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Ethanol differently affects stress protein and HERG K^+ channel expression in SH-SY5Y cells

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Abstract

Ethanol is known to be neurotoxic. Protective mechanisms, however, are activated upon ethanol induction of the glucose-regulated stress proteins (GRPs), GRP78 and GRP94. These endoplasmic reticulum-residing chaperones are known to be involved in channel subunit assembly. The GRP and human-ether-à-gogo-related gene (HERG) K^+-channel expression were monitored in short- and long-term ethanol incubation experiments using the human neuroblastoma cell line SH-SY5Y. mRNA of the stress proteins and protein levels of the GRPs and HERG were determined using Northern and Western blot methods. Short-term ethanol incubation caused a transient increase of GRP transcripts. Protein levels of GRP94 decreased in chronic experiments, whereas GRP78 did not change. HERG followed the same kinetics as GRP94 with a constant down-regulation. The coordinate down-regulation of GRP94 and HERG implies the specific involvement of the endoplasmic reticulum chaperone GRP94 and HERG, but not GRP78, in a process of cell adaptation.

Keywords: GRP (glucose-regulated stress protein); K^+-channel HERG; SH-SY5Y; Neuroblastoma cell, human; Ethanol

1. Introduction

Ethanol, at concentrations expected to be reached by social drinkers, exerts multiple effects on neuronal cells. The main reason for this wide range of effects is the lack of specific ethanol receptors. Since ethanol can penetrate into cells, it may be expected that proteins located intracellularly as well as at the surface of the cell are vulnerable to the effects of ethanol. Furthermore, chronic exposure to ethanol causes adaptive changes in proteins, specifically in the composition of receptor subunits. Although the mechanisms are unknown yet, the intracellular machinery involved in the synthesis of proteins could be one of the targets of ethanol. A part of this synthesis (namely, protein binding and assembling) is carried out by the glucose-regulated protein (GRP) system, a group of stress proteins located in the endoplasmic reticulum. The GRP system is unusual in that it reacts to changes of the intracellular milieu, unlike most cellular transduction processes which respond to extracellularly generated stimuli. Such intracellular changes include, e.g. glucose starvation, hypoxia, perturbation of calcium stores and accumulation of misfolded proteins in the endoplasmic reticulum–events leading to cellular stress (Little et al., 1994; Kuznetsov and Nigam, 1998). Two components of the GRP system are the molecular chaperones GRP78 and GRP94 (Kim and Arvan, 1998). The expression of both chaperones is stimulated by acute ethanol exposure (Miles et al., 1994). Cellular stress, for example, through acute ethanol exposure causes not only increased levels of molecular chaperones in the endoplasmic reticulum but also of cytoplasmic proteins like the heat-shock protein HSC70 (Miles et al., 1991). Increased levels of chaperones do not necessarily indicate functional impairment because the increase could reflect the accumulation of misfolded proteins, with normally folded proteins remaining largely unaffected. To evaluate functional consequences, it is important to determine deviations in the expression of proteins that are assembled by aid of the GRPs and subsequently transported to the plasma membrane. Among

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these proteins are several of the K^+-channels (shaker channel, Schulteis et al., 1998; $K_v1.3$, Sheng et al., 1997). Little is known about the adaptive processes of GRPs after long-term exposure to ethanol and nothing is known about the alterations occurring in human-ether-à-gogo-related gene (HERG) channels. We investigated the acute and chronic changes in a human neuroblastoma cell line (SH-SY5Y), which constitutively expresses HERG channels (Taglialatela et al., 1998). We examined the hypothesis that GRPs are involved in adaptation processes induced by acute and chronic ethanol. HERG was used as an indicator for K^+-channel expression changes. The molecular chaperones play a pivotal role in the defense mechanism against cellular stress and allow cells to respond to adverse conditions in a way that will enhance their chance of survival (Little et al., 1994). Neurotoxic and cardiotoxic effects of chronic ethanol could be explained in part by suppression of this protective mechanism. The knowledge of intracellular events leading to ethanol-induced cell damage as well as increased cellular resistance during chronic ethanol exposure will be important for developing strategies for the prevention of ethanol-induced neurotoxic and cardiotoxic injuries.

2. Materials and methods

2.1. Cell culture and treatment

SH-SY5Y cells were routinely grown with minimal essential medium (MEM), Earl's salts and 10% fetal calf serum, as described by Lichtenberg-Kraag et al. (1997). For short-term experiments, confluent cells from a 175-cm^2 flask were split into 10 aliquots—five with addition of ethanol and five without. In ethanol treatments, ethanol was added to 30 ml of medium, yielding a final concentration of 100 mM. Cells were collected at 3, 6, 12, 24 and 72 h after initiation of the experiment by detachment from the surface with 1 mM ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS), centrifuged for 5 min at 2000 × *g*, washed once in PBS and stored at − 80 °C.

For long-term experiments with and without ethanol, equal aliquots of cells were used for subcultivation in 175-cm^2 flasks, starting from approximately 1×10^6 cells. Cultures were split in a 1:5 fashion once a week; one part of the culture was used for further cultivation, four parts were collected for either protein extraction and determination or RNA extraction. Medium was changed twice a week for both treatments, with and without ethanol. Cells were collected as described for short-term ethanol incubation. In general, during chronic experiments, cells were collected shortly before the second weekly ethanol addition (4 days after last medium change) during cell split in order to avoid acute drug effects on the cells. Throughout the time course, three independent samples per time point were collected. Ionomycin incubation of cells with 25 μM ionomycin was performed for 24 h, as described by Lichtenberg-Kraag et al. (1997).

2.2. RNA extraction and Northern hybridization

Total cellular RNA was extracted from frozen, PBS-washed cells by the acid guanidine thiocyanate/phenol/chloroform method of Chomczynski and Sacchi (1987). An aliquot of total RNA (20 μg) was subjected to electro-

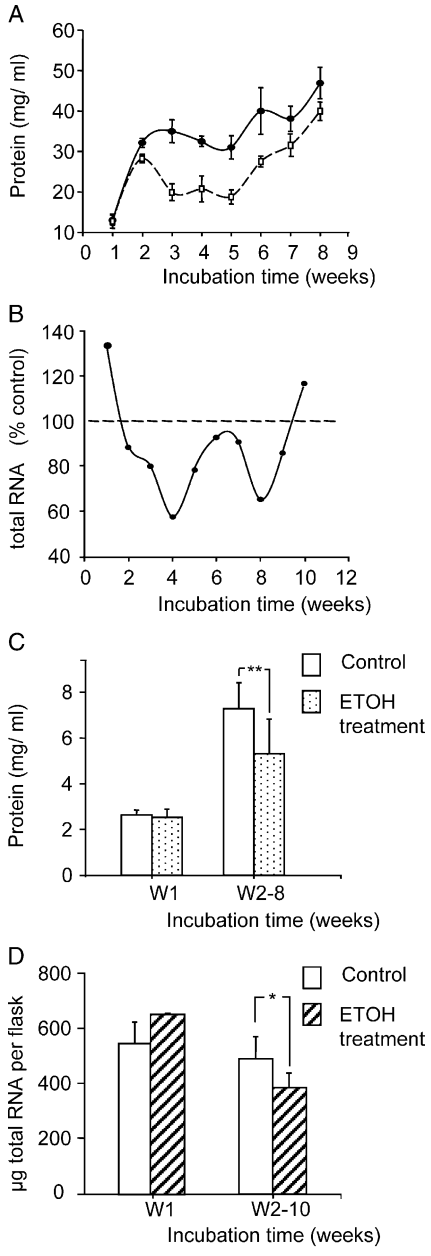

Fig. 1. Cell growth of SH-SY5Y cells during long-term chronic treatment with 100 mM ethanol in the cell culture medium. (A) Total cellular protein of controls (●) compared with cells treated with 100 mM ethanol (□) over the time period indicated (expressed in mg protein/ml per 175-cm^2 flask), means ± S.E.M. (B) Total cellular RNA of cells from one 175-cm^2 flask expressed as percent of control values. (C) Comparisons of average values of controls (open bars) versus ethanol-treated (stippled bars) total cellular protein levels at W1 and W2–W8 and (D) pooled data for total RNA in W1 and W2–W10 for controls (open bars) and ethanol treatment (hatched bars); means ± S.E.M. $*P<0.05$, $***P<0.001$ (Mann–Whitney *U*-test).

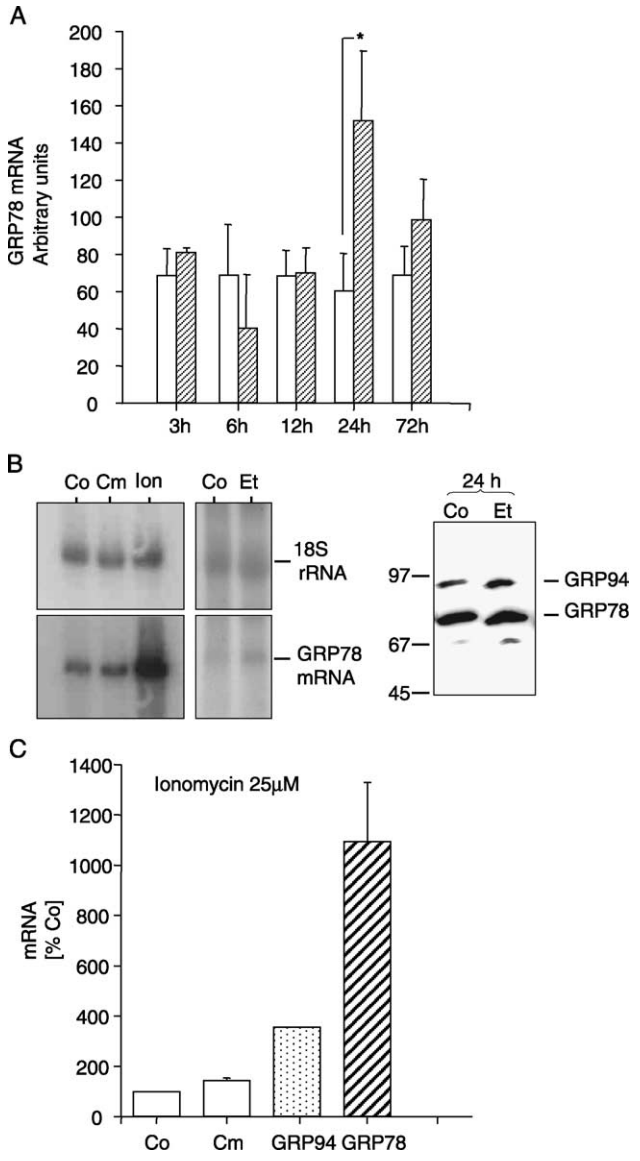

Fig. 2. GRP78 mRNA levels in SH-SY5Y neuroblastoma cells. (A) GRP78 mRNA control (open bars) and acute treatment levels with 100 mM ethanol (hatched bars) for the time periods indicated; means ± S.E.M of triplicate experimental repetitions are given, $*P<0.05$. (B) GRP78 mRNA signal intensity of a representative Northern blot after 24-h treatment of SH-SY5Y cells with 25 µM ionomycin (left panel). Co denotes signal of untreated cells and was defined as 100% signal intensity. Cm denotes signal of cells with vehicle methanol (0.18% v/v). Middle panel signal intensity of a Northern blot comparing GRP78 mRNA signal intensity of untreated (Co) and 24 h with 100 mM ethanol-treated (ET) SH-SY5Y cells. The right panel shows a Western blot comparing GRP78/GRP94 protein levels after 24 h of stimulation by 100 mM ethanol. Molecular weights are indicated in kilodaltons. (C) Quantification of the GRP78 and GRP94 mRNA signal from Northern blots after ionomycin treatment of SH-SY5Y cells; means ± S.E.M of $n=4$ blots for GRP78 and Cm, signal intensity of $n=1$ blot for GRP94 (nonparametric Mann–Whitney U-test).

phoresis on a 1.4% (w/v) agarose–formaldehyde gel, and transferred to a nylon membrane (Hybond N^+, Amersham Pharmacia Biotech, Freiburg, Germany) by capillary blotting. Ethidium bromide-stained gels were radiated at 254 nm for 2 min (UV-Crosslinker, BioRad Laboratories, Munich, Germany) before RNA transfer. RNA was fixed onto membranes by ultraviolet (UV) light radiation (40 s, 254 nm).

Blots were hybridized to random [^{32}P]-labeled deoxycytosinetriphosphate (dCTP) probes (Feinberg and Vogelstein, 1983) for HSC70, GRP94 and GRP78 mRNA, respectively. All cDNAs were obtained from the German Resource Center of the Human Genome Project at the Max-Planck Institute for Molecular Genetics, Berlin, Germany. The *Escherichia coli* clones were registered as IMAGp998H1071; IMAGp998G071425 and IMAGp998J092488. Labeling of cDNA

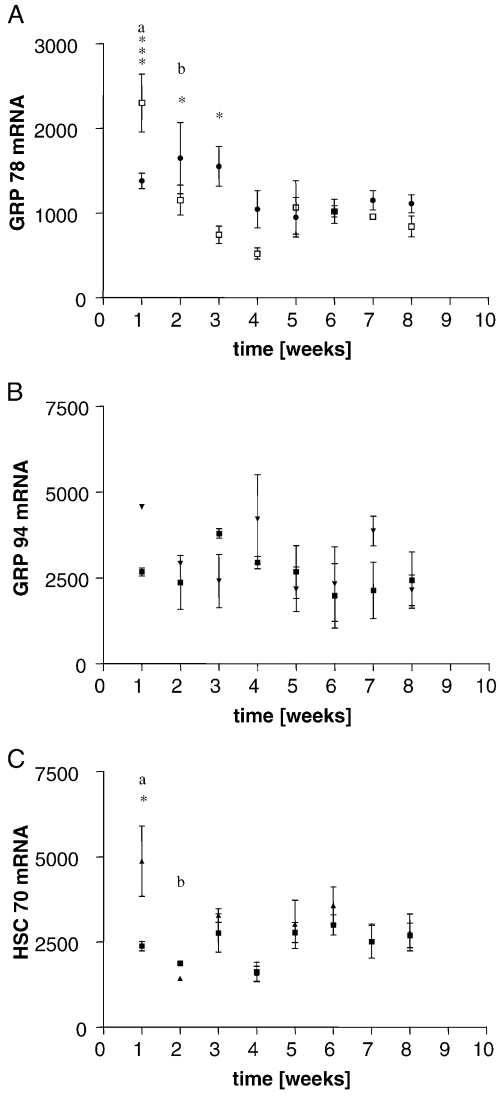

Fig. 3. GRP78 (A; ● control, □ ethanol), GRP94 (B; ■ control, ▼ ethanol) and HSC70 (C; ■ control, ▲ ethanol) mRNA levels in SH-SY5Y cells. Comparison of mRNA control levels and stimulated levels (in arbitrary units) during long-term incubation with 100 mM ethanol. The mRNA values of three samples at the time points indicated are expressed as means ± S.E.M. Statistical significance: $***P<0.001$, $*P<0.05$. Values denote comparison of controls versus ethanol-treated cells. a, b = group difference between time points ($P<0.05$; ANOVA followed by Bonferroni posttest analysis).

fragments proceeded as recommended by the supplier of the kit (Amersham) using 25 ng of cDNA and 50 μCi per reaction. Hybridization was carried out basically as described by Reiprich et al. (1995). In brief, hybridization proceeded overnight at 42 °C in 50% formamide, 5 × sodium saline citrate (SSC) buffer (0.15 M NaCl; 0.015 M Na_3 citrate, pH 7.0), 5 × Denhardt's solution, 1% sodium dodecyl sulfate (SDS) (w/v) and 200 μg/ml denatured salmon sperm DNA. Filters were washed twice with 1-fold concentrated SSC at 42 °C (30 min) and once with 0.2-fold concentrated SSC for 15 min at 60 °C. Quantitative autoradiography was performed by use of a phosphor imaging apparatus (Molecular Analyst System, BioRad) after 24 h of phosphor screen exposure. The mRNA signals were corrected by comparing with 18 S rRNA signal intensities after rehybridization of filters with an 18 S rRNA specific probe (Ambion, Woodward, TX, USA). All samples to be compared (from week 1 to week 8; W1–W8) were transferred to the same filter, hybridized and analyzed together. Mean values of three filters were pooled for comparison of controls and ethanol-incubated samples. Filters were repeatedly stripped by heating (95 °C, 15 min, 10 mM EDTA, pH 7.5) and rehybridized with the respective probes.

2.3. Western blotting

The antibodies used in hybridization experiments were obtained commercially. Anti-G-protein MS/1, beta subunit specific, from rabbit was purchased from NEN (Boston, MA, USA). Rabbit anti-HERG polyclonal antibody (anti-HERG amino acids 1118–1133) was obtained from Chemicon International (Temecula, CA, USA) and used in a 1:25 dilution. Anti-78 kDa glucose-regulated protein monoclonal antibody from mouse ascites was purchased from StressGen Biotechnologies (Victoria, Canada) and used in a 1:500 dilution. It was raised against lys-ser-glu-lys-asp-glu-leu of the carboxy-terminus of rat GRP78 and identifies GRP78 (the immunoglobulin heavy chain binding protein, BIP), GRP94 and an unknown protein of 40 kDa, containing a lysine aspartate/glutamate/leucine (KDEL, endoplasmic reticulum retention signal) sequence. Horseradish peroxidase-conjugated anti-rabbit immunoglobuline G (IgG) and horseradish peroxidase-conjugated anti-mouse IgG were purchased from Sigma (St. Louis, MO, USA).

After sonification of cell pellets in 200 μl of PBS buffer, protein for Western blots was extracted with chloroform/methanol (1:4) before the total protein concentration was determined (protein assay kit, BioRad). Then, 20 μg of the protein was dissolved in 20 μl of sample buffer and separated on a 4% (w/v) stacking gel and a 7% (w/v) SDS-polyacrylamide gel electrophoresis (PAGE) gel at 100 V for 1.5 h. For HERG determination, 40 μg of protein was subjected to SDS-PAGE separation and blotting. Electroblotting proceeded overnight at 50 mA in an electroblotting chamber. Polyvinylidenedifluoride (PVDF) membranes (Schleicher & Schuell, Dassel, Germany) were used throughout the experiments. Enhanced chemoluminescence (ECL) detection of immunoreactive material proceeded according to the manufacturer's description

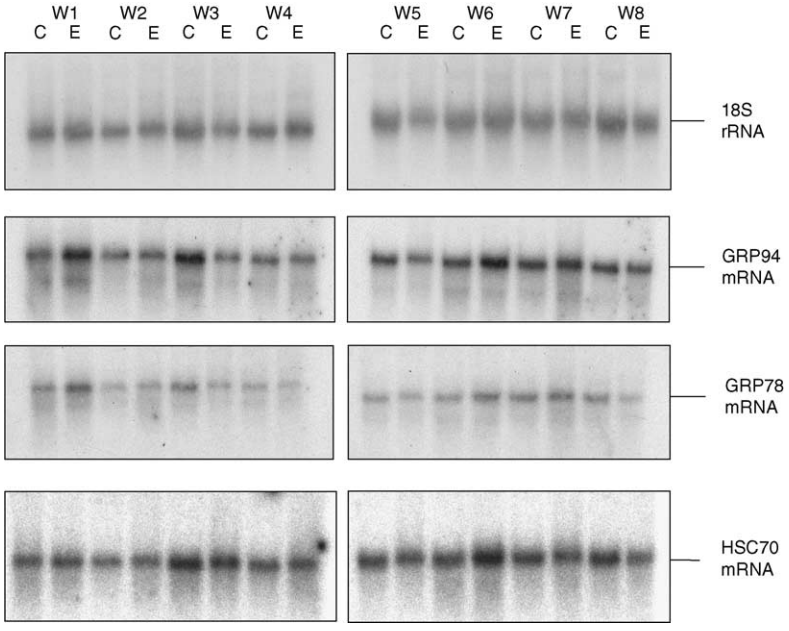

Fig. 4. Representative Northern blots demonstrating signals of 18S rRNA (standard) and GRP94, GRP78 and HSC70 mRNA of cell samples during long-term incubation with 100 mM ethanol (C, control; E, ethanol treatment).

(Boehringer Mannheim/Roche Biochemicals, Germany). A scanning device (BioRad) was used for quantification of signals. All samples to be compared were run on the same gel and assayed simultaneously. Average values of at least triplicate repetitions were evaluated.

2.4. Statistical analysis

All values are expressed as means ± standard error of the mean (S.E.M.). Comparisons of data from control and ethanol-incubated cells of different time points were performed by using two-way ANOVA (analysis of variance) followed by Bonferroni posttests for statistical significance and the statistical package for the social sciences (SPSS, version 9.01 for windows), or a nonparametric Whitney–Mann *U*-test as indicated.

3. Results

3.1. Growth characteristics

SH-SY5Y neuroblastoma cells grow in clusters; this makes direct counts of viable cells extremely difficult. We found that the addition of trypsin-containing solution causes the onset of cell mortality after about 2 min, in contrast to the findings of others (Luo and Miller, 1997). Therefore, we used total protein or total RNA as indirect indicators of cell growth.

When SH-SY5Y cells were incubated with 100 mM ethanol, no difference in total cellular protein was determined after 1 week (W1) between ethanol-incubated and control cells (Fig. 1A and C). During the following week, however, the amount of protein per flask (in mg/ml), more than doubled (2.5-fold, control; 2.3-fold, ethanol incubation); $n=3$ determinations (Fig 1A). A decreased amount of protein in ethanol-exposed cells, compared to control samples, suggesting a reduced number of cells, was clearly detectable between W3 and W5. The maximum difference between control and ethanol-incubated cells was 3 mg protein per flask (43% below control level; W3). After the fifth week, cells grown in the presence of ethanol seemed to adapt to the treatment and grew as fast as controls (W6–W8). At the end of the observation period (W8), protein levels differed only slightly (14.8% below control level). Pooled data for W2–W8 showed a significant effect of 100 mM ethanol on protein level ($P<0.001$, $n=7$; Fig. 1C). The average protein level was 21.3% lower in the ethanol-exposed cells.

Total RNA was monitored as another indicator of cell growth over a 10-week period with and without ethanol (100 mM). Total RNA decreased initially with a maximum difference of 55% between control and ethanol-incubated cells at W4 (Fig. 1B). Thereafter, the cells recovered until W7. After a second minimum at W8, the levels recovered again and reached a total RNA value slightly (15%) above control level. Pooled data for W2–W10 showed a significant negative effect on growth as expressed by total RNA values ($P<0.05$, $n=9$; Fig. 1D).

3.2. Quantitative northern hybridization

3.2.1. Acute ethanol-exposure experiments

Quantification of GRP78 mRNA levels after hybridization and 18S rRNA normalization of three separate experiments revealed a considerable (2-fold) increase in mRNA after 24 h, but not earlier (Fig. 2A; $P<0.05$; Whitney–

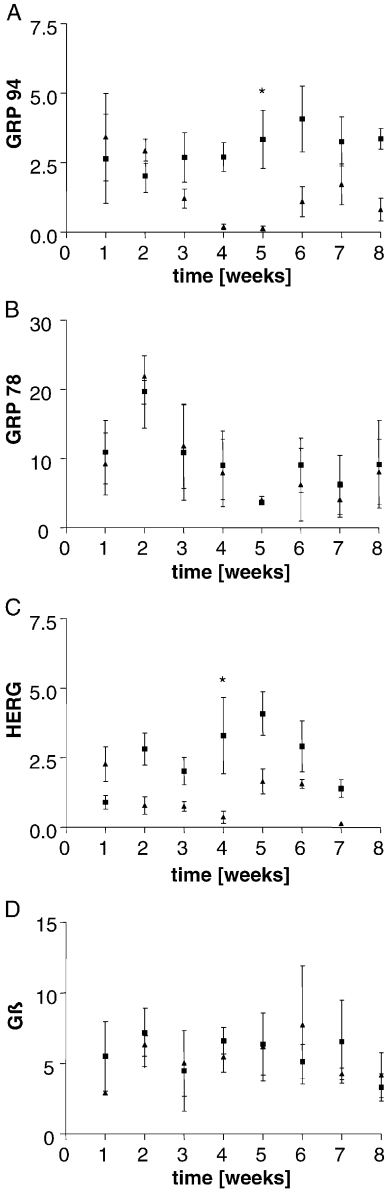

Fig. 5. Quantitative Western blot analysis of (A) GRP94, (B) GRP78, (C) HERG and (D) G_{β} during long-term incubation of SH-SY5Y cells with 100 mM ethanol (control ■ and treatment ▲). Average values ± S.E.M (arbitrary densitometric units) of $n=3$ experimental repetitions per time point between W1 and W8 are given. *Significant differences between treated and control batches at $P<0.05$ (ANOVA followed by Bonferroni posttest analysis).

Mann *U*-Test). After 72 h, levels were still higher than controls but did not reach statistical significance. Effects of the ionophore ionomycin (25 μmol/l) were much more pronounced (Fig. 2B and C) compared to ethanol effects on transcript abundancy (Fig. 2B; middle panel). An incubation lasting 24 h caused a 10-fold increase of GRP78 mRNA ($n=3$ repetitions of blotting) and a 4-fold increase of GRP94 mRNA (Fig. 2C, data from one blot). The effect of ionomycin was specific for the GRPs, and was not observed with the heat-shock proteins (HSP) HSP90α or HSP90β mRNA (data not shown). Furthermore, cells incubated with 0.18% (v/v) methanol (vehicle of the experiments with ionomycin) did not affect the expression of GRP94 (Fig. 2C). In Fig. 2B (right hand panel), the GRP78/GRP94 protein pattern of a Western blot experiment with ethanol-treated cells indicates the occurrence of two prominent bands at approximately 94 and 78 kDa molecular weights which were simultaneously visible in blotting experiments. The increase in GRP78 signal intensity of this blot was 1.6-fold and 1.8-fold for GRP94.

3.2.2. Chronic ethanol exposure experiments

Samples of long-term incubated neuroblastoma cells showed strong mRNA up-regulation of HSC70, GRP78 and GRP94 (104%, 67% and 96%, respectively; triplicate determinations of $n=3$ experiments with eight time points; Fig. 3A–C) at W1 which was significant for GRP78 and HSC70 only ($P<0.001$ and <0.05). Comparison of W1 versus W2 samples indicated a significant time effect for GRP78 and HSC70 transcript levels ($P<0.05$). The up-regulation of GRP78, GRP94 and HSC70 mRNA as effect of ethanol treatment at W1 was also visible on a representative Northern blot (Fig. 4) demonstrating single transcripts for GRP94, GRP78 and HSC70 mRNA. The 18S rRNA signal (Fig. 4, top), used for standardization of signals, indicates equal RNA quality of all batches. For GRP78 mRNA, W2 and W3 comparisons of control and treated groups were significant at the $P<0.05$% level (Fig. 3A). There was a significant overall time effect for GRP78 ($P<0.05$ and HSC70 ($P<0.05$) and a drug effect for HSC70 ($P<0.05$; statistical evaluation by ANOVA followed by Bonferroni posttest).

3.3. Western blotting

A GRP78/94-specific antibody revealed a band at approximately 78 kDa molecular weight (Fig. 6, top panel). No strong difference between controls and ethanol-incubated samples was determined for GRP78, with the exception of W6. However, a second band at approximately 90 kDa, a putatively GRP94-specific band, was strongly reduced in ethanol-incubated cells between W3 and W8. HERG was detected as a double band of approximately 130 and 150 kDa molecular sizes (Fig. 6, middle panel). The smaller protein was described as an immature precursor by Zhou et al. (1998) and was not considered for quantification in this study. The pattern of HERG protein levels in cell extracts of ethanol-incubated cells was similar to that observed for GRP94, whereby a decrease was less pronounced (Fig. 6, middle panel). A clear signal reduction could be demonstrated between W3 and W8 in ethanol-incubated cells (Fig. 6, middle panel).

The quantification of average GRP94 protein levels of $n=3$ experiments indicates a down-regulation of GRP94 protein levels from W3 onwards during ethanol incubation. Quantification of GRP94 ECL signals from ethanol-incubated samples revealed a minimum at W5, with a 95 % reduction of the signal (Fig. 5A). This effect, which was significant at W5 ($P<0.05$), lasted throughout the observation time. Comparing data from controls and ethanol-incubated cells after quantification of ECL signal intensities showed that the difference in signal intensity was not significant between W1 and W8 for GRP78 (Fig. 5B). When Western blots were performed with an HERG-specific antibody, expression was reduced in ethanol-incubated cells monitored between W2 and W7. The ANOVA analysis followed by Bonferroni posttest of treated versus control level showed a significant difference for W4 comparison

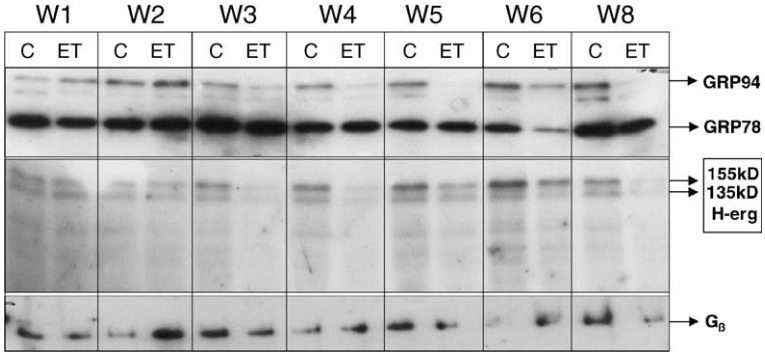

Fig. 6. Representative Western blots (HRP-ECL detection technique) demonstrating GRP94/GRP78 (top), HERG (middle) and G_β (bottom) expression during long-term exposure of SH-SY5Y neuroblastoma cells with 100 mM ethanol. Extracts from controls (C; 20 μg) for each week are shown adjacent to the same amount of extracts of treated samples (ET) for direct comparison. The GRP78/GRP94 antibody detects both proteins simultaneously (top). The HERG-specific antibody detects both a 135-kDa precursor and a 155-kDa mature protein (middle).

($P < 0.05$) with lower levels in ethanol-incubated batches, a 68.2% reduction of the HERG signal in comparison to controls at W4 (Fig. 5C). In contrast, the Gβ-protein levels were not significantly different between controls and ethanol-incubated cells (Figs. 6, bottom, and 5D). ANOVA calculations indicated a general significant effect of ethanol treatment ($P < 0.001$) on HERG and GRP94 protein levels ($P < 0.001$) but not for GRP78 and Gβ.

4. Discussion

Numerous clinical studies have reported the neurodegenerative and cardiotoxic effects of alcohol in human abusers, but little is known about the mechanisms involved in damage and/or death of the affected cells. The injury of cellular defense mechanisms by ethanol could be one explanation. The molecular chaperones, which are involved in protein folding, assembling and protein translocation (Hendershot et al., 1996; Beggah et al., 1996; Nagaya and Papazian, 1997), are components of the defense mechanisms against physiological stress (Little et al., 1994). One common effect of stress conditions is that they interfere with protein folding and modification. Subsequent accumulation of misfolded proteins in the endoplasmic reticulum induces a signal transduction pathway that leads to coordinated expression of GRP genes (Chang et al., 1989; Kuznetsov et al., 1996). Evidence is emerging that these stress proteins represent a novel class of apoptosis regulators that, when expressed at high levels, can protect the host cell against cell death (Little et al., 1994; Mehlen et al., 1996; Mosser et al., 1997; McMillan et al., 1998; Miyake et al., 2000).

Two of the glucose-regulated stress proteins (GRP78 and GRP94) and the heat-shock protein HSC70 were reported to be ethanol responsive in NG 108-15 neuroblastoma × glioma cells (Miles et al., 1991; Hsieh et al., 1996) within 24 h of incubation. Ethanol did not induce the related HSP70 gene (Miles et al., 1991). The ethanol responsiveness of GRP78 and HSC70 was confirmed in a hybridoma cell line (Mühlbauer et al., 2001). In the present study, we found short-term effects of ethanol upon GRP78 and GRP94 expression within 24 h, as indicated by increased mRNA levels in human neuroblastoma SH-SY5Y cells. The increased mRNA levels were also observed during the first week of incubation in experiments with chronic exposure. As verified by this study and as published earlier, GRPs, located in the endoplasmic reticulum as well as the cytoplasmic HSC70, are activated by ethanol in accordance to results for NG 108-15 and hybridoma cells (Miles et al., 1991; Mühlbauer et al., 2001).

Since it is well known that disturbances of Ca^{2+} concentrations trigger GRP78/94 up-regulation (Resendez et al., 1985), we incubated the cells with the calcium ionophore ionomycin. It was observed that the increase of GRP78 and GRP94 mRNA exceeded the ethanol effects several fold. Thus, the induction of the endoplasmic reticulum chaperones by ethanol does not reach the limits of responsiveness of the chaperones. This also shows that SHSY-5Y neuroblastoma cells belong to a cell type responding to depletion of calcium stores by GRP up-regulation in contrast to observations in other cell types (e.g. lymphoma cells; Lee, 2001).

This implicates a role for GRP78 and GRP94 for protecting against cell stress leading to apoptosis. Glycosylation defects seem to play a minor role for GRP induction. It has yet to be determined whether GRPs directly interact with cell death components.

During chronic ethanol exposure, both GRPs underwent a period of seemingly coordinated mRNA down-regulation (up to about W5), followed by normalization of both mRNAs between W6 and W8. To the best of our knowledge, this is the first report of down-regulation of both GRP mRNAs. Therefore, it was of great importance to investigate whether the GRP proteins decrease simultaneously. Surprisingly, protein levels were affected dichotomously. GRP94 was continuously down-regulated over the entire observation period from W2 on. This discrepancy between mRNA and protein level indicates translational and/or posttranslational, but only minor transcriptional effects of long-term ethanol influence and was also reported by others for GRP94 (Mengesdorf et al., 2001) in neuronal primary cells. In contrast, GRP78 was only slightly affected by chronic incubation with ethanol. This leads to speculation on specific functions and differences in the regulation of GRP94 in comparison to GRP78. Two specific GRP94 functions have recently been identified: calcium binding (Reddy et al., 1999) and high-density lipoprotein binding (De Crom et al., 1999). The latter authors speculated about possible functions of GRP94 also outside the endoplasmic reticulum, since both a KDEL signal for endoplasmic reticulum retention as well as one for translocation were identified. De Crom et al. (1999) presented evidence of plasma membrane-associated functions in liver cells. Their results substantiated earlier reports that the p185erbB2 receptor, a tyrosine kinase, was bound to GRP94 in the cytoplasmic membrane (Mimnaugh et al., 1996). Reddy et al. (1999) showed that the level of GRP94, but not GRP78, was diminished through proteolytic cleavage by calpain after etoposide-induced apoptosis. In two cell lines, the level of GRP94 was reduced to about 40% within 4 h. A residual amount of GRP94 remained uncleaved even after 6 h of etoposide treatment. It has been reported that apoptosis occurs when cells are exposed to ethanol (Bhave et al., 1999). Although such mechanisms may contribute to the down-regulation of GRP94 during chronic ethanol exposure, adaptive changes allow a return to normal cell growth (see Fig. 1), indicating that other overriding mechanisms are activated.

That GRP78 and 94 may play a neuroprotective role was postulated previously by Little et al. (1996) as a result of studies on rat brain after epileptic seizures. The authors observed that GRP78/94 mRNA was up-regulated indicating induction of a neuroprotective response.

Together with GRP78 and calreticulin GRP94 protects cells against oxidative damage by acting as Ca^{2+}-buffering molecules (Liu et al., 1998).

On the basis of our investigations of several proteins assembled in the endoplasmic reticulum by aid of GRP chaperones, the K^+-channel HERG was shown to be down-regulated almost simultaneously with GRP94. Drops to 20% of control levels were measured. Recovery was not recorded until after W7 of ethanol exposure (Fig. 5). These coordinated changes suggest that in SH-SY5Y cells, where HERG is expressed in relatively high amounts (Taglialatela et al., 1998), the GRP94 chaperone is co-regulated with this channel during ethanol induced cell stress whereas GRP78 is not. It is still unclear at the moment whether HERG down-regulation is due to transcriptional or translational effects and whether a direct GRP94/HERG interaction occurs.

Whereas both the cell growth and the GRPs mRNA data of the long-term study suggested adaptation to ethanol after decreased growth during the initial phase, the results on the protein level were quite different. Neither HERG nor GRP94 returned to control levels by the end of the first week of incubation and indicate posttranscriptional effects to act on GRP94 protein levels. One might speculate that reduced cell mortality instead of increased cell proliferation (which is HERG coupled, Arcangeli et al., 1995) contributed to the adaptation processes observed. An interesting finding was the unchanged level of $G_{\beta\gamma}$-protein which is in accord with data from Lichtenberg-Kraag et al. (1995). These authors found ethanol to have no effect on $G_{\beta\gamma}$-levels in platelets of intoxicated alcoholics, suggesting resistance of $G_{\beta\gamma}$-proteins towards ethanol. $G_s\alpha$, in contrast, is known to be down-regulated in a desensitization process after 5 days of incubation with 100 mM ethanol in N1E-115 neuroblastoma cells (Charness et al., 1988).

It is well known that high K^+-channel HERG expression is a feature of tumors (Bianchi et al., 1998) and possibly important in tumorigenesis. Furthermore, mutations of the HERG gene are associated with the long QT syndrome (LQT-2) due to HERG channel dysfunction (Zhou et al., 1998). HERG channels are known to regulate the duration of the heart action potential. Increased turnover of defective (misfolded) channel proteins in situations of artificially introduced dominant negative LQT-2 mutations was observed (Kagan et al., 2000). Interestingly these authors found evidence for a negative feedback effect of misfolded HERG protein on the expression of the wild-type gene. One might speculate that misfolding caused by ethanol is the underlying mechanism for the reduced HERG protein content of SH-SY5Y cells during ethanol incubation, however, transcriptional processes cannot be ruled out. It, thus, remains to be rigorously analysed whether the HERG protein down-regulation observed is due to channel subunit misassembly and/or misfolding as the consequence of ethanol influence on cells or whether ethanol directly leads to changed transcription and/or transcript turnover. It would be important to know if chaperones and their action might override the consequences of misassembled or malfunctioning K^+-channel proteins during cellular adaptation. Further research will be aimed at elucidating the chaperones responsible for HERG potassium channel subunit folding, quality control and assembly and will address the question whether glucose-regulated stress protein chaperones are directly involved in these events.

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