INSULIN-LIKE GROWTH FACTOR-I DECREASES APOPTOTIC CELL DEATH, BUT NOT PROAPOPTOTIC PROTEIN EXPRESSION IN A TRANSIENT FOREBRAIN ISCHEMIA-REPERFUSION MODEL IN THE RAT

Nazire Afşar, M.D.* / Sevinç Aktan, M.D.* / Dilek İnce-Günal, M.D.*
Çiğdem Çelikel, M.D. / Tamer Coşkun, M.D.*** / Berrak Yeğen, M.D.*****

** Department of Neurology, School of Medicine, Marmara University, Istanbul, Turkey.*
*** Department of Pathology, School of Medicine, Marmara University, Istanbul, Turkey.*
**** Department of Physiology, School of Medicine, Marmara University, Istanbul, Turkey.*

ABSTRACT

Objective: Cerebral ischemia results in both necrotic and apoptotic cell death. It has been suggested that approaches directed at disrupting the apoptotic process and expression of proapoptotic proteins might be beneficial for preserving functional neuronal tissue after an ischemic insult. The aim was to evaluate the presence of apoptotic cell death and the pattern of expression of proapoptotic protein (bax) in a transient forebrain cerebral ischemia model and to observe the potential benefits of a neurotrophic factor IGF-I on these parameters.

Methods: Female/male Wistar rats weighing 200-240g were subjected to transient forebrain ischemia by bilateral carotid artery occlusion combined with systemic hypotension for 10 minutes. Three reperfusion periods were performed as 1h, 24h and 7 days. The experiment was then conducted in two arms: in group I (n=6 for each reperfusion group), intracisternal injection of vehicle or 10 μg/rat of IGF-I was performed at all reperfusion periods, and these rats were evaluated for the presence of apoptosis and bax protein expression. Group II (n=4 for each reperfusion group) was evaluated for protein oxidation at the three reperfusion periods.

Results: Apoptosis was significantly higher ($p<0.01$) in the vehicle group compared to the sham group, and IGF-I treatment resulted in a significant decrease of apoptosis compared to the vehicle treated group at 24 hour reperfusion. Moreover, a peak in apoptotic cell death at 24 hour reperfusion was observed, however remaining just short of significance ($p = 0.0730$). No difference in bax protein expression and protein oxidation could be demonstrated between reperfusion periods and after IGF-I use.

Conclusion: 10μg/rat of IGF-I produces a significant suppression in apoptotic cell death at 24 hours reperfusion following transient forebrain ischemia.

Key Words: IGF-I, Apoptosis, Rat, Forebrain ischemia.

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Correspondance to: Nazire Afşar, M.D. - Department of Neurology, Marmara University Hospital, Tophanelioğlu Cad. 81190 Altunizade - Istanbul, Turkey.

e.mail address: nazire.afsar@marmara.edu.tr

INTRODUCTION

Cerebral ischemia results in both necrotic and programmed neuronal death (1-3). The programmed steps leading to apoptosis may be subject to modulation by extracellular conditions and thus may provide a potential for treatment in the setting of acute ischemic stroke (1, 3, 4). On the other hand, neurotrophic factors and pro- and antiapoptotic proteins have been reported to modulate apoptosis (5, 6).

One of the hypotheses concerning the occurrence of apoptosis is that the death of cells may result from the loss of survival signals such as trophic factors following injury . Moreover, oxidative stress can induce apoptosis along with necrosis (7). Antioxidants are known to prevent programmed cell death (8), whereas neurotrophic factors have been reported to enhance neural resistance to free radical-induced damage (9) and to promote neuronal survival (10). Thus a neurotrophic factor as insulin-like growth factor (IGF-I) may provide trophic support during the critical period and prevent the induction of mechanisms of programmed cell death (10-12).

The aims of this study were to demonstrate the presence of apoptotic cell death and the pattern of expression of proapoptotic protein (bax) at different reperfusion times after transient forebrain cerebral ischemia and to observe the potential benefits of a neurotrophic factor IGF-I on these parameters. A second purpose was to assess the correlation between these parameters and the oxidative injury occurring during the reperfusion period.

MATERIALS AND METHODS

Two independent studies were conducted (see experimental groups): in both arms vehicle or insulin-like growth factor I (IGF-I) was injected intracisternally after ten minutes of transient forebrain ischemia, and the histological outcome was analyzed 1 hour, 24 hours or 7 days later. The study was approved by the Institutional Ethics Committee.

Ischemia Model:

Transient forebrain ischemia was induced in adult Wistar albino rats (200 to 240 g body weight) of both sexes by bilateral carotid artery occlusion followed by hypotension (13). The animals were anesthetized by intraperitoneal injection (i.p.) of ketamine (100mg/kg) and chlorpromazine (0.75 mg/kg). Atropine (0.1mg/kg i.p.) was used to control pulmonary and gastric secretions. The carotid arteries were exposed via a midline ventral incision and temporarily ligated with a 3/0 silk; meanwhile the femoral artery was exposed and cannulated to produce a systemic hypotension of 50mmHg (Nihon-Kohden, Model RM 6100). After ten minutes of ischemia, the blood flow in the carotid arteries was restored and blood pressure was normalized with an autologous blood transfusion. Sham-operated animals received the same anesthesia and surgery but did not undergo ischemia.

Experimental Groups:

The study comprised two arms with the same design and consisted of a transient forebrain ischemia of ten minutes, followed by three different reperfusion periods, namely 1 hour, 24 hours and 7 days. Rats in each reperfusion subgroup received either intracisternal injection using a Hamilton syringe (50 µl) of vehicle or 10 µg/rat of IGF-I, or were sham operated. Group I (n=6 for each reperfusion and sham group) was designed for the demonstration of apoptosis and bax protein expression; whereas group II (n=4 for each reperfusion group) was destined to demonstrate the degree of oxidative injury by way of measuring protein oxidation.

Fixation and Brain Processing:

Group I rats were given an overdose of ether anesthesia at one of the three prespecified reperfusion periods (see experimental groups). Rat brains were fixed by transaortal perfusion and immersion in 10% buffered formalin phosphate (14, 15). Their brains were removed and embedded in paraffin. Three consecutive coronal sections at the level of the dorsal hippocampus, each of 5 µm in thickness, were performed and were destined for hematoxylin and eosin (H&E) staining; immunohistochemistry

for bax protein expression and for a molecular biological-histochemical system specific for staining fragmented DNA and apoptotic bodies (2, 15, 16). The evaluation was performed by one of the investigators blinded to the treatment and reperfusion groups.

Group II animals were subjected to lethal doses of ether anesthesia, and following immediate decapitation brains were stored at –70°C until determination of protein oxidation.

Immunohistochemistry:

Tissue sections were deparaffinized, rehydrated, and pretreated with microwave at 750 watts for 3 minutes. Staining was performed with primary antibody raised against bax protein (Biogenex) for 30 minutes at room temperature. After washing with tris-buffered saline (TBS) slides were incubated with rabbit animal detection kit (Biogenex) at room temperature. Diaminobenzidine was used as chromogen after washing with TBS. Sections were washed, counterstained with Mayer-hematoxyline, dehydrated and mounted with aqueous mounting media.

In situ labeling of fragmented DNA:

Apoptosis was identified histologically by a pathologist using the in situ end labeling procedure for the detection of nucleosomal DNA fragmentation, which uses the terminal transferase reaction (TUNEL, ApopTag kit, Oncor 7100). Negative controls for the apoptotic staining consisted of consecutive sections of each case in which the tdt enzyme was omitted.

Evaluation for bax protein and apoptosis expression was performed by the pathologist by light microscopy at x400 magnification by counting at least 50 neurons. The results were interpreted as percentage of neurons presenting nuclear staining among the total number scored.

Protein Oxidation:

Protein oxidation was determined by reaction of the released carbonyl groups with 2,4-dinitrophenylhidrazine (DNP) yielding 2,4-dinitrophenylhidrazone, which was in turn measured by way of spectrophotometry at an absorbency of 360 nm (17). At first the tissue protein contents were measured using the modified Lowry method (18). Brain tissue was homogenized into 20 mM K_2HPO_4 solution (1/10 weight/volume). Ten percent streptomycin sulfate (into 50 mM Hepes, pH=7.2) was then added to the homogenate at a proportion of 1/10, and after 15 minutes was subjected to 10 minutes centrifugation at 10,000g. Thereafter, 0.5ml of 20% tri-chloroacetic acid (TCA) was added to 0.5ml supernatant. The protein precipitate thus obtained was allowed to react with 10 mM DNP (in 2 N HCL) for one hour in a dark chamber at 37°C. Proteins were then denatured a second time with 0.5ml 20% TCA. The precipitate was then washed two times with 1ml solution of ethanol : ethyl acetate (1:1) to eliminate the non reacted part of DNP. Finally proteins were diluted into 1ml 1 N NaOH and were determined by spectrophotometry at 360 nm absorbency. The values thus obtained were expressed as carbonyl amount (nmol) per mg tissue protein by using a 22,000 $M^{-1}cm^{-1}$ coefficient.

Statistical evaluation:

Kruskal-Wallis non-parametric ANOVA was used to compare protein oxidation values between reperfusion subgroups. Logarithmic transformation of data for apoptosis and bax expression was performed in order to allow more accurate analysis. Bax protein and apoptosis expressions between reperfusion subgroups were compared by use of a parametric ANOVA test. Parametric repeated measures ANOVA test was used to compare bax protein and apoptosis expression in IGF-I and vehicle treated animals, as well as sham operated animals within each reperfusion group. A p value < 0.05 was considered significant.

RESULTS

The results of the group I for apoptotic cell and bax protein expression are given in Table I. A significant increase in apoptotic cell expression at 24 hour reperfusion was observed in the vehicle treated group as compared to the sham operated animals ($p < 0.01$). Moreover, exposure to IGF-I after ischemia produced a decrease in apoptotic cell expression at 24 hour reperfusion compared to the vehicle treated group ($p < 0.01$), while no difference with the sham operated group

was observable ($p > 0.05$). Comparison of different reperfusion groups for the vehicle treated animals showed a slight increase of apoptotic staining at 24 hour reperfusion, however remaining just short of significance ($p = 0.0730$). In contrast to apoptosis, no difference in bax protein expression could be detected at different reperfusion times and after the use of IGF-I as compared to the sham operated animals ($p > 0.05$).

Group II results for protein oxidation are given in Table II. A trend toward an increase in the amount of carbonyl group was observed with longer reperfusion periods. However no effect of IGF-I could be demonstrated ($p > 0.05$).

Table I.: Group I, Pathology Results

Reperfusion periods	Treatment (n=6)	Mass (g) (mean ± SD)	Apoptosis (median-% cell)	Apoptosis - log (mean ± SD)	Bax* (median-% cell)	Bax - log (mean ± SD)
1 hour	Sham	220 ± 8.8	1.0	0.46 ± 0.71	6.5	0.84 ± 0.20
	Vehicle	224 ± 11.6	20.5	0.96 ± 0.79	4.0	0.67 ± 0.46
	IGF-I	205 ± 19.4	1.0	0.30 ± 0.40	6.0	0.79 ± 0.32
24 hours	Sham	204 ± 20.2	1.0	0.15 ± 0.37	4.5	0.69 ± 0.23
	Vehicle	206 ± 7.3	45.5	1.40 ± 0.69 **	9.5	0.89 ± 0.33
	IGF-I	212 ± 10.7	1.0	0.27 ± 0.54 **	10.5	0.92 ± 0.29
7 days	Sham	218 ± 18.8	2.5	0.49 ± 0.58	5.5	0.71 ± 0.27
	Vehicle	212 ± 15.9	1.0	0.50 ± 0.77 †	10.5	0.87 ± 0.40
	IGF-I	211 ± 14.3	4.0	0.50 ± 0.55	4.0	0.62 ± 0.27

The results are given as median percentage of apoptotic cells or bax immunostaining cells among the total counted.

* Proapoptotic protein

** $p < 0.01$ (vehicle vs. Sham and IGF-I vs. vehicle)

† $p = 0.0730$

Table II.: Group II, Protein Oxidation Results

Reperfusion periods	Treatment (n=4)	Mass (g) (mean ± SD)	Carbonyl (nmol/ mg tissue protein) (median)*
1 hour	Vehicle	208 ± 14.2	4.34
	IGF-I	198 ± 6.9	3.50 †
24 hours	Vehicle	200 ± 13.3	5.36
	IGF-I	192 ± 8.8	5.10 †
7 days	Vehicle	214 ± 17.4	6.28
	IGF-I	208 ± 12.6	6.13 †

* Prtoein oxidation is expressed as the amount of carbonyl group per milligram tissue protein.

† non-significant.

DISCUSSION

The penumbra is now widely considered as an area that is potentially salvageable, in order to prevent further enlargement of the originally infarcted area (19, 20). Studies have suggested apoptotic cell death as a mechanism of the delayed neuronal loss occurring in focal and global cerebral ischemia (1, 2, 4, 19, 21-23) . Moreover, the limitation of delayed neuronal death by the use of protein synthesis inhibitors demonstrated earlier, was further considered to be a clue to the active process occurring after ischemia (3, 24-26). DNA fragmentation, a morphological feature of apoptosis, has been demonstrated as early as 0.5 to 1 hour in focal ischemia models, increasing in extent until 24 or 48 hours (1), and persisting for up to 4 weeks (2). Whereas, in a transient forebrain ischemia model, similar to ours, apoptotic nuclear staining has been found to be prominent between 48 and 72 hours of reperfusion (23). In the present study using a transient forebrain ischemia-reperfusion model apoptosis has been demonstrated to be evident at 24 hours of reperfusion. We were thus able to show the appearance of features indicating apoptotic cell death somewhere in the first 24 hours, without being able to provide any data as to its limits in time, probably because of a too long reperfusion period maintained.

Along with the occurrence of apoptosis, the proapoptotic bax protein has been shown to be upregulated both in global ischemia (27) and focal ischemia (28, 29). The temporal profile of bax upregulation differed in various studies. Increased bax expression was associated with apoptosis and has been shown as early as 0.5 to 3 hours in global ischemia (4). It was reported to last up to 72 to 96 hours (16) following transient forebrain ischemia and from 46 hours (29) to 3 days (28) following focal ischemia.

Yet, we were unable to show an increased staining for bax protein at 1 or 24 hours or 7 days of reperfusion compared to sham animals, and thus could not correlate the occurrence of apoptosis with the expression of a proapoptotic protein. Isenmann et al (28) have recently reported the occurrence of two areas at the border of the ischemic lesion, with differential immunoreactivity for bcl-2 and bax proteins associated with apoptosis. This difference in localization of pro- and antiapoptotic protein expression might be at the origin of the failure in the present study to correlate apoptosis with an increase in bax protein immunostaining. On the other hand Deshpande et al. (30) have postulated that cell death following transient cerebral ischemia was lacking morphological characteristics of apoptosis. Iwai et al. (31) demonstrated that DNA fragmentation occurred after structural damage of the neurons. More recently, Colbourne et al. (32) reported that electron microscopic evidence was against the presence of apoptotic cell death following global ischemia. Therefore a potential cause for the lack of correlation between apoptotic and bax protein staining observed in this study may be the absence of an apoptotic activity. However, this hypothesis can be tested with electron microscopy testing along with the immunostaining.

Neurotrophic factors, along with preventing apoptosis (12), may protect neurons from death by counteracting other mechanisms such as free-radical induced damage (9) or excitotoxic damage (33-35). Their action is thought to be through induction of antiapoptotic genes expression, such as bcl-2 (36), which together with preventing apoptotic cell death, were reported to act on necrotic cell death by having an antioxidant effect (35). Insulin-like growth factor-I (IGF-I) has been demonstrated to be neuroprotective when given as a continuous infusion (10) or as intracerebro-ventricular injection (12) following hypoxic-ischemic brain injury and with no effect in a transient global ischemia model (14).

In this study, we could only demonstrate a significant decrease of apoptotic cell staining with use of post-ischemic IGF-I, without any effect on bax protein expression or oxidative process following ischemia-reperfusion injury. This could either be due to the low dose of IGF-I used (10μg/rat), or a more dense ischemic insult induced in the present model resulting in necrotic cell death rather than apoptosis. Nevertheless, considering the encouraging results so far obtained with neurotrophic factors, and the potential to interfere with both apoptotic and necrotic cell death, we think that this field of neuroprotection merits still more research.

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REFERENCES

1. Charriaut-Marlangue C, Pollard H, Ben-Ari Y. Is ischemic cell death of the apoptotic type? In: Siesjö BK, Tadeusz W, eds. Advances in Neurology, vol. 71; Cellular and Molecular Mechanisms of Ischemic Brain Damage. Philadelphia: Lippincott-Raven Publishers, 1996: 425-430.
2. Li Y, Chopp M, Powers C, Jiang N. Apoptosis and protein expression after focal cerebral ischemia in rat. Brain Res 1997;765:301-312.
3. Linnik MD, Zobrist RH, Hatfield MD. Evidence supporting a role for programmed cell death in focal cerebral ischemia in the rats. Stroke 1993;24:2002-2009.
4. Schmidt-Kastner R, Fliss H, Hakim AM. Subtle neuronal death in striatum after short forebrain ischemia in rats detected by in situ end-labeling for DNA damage. Stroke 1997;28:163-170.
5. Krajewski S, Krajewska M, Shabaik A, Miyashita T, Wang HG, Reed JC. Immunohistochemical determination of in vivo distribution of bax, a dominant inhibitor of bcl-2. Am J Pathol 1994;145:1323-1336.
6. Krajewski S, Mai JK, Krajewska M, Sikorska M, Mossakowski MJ, Reed JC. Upregulation of bax protein levels in neurons following cerebral ischemia. J Neurosci 1995;15:6364-6376.
7. Nicotera P, Ankarcrona M, Bonfoco E, Orrenius S, Lipton SA. Neuronal necrosis and apoptosis: two distinct events induced by exposure to glutamate or oxidative stress. In: Seil FJ, ed. Advances in Neurology, vol. 72; Neuronal Regeneration, Reorganization, and Repair. Philadelphia: Lippincott-Raven Publishers, 1997: 95-101.
8. Oppenheim RW. Related mechanisms of action of growth factors and antioxidants in apoptosis: an overview. In: Seil FJ, ed. Advances in Neurology, vol. 72; Neuronal Regeneration, Reorganization, and Repair. Philadelphia: Lippincott-Raven Publishers, 1997: 69-78.
9. Nikolics K, Hefti F, Thomas GR, Gluckman PD. Trophic factors and their role in the postischemic brain. In: Siesjö BK, Tadeusz W, eds. Advances in Neurology, vol. 71; Cellular and Molecular Mechanisms of Ischemic Brain Damage. Philadelphia: Lippincott-Raven Publishers, 1996: 389-402.
10. Zhu CZ, Auer RN. Intraventricular administration of insulin and IGF-1 in transient forebrain ischemia. J Cereb Blood Flow Metab 1994;14:237-242.
11. Gluckman P, Klempt N, Guan J, et al. A role for IGF-1 in the rescue of CNS neurons following hypoxic-ischemic injury. Biochem Biophys Res Commun 1992;182:593-599.
12. Guan J, Williams C, Gunning M, Mallard C, Gluckman P. The effects of IGF-1 treatment after hypoxic-ischemic brain injury in adult rats. J Cereb Blood Flow Metab 1993;13:609-616.
13. Ginsberg MD, Busto R. Rodent models of cerebral ischemia. Stroke 1989;20:1627-1642.
14. Bergstedt K, Wieloch T. Changes in insulin-like growth factor receptor density after transient cerebral ischemia in the rat. Lack of protection against ischemic brain damage following injection of insulin-like growth factor 1. J Cereb Blood Flow Metab 1993;13:895-898.
15. Li Y, Sharov VG, Jiang N, Zaloga C, Sabbah HN, Chopp M. Ultrastructural and light microscopical evidence of apoptosis after middle cerebral artery occlusion in the rat. Am J Pathol 1995;146:1045-1051.
16. Hara A, Iwai T, Niwa M, et al. Immunohistochemical detection of Bax and Bcl-2 proteins in gerbil hippocampus following transient forebrain ischemia. Brain Res 1996;711:249-253.
17. Levine RL, Garland D, Oliver CN. Determination of carbonyl content in oxidatively modified proteins. Meth Enzym 1990;186:464-475.
18. Upret GS, Rosemary AR, Riches RC. Protein estimation in tissues containing high levels of lipid. Modification to Lowry's method of protein determination. Analytical Biochem 1988;168:421-427.
19. Fisher M, Garcia JH. Evolving stroke and the ischemic penumbra. Neurology 1996;47:884-888.
20. Hakim AM. Ischemic penumbra: the therapeutic window. Neurology 1998;51(Suppl 3):S44-S46.
21. Héron A, Pollard H, Dessi F, et al. Regional variability in DNA fragmentation after global

ischemia evidenced by combined histological and gel electrophoresis observations in the rat brain. J Neurochem 1993;6:1973-1976.

22. Nitatori T, Sato N, Waguri S, et al. Delayed neuronal death in the CA1 pyramidal cell layer of the gerbil hippocampus following transient ischemia is apoptosis. J Neurosci 1995;15:1001-1011.
23. Guglielmo MA, Chan PT, Cortez S, et al. The temporal profile and morphologic features of neuronal death in human stroke resemble those observed in experimental forebrain ischemia: the potential role of apoptosis. Neurol Res 1998;20:283-296.
24. Shigeno T, Yamasaki Y, Kato G, et al. Reduction of delayed neuronal death by inhibition of protein synthesis. Neurosci Lett 1990;120:117-119.
25. Goto K, Ishige A, Sekiguchi K, et al. Effects of cycloheximide on delayed neuronal death in rat hippocampus. Brain Res 1990;534:299-302.
26. Papas S, Crépel V, Hasboun D, Jorquera P, Chinestra P, Ben-Ari Y. Cycloheximide reduces the effects of anoxic insult in vivo and in vitro. Eur J Neurosci 1992;4:758-765.
27. MacManus JP, Linnik MD. Gene expression induced by cerebral ischemia: an apoptotic perspective. J Cereb Blood Flow Metab 1997;17:815-832.
28. Isenmann S, Stoll G, Schroeter M, Krajewski S, Reed JC, Bahr M. Differential regulation of bax, bcl-2, and bcl-x proteins in focal cortical ischemia in the rat. Brain Pathol 1998;8:49-62.
29. Li Y, Chopp M, Powers C, Jiang N. Apoptosis and protein expression after focal cerebral ischemia in rat. Brain Res 1997;765:301-312.
30. Deshpande J, Bergstedt K, Linden T, Kalimo H, Wieloch T. Ultrastructural changes in the hippocampal CA1 region following transient cerebral ischemia: evidence against programmed cell death. Exp Brain Res 1992;88:91-105.
31. Iwai T, Hara A, Niwa M, et al. Temporal profile of nuclear DNA fragmentation in situ in gerbil hippocampus following transient forebrain ischemia. Brain Res 1995;671:305-308.
32. Colbourne F, Sutherland GR, Auer RN. Electron microscopic evidence against apoptosis as the mechanism of neuronal death in global ischemia. J Neurosci 1999;19:4200-4210.
33. Finklestein SP, Kemmou A, Caday CD, Berlove DJ. Basic fibroblast growth factor protects cerebrocortical neurons against excitatory amino acid toxicity in vitro. Stroke 1993;24 (suppl I):I-141-I-143.
34. Mattson MP, Cheng B. Growth factors protect neurons against excitotoxic/ischemic damage by stabilizing calcium homeostasis. Stroke 1993;24 (suppl I):I-136-I-140.
35. Mattson MP, Mark RJ. Excitotoxicity and excitoprotection in vivo. In: Siesjö BK, Tadeusz W, eds. Advances in Neurology, vol. 71; Cellular and Molecular Mechanisms of Ischemic Brain Damage. Philadelphia: Lippincott-Raven Publishers, 1996:1-20.
36. Yuan J. The genes that regulate programmed cell death: from worm to mammal. In: Moskowitz MA, Caplan LR, eds. Cerebrovascular Diseases, 19th Princeton Stroke Conference. New York: Butterworth-Heinemann, 1995:199-216.