Enterolactone Induces Heme Oxygenase-1 Expression through Nuclear Factor-E2-Related Factor 2 Activation in Endothelial Cells

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Abstract

Enterolactone is a lignan formed by enterobacteria from precursors in plant foods. Due to its phenolic structure, it can act as an antioxidant, e.g. via direct scavenging of hydroxyl radical. Moreover, many, but not all, phenolic compounds can have indirect antioxidative effects through induction of heme oxygenase-1 (HO-1), which has antiinflammatory functions via production of antioxidants bilirubin and biliverdin as well as carbon monoxide, thereby contributing to cardiovascular health. Our aim was therefore to assess whether enterolactone has indirect antioxidative effects via induction of HO-1 in endothelial cells. The effect of enterolactone on HO-1 mRNA and protein expression in human umbilical vein endothelial cells (HUVEC) was analyzed by quantitative real-time PCR and western blot. The role of nuclear factor-E2-related factor 2 (Nrf2) in HO-1 induction by enterolactone was studied using small interfering RNA (siRNA) and chromatin immunoprecipitation (ChIP) methods. Our results showed that enterolactone induced HO-1 in HUVEC in a time- and concentration-dependent manner. The induction appeared to be mediated via the transcription factor Nrf2, as Nrf2 siRNA abolished the HO-1 induction by enterolactone. We also showed using ChIP that exposure to enterolactone increased the binding of Nrf2 to the promoter region of HO-1. In conclusion, enterolactone increases the expression of HO-1 via Nrf2, which may contribute to its vasculoprotective effects. J. Nutr. 138: 1263–1268, 2008.

Introduction

Polyphenols are a group of chemical substances found in plants, characterized by the presence of ≥1 phenol group per molecule. They are the most abundant antioxidants in plant foods. There are over 8000 known phenolic compounds, but the active ones are more likely metabolites than the native compounds found in food, which are most often tested in vitro studies (1). Health effects of dietary polyphenols have received a lot of attention in recent years and current evidence supports their role in the prevention of diseases such as cardiovascular disease (2,3).

Plant lignans are a group of phenolic compounds that can be found in diets rich in fiber. Enterolactone is a breakdown product of plant lignans. The production of mammalian lignans from the dietary precursors by intestinal bacteria occurs mainly in the large intestine. After removal of methyl and hydroxyl groups in precursors, enterolactone is absorbed from the gut to the circulation and then excreted in urine where enterolactone primarily exists as glucuronides (4). Recent studies have shown that high serum enterolactone levels reduce LDL peroxidation in vivo assessed by serum isoprostane levels (5). Enterolactone also reduced lipid peroxidation in vitro via direct scavenging of hydroxyl radical (6). This association implies a protective role of enterolactone against oxidative injury. In addition, estrogen-like biological effects of enterolactone have been reported, which may also result in protection against coronary heart disease (7,8).

Different chemical and biological properties of dietary polyphenols are involved in protection against cardiovascular diseases. Due to their direct antioxidative effects, polyphenols are potent inhibitors of LDL oxidation (1,9,10). In the vasculature, oxidized LDL promotes atherogenesis via, e.g., increasing the migration of monocytes to the vessel wall and their transformation to lipid laden macrophages, or foam cells (11,12). Dietary polyphenols may also prevent the development of atherosclerosis by increasing formation of nitric oxide, scavenging radical species, and inhibiting the angiogenic process and proliferation and migration of vascular cells (3,9,13).

In addition to direct antioxidative effects, some phenolic compounds can have indirect antioxidative effects, e.g. via induction of heme oxygenase-1 (HO-1) (13). HO-1 is a stress-
inducible enzyme catalyzing degradation of heme to bilirubin, biliverdin, and CO, which mediate the antioxidant and antiinflammatory actions of HO-1 in the vasculature (15–17).

Products of HO-1 can prevent smooth muscle cell proliferation (15,17), neointimal hyperplasia and atherosclerotic lesion formation after vascular injury (15,18), expression of proinflammatory cytokines and chemokines (15), LDL oxidation (17), and apoptosis of endothelial cells (19). HO-1 also contributes to cellular iron homeostasis (20,21) and vasodilatation (15).

Regulation of HO-1 occurs mainly at the transcriptional level, involving the Janus kinase/ signal transducer and activator of transcription pathway, the p38β mitogen-activated protein kinase signaling pathway, and the extracellular signal regulated protein kinase 1/2. Also, the genetic polymorphisms in the promoter of HO-1 modulate its transcriptional activity (15).

The HO-1 gene has several regulatory domains that serve as binding sites for different transcription factors. Both human and mouse HO-1 genes have 2 important distal enhancer regions, E1 and E2, located 4 and 10 kbp upstream of the transcription start site. The dominant element in the E1 and E2 regions is the stress-responsive element (StRE), which mediates transcriptional activation in response to almost all HO-1 inducers tested. StRE represents binding sites of several transcription factors such as nuclear factor-E2-related factor 2 (Nrf2), cAMP responsive stress-responsive element (StRE), which mediates transcriptional start site. The dominant element in the E1 and E2 regions is the stress-responsive element (StRE), which mediates transcriptional activation in response to almost all HO-1 inducers tested. StRE represents binding sites of several transcription factors such as nuclear factor-E2-related factor 2 (Nrf2), CAMP responsive element-binding protein 1, Maf, Jun, Fos, and ATF (22,23). Nrf2 belongs to the cap ‘n’ collar family of b-Zip transcription factors that play a critical and dominant role in HO-1 activation (22,24). Under basal conditions, Nrf2 is in the cytoplasm bound to Kelch-like ECH-associated protein 1, an adaptor molecule linking Nrf2 to Cullin-3-based ubiquitin ligase complex, thereby directing Nrf2 to proteosomal degradation (25). After treatment with electrophilic compounds, Nrf2 is released from Keap1 and translocates to the nucleus, where it can activate transcription of numerous detoxifying and antioxidant genes, such as HO-1 (25–28). Another cap ‘n’ collar-bZIP family member, Bach1, is a negative regulator of HO-1 transcription. Bach1 lacks a transcription domain and functions as a repressor competing with Nrf2 for binding at the StRE (23).

The aim of this study was to assess whether enterolactone has indirect antioxidative effects via HO-1 induction in human umbilical vein endothelial cells (HUVEC). Moreover, we examined the mechanism of regulation of HO-1 by enterolactone.

Materials and Methods

Cell culture. HUVEC were isolated from umbilical veins donated from the maternity ward of the University Hospital of Kuopio. All procedures were approved by the Kuopio University Hospital Ethics Committee. Cells were grown on fibronectin gelatin-coated flasks in endothelial cell growth medium, EGM (0.1% human epidermal growth factor, 0.1% hydrocortisone, 0.1% Gentamicin/Amphotericin-B, 0.4% bovine brain extract, 2% fetal bovine serum, Clonetics).

Enterolactone (Fig. 1) was purchased from Cayman Chemical. It was supplied as a solution in methyl acetate. The solvent was evaporated and changed into ethanol. Confluent HUVEC grown on 6-well plates were treated with different concentrations of enterolactone for 6 h for quantitative RT-PCR analysis and for 16 h for western blot analysis. For time course analysis, HUVEC were treated with 150 μmol/L enterolactone for 3–48 h for quantitative RT-PCR and western blot analysis.

RNA isolation and quantitative PCR. RNA was isolated with Trizol reagent (Invitrogen). One microgram of total RNA was used for the cDNA synthesis using random hexamer primers (Promega) and Moloney murine leukemia virus RT (Finzymes). The relative expression levels of mRNA encoding HO-1 or human β-2 microglobulin (B2M) in HUVEC were measured according to manufacturer’s protocol with quantitative RT-PCR (ABI PRISM 7700 Sequence detector, Applied Biosystems) using specific Assays-on-Demand (Applied Biosystems) target mixes (Hs00157965 and Hs00187842). The expression level of HO-1 was normalized to B2M and presented as -fold of untreated control.

Western blot. Total protein concentration was measured with BCA assay (Pierce). A total of 15 μg of protein was used for electrophoresis. The proteins were transferred to nitrocellulose membrane, blocked with 5% milk in Tris buffered saline/Tween-20, and incubated with rabbit polyclonal anti-HO-1 (Stressgen) or rabbit polyclonal anti-β-actin antibody (Cell Signaling). Blots were visualized using horseradish peroxidase-conjugated secondary antibodies and Supersignal chemiluminescence substrate (Pierce). Protein expression was quantified with ImageQuant TL 7.0 software (GE Healthcare).

Gene silencing with small interfering RNA. Nrf2-specific small interfering RNA (siRNA) oligonucleotide and a nonspecific RNA control (ON-TARGETplus SMARTpool siRNA reagent) were obtained from Dharmacon. HUVEC were divided on 6-well plates at the density of 150,000 cells per well and allowed to grow for 24 h. Cells were transfected with 100 nmol/L siRNA oligonucleotides using Oligofectamine (Invitrogen) for 24 h. Cells were treated with 150 μmol/L enterolactone for 16 h for western blot analyses.

Chromatin immunoprecipitation. HUVEC grown on 10-cm dishes were treated with 150 μmol/L enterolactone for 4 h. DNA and proteins of the treated cells were cross-linked by incubating cells in 1% formaldehyde for 10 min in room temperature. Cross-linking was stopped by 10-min incubation with 0.125 mol/L glycine. Cells were washed with cold PBS and lysed with 1 mL chromatin immunoprecipitation (ChIP) lysis buffer (50 mmol/L HEPES-KOH, 1 mmol/L EDTA, 0.5 mmol/L EGTA, 140 mmol/L NaCl, 10% glycerol, 0.5% Nonidet-P40, 0.25% Triton X-100) with protease inhibitors (Roche). Lysates were incubated 10 min on ice and centrifugated (700 × g; 5 min at 4°C) to pellet the nuclei. Pellets were resuspended in 1 mL ChIP wash buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, 0.5 mmol/L EGTA, 200 mmol/L NaCl) with protease inhibitors and incubated for 10 min on ice. After centrifugation, pellets were suspended in 1 mL ChIP-RIPA buffer (10 mmol/L Tris-HCl; 1% Triton X-100; 0.1% SDS; 0.1% sodiumdeoxycholate; 1 mmol/L EDTA; 0.5 mmol/L EGTA; 140 mmol/L NaCl) with protease inhibitors. Chromatin was sonicated to 300- to 1000-bp fragments on ice. Nuclear debris was removed by centrifugation (16,100 × g; 15 min, 4°C) and samples were preimmunoprecipitated with normal rabbit serum (Vector laboratories) and Protein A-Sepharose beads (Amersham Bioscience) for 2 h at 4°C. After centrifugation, 100 μL of each sample was separated for input control and the remaining sample was immunoprecipitated with 3 μg of specific Nrf2 antibody (sc-722, Santa Cruz Biotechnology), nonspecific IgG (anti-rabbit IgG, Vector laboratories), or water overnight at 4°C. Immune complexes were precipitated with salmon sperm DNA (Sigma-Aldrich) and Protein A-Sepharose beads for 2 h at 4°C. Sepharose beads were washed twice with RIPA buffer and once with TSE 1 (1% Triton X-100, 0.1% SDS, 2 mmol/L EDTA, 20 mmol/L Tris-HCl, 150 mmol/L NaCl), TSEII (1% Triton X-100, 0.1% SDS, 2 mmol/L EDTA, 20 mmol/L Tris-HCl, 500 mmol/L NaCl), and LiCl buffers and 3 times with TE buffer (29).

FIGURE 1 The structure of enterolactone.
protein complexes were eluted from Protein A-Sepharose beads with an elution buffer (1% SDS, 100 mmol/L NaHCO3). Cross-linking was reversed at 65°C overnight and DNA was extracted using QiAamp DNA mini kit (Qiagen) according to the manufacturer’s protocol for cultured cells. PCR was performed against the distal antioxidant response element (ARE) element of the HO-1 (primers 5'-CATCTGCGCGCTCTGC-3' and 5'-GAGCAGCTGGAATCTGAGGA-3') promoter in 25-μL reaction mixtures containing 25 pmol primers, 200 μmol/L deoxynucleoside triphosphates, 0.8 U Dynazyme DNA polymerase, 2 mmol/L MgCl2, and 2.5 μL 10× reaction buffer (all reagents from Finnzymes Diagnostic). Initial denaturation (5 min at 95°C) was followed by 45 cycles for 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C. Dynazyme DNA polymerase was added to the reaction mixtures after the initial denaturation step. PCR was completed by 10 min at 72°C and 1 min at 95°C and products were separated on 1.0% agarose gel (29).

**Statistical analysis.** Statistical analysis was performed with GraphPad Prism and the data were analyzed by 1-way ANOVA with Bonferroni’s post hoc comparisons. Data are expressed as means ± SEM and differences were considered significant at P < 0.05.

**Results**

**Enterolactone induces HO-1 time and dose dependently.** Treatment of HUVEC with 0–150 μmol/L enterolactone increased HO-1 expression concentration dependently. At 75 μmol/L, enterolactone increased the expression of HO-1 mRNA (Fig. 2A) and protein (Fig. 2B) significantly compared with the control.

To examine the temporal changes in HO-1 expression upon exposure to enterolactone, cells were incubated with 150 μmol/L enterolactone, the concentration showing the greatest effects in the previous experiment. The maximal mRNA induction was seen at 6–12 h (Fig. 3A). At the protein level, response was slower, with HO-1 expression increasing at 12–24 h and returning to the baseline level at 48 h (Fig. 3B). Concentrations used did not cause any obvious cytotoxicity.

**Enterolactone induces HO-1 expression through Nrf2 activation.** In HUVEC, a 95% decrease in Nrf2 mRNA was observed after 24 h when cells were transfected with 100 nmol/L Nrf2-specific siRNA compared with control siRNA (data not shown). To assess the effect of Nrf2 siRNA on HO-1 expression, cells transfected with Nrf2 siRNA or nonspecific siRNA were exposed to 150 μmol/L enterolactone and the protein expression was examined. The cells transfected with control siRNA showed an increase in HO-1 protein, whereas specific siRNA against Nrf2 inhibited HO-1 protein induction (Fig. 4). We used ChIP to assess whether Nrf2 binds directly to the promoter of the HO-1 gene. In Nrf2-immunoprecipitated samples, a clear band was observed when the cells were exposed to 150 μmol/L enterolactone, suggesting increased binding of Nrf2 to the HO-1 promoter upon exposure to enterolactone (Fig. 5).

**Discussion**

Flaxseed is a good source of plant lignan secoisolariciresinol diglycoside, which can be converted to its mammalian metabolites such as enterolactone by colonic bacteria (7,30). Previous studies have shown the beneficial role of enterolactone in cardiovascular diseases and cancer in both in vivo (5,31–33) and in vitro models (6). Vanharanta et al. (5) reported that enterolactone serum levels are inversely correlated with in vivo lipid peroxidation. Recently, Lee et al. (34) reported that dietary flaxseed supplementation increases the expression of Nrf2 and its target genes, NQO1 and HO-1, in vivo. They also showed that flaxseed supplementation protected mice from lung ischemia reperfusion injury in a manner partly dependent on HO-1 activity. However, the active metabolite responsible for the induction was not identified. Herein, we show that HO-1 can be induced by a plant lignan metabolite enterolactone.

In endothelial cells, several protective functions of HO-1 have been reported. HO-1 attenuates reactive oxygen species and tumor necrosis factor α-mediated apoptosis of endothelial cells and reduces the cytotoxic effects of hemin, an iron-containing porphyrin, as well as hydrogen peroxide. HO-1 also promotes cell growth and reendothelialization, increases production of nitric oxide, and decreases expression of cell adhesion molecules, thus limiting the attachment of inflammatory cells to the vessel wall (15,17). HO-1 expression is beneficial in vascular diseases in which inflammation plays a role. In addition to the protective role of HO-1 in vitro, it is also beneficial in vivo in animal models of atherosclerosis and restenosis (15). HO-1 gene polymorphisms in humans have been related to coronary heart disease in diabetic patients and smokers (35,36). A number of therapeutic agents induce HO-1, which can be regarded as a “therapeutic funnel” mediating the beneficial effects attributed to these molecules. For example, probucol, a synthetic polyphenolic antioxidant used as a cholesterol-lowering drug, has been
reported to protect against atherosclerotic disease in a manner dependent on HO-1 induction (15,37).

We also studied the mechanism behind the HO-1 induction after treatment with enterolactone and showed that the effect was dependent on Nrf2 (Figs. 4 and 5). In cytoplasm, Nrf2 is bound to Keap1, which contains highly reactive cysteine groups postulated to be the primary sensors that recognize and react with inducer molecules such as polyphenolic compounds, curcuminoids, and bis(benzylidene)acetones, leading to direct alkylation of these or the formation of protein disulfide linkages, disruption of the Nrf2-Keap1 complex, and nuclear translocation of Nrf2 (38,39). The most reactive cysteine residues of Keap1, C273 and C288, are located in the intervening region of Keap1 (39,40). Inducers that cause direct alkylation of cysteine groups are electrophilic in nature. However, enterolactone is not highly electrophilic and the only electrophilic site in the molecule is a ketone group in a lactone ring (Fig. 1). Therefore, the mechanisms by which enterolactone triggers Nrf2 activation occurs more likely via its participation in redox reactions, as has been suggested for some flavonoids (41). The ability of enterolactone to scavenge radicals has been shown in a deoxyribose assay (6), in liposome assay with 2,2′-azobis-amidinopropane hydrochloride initiation, and by measuring plasmid DNA damage (42), although the effective concentrations are much higher than for flavonoids (43). In addition to Keap1 modifications, phenolic compounds have been shown to trigger the activation of signal transduction pathways such as p38 mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and protein kinase C, which may contribute to the activation of Nrf2 (14,38). It is therefore possible that enterolactone would affect these cell signaling pathways, thereby modifying the Nrf2 response.

In the nucleus, Nrf2 binds to the ARE on the promoters of its target genes, activating their expression (26,27). However, HO-1 gene regulation is highly complex at the transcriptional level and it has several regulatory domains that serve as binding sites for different transcription factors such as Jun, Fos, and cAMP responsive element-binding protein 1 (22,23). Interestingly, the induction of HO-1 by probucol has been reported to be ARE

**FIGURE 3** Enterolactone (150 μmol/L) induced HO-1 mRNA (A) and protein (B) expression in a time-dependent manner in HUVEC, which were treated with enterolactone for 0–48 h and the expression of HO-1 was analyzed by quantitative real-time PCR (A) and western blot (B). The mRNA expression of HO-1 in A was normalized to B2M. The western blot is representative of 3 independent experiments. In the densitometric analysis of the western data, HO-1 expression was normalized to β-actin and the untreated control was set to 1. Each bar represents the mean ± SEM, n = 3 (A) or n = 4 (B). Means without a common letter differ, P < 0.05.

**FIGURE 4** Inhibition of Nrf2 by siRNA (100 nmol/L) attenuates enterolactone (150 μmol/L)-mediated induction of HO-1 in HUVEC, which were transfected with control or Nrf2 siRNA for 24 h followed by treatment with enterolactone for 16 h prior to the measurement of the expression level of HO-1 by western blot. The blot is representative of 3 independent experiments. In the densitometric analysis of the western data, HO-1 expression was normalized to β-actin and control was set to 1. Each bar represents the mean ± SEM, n = 6. Means without a common letter differ, P < 0.05.

**FIGURE 5** Enterolactone (150 μmol/L) induces binding of Nrf2 to the promoter of HO-1 in HUVEC, which were treated with enterolactone for 4 h. The binding of Nrf2 to the promoter region of HO-1 was determined with ChIP. Samples are representative of 3 independent experiments.
likely that glucuronidation at one of the phenolic rings would enterolactone are distant from each other (Fig. 1), it is therefore possible that glucuronidation occurs at one of the omnivores, vegetarians, and macrobiotics, the excreted amount when the urinary excretion of enterolactone was studied in rabbits, and higher (Fig. 2). HO-1 expression in HUVEC at the concentration of 75 μmol/L and higher (Fig. 2A,B). Although the effective concentration in our study appeared to be high, enterolactone concentrations in the μmol/L range have been reported for vegetarians (44). Also, the urinary excretion of enterolactone was studied in omnivores, vegetarians, and macrobiotics, the excreted amount of enterolactone was ~1.5 times higher in vegetarians and 5 times higher in macrobiotics than in omnivores (45). So, depending on the diet consumed, there are great differences in enterolactone concentrations between human subjects. In addition, glucuronic acid conjugates of enterolactone may also possess biological activity. Glucuronidation occurs at one of the hydroxyl moieties of the molecule. As the phenolic rings of enterolactone are distant from each other (Fig. 1), it is therefore likely that glucuronidation at one of the phenolic rings would not interfere with the biological activity of the other. In a recent study by Chen et al. (46), 50–100 μmol/L enterolactone was shown to induce apoptosis in human prostate carcinoma LNCaP cells via a mitochondria-mediated and caspase-dependent pathway (46). However, cytotoxicity caused by these doses of enterolactone appears to be a cell type-specific effect, because no cytotoxicity was observed in human nontumorigenic CRL-2221 prostate epithelial cells (46). The concentrations used in our present study did not cause any obvious cytotoxicity assessed by cell morphology (results not shown).

In conclusion, enterolactone, a mammalian metabolite of dietary plant lignans, induced HO-1 expression via Nrf2 activation in human umbilical vascular endothelial cells. We conclude that enterolactone may have a protective role in vascular cells through this mechanism.

### Literature Cited


