Heme Oxygenase Attenuates Angiotensin II–Mediated Increase in Cyclooxygenase-2 Activity in Human Femoral Endothelial Cells

Giovanni Li Volti, Francesca Seta, Michal L. Schwartzman, Alberto Nasjletti, Nader G. Abraham

Abstract—Heme oxygenase (HO) regulates cellular heme levels and catalyzes the formation of bilirubin and carbon monoxide. We hypothesize that the status of the endothelial HO system influences the angiotensin (Ang) II–induced increase in the endothelial production of prostaglandin I₂ (PGI₂) (measured as 6-keto-PGF₁₀α) and prostaglandin E₂ (PGE₂), eicosanoids that modulate the vascular actions of Ang II. In the present study, we determined the effect of interventions that suppress HO activity or induce HO-1 gene expression on Ang II–mediated increase in 6-keto-PGF₁₀α and PGE₂ in cultures of human femoral artery endothelial cells. Incubation of endothelial cells with Ang II (100 ng/mL) for 24 hours increased the levels of both 6-keto-PGF₁₀α and PGE₂ in the culture media. This effect of Ang II on prostaglandin production by endothelial cells was attenuated in cells treated with SnCl₂ (10 μmol/L), an inducer of HO-1, but was magnified in cells treated with the HO inhibitor ZnDPP or heme. Upregulation of HO-1 gene expression by retrovirus-mediated delivery of the human HO-1 gene also attenuated heme and Ang II–induced prostaglandin synthesis. Of note, prostaglandin synthesis by lysates of endothelial cells stimulated with heme or Ang II appear to involve COX-2, because it was blunted by NS-398, which is presumed to inhibit COX-2 specifically. These results indicate that overexpression of the HO system exerts an inhibitory influence on Ang II–induced synthesis of prostaglandins by endothelial cells. (Hypertension. 2003;41[part 2]:715-719.)

Key Words: heme oxygenase | cyclooxygenase activity | prostaglandins | angiotensin II | endothelial cell | gene transfer

The vascular endothelium manufactures prostaglandin E₂ (PGE₂) and/or I₂ (PGI₂) via a pathway that involves oxygenation of arachidonic acid by cyclooxygenase (COX)-1 and -2. Angiotensin (Ang) II acutely stimulates prostaglandin synthesis in endothelial cells by promoting phospholipase-catalyzed deacylation of arachidonic acid, and thus increasing the amount of arachidonic acid available to COX, and also may induce COX-2 expression. As prostaglandins produced by the vascular endothelium subserve mechanisms that counteract the actions of Ang II on the vasculature, the interplay between endothelium-derived prostaglandins and Ang II has great functional relevance.

COX-1 and COX-2 are heme proteins, and it is well documented that the heme prosthetic group of both COX isoforms is essential for the expression of catalytic activity. According to a recent study, prostaglandin synthesis in rabbit coronary and human endothelial cells decreases in response to interventions that upregulate the expression of heme oxygenase (HO)-1. HO isoforms -1 and -2 catalyze the metabolisms of heme to biliverdin and carbon monoxide (CO), respectively, an antioxidant and a vasoactive media-
tor. HO isoforms, particularly HO-1, play a critical role in the regulation of cellular heme levels, which in turn may impact on the expression of catalytically active COX isoforms.

Previous studies have documented induction of vascular and renal HO-1 in response to Ang II in vivo. It is conceivable, then, that Ang II–mediated upregulation of HO-1 creates a setting that does not favor Ang II–induced stimulation of prostaglandin synthesis. We tested this hypothesis by examining the effect of interventions that suppress HO activity, or induce HO-1 gene expression, on Ang II–induced prostaglandin synthesis in cultures of human femoral endothelial cells.

Methods

Cell Culture Conditions

Human femoral artery endothelial cells were obtained from ATCC (Manassas, VA) and were grown in MCDB131 medium (GIBCO-BRL; passages 12 to 25) supplemented with 10% fetal bovine serum, 10 ng/mL endothelial growth factor (Sigma), and 1 μg/mL hydrocortisone (Sigma). The cells were incubated at 37°C in a 5%
CO2–humidified atmosphere and maintained at subconfluence by passing with trypsin-EDTA (GIBCO-BRL).

Transduction of Endothelial Cells With Human HO-1 Gene Using Retrovirus Vector

The human HO-1–expressing replication-deficient retrovirus vector LSN-hHO-1 was constructed with the use of the backbone LXSNS vector as previously described.14 Briefly, PA317 retroviral packaging cells (3×10^6/mL) were seeded in 60-mm tissue culture dishes and incubated for 24 hours. Attached cells were washed twice with serum-reduced Opti-MEM (GIBCO-BRL). The cells were combined with 5 μg of retroviral vector (LSN-human HO-1 sense) as described previously14 by using 20 μL of lipofectamine reagent (GIBCO-BRL). The cells were selected for neomycin resistance (neo') in a medium containing G418 (600 μg/mL). After 14 days, the clone was selected, and stable transduced endothelial cells expressing HO-1 were obtained. Positive colonies were pooled, subcultured, and used in all subsequent studies. Total RNA was extracted and hybridized with the human HO-1 cDNA probe. Only transduced cells overexpressing HO-1 mRNA and HO-1 protein were used, as measured by Northern blot and immunohybridization, respectively.

Western Blot Analysis for HO-1 and HO-2 and HO Activity

Cells were harvested by using cell lysis buffer as previously described.16 The lysate was collected for Western blot analysis, and protein levels were visualized by immunoblotting with antibodies against human HO-1 or HO-2 (Stressgen Biotechnologies Corp). The antibodies used for HO-1 do not cross-react with HO-2. Similarly, the antibodies for HO-2 do not cross-react with HO-1 protein. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis immunoblots were also compared with the corresponding molecular weight band on the membranes. HO activity was assayed in cell lysates as previously described.17 The amount of bilirubin generated was determined by scanning spectrophotometer (Lambda 17 UV/VIS, Perkin-Elmer Cetus Instruments) and was defined as the difference between 460 and 530 nm (absorption coefficient, 40 mmol/L per centimeter for bilirubin). Results were expressed as nanomoles of bilirubin per milligram of protein per hour.

Measurement of PGE2 and 6-Keto-PGF1α

The levels of PGE2 and the stable metabolite of PGH2, 6-keto-PGF1α, were determined in the culture medium by using an enzyme-linked immunosorbent assay (ELA). Endothelial cells were counted and seeded in 24-well plates (1.2×10^5 cells/wells). Cells were treated with zinc deuteroporphyrin 2,4-bis glycol (ZnDPP) (10 μmol/L), Ang II (100 ng/mL), SnCl2 (10 μmol/L), or heme (10 μmol/L) for 24 hours, after which the media were removed and stored at −80°C. In some experiments, cells treated and not treated with HO-1 inducers, aliquots of cell lysate (300 μg of protein) were incubated (30 minutes at 37°C) with 100 μmol/L of arachidonic acid in 300 μL of incubation buffer (100 mmol/L phosphate buffer at pH 7.4, 250 mmol/L sucrose, 0.1% NP-40, 1 mmol/L EDTA and 1 mmol/L MgCl2) containing or not containing NS-398, a selective inhibitor of COX-2; SC-560, a specific inhibitor of COX-1; or indomethacin, a nonspecific inhibitor of COX activity. Reactions were terminated with addition of 2 mmol/L formic acid, and the 6-keto-PGF1α generated was determined by an ELA kit as previously described.18 All samples were run in 2 dilutions and triplicate.

Statistical Analyses

The data are presented as mean±SE for the number of experiments. Statistical significance (P<0.05) between the groups was determined by the Fisher method of analysis of multiple comparisons. For comparisons between treatment groups, the null hypothesis was tested by a single-factor ANOVA for multiple groups or unpaired t test for 2 groups.

Modulation of HO Protein Expression and Activity

Western blot analysis demonstrated that endothelial cells contain low basal levels of HO-1 protein (Figure 1). The addition of heme or SnCl2 for 24 hours markedly increased the protein levels of the HO-1 isofrom compared with control. HO-2 protein was not significantly altered after treatment with either SnCl2 or heme. The addition of ZnDPP and Ang II resulted in upregulation of HO-1 protein but had no effect on HO-2 protein levels. As shown in Figure 2, HO activity was increased by 2- and 6-fold, respectively, in endothelial cells treated with heme or SnCl2, and was inhibited by treatment with ZnDPP (2.4-fold). HO activity in cells treated with Ang II increased 1.91-fold (P<0.05) when compared with control, and was decreased by concurrent treatment with ZnDPP. Opposite results were obtained when Ang II–treated endothelial cells were concurrently treated with heme or SnCl2.

Effect of HO Induction and Inhibition on Prostaglandin Levels

Under basal conditions, PGE2 levels in the culture medium of endothelial cells were approximately 20 times higher than...
respectively, which is indicative of enhanced prostaglandin synthases by lysates of endothelial cells treated with Ang II.

Interestingly, the synthesis of 6-keto-PGF$_1\alpha$ in endothelial cells exposed to Ang II.

Overexpressing Human HO-1

Prostaglandin Levels in Endothelial Cells

Effect of ZnDPP on HO Activity of Endothelial Cells Transduced With HO-1 Gene Using Retrovirus Vector

HO was assayed as described in Methods. ZnDPP was added at a final concentration of 10 μmol/L in the incubation mixture as indicated. Results are expressed as mean±SE of 4 different experiments.

*P<0.05.
Discussion

A previous study in rabbit coronary microvessel endothelial cells demonstrated that upregulation of HO-1 leading to reduction in cellular heme levels decreases basal prostaglandin synthesis. Our present study shows that prostaglandin production is decreased in human femoral artery endothelial cells overexpressing HO-1 owing to treatment with SnCl2, a chemical HO-1 inducer, or to transfection with the human HO-1 gene, both of which interventions are documented to reduce heme levels in endothelial cells. On the other hand, a reciprocal relationship between HO-1 overexpression and prostaglandin production is not obtained in endothelial cells treated with heme or with ZnDPP.

As previously reported, and confirmed in our study, heme and HO inhibitors such as ZnDPP are powerful inducers of HO-1 protein. Importantly, the upregulation of HO-1 expression produced by these agents is unlikely to produce depletion of cellular heme, which could explain our finding that heme treatment increases and ZnDPP treatment is without effect on prostaglandin production in endothelial cells. Collectively, these observations support the notion that depletion of cellular heme is the connecting link between overexpression of HO-1 and diminished prostaglandin production in endothelial cells treated with SnCl2 or transfected with the human HO-1 gene. In this regard, it is known that heme bound to histidine residues of the peroxidase binding site of COX isoforms is required for catalytic activity. It is plausible that downregulation of prostaglandin production in human femoral endothelial cells overexpressing HO-1 is owing to a reduction in catalytically active COX because of suboptimal levels of cellular heme.

The present study examining interactions between Ang II, the heme–HO system, and prostaglandins reveals a novel mechanism by which Ang II–stimulated upregulation of HO-1 prevents the full expression of Ang II–induced production of prostaglandins in human femoral artery endothelial cells. Three key observations substantiate this conclusion. First, HO-1 protein expression and HO activity are enhanced in endothelial cells cultured in media containing Ang II. Second, Ang II–induced stimulation of prostaglandin production is magnified in endothelial cells treated with ZnDPP to prevent HO-mediated metabolism of endogenous heme; it is also magnified in cells treated with exogenous heme to compensate for reductions in cellular heme levels owing to HO-1 overexpression. Third, Ang II–induced stimulation of prostaglandin production is attenuated in endothelial cells treated with SnCl2 to further upregulate HO-1 and reduce cellular heme. Collectively, these observations suggest that the stimulation of prostaglandin production elicited by Ang II in endothelial cells is limited by the accompanying overexpression of HO-1, leading to lowering of cellular heme to a level below that which is required for optimal expression of Ang II–induced prostaglandin production.

We found that NS-398 and indomethacin, a putative specific inhibitor of COX-2 and a nonselective inhibitor of COX-1 and COX-2, respectively, were equally effective in suppressing prostaglandin synthesis during incubation of arachidonic acid with lysates prepared from endothelial cells treated with Ang II or heme. This implies that COX-2 rather than COX-1 is responsible for prostaglandin synthesis in the human femoral endothelial cells used in the study. Previous studies also have documented significant COX-2 expression in cultured rabbit coronary endothelial cells and bovine aortic endothelial cells. That COX-2 rather than COX-1 is responsible for prostaglandin synthesis in endothelial cells treated with Ang II may be relevant to our finding that overexpression of HO-1 in such a setting prevents the full expression of Ang II–induced prostaglandin production. For example, consideration should be given to the possibility that hemes bound to COX-1 and COX-2 differ in their vulnerability to degradation by HO-1.

Perspectives

In conclusion, the present study documents a novel and potentially important interaction among Ang II, the heme–HO system and prostaglandin synthesis in human endothelial cells. We found that the stimulation of prostaglandin production elicited by Ang II in endothelial cells is limited by the accompanying overexpression of HO-1. In as much as heme is required for the expression of catalytically
active COX, overexpression of HO-1 in response to Ang II may lower cellular heme to a level below that which is required for optimal expression of Ang II–induced prostaglandin synthesis.

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