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Role of Endoplasmic Reticulum Calcium Disequilibria in the Mechanism of Homocysteine-Induced ER Stress

JEFFREY G. DICKHOUT, SUDESH K. SOOD, and RICHARD C. AUSTIN

ABSTRACT

Our laboratory demonstrated that hyperhomocysteinemia accelerates atherosclerosis in mouse models through ER stress and activation of the unfolded protein response (UPR). In this study, we tested the hypothesis that homocysteine-induced ER stress may arise from ER-Ca^{2+} disequilibria. We found that homocysteine-induced cytosolic Ca^{2+} transients in T24/83 cells and human aortic smooth muscle cells (HASMCs). These calcium effects occurred at concentrations of homocysteine in the external medium (1–5 m*M*) that increase intracellular homocysteine in these cell types. Prolonged homocysteine treatment (5 h) at these exogenous concentrations reduced ER-Ca^{2+} emptying evoked by thapsigargin. However, these homocysteine-induced effects on ER-Ca^{2+} emptying were of a much smaller magnitude than those evoked by A23187 or thapsigargin (ER stressors known to induce ER stress through ER-Ca^{2+} depletion). T24/83 cells stably overexpressing the Ca^{2+}-binding ER chaperone GRP78 showed diminished cytosolic Ca^{2+} transients induced by homocysteine and reduced ER-Ca^{2+} emptying evoked by thapsigargin. Prevention of the homocysteine-induced UPR by cycloheximide pretreatment normalized GRP78 expression and ER-Ca^{2+} emptying evoked by thapsigargin. These results are inconsistent with a mechanism of ER stress induction by homocysteine through ER-Ca^{2+} depletion. *Antioxid. Redox Signal.* 9, 1863–1873.

INTRODUCTION

The endoplasmic reticulum (ER) is the cellular organelle primarily responsible for the production of secretory, transmembrane, and ER-resident proteins (21). This production involves the proper folding of the polypeptide chain with the aid of molecular chaperones into its tertiary structure. However, impaired folding of proteins causes ER stress and leads to an evolutionary conserved response, the unfolded protein response (UPR), involving the upregulation of protein-folding chaperones, including GRP78 (19). One critical aspect of maintaining the three-dimensional structure of proteins transiting the ER is the formation of disulfide bonds. This occurs in the ER because the luminal environment allows the oxidation of proteins in the presence of specific enzyme foldases, including protein disulfide isomerase (13). A sustained UPR in response to an inadequate protein-folding capacity within the ER leads to the accumulation of reactive oxygen species and apoptotic cell death (3).

As well as being of importance in protein folding, the ER is also the site of intracellular calcium storage and the origin and regulator of intracellular calcium signaling (8). Cellular calcium signaling modulates numerous cellular functions, including the release of secretory proteins (34). However, release of calcium from the ER sufficient to impair protein folding leads to ER stress and activation of the UPR (5). Cytoplasmic calcium levels are much lower than the extracellular fluid, and prolonged cytoplasmic calcium elevation leads to cytotoxicity (31). Further, ER calcium depletion leads to ER stress-induced UPR activation and apoptosis (18).

The thiol-containing amino acid homocysteine has been implicated as an independent risk factor for cardiovascular disease (9, 25). Elevated levels of plasma homocysteine, as seen in patients with cystathionine β-synthase deficiency, can be reduced in many cases by B-vitamin therapy and methionine dietary restriction, thereby reducing the risk of cardiovascular disease (27, 37). The mechanism by which homocysteine increases

Department of Pathology and Molecular Medicine, McMaster University, and the Henderson Research Centre, Hamilton, Ontario, Canada.

cardiovascular disease risk has not been fully elucidated. Several competing theories involve the modification of redox state and signal-transduction pathways (42). These include the effects of homocysteine to induce endothelial damage/dysfunction and reduce the bioavailability of nitric oxide (22), to generate reactive oxygen species or downregulate antioxidant enzymes (14), the specific ability of homocysteine to modify protein structures (17), and the action of homocysteine to induce ER stress (2). These competing theories are not mutually exclusive. The first known indication that elevations of homocysteine impair protein folding was reported in the endothelial cell secretory pathway, suggesting that it may lead to ER stress (23, 24). Previous studies in our laboratory have shown that homocysteine induces ER stress and increases expression of Ca^{2+}-binding ER resident molecular chaperones, including GRP78 and GRP94 (29, 36, 40). Many ER stressors such as calcium ionophores (33) and the ER-Ca^{2+} ATPase-inhibitor thapsigargin (38) are known to disrupt calcium homeostasis and induce ER stress through ER-Ca^{2+} depletion. How homocysteine acts to induce ER stress remains unknown.

Homocysteine has been shown to induce changes in intracellular calcium handling. In ECV304 cells, shown to be identical to T24/83 by genetic analysis (6), homocysteine pretreatment was found to reduce thapsigargin-induced capacitative calcium entry (39). Homocysteine was also shown to induce intracellular calcium transients in rat vascular smooth muscle cells because of calcium release from intracellular stores (28). Homocysteine has been found to inhibit rat myocardial mitochondrial Ca^{2+}-ATPase activity significantly (7), to induce excitotoxic neuronal injury through elevations of intracellular free Ca^{2+} (41), and to inhibit iberiotoxin-sensitive, Ca^{2+}-activated K^+ channels in smooth muscle (1). Because of the demonstrated effects of homocysteine on calcium handling in the cell and the critical role of cellular calcium handling for ER protein folding, we hypothesized that homocysteine induces ER stress by disrupting intracellular calcium homeostasis. To test this hypothesis, we applied dose ranges of exogenous homocysteine to T24/83 cells and HASMCs. These cell types were studied because they were previously shown to respond to homocysteine treatment with ER-based calcium effects. Further, they are known to undergo ER stress in response to homocysteine treatment. We examined the effects of doses of exogenous homocysteine known to cause ER stress on intracellular homocysteine concentrations, evocation of Ca^{2+} transients, and thapsigargin-sensitive Ca^{2+} release from ER Ca^{2+} stores. Further, we assessed these responses of homocysteine in reference to other known ER stress inducers to determine whether homocysteine-induced changes in intracellular calcium dynamics were a cause or consequence of ER stress.

METHODS AND MATERIALS

Cell culture

T24/83 cells (ATCC, Manassas, VA) were grown in medium 199 (Gibco, Carlsbad, CA) supplemented with 10% FBS (Sigma, St. Louis, MO) and 1× penicillin/streptomycin antibiotic (Gibco). HASMCs were obtained from Cascade Biologics and grown in 231 medium (Cascade Biologics, Portland, OR) containing 5% FBS, 2 ng/ml human basic fibroblast growth factor, 0.5 ng/ml human epidermal growth factor, 5 ng/ml heparin, 5 μg/ml insulin, and 0.2 μg/ml BSA. T24/83 cell lines were generated by transfection of T24/83 cells at 30% of confluence with the pcDNA3.1 expression plasmid encoding the human open reading frame for GRP78. Vector controls for these cell lines were generated by transfecting T24/83 cells with the pcDNA3.l plasmid alone. Stable cell lines were then selected with G418 at 1.2 mg/ml, and once the cell lines were established, the cells were cultured in medium 199 containing 10% FBS, 1× penicillin/streptomycin antibiotic, and G418 at a maintenance dose of 500 μg/ml. These cell lines were characterized earlier for their expression of GRP78 (35).

Intracellular homocysteine measurement

Intracellular homocysteine was measured by using the Abbott Laboratories IMx assay system to determine cellular homocysteine uptake produced by exogenous homocysteine loading. In brief, cells were incubated with L-homocysteine prepared from L-homocysteine thiolactone by the modified method of Duerre *et al.* (12). L-Homocysteine was then added to the medium at the appropriate dose for 4 h. Cells were washed three times in growth medium, three times in PBS to remove extracellular homocysteine, trypsinized, and subjected to freeze/thaw lysis. Total homocysteine was measured as well as total soluble protein. Protein measurements were based on the Bradford assay (BioRad) and used to normalize intracellular homocysteine concentrations per amount of cellular protein.

Indirect immunofluorescence

GRP78-overexpressing stable cell lines and pcDNA3.1 vector controls were subjected to indirect immunofluorescence to determine the level of GRP78 overexpression. Cells were grown on 1% gelatin-coated glass coverslips (Sigma) and at 50% confluence, washed in PBS and fixed with 4% paraformaldyde at 4°C overnight. The cells were washed in PBS and permeabilized with 0.025% Triton-X, blocked for nonspecific antibody binding in 3% BSA, and incubated with the goat polyclonal anti-GRP78 antibody (Santa Cruz, Santa Cruz, CA) for 1 h. The cells were then washed in PBS and incubated with the secondary antibody donkey anti-goat Alexa 488 (Molecular Probes, Inc., Carlsbad, CA), washed and mounted in anti-fade (Molecular Probes) for imaging under a Zeiss 510 laser-scanning confocal microscope.

Cytosolic Ca^{2+} measurement

Homocysteine and three other ER stress-inducing agents, the calcium ionophore A23187, the ER-Ca^{2+} ATPase inhibitor thapsigargin, as well as the N-linked glycosylation inhibitor tunicamycin were used. Their effects on cytosolic free Ca^{2+} levels in T24/83 and HASMCs were measured on a Molecular Devices SpectraMax Gemini Spectrofluorometer. Measurements were made at 37°C with the Ca^{2+}-sensitive dye Fura-2-AM (Molecular Probes) loaded for 30 min at 5 μM, ratiometrically (dual-wavelength excitation (340 nm/380 nm), 510 emission). Pluronic F127 (Molecular Probes) was used to dissolve the Fura-2-AM dye to prevent dye compartmentalization. The cal-

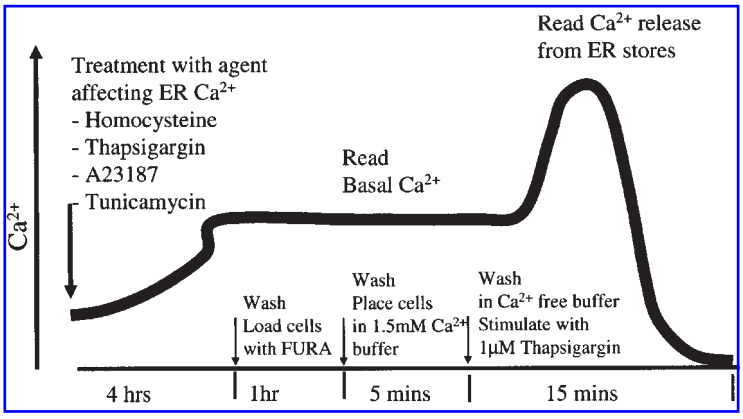

FIG. 1. Procedure for the measurement of ER-Ca^{2+} content in T24/83 and HASMCs. T24/83 and HASMCs were exposed to homocysteine and other ER-stress–inducing compounds for a period of 4 h, loaded with the calcium reporting dye Fura-2 over a 1-h period, and subjected to thapsigargin-stimulated ER-Ca^{2+} release in a Ca^{2+}-free buffer to measure ER-Ca^{2+} content.

cium-measurement buffer was Hanks' balanced salt solution (Gibco) with 20 m*M* HEPES, adjusted to pH 7.4 (HHBSS). Cells grown to confluence in 96-well black flat-bottom plates (Greiner bio-one, Longwood, FL) were washed free of growth medium, and the dye was loaded in the HHBSS buffer for 30 min at 37°C and then washed to remove excess dye. Fura-2 radiometric responses were calibrated intracellularly by using the calcium ionophore 4-bromo-A23187 (10 μM) (Molecular Probes) to measure maximum (ionophore + 2.5 m*M* Ca^{2+} buffer) and minimum (ionophore + 0 m*M* Ca^{2+} buffer with 100 μM EGTA) Fura responses, as in our previous studies (10, 11).

ER Ca^{2+} measurement

The thapsigargin-sensitive calcium content of ER stores was assessed after 5-h treatment with homocysteine or

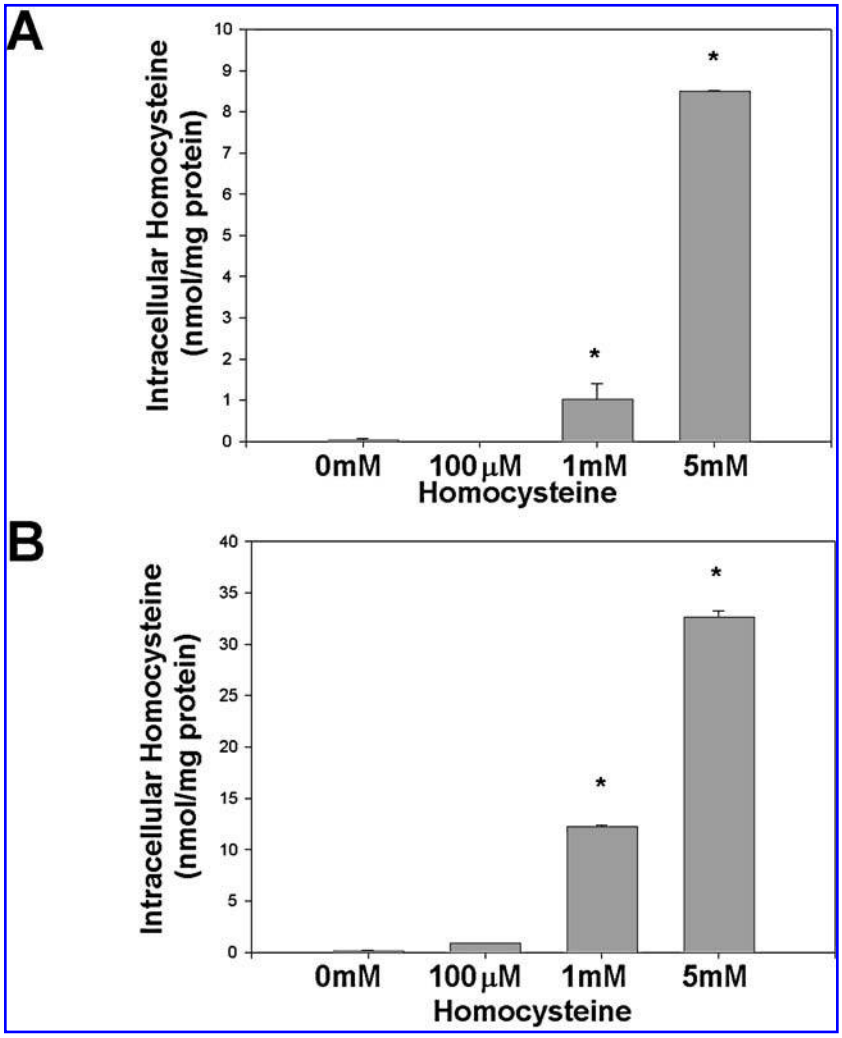

FIG. 2. Exogenously added homocysteine increases intracellular homocysteine concentrations. Intracellular homocysteine concentrations were measured in T24/83 (**A**) and HASMCs (**B**) with increasing doses of exogenously added homocysteine for 4 h. It was determined in both T24/83 and HASMCs that a threshold dose of 1 m*M* homocysteine or greater was required to increase intracellular homocysteine concentrations significantly(*$p < 0.05$ over control).

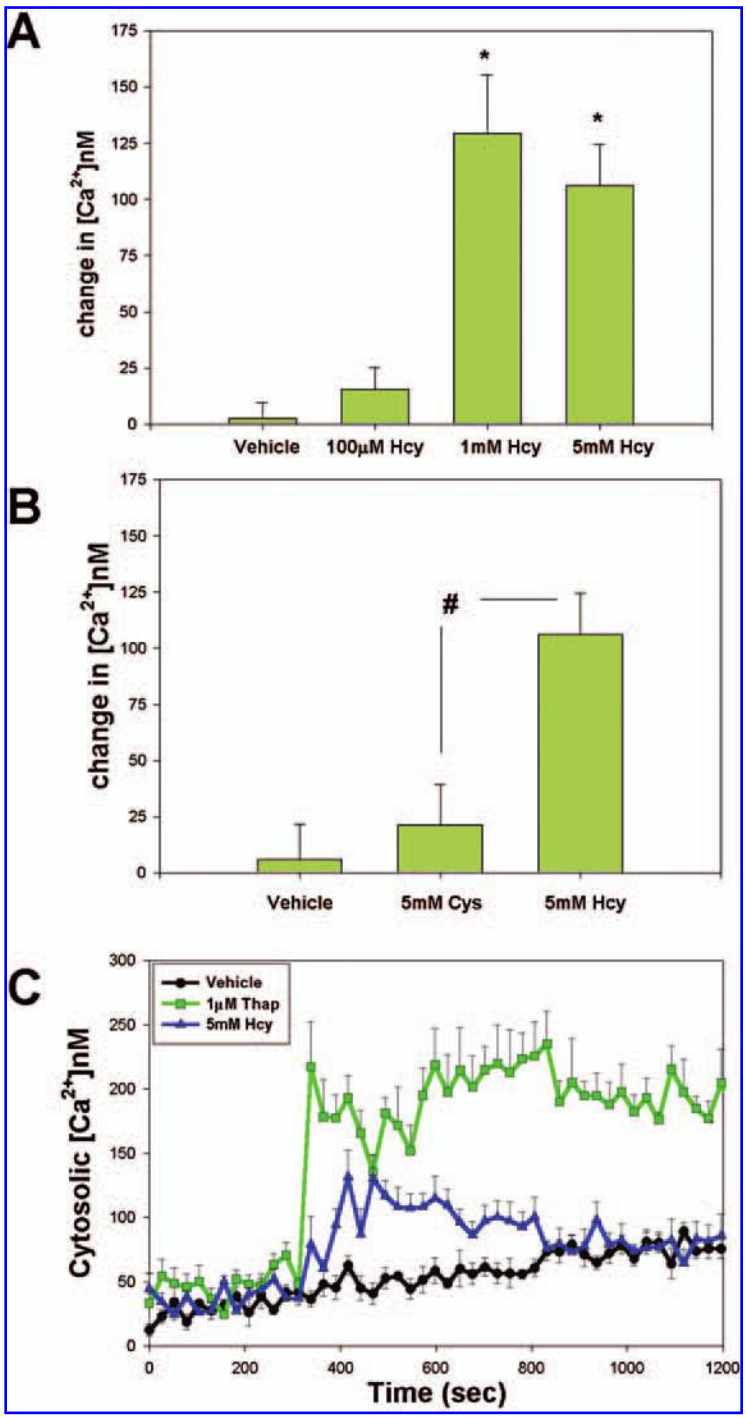

FIG. 3. Effect of homocysteine on cytosolic calcium concentrations. Cytosolic free Ca^{2+} measurements were made at 2 min after stimulation in T24/83 (**A** and **B**) and HASMCs (**C**) with increasing doses of homocysteine applied to the medium. (**A**) Significant increase in cytosolic Ca^{2+} occurred at $\geq$1 m*M* homocysteine (Hcy) dose ($*p < 0.05$). (**B**) This effect was specific for homocysteine (Hcy), as equimolar amounts of cysteine (Cys) did not elicit a response ($\#p < 0.05$). (**C**) A similar pattern of response was seen in HASMCs with 5 m*M* homocysteine (Hcy), shown inducing cytosolic Ca^{2+} transient in comparison to vehicle control. The response of HASMCs to thapsigargin (Thap) (**C**) is also depicted, showing the evoked cytosolic Ca^{2+} transient followed by sustained elevation of Ca^{2+}. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars)

other ER-stress–inducing agents, including A23187, thapsigargin, and tunicamycin, or in GRP78-overexpressing T24/83 cells. To achieve this, 1 μM thapsigargin was used to release ER-Ca^{2+} content to the cytosol while the cells were incubated in a Ca^{2+}-free medium to prevent Ca^{2+} influx from the bathing medium through store-operated Ca^{2+} channels (Fig. 1). The response, increase in Fura-2 ratio, represents only the ER-Ca^{2+} traversing through the cytosol. The area under the response curve represents the total ER-Ca^{2+} content.

Western blot analysis

Total protein lysates in SDS-PAGE sample buffer were separated on 10% SDS-polyacrylamide gels under reducing conditions and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The primary antibody to GRP78 (Anti-KDEL, StressGen, San Diego, CA) was conjugated with a horseradish peroxidase–conjugated secondary antibody (DAKO, Glostrup, Denmark). Membranes were developed by using the Renaissance Western blot chemiluminescence reagent, as in our previous work (10).

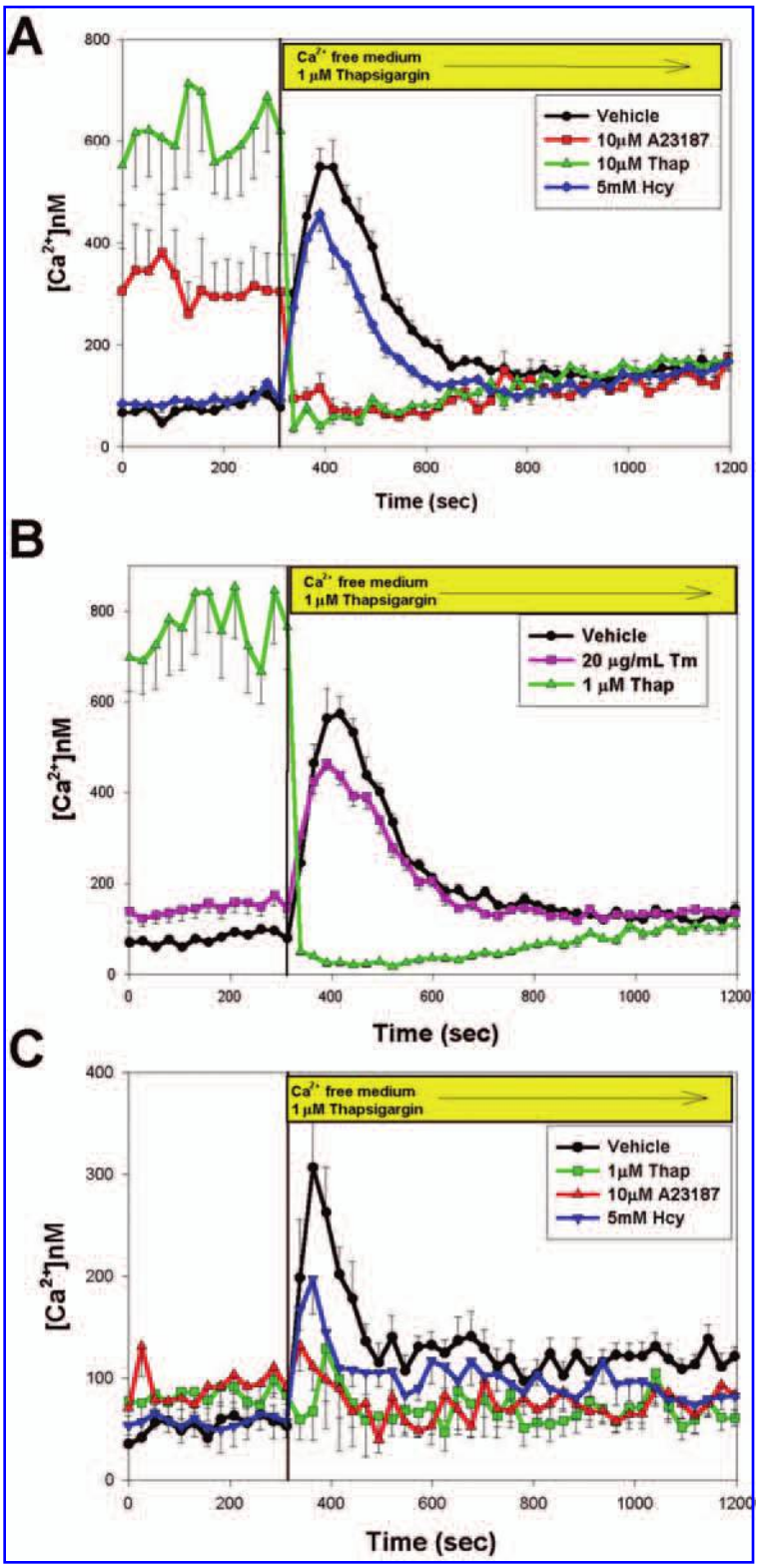

FIG. 4. Effect of homocysteine on release of calcium from intracellular stores. ER-Ca^{2+} release measurements were made on T24/83 (**A** and **B**) and HASMCs (**C**) 5 h after treatment with homocysteine (Hcy) or other ER-stress–inducing agents, A23187, thapsigargin (Thap), and tunicamycin (Tm). It was found that 5 m*M* homocysteine (Hcy) treatment significantly reduced Ca^{2+} release from ER stores in both T24/83 (**A**) and HASMC (**C**). A23187 and thapsigargin (Thap) treatment abolished Ca^{2+} release from ER stores in both T24/83 (**A**) and HASMC (**C**), and tunicamycin (Tm) significantly reduced responses in T24/83 cells (**B**). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars)

Statistical analysis

Values are expressed as mean ± standard error of the mean. Comparison between the means of treatment groups was performed with Student's unpaired *t* test. Significance was recognized at the 95% level.

RESULTS

Intracellular homocysteine concentrations

Intracellular concentrations of homocysteine were measured and shown to increase dose-dependently with homocysteine

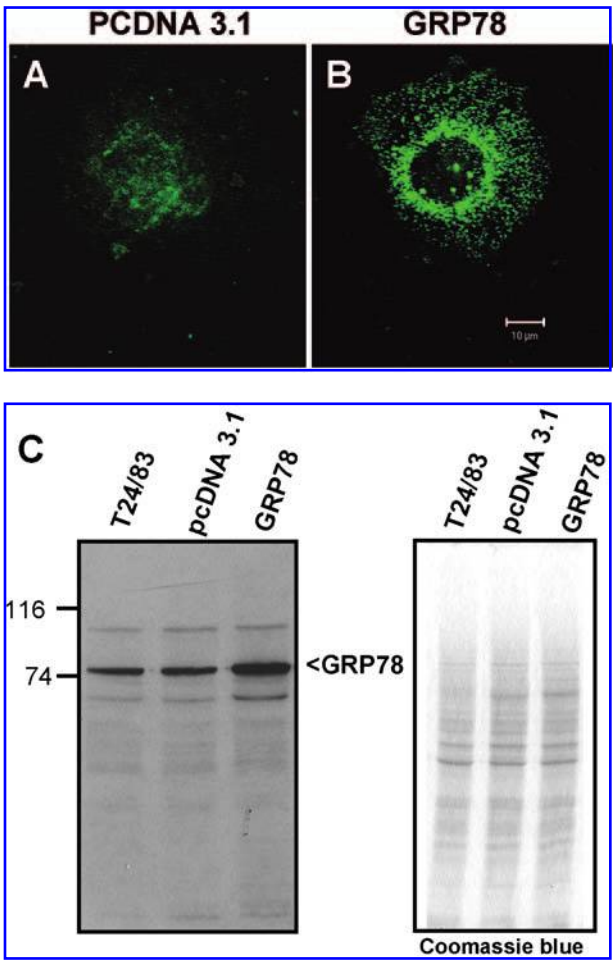

FIG. 5. Stable overexpression of GRP78 in T24/83 cells. Overexpression of GRP78 in T24/83 cells is shown by indirect immunofluorescence. GRP78 staining in cells stably expressing the pcDNA3.1 plasmid control (**A**) or the pcDNA3.1 plasmid containing the open reading frame for human GRP78; bar equals 10 μM (**B**). Western blotting for GRP78 (KDEL antibody) in wild-type T24/83 cells, cells stably transfected with the expression plasmid or the expression plasmid containing the open reading frame for human GRP78. Gels containing total protein lysates were stained with Coomassie Brilliant Blue to show equal protein loading (**C**). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars)

loaded into the extracellular medium. This occurred in T24/83 (Fig. 2A) and HASMCs (Fig. 2B) above a threshold dose of 100 μM, with 1 and 5 m*M* homocysteine showing significant elevations in intracellular homocysteine in T24/83 and HASMCs.

Cytosolic Ca^{2+} response to homocysteine

The effect of exogenously added homocysteine on cytosolic free Ca^{2+} concentration was assessed in T24/83 and HASMCs. Homocysteine caused a dose-dependent increase in cytosolic free Ca^{2+} concentration in T24/83 cells. Doses of 1–5 m*M* were required to produce statistically significant elevations at 2 min after stimulation (Fig. 3A). Homocysteine had similar effects in HASMCs by dose dependently increasing cytosolic free Ca^{2+} concentration, with 1 and 5 m*M* concentration producing significant elevations at 2 min after stimulation (data not shown). This effect was specific for homocysteine, because the thiol-containing amino acid cysteine did not produce similar effects (Fig. 3B). Homocysteine also evoked calcium transients in HASMC (Fig. 3C). These transients were smaller in magnitude than those evoked by 1 μM thapsigargin and did not show the sustained elevation of Ca^{2+} concentration produced by thapsigargin treatment (Fig. 3C).

ER-Ca^{2+} content

To determine whether homocysteine-induced ER stress is the result of ER-Ca^{2+} depletion similar to the ER stress induced by thapsigargin and A23187 but differing from the ER stress induced by tunicamycin, measurements of ER-Ca^{2+} content were made. T24/83 cells were treated with the ER stress–inducing agents A23187 (10 μM), thapsigargin (10 μM) or homocysteine (5 m*M*) for 5 h, and subsequent thapsigargin evoked ER-Ca^{2+} release measured in a Ca^{2+}-free buffer in relation to cells treated with drug vehicle alone (Fig. 4A). Measurement of cytosolic Ca^{2+} before evoking ER-Ca^{2+} release showed the sustained elevations of Ca^{2+} produced by A23187 and thapsigargin treatment. This sustained elevation of Ca^{2+} was absent with homocysteine or drug vehicle treatment (Fig. 4A). Evocation of ER-Ca^{2+} release after 5-h A23187 and thapsigargin treatments showed a complete depletion of thapsigargin-sensitive ER-Ca^{2+} stores (no Ca^{2+} increase to Ca^{2+}-free buffer/1 μM thapsigargin stimulation) (Fig. 4A). Five-hour treatment with 5 m*M* homocysteine resulted in a reduction in ER-Ca^{2+} release in comparison to the drug vehicle alone (Fig. 4A). The N-linked glycosylation inhibitor tunicamycin (20 μg/L) also showed a similar reduction in thapsigargin-evoked ER-Ca^{2+} release to that of homocysteine (5 m*M*) (Fig. 4B). The pattern of A23187 and thapsigargin treatment effects in HASMC was similar to those in T24/83 cells, characterized by the complete elimination of thapsigargin-evoked ER-Ca^{2+} release. Homocysteine (5 m*M*) treatment produced a reduction in ER-Ca^{2+} release in comparison to drug vehicle (Fig. 4C).

Effect of GRP78 overexpression on cytosolic Ca^{2+} responses

A hallmark feature of ER stress is the UPR response involving the upregulation of ER chaperones including GRP78. To determine whether the levels of GRP78 had any effect on calcium responses to the ER stress–inducing agents studied, experiments were performed in T24/83 cell lines stably overexpressing GRP78 and in pcDNA3.1 plasmid control cells. The level of GRP78 overexpression in these cells was visualized through indirect immunofluorescence in pcDNA3.1 plasmid controls and cells stably overexpressing the open reading frame of human GRP78. GRP78-overexpressing cells showed dense GRP78 staining in the paranuclear region, consistent with ER expression, and many cytoplasmic vesicles containing GRP78 in comparison to the pcDNA3.1 controls (Fig. 5A and B). These cell lines were also subjected to Western blot analysis, illustrating the overexpression of GRP78 in comparison to wild-type and pcDNA3.1 plasmid controls (Fig. 5C). GRP78 overexpression in T24/83 cells significantly reduced the cytosolic

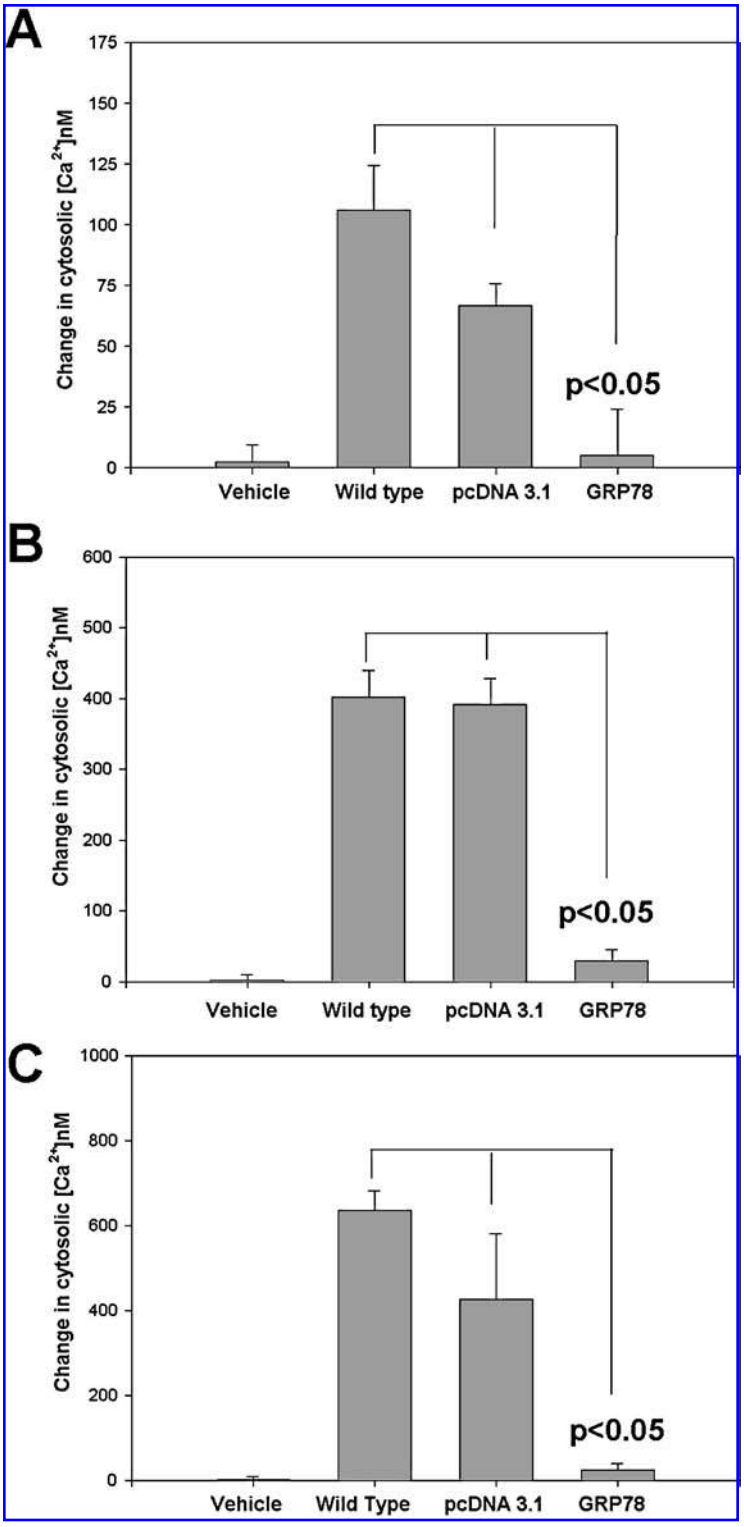

FIG. 6. Effect of GRP78 overexpression on homocysteine-induced calcium transients. Cytosolic free Ca^{2+} measurements were made on wild-type T24/83 cells or T24/83 cells stably transfected with either expression plasmid (pcDNA3.1) or expression plasmid encoding the open reading frame for human GRP78 (GRP78). GRP78 overexpressing T24/83 cells showed a significantly reduced cytosolic Ca^{2+} response ($p < 0.05$) to homocysteine (5 m*M*) (**A**), thapsigargin (1 μM) (**B**), or A23187 (10 μM) (**C**) in comparison with wild-type and pcDNA 3.1 plasmid controls.

Ca^{2+} transients measured at 2 min after stimulation evoked by 5 m*M* homocysteine in comparison with wild-type and vector-transfected controls (Fig. 6A). A similar significant reduction in cytosolic Ca^{2+} transients in response to 1 μM thapsigargin (Fig. 6B) and 10 μmol/L A23187 treatment was also found (Fig. 6C).

Effect of GRP78 overexpression on ER-Ca^{2+} release

To determine whether overexpression of GRP78 leads to ER-Ca^{2+} retention, thapsigargin-evoked ER-Ca^{2+} release was performed in Ca^{2+}-free buffer in wild-type T24/83 cells, T24/83

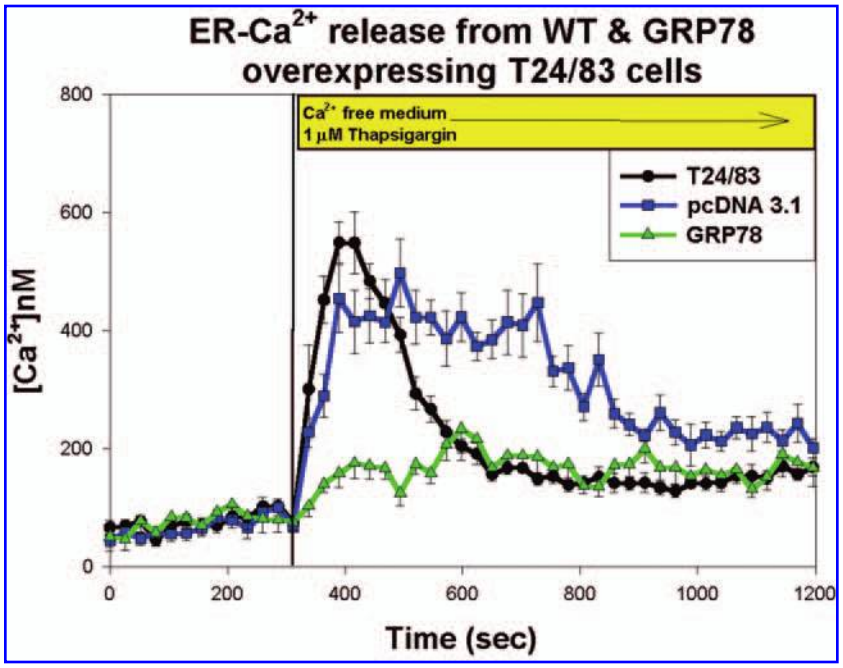

FIG. 7. Effect of GRP78 overexpression on ER-Ca^{2+} release. ER-Ca^{2+} responses were significantly reduced in T24/83 cells stably overexpressing GRP78 protein in comparison with both wild-type and pcDNA 3.1 plasmid controls. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars)

cells stably overexpressing GRP78, and pcDNA3.1 vector controls. It was determined that GRP78 overexpression in T24/83 cells led to ER-Ca^{2+} retention by diminishing thapsigargin-evoked ER-Ca^{2+} release in comparison to wild-type and pcDNA3.1 vector controls (Fig. 7).

Inhibition of GRP78 overexpression by cycloheximide pretreatment normalizes ER-Ca^{2+} release

Reduction in ER-Ca^{2+} release mediated by 5-h homocysteine or tunicamycin treatment may have resulted from the stimulation of the UPR. This in turn would lead to the upregulation of ER-Ca^{2+} binding chaperones and ER-Ca^{2+} retention. To determine whether this was indeed the case, inhibition of the UPR response was performed by 1-h cycloheximide pretreatment before homocysteine or tunicamycin ER-stress treatment. One-hour cycloheximide pretreatment reduced GRP78 expression levels in comparison with homocysteine or tunicamycin treatment alone (Fig. 8A). One-hour cycloheximide pretreatment also normalized the reduction in ER-Ca^{2+} release evoked by thapsigargin due to tunicamycin (20 μg/ml) or homocysteine (5 m*M*) treatment (Fig. 8B and C).

DISCUSSION

These results demonstrate that homocysteine has effects on intracellular calcium homeostasis. Doses of homocysteine able to produce an elevation of intracellular homocysteine in T24/83 and HASMCs also evoke cytosolic free-Ca^{2+} transients. Longer-term homocysteine treatment also reduced the Ca^{2+} response produced by thapsigargin in a Ca^{2+}-free buffer, indicating that increases in intracellular homocysteine diminished the release of Ca^{2+} from intracellular stores. These effects were also found with the Ca^{2+} ionophore A23187 and the ER-Ca^{2+} ATPase inhibitor thapsigargin. However, the effects induced by A23187 and thapsigargin were of a much greater magnitude than those evoked by homocysteine. Further, longer-term treatment with A23187 and thapsigargin produced sustained elevations of cytosolic free Ca^{2+} in a 1.5 m*M* Ca^{2+}-containing buffer (HHBSS). These sustained elevations of Ca^{2+} are referred to as capacitative calcium entry (CCE) and serve to replenish the ER with Ca^{2+} by allowing influx through the plasma membrane into the cytosol (32). These effects were not seen with long-term homocysteine treatment. This suggests that homocysteine, unlike A23187 and thapsigargin, did not produce a level of ER-Ca^{2+} depletion necessary to induce CCE. An additional ER-stress agent, tunicamycin, was tested and found not to evoke CCE but to diminish thapsigargin-induced ER-Ca^{2+} release similar to homocysteine. Because tunicamycin is an inhibitor of glycoprotein synthesis (20) and generates ER stress through the accumulation of proteins in the ER, the similarity of the cellular response to long-term treatment with homocysteine suggests that homocysteine induces ER stress through mechanisms other than ER-Ca^{2+} depletion.

Homocysteine has shown various effects on calcium handling by the cell. In rat vascular smooth muscle cells, homocysteine was found to induce intracellular calcium transients with an EC_{50} of 60 n*M* (28). We found similar effects in HASMCs, but at a greatly increased dose. At doses <100 μM homocysteine, we find virtually no acute effect of homocysteine on cytosolic Ca^{2+} levels in HASMCs or T24/83 cells. Further, at doses <100 μM homocysteine, no elevation of intracellular homocysteine was found in T24/83 and HASMCs induced by loading of homocysteine in the external medium. Homocysteine-induced cytosolic calcium transients may not require increases in intracellular homocysteine. They may be induced by redox changes resulting from homocysteine loading in the external medium,

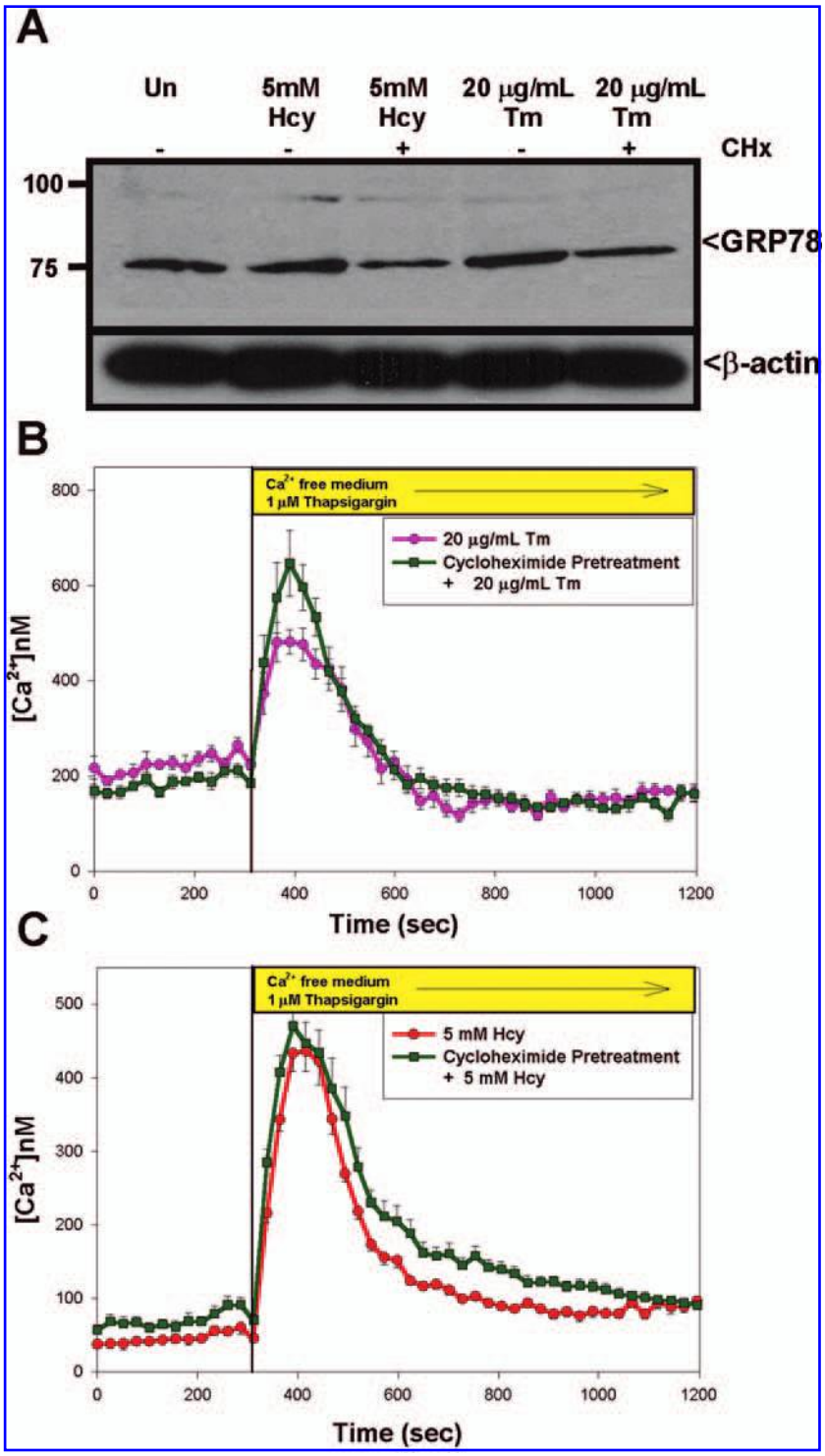

FIG. 8. Role of homocysteine-induced UPR in ER-Ca^{2+} retention. Western blot analysis demonstrated that cycloheximide (CHx) pretreatment of cells 1 h before 5-h homocysteine (Hcy) and tunicamycin (Tm) treatment prevented the overexpression of the GRP78 protein, (Un)-untreated control, β-actin used as loading control (**A**). Cycloheximide pretreatment also prevented ER-Ca^{2+} response reduction due to 5-h homocysteine (Hcy) (**B**) and tunicamycin (Tm) treatment (**C**). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars)

thereby exerting effects on resting membrane voltage potential stimulating the opening of voltage-dependent Ca^{2+} channels (13). This, however, seems unlikely, because the thiol-containing amino acid cysteine was unable to induce similar calcium transients at similar doses observed for homocysteine. Homocysteine at 10 μM in microvascular endothelial cells induced Ca^{2+} transients that were linked to the activation of a pertussis toxin–sensitive ERK1/2 signaling pathway (26). Homocysteine at 50 μM was also found to induce Ca^{2+} transients in rat mesangial cells, stimulating ERK activity (16). These results suggest a receptor-mediated mechanism for the evocation of homocysteine-induced Ca^{2+} transients and appear to be consistent with the results we have obtained in HASMCs and T24/83 cells. However, because our results indicate the stimulation of homocys-

teine-induced Ca^{2+} transients only at doses at which external homocysteine loading was capable of increasing intracellular homocysteine, homocysteine in HASMCs and T24/83 cells may be acting directly on calcium-releasing receptors on the ER membrane as opposed to the plasma membrane. Inositol 1,4,5-trisphosphate and ryanodine receptors are known to exist on the ER and lead to Ca^{2+} transients similar to those induced by homocysteine (4).

Elevations of intracellular homocysteine may have direct effects on the ER, leading to Ca^{2+} release, store depletion, or ER-Ca^{2+} retention. In ECV304 cells, extracellular homocysteine loading in the 100- to 500-μM dose range led to diminishment of thapsigargin-evoked Ca^{2+} release in Ca^{2+}-free medium and reduction in CCE (39). These results are similar to our observations in T24/83 and HASMCs. Homocysteine has also been found to have an inhibitory effect on mitochondrial calcium uptake by inhibiting mitochondrial Ca^{2+}-ATPase activity in the 500- to 1,000-μM dose range (7). If homocysteine had similar effects on the ER Ca^{2+}-ATPase, this might explain our results of diminished thapsigargin-stimulated Ca^{2+} release after long-term homocysteine treatment. However, other perturbations of the ER resulting from increases in intracellular homocysteine may be responsible for the reduction in thapsigargin-induced ER-Ca^{2+} release after long-term homocysteine treatment.

UPR activation has been shown to be induced by long-term homocysteine treatment in the dose range used in these experiments (2, 15, 30). UPR induction may have effects on ER-Ca^{2+} homeostasis, because many ER chaperones are also ER-Ca^{2+} binding proteins, including GRP78 (13). Our experiments with T24/83 cells have demonstrated that GRP78 overexpression modified calcium responses evoked by homocysteine. GRP78 overexpression reduced the calcium transients induced by homocysteine and reduced the ER-Ca^{2+} release evoked by thapsigargin. These results are consistent with the notion that GRP78 overexpression results in ER-Ca^{2+} retention. The action of other known ER-Ca^{2+} releasers, A23187 and thapsigargin, were similarly reduced by GRP78 overexpression in T24/83 cells. To determine whether these effects of GRP78 or other UPR-induced ER-Ca^{2+} binding proteins were responsible for the reduced ER-Ca^{2+} release resulting from long-term homocysteine treatment, cycloheximide pretreatment was used. Cycloheximide is a general protein synthesis inhibitor that prevents translational elongation. Thus, our cycloheximide pretreatment protocol prevented new protein synthesis during stimulation with the UPR agonists, homocysteine and tunicamycin, resulting in the prevention of UPR-induced protein upregulation, including GRP78. This cycloheximide pretreatment protocol was shown to reduce GRP78 expression in T24/83 cells subjected to long-term homocysteine treatment and to normalize thapsigargin-evoked ER-Ca^{2+} release in these cells. Cycloheximide pretreatment also normalized thapsigargin-evoked ER-Ca^{2+} release due to long-term tunicamycin treatment.

Taken together, these results suggest that homocysteine does not induce ER stress through a mechanism of ER-Ca^{2+} depletion. Its effects on ER calcium disequilibria were markedly different from those of thapsigargin and A23187, substances known to induce ER stress through ER-Ca^{2+} depletion. The effects of long-term treatment of homocysteine were similar in terms of ER-Ca^{2+} release to that of tunicamycin, an ER stress-inducing agent known to impair glycosylation without affecting ER-Ca^{2+} depletion. The effects of long-term treatment with both homocysteine and tunicamycin on ER calcium release appear to be related to their ability to induce the UPR and that of UPR-induced ER-Ca^{2+} retention.

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ABBREVIATIONS

ATPase, adenosine triphosphatase; BSA, bovine serum albumin; CCE, capacitative calcium entry; CHx, cycloheximide; Cys, cysteine; ddH2O, double distilled H_2O; EGTA, ethylene glycol tetraacetic acid; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GRP78, glucose-regulated protein, 78-kDa; HASMCs, human aortic smooth muscle cell; Hcy, homocysteine; HHBSS, HEPES-buffered Hank's balanced salt solution; PBS, phosphate-buffered saline; SDS, sodium dodecylsulfate; Thap, thapsigargin; Tm, tunicamycin; UPR, unfolded protein response.

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Address reprint requests to:
Richard C. Austin, Ph.D.
Henderson Research Centre
711 Concession Street
Hamilton, Ontario L8V 1C3, Canada

E-mail: raustin@thrombosis.hhscr.org

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