

## Aprotinin Inhibits Vascular Smooth Muscle Cell Inflammation and Proliferation via Induction of HO-1

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Aprotinin is used clinically in cardiopulmonary bypass surgery to reduce transfusion requirements and the inflammatory response. The mechanism of action for the anti-inflammatory effects of aprotinin is still unclear. We examined our hypothesis whether inhibitory effects of aprotinin on cytokine-induced inducible nitric oxide synthase (iNOS) expression (IL-1 $\beta$  plus TNF- $\alpha$ ), reactive oxygen species (ROS) generation, and vascular smooth muscle cell (VSMC) proliferation were due to HO-1 induction in rat VSMCs. Aprotinin induced HO-1 protein expression in a dose-dependent manner, which was potentiated during inflammatory condition. Aprotinin reduced cytokine mixture (CM)-induced iNOS expression in a dose dependent manner. Furthermore, aprotinin reduced CM-induced ROS generation, cell proliferation, and phosphorylation of JNK but not of P38 and ERK1/2 kinases. Aprotinin effects were reversed by pre-treatment with the HO-1 inhibitor, tin protoporphyrin IX (SnPPIX). HO-1 is therefore closely involved in inflammatory-stimulated VSMC proliferation through the regulation of ROS generation and JNK phosphorylation. Our results suggest a new molecular basis for aprotinin anti-inflammatory properties.

**Key Words:** Aprotinin, Inflammation, Vascular smooth muscle cell, Proliferation, HO-1, iNOS

### INTRODUCTION

Aprotinin is a broad-spectrum serine protease inhibitor extracted from bovine lung tissue. It was initially introduced into clinical practice for the treatment of pancreatitis in the 1950s, and is widely used in cardiovascular surgery as a hemostatic agent (Westaby, 1993). The hemostatic effects of aprotinin are mediated by the preservation of platelet function and reduced fibrolysis through inhibition of multiple proteases, including plasmin, plasminogen, and plasminogen activator (Poullis et al., 2000; Landis et al., 2001). Cardiac surgery with cardiopulmonary bypass (CBP) is associated with the development of a systemic inflammatory response that frequently leads to major organ dysfunction (Landis et al., 2001). Some reports have suggested that aprotinin therapy during CBP may have anti-inflammatory effects through reduction of complement and neutrophil activation (Wachtfogel et al., 1993; Asimakopoulou et al., 2000). We examined a mechanism of action for the simultaneous anti-thrombotic and anti-inflammatory effects of aprotinin.

Heme oxygenase (HO) is the rate-limiting enzyme that catalyzes heme degradation into biliverdin, carbon monoxide (CO), and iron (Tenhunen et al., 1968). HO is a microsomal enzyme with the inducible isoform HO-1 and the constitutive forms HO-2 and HO-3 (McCoubrey et al., 1997;

Maines, 1998). The 32 kDa HO-1 protein is expressed after cellular exposure to different stimuli (Keyse and Tyrrell, 1989). HO-1 expression has been induced both *in vitro* and *in vivo* by the anti-inflammatory molecules simvastatin (Lee et al., 2004) and resveratrol (Juan et al., 2005). HO-1 has also been induced in human and rat smooth muscle cells by IL-10 in lipopolysaccharide (LPS)-activated macrophages (Lee and Chau, 2002) and by 15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub> (15-deoxy- $\Delta^{12,14}$  PGJ<sub>2</sub>) in LPS-activated macrophages (Lee et al., 2003). All HO isoforms are inhibited by the metalloprotoporphyrins zinc protoporphyrin IX (ZnPP) and tin protoporphyrin IX (SnPP) (Yoshinaga et al., 1982).

Both HO-1 over-expression and induction have been associated with anti-inflammatory events. Transgenic mice with HO-1 over-expression in the lungs protected against pulmonary inflammation (Minamino et al., 2001). A human patient with HO-1 deficiency exhibited a fatal severe phenotype of general inflammation (Yachie et al., 1999), and similar observations were noted in HO-1 null mice (Poss and Tonegawa, 1997; Kapturczak et al., 2004). HO-1 also confers significant cytoprotective effects on vascular cells. HO-1 induction in vascular cells led to increased resistance to oxidative stress, while HO-1 deficiency resulted in enhanced cell injury (Motterlini et al., 1996; Yachie et al., 1999; Clark et al., 2000). Bilirubin is a product of biliverdin

**ABBREVIATIONS:** HO-1, heme oxygenase-1; VSMC, vascular smooth muscle cells; SHR, spontaneously hypertensive rats; WKY, wistar Kyoto rats; CO, carbon monoxide; CDK, cyclin-dependent kinase; MTT, 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide thiazolyl blue; DMEM, Dulbecco's modified eagle media; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide.

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degradation, and HO-1 cytoprotective effects are dependent on bilirubin generation (Motterlini et al., 1996; Clark et al., 2000). HO-1 also modulates interactions between platelets and vessel walls. HO-1 induction in vascular smooth muscle cells (VSMC) inhibited platelet aggregation in a platelet-VSMC co-incubation system, suggesting a potentially important antithrombotic role for HO-1 (Wagner et al., 1997). HO-1-mediated effects were mediated by CO release (Johnson et al., 1995; Motterlini et al., 1998), and HO-induced CO had anti-atherosclerotic effects through inhibition of VSMC proliferation in another study (Otterbein et al., 2003).

These results suggest that HO-1 is involved in the modulation of inflammation. We examined our hypothesis that aprotinin inhibits iNOS and proliferation during inflammation in VSMCs through HO-1 gene induction. To mimic VSMC inflammation, we stimulated VSMCs with mixture of two representative cytokines (TNF- $\alpha$  and interleukin-1 $\beta$ ).

## METHODS

### Reagents

Cell culture reagents were purchased from Hyclone, acrylamide and western blot reagents were purchased from Bio-Rad, and signal transduction antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA), Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), and Upstate Biotechnology (Lake placid, NY, USA). The anti-HO-1 antibody was purchased from Stressgen Bioreagents (Victoria, BC), and the anti-iNOS antibody was purchased from BD Transduction (Franklin Lakes, NJ). PRO-PREP protein extract solution was purchased from iNtRON Biotechnology (Seongnam, Korea), the ECL western blotting detection kit was purchased from NEURONEX, and aprotinin, PD98059, SB203580, JNK inhibitor and tin protoporphyrine (SnPP) were purchased from Calbiochem (La Jolla, CA, USA). Lipofectamine 2000, IL-1 $\beta$ , and TNF- $\alpha$  were purchased from R&D Systems (Minneapolis, MN, USA), and 2',7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFHDA) was obtained from Molecular Probe (Eugene, OR). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

### Rats

Male Sprague-Dawley rats 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 the Yeungnam Medical Center.

### Cell culture

Rats were anesthetized by pentobarbital (50 mg/kg). Thoracic aortae were isolated and connective tissues were removed. VSMCs were processed using a chop setting (1 mm) in a 10 cm culture dish, and were cultured with DMEM containing 50% fetal bovine serum and 1% antibiotics (penicillin 10,000 U/ml, amphotericin B 25  $\mu$ l/ml, streptomycin 10,000  $\mu$ l/ml) and were incubated in a CO<sub>2</sub> incubator (95% O<sub>2</sub>/5% CO<sub>2</sub>, 37°C) for 7 days. Cells from pas-

sages 4 to 10 were used for the experiments.

### Cell proliferation

VSMCs were seeded at a 1 $\times$ 10<sup>4</sup> cell/ml density to measure proliferation. Cell numbers were determined after 0, 1, 2, 3, and 4 days in response to stimulation with 10% FBS by a hemocytometer. Cell viability was analyzed with the MTT assay. VSMCs were seeded on 24-well plates (1 $\times$ 10<sup>4</sup> cells/well) and were cultured for 3 days in DMEM containing 10% FBS. VSMCs were serum starved for 48 hours and stimulated with or without reagents. MTT aliquots (50  $\mu$ l of the 1 mg/ml solution) were added to each well (0.1 mg/ml) after treatment and were incubated for 4 hours. Supernatants were aspirated, crystals were dissolved in 200  $\mu$ l dimethyl sulfoxide (DMSO), and were placed in 96-well plates (100  $\mu$ l/well). Light absorbance at 570 nm was read on a microplate reader (Bio-Rad). Experiments were repeated three times in triplicate.

### Western blot analysis

Cells were lysed in the PRO-PREP protein extract solution. Samples were centrifuged at 13,000 g for 5 minutes at 4°C and protein concentrations were determined by the Bradford method. Equal volumes of 2 $\times$  sample buffer were added to supernatant fraction aliquots from lysates. The mixes were boiled for 5 minutes. Proteins (30  $\mu$ g) were loaded onto each lane and resolved by 10% SDS-PAGE for 90 minutes at 30 mA. The separated proteins were transferred to PVDF membranes (Millipore) for one hour at 100 V with a semi-dry transfer cell (Bio-Rad). Membranes were blocked with 5% skim milk in 1 $\times$  PBS and 0.05% tween 20 (PBS-T) for 60 minutes at room temperature. Membranes were incubated with antibodies against HO-1, iNOS, JNK, and p-JNK. Proteins were detected with a horseradish peroxidase-coupled secondary antibody with the ECL system.

### RT-PCR

Total RNA was extracted with the TRIzol<sup>®</sup> reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA was reverse transcribed into single-stranded cDNA with a Maxime RT premix kit (iNtRON Biotechnology, Sungnam, Korea). Samples were incubated for 10 minutes at 30°C, 30 minutes at 42°C, and 5 minutes at 99°C in a final volume of 20  $\mu$ l. RT products (1  $\mu$ l apiece) were transferred to an Accupower<sup>®</sup> PCR premixed tube using the Accupower<sup>®</sup> PCR premix kit (Bioneer Inc, Alameda CA). PCR amplification was performed with cDNAs equivalent to 10 ng of initial mRNA samples. Amplification was performed with specific oligonucleotide primers for HO-1 (forward, 5'-ACTTTCAGAAGGGTCAGGTGTCC-3' and reverse, 5' TTGAGCAGGAAGCGGTCTTAG-3'), and  $\beta$ -actin (forward, 5'-TAGGCAGGCCTCTTTTCTCA-3' and reverse 5'-AGAGGGGACCTGGGTTTAGA-3'). The cDNAs were heated for 5 minutes at 95°C. HO-1 cycling conditions included 27 cycles of amplification (94°C for 45 sec, 55°C for 1 minutes, 72°C for 1 minutes) followed by an extension step of 5 minutes at 72°C. PCR products were separated on 1.2% ethidium bromide stained agarose gels.

### Determination of nitrite concentration

Accumulated nitrite is a stable breakdown product of NO.

We used the Griess reagent to detect the presence of accumulated nitrite in the culture media. In brief, an aliquot of the cell culture medium was mixed with an equal volume of Griess reagent and incubated at room temperature for 10 minutes. The azo dye production was analyzed by spectrophotometer with absorbance set at 550 nm (Biorad microplate reader). Sodium nitrite was used as a standard.

#### Detection of ROS generation

Intracellular ROS production was measured with 2', 7'-dichlorodihydrofluorescein fluorescence using live image microscopy and fluorescence activated cell sorting (FACS) analysis. Cells were incubated in sodium acetate-free media containing 10  $\mu$ M of 2', 7'-dichlorodihydrofluorescein deacetate (H<sub>2</sub>DCFHDA) for 1 hour for live image microscopy. Intercellular fluorescence was monitored using a temperature-regulated (37°C) live image microscope (Leica, Wetzlar, Germany). Cells were incubated in sodium acetate-free medium with 10  $\mu$ M of 2', 7'-dichlorodihydrofluorescein deacetate (H<sub>2</sub>DCFHDA) for 1 hour for FACS analysis. Cells were collected by scraping after drug treatment, were washed twice with phosphate buffered saline (PBS), and were resuspended in 1 ml PBS. Emission of the trapped oxidized DCFH was analyzed on a FACS system (Becton-Dickinson).

#### Statistical analysis

Results were expressed as mean±standard error of the mean (SEM) from at least three independent experiments. Statistical significance was tested by the Mann-Whitney test using the SPSS 12.0 version for comparisons between multiple groups.

## RESULTS

### Aprotinin induces HO-1 on cytokine-stimulated VSMC

To test whether aprotinin could induce HO-1, aprotinin was treated in VSMCs. Aprotinin stimulated marked increase in HO-1 mRNA and protein levels in a concentration-dependent manner with cytokines (IL-1 $\beta$  plus TNF- $\alpha$ , I+T) (Fig. 1A, B). In contrast, treatment of VSMCs with cytokines did not stimulate HO-1 mRNA and protein compared with untreated controls. To confirm these effects of aprotinin were related with HO-1 induction, we treated with HO-1 inhibitor. HO-1 mRNA and protein augmented by increasing concentration of aprotinin (Fig. 1). In contrast, VSMC incubation with the HO-1 inhibitor SnPPIX blocked aprotinin-mediated increase in HO-1 mRNA and protein.

### Aprotinin inhibits cytokine-induced iNOS expression in VSMC through HO-1

We additionally examined the anti-inflammatory, anti-proliferative, and anti-oxidant effects of aprotinin. The effects of aprotinin on CM-induced iNOS protein expression and nitrite production were evaluated. Aprotinin significantly inhibited CM-induced iNOS protein and NO production (Fig. 2). To confirm HO-1 mediates these inhibitory effects, SnPPIX was included in this system. As expected, SnPPIX markedly blocked iNOS protein expression in CM-activated-VSMCs, suggesting that aprotinin has potential anti-inflammatory effects in VSMCs through HO-1 induction.

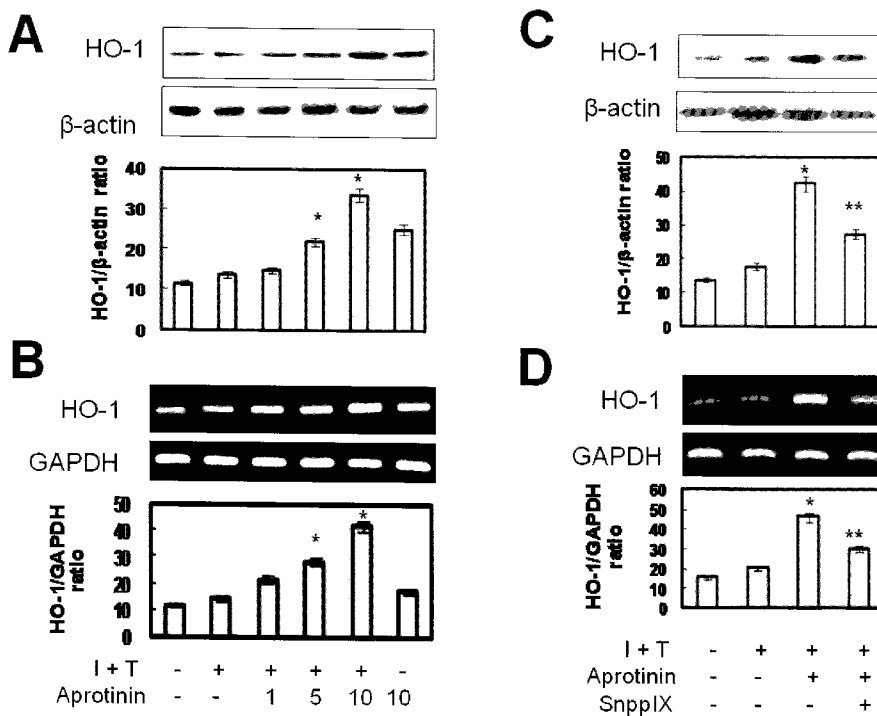
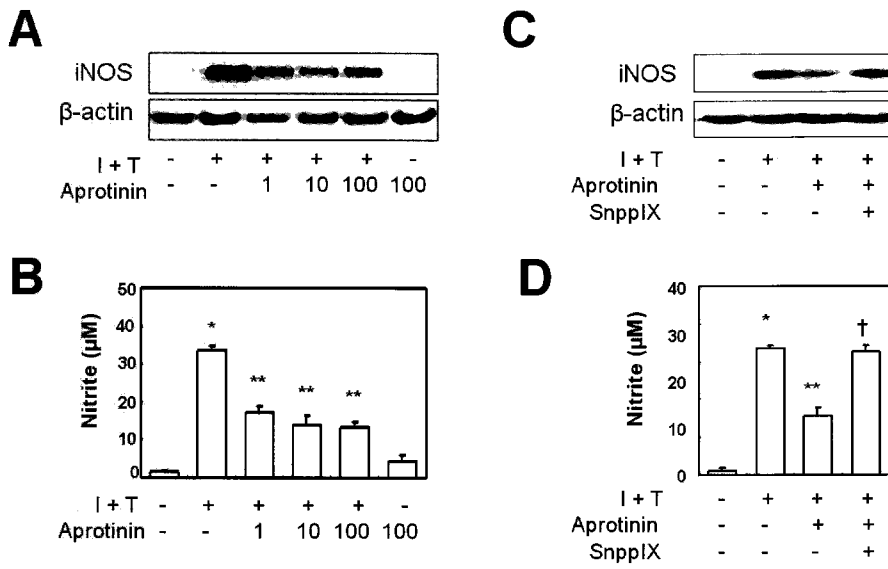
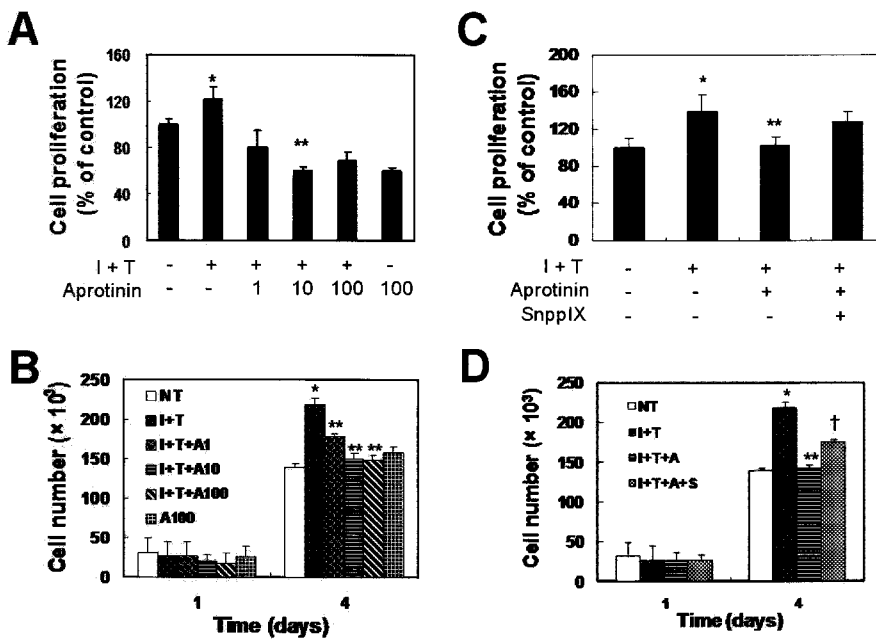


Fig. 1. Aprotinin induces HO-1 on cytokine-stimulated VSMCs. VSMCs were pre-treated with aprotinin (1~100  $\mu$ M) for 1 hr and treated with cytokine (10 ng/ml IL-1 $\beta$  plus 25 ng/ml TNF- $\alpha$ , I+T) for 12 hrs prior to measurement of HO-1 proteins by Western blotting (A, C). Cells were pre-treated with aprotinin (10  $\mu$ M) and SnPPIX (1  $\mu$ M) for 1 hr, and were treated with cytokine for 12 hrs prior to measurement of HO-1 mRNA by RT-PCR (B, D). Results represent three independent experiments. \*p value < 0.001 compared with control, \*\*p value < 0.001 compared with I+T plus aprotinin.



**Fig. 2.** Aprotinin inhibits cytokine-induced iNOS expression in VSMCs through HO-1. VSMCs were pre-treated with aprotinin (1~100 μM), were treated with cytokine (I+T) for 12 hrs prior to measurement of iNOS protein expression (A), and for 24 hrs prior to measurement of nitrite production (B). Cells were pre-treated with aprotinin (10 μM) and SnPPIX (1 μM), and were treated with cytokine for 12 hrs prior to measurement of iNOS protein expression (C) and nitrite production (D). Data represent mean±SD values of four independent experiments. \*p value<0.001 compared with control, \*\*p value<0.001 compared with I+T, †p value<0.001 compared with aprotinin.



**Fig. 3.** Aprotinin inhibits cytokine-induced VSMC proliferation through HO-1. VSMCs were pretreated with aprotinin (1~100 μM) for 1 hr, and were treated with cytokine (I+T) for 48 hrs prior to measurement of cell proliferation by the MTT assay (A) and for 4 days prior to counting cell numbers (B). Cells were pre-treated with aprotinin (10 μM) and SnPPIX (1 μM) for 1 hr, and were treated with cytokines for 48 hrs prior to measurement of cell proliferation by the MTT assay (C) and for 4 days prior to counting cell numbers (D). Data represent the mean±SD values of four independent experiments.

**Aprotinin inhibits cytokine-induced VSMC proliferation through HO-1**

The effects of aprotinin on CM-induced proliferation were evaluated by MTT assay and cell counts. Aprotinin decreased CM-induced VSMC proliferation in a concentration-dependent manner (Fig. 3). To confirm HO-1 mediates this inhibitory effect of aprotinin, we treated with SnPPIX (HO-1 competitive inhibitor). SnPPIX markedly blocked cell proliferation in VSMCs activated with CM. These findings suggested that aprotinin has potential anti-proliferative VSMC effects through HO-1 induction.

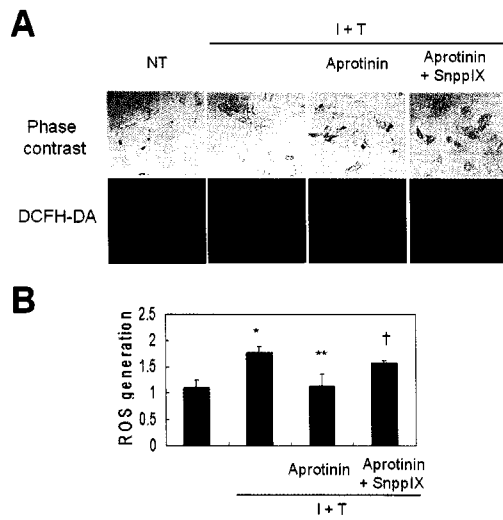
**Aprotinin inhibits cytokine-induced ROS production in VSMC through HO-1**

We demonstrated the effects of aprotinin on CM-stimulated ROS production by live image microscopy and FACS analysis. Aprotinin significantly inhibited ROS generation in the presence of CM in VSMCs (Fig. 4). We again treated with SnPPIX to confirm that HO-1 mediates effect of aprotinin. SnPPIX inhibited ROS production in CM-activated VSMCs when compared to the control. These facts suggested that aprotinin has potential anti-oxidant effects in VSMCs through HO-1 induction.

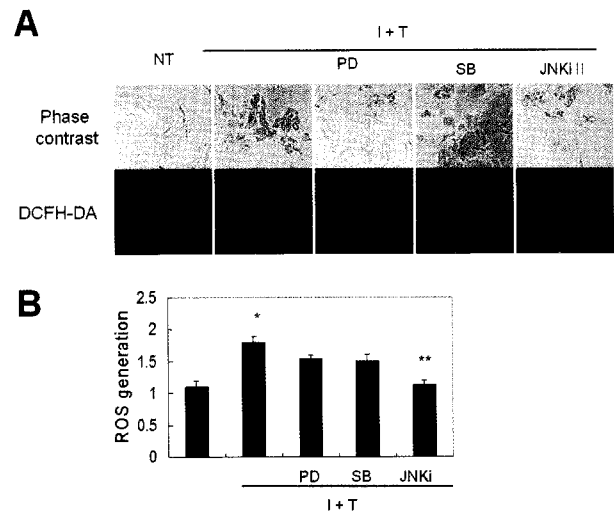
**Aprotinin inhibits cytokine-induced JNK phosphorylation through HO-1**

We investigated the association between aprotinin and mitogen-activated protein kinase (MAPKs) pathways in inflammatory responses. We examined the involvement of MAPKs pathways on iNOS protein expression, proliferation, and ROS generation in CM-activated VSMCs by treatment with the MEK inhibitor PD98059, p38 inhibitor SB203580,

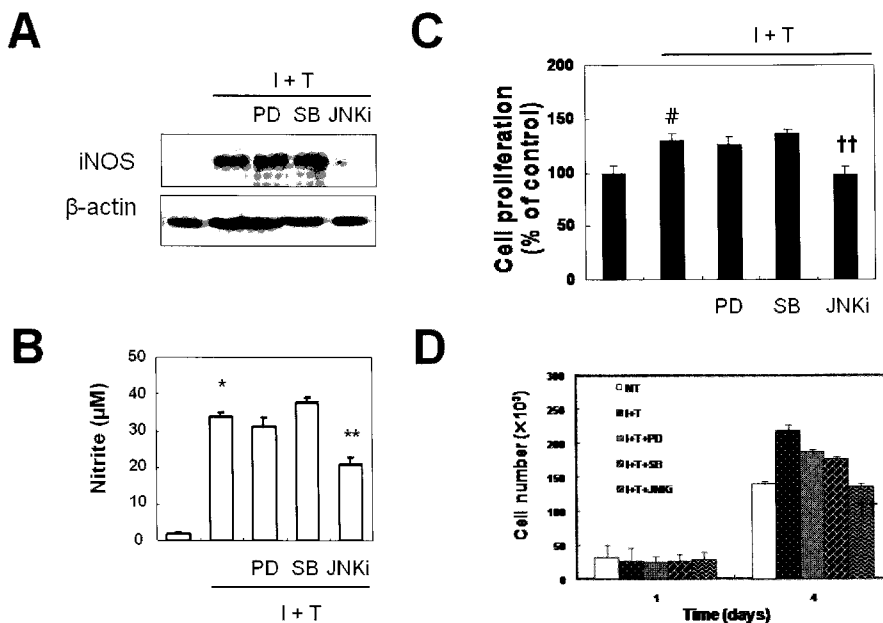
and JNK inhibitor CM-induced iNOS protein expression, nitrite production, and proliferation were blocked by the JNK inhibitor, but not by the ERK or p38 inhibitors (Fig. 5). CM-induced ROS generation was also blocked by the JNK inhibitor, which was not blocked by either ERK or p38 inhibitors (Fig. 6). When examined iNOS expression, ROS production and proliferation with JNK siRNA-transfected VSMCs, transfection of VSMC with JNK siRNA but not scrambled siRNA inhibited CM-induced iNOS protein expression, nitrite production, and proliferation (Data not



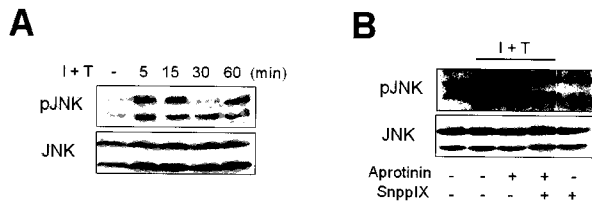
**Fig. 4.** Aprotinin inhibits cytokine-induced ROS generation in VSMCs through HO-1. VSMCs were pre-treated with aprotinin (10  $\mu$ M) and SnppIX (1  $\mu$ M) for 1 hr, and were treated with cytokine for 12 hrs prior to measurement of ROS generation by live image microscopy (A) and FACs analysis (B). ROS was detected using H<sub>2</sub>DCFHDA. Data represent the mean $\pm$ SD values of four independent experiments.



**Fig. 6.** JNK plays a role in the prevention of cytokine-induced ROS generation in VSMCs. VSMCs were pre-treated with MEK inhibitor (PD98059, 2.5  $\mu$ M), p38 inhibitor (SB203580, 10  $\mu$ M), and JNK inhibitor (10  $\mu$ M) for 1 hr, and were treated with cytokine for 12 hrs prior to measurement of ROS generation by live image microscopy (A) and by FACs analysis (B). Data represent the mean $\pm$ SD values of four independent experiments.



**Fig. 5.** JNK plays a role in the prevention of cytokine-induced iNOS expression and proliferation. VSMCs were pre-treated with the MEK inhibitor (PD98059, 2.5  $\mu$ M), p38 inhibitor (SB203580, 10  $\mu$ M), and JNK inhibitor (10  $\mu$ M) for 1 hr, and were treated with cytokines for 12 hrs prior to measurement of iNOS protein expression (A), for 24 hrs prior to measurement of nitrite production (B), for 48 hrs prior to measurement of cell proliferation by MTT assay (C), and for 4 days prior to measurement of cell proliferation by cell counts (D). Data represent the mean $\pm$ SD values of four independent experiments. \*p value < 0.0001 compared with control, \*\*p value < 0.0001 compared with I+T.



**Fig. 7.** Aprotinin inhibits cytokine-induced JNK phosphorylation through HO-1. Phosphorylated JNK was measured in cells treated with cytokines (I+T) for 1 hr by Western blotting (A). Cells were pre-treated with aprotinin (10  $\mu$ M) and SnPPiX (1  $\mu$ M) for 1 hr, and were treated with cytokines for 5 minutes prior to measurement of phosphorylated JNK by Western blotting (B). Results are representative of three experiments.

shown).

We also evaluated whether HO-1 mediate the effects of aprotinin on JNK phosphorylation. VSMCs were treated with CM and phosphorylation of JNK was examined. Phosphorylation of JNK levels peaked within 5 minutes and it declined to basal levels (Fig. 7A). Treatment with aprotinin abolished CM-induced JNK phosphorylation (Fig. 7B). In contrast, the HO-1 competitive inhibitor SnPPiX reversed the effects of aprotinin. These results suggested that aprotinin-induced HO-1 is involved in the CM-induced phosphorylation of JNK in VSMCs.

## DISCUSSION

Aprotinin is widely used as a hemostatic agent in various types of open heart surgery, and it effectively attenuates the post-bypass systemic inflammatory responses. The anti-inflammatory properties of aprotinin in CBP have been recognized for more than 10 years, although its molecular mechanisms are unclear. We demonstrated that aprotinin increased HO-1 expression through activation of JNK phosphorylation and it significantly decreased CM-induced iNOS expression in VSMCs. Aprotinin is clinically associated with decreased NO production during CBP (Hill et al., 1997).

Aprotinin-induced HO-1 may protect VSMCs from oxidative stress in inflammatory condition. However, no studies have suggested that aprotinin induces HO-1 expression during inflammatory conditions in VSMCs. We used representative proinflammatory cytokines such as IL-1 $\beta$  and TNF- $\alpha$  to mimic inflammatory condition in VSMC. They can be powerful stimulatory signals for NO production and ROS production in VSMC (Guikema et al., 2005). It has been reported that NO can induce HO-1 gene expression in VSMC (Durante et al., 1997). However, we showed that aprotinin is able to inhibit inflammatory key mediator, iNOS expression and nitrite production. Indeed, aprotinin-induced HO-1 inhibited production of NO from iNOS. We evaluated the protective effects of aprotinin against oxidative stress (cytokines) in VSMCs are due to HO-1 induction by using with SnPPiX. Anti-oxidative effects of aprotinin against CM-induced stress in VSMCs were examined with live image microscopy and FACs analysis, in which aprotinin promoted VSMC protection against oxidative stress through HO-1 dependent mechanisms.

CBP is associated with significant thrombin generation in the bypass circuit (Brister et al., 1993). Thrombin is gen-

erated in significant quantities during CBP and mediates platelet aggregation and pro-inflammatory responses. Aprotinin used clinically to reduce both transfusion requirements and the inflammatory response to bypass (Day et al., 2006). Induction of HO-1 in VSMCs inhibited platelet aggregation in a platelet-VSMC co-incubation system, suggesting that HO-1 induction in VSMCs may possess antithrombotic activity (Wagner et al., 1997). VSMCs from HO-1-deficient mice displayed enhanced DNA synthesis, enhanced growth, and greater intimal thickening than control mice (Duckers et al., 2001). HO-induced CO has anti-atherosclerotic effects through the inhibition of VSMC proliferation (Otterbein et al., 2003). Aprotinin increased HO-1 expression by activation of JNK phosphorylation and significantly decreased cytokine induced VSMC proliferation in the present study. This provides molecular evidence for an association between HO-1 induction and aprotinin-mediated inhibition of VSMC proliferation. HO-1 induction and inhibition of VSMC proliferation by aprotinin were abrogated by the SnPPiX inhibitor.

Our results suggested that aprotinin induces HO-1 mRNA and protein expression in VSMC through JNK phosphorylation. This HO-1 induction may have contributed to aprotinin's ability to inhibit VSMC proliferation and inflammation. Thus, induction of HO-1 by aprotinin may serve as a therapeutic target for the alleviation of systemic inflammatory responses, particularly vascular inflammation, during CBP.

## ACKNOWLEDGEMENTS

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