

# Haem oxygenase 1 gene induction by glucose deprivation is mediated by reactive oxygen species via the mitochondrial electron-transport chain

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Glucose depletion results in cellular stress and reactive oxygen species (ROS) production, which evokes adaptive and protective responses. One such protective response is the induction of haem oxygenase 1 (HO-1), which catalyses the rate-limiting step in haem degradation, liberating iron, CO and biliverdin. The present study evaluated the role of ROS and the mitochondrial electron-transport chain in the induction of HO-1 by glucose deprivation in HepG2 hepatoma cells. Either *N*-acetylcysteine, an antioxidant, or deferoxamine, an iron chelator, resulted in a dose-dependent inhibition of HO-1 mRNA and protein induction during glucose deprivation, suggesting a redox- and iron-dependent mechanism. Inhibitors of electron-transport chain complex III, antimycin A and myxothiazol, the ATP synthase inhibitor oligomycin and ATP depletion with 2-deoxyglucose or glucosamine also blocked HO-1

induction. To address the involvement of ROS further, specifically H<sub>2</sub>O<sub>2</sub>, we showed that overexpression of catalase completely blocked HO-1 activation by glucose deprivation. In contrast, inhibition of nuclear factor  $\kappa$ B, mitogen-activated protein kinase (MAPK), protein kinase A, protein kinase C, phosphoinositide 3-kinase, cyclo-oxygenase or cytosolic phospholipase A<sub>2</sub>, did not prevent HO-1 induction. These results demonstrate that activation of the HO-1 gene by glucose deprivation is mediated by a 'glucose metabolic response' pathway via generation of ROS and that the pathway requires a functional electron-transport chain.

**Key words:** antioxidant, hydrogen peroxide, mitochondrial respiration, oxidant stress.

## INTRODUCTION

Metabolic stressors such as glucose deprivation and hypoxia trigger signal-transduction pathways that alter gene expression. It is well known that a decrease in cellular glucose level causes the transcriptional regulation of a number of genes that encode proteins associated with endoplasmic reticulum (ER) stress, a pathway called the unfolded protein response (UPR) or the ER stress response [1,2]. We have recently reported that glucose deprivation induces the haem oxygenase 1 (HO-1) gene by transcriptional activation and that such induction occurs via a pathway independent of the UPR [3]. HO-1, a 32 kDa ER enzyme, catalyses the rate-limiting step in haem degradation, yielding equimolar quantities of biliverdin, iron and CO [4]. HO-1 is induced by a wide variety of stimuli that impose a significant shift in cellular redox, such as haem, UV radiation, H<sub>2</sub>O<sub>2</sub>, cytokines, endotoxin, growth factors, oxidized low-density lipoprotein and NO [5,6]. In addition to the classical antioxidant enzymes [e.g. superoxide dismutase (SOD), catalase and glutathione peroxidase], studies have identified HO-1 as another important enzyme with antioxidant function.

The physiological relevance of HO-1 induction has been demonstrated in several disease states, wherein induction of HO-1 confers cytoprotection. These include atherosclerosis, renal failure, transplant rejection, sepsis, acute lung injury and ischaemia-reperfusion injury in several organ systems (reviewed in [6]). The phenotype of a HO-1 knock-out mouse with chronic

inflammation, anaemia, tissue iron deposition and increased susceptibility to cell injury, as well as the recent description of a HO-1-deficient patient with similar phenotypic features, further highlights the biological importance of this enzyme [7,8]. The beneficial effects of HO-1 induction may occur via several postulated mechanisms. Increased HO-1 activity results in degradation of the haem moiety, a potentially toxic pro-oxidant [9], and generates bilirubin, an antioxidant capable of scavenging peroxy radicals and inhibiting lipid peroxidation [10,11]. Another product, CO, has also received considerable attention as a signalling molecule, similar to NO, with vasodilatory effects mediated via cGMP, as well as anti-apoptotic and anti-inflammatory effects [12,13]. Ferritin, an intracellular iron repository, is often co-induced with HO-1, thus allowing safe sequestration of unbound iron liberated from haem degradation [14]. Modulation of intracellular iron stores and increased iron efflux has been suggested as a mechanism for the cytoprotective effects of HO-1 [15].

Several lines of evidence suggest a prominent role of oxidant stress in the regulation of the HO-1 gene [16,17]. Glucose deprivation also causes perturbations in cellular sensitivity to oxidative stress, which is mediated by increased generation of pro-oxidants and decreased scavenging of free radicals, presumably via reduced formation of pyruvate and NADPH [18–21]. The enzymic source of reactive oxygen species (ROS) is not conclusive, but mitochondria, consuming about 90% of the inhaled oxygen, are a particularly rich source of endogenous

Abbreviations used: cPLA<sub>2</sub>, cytoplasmic phospholipase A<sub>2</sub>; 2-DOG, 2-deoxyglucose; DFO, deferoxamine mesylate; ER, endoplasmic reticulum; ERK, extracellular-signal-regulated kinase; FBS, foetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GRP78, glucose-regulated protein of 78 kDa; HO-1, haem oxygenase 1; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEM, minimal essential medium; NAC, *N*-acetylcysteine; PI 3-kinase, phosphoinositide 3-kinase; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF, tumour necrosis factor; UPR, unfolded protein response.

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ROS generated via the electron-transport chain [21–23]. In the absence of glucose, an important source of an oxidizable fuel for the mitochondria is glutamine, which has been shown to enhance mitochondrial production of ROS after stimulation of the tumour necrosis factor (TNF) [24]. It is known that cellular ATP production by glutamine oxidation increases with decrease in glucose levels [25]. In our previous study, we demonstrated that induction of HO-1 by glucose deprivation involves a glutamine-dependent mechanism [3]. Based on these studies, it was hypothesized that the generation of ROS via substrate oxidation by the tricarboxylic acid cycle results in HO-1 gene induction [3]. During glucose deprivation, an important source for pro-oxidant production is the mitochondrial electron-transport chain with activation of the tricarboxylic acid cycle [18–21]. The purpose of the present study was to evaluate the role of ROS and the mitochondrial electron-transport chain on the induction of HO-1 gene by glucose deprivation and investigate the signalling pathway(s) involved.

## EXPERIMENTAL

### Reagents

Tissue culture media, foetal bovine serum (FBS) and media supplements were obtained from Cellgro (Herndon, VA, U.S.A.). *N*-Acetylcysteine (NAC), deferoxamine mesylate (DFO), indomethacin, haemin, 2-deoxyglucose (2-DOG) and glucosamine were purchased from Sigma. Bay 11-7082 was purchased from Biomol Research Labs (Plymouth Meeting, PA, U.S.A.). PD98059, SB203580, SB202190, Gö6976, AACOCF<sub>3</sub>, genistein, KT5720, LY294002, oligomycin, myxothiazol and antimycin A were obtained from Calbiochem (San Diego, CA, U.S.A.). Zeocin was obtained from Invitrogen (Carlsbad, CA, U.S.A.). Anti-HO-1 antibody (SPA-895) was obtained from StressGen Biotechnologies Corp. (Victoria, BC, Canada), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody from Biogenesis (Kingston, NH, U.S.A.) and anti-catalase antibody from Calbiochem.

### Cell culture and treatments

Human hepatoma (HepG2) cells were cultured in minimal essential medium (MEM, pH 7.4), supplemented with NaHCO<sub>3</sub> (25 mM), glutamine (2 mM), streptomycin sulphate (10 µg/ml), penicillin G (100 µg/ml), gentamicin (28.4 µg/ml), *N*-butyl-*p*-hydroxybenzoate (0.23 µg/ml), BSA (0.2%) and FBS (10%, v/v). Cells were maintained at 37 °C in 95% air and 5% CO<sub>2</sub> 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 twice with PBS and then incubated in complete MEM or glucose-free MEM medium (pH 7.4), as described above but with 10% dialysed FBS. Cells were pretreated with NAC (0–10 mM), 2-DOG (0–10 mM), glucosamine (0–4 mM), LY294002 (0–20 µM), indomethacin (0–100 µM), Bay 11-7082 (10 µM), PD98059 (50 µM), SB203580 and SB 202190 (1 and 10 µM), Gö6976 (1 µM), AACOCF<sub>3</sub> (10 µM), genistein (30 µM), KT5720 (1 µM), oligomycin (0–50 nM), myxothiazol (0–10 ng/ml) and antimycin A (0–50 nM) for 30 min before the addition of complete MEM or glucose-free MEM. For iron chelation, cells were pretreated with DFO (0–1.0 mM) overnight and incubated with complete MEM or glucose-free MEM in the presence of DFO. For RNA and protein analyses, cells were incubated for 12 and 20 h respectively, time points at which induction of HO-1

mRNA and protein after glucose deprivation were observed previously [3].

### Northern-blot analysis

Total cellular RNA was extracted from cultured cells grown in 60 mm plates using the method described by Chomczynski and Sacchi [26] and purified using RNeasy mini kits (Qiagen, Valencia, CA, U.S.A.). RNA (15 or 20 µg) was electrophoresed on a 1% (w/v) agarose gel containing formaldehyde, electrotransferred on to a nylon membrane and hybridized with a <sup>32</sup>P-labelled 1.0 kb human HO-1 cDNA probe. The cDNA probe for glucose-regulated protein of 78 kDa (GRP78) was a 1498 bp sequence between the *Pst*I and *Eco*RI sites. The nylon membranes were stripped and rehybridized with a human GAPDH or ribosomal protein L7a cDNA probe to control loading of RNA. All experiments were repeated with at least additional 2–3 independent RNA preparations to show reproducibility. To quantify expression levels, autoradiographs were scanned on a Hewlett-Packard Scanjet 4C using Deskscan II software, and densitometry was performed using NIH image 1.63 software. Experiments were adjusted for loading using L7a or GAPDH mRNA quantification, then normalized and expressed in arbitrary units.

### Immunoblot analysis

For HO-1 immunoblots, cells were incubated in MEM or glucose-free MEM (each supplemented with 10% dialysed FBS) for 20 h in the presence or absence of various inhibitors. HepG2 cells treated with haemin (5 µM) for 16 h were used as positive controls. Cells were then washed twice with ice-cold PBS and lysed in a buffer containing a broad-spectrum mixture of protease inhibitors, consisting of aprotinin (10 µg/ml), EDTA (5 mM), leupeptin (1 µg/ml), pepstatin A (0.7 µg/ml), PMSF (1 mM) and 1% (v/v) Triton X-100. Protein concentration of lysates was assessed by the bicinchoninic acid assay (Pierce, Rockford, IL, U.S.A.). Samples were separated on SDS/polyacrylamide [10% (w/v) gel] and then transferred on to a PVDF membrane. The membranes were incubated for 1.5 h with the anti-HO-1 antibody (1:500 dilution) followed by incubation with peroxidase-conjugated goat anti-rabbit IgG antibody (1:10 000 dilution) for 1 h. To verify protein loading, the blots were stripped and re-probed with an anti-GAPDH antibody (1:1000 dilution). Labelled protein bands were examined by chemiluminescence according to the manufacturer's recommendation (Pierce).

### Stable transfection of catalase

Plasmid pZeoSV-CAT (2 µg) was transfected into human HepG2 cells using SuperFect reagent (Qiagen) according to the manufacturer's instructions. This expression plasmid contains a 1.6 kb human catalase cDNA cloned into the *Hind*III and *Not*I sites of pZeoSV2+ as described previously [27]. Cells transfected with the empty vector, pZeoSV2+ (Invitrogen), were used as controls. Cells were cultured for 48 h and then the antibiotic zeocin (1 mg/ml) was added. Medium containing 1 mg/ml zeocin was replaced on alternate days for approx. 6 weeks. Zeocin-resistant colonies were clonally selected and screened for catalase overexpression by immunoblot analysis using the anti-catalase antibody (1:500 dilution).

## RESULTS

### Essential role of ROS on induction of HO-1 mRNA and protein by glucose deprivation

Previous studies have demonstrated that a common denominator for most stimuli that induce HO-1 is the imposition of significant oxidant stress [16,17]. It is also recognized that iron plays an important role in regulating HO-1 induction [28–32] by its ability to potentiate oxidant stress [33]. We first evaluated the effects of a general antioxidant, NAC, and an iron chelator, DFO, in glucose-deprivation-mediated HO-1 induction. We and others have shown that the iron chelator, DFO, blocks HO-1 induction by hyperoxia, hypoxia and oxidized low-density lipoprotein, but does not block transforming growth factor- $\beta$  or curcumin-mediated HO-1 induction [28–32]. Similarly, NAC, which scavenges free radicals and increases cellular glutathione levels, blocks NO-mediated HO-1 induction [34]. Therefore these agents were used to investigate the possible involvement of oxidants in the induction of HO-1 mRNA after glucose deprivation. When HepG2 cells were incubated in a medium lacking glucose there was an approx. 20-fold increase in HO-1 mRNA content, but in the presence of NAC, induction of HO-1 mRNA was dose-dependently inhibited (Figures 1A and 1C). In addition, DFO also resulted in the inhibition of HO-1 induction during glucose deprivation (Figures 1A and 1D). Inhibition of HO-1 mRNA by NAC and DFO during glucose deprivation was also associated with a parallel decrease in HO-1 protein (Figure 1B). Interestingly, the induction of GRP78 mRNA, the result of activation of the UPR pathway, was not affected by either NAC or DFO (Figure 1). These results demonstrate that induction of HO-1 mRNA level by glucose deprivation is related to changes in cellular redox and iron, and confirms our observation [3] that induction occurs via a different mechanism than that for GRP78, i.e. the UPR.

### Nuclear factor $\kappa$ B, mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI 3-kinase), cyclo-oxygenase and cytoplasmic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>) are not necessary for the induction of HO-1 mRNA by glucose deprivation

The transcription factor, nuclear factor  $\kappa$ B, is stimulated by ROS and a number of conditions that perturb the ER function, eventually leading to serine phosphorylation and subsequent proteosomal degradation of the inhibitory  $\kappa$ B subunit. MAPKs are a family of serine–threonine protein kinases that are activated by a variety of extracellular stimuli and are important in the signal transduction of HO-1 induction by other stimuli [35,36]. Activation of protein kinase C is involved in the induction of HO-1 gene expression by cytokines in human endothelial cells [37]. Activation of protein kinase A and protein tyrosine kinase also induces HO-1 gene expression [37–39]. Therefore inhibitors of these pathways were used to evaluate the effects on induction of HO-1 mRNA after glucose limitation. Concentrations of the inhibitors used in these studies are based on previous studies in the literature that have established effective levels, mostly involving HepG2 cells [40–45]. HepG2 cells were subjected to glucose deprivation in the presence of 10  $\mu$ M Bay 11-7082 (an inhibitor of inhibitory  $\kappa$ B- $\alpha$  phosphorylation and degradation), 50  $\mu$ M PD98059 [extracellular-signal-regulated kinase (ERK) inhibitor], SB203580 and SB202190 (p38 MAPK inhibitors, both tested at 1 and 10  $\mu$ M), 1  $\mu$ M Gö6976 (protein kinase C inhibitor) (results not shown) and 0, 2.5, 10 and 20  $\mu$ M PI 3-kinase inhibition (LY294002) (Figures 2A and 2B). In each case, HO-1 levels were not inhibited, suggesting that these pathways were not required in the induction of HO-1 by glucose

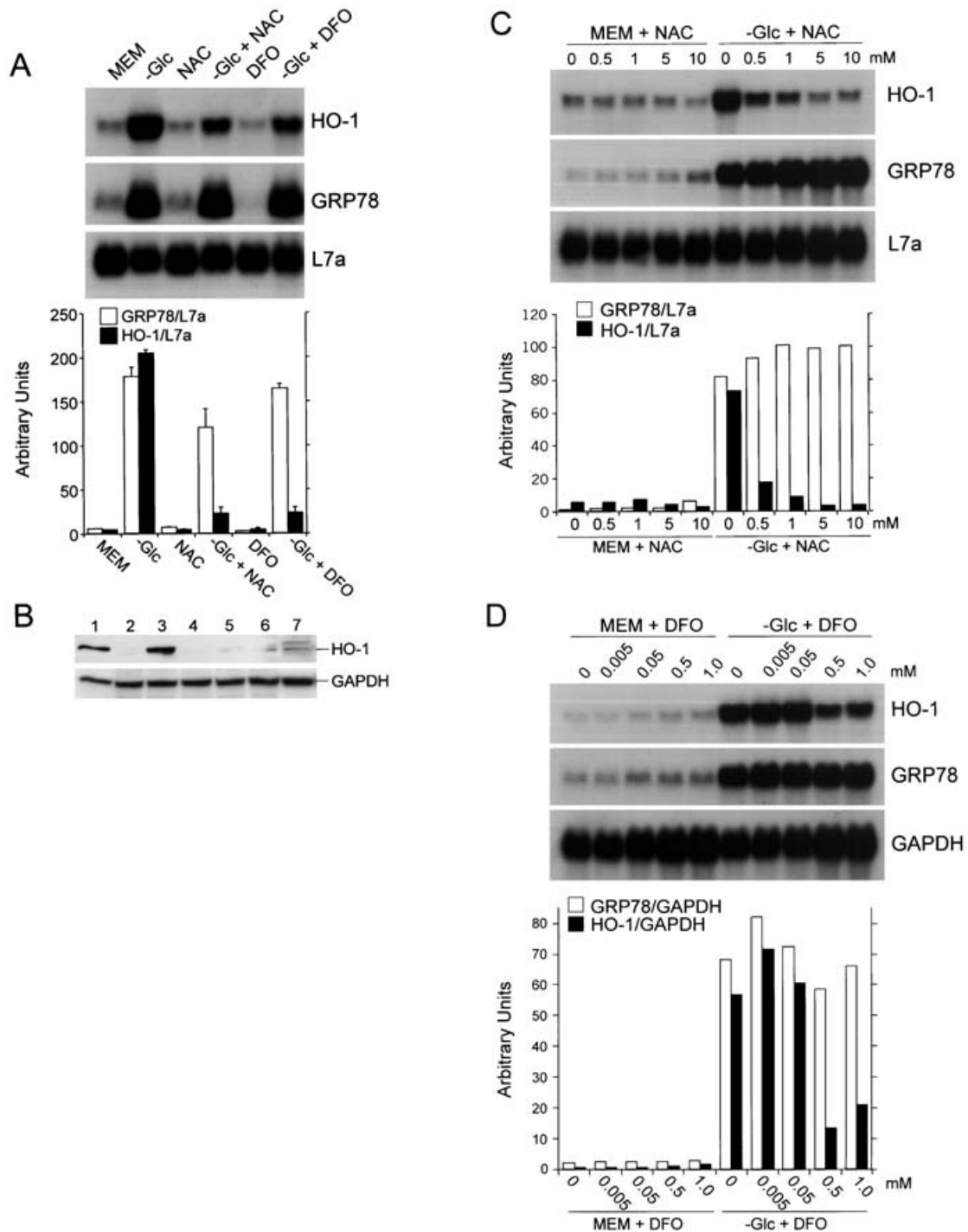
deprivation. We also evaluated the involvement of protein kinase A (1  $\mu$ M KT5720), protein tyrosine kinase (30  $\mu$ M genistein), cyclo-oxygenase (0–100  $\mu$ M indomethacin) and cPLA<sub>2</sub> (10  $\mu$ M AACOCF<sub>3</sub>) in glucose-deprivation-mediated HO-1 induction, but none of these inhibitors blocked the increase in cellular HO-1 mRNA (results not shown).

### Induction of glucose-deprivation-mediated HO-1 mRNA and protein is inhibited by 2-DOG and glucosamine in a dose-dependent manner

The glucose analogue 2-DOG suppresses ROS production, stabilizes mitochondrial transmembrane potential and calcium homeostasis in oxidative stressed neurons [46]. 2-DOG is phosphorylated, but in most tissues further metabolism is poor and therefore depletes the level of ATP in the cells [47]. To investigate the responsiveness of the HO-1 gene to ATP depletion, HepG2 cells were incubated in MEM or MEM lacking glucose, supplemented with the indicated concentrations of 2-DOG (Figure 3A). Whereas the basal HO-1 mRNA content was unaffected, the induction of HO-1 mRNA by glucose deprivation was dose-dependently attenuated by the addition of 2-DOG to the medium. Similarly, induction of HO-1 protein was also attenuated by 2-DOG (Figure 3B). The pentose phosphate cycle is the major source of NADPH, and glucosamine reduces the cellular NADPH concentration by inhibiting this pathway as a competitive inhibitor of glucose-6-phosphate dehydrogenase [48]. Similar to 2-DOG, glucosamine is taken up rapidly and phosphorylated by hexokinase at the expense of ATP, resulting in a decrease in cellular ATP levels [49]. Therefore ATP is not easily replenished in cells that are incubated in glucose-free medium. To test the effect of glucosamine on induction of HO-1 mRNA, HepG2 cells were incubated in MEM or MEM-lacking glucose, supplemented with indicated concentrations of glucosamine. The presence of glucosamine dose dependently inhibited the induction of HO-1 mRNA and protein by glucose deprivation (Figures 3C and 3D), suggesting a contribution of ATP or NADPH depletion, in the regulation of HO-1 gene expression by glucose levels.

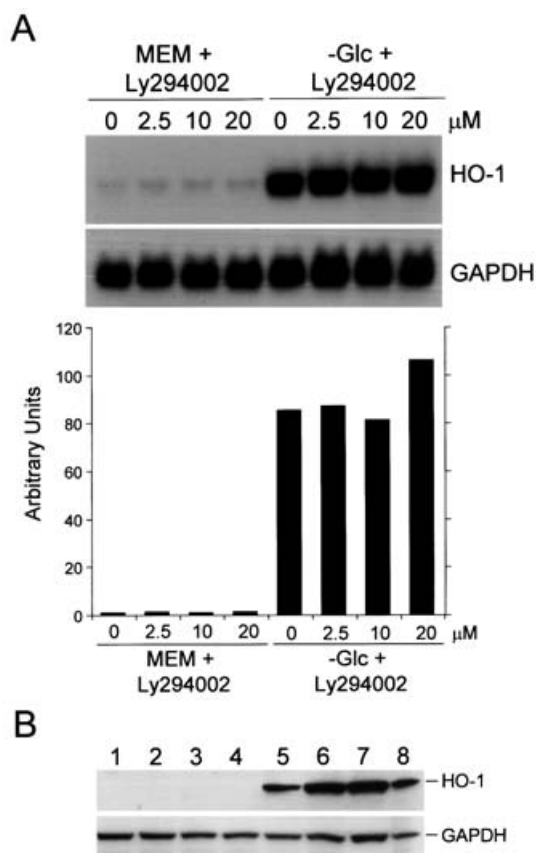
### Mitochondrial electron-transport inhibitors modulate glucose-deprivation-mediated HO-1 mRNA and protein induction

Recently, Rogers et al. [50] suggested that ROS could be a potential signal transducer of putative mitochondria-to-nucleus signalling pathways. Production of ROS in the mitochondria occurs via the electron-transport chain. In the absence of glucose, oxidation of glutamine and other fuels will occur via the tricarboxylic acid cycle and cause increased electron-transport chain activity. To evaluate the role of mitochondrial electron-transport chain on the induction of HO-1 mRNA by glucose deprivation, HepG2 cells were incubated for 12 h in MEM or MEM lacking glucose, supplemented with the indicated concentrations of antimycin A (Figure 4A) and myxothiazol (Figures 4B and 4C), inhibitors of complex III of the electron-transport chain, as well as the mitochondrial ATP synthase inhibitor oligomycin (Figures 4D and 4E). The increase in HO-1 mRNA content after glucose limitation was blocked by these inhibitors, suggesting the requirement of electron-transport chain activity for HO-1 induction. HO-1 protein levels were also examined after the incubation of HepG2 cells for 20 h in MEM or MEM lacking glucose in the presence of myxothiazol (Figure 4C) and/or oligomycin (Figure 4E). Similar to the results observed with HO-1 mRNA, the induction of HO-1 protein by glucose deprivation was inhibited by both myxothiazol and oligomycin. We were not able to evaluate HO-1 protein levels after treatment



**Figure 1** Effect of NAC and DFO on HO-1 mRNA and protein induction during glucose deprivation

(A) HepG2 cells were cultured in MEM containing 10% FBS and grown to 80% confluence in 60 mm dishes. Cells were incubated for 12 h in MEM or glucose-free MEM, each supplemented with 10% dialysed FBS, NAC (5 mM) and/or DFO (0.5 mM) as described in the Experimental section. RNA was isolated and subjected to Northern-blot analysis with a  $^{32}\text{P}$ -labelled cDNA specific for HO-1, GRP78 and L7a or GAPDH. A densitometric analysis of the autoradiograms corrected for the internal control (L7a or GAPDH) is also shown. The open bars represent GRP78/L7a or GAPDH and the black bars represent HO-1/L7a or GAPDH expressed in arbitrary units. The results are representative of three independent experiments (means  $\pm$  S.E.M.) (B) HepG2 cells were incubated for 16 h in complete MEM with 5  $\mu\text{M}$  haemin (lane 1, positive control), or for 20 h in complete MEM (lane 2), glucose-free MEM (lane 3), complete MEM with NAC (5 mM; lane 4), glucose-free MEM with NAC (lane 5), complete MEM with DFO (0.5 mM; lane 6) and glucose-free MEM with DFO (lane 7). Immunoblot analysis was performed as described in the Experimental section. HO-1 and GAPDH are identified as positive bands at masses approx. 32 and 36 kDa respectively. A dose-response using NAC and DFO at the indicated concentrations in MEM or glucose-free MEM is shown in (C) and (D) respectively. The results are representative of two independent experiments.



**Figure 2** Effect of PI 3-kinase pathway inhibition on glucose-deprivation-mediated HO-1 mRNA and protein induction

HepG2 cells were cultured in MEM containing 10% FBS and grown to 80% confluence in 60 mm dishes. Cells were washed and replaced with MEM or glucose-free MEM (with 10% dialysed FBS) containing the indicated concentrations of LY294002 and incubated for 12 h (for mRNA) (A) and for 20 h (for protein) (B) respectively. RNA was isolated and subjected to Northern-blot analysis with a  $^{32}\text{P}$ -labelled cDNA specific for HO-1 and GAPDH. A densitometric analysis of the autoradiograms corrected for the internal control (GAPDH) is shown in arbitrary units. (B) Immunoblot of HepG2 cells incubated with complete MEM (lanes 1–4) or glucose-free MEM (lane 5–8), supplemented with 2.5  $\mu\text{M}$  (lanes 2 and 6), 10  $\mu\text{M}$  (lanes 3 and 7) and 20  $\mu\text{M}$  (lanes 4 and 8) LY294002 respectively. HO-1 and GAPDH are identified as positive bands at masses approx. 32 and 36 kDa respectively. Results are representative of two independent experiments.

with antimycin A, since glucose deprivation with antimycin A for 20 h was associated with visible cell injury. No detectable cell injury was observed at the 12 h period used for the Northern-blot analysis.

#### Overexpression of catalase inhibits glucose-deprivation-mediated induction of HO-1

Previous studies have reported that intracellular oxidants such as  $\text{H}_2\text{O}_2$  generated via the electron-transport chain are increased during glucose deprivation [18–21]. If  $\text{H}_2\text{O}_2$  generation plays a role in the cellular pathway for detection and response to glucose deprivation, then elimination of  $\text{H}_2\text{O}_2$  by catalase should perturb the pathway. To test this hypothesis, we generated HepG2 cells that stably overexpress catalase, as confirmed by immunoblot analysis (Figure 5A). Cells transfected with either the vector alone or catalase cDNA were incubated with MEM or MEM-lacking glucose medium for 24 h (Figure 5A) and/or 12 h (Figure 5B). Overexpression of catalase completely blocked induction of HO-1

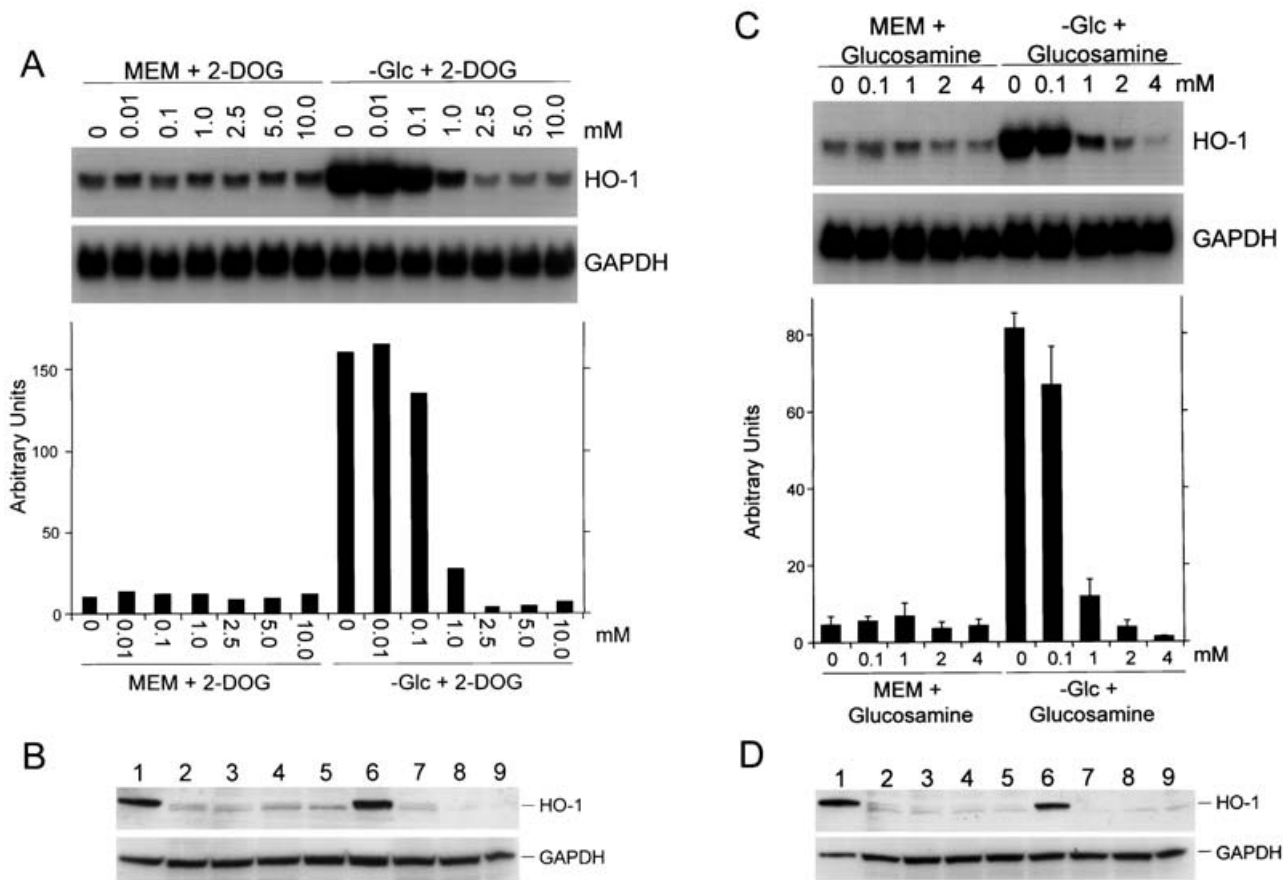
mRNA (Figure 5B) and protein (Figure 5A), strongly suggesting a role for  $\text{H}_2\text{O}_2$  as a signalling molecule in HO-1 induction by glucose deprivation.

#### DISCUSSION

The results of the present study demonstrate that during glucose limitation of HepG2 cells the mitochondrial generation of ROS,  $\text{H}_2\text{O}_2$  in particular, plays an important role in HO-1 induction. Although these studies are consistent with previous observations that change in cellular redox is a common feature that regulates HO-1 gene expression [16,17], a direct link between electron-transport-chain activity and HO-1 gene induction has not been reported. Several lines of evidence support our conclusion that such a link exists. First, general antioxidants such as NAC and the iron chelator, DFO, blocked glucose-deprivation-mediated HO-1 induction, but did not affect GRP78 induction via the UPR pathway. Secondly, inhibitors of complex III of the mitochondrial electron-transport chain inhibited HO-1 induction. Mitochondria are the major cellular organelles that consume oxygen and yield significant amounts of ROS from complexes I and III of the electron-transport chain [21]. Finally, overexpression of catalase, to prevent  $\text{H}_2\text{O}_2$  accumulation, attenuated glucose-deprivation-mediated HO-1 induction.

Our results also document that cellular ATP depletion with 2-DOG or glucosamine, inhibited HO-1 induction by glucose deprivation. Previous studies have shown that 2-DOG, in the absence of metabolic substrates, results in a reduction of cellular ATP content to < 5% of control values within 5 min [47], suppresses ROS production and stabilizes mitochondrial function [46]. Glucosamine also results in cellular ATP depletion [49]. A product of the hexosamine biosynthetic pathway, glucosamine, inhibits the pentose cycle by competing with glucose-6-phosphate dehydrogenase [48]. The pentose cycle is the major source of NADPH, which is required for providing reducing equivalents to the glutathione–glutathione peroxidase–glutathione reductase system [21]. Our findings of glucosamine-mediated inhibition of HO-1 induction are similar to the effects of glucosamine for the inhibition of NO synthesis, where cellular availability of NADPH has been suggested as a metabolic basis for these effects [48]. The mechanisms by which ATP depletion leads to the inhibition of HO-1 induction during glucose deprivation are not entirely clear at present. It is unlikely that ATP depletion results in non-specific effects consequent to decreased cellular energy levels, since GRP78 induction via the UPR would also require energy, but is not affected by 2-DOG [3] and glucosamine (results not shown). In fact, 2-DOG and glucosamine are actually activators of the UPR pathway [2], but do not induce HO-1. Thus glucosamine-mediated inhibition of HO-1 induction during glucose deprivation could be attributed to decreased cellular ATP or NADPH levels, which in turn would lead to decreased ROS production and, thereby, inhibit HO-1 induction.

Our studies demonstrate an important link between mitochondrial electron-transport-chain activity and HO-1 induction by glucose deprivation, an observation that has not been previously reported. In aerobic cells, mitochondrial respiration accounts for most of the ROS generation, particularly superoxide. Inhibitors of mitochondrial electron-transport chain have been shown to decrease intracellular ROS levels and alter gene expression [50,51]. Ebert et al. [51] have reported that expression of the glucose transporter, GLUT1, is enhanced by hypoxia and mitochondrial inhibitors. Rogers et al. [50] have shown that inhibition of electron-transport chain with antimycin A, myxothiazol or oligomycin inhibits TNF- $\alpha$ -mediated induction



**Figure 3** Effect of 2-DOG (A, B) and glucosamine (C, D) on the induction of HO-1 mRNA and protein

HepG2 cells were cultured in MEM containing 10% FBS and grown to 80% confluence in 60 mm dishes. Cells were incubated for 12 h in MEM or glucose-free MEM, each supplemented with 10% dialysed FBS, 2-DOG (A) and glucosamine (C) at the indicated concentrations. RNA was isolated and subjected to Northern-blot analysis with a  $^{32}\text{P}$ -labelled cDNA specific for HO-1 and GAPDH. A densitometric analysis of the autoradiograms corrected for the internal control (GAPDH) is also shown. The black bars represent HO-1/GAPDH expressed in arbitrary units. Results are derived from two independent experiments for 2-DOG and three independent experiments for glucosamine (means  $\pm$  S.E.M.). (B, D) Representative immunoblots of HepG2 cells incubated for 16 h in complete MEM, containing 5  $\mu\text{M}$  haemin (lane 1), or for 20 h in complete MEM (lanes 2–5) or glucose-free MEM (lanes 6–9). In (B), cells were supplemented with 1 mM (lanes 3 and 7), 5 mM (lanes 4 and 8) or 10 mM (lanes 5 and 9) 2-DOG respectively. In (D), cells were supplemented with 1 mM (lanes 3 and 7), 2 mM (lanes 4 and 8) or 4 mM (lanes 5 and 9) glucosamine respectively. HO-1 and GAPDH are identified as positive bands at masses approx. 32 and 36 kDa respectively.

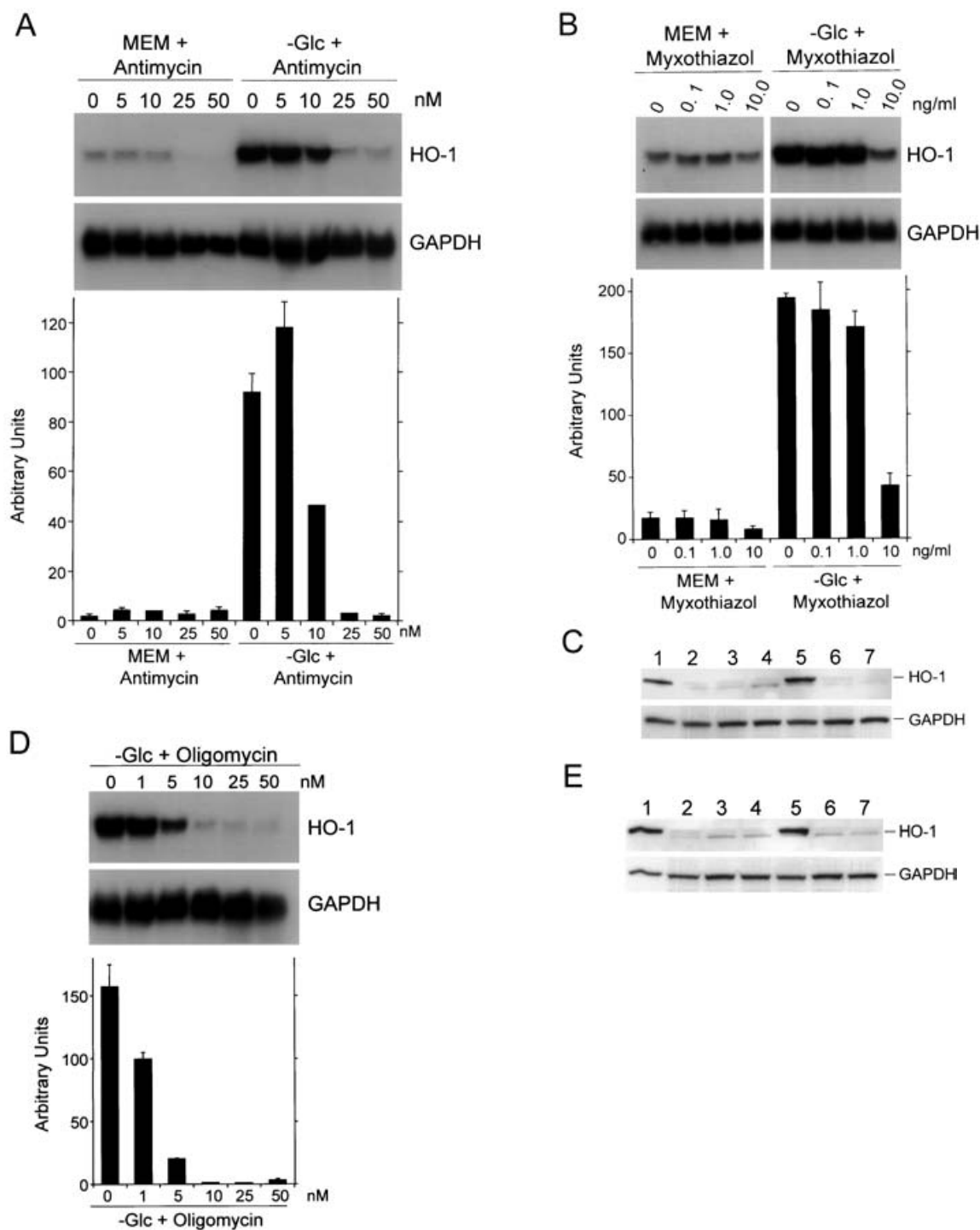
of the MnSOD gene. In those studies, the induction of MnSOD by TNF- $\alpha$  was also inhibited by the addition of NAC [50]. In this regard, our observations are similar to the induction of MnSOD by TNF- $\alpha$ , where electron-transport inhibitors and NAC inhibit HO-1 induction by glucose deprivation.

Small quantities of normally produced ROS can be detoxified by the endogenous scavenging mechanisms such as MnSOD, catalase and glutathione peroxidase. Previous studies have reported increased  $\text{H}_2\text{O}_2$  production and activation of the MAPK and c-Jun N-terminal kinase (JNK) pathways in multidrug-resistant human breast carcinoma MCF7/ADR cells after glucose deprivation [18–21]. Increase in  $\text{H}_2\text{O}_2$  levels and cytotoxicity by glucose deprivation were both reversed by the addition of glutamate and pyruvate [18]. Glutamate, derived from the action of glutaminase on glutamine, enters the tricarboxylic acid cycle during glucose deprivation and increases the formation of pyruvate, which can scavenge hydroperoxides [21]. The addition of glutamine did not decrease cytotoxicity as observed in the studies of Lee et al. [18], and it was suggested that MCF7/ADR cells are deficient in glutaminase and, hence, the contrasting results with glutamate and glutamine. In addition, the inclusion of NAC rescued cells from glucose-deprivation-

induced cytotoxicity. They suggested that one common mechanism of glucose-deprivation-induced toxicity is metabolic oxidative stress. These studies are supportive of our results suggesting a prominent role of oxidative stress following glucose deprivation as a mechanism for HO-1 induction.

Since previous studies have shown activation of the MAPK and JNK pathways after glucose deprivation [18,19], we also examined the effects of chemical inhibition of JNK using the JNK inhibitor, SP600125 (1, 10, 50, 100 and 200  $\mu\text{M}$ ), an anthrapyrazolone inhibitor of Jun N-terminal kinase [52], as well as a JNK-1 dominant-negative MCF-7/ADR cell line (kindly provided by Dr Y. J. Lee, University of Pittsburgh, Pittsburgh, PA, U.S.A.) and observed no inhibition of HO-1 induction by glucose deprivation (results not shown).

Although the excessive generation of ROS is detrimental to cell viability, subtoxic concentrations have been shown to initiate signalling cascades [53]. Recent studies have suggested that  $\text{H}_2\text{O}_2$  functions as an intracellular second messenger [54].  $\text{H}_2\text{O}_2$  readily diffuses across cellular membranes, causing activation of multiple signalling pathways, and depending on the concentration can induce cell injury or promote cell proliferation [55]. Previous studies have shown that  $\text{H}_2\text{O}_2$  is a powerful inducer of HO-1



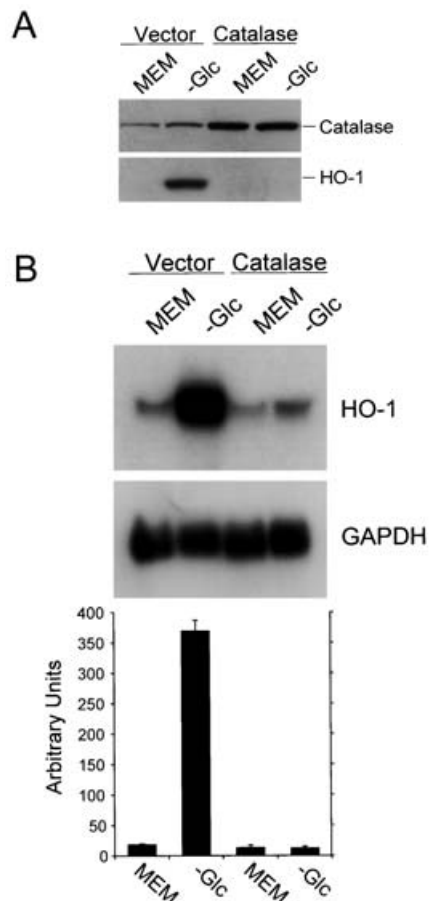
**Figure 4** Effect of mitochondrial electron-transport chain and ATP synthase inhibitors on the induction of HO-1 during glucose deprivation

HepG2 cells were cultured in MEM containing 10% FBS and grown to 80% confluence in 60 mm dishes. Cells were incubated in MEM or glucose-free MEM (containing 10% dialysed FBS) with antimycin A (**A**), myxothiazol (**B**) or oligomycin (**D**) at the indicated concentrations. RNA was isolated and subjected to Northern-blot analysis with a  $^{32}\text{P}$ -labelled cDNA specific for HO-1 and GAPDH. A densitometric analysis of the autoradiograms corrected for the internal control (GAPDH) is also shown. The black bars represent HO-1/GAPDH expressed in arbitrary units. Results are representative of three independent experiments (means  $\pm$  S.E.M.). (**C**, **E**) Representative immunoblots of HepG2 cells incubated for 16 h in complete MEM containing 5  $\mu\text{M}$  haemin (lane 1), or for 20 h in complete MEM (lanes 2–4) or glucose-free MEM (lanes 5–7). In (**C**), cells were supplemented with 5 ng/ml (lanes 3 and 6) or 10 ng/ml (lanes 4 and 7) myxothiazol respectively. In (**E**), cells were supplemented with 5 nM (lanes 3 and 6) or 25 nM (lanes 4 and 7) oligomycin respectively. HO-1 and GAPDH are identified as positive bands at masses approx. 32 and 36 kDa respectively.

[17]. The present study, in conjunction with the studies described above that demonstrate increased  $\text{H}_2\text{O}_2$  during glucose deprivation [18–21], suggests that  $\text{H}_2\text{O}_2$  plays a critical role in the induction of HO-1 after glucose limitation. Consistent with this hypothesis, the

present report documents that overexpression of catalase inhibits HO-1 induction.

To elucidate the signalling pathway of induction of HO-1 gene by glucose deprivation, we used specific inhibitors of predicted



**Figure 5** Effect of catalase overexpression on induction of HO-1 by glucose deprivation

HepG2 stable cell lines transfected stably with either vector alone (vector) or catalase were cultured in MEM containing 10% FBS and grown to 80% confluence in 60 mm dishes. **(A)** For immunoblot analysis, cells were incubated in MEM or glucose-free MEM for 24 h. After incubation, total cellular protein was extracted and immunoblot analysis performed as described in the Experimental section. Catalase and HO-1 were identified as positive bands at masses approx. 52 and 32 kDa respectively. **(B)** For Northern-blot analysis, vector alone and catalase overexpressing cells were incubated in MEM or glucose-free MEM containing 10% dialysed FBS for 12 h. RNA was isolated and subjected to Northern-blot analysis with a  $^{32}\text{P}$ -labelled cDNA specific for HO-1 and GAPDH. A densitometric analysis of the autoradiograms corrected for the internal control (GAPDH) is also shown. Results are representative of three independent experiments (means  $\pm$  S.E.M.).

signal pathways. The reason for choosing these pathways is that several of them have been involved in HO-1 induction by one or more stimuli (e.g. cadmium, hyperoxia, sodium arsenite). When HepG2 cells were subjected to glucose deprivation in the presence of these pathway inhibitors, induction of HO-1 was not blocked, suggesting that these pathways were not required. In addition, inhibition of cyclo-oxygenase (shown to be involved in UV-radiation-mediated HO-1 induction) [56] and cPLA<sub>2</sub> (involved in TNF- $\alpha$ -mediated induction of HO-1) [37] did not prevent increased HO-1 mRNA content, suggesting that these pathways were also not involved. Our results with the MAP kinase/ERK kinase-1 (ERK pathway) inhibitor, PD98059, showed induction of HO-1, whereas no inhibition of the increase after glucose deprivation was observed. These results are consistent with similar findings with both PD98059 and dominant-negative MAP kinase/ERK kinase inhibition in MCF7 cells [36], suggesting that

the ERK pathway may negatively regulate HO-1 expression in HepG2 cells as well.

In summary, our results suggest a 'glucose metabolic response' pathway involving ROS (specifically H<sub>2</sub>O<sub>2</sub>) and the mitochondrial electron-transport chain, in the induction of HO-1 during glucose limitation. Given the physiological role of HO-1 as a cytoprotective gene, understanding the signal pathways involved in glucose-deprivation-mediated HO-1 induction is therefore important and relevant.

This work was supported by grants from the National Institutes of Health (grant nos K08 DK02446 and R01 DK59600 to A.A.) and (grant nos DK-52064 and DK-59315 to M.S.K.). S.-H.C. was partially supported by the College of Medicine (Gyeongsang National University, South Korea). We thank Dr Tatsuo Tanaka (University of Ryukus, Japan) for the L7a cDNA.

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