Nuclear Heme Oxygenase-1 (HO-1) Modulates Subcellular Distribution and Activation of Nrf2, Impacting Metabolic and Anti-oxidant Defenses*

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With oxidative injury as well as in some solid tumors and myeloid leukemia cells, heme oxygenase-1 (HO-1), the anti-oxidant, anti-inflammatory, and anti-apoptotic microsomal stress protein, migrates to the nucleus in a truncated and enzymatically inactive form. However, the function of HO-1 in the nucleus is not completely clear. Nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor and master regulator of numerous antioxidants and anti-apoptotic proteins, including HO-1, also accumulates in the nucleus with oxidative injury and in various types of cancer. Here we demonstrate that in oxidative stress, nuclear HO-1 interacts with Nrf2 and stabilizes it from glycogen synthase kinase 3β (GSK3β)-mediated phosphorylation coupled with ubiquitin-proteasomal degradation, thereby prolonging its accumulation in the nucleus. This regulation of Nrf2 post-induction by nuclear HO-1 is important for the preferential transcription of phase II detoxification enzymes such as NQO1 as well as glucose-6-phosphate dehydrogenase (G6PDH), a regulator of the pentose phosphate pathway. Using Nrf2 knock-out cells, we further demonstrate that nuclear HO-1-associated cytoprotection against oxidative stress depends on an HO-1/Nrf2 interaction. Although it is well known that Nrf2 induces HO-1 leading to mitigation of oxidative stress, we propose a novel mechanism by which HO-1, by modulating the activation of Nrf2, sets an adaptive reprogramming that enhances antioxidant defenses.

The nuclear factor erythroid 2-related factor 2 or Nrf2,2 a member of the cap’n’collar family of basic leucine zipper tran-
scription factors, plays a crucial role in antioxidant defenses (1, 2). Nrf2 is constitutively expressed in the cytoplasm, and its accumulation and activation in the nucleus are favored in oxidative injury and also in cancer cells (3–6). In the nucleus, Nrf2 in a heterodimer with small Maf proteins binds to the antioxidant response elements (ARE) in the promoter regions of Nrf2 target genes. This signaling facilitates transcription of numerous antioxidant enzymes and growth factors, thereby promoting tolerance to oxidative stress (2, 6, 7). In addition, Bach1, which competes with Nrf2 for binding to the ARE, suppresses ARE-mediated gene transcription (8). One important step in Nrf2 signaling is stabilization of the constitutive Nrf2 from Kelch-like ECH-associated protein 1 (KEAP1), which acts as an adaptor protein between Nrf2 via the Neh2 domain at the N terminus and Cul3 E3 ligase targeting Nrf2 for ubiquitin-proteasomal degradation. Many studies show that, with oxidative or electrophilic stress, both Nrf2 and Keap1 undergo post-translational modification, favoring release of Nrf2 by the degradation of Keap1 thereby facilitating stabilization and nuclear transport of Nrf2 (9–13). However, the regulation of Nrf2 is complex and involves a network of intricate mechanisms beyond Keap1 (14–19). More recently it was reported that GSK3β-mediated phosphorylation of Ser344 and Ser347 within the phosphodegron motif of the Neh6 domain targets Nrf2 protein for proteasomal degradation by another E3 ligase, β-TrCP, in a Keap1-independent manner (20–22). This process is thought to occur in the nucleus post-induction.

It is well documented that heme oxygenase (HO)-1, a 32-kDa protein, is positively regulated by Nrf2 (1, 23). As a protective and adaptive response, most tissues exhibit robust activation of the highly inducible HO-1 (24–28). The beneficial effects of HO-1 in cytoprotection have long been attributed to its enzy-

* This work was supported, in whole or in part, by National Institutes of Health Grants HL-58752 and HL-11190 from the National Institutes of Health (to P. A. D.)
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2 The abbreviations used are: Nrf2, nuclear factor erythroid 2-related factor 2; ARE, antioxidant response element; HO-1, heme oxygenase-1; HO-1-TR, truncated HO-1, HO-1-FL, full-length HO-1; V, empty vector; MEF, mouse embryonic fibroblast; IP, immunoprecipitation; NQO1, NAD(P)H dehydrogenase, quinone 1; G6PDH, glucose-6-phosphate dehydrogenase; GSK3β, glycogen synthase kinase 3β; SOD2, superoxide dismutase 2; DHE, dihydroethidium; Keap1, Kelch-like ECH-associated protein 1; Bis-Tris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)propane-1,3-diol; DMSO, dimethyl sulfoxide; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide inner salt; TR, truncated; p, phosphorylated.

26882 JOURNAL OF BIOLOGICAL CHEMISTRY VOLUME 289 • NUMBER 39 • SEPTEMBER 26, 2014
motic action in heme degradation and also to the catalytic
byproducts including carbon monoxide and biliverdin (29).
More recently subcellular localization of HO-1 has been stud-
ied in the context of oxidative stress and cancer (30). Although
HO-1 is an integral protein of the smooth endoplasmic reticu-
ulum (31, 32), it can localize to other compartments including
caveolae (33), mitochondria (34), and the nucleus (35, 36),
where it can mediate signaling functions (30).

We have previously described a more rapidly migrating iso-
form of HO-1 in the nuclear extracts of hypoxia-exposed cells
transfected with N-terminally FLAG-tagged HO-1 (30). A neg-
ative reactivity for an antibody directed to the C terminus iden-
tified this isoform as a 28-kDa fragment of HO-1 missing 52
amino acids from the C terminus (36). This nuclear HO-1 was
also found to be enzymatically inactive; however, it was associ-
ated with enhanced activation of transcription factor AP-1 (37).
Earlier Hori et al. (38) showed that a mutated, enzymatically inac-
tive form of HO-1 was still able to protect against oxidative injury.

In addition, a HO-1 mutant with a deletion of the C-terminal
amino acids was shown to bind to heme but could not degrade it to
biliverdin (39). We documented that the enzymatically inactive
HO-1 can alter its own transcription through activation of AP-1
(36, 37). Because an AP-1 consensus sequence is found within the
ARE, and since both ARE and API sequences are found on the
sequences of this activation.

To evaluate this, we used hypoxia exposure, a clinically
relevant oxidative stress known to activate Nrf2 signaling (41–
43) and induce HO-1, resulting in its migration to the nucleus
(42). In the present study, mouse embryonic fibroblasts (MEFs)
were used, as was a prostate cancer cell line (LnCap) because
this cell line and human prostate cancer tissues have enhanced
nuclear localization of HO-1 when compared with the normal
prostate tissue (35). The present study demonstrates that nuclear
HO-1 specifically interacts with Nrf2 in the nucleus to
facilitate its sustained stabilization from GSK3β-mediated pro-
teolytic degradation. This leads to preferential activation of
cytoprotective pathways for sustained tolerance against oxida-
tive injury, as well as cell survival.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—MEFs, HO-1-knock-out (HMOX1−/−, KO) and
wild type (HMOX1+/+, WT), were generated from embryonic
day 13.5 embryos of the same litters. These cells were
immortalized by transfection of plasmids pSV3-neo (ATCC
37150) expressing the simian virus 40 (SV40) large T-antigen
immortalized by transfection of plasmids pSV3-neo (ATCC
day 13.5 embryos of the same littermates. These cells were

**Stable Knockdown of HO-1 with shRNA**—To knock down
endogenous HO-1 expression, lentiviruses carrying shRNA tar-
targeting HO-1 mRNA were produced as per the manufacturer’s
instructions (RMM4534-NM-010442, Thermo Fisher Sci-
entific Open Biosystems products). Based on the highest knock-
down efficiency, clone 6 (7175B) was chosen for the knockdown
experiments, and clone 11, which did not reduce HO-1 expres-
sion, was used as a control (nonspecific) shRNA.

**Hyperoxia Exposure**—Cells were seeded at 0.8 × 10⁶/10-cm
dish. After overnight culture, cells were transferred to fresh
medium and exposed to hyperoxia (O₂: 95% O₂/5% CO₂) or
normoxia (air: 21% O₂/5% CO₂) for 18 h (42). In some experi-
ments, cells were exposed to air or O₂ for 2, 6, and 18 h.

**Preparation of Cell Lysates**—Cells were harvested and homo-
genized in the presence of protease and phosphatase inhibitors
either to extract whole cell homogenate using the M-PER mam-
malian protein extraction reagent (78501) or to extract cyto-
plasmic and nuclear fractions using NE-PER nuclear and cyto-
plasmic extraction reagents (78835). Both extraction reagents
were obtained from Thermo Fisher Scientific.

**Protein Content Estimation**—The protein content in the whole
cell lysate or nuclear and cytosolic fractions was measured by a
96-well plate based Bradford assay.

**Western Blotting**—Proteins (15–30 µg) either from whole
cell lysate or from the fractions of cytoplasm and nucleus were
resolved by SDS-PAGE on a precast 4–12% Bis-Tris gels (Invit-
rogen), electrotransferred to PVDF membrane (Millipore,
IPVH00010), and assessed for the immunosignal of prot eins,
HO-1 (Enzo, ADI-SPA-896), FLAG (Sigma, F-7425), Myc
(Santa Cruz Biotechnology, sc789), Nrf2 (Santa Cruz Biotech-
nology, sc-722), pSer⁴⁰-Nrf2 (Epitomics, 2073-1), HA (Santa
Cruz Biotechnology, sc805), polyubiquitin (Santa Cruz Bio-
technology, sc8017), AKT (Cell Signaling, 9322), pSer⁴³⁷-AKT
(Cell Signaling, 4058), GSK3β (Cell Signaling, 9832), pSer⁴⁹-
GSK3β (Cell Signaling, 3222), calnexin (Enzo, ADI-SPA-860),
and SOD2 (Stress-Gen, ADI-SOD-110), and HA (Santa Cruz Biotechnology,
sc-805), using the respective antibodies as indicated in the
parentheses. Protein immunosignals are shown with the load-
ing controls. Simultaneously, the signals were plotted as bar
graphs after normalization against the respective loading
controls.
Cells (1 x 10^4/well in 2 ml) were seeded on coverslips placed in 6-well plates and grown overnight. The cells on coverslips after fixation with 3% paraformaldehyde were detected for compartmental localization of proteins as described previously (41, 42) and visualized by fluorescent microscopy. Immunoprecipitation (IP)—IP of FLAG fusion HO-1-FL and HO-1-TR from O2 or air-exposed stably infected MEF cells was performed using the anti-FLAG M2 affinity gel (Sigma, A2220) following the manufacturer's protocol. In brief 100–200 μg/200–300 μl of total protein either from whole cell lysate or from cytoplasmic and nuclear fractions was incubated overnight with 20 μl of packed volume of M2 beads in a cold room under slow rotation. After washing repeatedly we used 3×FLAG peptide (Sigma, F4799) at a final concentration of 150 ng/μl in 50 μl of TBS to elute the proteins. IP of endogenous Nrf2 was performed by overnight incubation with anti-Nrf2 (Santa Cruz Biotechnology, sc-772) and anti-pSer40-Nrf2 (Epitomics, 2073-1) antibodies at a concentration of 2–3 μg/100 μg of total protein. This was followed by further incubation with protein A-agarose beads (Invitrogen, 15918-014) at room temperature for an hour. After washing the beads repeatedly, the bound proteins from the beads were eluted with 0.1M glycine HCl, pH 3.5, and immediately restored in neutral pH by adding 0.5M Tris-HCl, pH 7.4, containing 1.5M NaCl. Similarly, IP of recombinant HA-tagged Nrf2 in the nuclear fractions was performed by incubation with anti-HA antibody (Santa Cruz Biotechnology, sc-805) followed by protein-A-agarose beads, and the elution was performed by low pH glycine as described above.

Expression and Purification of Recombinant GST Fusion HO-1-FL and HO-1-TR—GST fusion constructs of HO-1-FL and HO-1-TR were expressed in competent *Escherichia coli* strain BL21 (Invitrogen) as described previously (36). Bacteria were grown to an optical density of 0.6–0.8 at 600 nm. The fusion proteins thereafter were induced in the presence of 100 μg/ml isopropyl β-D-1-thiogalactoside (IPTG) and harvested after 4 h. The GST fusion protein was purified using glutathione-Sepharose resin (GE Healthcare) according to the manufacturer’s protocol.
The fusion proteins in the bacterial lysate were purified using a GST purification module (GE Healthcare, catalog number 18-1128-13AC). The eluted proteins were evaluated for protein content and stored in aliquots at −80 °C until used.

In Vitro Translation of Full-length Nrf2 and C-terminally Truncated Nrf2 Fragments (C1 to C4)—Mice Nrf2 ORF cloned in Pmx-Puro vector were used as a template to generate deletion mutants of Nrf2 (C1 to C4, see Fig. 4), serially truncated from the C terminus. The specific forward primer containing T7 promoter and the reverse primers are shown in a table (see Fig. 4D). DNA constructs were amplified by PCR. The PCR products of Nrf2 and the Nrf2 fragments were verified on agarose gel and purified using the QIAquick PCR purification kit (Qiagen, catalog number 28106) following the manufacturer’s instructions. The pure PCR-generated constructs were transcribed in vitro by using the T7 high yield RNA synthesis kit (New England Biolabs, catalog number E2050) in the presence of T7 promoter enhancer according to the manufacturer’s instructions, and the transcripts were detected on agarose gel. For in vitro translation, the transcripts were added to an aliquot of the TNT T7 coupled reticulocyte lysate system, (Promega, catalog number L4610) and incubated in a 50-μl reaction volume containing [35S]methionine instead of methionine for 60–90 min at 30 °C. After being resolved on SDS-PAGE, the radiolabeled Nrf2 and Nrf2 fragments were detected on the dried gel by autoradiogram.

An in vitro translation reaction in the absence of DNA template was also carried out as a specificity control.

In Vitro Binding of Nrf2 to HO-1 Captured on GST SpinTrap Columns—Equal amounts of the purified GST fusion HO-1-FL and HO-1-TR were captured on several GST SpinTrap columns (GE Healthcare, catalog number 18-1128-13AC). The
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columns were washed repeatedly with phosphate buffer to remove any unbound protein. Each of these columns was then incubated for 10 min individually with 20 μl of 35S-labeled Nrf2 and fragments (ΔC1–ΔC4). The columns were then washed with copious amounts of phosphate buffer. The Nrf2 and Nrf2 fragments bound to trapped GST HO-1-FL or HO-1-TR were then eluted using 30 μl of 10 mM reduced glutathione (supplied with the SpinTrap). Once the eluates were resolved on SDS-PAGE, the Nrf2 signal was evaluated by autoradiogram of dried gel. The nonspecific binding was verified by incubating radiolabeled Nrf2 on a GST SpinTrap without any HO-1 bound to it.

Quantitative Real Time PCR—Using the TaqMan gene expression assay (Applied Biosystems), mRNA levels were analyzed and further normalized to 18s gene product. The primers used from Applied Biosystems were HO-1 (Mm00516004), Nrf2 (Mm00477784), NQO1 (Mm01253560), G6PDX (Mm00656735), SOD2 (Mm01313000), and 18s (HS99999901).

Use of Inhibitors—The cells were exposed to O2 for 18 h followed by an additional 30-min incubation with an inhibitor of AKT (MK2206, ChemieTek, catalog number CT-MK2206) alone at 1.0 μM or after co-incubation with an inhibitor of GSK3β (SB 216763, Sigma-Aldrich, catalog number S3442) at 10 μM. The cell pellets were evaluated for Nrf2 and HO-1 signals by Western blot.

Evaluation of Oxidative Stress—After air or O2 exposure, cells were incubated with 10 μM dihydroethidium (DHE; Molecular Probes) for 30 min. The amount of oxethidium generated was measured by fluorescent microscopic analysis. The number of fluorescence-positive cells was counted in an average of 10 high power fields and expressed as a ratio of the total number of cells.

Evaluation of Protein Carbonyls—The cell lysates were also analyzed for the protein carbonyls using Oxiselect protein carbonyl immunoblot kit (Cell Biolabs Inc., STA 309) as per the manufacturer’s instructions. In brief, the carbonyl groups were derivatized by dinitrophenylhydrazine followed by immunoblotting with an anti-dinitrophenyl antibody.

G6PDH Enzyme Activity—Constitutive activity of glucose-6-phosphate dehydrogenase (G6PDH) in whole cell lysate was detected in a 96-well plate using a kit (Sigma, MAK015). In brief, 10 μl of lysate from 1 × 10⁸ cell homogenate in 100 μl of PBS was transferred in triplicates in 40 μl/well of master reaction mix. This assay detects oxidation of glucose 6-phosphate generating NADH, which is measured every 5 min colorimetrically at 450 nm using a standard curve of NADH. After subtracting the values from the background, the nmol amounts of NADH obtained between Tinitial and Tfinal were finally expressed in milliunits/ml by employing the following formula

\[
\text{G6PDH activity} = \frac{B \times \text{sample dilution factor}}{(\text{Reaction time}) \times V} \quad \text{(Eq. 1)}
\]

where B = amount (nmol) of NADH generated between Tinitial and Tfinal, Reaction time = Tfinal – Tinitial (minutes), and V = sample volume (ml) added to the well.

Measurement of Cell Viability by XTT Assay—The XTT assay (Biotium, 30007) was performed following the manufacturer’s instructions to measure the proliferation rate/metabolic activity of cells over a period of 6 h. Briefly the V, FL, and TR cells (20,000/well) in a 96-microwell plate were grown in DMEM with high glucose (25 mM) or without any energy substrate in the presence of XTT reagent. XTT is a tetrazolium analog with a negatively charged inner salt that more readily enters cells. In metabolically active cells XTT is rapidly reduced by NAD(P)H-dependent oxidoreductases and dehydrogenases and produces solubilized formazan that is colorimetrically measured at 405 nm (45, 46).

Statistical Analysis—The values in figures were expressed as means ± S.E. Unpaired t tests were used to compare two groups. Values of p < 0.05 and p < 0.005 were considered as statistically significant.

RESULTS

Hyperoxia Induces HO-1, Leading to Nuclear Localization of a 28-kDa Isoform and Simultaneous Accumulation of Nrf2 in the Nucleus—The WT MEF cells exposed to air or O2 were analyzed for HO-1 and Nrf2 proteins in Western blot (Fig. 1). In air, HO-1 in the whole cell lysate appeared as a single band at 32 kDa, whereas in O2, an additional faster migrating band also appeared at 28 kDa, which was accompanied with a simultaneous increase of Nrf2 (Fig. 1A). The 32-kDa isoform constitu-
tively was predominant in the cytoplasm, whereas in O2, the 28-kDa HO-1 was predominant and primarily localized to the nucleus. The latter was accompanied with a concomitant increase of nuclear Nrf2 (Fig. 1, B and C). The immunohistochemistry further verified an O2-driven nuclear enrichment of HO-1 (Fig. 1D).

N-Acetyl-L-cysteine, an antioxidant, reversed the O2-mediated nuclear shift of HO-1, suggesting that the migration of HO-1 to the nucleus is driven by oxidative stress. Because we observed a coordinated nuclear enrichment of Nrf2 along with the enrichment of the 28-kDa HO-1, the latter was silenced (Fig. 1E). This in turn significantly reduced both basal and O2-induced Nrf2 (Fig. 1, E and F). Furthermore, nuclear HO-1 increased in a time-dependent manner with exposure to O2 being maximal at 18 h (Fig. 1G). These data indicate that HO-1 modulates stabilization and nuclear accumulation of Nrf2.

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Nuclear HO-1 Physically Interacts with Nrf2—Nrf2 accumulation is predominantly regulated by post-translational mechanisms (17, 44, 47). To understand whether the nuclear accumulation of Nrf2 was induced by nuclear HO-1 was dictated by a physical interaction between the two proteins, HO-1-KO-MEF cells reconstituted with HO-1-FL or HO-1-TR and the empty vector (V) were used (Fig. 2A). The HO-1-TR was expressed in the nucleus due to the presence of three nuclear localization sequences, whereas the HO-1-FL expression was restricted to the cytoplasm (Fig. 2B). The lack of migration of FL protein to the nucleus could have been due to a resistance to proteolytic cleavage of the protein from the C terminus and release from the membrane. Alternatively, this may have resulted from a structural change due to the presence of the N-terminal Myc and C-terminal FLAG tags. The cytoplasmic and nuclear compartment-specific expression of HO-1-FL and HO-1-TR, respectively, was further documented by immunostaining using three different antibodies directed at HO-1, N-terminal Myc, and C-terminal FLAG tags (Fig. 2C).

The Nuclear HO-1-Nrf2 Complex Appears Late in Oxidative Stress—After exposure of the FL, TR, and the control (V) cells to air or O2 for 2, 6, and 18 h, the FLAG fusion proteins were immunoprecipitated from whole cell lysate. In comparison with the air-exposed cells, the IP of the O2-exposed cells showed increased abundance of both HO-1-FL and HO-1-TR at all time points (Fig. 2D). Counterstaining indicated co-purification of Nrf2 signal from in vitro translation and a translation reaction in the absence of any transcript representing specificity control (far right lane) are shown. G, the autoradiogram signals of Nrf2 and the Nrf2 fragments bound to GST-HO-1 (TR: lanes 1–5 and FL: lanes 7–11) on GST SpinTrap columns are represented; lane 6 shows the eluate from a GST SpinTrap without any HO-1 and serves as a control for the specificity.

Nuclear HO-1 Interacts with Nrf2 via transactivation domain, Neh4. A, the GST fusion HO-1-FL and HO-1-TR purified from bacterial lysate and a purification from uninduced bacterial culture (Control) are shown. B, schematic presents clones of Nrf2 and C-terminal deleted Nrf2 fragments (Δ1 to Δ4). C and D, primer sequences (C) and the amplified PCR products of the Nrf2 and the Nrf2 fragments on a 1% agarose gel (D) are shown. E, the transcripts obtained by reverse transcription of the PCR products are shown. F, the [35S]methionine-radiolabeled Nrf2 signal from in vitro translation and a translation reaction in the absence of any transcript representing specificity control (far right lane) are shown. G, the autoradiogram signals of Nrf2 and the Nrf2 fragments bound to GST-HO-1 (TR: lanes 1–5 and FL: lanes 7–11) on GST SpinTrap columns are represented; lane 6 shows the eluate from a GST SpinTrap without any HO-1 and serves as a control for the specificity.
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The HO-1/Nrf2 Complex Localizes to the Nucleus—To further assess compartment-specific localization of the nuclear HO-1/Nrf2 complex, FLAG IP from cytoplasmic and nuclear fractions of cells exposed to O2 for 18 h was performed. The co-precipitation of Nrf2 with HO-1-TR, indicating localization of HO-1-TR/Nrf2 complex predominantly in the nucleus (Fig. 2E). The IP of HO-1-FL, abundant in the cytoplasm, weakly counterstained with Nrf2, and the control (V) cells were completely devoid of Nrf2 signal. This further supported the preferential interaction between 28-kDa nuclear HO-1 with Nrf2 and predominant localization of the complex in the nucleus. A reverse IP of the endogenous Nrf2 using anti-Nrf2 antibody (sc772) further confirmed this preferential interaction between Nrf2 and nuclear HO-1 (Fig. 2E). We did observe a considerable amount of HO-1-TR in the cytoplasm, as shown by its co-precipitation with Nrf2 and in the input lane as well. Although this may have resulted from contamination of cytoplasm with the nuclear portion during fractionation, this could also suggest that Nrf2 interacts with HO-1 in the cytoplasm prior to migration to the nucleus as a complex. An IP of HA-tagged Nrf2 (HA-N) ectopically expressed in Nrf2-KO MEF cells (Fig. 2F) further confirmed the interaction of endogenous 28-kDa HO-1 with Nrf2 (Fig. 2G). As expected, this interaction was absent in Nrf2-KO MEF cells.

The LnCap Prostate Cancer Cells Constitutively Are Enriched with the 28-kDa HO-1 That Resides in the Nucleus and Forms a Complex with Nrf2—To further evaluate whether the HO-1/Nrf2 interaction is physiological, we used LnCap prostate adenocarcinoma cell lines. The whole cell lysate exhibited predominately a 28-kDa HO-1 isoform (Fig. 3, A and B), which upon fractionation of cytoplasm and nucleus exclusively localized to the nucleus (Fig. 3, C and D). The Nrf2 signal as well was significantly increased in the nucleus when compared with cytoplasm (Fig. 3, C and D). The Nrf2 IP from LnCap cells showed co-precipitation of HO-1 particularly of the 28 kDa (Fig. 3E). Because phosphorylation of Nrf2 at Ser40 is known to modulate Nrf2 activity (17), an antibody specific to pSer40 (Epitomics, 2073-1) was used to immunoprecipitate Nrf2. Although the IP showed abundant Nrf2, HO-1 co-precipitation was absent (Fig. 3E). These data indicated that Nrf2, if phosphorylated at Ser40, does not interact with nuclear HO-1 and hence does not participate in Ser40-mediated Nrf2 signaling.

Nuclear HO-1 Interacts with Nrf2 via the Neh4 Transactivation Domain—The purified GST fusion HO-1-FL and HO-1-TR proteins expressed in bacteria are presented in Fig. 4A. Nrf2 and Nrf2 fragments (AC1 to ΔC4) generated with an in vitro translation system are shown stepwise in Fig. 4, B–F. The binding of Nrf2 and Nrf2 fragments with HO-1 was verified with a binding assay using purified GST fusion HO-1-FL and HO-1-TR proteins trapped on GST columns (Fig. 4G). The binding of the [35S]methionine-radiolabeled Nrf2 fragments to GST-HO-1 revealed that Nrf2 could bind to HO-1-TR and that this binding

between nuclear HO-1 and Nrf2. The absence of the Nrf2 signal at early time points (2 and 6 h) further indicated that this interaction occurs late during oxidative stress.

The HO-1/Nrf2 Complex Localizes to the Nucleus—To further assess compartment-specific localization of the nuclear HO-1/Nrf2 complex, FLAG IP from cytoplasmic and nuclear fractions of cells exposed to O2 followed by an additional 30 min of incubation with MK2206 (5 μM). The immunosignal of p-AKT (pSer473, Cell Signaling, 9272), p-GSK3β (pSer9, Cell Signaling, 9832), and corresponding total Nrf2 levels (sc722) are shown in whole cell lysates. C. The whole cell lysate of FL and TR cells exposed to O2 followed by an additional 30 min incubation with DMSO or MK2206 (5 μM) or SB216763 (10 μM) alone or in co-incubation were assessed for levels of Nrf2 and calnexin. D, the Nrf2 signal quantitated after normalization to calnexin expressed in bar diagrams. MK, MK2206; SB, SB216763. E, cell lysates of V, FL, and TR exposed to air or O2 were assessed for HO-1 pulled down by FLAG-IP (upper panel), Nrf2 co-precipitation (middle panel), and polyubiquitination signal (lower panel). On the left, 5% input shows HO-1, polyubiquitinated proteins (Poly-Ub), Nrf2, and calnexin. *, p = 0.05; n = 3. Values are the mean ± S.E. of three separate measurements.

FIGURE 5. A, diagram of Nrf2 depicting domains and possible phosphorylation (P) and ubiquitination (Ub) sites (adapted from Refs. 20 and 21). ab sc772, antibody sc722, affinity purification (IP) of HA-tagged Nrf2 (HA-N) ectopically expressed in Nrf2-KO MEF cells (Fig. 2G). As expected, this interaction was absent in Nrf2-KO MEF cells.
did not occur with HO-1-FL. Furthermore, the presence of binding signal for ΔC1 to ΔC3 and the absence of this signal with ΔC4 indicated that the transactivation domain Neh4 is crucial for the interaction between Nuclear HO-1 and Nrf2.

Nuclear HO-1 Stabilizes Nrf2 from GSK3β-mediated Degradation—Active GSK3β phosphorylates serine moieties (343 and 347) in the Neh6 domain of Nrf2 (Fig. 5A) and targets Nrf2 for ubiquitination and degradation (20). To understand whether HO-1-TR protects Nrf2 from GSK3β-mediated proteolytic degradation, AKT, the negative regulator of GSK3β, was inhibited by MK2206. This in turn results in the activation of GSK3β by preventing phosphorylation at Ser-9 (48, 49). The phosphorylation signals of AKT and GSK3β were verified with specific antibodies (pSer473-AKT, Cell Signaling, 9272; and pSer9-GSK3β, Cell Signaling, 9832) in FL and TR cells after exposure to O₂ followed by an additional 30-min incubation with MK2206 (Fig. 5B). In the presence of MK2206, when GSK3β activity is increased, Nrf2 signal was reduced in FL, whereas it remained unaltered in TR. This observation was further verified in the next experiment where FL and TR cells exposed to O₂ were treated similarly with MK2206 alone or in co-incubation with SB16763, an inhibitor of GSK3β. When compared with the Nrf2 level in FL cells incubated only with DMSO (Fig. 5, C and D), the FL cells incubated with MK2206 showed significant loss of Nrf2, indicating that activation of GSK3β results in degradation of Nrf2. Restoration of this loss by co-incubation with SB16763 verifies that this effect is dependent on GSK3β. On the contrary, in TR cells, Nrf2 level was relatively higher and retained regardless of inhibitor treatment (Fig. 5, C and D).
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This validates the role of HO-1-TR in rescuing Nrf2 from GSK3β-mediated degradation.

Because ubiquitination is a general mechanism to target proteins for proteasomal degradation, this was evaluated in FL, TR, and control cells (Fig. 5E). Interestingly, maximum Nrf2 co-precipitation signal with FLAG-HO-1-TR IP was associated with minimal polyubiquitination signal, and the FLAG IP from FL and control cells showed less Nrf2 co-precipitation yet maximum polyubiquitination signal. The input samples of total cell lysates from TR also exhibited less polyubitin signal when compared with FL and control cells (Fig. 5E). These data could reflect sustenance of protein homeostasis and limited ubiquitination in TR. Collectively, these data support the hypothesis that HO-1-TR prevents Nrf2 ubiquitination by physically interacting with the protein and thus preventing it from GSK3β-mediated degradation, thereby facilitating Nrf2 accumulation in the nucleus.

Nuclear HO-1-mediated Stabilization of Nrf2 Regulates Transcription of Specific Downstream Antioxidants and Metabolic Genes—To understand the physiologic importance of HO-1-mediated nuclear accumulation of Nrf2, we evaluated steady state mRNA levels of HO-1, Nrf2, and a group of Nrf2 downstream target genes including NQO1 and G6PDH in WT-MEF and HO-1-silenced MEF cells (Fig. 5E). As expected, after exposure to air or O2, HO-1 mRNA was suppressed in the HO-1-silenced cells. In O2-exposed WT cells, levels of NQO1 mRNA and G6PDH mRNA increased concomitantly with the increase in HO-1. Disruption of this signal in HO-1-silenced cells indicated that regulation of the Nrf2 downstream genes is dependent on HO-1. Of note, Nrf2 mRNA induction was also increased in O2 exposure, indicating that nuclear HO-1 may influence Nrf2 transcription as well (Fig. 6A). In addition, in HO-1-TR cells, there was significant induction of NQO1 and G6PDH mRNA levels both at baseline and after O2 exposure when compared with control cells (Fig. 6B).

Cells Enriched with Nuclear HO-1 Exhibit Increased G6PDH Activity—Because HO-1-TR was associated with enhanced transcription of G6PDH in TR cells and disruption of HO-1 in WT cells diminished it, constitutive G6PDH activity was assessed in these cells. In fact, the basal G6PDH activity observed was indeed decreased significantly with the disruption of HO-1 by shRNA (Fig. 6C). Additionally, G6PDH activity was significantly higher in TR when compared with FL and control cells (Fig. 6D). These data further indicate that modulation of G6PDH activity by Nrf2 is particularly influenced by nuclear HO-1.

Cells Enriched with Nuclear HO-1 Are Metabolically More Viable in Glucose-enriched Medium—Increased activity of G6PDH should improve growth and proliferation of cells in glucose medium. This was tested in V, FL, and TR cells. Cells grown in medium without any energy substrate as expected did not grow (Fig. 6E). In glucose-enriched (25 mM) medium, TR cells showed proliferation significantly at a higher rate when compared with FL and control cells (Fig. 6F).

Nrf2 Requires Participation of Nuclear HO-1 to Induce Antioxidant Defenses—Because NQO1 by utilizing NAD(P)H and NADH plays a major role in scavenging toxic oxidized substrates, we measured the oxidation burden in both air-exposed...
and O₂-exposed V, FL, and TR cells by measuring the intensity of 2-hydroxyethidium fluorescence in cells after incubation with DHE and also assessed protein carbonyls as indices of protein oxidation. All cell groups showed low DHE fluorescence in air, whereas in O₂, only TR showed low levels of DHE fluorescence when compared with V and FL cells (Fig. 7, A and B).

To understand whether the enhanced anti-oxidant protection in the TR cells was by virtue of nuclear HO-1 interaction with Nrf2, we used Nrf2-WT and KO cells as such and also infected with cDNA constructs of V, FL, and TR. Fig. 7, C and D, indicate the levels of HO-1-TR and HO-1-FL expression in both Nrf2 WT and KO cells. We then evaluated levels of carbonylated proteins in these cells after exposure to air or O₂ (Fig. 7E). The protein carbonyls did not differ much between the types of cells in air. However, in O₂, Nrf2-WT cells with HO-1-TR showed reduced levels of carbonylated proteins when compared with those having HO-1-FL and empty vector, whereas the Nrf2-KO cells exhibited enhanced protein carbonyls, which were not reversed even by HO-1-TR or HO-1-FL.

Furthermore, Nrf2-WT cells containing HO-1-TR showed significantly increased transcription of NQO1 (Fig. 8A) and G6PDH (Fig. 8C) when compared with those with HO-1-FL and empty vector. The fact that NQO1 and G6PDH transcription was lower in Nrf2-KO background even in the presence of HO-1-TR (Fig. 8, B and D) further supports our hypothesis that nuclear HO-1 partners with Nrf2 or vice versa to enhance antioxidant defenses. Nevertheless, steady state levels of SOD2 were not modulated by the interaction of nuclear HO-1 and Nrf2 (Fig. 8, E and F).

DISCUSSION

We demonstrate in various ways that oxidative stress mediates induction of HO-1 along with nuclear localization of a 28-kDa isoform of HO-1, which physically interacts with Nrf2. This interaction results in the stabilization of Nrf2 in the nucleus. We further established that under physiologic conditions, Nrf2 interacts with the 28-kDa endogenous nuclear HO-1. Many have described how Nrf2 results in the induction of HO-1 via binding at the ARE (25, 50), but this is the first report of the converse, that is, the modulation of Nrf2 function by HO-1.

Participation of various kinase signaling pathways has been documented in ARE-mediated transcription (52). In particular, phosphorylation of Ser⁴⁰ by protein kinase C has been implied for the modulation of Nrf2 in a Keap1-dependent manner (17, 18). In the present study, neither isoform of HO-1 interacted with Nrf2-pSer⁴⁰. Nevertheless, activation of PI3K/Akt is well documented to provide protection from oxidative stress-medi-
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activated injury via Nrf2 activation (1). The PI3K-Akt-GSK3β axis may regulate Nrf2 activation in several ways. Active GSK3β can directly phosphorylate the phosphodegron motif in the Neh6 domain of Nrf2 protein, leading to Keap1-independent and β-TrCP-dependent proteasomal degradation of Nrf2 protein (20–22). Alternatively, GSK3β can phosphorylate and activate Fyn kinase, leading to phosphorylation and nuclear exclusion of Nrf2 protein (53). Therefore, phosphorylation of Nrf2 might dictate its accumulation and nucleocytoplasmic trafficking (52).

A cellular model where the truncated form of HO-1 is constitutively overexpressed in the nucleus of cells devoid of endogenous HO-1 allowed for documentation of a distinct function of nuclear HO-1, specifically that it preferentially binds to Nrf2 and influences its nuclear abundance. Accumulation of nuclear HO-1 and consequent interaction with Nrf2 may take up to 18 h, indicating a specific role for nuclear HO-1 in the regulation of Nrf2 post-induction. Nuclear enrichment of Nrf2 was observed in TR cells even with GSK3β activation, whereas in FL cells lacking nuclear HO-1, this resulted in loss of Nrf2. These data document specifically that by interacting with Nrf2, nuclear HO-1 stabilizes it from GSK3β-mediated proteolytic degradation. The observed decreased polyubiquitin signal in the lysates of O2-exposed TR cells could reflect sustained protein homeostasis by limited ubiquitination, although this would need to be systematically evaluated.

HO-1 has long been identified as proto-oncogenic due to its anti-apoptotic and pro-angiogenic properties (54, 55), and there is a positive relationship between high nuclear HO-1 content and severity of prostate cancer (35). Nonetheless, the precise role of HO-1 in cancer biology is far from being completely understood. Cancer cells display altered metabolic circuitry and nutrient uptake to increase proliferation preferentially, leading to glucose-dependent ATP production (Warburg theory). Increased expression and activity of G6PDH are frequently observed in various cancers (56–58). It is the first key rate-limiting enzyme in the pentose phosphate pathway, which provides a readily available supply of pentose sugars for RNA and DNA synthesis (59, 60). Also, by regulating the pentose phosphate pathway, G6PDH generates NAD(P)H, which provides reducing equivalents for the maintenance of a pool of reduced glutathione to balance redox state (61). Additionally, by utilizing NAD(P)H, NQO1 scavenges a wide variety of oxidized and toxic substrates such as quinones (62, 63). This should provide protection against oxidative stress. In accordance, the cells enriched with nuclear HO-1 show increased NQO1 transcripts and decreased oxidative damage of proteins and an increased G6PDH activity as well, and this indicates that these effects are attained by the interaction of nuclear HO-1 with Nrf2.

Interestingly, nuclear HO-1-mediated activation of Nrf2 led to the transcription of specific downstream genes including Nrf2 itself. This is explained by the fact that the pattern of resultant downstream genes depends on the alignment of the ARE and AP1 primary core sequences that Nrf2 binds to (51, 64). How the binding of Nrf2 to HO-1 mediates preferential transcription of NQO1 and G6PDH is still not clear. In addition, the fact that nuclear HO-1 may also regulate the transcription of Nrf2 needs further verification.

In summary, we demonstrate that with oxidative stress, there is increased expression of nuclear HO-1. This regulates subcellular distribution and activation of Nrf2 post-induction, resulting in transcriptional regulation of late phase II antioxidant enzymes and enabling a feed forward adaptive reprogramming for recovery and a survival advantage in oxidative stress.

Acknowledgments—We thank Drs. Melpo Christofidou-Solomidou and Dr. William J Fredericks, University of Pennsylvania for supplying us with the Nrf2-KO cells, and LnCap cell lines, respectively. We also thank Dr. Alan Diehl, University of Pennsylvania, for valuable suggestions and providing us with the HA-tagged Nrf2 cDNA. We acknowledge Prof. Yair Argon, University of Pennsylvania for valuable comments.

REFERENCES

1. Chen, H. H., Chen, Y. T., Huang, Y. W., Tsai, H. I., and Kuo, C. C. (2012) 4-Ketopinoneisol, a novel naturally occurring ARE activator, induces the Nrf2/HO-1 axis and protects against oxidative stress-induced cell injury via activation of PI3K/AKT signaling. Free Radic. Biol. Med. 52, 1054–1066

2. Moi, P., Chan, K., Asunis, I., Cao, A., and Kan, Y. W. (1994) Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the β-globin locus control region. Proc. Natl. Acad. Sci. U.S.A. 91, 9926–9930

3. Shelton, P., and Jaiswal, A. K. (2013) The transcription factor NF-E2-related factor 2: a protooncogene? FASEB J. 27, 414–423

4. Jaramillo, M. C., and Zheng, D. D. (2013) The emerging role of the Nrf2-Keap1 signaling pathway in cancer. Genes Dev. 27, 2179–2191

5. Sporn, M. B., and Liby, K. T. (2012) Nrf2 and cancer: the good, the bad and the importance of context. Nat. Rev. Cancer 12, 564–571

6. Nguyen, T., Nioi, P., and Pickett, C. B. (2009) The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. J. Biol. Chem. 284, 13291–13295

7. Malhotra, D., Portales-Casamar, E., Singh, A., Srivastava, S., Arenillas, D., Happel, C., Shyr, C., Wakabayashi, N., Kessler, T. W., Wasserman, W. W., and Biswal, S. (2010) Global mapping of binding sites for Nrf2 identifies novel targets in cell survival response through ChIP-Seq profiling and network analysis. Nucleic Acids Res. 38, 5718–5734

8. Dkhachimamourathy, S., Jain, A. K., Bloom, D. A., and Jaiswal, A. K. (2005) Bach1 competes with Nrf2 leading to negative regulation of the antioxidant response element (ARE)-mediated NAD(P)H:quinone oxidoreductase 1 gene expression and induction in response to antioxidants. J. Biol. Chem. 280, 16891–16900

9. Villeneuve, N. F., Lau, A., and Zhang, D. D. (2010) Regulation of the Nrf2-Keap1 antioxidant response by the ubiquitin proteasome system: an insight into Cullin-ring ubiquitin ligases. Antioxid. Redox Signal 13, 1699–1712

10. Kobayashi, A., Kang, M. I., Watai, Y., Tong, K. I., Shibata, T., Uchida, K., and Yamamoto, M. (2006) Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1. Mol. Cell. Biol. 26, 221–229

11. Velichkova, M., and Hasson, T. (2005) Keap1 regulates the oxidation-sensitive shuttling of Nrf2 into and out of the nucleus via a Crm1-dependent nuclear export mechanism. Mol. Cell. Biol. 25, 4501–4513

12. Cullinan, S. B., Gordan, J. D., Jin, J., Harper, J. W., and Diehl, J. A. (2004) The Keap1-βTrCP protein is an adaptor that bridges Nrf2 to a Cul3-based E3 ligase: oxidative stress sensing by a Cul3-Keap1 ligase. Mol. Cell. Biol. 24, 8477–8486

13. Ichimura, Y., Waguri, S., Sou, Y. S., Kageyama, S., Hasegawa, J., Ishimura, R., Saito, T., Yang, Y., Kouno, T., Fukutomi, T., Hoshii, T., Hiroa, A., Takagi, K., Mizushima, T., Motohashi, H., Lee, M. S., Yoshimori, T., Tanaka, K., Yamamoto, M., and Komatsu, M. (2013) Phosphorylation of p62 activates the Keap1-Nrf2 pathway during selective autophagy. Mol. Cell 51, 618–631
Nuclear HO-1 Modulates Nrf2 Function

D. S., Kauffman, H. F., and Choi, A. M. (2007) Mitochondrial localization and function of heme oxygenase-1 in cigarette smoke-induced cell death. *Am. J. Respir. Cell Mol. Biol.* 36, 409–417

Saccà, P., Meiss, R., Casas, G., Mazza, O., Calvo, J. C., Navone, N., and Vazquez, E. (2007) Nuclear translocation of heme oxygenase-1 is associated to prostate cancer. *Br. J. Cancer* 97, 1683–1689

Lin, Q., Wei, S., Yang, G., Weng, Y. H., Helston, R., Rish, K., Smith, A., Bordner, J., Polte, T., Gnauzit, F., and Denney, P. A. (2007) Heme oxygenase-1 protein localizes to the nucleus and activates transcription factors important in oxidative stress. *J. Biol. Chem.* 282, 20621–20633

Lin, Q. S., Wei, S., Yang, G., Zhuang, T., Abate, A., and Denney, P. A. (2008) Catalytic inactive heme oxygenase-1 protein regulates its own expression in oxidative stress. *Free Radic. Biol. Med.* 44, 847–855

Hori, R., Kashiba, M., Toma, T., Yachie, A., Goda, N., Makino, N., Soejima, A., Nagasawa, T., Nakabayashi, K., and Suematsu, M. (2002) Gene transfection of H25A mutant heme oxygenase-1 protects cells against hydroperoxide-induced cytotoxicity. *J. Biol. Chem.* 277, 10712–10718

Wilks, A., and Ortiz de Montellano, P. R. (1993) Rat liver heme oxygenase. High level expression of a truncated soluble form and nature of the mesohydroxylating species. *J. Biol. Chem.* 268, 22357–22362

Soriano, F. X., Baxter, P., Murray, L. M., Sporn, M. B., Gillingwater, T. H., and Hardingham, G. E. (2009) Transcriptional regulation of the AP-1 and HIF-1 target gene sulfiredoxin. *Mol. Cells* 27, 279–282

Ishikawa, K., and Sato, M. (1991) Degradation of heme by a heme oxygenase in microsomes. *J. Biol. Chem.* 266, 7629–7640

Huang, H. C., Nguyen, T., and Pickett, C. B. (2002) Phosphorylation of Nrf2 at Ser-40 by protein kinase C regulates antioxidant response element-mediated transcription. *J. Biol. Chem.* 277, 42769–42774

Chowdhry, S., Zheng, Y., McMahan, M., Sutherland, C., Cuadrado, A., and Hayes, J. D. (2013) Nrf2 is controlled by two distinct β-TrCP recognition motifs in its Neh6 domain, one of which can be modulated by GSK-3 activity. *Oncogene* 32, 3765–3781

Rada, P., Rojo, A. I., Evrard-Todeschi, N., Innamorato, N. G., Cotte, A., Jaworski, T., Tobo´n-Velasco, J. C., Devijver, H., García-Mayoral, M. F., Keyse, S. M., and Tyrrell, R. M. (1989) Heme oxygenase is the major role of heme oxygenase-1. *Biochem. Biophys. Res. Commun.* 16502–16510

Cuadrado, A. (2011) SCF/β-TrCP promotes glycogen synthase kinase-3β phosphorylation by sodium valproate and lithium. *Redox Biol.* 1, 127–152

Huang, H. C., Nguyen, T., and Pickett, C. B. (2002) Phosphorylation of Nrf2 at Ser-40 by protein kinase C regulates antioxidant response element-mediated transcription. *J. Biol. Chem.* 277, 42769–42774

Rada, P., Rojo, A. I., Chowdhry, S., McMahan, M., Hayes, J. D., and Cuadrado, A. (2011) SCF/β-TrCP distinguishes the DNA binding motif of the Neh2 domain of Nrf2 from that of Keap1. *Mol. Cell. Biol.* 31, 1121–1133

Kilic, U., Kilic, E., Tuzcu, Z., Tuzcu, M., Ozercan, I. H., Yilmaz, O., Sahin, F., and Sahin, K. (2012) Melatonin suppresses cisplatin-induced nephrotoxicity via activation of Nrf2/HO-1 pathway. *Nutr. Metab. (Lond.)* 10, 7

Abraham, N. G., Adema, G., van Kooyk, Y., de Witte, T., and Figdor, C. G. (2001) Heme is a potent inducer of inflammation in mice and is counteracted by heme oxygenase. *Blood* 98, 1802–1811

Alam, J., Shibahara, S., and Smith, A. (1989) Transcriptional activation of the heme oxygenase gene by heme and cadmium in mouse hepatoma cells. *J. Biol. Chem.* 264, 6371–6375

Applegate, L. A., Luscher, P., and Tyrrell, R. M. (1991) Induction of heme oxygenase: a general response to oxidant stress in cultured mammalian cells. *Cancer Res.* 51, 974–978

Keyse, S. M., and Tyrrell, R. M. (1989) Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite. *Proc. Natl. Acad. Sci. U.S.A.* 86, 99–103

Denney, P. A. (2000) Regulation and role of heme oxygenase in oxidative injury. *Curr. Top. Cell Regul.* 36, 181–199

Agarwal, A., and Bosl, S. E. (2013) Adaptive responses to tissue injury: role of heme oxygenase-1. *Trans. Am. Clin. Climatol. Assoc.* 124, 111–122

Denney, P. A. (2014) Signaling function of heme oxygenase proteins. *Antioxid. Redox Signal.* 20, 1743–1753

Yoshida, T., and Sato, M. (1989) Posttranslational and direct integration of heme oxygenase into microsomes. *Biochem. Biophys. Res. Commun.* 163, 1086–1092

Yoshida, T., Ishikawa, K., and Sato, M. (1991) Degradation of heme by a soluble peptide of heme oxygenase obtained from rat liver microsomes by mild trypsinization. *Eur. J. Biochem.* 199, 729–733

Jung, N. H., Kim, H. P., Kim, B. R., Cha, S. H., Kim, G. A., Ha, H. N., Y. E., and Cha, Y. N. (2003) Evidence for heme oxygenase-1 association with caveolin-1 and -2 in mouse mesangial cells. *IUBMB Life* 55, 525–532

Slobos, D. J., Ryter, S. W., van der Toorn, M., Liu, F., Guo, F., Baty, C. J., Karlsson, J. M., Watkins, S. C., Kim, H. P., Wang, X., Lee, J. S., Postma,
nase-1 induced by nitric oxide in experimental solid tumour. *Br. J. Cancer* 88, 902–909

55. Sunamura, M., Duda, D. G., Ghattas, M. H., Lozonschi, L., Motoi, F., Yamauchi, J., Matsuno, S., Shibahara, S., and Abraham, N. G. (2003) Heme oxygenase-1 accelerates tumor angiogenesis of human pancreatic cancer. *Angiogenesis* 6, 15–24

56. Koudstaal, J., Makkink, B., and Overdiep, S. H. (1975) Enzyme histochemical pattern in human tumours. II. Oxidoreductases in carcinoma of the colon and the breast. *Eur. J. Cancer* 11, 111–115

57. Baba, M., Yamamoto, R., Iishi, H., Tatsuta, M., and Wada, A. (1989) Role of glucose-6-phosphate dehydrogenase on enhanced proliferation of pre-neoplastic and neoplastic cells in rat liver induced by N-nitrosomorpholine. *Int. J. Cancer* 43, 892–895

58. Pretlow, T. G., 2nd, Harris, B. E., Bradley, E. L., Jr., Bueschen, A. J., Lloyd, K. L., and Pretlow, T. P. (1985) Enzyme activities in prostatic carcinoma related to Gleason grades. *Cancer Res.* 45, 442–446

59. Pretlow, T. G., 2nd, Whitehurst, G. B., Pretlow, T. P., Hunt, R. S., Jacobs, J. M., McKenzie, D. R., McDaniel, H. G., Hall, L. M., and Bradley, E. L., Jr. (1982) Decrease in creatine kinase in human prostatic carcinoma compared to benign prostatic hyperplasia. *Cancer Res.* 42, 4842–4848

60. Boros, L. G., Puigjaner, J., Cascante, M., Lee, W. N., Brandes, J. L., Bassilian, S., Yusuf, F. I., Williams, R. D., Muscarella, P., Melvin, W. S., and Schirmer, W. J. (1997) Oxythiamine and dehydroepiandrosterone inhibit the non-oxidative synthesis of ribose and tumor cell proliferation. *Cancer Res.* 57, 4242–4248

61. Kuo, W. Y., and Tang, T. K. (1998) Effects of G6PD overexpression in NIH3T3 cells treated with tert-butyl hydroperoxide or paraquat. *Free Radic. Biol. Med.* 24, 1130–1138

62. Ross, D., Kepa, J. K., Winski, S. L., Beall, H. D., Anwar, A., and Siegel, D. (2000) NAD(P)H:quinone oxidoreductase 1 (NQO1): chemoprotection, bioactivation, gene regulation and genetic polymorphisms. *Chem. Biol. Interact.* 129, 77–97

63. Cadenas, E., and Ernst, L. (1990) Quinoid compounds: high-performance liquid chromatography with electrochemical detection. *Methods Enzymol.* 186, 180–196

64. Wasserman, W. W., and Fahl, W. E. (1997) Comprehensive analysis of proteins which interact with the antioxidant responsive element: correlation of ARE-BP-1 with the chemoprotective induction response. *Arch. Biochem. Biophys.* 344, 387–396