Linoleyl Hydroperoxide Transcriptionally Upregulates Heme Oxygenase-1 Gene Expression in Human Renal Epithelial and Aortic Endothelial Cells

ANUPAM AGARWAL,* FUMIE SHIRAISHI,* GARY A. VISNER,† and HARRY S. NICK*‡†
Departments of *Medicine, †Pediatrics, and ‡Neuroscience, University of Florida, Gainesville, Florida.

Abstract. Atherogenic lipoproteins such as oxidized LDL are implicated in the pathogenesis of atherosclerosis and renal disease. Fatty acid hydroperoxides and phospholipids such as linoleyl hydroperoxide (LAox or 13-HPODE) and lysophosphatidylcholine (lyso-PC), abundant components of oxidized LDL, mediate the effects of atherogenic lipids. Oxidized LDL has been shown to induce heme oxygenase-1 (HO-1), a microsomal enzyme that is involved in heme detoxification and is a major endogenous source of carbon monoxide. HO-1 is also induced by many other stimuli that shift cellular redox. To identify the constituents and molecular mechanisms of oxidized LDL-mediated HO-1 induction, human renal epithelial cells and aortic endothelial cells were exposed to LAox and lyso-PC. Exposure to LAox (25 μM) showed an approximately 16-fold induction of HO-1 mRNA, whereas exposure to lyso-PC (25 μM) showed only an approximate 2.6-fold increase. Treatment with actinomycin-D (4 μM), a transcriptional inhibitor, as well as nuclear run-on assays, demonstrated that LAox-mediated HO-1 gene induction is dependent on de novo transcription. Cycloheximide did not affect LAox-mediated HO-1 mRNA induction, suggesting that new protein synthesis is not required for transcriptional induction. Transfection of a human HO-1 promoter-reporter gene construct showed that LAox upregulation of HO-1 occurs via mechanisms different from those of known inducers, heme and cadmium. These studies are the first demonstration that LAox induces HO-1 by transcriptional mechanisms and may have implications in the pathogenesis of cell injury in atherosclerosis and progressive renal disease.

Atherogenic lipoproteins such as oxidized LDL have been implicated in the pathogenesis of atherosclerosis and progressive renal disease (1–3). Oxidized LDL is a complex structure consisting of several chemically distinct pro-oxidant components (1,4). Fatty acid hydroperoxides, such as linoleyl hydroperoxide (LAox or 13-HPODE), and phospholipids, such as lysophosphatidylcholine (lyso-PC), are abundant components of oxidized LDL serving as specific redox signals in the expression of cell adhesion molecules and growth factors in endothelial cells (5,6). In addition, recent studies using antibodies to LAox have demonstrated an abundance of fatty acid hydroperoxides in oxidized LDL as well as in human atherosclerotic plaques (7,8). Although oxidation of LDL in the subintimal space of the artery has received the most attention, there is evidence to suggest that oxidation of LDL occurs at many other sites of inflammation, including the kidney (1,9).

In proteinuric renal disorders, renal tubular epithelial cells are exposed to a diverse array of macromolecules, including lipoproteins such as LDL; these cells mimic endothelial cells in their response to the injurious effects of oxidized LDL (10,11). LDL and antigens closely related to oxidized LDL have been identified in renal biopsies of patients with renal disease (9,12–14). Recent studies also demonstrate the release of proinflammatory cytokines such as interleukin-6 and transforming growth factor-β in proximal tubular cells exposed to LDL (15). Thus, renal tubular injury and the pathogenesis of progressive renal disease are very analogous to endothelial injury in atherosclerosis and offer an impetus for the present study in both human renal epithelial and endothelial cells.

Of importance to renal injury and atherosclerosis is the documented induction of heme oxygenase-1 (HO-1) by oxidized LDL (10,16). HO-1, a 32-kD microsomal enzyme, catalyzes the rate-limiting step in the degradation of heme, forming biliverdin and subsequently bilirubin (17). Important by-products of this reaction include the release of iron and carbon monoxide (CO), which has received considerable attention as a signaling molecule similar to nitric oxide (18–20). Two isoforms of heme oxygenase, HO-1 and HO-2, have been described (17). The two isozymes differ in their molecular and biochemical properties in that HO-1 is inducible, whereas HO-2 is refractory to most stimuli. A third isoform, HO-3, with properties similar to HO-2, has recently been characterized (21).

HO-1 is induced by various stimuli, including hydrogen peroxide, ultraviolet radiation, heat shock, heavy metals, hyp-
oxia, hyperoxia, shear stress, endotoxin, and cytokines, all of which provoke oxidant stress as a common denominator (22-24). Induction of HO-1 is also considered an adaptive and beneficial response to oxidative stress in a wide variety of cells (17,22,25). The beneficial effects of increased HO-1 activity occur by the following potential mechanisms. HO-1 degrades the toxic heme moiety and also generates bilirubin, an antioxidant capable of scavenging peroxo radicals and inhibiting lipid peroxidation (26). Coupled induction of ferritin, a dominant intracellular repository for iron, occurs with the induction of heme oxygenase (27). This response allows safe sequestration of the iron liberated from the HO-1 catalyzed reaction and serves a physiologically important cytoprotective function (27). Finally, the vasodilatory properties of CO mediated via cGMP may be beneficial in the setting of an abnormal atherosclerotic vessel (17).

The *in vivo* relevance of HO-1 induction is suggested by the identification of an abundance of HO-1 mRNA in human and animal atherosclerotic plaques by *in situ* hybridization (10,16). Recent work also suggests that HO-1 has a role in the inflammatory response in atherosclerosis, because induction of HO-1 inhibits monocyte chemotaxis induced by mildly oxidized LDL (16). Therefore, we present studies to evaluate the molecular mechanism of HO-1 induction by constituents of oxidized LDL in both human renal epithelial and aortic endothelial cells. This study is the first demonstration that fatty acid hydroperoxides such as LAox transcriptionally upregulate the HO-1 gene in human cultured cells with potential implications in the pathogenesis of atherosclerosis, renal tubular injury in proteinuric diseases, and in the molecular mechanisms controlling HO-1 gene expression.

Materials and Methods

**Reagents**

Tissue culture media, serum, and supplements were obtained from Clonetics (Walkersville, MD). Linoleic acid, lipoxygenase (type V, soybean), hemin, actinomycin-D, and cycloheximide were obtained from Sigma Chemical Co. (St. Louis, MO). Lα-Phosphatidylcholine (PC) and lyso-PC were purchased from Calbiochem-Novabiochem (La Jolla, CA).

**Cell Culture**

Human renal proximal tubular cells (HPTC) (Clonetics) were grown in renal epithelial basal medium supplemented with fetal bovine serum (1%), gentamicin (50 μg/ml), amphotericin B (50 μg/ml), insulin (5 μg/ml), transferrin (10 μg/ml), triiodothyronine (6.5 ng/ml), hydrocortisone (0.5 μg/ml), epinephrine (0.5 μg/ml), and human epidermal growth factor (10 ng/ml) at 37°C in 95% air and 5% carbon dioxide. Human aortic endothelial cells (HAEC) were derived from segments of human aorta obtained from heart transplant donors. These cells stained positively for factor VIII antigen and acetylated low-density lipoprotein (16). These cells were characterized by methods described previously (17). HAEC were grown in endothelial basal medium (Clonetics) supplemented with 10% fetal bovine serum, gentamicin (50 μg/ml), amphotericin B (50 μg/ml), hydrocortisone (1 μg/ml), human epidermal growth factor (10 ng/ml), and bovine brain extract (6 μg/ml) at 37°C in 95% air and 5% carbon dioxide. Studies were performed on cultures over a range of no more than four to five passages. Cells were grown in 10-cm tissue culture plates and studied as a confluent monolayer in all experiments.

**Preparation of LAox and lyso-PC**

Linoleic acid (100 mM stock solution), PC (10 mM stock solution), and lysophosphatidylcholine (10 mM stock solution) were dissolved in ethanol. In preliminary experiments, ethanol carrier alone tested at the highest final concentration (0.5%) was without effect. LAox was prepared by the oxidation of linoleic acid (200 μM in phosphate-buffered saline [PBS], pH 7.4), using soybean lipoxygenase (1000 U) as described previously (5,29). Oxidation was monitored continuously on a spectrophotometer at 234 nm as an increase in conjugated diene formation. Unoxidized linoleic acid and lipoxygenase alone were used as controls. The samples were made fresh each time and used immediately in the experiments. After oxidation, the preparation was added to the cells (1 to 25 μM) in serum-free media before RNA extraction or Western analysis. Incubation of cells with the above concentrations of LAox for 8 to 12 h did not result in any appreciable decrease in cell viability as assessed by morphology or lactate dehydrogenase release. PC (25 μM) and lyso-PC (1 to 25 μM) were added to cells in media containing 1% serum to preserve viability of the cells as reported previously (6). Although no appreciable cytotoxicity was observed with these doses for up to 16 h of incubation, higher concentrations of lyso-PC (>50 μM) resulted in significant cytotoxicity, as has been reported by others (30).

**Northern Analysis**

Total cellular RNA was extracted from cultured cells in 10-cm plates, after 4 h of incubation, using the method of Chomczynski and Sacchi (31). The RNA was electrophoresed on 1% agarose-formaldehyde gels, blotted onto nylon membranes, and hybridized to a 32P-labeled cDNA probe for human HO-1. To control for loading and transfer of RNA, the blots were reprobed with a cDNA probe for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Autoradiography was performed using intensifying screens at ~80°C.

**Western Blot Analysis**

For HO-1 immunoblots, cells were washed twice with ice-cold PBS and lysed in Triton lysis buffer. Protein concentration of lysates was determined by bicinchoninic acid assay (Pierce, Rockford, IL). Samples (20 μg) were separated in a 10% sodium dodecyl sulfate-polyacrylamide gel and then transferred onto a polyvinylidene difluoride membrane. The membranes were incubated for 1.5 h with rabbit polyclonal antibody against rat HO-1 (1:500 dilution) (StressGen Biotech Corp., Vancouver, British Columbia, Canada) followed by incubation with peroxidase-conjugated goat anti-rabbit IgG antibody (1:10,000 dilution) for 1 h (32). Labeled protein bands were examined using a chemiluminescence method (Pierce).

**Nuclear Run-On Transcription Assay**

Cells were grown in 15-cm cell culture plates and exposed to media containing PBS (control), LAox (25 μM), or hemin (5 μM) for 2 h. Nuclei were isolated and transcriptional rates were measured directly by nuclear run-on assay as described previously (33). Briefly, 0.2 ml of the nuclear suspension was labeled with 100 μCi of α-32P-UTP and incubated for 45 min at 30°C in incubation buffer (10 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 300 mM KCl, 1 mM dithiothreitol, 1 mM each ATP, GTP, and CTP, 8.8 mM creatine phosphate, 8 U creatine kinase, and 80 U RNase inhibitor). The 32P-labeled nuclear RNA was purified by a single extraction (31), and equal counts of labeled RNA (2 × 106 cpm/hybridization reaction) were hybridized to cDNA inserts for human HO-1 and human GAPDH, which were denatured and immobilized to nitrocellulose membranes using a dot-blot apparatus. After
hybridization at 60°C for 48 h, filters were washed and exposed to x-ray film at –80°C for 1 to 2 wk.

**Plasmid Construct and Promoter Analysis**

An approximately 4.5-kb fragment of the 5′ flanking region of the human HO-1 gene, extending from –4.5 kb to +80-bp position within exon 1, including the transcription initiation site, was generated by long-range PCR performed on human genomic DNA using rTth DNA polymerase (Perkin-Elmer, Foster City, CA). The 4.5-kb PCR product was ligated into TA cloning vector (Invitrogen, Carlsbad, CA) and verified by restriction analysis and sequencing (34). BamHI sites were incorporated in the primers to enable subcloning into a promoterless human growth hormone (hGH) vector. This promoter-reporter gene construct (pHHOGH) was used for transient expression studies. The promoter fragment in the reverse orientation (3′ to 5′) was used as a control and demonstrated no significant increase in reporter gene function in response to hemin or cadmium, known inducers of HO-1 (data not shown). A batch transient transfection protocol was performed in 15-cm plates, using diethylaminoethyl-dextran (35) for HAEC. After transfection, cells were split into four separate 10-cm plates and stimulated with media containing PBS (control), hemin (5 μM), cadmium (5 μM), and LAox (25 μM) for 4 h. Total RNA or media was collected at 12 and 72 h, respectively. hGH mRNA levels were evaluated by Northern analysis using a partial hGH cDNA probe. In addition to hGH mRNA levels, secreted hGH protein was also measured using a commercially available RIA kit (Nichols Institute, San Juan Capistrano, CA). The hGH reporter gene system is a highly sensitive, stable, straightforward, and rapid assay. Being a genomic clone, this system is not affected by extensive cloning manipulations and undergoes modifications such as capping, polyadenylation, and splicing analogous to the endogenous HO-1 gene (36).

**Statistical Analyses**

Data are expressed as mean ± SEM. Statistical analyses of the hGH protein levels in the different groups were performed using ANOVA and the Student-Newman-Keuls test. All results are considered significant at \( P < 0.05 \).

**Results**

**Induction of HO-1 by LAox and lyso-PC**

We have previously reported the induction of HO-1 mRNA and enzyme activity after exposure to oxidized LDL in renal epithelial and endothelial cells (10). Oxidized LDL is a complex particle consisting of several chemically distinct components (1,4) such as LAox and lyso-PC, which modulate the expression of several genes, including adhesion molecules and growth factors (5,6). To determine whether these chemical constituents of oxidized LDL were responsible for HO-1 gene induction, we exposed confluent monolayers of HPTC and HAEC to media containing PBS (control), lyso-PC (25 μM), LAox (25 μM), or ethanol (EtOH 0.5%) (Figure 1, A and B). Cells were exposed to lyso-PC for 8 h and LAox for 4 h, time...

![Figure 1](image-url)

**Figure 1.** Differential induction of heme oxygenase-1 (HO-1) mRNA with lysophosphatidylcholine (lyso-PC) and linoleyl hydroperoxide (LAox). Confluent monolayers of human renal proximal tubular cells (HPTC; A) and human aortic endothelial cells (HAEC; B) were incubated with media containing phosphate-buffered saline (PBS; control), lyso-PC (25 μM) for 8 h, LAox (25 μM) for 4 h, or carrier (ethanol [EtOH] 0.5%) as indicated. In separate experiments, cells were exposed to phosphatidylcholine (PC) (25 μM) or lyso-PC (25 μM) for 8 h (lower panel). RNA was isolated, electrophoresed, transferred to a nylon membrane, and hybridized with a \( ^{32} \)P-labeled human HO-1 cDNA probe. The membrane was stripped and reprobed with a human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe to control for loading and transfer. The results are representative of several experiments performed in duplicate.
points at which maximal induction of HO-1 was seen (data not shown). As shown in Figure 1, incubation with lyso-PC (25 μM) resulted in an approximately 6.5-fold increase in HO-1 mRNA, whereas exposure to LAox (25 μM) resulted in a much higher induction (approximately 16-fold) of HO-1 mRNA. The parent phospholipid compound PC (25 μM) did not induce HO-1 mRNA (Figure 1, lower panel). Thus, LAox mediates significant induction of HO-1 mRNA in HPTC and HAEC.

We also performed experiments in which HPTC and HAEC were incubated with media containing PBS (control), LAox (1, 10, and 25 μM), unoxidized linoleic acid (LA 25 μM), or lipoygenase alone (Lip 100 U) for 4 h. These concentrations of LAox are approximately equivalent to their abundance in 100 μg/ml oxidized LDL (5). As shown in Figure 2, significant induction of HO-1 mRNA and protein occurred with LAox in both HPTC (Figure 2, A and B) and HAEC (Figure 2, C and D). In HPTC, 10 and 25 μM LAox increased HO-1 mRNA by approximately fivefold and approximately 16-fold, respectively (Figure 2A). In HAEC, a similar induction of HO-1 mRNA occurred (Figure 2C). This increase in HO-1 mRNA was accompanied by significant induction of HO-1 protein (approximately 32 kD size), as measured by Western blot analysis (Figure 2, B and D). Linoleic acid and lipoygenase alone did not induce HO-1 mRNA or protein.

**Transcriptional Activation of HO-1 by LAox**

To evaluate the importance of de novo transcription, we performed a nuclear run-on analysis to directly measure the rate of HO-1 gene transcription after stimulation with LAox. Nuclei were isolated from HAEC treated with media containing PBS (control), LAox (25 μM), or hemin (5 μM) for 2 h. As shown in Figure 4, LAox augmented activation of HO-1 gene transcription by approximately 10-fold over control nuclei. Hemin, a potent activator of HO-1, increased HO-1 gene transcription by approximately 20-fold and was used as a positive control in this experiment. Similar results were obtained in HPTC (data not shown). Thus, the induction of HO-1 by LAox occurs by direct responses at the level of HO-1 gene transcription.

**Upregulation of HO-1 by LAox Requires RNA Synthesis**

To explore the dependence of LAox-mediated induction of HO-1 on new RNA or protein synthesis, we coincubated HPTC and HAEC with a transcriptional inhibitor, actinomycin-D (4 μM), or a translational inhibitor, cycloheximide (20 μM), in combination with LAox. Hemin (5 μM) was used as a positive control. As shown in Figure 3, actinomycin-D treatment completely abolished LAox-mediated induction of HO-1 mRNA, whereas cycloheximide had no effect on the induction. These data suggest that LAox-mediated HO-1 gene induction is dependent on RNA synthesis and does not require new protein synthesis for transcriptional induction.

**Upregulation of HO-1 by LAox Is Via Mechanisms Different from Those of Heme or Cadmium**

To identify promoter elements in the human HO-1 gene that mediate its transcription after stimulation with LAox, we performed experiments in which HPTC and HAEC were incubated with media containing PBS (control), LAox (1, 10, and 25 μM), or lipoygenase alone (Lip 100 U) for 4 h. These concentrations of LAox are approximately equivalent to their abundance in 100 μg/ml oxidized LDL (5). As shown in Figure 2, significant induction of HO-1 mRNA occurred (Figure 2C). This increase in HO-1 mRNA was accompanied by significant induction of HO-1 protein (approximately 32 kD size), as measured by Western blot analysis. Linoleic acid and lipoygenase alone did not induce HO-1 mRNA or protein.

**Figure 2. Induction of HO-1 in HPTC and HAEC with LAox.** Confluent monolayers of HPTC (A) and HAEC (C) were incubated with media containing PBS (control), LAox, unoxidized linoleic acid (LA), or lipoygenase (Lip) for 4 h in serum-free media. RNA was isolated, electrophoresed, transferred to a nylon membrane, and hybridized with a 32P-labeled human HO-1 cDNA probe. The membrane was stripped and reprobed with a human GAPDH cDNA probe to control for loading and transfer. Confluent monolayers of HPTC (B) and HAEC (D) were incubated with media containing PBS (control) or LAox (25 μM) for 4 h and then replaced with media alone for an additional 8 h. Western blot analysis was performed using a polyclonal anti-HO-1 antibody (StressGen Biotech Corp.), as described in Materials and Methods. HO-1 is identified as a 32-kD size protein.
Figure 3. HO-1 mRNA induction by LAox is blocked by the transcriptional inhibitor actinomycin-D. Confluent monolayers of HPTC (A) and HAEC (B) were incubated for 4 h with serum-free media containing PBS (control), LAox, actinomycin-D (4 μM) alone, actinomycin-D + LAox, cycloheximide (20 μM) alone, or cycloheximide + LAox. The concentration of LAox was 10 μM for HPTC and 25 μM for HAEC in these experiments. Hemin (5 μM) was used as a positive control in this experiment. RNA was isolated, electrophoresed, transferred to a nylon membrane, and hybridized with a 32P-labeled human HO-1 cDNA probe. The membrane was stripped and reprobed with a human GAPDH cDNA probe to control for loading and transfer. The results are representative of two separate experiments performed in duplicate.

Figure 4. Transcriptional regulation of LAox-mediated induction of HO-1. Nuclear run-on assay 2 h after exposure of HAEC to media containing PBS (control), LAox (25 μM), or hemin (5 μM). Nuclei were isolated, and transcriptional rates were measured directly by nuclear run-on assay as described in Materials and Methods. 32P-labeled RNA were hybridized to filter-bound DNA of human HO-1 and GAPDH. Hemin was used as a positive control in this experiment. Results shown are representative of two independent experiments.

formed transient transfection experiments in HAEC using an approximately 4.5-kb promoter fragment of the human HO-1 gene that was cloned into a promoterless hGH reporter gene. This construct (pHOGH) (Figure 5A) demonstrated significant promoter activity, as measured by hGH mRNA and protein, with known inducers of the HO-1 gene, hemin, and cadmium (Figure 5, B and C). Interestingly, no significant response was observed after stimulation by LAox on this 4.5-kb promoter fragment as assessed by both reporter gene mRNA or protein expression (Figure 5, B and C), suggesting that upregulation of HO-1 by LAox occurs by mechanisms different from that of heme or cadmium and requires sequences outside of this 4.5-kb promoter fragment.

Discussion

Considerable interest in the enzyme HO-1 has been generated due to its regulation by a diverse array of stimuli, all of which impose a significant shift in cellular redox. In addition to its role in degrading heme, recent attention has focused on HO-1 as a major endogenous source of the gaseous molecule CO, a potent vasodilator and a second messenger with many properties akin to nitric oxide (18-20). Induction of HO-1 is considered a protective and adaptive response in cells exposed to oxidant stress (22,23,25). Most recently, it has been suggested that HO-1 plays an important role in the inflammatory response as well (16,37). Studies in HO-1 knockout mice further corroborate these observations in that mice lacking HO-1 develop a progressive inflammatory disease characterized by splenomegaly, lymphadenopathy, leukocytosis, hepatic peribiliary inflammation, and glomerulonephritis (37). HO-1 has also been implicated in several clinically relevant disease states including atherosclerosis (10,16), hypertension (38), acute renal injury in rhabdomyolysis (39), toxic nephropathy (32), transplant rejection (20), endotoxic shock (40), and Alzheimer’s disease (41), as well as others (42-44).

Overexpression of HO-1 or prior induction of HO-1 is cytoprotective in both in vitro and in vivo models of injury (39,45,46). Abraham and coworkers (45), using transfection studies resulting in the overexpression of HO-1 in coronary microvascular endothelial cells, demonstrated cytoprotective effects against heme and hemoglobin toxicity. Vile et al. (46) have demonstrated a beneficial role of HO-1 in ultraviolet A radiation-induced cell injury. Inhibition of HO-1 activity by tin
Figure 5. HO-1 promoter-reporter gene analysis in response to hemin, cadmium, and LAox. (A) A promoter-reporter gene construct using a 4.5-kb promoter fragment of the human heme oxygenase (HHO) gene and a promoterless human growth hormone (hGH) plasmid was made. This construct was transfected into HAEC by the diethylaminoethyl-dextran method as described in Materials and Methods. (B) Cells were exposed to stimuli (PBS as control, hemin [5 μM], cadmium [5 μM], or LAox [25 μM]), and media were assayed for secreted growth hormone (hGH ng/ml) by an RIA kit as described in Materials and Methods. *P < 0.05 versus control and LAox. (C) In separate experiments, RNA was isolated from transfected cells after stimulation, electrophoresed, transferred to a nylon membrane, and hybridized with a 32P-labeled hGH cDNA probe. The membrane was stripped and reprobed with a human GAPDH cDNA probe to control for loading and transfer. The results are representative of three separate experiments and are shown as optical density units corrected for those of GAPDH mRNA.

protoporphyrin, an enzymatic inhibitor, or by antisense oligonucleotides abolished the protective effect of preirradiation, implicating HO-1 in the mechanism of cellular protection. We have also demonstrated that prior induction of HO-1 or inhibition with tin protoporphyrin plays a functional role in cisplatin-mediated toxicity both in vivo (32) and in HPTC (47). Furthermore, overexpression of HO-1 facilitates cellular protection against hyperoxia in lung epithelial cells (48). Therefore, the molecular mechanisms controlling the induction of HO-1 after exposure to heavy metals, heme, ultraviolet A radiation, cytokines, or altered oxygen tension are extremely relevant to the progression of many diseases. Keyse et al. (49) have previously shown that treatment of skin fibroblasts with ultraviolet A radiation, hydrogen peroxide, and sodium arsenite causes a dramatic increase in the rate of HO-1 RNA transcription measured directly by nuclear run-on assays with no effect on mRNA stability. Cadmium and cytokines also induce the human HO-1 gene via transcriptional mechanisms (34,50). Our results demonstrate that LAox induces HO-1 in human aortic endothelial and renal tubular epithelial cells by directly increasing HO-1 gene transcription. This oxidized C:18-containing fatty acid component of oxidized LDL may serve as a signal for induction of this cytoprotective protein during the events associated with atherosclerosis or renal tubular injury.

Our findings of HO-1 induction by LAox being significantly higher than by lyso-PC demonstrate the differential effects of the components of oxidized LDL. The reason for such differential upregulation by these two components may underlie the molecular mechanism controlling this induction. With regard to oxidized LDL, previous studies report induction of adhesion molecules such as intercellular adhesion molecule-1, vascular cell adhesion molecule-1, growth factors, endothelial nitric oxide synthase, and cyclo-oxygenase-2 by LAox and/or lyso-PC (5,6,51). Oxidized arachidonic acid-containing phospholipids also induce HO-1, but to a much lesser extent than oxidized LDL (16). It is interesting to speculate whether the diverse effects of oxidized LDL on many cellular molecules are mediated through specific components such as LAox.
Fang et al. (30) have recently elucidated the signal transduction pathway for the effects of lyso-PC and showed stimulation of activator protein-1 (AP-1) and c-Jun N-terminal kinase (JNK) activity, suggesting that the stimulated JNK and AP-1 activities may mediate some of the bioactive effects of lyso-PC. The molecular pathways for LAox-mediated HO-1 induction are not known. Consensus-binding sites for nuclear factor-kB, AP-2, and interleukin-6-responsive elements, as well as other transcription factors, have been identified in the promoter region of the HO-1 gene (43,52), suggesting a potential role for these trans-acting factors in modulating HO-1 gene expression.

Regarding the cis-acting elements that modulate HO-1 gene expression, a potential cadmium response element has been identified between -4.5 and -4 kb 5' to the transcriptional initiation site of the human HO-1 gene (34). We have shown that this 4.5-kb human HO-1 gene promoter fragment, which responds to known inducers of the gene such as heme and cadmium, does not contain the cis-acting elements necessary for LAox-dependent gene induction. Our findings of the absence of a response with this promoter fragment to LAox is further corroborated by the failure of this fragment to respond to other stimuli that directly increase de novo HO-1 gene transcription, i.e., hyperoxia and iron/hyperoxia (53). In the mouse HO-1 gene, Alam et al. (54) have described two distal enhancers, one at -4.0 and another at -10 kb, that are required for induction of HO-1 in response to heme, heavy metals, hydrogen peroxide, and sodium arsenite. It is possible, therefore, that the human HO-1 gene also requires distal enhancer element(s) similar to the mouse HO-1 gene for induction with LAox. Further experiments using additional promoter transfections, chromatin structure analysis, and in vivo footprinting are currently under way in our laboratory to delineate the region of the HO-1 gene that controls oxidized lipid-mediated induction.

In summary, our studies demonstrate that LAox is the major component of oxidized LDL that induces HO-1 by transcriptional mechanisms and is different from that of known stimulants such as heme and cadmium. LAox, an oxidized C:18 fatty acid, may serve as a signal for HO-1 gene induction in response to oxidized LDL. Further understanding of the molecular mechanism of HO-1 gene expression in response to oxidized LDL will be important in designing interventional strategies in the pathogenesis of endothelial injury in atherosclerosis and renal tubular injury seen in proinflammatory states. Studies to evaluate the cytoprotective effects of selective overexpression or blockade of the HO-1 gene will elucidate the functional significance of this gene in oxidized lipid-mediated cell injury.

Acknowledgments
This work was supported by National Institutes of Health Grants K08 DK02446 (to Dr. Agarwal) and HL39593 (to Dr. Nick) and funding from the American Lung Association of Florida (to Dr. Visner). Dr. Shiraiishi is supported by a research fellowship from the National Kidney Foundation of Florida. We thank Jane Mellot, Leigh Truong, and Laurie Archbould for excellent technical assistance. We are also grateful to C. Craig Tisher, M.D., Professor and Chief, Division of Nephrology, Hypertension and Transplantation, University of Florida, for critical review of this manuscript.

References


