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All-*trans*-Retinoic Acid Intensifies Endoplasmic Reticulum Stress in *N*-Acetylglucosaminyltransferase V Repressed Human Hepatocarcinoma Cells by Perturbing Homocysteine Metabolism

Ying-Ying Xu,[1] Dong-Yin Guan,[1] Min Yang,[1] Hao Wang,[2] and Zong-Hou Shen[1*]

[1]*Department of Biochemistry and Molecular Biology, Shanghai Medical College, Fudan University, 130 Dongan Road, Shanghai 200032, China*

[2]*Department of Anesthesiology, Zhongshan Hospital, Fudan University, 180 Fenglin Rd., Shanghai 200032, China*

ABSTRACT

We previously reported that all-*trans*-retinoic acid (ATRA) induced apoptosis in *N*-acetylglucosaminyltransferase V (GnT-V) repressed human hepatocarcinoma 7721 (GnT-V-AS/7721) cells via endoplasmic reticulum (ER) stress. In addition to confirming these findings, we further found that ATRA repressed the expression of betaine-homocysteine methyltransferase (BHMT) and cystathionine-β-synthase (CBS), which are key enzymes that are involved in homocysteine metabolism, increased the level of intracellular homocysteine, and decreased the glutathione (GSH) level in GnT-V-AS/7721 cells. To investigate the effect of ATRA on homocysteine metabolism, cells were challenged with exogenous homocysteine. In GnT-V-AS/7721 cells with ATRA treatment, a significant elevation of intracellular homocysteine levels suggests that ATRA perturbs homocysteine metabolism in GnT-V-AS/7721 cells and, therefore, sensitizes the cells to homocysteine-induced ER stress. An obvious increase in the levels of GRP78/Bip protein and spliced XBP1 mRNA were observed. Furthermore, we observed that ATRA blunted the homocysteine-induced increase of GSH only in GnT-V-AS/7721 cells. These results demonstrate that ATRA intensifies ER stress and induces apoptosis in GnT-V-AS/7721 cells by disturbing homocysteine metabolism through the down-regulation of CBS and BHMT, depleting the cellular GSH and, in turn, altering the cellular redox status. In addition, we showed that ATRA did not trigger ER stress, induce apoptosis, or affect homocysteine metabolism in L02 cells, which is a cell type that is derived from normal liver tissue. These results provide support for the hypothesis that ATRA is an anticancer agent. J. Cell. Biochem. 109: 468–477, 2010.

KEY WORDS: ALL-*TRANS*-RETINOIC ACID; *N*-ACETYLGLUCOSAMINYLTRANSFERASE V; ENDOPLASMIC RETICULUM STRESS; HOMOCYSTEINE; GLUTATHIONE

The endoplasmic reticulum (ER) is an essential eukaryotic organelle that provides a unique environment for protein folding [Kaufman, 1999]. All secreted and membrane proteins are post- or co-translationally translocated into the lumen of the ER, where they are modified, folded, and assembled correctly prior to transport to the Golgi compartment [Shen et al., 2004]. When the functions of the ER are perturbed by stimuli, such as alterations in the redox state, calcium homeostasis, or improper post-translational modifications, impaired folding of proteins occurs and causes ER stress. To cope with ER stress, eukaryotic cells activate a series of signaling pathways that are referred to as the unfolded protein response (UPR). The UPR in mammalian cells has evolved into a set of co-ordinated signaling cascades that involve procession of the transcription factor, activating transcription factor 6 (ATF6), the up-

Abbreviations used: ATF6, activating transcription factor 6; ATRA, all-trans-retinoic acid; BHMT, betaine-homocysteine methyltransferase; CBS, cystathionine-β-synthase; CHOP, C/EBP homologous protein; DMSO, Dimethyl sulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GnT-V, N-acetylglucosaminyltransferase V; GRP78/Bip, glucose regulated protein 78/immunoglobulin chain binding protein; MS, methionine synthase; PARP, Poly (ADP-ribose) polymerase; XBP1, X box binding protein 1.

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*Correspondence to: Zong-Hou Shen, Department of Biochemistry and Molecular Biology, Shanghai Medical College, Fudan University, 130 Dongan Rd., Shanghai 200032, China. E-mail: zhshen@shmu.edu.cn

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regulation of glucose-regulated protein 78/immunoglobulin chain binding protein (GRP78/Bip) and of C/EBP homologous protein (CHOP), and the splicing of X box binding protein 1 (XBP1) mRNA [Yoshida et al., 2001]. The early UPR dampens ER stress and ensures cell survival. However, prolonged or sustained UPR provokes a complex network of interacting and parallel responses that lead to apoptotic cell death [Xu et al., 2005; Boyce and Yuan, 2006]. Although ER stress has been thought to be associated with liver disorders, its specific contribution to liver diseases has just recently been identified [Ji, 2008].

In the ER, the redox balance is in a very sensitive and carefully maintained equilibrium [Papp et al., 2003, 2006]. This unique oxidizing environment is required for proteins to be folded into their native structure. The most important redox buffer is the glutathione (GSH)/oxidized GSH (GSSG) pair. Alteration in the cellular redox status is an important factor that leads to ER stress [McCullough et al., 2001; Gilmore and Kirby, 2004]. Links between protein folding defects and cellular redox changes in various diseases have been receiving revived attention these days. ER stress has been found to be a common feature of diabetes [Nardai et al., 2005; Bakker et al., 2009], Alzheimer's disease [Unterberger et al., 2006], Parkinson's disease [Andersen, 2004; Lindholm et al., 2006], and many other diseases that are related to oxidative changes [Andersen, 2004; Unterberger et al., 2006; Bakker et al., 2009].

Homocysteine, a sulfur-containing amino acid that functions as a key intermediate in the methionine cycle, has been shown to induce changes in the cellular redox balance and to trigger ER stress in several human cell lines, such as endothelial cells [Outinen et al., 1999; Zhang et al., 2001], HepG2 cells [Werstuck et al., 2001], primary neuronal cells [Althausen and Paschen, 2000], and neuroblastoma cells [Kim et al., 2008]. The fates of intracellular homocysteine are remethylation to methionine, *trans*-sulfuration to cystathionine, or transport from the cell. In most tissues, the primary remethylation pathway for homocysteine is catalyzed by the vitamin B12-dependent enzyme methionine synthase (MS). In the liver and kidney, a second remethylation pathway, catalyzed by the enzyme betaine-homocysteine methyltransferase (BHMT), is present. *Trans*-sulfuration is an irreversible process that results in the production of cysteine and other sulfur-containing compounds, including GSH, and involves cystathionine-β-synthase (CBS) and γ-cystathionase. The liver is the main place for the metabolism of homocysteine. It was reported that the mRNA levels of the enzymes involved in methionine metabolism, including MS, BHMT, and CBS, were markedly reduced in hepatocellular carcinoma (HCC) tissues [Avila et al., 2000].

N-Acetylglucosaminyltransferase V (GnT-V) is a key enzyme in the processing of asparagine-linked glycans (*N*-glycans) during the synthesis of glycoproteins. We previously reported that ER stress was triggered in human hepatocarcinoma 7721 cells that were transfected with the antisense cDNA of GnT-V (GnT-V-AS/7721) [Fang et al., 2006] and that all-*trans*-retinoic acid (ATRA) intensified this ER stress, even inducing apoptosis in GnT-V-AS/7721 cells [Xu et al., 2007]. However, knowledge of the molecular basis of the ER stress is limited. In this study, we demonstrate that ATRA intensifies the ER stress in GnT-V-AS/77221 cells by perturbing homocysteine metabolism and the cellular redox balance.

MATERIALS AND METHODS

CELL CULTURE AND TREATMENT

Human hepatic L02 cells are normal hepatocytes from adult liver tissue and have a distinct ultrastructure compared with hepatic carcinoma cells. The cells were obtained from the Institute of Cell Biology, Academia Sinica [Wang et al., 2008]. Human hepatocarcinoma 7721 cells were obtained from the Institute of Cell and Biochemistry Research of the Chinese Academy of Science. The GnT-V-AS/7721 cell line (shown as AS) was constructed by transfecting the expression plasmid of antisense GnT-V (pcDNA3/GnT-V-AS) into 7721 cells, as previously described [Guo et al., 1999]. The cells that were transfected with pcDNA3 plasmid as a control were termed mock cells. Cells were cultured in RPMI-1640 medium (Life Technologies, Inc., Gibco BRL, Gaithersburg, MA) supplemented with 10% new born bovine serum (Life Technologies, Inc., Gibco BRL) and 1% penicillin and streptomycin at 37°C, in a humidified atmosphere of 5% CO_2.

ATRA (Sigma, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) (Sigma), sterilized by filtration, and added to the cell cultures. Cells were stimulated by 80 μM ATRA or DMSO for 24 h [Guo et al., 1999]. The final concentration of DMSO in all samples was <0.3% (v/v). D,L-Homocysteine (Sigma) was prepared freshly in culture medium, sterilized by filtration, and added to the cell cultures that had been pretreated with 80 μM ATRA or DMSO for 18 h. Cells were exposed to 1, 2, 5, or 10 mM homocysteine for 6 h. The range of homocysteine concentrations and the period of exposure time were selected based on prior publications [Althausen and Paschen, 2000; Werstuck et al., 2001; Ji et al., 2007].

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA was extracted from L02, mock, and GnT-V-AS/7721 cells with Trizol reagent (Life Technologies, Inc., Gibco BRL) according to the manufacturer's instructions and was used as templates for cDNA synthesis. Reverse transcription was carried out using M-MuLV (Promega Co., Madison, WI), and the resulting cDNA was then amplified by PCR. The primers used for the PCR reactions were: GnT-V, GGCAGAAAAGCAGAACCTTG and AGCATGCACTGGTAATGAACC [Kemmner et al., 2003]; XBP1, CCTTGTGTAGTTGAGAACCAGG and GGGGCTTGGTATATATGTGG [Yoshida et al., 2001]; BHMT, CTGTGTGGGCAGTTGAAACC and TGCTGCTCAGTTGTGGCTTC; CBS, GCGGCTGAAGAACGAAATCC and GCGTCACCATTCCCAGGATT; MS, ATGTCACCCGCGCTCCAAGAC and TCCAGAAGTCCTTTGGCCTGC [Ji et al., 2007]; β-actin, TGGGCATGGGTCAGAAGGAT and AAGCATTTGCGGTGGACGAT. The primers for XBP1 were designed to permit the detection of both spliced and unspliced (intron-containing) mRNAs. These two bands were 442 and 416 bp, respectively, representing the unspliced and spliced mRNA of XBP1. The PCR products were electrophoresed on an agarose gel and visualized by ethidium bromide (EB) staining. β-Actin was used as the loading control. The relative levels of the mRNA were determined by quantification of the PCR bands using densitometry and were normalized to the density of β-actin.

REAL-TIME QUANTITATIVE REVERSE TRANSCRIPTASE

Total RNA was isolated from cells by use of Trizol (Life Technologies, Inc., Gibco BRL). First-strand cDNA was synthesized from 2 μg RNA according to the manufacturer's protocol. Real-time quantitative RT-PCR was performed for the candidate genes and for glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) as the internal control. Primers used were as follows: GnT-V, GAGCAGATCCTGGACCTCAG and GCTGTCATGACTCCAGCGTA; GAPDH, CAGCCTCAAGATCATCAGCA and GTCTTCTGGGTGGCAGTGAT [Guo et al., 2007]. Quantitative real-time PCR was performed in an ABIPRISM 7300 Sequencing Detector (Applied Biosystems, Foster City, CA) for SYBR green PCR master mix.

WESTERN BLOT

Cells were lysed in 1× SDS buffer with 1% phenylmethylsulfonyl fluoride (PMSF). The protein was applied to SDS–polyacrylamide gels for electrophoresis and then transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with TBS containing 0.1% (v/v) Tween-20 and 5% (w/v) nonfat dried milk for 1 h at room temperature and were then incubated at 4°C overnight with the following primary antibodies: rabbit anti-GRP78, anti-PARP, and anti-caspase 3 (Cell Signaling Technology, Danvers, MA); mouse anti-ATF6 (Imgenex Corporation, San Diego, CA), anti-BHMT (Abcam, Cambridge, UK), and anti-CBS (Abnova, Taipei, Taiwan); rabbit anti-CHOP (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-GAPDH (Kang-Chen Biotech, Shanghai, China). After incubation with the primary antibody, the membranes were washed and incubated with a horseradish peroxidase (HRP) conjugated secondary antibody (Kang-Chen Biotech) for 2 h at room temperature. The membranes were washed, and antibody binding was detected using an enhanced chemiluminescent (ECL) reagent (Pierce, Rockford, MA) and exposure to X-ray film. GAPDH was used as the loading control. The relative level of the proteins was quantified using densitometry and normalized to the expression of GAPDH.

HOECHST STAINING

After treatment with 80 μM ATRA for 24 h, cells were grown on glass cover slips and fixed with methanol/glacial acetic acid (3:1) at 4°C for 5 min. After washing three times with distilled water, the cells were stained with Hoechst 33258 at room temperature for 10 min. The stained cells were fixed with citrate buffer (20 mM citrate, 50 mM monosodium phosphate, 50% glycerol, pH 5.5) and observed using a Nikon phase-contrast microscope with a fluorescence attachment.

DETERMINATION OF INTRACELLULAR HOMOCYSTEINE LEVELS

Cells were washed three times in PBS and lysed in water by three freeze/thaw cycles. Cellular debris was then removed by centrifugation. The homocysteine levels in the cellular lysates were determined with the human Homocysteine Elisa kit from USCNLIFE (Wuhan, China) according to the manufacturer's instructions. The microtiter plate provided in the kit had been pre-coated with an antibody specific to homocysteine. Standards or samples were then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation that was specific for homocysteine. Next, avidin conjugated to HRP was added to each microplate well and incubated. A substrate solution was then added to each well, and the color change was measured at a wavelength of 450 nm. The concentration of homocysteine in the samples was then determined by comparing the OD of the samples to the standard curve.

DETERMINATION OF INTRACELLULAR LEVELS OF GSH AND GSSG

Cells were lysed by freezing and thawing three times. GSH and GSSG levels in the supernatant were determined with a quantification kit (Beyotime Institute of Biotechnology, Jiangsu, China) according to the manufacturer's protocol by measuring absorbance at 405 nm.

STATISTICAL ANALYSIS

The studies were replicated three times, with the data shown as the means ± the standard error of the mean. A statistical analysis was performed with a Student's *t*-test for unpaired data or an analysis of variance and post hoc Tukey–Kramer multiple-comparison test. A *P*-value less than 0.05 was considered to be significant.

RESULTS

ATRA INTENSIFIES THE ER STRESS AND INDUCES APOPTOSIS IN GNT-V-AS/7721 CELLS

The expression of GnT-V at the transcriptional level was significantly increased in mock cells compared with L02 cells (Fig. 1A,B). In GnT-V-AS/7721 cells, the GnT-V expression at the mRNA level was restored to a level comparable to that of L02 cells. The addition of ATRA decreased the expression of GnT-V slightly in mock cells and obviously in GnT-V-AS/7721 cells. To confirm ER stress in GnT-V-AS/7721 cells, we measured the expression of ER stress response genes. The results showed that ATRA greatly increased the expression of GRP78/BiP, CHOP, processed ATF6, and the spliced isoforms of XBP1 mRNA in GnT-V-AS/7721 cells (Fig. 1C). Caspase-3 plays a key role in apoptotic cell death. Poly(ADP-ribose) polymerase (PARP) is a nuclear enzyme involved in DNA repair and is a well-known substrate for caspase-3 cleavage. We detected the cleaved caspase-3 (17 kDa) and PARP (85 kDa) only in GnT-V-AS/7721 cells that were treated with ATRA (Fig. 1C), implying that ATRA induced apoptosis in GnT-V-AS/7721 cells. Furthermore, apoptotic morphology, exemplified by bright blue fluorescent condensed nuclei and nuclear fragments, was observed in GnT-V-AS/7721 cells with ATRA treatment (Fig. 1D). These data suggest that 80 μM ATRA intensifies the ER stress and specifically induces apoptosis in GnT-V-AS/7721 cells.

ATRA REPRESSES THE ENZYMES INVOLVED IN HOMOCYSTEINE METABOLISM

In agreement with previous data showing that the mRNA levels of the enzymes involved in methionine metabolism were reduced in HCC tissues [Avila et al., 2000], the endogenous expression of BHMT and CBS at both the mRNA and protein levels in 7721 and mock cells were significantly lower than those in L02 cells (Fig. 2A–D). However, there was no difference in the endogenous expression of MS at the mRNA level (the antibody against MS was commercially unavailable) in L02, 7721, and mock cells (Fig. 2A,B). The repression of GnT-V reduced the endogenous expression of BHMT, CBS, and

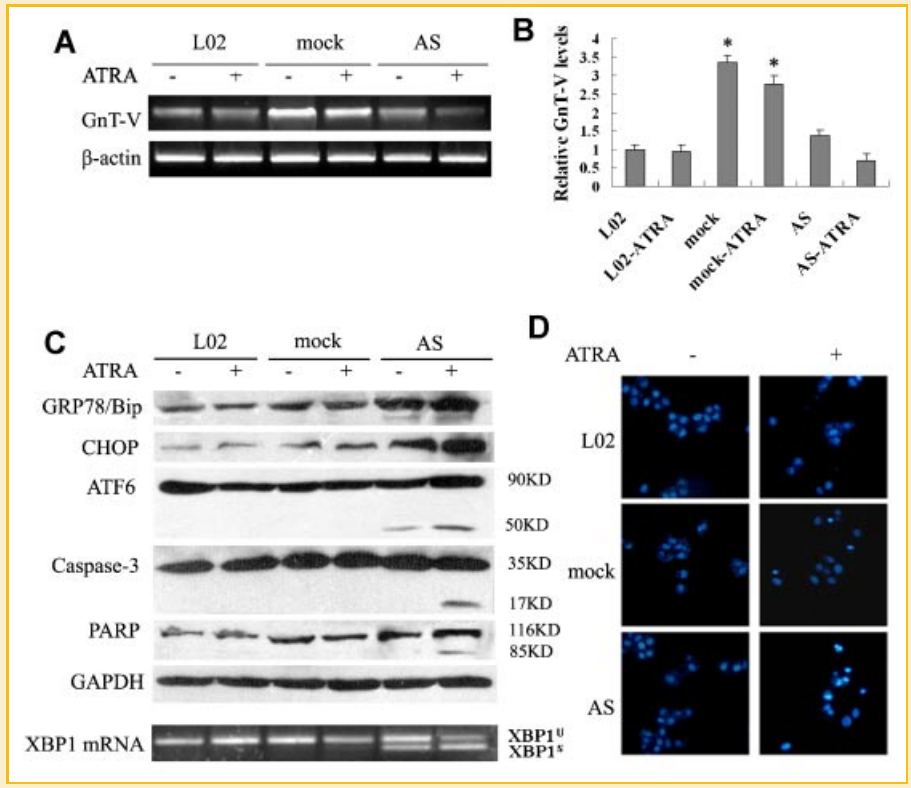

Fig. 1. ATRA intensifies ER stress and induces apoptosis in GnT-V-AS/7721 cells. L02, mock, and GnT-V-AS/7721 cells were cultured with 80 μM ATRA or DMSO for 24 h. A: Total RNA from these cells was analyzed by RT-PCR for GnT-V and β-actin mRNA. B: Relative GnT-V mRNA levels normalized to β-actin. $^{*}P < 0.05$ versus L02 cells. C: An equal amount of the whole cell lysates was analyzed by Western blot using antibodies against GRP78/Bip, CHOP, ATF6, caspase-3, PARP, and GAPDH, respectively. Total RNA from these cells was analyzed by RT-PCR for XBP1 and β-actin mRNA as described in the Materials and Methods. $XBP1^{U}$: unspliced XBP1 mRNA, $XBP1^{S}$: spliced XBP1 mRNA. D: The cells were stained with Hoechst 33258 and examined under a fluorescence microscope. AS, GnT-V-AS/7721. L02, mock, and GnT-V-AS/7721 cells with ATRA treatment were shown as L02-ATRA, mock-ATRA and AS-ATRA, respectively. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

MS in 7721 cells (Fig. 2A,B). The addition of ATRA down-regulated the expression of BHMT and CBS moderately in mock cells and significantly in GnT-V-AS/7721 cells, whereas the mRNA expression of MS was unaffected (Fig. 2E–H). The effect of ATRA on the expression of BHMT, CBS, and MS in L02 cells was not obvious (Fig. 2I).

ATRA DISTURBS THE HOMOCYSTEINE METABOLISM IN GNT-V-AS/7721 CELLS

The basal level of intracellular homocysteine was the highest in GnT-V-AS/7721 cells and the lowest in L02 cells (Fig. 3A). The addition of ATRA caused a significant increase of intracellular homocysteine in GnT-V-AS/7721 cells but had no obvious effect on L02 cells (Fig. 3A). To examine homocysteine metabolism in the cells, cell cultures were exposed to 1, 2, 5, and 10 mM homocysteine for 6 h and then analyzed to determine their intracellular homocysteine levels. The addition of exogenous homocysteine elevated the intracellular homocysteine in a dose-dependent manner in all cell types (Fig. 3B). The addition of ATRA increased the values of intracellular homocysteine in mock and GnT-V-AS/7721 cells, most significantly in GnT-V-AS/7721 cells. These results indicate that ATRA interferes with the homocysteine metabolism in GnT-V-AS/7721 cells that have been impaired, leading to the accumulation of intracellular homocysteine.

ATRA SENSITIZES GNT-V-AS/7721 CELLS TO HOMOCYSTEINE-INDUCED ER STRESS

To observe the homocysteine-induced ER stress, the levels of two key markers of ER stress, which are the GRP78/Bip protein and XBP1 mRNA, were analyzed after the addition of exogenous homocysteine. Exogenous homocysteine increased the levels of GRP78/Bip protein and spliced XBP1 mRNA in a dose-dependent manner in L02, mock, and GnT-V-AS/7721 cells (Fig. 4). The addition of ATRA elicited a homocysteine-induced increase in the levels of GRP78/Bip protein and spliced XBP1 mRNA in mock and GnT-V-AS/7721 cells (Fig. 4A–D). A marked increase in the level of GRP78/Bip protein and the complete splicing of XBP1 mRNA into a single isoform were both observed in GnT-V-AS/7721 cells with ATRA treatment, suggesting that ATRA significantly sensitizes GnT-V-AS/7721 cells to homocysteine-induced ER stress. L02 cells were not sensitive to the homocysteine-induced increase in GRP78/Bip protein and spliced XBP1 mRNA, and the addition of ATRA had no obvious effect on L02 cells (Fig. 4E,F).

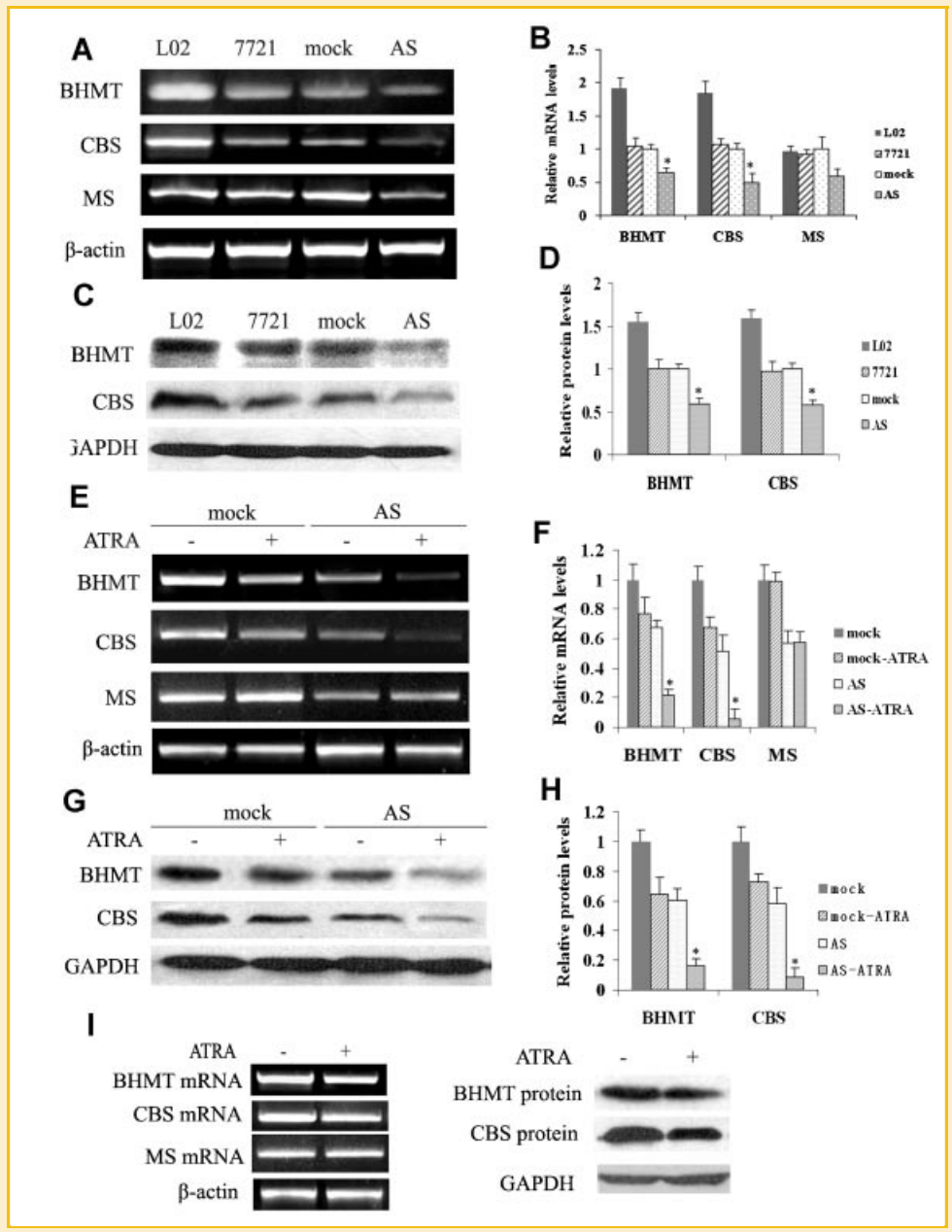

Fig. 2. The expression of the enzymes BHMT, CBS, and MS. L02, 7721, mock, and GnT-V-AS/7721 cells were cultured with 80 μM ATRA or DMSO for 24 h. The endogenous expression of BHMT, CBS, and MS was detected by RT-PCR (A) and Western blot (C). The relative mRNA levels normalized to β-actin (B) and the relative protein levels normalized to GAPDH (D) were shown. $^{*}P < 0.05$ versus L02 cells. To observe the effect of ATRA on the expression of BHMT, CBS, and MS proteins, L02, mock, and GnT-V-AS/7721 cells were pretreated with 80 μM ATRA or DMSO for 18 h, followed by the addition of exogenous homocysteine to the culture medium. Cells were exposed to the indicated concentrations of homocysteine for 6 h, followed by the analysis of RT-PCR and Western blot. E: RT-PCR of samples from mock and GnT-V-AS/7721 cells. F: Relative mRNA levels normalized to β-actin. $^{*}P < 0.05$ versus mock cells. G: Western blot of samples from mock and GnT-V-AS/7721 cells. H: Relative protein levels normalized to GAPDH. $^{*}P < 0.05$ versus mock cells. I: RT-PCR and Western blot of samples from L02 cells.

ATRA ALTERS CELLULAR REDOX STATUS IN GNT-V-AS/7721 CELLS BY DEPLETING GSH

The GSH level was highest in L02 cells and lowest in GnT-V-AS/7721 cells, and the addition of ATRA significantly decreased the GSH level in GnT-V-AS/7721 cells (Fig. 5A). The GSH/GSSG ratio, which gives an indication of the overall redox status of the cell, was consistent with the GSH levels. This might be attributed to the similar levels of GSSG in all cell types, regardless of ATRA treatment (Fig. 5A,B). The exogenous homocysteine-induced elevations of the GSH and GSSG levels were positively correlated with the concentration of exogenous homocysteine that was added to all cell types, regardless of ATRA treatment (Fig. 5C). In

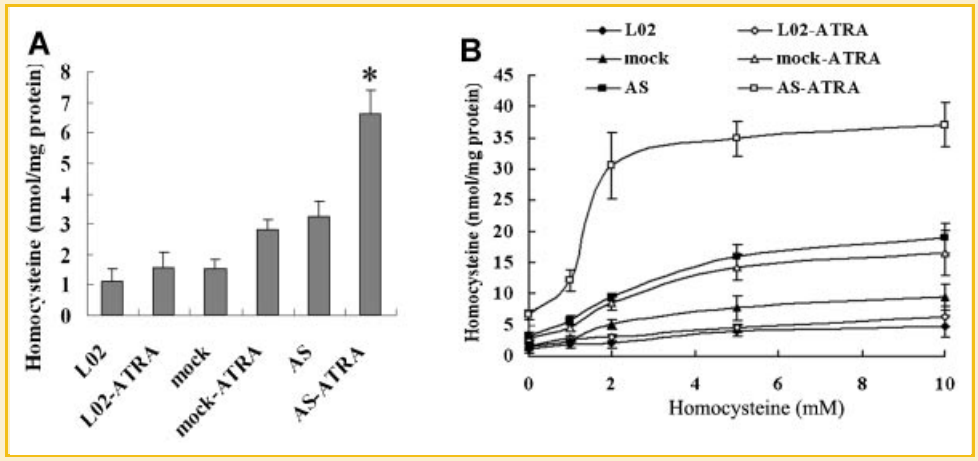

Fig. 3. The effect of ATRA on intracellular homocysteine. A: L02, mock, and GnT-V-AS/7721 cells were cultured with 80 μM ATRA or DMSO for 24 h. The basal levels of intracellular homocysteine were determined. $^{*}P < 0.05$ versus L02 cells. B: Cells were pretreated with 80 μM ATRA or DMSO for 18 h, followed by the addition of exogenous homocysteine to the culture medium. Cells were exposed to the indicated concentrations of homocysteine for 6 h, followed by analysis of the levels of intracellular homocysteine.

addition, the trends for the GSSG levels were similar (Fig. 5D). Remarkably, the exogenous homocysteine-induced elevation of the GSH levels was almost undetected in GnT-V-AS/7721 cells that were treated with ATRA (Fig. 5C), implying that GSH synthesis was inhibited.

DISCUSSION

We previously reported that 80 μM ATRA intensified ER stress that was triggered in GnT-V-AS/7721 cells [Xu et al., 2007]. Our present study confirmed these results, demonstrating a significant increase in the levels of GRP78/Bip, CHOP, and processed ATF6 proteins and that of spliced XBP1 mRNA. We also demonstrated that ATRA disturbs homocysteine metabolism by repressing the expression of BHMT and CBS and alters the cellular redox status through a depletion of GSH, which results in intensified ER stress and apoptosis in GnT-V-AS/7721 cells.

Our investigation showed that ER stress was triggered only when the levels of intracellular homocysteine were sufficiently high and that ER stress was intensified along with the elevation of intracellular homocysteine levels. The additional observation that ATRA sensitized GnT-V-AS/7721 cells to the exogenous homocysteine-induced ER stress was correlated to a significant elevation in the levels of intracellular homocysteine. These results suggest that accumulated intracellular homocysteine is an inducer of ER stress, which is in agreement with previous findings that homocysteine induces ER stress [Outinen et al., 1999; Althausen and Paschen, 2000; Werstuck et al., 2001; Zhang et al., 2001; Kim et al., 2008]. These findings support the hypothesis that ATRA intensifies the ER stress that is triggered in GnT-V-AS/7721 cells by increasing the level of intracellular homocysteine.

The concentration of homocysteine can be modulated by changes in its metabolism by either the *trans*-sulfuration or the remethylation pathway. Impaired homocysteine metabolism can result in the accumulation of intracellular homocysteine. In the liver, homocysteine is metabolized by BHMT and MS to produce methionine and by CBS to produce castathionine. Here, we show that homocysteine metabolism is impaired in GnT-V-AS/7721 cells, as demonstrated by the obvious accumulation of intracellular homocysteine after the addition of exogenous homocysteine. This likely attributes to the repressed expression of CBS, BHMT, and MS. ATRA has been shown to alter the expression and activity of enzymes that are involved in methionine metabolism [Ozias and Schalinske, 2003; Nieman et al., 2004]. We found that ATRA aggravated the impaired homocysteine metabolism in GnT-V-AS/7721 cells by down-regulating the expression of CBS and BHMT, resulting in rapidly elevated levels of intracellular homocysteine and, therefore, a significant increase in levels of GRP78/Bip protein and spliced XBP1 mRNA in GnT-V-AS/7721 cells that were exposed to exogenous homocysteine. Therefore, we postulate that ATRA elevates the level of intracellular homocysteine by disturbing homocysteine metabolism, and, thus, the ER stress triggered in GnT-V-AS/7721 cells becomes intensified. In particular, L02 cells exhibited a low sensitivity to the exogenous homocysteine-induced ER stress, which is likely due to their high expression of CBS, BHMT, and MS.

It was reported that alteration of cellular redox status is an inducer of ER stress [McCullough et al., 2001; Gilmore and Kirby, 2004]. The present data showed that the GSH/GSSG ratio, which is an indicator of the cellular redox status, was decreased in GnT-V-AS/7721 cells and that this contributed to ER stress in the cells. ATRA decreased the GSH/GSSG ratio in GnT-V-AS/7721 cells and, therefore, intensified the ER stress in the cells.

An important finding of this study is that ATRA treatment results in GSH depletion in GnT-V-AS/7721 cells. Direct measurement of cellular GSH and GSSG levels indicated that the low GSH/GSSG ratio in GnT-V-AS/7721 cells could be attributed to the low level of

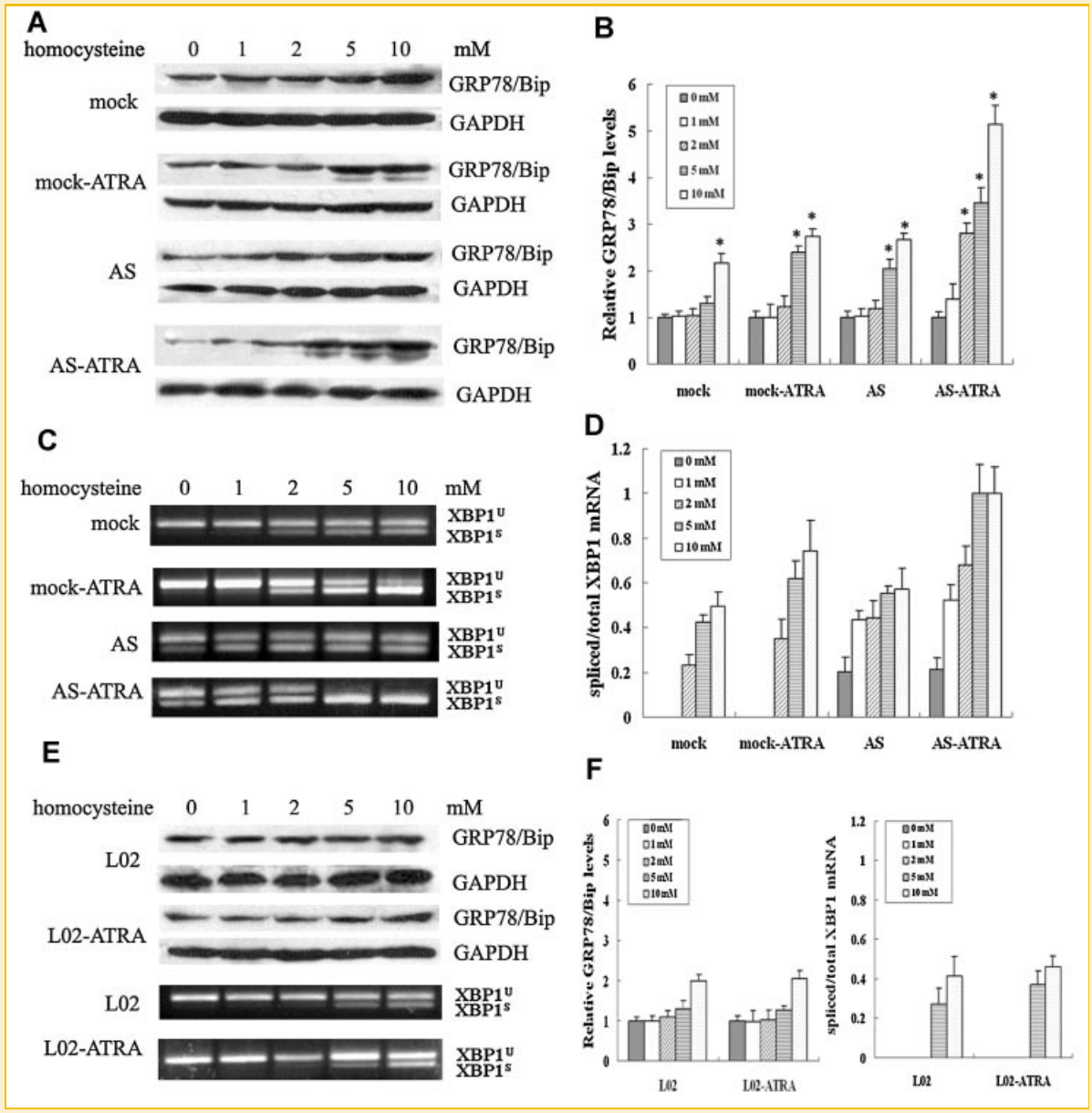

Fig. 4. Homocysteine-induced ER stress. Cells were pretreated with 80 μM ATRA or DMSO for 18 h, followed by the addition of exogenous homocysteine to the culture medium. Cells were exposed to the indicated concentrations of homocysteine for 6 h, followed by analysis of ER stress response genes. A: Western blot of GRP78/Bip in mock and GnT-V-AS/7721 cells. B: Relative GRP78/Bip protein levels normalized to GAPDH. $^{*}P < 0.05$ versus 0 mM cells. C: RT-PCR of XBP1 mRNA in mock and GnT-V-AS/7721 cells. D: Spliced XBP1 mRNA levels normalized to the total XBP1 mRNA. E: Western blot of GRP78/Bip, and RT-PCR of XBP1 mRNA in L02 cells. F: Relative GRP78/Bip protein levels normalized to GAPDH, and spliced XBP1 mRNA levels normalized to the total XBP1 mRNA. $XBP1^{U}$: unspliced XBP1 mRNA, $XBP1^{S}$: spliced XBP1 mRNA.

GSH. Similarly, ATRA severely depleted the GSH pool in GnT-V-AS/7721 cells, resulting in a decreased GSH/GSSG ratio. GSH has a number of vital functions in the cell. In addition to its role in detoxifying electrophiles and scavenging free radicals, GSH was proven to play an important role in maintaining the redox balance [Kern and Kehrer, 2005]. Intracellular GSH concentrations vary considerably depending on its synthesis, utilization, and export. Our GSH measurements in the cells with homocysteine treatment reveal that ATRA inhibits GSH synthesis in GnT-V-AS/7721 cells, as demonstrated by the almost unchanged GSH levels. CBS is involved in the *trans*-sulfuration pathway, in which GSH is converted to a final product of homocysteine. The ATRA-induced down-regulation of CBS likely contributes to the inhibition of GSH synthesis in GnT-V-AS/7721 cells. Additionally, thiol depletion has been shown to sensitize cells to cell death caused by a variety of stimuli, and, in some cell types, it is sufficient to induce apoptosis

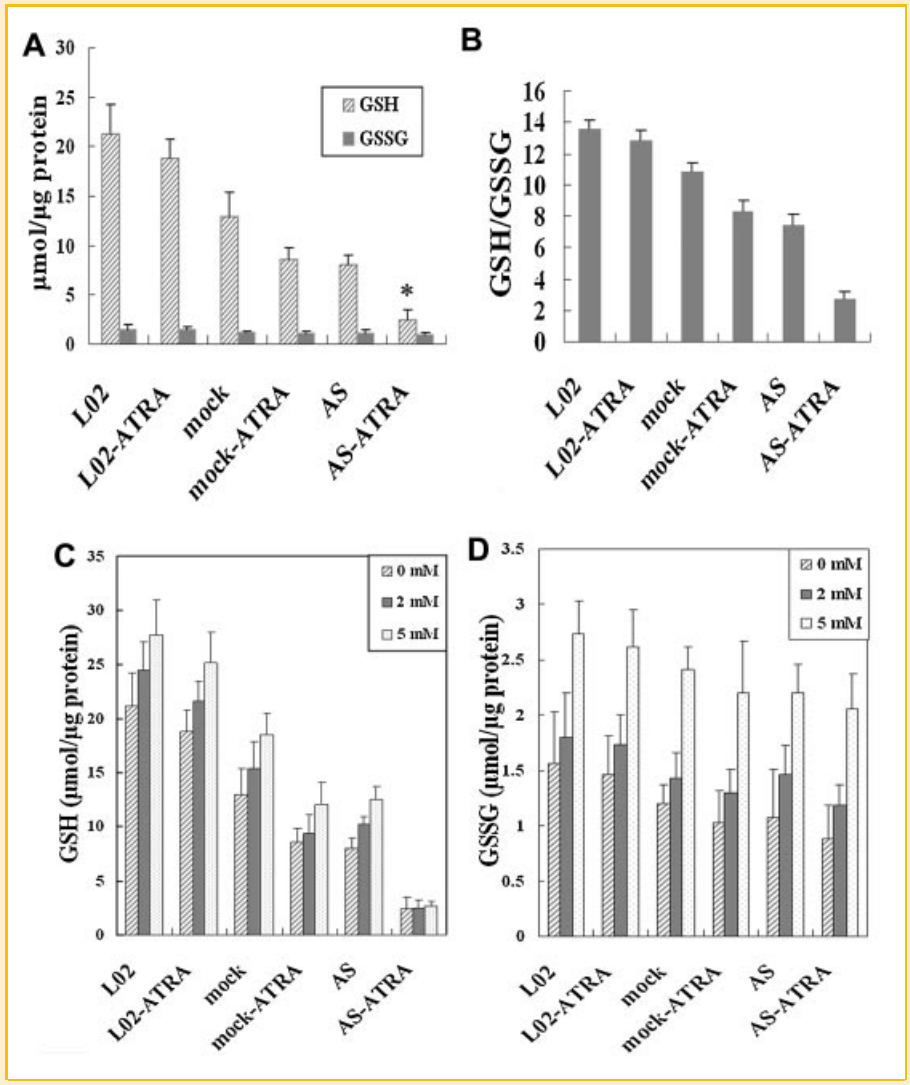

Fig. 5. The effect of ATRA on GSH and GSSG levels. A: L02, mock, and GnT-V-AS/7721 cells were cultured with 80 μM ATRA or DMSO for 24 h. The basal levels of GSH and GSSG were determined. $^{*}P < 0.05$ versus L02 cells. B: The ratio of GSH/GSSG is reported. $^{*}P < 0.05$ versus L02 cells. C: Cells were pretreated with 80 μM ATRA or DMSO for 18 h, followed by the addition of exogenous homocysteine to the culture medium. Cells were exposed to the indicated concentrations of homocysteine for 6 h, followed by analysis of cellular GSH and GSSG levels.

[Chiba et al., 1996; Wang et al., 1996]. Thus, GSH depletion may contribute to ATRA-induced apoptosis in GnT-V-AS/7721 cells.

In both our previous and current work, we demonstrated that GnT-V repression sensitized human 7721 cells to ATRA-induced apoptosis. GnT-V is located in the *trans*-Golgi apparatus, and, like other GlcNAc transferases, it regulates branch formation in complex-type N-linked oligosaccharides. The activity of GnT-V is low in normal hepatic tissue but increases in HCC tissue, which is correlated with its cancerous progression [Dennis et al., 1999]. The defective function of GnT-V can change *N*-glycosylation status, which impairs the assembly, maturation, and selectively degradation of proteins. We found that GnT-V repression impaired homocysteine metabolism and triggered ER stress in GnT-V-AS/7721 cells, which sensitized the cells to ATRA-induced apoptosis. ATRA is a promising anticancer agent. Here, we establish a novel mechanism by which ATRA induces apoptosis. By increasing intracellular homocysteine levels through the down-regulation of BHMT and CBS and by depleting the cells of GSH, ATRA intensifies ER stress and induces apoptosis in GnT-V-AS/7721 cells. The same mechanism likely explains the ER stress triggered in GnT-V-AS/7721 cells. Although similar but moderate changes, including the repressed expression of BHMT and CBS, elevated levels of intracellular homocysteine, and decreased levels of GSH, were found in mock cells with ATRA treatment, neither ER stress nor apoptosis was observed in these cells. L02 cells are normal hepatocytes from adult liver tissue, in which the expression of GnT-V physiologically remains a low level. Although the cells contained the similar level of

GnT-V as the GnT-V-AS/7721 cells, L02 cells were not sensitive to ATRA-induced apoptosis and homocysteine-induced ER stress, which might be attributed to their ability to metabolize homocysteine and maintain the cellular redox status. These findings demonstrate that ATRA specifically induced apoptosis in GnT-V repressed human 7721 cells but had no effect on L02 cells. Taken together, these observations provide further support for the use of ATRA as an anticancer agent. ATRA treatment, along with GnT-V repression, may work synergistically to induce apoptosis in hepatocarcinoma cells, representing a potential treatment for HCC.

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REFERENCES

Althausen S, Paschen W. 2000. Homocysteine-induced changes in mRNA levels of genes coding for cytoplasmic- and endoplasmic reticulum-resident stress proteins in neuronal cell cultures. Brain Res Mol Brain Res 84:32–40.

Andersen JK. 2004. Oxidative stress in neurodegeneration: Cause or consequence? Nat Med 10 (Suppl): S18–S25.

Avila MA, Berasain C, Torres L, Martin-Duce A, Corrales FJ, Yang H, Prieto J, Lu SC, Caballeria J, Rodes J, Mato JM. 2000. Reduced mRNA abundance of the main enzymes involved in methionine metabolism in human liver cirrhosis and hepatocellular carcinoma. J Hepatol 33:907–914.

Bakker W, Eringa EC, Sipkema P, van Hinsbergh VW. 2009. Endothelial dysfunction and diabetes: Roles of hyperglycemia, impaired insulin signaling and obesity. Cell Tissue Res 335:165–189.

Boyce M, Yuan J. 2006. Cellular response to endoplasmic reticulum stress: A matter of life or death. Cell Death Differ 13:363–373.

Chiba T, Takahashi S, Sato N, Ishii S, Kikuchi K. 1996. Fas-mediated apoptosis is modulated by intracellular glutathione in human T cells. Eur J Immunol 26:1164–1169.

Dennis JW, Granovsky M, Warren CE. 1999. Glycoprotein glycosylation and cancer progression. Biochim Biophys Acta 1473:21–34.

Fang H, Huang W, Xu YY, Shen ZH, Wu CQ, Qiao SY, Xu Y, Yu L, Chen HL. 2006. Blocking of N-acetylglucosaminyltransferase V induces cellular endoplasmic reticulum stress in human hepatocarcinoma 7721 cells. Cell Res 16:82–92.

Gilmore WJ, Kirby GM. 2004. Endoplasmic reticulum stress due to altered cellular redox status positively regulates murine hepatic CYP2A5 expression. J Pharmacol Exp Ther 308:600–608.

Guo HB, Liu F, Chen HL. 1999. Increased susceptibility to apoptosis of human hepatocarcinoma cells transfected with antisense N-acetylglucosaminyltransferase V cDNA. Biochem Biophys Res Commun 264: 509–517.

Guo HB, Randolph M, Pierce M. 2007. Inhibition of a specific N-glycosylation activity results in attenuation of breast carcinoma cell invasiveness-related phenotypes: Inhibition of epidermal growth factor-induced dephosphorylation of focal adhesion kinase. J Biol Chem 282:22150–22162.

Ji C. 2008. Dissection of endoplasmic reticulum stress signaling in alcoholic and non-alcoholic liver injury. J Gastroenterol Hepatol 23 (Suppl 1): S16–S24.

Ji C, Shinohara M, Kuhlenkamp J, Chan C, Kaplowitz N. 2007. Mechanisms of protection by the betaine-homocysteine methyltransferase/betaine system in HepG2 cells and primary mouse hepatocytes. Hepatology 46:1586–1596.

Kaufman RJ. 1999. Stress signaling from the lumen of the endoplasmic reticulum: Coordination of gene transcriptional and translational controls. Genes Dev 13:1211–1233.

Kemmner W, Roefzaad C, Haensch W, Schlag PM. 2003. Glycosyltransferase expression in human colonic tissue examined by oligonucleotide arrays. Biochim Biophys Acta 1621:272–279.

Kern JC, Kehrer JP. 2005. Free radicals and apoptosis: Relationships with glutathione, thioredoxin, and the BCL family of proteins. Front Biosci 10: 1727–1738.

Kim HJ, Cho HK, Kwon YH. 2008. Synergistic induction of ER stress by homocysteine and beta-amyloid in SH-SY5Y cells. J Nutr Biochem 19:754–761.

Lindholm D, Wootz H, Korhonen L. 2006. ER stress and neurodegenerative diseases. Cell Death Differ 13:385–392.

McCullough KD, Martindale JL, Klotz LO, Aw TY, Holbrook NJ. 2001. Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. Mol Cell Biol 21:1249–1259.

Nardai G, Stadler K, Papp E, Korcsmaros T, Jakus J, Csermely P. 2005. Diabetic changes in the redox status of the microsomal protein folding machinery. Biochem Biophys Res Commun 334:787–795.

Nieman KM, Rowling MJ, Garrow TA, Schalinske KL. 2004. Modulation of methyl group metabolism by streptozotocin-induced diabetes and all-trans-retinoic acid. J Biol Chem 279:45708–45712.

Outinen PA, Sood SK, Pfeifer SI, Pamidi S, Podor TJ, Li J, Weitz JI, Austin RC. 1999. Homocysteine-induced endoplasmic reticulum stress and growth arrest leads to specific changes in gene expression in human vascular endothelial cells. Blood 94:959–967.

Ozias MK, Schalinske KL. 2003. All-trans-retinoic acid rapidly induces glycine N-methyltransferase in a dose-dependent manner and reduces circulating methionine and homocysteine levels in rats. J Nutr 133:4090–4094.

Papp E, Nardai G, Soti C, Csermely P. 2003. Molecular chaperones, stress proteins and redox homeostasis. Biofactors 17:249–257.

Papp E, Szaraz P, Korcsmaros T, Csermely P. 2006. Changes of endoplasmic reticulum chaperone complexes, redox state, and impaired protein disulfide reductase activity in misfolding alpha1-antitrypsin transgenic mice. FASEB J 20:1018–1020.

Shen X, Zhang K, Kaufman RJ. 2004. The unfolded protein response—A stress signaling pathway of the endoplasmic reticulum. J Chem Neuroanat 28:79–92.

Unterberger U, Hoftberger R, Gelpi E, Flicker H, Budka H, Voigtlander T. 2006. Endoplasmic reticulum stress features are prominent in Alzheimer disease but not in prion diseases in vivo. J Neuropathol Exp Neurol 65:348–357.

Wang XZ, Lawson B, Brewer JW, Zinszner H, Sanjay A, Mi LJ, Boorstein R, Kreibich G, Hendershot LM, Ron D. 1996. Signals from the stressed endoplasmic reticulum induce C/EBP-homologous protein (CHOP/GADD153). Mol Cell Biol 16:4273–4280.

Wang H, Xue Z, Wang Q, Feng X, Shen Z. 2008. Propofol protects hepatic L02 cells from hydrogen peroxide-induced apoptosis via activation of extracellular signal-regulated kinases pathway. Anesth Analg 107:534–540.

Werstuck GH, Lentz SR, Dayal S, Hossain GS, Sood SK, Shi YY, Zhou J, Maeda /SNM> N, Krisans SK, Malinow MR, Austin RC. 2001. Homocysteine-induced endoplasmic reticulum stress causes dysregulation of the cholesterol and triglyceride biosynthetic pathways. J Clin Invest 107: 1263–1273.

Xu C, Bailly-Maitre B, Reed JC. 2005. Endoplasmic reticulum stress: Cell life and death decisions. J Clin Invest 115:2656–2664.

Xu YY, Lu Y, Fan KY, Shen ZH. 2007. Apoptosis induced by all-trans retinoic acid in N-acetylglucosaminyltransferase V repressed human hepatocarcinoma cells is mediated through endoplasmic reticulum stress. J Cell Biochem 100:773–782.

Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K. 2001. XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. Cell 107:881–891.

Zhang C, Cai Y, Adachi MT, Oshiro S, Aso T, Kaufman RJ, Kitajima S. 2001. Homocysteine induces programmed cell death in human vascular endothelial cells through activation of the unfolded protein response. J Biol Chem 276: 35867–35874.