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Glucose Deprivation Induces Heme Oxygenase-1 Gene Expression by a Pathway Independent of the Unfolded Protein Response*

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Se-Ho Chang‡§, Ione Barbosa-Tessmann¶‖, Chin Chen¶, Michael S. Kilberg¶, and Anupam Agarwal‡¶****

From the ‡Department of Medicine, Division of Nephrology, Hypertension & Transplantation and ¶Department of Biochemistry and Molecular Biology, University of Florida, Gainesville, Florida 32610

Nutrients such as glucose regulate the expression of genes that are involved in plasma membrane transport, metabolic functions, and protein trafficking in the endoplasmic reticulum. Depletion of nutrients results in cellular stress, which evokes adaptive and protective responses, one of which is the induction of heme oxygenase-1 (HO-1), a 32-kDa endoplasmic reticulum enzyme that catalyzes the rate-limiting step in heme degradation. Incubation of HepG2 human hepatoma cells in glucose-free medium resulted in an increased HO-1 mRNA content, reaching a maximum of ~25-fold over control cells after 12 h. The glucose-dependent induction of HO-1 mRNA was concentration-dependent ($k_{1/2}$ ~0.5 mM) and was attenuated by fructose, galactose, mannose, and 2-deoxyglucose, but not by the non-metabolizable glucose analog, 3-*O*-methylglucose. Tunicamycin, thapsigargin, or azetidine 2-carboxylate, each of which activates the unfolded protein response pathway, did not induce HO-1 mRNA expression, whereas glucose-regulated protein 78 mRNA was increased. These results demonstrate that glucose availability regulates transcription of the HO-1 gene via a pathway that is different from the unfolded protein response. The induction of HO-1 may serve as a protective response in hypoglycemic circumstances and underscores the importance of understanding nutrient control of the HO-1 gene.

Regulation of gene expression by nutrients is an important mechanism in the adaptation of mammals to their environment (1, 2). Starvation for amino acids is known to activate many genes involved in amino acid biosynthesis and other cellular processes (2). Additionally, a decrease in cellular carbohydrate levels causes the transcriptional regulation of a number of genes that encode proteins associated with endoplasmic reticulum stress (1), for example, the glucose-regulated proteins (GRP)[1] 78 and 94 and the transcription factor GADD153/CHOP (for growth arrest and DNA damage-inducible gene, C/EBP-homologous protein; reviewed in Ref. 3). Collectively, the changes following glucose starvation are the result of a cellular recognition of abnormal protein accumulation within the endoplasmic reticulum (ER), called the unfolded protein response (UPR) in yeast, also known as the ER stress response in mammalian cells (3). The transcription of this same set of genes can be increased by treatment of cells with other agents that cause ER stress, the glycoprotein biosynthesis inhibitor tunicamycin (4), the calcium ATPase inhibitor thapsigargin (5), or with amino acid analogs that incorporate and cause improper folding, such as the proline analog azetidine-2-carboxylate (Aze) (6).

Cellular heme derived from ubiquitously disposed heme-containing proteins, such as hemoglobin, myoglobin, cytochromes, peroxidases, catalase, nitric-oxide synthases, and respiratory burst enzymes, is degraded by the heme oxygenase enzyme (7–9). In the presence of O_2 and NADPH, the heme oxygenase enzyme opens the heme ring liberating equimolar quantities of biliverdin, iron, and carbon monoxide (CO). Three isoforms of heme oxygenase, HO-1, HO-2, and HO-3, have been characterized. HO-1 and HO-2 are known to share ~43% amino acid homology (10), and HO-3 shares ~90% amino acid homology with HO-2 and exhibits only low enzyme activity (11). HO-1 is inducible and ubiquitously distributed in mammalian tissues, and HO-2 is constitutively expressed in several tissues (7–9). It has been suggested that HO-2 may function as a physiologic regulator of cellular function, whereas HO-1 plays a role in modulating tissue responses to injury in pathophysiologic states (12). HO-1 is induced by heme products and a wide variety of non-heme stimuli, which include hydrogen peroxide, ultraviolet A radiation, heavy metals, endotoxin, cytokines, growth factors, oxidized low density lipoprotein, and others (9). The induction of HO-1 is thought to be a beneficial response that offers cytoprotection to cells/tissue against oxidant stress (13, 14).

The purpose of this study was to evaluate the effect of glucose availability on the expression of HO-1. We observed that glucose deprivation, over a physiological range of concentrations, resulted in a significant induction of HO-1 mRNA content in HepG2 human hepatoma cells. Induction of HO-1 by glucose deprivation was reversed by the presence of specific sugars including 2-deoxyglucose (2-DOG), but not by the non-metabolizable glucose analog, 3-*O*-methylglucose (3-OMG). Activation of the UPR pathway by tunicamycin, thapsigargin, or

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§ Assistant Professor of Internal Medicine at, and partially supported by, the College of Medicine, Gyeongsang National University, Chinju, Korea.

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** To whom correspondence should be addressed: Division of Nephrology, Hypertension and Transplantation, Box 100224 JHMHC, 1600 S.W. Archer Rd., University of Florida, Gainesville, FL 32610. Tel.: 352-392-4008; Fax: 352-392-3581; E-mail: agarwal@nersp.nerdc.ufl.edu.

[1] The abbreviations used are: GRP, glucose-regulated protein; GRP*n*, glucose-regulated protein of *n* kDa; ANOVA, analysis of variance; Aze, azetidine-2-carboxylate; 2-DOG, 2-deoxyglucose; ER, endoplasmic reticulum; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HO, heme oxygenase; MEM, minimum essential medium; 3-OMG, 3-*O*-methylglucose; PBS, phosphate-buffered saline; UPR, unfolded protein response.

Aze caused significant up-regulation of GRP78 mRNA, but did not induce HO-1. Removal of glutamine from the medium blocked the induction of HO-1 by glucose deprivation, suggesting that the generation of reactive oxygen species via intermediates of the tricarboxylic acid cycle may result in HO-1 induction. These results demonstrate that glucose availability regulates expression of HO-1 and that glucose deprivation can activate transcription via a pathway that is different from the UPR. We speculate that the induction of HO-1 may serve as a protective response in circumstances wherein cells are deprived of glycolytic substrates, such as glucose, and underscores the importance of further studies to understand the nutrient control of the HO-1 gene.

EXPERIMENTAL PROCEDURES

Reagents—Tissue culture media, fetal bovine serum (FBS), and media supplements were obtained from Invitrogen and Cellgro (Herndon, VA). Tunicamycin, Aze, 2-DOG, 3-OMG, fructose, mannose, and galactose were purchased from Sigma. Thapsigargin was obtained from Calbiochem (San Diego, CA). Anti-HO-1 antibody (SPA-895) was purchased from StressGen (Victoria, British Columbia, Canada). The anti-GRP78 antibody and cDNA was a kind gift from Dr. Susan Frost (University of Florida, Gainesville, FL).

Cell Culture and Treatments—Human hepatoma (HepG2) cells were obtained from American Type Culture Collection and were cultured in minimal essential medium (MEM), pH 7.4, supplemented with $NaHCO_3$ (25 mM), glutamine (4 mM), streptomycin sulfate (10 μg/ml), penicillin G (100 μg/ml), gentamicin (28.4 μg/ml), *N*-butyl-*p*-hydroxybenzoate (0.23 μg/ml), bovine serum albumin (0.2%), and FBS (10%). Cells were maintained at 37 °C in 95% air and 5% carbon dioxide in T-75 flasks. To induce expression of the HO-1 gene, cells were transferred to either 60- or 100-mm dishes, cultured to near 80% confluence, rinsed two times with PBS, and then incubated in complete MEM or glucose-free MEM, pH 7.4, each supplemented with $NaHCO_3$ (25 mM), glutamine (4 mM), streptomycin sulfate (10 μg/ml), penicillin G (100 μg/ml), gentamicin (28.4 μg/ml), *N*-butyl-*p*-hydroxybenzoate (0.23 μg/ml), and 10% dialyzed FBS. When cells were incubated with tunicamycin, thapsigargin, Aze, 2-DOG, fructose, mannose, galactose, or actinomycin D, they were pretreated for 30 min prior to the addition of complete MEM or glucose-free MEM.

Northern Analysis—Total cellular RNA was extracted from cultured cells grown in 60-mm plates using the method described by Chomczynski and Sacchi (15) and purified using RNeasy mini kits (Qiagen, Valencia, CA). The RNA (15–20 μg) was electrophoresed on a 1% agarose gel containing formaldehyde, electrotransferred to a nylon membrane, and hybridized with a ^{32}P-labeled 1.0-kb human HO-1 cDNA probe (16). The cDNA probe for GRP78 was a 1498-bp sequence between the *Pst*I and *Eco*RI sites (17). The cDNA probe for the ribosomal protein L7a was a 600-bp sequence between two *Pst*I sites that covered a portion of the coding and untranslated regions within the 3′ half of the full-length cDNA. The cDNA probe for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was a 983-bp fragment generated by reverse transcription-PCR using primers from CLONTECH laboratories (Palo Alto, CA). The nylon membranes were stripped and rehybridized with a human L7a cDNA probe or a human GAPDH cDNA probe to control for RNA loading. All experiments were repeated with at least two or three additional independent RNA preparations to show reproducibility. To quantitate expression levels, autoradiographs were scanned on a Hewlett-Packard Scanjet 4C using Deskscan II software and densitometry was performed using NIH Image 1.60 software. Experiments were adjusted for loading using L7a or GAPDH mRNA quantification, then normalized and expressed as arbitrary units.

Immunoblot Analysis—For HO-1 immunoblots, cells were treated with stimulus at the concentrations and times indicated in each figure legend. Cells were then washed twice with ice-cold PBS and lysed in a buffer containing a mixture of protease inhibitors consisting of aprotinin (10 μg/ml), EDTA (5 mM), leupeptin (1 μg/ml), pepstatin A (0.7 μg/ml), phenylmethanesulfonyl fluoride (1 mM), and 0.1% Triton X-100. Protein concentration of lysates was assessed by the bicinchoninic acid assay (Pierce). Samples were separated in a 10% SDS-polyacrylamide gel and then transferred onto a polyvinylidene difluoride membrane. The membranes were incubated for 1.5 h with a 1:500 dilution of the anti-HO-1 antibody, followed by incubation with a 1:10,000 dilution of peroxidase-conjugated goat anti-rabbit IgG antibody (Sigma) for 1 h. Membranes were also probed with an anti-GRP78 antibody (1:10,000 dilution) for 1.5 h, followed by incubation with a 1:10,000 dilution of peroxidase-conjugated goat anti-rabbit IgG antibody. Labeled protein bands were revealed using a chemiluminescence method according to the manufacturer's recommendation (Pierce).

Plasmid Construct, Transient Transfection, and Promoter Analysis—A ~4.5-kb fragment of the 5′-flanking region of the human HO-1 gene, extending from −4.5 kb to +80 bp, was generated by long range PCR performed on human genomic DNA using rTth DNA polymerase (PerkinElmer Life Sciences). The 4.5-kb PCR product was ligated into the TA cloning vector (Invitrogen) and verified by restriction analysis and sequencing (16). *Bam*HI sites were incorporated in the primers to enable subcloning into the *Bgl*II site of the pGL3 basic luciferase vector (Promega, Madison, WI). This promoter-reporter gene construct (pGL3/−4.5) was used for transient expression studies in HepG2 cells grown to ~75% confluence. A plasmid containing *Renilla* luciferase (Promega) was used as a control for transfections. A ratio of 6 μg of DNA (4 μg of −4.5 kb HO-1 and 2 μg of *Renilla* luciferase) per 36 μl of SuperFect reagent (Qiagen) per 6.6×10^6 cells/100-mm dish was constant in each transfection. The cells were washed once with PBS and then incubated with the transfection mixture for 3 h at 37 °C in 5.0 ml of MEM containing 10% FBS and antibiotics. After transfection, cells were washed once with PBS, fresh culture medium was added, and the cells were cultured for 24 h. The 100-mm dishes of HepG2 cells were then split into 12-well plates, and the next day the cells were incubated in either glucose-containing or glucose-free MEM for 16 h. Luciferase activity was measured using a Dual-Luciferase® reporter assay system (Promega).

Agarose Gel Electrophoresis for DNA Fragmentation—DNA fragmentation was performed as a measure of cell injury. HepG2 cells incubated with complete MEM, MEM lacking glucose, or MEM lacking glucose and glutamine for 0, 12, and 24 h in 100-mm plates were collected by scraping and centrifugation at $1000 \times g$ for 5 min at room temperature. Cell pellets were resuspended in 50 μl of TE (10 mM Tris, 10 mM EDTA, pH 8.0), followed by the addition of 900 μl of lysis buffer (TE plus 0.5% Triton X-100), and then incubation on ice for 15 min. The samples were subjected to centrifugation for 10 min and supernatants treated with 50 μg/ml RNase A for 1 h at 37 °C. Proteinase K (100 μg/ml) and 0.5% SDS were added and allowed to incubate for 2 h at 50 °C. Fragmented DNA was extracted using equal volumes of phenol/chloroform and ethanol precipitation. The nucleic acid pellets were resuspended in 30 μl of TE and subjected to 2% agarose gel electrophoresis at 100 V for 1 h.

Data Analysis—Data are expressed as the means ± S.E. Statistical analysis was performed using the Student *t* test or ANOVA and the Student-Newman-Keuls test. All results are considered significant at $p < 0.05$.

RESULTS

Time Course of HO-1 mRNA Induction—HO-1 gene expression is responsive to a wide variety of stress-related stimuli (8, 9). Given the ER stress response induced by glucose deprivation (3, 4), experiments were performed to test the effect of this nutritional stress on HO-1 expression. HepG2 cells were incubated in MEM in the presence or absence of 5.5 mM glucose for 0, 2, 4, 8, and 12 h (Fig. 1*A*) or 0, 4, 12, 18, and 24 h (Fig. 1*B*) before isolation of RNA for Northern analysis. Glucose deprivation-induced HO-1 mRNA content was detectable at 12 h (~25-fold) and remained elevated at 18 and 24 h, respectively (Fig. 1*C*). The blots were reprobed with cDNA probes for GRP78 (as a positive control) and the ribosomal protein L7a (as a negative control). The GRP78 mRNA content was also increased following glucose deprivation (Fig. 1), consistent with previous studies on induction of GRP78 mRNA in HepG2 cells (18). These data illustrate the responsiveness of HO-1 expression to glucose availability, and the lack of a change in L7a mRNA level illustrates that the increased content of HO-1 and GRP78 mRNA was a specific response.

HO-1 Protein Content—To establish whether or not the HO-1 protein content was also increased following glucose deprivation, HepG2 cells were incubated in MEM in the presence or absence of 5.5 mM glucose for 12, 16, 20, or 24 h (Fig. 2). Cells incubated for 20 or 24 h in the absence of glucose had a significant increase in the amount of HO-1 protein. The data clearly show that the increased HO-1 mRNA following glucose

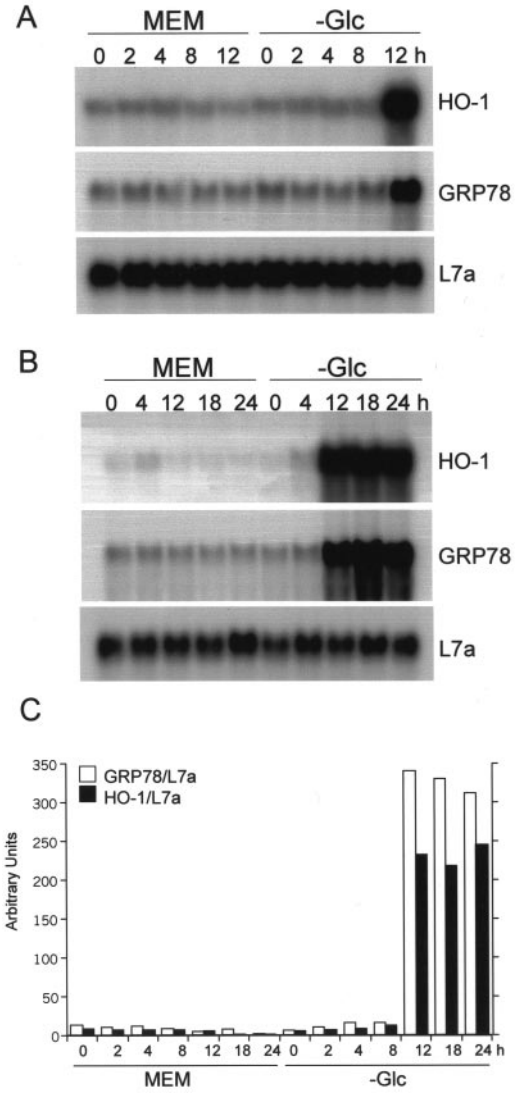

FIG. 1. **Time course of HO-1 mRNA induction in HepG2 cells cultured in the presence or absence of glucose.** HepG2 cells were cultured in supplemented MEM containing 10% FBS and grown to 80% confluence in 60-mm dishes, as described under "Experimental Procedures." At time 0, cells were transferred to glucose-containing MEM (5.5 mM) or glucose-free MEM (*−Glc*), and total RNA was isolated at 0, 2, 4, 8, and 12 h (*A*), or at 0, 4, 12, 18, and 24 h (*B*). Northern blot analysis (20 μg/lane) was performed with a ^{32}P-labeled human HO-1 cDNA probe, as described under "Experimental Procedures"; the blot was also probed with a cDNA for GRP78 and the ribosomal protein L7a. *C*, a densitometric analysis of the autoradiograms in *A* and *B* corrected for the internal control (L7a) is shown in the *graph*. *Open bars* represent GRP78/L7a, and the *dark bars* represent HO-1/L7a. Results are representative of two independent experiments.

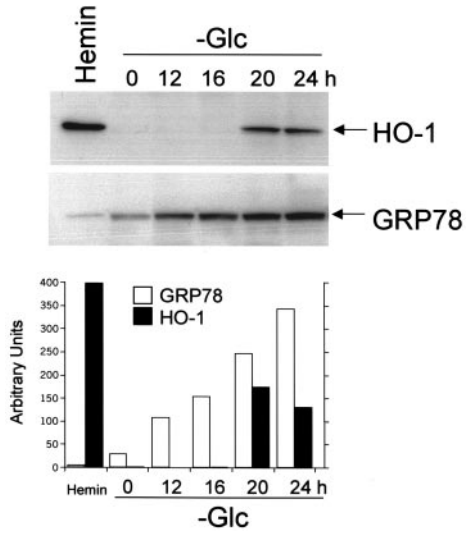

FIG. 2. **HO-1 protein content of glucose-deprived HepG2 cells.** HepG2 cells were cultured in MEM containing 10% FBS and grown to 80% confluence in 60-mm dishes. Cells treated with hemin (5 μM) for 16 h were used as a positive control. After incubation with glucose-free MEM (*−Glc*), total cellular protein was extracted and immunoblot analysis performed as described under "Experimental Procedures." HO-1 and GRP78 were identified as positive bands at ~32 and 78 kDa sizes, respectively. A densitometric analysis of the autoradiograms is shown in the *graph below*. *Open bars* represent GRP78, and the *dark bars* represent HO-1. Results are representative of two independent experiments.

deprivation is translated into protein and, as expected, the increase in protein temporally lagged behind that of the mRNA, In contrast to HO-1, immunoblot analysis for GRP78 showed accumulation as early as 12 h, reaching a maximal level by 24 h (Fig. 2).

Glucose Concentration—To determine the glucose concentration range over which the HO-1 mRNA content was altered, HepG2 cells were incubated for 12 h in MEM containing 0.001–15 mM glucose (Fig. 3). Between 0.5 and 5 mM, the HO-1 mRNA content was inversely proportional to glucose concentration. The HO-1 mRNA content was slightly more suppressed than GRP78 at glucose concentrations of 1 mM or more. The $k_{1/2}$ (concentration causing a half-maximal response) of glucose-dependent repression of HO-1 mRNA was ~0.5 mM, whereas that of GRP78 mRNA was 1 mM.

Sugar Specificity—Whether or not mammalian cells use specific sugar molecules to detect the availability of carbohydrate has not been confirmed conclusively. Data have been reported that phosphorylated glucose intermediates, such as glucose 6-phosphate, or metabolites within the pentose phosphate pathway are putative candidates for genes activated by the presence of carbohydrate (19). To investigate the responsiveness of the HO-1 gene to the presence of specific carbohydrates, HepG2 cells were incubated for 12 h in MEM lacking glucose or MEM supplemented with specific metabolites (Fig. 4). As expected from the previous results, HO-1 mRNA content was increased by complete glucose deprivation. The presence of 5.5 mM glucose, fructose, galactose, mannose, or 2-DOG prevented an increase in the HO-1 mRNA content. In contrast, including the non-metabolizable glucose analog, 3-OMG, did not block induction of the HO-1 mRNA content (Fig. 4). The changes in HO-1 mRNA paralleled changes in GRP78 expression, with the exception of 2-DOG, which failed to repress glucose deprivation-mediated GRP78 mRNA induction. These results eliminate free glucose or osmotic changes as the sensor for carbohydrate availability. Furthermore, the 2-DOG sensitivity reveals a striking difference between HO-1 and GRP78 regulation.

Analysis of Transcription and mRNA Stability in Glucose Deprivation-induced HO-1 Expression—To determine whether changes in HO-1 mRNA content in response to glucose deprivation involved transcriptional control of the gene, a 4.5-kb human HO-1 proximal promoter fragment was evaluated in transient transfection experiments. We have reported previously that this fragment mediates increased transcription in response to heme and cadmium, known inducers of the HO-1 gene (16). This HO-1 promoter fragment demonstrated a significant increase (~1.7-fold) in transcriptional activity, as measured by luciferase expression (Fig. 5*A*), suggesting that transcriptional mechanisms contribute, at least in part, to glucose deprivation-mediated HO-1 mRNA induction. Interestingly, the degree of luciferase reporter gene expression did not recapitulate the level of HO-1 induction seen at the Northern

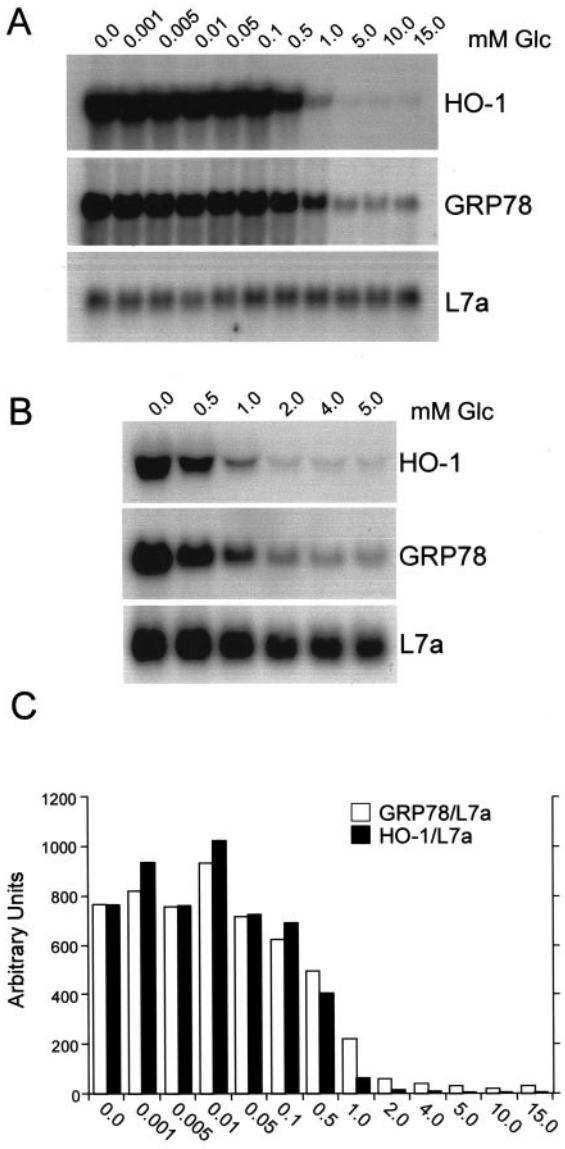

FIG. 3. **Effect of glucose concentration on HO-1 mRNA content.** HepG2 cells were cultured in MEM containing 10% FBS and grown to 80% confluence in 60-mm dishes. Cells were washed and the medium replaced with MEM containing the indicated glucose concentrations and incubated for 12 h (*A* and *B*). RNA was isolated and subjected to Northern blot analysis (20 μg/lane) with ^{32}P-labeled cDNA probes specific for HO-1, GRP78, or L7a. *C*, a densitometric analysis of the autoradiograms in *A* and *B* corrected for the internal control (L7a) is shown in the graph *below*. *Open bars* represent GRP78/L7a, and the *dark bars* represent HO-1/L7a. Results are representative of two independent experiments.

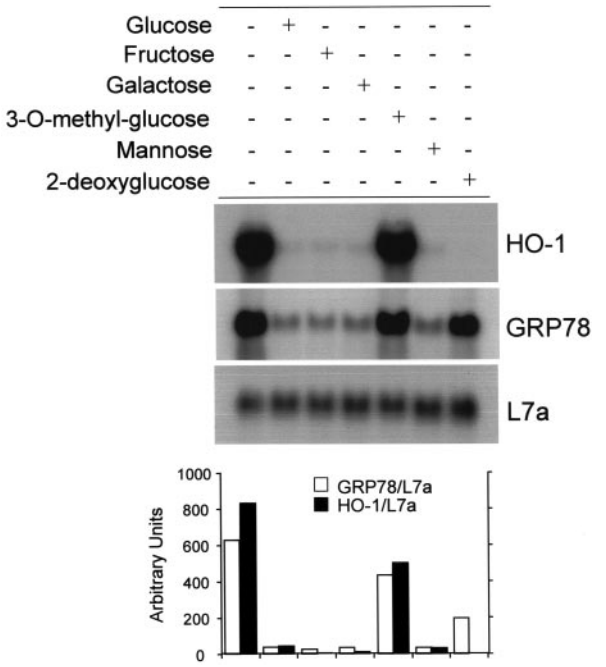

FIG. 4. **Effect of specific sugar molecules on the content of HO-1 mRNA.** HepG2 cells were cultured in MEM containing 10% FBS and grown to 80% confluence in 60-mm dishes. Cells were washed and the medium replaced with MEM containing indicated sugars (5 mM) and incubated for 12 h. RNA was isolated and subjected to Northern blot analysis (20 μg/lane) with ^{32}P-labeled cDNA probes specific for HO-1, GRP78, or L7a. A densitometric analysis of the autoradiograms corrected for the internal control (L7a) is shown in the *graph below*. *Open bars* represent GRP78/L7a, and the *dark bars* represent HO-1/L7a. Results are representative of two independent experiments.

level, suggesting that additional sequences outside of the 4.5-kb promoter region or mRNA stability may be required for complete transcriptional activation of the HO-1 gene in response to glucose deprivation.

To test for a possible role of mRNA stability in the induction of HO-1 mRNA following stimulation with glucose-free MEM, the half-life of HO-1 mRNA in HepG2 cells was measured. Confluent HepG2 cells were exposed to glucose-free MEM for 12 h, and then placed in complete MEM or glucose-free MEM both containing actinomycin D (4 μM) (Fig. 5*B*). Total cellular RNA was collected at the indicated time points and Northern analysis performed. In the absence of *de novo* RNA synthesis, the half-life of HO-1 mRNA was ~4 h and did not increase but, in fact, was somewhat shorter following additional glucose deprivation. These results indicate that increased mRNA stability is not responsible for the induction of HO-1 mRNA by glucose deprivation.

HO-1 mRNA Is Not Regulated by the UPR Pathway—The UPR pathway controls the transcription of several glucose-regulated genes (3, 4). Given the increase in HO-1 mRNA in response to glucose deprivation, the UPR pathway was a candidate for the mechanism by which this process was mediated. To test whether or not the HO-1 gene responds to other recognized activators of the UPR pathway, HepG2 cells were incubated in the presence of tunicamycin (5 μg/ml), thapsigargin (300 nM), or Aze (5 mM). When cells were incubated in complete MEM containing these UPR activators, HO-1 mRNA was not induced, whereas significant induction of GRP78 was observed (Fig. 6, *A–C*). These results document that activation of the UPR pathway does not lead to induction of the HO-1 gene; thus, a previously unidentified signal transduction pathway is triggered following glucose deprivation in HepG2 cells.

Relation between HO-1 mRNA Content and Oxidation via the Tricarboxylic Acid Cycle—Although most cells in culture are highly glycolytic, following removal of glucose from the culture medium, cells are dependent on energy via the tricarboxylic acid cycle. As a result, amino acids, such as glutamine, serve as the primary substrates for ATP synthesis via oxidation by the tricarboxylic acid cycle. Indeed, glutamine is usually included in tissue culture media at 2–4 mM as a primary fuel. To determine the role of oxidation of carbon skeletons via the tricarboxylic acid cycle, HO-1 mRNA content was measured in HepG2 cells incubated for 12 h in glucose-free MEM containing 4 mM glutamine or glucose- and glutamine-free MEM. The induction of HO-1 mRNA by glucose deprivation was completely attenuated by the simultaneous removal of glutamine from the medium, whereas GRP78 mRNA was only partially blocked (Fig. 7*A*). As shown in Fig. 7*B*, the induction of HO-1 by

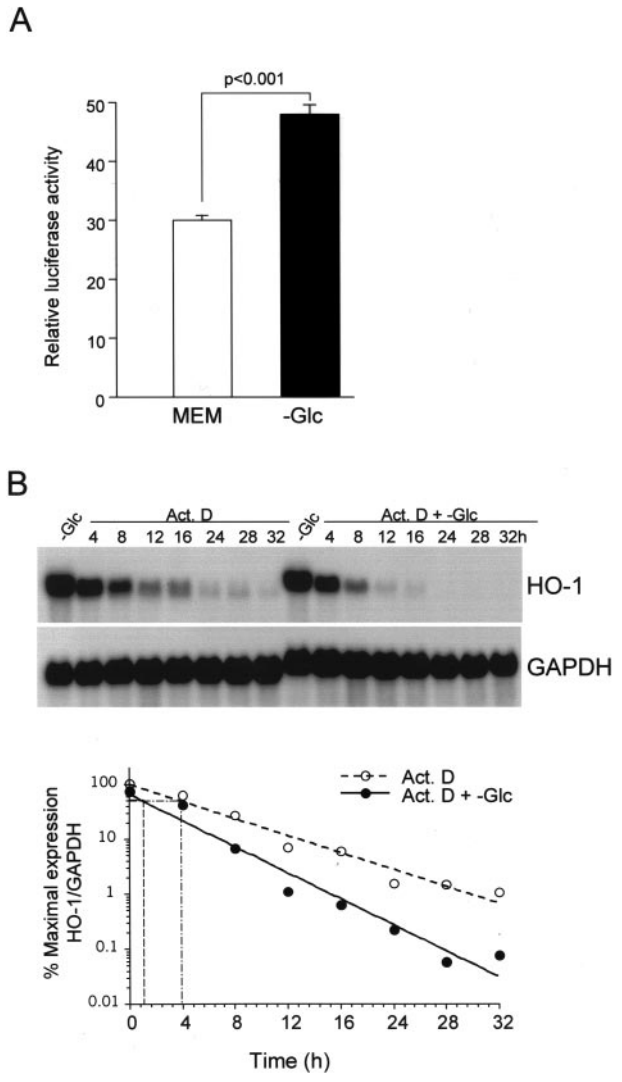

FIG. 5. **Contribution of transcription (*A*) and mRNA stability (*B*) in glucose deprivation-induced HO-1 expression.** *A*, HepG2 cells were co-transfected with a ~4.5-kb human HO-1 promoter construct cloned in pGL3 Firefly luciferase basic vector and a *Renilla* luciferase plasmid, as a control for transfection efficiency. Transient transfection was performed using SuperFect reagent, as described under "Experimental Procedures." Cells were incubated in complete MEM (*open bars*) ($n = 8$) or glucose-free MEM (*−Glc*) (*dark bars*) ($n = 8$) for 16 h, and luciferase activities determined using the Dual-Luciferase® reporter assay system. Results are expressed as relative luciferase units, and the p value is indicated. *B*, HepG2 cells were cultured in MEM containing 10% FBS and grown to 80% confluence in 60-mm dishes. Cells were exposed to −Glc for 12 h, and then placed in complete MEM or −Glc, both containing actinomycin D (*Act. D*) (4 μM). Total cellular RNA was collected at the indicated time points and subjected to Northern blot analysis (20 μg/lane) with ^{32}P-labeled cDNA probes specific for HO-1 or GAPDH. A densitometric analysis of the autoradiograms corrected for the internal control (GAPDH) is shown in the *graph below*. *Open circles* represent cells treated with actinomycin D alone, and *dark circles* represent cells treated with actinomycin D and additional glucose deprivation. Results are representative of three independent experiments.

glucose deprivation requires a concentration of at least 4.0 mM glutamine, whereas enhanced GRP78 expression was not affected until the concentration of glutamine was reduced to 0.1 mM or lower, at which expression was decreased by ~35%.

To demonstrate that incubation of HepG2 cells with MEM lacking either glucose alone or glucose and glutamine did not result in cell damage, we performed DNA fragmentation studies as well as observed the cells by phase contrast microscopy. Neither DNA fragmentation (Fig. 7*C*) nor evidence of cell injury (cell rounding, detachment, vacuolization) was observed in cells incubated with glucose-free MEM for up to 24 h and up to 12 h in cells incubated in MEM lacking both glucose and glutamine. These data demonstrate that glucose deprivation-mediated HO-1 induction is dependent on glutamine and that these changes in gene expression are not because of nonspecific effects of cell injury *per se*.

DISCUSSION

The present data demonstrate that glucose deprivation induces expression of HO-1, a critical enzyme in heme metabolism that exhibits antioxidant and cytoprotective effects. The second novel contribution is that the pathway by which this activation occurs is different from the UPR pathway, the only previously recognized mechanism for transcriptional activation by glucose deprivation. The third important observation is that the glucose deprivation-mediated HO-1 induction involves a glutamine-dependent mechanism. The beneficial effects of heme oxygenase activity are a consequence of its robust induction as an adaptive response in cells/tissues exposed to a wide variety of injurious stimuli. These include excess heme, ultraviolet radiation, hydrogen peroxide, cytokines, endotoxin, growth factors, heavy metals, oxidized low density lipoprotein, shear stress, hyperoxia, nitric oxide (NO), and NO donors: all stimuli imposing a significant shift in cellular redox (8, 9).

The responsiveness of HO-1 mRNA content to glucose concentration qualitatively paralleled that of GRP78, but HO-1 appeared to be slightly more responsive to glucose. The observation that the extracellular glucose concentration must be below 1 mM for significant gene induction is consistent with previous studies with GRP78 and asparagine synthetase expression (18). Given the rapid metabolism of glucose within the cell, the intracellular level of glucose will be substantially less. Furthermore, the lack of repression by 3-OMG indicates that the concentration of a metabolite, rather than free glucose, will be the critical factor. However, our studies demonstrate that cells do respond to extracellular fluctuations in glucose concentrations with extensive adaptive responses, such as induction of the UPR pathway and the HO-1 pathway, suggesting that this is a physiological response.

The contribution of mRNA stability in the induction of HO-1 mRNA by glucose deprivation was analyzed, and the data documented that the estimated half-life was not increased by glucose starvation (indeed, it was slightly shortened). Therefore, mRNA stability is not responsible for the increase in HO-1 mRNA. This result is consistent with the accepted belief that, for most of the previously studied stimuli, regulation of HO-1 expression occurs at the transcriptional level (20–23). Consistent with this belief, studies using a 4.5-kb upstream fragment that contains the human HO-1 promoter indicated that transcriptional activation occurs following glucose deprivation. A number of genes are transcriptionally activated following glucose deprivation as the result of abnormal protein accumulation within the ER and subsequent activation of the UPR signal transduction pathway (3, 4). Treatment of cells with the glycoprotein biosynthesis inhibitor tunicamycin, or with thapsigargin, an inhibitor of microsomal calcium-ATPase, or with amino acid analogues that incorporate and cause improper folding, such as Aze, causes activation of the UPR pathway (4–6). The present results demonstrate that, in contrast to GRP78, induction of the HO-1 gene does not occur in response to any of these activators, documenting that HO-1 induction following glucose deprivation occurs via a pathway completely different from the UPR pathway. Consistent with these experimental results, computer analysis of a BAC clone sequence (accession no. Z82244), containing the entire human HO-1 gene and >20 kb of

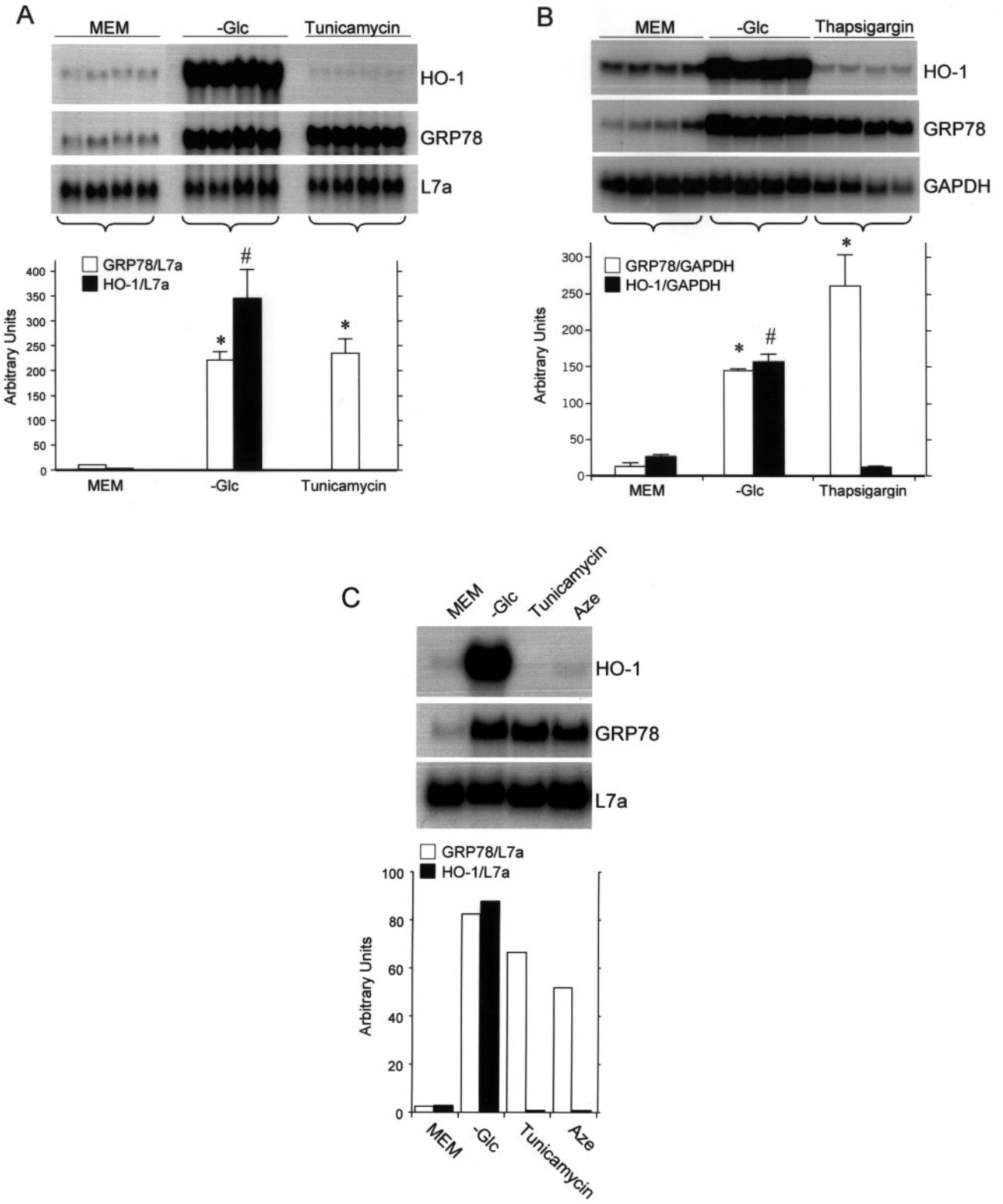

FIG. 6. **HO-1 mRNA is not regulated by the UPR pathway.** HepG2 cells were cultured in complete MEM containing 10% (v/v) FBS and grown to 80% confluence in 60-mm dishes. Cells were incubated in MEM, glucose-free MEM (−*Glc*), or in complete MEM containing tunicamycin (5 μg/ml) (*A*), thapsigargin (300 nM) (*B*), or Aze (5 mM) (*C*) for 12 h. RNA was isolated and subjected to Northern blot analysis (20 μg/lane) with ^{32}P-labeled cDNA probes specific for HO-1, GRP78, or L7a/GAPDH, as shown. A densitometric analysis of the autoradiograms corrected for the internal control (L7a/GAPDH) is shown in the *graph below*. Results are representative of three independent experiments. *, $p < 0.001$; #, $p < 0.001$ (ANOVA and Student-Newman-Keuls test).

5′-flanking region, did not reveal a mammalian UPR element (5′-CCAAT-N_9-CCACG-3′).

To investigate the role of glucose metabolism on HO-1 mRNA induction, the ability of specific sugars and glucose analogs to maintain the basal HO-1 mRNA content was tested. The data illustrate that fructose, galactose, mannose, or 2-DOG repressed the induction of HO-1 mRNA under conditions of glucose deprivation. In contrast, the glucose analog, 3-OMG, which is transported into cells, but is not phosphorylated, was unable to repress the induction of HO-1. The 2-DOG, which is

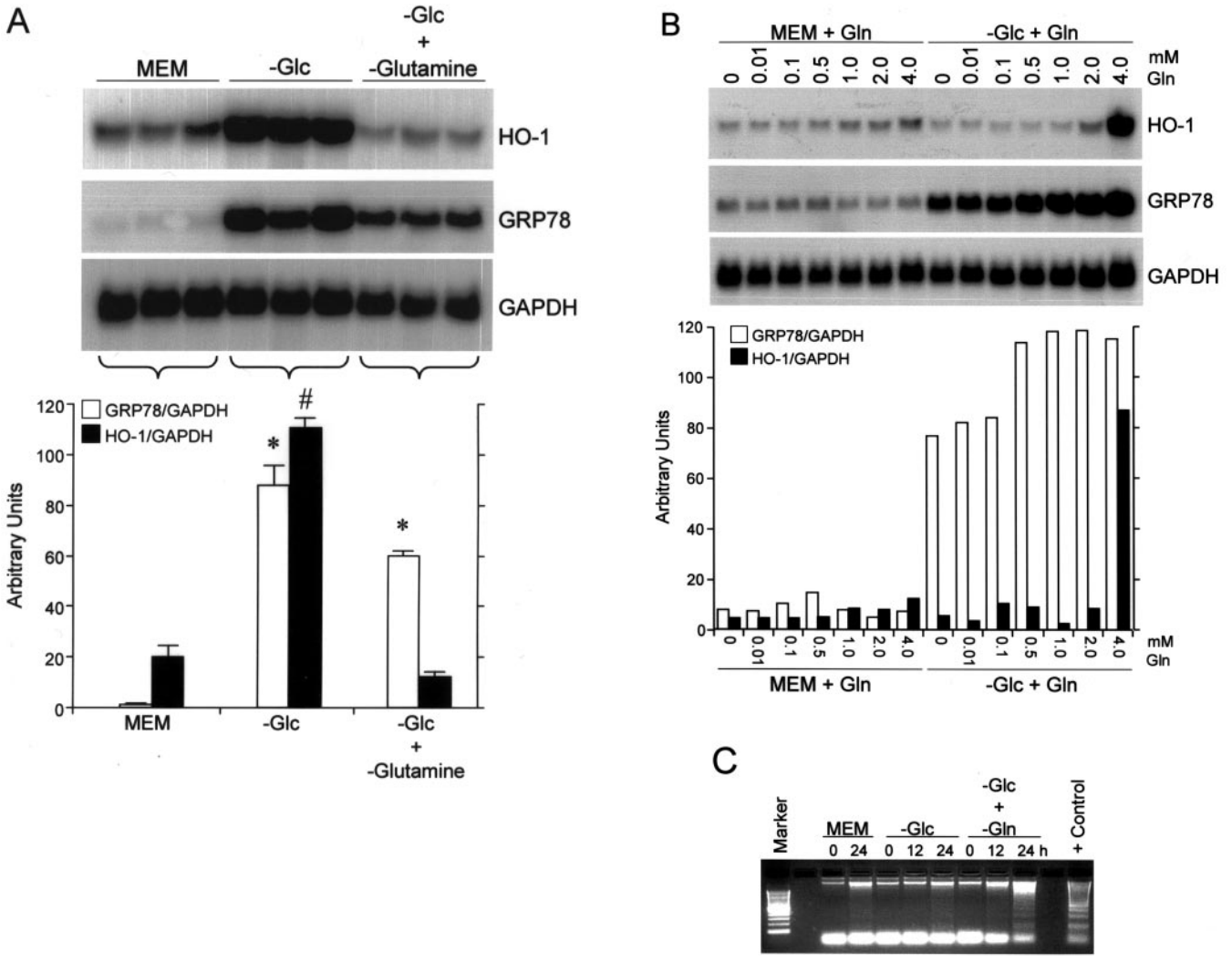

FIG. 7. **Effect of the glutamine on the induction of HO-1 mRNA.** *A*, HepG2 cells were cultured in MEM containing 10% FBS and grown to 80% confluence in 60-mm dishes. Cells were incubated in MEM, glucose free-MEM (*−Glc*), or glucose and glutamine free-MEM (*−Glc + −Gln*) for 12 h. RNA was isolated and subjected to Northern blot analysis with ^{32}P-labeled cDNA probes specific for HO-1, GRP78, or GAPDH. A densitometric analysis of the autoradiograms corrected for the internal control (GAPDH) is shown in the *graph below*. *Open bars* represent GRP78/GAPDH, and the *dark bars* represent HO-1/GAPDH. Results are representative of three independent experiments. *, $p < 0.001$; #, $p < 0.001$ (ANOVA and Student-Newman-Keuls test). *B*, HepG2 cells were cultured in MEM or glucose-free MEM (*−Glc*) for 12 h with increasing concentrations of glutamine (*Gln*) as indicated. RNA was isolated and subjected to Northern blot analysis as in *A*. A densitometric analysis of the autoradiograms corrected for the internal control (GAPDH) is shown in the *graph below*. *Open bars* represent GRP78/GAPDH, and the *dark bars* represent HO-1/GAPDH. *C*, confluent monolayers of HepG2 cells were cultured in MEM, glucose-free MEM (*−Glc*), or glucose and glutamine-free MEM (*−Glc + −Gln*). Cells were collected at the indicated time points, and gel electrophoresis for DNA fragmentation was carried as described under "Experimental Procedures." A 100-bp DNA ladder was used as a marker. HepG2 cells exposed to cisplatin (20 μM) for 24 h were used as a positive control. Results are representative of two independent experiments.

phosphorylated but not readily metabolized further, blocked HO-1 induction completely, but had no effect on induction of GRP78. These data for GRP78 are consistent with previously published reports (18, 24) and illustrate two important points: (i) the sensing mechanism for glucose metabolites of the new signal transduction pathway differs from the UPR, and (ii) free glucose is not the repressive signal molecule for HO-1 gene expression, but glucose 6-phosphate might be detected.

During glucose deprivation, steady state levels of intracellular pro-oxidants, such as hydroperoxides, increase immediately (25). This observation suggests that hydroperoxides are produced by non-glycolytic metabolic processes and that their rate of production is increased or their metabolic decomposition is compromised by the removal of glucose, possibly via a decrease in intracellular NADPH and pyruvate (26, 27). Recently, it has been suggested that, in the absence of glucose, increased pro-oxidant production occurs via the mitochondrial electron transport chain (27) because fatty acid and amino acid carbon skeletons provide the primary fuel as substrates for the tricarboxylic acid cycle. Foremost among these is glutamine, an essential amino acid for growth of cells in culture because of its importance as an oxidative fuel. The present results demonstrate a pivotal role for glutamine oxidation via the tricarboxylic acid cycle as a prerequisite for HO-1 mRNA induction following glucose deprivation and suggest that pro-oxidant molecules, arising from increased electron transport activity, may contribute to activation of the HO-1 gene.

Recent evidence has demonstrated the critical importance of HO-1 expression in mediating anti-oxidant, anti-inflammatory, and anti-apoptotic effects (28–31). Furthermore, induction of HO-1, by chemical inducers (28, 32) or selective overexpression (28, 33, 34), is cytoprotective both *in vitro* and *in vivo*. In addition, the phenotype of the HO-1 knock-out mouse, characterized by chronic renal and hepatic inflammation, tissue iron deposition, anemia, and increased sensitivity to oxidant stress, underscores the functional and biological significance of HO-1

(31, 35). The beneficial effects of HO-1 induction occur via several postulated mechanisms. Increased HO-1 activity results in degradation of the heme moiety, a potentially toxic pro-oxidant (36), and generates bilirubin, an antioxidant capable of scavenging peroxy radicals and inhibiting lipid peroxidation (37, 38). Another product, CO, has also received considerable attention as a signaling molecule, similar to NO, with vasodilatory effects mediated via cGMP, as well as anti-apoptotic and anti-inflammatory effects (8, 9, 29, 30). Ferritin, an intracellular iron repository, is often co-induced with HO-1, thus allowing safe sequestration of unbound iron liberated from heme degradation (39). Modulation of intracellular iron stores and increased iron efflux has recently been suggested as a mechanism for the cytoprotective effects of HO-1 (40).

Collectively, the results indicate that the induction of HO-1 mRNA serves as an adaptive, and likely protective, response in circumstances wherein cells are deprived of glucose. Prior to these studies, the only known signaling pathway to initiate a cellular response to glucose limitation was the UPR (3, 4). That the induction of HO-1 following glucose deprivation occurs via a novel signal transduction pathway, different from the UPR, provides an exciting new avenue for research on nutrient control of gene expression.

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