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Induction of heme oxygenase-1 is involved in anti-proliferative effects of paclitaxel on rat vascular smooth muscle cells

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Abstract

In this study, we evaluated the possibility that the anti-proliferative effects of paclitaxel on vascular smooth muscle cells (VSMCs) of the rat might be due to the induction of HO-1 gene expression. Treatment of the cells with paclitaxel resulted in marked time- and dose-dependent inductions of HO-1 mRNA, followed by corresponding increases in HO-1 protein expression and HO enzymatic activities. Furthermore, paclitaxel rapidly activated the JNK, ERK, and p38 mitogen-activated protein kinase pathways. A specific inhibitor of JNK, SP600125, abolished paclitaxel-induced HO-1 mRNA expression, whereas PD98059, a specific inhibitor of ERK, and SB203580, a specific inhibitor of p38, had no significant effect. Finally, the suppression of platelet-derived growth factor induced VSMC proliferation was abolished by the HO inhibitor, ZnPP, as well as by the CO scavenger, hemoglobin. These results demonstrated that paclitaxel induces the expression of HO-1 via the JNK pathway in VSMC and that HO-1 expression might be responsible for the anti-proliferative effect of paclitaxel on VSMC.
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The proliferation of vascular smooth muscle cells (VSMCs) is a central event in the pathogenesis of vascular lesions, including postangioplasty restenosis, transplant arteriosclerosis, and vein graft occlusion [1]. Paclitaxel has several properties that make it a good candidate for local drug therapy of excessive arterial smooth muscle cell proliferation in restenosis after balloon angioplasty or stent implantation. These properties have been tested in vitro [2], in animal models [3], and in clinical studies [4]. An anti-proliferative effect of paclitaxel on vascular cells has been shown in vitro in rat VSMCs as well as in vivo in the rat carotid artery injury model [5]. Paclitaxel was found to interfere with VSMC proliferation and migration at nanomolar levels in vitro and to prevent neointimal VSMC accumulation in the carotid artery in vivo [6].

Heme oxygenases (HOs) are the rate-limiting enzymes in heme degradation, catalyzing the cleavage of the heme ring to form ferrous iron, carbon monoxide (CO), and biliverdin [7]. Three distinct isoforms of HOs have been cloned [8–10]. Both HO-2 and HO-3 are constitutively expressed isoforms that are present in high concentrations in selected mammalian tissues [9,10]. By contrast, the HO-1 is strongly induced by a

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variety of physiologic and pathophysiologic stimuli, including heme, heavy metals, endotoxin, inflammatory cytokines, and nitric oxide [8,11,12].

HO-derived CO release has been shown to play a significant physiological role in the circulation [13]. In systemic blood vessels, CO may have an important homeostatic role where, like its better-studied counterpart nitric oxide, it is emerging as a vasodilator [14] and an inhibitor of cell proliferation [15,16]. Recently, Motterlini et al. [17] have reported the ability of a series of transition metal carbonyls, which they have termed carbon monoxide-releasing molecules (CO-RM), to liberate carbon monoxide and mimic the effects of endogenous heme oxygenase-1 induction in the systemic circulation of the rat. In addition to regulating SMC function, CO modulates platelet reactivity. Both exogenously administered and vascular cell-derived CO inhibits platelet aggregation [18,19]. All these biological effects of CO are mediated via the activation of soluble guanylate cyclase and the consequent rise in intracellular cGMP levels in target tissues [18–20]. Growing evidence suggests that paclitaxel may have an inhibitory role in cellular proliferation [5,6]. Recently, HO-1 or one of its products, CO, has also been shown to inhibit VSMC proliferation in response to an injury [20,21].

Thus, the present study examined the possibility that the anti-proliferative effects of paclitaxel may include the induction of HO-1. We now report that paclitaxel induces HO-1 gene expression and CO release in VSMCs. We also demonstrate that paclitcxel-mediated increases in HO activity contribute to the anti-proliferative effects of paclitaxel.

Materials and methods

Materials. Paclitaxel, tubulozol, nocodazol, colchicines, platelet-derived growth factor (PDGF), and tricarbonyldichlororuthenium (II) dimer (CO-releasing molecule, CO-RM) were purchased from Sigma–Aldrich (St. Louis, MO). Zinc protoporphyrin (ZnPP), an inhibitor of heme oxygenase activity, was from Porphyrin Products (Logan, UT), and the Cell Proliferation Kit II (XTT) was obtained from Boehringer Mannheim. Anti-HO-1, ERK, p38, and JNK antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The inhibitors of mitogen-activated protein kinases (MAPKs), PD98059, SB203580, and SP600125 were from Calbiochem (San Diego, CA). Unless indicated otherwise, all other chemicals were obtained from Sigma–Aldrich (St. Louis, MO).

Cell culture. Primary cultures of rat VSMC were prepared and cultured as previously described [22] and used for experiments as completely confluent monolayers at passages 5–10. Cells were maintained in Dulbecco's modified Eagle's medium (Gibco-BRL, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) and gentamicin (50 μg/mL) at 37 °C in a humidified incubator with 5% CO_2.

Reverse transcriptase-polymerase chain reaction analysis. RT-PCR was conducted as described previously [23]. PCR conditions for HO-1 and β-actin were as follows: 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s. The primer pairs were as follows: HO-1, 5′-ACTTT CAGAAGGGTCAGGTGTCC-3′ and 5′-TTGAGCAGGAAGGCG GTCTTAG-3′ (524 bp); β-actin, 5′-CCTTCTACAATGAGC-3′ and 5′-ACGTCACACTTCATG-3′ (594 bp). Amplification products were resolved by 1.2% agarose gel electrophoresis, stained with ethidium bromide, and photographed under ultraviolet light.

Western blot analysis. Western blot analysis was performed as follows. Briefly, cells were harvested, washed twice with ice-cold phosphate-buffered saline (PBS), and resuspended in 20 mM Tris–HCl buffer (pH 7.4) containing a protease inhibitor mixture (0.1 mM phenylmethylsulfonyl fluoride, 5 μg/mL aprotinin, 5 μg/mL pepstatin A, and 1 μg/mL chymostatin). Protein concentration was determined with the Lowry protein assay kit (P5626, Sigma). An equal volume of 2× SDS–sample buffer was added, and the samples were boiled for 5 min. Samples (40 μg) were subjected to electrophoresis in a 12% SDS–polyacrylamide gel for 2 h at 20 mA and then transferred to nitrocellulose. The membranes were incubated for 1 h in 5% (wt/vol) dried milk protein in PBS containing 0.05% (vol/vol) Tween 20. The membranes were washed in PBS containing 0.05% (vol/vol) Tween 20 and incubated for 1 h in the presence of primary antibody (1:1000). The membranes were washed extensively and then incubated for 1 h with antigoat IgG conjugated to HRP (1:4000). The membranes were washed extensively again and the protein bands were visualized using chemiluminescent reagents according to the manufacturer's instructions (Supersignal Substrate; Pierce).

Assay for HO activity. HO enzyme activity was measured by previously described method [24]. Briefly, microsomes from harvested cells were added to a reaction mixture containing NADPH, rat liver cytosol as a source of biliverdin reductase and the substrate hemin. The reaction was carried out in the dark for 1 h at 37 °C, terminated by the addition of 1 mL chloroform, and bilirubin extracted was calculated by the difference in absorbance between 464 and 530 nm.

Cell proliferation. Proliferation activity was evaluated with the tetrazolium-based assay, XTT, according to manufacturer's instructions. VSMCs were incubated in 96-well plates in cell culture media with or without PDGF (50 ng/mL), paclitaxel (200 μM), or ZnPP (10 μM). XTT at a concentration of 0.3 mg/mL was added to each well for 8 h at 37 °C in a humidified incubator with 5% CO_2 and determination of absorbance at 450 or 490 nm.

Statistical analysis. Differences in the data among the groups were analyzed by one-way analysis of variance combined with Bonferroni's test, and all values were expressed as means ± SD. The differences between groups were considered to be significant at $p < 0.05$.

Results

Paclitaxel induces HO-1 expression and HO activity in VSMCs.

To examine whether the anti-proliferative effects of paclitaxel are mediated by HO-1, we first evaluated HO-1 expression in response to paclitaxel in VSMCs. RT-PCR analyses were performed to examine the steady-state levels of HO-1 mRNA in primary cultures of rat VSMCs after exposure to the paclitaxel. Total RNA was isolated at 2, 4, 8, 12, and 24 h after 200 nM paclitaxel treatment and analyzed for HO-1 mRNA expression (Fig. 1A). HO-1 mRNA expressions were induced in a time-dependent manner. HO-1 mRNA levels showed an initial rise at 4 h, a peak induction at 12 h, and a decrease by 24 h. Induction of the HO-1 mRNA in VSMCs is also dose dependent, as evidenced by linear increase in mRNA expression by doses of paclitaxel

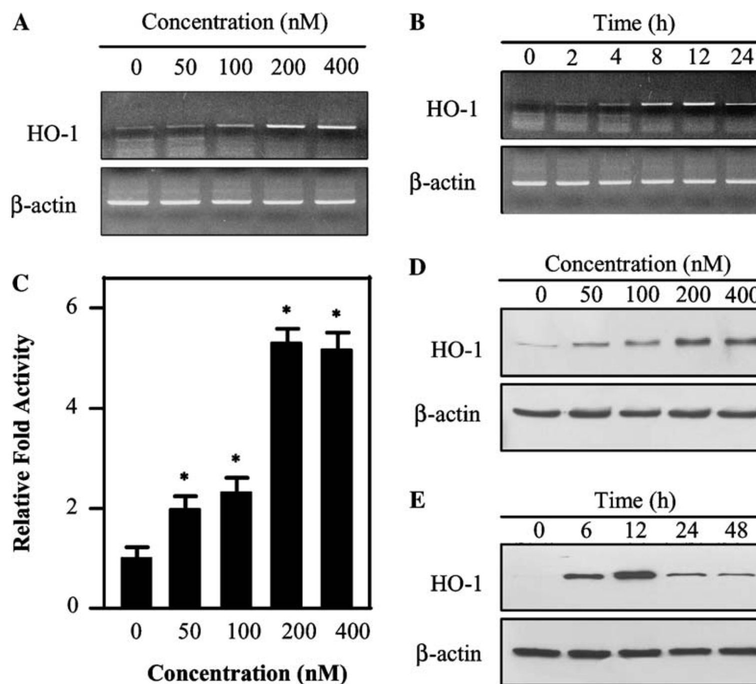

Fig. 1. Induction of HO-1 expression by paclitaxel in VSMCs. Expression of HO-1 mRNA in VSMCs treated with the indicated concentrations of paclitaxel for 8 h (A) and with 200 nM paclitaxel for the indicated times (B). Total RNA was isolated and analyzed for HO-1 mRNA expression by RT-PCR. β-Actin was used as the loading control. HO-1 activity (C) and HO protein expression (D) were measured in VSMCs at 12 h after treatment of the cells with various concentrations of paclitaxel. The cells were treated with 200 nM of paclitaxel, and HO-1 protein expression (E) was measured at various time points indicated in the figures. Total cellular proteins were isolated from the cells treated with paclitaxel and Western blot analysis was performed using specific antibodies for HO-1 and β-actin. Each bar represents the mean ± SD of three independent experiments. *$P < 0.05$ vs. control.

ranging from 50 to 400 nM (Fig. 1B). HO-1 mRNA levels showed an initial rise at 50 nM and a peak induction at 200 nM.

We examined the effects of paclitaxel on the HO activity and HO-1 protein expression in VSMCs. As shown in Fig. 1C, exposure of the cells to paclitaxel (50–400 nM) for 12 h resulted in dose-dependent increases in HO activity. The increase was significantly different from control (untreated cells, $p < 0.05$), with a maximal enzymatic activity at 200 nM paclitaxel (Fig. 1C). Western blot analysis revealed that enhanced HO activities by paclitaxel treatments were directly correlated with HO-1 protein levels (Fig. 1D). Because the concentration of paclitaxel at 200 nM was capable of inducing maximal expression of HO in this cells, all subsequent experiments involving paclitaxel were performed using a concentration of 200 nM paclitaxel. Treatment of the cells with paclitaxel resulted in a time-dependent increase in HO-1 protein expression. Using 200 nM of paclitaxel, the HO-1 protein expression was evident as early as 6 h and reached a maximum at 12 h after treatment of the cells with paclitaxel (Fig. 1E). Paclitaxel in the range of 50–400 nM had no significant effect on cell viability under these conditions (data not shown).

To eliminate the possibility that the HO-1 induction was occurring in response to a unique element of the paclitaxel rather than the inhibitors of microtubule assembly, we stimulated VSMC with three additional widely used inhibition of microtubule assembly, tubulozol, nocodazol, and colchicine. All of these inhibitors of microtubule assembly induced HO-1 expressions similar to those observed in the cells treated with paclitaxel (Fig. 2).

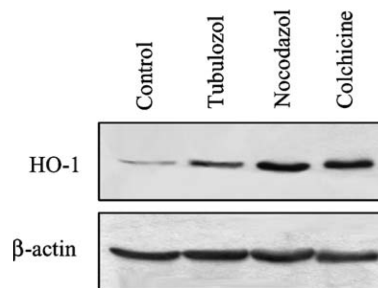

Fig. 2. Effects of inhibitors of microtubule assembly on HO-1 mRNA expression in VSMCs. Cells were treated for 12 h with the various indicated inhibitors of microtubule assembly (100 nM of tubulozol, nocodazol, or colchicine), at which time protein was isolated and analyzed for HO-1 protein expression by Western blot analysis. β-Actin is shown as a normalization control. Data shown are representative of three independent experiments.

Involvement of the JNK pathway in the induction of HO-1 expression by paclitaxel in VSMCs

Recently, studies on HO-1 induction by stress stimuli have shown that pathways involving MAPKs are

responsible for the transduction of signals to initiate gene activation [25–27]. To determine whether a similar signal mechanism is responsible for the upregulation of HO-1 expression by paclitaxel in VSMCs, we examined the activation states of three MAPK subfamilies, JNK1/2, ERK1/2, and p38, in VSMCs. Cells were exposed to paclitaxel and then immunoblots were performed using anti-phospho JNK1/2, ERK1/2, and p38. As shown in Fig. 3A, phosphorylated JNK1/2, ERK1/2, and p38, indicating activation, were all increased by paclitaxel. The same blots were probed with antibody to total JNK1/2, ERK1/2, or p38 as protein loading controls. The paclitaxel-mediated increase in HO-1 protein expression was completely blocked by SP600125, a specific inhibitor of JNK, whereas similar concentrations of PD98059, a specific inhibitor of ERK, and SB203580, a specific inhibitor of p38, had no significant effect (Fig. 3B).

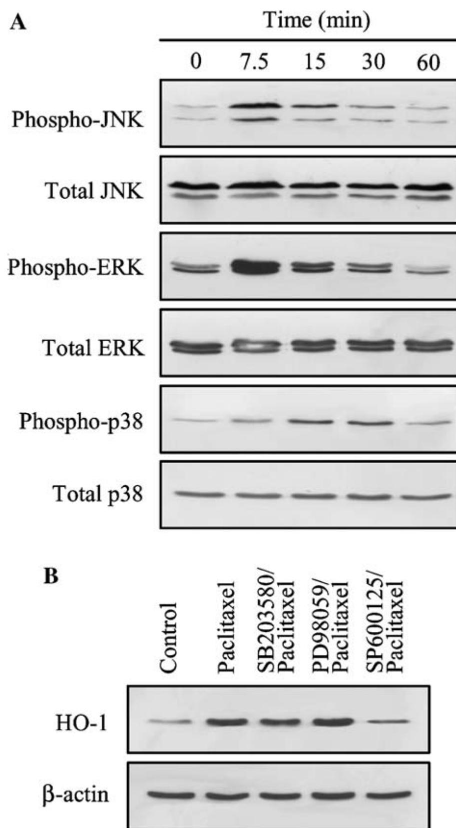

Fig. 3. Effects of paclitaxel on phosphorylations of the MAPKs in VSMCs. (A) The cells incubated in the absence or in the presence of paclitaxel (200 nM) for the indicated times were subjected to Western blot analysis using phospho-specific antibodies to JNK, ERK, or p38. As controls, the same cell lysates were subjected to Western blot analysis using corresponding non-phospho-specific antibodies to detect total JNK, ERK, or p38. (B) Cells were pretreated with or without SB203580 (20 μM), PD98059 (20 μM), or SP600125 (40 mM), as indicated, and then incubated in the absence or presence of paclitaxel for 12 h. Western blot analysis was performed using specific antibodies for HO-1 and β-actin. Data shown are a representative of three independent experiments.

These results indicated that kinases of the JNK pathway might be involved in the regulation of HO-1 expression by paclitaxel.

HO-1 expressed by paclitaxel inhibits VSMC proliferation

Growing evidence suggests that paclitaxel may have an inhibitory role in cellular proliferation [5,6]. In addition, HO-1 or one of its products, CO, has also been shown to inhibit VSMC proliferation in response to an injury [20,21]. Thus, we asked whether the inhibitory effects of paclitaxel on PDGF-dependent VSMC proliferation could be mediated by its induction of HO-1 expression. Like previous studies, we found that paclitaxel suppressed PDGF-dependent cell proliferation. We exposed VSMC to ZnPP, an inhibitor of HO activity, in the presence of 200 nM paclitaxel. Pretreatment of the cells with 20 μM ZnPP IX abrogated the inhibitory effects of paclitaxel on PDGF-dependent VSMC proliferation (Fig. 4A).

To determine that CO, one of HO-1 products, might be responsible for the inhibitory effects of paclitaxel on VSMC proliferation, we examined the effects of hemoglobin (a CO scavenger) and CO-RM (a CO donor) on PDGF-dependent VSMC proliferation. As shown in Fig. 4B, the addition of hemoglobin resulted in a loss of the inhibitory effects of paclitaxel on PDGF-induced cell proliferation. Also, we found that CO-RM, at concentrations of 50 μM, effectively blocked PDGF-induced cell proliferation (Fig. 4C). These results suggest that HO-1 or one of its products, CO, might be involved in the suppressive effects of paclitaxel on PDGF-dependent VSMC proliferation.

Discussion

The present study demonstrates for the first time that paclitaxel is a potent inducer of HO-1 gene expression in VSMCs. We show that the levels of both HO-1 mRNA and HO-1 protein are increased in the VSMCs by the treatment of the cells with paclitaxel (Fig. 1). We further confirmed that the paclitaxel-induced increases in HO-1 mRNA and protein are accompanied by corresponding increases in HO enzymatic activities. We have also found that inhibitors of microtubule assembly caused inductions of HO-1 expressions (Fig. 2), suggesting that HO-1 expression by paclitaxel could be due to its effect on microtubule assembly.

Recently, studies on HO-1 induction by stress have shown that pathways involving MAPKs are responsible for the transduction of signals to initiate the gene expression [25–27]. The roles of MAPKs have previously been demonstrated in various cell culture systems [27–30], and contradictory results on the regulatory role of

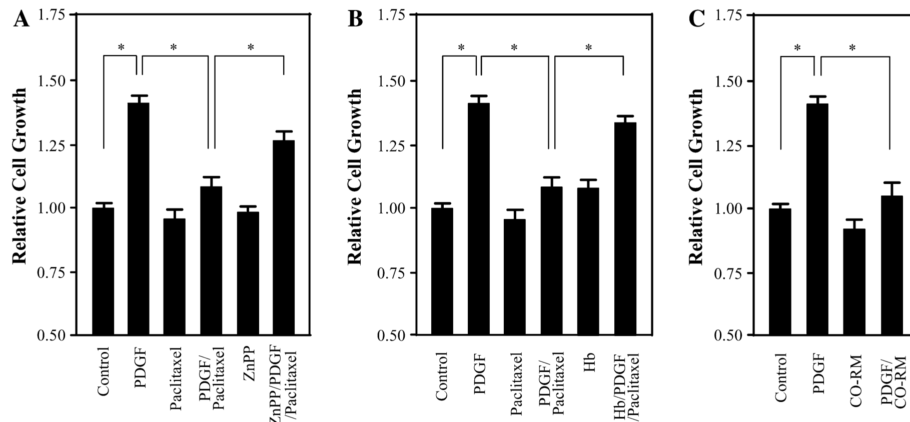

Fig. 4. Effects of paclitaxel-induced HO-1 on the VSMC proliferations. (A) The cells were incubated with or without PDGF (50 ng/mL), paclitaxel (200 nM), or ZnPP (10 μM). (B) The cells were incubated with or without PDGF, paclitaxel, or hemoglobin (Hb, 40 μg/mL). (C) The cells were incubated with or without PDGF or CO-RM (40 μM). Each bar represents relative increases in numbers of treated cells compared with those of control cells based on tetrazolium-based XTT assay. Each bar represents the mean ± SD of three independent experiments. $*P < 0.05$.

different MAPK pathways for HO-1 expression were reported. Chen and Maines [25] have demonstrated that exposure of HeLa cells to NO increases HO-1 mRNA via ERK and p38 pathways, but not SAPK/JNK pathway. In other human cell line, HepG2, overexpression of MEKK1, led to the induction of HO-1 protein expression [30]. On the other hand, Masuya et al. [31] reported that the induction of HO-1 expression by cadmium, arsenite, and hemin was not mediated via MAPKs in HeLa cells. However, data from our study show that kinases of the JNK pathway are involved in the induction of HO-1 expression by paclitaxel in VSMCs (Fig. 3). Similar to our results, the induction of HO-1 expression by phorone or sodium arsenite is mediated via the JNK pathway [28,29]. A major reason for these regulatory discrepancies could be cell- or inducer-specific variations that may affect the regulation of HO-1 expression via MAPKs.

The antiproliferative effect of the HO-1 pathway in smooth muscle cells was described in rat VSMCs in vitro [15,20] and then confirmed in vivo in animal models of vascular remodeling after balloon injury [32]. Also, the HO-1-catalyzed release of CO may provide an important adaptive mechanism to maintain homeostasis at sites of vascular injury. Paclitaxel was also reported to interfere with VSMC proliferation and migration at nanomolar levels in vitro and to prevent neointimal VSMC accumulation in the carotid artery in vivo [6]. In this study, we provide the evidence that HO-1 might be involved in the suppressive effects of paclitaxel on VSMC proliferation (Fig. 4). This suppressive effects were abrogated in the presence of the HO inhibitor, ZnPP IX, suggesting that HO induced by paclitaxel may mediate the inhibitory effects of paclitaxel on PDGF-dependent VSMC proliferation (Fig. 4). Similar to our data, the antiproliferative effects of rapamycin were reported to be mediated via the induction of HO-1 expression [33]. In addition, previous studies have demonstrated that proliferation of VSMCs is inhibited by CO [20,21]. Our results also confirm and expand the involvement of HO-1-derived CO in the inhibition of VSMC proliferation by the treatment of the cells with paclitaxel (Fig. 4).

In conclusion, our data demonstrate that paclitaxel induces the expression of HO-1 and that HO-1 expression might be responsible for the anti-proliferative effect of paclitaxel on VSMCs.

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