Valproate protects cells from ER stress-induced lipid accumulation and apoptosis by inhibiting glycogen synthase kinase-3

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Accepted 24 September 2004
Journal of Cell Science 118, 89-99 Published by The Company of Biologists 2005
doi:10.1242/jcs.01562

Summary

A wide range of agents and conditions are known to disrupt the ability of the endoplasmic reticulum (ER) to fold proteins properly, resulting in the onset of ER dysfunction/stress. We and others have shown that ER stress can induce intracellular lipid accumulation through the activation of the sterol responsive element binding proteins (SREBPs) and initiate programmed cell death by activation of caspases. It has been suggested that ER stress-induced lipid accumulation and cell death play a role in the pathogenesis of disorders including Alzheimer's disease, Parkinson's disease, type-1 diabetes mellitus and hepatic steatosis. Here we show that exposure of HepG2 cells to the branch chain fatty acid, valproate, increases cellular resistance to ER stress-induced dysfunction. Two distinctly different potential mechanisms for this protective effect were investigated. We show that exposure to valproate increases the expression of chaperones that assist in the folding of proteins in the ER including GRP78/BiP, GRP94, PDI and calreticulin as well as the cytosolic chaperone, HSP70. However, exposure of HepG2 cells to valproate does not decrease the apparent ER stress response in cells challenged with tunicamycin, A23187 or glucosamine, suggesting that valproate-conferred protection occurs downstream of ER dysfunction. Finally, we demonstrate that valproate directly inhibits the glycogen synthase kinases (GSK)-3α/β. The ability of lithium, another inhibitor of GSK3α/β to protect cells from ER stress-induced lipid accumulation suggests that GSK3 plays a central role in signaling downstream effects of ER stress. Strategies to protect cells from agents/conditions that induce ER stress may have potential in the treatment of the growing number of diseases and disorders linked to ER dysfunction.

Key words: Valproate, Endoplasmic reticulum stress, Apoptosis, Lipid accumulation, Glycogen synthase kinase

Introduction

In eukaryotic cells, the endoplasmic reticulum (ER) provides a contained environment for the synthesis and modification of membrane proteins and proteins destined to be secreted. These co- and post-translational modifications, including disulfide bond formation and N-linked glycosylation, play an important role in the subsequent folding and oligomeric assembly of proteins (Helenius, 1994). To assist in the folding of nascent polypeptides and to prevent aggregation of folding intermediates, the ER contains a high concentration of protein chaperones including the glucose-regulated proteins (GRPs), calnexin, calreticulin, protein disulphide isomerase and Erp72 (Gething and Sambrook, 1992; Ruddon and Bedows, 1997). In addition to their role in folding, some of these chaperones are postulated to act as a quality control system to ensure that only correctly folded proteins proceed to the Golgi for further processing and secretion (Kuznetsov et al., 1997). Misfolded polypeptides are retained in the ER and subsequently targeted for degradation (Travers et al., 2000; Friedlander et al., 2000). Disruption of the ER protein processing system by pathogens, mutations or specific deficiencies in the protein folding machinery have been implicated in the progression of several human diseases including Alzheimer's, Huntington's, Parkinson's and type-1 diabetes (Katayama et al., 2001; Nishitoh et al., 2002; Ryu et al., 2002; Oyadomari et al., 2002a) (reviewed by Aridor and Balch, 1999).

Agents or conditions that adversely affect ER protein folding cause the accumulation of unfolded or misfolded proteins in the ER, a condition defined as ER stress. ER stress can be induced by agents that interfere with protein glycosylation (i.e. glucose starvation, tunicamycin, glucosamine), disulfide bond formation (i.e. DTT, homocysteine), Ca^{2+} balance (A23187, thapsigargin, EGTA) and/or the general overloading of the ER with proteins (i.e. viral and non-viral oncogenesis) (reviewed by Pahl, 1999; Kaufman, 1999; Lee, 2001). Conditions of ER stress trigger the cellular unfolded protein response (UPR). In mammals the UPR is activated through three distinct signaling pathways. Activation

of the ER-resident PKR-ER-related kinase (PERK) promotes the phosphorylation/inhibition of the translation initiation factor, eIF2α (Harding et al., 2000). The resulting attenuation of general protein synthesis prevents further overloading of the ER folding machinery. Activation of ER resident proteins IRE1α and ATF6 promote the expression of the ER chaperones thereby increasing the capacity of the ER to fold the accumulated polypeptides (Wang et al., 1998; Haze et al., 1999). These ER chaperones also function as part of the quality control system that targets irrevocably misfolded proteins for ER-associated protein degradation (ERAD) (Travers et al., 2000). Thus, the UPR acts to alleviate ER stress by increasing folding capacity, inhibiting general protein translation and promoting the degradation of misfolded proteins.

Under conditions of severe ER stress, or when the UPR has been compromised, the cell may be incapable of maintaining ER homeostasis. In this case the detrimental effects of ER disruption can activate intracellular pathways that lead to programmed cell death (Nakagawa et al., 2000; Oyadomari et al., 2002b). ER stress has been shown to promote apoptosis in pancreatic β cells (Oyadomari et al., 2002a), neurons, (Nishitoh et al., 2002) and endothelial cells (Hossain et al., 2003). Although the molecular mechanisms by which ER stress activates the apoptosis are not clear, several proteins have been implicated in this pathway including the transcription factor GADD153/CHOP (growth arrest and DNA damage, also called CHOP) (Zinszner et al., 1998; Oyadomari et al., 2002a), the ER-resident cysteine protease, caspase 12 (Nakagawa et al., 2000), and the T-cell death-associated gene TDAG51 (Hossain et al., 2003; Scheuner et al., 2001).

In recent years additional cellular responses to ER stress have been identified. We have shown that homocysteine-induced ER stress can affect lipid metabolism through the activation and dysregulation of the sterol regulatory element binding proteins (SREBPs) (Werstuck et al., 2001). SREBPs promote cholesterol and fatty acid biosynthesis and also induce LDL receptor expression and activity (Brown and Goldstein, 1999; Horton and Shimomura, 1999). Thus, ER stress-induced SREBPs promote lipid accumulation in human aortic smooth muscle cells and hepatocytes (Werstuck et al., 2001). The specific cellular response elicited by an ER stress-inducing agent appears to be dependent upon cell type and the severity of the stress. The signaling mechanisms that link ER stress to the apoptotic and lipid biosynthetic pathways have yet to be delineated.

The potential role of ER stress-induced cellular dysfunction in the development and/or progression of human disease make this pathway a plausible therapeutic target. In this paper we demonstrate the ability of a small molecule drug, valproate (2-propylpentanoic acid), to increase the resistance of HepG2 cells to ER stress-induced lipid accumulation and apoptosis. Valproate is a widely prescribed drug in the treatment of epilepsy and bipolar disorder although the mechanisms by which valproate alleviate convulsions and modify behavior remain controversial (Penry et al., 1989; Bowden et al., 1994). To determine the molecular mechanisms by which valproate protects cells from ER stress-induced dysfunction we have investigated the previously reported capacity of this molecule to (1) increase intracellular ER chaperone levels (Wang et al., 1999; Brown et al., 2000), and (2) inhibit the serine-threonine kinase, glycogen synthase kinase 3α/β (GSK3α/β) (Chen et al., 1999a; Tatebayashi et al., 2004). Our results clearly show that, although valproate can increase intracellular protein levels of ER chaperones in HepG2 cells, the protection against ER stress-induced cellular dysfunction occurs as a result of GSK3 inhibition. We propose that the strategy of protecting cells from ER stress-associated complications with small molecule drugs may be useful in the treatment of disorders associated with ER dysfunction.

Materials and Methods

Cell culture and treatment conditions

The human hepatocarcinoma cell line, HepG2, was obtained from the American Type Culture Collection (ATCC; Rockville, Maryland, USA) and cultured in DMEM (Life Technologies, Burlington, Ontario) containing 10% fetal bovine serum. Cells were maintained in a humidified incubator at 37°C with 5% CO_2. Sodium valproate, A23187, tunicamycin, glucosamine, H_2O_2 and filipin were purchased from Sigma (Oakville, Ontario). GSK inhibitor II, 3-(3-carboxy-4-chloroanilino)-4-(3-nitrophenyl) maleimide, was purchased from CalBiochem (La Jolla, California, USA). All compounds were prepared fresh in culture medium, sterilized by filtration and added to the cell cultures.

Cell death assays

HepG2 cells were pretreated with 0 or 0.5 mM valproate for 18 hours after which cells were washed and cultured in DMEM without serum. Cells were then challenged with 0 to 10 μM A23187 or 0 to 10 μg/ml tunicamycin. After 6 hours, media were removed from each well and lactate dehydrogenase (LDH) activity was determined using a cytotoxicity assay kit (Roche Diagnostic, Laval, Quebec). Total LDH activity was determined by lysing cells with 0.5% Triton X-100 prior to media sampling. To monitor caspase 3 activation, HepG2 cells were cultured and challenged with ER stress agents as described above. After 6 hours, cells were harvested, lysed and cell lysates were assayed for caspase 3 activity using the colorimetric CaspACE Assay System (Promega, Madison, Wisconsin).

Free-cholesterol staining

The accumulation of free cholesterol was determined by filipin staining (Kruth, 1984). HepG2 cells, grown on coverslips, were treated with 0.5 mM valproate, 5 mM lithium or 20 μM GSK3 inhibitor II for 2 hours and then challenged with A23187 (5 μM), tunicamycin (2 μg/ml) or glucosamine (5 mM) for an additional 18-24 hours. Cells were washed three times with Medium 1 (150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 20 mM HEPES pH 7.4, 2 g/l glucose), fixed with 3% paraformaldehyde and then incubated for 2 hours at room temperature with 50 μg/ml filipin in Medium 1. Cells were again washed three times with Medium 1 and then filipin-free cholesterol complexes were visualized by fluorescence microscopy with excitation at 335-385 nm (emission at 420 nm). Relative fluorescence was quantified using Sigma Scan Pro software and results were normalized to total cell area.

Analysis of protein synthesis

HepG2 cells were pretreated with 0.5 mM valproate for 48 hours and then challenged with 0-10 μg/ml tunicamycin or 0-10 μM A23187. Cells were washed in 1×PBS, transferred into methionine/cysteine-free DMEM for 30 minutes then labeled with Express Mix [^{35}S]Met/Cys (100 μCi/ml; 1000 Ci/mmol; Perkin Elmer, Boston, Massachusetts) for 30 minutes. After washing with PBS, cell extracts were prepared and analyzed by SDS-PAGE.

Northern blot analysis

Total RNA was isolated from cultured cells using the RNeasy total RNA kit (Qiagen, Mississauga, Ontario) and resuspended in diethyl pyrocarbonate-treated water. Quantification and purity of the RNA was assessed by A_{260}/A_{280} absorption, and RNA samples with ratios above 1.6 were stored at –70°C for further analysis. Total RNA (10 μg/lane) was size-fractionated on 2.2 M formaldehyde/1.2% agarose gels, transferred to Zeta-Probe GT nylon membranes (BioRad Laboratories, Toronto, Ontario) and hybridized using radiolabeled cDNA probes as described previously (Outinen et al., 1999). Signal intensities were quantified by densitometric scanning of the autoradiograms using the ImageMaster VDS and Analysis Software (Amersham Pharmacia Biotech, Baie d'Urfé, Québec) or using a Typhoon 9410 phosphoimaging system. To correct for differences in gel loading, integrated optical densities were normalized to human glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The ERdj4 cDNA probe was purchased from ATCC (IMAGE 1920927). The cDNA probes encoding GRP78/BiP or GADD153/CHOP have been described previously (Outinen et al., 1999).

Immunoblot analysis

Antibodies to calreticulin (SPA-600) and the anti-KDEL monoclonal antibody (SPA-827), which recognizes GRP78/BiP, HSP47 and GRP94, were purchased from StressGen Biotechnologies (Victoria, British Columbia). Anti-GADD153/CHOP (sc-575), anti-eIF2α (sc-7629) and anti-ATF4 (sc-200) polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, California). Antibodies against the phosphorylated form of eIF2α (RG0001) were purchased from ResGen (Huntsville, Alabama). Anti-β actin (AC-15) antibodies were purchased from Sigma. Total protein lysates (40 μg/ml) from cultured cells were solubilized in SDS-PAGE sample buffer and separated on SDS-polyacrylamide gels under reducing conditions. After incubation with the appropriate primary and horseradish peroxidase (HRP)-conjugated secondary antibodies (Life Technologies, Burlington, Ontario), the membranes were developed using the SuperSignal chemiluminescent substrate (Pierce, Rockford, Illinois).

GSK3 Activity

GSK3 was immunoprecipitated from freshly prepared HepG2 lysates with antibodies directed against the alpha (5 μg, Upstate Biotech, Charlottesville, Virginia) or beta (2 μg, BD Bioscience, Mississauga, Ontario) using a Catch and Release IP Kit (UpState). The immobilized immune complexes were washed twice with lysis buffer (1% NP40, 0.25% deoxycholic acid, pH 7.4) and once with 4× kinase buffer (8 mM MOPS, 0.2 mM EDTA, 10 mM Magnesium acetate, pH 7.4) before elution. Kinase activity was measured by adding 15 μM substrate (phosphoglycogen synthase peptide-2, pGS-2; Upstate) and 0.5 μCi/μl [γ-^{32}P]ATP to recombinant or immunoprecipitated GSK3α/β (New England Biolabs and UpState Biotech) in a reaction mixture containing 20 mM MOPS, 50 μM EDTA, 0.25 mM Mg acetate, 5 mM $MgCl_2$, 5 mM β-glycerol phosphate, 1 mM EGTA, 0.25 mM Na_3VO_4, 0.25 mM DTT and 35 μM ATP in a total volume of 40 μl. Lithium (0-20 mM) or valproate (0-5 mM) was added to GSK3 before the substrate and ATP were added. After 30 minutes at 30°C samples were placed on ice, then spotted onto Whatman P81 phosphocellulose paper and washed three times with 0.75% *o*-phosphoric acid and once with acetone. ^{32}P incorporation onto the substrate was determined by scintillation counting and total counts minus background were normalized to the total protein concentration of each cell lysates before immunoprecipitation.

Statistical analysis

Results are presented as the mean±s.d. Significant differences in treatment groups were determined using the unpaired Student's *t*-test. For all analyses, $P<0.05$ was considered statistically significant.

Results

Exposure to valproate can protect HepG2 cells from ER stress-induced apoptosis

The use of valproate as an anti-convulsant or anti-epileptic drug has been associated with hepatotoxicity in humans (Suchy et al., 1979). The mood-stabilizing effects of valproate occur at plasma concentrations over 0.35 mM and toxicity is observed at concentrations over 1.4 mM (Bowden et al., 1994; McElroy and Keck, 1995). We first investigated the cytotoxic properties of valproate in HepG2 cells (Fig. 1A). Concentrations of valproate between 0.25-2.0 mM had no cytotoxic effect on HepG2. Growth and proliferation of HepG2 cells were not significantly affected by concentrations of valproate below 2 mM (data not shown).

Our previous studies, and those of other labs, have demonstrated that ER stress-inducing agents can cause cellular dysfunction including apoptosis and disrupted lipid metabolism (Ng et al., 2000; Shank et al., 2001; Werstuck et al., 2001; Hossain et al., 2003). It has been suggested that the physiological benefits of valproate in individuals with bipolar disease and epilepsy may result from neural protective properties of this drug. Previous reports have suggested that valproate can have pro- or anti-apoptotic characteristics (Mora et al., 1999; Bittigau et al., 2002). We examined the effect of valproate treatment on cell survival in HepG2 cells exposed to agents that cause ER stress including tunicamycin and the calcium ionophore A23187. Results show that exposure of HepG2 cells to tunicamycin or A23187 for 6 hours resulted in the dose-dependent release of lactate dehydrogenase (LDH) activity into the culture media, an indication of cytotoxicity. Pretreatment with 0.5 mM valproate significantly decreased the release of LDH after exposure to either ER stress agent (Fig. 1B). The protective effect conferred by valproate appeared to be specific for ER dysfunction because pretreatment with valproate did not alter the sensitivity of HepG2 cells to oxidative stress-inducing agents such as hydrogen peroxide (H_2O_2) that do not cause ER stress. We conclude that exposure to valproate decreases the sensitivity of HepG2 cells to ER stress-induced cell death.

To determine if valproate specifically protects cells from ER stress-induced apoptosis we measured the activity of the pro-apoptotic cysteine protease, caspase 3, in cells challenged with tunicamycin. HepG2 cells were incubated in the presence or absence of 0.5 mM valproate, challenged with tunicamycin (20 μg/ml) for 6 hours and caspase 3 activity was determined in cell lysates. Data indicate that valproate pretreatment had no significant effect on caspase 3 activity in unstressed HepG2 cells (Fig. 1C), a result consistent with previous findings showing that 0.5 mM valproate has no detrimental effect on HepG2 cell growth and viability (Fig. 1A,B). Exposure to tunicamycin elevated the level of caspase 3 activity in control cells, indicative of an early stage of ER stress-induced apoptosis. Caspase 3 activity was significantly attenuated in cell lysates containing the general caspase inhibitor, Z-VAD as well as in cells pretreated for up to 48 hours with 0.5 mM valproate (Fig. 1C). These findings are consistent with the results from the LDH assay and further support our hypothesis

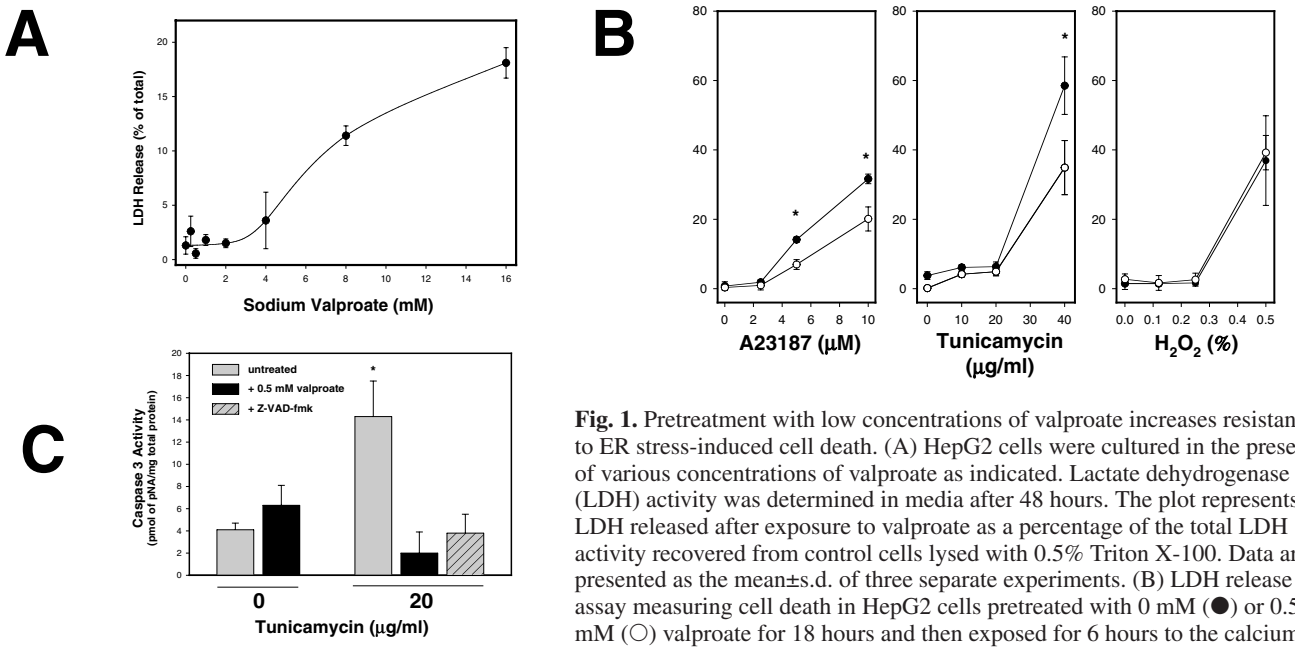

Fig. 1. Pretreatment with low concentrations of valproate increases resistance to ER stress-induced cell death. (A) HepG2 cells were cultured in the presence of various concentrations of valproate as indicated. Lactate dehydrogenase (LDH) activity was determined in media after 48 hours. The plot represents LDH released after exposure to valproate as a percentage of the total LDH activity recovered from control cells lysed with 0.5% Triton X-100. Data are presented as the mean±s.d. of three separate experiments. (B) LDH release assay measuring cell death in HepG2 cells pretreated with 0 mM (●) or 0.5 mM (○) valproate for 18 hours and then exposed for 6 hours to the calcium ionophore, A23187 (0-10 μM), tunicamycin (0-40 μg/ml) or H_2O_2 (0-0.5%) as indicated. Results are expressed as the percentage of the total LDH present in untreated cells and depicted as mean±s.d. from three separate experiments. **P*<0.01 when compared to levels in the corresponding controls. (C) Determination of caspase 3 activation in HepG2 cells pretreated for 18 hours in the absence or presence of 0.5 mM valproate and then exposed to 0 or 20 mg/ml tunicamycin for 6 hours as indicated. Hatched bars represent cells that were treated with the caspase 3 inhibitor, Z-VAD, in addition to tunicamycin. Caspase 3 activity was determined in the cell lysates and normalized to total protein concentrations. Data are presented as the mean±s.d. of three separate experiments. **P*<0.01 when compared to the activity in the corresponding control.

that valproate treatment increases the resistance of HepG2 cells to ER stress-induced cell death.

Valproate pretreatment blocks ER stress-induced lipid accumulation

We and others have demonstrated that ER stress can cause cellular dysfunction through the dysregulation of cholesterol and fatty acid metabolism in HepG2 cells (Werstuck et al., 2001; Shank et al., 2001). Specifically, we have shown that ER stress agents can activate SREBPs, and thereby increase endogenous lipid biosynthesis as well as lipid uptake resulting in the accumulation of lipids in specific cell types including HepG2s (Werstuck et al., 2001). To determine if valproate can attenuate ER stress-induced lipid accumulation we monitored intracellular free cholesterol by staining cells with filipin (Kruth, 1984). Treatment of cells with ER stress agents tunicamycin (2 μg/ml), A23187 (5 μM) or glucosamine (5 mM) for 24 hours significantly increased the free cholesterol content of HepG2 cells (1.9-, 1.7- and 2.6-fold, respectively; Fig. 2A,B), a result consistent with our previous findings (Werstuck et al., 2001). Free cholesterol accumulation was especially evident around the nuclei of the stressed cells in what appears to be the ER membrane. This observation is consistent with the increased endogenous cholesterol biosynthesis that occurs in the smooth ER. Pretreatment of HepG2 cells with valproate significantly decreased free cholesterol accumulation associated with exposure to tunicamycin and glucosamine (Fig. 2B). Treatment with the HMG-CoA reductase inhibitor, mevastatin, also reduced ER stress-induced intracellular cholesterol accumulation. Immunoblot analysis shows that HepG2 cells exposed to 5 mM glucosamine have increased levels of the mature/active form of SREBP-2 (68 kDa) (Fig. 2C). In cells pretreated with valproate, the active form of SREBP-2 is barely detectable. These results suggest that valproate blocks lipid accumulation by inhibiting ER stress-induced activation of SREBPs.

Valproate induces ER chaperone expression independent of the PERK pathway

We have demonstrated that overexpression of the ER-resident chaperone GRP78/ BiP can protect HepG2 cells from ER stress-induced lipid accumulation and cell death (Werstuck et al., 2001). It has been shown that valproate can induce the expression of the ER chaperones GRP78/BiP and calreticulin in the cerebral cortex and hippocampus of the rats injected with valproate as well as in cultured rat brain glioma cells (Wang et al., 1999; Brown et al., 2000). To determine if valproate protects HepG2 cells from ER stress-induced apoptosis and lipid accumulation by promoting GRP78/BiP expression, we examined the ability of valproate to affect ER chaperone expression levels in these cells. Our results indicate that exposure to 0.5 mM valproate for 48 hours induces the steady state mRNA levels of GRP78/BiP but not the ER stress-inducible transcription factor, GADD153/CHOP relative to the mRNA transcript level of the 'housekeeping' gene GAPDH (Fig. 3A). Furthermore, exposure of HepG2 cells to 0.5 mM valproate for 48 hours results in increased protein levels of the ER chaperones, GRP78/BiP, HSP47, calreticulin and PDI as well as the cytosolic chaperone, HSP70 (Fig. 3B). Typically, ER chaperones are induced as a response to the accumulation

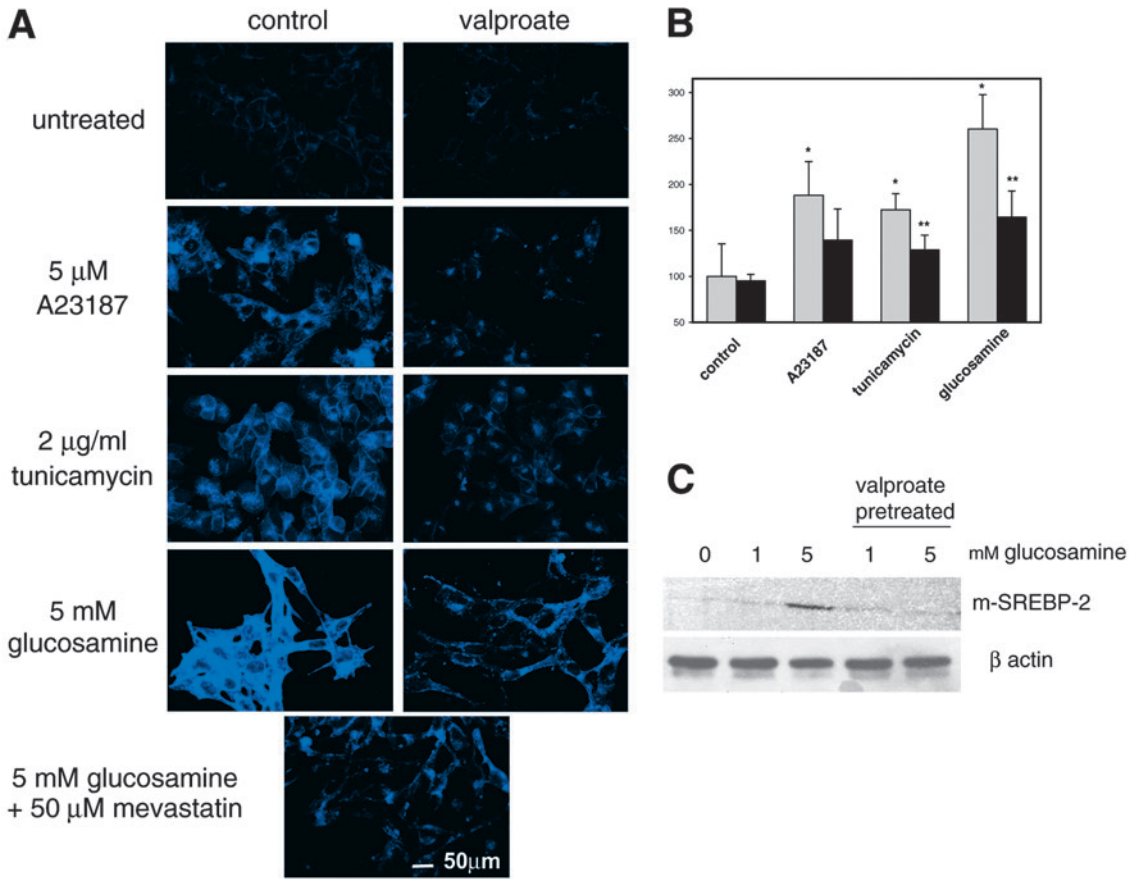

Fig. 2. Valproate blocks ER stress-induced activation of SREBP and accumulation of free cholesterol in HepG2 cells. (A) Filipin staining of HepG2 cells pre-cultured for 2 hours in the presence or absence of 0.5 mM valproate and then exposed to 5 μM A23187, 2 μg/ml tunicamycin or 5 mM glucosamine as indicated. As a control, HepG2 cells were exposed to the HMG CoA reductase inhibitor, mevastatin (50 μM) and 5 mM glucosamine as described above. After 24 hours, cells were washed, fixed in paraformaldehyde and stained with filipin. Intracellular filipin-cholesterol complexes were visualized by fluorescence microscopy and images were captured with a digital camera. Representative images are shown. (B) Median fluorescence of filipin-stained cells treated as described in A. *P<0.01 when compared with the fluorescence in the corresponding control. **P<0.05 compared to the relative fluorescence under the same conditions without valproate. (C) Immunoblot analysis of mature SREBP-2 protein levels in HepG2 cells pretreated with 0.5 mM valproate for 2 hours and then exposed to 0, 1 or 5 mM glucosamine for 24 hours as indicated. Total protein lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunostained with antibodies against SREBP-2.

of unfolded or misfolded proteins in the ER, a condition known as ER stress. However, the observation that valproate does not induce the expression of the ER stress-response gene, GADD153/CHOP, indicates that the valproate-dependent induction of ER chaperone expression may occur in the absence of ER stress. To investigate this possibility further we examined diagnostic markers of ER stress in valproate-treated cells.

A well-characterized pathway of the ER stress response involves the activation of PERK, an ER-resident kinase that phosphorylates eukaryotic initiation factor 2 alpha (eIF2α) on serine52 thereby blocking translation initiation and relieving the burden of de novo protein synthesis on the ER (Harding et al., 2000). By monitoring [^{35}S]methionine/cysteine incorporation into newly expressed proteins, we have demonstrated that exposure of HepG2 cells to 10 μM A23187 for 1 hour results in an 80% decrease in the rate of general protein synthesis but 0.5 mM valproate for 0-48 hours has no significant effect (Fig. 4A). Using antibodies directed against eIF2α and specifically against the phosphorylated form, eIF2α-P (ser52), we have shown that neither the total cellular concentration nor the phosphorylation status of eIF2α are affected in HepG2 cells exposed to 0.5 mM valproate for up to 48 hours (Fig. 4B). We further examined the expression of ER stress-response proteins, GADD153/CHOP and ATF4, which lie downstream of PERK/eIF2α (Harding et al., 2000). Although tunicamycin and A23187 promote the expression of GADD153/CHOP and ATF4, valproate had no effect on protein levels after 24 hours and may cause a decrease in GADD153/CHOP after 72 hours (Fig. 4C), a result consistent with mRNA levels (Fig. 3A). To investigate effects on other ER stress pathways we examined the IRE-1 dependent ERdj4 transcript (Lee et al., 2003). In contrast to tunicamycin, valproate treatment has no effect on ERdj4 expression (Fig. 4D). ATF6 expression and activation were also unaffected in valproate-treated cells (not shown). Together, these results show that valproate does not activate the ER stress-induced PERK pathway and therefore, contrary to previous reports

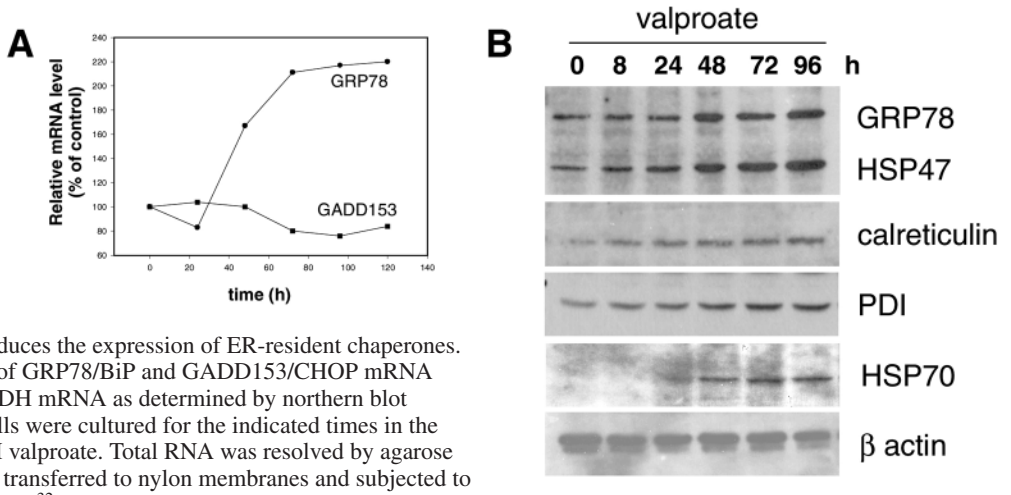

Fig. 3. Valproate induces the expression of ER-resident chaperones. (A) Relative levels of GRP78/BiP and GADD153/CHOP mRNA normalized to GAPDH mRNA as determined by northern blot analysis. HepG2 cells were cultured for the indicated times in the presence of 0.5 mM valproate. Total RNA was resolved by agarose gel electrophoresis, transferred to nylon membranes and subjected to blot hybridization with ^{32}P-radiolabeled cDNA probes encoding human GRP78/BiP and GADD153/CHOP. Relative mRNA levels were quantified using a Typhoon 9410 phosphoimaging system and normalized to GAPDH (loading control) RNA levels. (B) Immunoblot analysis of protein chaperone levels in HepG2 cells cultured for the indicated time in the presence of 0.5 mM valproate. Total protein lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunostained with antibodies against GRP78/BiP, HSP47, calreticulin, PDI or HSP70 as indicated. As a loading control, an identical blot was immunostained with an antibody against β-actin.

(Lee, 2001), valproate does not appear to cause ER stress. Furthermore, the induction of ER-resident chaperone expression by valproate appears to be independent of ER stress.

ER stress agents activate the UPR in valproate-treated cells

We and others have shown, using transgene systems, that overexpression of specific ER-resident chaperones can protect cells from ER stress and ER stress-related cellular dysfunction (Liu et al., 1997; Johnson et al., 1998; Werstuck et al., 2001). The ability of valproate to induce the expression of several ER chaperones, including GRP78/BiP, HSP47, calreticulin and PDI independent of ER dysfunction (Figs 3 and 4) suggests that pretreatment of cells with valproate may confer resistance to ER stress agents. To test this possibility we challenged control and valproate-pretreated cells with the ER stress agents tunicamycin and A23187. As previously observed (Fig. 3), exposure of HepG2 cells to 0.5 mM valproate increases the steady-state levels of mRNAs encoding GRP78/BiP but not GADD153/CHOP (Fig. 5A). As expected, in the absence of valproate, HepG2 cells exposed to an ER stress agent exhibited a typical ER stress response with an increase in both GRP78/BiP and GADD153/CHOP mRNA levels. Pretreatment with valproate for 48 hours before challenge with 2 and 10 μg/ml tunicamycin or 2 and 10 μM A23187 did not diminish the ER stress response as measured by the level of induction of GADD153/CHOP mRNA. In support of this observation, the pretreatment of cells with valproate did not attenuate the ER stress-induced expression of GADD153/CHOP protein or the increased phosphorylation of eIF2α (Fig. 5B). Furthermore, pretreatment with valproate did not alleviate the A23187-induced translation block that results from eIF2a phosphorylation (data not shown). Together these results indicate that conditions of valproate exposure that confer significant protection from ER stress-induced apoptosis and lipid accumulation do not affect the ability of ER stress agents to elicit an ER stress response. This finding suggests that valproate does not alleviate the ER stress levels in HepG2 cells but rather acts at some point subsequent to ER stress to block pathways associated with apoptosis and lipid metabolism.

Valproate protects cells from ER stress-induced dysfunction by inhibiting GSK3

Possible downstream targets of valproate include GSK3α and -β. Valproate has been reported to inhibit GSK3β (Chen et al., 1999a; Tatebayashi et al., 2004) although conflicting data have been published (Phiel et al., 2001). To the best of our knowledge, the effects of ER stress and valproate on GSK3α have not been determined. It has previously been reported that the ER stress agent thapsigargin induces apoptosis by enhancing GSK3β activity in human neuroblastoma SH-SY5Y cells (Song et al., 2002). We directly assayed GSK3α and -β activity by immunoprecipitating each kinase from lysates of HepG2 cells and measuring its activity in vitro. In HepG2 cells we observed no increase in GSK3α or -β activity upon treatment with tunicamycin or A23187 (Fig. 6A). These findings are consistent with our observation that the phosphorylation status of Akt (ser473) and GSK3 (ser9), sites important in the regulation of kinase activity (Song et al., 2002), were not affected in cells exposed to ER stress in the presence or absence of valproate (not shown). To determine if valproate directly inhibits GSK3α and/or GSK3β, 0-5 mM valproate or 0-50 mM lithium, a selective inhibitor of GSK3α/β (Stambolic et al., 1996) were added to purified recombinant GSK3α or -β and kinase activity was determined. Results show that 20 mM lithium significantly inhibited both isoforms whereas valproate preferentially inhibited the β form of the kinase (Fig. 6B). Next, the ability of valproate to inhibit intracellular GSK3 was compared to inhibition in vitro. GSK3α and -β were immunoprecipitated from HepG2 cells that had been pretreated for 2 hours in the presence or absence of 0.5 mM valproate. As observed with the recombinant

enzymes, valproate was a more effective inhibitor of GSK3β (Fig. 6C). However, significant inhibition of GSK3α was observed in vivo suggesting that valproate indirectly affects GSK3 activity in addition to its ability to inhibit kinase activity directly. Similar patterns of inhibition were observed for GSK3 immunoprecipitated from cells exposed to ER stress-inducing agents (not shown).

If valproate protects HepG2 cells from ER stress-induced cellular dysfunctions including lipid accumulation and apoptosis by virtue of its ability to inhibit GSK3β, then other inhibitors of this kinase should confer similar protection. We investigated the ability of lithium to inhibit caspase 3 activation by tunicamycin in HepG2 cells. We found that 20 mM lithium attenuates the ER stress-induced caspase 3 activity in HepG2 cells in a manner similar to valproate (Fig. 7A). To investigate the potential role of GSK3 in ER stress-induced lipid accumulation, HepG2 cells were pretreated with lithium or GSK inhibitor II (CalBiochem) and then challenged with 5 mM glucosamine for 24 hours. Both GSK inhibitors significantly decreased free cholesterol levels in HepG2 cells as determined by filipin staining (Fig. 7B,C). To the best of our knowledge, these are the first experiments that link GSK3 activity to lipid metabolism. Together these results suggest that valproate protects cells from ER stress-induced lipid accumulation and apoptosis by blocking the activation of GSK3β by conditions of ER stress. At the present time we cannot rule out the possibility that HDAC inhibition or other effects contribute to the observed cytoprotective role of valproate.

Discussion

Disruption of ER function has been linked to the development of several disorders including Alzheimer's, Huntington's and Parkinson's diseases, type-1 diabetes mellitus and hepatic steatosis (Werstuck et al., 2001; Siman et al., 2001; Harding et al., 2001; Ryu et al., 2002; Nishitoh et al., 2002). Although in most cases, a causative role has yet to be clearly demonstrated, possible mechanisms by which ER dysfunction could initiate or promote a disease state have been proposed. For example, several independent studies have demonstrated that ER stress can promote apoptosis through caspase 7-mediated caspase 12 activation and/or through the activation of caspase 8 (Rao et al., 2001; Jimbo et al., 2003). It has been postulated that ER stress promotes dopaminergic neuronal cell death in Parkinson's disease (Ryu et al., 2002), cortical neuronal cell death in Alzheimer's disease (Nakagawa et al., 2000) and in pancreatic β cell death associated with the Wolcott-Rallison syndrome of infantile diabetes (Harding et al., 2001). In addition to inducing apoptosis, we have demonstrated that ER stress can lead to the activation and dysregulation of the sterol-responsive element binding proteins, transcription factors that control lipid biosynthesis and uptake. In human aortic smooth muscle cells, HepG2 cells and in some plant cells, this can result in lipid accumulation (Werstuck et al., 2001; Shank et al., 2001). Hepatic steatosis associated with severe hyperhomocysteinemia may represent one physiological manifestation of ER stress-induced dysregulation of lipid metabolism.

The potential role of ER stress in the pathogenesis of different diseases supports the therapeutic relevance of strategies that either (1) alleviate the burden of unfolded proteins in the ER, or (2) block the signaling pathways from the dysfunctional ER that trigger detrimental downstream events. Support for the first strategy has come from experiments in which our lab, and others,

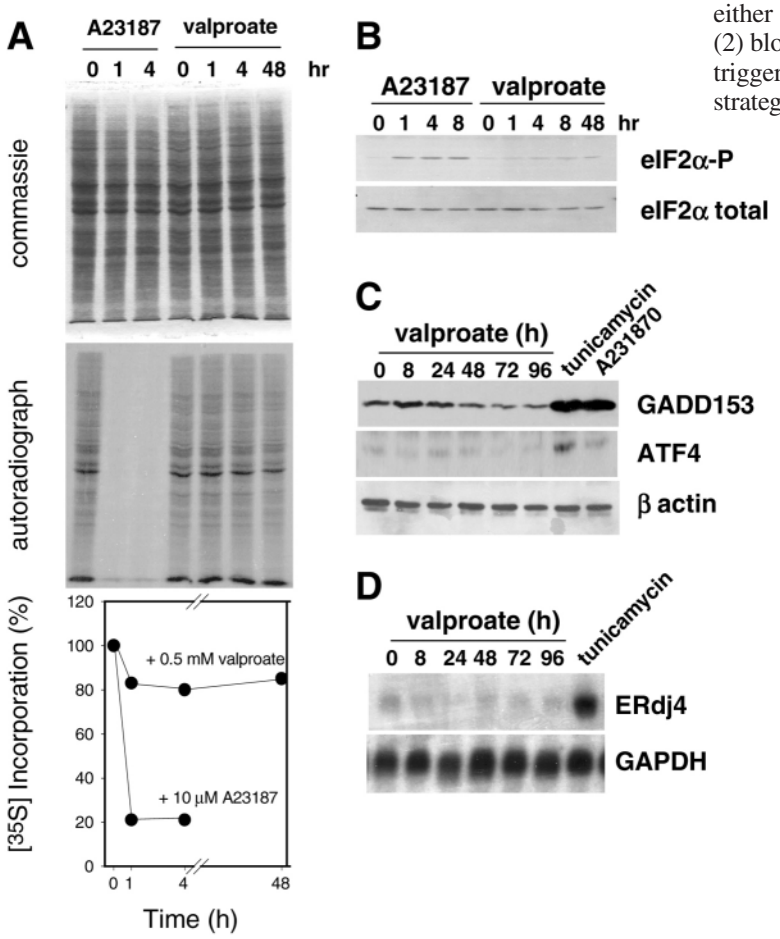

Fig. 4. Valproate does not induce an ER stress response in HepG2 cells. (A) Protein synthesis rates measured by the incorporation of [^{35}S]methionine/cysteine into proteins during a 15-minute pulse of labeling of HepG2 cells that were pre-exposed to 10 μM A23187 or 0.5 mM valproate for the indicated times. Total protein extracts were resolved by SDS-PAGE and visualized by Coomassie Blue staining (top panel) or autoradiography (middle panel). Total ^{35}S incorporation in each of the tested conditions was determined by scintillation counting and plotted as a percentage of the control count (bottom panel). (B) Immunoblot analysis of protein extracts treated as described in A. Total protein lysates were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were immunostained with antibodies against total eIF2α or the ser-52 phosphorylated form of eIF2α. (C) Immunoblot analysis of HepG2 cells cultured for the indicated times in the presence of 0.5 mM valproate or in the presence of 10 μg/ml tunicamycin or 10 μM A23187 for 8 hours. Total protein lysates were resolved and transferred to nitrocellulose membranes. Membranes were immunostained with antibodies against total GADD153/CHOP, ATF4 or β-actin (loading control). (D) Analysis of ERdj4 mRNA levels in HepG2 cells cultured for the indicated times in presence of 0.5 mM valproate or for 8 hours with 10 μg/ml tunicamycin. Total RNA was resolved by agarose gel electrophoresis, transferred to nylon membranes and subjected to blot hybridization with ^{32}P-radiolabeled cDNA probes encoding ERdj4 and GAPDH (loading control).

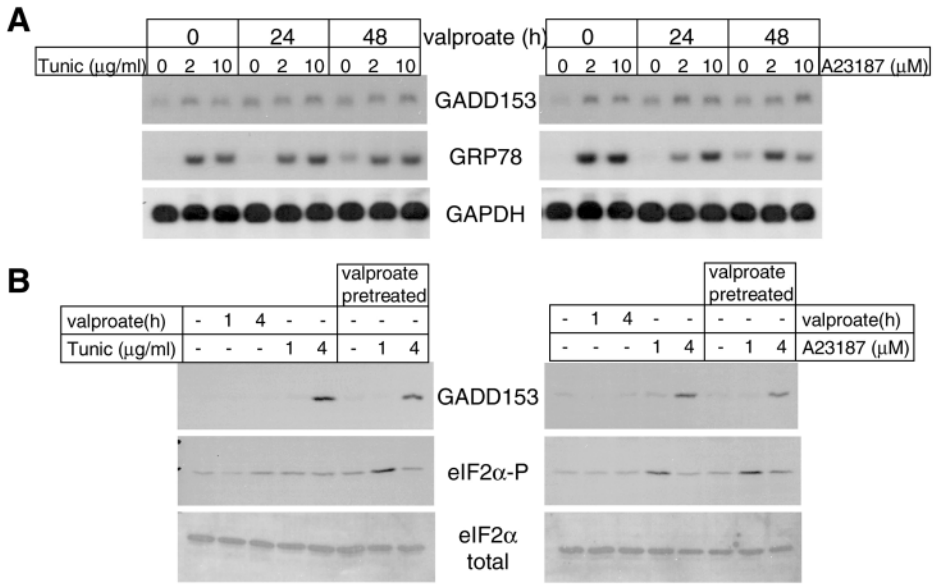

Fig. 5. Valproate pretreatment does not abrogate an ER stress response. (A) Northern blot analysis of HepG2 cells pretreated for 0, 24 or 48 hours with 0.5 mM valproate and then challenged with 0, 2 or 10 μg/ml tunicamycin (left panel) or 0, 2, 10 μM A23187 (right panel) for 4 hours as indicated. Total RNA was resolved by agarose gel electrophoresis, transferred to nylon membranes and hybridized to radiolabeled cDNA probes encoding human GRP78/BiP or GADD153/CHOP. Control for equivalent RNA loading was assessed using a radiolabeled GAPDH cDNA probe. (B) Immunoblot analysis of HepG2 cells treated with valproate, tunicamycin and A23187 as described above. Total protein lysates were resolved and transferred to nitrocellulose membranes. Membranes were immunostained with antibodies against GADD153/CHOP, the ser9-phosphorylated form of eIF2α (eIF2α-P) or total eIF2α as indicated.

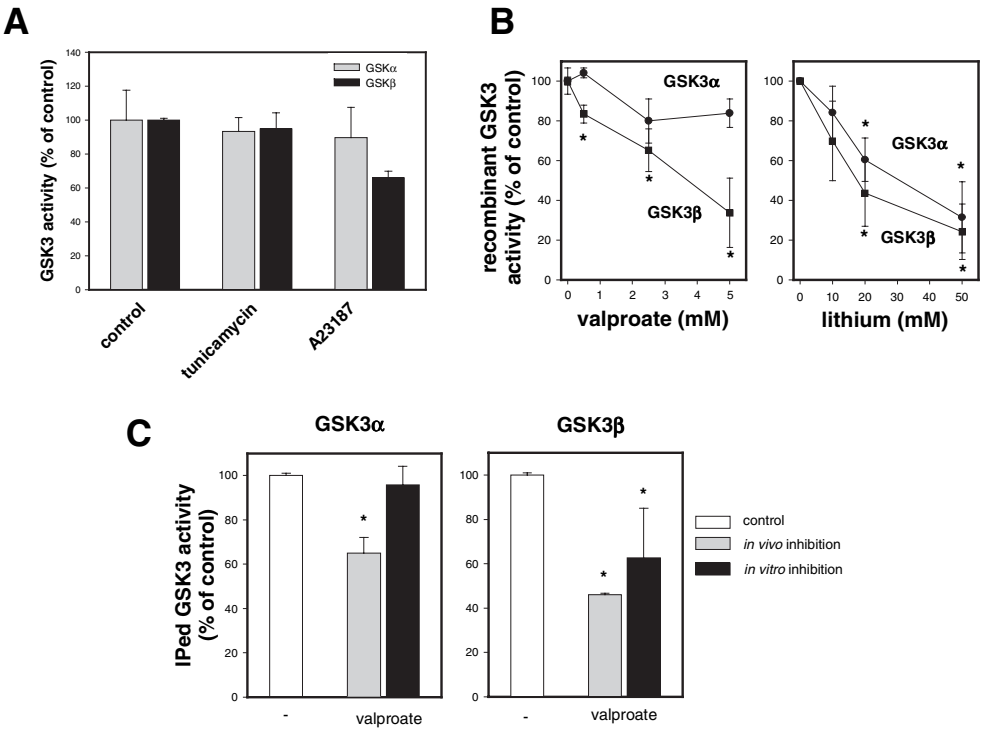

Fig. 6. Effects of ER stress-inducing agents and valproate on GSK3α/β activity. (A) GSK3α and -β activity immunoprecipitated from cells exposed to 10 μg/ml tunicamycin or 10 μM A23187 for 2 hours. Quantitative values are expressed as a percentage of GSK3α or -β activity in untreated cells. Data are expressed as the mean±s.d. (n=3). (B) Recombinant GSK3α and -β activity in the presence of 0-5 mM valproate or 0-50 mM lithium. Quantitative values are expressed as a percentage of recombinant GSK3α or -β activity in the absence of inhibitor. Data are expressed as the mean±s.d. (n=3). *P<0.05 relative to activity in the corresponding control. (C) GSKα and -β activity immunoprecipitated from HepG2 cells. Valproate (0.5 mM) was added in vivo to the cells 2 hours prior to harvesting and immunoprecipitation or to the immunopurified GSK3 during the kinase assay as indicated. Quantitative values are expressed as a percentage of GSK3α or β activity in untreated cells. Data are expressed as the mean±s.d. (n=3). *P<0.05 relative to levels in the corresponding control.

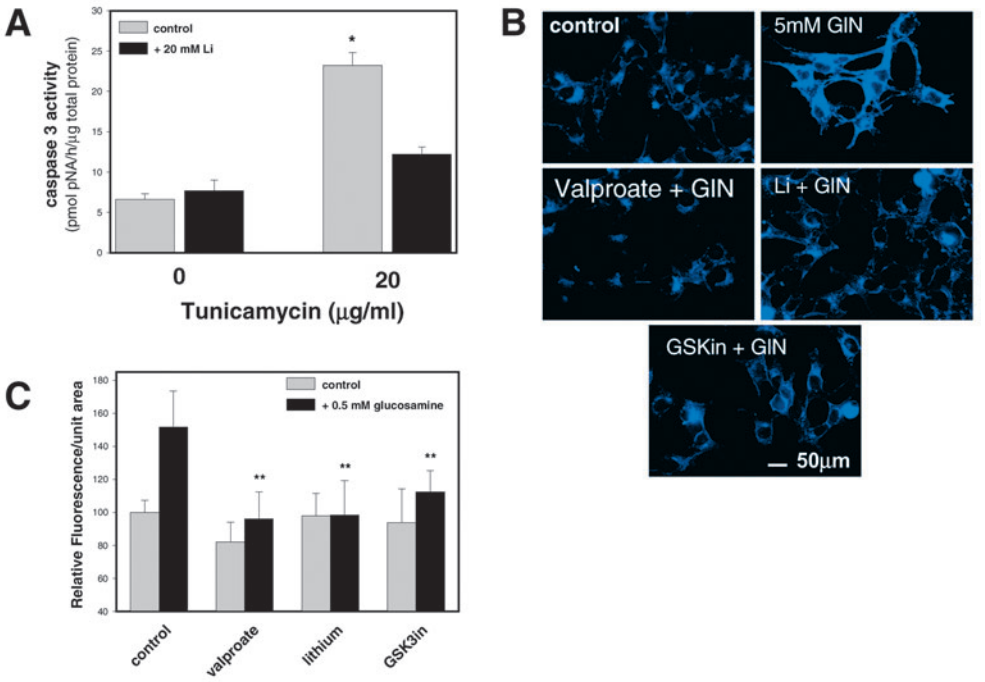

Fig. 7. Agents that inhibit GSK3 activity also block ER stress-induced lipid accumulation and apoptosis. (A) Determination of caspase 3 activity in HepG2 cells pretreated for 1 hour in the presence or absence of 20 mM LiCl and then exposed to 0 or 20 µg/ml tunicamycin for 6 hours as indicated. Caspase 3 activity was determined in cell lysates and normalized to total protein concentrations. Data are presented as the mean±s.d. of three independent experiments. *P<0.01 when compared to the corresponding control. (B) Filipin staining of HepG2 cells pretreated for 1 hour in the presence of 0.5 mM valproate, 20 mM LiCl or 20 µM GSK inhibitor II (GSKin) and then challenged with 0 or 5 mM glucosamine as indicated. After 24 hours, cells were washed, fixed in paraformaldehyde and stained with filipin. Intracellular filipin-cholesterol complexes were visualized by fluorescence microscopy. (C) Median fluorescence of filipin-stained cells treated as described in B. *P<0.01 when compared to the corresponding control. **P<0.01 compared to relative fluorescence in the absence of GSK3 inhibition.

have demonstrated that the overexpression of GRP78/BiP can increase the resistance of cells to ER stress-induced apoptosis and SREBP activation (Liu et al., 1997; Werstuck et al., 2001; Rao et al., 2002). However, the transgene strategy to block ER stress is limited by our ability to stably transfect and maintain ER chaperone-overexpressing cells, especially in vivo. Verification of the efficacy of the second strategy, to block the signals from the ER that trigger detrimental downstream events, has been impeded by our lack of knowledge of the pathways linking ER dysfunction to specific cellular responses.

Valproate is a potent and widely prescribed drug that acts both as an anti-convulsant in the treatment of epilepsy and as a mood-stabilizer to control bipolar disorder (Bowden et al., 1994; Penry and Dean, 1989). Exposure to valproate can induce a variety of cellular responses that may be responsible for its clinical efficacy. For example, it has been reported that valproate potentiates GABA-mediated postsynaptic inhibition, an effect that could play a direct role in the prevention of seizures (MacDonald and Bergey, 1979). Valproate and other anti-convulsant drugs have been shown to stabilize neuronal growth cones by depleting intracellular inositol concentrations (Williams et al., 2002). In addition, valproate has been shown to modulate gene expression by activating the AP-1 family of transcription factors as well as through the inhibition of histone deacetylases (HDACs) (Chen et al., 1999b; Phiel et al., 2001). Finally, valproate and another mood modifier, lithium, have been shown to inhibit GSK3, a kinase involved in a diverse number of signaling pathways including those controlling neuronal gene expression and survival (Chen et al., 1999a; De Sarno et al., 2002). It is not known which if any of these characteristics of valproate contribute to its physiological properties in the treatment of epilepsy and bipolar disorder.

It has recently been demonstrated that GRP78/BiP protein and mRNA levels are increased in the cerebral cortex of rats injected with valproate as well as in rat C6 glioma cells treated with valproate (Wang et al., 1999; Brown et al., 2000). We have shown that in HepG2 cells, valproate increases protein levels of GRP78/BiP, GRP94, calreticulin, PDI as well as HSP70. The mechanism by which valproate enhances ER chaperone gene expression is not clear but appears to be independent of the UPR pathway of induction for two reasons. First, induction of GRP78/BiP by the UPR has been shown to require PERK-dependent phosphorylation of eIF2α (Scheuner et al., 2001). We have shown that valproate promotes the expression of GRP78/BiP and other chaperones in a manner that is independent of eIF2α phosphorylation (Fig. 4). Second, the induction of ER resident chaperones by valproate requires prolonged cellular exposure: 48 hours in HepG2 cells (Fig. 3) and up to 7 days in rat glioma cells (Brown et al., 2000). By contrast, activation of the UPR results in the rapid induction of ER chaperone expression that is evident within 4 hours of exposure to an ER stress-inducing agent (Figs 4 and 5). The differing kinetics of ER chaperone accumulation suggest that valproate acts indirectly on gene transcription through a pathway distinct from the UPR. The existence for such a pathway is supported by the ability of mitogens including interleukin 3

(IL3) and erythropoietin to activate GRP78/BiP and GRP94 expression in non-stressed myeloid FDC-P1.2 cells (Brewer et al., 1997). The significance of this pathway with regard to cellular function and response to unfolded proteins is not known.

Our observation that valproate could protect HepG2 cells from ER stress-induced lipid accumulation and apoptosis (Figs 1 and 2), led us to investigate the possibility that this protection was a result of the ability of valproate to increase ER chaperone levels and thereby relieve the burden of unfolded proteins in the ER. However, upon further examination, this mechanism was discounted for two reasons. First, valproate-conferred protection occurs at least 20 hours before the observed increase in ER chaperone levels (Figs 1-3). Second, cells pretreated with valproate, despite exhibiting increased resistance to disrupted lipid metabolism and apoptosis, mounted a similar ER stress response when challenged with agents that cause protein misfolding (Fig. 5). In these experiments an ER stress response was defined as the increased phosphorylation of eIF2α by activated PERK on serine52. This has been identified as one of the earliest events in the UPR and occurs within 60 minutes of addition of an agent such as tunicamycin or A23187 (Harding et al., 2000). Other indicators of ER stress including induction of GADD153 expression were also not effected by valproate pretreatment. These findings suggest that valproate does not directly protect HepG2 cells from ER stress but instead acts at some subsequent stage to block the onset of cellular complications that are induced by ER stress. The inability of valproate-induced ER chaperone protein levels to protect cells from ER stress is probably a result of the comparatively low levels of chaperone induction observed relative to the previously utilized GRP78/BiP-overexpressing cell lines (Werstuck et al., 2001). It is also possible that the increase in chaperone levels observed in valproate-treated cells is offset by a corresponding increase in the load of folding proteins in the ER under the conditions tested.

The ability of valproate to inhibit GSK3α/β (Fig. 6B) together with the observation that lithium and a synthetic GSK3 inhibitor can mimic the ability of valproate to block cholesterol accumulation in cells exposed to ER stress (Fig. 7B,C) suggests that GSK3 plays a central role in signaling from the ER. This observation is consistent with a previous report indicating that GSK3 plays a central role in ER stress-induced apoptosis (Song et al., 2002). GSK3α/β were initially identified as two homologous kinases that phosphorylate the rate-limiting enzyme in glycogen synthesis, glycogen synthase (Plyte et al., 2002). Over the last 20 years the list of GSK3 substrates has grown to encompass a broad array of proteins that are involved in several important regulatory and developmental pathways including translation initiation factor eIF2βε, transcription factors CREB, c-Jun, c-Myc, c-Myb, HSF-1, ATP-citrate lysase and tau (Doble and Woodgett, 2003). Valproate and lithium have previously been reported to inhibit GSK3β through direct (Chen et al., 1999a) and/or indirect mechanisms (De Sarno et al., 2002). Our results using both recombinant GSK3s as well as GSK3 immunoprecipitated from HepG2 cell lysates indicate that physiological concentrations of valproate directly inhibit GSK3β in vitro and GSK3α and -β activity in vivo (Fig. 6).

Together these findings indicate that in HepG2 cells ER dysfunction triggers cellular responses that can lead to lipid accumulation and/or apoptosis. Although it is still not clear if GSK3α/β activity is enhanced under conditions of ER stress, a role for GSK3α/β is implied by the ability of several different inhibitors of this kinase to block both ER stress-induced lipid accumulation and apoptosis. GSK3α/β has been recognized as a therapeutic target for the treatment of cancer and neurological disorders. These results suggest that this already diverse kinase may be involved in an even broader array of conditions and may represent a point of convergence between different pathways of disease progression. Further studies will be required in different cell types and in animal models to explore the potential efficacy of valproate on the development and progression of conditions associated with ER stress.

We would like to thank Jack Hirsh and Jeffrey Weitz for helpful comments and suggestions throughout the course of these studies. This work was supported by a Research Grant (MOP-62910) from the Canadian Institutes of Health Research (to G.H.W.).

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