Published in final edited form as:

Biochem J. 2009 April 15; 419(2): 427–436. doi:10.1042/BJ20081350.

The permissive role of mitochondria in the induction of haem oxygenase-1 in endothelial cells

Karina C. RICART^{*,†}, Subhashini BOLISETTY^{†,‡,§}, Michelle S. JOHNSON^{*,†}, Jessica PEREZ^{*,†}, Anupam AGARWAL^{†,‡,§}, Michael P. MURPHY^{||}, and Aimee LANDAR^{*,†,§,1}

^{*} Department of Pathology, University of Alabama at Birmingham, Birmingham, AL 35294-2180, U.S.A

[†] Center for Free Radical Biology, University of Alabama at Birmingham, Birmingham, AL 35294-2180, U.S.A

[‡] Department of Medicine, University of Alabama at Birmingham, Birmingham, AL 35294-2180, U.S.

[§] Nephrology Research and Training Center, University of Alabama at Birmingham, Birmingham, AL 35294-2180, U.S.A.

^{||} MRC-Dunn Human Nutrition Unit, Wellcome Trust/MRC Building, Hills Road, Cambridge CB2 2XY, U.K

Abstract

HO-1 (haem oxygenase 1) is an essential antioxidant enzyme in the cell that exerts its effects through removal of pro-oxidant haem groups and the formation of antioxidant molecules and carbon monoxide. The electrophilic cyclopentenone 15d-PGJ₂ (15-deoxy-Δ^{12,14}-prostaglandin J₂) induces the expression of HO-1 protein through the covalent modification of protein thiols. It has been shown that specific thiol residues of the redox-sensor Keap1 (Kelch-like ECH-associated protein 1) are modified by 15d-PGJ₂, leading to activation of the transcription factor Nrf-2 (nuclear factor-erythroid 2 p45 subunit-related factor 2) and up-regulation of genes under control of the electrophile-response element, including HO-1. However, 15d-PGJ₂ has also been shown to modify other proteins which comprise the electrophile-responsive proteome. Since 15d-PGJ₂ has been shown to localize to the mitochondria in endothelial cells, we hypothesized that mitochondrial protein modification may also be important in Keap1/Nrf-2 signal transduction, leading to HO-1 up-regulation. In order to determine the role of mitochondrial protein thiol modification in HO-1 induction, we used the mitochondrial-targeted thiol-reactive compound IBTP [(4-iodobutyl)triphenyl-phosphonium]. IBTP had no effect on basal HO-1 levels, but effectively blocked HO-1 induction by a variety of reagents including haemin, iodoacetamide and 15d-PGJ₂. Mechanistically, IBTP did not prevent the covalent modification of Keap1 by 15d-PGJ₂. However, IBTP prevented the 15d-PGJ₂-dependent increases in HO-1 mRNA and protein. Furthermore, IBTP prevented the nuclear accumulation of Nrf-2, suggesting cross-talk between mitochondria and antioxidant-response signal transduction. This effect was independent of reactive oxygen species formation or mitochondrial membrane potential. In addition, IBTP significantly enhanced the toxicity of high concentrations of 15d-PGJ₂, suggesting that loss of mitochondrial control of HO-1 leads to increased susceptibility to electrophilic stress in endothelial cells. The implications for these studies in understanding the balance between cytoprotection and cytotoxicity in the context of diseases such as atherosclerosis is discussed.

¹To whom correspondence should be addressed (email landar@uab.edu).

Keywords

15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂; haem oxygenase 1 (HO-1); Kelch-like ECH-associated protein 1 (Keap1); nuclear factor-erythroid 2-related factor 2 (Nrf-2); redox signalling; thiol

INTRODUCTION

HO (haem oxygenase)-1 has been shown to be an important mediator of cellular protection against oxidative stress in a number of pathologies, including atherosclerosis [1]. The enzyme catalyses the conversion of haem into biliverdin, iron and carbon monoxide [2]. This is important, since carbon monoxide has been shown in endothelial cells to be anti-apoptotic [3]. Another factor contributing to the anti-apoptotic properties of HO-1 is the direct translocation of the protein to the mitochondrion, where it may influence mitochondrially mediated apoptotic pathways [4]. In addition, biliverdin and its downstream product bilirubin have demonstrated antioxidant effects [5,6]. Interestingly, processed forms of HO-1 have also recently been shown to translocate to the nucleus and contribute to cellular protection against oxidative stress [7]. In atherosclerosis models, HO-1 induction has been shown to be protective, since HO-1-knockout mice exhibit more vascular lesions [8]. Thus up-regulation of HO-1 in endothelial cells in response to oxidized lipids may be an important factor in maintaining vascular tone and lesion stability [9,10].

HO-1 is an inducible protein, and its levels are up-regulated in response to signals such as haemin, ROS (reactive oxygen species) and RNS (reactive nitrogen species), and electrophiles such as curcumin and sulforaphane [11]. In addition, the endogenous reactive lipid 15d-PGJ₂ (15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂), a product of the cyclo-oxygenase pathway involved in the resolution of inflammation, is a potent inducer of HO-1 [12–15]. Previous reports have shown that 15d-PGJ₂ can induce HO-1 protein expression at low concentrations [12–15], whereas it is toxic at higher concentrations [16]. The precise concentrations at which these effects are observed depends on a number of factors including cell type, medium volume and presence of serum in the medium [14]. HO-1 protein levels are regulated primarily at the transcriptional level. This regulation of HO-1 induction is complex and is controlled primarily at the transcriptional level by multiple transcription factors and a repressor protein, allowing rapid induction of HO-1 during cellular stress [17,18]. One of the major mechanisms of HO-1 regulation by reactive lipids is by activation of the EpRE (electrophile-response element), also known as ARE (antioxidant-response element), by Nrf-2 (nuclear factor-erythroid 2-related factor 2). Nrf-2 is normally bound to Keap1 (Kelch-like ECH-associated protein 1), which allows for ubiquitination and proteasomal degradation of Nrf-2 under basal conditions [19]. Upon exposure to electrophilic stressors, including 15d-PGJ₂, critical cysteinyl thiols of Keap1 are modified, resulting in decreased proteasomal turnover of Nrf-2 [20–22]. Subsequent phosphorylation steps are then required for Nrf-2 to translocate to the nucleus and activate transcription via the EpRE [23].

In addition to Keap1, 15d-PGJ₂ modifies other thiol-containing proteins, referred to as the electrophile-responsive proteome [24,25]. Some members of this proteome have been identified, including a number of redox-sensitive transcription factors, kinases, structural proteins, thiol-metabolizing enzymes and proteins within the mitochondrion [26–28]. Some previous studies have shown that mitochondria may play a role in HO-1 induction in response to glucose deprivation and curcumin treatment, possibly through the induction of ROS from the mitochondrial respiratory chain [29,30]. We have shown previously that 15d-PGJ₂ itself localizes to the mitochondria, and causes mitochondrial ROS formation [13]. However, the cross-talk between the mitochondrial effects of 15d-PGJ₂ and Keap1 modification/EpRE up-regulation have not been explored and are the subject of the present study.

In the present study, we have sought to determine the role of mitochondria in the up-regulation of HO-1. In order to accomplish this, we have assessed the impact of $\Delta\psi$ (mitochondrial membrane potential), ROS formation and modification of mitochondrial protein thiols on HO-1 induction in endothelial cells [31].

MATERIALS AND METHODS

Cell culture

BAECs (bovine aortic endothelial cells) harvested previously from descending thoracic aortas were obtained and maintained (at 37°C in 5% CO₂) in DMEM (Dulbecco's modified Eagle's medium) (Gibco) supplemented with 10% FBS (fetal bovine serum), 1% penicillin/streptomycin and 4 mM glutamine. Cells used were between passages 5 and 9. At confluence, medium was changed to low-serum medium (DMEM with 0.5% FBS, 1% penicillin/streptomycin and 4 mM glutamine) to minimize lipid interactions with serum proteins. Cells were acclimated to low-serum medium for 20 h before all treatments.

Biotinylation of 15d-PGJ₂

The biotin group was linked to 15d-PGJ₂ (Cayman Chemicals), to form BT-15d-PGJ₂, via a carbodi-imide-mediated condensation reaction with 5-(biotinamido)pentylamine (Pierce), as described previously [20]. Biotinylation was confirmed by electrospray MS, and concentrations were determined spectrophotometrically at 306 nm using a molar absorption coefficient of 12 000 M⁻¹·cm⁻¹.

Measurement of $\Delta\psi$

The inner membrane $\Delta\psi$ was assessed using JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) (Invitrogen). Briefly, cells grown in 12-well culture plates were treated as described in the Figure legends, and 7.4 μ M JC-1 was added directly to BAECs in culture medium and incubated for 30 min. Next, cells were washed with PBS, and red/green fluorescence was measured using a fluorescence plate reader. Data are expressed as the ratio of red to green fluorescence.

Cytosolic and nuclear protein extraction

Cell lysates were prepared by rinsing plates with ice-cold PBS and incubating on ice for 5 min with 80 μ l of lysis buffer A [10 mM Tris/HCl (pH 7.4) and 1% Triton X-100, containing Mini-CompleteTM PIC (protease inhibitor cocktail) (Roche)]. Cell lysates were collected by scraping, and subjected to centrifugation at 12 500 rev./min for 10 min at 4°C using a Hermle 220.49V06 rotor. Supernatants were collected as the cytosolic protein fraction. Pellets were rinsed with lysis buffer A, and then centrifuged at 12 500 rev./min for 10 min at 4°C using a Hermle 220.49V06 rotor. The resultant nuclear pellet was resuspended, homogenized and incubated in 100 μ l of nuclear extraction buffer (0.1 M Tris/HCl, pH 6.8, 4% SDS, 10% glycerol, 0.2% Bromophenol Blue and 720 mM 2-mercaptoethanol). Finally, the nuclear suspension was centrifuged at 13 000 rev./min for 20 min using a Hermle 220.49V06 rotor to collect the supernatant as the nuclear fraction.

Western blot analysis

Cell lysate proteins were separated by SDS/PAGE (10 or 12.5% gels) and transferred on to nitrocellulose membranes (Bio-Rad Laboratories). Protein amounts were quantified using the Bradford method (Bio-Rad Laboratories), and equivalent amounts of protein were loaded in triplicate per gel in all experiments, except in the case of the biotin-affinity precipitation (where equivalent volumes of eluate were loaded). Uniform protein loading was verified by Ponceau S staining of the membranes, which showed no significant differences between samples. All

samples were performed in triplicate in the same gel. The membranes were blocked with 5% non-fat dried skimmed milk powder/TBS-T (Tris-buffered saline with Tween 20: 17.5 mM Tris/HCl, pH 7.4, 140 mM NaCl, 2.6 mM KCl and 0.05% Tween 20) solution for 1 h at room temperature (21°C), and then incubated overnight with primary antibody [anti-HO-1, 1/5000 dilution (Stressgen), or anti-Keap1 (E-20), 1/1000 dilution (Santa Cruz Biotechnology)] at 4°C. After washing with TBS-T, membranes were incubated with a horseradish-peroxidase-conjugated anti-rabbit secondary antibody for HO-1, 1/3000 dilution (GE Healthcare) or anti-goat secondary antibody for Keap1 (1/1000) (Santa Cruz Biotechnology) in TBS-T for 1 h at room temperature. After rinsing, membranes were developed using SuperSignal West Dura chemiluminescence substrate (Pierce) and imaged using a CCD (charge-coupled device) camera imaging system (AlphaInnotech). Where indicated in the Figure legends, membranes were stripped with re-blot buffer (Re-Blot Plus Mild, Chemicon) for 10 min and re-blocked with non-fat dried skimmed milk powder in TBS-T, followed by incubation with a rabbit polyclonal antibody against HO-2 protein, 1/1000 dilution (Stressgen), with further development as described above.

Cell viability assay

Cell viability was estimated by measuring LDH (lactate dehydrogenase) release from damaged cells into the culture media. Medium was collected, and cell lysates were prepared by rinsing culture plates with ice-cold PBS and incubating on ice for 5 min with 80 μ l of lysis buffer B (PBS, pH 7.4, and 0.1% Triton X-100, with PIC). Then, 20 μ l of cell lysates or 200 μ l of culture medium were incubated with NADH (300 μ M) and pyruvate (10 mM). The conversion of NADH into NAD was followed for 4 min at 340 nm in a spectrophotometer. The rate of NADH conversion is correlated with LDH activity levels. Relative LDH release was expressed as the percentage LDH activity in supernatants of cultured cells (medium) compared with total LDH activity present per well (medium+cell lysate). The total LDH activity (medium+cell lysate) was not statistically different among any of the treatment conditions (see Supplementary Figure S1 at <http://www.BiochemJ.org/bj/419/bj4190427add.htm>).

Affinity precipitation

Biotinylated proteins were affinity precipitated using 100 μ l of a 50% slurry of Neutravidin beads (Pierce), in chromatography columns. Then, beads were washed six times with 20 mM Tris/HCl (pH 7.4). Cell lysates were prepared as described above, and 500 μ g of total protein was added to the beads and incubated overnight at 4°C with continuous rotation. After rinsing six times with 500 μ l of 0.1 M glycine (pH 2.8), beads were washed with 1 ml of 20 mM Tris/HCl (pH 7.4). The beads were resuspended in 100 μ l of loading buffer (0.1 M Tris/HCl, pH 6.8, 4% SDS, 10% glycerol, 0.2% Bromophenol Blue and 2% 2-mercaptoethanol) and heated at 80°C for 10 min. After centrifugation at 12 500 rev./min for 10 min at 4°C using a Hermle 220.49V06 rotor, equivalent volumes of supernatants were separated by SDS/PAGE and immunoblotted for Keap1, as described above [14].

Confocal fluorescence microscopy

BAECs on coverslips were imaged with a laser-scanning confocal scan-head (Olympus FV300) mounted on an inverted fluorescence microscope (Olympus IX71) equipped with oil-immersion objectives [Olympus 60 $\times$ 1.2 NA (numerical aperture)]. Cells were loaded with 2.5 μ M H₂DCF-DA (2',7'-dichlorofluorescein diacetate) (Invitrogen) for 1 h before imaging. In addition, 0.5 μ M MitoTracker Deep Red (Invitrogen) was added to cells 15 min before imaging in order to ensure proper focusing and to confirm similar cell numbers among sample groups. DCF (dichlorofluorescein) fluorescence, arising from the reaction of H₂DCF-DA with ROS, was excited with an argon laser (488 nm), and its emission filtered at 510 $\pm$ 20 nm. MitoTracker fluorescence was excited with a helium/neon green laser (543 nm) and its emission filtered at

605 ± 20 nm; sequential imaging of double-labelled cells was achieved using a 570 nm dichroic mirror. Single images were scanned at 512 pixel×512 pixel resolution (1.96 s/frame) avoiding PMT (photomultiplier tube) saturation (400–500 V PMT voltage, 1× gain, zero offset), saved as TIFF files, and analysed using ImageJ (NIH). The average pixel intensity within single ROI (region-of-interest) defined around individual cells in DCF fluorescence images was used as a quantitative estimation of free radical levels. Fluorescence intensity is given as arbitrary units (AU), and was averaged across all ROIs within a coverslip.

HO enzyme activity

HO activity in BAECs was measured by bilirubin generation [32]. BAECs were treated with 15d-PGJ₂ (10 μM) for 4 h. Cells were washed once, scraped in 5 ml of HBSS (Hanks balanced salt solution) and centrifuged at 1000 rev./min for 10 min at 4°C using a Hermle 220.49V06 rotor. The cells were resuspended in 2 mM MgCl₂ and 100 mM potassium phosphate buffer (pH 7.4), and exposed to three cycles of freezing and thawing (−80°C for 15 min, 37°C for 5 min). The suspension was then sonicated on ice for 15 s and centrifuged at 10 500 rev./min for 10 min at 4°C using an Eppendorf 5415R centrifuge and F45-24-11 rotor. The supernatant was added to the reaction mixture (316 μl) containing rat liver cytosol (2 mg/ml protein), haemin (20 μM), glucose 6-phosphate (2 mM), glucose-6-phosphate dehydrogenase (0.2 unit) and NADPH (0.8 mM) for 1 h at 37°C in the dark. Bilirubin was extracted with chloroform and quantified by measuring the ΔD ($D_{464} - D_{530}$) and using an absorption coefficient of 40 mM^{−1}·cm^{−1}. HO activity was expressed as pmol of bilirubin formed/mg of protein per h. Protein concentration was measured from the supernatant using the Bradford method, and mouse spleen was used as positive control.

mRNA extraction and real-time PCR

cDNA was synthesized from approx. 5 μg of total mRNA isolated from BAECs using RNA STAT-60 (Tel-Test) according to the manufacturer's instructions. Reverse transcriptase and oligo(dT) were from Invitrogen. PCR was performed using Invitrogen PCR mix, using oligonucleotide primers: 5'-GATGGCGTCTTTGTACCACAT-3' and 5'-GTACAGCTGCTTGAACCTGGTG-3' for HO-1, and 5'-GAGGGACTTATGACCACTGTCC-3' and 5'-TAGCCTAGAATGCCCTTGAGAG-3' for GAPDH (glyceraldehyde-3-phosphate dehydrogenase), as internal standards. Primers were designed based on the bovine HO-1 and GAPDH sequence using Invitrogen software. The resulting PCR product was separated in 2% agarose gel with ethidium bromide to visualize two fragments: 397 bp (HO-1) and 314 bp (GAPDH). HO-1 mRNA expression was quantified by real-time PCR using the primers above and Sypro Green ER mix from Invitrogen. The *C_t* (cycle threshold) of each sample was automatically determined to be the first cycle at which a significant increase in optical signal above an arbitrary baseline was detected. Amplification of GAPDH cDNA in the same samples was used as an internal control. Relative mRNA expression was quantified using the $\Delta \Delta C_t$ method.

Statistical analysis

Data are expressed as means ± S.E.M. for triplicates from at least three independent experiments, and subjected to one-way ANOVA followed by Bonferroni's multiple comparison tests. *P* < 0.05 were considered statistically significant.

RESULTS

Effect of 15d-PGJ₂ or BT-15d-PGJ₂ on HO-1 induction

In order to define the concentrations necessary for HO-1 induction in BAECs which do not cause cell death, time-course and dose–response curves were established. First, BAECs were

exposed for 0–16 h to 10 μ M 15d-PGJ₂ or BT-15d-PGJ₂, a biotinylated form which can be used to follow protein adduct formation [20,33]. In all Western blotting experiments, uniform protein loading was verified by Ponceau S staining of the membranes, which showed no significant differences among samples. HO-1 protein levels were increased significantly after 4 h of incubation with 15d-PGJ₂ or BT-15d-PGJ₂, and continued to increase up to 16 h (Figure 1A, upper panel). However, levels of the non-inducible isoform, HO-2, were unchanged throughout the time course (Figure 1A, lower panel). The levels of HO-1 protein were similar after exposure to 15d-PGJ₂ or BT-15d-PGJ₂, indicating that the biotin tagging of the lipid does not affect its ability to induce HO-1. At concentrations of 10 μ M 15d-PGJ₂ or BT-15d-PGJ₂, a robust induction of HO-1 protein was observed. In some lysate preparations, we have observed a minor lower-molecular-mass band which is immunoreactive in the HO-1 Western blot. This band is consistent with a proteolytically cleaved form reported by Dennery and colleagues [7], and was included for quantification purposes when present. Concentrations of either lipid above 10 μ M were markedly less effective at inducing HO-1 (Figure 1B). Interestingly, the lack of HO-1 induction at the higher concentrations was also associated with decreased viability of the cells with both 15d-PGJ₂ and BT-15d-PGJ₂ (Figure 1C). Since 10 μ M 15d-PGJ₂ caused robust induction of HO-1 protein with minimal cytotoxicity, this concentration was used for further experiments.

To examine whether the induction of HO-1 occurs at the level of transcription or translation, we used actinomycin and cycloheximide to inhibit transcription and translation respectively. Figure 2(A) shows that the effect of 15d-PGJ₂ on HO-1 protein levels requires transcription. In addition, since it has been reported previously that induction of HO-1 protein does not necessarily correlate with increased HO enzymatic activity [34], we measured HO activity in response to 15d-PGJ₂. At 10 μ M 15d-PGJ₂, HO activity increased 4.3-fold when compared with control (224 ± 21 compared with 52 ± 5 pmol of bilirubin/mg of protein per h; Figure 2B). This increase in HO activity correlates well with the observed increase in HO-1 protein levels shown in Figure 1(A) (5.8 ± 0.5 -fold at 4 h). These results demonstrate that 15d-PGJ₂ induces HO-1 protein via transcriptional mechanisms, and that the induced HO-1 protein is functional.

Induction of HO-1 protein by IAM (iodoacetamide), but not IBTP [(4-iodobutyl) triphenylphosphonium]

Since we and others have shown previously that 15d-PGJ₂ modifies cytosolic protein thiols [20,35] and also localizes to the mitochondria [13], we compared the effects of thiol-alkylating agents which can discriminate between mitochondrial and non-mitochondrial thiols on HO-1 induction. To accomplish this, we first used an alkylating agent, IAM, which does not distinguish between protein thiols in the cytosol or in mitochondria. BAECs were incubated with concentrations of IAM (1–50 μ M) for 4 h (Figure 3A). The lowest IAM concentrations (1–5 μ M) increased HO-1 protein levels, but higher concentrations (10–50 μ M) failed to induce HO-1, an effect similar to that of 15d-PGJ₂ (see Figure 1B). To investigate the role of mitochondrial thiols in the induction of HO-1, we used the mitochondrial-targeted compound IBTP, which accumulates 100–500-fold in the organelle in response to the $\Delta\psi$ and selectively alkylates mitochondrial thiols [31,36]. Cells were incubated with 15d-PGJ₂ (10 μ M) as a positive control, or increasing concentrations of IBTP (0.01–15 μ M) for 4 h before measurement of HO-1 (Figure 3B). In contrast with IAM, IBTP over a wide concentration range did not increase HO-1 protein levels. These data are consistent with previous reports that modification of cytosolic protein thiols such as Keap1 results in HO-1 induction, and demonstrate that selective modification of mitochondrial protein thiols is not sufficient to increase HO-1 protein expression.

Effect of IBTP on 15d-PGJ₂-dependent HO-1 induction and cell viability

Next, we examined the consequence of IBTP treatment on BT-15d-PGJ₂-dependent HO-1 induction. BAECs were pre-treated with different concentrations of IBTP for 4 h, followed by exposure to BT-15d-PGJ₂ (10 μ M) for a further 4 h (Figure 4A). Interestingly, pre-treatment with IBTP significantly decreased BT-15d-PGJ₂-induced HO-1 protein up-regulation in a concentration-dependent manner. IBTP blocked the increase in mRNA observed after incubation with 15d-PGJ₂ (10 μ M) for 4 h (Figure 4B), indicating that the effect of IBTP is transcriptional. A mitochondrial-targeted analogue lacking the thiol-reactive moiety, TPMP (triphenylmethylphosphonium), did not significantly induce HO-1 alone (results not shown). However, TPMP had a slight, but statistically significant, effect on BT-15d-PGJ₂-induced HO-1 protein levels when compared with BT-15d-PGJ₂ alone (Figure 4C).

Given that increased levels of HO-1 enzymatic activity have been linked to protection against oxidative stress, we determined the effects of IBTP pre-incubation on the cytotoxicity dose-response of 15d-PGJ₂. BAECs were treated with 15d-PGJ₂ (10 μ M) in the presence of IBTP or TPMP (15 μ M) for 24 h, and LDH release was measured as an index of cell viability. In order to confirm that LDH activity was not affected by the treatment conditions, we verified that the total LDH activity in medium and lysates was not significantly different among experimental groups (see Supplementary Figure S1). Figure 5 shows that neither TPMP nor IBTP significantly decreases cell viability alone (0 μ M 15d-PGJ₂). As shown in Figure 1(C), 15d-PGJ₂ significantly decreases cell viability at the highest concentration tested (20 μ M). The addition of TPMP has no effect on the toxicity curve in the presence of 15d-PGJ₂. However, IBTP significantly decreases cell viability at 10 μ M 15d-PGJ₂, conditions which are consistent with decreased HO-1 levels (see Figure 4C).

Effect of IBTP on other inducers of HO-1

To determine whether IBTP inhibition of HO-1 up-regulation is specific to 15d-PGJ₂, BAECs were pre-incubated with or without IBTP (15 μ M) for 4 h, followed by exposure to haemin (Figure 6A), or IAM (Figure 6B), for a further 4 h. Concentrations of these agents were selected to result in a similar degree of HO-1 induction. For comparison in all panels, HO-1 protein levels are shown for untreated cells, cells treated with BT-15d-PGJ₂ (10 μ M), haemin (5 μ M) or IAM (3 μ M) alone, or cells treated with IBTP (15 μ M) for 4 h followed by BT-15d-PGJ₂, haemin (5 μ M) or IAM (3 μ M). From Figure 6, it is evident that IBTP prevents HO-1 induction in response to all of these compounds.

ROS formation does not correlate with HO-1 induction

Since 15d-PGJ₂ has been shown to cause ROS formation [13,37,38], we investigated the effect of IBTP on this process. For these experiments, the fluorescent probe H₂DCF-DA was used to detect peroxides in the presence of cellular peroxidases [39]. In the absence of 15d-PGJ₂, ROS formation was induced by both IBTP and the mitochondrial uncoupler FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone), whereas no significant ROS formation was detected with TPMP (Figure 7). In the presence of 10 μ M 15d-PGJ₂, ROS formation was also induced, and there was no significant increase or decrease with TPMP, IBTP or FCCP treatment.

Effect of $\Delta\psi$ on HO-1 induction

It is possible that complete loss of mitochondrial function could account for the inhibition of HO-1 induction. To test this, mitochondrial $\Delta\psi$ was measured using the fluorescence indicator JC-1 after a 30 min treatment with 15d-PGJ₂ (10 μ M), IBTP (15 μ M) or TPMP (15 μ M). The mitochondrial uncoupler FCCP (10 μ M) was included as a positive control to dissipate the mitochondrial $\Delta\psi$. Interestingly, no significant changes were observed in mitochondrial $\Delta\psi$

with IBTP or TPMP, whereas a slight, but significant, decrease was observed with 15d-PGJ₂ (Figure 8A).

We have reported recently that Keap1 adduct formation by 15d-PGJ₂ can be detected as early as 30 min after exposure to the electrophile [14]. To determine whether IBTP interferes with such early events, cells were co-incubated with IBTP (15 μ M) and BT-15d-PGJ₂ (10 μ M) for 4 h and compared with our previous protocol in which cells were incubated with IBTP before the addition of BT-15d-PGJ₂. In both cases, IBTP effectively inhibited HO-1 induction by the electrophile (Figure 8B; 0 and -4 h). In contrast, cells exposed to BT-15d-PGJ₂ (10 μ M) for 4 h, followed by IBTP (15 μ M) for another 4 h (total induction time = 8 h) showed no inhibitory effect on HO-1 protein levels (Figure 8B; +4 h). Thus the modification of mitochondrial thiols must occur before or simultaneously with BT-15d-PGJ₂ treatment in order to block HO-1 induction.

Modification of Keap1 by BT-15d-PGJ₂ is not altered by IBTP

BT-15d-PGJ₂ was used to monitor Keap1 modification in the presence and absence of IBTP. In these experiments, the biotin tag was used to affinity-precipitate proteins modified by BT-15d-PGJ₂, and Keap1 was detected by Western blot analysis [14]. As shown in Figure 9 (A), treatment with BT-15d-PGJ₂ results in the modification of Keap1. This modification was not altered by treatment with IBTP, indicating that the effects of IBTP on HO-1 induction are distal to Keap1 modification.

Inhibition of HO-1 induction is associated with loss of nuclear Nrf-2

In order to determine whether IBTP blocks Nrf-2 nuclear localization, BAECs were treated with IBTP (15 μ M) or BT-15d-PGJ₂ (10 μ M) alone or in combination, and nuclear extracts were prepared and probed by Western blot analysis for Nrf-2 protein. Under baseline conditions, a relatively small amount of Nrf-2 protein was present in the nuclear fraction, and, upon BT-15d-PGJ₂ treatment, an increase in nuclear-associated Nrf-2 was observed (Figure 9B). Interestingly, IBTP treatment did not alter baseline Nrf-2 levels in the nucleus, whereas IBTP treatment followed by BT-15d-PGJ₂ resulted in a dramatic decrease in nuclear Nrf-2 (Figure 9B). Furthermore, the non-thiol-reactive analogue TPMP did not significantly decrease Nrf-2 translocation in response to BT-15d-PGJ₂.

DISCUSSION

The regulation of antioxidant defences in the vasculature is a critical factor in determining the progression of atherosclerotic lesions. The antioxidant enzyme HO-1 is particularly important in this respect. For example, in experimental models of atherosclerosis, HO-1 overexpression has been shown to protect against disease progression, whereas HO-1 deficiency exacerbates the disease [1,40–42]. It is therefore important to determine the mechanisms of HO-1 regulation in vascular cells.

It has become clear that damage to the mitochondrion may not only have an impact on the bioenergetic status of vascular cells, but also on redox cell signalling, including the regulation of HO-1 levels [43]. The best-characterized regulatory sites on proteins which control redox signalling events are protein thiols, which then function as molecular switches [44]. A number of reports have shown that the induction of HO-1 is particularly susceptible to manipulation of intracellular thiols. For example, the thiol-regulatory enzyme thioredoxin reductase has been shown to be required for the induction of HO-1 in BAECs [45]. In addition, depletion of intracellular glutathione results in increased HO-1 expression [46].

A number of studies also suggest that mitochondria may be involved in the regulation of HO-1. Recently, the lipid lysophosphatidylcholine, which is increased in atherosclerosis, induces HO-1 in endothelial cells through a mitochondrially associated mechanism [47]. In addition, subjecting HepG2 cells to hypoglycaemic stress also increases HO-1 levels through a mitochondrial mechanism [29]. Taken together, these data suggest the hypothesis that mitochondrial thiols may play an important role in the regulation of HO-1.

In the present study, we investigated the mechanisms through which mitochondria contribute to the regulation of HO-1 levels in endothelial cells. For this purpose, the electrophilic cyclopentenone prostaglandin 15d-PGJ₂ was used to induce HO-1 at concentrations which did not cause significant cytotoxicity. Interestingly, cytotoxic concentrations of the lipids failed to induce HO-1. It is possible that, under conditions of extreme electrophilic stress, as may occur with environmental toxicants or at sites of inflammation, the attenuation of HO-1 expression may lead to increased cell death. It is tempting to speculate that overcoming this HO-1 suppression underlies the atheroprotective effect of HO-1 overexpression by adenoviral vectors [42].

Since 15d-PGJ₂ has the potential to interact with both cytosolic and mitochondrial protein thiols, we investigated the role of these two thiol pools in the regulation of HO-1 protein. Both of the two iodo-compounds used (IAM and IBTP) form stable thioether adducts with reactive cysteine residues through the same S_N2 chemical mechanism. IAM, which is not targeted to mitochondria, induces HO-1 at similar concentrations to 15d-PGJ₂. At higher concentrations, IAM did not induce HO-1, a result similar to 15d-PGJ₂. In order to selectively modify mitochondrial protein thiols, we used a targeted thiol-reactive compound, IBTP, which we have shown previously localizes to the mitochondria using electron microscopy and immunocytochemistry [31]. In addition, it has been demonstrated that protein adduct formation with IBTP in BAECs can be inhibited by the mitochondrial uncoupling agent FCCP [31]. In the present study, we have demonstrated that IBTP does not induce HO-1 over a concentration range which spanned over three orders of magnitude. These results demonstrate that the protein thiol modification caused by IBTP itself does not result in HO-1 induction.

Although IBTP did not induce HO-1, our data show that IBTP prevented HO-1 induction by 15d-PGJ₂ in a dose-dependent manner. Interestingly, the mitochondria-targeted non-thiol-reactive analogue, TPMP, could recapitulate approx. 50% of the inhibition of 15d-PGJ₂-dependent HO-1 induction that was observed with IBTP. Similarly to IBTP, TPMP did not induce HO-1 by itself. These data provide evidence of two contributing factors to the mitochondrial inhibition of HO-1 induction by 15d-PGJ₂: (i) the triphenylphosphonium group; and (ii) modification of thiol residues by the iodoalkyl group.

Numerous studies have established that HO-1 can protect cells against oxidative or electrophilic stress (reviewed in [1]). We therefore reasoned that the IBTP prevention of HO-1 induction should enhance the toxicity of high concentrations of 15d-PGJ₂. Whereas IBTP itself did not decrease cell viability, it significantly enhanced the cytotoxicity observed with 15d-PGJ₂. Unlike IBTP, TPMP did not affect cell viability alone or in the presence of 15d-PGJ₂. The fact that TPMP partially decreases HO-1 induction, but does not potentiate cytotoxicity could be due to a threshold effect for the level of HO-1 necessary to achieve cytoprotection. Alternatively, IBTP may have additional effects on mitochondrial proteins which contribute to cytotoxicity independently of HO-1, such as modification of respiratory proteins including Complex I [31]. Taken together, these data support the concept that mitochondrial thiols play an important role in the cytoprotective response to electrophilic stress.

To test whether mitochondrial thiols are required for HO-1 induction by other inducers of the enzyme, haemin and IAM were used. As found with 15d-PGJ₂, IBTP abrogated the increase

in HO-1 levels which occurred in response to these stimuli. These results suggest that mitochondrial thiols are permissive for the induction of HO-1 by both electrophilic and oxidant stressors. Since ROS have been reported to induce HO-1, and we have shown previously that 15d-PGJ₂ can induce mitochondrial ROS, we investigated the effect of IBTP on ROS production as measured by DCF fluorescence. In these studies, the mitochondrial uncoupler FCCP was used as a positive control, since it has been shown previously to promote ROS formation in intact endothelial cells [48]. Both IBTP and FCCP induced significant levels of ROS alone, whereas TPMP did not. In combination with 15d-PGJ₂, both IBTP and FCCP caused a slight, but not significant, effect on ROS production compared with 15d-PGJ₂ alone. TPMP did not result in increased levels of ROS with or without 15d-PGJ₂, indicating that the increased ROS formation by IBTP was not due to the triphenylphosphonium moiety. These data dissociate the production of ROS from HO-1 induction in several ways: (i) IBTP induces ROS formation to a similar level found with 15d-PGJ₂, but does not induce HO-1; (ii) IBTP does not decrease the 15d-PGJ₂-dependent formation of ROS, but inhibits the electrophile-dependent induction of HO-1; and (iii) TPMP has no significant effect on ROS formation, but has approx. 50% of the effect of IBTP on HO-1 induction by 15d-PGJ₂. It is important to note that these data do not rule out the possibility that the ROS produced by IBTP and 15d-PGJ₂ are in different locations within the mitochondria, and may therefore exert different effects on HO-1 induction.

The signalling events which lead to regulation of HO-1 levels in the cell occur rapidly upon exposure to the electrophilic stimulus. The persistence and duration of the signal is dependent on covalent adduct formation of the electrophile with Keap1 and the activation of Nrf-2. At the concentrations of 15d-PGJ₂ used in this study (10 μ M), adduct formation with Keap1 occurs over 15–60 min [14]. Accordingly, we tested the effects of TPMP, IBTP and 15d-PGJ₂ on mitochondrial $\Delta\psi$ at an early time point (30 min) and found that only 15d-PGJ₂ had a slight, but significant, depolarizing effect on mitochondrial $\Delta\psi$. These results suggest that IBTP does not cause rapid mitochondrial dysfunction leading to the inhibition of HO-1 induction. Importantly, we also showed that mitochondrial thiol modification by IBTP must occur before or concurrently with 15d-PGJ₂ exposure in order to inhibit up-regulation of HO-1.

The selective reaction of IBTP with mitochondrial proteins in cells has been demonstrated previously [31]. This is due in part to a 5–10-fold higher reactivity of IBTP with thiols in the mitochondrial matrix relative to the cytosol owing to the higher intramitochondrial pH [31]. In addition, IBTP is concentrated within the mitochondria up to 500-fold due to the mitochondrial $\Delta\psi$ (120–170 mV). However, there is also a slight, but significant, plasma membrane potential (approx. 60 mV) which could allow for an accumulation of IBTP in the cytosol of approx. 5–10-fold [49]. Thus there was a slight possibility that IBTP might also modify reactive thiols on cytosolic proteins, such as Keap1, thereby blocking the reaction with 15d-PGJ₂ in a competitive manner. However, our results show that Keap1 modification by BT-15d-PGJ₂ is not altered by IBTP.

Finally, we determined whether IBTP interfered with the apparent translocation of Nrf-2 to the nucleus. In these experiments, it is clear that nuclear Nrf-2 levels are significantly enhanced by BT-15d-PGJ₂, and that this effect is inhibited by IBTP. Importantly, IBTP alone had no effect on baseline nuclear Nrf-2 levels, consistent with its inability to induce HO-1 by itself. In addition, TPMP had no effect on 15d-PGJ₂-dependent Nrf-2 nuclear translocation, which suggests that its partial effect on HO-1 levels occurs through a mechanism that was independent of mitochondrial thiol modification.

In summary, modification of mitochondrial thiols with a specific mitochondrial thiol-alkylating reagent inhibited HO-1 induction by 15d-PGJ₂ and other HO-1 inducers. Interestingly, although a broad range of inducers of HO-1 have been identified, very few suppressors of

expression are known. The finding that mitochondrial thiol modification by IBTP can suppress HO-1 protein levels is of particular interest, since the process of inflammation can lead to mitochondrial oxidative stress and presumably protein thiol oxidation. Taken together, these data suggest that mitochondrial thiol modification is an important regulator in the cytoprotective signalling leading to HO-1 induction in the vasculature, and dysregulation of this pathway may be important in the development or progression of atherosclerosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by the American Heart Association [Scientist Development Grant 0635361N to A.L.].

Abbreviations

BAEC	bovine aortic endothelial cell
DCF	dichlorofluorescein
DMEM	Dulbecco's modified Eagle's medium
EpRE	electrophile-response element
FBS	fetal bovine serum
FCCP	carbonyl cyanide <i>p</i> -trifluoromethoxyphenylhydrazone
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
H₂-DCF-DA	2',7'-dichlorofluorescein diacetate
HO	haem oxygenase
IAM	iodoacetamide
IBTP	(4-iodobutyl)triphenylphosphonium
Keap1	Kelch-like ECH-associated protein 1
LDH	lactate dehydrogenase
Nrf-2	

	nuclear factor-erythroid 2-related factor 2
15d-PGJ₂	15-deoxy- $\Delta^{12,14}$ -prostaglandin J ₂
BT-15d-PGJ₂	biotinylated 15d-PGJ ₂
PIC	protease inhibitor cocktail
PMT	photomultiplier tube
ROI	region-of-interest
ROS	reactive oxygen species
TBS-T	Tris-buffered saline with Tween 20
TPMP	triphenylmethylphosphonium

References

1. Stocker R, Perrella MA. Heme oxygenase-1: a novel drug target for atherosclerotic diseases? *Circulation* 2006;114:2178–2189. [PubMed: 17101869]
2. Tenhunen R, Marver HS, Schmid R. Microsomal heme oxygenase: characterization of the enzyme. *J Biol Chem* 1969;244:6388–6394. [PubMed: 4390967]
3. Abraham NG, Kappas A. Heme oxygenase and the cardiovascular–renal system. *Free Radical Biol Med* 2005;39:1–25. [PubMed: 15925276]
4. Turkseven S, Drummond G, Rezzani R, Rodella L, Quan S, Ikehara S, Abraham NG. Impact of silencing HO-2 on EC-SOD and the mitochondrial signaling pathway. *J Cell Biochem* 2007;100:815–823. [PubMed: 17131376]
5. Ryter SW, Alam J, Choi AM. Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. *Physiol Rev* 2006;86:583–650. [PubMed: 16601269]
6. Stocker R. Antioxidant activities of bile pigments. *Antioxid Redox Signaling* 2004;6:841–849.
7. Lin Q, Weis S, Yang G, Weng YH, Helston R, Rish K, Smith A, Bordner J, Polte T, Gaunitz F, Dennery PA. Heme oxygenase-1 protein localizes to the nucleus and activates transcription factors important in oxidative stress. *J Biol Chem* 2007;282:20621–20633. [PubMed: 17430897]
8. Yet SF, Layne MD, Liu X, Chen YH, Ith B, Sibinga NE, Perrella MA. Absence of heme oxygenase-1 exacerbates atherosclerotic lesion formation and vascular remodeling. *FASEB J* 2003;17:1759–1761. [PubMed: 12958201]
9. Siow RC, Sato H, Mann GE. Heme oxygenase–carbon monoxide signalling pathway in atherosclerosis: anti-atherogenic actions of bilirubin and carbon monoxide? *Cardiovasc Res* 1999;41:385–394. [PubMed: 10341838]
10. True AL, Olive M, Boehm M, San H, Westrick RJ, Raghavachari N, Xu X, Lynn EG, Sack MN, Munson PJ, et al. Heme oxygenase-1 deficiency accelerates formation of arterial thrombosis through oxidative damage to the endothelium, which is rescued by inhaled carbon monoxide. *Circ Res* 2007;101:893–901. [PubMed: 17885218]
11. Cho HY, Reddy SP, Kleeberger SR. Nrf2 defends the lung from oxidative stress. *Antioxid Redox Signaling* 2006;8:76–87.

12. Lee TS, Tsai HL, Chau LY. Induction of heme oxygenase-1 expression in murine macrophages is essential for the anti-inflammatory effect of low dose 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂. *J Biol Chem* 2003;278:19325–19330. [PubMed: 12642589]
13. Landar A, Zmijewski JW, Dickinson DA, Le Goffe C, Johnson MS, Milne GL, Zannoni G, Vidari G, Morrow JD, Darley-Usmar VM. Interaction of electrophilic lipid oxidation products with mitochondria in endothelial cells and formation of reactive oxygen species. *Am J Physiol Heart Circ Physiol* 2006;290:H1777–H1787. [PubMed: 16387790]
14. Oh JY, Giles N, Landar A, Darley-Usmar V. Accumulation of 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ adduct formation with Keap1 over time: effects on potency for intracellular antioxidant defence induction. *Biochem J* 2008;411:297–306. [PubMed: 18237271]
15. Kitamura Y, Kakimura J, Matsuoka Y, Nomura Y, Gebicke-Haerter PJ, Taniguchi T. Activators of peroxisome proliferator-activated receptor- (PPAR) inhibit inducible nitric oxide synthase expression but increase heme oxygenase-1 expression in rat glial cells. *Neurosci Lett* 1999;262:129–132. [PubMed: 10203248]
16. Levonen AL, Dickinson DA, Moellering DR, Mulcahy RT, Forman HJ, Darley-Usmar VM. Biphasic effects of 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ on glutathione induction and apoptosis in human endothelial cells. *Arterioscler Thromb Vasc Biol* 2001;21:1846–1851. [PubMed: 11701476]
17. Kitamuro T, Takahashi K, Ogawa K, Uono-Fujimori R, Takeda K, Furuyama K, Nakayama M, Sun J, Fujita H, Hida W, et al. Bach1 functions as a hypoxia-inducible repressor for the heme oxygenase-1 gene in human cells. *J Biol Chem* 2003;278:9125–9133. [PubMed: 12511571]
18. Alam J, Cook JL. How many transcription factors does it take to turn on the heme oxygenase-1 gene? *Am J Respir Cell Mol Biol* 2007;36:166–174. [PubMed: 16990612]
19. Kobayashi A, Kang MI, Okawa H, Ohtsui M, Zenke Y, Chiba T, Igarashi K, Yamamoto M. Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. *Mol Cell Biol* 2004;24:7130–7139. [PubMed: 15282312]
20. Levonen AL, Landar A, Ramachandran A, Ceaser EK, Dickinson DA, Zannoni G, Morrow JD, Darley-Usmar VM. Cellular mechanisms of redox cell signalling: role of cysteine modification in controlling antioxidant defences in response to electrophilic lipid oxidation products. *Biochem J* 2004;378:373–382. [PubMed: 14616092]
21. Itoh K, Mochizuki M, Ishii Y, Ishii T, Shibata T, Kawamoto Y, Kelly V, Sekizawa K, Uchida K, Yamamoto M. Transcription factor Nrf2 regulates inflammation by mediating the effect of 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂. *Mol Cell Biol* 2004;24:36–45. [PubMed: 14673141]
22. Kobayashi A, Kang MI, Watai Y, Tong KI, Shibata T, Uchida K, Yamamoto M. Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1. *Mol Cell Biol* 2006;26:221–229. [PubMed: 16354693]
23. Jaiswal AK. Nrf2 signaling in coordinated activation of antioxidant gene expression. *Free Radical Biol Med* 2004;36:1199–1207. [PubMed: 15110384]
24. Ceaser EK, Moellering DR, Shiva S, Ramachandran A, Landar A, Venkartraman A, Crawford J, Patel R, Dickinson DA, Ulasova E, et al. Mechanisms of signal transduction mediated by oxidized lipids: the role of the electrophile-responsive proteome. *Biochem Soc Trans* 2004;32:151–155. [PubMed: 14748737]
25. Uchida K, Shibata T. 15-Deoxy- $\Delta^{12,14}$ -prostaglandin J₂: an electrophilic trigger of cellular responses. *Chem Res Toxicol* 2008;21:138–144. [PubMed: 18052108]
26. Satoh T, Lipton SA. Redox regulation of neuronal survival mediated by electrophilic compounds. *Trends Neurosci* 2007;30:37–45. [PubMed: 17137643]
27. Stamatakis K, Sanchez-Gomez FJ, Perez-Sala D. Identification of novel protein targets for modification by 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ in mesangial cells reveals multiple interactions with the cytoskeleton. *J Am Soc Nephrol* 2006;17:89–98. [PubMed: 16291835]
28. Landar A, Shiva S, Levonen AL, Oh JY, Zaragoza C, Johnson MS, Darley-Usmar VM. Induction of the permeability transition and cytochrome c release by 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ in mitochondria. *Biochem J* 2006;394:185–195. [PubMed: 16268779]
29. Chang SH, Garcia J, Melendez JA, Kilberg MS, Agarwal A. Haem oxygenase 1 gene induction by glucose deprivation is mediated by reactive oxygen species via the mitochondrial electron-transport chain. *Biochem J* 2003;371:877–885. [PubMed: 12585963]

30. McNally SJ, Harrison EM, Ross JA, Garden OJ, Wigmore SJ. Curcumin induces heme oxygenase 1 through generation of reactive oxygen species, p38 activation and phosphatase inhibition. *Int J Mol Med* 2007;19:165–172. [PubMed: 17143561]
31. Lin TK, Hughes G, Muratovska A, Blaikie FH, Brookes PS, Darley-USmar V, Smith RA, Murphy MP. Specific modification of mitochondrial protein thiols in response to oxidative stress: a proteomics approach. *J Biol Chem* 2002;277:17048–17056. [PubMed: 11861642]
32. Balla G, Jacob HS, Balla J, Rosenberg M, Nath K, Apple F, Eaton JW, Vercellotti GM. Ferritin: a cytoprotective antioxidant strategem of endothelium. *J Biol Chem* 1992;267:18148–18153. [PubMed: 1517245]
33. Oh J, Johnson MS, Landar A. Methods for determining the modification of protein thiols by reactive lipids. *Methods Cell Biol* 2007;80:417–434. [PubMed: 17445707]
34. Sardana MK, Kappas A. Dual control mechanism for heme oxygenase: tin(IV)-protoporphyrin potently inhibits enzyme activity while markedly increasing content of enzyme protein in liver. *Proc Natl Acad Sci USA* 1987;84:2464–2468. [PubMed: 3470805]
35. Itoh K, Tong KI, Yamamoto M. Molecular mechanism activating Nrf2–Keap1 pathway in regulation of adaptive response to electrophiles. *Free Radical Biol Med* 2004;36:1208–1213. [PubMed: 15110385]
36. Coulter CV, Kelso GF, Lin TK, Smith RA, Murphy MP. Mitochondrially targeted antioxidants and thiol reagents. *Free Radical Biol Med* 2000;28:1547–1554. [PubMed: 10927180]
37. Martinez B, Perez-Castillo A, Santos A. The mitochondrial respiratory complex I is a target for 15-deoxy- $\Delta^{12,14}$ -prostaglandin J_2 action. *J Lipid Res* 2005;46:736–743. [PubMed: 15654126]
38. Kondo M, Oya-Ito T, Kumagai T, Osawa T, Uchida K. Cyclopentenone prostaglandins as potential inducers of intracellular oxidative stress. *J Biol Chem* 2001;276:12076–12083. [PubMed: 11278531]
39. Tampo Y, Kotamraju S, Chitambar CR, Kalivendi SV, Keszler A, Joseph J, Kalyanaraman B. Oxidative stress-induced iron signaling is responsible for peroxide-dependent oxidation of dichlorodihydrofluorescein in endothelial cells: role of transferrin receptor-dependent iron uptake in apoptosis. *Circ Res* 2003;92:56–63. [PubMed: 12522121]
40. Ishikawa K, Sugawara D, Goto J, Watanabe Y, Kawamura K, Shiomi M, Itabe H, Maruyama Y. Heme oxygenase-1 inhibits atherogenesis in Watanabe heritable hyperlipidemic rabbits. *Circulation* 2001;104:1831–1836. [PubMed: 11591622]
41. Ishikawa K, Sugawara D, Wang X, Suzuki K, Itabe H, Maruyama Y, Lusis AJ. Heme oxygenase-1 inhibits atherosclerotic lesion formation in LDL-receptor knockout mice. *Circ Res* 2001;88:506–512. [PubMed: 11249874]
42. Juan SH, Lee TS, Tseng KW, Liou JY, Shyue SK, Wu KK, Chau LY. Adenovirus-mediated heme oxygenase-1 gene transfer inhibits the development of atherosclerosis in apolipoprotein E-deficient mice. *Circulation* 2001;104:1519–1525. [PubMed: 11571246]
43. Gutierrez J, Ballinger SW, Darley-USmar VM, Landar A. Free radicals, mitochondria, and oxidized lipids: the emerging role in signal transduction in vascular cells. *Circ Res* 2006;99:924–932. [PubMed: 17068300]
44. Ying J, Clavreul N, Sethuraman M, Adachi T, Cohen RA. Thiol oxidation in signaling and response to stress: detection and quantification of physiological and pathophysiological thiol modifications. *Free Radical Biol Med* 2007;43:1099–1108. [PubMed: 17854705]
45. Trigona WL, Mullarky IK, Cao Y, Sordillo LM. Thioredoxin reductase regulates the induction of haem oxygenase-1 expression in aortic endothelial cells. *Biochem J* 2006;394:207–216. [PubMed: 16209660]
46. Ewing JF, Maines MD. Glutathione depletion induces heme oxygenase-1 (HSP32) mRNA and protein in rat brain. *J Neurochem* 1993;60:1512–1519. [PubMed: 8455037]
47. Watanabe N, Zmijewski JW, Takabe W, Umezu-Goto M, Le Goffe C, Sekine A, Landar A, Watanabe A, Aoki J, Arai H, et al. Activation of mitogen-activated protein kinases by lysophosphatidylcholine-induced mitochondrial reactive oxygen species generation in endothelial cells. *Am J Pathol* 2006;168:1737–1748. [PubMed: 16651638]
48. Millar TM, Phan V, Tibbles LA. ROS generation in endothelial hypoxia and reoxygenation stimulates MAP kinase signaling and kinase-dependent neutrophil recruitment. *Free Radical Biol Med* 2007;42:1165–1177. [PubMed: 17382198]

49. Murphy MP, Smith RA. Targeting antioxidants to mitochondria by conjugation to lipophilic cations. *Annu Rev Pharmacol Toxicol* 2007;47:629–656. [PubMed: 17014364]

Figure 1. Effects of 15d-PGJ₂ or BT-15d-PGJ₂ on HO-1 protein levels and BAEC survival
 BAECs were incubated with 15d-PGJ₂ or BT-15d-PGJ₂ for the times and concentrations shown. **(A)** Time course for HO-1 induction by 10 μ M 15d-PGJ₂ (□) or BT-15d-PGJ₂ (◆), quantified by Western blotting and expressed as fold increase over vehicle control. Representative Western blot images of HO-1 and HO-2 are shown in the inset. **(B)** HO-1 levels induced by 10 or 20 μ M 15d-PGJ₂ and BT-15d-PGJ₂ after a 4 h exposure to the lipids. **(C)** Viability of cells exposed to 10 or 20 μ M 15d-PGJ₂ or BT-15d-PGJ₂ after 24 h, determined by LDH release assay. Results are mean $\pm$ S.E.M. for three independent experiments, each

performed in triplicate. * $P < 0.05$ compared with vehicle for both 15d-PGJ₂ and BT-15d-PGJ₂ (Veh = ethanol), or # $P < 0.05$ compared with 10 μ M 15d-PGJ₂ or BT-15d-PGJ₂ alone.

Figure 2. Effects of 15d-PGJ₂ on HO-1 mRNA and enzymatic activity

(A) BAECs were pre-incubated with 10 μM cycloheximide (CHX) or 1 μM actinomycin (ACT) for 30 min before the addition of 10 μM 15d-PGJ₂ for an additional 4 h, after which HO-1 protein levels were determined by Western blotting. (B) BAECs were exposed to 10 μM 15d-PGJ₂ for 4 h, and HO enzymatic activity was measured. Activity is expressed as pmol of bilirubin formed/mg of cell protein per h. Results are means ± S.E.M. for three independent experiments, each performed in triplicate. **P* < 0.05 compared with vehicle (Veh = ethanol), or #*P* < 0.05 compared with 15d-PGJ₂ alone.

Figure 3. Differential induction of HO-1 protein by IAM and IBTP

BAECs were exposed to the concentrations shown of thiol reactive reagents for 4 h, and HO-1 levels were determined by Western blotting. Insets show representative Western blot images. **(A)** HO-1 induction in response to 1–50 μ M IAM. **(B)** HO-1 induction in response to 0.01–15 μ M IBTP, with 10 μ M 15d-PGJ₂ included as a positive control. Results are means $\pm$ S.E.M. for three independent experiments, each performed in triplicate. * P < 0.001 compared with vehicle (Veh = ethanol).

Figure 4. Effects of IBTP and TPMP on 15d-PGJ₂-dependent HO-1 induction

(A) BAECs were pre-incubated with 0–15 μM IBTP for 4 h, followed by 10 μM BT-15d-PGJ₂ for 4 h, and HO-1 protein levels were determined by Western blotting. The levels of HO-1 after exposure to BT-15d-PGJ₂ were established as the maximal response (100%) and the effect of increasing concentrations of IBTP with (◆) and without (□) BT-15d-PGJ₂ are shown. (B) BAECs were pre-incubated with 15 μM IBTP alone for 4 h, or with 10 μM 15d-PGJ₂ with or without 15 μM IBTP for 4 h. The expression of HO-1 was examined using RT (reverse transcription)–PCR (insert) and the quantification analysis was performed using Q-PCR (quantitative PCR). GAPDH was used as a loading control. (C) BAECs were pre-

incubated with 15 μ M TPMP alone for 4 h, or 10 μ M BT-15d-PGJ₂ with or without 15 μ M IBTP for 4 h or 15 μ M TPMP for 4 h, and HO-1 protein levels were determined by Western blotting. Results are means $\pm$ S.E.M. for three independent experiments, each performed in triplicate. * P < 0.05 compared with vehicle (Veh =ethanol) or # P < 0.05 compared with 15d-PGJ₂ or BT-15d-PGJ₂ alone.

Figure 5. Effects of IBTP and TPMP on 15d-PGJ₂-dependent cell viability

Viability of cells pre-incubated with vehicle (ethanol) for 4 h (□), 15 μM TPMP for 4 h (●) or 15 μM IBTP for 4 h (◆), followed by 10 μM 15d-PGJ₂ for 24 h determined by measuring LDH release. Results are means ± S.E.M. for three independent experiments, each performed in triplicate. **P* < 0.05 compared with vehicle or #*P* < 0.05 compared with 15d-PGJ₂.

Figure 6. Effect of IBTP on the induction of HO-1 by haemin and IAM

BAECs were pre-incubated with 15 μ M IBTP for 4 h before the addition of 10 μ M BT-15d PGJ₂, 5 μ M haemin (A) or 3 μ M IAM (B) for an additional 4 h, after which HO-1 protein levels were determined by Western blotting. Results are the mean $\pm$ S.E.M. percentage of maximal HO-1 protein induction by BT-15d-PGJ₂ for three independent experiments, each performed in triplicate. * P < 0.001 compared with vehicle (ethanol), or # P < 0.001 compared with the respective inducer without IBTP.

Figure 7. Effects of mitochondria-targeted compounds on ROS production in BAECs
 BAECs were treated with 15 µM IBTP, 15 µM TPMP or 10 µM FCCP without or with 10 µM 15d-PGJ₂ for 4 h. Fluorescent indicators were added as described in the Materials and methods section. **(A)** DCF images were acquired by confocal microscopy. **(B)** Quantification of images in **(A)** expressed as percentage of DCF fluorescence intensity in 15d-PGJ₂ alone. Results are means ± S.E.M for three replicate wells from two independent experiments. **P* < 0.05 compared with vehicle (ethanol).

Figure 8. Effects of 15d-PGJ₂, IBTP and TPMP on $\Delta\psi$ in BAECs

(A) BAECs were exposed to 10 μ M FCCP, 10 μ M 15d-PGJ₂, 15 μ M IBTP or 15 μ M TPMP for 30 min, and $\Delta\psi$ was measured using JC-1. (B) HO-1 protein levels were determined after cells were pre-incubated (-4 h) or co-incubated (0 h) without (-) or with (+) 15 μ M IBTP, followed by exposure to 10 μ M BT-15d-PGJ₂ for an additional 4 h. BAECs were also treated with 10 μ M BT-15d-PGJ₂ for 4 h, followed by an additional 4 h incubation with (+) or without (-) 15 μ M IBTP as a 'post' incubation (+4 h). Results are mean $\pm$ S.E.M. percentages of the maximal response to BT-15d-PGJ₂ for 4 h for three independent experiments, each performed in triplicate. * P < 0.01 compared with vehicle (Veh = ethanol) (A), or # P < 0.01 compared with BT-15d-PGJ₂ for 4 h (B).

Figure 9. Effects of 15d-PGJ₂, IBTP and TPMP on Keap1 and Nrf-2 pathways

(A) BAECs were pre-incubated with 15 μM IBTP for 4 h followed by 10 μM BT-15d-PGJ₂ for an additional 4 h. Protein adducts were affinity-precipitated (IP), and eluates were subjected to SDS/PAGE and Western blot (WB) analysis using an anti-Keap1 antibody. (B) BAECs were treated with 15 μM IBTP, 15 μM TPMP or vehicle (ethanol) without (–) or with (+) BT-15d-PGJ₂ (10 μM) for 4 h. Nrf-2 levels in nuclear fractions were determined by SDS/PAGE and Western blot analysis using an anti-Nrf-2 antibody. Results are mean ± S.E.M. density expressed as a percentage of BT-15d-PGJ₂ alone for three independent experiments. **P* < 0.001 compared with vehicle or #*P* < 0.001 compared with BT-15d-PGJ₂ alone. NS; not significant.