Biochemical and Biophysical Research Communications **270,** 324–328 (2000)
doi:10.1006/bbrc.2000.2408, available online at http://www.idealibrary.com on IDEAL®

# Induction of Heme Oxygenase-1 Modulates *cis*-Aconitase Activity in Lens Epithelial Cells

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Received February 25, 2000

**Heme oxygenase-1 is the heme catabolic enzyme induced in human dermal fibroblasts by environmental stress. We report an increase of heme oxygenase-1 message in lens epithelial cells after exposure to UVA radiation, followed by a 10-fold increase of protein expression. The size of message was larger than previously demonstrated for fibroblasts. The relationship between heme oxygenase-1 activation and iron metabolism was investigated by measurement of activities of both cytosolic and mitochondrial *cis*-aconitase enzymes. A 2-fold increase in mitochondrial *cis*-aconitase activity in UVA-exposed cells coincided with the time of maximal heme oxygenase-1 expression. We propose that modulation of *cis*-aconitase activity at the translational level by an increase of cellular iron is an important consequence of heme oxygenase-1 activation. This might be a novel aspect of the protective role of heme oxygenase-1 in modulating the response of cells challenged with oxidative stress.** 

***Key Words:*** **lens epithelial cells; UVA radiation; heme oxygenase-1; *cis*-aconitase.**

Heme oxygenase-1 (HO-1), also known as low molecular weight heat shock protein Hsp 32 (1), is the inducible form of two isoenzymes, which are products of two separate genes (2). The induction of HO-1 at the transcriptional level has been observed as a response of cells to oxidative stress, produced by UVA radiation, oxidants (3), intense visible light (4), hyperbaric oxygen (5), or agents that deplete the level of intracellular antioxidant glutathione (6, 7). Part of this response of the cells to oxidative stress is due to the generation of free radicals. In the enzymatic reaction, HO-1, a key heme catabolism enzyme, in conjunction with NADPH-cytochrome P-450 reductase, produces iron, carbon monoxide and biliverdin, which together with bilirubin, its derivative and end product of the heme degradation pathway, are important cellular molecules with antioxidant properties (8). These data have led to a hypothesis which assumes that HO-1 has a protective role in cellular defense against the oxidative stress, due to degradation of the pro-oxidant molecule heme, and increase of antioxidants such as bilirubin, biliverdin, and the iron storage protein ferritin (9–11). However, this hypothesis does not account for the fact that the second product in the reaction catalyzed by HO-1, free iron, is a pro-inflammatory molecule per se, which has been shown to be an important catalyst in the generation of reactive oxygen species via the Fenton reaction (12). The cellular pool of free iron is very small, and the mechanism of regulation at the translational level of proteins involved in iron metabolism, namely ferritin, mitochondrial *cis*-aconitase (mAcon) (a Krebs cycle iron-sulfur enzyme that catalyses the conversion of citrate to isocitrate), erythroid δ-aminolevulinic acid synthetase and transferrin receptor, is controlled by iron regulatory protein 1 (IRP-1), which in its iron loaded version, is the *apo* form of cytosolic *cis*-aconitase (reviewed in 13). To examine the potential role of iron indirectly, we assessed the activities of two proteins, which are regulated by iron levels,

Abbreviations used: HO-1, heme oxygenase-1; UVA, ultraviolet A; Hsp 32, heat shock protein 32; IRE, iron response element, a *cis*-acting sequence in 5′-untranslated regions of mRNA, serving as a binding site for iron regulatory protein; IRP-1, iron regulatory protein 1; cAcon, cytosolic *cis*-aconitase; mAcon, mitochondrial *cis*-aconitase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SSC, sodium chloride, sodium phosphate buffer; SPEE, sodium chloride, sodium phosphate, ethylenediaminetetraacetic acid buffer; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

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0006-291X/00 $35.00

i.e. the iron response protein in its iron loaded version/ cytosolic *cis*-aconitase and mitochondrial *cis*-aconitase.

## MATERIALS AND METHODS

*Materials.* Rat HO-1 antibody (SPA895) was obtained from StressGen Biotechnologies Corp. (Victoria, BC, Canada). Murine cDNA probe for GAPDH was purchased from Ambion, Inc. (Austin, TX). Rapid-hyb buffer and $^{32}$P-ATP were from Amersham Life Sciences (U.K.). *Sn*-Protopophyrin IX was the product of Pophyrin Products Inc. (Logan, UT). Leupeptin, pepstatin, aprotinin desferrioxamine, $\beta$-nicotinamide-adenine dinucleotide phosphate and other chemicals were from Sigma (St. Louis, MO).

*Cell culture.* Cultured rabbit lens epithelial cells (cell line N/N 1003A (14)) were routinely grown in Eagles Minimum Essential Medium (Sigma) supplemented with 8% rabbit serum, L-glutamine and gentamicin as described previously (15). These cells have been shown to maintain normal ploidy and typical characteristics of lens epithelial cells through numerous population doublings (14).

*UVA radiation.* Irradiation experiments with a dose comparable to in vivo levels (16) were performed with confluent cells as described by Applegate *et al* (9). UVA radiation at 365 nm was obtained from a Hg-Xe arc lamp using a monochromator, focusing lens and beam turner to give a fluence rate of 0.007 W/cm$^2$ and a total fluence of 50.4 J/cm$^2$ unless indicated otherwise (18).

*Northern blot analysis.* Total RNA isolated using RNA-state 60 (Tel-test "B") was fractionated on 1% agarose-formaldehyde gels (30 $\mu$g per lane), transferred onto nylon fiters (Magna Charge) in 20× SSC buffer. Before transfer, the gels were washed in 15 mM glycine for 30 min, washed with 7.5 mM NaOH for 20 min and equilibrated in 20× SSC for 40 min. The RNA was fixed by incubation for 2 h at 80°C and exposure to UVC for 40 s.

Full-length probe (pRHO1, a generous gift from Dr. Shigeki Shibahara (19)) was radiolabeled with $^{32}$P-ATP by the random priming method. Hybridization was performed at 65°C in Rapid-hyb solution containing HO-1 probe overnight. To control variability in the quantity of RNA, the same membranes were stripped with 50% formamide and 6× SPEE for 30 min at 65°C and reprobed with the 316 bp *Sac*I-*Bam*H1 restriction fragment of GAPDH (glyceraldehyde 3-phosphate dehydrogenase) to determine the steady-state levels of GAPDH gene-related sequences. The detected bands were quantified with the Storm 860 phospho-imaging system (Molecular Dynamics) using the ImageQuant program. Relative mRNA levels detected were corrected for the signal for GAPDH for each sample condition and plotted as fold change from control, untreated cells.

*SDS–PAGE and Western blot analysis.* At the end of each post-radiation period N/N1003A cells were washed in PBS, detached with trypsin, and treated with endonuclease (Sigma) for 30 min to degrade DNA. Cells were incubated in lysis buffer containing protease inhibitors leupeptin, pepstatin and aprotinin (10 $\mu$g/ml each), 50 mM Tris–HCl, pH 7.45, 150 mM NaCl, 1% Triton X-100 and 0.5% sodium deoxycholate for 30 min on the ice as described previously (18). Protein was then quantitated and 25 $\mu$g was run on SDS-polyacrylamide gel. After transfer to Immobilon membranes (Millipore) Western blot analysis was carried out using the HO-1 antibody diluted 1:1000. $^{125}$IPA was used to detect primary antibody-antigen complex. The detected bands were quantified with the Storm 860 phospho-imaging system, and relative protein levels for each condition were plotted as fold change from the control, untreated cells.

*Enzyme assays.* *cis*-Aconitase activity was determined by the method of Rose and O'Connell (20) in cytosolic extracts prepared according to the method of Drapier *et al.* (21). After collecting the cytosolic fraction, the pelleted material containing intact mitochondria was washed twice with sucrose buffer, then lysed for 30 min on ice in buffer containing 50 mM Tris–HCl, pH 7.45, 150 mM NaCl, 1% Triton X-100 and 0.5% sodium deoxycholate. The lysates were passed 10 times through a syringe with a 23½ gauge needle. Insoluble material, containing mainly cytoskeletal proteins was spun down at 10,000 × g for 15 min and discarded. The membrane extract obtained was used as a source of mitochondrial *cis*-aconitase, localized exclusively in mitochondrial membranes. The cytosolic fraction was concentrated to a final volume of 20–30 $\mu$l using Centricon concentrators (Amicon). Protein concentration was measured by the Pierce BCA protein assay kit prior to each enzyme assay.

## RESULTS

*HO-1 induction.* The exposure to UVA radiation followed by a post-radiation recovery period increased the steady-state level of HO-1 message in N/N1003A rabbit lens epithelial cells in a time dependent manner. The maximal accumulation of the transcript was observed during 2–6 h of the post-radiation period (lanes 4–6, Fig. 1). As detected by the HO-1 probe, the size of the HO-1 message was estimated to be 4.3 kbp, significantly larger than that reported in fibroblasts (22, 23). Increase in HO-1 message was followed by enhanced expression of HO-1 protein, with maximum expression between 14 and 18 h of the post-radiation period (lanes 4–6, Fig. 2), which is parallel to the changes in enzymatic activity observed earlier in irradiated fibroblasts (17).

*Cytosolic cis-aconitase (cAcon).* The release of free iron as an immediate product of HO-1 enzymatic activity was monitored in this study, as an acquirement by IRP-1 (iron response protein) in a stable and irreversible process, a function of cytosolic *cis*-aconitase (cAcon). However no significant increase in cAcon activity was observed 18 h after UVA radiation of N/N1003A cells followed by 18 h of post-radiation period (Fig. 3). Unexpectedly, data from triplicate experiments showed a slight decrease close to the level observed after treatment of cells with HO-1 inhibitor, *Sn*-protoporphyrin IX (24) (Fig. 3). This observation suggests that the activation of IRP-1, due to binding of free iron and dissociation from iron binding site, is a fast process occurring in the early phase of HO-1 activation, earlier than our 18 h post-radiation time. The lower than control level of enzymatic activity might suggest the loss or initiation of an irreversible degradation process of cytosolic *cis*-aconitase.

*Mitochondrial cis-aconitase (mAcon).* In contrast to cAcon activity, at the 18 h post-radiation time we detected a two-fold increase in mAcon activity (Fig. 4). The increase of mAcon activity in rabbit lens epithelial cells is the direct result of up-regulation of HO-1 activity by exposure of cells to UVA radiation. Incubation with *Sn*-protoporphyrin IX, a heme oxygenase inhibitor, decreased mAcon activity to control levels (Fig. 4). Preincubation of N/N1003A cells for 48 h with a chelator of free iron, desferrioxamine, in control cells reduced mAcon activity to below the baseline levels which suggests that under normal conditions equilibrium between both IRE associated and dissociated

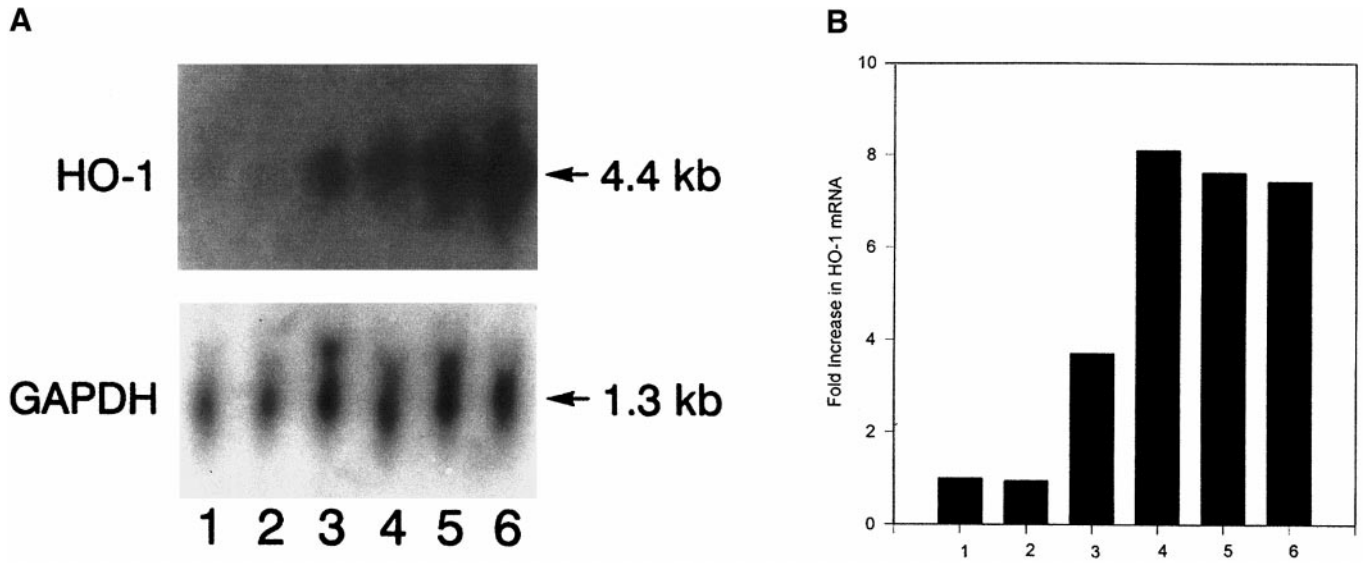


**FIG. 1.** (A) Northern blot analysis of RNA isolated from N/N1003A cells exposed to UVA radiation using the HO-1 cDNA probe (top panel) or GAPDH probe (bottom panel). Lane 1, control; lane 2, UVA-exposed; lanes 3–6, UVA-exposed and incubated for post-radiation times of 1, 2, 4 and 6 h, respectively. (B) Fold increase in HO-1 mRNA expression by UVA radiation. Sample numbers 1-6 correspond to the respective conditions listed in (A). The data are representative of three experiments.

forms of IRP-1 exists. However, desferrioxamine did not reverse the effect of UVA radiation on mAcon activity (data not shown). These data confirm earlier observations (11, 17) that iron chelators do not prevent HO-1 activation at transcriptional or translational levels.

## DISCUSSION

In a system of tight control of iron metabolism in cells, the release of free iron, in the reaction catalyzed by HO-1 seems unlikely to be left uncompensated. An increase in ferritin level, parallel to the increase of

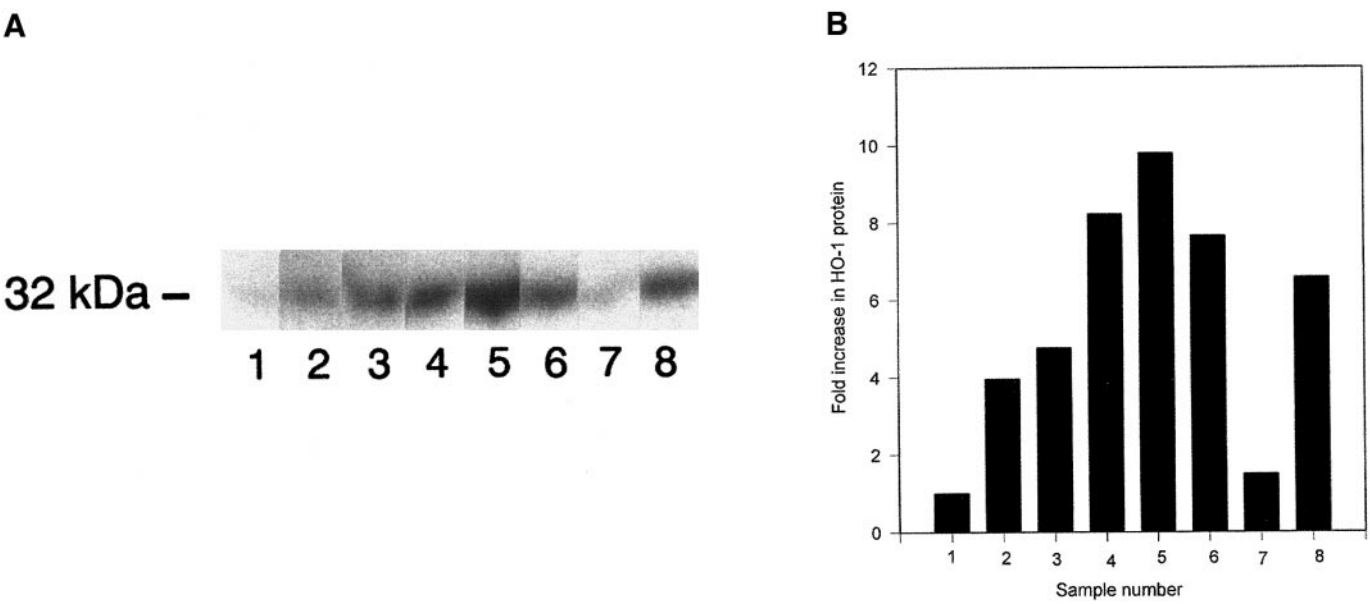


**FIG. 2.** (A) Western blot analysis of HO-1 protein in N/N1003A cells exposed to UVA radiation with an antibody specific to HO-1 protein. Lane 1, control; lanes 2–6, cells exposed to UVA and then incubated for post-radiation time 8, 10, 14, 18 and 24 h, respectively; lane 7, control cells preincubated with 100 $\mu$M desferrioxamine for 17 h; lane 8, cells preincubated with 100 $\mu$M desferrioxamine for 17 h, and then exposed to UVA and further incubated with desferrioxamine for a post-radiation time of 18 h. (B) Fold increase in HO-1 protein expression by UVA radiation. Sample numbers 1-8 correspond to the respective conditions listed in (A). The data are representative of three experiments.

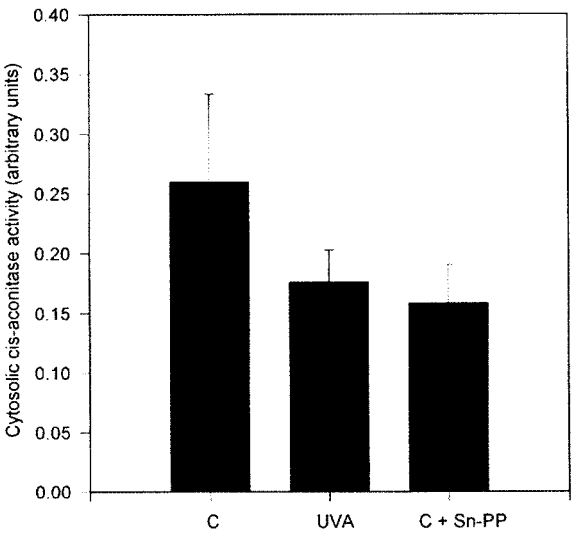


**FIG. 3.** Cytosolic *cis*-aconitase activity in N/N1003A lens epithelial cells. C, control cells; UVA, cells exposed to UVA radiation and incubated for a post-radiation time of 18 h at 37°C; C + Sn-PP, control cells treated with an inhibitor of HO-1, Sn-protoporphyrin IX for 2 h. Activity is expressed as the increase in absorbance at 340 nm ($A_{340}$) per mg protein per 60 min at 37°C, in the linear phase of the assay. The data represent mean of three experiments ± standard error.

HO-1 expression as the result of ultraviolet irradiation has been reported in human fibroblasts (17). In lens epithelial cells exposed to environmental levels of UVA radiation (considered to be one of the factors in cataract development (25)), the defense mechanism also involves the increase in steady-state message for HO-1 (Fig. 1), followed by an increase in the level of HO-1 protein (Fig. 2). Although we were unable to detect the increase of cAcon activity (as a consequence of the increase of free iron level produced by HO-1 and dissociation of iron-loaded version of IRP-1 from messages with iron-response elements, IREs), we have demonstrated an increase in mAcon activity (Fig. 4) which is reportedly due to its unblocked translation (13). Since the formation of cAcon is irreversible in vivo, the elevated level of mAcon activity accompanying HO-1 activation indicates that the processes triggered by iron release, including translation of the messages containing functional IRE, have not returned to the baseline level. This is consistent with the reported irreversible proteolysis of IRP-1-heme complex (reviewed in 13). Therefore it is more likely that cAcon may lose its enzyme activity, regaining its iron-binding properties in a feed-back mechanism regulated by free radicals (26). Such a phenomenon has been observed in fibroblasts exposed to $H_2O_2$ (27). It has been speculated that the potential role of mAcon might be to enhance the production of isocitrate and to speed up the Krebs cycle (reviewed in 13). The increase in free radical production could be the ultimate effect of augmentation of respiratory chain supplemented now with enhanced level of the Krebs cycle intermediate, isocitrate (as a result of mAcon activity). The production of isocitrate and the utilization of citrate can be important for cellular defense from oxidative stress, since both cAcon and mAcon will also likely respond, in concert, to changes in pH and thiols (28), important factors in oxidative stress defense. Early studies on heat shock phenomenon reported an increase in isocitrate respiration and retardation of gene activation in the presence of substrates of the citric acid cycle (29). The increase in activity of mitochondrial, non-Krebs cycle enzymes has also been demonstrated (30, 31).

The results of the present study suggest that bilirubin and biliverdin production is not the only mechanism by which HO-1 protects cells against oxidative stress. The regulation of iron homeostatsis and processes connected to it might be another role of HO-1. The sequence of the events in the cells exposed to oxidative stress seems to be in following order: (1) initial release of free iron by HO-1, (2) sequestration of free iron by IRP-1 with concomitant conversion of iron-loaded IRP-1 into cAcon (which occurs prior to the increased translation of mAcon), (3) the increase of translation of mAcon, regulated by release of IRP-1 from IRE, (4) inhibition of cAcon/IRP-1 with restoration of its IRE binding properties in the feedback mechanism by free radicals, which accompanies the activation of mAcon, and (5) enhancement of respiratory metabolism with isocitrate, and the production ATP. The increase of ferritin synthesis as iron depot, which is controlled by *cis*-aconitase may

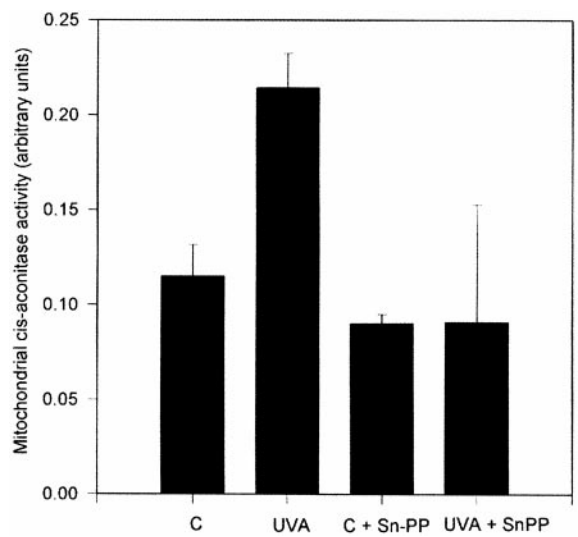


**FIG. 4.** Mitochondrial *cis*-aconitase activity in N/N1003A cells. C, control cells; UVA, cells exposed to UVA radiation and incubated for 18 h of postradiation time; C + Sn-PP, control cells treated with 100 $\mu$M Sn-protoporphyrin IX; UVA + Sn-PP, UVA exposed cells which had been pre-incubated with Sn-protoporphyrin IX for 2 h. Activity is expressed as the increase in absorbance at 340 nm ($A_{340}$) per mg protein per 60 min at 30°C, in the linear phase of the assay. The data represent mean of three experiments ± standard error.

be a final event, completing the reaction cycle. The importance of the role of iron in the regulation of both *cis*-aconitase activities for cellular protection against oxidative stress seems to be supported by the following observations. First, in the presence of high concentration of reductants, the IRP-1-iron complex rebinds to IRE, and its binding can be prevented by the addition of citrate. Second, the heme molecule itself can bind to IRP-1 and initiate ferritin synthesis. Finally, in cells treated with hemin, the complex IRP-heme is quickly degraded, when binding of iron results in the formation of cAcon (reviewed in 13). Chelating of free iron by desferrioxamine, known to protect against oxidative stress (11, 17) neither prevents HO-1 induction nor activation of mAcon (data not shown). This indicates that iron release from heme, catalyzed by HO-1 is a unique process, which does not interfere with the protective effect of desferroxiamine. An additional function of HO-1 may be the regulation of cell growth. Epoxygenases, which belong to the superfamily of fatty acid cytochrome P-450 monooxygenases, contain heme as the prosthetic group. Their products, epoxyeicosatrienoic acids have been shown to restore cell growth (32). Stimulation of HO-1 in rats treated with phenylhydrazine decreases the level of cytochrome P-450, without changes in the total heme level (33). The other indication suggesting the role of HO-1 in regulation of cell growth might be the high level of IRP-1 with stable *cis*-aconitase activity in non-proliferating cells (reviewed in 13). In summary, HO-1 may have a very complex and potent role in cellular defense. In addition to its well known role in preventing against oxidation by production of bilirubin and biliverdin, we propose that through free iron release, HO-1 may connect with iron homeostasis regulatory mechanisms including modulation of *cis*-aconitase activity, isocitrate production and enhancement of the Krebs cycle.

## ACKNOWLEDGMENTS

We thank Zheng Song for valuable help with Western blot analysis. This work is supported by National Institutes of Health Grants EY05681 (to U.P.A.) and EY02687 (Core grant), and RPB.

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