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NS-398, a selective COX-2 inhibitor, inhibits proliferation of IL-1β-stimulated vascular smooth muscle cells by induction of HO-1

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ABSTRACT

We investigated whether NS-398, a selective inhibitor of COX-2, induces HO-1 in IL-1β-stimulated vascular smooth muscle cells (VSMC). NS-398 reduced the production of PGE_2 without modulation of expression of COX-2 in IL-1β-stimulated VSMC. NS-398 increased HO-1 mRNA and protein in a dose-dependent manner, but inhibited proliferation of IL-1β-stimulated VSMC. Furthermore, SnPPIX, a HO-1 inhibitor, reversed the effects of NS-398 on PGE_2 production, suggesting that COX-2 activity can be affected by HO-1. Hemin, a HO-1 inducer, also reduced the production of PGE_2 and proliferation of IL-1β-stimulated VSMC. CORM-2, a CO-releasing molecule, but not bilirubin inhibited proliferation of IL-1β-stimulated VSMC. NS-398 inhibited proliferation of IL-1β-stimulated VSMC in a HbO_2-sensitive manner. In conclusion, NS-398 inhibits proliferation of IL-1β-stimulated VSMC by HO-1-derived CO. Thus, NS-398 may facilitate the healing process of vessels in vascular inflammatory disorders such as atherosclerosis.

Heme oxygenase (HO) catalyzes the rate-limiting step in the oxidative degradation of heme (a potent oxidant) to biliverdin (rapidly converted to bilirubin, an anti-oxidant), iron (sequestered by ferritin), and carbon monoxide (CO, a vasodilatory gas that has anti-inflammatory properties) [1]. Three distinct HO isoforms have been cloned. HO-1 is inducible [2,3], whereas HO-2 and HO-3 are constitutively expressed [4]. Unlike HO-2, HO-1 is ubiquitously distributed and strongly induced by oxidative, nitrosative, osmotic, hemodynamic, hyperthermic and endoplasmic reticulum stress [5–7]. HO-1 has many beneficial effects, with anti-oxidant, anti-apoptotic, and potent anti-inflammatory properties [1,8–10]. HO-1 inhibits vascular smooth muscle cell growth in atherosclerotic lesions and hypertension [11,12]. The absence of HO-1 facilitates the formation of atherosclerotic lesions and vascular remodeling [13].

Vascular diseases such as atherosclerosis and restenosis are the leading causes of worldwide mortality [14]. Atherosclerosis involves a multitude of pathophysiological processes including endothelial dysfunction, inflammation, vascular smooth muscle cell (VSMC) proliferation, and extracellular matrix alterations [15]. The abnormal accumulation of VSMC within the intima is one of the characteristic histopathological changes noted in vessels as a part of primary atheromatous plaque formation. Preventing VSMC proliferation is an important therapeutic approach for such disorders [16]. Our group recently reported that NS-398 protected brain cells from hypoxic injury by inducing HO-1 expression [17]. However, it is not known whether NS-398 induces HO-1 protein expression in VSMC as well. Therefore, the aim of the present study was to determine if NS-398 inhibits inflammatory cytokine-induced VSMC proliferation through HO-1.

Materials and methods

Reagents. Cell culture reagents were purchased from Hyclone. Acrylamide and western blot reagents were purchased from Bio-Rad. The antibody against HO-1 was purchased from Stressgen Bioreagents (Vitoria, BC). COX-2 antibody was purchased from Cayman (Michigan, MA). PRO-PREP protein extract solution was purchased from iNtRON Biotechnology (Sungnam, Korea). The ECL western blotting detection kit was purchased from NEURONEX. NS-398 and SnppIX were purchased from Calbiochem (La Jolla, CA). All other chemicals including Tricarbonyldichlororuthenium dimmer ($[Ru(CO)_3(Cl)_2]_2$, CORM-2, hemoglobin (Hb) and MTT were purchased from Sigma–Aldrich (St. Louis, MO).

Rats. Male Sprague–Dawley rats weighing 200–250 g were used in this study. All experimental animals received autoclaved food and bedding to minimize exposure to viral or microbial pathogens. The rats were cared for in accordance with the Guide for the Care and Use of Experimental Animals of Yeungnam Medical Center.

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Cell culture. Male Sprague–Dawley rats weighing 200–250 g were used in this study. Rats were anesthetized by pentobarbital (50 mg/kg). VSMC were isolated from thoracic aorta and the connective tissue was removed. VSMC were processed using a 1 mm chop setting in a 10 cm culture dish, and cultured with DMEM containing 50% FBS and 1% antibiotics, and incubated in a CO_2 incubator (95% CO_2 air, 37 °C) for 7 days. Cells from passage 4 to 10 were used for the experiments.

[^{3}H]thymidine incorporation assay. VSMC were plated into 24-well plates at a concentration of 3–4 × 10^4 cells/ml, in complete DMEM growth medium. After a 24 h serum-deprivation period, VSMC were treated with a drug solution that containing 10% FBS for 48 h. At the end of this time period, fresh DMEM containing 1 μCi/ml [^{3}H]thymidine was added for an additional 8 h incubation period. Medium was then removed, and each well was washed twice with 1 ml autoclaved 1× PBS and then treated with 0.1% SDS for 5 min, and transferred to vials containing 5 ml cocktail solution. Each individual experimental treatment was counted by a Liquid Scintillation Analyzer (Packard Bioscience).

RT-PCR. Total RNA was extracted using TRIzol® reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA was reverse transcribed into single-stranded cDNA by incubation for 10 min at 30 °C, 30 min at 42 °C, and 5 min at 99 °C in a final volume of 20 μl using a Maxime RT Premix kit (iNtRON Biotechnology, Sungnam, Korea). One microliter of RT product was transferred to an Accupower® PCR premixed tube using the Accupower® PCR premix kit (Bioneer Inc., Alameda CA). PCR amplification was carried out on cDNA equivalent to 10 ng of starting mRNA using specific oligonucleotide primers for HO-1 (forward, 5′-ACTTTCAGAAGGGTCAGGTGTCC-3′ and reverse, 5′ TTGAGCAGG AAGGCGGTCTTAG-3′), and β-actin (forward, 5′-TAGGCAGGCCTC TTTTCTCA-3′ and reverse 5′-AGAGGGGACCTGGGTTTAGA-3′). The cDNA was heated for 5 min at 95 °C, then amplified for 27 cycles for HO-1 (94 °C for 45 s, 55 °C for 1 min, 72 °C for 1 min), followed by 5 min of extension at 72 °C. The PCR products were electrophoresed on 1.2% ethidium bromide stained agarose gels.

Western blot analysis. Cells were lysed in PRO-PREP protein extract solution. The sample was centrifuged at 13,000*g* for 5 min at 4 °C. Protein concentrations were determined by the Bradford method. An equal volume of 2× sample buffer was added to aliquots of the supernatant fraction from the lysates and the mix was boiled for 5 min. Thirty micrograms of protein were loaded per lane, resolved by 10% SDS–PAGE for 1 h 30 min at 30 mA. The separated proteins were transferred to PVDF membranes (Millipore) for 1 h at 100 V with a SD Semi-dry Transfer Cell (Bio-Rad). The membranes were blocked with 5% Skim milk in 1× PBS containing 0.05% Tween 20 (PBS-T) for 1 h at room temperature. The membranes were then incubated with antibodies against COX-2 (No. 160106 Cayman), HO-1 (No. SPA-895, Stressgen), or β-actin (No. A5316, Sigma–Aldrich). Proteins were detected with a horseradish peroxidase-coupled secondary antibody by means of the ECL system.

Prostaglandin E_2 enzyme immunoassay. Primary VSMC were seeded into 24-well plates (1 × 10^4 cells/well) and cultured for a day in DMEM containing 10% FBS. Cells were serum starved for 24 h and then stimulated with or without reagents. After different treatments, the concentrations of prostaglandin E_2 (R&D systems, Minneapolis, MN, USA) in the culture supernatants were measured by ELISA according to the manufacturer's instructions. The optical density at 450 nm was measured within 30 min.

Statistical analysis. Results are expressed as mean ± SD from at least three independent experiments. For comparison between multiple groups, statistical significance was tested by Mann–Whitney test using SPSS 12.0 version.

Results and discussion

NS-398 induces HO-1 without affecting COX-2 expression and inhibits PGE_2 production in IL-1β-stimulated VSMC

Inflammation has emerged as a major driving force of atherosclerotic lesion development. It is now well established that from early lesion to vulnerable plaque formation, numerous cellular and molecular inflammatory components participate in the disease process. To investigate the effect of NS-398 on marker protein of inflammation, COX-2, VSMC cells were treated with IL-1β (10 ng/ml) and NS-398 (25 μM) for 24 h. Western blot analysis showed that IL-1β-induced COX-2 protein expression. However, strangely enough, treatment with NS-398 did not weaken the IL-1β-induced COX-2 protein expression (Fig. 1A), but reduced the production of PGE_2 significantly, confirming that NS-398 has a COX-2 inhibitory action. This is consistent with others in which they showed that NS-398 reduced the production of PGE_2 with no effect on COX-2 expression in RAW 264.7 cells [18] and colon cancer cells [19]. On the other hand, our group recently reported that HO-1 induction caused by a synthetic compound, YS 49, significantly reduced Ang II-induced VSMC proliferation [12] and NS-398-induced HO-1 induction in C6 glial cells under hypoxic conditions [17]. Thus, to investigate whether NS-398 induces HO-1 in VSMC under IL-1β stimulation, cells were treated with IL-1β and NS-398. We found that NS-398 up-regulated HO-1 mRNA and protein expression, in a concentration-dependent manner, in VSMC activated with IL-1β (Fig. 2A and B). Next, we asked how HO-1 induction relates with COX-2 protein expression, COX-2 activity and proliferation of

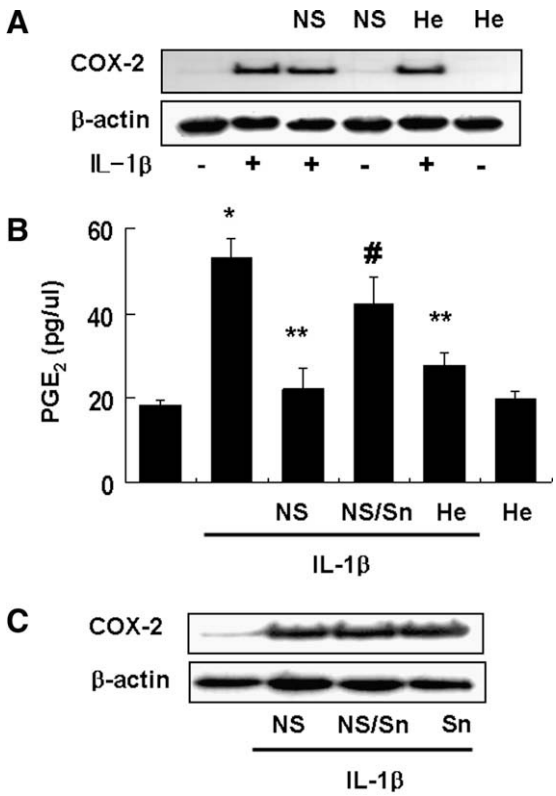

Fig. 1. Effect of NS-398, SnPPIX and hemin on COX-2 expression and PGE_2 production in IL-1β-stimulated VSMC. VSMC were pretreated with NS-398 (NS, 25 μM), h (He, 5 μM), SnPPIX (Sn, 1 μM) or NS-398 (NS, 25 μM) plus SnPPIX (Sn, 1 μM) for 1 h, then stimulated with 10 ng/ml of IL-1β for 24 h before measurement of COX-2 protein expression (A and C) and PGE_2 accumulation (B). Data represent means ± SD of three independent experiments ($^*P < 0.005$, compared to control; $^{**}P < 0.01$, compared to IL-1β; $^{\#}P < 0.01$, compared to NS-398).

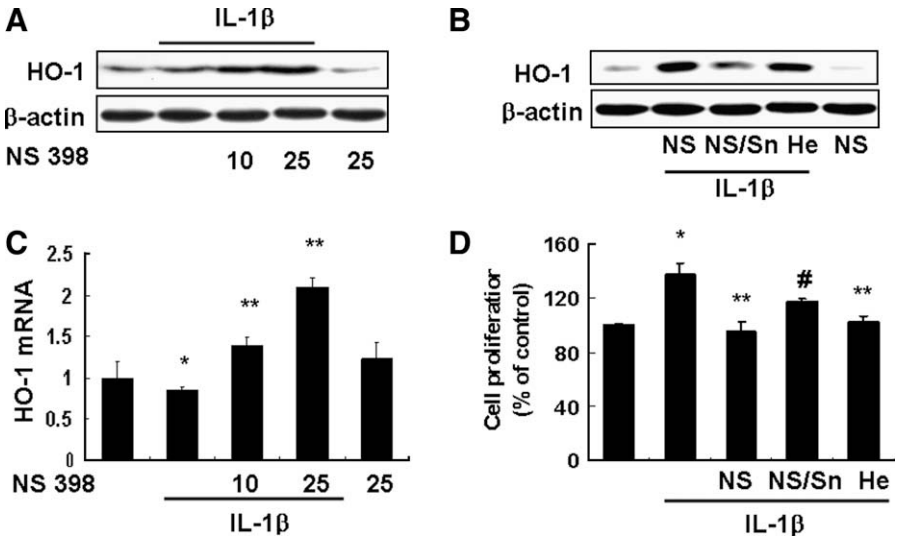

Fig. 2. Effect of NS-398 on HO-1 induction and proliferation by IL-1β-activated VSMC. VSMC were pretreated with NS-398 (10, 25 μM) and for 1 h, then stimulated with 10 ng/ml IL-1β at for 24 h before measurement of HO-1 protein (A) and mRNA (B). Cells were pretreated with NS-398 (NS, 25 μM), Hemin (He, 5 μM) or SnppIX (Sn, 1 μM) for 1 h, then stimulated with 10 ng/ml of IL-1β for 24 h before determination of cell proliferation by [^{3}H] thymidine incorporation (C) and HO-1 protein expression (D). Results are representative of three experiments. Data represent means ± SD of three independent experiments ($^{*}P < 0.05$, compared to control; $^{**}P < 0.01$, $^{\S}P < 0.005$, compared to IL-1β; $^{\#}P < 0.01$, compared to NS-398).

VSMC. Therefore, hemin, a well-known HO-1 inducer, was treated in VSMC and examined COX-2 expression and activity. To our surprise, hemin also did not reduce IL-1β-induced COX-2 protein expression (Fig. 1A), however, it significantly inhibited IL-1β-induced PGE_2 production (Fig. 1B), indicating that HO-1 induction can modulate COX-2 activity in VSMC. To clarify this phenomenon further, effect of SnppIX, HO-1 inhibitor, on PGE_2 production and COX-2 expression was investigated in IL-1β stimulated VSMC. As shown in figure, the reduced production of PGE_2 by NS-398 in IL-1β stimulated VSMC was significantly reversed by SnPPIX (Fig. 1B), and interestingly SnPPIX itself increased COX-2 expression (Fig. 1C). However, SnPPIX significantly inhibited not only expression of HO-1 but anti-proliferative effect by NS-398 in IL-1β stimulated VSMC (Fig. 2C and D). These findings suggest that HO-1 may modulate COX-2 activity somehow. Furthermore, this implies that NS-398 functions as a COX-2 enzyme inhibitor but not expression inhibitor. Actually, it is known that heme bound to histidine residues of the peroxidase binding site of COX isoforms

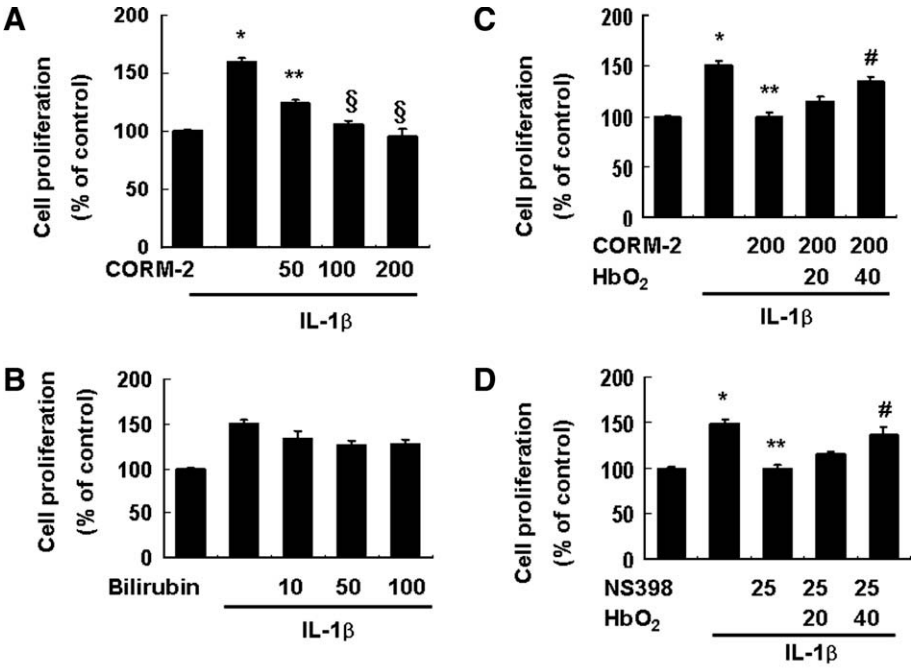

Fig. 3. Effects of CO and bilirubin on proliferation of IL-1β-activated VSMC. VSMC were pretreated with CORM-2 (50, 100, 200 μM) (A) or bilirubin (10, 50, 100 μM) (B) for 1 h, and then stimulated with 10 ng/ml of IL-1β for 24 h before measurement of cell proliferation by [^{3}H] thymidine incorporation. Cells were pretreated with HbO_2 (20, 40 μM), CORM-2 (C), and NS-398 (D) for 1 h, then stimulated with 10 ng/ml IL-1β for 24 h before measurement of cell proliferation by [^{3}H] thymidine incorporation. Results are representative of three experiments. Data represent means ± SD of three independent experiments ($^{*}P < 0.005$, compared to control; $^{**}P < 0.01$, $^{\S}P < 0.005$, compared to IL-1 β; $^{\#}P < 0.01$, compared to NS-398).

is required for catalytic activity [20]. We thus speculate that induction of HO-1 by NS-398 (or any HO-1 inducers) may lead to reduction of cellular heme contents, which may limit the action of catalytically active COX due to suboptimal level of cellular heme (Fig. 4). In this regard, it has been recently reported that HO-1 may participate in the anti-inflammatory effects of taurine possibly by inhibiting COX-2 activity and decreasing PGE_2 production in J774.2 macrophages [21]. Therefore, SnPPIX may antagonize effect of NS-398 on COX-2 inhibition by inhibition of HO-1, so it increases PGE_2 production again that was suppressed by NS-398 in IL-1β-activated VSMC. However, the mechanism by which NS-398 do not reduce COX-2 protein expression is still not known. Present study also shows that hemin, a HO-1 inducer, reduced PGE_2 secretion in IL-1β-activated VSMC.

HO-1-derived CO inhibits IL-1β-induced VSMC proliferation

HO-1 products, such as CO or bilirubin are known to protect many cells by anti-oxidant, anti-apoptotic, and anti-inflammatory functions [1,8,10]. Therefore, the induction of the HO-1 protein may be significant for general endogenous cellular protection during inflammation [22]. Fig. 3A shows that the increased VSMC proliferation caused by IL-1β was significantly reversed by the CO-releasing molecule, CORM-2. Depending on the concentration of the CO donor, cell proliferation was reduced relative to control levels (IL-1β treatment). In contrast, bilirubin (up to 100 μM) did not affect cell proliferation (Fig. 3B). Fig. 3C demonstrates that reduced cell proliferation caused by a CO donor was significantly antagonized by HbO_2. Likewise, the effect of NS-398 was reversed by the presence of HbO_2 (Fig. 3D). Here, we found that NS-398 significantly reduced IL-1β-induced VSMC proliferation. This inhibition was reversed by SnPPIX, a HO-1 inhibitor, and oxyhemoglobin (HbO_2), a CO scavenger. Consistent with these findings, a recent study observed that HO-1-derived CO is an autocrine inhibitor of VSMC growth [23]. These findings suggest that HO-1 expression and subsequent release of its product, CO, may play a major role in the inhibitory effect of NS-398 on IL-1β-induced VSMC proliferation. Of course, the possibility for the contribution of the COX-2 inhibitory effect of NS-398 on proliferation cannot be dismissed

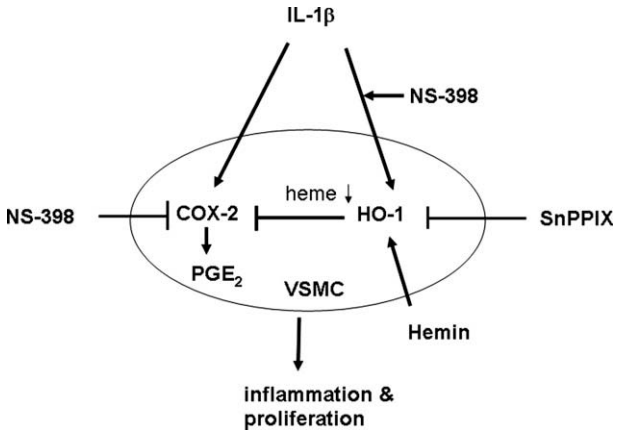

Fig. 4. Proposed effect of NS-398 against inflammation and proliferation in IL-1β-stimulated VSMC. Inflammatory cytokine, such as IL-1β activates VSMC resulting in induction of COX-2. NS-398, a selective COX-2 inhibitor, reduces the production of PGE_2 by inhibitory action of COX-2. It should be noted that NS-398 did not affect on COX-2 protein expression although the mechanism of action is still elusive. At the same time, NS-398 requires IL-1β for the induction of HO-1. The produced HO-1 by either NS-398 or hemin may inhibit COX-2 activity by depletion of available heme molecules, which limits heme binding to catalytic site of the enzyme. SnPPIX, a HO-1 inhibitor, reverses effect of hemin or NS-398. Thus, NS-398 reduces not only inflammation but also proliferation by dual mechanism in IL-1β-stimulated VSMC.

[19]. Finally, we reported that phosphorylation of Akt is one of the important signals in the induction of HO-1 by NS-398 in C6 cells [17]. Thus, this possibility is still open and needs further study. Since we showed the effect of NS-398 on HO-1 induction is strictly depended on IL-1β-stimulated VSMC, whether this effect is applied to all vascular inflammatory situations is not known. However, induction of the HO-1 gene in IL-1β-activated VSMC by NS-398, a selective COX-2 inhibitor, is of physiological and pharmacological significance for several reasons. COX inhibitors are widely and effectively used in clinical practices, in particularly, for the treatment of inflammatory disorders. Because the function of HO-1 is related to its cytoprotective role, it is highly conceivable that COX inhibitors can facilitate the healing process of damaged cells by inducing HO-1 protein expression. In conclusion, NS-398 increases HO-1 protein expression by VSMC after inflammatory cytokine stimulation. This action, in conjuction with COX-2 inhibition, may be beneficial in treating vascular inflammatory disorders such as atherosclerosis.

Acknowledgments

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