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Nrf2-Mediated Heme Oxygenase-1 Upregulation as Adaptive Survival Response to Glucose Deprivation-Induced Apoptosis in HepG2 Cells

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Abstract

Induction of heme oxygenase-1 (HO-1) represents an important cellular adaptive survival response to oxidative stress and other toxic insults. In the present study, HepG2 cells grown in glucose-free media underwent apoptotic cell death, but they exhibited elevated expression of HO-1 before apoptosis manifested. Treatment of HepG2 cells with $SnCl_2$, a HO-1 inducer, rescued these cells from glucose deprivation-induced apoptosis, while inhibition of the HO activity with zinc protoporphyrin IX exacerbated apoptosis under the same condition. HepG2 cells transfected with a dominant negative Nrf2 were more vulnerable to glucose deprivation-induced apoptosis compared to cells transfected with empty vector alone. To confirm the involvement of Nrf2 in the induction of HO-1 caused by glucose deprivation, we used embryonic fibroblasts prepared from *nrf2*$^{-/-}$, *nrf2*$^{+/-}$, and *nrf2*$^{+/+}$ embryos. Compared to the wild-type and the *nrf2*$^{+/-}$ embryonic fibroblasts, *nrf2*$^{-/-}$ cells were less prone to induce HO-1 expression upon glucose deprivation. Exposure of HepG2 cells to glucose-deprived media resulted in an elevated accumulation of reactive oxygen species (ROS). Pretreatment with N-acetylcysteine prevented the glucose deprivation-induced ROS accumulation and also the HO-1 expression. In conclusion, the Nrf2-mediated HO-1 upregulation upon glucose deprivation is mediated by ROS in HepG2 cells, and responsible for the adaptive survival response. *Antioxid. Redox Signal.* 13, 1639–1648.

Introduction

Metabolic stresses can cause the depletion of intracellular reducing equivalents, which activates signal transduction pathways inducing the expression of a battery of stress responsive genes (4). It is well known that a decrease in glucose supply, the principal source of cellular reducing equivalents, results in transcriptional modulation of a number of genes that encode proteins associated with redox signaling (15). Heme oxygenase-1 (HO-1), one of the well-known antioxidant enzymes, catalyzes the rate-limiting step in the degradation of free heme released upon oxidative stress, yielding equimolar quantities of biliverdin, iron, and carbon monoxide (CO) (17). HO-1 is induced by a wide variety of stimuli that impose a significant shift in cellular redox (21). Since HO-1 functions as a key component of cytoprotection against oxidative stress caused by free heme and other stressful insults in various cell types, we have investigated whether this antioxidant enzyme is induced and protects in HepG2 cells from cytotoxicity in response to glucose deprivation.

Upregulation of HO-1 expression is modulated by several transcriptional regulatory elements that respond to the activation of several redox-sensitive transcription factors, particularly nuclear factor-erythroid 2-related factor-2 (Nrf2) (9). Nrf2 is a member of the basic leucine zipper family of transcription factors. The transcriptional activity of Nrf2 is controlled by its interaction with the cytoskeleton-associated inhibitory protein called Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm. Modification of Keap1 upon exposure of cells to electrophilic or oxidative stimulus allows free Nrf2 to translocate into the nucleus. Once translocated into the nucleus, Nrf2 binds to the antioxidant response element (ARE), a *cis*-acting regulatory element that plays an important role in the transcriptional activation of genes encoding HO-1

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and several other cytoprotective proteins, such as NAD(P)H: quinone oxidoreductase 1 (NQO1), glutathione *S*-transferase (GST), and glutamate cysteine ligase (GCL) (2, 7, 18). Many studies have revealed that Nrf2 activation promotes cell survival under stressful conditions (12, 26). In the present study, we investigated the possible role of Nrf2-mediated HO-1 upregulation in cellular defense against glucose deprivation-induced oxidative stress in HepG2 cells. Here, we report that glucose deprivation enhances HO-1 expression through nuclear translocation and ARE binding of Nrf2.

Materials and Methods

Materials

Dulbecco's modified Eagle's media (DMEM) containing high glucose (25 m*M*), low glucose (5.6 m*M*), or no glucose, and fetal bovine serum were obtained from Gibco BRL (Grand Island, NY). Zinc protoporphyrin IX (ZnPP IX) was the product of OXIS International, Inc. (Portland, OR). [γ-^{32}P]ATP was purchased from PerkinElmer Life Sciences (Waltham, MA). *N*-Acetyl-L-cysteine (NAC), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Aldrich Co. (St. Louis, MO) and the lactate dehydrogenase (LDH) cytotoxicity assay kit was provided by Cayman Chemical Co. (Ann Arbor, MI). An antibody against HO-1 was obtained from Stressgen Biotechnologies Co. (Victoria, BC, Canada), whereas those against HO-2 and Nrf-2 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies for poly(ADP-ribosyl)polymerase (PARP) and cleaved PARP were provided by Cell Signaling Technology, Inc. (Beverly, MA). An antibody against lamin B1 and secondary antibodies used for Western blot analysis were obtained from Zymed Laboratories Inc. (San Francisco, CA). pEF (control vector), pEF-dominant-negative Nrf2 (DN-Nrf2), and reporter gene-fusion constructs for luciferase (pTi-luciferase), wild-type ARE, and GC mutant ARE were kindly provided by Dr. Jeffrey A. Johnson (University of Wisconsin-Madison, Madison, WI).

Cell culture and viability measurement

HepG2 cells were grown at 37°C in high glucose DMEM supplemented with 10% heat-inactivated fetal bovine serum in a humidified atmosphere of 10% CO_2 and 90% air. Cells were plated at an appropriate density according to the scale of each experiment, and the medium was changed at 2-day intervals. After incubation, cells were treated further with MTT solution (1 mg/ml final concentration) for 2 h to assess their viability. Dark blue formazan crystals formed as a consequence of reduction of MTT, were dissolved in DMSO and their absorbance was read at 570 nm using a microplate reader. Results are expressed as the percentage of MTT reduction obtained in the treated cells compared to the absorbance of control cells.

LDH cytotoxicity assay

Cytotoxicity was measured by the release of LDH after exposure of the cells to complete DMEM or glucose free DMEM, according to the manufacturer's instruction (Cayman Chem Co., 10008882). The evaluation of cytotoxicity was calculated from the typical absorbance curve of LDH standards that were included in the assay kit.

Preparation and culturing of mouse embryonic fibroblasts

Nrf2-null mice, in which the *nrf2* gene is disrupted by targeted gene knockout, were provided by Dr. Jeffery Johnson, University of Wisconsin, Madison, WI. The *nrf2*$^{-/-}$, *nrf2*$^{+/-}$, and wild-type mice were maintained in the animal quarters in accordance with the university guidelines for animal care and were housed in a 12-h light/dark cycle. They were fed standard rodent chow and given water *ad libitum*. Male and female *nrf2*$^{+/-}$ mice were paired and the pregnancies were monitored. Embryos were obtained at the day 13.5 after pairing under aseptic conditions. The heads of the embryos were used to confirm the *nrf2* genotype by PCR, and the embryo bodies were minced into small pieces and cultured in high glucose DMEM supplemented with 10% fetal bovine serum and kept at 37°C with 5% CO_2.

Western blot analysis

After treatment with glucose-deprived media or other agents, cells (1×10^5/10 ml in a 100-mm dish) were collected, washed with phosphate-buffered saline (PBS), and centrifuged. Collected cells were vigorously shaken in lysis buffer (Cell Signaling Technology) at 4°C for 30 min. Following centrifugation at 10,000 *g* for 15 min, the supernatant was collected and stored at −70°C until further analysis. The protein concentration was determined using the BCA protein assay kit. After mixing with loading buffer, protein samples were electrophoresed on a 12% SDS-polyacrylamide gel. Separated proteins were transferred to polyvinylidene difluoride membrane at 300 mA for 4 h. To prevent nonspecific antibody binding to the separated proteins, the polyvinylidene difluoride membrane was blocked for 1 h at room temperature using the blocking buffer (0.1% Tween 20 in Tris-buffered saline (pH 7.4) containing 5% nonfat dried milk). Dilutions of primary antibodies, such as anti-HO-1 (Stressgen, Victoria, Canada), and anti-HO-2 (Santa Cruz Biotechnology Inc.), anti-cleaved PARP (Cell Signaling Technology), anti-lamin B1 (Zymed Laboratories Inc., South San Francisco, CA), and anti-Nrf2 (Santa Cruz Biotechnology Inc.) were made in PBS containing 3% nonfat dried milk. Following three washes with PBS and 0.1% Tween 20, the blots were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature in PBS containing 3% nonfat dried milk. After three additional washes with PBS and 0.1% Tween 20, the blots were incubated for 1 min in ECL substrate solution (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions and were visualized by exposure to X-ray film.

Reverse transcriptase–polymerase chain reaction

Total RNA was isolated from HepG2 cells incubated in glucose-free media using Trizol (GibcoBRL, Grand Island, NY) according to the manufacturer's instructions. Reverse transcriptase–polymerase chain reaction (RT-PCR) primers used in this study were as follows: (forward and reverse, respectively): *HO-1*, 5′-CAG GCA GAG AAT GCT GAG TTC-3′ and 5′-GAT GTT GAG CAG GAA CGC T-3′, 555 bp; *GAPDH*, 5′-AAG GTC GGA GTC AAC GGA TT-3′ and 5′-GCAGTGGGTCTCTCTCCT-3′, 1,054 bp. All primers were purchases from Bionics (Seoul, South Korea). To amplify the

cDNA specific for HO-1, 25 cycles at 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min were carried out. The housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was used as an internal control, and was amplified under the following condition: 26 cycles at 94°C for 1 min, 56°C for 2 min, and 72°C for 2 min. These amplification cycles were followed by a final extension at 72°C for 10 min. Each condition of polymerization depends on the size of primers and target genes. Amplification products were separated using 1.2% agarose gel electrophoresis, stained with ethidium bromide, and photographed under UV light.

Electrophoretic mobility shift assay for assessment of Nrf2-ARE binding

A synthetic double-stranded oligonucleotide containing the Nrf2-binding domain (ARE) was labeled with [γ-^{32}P]ATP using a T4 polynucleotide kinase and separated from the free [γ-^{32}P]ATP by gel filtration using a NICK spin column (Amersham Biosciences). The sequences of oligonucleotides in the double strands used in this study were 5′-TTT TCT GCT GAG TCA AGG TCC G-3′ and 3′-AAA AGA CGA CTC AGT TCC AGG C-5′. The oligonucleotide was synthesized by Bionics. Prior to the addition of radiolabeled oligonucleotide (100,000 cpm), 10 μg of the nuclear extract was kept on ice for 15 min in gel shift binding buffer [4% glycerol, 1 m*M* EDTA, 1 m*M* dithiothreitol, 100 m*M* NaCl, 10 m*M* Tris-HCl (pH 7.5), and 0.1 mg/ml sonicated salmon sperm DNA]. The DNA protein complexes that formed were resolved on a 6% nondenaturing polyacrylamide gel at 145 V for 2 h and were subjected to autoradiography.

Measurement of intracellular ROS accumulation

The fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCF-DA) purchased from Invitrogen (Carlsbad, CA) was used to monitor the net intracellular accumulation of ROS. After treatment of HepG2 cells (1×10^4/ml on a 4-well chamber slide) with glucose-deprived media, the cells were rinsed with Krebs-Ringer solution and were loaded with 10 μM DCF-DA. After one 15 min incubation at 37°C, the cells were scanned with laser (488 nm, 200 milliwatt) by confocal microscope (Leica Inverted DM IRB, Bannockburn, IL).

Immunocytochemical analysis of Nrf2

To demonstrate the glucose-deprivation-dependent increase of Nrf2 nuclear translocation, immunocytochemistry was performed using the polyclonal antibody recognizing Nrf2. Cells treated with glucose deprived media (1×10^5 cells/600 μl in a 4-well chamber slide) were fixed in 10% neutral buffered formalin for 30 min at room temperature. After a rinse with PBS, the fixed cells were incubated in fresh blocking buffer (0.5% Tween-20 in PBS, pH 7.4, containing

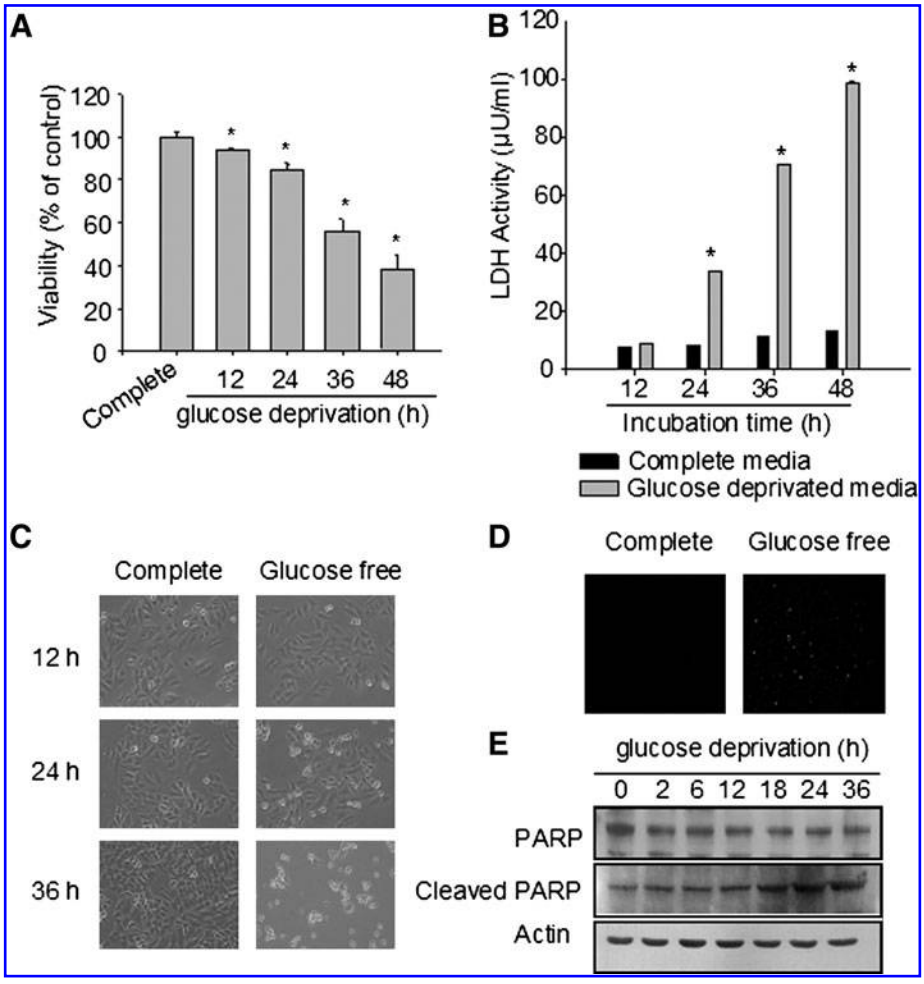

FIG. 1. Glucose deprivation-induced apoptotic cell death in HepG2 cells. (**A, B, C**) Cells were incubated for the indicated periods in glucose-free DMEM supplemented with 10% FBS. Incubation in glucose-free DMEM caused substantial cell death as determined by the MTT reduction assay (**A**), the LDH cytotoxicity assay (**B**), and by phase contrast microscopy (**C**). The results are presented as means $\pm$ S.D. ($n = 3$); *$p < .05$ (**D**) Internucleosomal DNA fragmentation was assessed by *in situ* terminal nick end-labeling (TUNEL). Cells were incubated for 36 h in complete DMEM or glucose-free DMEM. Cells exposed to glucose-free DMEM had increased internucleosomal DNA fragmentation. (**E**) Cells were cultured for the indicated times and harvested for immunoblot analysis of key proteins involved in apoptosis. Protein from cell lysates was resolved by SDS-PAGE and identified by Western blot analysis using antibodies against PARP and cleaved PARP. Incubation of cells in glucose-free DMEM resulted in an increased production of cleaved PARP. Actin levels were measured to ensure equal protein loading.

10% normal goat serum) for 1 h at room temperature. Upon addition of the anti-Nrf2 primary antibody solution (diluted 1:100 in PBS with 1% bovine serum albumin), the cells were then incubated overnight at 4°C. Afterward, the cells were washed three times with PBST (PBS containing 0.1% Tween-20) and incubated for 1 h at room temperature following addition of FITC-goat anti-rabbit IgG secondary antibody diluted (1:1,000) (Invitrogen) in PBST containing 3% bovine serum albumin. Cells were then rinsed with PBS, and the stained cells were analyzed under a confocal microscope (Leica, Bannaockburn, IL).

Luciferase assay for the determination of Nrf2 transcriptional activity

The luciferase activity was measured in HepG2 cells transfected with an ARE-luciferase reporter plasmid, as reported previously (16).

Statistical analysis

Where necessary, data are expressed as the means ± S.D., and Student's *t* test was used to perform statistical analysis for a single comparison. The criterion for statistical significance was $p < 0.05$.

Results

Glucose deprivation causes apoptosis in HepG2 cells

HepG2 cells were maintained in glucose-free media for 12, 24, 36, and 48 h, and their viability was measured by using the MTT assay. Compared with the cells grown in high-glucose complete media, cells which were treated with glucose-free media exhibited reduced viability (Fig. 1A). Cell viability was also measured by the LDH cytotoxicity assay. Cells incubated in glucose-free media for 48 h showed a significant increase of LDH activity more than 7-fold compared with cells which were incubated in complete media for the same duration. In addition, the cells grown in the glucose-free media exhibited distinct morphological changes. These cells were detached and had globular shapes (Fig. 1C). To determine whether glucose deprivation-induced cytotoxicity was associated with apoptotic death, we conducted TUNEL staining. After 36 h of incubation in glucose-free media, there was an increased proportion of TUNEL-positive apoptotic cells observed (Fig. 1D). Furthermore, cleavage of PARP, another hallmark of apoptosis, was evident in cells subjected to glucose deprivation (Fig. 1E).

Glucose deprivation induces HO-1 expression as an adaptive response

HO-1 is an important stress-responsive antioxidant enzyme known to be induced by a wide variety of oxidative or electrophilic stimuli. When HepG2 cells were incubated in media lacking glucose, there was a significant increase in the expression of HO-1, but the expression of the noninducible isoform HO-2 remained unchanged (Fig. 2A). However, expression levels of other antioxidant enzymes, such as GST, GCL, and NQO1, barely changed by glucose deprivation (data not shown). Upon exposure to glucose-deprived media, *HO-1* mRNA expression increased rapidly and preceded the HO-1 protein accumulation (Fig. 2B). To confirm the crucial

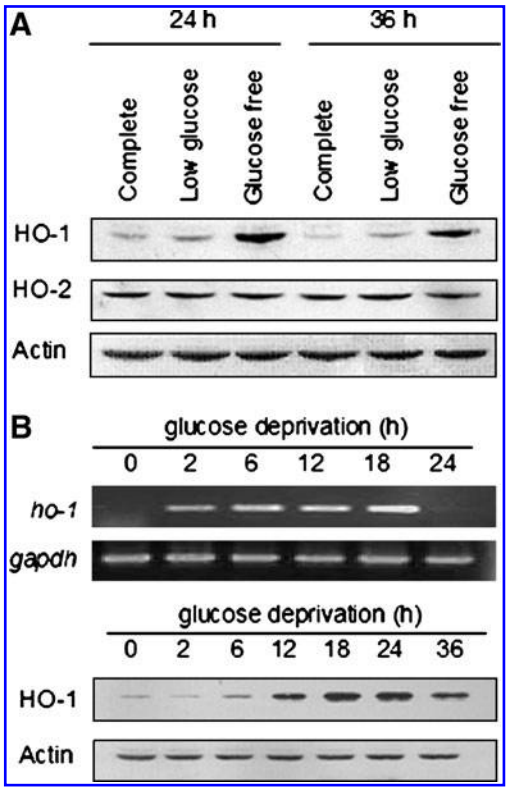

FIG. 2. Effect of glucose deprivation on HO-1 expression. (**A**) Cells were incubated in the indicated DMEM for either 24 h or 36 h. Protein in cell lysates was analyzed by Western blot using HO-1 and HO-2 specific antibodies. (**B**) Cells were cultured in glucose-free DMEM and harvested at various time intervals. RT-PCR and Western blot analyses were conducted to measure the levels of *HO-1* mRNA transcript and protein, respectively. Actin and *GAPDH* levels were measured to ensure equal amounts of protein and mRNA loaded, respectively.

cytoprotective role of HO-1 induction, cells were treated with a well-known HO inducer, $SnCl_2$, prior to being deprived of glucose. The elevation of HO-1 levels in $SnCl_2$-treated cells was confirmed by Western blot analysis (Fig. 3A). $SnCl_2$ treatment attenuated glucose deprivation-induced cell death in a dose-dependent manner (Fig. 3B). In contrast, treatment of HepG2 cells with ZnPP IX, an inhibitor of HO-1 activity, exacerbated the cytotoxicity induced by exposure to glucose-free media (Figs. 3C and 3D). The viability of cells maintained in complete glucose medium was also reduced by the ZnPP IX pretreatment. These results suggest that upregulation of HO-1 expression does play a role in cellular protection against cytotoxicity caused by glucose deprivation.

HO-1 upregulation induced by glucose deprivation is mediated by transient activation of Nrf2

The promoter region of the human *HO-1* gene harbors consensus ARE sequences that are essential for the induced expression of antioxidant enzymes and other cytoprotective proteins in response to oxidative stress. A number of conditions that perturb the intracellular redox status are known to activate the transcription factor Nrf2 and to promote its translocation into the nucleus, where it binds to ARE and

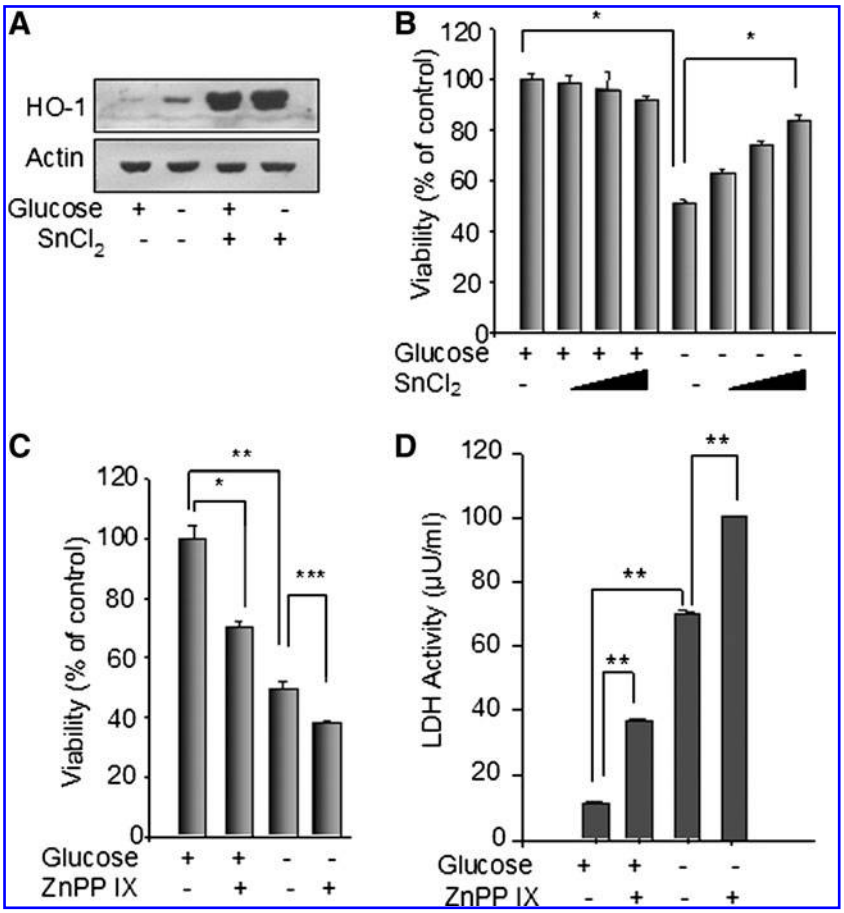

FIG. 3. Effect of HO-1 induction or inhibition of HO-1 activity on survival against glucose deprivation-induced cytotoxicity. (**A**) Cells were treated with 0 or 0.5 m*M* $SnCl_2$, a HO-1 inducer, for 24 h in complete or glucose-free DMEM. To measure induction of HO-1 protein expression, Western blot analysis was conducted. (**B**) Cells were treated with varying concentrations (0, 0.1, 0.25, or 0.5 m*M*) of $SnCl_2$ for 36 h in either complete DMEM or glucose-free DMEM. After 36 h incubation, the MTT reduction assay was performed for evaluating cell viability. $*p < 0.00005$. (**C, D**) Cells were pretreated with ZnPP IX (20 μM) for 12 h before incubation in glucose-free media. The cells were maintained in either complete DMEM or glucose-free DMEM for 36 h. Viability/cytotoxicity were assessed by the MTT reduction assay (**C**) and the LDH cytotoxcitiy assay (**D**). $*p < 0.00005$, $**p < 0.005$, $***p < 0.05$.

enhances the ARE-mediated expression of a battery of antioxidant and cytoprotective proteins. Glucose deprivation led to a transient increase in the levels of Nrf2 protein localized in the nuclear fraction (Figs. 4A and 4B), and also induced its binding to ARE (Fig. 4C). To examine the role of Nrf2 in inducing the ARE-dependent transcriptional activity, cells were transfected with a luciferase reporter construct harboring the ARE sequence. As a control, we also transfected cells with a Ti-Luc vector or GC-mutant vector. Exposure of cells transfected with an intact ARE-Luc vector to a medium lacking glucose resulted in a marked upregulation of ARE-luciferase activity at 12 h (Fig. 4D). To confirm that the upregulation of HO-1 expression in HepG2 cells treated with glucose-deprived media was mediated by Nrf2, we utilized mouse embryonic fibroblast (MEF) cells obtained from Nrf2 knockout mice (Fig. 5). In the Nrf2 wild-type MEF cells, prominent induction of HO-1 expression occurred by exposure to glucose-deprived media. However, in the MEFs prepared from the Nrf2 knockout ($nrf2^{-/-}$) embryos, upregulation of HO-1 expression did not occur regardless of the presence of glucose.

Glucose deprivation enhances ROS production responsible for Nrf2 activation and HO-1 induction

Previous studies have demonstrated that the majority of stimuli that induce HO-1 expression are caused by oxidative stress. Chang *et al*. suggested that the overproduction of ROS under oxidative stress conditions could serve as a crucial signaling messenger, leading to the induction of HO-1 expression under glucose deprivation conditions (5). Intracellular ROS accumulation resulting from exposure to glucose-deprived media was measured using DCF-DA, a membrane permeable fluorescent probe that produces fluorescence upon reaction with peroxides such as H_2O_2. When HepG2 cells were incubated in glucose-deprived media, there was a marked increase in intracellular ROS accumulation (Fig. 6A). Pretreatment of the cells with a thiol antioxidant NAC markedly reduced intracellular ROS accumulation (Fig. 6A) and Nrf2-ARE binding (Fig. 6B), as well as nuclear localization of Nrf2 (Fig. 6C) caused by glucose deprivation. Likewise, the upregulation of HO-1 expression induced by glucose deprivation was suppressed by NAC (Fig. 6D). These results suggest that glucose deprivation results in ROS production, which in turn upregulates HO-1 expression through activation of Nrf2 signaling.

To confirm that activation of functional Nrf2 accounts for the upregulation of HO-1 expression that provides the cytoprotection under glucose-deprived conditions, we examined apoptotic status of cells transfected with a dominant-negative (DN) mutant form of Nrf2 with a truncated N terminus (3). In the HepG2 cells transiently transfected with the plasmid harboring DN-Nrf2, glucose deprivation-induced apoptosis was augmented as determined by TUNEL positivity (Fig. 7).

Discussion

Nearly all types of tumor cells depend on glycolysis, which proceeds at a much higher rate than that in noncancerous tissues. Thus, tumor cells are exposed frequently and maybe constantly to glucose deprived conditions (27, 29, 30). In

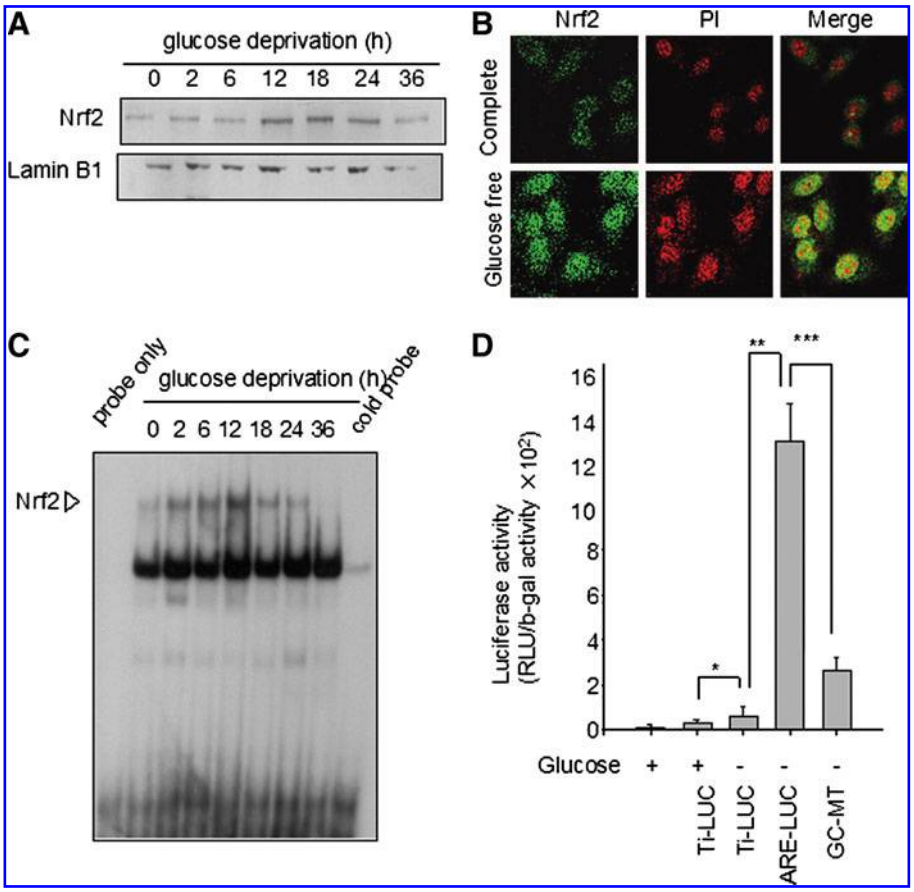

FIG. 4. Glucose deprivation-induced transient activation of Nrf2 in HepG2 cells. (**A**) Glucose deprivation-induced Nrf2 nuclear translocation was examined by Western blot analysis using an anti-Nrf2 antibody. The nuclear protein was extracted from HepG2 cells incubated in glucose-free DMEM for indicated times. (**B**) Cells were incubated in the indicated media for 12 h. Staining of Nrf2 and nucleus was done using an anti-Nrf2 antibody and PI, respectively. Cells were examined by confocal laser-scanning microscope. The immunocytochemical assay showed that glucose deprivation induced nuclear translocation of Nrf2. (**C**) The Nrf2-ARE-DNA binding activity was assessed by the electrophoretic mobility shift assay after incubation of HepG2 cell in glucose-free DMEM for the indicated times. Nuclear extracts were incubated with [γ-^{32}P]-labeled oligonucleotides harboring the ARE consensus sequence. (**D**) Glucose deprivation-induced Nrf2 transcriptional activity was measured by the luciferase reporter gene assay. HepG2 cells were transiently transfected with the plasmid containing the ARE-binding site-luciferase construct (ARE-LUC) or GC mutant or control vector (Ti-Luc). After incubation in glucose-free media for 12 h, cells were treated with reporter lysis buffer for the measurement of ARE promoter activity. The results are presented as means ± S.D. ($n = 3$); *$p > 0.05$; **$p < 0.001$; ***$p < 0.005$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

particular, the microenvironment of solid tumor tissues are often confronted with unfavorable conditions such as hypoxia and hypoglycemia (27, 29, 30). Thus, cancer cells tend to develop self-defense mechanisms to survive such adverse conditions. Several studies have revealed that Nrf2 signaling is constitutively activated in some cancerous tissues, such as hepatocarcinomas (8) and lung carcinomas (20, 24). As a consequence, the downstream genes of Nrf2 are abnormally upregulated in some cancer cell lines and human tumor specimens. This provides cancer cells with a survival advantage against oxidative cytotoxicity caused by many anticancer drugs and radiation therapy. In support of this notion, cancer cells with increased Nrf2 signaling have been shown to be more resistant to chemotherapeutic drugs, such as cisplatin, doxorubicin, and etoposide. Constitutive activation of Nrf2 is likely to be responsible for acquired chemoresistance to the

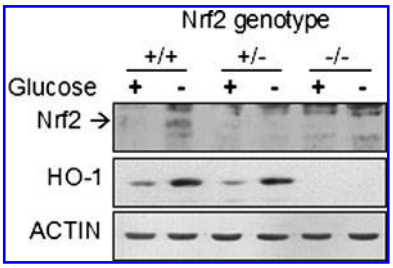

FIG. 5. Effect of glucose deprivation on HO-1 expression in Nrf2$^{-/-}$ MEFs. MEF cells were obtained from Nrf2 $^{-/-}$, $^{+/-}$ and $^{+/+}$ embryos. MEF cells of each genotype were incubated in complete or glucose-free media for 24 h. The Western blot analysis was performed from whole cell lysates.

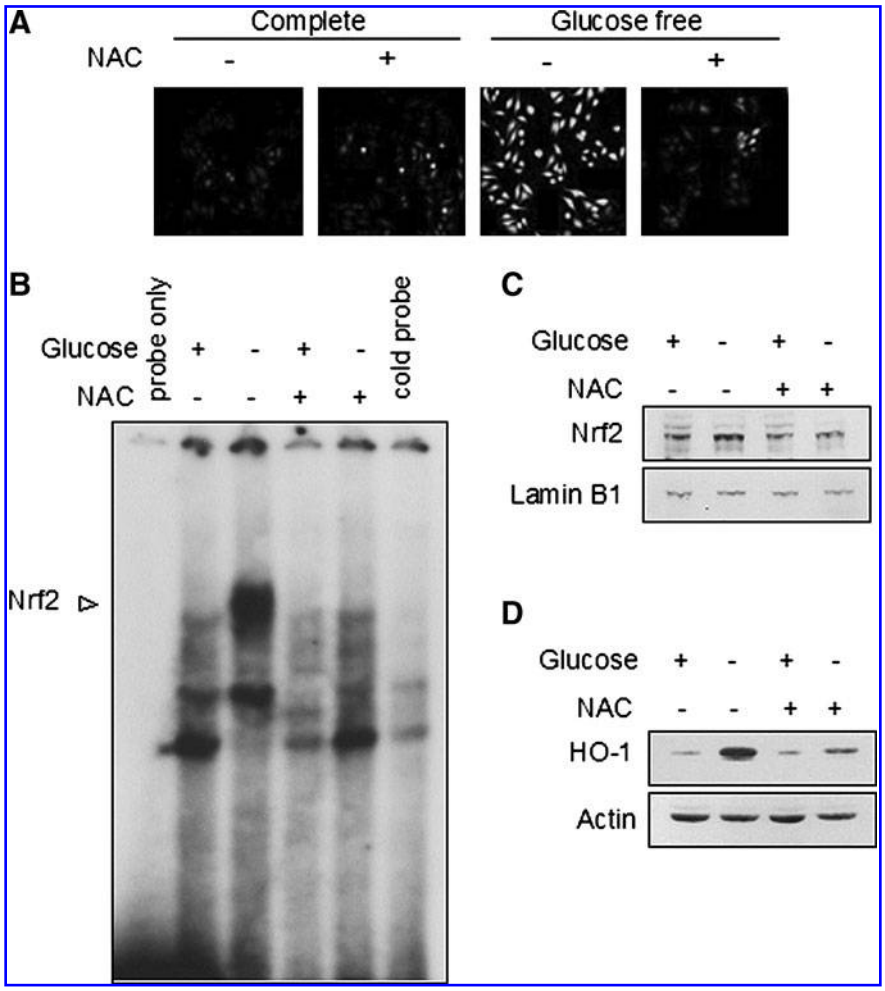

FIG. 6. Involvement of ROS accumulation in glucose deprivation-induced HO-1 expression through Nrf2 activation. (**A**) Intracellular ROS levels were determined based on the DCF-DA fluorescence. Cells were incubated in the indicated DMEM for 6 h with or without 5 m*M* NAC. Images were acquired by using a confocal laser-scanning microscope. (**B, C, D**) HepG2 cells were treated with 5 m*M* of NAC in glucose-free media for 12 h (**B**, **C**) or 24 h (**D**). Glucose deprivation-induced Nrf2 activation (**B**, **C**) and HO-1 expression (**D**) were inhibited by treatment with NAC.

anticancer drugs (19, 23, 26). Recently, Nrf2 has been recognized as a double-edged sword because not only does it protect normal cells from oxidants and electrophilic toxicants, but it also promotes the survival of cancer cells under anticancer therapeutic conditions (14).

In this study, we have focused on the induction of HO-1 expression in human hepatoma cells via the Nrf2 signaling pathway. Of the target genes whose expression is upregulated by Nrf2, HO-1 is the key enzyme that plays a crucial role in eliminating the free heme responsible for the hydroxyl radical-producing Fenton reaction, and hence promotes the survival of cells under oxidative stress (11). Several studies indicate that overexpressed HO-1 promotes cancer cell growth and survival, enhances cancer cell resistance to

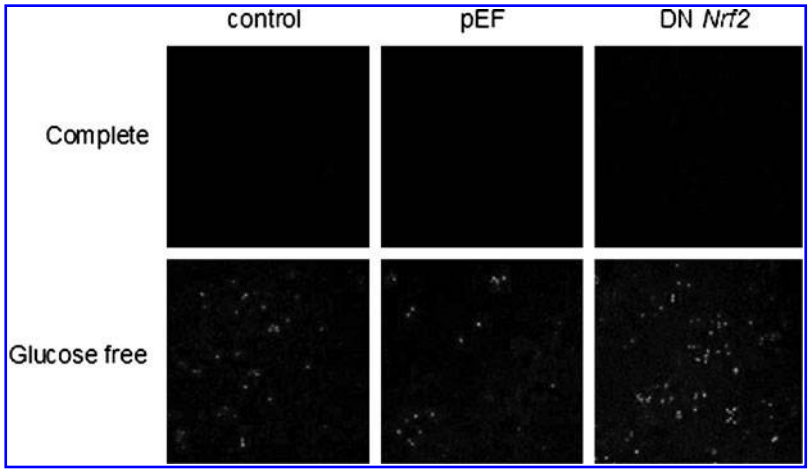

FIG. 7. Effect of Nrf2 on glucose deprivation-induced cytotoxicity as an adaptive response. Cells transfected with a dominant negative (DN) *Nrf2* expressing vector or with a control *pEF* plasmid were incubated in either complete DMEM or glucose-free DMEM for 24 h. Internucleosomal DNA fragmentation was assessed by TUNEL. Cells transfected with DN-*Nrf2* showed more prominent apoptotic cell death when compared with the cells transfected with the control vector (pEF).

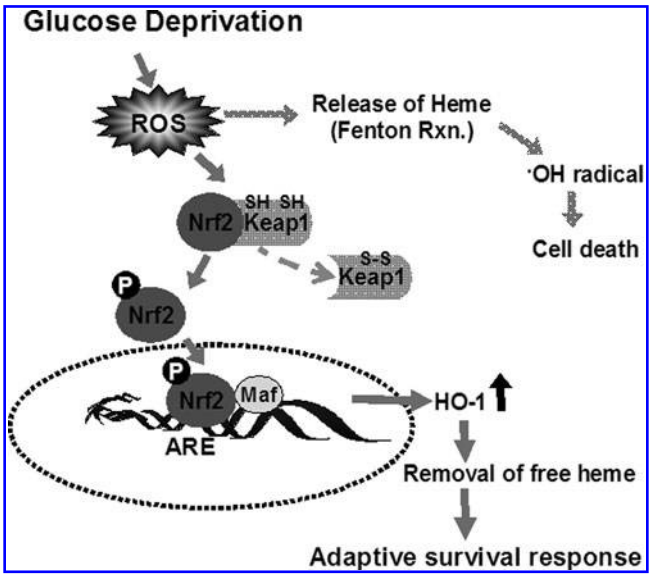

FIG. 8. A proposed pathway for glucose deprivation-induced Nrf2 activation and HO-1 expression, which confers an adaptive survival response in HepG2 cells.

apoptotic death under oxidative stress induced by anticancer drugs, and even stimulates metastasis and angiogenesis (14, 28). Although prolonged glucose deprivation induced apoptotic cell death in HepG2 cells, there was upregulated HO-1 expression before apoptosis manifested. HepG2 cells pretreated with $SnCl_2$ exhibited an increase in HO-1 expression and survival from apoptosis induced by glucose deprivation. In contrast, pharmacologic inhibition of HO-1 activity or functional inactivation of Nrf2 aggravated cell death upon exposure to glucose deprivation. These results indicate that induction of Nrf2-mediated HO-1 upregulation in glucose-deprived HepG2 cells confers an adaptive survival response before the manifestation of cell death.

In normal aerobic cells, mitochondrial respiration is the main source of ATP production and also the generation of ROS. The accumulation of intracellular ROS can be decreased by treatment with inhibitors of mitochondrial electron-transport chain. (6, 22). Normally, the small quantities of ROS produced can be eliminated or inactivated by endogenous antioxidant enzymes, such as glutathione peroxidase, at the expense of GSH oxidation causing mild oxidative stress. As the regeneration of reduced GSH serves as a critical modulator of maintaining cellular redox potential and NADPH provided by the glycolytic pentose phosphate pathway is utilized as a crucial reducing cofactor, constant and abundant supply of glucose is essential (13). Glucose-6-phophate dehydrogenase (G6PDH), the rate-limiting enzyme of the pentose phosphate pathway, controls the metabolism of glucose to generate an adequate amount of NADPH, an essential reducing molecule (13). Thus, some reports suggest that adequate supply of glucose and expression of G6PDH is essential for the maintenance of redox balance and protection of cells against oxidative stress (25). Therefore, aerobic cells subjected to glucose deprivation are unable to eliminate ROS and exhibit accumulated ROS levels, due to a limited supply of NADPH (1).

Induction of HO-1 expression observed upon glucose deprivation is mediated by excessive generation of ROS or lack of their elimination (5). Glucose deprivation-induced HO-1 expression appeared to be initiated by production of ROS, as this was abrogated by the addition of NAC, a cell-permeable cysteine that can scavenge and neutralize the ROS causing oxidative stress. NAC treatment attenuated the overproduction of ROS, thus, subsequently abolishing the nuclear translocation of Nrf2 and ARE-Nrf2 binding. Under normal physiological conditions, Kelch-like ECH associating protein (Keap1) sequesters and degrades Nrf2 in the cytoplasm (10). Keap1 is recognized as an essential sensor for oxidative signaling induced by extracellular stimulation, since it contains several cysteine residues that can undergo oxidation upon contact with ROS (10). As a consequence of oxidation of critical sensor SH residues in Keap1, free Nrf2 is able to translocate into the nucleus and bind to ARE-bearing genes that encode distinct antioxidant enzymes, including HO-1. This is schematically represented in Figure 8. It is likely that glucose deprivation-provoked ROS generation leads to the oxidation of critical cysteine residues on Keap1.

In summary, ROS produced by glucose deprivation stimulates dissociation of the Nrf2-Keap1 complex, presumably by oxidation of critical cysteine thiol residues present in Keap1. This promotes nuclear translocation of free Nrf2 and the binding of Nrf2 to the ARE, leading to the upregulation of HO-1 gene expression. Our findings support the hypothesis that activation of the Nrf2-HO-1 signaling pathway contributes to the survival of cancer cells under glucose-deprived conditions. The 'dark' side of Nrf2 activation is a newly emerging issue because of its implication for the resistance to cancer therapy (14). Better understanding of the mechanisms by which glucose deprivation causes Nrf2 activation and induces HO-1 expression will be useful in the development of novel therapeutic strategies for effective treatment of cancer.

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Author Disclosure Statement

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Abbreviations Used

ARE = antioxidant response element
DMEM = Dulbecco's modified Eagle's medium
HepG2 = human hepatoma cells
HO-1 = heme oxygenase-1
Keap1 = Kelch-like ECH-associating protein 1
MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC = *N*-acetyl-L-cysteine
NADPH = reduced form of nicotinamide adenine dinucleotide phosphate
Nrf2 = NF-E2-related factor2
PAGE = polyacrylamide gel electrophoresis
PBS = phosphate-buffered saline
ROS = reactive oxygen species
SDS = sodium dodecylsulfate

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