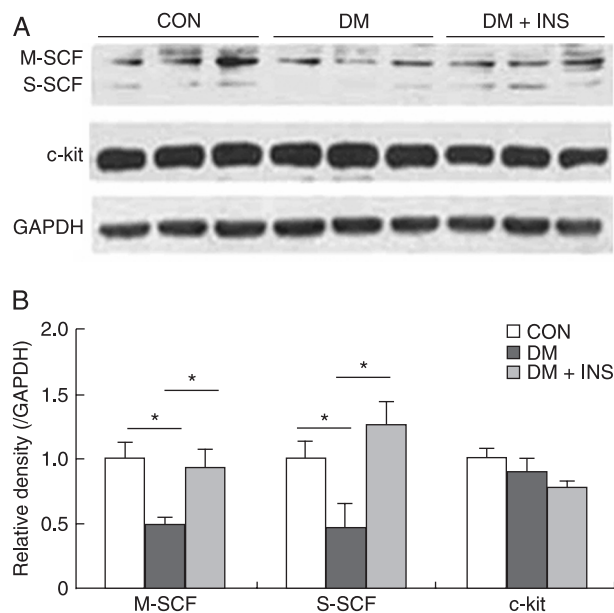


Figure 4. *A*, Expression of M-SCF, S-SCF and c-kit by Western blotting. *B*, Relative density of *A*. Expression of SCF (membrane-bound SCF, M-SCF and soluble SCF, S-SCF) in the DM group was lower than in the CON group; DM + INS increased the expression of SCF. There were no significant differences among the three groups in the expression of c-kit. * $P < 0.05$ (one-way ANOVA). For abbreviations, see legend to Figure 1.

repair (14,20-22). SCF protects against camptothecin and glutamate-induced neuronal cell death (13,14). SCF and Kit are responsible for the survival of a variety of cells including neuronal cells and hematopoietic lineages (23). Targeted disruption of the kit gene leads to marked defects in learning and memory (12), also a key manifestation of diabetic encephalopathy. In the present study, no abnormal expression of c-kit was found in the diabetic brain. Kit alteration may possibly follow alterations in SCF. Our model using milder diabetic damage suggests that pathological injury in the diabetic brain may first occur in the cortex and only later extend to the hippocampus. Our model further suggests that SCF/c-kit expression may be used to evaluate the severity of diabetic encephalopathy.

Neuroprotection in diabetes by SCF via activation of AKT/ERK

To further investigate which signaling pathway is involved in neuroprotection by SCF in diabetes, diabetic mice were pretreated with insulin, SCF or vehicle and the subsequent activation of MAPK/ERK1/2 and AKT in the cortex was determined by Western blotting, as described in Figure 5. The results indicated that there was less activation of ERK1/2 and AKT in the cortex of the diabetic brain than in the normal cortex. However, the addition of SCF or insulin to this model effectively activated MAPK and AKT. Both the MAPK/ERK and PI-3K/Akt signaling pathways are essential

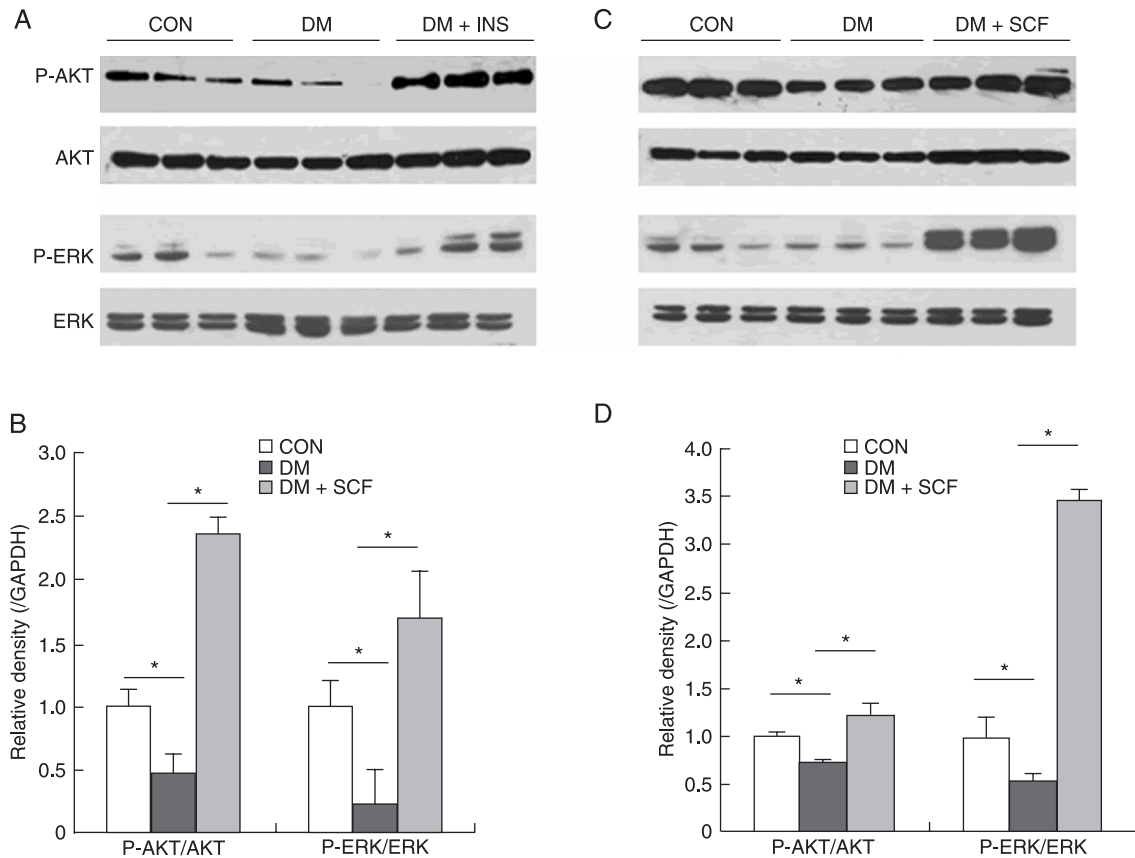


Figure 5. A, C, Expression of phosphorylated AKT (P-AKT) and ERK (P-ERK) by Western blotting. B, D, Relative density of A and C. There is decreased activation of AKT and ERK in DM; INS or SCF increased the expression of phosphorylated AKT and ERK. *P < 0.01 (one-way ANOVA). For abbreviations, see legend to Figure 1.

in the prevention of neuronal apoptosis and are also implicated in the protection of the brain from ischemic stroke and Alzheimer's disease (14,17). SCF also suppressed camptothecin and glutamate-induced neuronal apoptosis via MAPK/ERK and PI-3K/Akt activation. SCF-mediated signal transduction may involve a different cell signaling pathway (14). Interestingly, SCF-mediated cytoprotection in non-central nervous system cell types is also linked to activation of the AKT and/or the MAPK signaling pathways (24,25), suggesting that a similar mechanism of action of SCF/c-kit may be involved in neuroprotection and in the protection of other cell types.

References

- Bestetti G, Rossi GL. Hypothalamic lesions in rats with long-term streptozotocin-induced diabetes mellitus. A semiquantitative light- and electron-microscopic study. *Acta Neuropathol* 1980; 52: 119-127.
- Mukai N, Hori S, Pomeroy M. Cerebral lesions in rats with

Cortical neuronal apoptosis appeared in the diabetic brain due to reduced SCF expression and this apoptosis could be inhibited by SCF. The mechanism by which this occurred may be correlated to the activation of the ERK and AKT pathways. Accordingly, SCF may hold promise for development as a neuroprotectant for treating diabetes-related brain injury.

Acknowledgments

We thank Mrs. Marilyn White for editing the manuscript.

- streptozotocin-induced diabetes. *Acta Neuropathol* 1980; 51: 79-84.
- Mijnhout GS, Scheltens P, Diamant M, Biessels GJ, Wessels AM, Simsek S, et al. Diabetic encephalopathy: A concept in need of a definition. *Diabetologia* 2006; 49: 1447-1448.

4. Srinivasan S, Stevens M, Wiley JW. Diabetic peripheral neuropathy: evidence for apoptosis and associated mitochondrial dysfunction. *Diabetes* 2000; 49: 1932-1938.
5. Li ZG, Zhang W, Sima AA. C-peptide enhances insulin-mediated cell growth and protection against high glucose-induced apoptosis in SH-SY5Y cells. *Diabetes Metab Res Rev* 2003; 19: 375-385.
6. Kuemmerle JF. Endogenous IGF-I protects human intestinal smooth muscle cells from apoptosis by regulation of GSK-3 beta activity. *Am J Physiol Gastrointest Liver Physiol* 2005; 288: G101-G110.
7. Hsueh WA, Law RE. Insulin signaling in the arterial wall. *Am J Cardiol* 1999; 84: 21J-24J.
8. Horvath VJ, Vittal H, Lorincz A, Chen H, Almeida-Porada G, Redelman D, et al. Reduced stem cell factor links smooth myopathy and loss of interstitial cells of Cajal in murine diabetic gastroparesis. *Gastroenterology* 2006; 130: 759-770.
9. Ashman LK. The biology of stem cell factor and its receptor C-kit. *Int J Biochem Cell Biol* 1999; 31: 1037-1051.
10. Hollenbeck ST, Sakakibara K, Faries PL, Workhu B, Liu B, Kent KC. Stem cell factor and c-kit are expressed by and may affect vascular SMCs through an autocrine pathway. *J Surg Res* 2004; 120: 288-294.
11. Motro B, Wojtowicz JM, Bernstein A, van der Kooy D. Steel mutant mice are deficient in hippocampal learning but not long-term potentiation. *Proc Natl Acad Sci U S A* 1996; 93: 1808-1813.
12. Katafuchi T, Li AJ, Hirota S, Kitamura Y, Hori T. Impairment of spatial learning and hippocampal synaptic potentiation in c-kit mutant rats. *Learn Mem* 2000; 7: 383-392.
13. Manova K, Bachvarova RF, Huang EJ, Sanchez S, Pronovost SM, Velazquez E, et al. c-kit receptor and ligand expression in postnatal development of the mouse cerebellum suggests a function for c-kit in inhibitory interneurons. *J Neurosci* 1992; 12: 4663-4676.
14. Dhandapani KM, Wade FM, Wakade C, Mahesh VB, Brann DW. Neuroprotection by stem cell factor in rat cortical neurons involves AKT and NFkappaB. *J Neurochem* 2005; 95: 9-19.
15. Matsui J, Wakabayashi T, Asada M, Yoshimatsu K, Okada M. Stem cell factor/c-kit signaling promotes the survival, migration, and capillary tube formation of human umbilical vein endothelial cells. *J Biol Chem* 2004; 279: 18600-18607.
16. Biessels GJ, Gispen WH. The impact of diabetes on cognition: what can be learned from rodent models? *Neurobiol Aging* 2005; 26 (Suppl 1): 36-41.
17. Xu Y, Zhang W, Klaus J, Young J, Koerner I, Sheldahl LC, et al. Role of cocaine- and amphetamine-regulated transcript in estradiol-mediated neuroprotection. *Proc Natl Acad Sci U S A* 2006; 103: 14489-14494.
18. Li ZG, Zhang W, Grunberger G, Sima AA. Hippocampal neuronal apoptosis in type 1 diabetes. *Brain Res* 2002; 946: 221-231.
19. Sima AA, Li ZG. The effect of C-peptide on cognitive dysfunction and hippocampal apoptosis in type 1 diabetic rats. *Diabetes* 2005; 54: 1497-1505.
20. Kelly DJ, Zhang Y, Gow RM, Itescu S, Gilbert RE. Cells expressing the stem cell factor receptor, c-kit, contribute to neovascularization in diabetes. *Diab Vasc Dis Res* 2005; 2: 76-80.
21. Jin K, Mao XO, Sun Y, Xie L, Greenberg DA. Stem cell factor stimulates neurogenesis *in vitro* and *in vivo*. *J Clin Invest* 2002; 110: 311-319.
22. Sun L, Lee J, Fine HA. Neuronally expressed stem cell factor induces neural stem cell migration to areas of brain injury. *J Clin Invest* 2004; 113: 1364-1374.
23. Gore BB, Wong KG, Tessier-Lavigne M. Stem cell factor functions as an outgrowth-promoting factor to enable axon exit from the midline intermediate target. *Neuron* 2008; 57: 501-510.
24. Edling CE, Pedersen M, Carlsson L, Ronnstrand L, Palmer RH, Hallberg B. Haematopoietic progenitor cells utilise conventional PKC to suppress PKB/Akt activity in response to c-Kit stimulation. *Br J Haematol* 2007; 136: 260-268.
25. Kapur R, Chandra S, Cooper R, McCarthy J, Williams DA. Role of p38 and ERK MAP kinase in proliferation of erythroid progenitors in response to stimulation by soluble and membrane isoforms of stem cell factor. *Blood* 2002; 100: 1287-1293.