Expression of an HSP110 Family, Ischemia-Responsive Protein (irp94), in the Rat Brain after Transient Forebrain Ischemia

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The transcriptional expression of an ischemia responsive protein (irp94) in the hippocampus of rats was analyzed by Northern blotting. A transient forebrain ischemia was induced in the rats by temporary occluding of the bilateral common carotid arteries (CCAs) for various periods, and then reperfusion. Among the frontal, parietal, temporal and occipital lobes, and the cerebellum and hippocampus, the maximum mRNA expression of irp94 was at the occipital lobe, and the minimum was at the parietal lobe following ten min of forebrain ischemia. The irp94 mRNA expression reached a maximum fifteen min after the transient ischemia. From twenty min on after the ischemia its expression decreased. After a ten-min ischemia and the following reperfusion, irp94 mRNA expression gradually increased in the first twelve h, and then decreased. The expression pattern was like that of the endoplasmic reticulum chaperone, Erp72, but not that of the cytosol chaperone, hsp72. In addition, when intracellular ATP was depleted with antimycin A the mRNA level of irp94 increased in a thyrocyte cell culture model.

The results suggest that irp94, like a molecular chaperone, may play a role in protecting the cell against external stimulation, especially after a transient forebrain ischemia. Although future studies of irp94 will be required to clarify the interactions with other intracellular factors inducing ischemia or showing molecular chaperone activity, what is offered here is an insight into its functional role as a component of stress response in neurons that should be considered as a new therapeutic approach for the treatment of ischemia.

Introduction

Cerebral ischemia occurs either in the context of focal reductions in blood flow to regions of the brain or as a global deficit after cardiac arrest (Morimoto *et al.*, 1994). Until now the exact mechanisms of the ischemic cell injury of neurons are still a matter of debate. It has been generally accepted that a postischemic disturbance of calcium homeostasis plays a major role in the pathological process triggered by a transient cerebral ischemia (Siesjo, 1981). Recently, there has been further evidence that one of the major causes of ischemic cell damage is a secondary disturbance of the energy producing system triggered during the recovery phase (Paschen *et al.*, 1998). When ischemia and subsequent reperfusion are induced, several cellular events occur, including disruption of the tight junctions, subcortical actin cytoskeleton, cell-substratum interactions mediated by integrins, decreasing ATP levels disruption of the Na^+/K^+ ratio, intracellular pH, homeostasis of Ca^{2+}, and an altered intracellular redox state (Kristian and Siesjo, 1996).

It is also known that a member of the cytosolic stress proteins in the hsp family is associated with ischemia induction following cell damage and recovery (Chopp *et al.*, 1989; Nowak, 1991). Like other molecular chaperones, hsp70 participates actively in the ATP-dependent protein folding/assembly of newly synthesized proteins, and may play a role in the detection or degradation of un-/

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misfolded or un-/misassembled proteins (Gething and Sambrook, 1992; Harrub and Nowak, 1998). This process is believed to provide protection from further ischemic. In contrast, ischemic tolerance is relatively disregarded at neural stress responses including gene expression, signal transduction, and detection of novel gene isolation.

Recently, a mRNA differential display technique was used to isolate a cDNA encoding irp94 from the brains of rats. This protein has 840 amino acids, a predicated Mw of 94 kDa, 5.13 isoelectric points and -0.58 hydrophobicity (Yagita *et al.*, 1999). The protein sequence showed a high homology to the members of the hsp110 family, including apg-2 from the mouse testis (Kaneko *et al.*, 1997), hsp70RY from the human B cell line (Fathallah *et al.*, 1993), hamster hsp110 (Lee-Yoon *et al.*, 1995), mouse hsp105 (Yasuda *et al.*, 1995) and mouse apg-1 (Xue *et al.*, 1998). The expression of irp94 mRNA has already revealed two forms, where the difference is in the length of the 3′-UTR (untranslated region), at 3 kbp and 5 kbp (Yagita *et al.*, 1999). Mainly the irp94 mRNA is detected in tested tissues including the lung, stomach, liver, spleen, kidney, testis and especially the brain lobes, where the 5 kbp mRNA is dominantly expressed. The reason there are different mRNA expressions is that the UTR variation may affect the degradation process of unmaturated mRNA *in vivo*, which might yield a different stability and function of the irp94 mRNA (Shaw and Kamen, 1986).

While heat shock proteins, especially those in the hsp70 family, have been studied relatively thoroughly for their response to ischemia, the study of the hsp110 family when induced by ischemia is very limited, as far as the brain is concerned. Therefore, in this study, we have tested the expression of a member of hsp110, irp94 (Yagita *et al.*, 1999), in the rat brain after a transient forebrain ischemia, and its expression following reperfusion. There are a number of studies that cover the ischemia at a molecular level, but they have mainly focused on the functions of cytosolic hsp. Other kinds of hsp, local to other cellular compartments of the endoplasmic reticulum (ER), were much less investigated. While it is accepted that many of the proteins' functions are disrupted by ischemia, they are secretory proteins initially folded and assembled in the ER (Paschen *et al.*, 1996). Thus, we have studied the role of ATP on the induction of the irp94 expression using antimycin A, an ATP-depleting agent, in a cell culture model.

Materials and Methods

Animal models and RNA isolation

Adult male Sprague-Dawley rats at an average body weight of 250 g were anesthetized with halothane (1.5% in 70% N_2O and 30% O_2). The body temperature of the rats was maintained at 36~37 °C. A transient forebrain ischemia was induced by occlusion of the bilateral common carotid arteries (CCAs) using metal clips for ten minutes. Then the brains were removed as quickly as possible under anesthetized conditions. To investigate the localization of the irp94 expression, the brain was dissected into the different lobes frontal, parietal, temporal, hippocampus, occipital and cerebellum on ice. To check any changes in the irp94 expression in the hippocampus, CCAs were clamped for five min intervals until twenty min and the hippocampus of each group was removed and then placed on ice. To test the effects of reperfusion, the CCAs were clamped for ten min, and then unclamped removing both clips simultaneously for recirculation, for various intervals between one and 48 h each. After recovery from the anesthesia, the rats lived freely. Ultimately, the hippocampus of each group was removed on ice. Total RNA from each sample was isolated using a RNAzol™ Kit (Tel-Test, INC, Texas, USA) according to the manufacture's instructions.

Probes preparation by RT-PCR

For this study, DNA fragments of irp94 were acquired by RT-PCR against the rat brain total RNA. The conditions and primers used were the forward primer (5′-CAGGATTTGCCCTATCCAGA-3′) and the reverse primer (3′-GTCATTCCGTTCCTTCTCCA-5′). Both sequences were derived from the rat irp94 (GenBank accession No. AF077354) (Yagita *et al.*, 1999). One cycle (five min) at 94 °C, was performed, followed by 29 cycles (one min at 94 °C, one min at 58 °C, one min 72 °C), and then one cycle (nine min at 72 °C). PCR products (501 bp)

were cloned into the TA plasmid (Promega, Madison, USA) and amplified. Single stranded DNA was isolated and sequenced by the dideoxy-chain-termination method, using a Sequenase version 2.0 Kit (US Biochemical, Cleveland, OH), to confirm the irp94 DNA sequence.

Northern blot analysis (Sambrook *et al.*, 1989)

Total RNA samples were taken from each group (twenty micrograms each), electrophoresed on the 1% formaldehyde-agarose gels, and transferred onto a nylon membrane (Boehringer GmbH, Mannheim, Germany). After baking at 68 °C for two h, the membranes were hybridized with the ^{32}P-labeled irp94 cDNA probe generated by the random primer method using a kit (Amersham Life Science, Arlington Heights, USA) (Feinberg and Vogelstein, 1983). After washing at a final stringency of 1 × standard saline citrate (SSC) (150 mM NaCl, 15 mM NaH_2PO_4, pH 7.4) at 65 °C, the membranes were autoradiographed and left overnight at −80 °C. FRTL-5 cells (Fisher rat thyroid epithelial culture cell line) (Kohn and Valente, 1989) were incubated for 60 min in either a normal growth medium as a control or with antimycin A (1, 2.5 and 5 mM). Tunicamycin (10 mg/ml) was used as a positive control for maximal induction of the ER chaperone. Quantification of the exposed intensity on X-ray film was analyzed with an image analyzing system.

Results and Discussion

To investigate the localization of the irp94 mRNA expression in the rat brain, a transient forebrain ischemia was induced by clamping both CCAs for ten min under halothane (1.5% in 70% N_2O and 30% O_2) anesthetization. The brain was then dissected into the different lobes (frontal, parietal, temporal, occipital, and hippocampus and cerebellum) and total RNA was isolated as described in the Methods section of this report. Twenty-μg total RNA for each lobe was used in the Northern blotting. As shown in Fig. 1A, the resulting irp94 expression, like other heat shock proteins, was distributed through all the tested lobes. The maximum expression was observed in the occipital lobe and the minimum expression was in the parietal lobe.

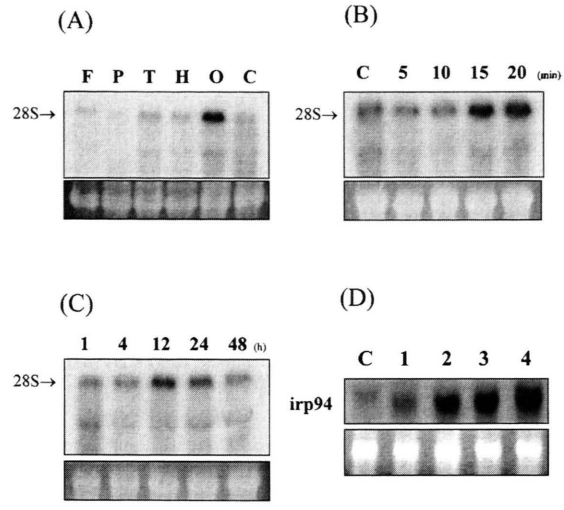

Fig. 1. Expression of irp94 mRNA in rat brain and in cultured cells.
Upper panel: Each lane contained 20 μg of total RNA. Lower panel: Ethidium bromide staining of the 28S rRNA was used to indicate the equivalence of the load on the gel. An arrow of 28S indicates the location of 28S rRNA used as a marker of mRNA size. The experiments above were performed with duplicate samples.
(A) Localization of the irp94 mRNA expression in rat brain after 10 min transient forebrain ischemia. Abbreviations: F = frontal lobe, T = temporal lobe, P = parietal lobe, H = hippocampus, O = occipital lobe, C = cerebellum.
(B) Expression of irp94 mRNA in rat hippocampus following various periods of transient forebrain ischemia. C indicates control, which is only operated but not clamped CCAs, lanes 5, 10, 15, 20 min of ischemia induction.
(C) Expression of irp94 mRNA in rat hippocampus following various reperfusion after 10 min of transient forebrain ischemia. Lanes 1, 4, 12, 24, 48 h of reperfusion after 10 min induction of transient forebrain ischemia.
(D) ATP depletion in cultured cells inducing an ischemia-like status. Upper panel: Each column contained 20 μg of total RNA from various conditions. FRTL-5, thyroid epithelial cells, grown up to 60 min in the absence (as a control, indicated by C) or presence of either 1, 2.5, 5 μM antimycin A (lanes 1, 2, 3) or 10 μg/ml tunicamycin (lane 4) as a positive control for maximal induction of hsp.

The irp94 mRNA expression in the occipital lobe was approximately 36 times higher than its expression in the parietal lobe and six times higher than its expression in the hippocampus. The Yagita group found different results showing the maximum irp94 mRNA expression in the cerebellum (Yagita *et al.*, 1999). We suggest that the different results may be due to an alternative way of induc-

ing the transient ischemia. Although the maximum observed expression was different, Yagita *et al.* also reported that the irp94 mRNA was expressed through the entire brain. The results of our study suggest that the brain was largely stimulated by the transient ischemic injury, but that the occipital lobe was the most sensitive to this particular injury and the parietal lobe was the least sensitive.

We have examined the irp94 mRNA expression in the hippocampus of rats, by varying the clamping times of both CCAs following a transient forebrain ischemia. As shown in Fig. 1B, after five min of induced ischemia, the irp94 mRNA expression began to increase gradually, reaching its maximum expression at fifteen min, and then decreasing by about 15% after a total of twenty min. The relative value of the maximum irp94 expression was about 2.5 times its minimum in the control group. The maximum expression of the irp94 at fifteen min was similar to that in Yagita's report, but his report does not show the decreased expression afterward fifteen min.

Considering the fact that irp94 belongs to the hsp110 family, the similar expression patterns are not a strong argument because, in general, the hsp expression increased rapidly only early on and for a short time after external stimulations (e.g. heat, heavy metal, metabolically toxic matters, or unfolded protein accumulation). It is well known that, early in the ischemic stimulation, hsp overexpression acts to protect the host cell or/and tissue to keep them physiologically homeostatic. In this study, we could not show any direct evidence that the irp94 mRNA expression was associated with ischemic tolerance. However, the results do suggest that in cases of induced transient forebrain ischemia, the irp94 mRNA expression might play a pivotal role in minimizing cell damage against rapid environmental changes of ATP and biofactors supplying blood flow to brain.

To understand whether the irp94 expression is associated with rehabilitating the damaged cells in the hippocampus of a rat that has had an induced transient forebrain ischemia, we have performed reperfusion experiments. These experiments consisted of clamping both CCAs of the rat for ten min, and then allowing reperfusion for several different durations ranging between one and 48 h. As shown in Fig. 1C, the expression of the irp94 mRNA in the hippocampus for reperfusion gradually increased during the first twelve h, showing its maximum expression at twelve hours after reperfusion, and decreasing thereafter. The relative maximum value of irp94 at twelve h was approximately three times higher than its expression at one h. The data suggest that irp94 might aid in the rehabilitation of cells that are damaged by ischemia in the rat hippocampus. This is due to occulted bilateral CCAs only and not bilateral vertebral arteries. It has already been reported in several cases that hsp performs such recovery functions.

Oh *et al.* demonstrated that hsp110 protected intracellular proteins from denaturation by heat stress more effectively than cytosol resident hsc70 (Oh *et al.*, 1997), and Yagita *et al.* (1999) reported that irp94 played a role in repairing thermotolerance in the brain. These data may suggest that irp94 plays a more direct role in protein folding and in repairing misfolded proteins in the ER.

To understand this better, we ran comparative experiments using a typical ER resident molecular chaperone, Erp72, which has protein folding and assembly functions in the ER. As shown in Fig. 1D, the results of the Northern blot analysis revealed that the Erp72 mRNA expression showed similar inducibility as irp94 in the hippocampus induced transient forebrain ischemia after reperfusions lasting one to 48 h. After clamping the CCAs for ten min and reperfusion, the irp94 mRNA expression reached a maximum at the expression of hsp72 was constant during the one to 48 h of reperfusion. The present data may be strong evidence that irp94 performs protein folding and assembly functions like the molecular chaperones in the ER, with the exception of cytosol because Erp72 exists only in the ER lumen and hsp72 is in the cytosol to play both proteins as a molecular chaperone.

The effects of ischemia on protein maturation are not yet well understood. During ischemia, there is a rapid decrease in ATP levels and an increase in ER molecular chaperones, among other things. The function of these depends largely on a constant intracellular ATP, which is thought to be required for at least the folding/refolding processes (Paschen, 1996). In ischemia, it is believed that intracellular ATP depletion leads to misfolding and accumulation of proteins, which leads to an extensive increase in the irp94 expres-

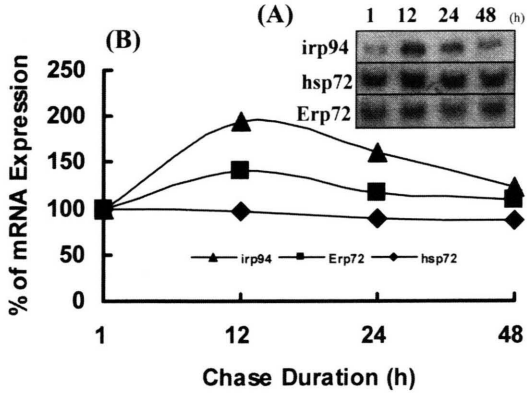

Fig. 2. Comparative expression of irp94 with other heat shock proteins of Erp72 and hsp72.
(A) Lanes 1, 12, 24, 48 h of reperfusion after 10 min of induced transient forebrain ischemia. Each column contained 20 μg of total RNA from the hippocampus. Northern blotting was performed using the ^{32}P-labeled probe for irp94, Erp72 and hsp72, respectively. (B) Quantification of the exposed intensity on X-ray film was analyzed by using image analyzing system. The experiments were performed with duplicate samples and the results were the averages of three repeated experiments. Data of a typical expt. are shown.

sion, like other ER chaperones that assist effective folding and/or degradation of misfolded proteins.

The present study investigated the effects of ER resident ATP depletion on the expression of the irp94 gene by examining the cultured cell models for the epithelial ischemic state. Antimycin A, the ATP-depleting agent, has been used extensively for creating an *in vitro* cell culture model for ischemia. Kuznetsov *et al.*, (1996) already demonstrated that rat thyrocytes of the FRTL-5 cell line show a very high sensitivity to antimycin A: One h of treatment with 2.5 μM concentration of antimycin A alone reduced intracellular ATP level to 2% of control. As described in the Method section, the cultured FRTL-5 cells were incubated for 60 min in either normal growth medium as a control, or with antimycin A (1, 2.5 and 5 μM). Tunicamycin (10 μg/ml) was used as a positive control so the ER chaperone would be induced with the greatest likelihood.

Fig. 2 shows the results of the Northern blot analysis and indicates that the expression of irp94 was triggered by a decrease in the ER ATP, and that the constant increase in the mRNA expression for irp94 was dependent on the concentration of antimycin A. The increase in the level of irp94 mRNA after treatment with a high concentration of antimycin A was basically the same as in the results of tunicamycin-treated cells. Thyrocytes proved to be extremely sensitive to the tunicamycin treatment and induced the rapid expression of the ER chaperones. This is attributed to thyroglobulin, a primary secretory glycoprotein, misfolding in the ER due to inhibited glycosylation. As expected, the irp94 mRNA expression was also very sensitive to the antimycin A treatment, suggesting that a transient forebrain ischemia induces disturbances of the ER functions, probably by depleting the stored ER resident ATP. In addition, irp94 may play the role of an ER chaperone by correcting for newly synthesized protein folding.

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Chopp M., Chen H., Ho K. L., Dereski M. O., Brown F. E., Hetzel F. W. and Welch K. M. A. (1989), Transient hyperthermia protects against subsequent forebrain ischemic cell damage in the rat. Neurology **39**, 1396–1398.

Fathallah D. M., Cherif D., Dellagi K. and Arnaout M. A. (1993), Molecular cloning of novel human hsp70 from a B cell line and its assignment to chromosome 5. J. Immunol. **151**, 810–813.

Feinberg A. P. and Vogelstein B. (1983), A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**, 6–13.

Gething M. J. and Sambrook J. (1992), Protein folding in the cell. Nature **355**, 33–45.

Harrub J. B. and Nowak T. S. Jr. (1998), Cryptic expression of the hsp72 in gerbil hippocampus after transient ischemia. Neurochem. Res. **23**, 703–708.

Kaneko Y., Kimura T., Kishishita M., Noda Y. and Fujita J. (1997), Cloning of apg-2 encoding member of heat shock protein 110 family. Gene **189**, 19–24.

Kohn L. D. and Valente W. A. (1989), FRTL-5 manual: A current guide. In: FRTL-5 Today (Perrild H. ed.). Elsevier Science Publishers, New York, pp. 243–262.

Kristian T. and Siesjo B. K. (1996), Calcium-related damage in ischemia, Life Sci. **59**, 357–367.

Kuznetsov G., Bush K. T., Zhang P. L. and Nigam S. K. (1996), Perturbations in maturation of secretory proteins and their association with endoplasmic reticulum chaperones in a cell culture model for epithelial ischemia Proc. Natl. Acad. Sci. USA. **93**, 8584–8589.

Lee-Yoon D., Easton D., Murawski M., Burd R. and Subjeck J. R. (1995), Identification of a major subfamily of large hsp70-like proteins through the cloning of the mammalian 110-kDa heat shock protein. J. Biol. Chem. **270**, 15725–15733.

Morimoto R. I., Tissieres A. and Georgopoulos C. (1994), The biology of heat shock proteins and molecular chaperones. In: Postischemic Stress Response in Brain (Nowak Jr. T. S. and Abe H. ed.). (Cold Spring Harbor Laboratory Press). pp. 553–575.

Nowak T. S. Jr. (1991), Localization of 70 kDa stress protein mRNA induction in gerbil brain after ischemia. J. Cereb. Blood Flow Metab. **11**, 432–439.

Oh H. J., Chen X. and Subjeck J. R. (1997), hsp110 protects heat denatured proteins and confers cellular thermoresistance. J. Biol. Chem. **272**, 31636–31640.

Paschen W. (1996), Disturbances in calcium homeostasis within the endoplasmic reticulum may contribute to the development of ischemic cell damage. Med. Hypoth. **47**, 283–288.

Paschen W., Doutheil J., Gissel C. and Treiman M. (1996), Depletion of neuronal endoplasmic reticulum calcium stores by thapsigargin effect on protein synthesis. J. Neurochem. **67**, 1735–1743.

Paschen W., Gissel C., Linden T. and Doutheil J. (1998), Erp72 expression activated by transient cerebral ischemia or disturbance of neuronal endoplasmic reticulum calcium stores. Metab. Brain Dis. **13**, 55–68.

Sambrook J., Fritsch E. F. and Maniatis T. (1989), Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Shaw G. and Kamen R. (1986), A conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell **46**, 659–669.

Siesjo B. K. (1981), Cell damage in the brain: a speculative synthesis, J. Cereb. Blood Flow Metab. **1**, 155–185.

Xue J. H., Fukuyama H., Nonoguchi K., Kaneko Y., Kido T., Fukumoto M., Fujibayashi Y., Itoh K. and Fujita J. (1998), Induction of apg-1, a member of the heat shock protein 110 family, following transient forebrain ischemia in the rat brain. Biochem. Biophys. Res. Commun. **247**, 796–801.

Yagita Y., Kitagawa K., Taguchi A., Ohtsuki T., Kuwabara K., Mabuchi T., Matsumoto M., Yanagihara T. and Hori M. (1999), Molecular cloning of novel member of the HSP110 family of genes, ischemia-responsive protein 94 kDa (irp94), expressed in rat brain after transient forebrain ischemia. J. Neurochem. **72**, 1544–1551.

Yasuda K., Nakai A., Hatayama T. and Nagata K. (1995), Cloning and expression of murine high molecular mass heat shock proteins, HSP105. J. Biol. Chem. **270**, 29718–29723.