Response of neurons to an irreversible inhibition of endoplasmic reticulum Ca^{2+}-ATPase: relationship between global protein synthesis and expression and translation of individual genes

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In the physiological state, there appears to be a regulatory link between endoplasmic reticulum (ER) Ca^{2+} homoeostasis and the initiation of neuronal protein synthesis. Exposing neuronal cell cultures to thapsigargin (Tg), an irreversible inhibitor of sarcoplasmic/ER Ca^{2+}-ATPase (SERCA), induced an almost complete suppression of protein synthesis, which recovered to approx. 60 % of control 24 h after Tg exposure. This is an experimental model where the regulatory link between the initiation of protein synthesis and ER Ca^{2+} homoeostasis recovers, despite an irreversible suppression of SERCA activity [Doutheil, Treiman, Oschlies and Paschen (1999) Cell Calcium **25**, 419–428]. The model was used to investigate the relationship between transcription and translation of various stress genes that respond to conditions causing graded suppression of protein synthesis. Expression patterns revealed three groups of genes. The mRNA levels of genes responding to conditions of ER stress (*grp78*, *grp94*, *gadd34* and *gadd153*) were increased up to 200-fold after Tg exposure, whereas those coding for ER-resident proteins (SERCA 2b and Bcl-2) were increased up to 6-fold in treated cultures, and those coding for cytoplasmic proteins (heat-shock protein 70 and p67) were increased only 2–4-fold. Analysis of translation of these mRNAs suggests an imbalance in the synthesis of apoptosis-inducing (GADD153) and tolerance-activating (GRP78 and Bcl-2) proteins after blocking of the ER Ca^{2+} pump. The observation that the relationship between Tg-induced changes in mRNA and protein levels varied considerably for the various genes studied implies that translation of the respective transcripts is differently regulated under conditions causing graded suppression of global protein synthesis. Detailed analysis of the response of neuronal cells to transient disturbance of ER Ca^{2+} homoeostasis may help to elucidate the mechanisms underlying neuronal cell injury in those neurological disorders in which an impairment of ER function is thought to contribute to the pathological process of deterioration.

Key words: GADD153, GRP78, heat-shock protein 70, neuronal cell culture, unfolded-protein response.

INTRODUCTION

The endoplasmic reticulum (ER) plays two central roles in cells: it contributes to cellular Ca^{2+} signalling and storage [1,2], and also provides the site for folding and processing of newly synthesized membrane and secretory proteins, reactions which require a high Ca^{2+} activity [3–5]. The pivotal role of these processes for proper cell function is indicated by the observation that under conditions associated with ER dysfunction (i.e. disturbance of ER Ca^{2+} homoeostasis or impairment of the folding and processing reactions), two highly conserved stress responses are activated, the ER-overload response (reviewed in [6]) and the unfolded-protein response (UPR; reviewied in [7]). The ER-overload response is characterized by an induction of the transcription factor, nuclear factor κB, which then activates the expression of target genes coding for pro-inflammatory proteins [6]. UPR is characterized by an activation of two divergent processes: a suppression of the initiation process of global protein synthesis, and an activation of the expression of genes encoding ER-resident proteins and the transcription factor GADD153 (growth arrest- and DNA damage-inducible gene 153) [7]. A similar response is induced in the brain in conditions associated with severe forms of metabolic stress, implying common underlying mechanisms. It has therefore been proposed that ER dysfunction contributes to neuronal cell injury in pathological states of the brain, including ischemia and Alzheimer's disease [8,9].

Depletion of ER Ca^{2+} stores by exposure of cells to thapsigargin (Tg), an irreversible inhibitor of the sarcoplasmic/ER Ca^{2+}-ATPase pump (SERCA) [10], has been shown to induce cell injury in various neuronal and non-neuronal cells (reviewed in [8]). However, the mechanisms underlying this pathological process remain to be established. The present series of experiments was designed to elucidate in neuronal cells the sequel of biochemical and molecular biological changes triggered by blocking the ER Ca^{2+} pump. Primary neuronal cell cultures were exposed to Tg for 30 min, followed by 1–24 h of recovery. We evaluated the temporal profile of Tg-induced inhibition and recovery of protein synthesis, and Tg-induced changes in mRNA levels (by quantitative PCR) and protein levels (by Western blotting) of the following groups of genes: (i) ER-resident chaperones, glucose-regulated protein (*grp78*) and *grp94*, growth arrest and DNA damage-inducible (*gadd*)*34* and *gadd153* (i.e. genes specifically expressed under conditions of ER stress); (ii)

Abbreviations used: eIF-2α, eukaryotic initiation factor 2α; ER, endoplasmic reticulum; MEM, minimal essential medium; SERCA, sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase; Tg, thapsigargin; UPR, unfolded-protein response; hsp70, heat-shock protein 70; PLSD, protected least-significant difference; GRP, glucose-regulated protein; GADD, growth arrest- and DNA damage-inducible gene..

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genes encoding other ER proteins, such as the ER Ca^{2+} pump, SERCA 2b, and the neuroprotective protein Bcl-2; and (c) genes encoding cytoplasmic proteins, such as heat-shock protein 70 (hsp70; a member of the cytoplasmic stress-response system), and p67, a highly glycolysated protein which associates with the eukaryotic initiation factor 2α (eIF-2α), thereby blocking eIF-2α phosphorylation and suppression of protein synthesis induced by activated eIF-2α kinases [11]. We also investigated the temporal profiles of Tg-induced changes in the phosphorylation state of eIF-2α.

EXPERIMENTAL

Cell culture

Primary neuronal cell cultures were prepared from the cortex of embryonic rat brains at days 16–17 of gestation [12]. Neurons were dissociated in minimal essential medium (MEM), supplemented with 30 mM glucose and 2 mM glutamine, DNase I and trypsin. The tissue was passed through a nylon mesh and enzymes were inactivated by the addition of horse serum. After washing, cells were suspended in MEM supplemented with 30 mM glucose, 2 mM glutamine, 100 i.u./ml penicillin, 10 ng/ml streptomycin and 5% horse serum. Cells were plated on polyethyleneimine-coated dishes and incubated in a humidified atmosphere containing 5% CO_2 for 10 days before starting experiments. Immunohistochemistry with antibodies specific for neurons and glial cells revealed that approx. 80% of cells were neurons.

Depletion of ER Ca^{2+} stores

ER Ca^{2+} stores were depleted by exposing cells to Tg, a specific irreversible inhibitor of SERCA [10]. Immediately before starting experiments, Tg stock solutions (1 mM) were freshly prepared in dry DMSO. Cells were exposed to Tg (1 μM) or the Tg solvent, DMSO (0.1%), for 30 min in MEM medium. Tg/DMSO solution was washed off and cells were transferred to MEM supplemented with 5% horse serum. After recovery periods of 0.25–24 h, cells were taken for analysis of Tg-induced changes in protein synthesis and measurement of mRNA levels and protein contents of genes relevant to the stress response of cells and to the regulation of the initiation of protein synthesis.

Protein synthesis

Protein synthesis was evaluated by measuring the incorporation of L-[4,5-^{3}H]leucine (10 μCi/ml) into proteins. After a 30 min incorporation period, cells were washed with warm and cold balanced salt solution. Trichloroacetic acid was then added and cells were homogenized. After repetitive washing with trichloroacetic acid and centrifugation, the final pellets were dissolved in NaOH. Aliquots were taken for determination of protein content [13] and measurement of radioactivity incorporated into proteins. To investigate whether the regulatory link between ER Ca^{2+} homoeostasis and protein synthesis recovered after Tg treatment, cells were exposed for 30 min to 0.07–0.7 mM 2-hydroxycarbazole, an agonist of the ryanodine receptor [14], 24 h after Tg treatment. Protein synthesis was evaluated as described above by adding L-[4,5-^{3}H]leucine to the 2-hydroxycarbazole-containing medium, followed by a 30 min incorporation period.

Immunoblotting

Tg-induced changes in the levels of proteins contributing to the stress response of cells were evaluated by immunoblotting. At the end of experiments, cells were washed with PBS solution. Cells were scraped off the dishes with lysis buffer containing 20 mM Hepes (pH 7.5), 1% Triton X-100, 150 mM NaCl, 10% glycerol and 1 mM EDTA, to which the following protease and phosphatase inhibitors had been added immediately before use: 5 mM sodium orthovanadate, 10 mM tetrasodium pyrophosphate, 100 mM NaF, 1 mM PMSF, 4 μg/ml aprotinin and 2 μg/ml pepstatin A. After measurement of the protein concentration of the samples using the Bradford technique [15], a standard amount of protein from each sample was mixed with loading buffer and loaded on to a Tris/HCl SDS/8% or 12% PAGE gel. Protein bands were transferred to nitrocellulose membranes (Hybond™; Pharmacia Biotech, Freiburg, Germany). Membranes were further processed by incubation with the appropriate primary antibody for 16 h at 4 °C, followed by visualization of bands using the enhanced chemiluminescence Western-blotting analysis system (Pharmacia Biotech). Membranes were incubated with the following secondary antibodies: anti-mouse/sheep or anti-rabbit/goat horseradish peroxidase conjugates (Pharmacia Biotech; 1:1000 dilution), or anti-goat/donkey horseradish peroxidase conjugate (Santa Cruz Biotechnology, Heidelberg, Germany; 1:5000 dilution) for 1 h. The following primary antibodies were used: anti-rabbit GRP78 (Affinity Bioreagents, Golden, CO, U.S.A.; 1 μg/ml dilution), anti-rat/mouse hybridoma, clone 9G10 GRP94 (Affinity Bioreagents Inc, Golden, CO., U.S.A.; dilution 1:500), anti-rabbit GADD34 (a gift from Dr B. Roizman, Chicago, IL, U.S.A.; 1:2500 dilution), anti-rabbit GADD153 (Santa Cruz Biotechnology; 1:100 dilution), anti-rabbit SERCA 2b (a gift from Dr F. Wuytack, Leuven, Belgium; 1:2000 dilution), anti-mouse Bcl-2 (Santa Cruz Biotechnology; 1:200 dilution), anti-mouse clone BRM-22, hsp70 (Sigma; 1:10000 dilution), anti-rabbit p67 (a gift from Dr Datta, Lincoln, NE, U.S.A., 1:2000 dilution), anti-goat eIF-2α (Santa Cruz Biotechnology; 1:100 dilution), and anti-rabbit eIF-2a-P (Research Genetics, Huntsville, AL, U.S.A.; 1:750 dilution).

Quantitative PCR

Tg-induced changes in mRNA levels were evaluated by quantitative PCR as described earlier [16]. In short, total RNA was isolated using the acid guanidinium thiocyanate/phenol/chloroform extraction technique [17] and reverse-transcribed into cDNA. PCR reactions were run in the presence of an internal standard, which had a non-matching sequence in the middle of the molecule and the sequence of interest at each end. Thus the internal standard could be amplified together with the specific cDNA using the same set of primers but yielding a smaller PCR product. Quantification was performed by image analysis using appropriate standard curves [16]. Standard curves were prepared by running PCR reactions with different concentration ratios of plasmids containing internal standard cDNA or the cDNA of interest as inserts. The following pairs of primers were used: (one upper and one lower): p67, 5′-GGGCGTGGAAGAGGCATCG-3′ and 5′-GGAACTGAGGGAGGGTCTG-3′; GADD34, 5′-GTCCATTTCCTTGCTGTCTG-3′ and 5′-AAGGCGTGCCCATGCTCTGG-3′; GADD153 (GenBank® accession number U30186), 5′-TCAGATGAAATTGGGGGCAC-3′ and 5′-TTTCCTCGTTGAGCCGCTCG-3′; bcl-2, 5′-AGGGATGGGGTGAACTGGGGG-3′ and 5′-TTGTTTGGGGCAGGTCTGCTG-3′; GRP78, 5′-GTTCTGCTTGATGTGTGTCC-3′ and 5′-TTTGGTCATTGGTGATGGTG-3′; GRP94, 5′-TCCCCCTTAATGTTTCCCGTG-3′ and 5′-TAGCCCTTCTTCAGAAGCCTC-3′; SERCA 2b, 5′-GGGAGTGGGGCAGTGGCAGC-3′ and 5′-CGTCTCTCTGGGCTGAGGGG-3′; hsp70, 5′-TGCTGACCAAGATGAAGG-3′ and 5′-AG-

AGTCGATCTCCAGGC-3′. For quantitative evaluation, the cDNA samples used as templates for PCR were diluted (if necessary) in such a way that the optical densities of bands derived from the sample and internal standard cDNA were within the same range.

Statistical evaluation

All values are presented as means ± S.D. Statistically significant differences between experimental groups and controls were evaluated by ANOVA, followed by Fisher's protected least-significant difference (PLSD) test to correct for multiple comparisons. A probability of 95 % was taken to indicate a significant difference between mean values of groups.

RESULTS

Protein synthesis

Exposure of primary neuronal cell cultures to Tg resulted in an almost complete suppression of protein synthesis (Figure 1A). After washing off Tg, and transfer of cells to medium supplemented with 5 % horse serum, protein synthesis gradually recovered to approx. 60 % of control 24 h after Tg treatment. To confirm that the regulatory link between ER Ca^{2+} levels and protein synthesis recovered 24 h after Tg exposure of neurons, cells were exposed to 0.07–0.7 mM 2-hydroxycarbazole, an agonist of the ryanodine receptor, and the effect on protein synthesis was evaluated (Figure 1B). In control cells exposed to the Tg solvent, DMSO, and in cells exposed to Tg and left to recover for 24 h, 2-hydroxycarbazole induced a concentration-dependent suppression of protein synthesis, indicating recovery of the regulatory link between the ER Ca^{2+} pool that responds to activation of the ryanodine receptor and protein synthesis [18]. The effect of 2-hydroxycarbazole on protein synthesis was, however, considerably more pronounced in cells investigated 24 h after Tg exposure than in control cells (Figure 1B).

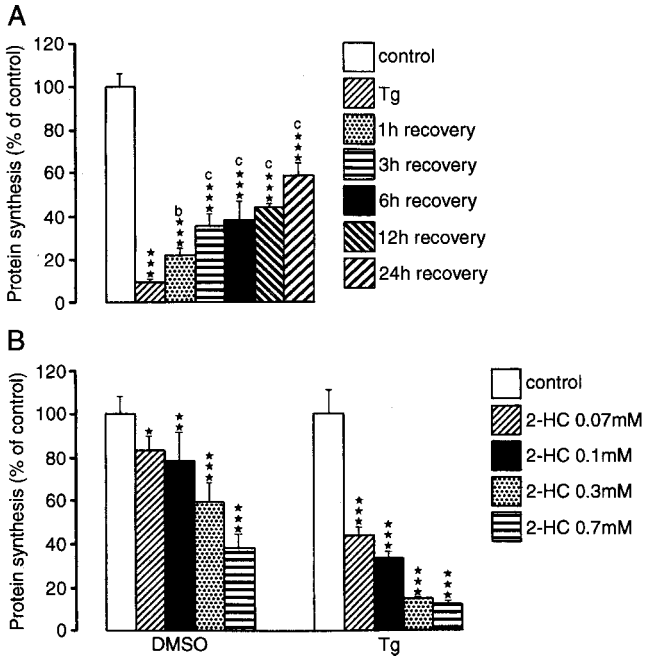

Figure 1 Control of protein synthesis by ER Ca^{2+} levels

Primary neuronal cell cultures were exposed to Tg (1 μM) for 30 min, followed by different recovery times. Protein synthesis was evaluated by measuring the incorporation of L-[4,5-^{3}H]leucine into proteins. Tg exposure induced an almost complete suppression of protein synthesis (**A**). At 24 h after Tg exposure, protein synthesis recovered to approx. 60% of control. b, $P < 0.01$; c, $P < 0.001$. In the physiological state, protein synthesis is controlled by levels of a Ca^{2+} pool sensitive to activation of the ryanodine receptor. (**B**) 2-Hydroxycarbazole (2-HC), a ryanodine receptor agonist, caused a concentration-dependent suppression of protein synthesis. This regulatory link between protein synthesis and ryanodine receptor activation was restored 24 h after exposure of cells to Tg. However, 2-hydroxycarbazole exposure produced a more pronounced suppression of protein synthesis 24 h after Tg exposure. Data are presented as means ± S.D. ($n = 4$ per group). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ for statistically significant differences between experimental groups and controls (ANOVA, followed by Fisher's PLSD test).

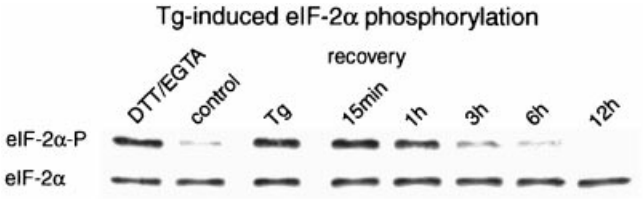

Figure 2 Phosphorylation of the eIF-2α by transient exposure of cells to Tg (1 μM)

Cells were exposed to Tg for 30 min, followed by recovery periods ranging from 15 min to 24 h. In addition, cells were exposed to 1 mM EGTA and 1 mM dithiothreitol (DTT) for 30 min, experimental conditions which are thought to induce maximal ER stress. At the end of the experiments, total protein was isolated, and Tg-induced changes in eIF-2α phosphorylation were evaluated by Western blotting with antibodies specific for eIF-2α protein and phosphorylated eIF-2α (see the Experimental section), using 20 μg of protein/lane. Total eIF-2α protein levels remained unchanged throughout the experiment. Directly after Tg exposure, eIF-2α was phosphorylated to a marked extent, similar to that observed in cultures exposed to EGTA/DTT. After 1h recovery, the extent of eIF-2α phosphorylation was less pronounced, and after 3 h of recovery levels of phosphorylated eIF-2α were similar to those found in control cells.

Phosphorylation of eIF-2α

Exposure of cells to Tg caused a marked phosphorylation of eIF-2α (Figure 2), which was maximal during exposure, and stayed high after 15 min of recovery. After 1 h of recovery, the extent of eIF-2α phosphorylation was reduced to approx. 50 % of the values observed during Tg exposure, and it returned almost to control levels after 3 h of recovery. The extent of eIF-2α phosphorylation measured during Tg exposure was similar to the phosphorylation rate induced in cells incubated under conditions known to cause maximal ER stress (Ca^{2+}-free medium supplemented with dithiothreitol) (Figure 2).

Tg-induced changes in mRNA and protein levels

Tg-induced changes in mRNA, and the respective protein levels, are shown in Figures 3–5. Cultures were exposed to MEM supplemented with 1 μM Tg for 30 min, after which the drug was washed off and the cells were transferred to fresh MEM supplemented with 5 % horse serum. Two different control cultures were used: plain control cells and cultures exposed for 30 min to the Tg solvent, DMSO, and then allowed to recover for 24 h in MEM supplemented with 5 % horse serum (24 h control). GRP78, GRP94, GADD34 and hsp70 mRNA levels were significantly increased in 24 h control cultures compared with plain control cells, i.e. 2-fold for GRP78 ($P < 0.05$), 1.3-fold for GRP94 ($P < 0.01$), 3.2-fold for GADD34 ($P < 0.001$) and 2.5-fold for hsp70

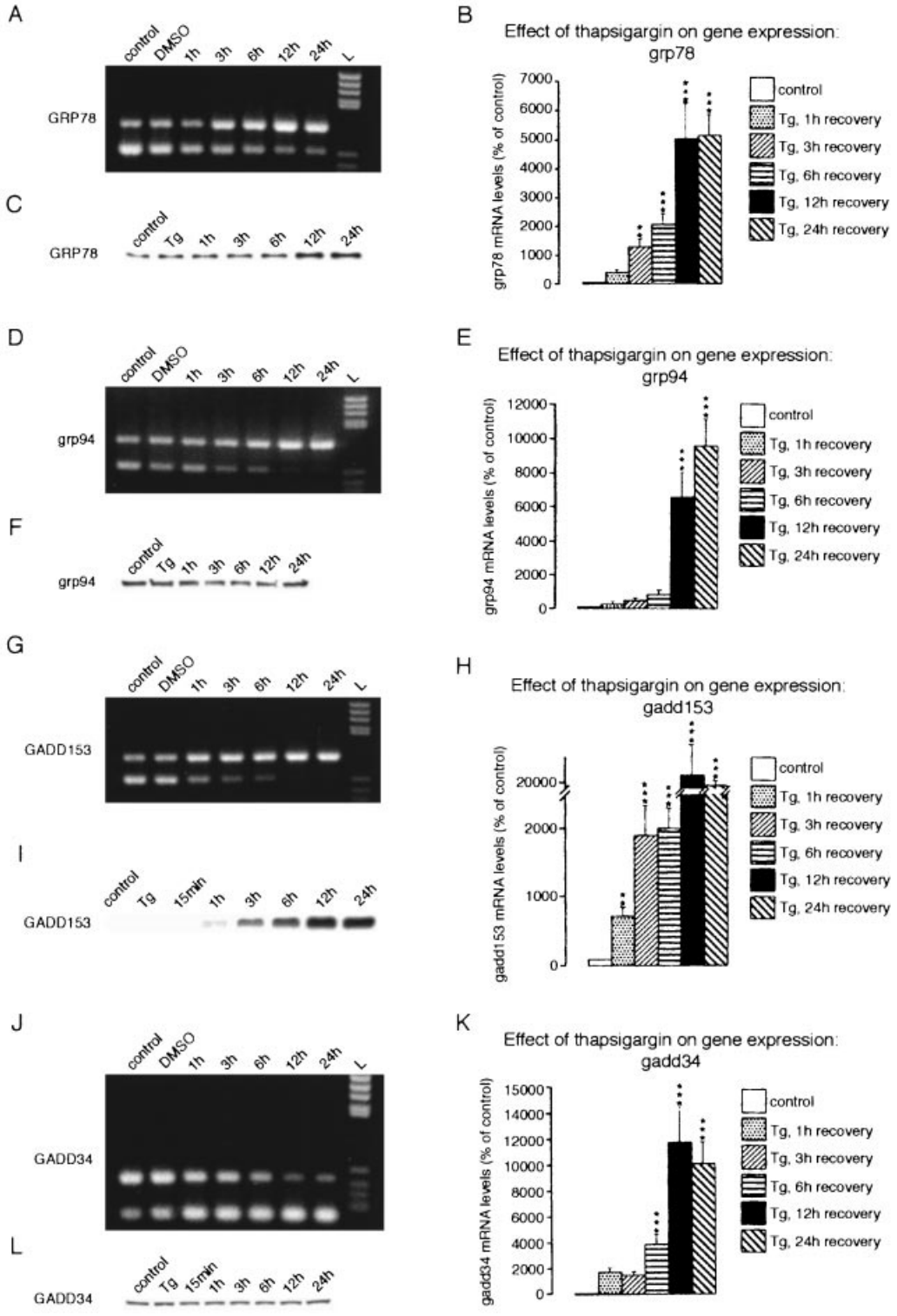

Figure 3 Tg-induced changes in expression and translation of GRP78 (A, B, C), GRP94 (D, E, F), GADD153 (G, H, I) and GADD34 (J, K, L)

Tg-induced changes in GRP78, GRP94, GADD153 and GADD34 expression were evaluated by quantitative PCR. PCR products were separated on an agarose gel (**A**, **D**, **G**, **J**) and changes in mRNA levels were assessed by image analysis (**B**, **E**, **H**, **K**). Tg-induced changes in protein levels were analysed by Western blotting, using 2 μg for GRP78 (**C**), 100 μg for GRP94 (**F**) or 10 μg for GADD153 (**I**) and GADD34 (**L**) of protein/lane. GRP78, GRP94, GADD153 and GADD34 mRNA levels rose sharply after Tg exposure. Tg-induced changes in protein levels varied considerably. Whereas GRP78 protein levels were increased to 4705 and 540% of control after 12 h and 24 h of recovery (**C**) respectively, GRP94 protein levels were transiently reduced to 51% of control after 3h recovery (**F**). GADD153 protein levels were dramatically increased starting at 3 h of recovery (**I**), whereas GADD34 protein levels remained unchanged (**L**). Tg-induced changes in mRNA levels (**B**, **E**, **H**, **K**) are presented as means $\pm$ S.D., with $n = 4$ per group. $^{**}P < 0.01$ and $^{***}P < 0.001$ for statistically significant differences between experimental groups and controls (ANOVA, followed by Fisher's PLSD test). Abbreviation: L, DNA molecular-mass-marker V (Boehringer Mannheim).

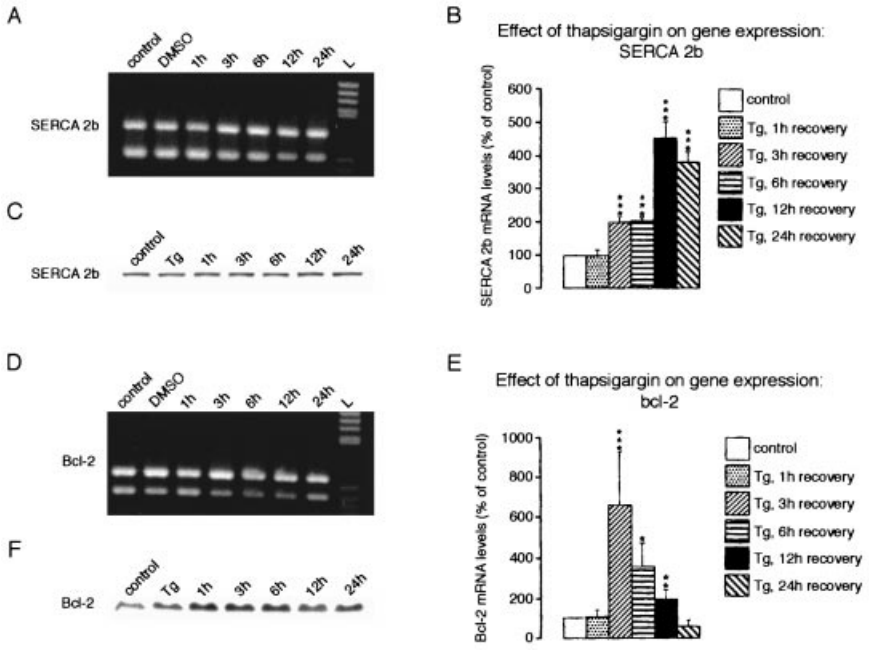

Figure 4 Tg-induced changes in SERCA 2b (A, B, C) and Bcl-2 (D, E, F) expression and translation

Tg-induced changes in SERCA 2b and Bcl-2 expression were evaluated by quantitative PCR. PCR products were separated on an agarose gel (**A**, **D**) and changes in mRNA levels were assessed by image analysis (**B**, **E**). Tg-induced changes in SERCA 2b and Bcl-2 protein levels were analysed by Western blotting, using 10 μg for SERCA-2b (**C**) or 50 μg for Bcl-2 (**F**) of protein/lane. SERCA 2b mRNA levels rose to approx. 400% of control 12 h and 24 h respectively after exposure of cells to Tg. Bcl-2 mRNA levels rose only transiently (to approx. 600% of control at 3 h of recovery). Tg exposure did not induce any change in SERCA 2b protein levels, but it induced a transient rise in Bcl-2 protein levels (to 300% of control after 3 h of recovery). Tg-induced changes in mRNA levels (**B**, **E**) are presented as means $\pm$ S.D., with $n = 4$ per group. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ for statistically significant differences between experimental groups and controls (ANOVA, followed by Fisher's PLSD test). Abbreviation: L, DNA molecular-mass-marker V.

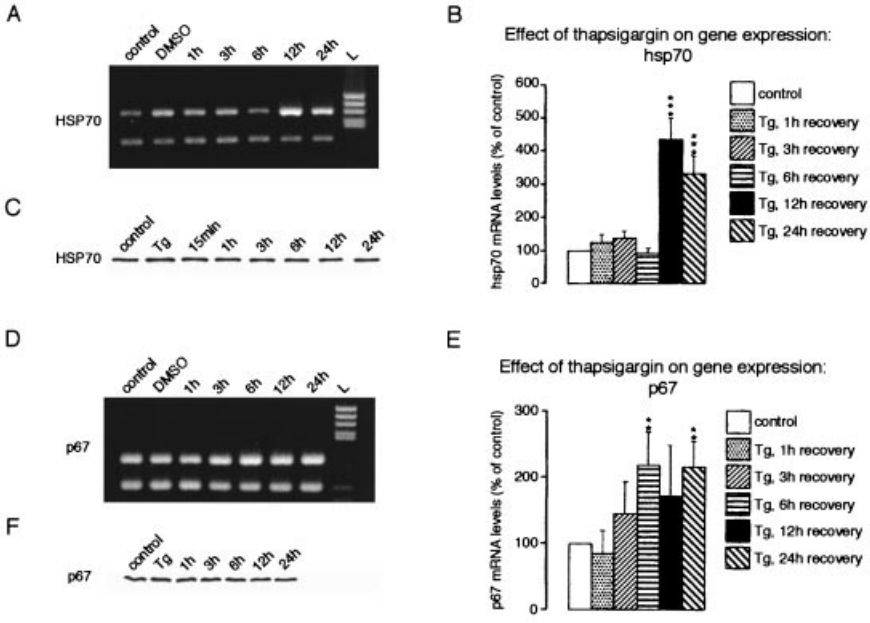

Figure 5 Tg-induced changes in hsp70 (A, B, C) and p67 (D, E, F) expression and translation

Tg-induced changes in hsp70 and p67 expression were evaluated by quantitative PCR. PCR products were separated on an agarose gel (**A**, **D**) and changes in mRNA levels were assessed by image analysis (**B**, **E**). Tg-induced changes in protein levels were analysed by Western blotting, using 10 μg for hsp70 (**C**) or 50 μg for p67 (**F**) of protein/lane. hsp70 mRNA levels rose only modestly (**B**), and p67 mRNA levels rose to approx. 200% of control 6 h and 24 h after exposure of cells to Tg (**E**). Tg exposure did not induce an increase in hsp70 or p67 protein levels. Tg-induced changes in mRNA levels (**B**, **E**) are presented as means $\pm$ S.D., with $n = 4$ per group. $^{*}P < 0.05$ and $^{**}P < 0.01$ for statistically significant differences between experimental groups and controls (ANOVA, followed by Fisher's PLSD test). Abbreviation: L, DNA molecular-mass-marker V.

($P < 0.001$). For quantitative evaluation, Tg-induced changes in mRNA levels were related to the mRNA levels found in plain control cultures.

Three different patterns of changes in mRNA levels were identified. In the first group, mRNA levels increased approx. 20–200-fold. This group included GADD34, GADD153, GRP78 and GRP94, i.e. genes encoding stress proteins, the expression of which is up-regulated under conditions associated with an activation of the UPR (see the Introduction) (Figure 3). In the second group, mRNA levels increased 5–6-fold; this group included mRNAs encoding other ER-resident proteins, such as SERCA 2b and Bcl-2 (Figure 4). In the third group, mRNA levels increased only 1.5–4-fold; this group included mRNAs encoding the cytoplasmic proteins hsp70 and p67 (Figure 5).

The temporal profile of changes of mRNA and protein levels of group 1 genes differed considerably (Figure 3). After 1 h recovery from Tg exposure of cells, mRNA levels rose 4.2-fold for GRP78 (Figures 3A and 3B), 3.2-fold for GRP94 (Figures 3D and 3E), 7.2-fold for GADD153 (Figures 3G and 3H) and 17.3-fold for GADD34 (Figures 3J and 3K). A steep increase in mRNA levels was observed between 6 h and 12 h of recovery, reaching 200 times control levels in the case of GADD153. The temporal profiles of changes in mRNA levels for bcl-2 and SERCA 2b diverged in a similar way; whereas SERCA 2b mRNA levels increased by only approx. 2-fold 3 h and 6 h after Tg exposure of cells, and the highest SERCA 2b mRNA levels were measured after 12 h and 24 h of recovery (Figures 4A and 4B), bcl-2 mRNA levels increased approx. 6-fold after 3 h of recovery and returned to control levels when the recovery period was extended (Figures 4D and 4E). hsp70 mRNA levels increased only slightly after exposure of cultures to Tg, reaching a first peak at 3 h recovery (Figures 5A and 5B; 1.4-fold increase) and a second one at 12 h of recovery (4.4-fold increase), whereas the highest p67 mRNA levels were observed after 6 h and 24 h of recovery (Figures 5D and 5E).

Tg-induced up-regulation of mRNA levels was not always paralled by a corresponding increase in protein levels. GRP78 protein levels increased after prolonged recovery from exposure of cells to Tg (to approx. 470 % and 540 % of control after 12 h and 24 h of recovery respectively; Figure 3C), and GRP94 protein levels were transiently down-regulated (to approx. 50 % of control between 3 h and 12 h of recovery) and reached control levels again after 24 h of recovery (Figure 3F). GADD153 protein levels were sharply up-regulated after exposure of cultures to Tg (Figure 3I). In control cells, we could not identify GADD153 protein on Western blots. A faint GADD153 band was found after 1 h of recovery, and GADD153 protein levels gradually increased until a strong band was present starting at 12 h after Tg exposure (Figure 3I). GADD34 protein levels did not change after Tg exposure (Figure 3L), despite a sharp increase in GADD34 mRNA levels (Figure 3K).

Although SERCA 2b and bcl-2 mRNA levels rose to a similar extent (approx. 6-fold) after Tg exposure, SERCA 2b protein levels did not change (Figure 4C), whereas Bcl-2 content rose transiently, peaking at 1–3 h of recovery (3-fold over control; Figure 4F). hsp70 and p67 protein levels did not change after exposure of cells to Tg (Figures 5C and 5F). This was not surprising, since we observed only a small increase in the hsp70 and p67 mRNA levels (Figures 5B and 5E).

DISCUSSION

In the control state, protein synthesis is closely affected by levels of ER Ca^{2+} stores (reviewed in [19]). Under conditions associated with disturbance of ER Ca^{2+} homoeostasis or an impairment of ER-resident protein folding or processing reactions, the initiation process of protein synthesis is suppressed, as indicated by a phosphorylation of eIF-2α and a disaggregation of polyribosomes [20–22]. Depletion of ER Ca^{2+} stores causes a slight transient increase in cytoplasmic Ca^{2+} activity [16], which may also affect protein synthesis. However, after an isolated increase in cytoplasmic Ca^{2+} activity, it is the elongation, and not the initiation step, which is blocked, leading to maximal aggregation and not to a disaggregation of polyribosomes [23]. The involvement of ER Ca^{2+} stores in the control of protein synthesis is also indicated by the observation that translation is suppressed by drawing Ca^{2+} out of the ER by Ca^{2+} chelation, an experimental paradigm which is associated with a decrease in cytoplasmic Ca^{2+} activity [24].

We have shown in earlier experiments [25] that in our neuronal cell cultures protein synthesis recovered partially after exposing cells to Tg. In the present study, we investigated for the first time possible mechanisms underlying the partial recovery of protein synthesis, by evaluating Tg-induced changes in eIF-2α phosphorylation and p67 expression, and we compared the effect of ryanodine receptor activation on protein synthesis in cultures 24 h after exposure to Tg or the Tg solvent, DMSO. Protein synthesis recovered partially, although ER Ca^{2+} pump activity did not recover [25], implying the development of a new system for the uptake of Ca^{2+} into the ER. At 24 h after exposure of cells to Tg, protein synthesis was more sensitive to an activation of the ryanodine receptor than control cells (Figure 1). This indicates that the ryanodine receptor-sensitive Ca^{2+} pool, and the regulatory link between the Ca^{2+} levels in this pool and protein synthesis, recovered at least partially; however, the newly developed uptake system was not able to counterbalance Ca^{2+} release induced by maximal receptor activation.

We do not know at present which mechanisms are responsible for the delayed recovery of protein synthesis observed in the present study. The phosphorylation state of eIF-2α did not correlate closely with the rates of protein synthesis; eIF-2α was almost completely de-phosphorylated 3 h after Tg exposure, although at this period protein synthesis was still severely inhibited (Figure 1). A possible candidate contributing to this recovery of protein synthesis could be the eIF-2α-associated 67 kDa glycoprotein, p67. p67, in its highly glycolysated form, reverses the inhibition of protein synthesis induced by phosphorylation of eIF-2α, even in the presence of activated eIF-2α kinase PKR [11], an enzyme which contributes to ER dysfunction-induced phosphorylation of eIF-2α and suppression of protein synthesis [26,27]. Indeed, it has been proposed that an up-regulation of p67 is largely responsible for the recovery of protein synthesis after serum withdrawal or heat-shock [28,29]. However, protein levels of highly glycolysated p67 did not change after Tg treatment (Figure 5), suggesting that the partial recovery of protein synthesis was not induced by an up-regulation of p67.

In the present study, we have evaluated for the first time in detail the relationship between transcription and translation of stress genes under conditions of graded suppression of protein synthesis induced by Tg exposure of cultures. Exposing cells to Tg had variable effects on expression and translation of genes responding to conditions associated with ER dysfunction. Whereas GADD153, GADD34 and GRP94 mRNA levels were all sharply increased 12 h to 24 h after Tg exposure, only GADD153 protein levels were markedly upregulated, whereas GADD34 protein levels stayed unchanged and GRP94 protein levels decreased transiently. The expression of genes encoding ER-resident stress proteins is activated by Tg-induced depletion of ER Ca^{2+} stores and not by the corresponding

increase in cytoplasmic Ca^{2+} activity. The expression is not blocked by cytoplasmic Ca^{2+} chelation [30], and it is also induced by drawing Ca^{2+} ions out of intracellular compartments through extracellular chelation [31], an experimental paradigm where depletion of ER Ca^{2+} stores is associated with a decrease in cytoplasmic Ca^{2+} activity [24]. Exposure of neuronal cell cultures to Tg induced different effects on translation of pro-apoptotic (GADD153) and anti-apoptotic (bcl-2 and GRP78) mRNAs. Results from various studies suggest that up-regulation of GADD153 expression is sufficient to induce apoptosis [32–34]. Bcl-2 and GRP78, in contrast, are anti-apoptotic proteins. Overexpression of Bcl-2 has been shown to protect cells from ER Ca^{2+} pool depletion and cell injury under various experimental conditions, including growth factor withdrawal, ER Ca^{2+} pool depletion, oxidative stress and transient ischaemia [35–38]. Recently [39], it has been shown that ER dysfunction-induced cytochrome *c* release and caspase 3 activation can be blocked by Bcl-2 targeted on the ER, pointing to a causal relationship between impairment of ER function and the release of apoptotic signals from mitochondria. The results of various studies [40–43] suggest that up-regulation of GRP78 helps to protect cells from injury, whereas GRP78 down-regulation reduces the tolerance of cells towards a severe form of stress. The observation that exposure of neuronal cell cultures to Tg induced a dramatic up-regulation of the pro-apoptotic GADD153 protein, but only a small increase in the anti-apoptotic Bcl-2 and GRP78 proteins, suggests that the imbalance between pro-apoptotic and anti-apoptotic signals induced by ER dysfunction may contribute to Tg-induced cell injury.

There is evidence to suggest that ER dysfunction contributes to neuronal cell injury in acute and chronic pathological states of the brain [8,9]. Blocking of ER function is sufficient to induce cell injury; however, whether the stress response activated under these conditions (the ER-overload response and UPR; see the Introduction) contributes to pathological processes remains to be established. The observations that cells are less resistant to ER stress after stable transfection with familial Alzheimer's disease-linked mutant presenilin-1 (which down-regulates UPR; [44]), and that GRP78 protein levels are decreased in the brains of Alzheimer's disease patients [44], point to a central role of GRP78 protein, and thus a shift in the balance towards anti-apoptotic signals, in protecting cells from injury in pathological states of the brain in which ER function is disturbed. The results of the present study suggest that detailed analysis of the response of neuronal cells to transient Tg exposure may help to elucidate the mechanisms underlying neuronal cell injury in those neurological disorders in which a disturbance of ER function is thought to contribute to the pathological process of deterioration.

The excellent technical assistance of Cordula Strecker and Änne Pribliczki is gratefully acknowledged. This work was supported by the Deutsche Forschungsgemeinschaft, Grant Pa 266/13-1.

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Received 17 October 2000/8 March 2001; accepted 30 March 2001