ENDOPLASMIC RETICULUM STRESS INCREASES THE EXPRESSION OF METHYLENETETRAHYDROFOLATE REDUCTASE THROUGH THE IRE1 TRANSDECER*

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Running Title: MTHFR up-regulation by ER stress

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Methylenetetrahydrofolate reductase (MTHFR), an enzyme in folate and homocysteine metabolism, influences many cellular processes including methionine and nucleotide synthesis, methylation reactions, and maintenance of homocysteine at nontoxic levels. Mild deficiency of MTHFR is common in many populations and modifies risk for several complex traits including vascular disease, birth defects, and cancer. We recently demonstrated that MTHFR can be up-regulated by NF-κB, an important mediator of cell survival that is activated by endoplasmic reticulum (ER) stress. This observation, coupled with the reports that homocysteine can induce ER stress, prompted us to examine the possible regulation of MTHFR by ER stress. We found that several well-characterized stress inducers (tunicamycin, thapsigargin and A23187) as well as homocysteine could increase Mthfr mRNA and protein in Neuro-2a cells. The induction of MTHFR was also observed after overexpression of inositol-requiring enzyme-1 (IRE1) and was inhibited by a dominant-negative mutant of IRE1. Since IRE1 triggers c-Jun signaling, we examined the possible involvement of c-Jun in up-regulation of MTHFR. Transfection of c-Jun and two activators of c-Jun (LiCl and sodium valproate) increased MTHFR expression whereas a reported inhibitor of c-Jun (SP600125) and a dominant-negative derivative of c-Jun N-terminal kinase-1 (JNK1) reduced MTHFR activation. We conclude that ER stress increases MTHFR expression and that IRE1 and c-Jun mediate this activation. These findings provide a novel mechanism by which the ER can regulate homeostasis and allude to an important role for MTHFR in cell survival.

The endoplasmic reticulum (ER) is a dynamic membranous organelle that plays a critical role in the folding, transport and processing of newly synthesized proteins. Numerous xenotoxic agents and adverse metabolic conditions interfere with protein folding in the ER leading to cellular stresses, known collectively as ER stress. For example, tunicamycin blocks N-glycosylation and leads to the accumulation of misfolded proteins in the ER. Thapsigargin inhibits the ER Ca2+-ATPase and is also a very potent ER stress inducer. The Ca2+ ionophore A23187 influences stress through depletion of Ca2+ stores in the ER. These chemicals, the most widely used agents to experimentally induce ER stress, activate a complex signaling pathway known as the unfolded protein response (UPR). The UPR represents a set of signaling cascades by which conditions within the ER are communicated to the protein translation machinery (to decrease ribosome activity and promote degradation of mRNAs for ER proteins) and to the nucleus (through transcription factors) in order to balance the folding capacity of the ER with the protein processing demand (1,2). Three ER-resident transmembrane proteins, inositol-requiring enzyme-1 (IRE1), pancreatic ER stress kinase (PERK), and activating transcription factor 6 (ATF6) have been identified as proximal sensors of ER stress (1). Activation of these transducers/transcription factors results in the up-regulation of genes encoding ER chaperone proteins such as GRP78 that facilitate protein folding and reduce protein aggregation. The UPR also affects proteins that are not
directly involved in ER function but have a role in cell survival after exposure to ER stress. Failure to counteract induced ER stress can result in activation of apoptosis.

Homocysteine (Hcy) is a thiol-containing amino acid generated by demethylation of methionine. Hcy can inhibit the biosynthesis of proteins normally secreted by some cells; this has been attributed to ER retention of proteins (3). The molecular targeting hypothesis for Hcy-induced damage (4) suggests that Hcy can form stable disulfide bonds with cysteine residues in proteins, thereby altering specific cellular processes and pathways. In vitro studies have provided experimental evidence for the effect of Hcy on expression of ER stress response genes (5,6).

Methylenetetrahydrofolate reductase (MTHFR) is a critical enzyme in Hcy metabolism. MTHFR catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. This reaction is the only source of 5-methyltetrahydrofolate, which serves as the methyl donor in the remethylation of Hcy to methionine. MTHFR first received medical recognition in reports of patients with homocystinuria (OMIM # 236250), an inborn error of metabolism that can be caused by deleterious mutations in the MTHFR gene (7). However, considerably greater interest has been generated in MTHFR through our identification of a common variant, 677C→T (A222V) (8), which increases risk for vascular disease, neural tube defects and possibly other birth defects (9). This variant may also increase risk for certain neoplasias, although it lowers risk of colorectal cancer when folate status is adequate. MTHFR may influence disease through elevation of plasma homocysteine, disruption in methionine or S-adenosylmethionine (SAM) synthesis or altered distribution of folate metabolites with consequent effects on nucleotide synthesis (9). Mthfr knockout mice have an elevation of plasma Hcy, a decrease of S-adenosylmethionine and DNA methylation, and altered folate distributions (10,11). The cerebellar pathology in Mthfr−/− mice is associated with increased apoptosis (12).

Studies on MTHFR regulation are few in number. SAM is an allosteric regulator of MTHFR and the posttranslational modification of MTHFR by phosphorylation leads to decreased activity and increased sensitivity of the enzyme to SAM (13). Phosphorylation of MTHFR has been demonstrated for the smaller MTHFR isoform (70 kDa); phosphorylation of the larger isoform (77 kDa) has not been examined (13). We recently characterized 2 promoters for MTHFR, each of which may direct the synthesis of one of the two isoforms through different transcriptional start site clusters and alternative splicing (14). We also demonstrated that MTHFR expression was enhanced by NF-κB through an effect on the downstream promoter (14). Since NF-κB is an important modulator of cell survival that is activated by ER stress and since MTHFR is involved in several critical cellular pathways, we examined the possibility that MTHFR could also participate in the response to ER stress. Our findings suggest that MTHFR is up-regulated by ER stress and that this effect is mediated by IRE1 and c-Jun. This link between the ER stress pathway and a new regulatory mechanism for MTHFR supports an important role for this enzyme in cellular homeostasis or survival.

**Experimental Procedures**

Cell culture conditions and transfection—Neuro-2a neuroblastoma cells and RAW264.7 macrophages were maintained in high glucose Dulbecco’s modified Eagle’s medium containing 10% serum at 37°C. For ER stress induction, the cells were grown to 70% confluence and treated with tunicamycin, thapsigargin, A23187 or DL-Hcy (all from Sigma, Oakville, Ontario) for the time and concentrations indicated in RESULTS. LiCl was from Fisher Scientific (Nepean, Ontario) and valproic acid, pyrrolidinethiocarbamate (PDTC), Bay11-7082, lipopolysaccharides from E. coli K-235 (LPS), SP600125, DL-cysteine and DL-methionine were from Sigma (Oakville, Ontario). Transfections were performed in OPTI-MEM (Invitrogen, Burlington, Ontario) using Lipofectamine 2000 Transfection Reagent (Invitrogen, Burlington, Ontario) according to the manufacturer’s protocol. All transfections were performed three times, in independent experiments, using different preparations of every tested plasmid.
Overexpression of MTHFR isoforms—Overexpression of DNA constructs harboring the short or long isoforms of MTHFR was performed in E. coli, as previously described (15). The inserts were transferred into the pCMV vector and transiently expressed in Neuro-2a cells after transfection.

Other plasmids—Various plasmids were kindly provided by investigators as indicated: mPERKWT9E10, mIRE1B, mIRE1B9E10 and mIRE1BdelC9E10 by Dr. David Ron, New York Univ. School of Medicine, New York, NY; pCGN-ATF6 (1-373) by Dr. Ron Prywes, Columbia Univ., New York, NY; pcDNA3-Flag-JNK1(APF) by Dr. Roger J. Davis, University of Massachusetts Medical School, Worcester, MA; pRJB/CJUN by Dr. Jawed Alam, Health Sciences Center, New Orleans, LA; and pCMV-CJUN by Dr. Michael J. Birrer, National Cancer Institute, Bethesda, MD.

RNA purification and real time RT-PCR—Total RNA extraction from cultured cells and real-time RT-PCR were performed as previously described (14). Platinum SYBR Green qPCR Supermix-UDG was from Invitrogen (Burlington, Ontario). Generation of single amplicons of the expected sizes was confirmed by polyacrylamide gel electrophoresis and denaturation curves also confirmed amplification of unique products. Specificity of amplifications was verified by cloning and sequencing representative products. No significant amplification was observed with the use of “minus RT controls” (reverse transcriptase omitted during RT) as well as in “no template controls” (omission of cDNA). The amplicon signal for each target cDNA strongly correlated with serial dilution of template (r>0.95). Oligonucleotides for quantitation of Mthfr, Nos2 and Gapdh were described in Pickell et al (14). For detection of Grp78, the PCR primers 5'-GGTTTCTCACTAAAATGAAGGAGA-3' (sense) and 5'-GTACAGTAACAACCTGCAATTTCAA-3' (antisense) were employed for amplification of a 74-bp specific segment. Data analysis and calculations were performed according to the Relative Quantitative Analysis method, using Gapdh as the normalizer target. The internal reference dye was ROX. All analyses were standard procedures of the MX4000 QPCR System (Stratagene, La Jolla, CA).

Analysis of Xbp1 mRNA processing—An RT-PCR assay was designed for visualization of Xbp1 splicing, using primers 5'-AGTACGGCTGGTGCGGGGTCT-3' (sense) and 5'-GAAGATGTCTGAGGGAGGTGAC-3' (antisense), that span the relevant Xbp1 splice site. Products were resolved on 8% polyacrylamide gels. The unspliced form generated an amplicon of 100 bp and a fragment of 74 bp was observed for the processed mRNA. Heteroduplexes were also observed, as seen in a similar experimental design (16).

Western blotting—Cells were collected by scraping in phosphate-buffered saline (PBS). Cell lysis was performed in a lysis buffer (1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS in PBS) in the presence of Complete Mini (protease inhibitor cocktail from Roche Applied Science, Laval, Quebec). After incubation on ice for 30 minutes, lysates were centrifuged (16000 g, 15 minutes, 4°C). Supernatants were collected and protein concentrations assessed. Unless otherwise indicated, 50 µg protein from each lysate were diluted with loading buffer, boiled and loaded onto SDS-polyacrylamide gels. Samples were electrophoresed and proteins were transferred onto nitrocellulose. Relevant antigens were visualized after incubation with rabbit polyclonal primary antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibody and detection as previously described (15). To ensure reproducibility of observations, all Western blot experiments were performed at least twice, using extracts from independent experiments. Antibodies against GRP78, FLAG and β-actin were from Sigma (Oakville, Ontario). Antibodies against eIF2α and phospho-eIF2α were kindly provided by Dr. Nahum Sonenberg, McGill University, Montreal, Quebec. Polyclonal antibody against MTHFR was previously described (15). Other antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

MTHFR enzyme assay—Cytosolic extracts and enzymatic assays were performed as in Tran et al (15). Activities were measured in duplicate and less than 6% variation was observed between repeats.
RESULTS

Up-regulation of Mthfr mRNA by ER stress. To determine whether several well-documented ER stress inducers could alter expression of MTHFR, we treated Neuro-2a cells with tunicamycin, thapsigargin and A23187 (Fig. 1A). As positive controls, we assessed Grp78 (Fig. 1B) and Nos2 (Fig. 1C) mRNA levels. GRP78 is a chaperone protein that is a standard marker of the ER stress response, whereas Nos2 activation can be attributed to activation of NF-κB signaling, since it is abolished when BAY11-7082 (Fig. 1C) or PDTC (data not shown) are administered with tunicamycin. Mthfr, Grp78 and Nos2 mRNA levels were all increased following treatment by the 3 stressors. Bay11-7082 (or PDTC, not shown) did not inhibit the up-regulation of Grp78 and Mthfr mRNAs by tunicamycin (Figs. 1A & B), indicating that these 2 genes are activated by ER stress independently of NF-κB. Similar results were obtained when NF-κB inhibitors were combined with tunicamycin or A23187 instead of tunicamycin (data not shown). We performed the same types of experiments with RAW264.7 cells and reached the same conclusions (data not shown). In addition to Grp78 induction, Xbp1 mRNA processing confirmed the effectiveness of the ER stressors in our experimental system (Fig. 1D).

Hcy induces ER stress and increases Mthfr mRNA levels. Since Hcy adversely affects ER function (5,17), we examined the effects of Hcy on ER stress and on Mthfr mRNA levels. Treatment of Neuro-2a cells with Hcy rapidly increased Grp78 mRNA (Fig. 2B), confirming the induction of ER stress by Hcy in this model system. Mthfr mRNA induction was also observed in response to Hcy treatment in a time- and dose-dependent manner (Fig. 2A). The pattern of up-regulation was similar for both Mthfr and Grp78, supporting their coordinate activation by Hcy-induced ER stress. Similar results were obtained with the Raw264.7 cell line (data not shown). We had observed in an earlier study that Mthfr expression can be induced by NF-κB (14). Cross-talk occurs between ER stress and NF-κB signaling (18), through mechanisms that involve IRE1 and PERK (19, 20). We looked for evidence/absence of involvement of NF-κB in the response of Mthfr to Hcy treatment. Nos2, a gene that is activated by NF-κB (14,21), was not affected by Hcy in these experiments (Fig. 2C), suggesting that Hcy-induced ER stress is not mediated through NF-κB. We verified that Nos2 mRNA levels were increased after LPS treatment of RAW264.7 cells, and that this increase was abolished by PDTC or Bay11-7082, two inhibitors of NF-κB (data not shown, see also 14). However, since the up-regulation of Mthfr and Grp78 by Hcy was not affected by the NF-κB inhibitors (data not shown) and Hcy did not alter expression of Nos2, we conclude that the Hcy-induced increase of ER stress and Mthfr is not mediated by NF-κB.

Up-regulation of MTHFR protein by ER stress. Since Mthfr mRNA levels were increased by ER stress, we questioned whether MTHFR protein levels were also affected. We used tunicamycin or thapsigargin to study the effect of ER stress on MTHFR protein levels, in Neuro-2a and in RAW264.7 cells (Fig. 3A). ER stress is significantly induced in these 2 cell lines in the presence of tunicamycin and thapsigargin, as verified by increased levels of GRP78 (Fig. 3A) and processing of Xpb1 (Fig. 3B). As expected, we obtained complex Western blot patterns for MTHFR since there are two possible protein isoforms (with apparent M.W. of 70 or 77 kDa (15)) and the shorter MTHFR isoform has been shown to undergo phosphorylation (13). The stress inducers clearly increased the amount of GRP78 as well as the intensity of the MTHFR protein (short isoform of 70 kDa, phosphorylated (SP) and non-phosphorylated (S) forms). The identity in Fig. 3A of the short MTHFR subunit, with or without phosphorylation, was deduced by overexpression of the short and long isoforms in Neuro-2A cells, or by mixing bacterial extracts containing the overexpressed short and long isoforms with the Neuro-2A cells (Fig. 3C & Supplemental Fig. 1A). We observed that overexpression of the short isoform after transfection into Neuro-2a cells generates
mainly the phosphorylated form (Fig. 3C, Mock+S & Supplemental Fig. 1A), with smaller amounts of the non-phosphorylated protein. Transfection of Neuro-2a cells with a construct expressing the long MTHFR isoform (Fig. 3C, Mock+L) demonstrated that the long isoform of 77 kDa migrates slightly above the phosphorylated form of the short isoform (Fig. 3C, Mock +S) and that this long isoform does not appear to be efficiently expressed in Neuro-2a cells. We also confirmed, as initially reported (13), the identity of the phosphorylated and non-phosphorylated 70 kDa isoforms by treatment with alkaline phosphatase (Fig. 3D).

Mixing of protein extracts from Neuro-2a cells with bacterial extracts containing the overexpressed short and long isoforms of MTHFR (Supplemental Fig. 1B) supported our conclusions on the banding pattern of the 2 isoforms and confirmed that the long MTHFR isoform was not expressed in the cell lines used in the present study. Occasionally we observed a band that migrated slower than the long isoform (labeled with an asterisk in Fig. 3D). It is possible that this faint band represents the phosphorylated form of the long isoform but phosphorylation of the long isoform has not been examined by any group thus far. We also occasionally detected a band that migrated faster than the non-phosphorylated small isoform (labeled with 2 asterisks in Fig. 3D). This unidentified band is likely to be a degradation product of MTHFR, since a band of similar size was observed in Yamada et al (13) and, in our studies, this protein appeared to increase in intensity under circumstances that increased the amount of the 70 kDa MTHFR protein (as shown in Fig. 3A).

Effect of individual ER stress transducers/ transcription factors. To determine which branch of ER stress - PERK, ATF6 or IRE1- was involved in MTHFR activation, we used plasmids that overexpressed the relevant transducers/transcription factors. Cells overexpressing PERK or IRE1 accumulate large quantities of these proteins, which then dimerize and are activated, even in the absence of ER-stress (22,23). We used a N-terminal derivative of ATF6, which lacks the transmembrane and C-terminal regions of the original protein. This truncated and activated form of ATF6 (aa 1-373) results in a protein that localizes entirely in the nucleus, even in the absence of ER stress (24).

We did not observe an increase in MTHFR expression after transfection of PERK and ATF6 constructs (Figs. 4A & B, respectively); confirmation of overexpression of the plasmids was performed by immunoblotting for the c-Myc and HA tags for PERK and ATF6, respectively. Furthermore, the induction of eIF2α phosphorylation (Fig. 4A) and the increased levels of GRP78 (Fig. 4B) confirmed the functionality of the expression plasmids for PERK (1) and ATF6 (25), respectively. In contrast, MTHFR protein levels increased when Neuro-2a cells were transfected with plasmids overexpressing IRE1 (Fig. 4C). Similar results were obtained whether native IRE1 or a c-Myc tagged IRE1 were transfected; overexpression of the latter plasmid was confirmed using a c-Myc antibody (Fig. 4C). The overexpression of IRE1 also increased levels of GRP78 and phosphorylated c-Jun (p-c-Jun, its active form; Fig. 4C), as previously reported (23, 26). The degree of increase in GRP78 and c-Jun expression varied depending on transfection conditions (Fig. 4D, +, ++ or +++ lanes). The optimal transfection conditions for c-Jun were also associated with higher levels of MTHFR expression.

A vector expressing a dominant-negative form of IRE1 (IRE1delC) partially abolished the effect of tunicamycin (Fig. 4E), as well as the effect of Hcy (Fig. 4F) on MTHFR protein levels. IRE1delC also attenuated the basal expression of MTHFR (Figs. 4E & F). Our assay for Xbp1 processing showed detectable levels of ER stress in our basal conditions (particularly visible in the top panels of Figs. 3B & 4G); this is likely to contribute to the decrease in basal MTHFR expression by IRE1delC. The dominant-negative IRE1 mutant efficiently eradicated the tunicamycin- and Hcy-induced increases of p-c-Jun (Figs. 4E & F, respectively). Evaluation of Xbp1 processing after Hcy treatment (Fig. 4G) demonstrated increased splicing in the presence of Hcy; this finding is consistent with the proposed IRE1 activation by Hcy since Xbp1 is downstream of IRE1 in ER signaling (1).
Hcy appeared to inhibit the phosphorylation of the short MTHFR isoform (Fig. 4F) since the Hcy-induced increase in MTHFR protein was largely limited to an increase in the non-phosphorylated band; this pattern is distinct from that seen with the other stress inducers or IRE1, which did not restrict activation to the non-phosphorylated short isoform (as shown in Figs. 3A, 4C & D). The Hcy-dependent decrease in MTHFR phosphorylation is consistent with the findings of Yamada et al. (13) who suggested that the phosphorylation of MTHFR is decreased by a low S-adenosylmethionine/S-adenosylhomocysteine ratio.

Effect of other regulators of p-c-Jun. To further address the involvement of c-Jun in the up-regulation of MTHFR, we investigated the effects of LiCl and valproate, which can increase the levels of active c-Jun (27,28). We also examined SP600125, a synthetic, classic inhibitor of SAPK/JNK (which activates c-Jun by phosphorylation (29)). Treatment of cells with LiCl and valproate enhanced the levels of p-c-Jun and MTHFR without an increase in GRP78 (Fig. 5A), suggesting that p-c-Jun can affect MTHFR expression without an increase in ER stress. These compounds in combination with tunicamycin increased both p-c-Jun and GRP78, with a concomitant increase in MTHFR. LiCl increased the amount of the non-phosphorylated MTHFR protein, as mentioned above for Hcy, suggesting that it might also affect phosphorylation of MTHFR. The protein sequence encompassing MTHFR phosphorylation sites (13) resembles a glycogen synthase kinase-3 (GSK-3) target sequence (30). LiCl is commonly used for inhibition of GSK-3β (31) but may be a potent inhibitor of other protein kinases (32); therefore a LiCl-sensitive kinase may be involved in MTHFR phosphorylation.

Incubation of Neuro-2a cells with SP600125, alone or in combination with tunicamycin and/or LiCl, decreased the amount of p-c-Jun, as expected, and also decreased the global amount of MTHFR protein (Fig. 5B). SP600125 also reduced the levels of Mthfr mRNA, in the presence and absence of tunicamycin (Fig. 5C). However, because this inhibitor also increased the ratio of non-phosphorylated/phosphorylated MTHFR (Fig. 5B), as did LiCl, it is possible that SP600125 affects the unidentified kinase(s) responsible for MTHFR phosphorylation, since it does not exclusively inhibit SAPK/JNK (32).

Hcy increases MTHFR protein levels and the non-phosphorylated/phosphorylated MTHFR ratio. As shown in Fig. 2 and Fig. 4F, Hcy induced ER stress and MTHFR expression in Neuro-2a cells, possibly through a c-Jun-dependent mechanism. To further explore the effects of Hcy, we first confirmed that the effect was specific to this amino acid, by examining other sulphur amino acids – D,L-cysteine and D,L-methionine. Incubation with these other amino acids did not increase GRP78 levels (Fig. 6A); L-cysteine and L-methionine also had no effect (data not shown). The increase in Hcydependent ER stress was accompanied by an increase in the non-phosphorylated MTHFR isoform. Additional experiments (Fig. 6B), comparing the effects of Hcy and LiCl separately and in combination, demonstrated that both compounds increased the non-phosphorylated:phosphorylated MTHFR ratio. The proposed decrease in MTHFR phosphorylation could be dissociated from an increase in ER stress, since LiCl affected MTHFR phosphorylation without an effect on GRP78.

The effects of Hcy and SP600125, alone or in combination, were also studied (Figs. 6C & D). Hcy alone induced ER stress (increased GRP78 levels), increased p-c-Jun protein levels, and increased MTHFR mRNA and protein; the increase in the non-phosphorylated:phosphorylated MTHFR ratio was also observed. SP600125 alone had no effect on ER stress but also increased the non-phosphorylated:phosphorylated MTHFR ratio, presumably by modulating the kinase that phosphorylates MTHFR. The combination of Hcy and SP600125 limited the p-c-Jun increase, blocked MTHFR induction at the mRNA and protein levels, and also affected MTHFR phosphorylation. These experiments supported our hypothesis that Hcy mediates ER stress and increases MTHFR expression through c-Jun. They also confirmed that Hcy, LiCl and SP600125 affect MTHFR phosphorylation.
although the nature of the kinase(s) remains to be determined.

The non-phosphorylated isoform of MTHFR was proposed to be more active than the phosphorylated isoform (13). Table I shows that MTHFR activity is increased by tunicamycin, LiCl and Hcy under the same conditions in which MTHFR protein levels were measured; these results indicate that the modulation of MTHFR protein levels by these compounds is consistent with their modulation of enzyme activity.

Regulation of p-c-Jun by transfection. To directly examine the regulation of MTHFR by c-Jun, Neuro-2a cells were transfected with 2 c-Jun plasmids, pRJB/CJUN or pCMV-CJUN (Fig. 7A). A marked increase in total c-Jun as well as in p-c-Jun (active form of the transcription factor) was observed. These findings suggest that Neuro-2a cells can phosphorylate large amounts of c-Jun protein. This is not surprising since the levels of activated SAPK/JNK enzyme are substantially higher in cells of neuronal origin or in the brain than in peripheral organs (27), even if total SAPK/JNK protein levels are similar (33). Transfection with the c-Jun plasmids resulted in an increase of MTHFR, more specifically in the phosphorylated isoform, since we did not add any of the previously-mentioned compounds that can inhibit MTHFR phosphorylation (Hcy, LiCl or SP600125). Transfection with these c-Jun constructs also increased Mthfr mRNA levels (Fig. 7B).

To confirm the role of c-Jun in MTHFR expression, we also used a nonphosphorylatable mutant JNK1, in which the canonical T-P-Y activation motif has been mutated to A-P-F (34). Overexpression of this dominant-negative mutant JNK1, confirmed by immunodetection of the FLAG epitope (Figs. 7E & F), resulted in a reduction of Mthfr mRNA levels (Figs. 7C & D) and limited Mthfr mRNA induction by tunicamycin (Fig. 7C) or Hcy (Fig. 7D). The kinase-inactive JNK also caused a marked decrease of MTHFR protein and limited the up-regulation of MTHFR protein by tunicamycin (Fig. 7E) or Hcy (Fig. 7F).

DISCUSSION

When mammalian cells are subjected to a variety of physiological stress conditions that target the ER, the cells respond by activating a defense mechanism referred to as the UPR. This response modulates several transcriptional and translational pathways, which include induction of stress-response genes, such as GRP78. In the present study, we identified ER stress as a novel inducer of Mthfr gene expression in Neuro-2a cells. ER stress induction causes an increase in Mthfr mRNA levels and in MTHFR protein levels. We propose that IRE1 is involved in the signal transduction after ER stress and that c-Jun acts downstream of IRE1 in the context of MTHFR regulation. This concept is based on several lines of evidence. We found that MTHFR was induced by diverse conditions that cause ER stress, such as exposure to Hcy, to tunicamycin (which inhibits N-linked glycosylation) and to thapsigargin or A23187 (which disrupt calcium homeostasis in the ER). Mthfr mRNA induction paralleled that of the ER stress gene Grp78. Overexpression of a dominant-negative IRE1 abolished the MTHFR response to tunicamycin or to Hcy treatment. Overexpression of IRE1 and c-Jun mimicked the effect of ER stress inducers on MTHFR levels. After treatment of cells with relevant agents (LiCl, tunicamycin, and valproate), the levels of active c-Jun correlated with levels of MTHFR. Inhibition of c-Jun signalling blocked the increase in MTHFR expression. Additional studies are required to determine whether c-Jun exerts its effect directly on the Mthfr promoter or through an indirect mechanism.

We cannot exclude the possibility that other branches of the UPR besides IRE1 may contribute to the MTHFR response to ER stress. Similarly, we cannot rule out intermediates other than c-Jun, downstream of IRE1, that might influence MTHFR expression.

Hcy has been shown to induce a pathway that can lead to apoptosis after activation of the UPR, through mediation by the transmembrane protein IRE1 (35). The cytoplasmic portion of IRE1 can interact with TRAF2 (26), an adaptor protein involved in activation of the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK). These results and the findings in Cai et al (36) suggest that Hey can increase the amount of p-c-
Jun. Several studies have described the physiological relevance of these observations. For example, Grp78 mRNA levels are elevated in vivo in hyperhomocysteinemic mice (37). In vitro as well as in vivo studies have shown that Hcy-induced ER stress leads to transcriptional activation of genes involved in lipogenesis; this association contributes to hepatic steatosis in hyperhomocysteinemia (38). Robert et al (39) reported that the SAPK/JNK transduction cascade is activated in vivo in the brains of hyperhomocysteinemic mice, resulting in a higher amount of p-c-Jun. Ramaswami et al (40) observed that curcumin (an inhibitor of c-Jun) blocks the Hcy-induced impairment of endothelium-dependent vasorelaxation.

Results from microarray analysis of Mthfr−/− mice suggested that neuronal damage by severe hyperhomocysteinemia involves disruption of intracellular Ca2+ (41). An elevation of Hcy can activate the SAPK/JNK cascade through overstimulation of glutamate receptors and a disturbance of calcium homeostasis (refs in 39). Our observations do not exclude the possibility that Hcy can activate the c-Jun pathway by mechanisms other than ER stress or that factors other than c-Jun (e.g. ATF6, PERK or XBP1) may be required for optimal MTHFR response to ER stress.

In addition to transcriptional regulation, cells under ER stress control the translational capacity, protecting against further accumulation of unfolded proteins in the ER but allowing synthesis of proteins essential for cell survival. Translational inhibition occurs through phosphorylation of the α-subunit of eukaryotic initiation factor 2, catalyzed by PERK (22). GRP78 mRNA can still be translated when general, cap-dependent translation of mRNAs is inhibited, indicating that cap-independent translation initiated at an internal ribosome entry site (IRES) is utilized (42). It would be interesting to address the molecular mechanisms that facilitate the translation of Mthfr. Its long, GC-rich 5'UTR, containing multiple upstream ATGs (15), might possess structural features that permit its effective translation during ER stress. Translation of methionine synthase, the enzyme that functions immediately downstream of MTHFR, by transferring the methyl group from 5-methyltetrahydrofolate to homocysteine for the synthesis of methionine, is influenced by a functional IRES element (43). It has also previously been reported that methionine synthase mRNA levels are increased by treatment of cultured cells with Hcy (44).

MTHFR phosphorylation sites lie in a protein sequence segment similar to that of a putative GSK-3 consensus motif (13). There is no strict consensus motif for substrate phosphorylation by GSK-3, but many GSK-3 substrates require prior phosphorylation to form the motif –S/T-X-X-X-S/T(P)– before phosphorylation by GSK-3 is possible (45). The downstream phosphorylated residue Thr-34 in MTHFR plays an important role as the priming phosphorylation site (13). We observed that MTHFR phosphorylation is sensitive to the GSK-3 inhibitor lithium (30), supporting the concept that the kinase(s) regulating MTHFR shares some properties with GSK-3. Protein phosphorylation is one of the most important modifications for cellular regulation. Greater understanding of the significance of MTHFR phosphorylation will require identification of the kinase(s) and phosphatase(s) responsible for its reversible modification. We observed that the ratio of non-phosphorylated/phosphorylated MTHFR is increased by Hcy, LiCl and SP600125. It is interesting to note that differential display analysis, in addition to revealing the increased expression of GRP78, identified RTP (reducing agents and tunicamycin-responsive protein) as a novel up-regulated gene after Hcy treatment of cultured human umbilical vein endothelial cells (17). RTP, a soluble protein induced by ER stress, was shown to be phosphorylated at several sites, and Hcy treatment of cells resulted in dephosphorylation of RTP (46). Since many of the features displayed by RTP were observed for MTHFR, it would be interesting to determine whether both enzymes are modified by the same kinase(s) and phosphatase(s).

It is not surprising that Hcy might increase MTHFR expression in an effort to reduce hyperhomocysteinemia and therefore limit the Hcy-induced stress. The increase in MTHFR expression by the more classic ER stress inducers is more intriguing and suggests that MTHFR, a protein that has no known direct involvement in ER function, has a role to play in
the UPR. Protein secretion irreversibly depletes intracellular amino acid pools and ER stress has already been noted to activate some genes involved in amino acid import and metabolism (47 and refs therein). Up-regulation of MTHFR by ER stress could contribute to amino acid sufficiency by supplying methionine for protein synthesis, to allow the cell to adapt to the metabolic consequences of high ER activity. Alternatively, increased MTHFR expression would increase methionine levels for production of SAM and methylation reactions. This increase could contribute to the general increase in DNA methylation induced by homocysteine (48) or to specific methylation changes associated with ER stress, such as the methylation modification of nucleosomes at the GRP78 promoter (49). ER stress can induce the UPR, which can lead to cell death as well as to prosurvival mechanisms.

The interplay between these processes has clinical implications in neurodegenerative diseases, atherosclerosis and cancer (38,50). MTHFR deficiency has been shown to modify risk for atherosclerosis, cancer, and birth defects; methyl group metabolism may also have a role to play in neurodegenerative diseases. Our finding of MTHFR up-regulation by ER stress through IRE1 is compatible with a role for MTHFR in the recovery from stress and in the fine balance that exists between cell death and survival. IRE1 signaling enhances cell viability shortly after the onset of ER stress (51). Cells with a deficiency in MTHFR may not be able to recover optimally from ER stress or other forms of cellular injury; this could contribute to the wide spectrum of clinical consequences of MTHFR deficiency.

REFERENCES


FOOTNOTES

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1 The abbreviations used are: ATF6, activating transcription factor 6; ER, endoplasmic stress; IRE1, inositol-requiring enzyme-1; IRES, internal ribosome entry site; LPS, lipopolysaccharide; MTHFR, methylenetetrahydrofolate reductase; PDTC, pyrrolidinethiocarbamate; PERK, pancreatic ER stress kinase; SAPK/JNK, stress-activated protein kinase/c-Jun N-terminal kinase; UPR, unfolded protein response; XBP1, X-box binding protein-1.

FIGURE LEGENDS

**FIG. 1. Mthfr mRNA is up-regulated by ER stress.** Neuro-2a cells were treated for 6 hours with tunicamycin (Tuni, 5 µg/ml), thapsigargin (Thapsi, 2 µM) or A23187 (3 µM). When Bay11-7082 (Bay) and tunicamycin were used in combination, cells were pre-incubated for 1 hour with Bay11-7082 (25 µM) before addition of tunicamycin. Relative quantitation of Mthfr (A), Grp78 (B) or Nos2 (C) mRNAs was performed by real-time RT-PCR, using Gapdh as the normalizer. Measurements were performed in quadruplicate in three independent experiments. Results are presented as means ± S.E. Asterisks indicate a significant difference (two-tailed t-test, *p<0.05 and **p<0.01) between treated cells and mock. Neuro-2a cells were treated as above, and Xbp1 mRNA processing was examined by RT-PCR (D). The positions of amplicons specific for the full-length (F) and processed (P) Xbp1 mRNA are indicated. The position of the heteroduplexes (H) is also shown. Gapdh amplification was performed as a control for these samples.

**FIG. 2. Mthfr mRNA is up-regulated by Hcy.** Neuro-2a cells were incubated with 0.2 mM (■), 1 mM (●) or 5 mM (▲) Hcy for the indicated times. Mthfr (A), Grp78 (B) or Nos2 (C) transcripts were measured by real-time RT-PCR and normalized to Gapdh. Measurements were performed in quadruplicate in three independent experiments. Results are presented as means ± S.E. Significant differences with mock (two-tailed t-test) are denoted by asterisks (*p<0.05, **p<0.01).

**FIG. 3. ER stress increases MTHFR protein levels.** A, Neuro-2a (N2a) or RAW264.7 cells were exposed to tunicamycin (Tuni, 5 µg/ml) or thapsigargin (Thapsi, 2 µM) for 18 hours and extracts were processed by Western blotting; mock-treated cells (Mock) were processed in parallel. Identity of bands, indicated on the right side of the figure as SP (short phosphorylated isoform) and S (short non-phosphorylated isoform) were deduced from analysis of immunoblots for MTHFR in Figs. 3C & D, and from Supplemental Fig. 1. Results shown for Neuro-2a and Raw264.7 cells are from different sections of the same blot. B, Xbp1 mRNA processing was tested as in Fig. 1D. The top panel shows a longer electrophoresis of the same samples, performed to reveal the two expected heteroduplexes (16). This image was inverted for increased resolution. C, Proteins from Neuro-2a cells were separated after mixture with 0.1 µg protein from another preparation of Neuro-2a cells that were mock-transfected or transfected with a plasmid overexpressing the MTHFR short (+ S) or long (+ L) isoform. 50 µg protein were loaded per lane. A longer exposure of the first lane is shown. D, 25 µg protein from tunicamycin-treated cells were compared to mock-treated cell extracts (50 µg) that were exposed or not to alkaline phosphatase. *, weak unidentified band; **, possible degradative product of MTHFR.
FIG. 4. **IRE1 is involved in the activation of MTHFR by ER stress.** A, Neuro-2a cells were mock-transfected or transfected with a vector expressing PERK (c-Myc tag). After 48 hours, cell lysates were prepared, separated on SDS-polyacrylamide gels and blotted for MTHFR, c-Myc, p-eIF2α, eIF2α and actin. B, Cells were treated as described above, but transfection was performed with a vector expressing ATF6 (HA epitope). For PERK and ATF6, similar results were obtained 24 or 72 hours after transfection. C, Cells were transfected with a vector expressing IRE1 (no tag) or c-Myc tagged IRE1. Indicated epitopes were analyzed by Western blotting. D, c-Myc tagged IRE1 was transfected at 1:10, 1:5 or 1:2.5 μg DNA: μl lipofectamine ratios and analysis was performed as in C. E, Cells were mock-transfected or transfected with a dominant-negative version of IRE1 (IRE1delC) tagged with c-Myc. 22 hours after transfection, cells were treated with 5 µM Hcy for a period of 7 hours. Cell lysates were analyzed by Western blotting as above. G, *Xbp1* processing was examined after Hcy treatment. The positions for full-length (F) *Xbp1*, processed (P) *Xbp1* and heteroduplexes (H) are indicated. Amplification of *Gapdh* is also presented as a control. Grouping of images from different sections of the same blots or gel is shown by a vertical dividing bar.

FIG. 5. **Regulators of the p-c-Jun pathway modulate MTHFR levels.** A, Neuro-2a cells were incubated with 20 mM LiCl (Li), 1 mM valproate (VPA) or 5 µg/ml tunicamycin (Tuni) for 18 hours. In combination treatments, LiCl or valproate was added one hour before the addition of tunicamycin. B, Neuro-2a cells were incubated with 50 µM SP600125 (SP600), 5 µg/ml tunicamycin or 20 mM LiCl for 18 hours. In combination treatments, SP600125 or LiCl was added 90 minutes or one hour, respectively, before the addition of tunicamycin. Non-adjacent lanes of the same blot are separated by a black line. C, Neuro-2a cells were treated for 6 hours with tunicamycin (Tuni, 5 µg/ml) or 50 µM SP600125 (SP600). SP600125 was introduced 90 minutes before tunicamycin. Relative quantitation of Mthfr mRNA was performed by real-time RT-PCR and normalized to *Gapdh*. Measurements were performed in quadruplicate in three independent experiments. Results are presented as means ± S.E. *p<0.05, **p<0.01, two-tailed t-test compared with mock.

FIG. 6. **Effect of Hcy on MTHFR levels.** A, Neuro-2a cells were incubated for 7 hours with 5 mM DL-Hcy (Hcy), DL-cysteine (Cys) or DL-methionine (Met). B, Neuro-2a cells were incubated with 5 mM DL-Hcy for 7 hours. The treatment with 20 mM LiCl (Li) was initiated one hour before the addition of Hcy. Non-adjacent lanes of the same blot are separated by a black line. C, Cells were exposed to 5 mM DL-Hcy or to 50 µM SP600125 (SP600). In the combination experiment, SP600125 was added one hour before the 7-hour incubation with Hcy. D, Neuro-2a cells were incubated with 5 mM Hcy for 2 hours or with 50 µM SP600125 (SP600). In the combination experiment, SP600125 was introduced one hour before Hcy. Mthfr transcripts were measured by real-time RT-PCR and normalized to *Gapdh*. Measurements were performed in quadruplicate in three independent experiments. Results are presented as means ± S.E. Significant differences with mock (two-tailed t-test) are indicated (*p<0.05, **p<0.01).

FIG. 7. **Effect of exogenous c-Jun and a dominant-negative JNK1 mutant on MTHFR expression.** A, Neuro-2a cells were transfected with the indicated plasmids and protein extraction was performed 48 hours post-transfection. B, Mthfr mRNA levels were analyzed 48 hours after transfection of Neuro-2a cells with the indicated plasmids. C, Neuro-2a cells were incubated for 6 hours with tunicamycin (Tuni, 5 µg/ml), 48 hours after transfection with pcDNA3-Flag-JNK1(APF), as indicated (DN). D, Neuro-2a cells were incubated for 2 hours with 5 mM Hcy, 48 hours after transfection with pcDNA3-Flag-JNK1(APF), as indicated (DN). Mthfr mRNA levels in panels B-D were normalized to *Gapdh* expression. Measurements were performed in quadruplicate in three independent experiments. Results are presented as means ± S.E. Significant differences with mock (two-tailed t-test) are shown by asterisks (*p<0.05, **p<0.01). E, Neuro-2a cells were incubated for 18 hours with 5 µg/ml tunicamycin (Tuni), 30 hours after
transfection with pcDNA3-Flag-JNK1(APF). The vertical line separates non-adjacent lanes of the same blot. F, Neuro-2a cells were incubated for 7 hours with 5 mM DL-Hcy. This treatment was initiated 41 hours after transfection with pcDNA3-Flag-JNK1(APF). In panels A, E and F, Western blot analyses were carried out using antibodies directed against the indicated proteins.

Supplemental FIG. 1. Analysis of MTHFR by Western blotting. A, Proteins from Neuro-2a cells treated with thapsigargin or mock-treated cells were analyzed. Proteins from mock-treated cells were mixed or not with 0.1 µg of a protein extract from Neuro-2a cells overexpressing the short MTHFR isoform [+S (transf.)]. B, Protein from Neuro-2a cells was mixed with 0.3 µg protein extracted from *E. coli* extracts overexpressing the short (S) and long (L) isoforms of human MTHFR (Mock +S+L). For direct comparison of bands, extracts from thapsigargin or tunicamycin–treated cells were processed in parallel. *, weak unidentified band; **, possible degradative product of MTHFR.
**TABLE 1**

MTHFR activity after treatment with tunicamycin, DL-Hcy or LiCl.

Neuro-2a cells were treated as described in legends to Figs. 4 and 5.

<table>
<thead>
<tr>
<th>Effect of tunicamycin(^a) and/or LiCl(^b)</th>
<th>Effect of Hcy(^c) and/or LiCl(^b)</th>
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<tr>
<td>Treatment</td>
<td>Activity(^d)</td>
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<tr>
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\(^a\) Neuro-2a cells were treated with tunicamycin (5 µg/ml) for 18 hours before extraction of cytosolic proteins.

\(^b\) LiCl (20 mM) was added one hour before the initiation of treatment with tunicamycin or Hcy.

\(^c\) Cell treatment with 5 mM DL-Hcy was performed for 7 hours.

\(^d\) MTHFR enzyme activity is expressed as nmol formaldehyde/mg protein/hr.
Figure 1

A

**Mdhfr**

Mock  | Tuni  | Thapsi | A23187 | Tuni+Bay | Bay

B

**Grp78**

Mock  | Tuni  | Thapsi | A23187 | Tuni+Bay | Bay

C

**Nos2**

Mock  | Tuni  | Thapsi | A23187 | Tuni+Bay | Bay

D

**Xbp1**

Blank  | Mock  | Tuni  | Thapsi | A23187 | Tuni+Bay | Bay | Blank

**Gapdh**
Figure 3

A  
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<tr>
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B  
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C  
|       |       |       |
|       | Mock (longer exp.) | Mock+L (transfec.) |

D  
<table>
<thead>
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<th>Alk. Phos.</th>
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Xbp1
Gapdh

*  
**