Integrated Stress Response Modulates Cellular Redox State via Induction of Cystathionine γ-Lyase

CROSS-TALK BETWEEN INTEGRATED STRESS RESPONSE AND THIOL METABOLISM*‡

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Background: The integrated stress response (ISR) maintains cellular homeostasis during aberrant protein folding (ER stress).

Results: The ISR enhances glutathione synthesis through up-regulation of cystathionine γ-lyase via the eIF2α-ATF4 pathway.

Conclusion: Cells undergoing the ISR induce cystathionine γ-lyase, thereby maintaining cellular homeostasis.

Significance: These findings link the cells response to ER stress and redox homeostasis through the ISR.

The integrated stress response mediated by eukaryotic translation initiation factor 2α (eIF2α) phosphorylation maintains cellular homeostasis under endoplasmic reticulum (ER) stress. eIF2α phosphorylation induces activating transcription factor 4 (ATF4), a basic leucine zipper transcription factor that regulates the expression of genes responsible for amino acid metabolism, cellular redox state, and anti-stress responses. Cystathionine γ-lyase (CSE) and cystathionine β-synthase are critical enzymes in the transsulfuration pathway, which also regulate cellular redox status by modulating glutathione (GSH) levels. To determine the link between the integrated stress response and the transsulfuration pathway, we used homocysteine (Hcy) as an inducer of eIF2α phosphorylation and ATF4 gene induction. Mouse embryonic fibroblasts (MEFs) lacking ATF4 (ATF4−/−) had reduced GSH levels and increased reactive oxygen species and were susceptible to apoptotic cell death under normal culture conditions. Further, ATF4−/− MEFs were more sensitive to Hcy-induced cytotoxicity and showed significantly reduced intracellular GSH levels associated with apoptosis. ATF4−/− MEFs could be rescued from 1-Hcy-induced apoptosis by β-mercaptoethanol medium supplementation that increases cysteine levels and restores GSH synthesis. ATF4−/− MEFs showed little or no CSE protein but did express cystathionine β-synthase. Further, ER stress-inducing agents, including tunicamycin and thapsigargin, induced the expression of CSE in ATF4−/−/− MEFs. Consistent with ATF4−/− MEFs, CSE−/− MEFs showed significantly greater apoptosis when treated with tunicamycin, thapsigargin, and 1-Hcy, compared with CSE−/+ MEFs. Liver and kidney GSH levels were also reduced in CSE−/− mice, suggesting that CSE is a critical factor in GSH synthesis and may act to protect the liver and kidney from a variety of conditions that cause ER stress.

Elevated levels of homocysteine (Hcy), termed hyperhomocysteinemia (HHcy), are positively associated with coronary heart disease and atherosclerosis (1, 2). This relationship has proven to be dose-dependent, independent of other risk factors, and biologically plausible (1, 2). However, prospective studies have been unable to demonstrate a consistent relationship between mild elevations of Hcy and cardiovascular risk (2). Furthermore, large randomized controlled trials in patients with elevated risk for cardiovascular disease who experienced Hcy lowering through B-vitamin and folic acid therapy showed no...
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significant reduction in their relative risk of subsequent cardiovascular events (3–5).

In these studies, Hcy plasma levels at base line before B-vitamin and folic acid therapy were in the normal to mild range. In contrast, homocysteine-lowering therapy in patients with severe homocystinuria, due to CBS deficiency resulted in a 10-fold reduction in relative risk of adverse vascular events (6). In mouse models of atherosclerosis, HHCy has been shown to enhance the atherogenic process (7–9). This proatherogenic effect, however, is dependent on other established cardiovascular risk factors, including enhanced proinflammatory response and elevation of VLDL cholesterol (10). A recently described mouse model of homocystinuria exhibits a hypercoagulative phenotype that responds to Hcy-lowering therapy with betaine (11).

Many competing theories exist to explain the pathophysiological effects of HHcy leading to cardiovascular risk. It has been demonstrated that HHcy results in endoplasmic reticulum (ER) stress and activation of the unfolded protein response (UPR) (12, 13). The addition of exogenous Hcy doses (100–500 μM) to cultured cells also leads to reactive oxygen species (ROS) generation (14). This may be the result of Hcy down-regulating the intracellular levels of antioxidant enzymes (15). Further, Hcy has been shown to modify protein structure through homocysteinylation, which could lead to protein misfolding and account for its cytotoxic effects (16).

Elevation of plasma Hcy levels accelerates atherosclerosis in mouse models through pathological processes that cause ER stress and activation of the UPR (17). Peroxynitrite, the oxidized product of nitric oxide, mediates protein nitration and co-localizes with ER stress induction in early atherosclerotic lesions (18). ER stress has also been shown to induce ROS generation, which may involve glutathione (GSH) depletion due to GSH-mediated reduction of unstable or improperly formed disulfide bonds (19). Thus, Hcy may exert its harmful effects in atherogenic lesions through ER stress-induced ROS generation and oxidation of nitric oxide to form peroxynitrite (18).

Specific components of the UPR pathway provide a prosurvival function in cells undergoing ER stress. The induction of protein folding chaperones, such as the 78-kDa glucose-regulated protein (GRP78) and the 94-kDa glucose-regulated protein (GRP94), has been shown to inhibit apoptosis (18, 20) induced by peroxynitrite (18, 20). Further, UPR activation through the protein kinase R-like ER kinase (PERK) pathway leads to the phosphorylation of eukaryotic translation initiation factor 2α (eIF2α), which in turn inhibits general translation initiation and reduces the cellular burden of unfolded proteins. However, activating transcription factor 4 (ATF4) is selectively translated (21). ATF4 increases the expression of a number of genes that modulate amino acid metabolism and transport, cellular redox status, and UPR activation as well as CHOP/ GADD153 expression (21). In addition to PERK, other kinases, namely GCN2 (general control non-repressed 2), HRI (heme-regulated eIF2α kinase), and protein kinase R, have also been shown to induce eIF2α phosphorylation and the preferential translation of ATF4. This pathway is referred to as the integrated stress response (ISR) because it maintains the balance between restoration of cellular homeostasis under physiologi- 

It was recently reported that ATF4-deficient (ATF4−/−) mouse embryonic fibroblasts (MEFs) are sensitive to oxidative stress and require amino acid supplementation and reducing equivalents added to the medium to survive (23). Hcy treatment has been shown to induce ER stress in both wild type (ATF4+/+) and ATF4−/− MEFs (24, 25). Because ER stress results in oxidative stress (26) and Hcy has been shown to induce oxidative stress directly (14), we hypothesized that ATF4 acts as a prosurvival factor by enhancing the cellular capacity for GSH synthesis via the transsulfuration pathway.

In this report, we show that ATF4−/− MEFs displayed increased apoptosis in a condition of excess ROS generation. These cells also demonstrated GSH depletion that could be rescued by high dose amino acid supplementation necessary for the synthesis of GSH (glycine, L-glutamic acid, and L-cysteine). Increased GSH levels using this supplementation also inhibited apoptotic cell death. The sensitivity of these ATF4+/− MEFs to GSH depletion appears to involve impairment in the transsulfuration pathway for L-cysteine synthesis due to very low or absent CSE expression. Cells in this state showed increased sensitivity to GSH depletion by Hcy treatment, resulting in greater apoptotic cell death. These findings imply that ATF4 can modulate intracellular levels of GSH by altering the expression of genes in the transsulfuration pathway. In support of the concept that CSE expression is a critical factor for GSH synthesis, CSE-deficient (CSE−/−) mice demonstrated down-regulation of GSH levels in liver and kidney. Moreover, CSE−/− MEFs are more sensitive to both Hcy and ER stress-induced apoptosis caused by thapsigargin (Tg) and tunicamycin (Tm). Taken together, these findings suggest that ATF4-dependent expression of CSE is an important cellular process that maintains cellular redox status during ER stress via the transsulfuration pathway.

EXPERIMENTAL PROCEDURES

Animal Studies—Wild type (CSE+/+) and CSE-deficient (CSE−/−) mice were used for kidney and liver studies (27). Experiments were conducted in compliance with the Care and Use of Laboratory Animals guidelines and approved by the Animal Care Committee of Lakehead University, Thunder Bay, Canada. All animals were housed in a controlled environment with unlimited access to food and water on a 12-h light/dark cycle. Mice were fed a standard rodent chow diet (Rodent RQ 22-5, Zelgler Bros Inc.). Livers and kidneys were extracted and snap frozen in liquid nitrogen. Male CSE+/+ and CSE−/− mice (n = 5) were used for the determination of kidney and liver GSH levels at 12–14 weeks of age. MEFs were derived from CSE+/+ and CSE−/− mice by methods previously described (24). GSH levels were determined using high performance liquid chromatography (HPLC), as described previously (28).

Cell Culture—MEFs were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 4.5 g/liter D-glucose and L-glutamine (Invitrogen), 10% FBS (Sigma), and 1 × penicillin/streptomycin antibiotic (Invitrogen). The ATF4−/− MEFs and the CSE−/− MEFs were cultured in the base medium supplemented with 1 × non-essential amino acids (NEAA) containing the amino acids (glycine, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline, and L-serine) (Invitrogen) and
55 μM β-mercaptoethanol (β-Mer) (Invitrogen) (DM+). Supplementation provided additional amino acids that could not be synthesized by ATF4−/− MEFs as well as reducing equivalents to counter oxidative stress, as first described by Harding et al. (23). For experimentation, this supplementation was removed to allow comparison with ATF4+/+ MEFs for periods of time from 2 to 48 h.

**Western Blot Analysis of Unfolded Protein Response in ATF4−/− MEFs**—Total protein lysates were solubilized in SDS-PAGE sample buffer, separated on 10% SDS-polyacrylamide gels under reducing conditions, and transferred to nitrocellulose membranes (Bio-Rad). Primary antibodies to phospho-eIF2α (9721, Cell Signaling), CHOP/GADD153 (sc-7351, Santa Cruz Biotechnology, Inc. (Santa Cruz, CA)), and ATF4 (sc-200, Santa Cruz Biotechnology, Inc.) were recognized with the appropriate horseradish peroxidase-conjugated secondary antibody (DAKO). Membranes were developed using the Renaissance Western blot chemiluminescent reagent as described in our previous work (18). Blots were probed for β-actin to normalize for protein loading. Densitometry was conducted using ImageJ software (National Institutes of Health, Bethesda, MD).

**Protein Gel Electrophoresis and Quantitative Western Blotting for CBS and CSE from MEFs**—Cell pellets were resuspended in lysis buffer containing 100 mM KPi, pH 7.4, 1 mM EDTA, and 1:100 (v/v) protease inhibitor mixture (Sigma). Cells were disrupted by sonication, and the cell debris was removed by centrifugation at 20,000 × g for 20 min. Protein concentration of the total cell lysates was determined by the Bradford method using bovine serum albumin as a standard (29). Following heat denaturation, 120 μg of the protein lysates were separated by SDS-PAGE using a 9% separating gel with a 4% stacking gel under reducing conditions (30). Proteins were transferred onto PVDF membrane using a semidry transfer cell (Bio-Rad). Resulting blots were probed with primary antibodies to CBS (H00000875-A02, Abnova), CSE (H0001491-M02, Abnova), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (G9545, Sigma). Signals were detected using a Typhoon 9400 imager (Amersham Biosciences) after incubation with the appropriate fluorescein- or Texas Red-conjugated secondary antibodies (Vector Laboratories) or Alexa Fluor 647-conjugated secondary antibody (Invitrogen). The relative intensities of protein bands were quantified using Quantity One version 4.6.5 software (Bio-Rad).

**Cell Death Assays**—The Cytotoxicity Detection Kit (Roche Applied Science) was used to measure MEF cell death by detecting lactate dehydrogenase (LDH) enzyme activity in the culture medium. Cells were seeded into 6-well plates (1.0 × 10⁶ cells per ml) with DMEM containing 1% FBS and 1 × antibiotics. CSE−/− or CSE+/− MEFs were treated with tunicamycin (Tm; 2 μg/ml, 5 μg/ml) or thapsigargin (Tg; 400 nM, 1 μM) for 24 or 48 h. LDH was also measured in ATF4−/− and ATF4+/− MEFs as well as ATF4−/− supplemented with 55 μM β-Mer and 1 × NEAA. LDH activity was measured at 492 nm using a VERSAmax microplate reader (Molecular Devices) where cytotoxicity (%) = ((experimental value − low control)/(high control − low control)) × 100%. A terminal deoxynucleotidyltransferase biotin-dUTP nick end labeling (TUNEL) assay (Roche Applied Science) was used as a measure of apoptosis in ATF4−/− and ATF4+/− MEFs with or without l-Hcy (0, 100 μM, 1 mM) treatment for 48 h in the presence or absence of 1 × NEAA and 55 μM β-Mer. l-Hcy was prepared as described previously (24). Further, CSE−/− and CSE+/− MEFs were treated with Tg (1 μM), Tm (5 μg/ml), or l-Hcy (100 μM, 1 mM) to calculate the percentage of TUNEL-positive cells. DNase I digestion (100 units) served as positive control for TUNEL staining, and the TdT enzyme was omitted as a negative control.

**Reactive Oxygen Species Measurement**—5- (and -6)-Carboxy-2′,7′-dichlorodihydrofluorescein diacetate (Molecular Probes) was used to measure ROS in MEFs at the 48 h time point. 5- (and -6)-Carboxy-2′,7′-dichlorodihydrofluorescein diacetate measurements were made after loading cells with the dye for 30 min at 37 °C in HEPES-buffered Hanks’ buffered salt solution with or without the addition of 1 × NEAA and 55 μM β-Mer. Methods were adapted from Dickhout et al. (31).

**GSH Measurement**—GSH was measured either with the GSH-400 assay kit (Oxis Research) according to manufacturer’s instructions or via HPLC, as described previously (32). Briefly, adherent MEFs were washed in PBS and removed from the plates with 1 × trypsin/EDTA (Invitrogen). Cells were placed on ice and spun at 1000 × g at 4 °C, and the cell pellet was lysed in ice-cold metaphosphoric acid. Lysates were subjected to the colorimetric assay procedure, and reaction product absorbance was read at 400 nm on a VERSAmax microplate reader (Molecular Devices). Absorbance values were fit to a standard curve for calculation of GSH concentration and normalized to cell number as described previously (33). Cells were treated with l-Hcy or propargylglycine (PPG) as an inhibitor of CSE. Further, in liver and kidney, GSH was measured using HPLC, and a similar procedure was followed, where GSH and GSSG concentration were measured from cell lysates, as described previously (32). The results were normalized to the protein concentration in each sample.

**Statistical Analysis**—Values are expressed as means ± S.E. Comparisons of the means of treatment groups were performed by Student’s unpaired t test. Significance was recognized at the 95% level.

**RESULTS**

**Role of ATF4 in Unfolded Protein Response and Hcy Induction of Integrated Stress Response**—In order to define our experimental system, we investigated the general effects of ATF4 deletion upon the induction of the UPR in ATF4−/− MEFs. Lack of ATF4 was demonstrated in ATF4−/− MEFs in the non-treated state as well as with ER stress induction by Tm (5 μg/ml) or Tg (1 μM) at 4 and 6 h (Fig. 1A). ATF4 was, however, up-regulated in ATF4−/− MEFs in response to the ER stressors (Fig. 1A). The ability of ATF4 to induce CHOP/GADD153 under conditions of ER stress was examined in ATF4−/− and ATF4+/− MEFs (Fig. 1, B and C). Experiments with ATF4−/− MEFs demonstrated that ER stress-induced UPR activation with Tg (400 nM) resulted in phosphorylation of eIF2α (upstream of ATF4) comparable with that of ATF4+/− cells. However, CHOP/GADD153, a downstream target of ATF4, showed either no induction (Tg, 400 nM; Fig. 1B) or a highly blunted response (Tm, 2 μg/ml; Fig. 1C) to ER stress. The UPR-mediated up-regulation of the ER luminal chaperone GRP78
showed a normal response following treatment of MEFs with Tm (2 μg/ml) (Fig. 1C). ATF4+/− and ATF4−/− MEFs were used to determine if Hcy treatment would induce the ISR, as shown by eIF2α phosphorylation. ISR induction was demonstrated in both ATF4+/− and ATF4−/− MEFs after treatment with 100 μM and 1 mM L-Hcy for 4 h. It appeared that eIF2α phosphorylation was enhanced in ATF4−/− MEFs, in response to all treatments (Fig. 1, D and E).

**Effect of ATF4 Deficiency on GSH Synthesis and Cell Death**—GSH is a tripeptide synthesized from the amino acids glycine, l-glutamic acid, and l-cysteine that plays a crucial role in maintaining a reducing environment in the cytoplasm of the cell. To determine the role of ATF4 in GSH synthesis and the maintenance of a reducing environment in the cytoplasm, intracellular GSH levels in ATF4+/− MEFs were measured using the GSH-400 assay kit (Fig. 2). GSH levels were significantly reduced in the ATF4−/− MEFs in comparison with ATF4+/− MEFs when grown for 48 h in the absence of NEAA and β-Mer supplementation (Fig. 2A). These reduced levels of GSH in ATF4−/− MEFs were significantly restored via supplementation with DM++ (Fig. 2B). ATF4+/− or ATF4−/− MEFs grown in the supplemented DMEM (DM++) showed no significant difference in the measured levels of intracellular GSH (Fig. 2C). Further, DMEM supplementation normalized cell growth in ATF4−/− MEFs (data not shown). An LDH release assay was used to assess the influence of DM++ supplementation on cell death at 24 h (Fig. 2D). Cell death was significantly increased in the ATF4−/− cells with reduced GSH levels, compared with ATF4+/− cells. Furthermore, supplementation of the medium with DM++, which increases GSH levels (Fig. 2B), decreased cell death to levels observed in the ATF4+/− MEFs (Fig. 2D). Using an HPLC method that distinguishes between oxidized and reduced forms of GSH, the decrease in GSH levels observed in ATF4−/− cells was not found to be due to oxidation of GSH to GSSG. ATF4−/− MEFs showed no significant change in GSSG levels in comparison with the ATF4+/− cells (Table 1). Taken together, these results demonstrate the importance of GSH synthesis in the prosurvival function of ATF4 in the ISR.

**ATF4 Influence on Apoptotic Cell Death**—Cell death and apoptosis assays were used to determine if ATF4−/− decreases...
TABLE 1
Loss of ATF4 expression decreases GSH levels but does not increase oxidation of GSH to GSSG

|          | GSH  | GSSG |
|----------|------|------|
| DM       | 123.8 ± 8.13 | 1.15 ± 0.7 |
| ATF4−/−  | 3.9 ± 0.44   | 0.05 ± 0.0 | 5.09 ± 2.32 |
| p value  | < 0.0001 | p = 0.09  | p = 0.08       |
| DM++     | 80.79 ± 19.4 | 0.03 ± 0.37 |
| p value  | p = 0.044 | p = 0.004  | p = 0.037       |

ATF4 Deficiency Increases Reactive Oxygen Species Generation—Because redox signaling has been shown to be a factor in the induction of apoptosis (34), we investigated whether the loss of the ATF4 gene was associated with increased production of ROS. ROS production, as measured by H$_2$DCF, was significantly elevated in ATF4−/− MEs, whereas supplementation of DMEM with DM++ significantly reduced H$_2$DCF signal in both ATF4+/+ and ATF4−/− MEs (Fig. 3D). These findings demonstrate that loss of the ATF4 gene leads to an increase in ROS generation, as shown previously by Harding et al. (23).

Effect of ATF4 Deficiency on Transsulfuration Pathway and GSH Biosynthesis—Previous studies have suggested that significant levels of GSH can be generated via the transsulfuration pathway (35–37). To investigate whether the impaired conversion of Hcy to cysteine through the transsulfuration pathway (35–37). 

![Figure 2](image_url)

**FIGURE 2. Effect of ATF4 deficiency on intracellular glutathione levels in MEs.** A, intracellular GSH levels were measured using the GSH-400 assay kit and were found to be significantly reduced (*, p < 0.05) in ATF4−/− MEs, compared with ATF4+/+ MEs when grown in DMEM containing 10% FBS and 1× penicillin/streptomycin antibiotics. B, intracellular GSH levels were measured as in A. GSH levels in ATF4+/+ MEs were significantly increased in DM++ for 48 h (*, p < 0.05). C, ATF4+/+ and ATF4−/− MEs grown in DMEM containing 10% FBS and 1× antibiotics with DM++ for 48 h resulted in no significant difference in the measured levels of intracellular GSH. GSH levels were measured as in A. D, LDH release assay showed significantly increased cell death in ATF4−/− MEs versus ATF4+/+ MEs 24 h after removal of DM++ supplementation (*, p < 0.05). This increase in cell death in the ATF4−/− cells was significantly decreased by the addition of DM++ supplementation (**, p < 0.05). Error bars, S.E.
pathway was responsible for the reduced levels of GSH found in the ATF4−/− MEFs, Western blot analysis was used to assess the levels of CBS and CSE, critical enzymes in the transsulfuration pathway. Given that CBS activity is regulated by proliferation (38, 39), we determined CBS and CSE protein levels in both actively dividing (70% confluence) and quiescent cells (100% confluence). Protein levels of CBS, the first enzyme in the transsulfuration pathway that condenses serine and Hcy to form cystathionine, were higher in the ATF4+/− MEFs, compared with ATF4+/− MEFs (Fig. 4A). Densitometry analysis of the Western blots showed a significant increase in the relative amount of CBS protein in the ATF4+/− cells grown to 70 and 100% confluence (Fig. 4B). In contrast to CBS, protein levels of CSE, which converts cystathionine to cysteine, were undetectable in the ATF4+/− MEFs (Fig. 4A). Using the GSH-400 assay, data from the ATF4+/− cells showed that inhibition of CSE with 2.5 mM PPG resulted in a significant reduction of intracellular GSH levels in ATF4+/− MEFs (Fig. 4C). This is consistent with our finding that ATF4−/− MEFs have reduced CSE expression levels.

**ATF4 Deficiency Decreases GSH Synthesis and Increases Apoptosis in MEFs Treated with Hcy**—The transport of extracellular cystine across the plasma membrane leads to the reduction and generation of cysteine. This transport can be accomplished by the cystine/glutamate antiporter system (xCT) (40). We have demonstrated that xCT protein expression is reduced in ATF4+/− MEFs (supplemental Fig. 1). The reduction in the xCT protein expression levels found in the ATF4+/− MEFs may also contribute to the reduced GSH synthesis demonstrated in this cell type. These results are consistent with previous findings showing that xCT expression is regulated by ATF4 through its promoter (40). Hcy treatment may lead to the generation of ROS directly or through the induction of ER stress. To counteract this effect, ATF4 up-regulation via eIF2α phosphorylation and activation of the transsulfuration pathway may provide the necessary reducing equivalents through increased GSH synthesis. In this context, l-Hcy would act as a substrate for both l-cysteine and GSH synthesis through the transsulfuration pathw
result, cells would be susceptible to Hcy-induced apoptosis because intracellular GSH levels would become limited. To determine if this was indeed the case, ATF4+/+ and ATF4−/− MEFs without DM++ supplementation were grown for 24 h and treated with 0, 100 μM, or 1 mM l-Hcy. Intracellular GSH levels, as measured by the GSH-400 assay, were found to be significantly lower at this time without l-Hcy treatment (0 l-Hcy) (Fig. 5A). GSH levels were significantly reduced in ATF4−/− MEFs with 100 μM l-Hcy treatment, whereas ATF4+/+ MEFs were unaffected by this dose (Fig. 5A). However, 1 mM l-Hcy treatment also reduced the levels of GSH in both ATF4+/+ and ATF4−/− MEFs (Fig. 5A). The effect of l-Hcy treatment on apoptosis was determined after 48 h. The TUNEL assay showed a significantly increased percentage of TUNEL-positive cells in ATF4−/− MEFs over ATF4+/+ controls treated with 100 μM or 1 mM l-Hcy (Fig. 5B). Furthermore, treatment of ATF4−/− MEFs with 1 mM l-Hcy both decreased GSH levels and increased apoptotic cell death. Supplementation of ATF4−/− MEFs with DM++ significantly reduced the percentage of TUNEL-positive cells induced by Hcy (Fig. 5B). ATF4+/+ cells where supplementation (DM++) was maintained did not show any significant difference in the percentage of TUNEL-positive cells, compared with ATF4−/− cells where supplementation was withdrawn (data not shown).

CSE Is Protective against ER Stress-induced Apoptosis and Helps Maintain GSH Synthesis—ATF4+/+ MEFs treated with ER stress inducers Tm (5 μg/ml) and Tg (1 μM) for 18 h showed significant increases in CSE protein content, demonstrating that CSE is an ER stress-inducible gene (Fig. 6). To determine if the loss of CSE protein expression, as seen in ATF4−/− MEFs, would result in GSH depletion, also observed in the ATF4−/− MEFs, CSE−/− MEFs were derived from CSE-deficient embryos (27). As expected, immunoblot analysis showed a complete absence of CSE protein in CSE−/− MEFs, compared with CSE+/+ MEFs (Fig. 7A). To determine whether MEFs lacking CSE were more sensitive to ER stress-induced apoptosis, LDH and TUNEL assays were performed. LDH analysis showed that both Tm (5 μg/ml) and Tg (1 μM) led to significant increases in cytotoxicity in CSE−/− MEFs, compared with the CSE+/+ MEFs (Fig. 7, B and C). To determine if cell death

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**FIGURE 4. Effect of ATF4 deficiency on the expression of enzymes in the transulfuration pathway.** A, multiple samples of untreated ATF4+/+ (n = 3) and ATF4−/− (n = 4) MEFs were subject to Western blotting analysis. Increased levels of CBS protein were observed in ATF4−/− MEFs, compared with ATF4+/+ MEFs at 100% confluence. In contrast, levels of CSE protein were reduced in ATF4−/− MEFs when compared with ATF4+/+ MEFs at 100% confluence. Blots were probed for GAPDH as a control for protein loading. B, densitometry showed a significant increase in the relative amounts of CBS protein in ATF4−/− MEFs when compared with ATF4+/+ cells (*, p < 0.01) at both 70 and 100% confluence. C, GSH was measured using the GSH-400 assay kit. Inhibition of CSE activity with 2.5 mM proparglyglycine (+ 2.5 mM PPG) for 48 h resulted in a significant reduction of intracellular GSH levels (*, p < 0.05) in ATF4−/− MEFs in comparison with untreated control MEFs. Error bars, S.E.

**FIGURE 5. Homocysteine treatment reduces intracellular GSH synthesis and increases apoptosis in ATF4−/− MEFs.** A, GSH was measured using the GSH-400 assay kit. ATF4+/+ and ATF4−/− MEFs were grown without DM++ supplementation for 24 h to determine the response to l-Hcy treatment in intracellular GSH levels. GSH levels were significantly reduced initially in ATF4−/− without l-Hcy dosing (0 l-Hcy). Treatment with 100 μM l-Hcy did not reduce the levels of GSH in ATF4+/+ MEFs, whereas GSH is significantly reduced (~2-fold) in ATF4−/− MEFs (*, p < 0.05). Treatment with 1 mM l-Hcy reduced GSH levels in both ATF4+/+ and ATF4−/− MEFs. B, the effect of 100 μM and 1 mM l-Hcy treatment on apoptotic cell death was determined after 48 h, indicating a significant increase in the percentage of TUNEL-positive cells in ATF4−/− MEFs in comparison with ATF4+/+ MEFs at all l-Hcy doses (*, p < 0.05). Supplementation of ATF4−/− MEFs with DM++ resulted in significant reductions of the percentage of TUNEL-positive cells in comparison with unsupplemented ATF4−/− MEFs (p < 0.05). Further, supplemented ATF4−/− MEFs showed a significantly lesser percentage of TUNEL-positive cells than wild type controls following treatment with 1 mM l-Hcy (&, p < 0.05). Error bars, S.E.
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![Graph showing ER Stress Pathway Modulation](image)

**FIGURE 6.** CSE is an ER stress-inducible gene. Multiple samples (n = 3) of untreated (NT) or treated ATF4<sup>−/−</sup> MEFs were subject to Western blot analysis. CSE protein expression was significantly increased (*, p < 0.05) in cells treated with the ER stress inducer Tm or Tg for 18 h. Western blots were probed for β-actin as a control for protein loading. Error bars, S.E.

resulted from apoptosis, the TUNEL assay was used to examine the cells under the same treatment conditions. CSE<sup>−/−</sup> MEFs treated with both Tm (5 μg/ml) and Tg (1 μM) for 48 h displayed a significantly higher percentage of TUNEL positive cells, compared with CSE<sup>+/+</sup> controls (Fig. 7, D and E), indicating that MEFs deficient in CSE are more sensitive to ER stress-induced apoptosis.

To determine whether CSE deficiency increases sensitivity to l-Hcy-induced apoptosis, TUNEL analysis was performed on MEFs treated with l-Hcy (100 μM and 1 mM) for 48 h. TUNEL analysis showed that l-Hcy (100 μM and 1 mM) caused significantly more apoptosis in CSE<sup>−/−</sup> MEFs, compared with the CSE<sup>+/+</sup> controls.

Previous studies in multiple mouse models of severely elevated Hcy have shown that the liver and kidney actively synthesize cysteine through the transsulfuration pathway and incur significantly decreased levels of cysteine and GSH in its absence. Other tissues, such as heart and muscle, did not show any significant depletion in these metabolites (36, 41). Based on these findings, we investigated the role of CSE in GSH synthesis in the liver and kidney. Using HPLC to measure reduced GSH levels, samples of kidney and liver from CSE<sup>−/−</sup> mice were examined. Both livers and kidneys of CSE<sup>−/−</sup> mice displayed significant lower reduced GSH levels, demonstrating the importance of this enzyme in maintaining GSH synthesis in these organs (Fig. 8).

**DISCUSSION**

Previous work has demonstrated that activation of the UPR by ER stress is considered to be a pro-survival response (18, 20, 42, 43). Specifically, the up-regulation of ER chaperones has been shown to mediate this prosurvival function (20). This has been demonstrated by the up-regulation of GRP78 in vascular endothelium (18). eIF2α phosphorylation and activation of the ISR (23) have also been shown to promote resistance to cellular stresses (43).

In this study, we demonstrate the prosurvival function of the ISR response gene ATF4 through its role in the regulation of de novo GSH synthesis via the induction of CSE and the transsulfuration pathway. Studies on the regulation of the CBS promoter regions have indicated that expression of the CBS gene is not induced by Hcy but can be up-regulated by depleting glutathione using buthionine sulfoximine. This finding provides a possible explanation for why CBS expression is induced in the ATF4<sup>−/−</sup> MEFs, whereas CSE is clearly repressed and glutathione is significantly depleted.

GSH is a key intracellular reductant and can reduce cell injury caused by oxidative stress. In MEFs, cysteine synthesis through CSE played a key role in maintaining intracellular GSH levels. Disruption of the ATF4 gene resulted in a critical down-regulation of CSE protein, thereby leading to diminished intracellular levels of GSH and a corresponding increase in ROS production. These findings confirm and extend the previous work of Harding et al. in ATF4<sup>−/−</sup> MEFs (23). We found that ATF4-deficient MEFs were more sensitive to Hcy-induced cytotoxicity, resulting in apoptotic cell death. Similarly, in cultured human astrocytes, CSE inhibition via PPG reduces cell viability under oxidizing conditions (37). Prolonged or severe ER stress has been shown to result in apoptosis (44, 45). Moreover, the generation of ROS due to ER stress may be another mediating factor in the induction of the apoptotic pathway. ER stress itself has been shown to produce ROS in cells through the accumulation of misfolded proteins (26). In support of these findings, ATF4<sup>−/−</sup> MEFs were much more sensitive to Hcy-induced apoptotic cell death than ATF4<sup>+/+</sup> controls and displayed diminished GSH levels. ATF4 may play a critical role in combating ER stress-induced oxidative stress through increased GSH synthesis. GSH has been shown to be one of the major intracellular scavenging systems, limiting ROS-induced cell death via the mitochondrially mediated caspase-9 activation pathway (46). Our findings demonstrate that in Hcy-induced cytotoxicity of ATF4<sup>−/−</sup> MEFs, GSH levels are significantly decreased, and apoptotic cell death ensues.

GSH depletion may be the consequence of ROS generation induced by a cytotoxic challenge, such as Hcy treatment with ATF4<sup>−/−</sup> MEFs lacking the ability to maintain GSH levels. Further, partially preventing the UPR, due to ATF4 knock-out, may lead to a greater accumulation of unfolded proteins. This condition would consume GSH, causing its oxidation to GSSG to reduce improperly paired disulfide bonds within misfolded proteins (26). As such, even reduction of oxidative stress by the DM<sup>++</sup> supplementation may not decrease the level of GSSG to fully reflect the non-oxidative conditions in the cell. Our data appear to show this phenomenon because GSSG concentrations were elevated in ATF4<sup>−/−</sup> MEFs despite DM<sup>++</sup> supplementation, which reduced H<sub>2</sub>DCF measured oxidative stress below levels in the un-supplemented ATF4<sup>++</sup> cells.

ER stress-induced apoptosis may result from the up-regulation of pro-apoptotic genes, such as CHOP/GADD153, or from the accumulation of ROS generated by ER stress. CHOP/GADD153 expression is reduced in ATF4<sup>−/−</sup> MEFs, indicating that oxidative stress may be the main proapoptotic signal. In this case, ATF4<sup>−/−</sup> MEFs should undergo apoptosis in non-ER stress conditions, because ROS are elevated and GSH synthesis is significantly reduced. We observed in ATF4<sup>−/−</sup> MEFs

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5 K. Maclean, unpublished data.
increased apoptosis under normal growth conditions. This implies a general effect of ATF4 deficiency on cellular viability rather than merely in conditions of ER stress. Further, the doses of L-Hcy used in this study (100 μM to 1 mM) are similar to levels observed in the blood of patients with homocystinuria (50–300 μM) (47). Our results indicate that the ISR, through the action of ATF4, may be a critical factor in protecting cells and tissues from Hcy toxicity.

Cysteine synthesis or bioavailability is the main rate-limiting factor in the synthesis of GSH. ATF4−/− MEFs displayed increased eIF2α phosphorylation as a response to amino acid deprivation (23). ATF4 is a central downstream regulator of the eIF2α kinase stress response, and the protein levels of ATF4 are rapidly increased in response to ER stress through translational control (48). It has been reported that several genes involved in the metabolism of sulfur-containing amino acids were

FIGURE 7. CSE provides protection against ER stress-induced apoptosis. A, Western blot analysis demonstrated a lack of CSE immunostaining in the total cell lysates from CSE-deficient (CSE−/−) MEFs. B, LDH release assays were used to measure cell cytotoxicity in response to ER stress in both CSE+/+ and CSE−/− MEFs. In response to Tm treatment (5 μg/ml), there was a significant increase in cell death in the CSE−/− cells, compared with the CSE+/+ cells (*, p < 0.05). C, Tg (1 μM) showed a similar increase in cell cytotoxicity in the CSE−/− MEFs, compared with the CSE+/+ cells (*, p < 0.05). D, TUNEL assay was used to measure cell apoptosis induced by ER stress after 48 h in CSE−/− and CSE+/+ MEFs. In response to Tm treatment (5 μg/ml), there was a significant increase in apoptosis in both the CSE−/− and CSE+/+ MEFs (*, p < 0.05); however, the increase was significantly greater in the CSE−/− cells compared with the wild type control (#, p < 0.05). E, Tg (1 μM) showed a similar increase in apoptosis in the CSE−/− MEFs compared with CSE+/+ cells (*, p < 0.05). However, there was a significantly greater degree of apoptosis in CSE−/− cells compared with the CSE+/+ MEFs at both doses of L-Hcy (#, p < 0.05). Error bars, S.E.
ER Stress Pathway Modulates Cellular Redox State

FIGURE 8. Effect of CSE deficiency on kidney and liver glutathione levels. Levels of reduced GSH in the liver and kidney were measured using HPLC. Both liver and kidney homogenates prepared from CSE knock-out mice displayed a significant decrease in reduced GSH levels, compared with wild type controls (*, p < 0.05). Error bars, S.E.

repressed in ATF4−/− MEFs, including the glycine transporter, which supports the synthesis of GSH; methylenetetrahydrofolate dehydrogenase, an important enzyme in embryonic glycine synthesis (49); and CSE, the final enzyme for the synthesis of cysteine in the transsulfuration pathway (23). Further, the expression of the light chain, xCT, of the cystine/glutamate antiporter system mediating cystine transport into the cell was found to be down-regulated (supplemental Fig. 1) as bands for xCT at 55 and 35 kDa were reduced in ATF4−/− MEFs. In, ATF4−/− MEFs, a new immunoreactive band at 30 kDa appears, which may be a degradation product of xCT. These results are consistent with our finding that supplementation with β-Mer, which reacts with l-cystine incorporated in the DMEM to produce l-cysteine (50), and NEAA containing glycine and l-glutamic acid rescues ATF4−/− MEFs. These three amino acids (glycine, l-glutamic acid, and l-cysteine) that are required for GSH synthesis are also necessary for ATF4−/− MEF survival. Hcy supplementation was not able to replace β-Mer supplementation and resulted in apoptotic cell death. This effect is indicative of the block in the transsulfuration pathway due to very low CSE expression, which would prevent l-cysteine synthesis from Hcy. In contrast, high dose l-cysteine supplementation (2 mM) could substitute for the reductant, β-Mer, normalizing growth in ATF4−/− MEFs (data not shown). As reported previously, both NEAA and a reductant were required to normalize growth in ATF4−/− MEFs; when NEAA or a reductant were used separately, cell growth was improved but not normalized (23).

As we have demonstrated, ATF4 modulates the expression of CHOP/GADD153. CHOP/GADD153 has several proapoptotic functions in the UPR. However, under hypoxic conditions, it has been shown that the prosurvival influence of the PERK pathway, including the actions of ATF4, can override the proapoptotic functions of CHOP/GADD153 (51). In our experiments, ATF4−/− cells showed dramatically reduced levels of CHOP/GADD153 in response to eIF2α phosphorylation; however, these cells still experienced higher levels of apoptotic cell death when exposed to Hcy. The deleterious effects of ablating ATF4 appear to outweigh the possible survival advantage of the resultant blocking of CHOP/GADD153 in MEFs. Further, the PERK/eIF2α/ATF4 arm of the UPR has been shown to have a prosurvival function during hypoxia in tumor cells (52). This is consistent with our results in MEFs. ATF4 also regulates the dephosphorylation of eIF2α through increased transcriptional activation of GADD34 (48). This is a critical step in the resumption of protein synthesis following ER stress. Conversely, ATF4−/− neurons were protected from oxidative stress-induced cell death, stimulated by the glutamate analog HCA (53). The mechanism is unclear but may involve ATF4-mediated CHOP/GADD153 activation.

Our finding that CSE is induced by ER stress is in agreement with microarray analysis data, which show that CSE is up-regulated by Tm and Tg (54). CSE expression appears to be a critical component of the cytoprotective ATF4 transcriptional response. Our novel finding that CSE deficiency leads to a greater sensitivity to apoptosis induced by ER stressors as well as l-Hcy indicates the importance of GSH up-regulation through the transsulfuration pathway to promote cell survival. Accumulation of unfolded proteins in the ER is capable of generating ROS (26). We have shown that the PERK-eIF2α/ATF4 pathway, through the regulation of CSE, provides protection against oxidative stress and GSH depletion caused by protein misfolding.

CSE deficiency in humans leads to cystathioninuria (55–58), which has been linked to elevated plasma homocysteine levels (58). In mice, CSE deficiency resulted in decreased GSH levels in the aorta and in the mesenteric artery beds (27). Further characterization of these CSE-deficient mice showed that they were hyperhomocysteinemic and displayed systemic vulnerability to oxidative injury, and hepatocytes derived from these mice required cystine supplementation to survive (59). This is similar to the results we obtained in CSE−/− MEFs, which showed reduced growth in comparison with CSE+/+ MEFs that was improved by DM supplementation (data not shown). As well, CSE-deficient MEFs displayed greater sensitivity to apoptosis induced by ER stress agents and l-Hcy, which have been shown to cause oxidative injury (19, 26).

It has been shown that half of the intracellular GSH pool in human liver cells is derived from the transsulfuration pathway through CSE (35). The transsulfuration pathway is responsible for cysteine synthesis, the rate-limiting amino acid in GSH synthesis (60). CSE is expressed in a tissue-specific manner and is found mainly in liver and kidney, where the transsulfuration pathway plays an important role in GSH production (61). The other important enzyme in the transsulfuration pathway, CBS, is also expressed in liver, kidney, and brain (36). mRNA levels, protein levels, and activity of CSE were significantly reduced in both the liver and kidney of the CSE-deficient mouse (59). CSE levels in the liver and kidney increase into adulthood (61). Furthermore, CSE expression and activity increase dramatically with the fetal-to-neonatal transition, and moderate oxidative stress may be a signal to up-regulate CSE expression during that transition (62).

In conclusion, ATF4 deletion led to a disruption of GSH synthesis, resulting in the generation of ROS in cultured MEFs. l-Hcy treatment resulted in increased apoptosis when the ISR was disrupted by ATF4 deletion. This increased apoptosis due to l-Hcy treatment was found to occur by blocking the transsulfuration pathway at the point of CSE expression. Thus, GSH
synthesis is directly coupled to the ISR, and loss of ATF4 impairs GSH production by inhibiting the expression of CSE.

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