Acta Med. Okayama, 2001
Vol. 55, No. 1, pp. 25-30

Acta Medica
Okayama
http://www.lib.okayama-u.ac.jp/www/acta/

Original Article

Reduction of Ischemic Damage by Application of Insulin-like Growth Factor-1 in Rat Brain after Transient Ischemia

Jiang Ming Wang[a,b], Takeshi Hayashi[a], Wen Ri Zhang[a], Feng Li[a], Masanori Iwai[a], and Koji Abe[a*]

[a]*Department of Neurology, Okayama University Medical School, Okayama 700-8558, Japan, and*
[b]*Department of Neurology, Peking Union Medical College Hospital, Beijing, China*

In order to investigate a possible effect of insulin-like growth factor-1 (IGF-1) on ischemic brain injury, IGF-1 was applied topically on the brain surface of reperfused rat brain after 60 min of transient middle cerebral artery occlusion. In contrast to the cases treated with vehicle, the infarct volume was greatly reduced at 24 h of reperfusion by the treatment with IGF-1. Immunohistochemical analysis in the middle cerebral artery territory showed that Caspase-3 staining was markedly reduced in the cases with IGF-1 treatment, but 72-kDa heat shock protein staining remained almost unchanged. The present results suggest that treatment with IGF-1 exerts a significant effect on ameliorating brain injury after transient focal brain ischemia. Moreover, this effect is greatly associated with the reduction of Caspase-3 staining, but is only minimally associated with a decreasd stress response at the cellular level.

Key words: Caspase-3, cerebral ischemia, 72-kDa heat shock protein

Insulin-like growth factor-1 (IGF-1), a single-chain polypeptide structurally similar to insulin, is known to mediate many cellular and molecular effects and is considered a member of the class of growth factors. IGF-1 plays important roles not only in the proliferation of granule cell progenitors *in vitro* [1, 2], but also in the survival and recovery of central and peripheral nervous systems from various injuries *in vivo* [3-8]. Many studies showed that central administration of IGF-1 to adult rats reduced both the neuronal loss and the incidence of cortical infarct after experimental hypoxic ischemia [4] and transient cerebral ischemia [6, 9]. These studies demonstrate the action of IGF-1 in inhibiting apoptotic, hypoglycemic, or cycloheximide-induced cell death. However, the primary mechanism whereby IGF-1 acts to promote neuronal rescue after hypoxic or ischemic brain injury remains incompletely understood.

Most effects of IGF-1 are mediated by the IGF-I receptor (IGF-1R), a heterotetramer that has tyrosine kinase activity and phosphorylates insulin receptor substrates (IRS-1 and 2), which leads to the activation of 2 downstream signaling cascades: the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol-3 kinase (P3K) cascade [10, 11]. Recent data suggest that the activated IGF-I/IGF-IR system displays anti-apoptotic properties in various cell types by stimulating distinct intracellular signaling pathways [11, 12]. Binding of IGF-I to its tyrosine kinase receptor (IGF-IR) leads to receptor dimerization and autophosphorylation, and initiates a cascade of cellular signal transduction events which activate the P3K and MAPK pathways [13, 14]. The protective effects of activated IGF-1/IGF-1R against cell death have been understood for several years, particularly those of the central nervous system [15, 16]. The protection from apoptosis by the IGF-1/IGF-1R system mainly depends on the regulation of the main

Received August 10, 2000; accepted October 10, 2000.
*Corresponding author. Phone: +81-86-235-7365; Fax: +81-86-235-7368
E-mail: abek@cc.okayama-u.ac.jp (K.Abe)

elements in the apoptotic cascade. In this context, the IGF-1/IGF-1R mediated inhibition of Caspase-3 appears to be crucial [17]. Caspase-3 plays a key role in the apoptotic process as the main effector of enzymatic cleavage. Apoptotic process is controlled by certain other proteins, such as the pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins [18]. Caspase-3 initiates certain biochemical and morphological characteristics of programmed cell death. The IGF-1/IGF-1R system is indirectly able to inhibit Caspase-3 activity by P3k-mediated signalling, activation of Bcl-2 and inhibition of Bax *in vitro* [9, 19]. On the other hand, 72-kDa heat shock protein (HSP72) is a well-known molecular chaperone and a marker of injury that is induced under stressful conditions such as ischemia; induction has been shown to decrease in the presence of cell protective agent [20, 21]. Thus, a possible reduction of ischemic brain injury by treatment with IGF-1 was examined in relation to a possible modification of immunoreactivity for Caspase-3 and HSP72 by a topical application after transient focal cerebral ischemia in rats.

Materials and Methods

Adult male Wistar rats weighing 250–280 g were used for the experiments. They were anesthetized with an intraperitoneal injection of pentobarbital (40 mg/kg), and positioned in a stereotaxic operating apparatus. A burr hole with a diameter of 2 mm was carefully made in the skull using an electric dental drill to avoid traumatic brain injury. The location of the burr hole was 3 mm dorsal and 5 mm lateral to the right from the bregma, which is located in the upper portion of the middle cerebral artery (MCA) territory. The dura mater was preserved at this time. The animals were allowed to recover in an ambient atmosphere (21 to 24 °C).

Approximately 24 h after the drilling, the rats were anesthetized by inhalation of a nitrous oxide/oxyten/halothane (69%:30%:1%) mixture; a face mask was used for the surgical preparation. After stopping the inhalation of anesthetics, the origin of the right MCA was occluded by an insertion of 4–0 surgical nylon thread with silicone coating through the common carotid artery, performed as described in our previously reports [22, 23]. Body temperature was monitored with a rectal probe, and was maintained at 37 °±0.3 °C using a heating pad during the surgical procedure for MCA occlusion (MCAO). After 60 min of MCAO, the cerebral blood flow (CBF) was restored by a removal of the nylon thread. Immediately after the restoration of CBF, a part of the dura mater was carefully removed (diameter: 2 mm) through the drilling hole. A small piece (8 mm^3) of gelfoam (Upjohn, Kalamazoo, IL, USA) presoaked in 9 μl of normal saline or IGF-1 (20 μg in 9 μl of saline) was placed in contact with the surface of the cerebral cortex, as described in our previous reports [20, 22]. The gelfoam was buried in the skull bone. The surface of the skull bone was then covered with a vinyl tape, and the scalp was sewed. It is reported that application of growth factor in gelfoam is as effective as direct infusion [24]. The above operations were performed in a sterile environment. Sham-operated control animals underwent burr hole surgery, exposure of the common carotid artery without MCAO, and placement of gelfoam presoaked in vehicle. The animals recovered at an ambient temperature (21–24 °C) until sampling. Blood samples (90 μl) were collected before and after MCAO from the ventral tail artery for measurement of PO_2, PCO_2, and pH (blood gas analyzer model ABL300, Radiometer). The rectal temperature was measured before and after MCAO. The regional CBF of the left frontoparietal cortex region was measured before and after MCA occlusion through the burr hole using a laser blood flowmeter (Flo-Cl, Omegawave). The experiment protocol and procedures were approved by the Animal Committee of the Okayama University School of Medicine, Japan.

In order to investigate the possible effect of IGF-1 on infarct size after transient MCAO, rats were decapitated under deep anesthesia, and the rat forebrains were removed and divided into 6 coronal (2 mm thickness) sections at 24 h after reperfusion with vehicle or IGF-1 treatment (n = 9 for each group). The coronal sections were stained with saline containing 2% 2, 3, 5-triphenyltetrazolium chloride (TTC) at 37 °C for 30 min, after fixation in 10% neutralized formalin. The infarct area of each section was calculated using NIH Image software, version 1.62a, and then infarct areas on each slice were summed and multiplied by slice thickness to give the infarct volume.

To examine the possible effects of IGF-1 on Caspase-3 and HSP72 staining, the rat forebrains were removed and quickly frozen at 24 h of reperfusion with vehicle or IGF-1 treatment (n = 3 for each group). Coronal sections at the caudate and dorsal hippocampal levels were cut on a cryostat at − 20 °C with 10 μm thickness. Sham control sections (n = 2) were also obtained. Immunohis-

tochemical analysis for Caspase-3 and HSP72 was performed according to previous reports [20, 23]. In brief, the sections were fixed in ice-cold acetone for 10 min and air-dried, followed by a rinse in phosphate-buffered saline. After blocking with 10% normal rabbit or horse serum for Caspase-3 or HSP72 staining, respectively, the slices were incubated with primary antibodies and 0.3% Triton X-100 for about 16 h at 4 °C. The primary antibodies used and each dilution were as follows: goat polyclonal antibody against Caspase-3 (CPP32 P20, L-18) (sc-1225, Santa Cruz Biotechnology Inc., CA, USA) at 1:100, and mouse monoclonal antibody against HSP72 (RPN1197, clone C92, Amersham, London, UK) at 1:400. The slides were washed and then incubated for 3 h with the biotinylated anti-goat IgG (PK-6105, Vector Laboratories) for Caspase-3, or anti-mouse IgG (PK-6102, Vector Laboratories), at 1:200 dilution in PBS. Subsequently, the slides were incubated with avidin-biotin-horseradish peroxidase complex for 30 min, and then were developed with diaminobenzidine. The reaction was stopped by washing the slides in distilled water.

The sections were examined by light microscope, and the number of positively stained cells in the MCA boundary area was counted as our previous report [22]. The staining was then categorized into 5 grades: no staining, a few cells (2–10 or so), or a small (20–50), moderate (50–200), or a large (200–500 or more) number of stained cells; respective categorizations were assigned as follows: (−), (c), (+), (2+), and (3+).

All data are expressed as means ± SD. Statistical analyses were performed using the 2-tailed unpaired Student's *t*-Test.

Results

The body weight, blood gas, and rectal temperature were comparable in the vehicle-treated and IGF-1-treated group. Physiological parameters are shown in Table 1. There was also no significant difference in the regional CBF between the 2 groups before and after MCA occlusion or restoration (Table 2).

The infarct area of 3 coronal sections (6, 8, and 10 mm caudal from the frontal pole) from the IGF-1-treated group were significantly smaller than those of the vehicle group (Table 3). The infarct volume of the vehicle and IGF-1-treated group at 24 h of reperfusion after 60 min transient MCAO were 225.4 ± 59.5 mm^3 (mean ± SD, n = 9) and 139.7 ± 48.2 mm^3 (n = 9; P = 0.004 against the vehicle group), respectively (Table 3). Application of IGF-1 produced a significant reduction in infarct volume.

Immunoreactivity for Caspase-3 was not detectably present in the sham control brain sections (Fig. 1a).

Table 1 Physiological parameters in MCA occluded rats

	Vehicle group		IGF-1 group	
	Pre-MCAO	Post-MCAO	Pre-MCAO	Post-MCAO
Body weight (g)	266.6 ± 8.6		269.1 ± 7.4	
Blood gas: pH	7.45 ± 0.02	7.50 ± 0.01	7.45 ± 0.03	7.40 ± 0.01
PO2 (mmHg)	119.0 ± 13.9	114.6 ± 9.6	121.8 ± 15.4	118.2 ± 11.9
PCO2 (mmHg)	38.9 ± 3.9	37.3 ± 4.7	41.6 ± 3.9	43.3 ± 5.7
Rectal temperature (°C)	36.7 ± 0.1	36.9 ± 0.2	36.8 ± 0.1	36.9 ± 0.2

Data is expressed mean ± SD.

Table 2 Changes of regional CBF of both IGF- and vehicle-treated groups during MCA occlusion and restoration

	Regional CBF			
	Pre-MCAO	Post-MCAO	Pre-restoration	Post-restoration
Vehicle group	100	18.8 ± 5.8	15.9 ± 4.0	92.4 ± 8.3
IGF-1 group	100	16.9 ± 14.3	15.9 ± 3.9	92.0 ± 7.1

The data is compared to that of initial CBF, and is presented as the percentage of mean ± SD.

Table 3 Infarct areas of 5 coronal sections and infarct volume at 24 h of reperfusion after 60 min of transient MCAO

	Vehicle	IGF-I
Infarct area (mm^2)		
Section 1	14.4 ± 6.5	8.6 ± 5.6
Section 2	38.8 ± 5.6	29.4 ± 8.7*
Section 3	36.5 ± 6.3	22.1 ± 5.2**
Section 4	17.1 ± 8.3	8.3 ± 5.6*
Section 5	5.8 ± 7.8	1.4 ± 3.0
Infarct volume (mm^3)	225.4 ± 59.5	139.7 ± 48.2**

Data are expressed as mean ± SD (n = 9 in vehicle group and IGF-I group). *$P < 0.05$ and **$P < 0.01$ against the vehicle group. The distance of 5 coronal sections were 4, 6, 8, 10, and 12 mm caudal from frontal pole, respectively.

Table 4 Number of immunopositive neurons for Caspase-3 and HSP72 with or without IGF-I treatment after transient MCA occlusion

	Animal	Caspase-3	HSP72
Sham control	1	−	−
	2	−	−
Vehicle group	1	+	2+
	2	+	+
	3	2+	+
IGF-I group	1	±	+
	2	−	+
	3	±	+

The staining was categorized into the following 5 grades: no staining, a very small number of cells (2-10 or so), few cells (20-50), moderate number of (50-200), or a large (200-500 or more) number of stained cells; the respective categories are designated as (−), (±), (+), (2+), and (3+). Sham control group, n = 2; Vehicle and IGF-I group, n = 3.

However, it became markedly present in the neuronal cytoplasm of the cerebral cortex, especially in the inner boundary zone of the occluded MCA territory (Fig. 1b, at left) in the vehicle group, while it was only slightly present in the IGF-1-treated group. The number of positive cells was markedly reduced in the IGF-1 treated cases. Caspase-3 staining was negative in the brain sections of the ischemic dorsal caudate in both the vehicle and IGF-1-treated group (data not shown).

As regards HSP72, this protein was not recognized in the neurons of the cerebral cortex (Fig. 1d) nor in the dorsal caudate in the sham control brain. However, heavy staining was seen in the ischemic cerebral cortex of vehicle group at 24 h after CBF restoration (Fig. 1e). In brain sections of the IGF-1-treated group, immunoreactivity of HSP72 was minimally reduced in the ischemic cerebral cortex (Fig. 1f). Although the number of positive cells was slightly reduced in this group, the dense stained neurons were few in number, in contrast to vehicle group. HSP72 staining was also negative in the brain sections of the ischemic dorsal caudate in both groups. The grades of immunoreactivity for Caspase-3 and HSP72 are summarized in Table 4.

Discussion

The present results show that topical application of IGF-1 significantly reduced the infarction volume of the MCA region after induction of transient focal brain ischemia. The protective effect of IGF-1 was greatly associated with a reduction of Caspase-3 staining. Apoptotic neurons were localized primarily in the inner boundary zone of the infarct, whereas necrotic cells were mainly distributed in the ischemic core after transient focal ischemia was induced in rats [25, 26]. Caspases, which play a large role in the process of apoptotic cell death, were induced and mainly located in the ischemic penumbra, suggesting that the expansion of neuronal cell damage in this area may occur primarily by an apoptostic mechanism. In this transient MCAO model, less than 1 h of ischemic period produces only reversible neuronal damage, though 3 h of ischemia produces secondary damage to the anterior cerebral artery-perfused area, a finding which is in accordance with previous reports from our laboratory [12]. Immunoreactivity for Caspase-3 was markedly reduced in brain sections in the IGF-1 treated group in contrast to the vehicle treated group. Thus, the great reduction of infarct volume accompanied by that of Caspase-3 staining in the IGF-1 treatment group suggests that the protective effects of IGF-1 could be due to on the amelioration of the apoptotic mechanisms of neuronal death through inhibiting the Caspase-3 activity.

It is of note that the amelioration of ischemic brain injury by IGF-1 was greatly associated with a reduction in Caspase-3 staining, but was minimally associated with the stress response of the cell. In the CNS, HSP72 is induced by various injuries, and the degree of the stress is proportional with the induction of HSP72 [21]. Although, in the present study, immunoreactivity for

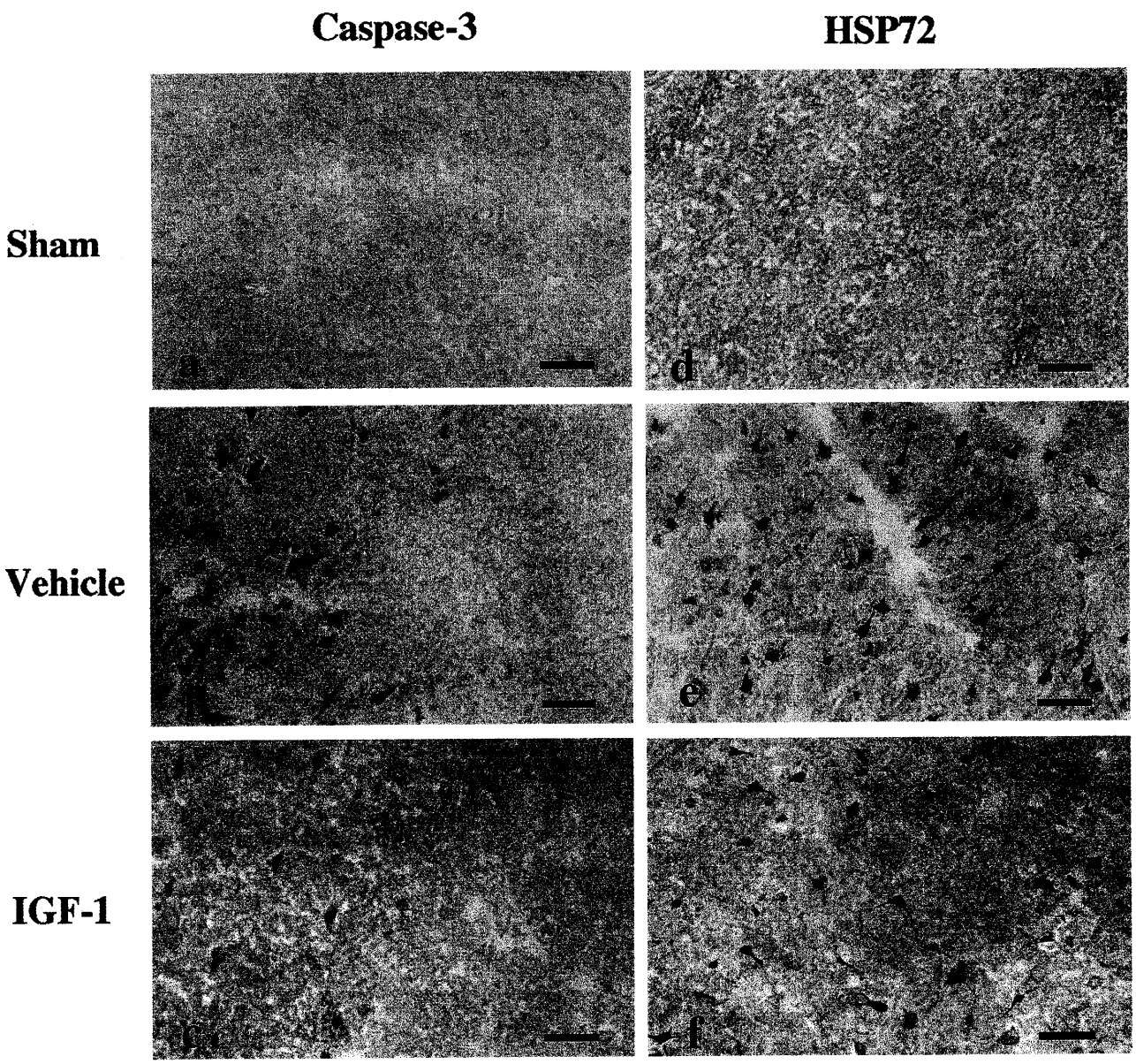

Fig. 1 Representative photomicrographs of immunohistochemistry for Caspase-3 (a-c) and HSP72 (d-f). Note that no neurons are positive for Caspase-3 (a) or for HSP72 (d) in the cerebral cortex in the brain sections from controls. Many neurons were positive for Caspase-3 in the inner boundary zones of the occluded MCA territory in the vehicle group (b, left portion of figure); only a few cells were positive for Caspase-3 in the IGF-1-treated group (c, left part). However, the immunoreativity for HSP72 was only slightly reduced in the neurons of the ischemic cerebral cortex in the IGF-1 treated group (f), in contrast to that of vehicle group (e). Scale Bars: 50 μm.

HSP72 was slightly reduced in the IGF-1-treated group, the dense stained neurons were not numerous (Fig. 1f) in contrast to the vehicle group (Fig. 1e). Because HSP72 in the cytosol migrates into the nucleus during severe stresses and prevents protein malfolding in the nucleus [27], the marked reduction of immunoreactive HSP72 in the nuclei may also support the reduction of ischemic damage by IGF-1 application. In addition, HSP72 protein protected neuronal cells from thermal stress but not from programmed cell death [28]. A previous report suggested that subpopulations of neuronal cells stained with TUNEL or HSP72 immunohistochemistry were

essentially different, and that only 5% to 7% of the cells were overlapping [29]. This phenomenon was also found in the present study.

Acknowledgments. This work was partly supported by Grants-in-Aid for Scientific Research Nvs. 09470151 and 10176204 from the Ministry of Education, Japan, and from the Kobayashi Memorial Foundation, the Terumo Scientific Foundation, the Grant from the Japan Research Foundation for Clinical Pharmacology, and from the Japan Heart Foundation Dr. Hiroshi Irisawa Commemorative Research Grant.

References

1. Cui H, Meng Y and Bulleit RF: Inhibition of glycogen synthase kinase 3β activity regulates proliferation of cultured cerebellar granule cells. Dev Brain Res (1998) **111**, 177-188.
2. Lin X and Bulleit RF: Insulin-like growth factor I (IGF-I) is a critical trophic factor for developing cerebellar granule cells. Dev Brain Res (1997) **99**, 234-242.
3. Contreras PC, Steffler C, Yu E, Callison K, Stong D and Vaught JL: Systemic administration of rhIGF-I enhanced regeneration after sciatic nerve crush in mice. J Pharmacol Exp Ther (1995) **274**, 1443-1449.
4. Johnston BM, Mallard EC, Williams CE and Gluckman PD: Insulin-like growth factor-I is a potent neuronal rescue agent after hypoxic-ischemic injury in fetal lambs. J Clin Invest (1996) **97**, 300-308.
5. Saatman KE, Contreras PC, Smith DH, Raghupathi R, McDermott KL, Fernandez SC, Sanderson KL, Voddi M and McIntosh TK: Insulin-like growth factor-I (IGF-I) improves both neurological motor and cognitive outcome following experimental brain injury. Exp Neurol (1997) **147**, 418-427.
6. Tagami M, Ikeda K, Nara Y, Fujino H, Kubota A, Numano F and Yamori Y: Insulin-like growth factor-I attenuates apoptosis in hippocampal neurons caused by cerebral ischemia and reperfusion in stroke-prone spontaneously hypertensive rats. Lab Invest (1997) **76**, 613-617.
7. Vergani L, Finco C, Di Giulio AM, Muller EE and Gorio A: Effects of low doses of glycosaminoglycans and insulin-like growth factor-I on motor neuron disease in wobbler mouse. Neurosci Lett (1997) **228**, 41-44.
8. Zhu CZ and Auer RN: Intraventricular administration of insulin and IGF-I in transient forebrain ischemia. J Cereb Blood Flow Metab (1994) **14**, 237-242.
9. Wang L, Ma W, Markovich R, Lee WL and Wang PH: Insulin-like growth factor I modulates induction of apoptotic signaling in H9C2 cardiac muscle cells. Endocrinology (1998) **139**, 1354-1360.
10. Butt AJ, Firth SM and Baxter RC: The IGF axis and programmed cell death. Immunol Cell Biol (1999) **77**, 256-262.
11. Cross DA, Alessi DR, Cohen P, Andjelkovich M and Hemmings BA: Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature (1995) **378**, 785-789.
12. Abe K, Kawagoe J, Araki T, Aoki M and Kogure K: Differential expression of heat shock protein 70 gene between the cortex and caudate after transient focal cerebral ischaemia in rats. Neurol Res (1992) **14**, 381-385.
13. Kulik G and Weber MJ: Akt-dependent and -independent survival signaling pathways utilized by insulin-like growth factor I. Mol Cell Biol (1998) **18**, 6711-6718.
14. McCarthy MJ, Rubin LL and Philpott KL: Involvement of caspases in sympathetic neuron apoptosis. J Cell Sci (1997) **110**, 2165-2173.
15. D'Mello SR, Galli C, Ciotti T and Calissano P: Induction of apoptosis in cerebellar granule neurons by low potassium: Inhibition of death by insulin-like growth factor I and cAMP. Proc Natl Acad Sci USA (1993) **90**, 10989-10993.
16. Gluckman P, Klempt N, Guan J, Mallard C, Sirimanne E, Dragunow M, Klempt M, Singh K, Williams C and Nikolics K: A role for IGF-I in the rescue of CNS neurons following hypoxic-ischemic injury. Biochem Biophys Res Commun (1992) **182**, 593-599.
17. Grothey A, Voigt W, Schober C, Muller T, Dempke W and Schmoll HJ: The role of insulin-like growth factor I and its receptor in cell growth, transformation, apoptosis, and chemoresistance in solid tumors. J Cancer Res Clin Oncol: (1999) **125**, 166-173.
18. Villa P, Kaufmann SH and Earnashaw WC: Caspases and caspase inhibitors. Trends Biochem Sci (1997) **22**, 388-393.
19. Singleton JR, Dixit VM and Feldman EL: Type I insulin-like growth factor receptor activation regulates apoptotic proteins. J Biol Chem (1996) **271**, 31791-31794.
20. Abe K, Hayashi T and Itoyama Y: Amelioration of brain edema by topical application of glial cell line-derived neurotrophic factor in reperfused rat brain. Neurosci Lett (1997) **231**, 37-40.
21. Gonzalez MF, Shiraishi K, Hisanaga K, Sagar SM, Mandabach M and Sharp FR: Heat shock proteins as a markers of neural injury. Mol Brain Res (1989) **6**, 93-100.
22. Hayashi T, Abe K and Itoyama Y: Reduction of ischemic damage by application of vascular endothelial growth factor in rat brain after transient ischemia. J Cereb Blood Flow Metab (1998) **18**, 887-895.
23. Kitagawa H, Hayashi T, Mitsumoto Y, Koga N, Itoyama Y and Abe K: Reduction of ischemic brain injury by topical application of glial cell line-derived neurotrophic factor after permanent middle cerebral artery occlusion in rats. Stroke (1998) **29**, 1417-1422.
24. Otto D, Frotscher M and Unsicker K: Basic fibroblast growth factor and nerve growth factor administered in gel foam rescue medial septal neurons after fimbria fornix transection. J Neurosci Res (1989) **22**, 83-91.
25. Li Y, Chopp M, Jiang N, Yao F and Zaloga C: Temporal profile of *in situ* DNA fragmentation after transient middle cerebral artery occlusion in the rat. J Cereb Blood Flow Metab (1995) **15**, 389-397.
26. Charriaut-Marlangue C, Margaill I, Represa A, Popovici T, Plotkine M and Ben-Ari Y: Apoptosis and necrosis after reversible focal ischemia: An *in situ* DNA fragmentation analysis. J Cereb Blood Flow Metab (1996) **16**, 186-194.
27. Gething MJ and Sambrook J: Protein folding in the cell. Nature (1992) **355**, 33-45.
28. Mailhos C, Howard MK and Latchman DS: Heat shock proteins hsp90 and hsp70 protect neuronal cells from thermal stress but not from programmed cell death. J Neurochem (1994) **63**, 1787-1795.
29. States BA, Honkaniemi J, Weinstein PR and Sharp FR: DNA fragmentation and HSP70 protein induction in hippocampus and cortex occurs in separate neurons following permanent middle cerebral artery occlusions. J Cereb Blood flow Metab (1996) **16**, 1165-1175.