Heme Oxygenase-1 Attenuates Interleukin-1β-Induced Nitric Oxide Synthase Expression in Vascular Smooth Muscle Cells

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Key Words
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Abstract
Heme oxygenase-1 (HO-1) has been implicated in antioxidant and anti-inflammatory actions. To characterize the role of HO-1 in the vascular inflammatory response, we examined the effect of HO-1 on the expression of inducible nitric oxide synthase (iNOS) induced by interleukin-1β (IL-1β) in rat vascular smooth muscle cells (VSMCs). Western blot analysis demonstrated that IL-1β-induced iNOS expression was significantly reduced by hemin cotreatment or adenovirus-mediated HO-1 gene transfer. Scavenging carbon monoxide (CO), one of the by-products of heme degradation by HO-1, significantly attenuated HO-1-mediated suppression of iNOS gene induction as revealed by Northern blot analysis. Exposure of cells to CO or a CO donor, the tricarbonyldichlororuthenium(II) dimer, also markedly inhibited IL-1β-induced iNOS expression. Transient transfection experiments with a reporter gene construct carrying the rat iNOS gene promoter demonstrated that IL-1β-induced promoter activity was substantially reduced by cotreatment with CO or a CO donor. Furthermore, the effects of CO on iNOS gene promoter activity and protein expression were diminished by cotreatment with the specific guanylate cyclase inhibitor, 1H-[1,2,4]oxadiazolo-(4,3-a)quinoxalin-1-one. These data support the finding that HO-1 attenuates IL-1β-induced iNOS gene expression in VSMCs. CO appears to mediate the suppressive effect of HO-1, at least in part, through downregulating transcriptional activation of the iNOS gene via a cGMP-dependent pathway.

Introduction
Heme oxygenase (HO) is a key enzyme responsible for the catabolism of heme in mammalian cells [27]. Degradation of heme by HO leads to the liberation of free iron, carbon monoxide (CO) and biliverdin, the last of which is subsequently converted to bilirubin by biliverdin reductase. Three HO isoforms have been identified, one of which, HO-1, is a stress-inducible protein [1, 35]. Over the past decade, numerous studies have demonstrated the importance of HO-1 in the cytoprotective defense response following various oxidative insults both in vitro and in vivo [1, 35]. In the vascular system, the HO-derived CO, like nitric oxide (NO), can activate the guanylate cyclase-cGMP pathway [13], and in turn elicit vasodilation and inhibit platelet activation [3, 29]. The other end product, bilirubin, acts as an antioxidant and inhibits monocyte adhesion and transmigration through the endothelium [15, 19], which is one of the crucial events in atherogenesis. Furthermore, HO-1 overexpression appears to facilitate iron mobilization and reduces iron overload in vascular cells [21]. More recently, increasing evidence has also accumulated to support a potent anti-inflammatory action for HO-1 [34]. It has been shown...
that CO mediates the suppressive effect of HO-1 on tumor necrosis factor-α (TNF-α) production in lipopolysaccharide-activated macrophages through modulating the activation of p38 mitogen-activated protein kinase [34], although the detailed mechanism remains to be clarified. Studies from another laboratory and ours have revealed that HO-1 is induced in atherosclerotic lesions of humans and experimental animals [32, 38]. Overexpression of HO-1 in arterial walls reduces lesion formation [20, 21] as well as intimal hyperplasia subsequent to vascular injury in animals [2, 36, 37], and thus supports its vasoprotective function in vivo.

Expression of inducible NO synthase (iNOS) has been localized in atherosclerotic lesions and represents a major inflammatory response in the course of disease development [4, 26, 40]. High levels of NO produced by iNOS can react with superoxides to form peroxynitrite, which is a strong oxidant that exacerbates inflammation and oxidative injury at sites of vascular lesions [4, 18]. The detrimental role of iNOS has been further documented by the observation that iNOS deficiency significantly reduces the extent of atherosclerosis in apolipoprotein E-deficient mice [8]. In vitro studies have demonstrated that peroxynitrite and other reactive nitrogen species are capable of inducing HO-1, which can be regarded as an adaptive response to the nitroactive stress [11, 12, 33]. Furthermore, increased iNOS expression has been shown to mediate the induction of HO-1 in vitro and in vivo [23, 24, 31]. Conversely, whether HO-1 influences the expression of iNOS and the production of NO is not yet well characterized. To address this issue, in the present study, we tested the effect of HO-1 induced by hemin treatment or adenovirus (Adv)-mediated gene transfer on iNOS gene expression stimulated by cytokine in rat vascular smooth muscle cells (VSMCs). We found that HO-1 overexpression significantly suppressed the induction of iNOS expression in VSMCs following interleukin-1β (IL-1β) treatment. CO appears to mediate the inhibitory effect of HO-1 at least in part through downregulating the IL-1β-induced transcriptional activation of the iNOS gene via a cGMP-dependent pathway. This finding reveals an additional mechanistic pathway underlying the vasoprotective effects of HO-1.

**Methods**

**Cell Culture**

Rat VSMCs were isolated from thoracic aortas of Sprague-Dawley rats and cultured in DMEM containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 0.1 mg/ml streptomycin as described previously [25]. Cells were changed to medium containing 1% FBS prior to treatment with various agents for selected intervals.

**Infection with Recombinant Adv**

Replication-defective empty Adv and recombinant Adv carrying the human HO-1 gene (Adv-HO-1) were constructed and prepared as described elsewhere [21]. Cells were subcultured in complete medium containing 10% FBS for 12 h, and then changed to serum-free DMEM containing the indicated titers of adenoviral vectors. Following 2 h of incubation, an equal volume of complete medium was added to each culture, and the incubation was continued for an additional 22 h.

**Western Blot Analysis**

After 2 rinses with ice-cold phosphate-buffered saline ( PBS), cells were lysed in 25 mM Tris-HCl, pH 7.4, containing 1 mM phenylmethylsulfonyl fluoride and 1 mM EDTA, sonicated and centrifuged at 1,000 g for 5 min at 4 °C. The supernatant was removed, and the protein concentration was determined using a Bio-Rad (Hercules, Calif., USA) protein assay. Cell lysates (30 μg) were electrophoresed on 10% SDS-polyacrylamide gels and transblotted onto Immobilon-P membranes (Millipore, Bedford, Mass., USA). The blot was blocked with 5% skim milk in PBS containing 0.1% Tween-20 (PBST) for 1 h at room temperature. This was followed by incubation with an anti-HO-1 antibody (StressGen, Victoria, Canada; diluted 1:1,000) or anti-iNOS antibody (Transduction Laboratories, Lexington, Ky., USA; diluted 1:1,000) for another hour in PBST containing 1% skim milk. After 3 washes, the blot was incubated with horseradish peroxidase-conjugated goat secondary antibody IgG (diluted 1:2,000) for an additional 1 h. The antigen-antibody complex was detected by a chemiluminescence system.

**Northern Blot Analysis**

A cDNA fragment (0.55 kb) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was prepared as described previously [5]. A rat iNOS cDNA fragment (0.4 kb) was prepared by RT-PCR using total RNA isolated from IL-1β-treated VSMCs. The iNOS-specific primers used were as follows: sense 5'-CCCTGGTGACCTCCGCAGAGTTGGATGTCGAAGCAAAC-3', and antisense 5'-CAGTAGCTGCCACTCTCATCTAGAACCTCC-3'. The amplified cDNA fragments were verified by sequence analysis. Northern blot was performed as described previously [38].

**HO-1 Activity Assay**

After 2 rinses with ice-cold PBS, cells were harvested by centrifugation at 1,000 g for 2 min at 4 °C. The cell pellet was resuspended in 0.1 M potassium phosphate buffer (pH 7.4) containing 2 mM MgCl2 and freeze-thawed 3 times. After sonication, the cell homogenate was centrifuged at 18,800 g for 10 min at 4 °C. The supernatant was added to 0.1 M phosphate buffer (pH 7.4) containing 2 mg of rat liver cytosol, 20 μM hemin, 0.8 mM NADPH, 2 mM glucose-6-phosphate and 0.2 units of glucose-6-phosphate dehydrogenase, and incubated in the dark for 1 h at 37 °C. Bilirubin was then extracted with chloroform and quantitated by the absorbance difference between 464 and 530 nm with an extinction coefficient of 40/mM·cm.