Ischemia-Responsive Protein (irp94) Gene Expression in Neurons

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An increased expression of the ischemia-responsive protein gene (irp94) was detected in a Mongolian gerbil brain after an ischemic injury, particularly in the cerebral cortex and hippocampus. In a rat phaeochromocytoma tumour cell line (PC12 cells), actinomycin D blocked the irp94 gene expression but cycloheximide did not. This indicates that irp94 gene expression is transcriptionally controlled. The half-life of irp94 mRNA was estimated to be approx. 5 h using 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB). In addition, irp94 expression was enhanced by either endoplasmic reticulum (ER)-stress-inducible drugs or protease inhibitors. This suggests that irp94 gene expression is strongly associated with the unfolded protein response (UPR) in neurons.

Key words: Ischemia-Responsive Protein (irp94) Gene, Mongolian Gerbil, PC12 Cells

Introduction

The cDNA that encodes a 94 kDa ischemia-responsive protein (irp94) from the rat hippocampal was isolated using a mRNA differential display technique in the four-vessel occlusion model in rats. The Yagita group characterized irp94 at the molecular level (Yagita *et al*., 1999). Sequence analysis showed that rat irp94 shared a high homology with mouse apg-2 ($> 90\%$), human heat shock protein (hsp) 70RY and a member of the HSP110 family (approx. 60%). The irp94 mRNA was constitutively expressed in the normal hippocampus. However, its expression is mainly enhanced under ischemic injury for 10 min (1.9-fold increase) and 15 min (3.4-fold increase) but not by heat shock. This suggests that irp94 expression was enhanced and not associated with the heat shock signaling mechanism (Yagita *et al*., 1999). Irp94, a novel member of the HSP110 family, plays an important role in altering neuronal functions. In addition, the localization of irp94 mRNA was determined in the endoplasmic reticulum using *in situ* hybridization histochemistry (Yagita *et al*., 2001).

It was also suggested that irp94, like a molecular chaperone, plays a role in protecting the cell against external stimulation, particularly after transient forebrain ischemia in a rat (Koh *et al*., 2000). On the other hand, in a thyroid cell culture model, FRTL-5, the irp94 expression pattern was similar to that of the endoplasmic reticulum chaperone, Erp72, but not to the cytosol chaperone, hsp72. The functional role of irp94 was suggested to be a component of the stress response in neurons. In addition, its enhanced expression was detected when the cells were incubated with the thyroid-stimulating hormone (Kim *et al*., 2001). This study demonstrates the molecular properties of irp94 expression such as the mRNA half-life, response to ER (endoplasmic reticulum) stresses and transcriptional control. As a step toward elucidating the function of the irp94 in neurons, it is essential to know how the irp94 gene is controlled under different conditions.

Materials and Methods

Male Mongolian gerbils (*Meriones unguiculatus*; 60–80 g) were anesthetized with halothane (1.5% in 70% N_2O and 30% O_2) and given an ischemic injury. Both the common carotid arteries (CCAs) were exposed by a ventral midline incision and separated carefully from the adjacent veins and nerves. After scarification, the brain tissues were removed as quickly as possible under standard

conditions. The total RNA was isolated and examined by Northern blot analysis (Giuffrida *et al.*, 1992).

Rat phaeochromocytoma PC12 cells were routinely cultured in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% horse serum and 5% fetal calf serum on collagen-coated dishes in a humidified 5% CO_2 atmosphere at 37 °C (De Leon *et al.*, 1994). The total RNA was extracted using a SV Total RNA isolation system (Promega, Medison, USA). Each 10 μl aliquot of the total RNA was separated on denaturing agarose gel (2.5% agarose containing 2.2 M formaldehyde), transferred onto a nylon blotting membrane (Schleicher & Schuell, New Hampshire, USA), and the resulting membrane was hybridized with a high concentration SDS buffer [7% SDS, 50% formamide, 5 × SSC (43.8 g NaCl, 22 g sodium citrate/l water), 2% blocking reagent, and 50 mM sodium phosphate] at 50 °C overnight and with the [α-^{32}P]dCTP-labelled irp94 DNA probe (501 bp), which was acquired by RT-PCR using the forward primer (5′-CAGGATTTGCCCTATCCAGA-3′) and reverse primer (3′-GTCATTCCGTTCCTTCTCCA-5′) derived from rat irp94 (GenBank accession No. AF077354). Both RT-PCR primers were supplied by Bioneer Co., Korea. The membrane was rinsed twice with 2 × SSC and 0.1% SDS, and exposed to a X-ray film at −70 °C for 5–24 h. The ER-stress-inducible drugs tunicamycin, A23187 and DTT, the proteasome inhibitors ALLN (*N*-acetyl-L-leucinyl-L-leucinyl-norleucinal) and MG 132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal), unless otherwise noted, were purchased from Sigma (St. Louis, USA).

Results and Discussion

Northern blot analysis of irp94 mRNA in Mongolian gerbil brain, where only common carotid arteries (CCAs) exist without a vertebral artery, was carried out to determine the expression level of the irp94 gene induced by the CCAs. After inducing an occlusion for 30 min followed by reperfusion for 3 h, the brain was dissected into the cerebral cortex, cerebellum, hippocampus, and spinal cord. The total RNA was isolated and 20 μg of each sample were used as described in Materials and Methods. Strong irp94 mRNA expression was observed at both the cerebral cortex (approx. 3.5 times) and hippocampus (approx. 4 times higher) in the experimental group but not in the control group (Fig. 1). It was already reported that the induction of GRP94 gene expression by the CCAs is mainly detected at the occipital lobe (Kim *et al.*, 2003). However, in this case, irp94 was expressed differently at the cerebral cortex and hippocampus.

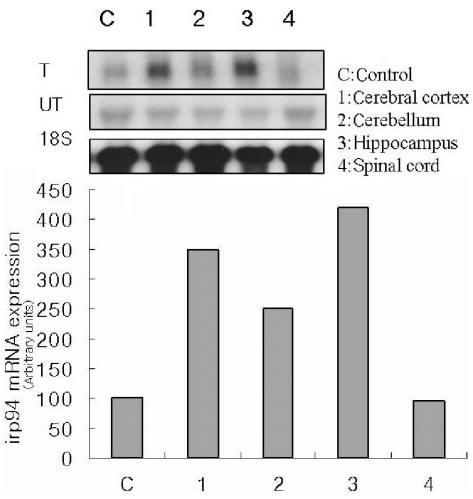

Fig. 1. Northern blot analysis of irp94 mRNA in Mongolian gerbil brain. The upper panel in the Northern blot shows irp94 mRNA resulting from ischemia (T), the middle panel shows the expression without the induction of ischemia (UT), and the left lower panel shows rat 18S rRNA as a loading control. The irp94 band intensities were measured using the ImageQuant software package. The experiments were performed in duplicate and the average result is indicated in the bar graph. 18S (18S rRNA) was used to indicate the equivalence of the load on the gel. Northern blotting conditions are described in Materials and Methods.

PC12 cells were treated with actinomycin D (AD; transcription inhibitor) and cycloheximide (CH; translation inhibitor) for the indicated times in order to determine if irp94 mRNA expression is regulated at the transcriptional or translational steps, respectively (Park *et al.*, 2001). Fig. 2 shows the Northern blotting results. When the cells were treated with 0.2 μg/ml AD for 2 h and 0.2 μg/ml AD for 10 h, the resulting signal of irp94 mRNA almost vanished. On the other hand, for the cells treated with 0.2 μg/ml CH (2 h), the expression pattern was similar to that of the control, even though its expression was slightly weaker. A comparatively weaker irp94 mRNA expression was observed in the cells treated with 2 μg/ml CH for 10 h comparing its expression with the control group. This was attributed to a side effect of the depressed degree of translation. It is suggested

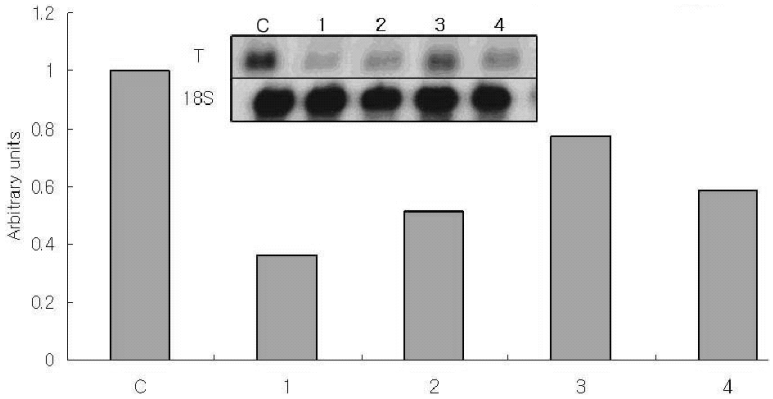

Fig. 2. Effects of actinomycin D (AD) and cycloheximide (CH) on irp94 mRNA. Confluent PC12 cells were treated with either actinomycin D or cycloheximide for the indicated times and doses, respectively. C, control; lane 1, 0.2 μg/ml AD for 2 h; lane 2, 0.2 μg/ml AD for 10 h; lane 3, 2 μg/ml CH for 2 h; lane 4, 2 μg/ml CH for 10 h; T, ischemia induction. Each lane equivalent total RNA was used in Northern blotting and the irp94 band intensities were measured using the ImageQuant software package. All experiments were carried out in duplicate and the average result is indicated in the bar graph.

that irp94 gene expression in the neurons would be controlled in the transcription step not in the translation step.

The half-life of irp94 mRNA in a neuron was estimated using DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole), which is a specific inhibitor of RNA polymerase II (Sinn and Sigmund, 1999). DRB treatment prevents new mRNA synthesis and allows to monitor intracellular residue mRNAs. As shown in Fig. 3, the expression level of irp94 mRNA in the neurons gradually weakened with time, the basal signal being detected after 6 h. This means that the previously aggravated irp94 mRNA had almost decayed. Finally, the half-life of irp94 mRNA in the neurons was estimated to be approx. 5 h, when half of the irp94 mRNA in the neurons had been destroyed.

As shown in Fig. 4, the PC12 cells were treated with some types of ER-stress-inducible drugs such as the calcium ionophore A23187, BFA (brefeldin A), tunicamycin, DTT (dithiothreitol) and H_2O_2. Of these, the maximal response of irp94 mRNA expression against the ER-stress-inducible drugs was observed with tunicamycin, which inhibited *N*-glycosylation on the newly synthesized secretory and membrane protein in the ER. None of the other drugs, except for H_2O_2, produced any significant induction of irp94 mRNA. This shows that irp94 gene expression is ER-stress-inducible. In particular, the expression is susceptible to tunicamycin. It means unfolded/misfolded proteins causing a deficiency in *N*-glycosylation are main

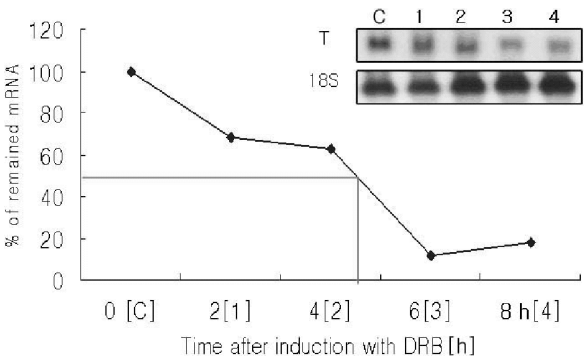

Fig. 3. Estimation of the irp94 mRNA level in a cell using 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB). Confluent PC12 cells were treated with DRB (25 μg/ml) for the indicated times. C, control; lane 1, after 2 h; lane 2, after 4 h; lane 3, after 6 h; lane 4, after 8 h. The total RNA was isolated at various times after washing out the old medium. Each lane equivalent total RNA was used in Northern blotting and the irp94 band intensities were measured using the ImageQuant software package. All experiments were carried out in duplicate and the average result is indicated in the bar graph.

triggers of irp94 mRNA for the post-translational modification of the secretory glycoproteins compared with other ER stresses (Park *et al.*, 2005).

According to the up-dated study when inducing ER stress, the concept of endoplasmic reticulum-associated degradation (ERAD) has been used to show that the abnormal protein integrated in ER is broken down at a steady rate (Meusser *et al.*, 2005). Proteins destined for degradation in the ER

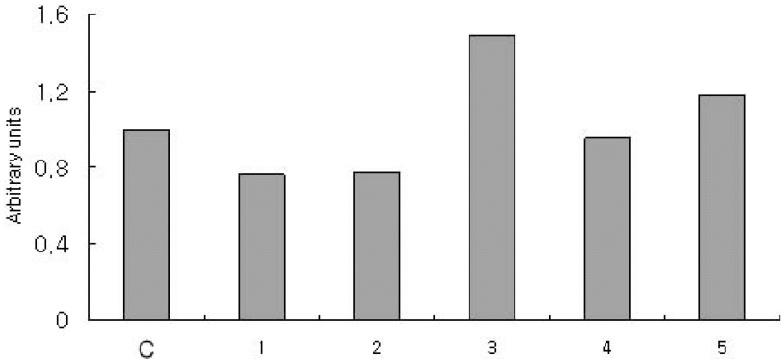

Fig. 4. The expression of irp94 against ER-stress-inducible drugs. Confluent PC12 cells were treated with ER-inducible drugs for 3 h. C, control; lane 1, 10 μM Ca^{2+} ionophor A23187; lane 2, 10 μg/ml BFA; lane 3, 20 μg/ml tunicamycin; lane 4, 3 mM DTT; lane 5, 10 mM H_2O_2. Each lane equivalent total RNA was used in Northern blotting and the irp94 band intensities were measured using the ImageQuant software package. All experiments were carried out in duplicate and the average result is indicated in the bar graph.

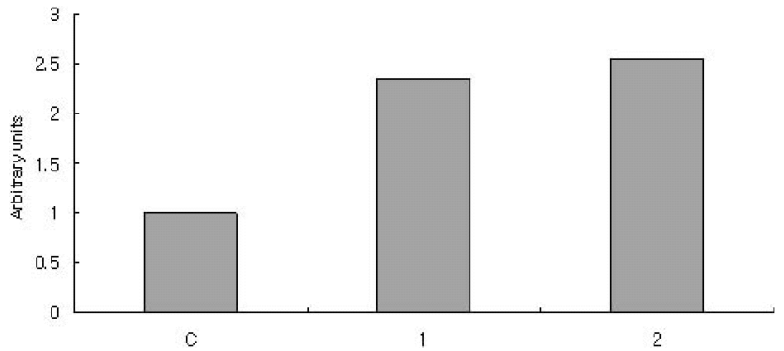

Fig. 5. The expression of irp94 against the proteasome inhibitors. Confluent PC12 cells were treated with the proteasome inhibitors for 2 h. C, control; lane 1, 25 μM ALLN; lane 2, 25 μM MG 132. Each lane equivalent total RNA was used in Northern blotting and the irp94 band intensities were measured using the ImageQuant software package. All experiments were carried out in duplicate and the average result is indicated in the bar graph.

lumen must be transported across the ER membrane to the cytosol, where they can be degraded through the assistance of the 26S proteasome. It was already reported that the inhibition of the 26S proteasome enhances the expression of the ER-resident chaperone, which is in this case ERSD (ER storage disease) (Kim and Arvan, 1998). This study examined whether or not irp94 expression is inducible when the cells are treated with proteasome inhibitors. Strong irp94 expression was detected after each ALLN and MG 132 treatment (Fig. 5). This shows that the expression pattern of irp94 is similar to the typical ER-stress-inducible chaperones. This suggests that irp94 may act through a process that directly removes the protein from the folding environment of the ER.

In summary, a significant increase in the ischemia-responsive protein (irp94) gene expression was detected in both cerebral cortex and hippocampus in an *in vivo* Mongolian gerbil brain ischemic injury model. In PC12 cells, irp94 gene expression was transcriptionally controlled because actinomycin D (transcription inhibitor) blocked the irp94 expression while cycloheximide (translation inhibitor) did not. The half-life of irp94 mRNA was estimated to be approx. 5 h by DRB. In addition, the expression of irp94 was enhanced by either ER-stress-inducible drugs such as calcium ionophore A23187, BFA (brefeldin A), tunicamycin, DTT and H_2O_2, and protease inhibitors like ALLN and MG 132. These results have potential implications in brain ischemia. This is because

irp94 expression is strongly associated with the unfolded protein response (UPR), which is conserved in all eukaryotic cells and participates in the progression of brain ischemia. Future studies, relating irp94 activation to brain ischemia protection, as well as studies examining the roles of other branches of the UPR during ischemia damages, will be needed to further illuminate the potential involvement of irp94 expression in ER-stress-associated disease and pathology.

Acknowledgement

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