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Malabaricone C Inhibits PDGF-Induced Proliferation and Migration of Aortic Smooth Muscle Cells Through Induction of Heme Oxygenase-1

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ABSTRACT

Malabaricone C (Mal-C), isolated from nutmeg, is known to exert a variety of pharmacological activities. However, the effect of Mal-C on vascular smooth muscle cells (VSMCs) is unknown. This study examined the effect of Mal-C on proliferation and migration of primary rat aortic smooth muscle cells (RASMCs) as well as its underlying mechanisms. Treatment of RASMCs with Mal-C induced both protein and mRNA expression of heme oxygenase-1 (HO-1) in a dose- and time-dependent manner. Mal-C-mediated HO-1 induction was inhibited by treatment with actinomycin D or by cycloheximide. SB203580 (a p38 inhibitor), SP600125 (a JNK inhibitor), U0126 (a MEK inhibitor), and N-acetylcysteine (NAC, an antioxidant) did not suppress Mal-C-induced HO-1 expression. In contrast, LY294002 (a PI3K inhibitor) blocked Mal-C-induced HO-1 expression. Moreover, RASMCs treated with Mal-C exhibited activation of AKT in a dose- and time-dependent manner. Treatment of RASMCs with Mal-C increased nuclear translocation of nuclear factor-E2-related factor 2 (Nrf2), which is a key regulator of HO-1 expression, and this translocation was also inhibited by LY294002. Consistent with the notion that HO-1 has protective effects against VSMCs, Mal-C remarkably inhibited platelet-derived growth factor (PDGF)-induced proliferation and migration of RASMCs. However, inhibition of HO-1 significantly attenuated the inhibitory effects of Mal-C on PDGF-induced proliferation and migration of RASMCs. Taken together, these findings suggest that Mal-C could suppress PDGF-induced proliferation and migration of RASMCs through Nrf2 activation and subsequent HO-1 induction via the PI3K/AKT pathway, and may be a potential HO-1 inducer for preventing or treating vascular diseases. J. Cell. Biochem. 113: 2866–2876, 2012.

KEY WORDS: MALABARICONE C; HEME OXYGENASE-1; VASCULAR SMOOTH MUSCLE CELL; PROLIFERATION; MIGRATION

Abnormal vascular smooth muscle cell (VSMC) proliferation and migration play fundamental roles in the pathogenesis of vascular diseases, such as atherosclerosis and restenosis [Owens et al., 2004; Lüscher et al., 2007]. The initiation and progression of intimal thickening in arterial walls is largely due to migration and subsequent proliferation of VSMCs in the subintimal space in response to various stimuli including oxidized low-density lipoprotein, circulating growth factors, and inflammatory cytokines [Schwartz, 1997; Kher and Marsh, 2004]. Of these, PDGF, a growth factor released by vascular SMC, endothelial cells, and platelets, has been reported as the most potent inducer of VSMC migration within the injured area of the vascular wall [Raines, 2004].

Heme oxygenases (HOs) catalyze the oxidation of heme to biologically active products including carbon monoxide (CO), biliverdin, and ferrous iron. Two distinct variants of HOs have been described in humans and rodents, each encoded by a different gene:

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HO-2, which is constitutively expressed, and *HO-1*, which is potently induced in many cell types by heme, inflammatory cytokines, and oxidative stress-related factors [Maines and Panahian, 2001]. The protective actions of HO-1 likely relates to the production of its enzymatic products, biliverdin, bilirubin, and carbon monoxide (CO) [Ollinger et al., 2007; Ryter et al., 2007; Peterson et al., 2009]. HO-1 has cellular and tissue protective effects in vascular injury and disease [Idriss et al., 2008; Peterson et al., 2009]; it inhibits VSMC proliferation and migration in vitro as well as neointima formation [Morita, 2005; Nakao et al., 2005; Rodriguez et al., 2010]. HO-1 expression is regulated mainly at the transcriptional level through the signaling pathways involving phosphoinositide 3-kinase (PI3K)/AKT and mitogen-activated protein kinases (MAPKs) such as c-Jun NH2-terminal kinase (JNK), extracellular signal regulated kinase-1/2 (Erk1/2) and p38 kinase [Morse et al., 2009; Paine et al., 2010]. The transcription factor nuclear factor-E2-related factor 2 (Nrf2) plays a predominant role in HO-1 expression [Paine et al., 2010]. Recently, induction of HO-1 exerts beneficial effects on vascular injury and diseases [Chen et al., 2011; Feng et al., 2011] and inhibits PDGF-BB-induced proliferation and migration of VSMC [Kim et al., 2009; Rodriguez et al., 2010; Seidel et al., 2010; Roos et al., 2011]. Therefore, targeting of HO-1 by natural phytochemicals may be a valuable strategy for preventing or treating vascular diseases and restenosis after angioplasty.

The seed of *Myristica fragrans* Houtt (Myristicaceae), which is commonly known as nutmeg, has been used as a spice in eastern Asia. Both in vitro and in vivo studies have resulted in a wide array of pharmacological actions attributed to nutmeg including antiinflammatory [Olajide et al., 1999] and hepatoprotective [Morita et al., 2003] activities. Malabaricone C (Mal-C), which is isolated from nutmeg, has been known to have anticancer [Patro et al., 2010], antiulcer [Banerjee et al., 2008], antioxidant [Patro et al., 2005], and nematocidal activities [Hosoi et al., 1999]. However, the effect of Mal-C on VSMCs has not been studied yet.

In the present study, we investigated the effect of Mal-C on PDGF-BB-induced proliferation and migration of rat aortic smooth muscle cells (RASMCs). We found that Mal-C inhibits the proliferation and migration of RASMCs through induction of HO-1 via the activation of AKT/Nrf2 pathway.

MATERIALS AND METHODS

ISOLATION AND CULTURE OF RASMCs

The investigation is approved by the Animal Research Committee of Kangwon National University. RASMCs were isolated from the thoracic and upper parts of the abdominal aorta of 4- to 5-week-old male Spraque–Dawley rats as previously described [Wang et al., 1995] with minor modifications. Briefly, the stripped aorta was prepared from the anesthetized rat and cut into 2-mm pieces, which were treated with type II collagenase (1 mg/ml, Invitrogen, Carlsbad, CA) for 1 h to remove the endothelial cells. The de-endothelialized aortic pieces were incubated with culture medium on gelatin (0.1%)-coated culture dish for approximately 10 days. The purity of the RASMCs cultures was >95%, as confirmed by immunocytochemical staining of α-smooth muscle actin. RASMCs were prepared from three rats. Experiments were performed with cells from passages 4 through 8. The cells were cultured in DMEM supplement with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 8 mM HEPES and 2 mM glutamine at 37°C in a humidified 5% CO_2 incubator.

ISOLATION OF MALABARICONE C

Mal-C was isolated from the seeds of *M. fragrans* as described previously [Orabi et al., 1991]. Mal-C was obtained as white powder, showed a $[M]^+$ peak at m/z 358 in the ESI-MS, corresponding to a molecular formula of $C_{21}H_{26}O_5$. Its structure is shown in Figure 1. The purity of Mal-C was checked by ^1H and ^{13}C NMR spectra, and its spectra showed highly pure signals without any other impurities [Cuong et al., 2011]. Mal-C was solubilized in 100% dimethyl sulfoxide and used at a final concentration of less than 0.05% dimethyl sulfoxide.

ANTIBODIES AND REAGENTS

Recombinant rat PDGF-BB was purchased from R&D Systems (Minneapolis, MN). Antibodies for phospho-specific AKT, AKT, and poly ADP-ribose polymerase (PARP) were from Cell Signaling Technology (Beverly, MA). Antibodies for α-smooth muscle actin, HO-1, Nrf2, and glyceraldehydes 3-phosphate dehydrogenase (GAPDH) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Cyclohexamide, actinomycin D, N-acetyl-cysteine (NAC), and antibody for α-tubulin were from Sigma-Aldrich (St. Louis, MO). U0126, SB203580, SP600125, and LY294002 were purchased from Calbiochem (San Diego, California). Tin protoporphyrin IX (SnPP) and copper protoporphyrin IX (CuPP) were from Porphyrin Products Inc. (Logan, UT). DAPI (4′,6-diamidino-2-phenylindole) and goat anti-rabbit secondary AlexaFluor 546 were from Molecular Probes (Invitrogen).

SMALL INTERFERING RNA AND TRANSFECTION

Scramble small interfering RNA (siRNA), Nrf2 siRNA, HO-1 siRNA were purchased from Santa Cruz Technology. RASMCs (1×10^5) were seeded in 60 mm plates. After 12 h incubation, the cells were transfected with Nrf2 siRNA, HO-1 siRNA, or scramble siRNA using Lipofectamine Plus reagent according to the manufacturer's instructions (Invirogen). After transfection for 48 h, the cells were used for experiments.

CELL PROLIFERATION ASSAY

The proliferation of RASMCs was measured by DNA synthesis using bromodeoxyuridine (BrdU) proliferation assay kit according to the manufacturer's instructions (Millipore, Billerica, MA). In brief, both the vehicle and Mal-C-treated cells were labeled with BrdU for 4 h prior to incubation with anti-BrdU-peroxidase. The immune complex was detected following the addition of trimethyl benzidine substrate and measured at 450 nm using an ELISA reader. The number of proliferating cells is represented by the level of BrdU incorporation which directly correlates to the color intensity and the absorbance values. Cell proliferation was expressed as the % BrdU incorporation.

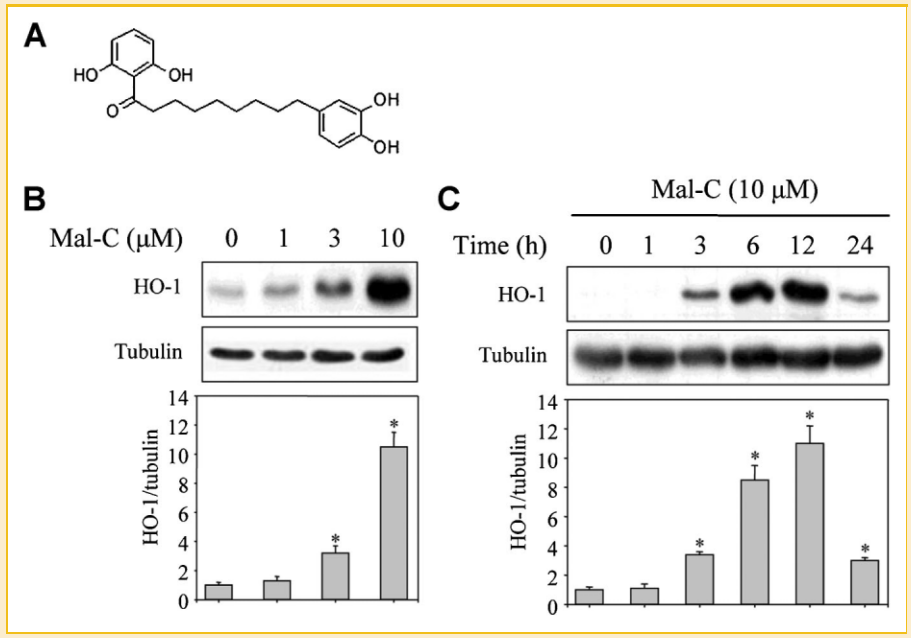

Fig. 1. Mal-C induces HO-1 protein expression in RASMCs. A: Chemical structure of Mal-C. B: RASMCs were treated for 6 h with indicated concentrations of Mal-C. The expression levels of HO-1 were determined by Western blot analysis. C: RASMCs were treated with Mal-C for indicated periods of time. The expression levels of HO-1 were determined by western blot analysis. Data are expressed as the mean ± SD of three independent experiments. $^{*}P < 0.05$ versus vehicle-treated control.

WESTERN BLOTTING

To prepare whole cell lysates, cells were lysed with a buffer [50 mM Tris-HCl (pH 7.5), 1% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, 2 mM benzamidine, 50 mM NaF, 5 mM sodium orthovanadate, and 150 mM NaCl]. Nuclear and cytoplasmic fractions were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Thermo Fisher Scientific, Rockford, IL) following the manufacturer's instruction. Equal amounts of proteins were separated onto SDS–PAGE and transferred to a Hybond-P membrane (Amersham Biosciences, Buckinghamshire, UK). Membranes were blocked with 5% skim milk at room temperature for 1 h, and then incubated for 2 h with primary antibodies. After washing, membranes were incubated with the appropriate secondary antibody conjugated to horseradish peroxidase. The signal was detected using the enhanced chemiluminescence system (Intron, Seongnam, Korea).

CELL MIGRATION ASSAYS

The effect of Mal-C on RASMCs migration was measured with a modified Boyden chamber assay as previously described [Hwangbo et al., 2010]. Briefly, RASMCs were pretreated with various concentrations of Mal-C for 4 h and washed with serum-free media for three times. The cells were seeded in triplicate at a density of 3×10^4 cells/well on the upper part of each chamber in serum-free medium containing 0.2% BSA. PDGF-BB (40 ng/ml) was added only to the lower compartment of the chamber. The chamber was incubated at 37°C in 5% CO_2 humidified atmosphere for 4 h. Cells that had migrated to the lower surface of the filter were fixed with methanol. The cells were then stained and counted in at least five randomly selected microscopic fields (× 100 magnification) per filter.

IMMUNOFLUORESCENCE AND CONFOCAL MICROSCOPY

Cells were rinsed once in PBS, fixed in fresh 4% paraformaldehyde for 5 min at room temperature, and permeabilized in 0.5% Triton X-100. Nonspecific sites were blocked by incubation with PBS containing 1% goat serum before incubating the cells with an antibody against Nrf2. After four washes in PBS, cells were incubated goat anti-rabbit secondary AlexaFluor 546 for 3 h at room temperature, washed, stained with DAPI, and mounted. Confocal images were acquired using an OLYMPUS FV1000 inverted laser scanning confocal microscope equipped with an external argon, HeNe laser Green, and HeNe laser Red. Using a UPLSAPO 60X NA1.35 oil immersion objective (OLYMPUS), images were captured at the colony midsection.

REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION (RT-PCR)

The cells were harvested and total RNA was isolated using RNeasy Mini Kits according to the manufacture's instructions (Qiagen, Santa Clarita, CA). Two microgram of total RNA was used to synthesis 1st stranded cDNA using RT-PCR kit (Invitrogen, Carlsbad, CA). For amplification of rat HO-1, the following primers were used: HO-1, 5′-CAT CTC CTT CCA TTC CAG AG-3′(sense) and 5′-CTG CTA GCC TGG TTC AAG ATA-3′(antisense). The cDNA for GAPDH was also amplified as a control in a similar way using the following primers: 5′-AAT GCA TCC TGC ACC ACC AAC TGC-3′ (sense) and 5′-GGA GGC CAT GTA GGC CAT GAG GTC-3′ (antisense). For PCR

amplification, the following conditions were used: 94°C for 5 min for one cycle, and then 94°C for 1 min, 56°C for 30 s and 72°C for 1 min for 25 cycles. The amplified PCR products were separated with 1.5% agarose gel, and then stained with EtBr.

ANNEXIN-V AND PROPIDIUM IODIDE DOUBLE STAINING ASSAY

Annexin-V and propidium iodide (PI) labeling for cell death detection was carried out using an Annexin-V-FLUOS staining kit according to the manufacturer's instructions (BD Biosicences, San Jose, CA). Briefly, RASMCs were treated with various concentrations of Mal-C for 24 h. The cells were then trypsinized, collected, rinsed twice with cold PBS, resuspended in 100 μl of a binding buffer, and incubated with 5 μl FITC-labeled annexin-V and 5 μl PI (stock solution 50 μg/ml) for 20 min in the dark at room temperature. The RASMCs were analyzed by FACS Calibur (Becton & Dickinson Co.) and the percentage of annexin-V-positive staining was determined.

STATISTICAL ANALYSIS

Data are expressed as the mean ± SD, unless otherwise specified. Statistical significance was assessed by two-tailed unpaired Student's *t*-test and $P < 0.05$ was considered statistically significant.

RESULTS

Mal-C INDUCES HO-1 EXPRESSION IN RASMCs

To investigate the effect of Mal-C on RASMCs, we first determined whether Mal-C induces HO-1 expression. RASMCs were treated with increasing concentrations of Mal-C for 6 h and expression of HO-1 was determined by western blot analysis (Fig. 1B). Mal-C increased the expression level of HO-1 in a dose-dependent manner. A time course experiment of HO-1 induction with 10 μM Mal-C revealed that HO-1 protein was increased 3 h after treatment and that its level continued to increase steadily even at 12 h (Fig. 1C).

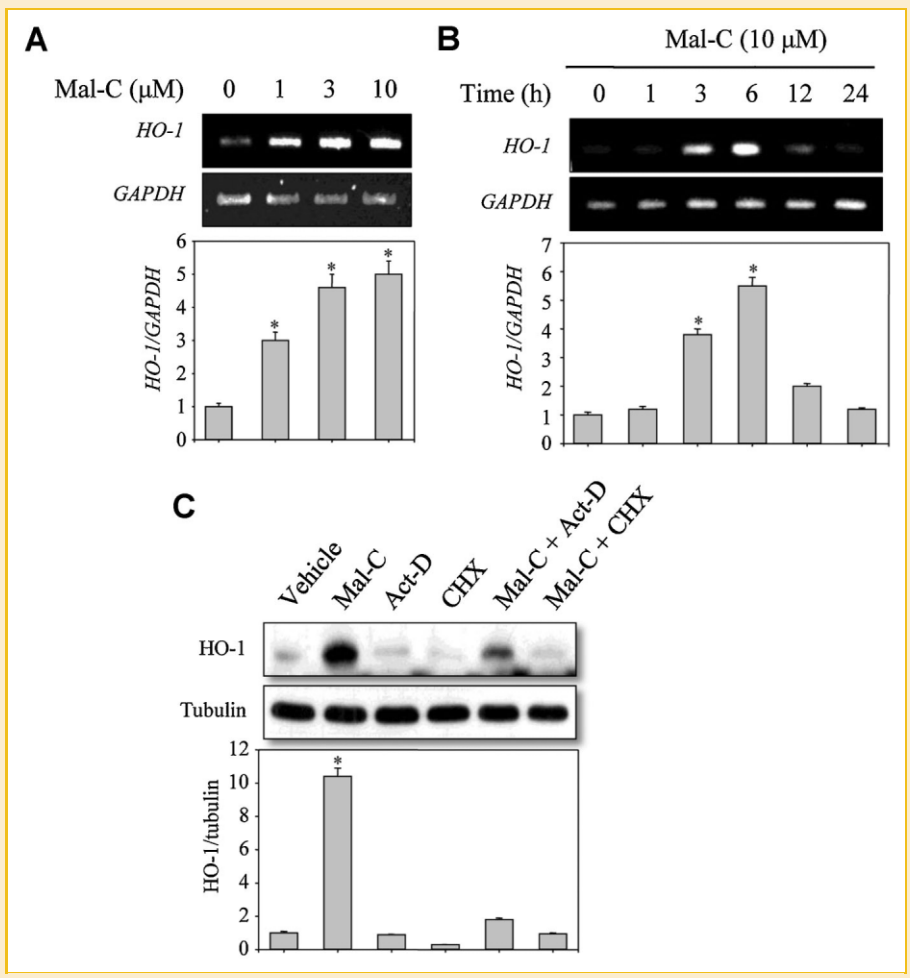

Fig. 2. Mal-C up-regulates HO-1 expression at transcriptional level. A: RASMCs were treated for 3 h with indicated concentrations of Mal-C. The mRNA expression levels of HO-1 were determined by RT-PCR analysis. B: RASMCs were treated with Mal-C for indicated periods of time. The mRNA expression levels of HO-1 were determined by RT-PCR analysis. C: RASMCs were treated with Mal-C (10 μM) alone or in the presence of actinomycin D (Act-D, 50 ng/ml), or cyclohexamide (CHX, 100 ng/ml) for 6 h. The expression levels of HO-1 were determined by western blot analysis. Data are expressed as the mean ± SD of three independent experiments. $^{*}P < 0.05$ versus vehicle-treated control.

Mal-C INDUCES HO-1 EXPRESSION AT THE TRANSCRIPTIONAL LEVEL

We next investigated whether Mal-C induced the expression of HO-1 at the transcriptional level. Treatment of RASMCs with increasing concentrations of Mal-C for 3 h increased the mRNA expression level of HO-1 in a dose-dependent manner, as assessed by RT-PCR analysis (Fig. 2A). At 10 μM Mal-C, a time course experiment of HO-1 induction revealed that the expression level of HO-1 mRNA was increased by 3 h and reached a peak at 6 h, and then this induction returned to the basal levels 12 h after treatment (Fig. 2B).

We then attempted to determine the effect of actinomycin D and cycloheximide on Mal-C-mediated HO-1 expression (Fig. 2C). Cotreatment of RASMCs with Mal-C and actinomycin D, a transcriptional inhibitor, significantly suppressed Mal-C-mediated HO-1 expression, confirming that Mal-C may induce HO-1 expression at the transcriptional level. Cycloheximide, a translational inhibitor, also blocked HO-1 expression induced by Mal-C. These results suggested that Mal-C induced the expression of HO-1 at the transcriptional level.

Mal-C INCREASES EXPRESSION OF HO-1 VIA ACTIVATION OF THE AKT PATHWAY

Since it is known that HO-1 expression is regulated mainly at the transcriptional level through the signaling pathways involving AKT and MAPKs such as c-Jun NH_2-terminal kinase (JNK), extracellular signal regulated kinase-1/2 (Erk1/2) and p38 kinase [Morse et al., 2009; Paine et al., 2010], we determine whether inhibition of AKT or MAPKs affected Mal-C-mediated HO-1 induction (Fig. 3A,B). Cotreatment of U0126 (a MEK inhibitor), SB203580 (a p38 inhibitor), or SP600125 (a JNK inhibitor) failed to modulate both Mal-C-induced mRNA and protein expression of HO-1. In contrast, LY294002 (a PI3K inhibitor) completely blocked Mal-C-induced mRNA and protein expression of HO-1, suggesting that the PI3K/AKT pathway could play a critical role in Mal-C-mediated HO-1 induction. We also determined whether NAC, an antioxidant, modulates Mal-C-induced HO-1 expression (Fig. 3C). Cotreatment of RASMCs with Mal-C and NAC did not change HO-1 expression induced by Mal-C, suggesting that oxidative stress is not involved in the induction of HO-1 by Mal-C.

Since LY294002 blocked Mal-C-induced HO-1 expression, we focused our study on the role of PI3K/AKT in HO-1 induction. We determined whether Mal-C induced AKT activation. A time course experiment of AKT phosphorylation with 10 μM Mal-C revealed that phosphorylation of AKT was increased within 15 min and returned to basal levels 120 min after treatment (Fig. 4A). Treatment of RASMCs with Mal-C for 30 min increased phosphorylation levels of AKT in a dose-dependent manner (Fig. 4B). These results suggested Mal-C may mediate HO-1 induction through the activation of AKT.

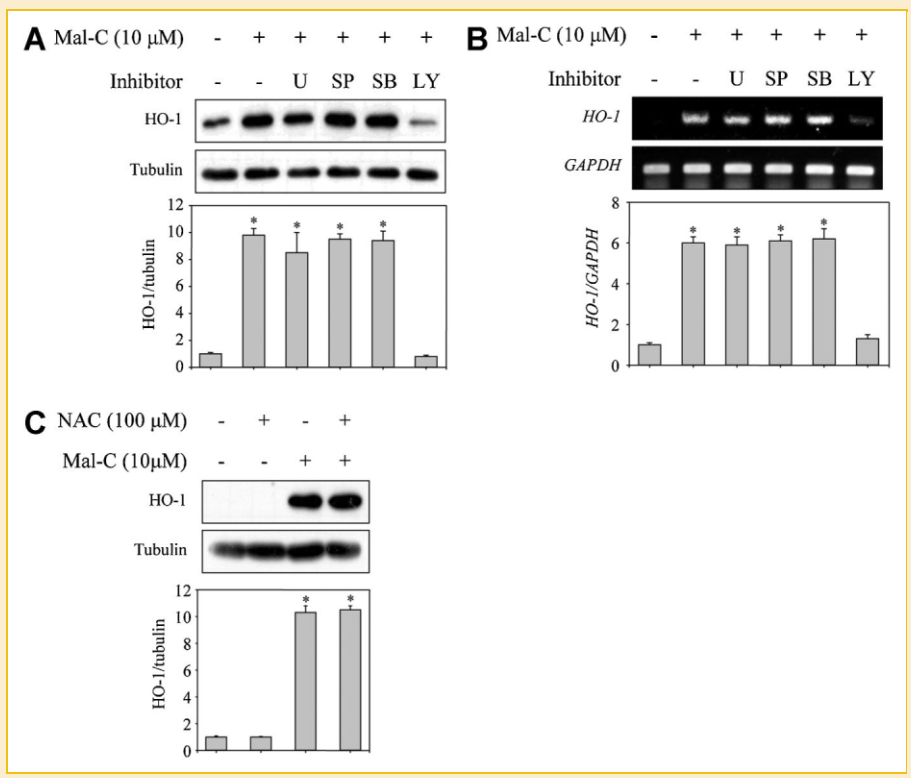

Fig. 3. Mal-C increases expression of HO-1 via activation of the AKT pathway. A, B: RASMCs were treated with Mal-C alone or in the presence of U0126 (U, 10 μM), SP600125 (SP, 10 μM), SB203580 (SB, 10 μM), or LY294002 (LY, 10 μM). The protein and mRNA expression levels of HO-1 were determined by western blot (A) and RT-PCR (B) analyses, respectively. C: Effect of N-acetylcysteine (NAC) on Mal-C-induced HO-1 expression. RASMCs were treated for 6 h with Mal-C alone or in the presence of NAC (100 μM). The protein expression levels of HO-1 were determined by western blot analysis. Data are expressed as the mean ± SD of three independent experiments. $^{*}P < 0.05$ versus vehicle-treated control.

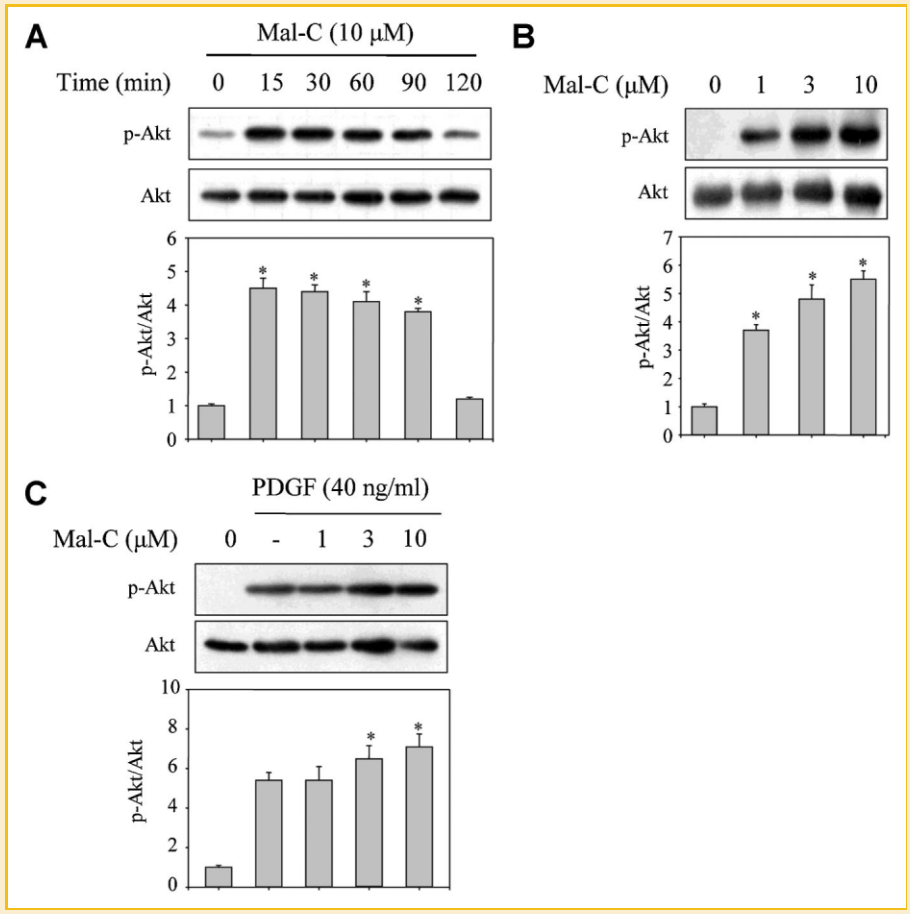

Fig. 4. Mal-C induces AKT activation. A: RASMCs were treated with Mal-C for indicated periods of time. B: RASMCs were treated for 30 min with indicated concentrations of Mal-C. C: Serum-starved RASMCs were stimulated with PDGF-BB for 15 min in the presence of the indicated concentrations of Mal-C. The expression levels of p-AKT were determined by western blot analysis. Data are expressed as the mean ± SD of three independent experiments. $^{*}P < 0.05$ versus vehicle-treated control.

We next determined whether Mal-C affects PDGF-BB-induced activation of AKT. Cotreatment of RASMCs with Mal-C and PDGF increased the level of AKT phosphorylation (Fig. 4C).

Mal-C INDUCES ACTIVATION OF Nrf2

Nrf2 has been reported to play a central role in the induction of HO-1 [Alam et al., 1999]. Therefore, we examined whether Mal-C can activate Nrf-2 in RASMCs. Treatment of RASMCs with Mal-C increased the nuclear translocation of Nrf2 in a dose-dependent manner, whereas LY294002 blocked this translocation (Fig. 5A). We confirmed this result with immunofluorescence, where Mal-C again induced nuclear translocation of Nrf2 and LY294002 blocked Mal-C-induced nuclear translocation of Nrf2 (Fig. 5B). We next examined whether knockdown of Nrf2 impaired Mal-C-mediated HO-1 induction (Fig. 5C). Transfection of RASMCs with control siRNA did not suppress Mal-C-induced HO-1 expression. In contrast, Nrf2-targeted siRNA significantly suppressed this induction. Taken together, these results suggested that Mal-C induces HO-1 expression through the activation of Nrf2 via activation of the PI3K/AKT pathway.

Mal-C INHIBITS PDGF-BB-INDUCED PROLIFERATION AND MIGRATION OF RASMCs

Since Mal-C was able to induce HO-1 expression, we next determined the effect of Mal-C on the proliferation and migration of RASMCs. Stimulation of RASMCs with 40 ng/ml of PDGF-BB potently increased BrdU incorporation into cells (Fig. 6A). Mal-C significantly inhibited PDGF-BB-induced BrdU incorporation in a concentration-dependent manner. The percentage of inhibition at concentrations of 1, 3, 10 μM was 25.8 ± 3.2% ($P < 0.05$), 56.9 ± 2.4% ($P < 0.01$) and 99.3 ± 1.5% ($P < 0.01$), respectively. To determine the effect of Mal-C on PDGF-BB-induced migration, RASMCs were pretreated with Mal-C for 4 h to induce HO-1 and migration assays were conducted. Mal-C also potently inhibited PDGF-BB-induced migration of RASMCs in a dose-dependent manner (Fig. 6B).

In order to verify if the induction of cell death occurred during Mal-C treatment, the presence of apoptotic or necrotic cells was determined by double staining with annexin-V and PI. Flow cytometric assays revealed that the proportions of apoptotic or necrotic cells did not significantly increase in Mal-C treated RASMCs up to 10 μM (Fig. 6C). Taken together, these results

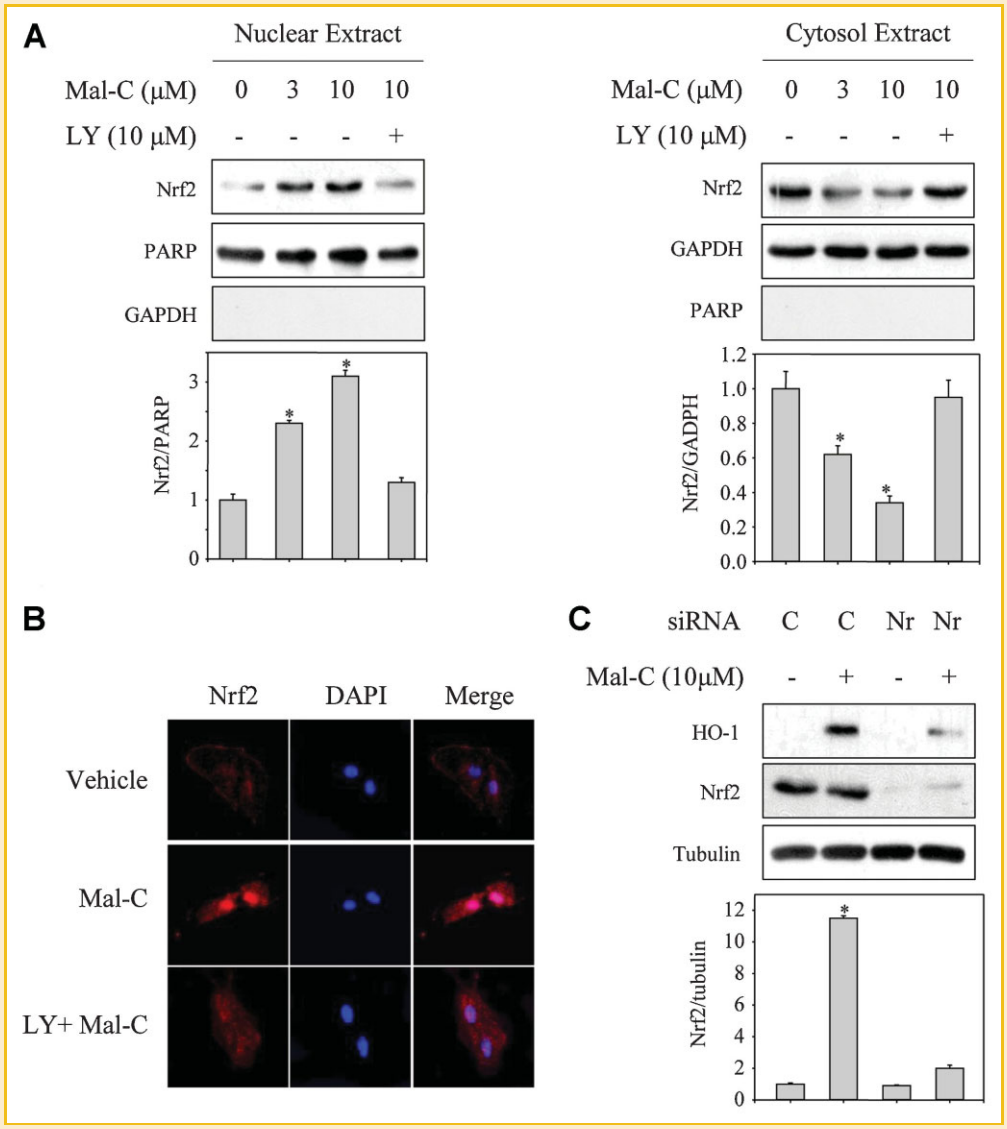

Fig. 5. Mal-C induces HO-1 expression via Nrf2 activation. A: RASMCs were treated for 2 h with indicated concentrations of Mal-C alone or in the presence of LY294002 (LY). Nuclear and cytosol extracts were subjected to western blot analysis to determine the level of Nrf2. PARP was used as the nuclear marker and GAPDH as the cytosol protein marker. Data are expressed as the mean ± SD of three independent experiments. B: RASMCs were treated for 2 h with Mal-C (10 μM) alone or in the presence of LY294002 (LY, 10 μM). Cells were immunostained with Nrf2 antibody. DAPI stains nuclei of cells. C: RASMCs transfected with the control siRNA (C) or Nrf2-targeted siRNA (Nr) were treated with Mal-C for 6 h. Whole cell lysates were subjected to western blot analysis to determine the expression levels of HO-1. $^{*}P < 0.05$ versus vehicle-treated control. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

suggested that Mal-C suppresses PDGF-BB-induced proliferation and migration of RASMC without inducing cell death.

INHIBITION OF HO-1 ATTENUATED THE INHIBITORY EFFECTS OF Mal-C ON PROLIFERATION AND MIGRATION OF RASMCs

We examined whether Mal-C-mediated HO-1 induction could be responsible for the inhibitory effects of Mal-C on the proliferation and migration of RASMCs. To test this, we utilized a specific HO-1 siRNA. Transfection of RASMCs with HO-1 siRNA significantly suppressed Mal-C-induced HO-1 expression (Fig. 7A). Inhibition of HO-1 by siRNA significantly reversed Mal-C-mediated suppression of PDGF-BB-induced proliferation and migration of RASMCs (Fig. 7B,C). In contrast, scrambled siRNA showed no effect. Similar results were obtained with SnPP, a specific HO-1 inhibitor, that dramatically reversed Mal-C-mediated suppression of PDGF-BB-induced proliferation and migration of RASMCs (Fig. 7D,E). In contrast, CuPP, an inactive compound, showed no effect. Taken together, these results suggest that Mal-C-mediated HO-1 induction may be responsible for the inhibitory effects of Mal-C on the proliferation and migration of RASMCs.

DISCUSSION

The abnormal growth of VSMCs is a prominent feature of vascular disease, including atherosclerosis and postangioplasty restenosis

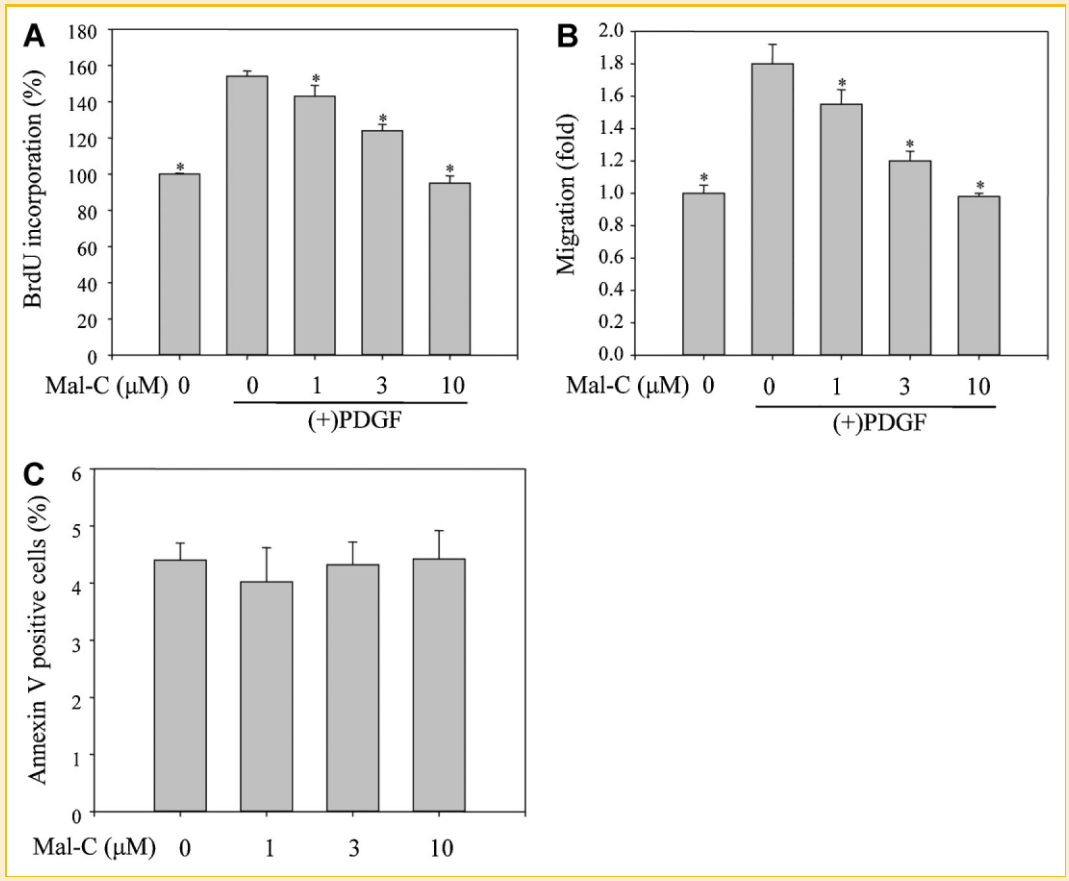

Fig. 6. Mal-C suppresses the proliferation and migration of RASMCs. A: RASMCs were serum-starved for 24 h, and then stimulated with PDGF-BB (40 ng/ml) in the presence of the indicated concentrations of Mal-C for 24 h. The incorporation of BrdU was determined. B: RASMCs were pretreated with indicated concentrations of Mal-C for 4 h. The cells were washed with serum-free medium three times and then subjected to migration assays in the presence of PDGF-BB (40 ng/ml). C: RASMCs were treated with indicated concentrations of Mal-C for 24 h, and subsequently stained with annexin-V-FITC and PI, followed by analysis using a flow cytometer. The percentages of apoptotic cells were indicated by annexin-V positive cells. Data are expressed as the mean ± SD of three independent experiments. $^{*}P < 0.05$ versus vehicle-treated control.

[Ross, 1993]. Neointimal thickening is mainly due to VSMCs, which proliferate and migrate from the vascular media. Inhibition of VSMC proliferation and migration represents a potentially important therapeutic strategy for the treatment of cardiovascular diseases [Schwartz, 1997]. In the present study, we investigated the inhibitory effect of Mal-C, a natural compound isolated from nutmeg, on PDGF-BB-induced proliferation and migration, and its underlying mechanism in cultured RASMCs. To our knowledge, we have shown for the first time that Mal-C inhibited PDGF-BB-induced proliferation and migration of RASMCs via induction of HO-1 through the activation of Nrf2.

We showed that Mal-C inhibited the proliferation and migration of RASMCs through induction of HO-1. HO-1 may be involved in the pathophysiology of atherosclerosis and targeting HO-1 may be useful for vascular disease [Durante, 2010]. HO-1 is upregulated in atherosclerotic lesions [Wang et al., 1998; Cheng et al., 2009]. Overexpression of HO-1 significantly attenuates the development of atherosclerosis in apoE-deficient mice, whereas the absence of HO-1 aggravates atherosclerotic lesion formation compared with wild-type mice [Juan et al., 2001; Yet et al., 2003]. Additionally, HO-1 plays an important role in maintaining vascular homeostasis by regulating migration and proliferation of VSMCs [Guan et al., 2008; Loboda et al., 2008]. Several lines of evidence have been shown that pharmacological induction of HO-1 has therapeutic effect against vascular disease. For example, sulfasalazine suppresses VSMC growth and prevents neointimal hyperplasia via HO-1 induction [Kim et al., 2009]. Ginkgo biloba extract exerts its anti-atherogenesis and vascular protective effects by inducing vascular HO-1 expression [Chen et al., 2011]. Oleanolic acid protected VSMCs from hydrogen peroxide-induced apoptotic cell death by upregulation of HO-1 [Feng et al., 2011]. In this study, we also showed that Mal-C is a potent inducer of HO-1 expression in VSMCs. Thus, Mal-C may be a useful compound for the treatment of vascular diseases such as atherosclerosis and restenosis after angioplasty.

The HO-1 gene can be transcriptionally activated by Nrf2, nuclear factor-kappa B, and activator protein-1 [Paine et al., 2010]. Among them, Nrf2 is considered to play a major role in HO-1expression. Under basal conditions, the Kelch-like ECH-associated protein (Keap1) binds to Nrf2 and sequesters it in the cytoplasm, which results in a lower accumulation of Nrf2 in the nucleus and reduced transcription of the HO-1 gene [Itoh et al., 1999; Kang et al., 2004; Cho et al., 2006]. Oxidation of redox-sensitive cysteines within

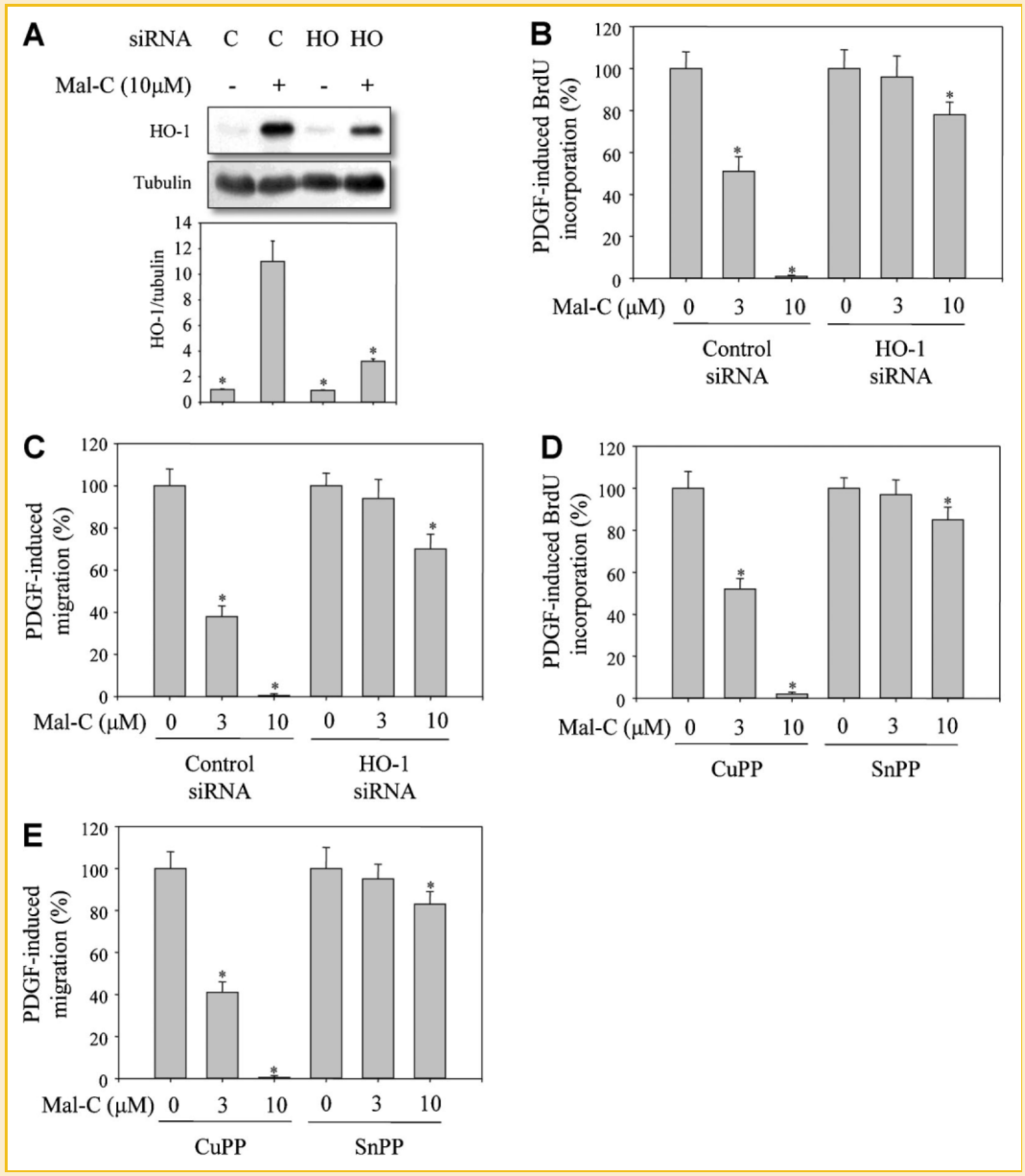

Fig. 7. Inhibition of HO-1 attenuates the inhibitory effects of Mal-C on the proliferation and migration of RASMCs. A: RASMCs transfected with the control siRNA (C) or HO-1-targeted siRNA (HO) were treated with Mal-C for 6 h. Whole cell lysates were subjected to western blot analysis to determine the expression levels of HO-1. B: RASMCs transfected with siRNA as described in (A) were stimulated with PDGF-BB (40 ng/ml) in the presence of Mal-C for 24 h. The incorporation of BrdU was determined. C: RASMCs transfected with siRNA as described in (A) were pretreated with indicated concentrations of Mal-C for 4 h. The cells were washed with serum-free medium three times and then subjected to Transwell migration assays in the presence of PDGF-BB (40 ng/ml). D: RASMCs treated with indicated concentrations of Mal-C with SnPP (10 μM) or CuPP (10 μM) were stimulated with PDGF-BB (40 ng/ml) for 24 h. The incorporation of BrdU was determined. E: RASMCs were treated with indicated concentrations of Mal-C for 4 h. The cells were washed with serum-free medium three times and then PDGF-induced migration was determined in the presence of SnPP (10 μM) or CuPP(10 μM). Data are expressed as the mean ± SD of three independent experiments. $^{*}P < 0.05$ versus vehicle-treated control.

Keap1 releases Nrf2, and Nrf2 then translocates from the cytosol to the nucleus and binds to HO-1 gene. In the present study, we showed that NAC, a potent antioxidant, did not block Mal-C-mediated induction of HO-1, suggesting that reactive oxygen species are not involved in this induction. PI3K/AKT also serves as an important signaling pathway in the induction of HO-1. Several lines of evidence have shown that HO-1 expression is regulated by a PI3K/AKT-Nrf2-dependent pathway. For example, HO-1 promoter activity is preceded by an increase in AKT phosphorylation, while AKT knockdown with siRNA nearly suppresses HO-1 expression by blocking Nrf2 activation in VSMCs [Brunt et al., 2006]. Carnosol and fluvastatin induce HO-1 expression through the activation of Nrf2 via the PI3K/AKT pathway [Martin et al., 2004; Makabe et al., 2010]. In this study, we also showed that Mal-C induced the activation of AKT in RASMCs and inhibition of AKT by a PI3K inhibitor completely blocked Mal-C-induced both HO-1 expression and Nrf2

activation, further suggesting that HO-1 expression is regulated by PI3K/AKT-Nrf2 pathway.

It has been known that HO-1 suppressed PDGF-stimulated proliferation and migration of VSMCs [Kim et al., 2009; Rodriguez et al., 2010; Seidel et al., 2010; Roos et al., 2011]. For example, adenovirus-mediated expression of HO-1 in RASMC inhibits PDGF-induced migration via inhibition of Nox1 [Rodriguez et al., 2010]. We found that Mal-C increased the levels of HO-1 mRNA and protein expression in RASMCs. Moreover, inhibition of HO-1 by siRNA or a HO inhibitor significantly abrogated the inhibitory effects of Mal-C on PDGF-BB-induced proliferation and migration of RASMCs, indicating that these inhibitory effects of Mal-C were mediated by HO-1. In addition, although the present study was conducted in cell culture, these findings should provide some useful molecular mechanisms for the beneficial effects of Mal-C in vascular diseases. In the future, we will test whether these observations can be confirmed in in vivo experiments.

Activation of PI3K/AKT plays a pivotal role in cell migration, proliferation, and anti-apoptotic events in various types of cells including VSMCs [Goncharova et al., 2002; Muto et al., 2007] and inhibition of PI3K/AKT has been shown to inhibit PDGF-induced proliferation and migration of VSMCs [Yellaturu et al., 2002; Park et al., 2010]. However, we showed that Mal-C inhibited PDGF-mediated proliferation and migration of RASMCs through HO-1 induction via activating PI3K/AKT. Moreover, Mal-C increased PDGF-BB-induced phosphorylation of AKT. These findings raise intriguing questions as to how Mal-C inhibits PDGF-induced proliferation and migration of VSMCs although it activates the PI3K/AKT pathway. Fluvastatin induces HO-1 expression through the upregulation of Nrf2 via the PI3K/AKT pathway and inhibits PDGF-induced proliferation of human smooth muscle cells [Nègre-Aminou et al., 1997; Skaletz-Rorowski et al., 2003; Makabe et al., 2010]. Our results also showed that Mal-C induced HO-1 through the activation of Nrf-2 via the PI3K/AKT pathway and inhibited PDGF-induced proliferation and migration of RASMCs. It has been known that HO-1 and its enzymatic product carbon monoxide (CO) suppressed PDGF-stimulated signaling pathways [Rodriguez et al., 2010]. Thus, it is likely that one of the signaling mechanisms by which PI3K/AKT activates Nrf2 may induce HO-1 expression, which in turn suppresses PDGF-induced proliferation and migration of RASMCs. Further studies are required to assess the role of HO-1 in controlling PDGF-stimulated signaling pathways.

In summary, our results demonstrate that Mal-C leads to the activation of the PI3K/AKT pathway, which is involved in Nrf2 nuclear localization and subsequent upregulation of HO-1 expression. Additionally, induction of HO-1 by Mal-C may inhibit PDGF-BB-induced proliferation and migration of VSMCs. Therefore, Mal-C may be a potential candidate for preventing or treating vascular diseases and restenosis after angioplasty.

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