α-Lipoic Acid–Induced Heme Oxygenase-1 Expression Is Mediated by Nuclear Factor Erythroid 2-Related Factor 2 and p38 Mitogen-Activated Protein Kinase in Human Monocytic Cells

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Objective—Heme oxygenase-1 (HO-1), the rate-limiting enzyme in heme catabolism, plays a protective role in the vascular system. HO-1 induction inhibits cytokine production in macrophages. Antioxidants induce HO-1 expression in various cell types. α-lipoic acid (ALA), a thiol-containing dietary antioxidant, exhibits protective effects in vascular disease and induces anti-inflammatory effects in monocytes. This study examined the effects of ALA on HO-1 expression in human monocytic cells.

Methods and Results—ALA time and dose-dependently induced HO-1 mRNA expression in THP-1 cells, with peak expression at 4 hours and returning to baseline by 24 hours. This correlated with an increase in HO-1 protein expression. ALA stimulated translocation of the transcription factor nuclear factor-erythroid 2–related factor 2 (Nrf2) into the nucleus and binding to a human HO-1 antioxidant response element (ARE) by 30 minutes. A dominant-negative Nrf2 inhibitor reduced ALA-induced HO-1 mRNA expression by 66%. Pretreatment with SB203580, a p38 mitogen-activated protein kinase inhibitor, reduced ALA-induced HO-1 mRNA expression by 75% and inhibited ALA-induced Nrf2 binding to the HO-1 ARE.

Conclusions—These results demonstrate that ALA induces HO-1 expression in THP-1 monocytic cells via Nrf2 and p38. Further studies are required to investigate whether the protective effects of ALA in monocytes are mediated by HO-1. (Arterioscler Thromb Vasc Biol. 2005;25:2100-2105.)

Key Words: α-lipoic acid ■ monocyte ■ heme oxygenase-1 ■ Nrf2 ■ p38 MAPK

Heme oxygenase (HO) catalyzes the degradation of the pro-oxidant heme to biliverdin, free iron, and carbon monoxide. Biliverdin is converted to bilirubin, a potent chain-breaking antioxidant, by biliverdin reductase, whereas free iron is sequestered by ferritin.1–3 Three isoforms of HO have been identified in mammalian cells. HO-1 is inducible in many cell types in response to a wide variety of stimuli, including heavy metals, antioxidants, and prostaglandins.2 In contrast, HO-2 and HO-3 are constitutively expressed in many tissues including brain, liver, and spleen.3

The pathogenesis of vascular disease is a multifactorial process that is propagated by oxidative stress and proinflammatory mediators. HO-1 and the metabolites produced from its action on heme play a key role in protection against the oxidative stress and inflammation associated with vascular disease. These metabolites, including bilirubin and carbon monoxide, exert antioxidative and anti-inflammatory effects in the vascular system in vitro and in vivo.4–8 HO-1 induction inhibits cytokine production in macrophages and monocyte adherence to the endothelium.9–11 Studies in animal models of atherosclerosis also implicate a protective role for HO-1. In low-density lipoprotein receptor–knockout mice, hemin-induced HO-1 expression results in reduced atherosclerotic lesion formation.12 Double knockout mice, deficient in apolipoprotein E and HO-1, experience rapid atherosclerotic lesion formation and demonstrate increased sensitivity to oxidative stress compared with single apolipoprotein E knockout controls.13 In addition, human HO-1 deficiency results in a lethal phenotype that includes marked endothelial damage and abnormal clotting.14 Finally, polymorphisms in the 5′-flanking region of the human HO-1 gene have been identified that result in HO-1 functional defects. The incidence of these polymorphisms is higher in a variety of cardiovascular diseases, including diabetes-associated coronary artery disease, abdominal aortic aneurysms, restenosis, and patients with pre-existing risk factors.15 Together, these studies highlight a key protective role for HO-1 in vascular disease initiation and progression.

The 5′-flanking region of the human HO-1 gene contains many cis-acting transcription regulatory elements, termed the
antioxidant response element (ARE), with the core sequence nGATGACnnnGCn.16 The ARE is present in the promoters of a variety of cytoprotective and detoxification genes. Several transcription factors, including the nuclear factor erythroid 2 (NF-E2)–related factor (Nrf) family of transcription factors, bind to the ARE. The Nrf family comprises basic leucine zipper, cap–n-collar transcription factors, and includes NF-E2, Nrf1, Nrf2, Nrf3, Bach1, and Bach2. Nrf2 is emerging as a key regulator of ARE-dependent gene expression. Under basal conditions, Nrf2 is retained in the cytosol by its interaction with the inhibitory protein Keap-1. On stimulation, Nrf2 dissociates from Keap-1 and translocates to the nucleus, where it heterodimerizes with a variety of transcription factors, including members of the small Maf family.17

In monocytes and macrophages, HO-1 is induced by the dietary antioxidants tert-butyldihydroquinone (t-BHQ), quercetin, and curcumin.18–21 α-Lipoic acid (ALA) is a thiol antioxidant found in the diet, including broccoli, spinach, and tomatoes.22 ALA inhibits cytokine secretion, adhesion molecule expression, and NO synthesis in monocytes.23,24 ALA supplementation results in reduced blood pressure and reduced aortic advanced glycation end product content, heart mitochondrial superoxide production, and insulin resistance in hypertensive and diabetic animal models.25,26 These studies highlight an important beneficial role for ALA in protection against vascular disease. However, it remains to be established whether ALA induces expression of HO-1; thus, the present study was designed to examine this in human monocytes and to investigate the intracellular pathways involved.

Methods

Materials

SB203580, Ro-31-8220, and PD98059 were purchased from Calbiochem. (2)-ALA, curcumin, resveratrol, t-BHQ, and all other chemicals were obtained from Sigma.

Cell Culture

THP-1 monocytic cells, a human leukemia cell line,27 were purchased from ECACC and cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mmol/L L-glutamine (Biowhittaker Ltd), and 2-mercaptoethanol. Cells were maintained in a humidified atmosphere at 37°C and 5% CO2. ALA was prepared as described previously.22 Cytotoxicity was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.28

Western Immunoblotting

Cells were unstimulated or stimulated with ALA for various times. Whole cell lysates were prepared by boiling in sodium dodecyl sulfate–containing sample buffer for 5 minutes (Invitrogen). Cytosolic and nuclear extracts were prepared as described previously.29 Proteins were separated by SDS-PAGE on either a 4% to 12% or 10% polyacrylamide gel and transferred to polyvinylidene fluoride membrane (Invitrogen). Immunoblotting was performed using mouse anti-human HO-1 antibody (Stressgen Biotechnologies Corporation), rabbit anti-human phosphorylated p38 mitogen-activated protein kinase (MAPK) antibody, mouse anti-human p38 MAPK antibody (Cell Signaling Technology), and goat anti-mouse and goat anti-rabbit secondary antibodies (Santa Cruz Biotechnology). Proteins were detected using the ECL (enhanced chemiluminescence) Western blotting system (Amersham Pharmacia Biotech).

Real-Time Polymerase Chain Reaction

Cells were unstimulated or stimulated with ALA for various times at 37°C. In kinase experiments, cells were pretreated with kinase inhibitors for 30 minutes before ALA stimulation. Total RNA was extracted from 1×10⁶ cells using RNAwiz according to manufacturer instructions (Ambion). Reverse transcription was performed using the RNA polymerase chain reaction (PCR) core kit (Applied Biosystems). Real-time PCR primers for 18s and HO-1 were purchased from Sigma-Genosys, and for Nrf2 and GAPDH, they were purchased from Applied Biosystems. Quantitative real-time PCR used SYBR green technology (for HO-1 and 18s) and Taqman technology (for Nrf2 and GAPDH; Applied Biosystems) on cDNA generated from the reverse transcription of purified RNA (for sequences and cycle times please see the online supplement, available at http://atvb.ahajournals.org). HO-1 mRNA expression was normalized against 18s ribosomal subunit and Nrf2 mRNA normalized against GAPDH mRNA expression using the comparative cycle threshold method as per manufacturer instructions (Applied Biosystems).

Electrophoretic Mobility Shift Assays

A biotinylated oligonucleotide probe containing a human HO-1 ARE site (in italics), 5′-GCATTCTCTGCTGGTCATGTGGGAGG-3′, was manufactured by Sigma-Genosys. For competition binding, the same sequence was manufactured without the biotin label. Nuclear extracts were prepared from 5×10⁶ THP-1 cells as described previously29 and incubated with the biotin-labeled probes using the LightShift Chemiluminescent Electrophoretic Mobility Shift Assay (EMSA) kit (Pierce Biotechnology) as per manufacturer instructions. For supershift analysis, nuclear extracts from ALA-treated THP-1 cells were preincubated with 1 μg of either anti-human Nrf2 or anti-human p65 supershift antibodies (Santa Cruz Biotechnology) for 20 minutes before gel shift analysis.30

Transfection of THP-1 Cells

A dominant-negative mutant of Nrf2 (pcDNA3DN-Nrf2; a kind gift from Dr X. Chen, Atherogenics Inc, Alpharetta, Ga)31 or control DNA (pcDNA3) were transiently transfected into 2×10⁶ THP-1 cells using Effectene transfection reagent for 48 hours as per manufacturer instructions (Qiagen Inc). After this, transfected cells were left unstimulated or stimulated with ALA for 4 hours, RNA extracted, and real-time PCR analysis performed to detect HO-1 mRNA expression. The transfection efficiency using this method is 41.4±3.8% (mean±SD; n=3), determined using a green fluorescence protein reporter plasmid transfected into THP-1 cells, and fluorescence detected by flow cytometry.

Statistical Analyses

Student t test was performed to assess statistical significance from controls. Results with P<0.05 were considered statistically significant. Results are mean±SD of 3 independent experiments. For Western blotting and EMSA experiments, data are representative of 3 independent experiments.

Results

ALA Induces HO-1 Expression in THP-1 Monocytic Cells

THP-1 cells, treated with ALA for 4, 8, and 24 hours, exhibited a time- and dose-dependent increase in HO-1 mRNA expression, with peak induction detected at 4 hours (Figure 1a). This correlated with an increase in HO-1 protein expression, which was maximal at 8 hours and declined by 24 hours after ALA stimulation (Figure 1b). MTT assay confirmed that up to 1 mmol/L ALA was not cytotoxic to THP-1 cells (data not shown). The effects of ALA were compared with other antioxidants including resveratrol, t-BHQ, and curcumin, a
potent HO-1 inducer. Antioxidants were added at concentrations that did not affect cell viability and that have been reported previously to induce HO-1 expression in other cell types. Curcumin increased HO-1 mRNA expression in THP-1 cells, with a peak induction detected at 4 hours (1 mmol/L, 1.59 ± 0.06; 5 mmol/L, 25.63 ± 0.06; 15 mmol/L, 36.63 ± 0.51, mean fold increase above unstimulated control ± SD). In contrast, t-BHQ (40 µmol/L) induced a slower and weaker increase in HO-1 mRNA expression, peaking at 8 hours (2.4 ± 0.2; mean ± SD; P = 0.03). Resveratrol (25 to 100 µmol/L) did not increase HO-1 mRNA expression in THP-1 cells (data not shown).

ALA Stimulates Nrf2 Nuclear Translocation and ARE Binding in THP-1 Monocytic Cells

Nuclear extracts were prepared from unstimulated and ALA-treated THP-1 cells. ALA stimulated Nrf2 protein accumulation in the nucleus, detected by 30 minutes (Figure 2a). Densitometric analysis using β-actin for normalization revealed that this was a 2.5-fold increase in Nrf2 protein over unstimulated cells. Longer incubations with 1 mmol/L ALA revealed that Nrf2 was maximally present in the nucleus by 2 hours, with a 3.5-fold increase over unstimulated cells, and was still present at 4 hours (data not shown). ALA did not significantly increase Nrf2 mRNA expression in THP-1 cells (Figure I, available online at http://atvb.ahajournals.org). Nuclear extracts from THP-1 cells treated with 1 mmol/L ALA for 30 minutes were also analyzed by EMSA. No protein–DNA complex was detected in unstimulated cells. After ALA treatment, a complex bound to the human HO-1 ARE site within 30 minutes (Figure 2a). The specificity of the oligonucleotide in the EMSA was confirmed by the addition of unbiotinylated probe (Figure 2b). Nrf2 was confirmed to be present in the complex by supershift analysis, using anti-Nrf2 antibodies (Figure 2b). Anti-p65 (NF-κB) antibodies were used as a negative control. These results suggest that ALA stimulates Nrf2 activation in THP-1 cells.

ALA-Induced HO-1 mRNA Expression Is Reduced by a Dominant-Negative Nrf2 Inhibitor

The role of Nrf2 in regulating ALA-induced HO-1 expression was confirmed using a dominant-negative mutant of Nrf2. This lacks the transcription activation domains of Nrf2 but contains the cap’n’collar homology region and the basic leucine-zipper domain, required for DNA binding and protein dimerization (amino acids 399 to 598). This DNA fragment was cloned into pcDNA3 (pcDNA3DN-Nrf2). When transfected into THP-1 cells, pcDNA3DN-Nrf2 expresses the mutated Nrf2, which blocks wild-type Nrf2-mediated transcriptional activation.31,32 THP-1 cells were transfected with either pcDNA3 (control vector) or pcDNA3DN-Nrf2 before treatment with 1 mmol/L ALA for 4 hours. Transfection with pcDNA3DN-Nrf2 resulted in a 66% reduction in ALA-induced HO-1 mRNA expression compared with control (pcDNA3; P < 0.05; Figure 2c). Together, these results indicate a key role for Nrf2 in regulating ALA-induced HO-1 expression in THP-1 cells.

ALA-Induced HO-1 mRNA Expression and ARE Binding Are Regulated by p38 MAPK

A range of inhibitors was used to examine kinases regulating ALA-induced HO-1 mRNA expression. Ro-31-8220 (5 µmol/L), a pan–protein kinase C (PKC) inhibitor, and PD98059 (25 µmol/L), an extracellular signal-regulated ki-
nase (ERK) pathway inhibitor, partially reduced ALA-induced HO-1 mRNA expression in THP-1 cells, but these reductions were not statistically significant (Figure 3a). In contrast, SB203580 (5 μmol/L), a p38 MAPK inhibitor, significantly reduced ALA-induced HO-1 mRNA expression by 75% (P<0.05). The effect of SB203580 on ALA-induced HO-1 mRNA expression was dose dependent (Figure 3b). After ALA treatment, p38 became phosphorylated by 5 minutes and still remained at 8 hours in THP-1 cells (Figure 3c). To confirm equal loading, blots were reprobed with anti-p38 MAPK antibodies. SB203580 also inhibited Nrf2-ARE binding in response to ALA (Figure 4).

**Discussion**

Monocytes play an essential role in the pathogenesis of vascular disease. HO-1 is an important intracellular defense against the oxidative stress and inflammation associated with vascular disease. In addition, ALA supplementation has been reported to be beneficial in models of vascular disease.25,26 The present study demonstrates for the first time that in human monocytes, ALA increases HO-1 expression. The present study demonstrates for the first time that in human monocytic cells, ALA increases HO-1 expression.

Furthermore, this is mediated via p38 MAPK, upstream of Nrf2 binding to ARE sites in the regulatory region of the human HO-1 gene.

Several structurally diverse dietary antioxidants including resveratrol, curcumin, sulforaphanes, carnosol, and t-BHQ induce HO-1 expression in a variety of cell types.20 However, few studies to date have been performed in human cells. In the present study, we demonstrate that ALA, a thiol-containing antioxidant, induced up to a 40-fold increase in HO-1 expression by 4 hours in human THP-1 monocytic cells. Curcumin, a polyphenolic antioxidant and major component of the spice turmeric, induced HO-1 mRNA expression with similar strength and kinetics as ALA in these cells. In contrast, t-BHQ, which is considered to be a potent stimulator of HO-1 and ARE-mediated gene expression,2 only weakly induced HO-1 expression in THP-1 cells. Resveratrol (25 to 100 μmol/L), which has been reported recently to increase HO-1 mRNA in a murine phaeochromocytoma cell line and in human aortic vascular smooth muscle cells,33,34 had no effect on HO-1 mRNA expression in THP-1 cells, suggesting that there are cell-specific differences in response to antioxidants. Other dietary antioxidants, including α-tocopherol and ascorbic acid (25 to 100 μmol/L), also do not increase HO-1 mRNA expression in THP-1 cells (our unpublished data, 2005).

The concentrations of ALA used in this study (0.25 to 1 mmol/L) had no cytotoxic effects in THP-1 cells. These concentrations were similar or lower than those used in other in vitro studies, in which ALA has been reported to inhibit cytokine secretion, adhesion molecule expression, and NO synthesis in monocytes and endothelial cells.23,24,35 A total of 250 μmol/L ALA significantly increased HO-1 expression in THP-1 cells, a dose within the clinically relevant range.35 Pharmacokinetic studies of ALA demonstrate that after a single orally administered dose of 10 mg/kg body weight, the plasma concentration reaches up to 70 μmol/L, and that higher plasma concentrations can be achieved if ALA is administered intravenously.36,37 However, the half-life of ALA in plasma is short (30 minutes), suggesting that it is rapidly taken up into tissues or metabolized, and it is therefore plausible that higher concentrations may accumulate in target tissues. Extrapolation of oral lethal dose studies in rodents suggests that humans can tolerate several grams of ALA given orally.38 ALA (200 to 1800 mg) has been used safely in several clinical trials for human therapy to treat complications associated with diabetes.39,40 However, studies are required in humans to confirm other beneficial effects in cardiovascular disease and to determine optimum dosage, form of administration, or preferred type of ALA.22

HO-1 belongs to a family of cytoprotective and detoxification genes that possess AREs in their regulatory regions. The Nrf family of transcription factors can bind the ARE.17 Recently, carnosol, a dietary antioxidant derived from rosemary, has been reported to induce HO-1 expression in PC12 cells via Nrf2 activation.41 In addition, curcumin induces Nrf2-dependent HO-1 promoter activity in porcine renal epithelial cells,42 and we recently found that it also induces Nrf2 activation in primary human monocytes and THP-1 cells (our unpublished data, 2005). These studies suggest that Nrf2...
plays a key role in antioxidant-induced HO-1 expression. In this study, ALA induced Nrf2 nuclear translocation and binding of Nrf2 to the HO-1 ARE by 30 minutes. In addition, a dominant-negative mutant of Nrf2 transiently transfected into THP-1 cells to quench wild-type Nrf2 activity inhibited ALA-induced HO-1 mRNA expression, suggesting that Nrf2 is indeed important for ALA-induced HO-1 expression in monocytic cells.

Several studies have reported that in resting cells, Nrf2 has a rapid turnover (half-life of <30 minutes) in the cytosol because of its continuous targeted degradation by Keap 1, and that on stimulation, Nrf2 stabilization occurs. In macrophages, increased nuclear Nrf2 protein expression is the result of post-transcriptional stabilization of Nrf2 protein because induction of nuclear Nrf2 protein occurs without a significant increase in Nrf2 mRNA expression. Similarly, ALA did not significantly increase Nrf2 mRNA expression in THP-1 cells, suggesting a similar mechanism of action in human monocytic cells.

Several kinases may play a role in the intracellular pathways involved in antioxidant-mediated HO-1 expression, including PKC, ERK MAPK, and p38 MAPK. Previous studies have reported that PKC phosphorylates Nrf2 on the Ser40 residue, and that this is required for its release from Keap-1 in HepG2 cells. In this study, Ro-31-8220 inhibited ALA-induced Nrf2 binding to the ARE (data not shown), suggesting that PKC may play a role in ALA-induced Nrf2 activation in THP-1 cells. However, Ro-31 to 8220 only partially inhibited ALA-induced HO-1 mRNA expression in these cells, a result that was not statistically significant, suggesting that PKC does not play a major role in the induction of HO-1 by ALA. t-BHQ induces HO-1 expression via ERK MAPK activation in THP-1 cells. In this study, PD 98059, an ERK pathway inhibitor, only partially inhibited ALA-induced HO-1 mRNA expression in THP-1 cells and had no effect on ALA-induced ARE-binding (data not shown). However, the p38 inhibitor SB203580 significantly reduced ALA-induced HO-1 expression (P<0.05). ALA treatment also resulted in p38 phosphorylation in THP-1 cells within 5 minutes and was sustained for 8 hours. Similarly, in adipocytes and muscle cells, ALA stimulates p38 MAPK phosphorylation, and the p38 inhibitor SB203580 inhibits ALA-induced glucose uptake in these cells. In other antioxidant studies, p38 MAPK has been reported to mediate curcumin-induced HO-1 expression in renal epithelial cells and t-BHQ-induced HO-1 expression in THP-1 cells. SB203580 also inhibited ALA-induced Nrf2 binding to the ARE in THP-1 cells. p38 MAPK is rapidly phosphorylated in response to ALA in THP-1 cells, which fits with the Nrf2–ARE binding kinetics observed in this study. In addition, p38 MAPK phosphorylation is still detectable at 4 and 8 hours, the time when maximal HO-1 mRNA and protein is increased.

In summary, these results demonstrate that ALA induces HO-1 expression via Nrf2 and p38 in human monocytic cells. The consequences of HO-1 induction by ALA in monocytes are potentially important for vascular disease. Monocytes and macrophages are continually exposed to oxidative stress and are central to the development of atherosclerotic processes.

ALA is an antioxidant that exerts anti-inflammatory effects in monocytes, inhibiting tumor necrosis factor production and adhesion molecule expression. HO-1 induction also exerts anti-inflammatory effects in vascular cells, including inhibition of adhesion molecules and proinflammatory cytokine secretion. For example, HO-1 overexpression inhibits vascular cell adhesion molecule-1 expression in human endothelial cells. In addition, in mouse macrophages, the anti-inflammatory mediators interleukin-10 and 15-deoxy-Δ12,14-prostaglandin J2 inhibit lipopolysaccharide-induced tumor necrosis factor-α production via HO-1. Similarly to ALA, HO-1 induction by interleukin-10 and 15-deoxy-Δ12,14-prostaglandin J2 requires p38, suggesting a similar pathway of activation. Further studies are currently being undertaken to determine whether ALA-induced HO-1 expression inhibits oxidative stress, cytokine production, and adhesion molecule expression in these cells and whether the metabolites of the action of HO-1 on heme could be responsible for its anti-inflammatory effects in monocytes.

Acknowledgments

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Supplemental Data

Methods

Real-time PCR

The real-time PCR primer sequences were:

HO-1 forward primer, 5’- ATGGCCTCCCTGTACCACATC-3’
HO-1 reverse primer, 5’-TGTTGCGCTCAATCTCCTCCT-3’
18s forward primer, 5’-CGGCTACCACATCC AAGGAA-3’
18s reverse primer, 5’-GGCTGCTGGCACCAGACTT-3’
Nrf2 forward primer, 5’-CCTCAACTATAGCGATGCTGAATCT-3’
Nrf2 reverse primer, 5’-AGGA GTTGGGCATGAGTGAGTAG-3’
Nrf2 minor groove binding probe, 5’-CGCAG CGAATATG-3’
GAPDH forward primer, 5’-CTTCCGTGTCCCCCACTGC-3’
GAPDH reverse primer, 5’-GATGTCATCATATTTGCGAGGTTTT-3’
GAPDH minor groove binding probe, 5’- CCACCACTGACACGTT-3’

The PCR reactions were pre-amplified at 95 ºC for 10 min and then amplified for 40 cycles (95 ºC for 15 sec, 57 ºC for 20 sec and 60ºC for 1 min) on an ABI Prism 7000 Sequence Detection System (Applied Biosystems).
Figure I: ALA does not induce Nrf2 mRNA expression in THP-1 cells.
THP-1 cells were treated with 1mM ALA for indicated times, RNA extracted and real-time PCR performed as described in methods.