Ischemia-Responsive Protein (irp94) Is Up-Regulated by Endoplasmic Reticulum Stress

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The expression of the ischemia-responsive protein (irp94) was enhanced by endoplasmic reticulum (ER) stress inducing drugs such as brefeldin A (BFA), calcium ionophor A23187, dithiothreitol (DTT) and tunicamycin in fisher rat thyroid epithelial cell line (FRTL-5 cells). In particular, irp94 mRNA expression was increased dose dependently by tunicamycin, and there was increased irp94 expression when the cells were incubated with the thyroid-stimulating hormone (TSH) together.

Introduction

Recently, the cDNA encoding irp94 from rat brains was isolated using a mRNA differential display technique (Yagita *et al.*, 1999). Irp94 transcriptional expression has been previously reported, in which after 10 min. of ischemia, the highest level of mRNA expression was found to be at the occipital lobe and the lowest level was located at the parietal lobe. After 10 min ischemia followed by reperfusion, the irp94 mRNA expression level gradually increased in the first 12 h, then decreased. In addition, the irp94 mRNA expression pattern was similar to the expression of the endoplasmic reticulum chaperone, Erp72, after intracellular ATP depletion using antimycin A in a thyrocyte cell culture model (Koh *et al.*, 2000). This suggested that irp94 may play a role in protecting cells against ER stress. In this report irp94 expression, whether or not in response to ER stress using ER stress inducible drugs such as BFA (blocking secretory protein transfer from the ER to the Golgi complex), the calcium ionophor A23187 (depletion of calcium stores), DTT (a disruption of proper disulfide bond formation) and tunicamycin (a N-glycosylation inhibitor) in the FRTL-5 cells, was confirmed.

Materials and Methods

FRTL-5 cells (American Type Culture Collection, CRL #8305) were cultured in Coon's medium, containing 5% calf serum, 10^{-3} μM TSH, 5 μg/ml transferrin, 1 μg/ml insulin and 10 nM hydrocortisone (Kwon *et al.*, 1999). Following treatment, the FRTL-5 cells were scraped into 10 ml of cold PBS and centrifuged for 3 min at 2000×*g*. The pellet was homogenized in 15 ml of a lysis buffer [10 mM Tris (hydroxymethyl) aminomethane-HCl pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 0.25% Triton X-100]. To obtain the total RNA, the suspension was layered onto a 1.2-ml cushion of 5.7 M CsCl in 0.1 M EDTA, and centrifuged at 15 °C for 24 h at 120,000 *g* in a Beckman SW55 rotor. Equal amounts of RNA (20 μg) were separated on a denaturing agarose gel (2.5% agarose containing 2.2 M formaldehyde), then transferred to a nylon membrane (Boehringer GmbH, Germany). After the membrane was UV-crosslinked (UV-Stratalinker 1800, Stratagene), it was prehybridized in a high concentration SDS buffer [7% SDS, 50% formamide, 5 × SSC (43.8 g NaCl, 22.05 g sodium citrate/1 l water), 2% blocking reagent, and 50 mM sodium phosphate] at 50 °C for 2 h. Overnight hybridization at 50 °C was performed using the same SDS buffer containing a [α-^{32}P]-dCTP-labeled irp94 DNA probe (501 bp), which was acquired by RT-PCR using the forward primer (5′-CAGGATTTGCCCTATCCAGA-3′) and the reverse primer (3′-GTCATTCCGTTCCTTCTCCA-5′) derived from rat irp94 (GenBank accession No. AF077354) (Yagita *et al.*, 1999). The membrane was rinsed twice with 2 × SSC and 0.1% SDS then exposed to X-ray film at −70 °C for 5–24 h. Drugs of BFA, calcium ionophor A23187, DTT and tunicamycin were from Sigma Chemical Co. (St. Louis, MO).

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Results and Discussion

While a number of cytosolic stress proteins involved in ischemia induction following cell damage and/or recovery have been well studied, data on the endoplasmic reticulum (ER) stress proteins associated with ischemia are relatively limited (Nowak, 1991). It has been previously demonstrated that irp94 mRNA expression is enhanced by ischemia stress and ATP depletion (Koh *et al.*, 2000). However, the effect of ER stress on irp94 - gene expression still unclear. As a step toward elucidating the function of irp94 against the unfolded protein response (UPR) in the ER, it was necessary to determine whether or not the irp94 gene is controlled by ER stresses. With this in mind, irp94 mRNA expression was investigated under various ER stress conditions by Northern blot analysis. A previous report on irp94 mRNA expression in the FRTL-5 cells revealed two different transcriptional forms, the shorter (3 kb) and the longer (5 kb). The reason why the two kinds of irp94 mRNA originate from the one gene is not clear. However, it has been suggested that they affect the degradation process of irp94 mRNA for the different functions (Koh *et al.*, 2000). While irp94 expression by ischemia stress in a previous study showed mainly the 5 kb transcript, the shorter 3 kb transcript was mainly induced by ER stresses in this study. This suggests that alternative irp94 mRNA splicing occurs by different intracellular stress, which might yield a different stability *in vivo*.

As shown Fig. 1A, the FRTL-5 cells were treated with various ER-stress inducible drugs such as BFA, calcium ionophor A23187, DTT and tunicamycin. After 3 h treatment with BFA, the calcium ionophor A23187 and DTT, compared to the control (0 h), irp94 mRNA expression peaked then decreased thereafter (8 h). However, irp94 expression in the cells treated with tunicamycin increased gradually and the maximum by chase periods for 8 h. In general, other typical ER stress proteins (Bip, GRP94, Erp72) were also expressed, which reached a peak after 8 h (Welihinda *et al.*, 1999). This indicates that irp94 mRNA is ER stress-inducible mRNA, like other ER chaperones, in that its expression is susceptible to tunicamycin. Subsequently the effect of different tunicamycin concentrations on irp94 mRNA expression over 5 h was examined (Fig. 1B). Although weak irp94 expression was detected when the cells were not treated with tunicamycin, its expression was increased gradually by increasing the tunicamycin concentrations for 5 h. This suggests that tunicamycin inhibits N-glycosylation on newly synthesized secretory proteins in the ER, resulting in the accumulation of unfolded/misfolded proteins, which may induce irp94 expression for post-translational modification of secretory proteins in the ER. To investigate the effect of TSH on irp94 mRNA expression under ER stress by tunicamycin, after preincubation for 2 days in the absence of TSH to remove the effection of TSH, the FRTL-5 cells were treated for 8 h with tunicamycin in the presence or absence of 10^{-3} μM TSH. As shown in Fig. 1C, irp94 expression in cells

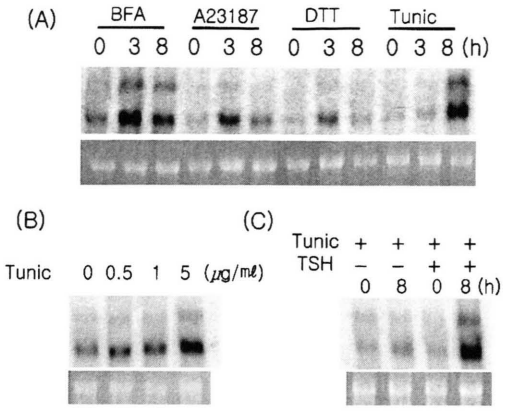

Fig. 1. Expression of irp94 under ER stresses and TSH.
(A) Various ER stress induced irp94 mRNA expression. Upper panel: The cells were incubated with 10 μg/ml brefeldin A (BFA), 7 μM A23187, 3 mM dithiothreitol (DTT), and 2 μg/ml tunicamycin for indicated periods (0, 3, 8 h). Each column contained 20 μg of total RNA. Northern blotting was performed using the [α-^{32}P]-dCTP-labeled irp94 DNA probe acquired by RT-PCR. The experiments were performed in duplicate. Lower panel: Ethidium bromide staining of the 28S rRNA was used to indicate the equivalence of the load on the gel.
(B) Tunicamycin dose dependent expression of irp94 mRNA.
The cells were incubated with different tunicamycin concentrations (0, 0.5, 1, 5 μg/ml). The conditions of Northern blotting and equivalence of the loading total RNA on the gel were same with upper (A).
(C) Thyroid-stimulating hormone (TSH) accelerated induction of irp94 mRNA expression.
The cells were incubated with 2 μg/ml tunicamycin and/or 10^{-3} μM TSH after preincubation for 2 days in the absence of TSH. The conditions of Northern blotting and the equivalence of the total RNA load on the gel were the same with the upper (A).

cell treated with tunicamycin in the absence of TSH for 8 h was weak compared with the cells treated with tunicamycin and TSH. Although some reports show that some genes in FRTL-5 cells express TSH dependently (Kwon *et al.*, 2000; Park *et al.*, 2001; Shong *et al.*, 1999), this is the first report suggesting that irp94 mRNA is TSH dependently expressed, and is particularly enhanced under ER stress.

In conclusion, irp94 expression in the thyrocytes of FRTL-5 cells was enhanced under ER stress conditions. This is the first report that irp94 mRNA is an ER stress inducible mRNA. In addition, TSH enhances its expression. It is recommended that the functional role of irp94 as a chaperone in the thyrocytes, in that it might help the folding and assembly of secretory proteins in the ER, be further investigated.

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