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Promotion of Endothelial Wound Healing by the Chalcones 4-hydroxyderricin and Xanthoangelol, and the Molecular Mechanism of This Effect

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The two chalcones xanthoangelol (XA) and 4-hydroxyderricin (4HD) are major functional polyphenolic compounds in the edible herb *Angelica keiskei*, which is native to Japan. The compounds XA and 4HD are known to have anti-inflammatory and anti-diabetic functions although their beneficial effect on vascular disease is not clear. Atherosclerosis induced by lifestyle-related diseases such as obesity and hypertension is a serious vascular disease in which endothelial injury plays a major pathogenic role. Therefore, the healing of endothelial injury is considered important in preventing atherosclerosis. The present study examined whether XA and 4HD promote the wound healing of cultured porcine vascular endothelial cells (ECs) and analyzed the molecular mechanisms of their effect. Both compounds promoted endothelial wound healing, induced nitric oxide (NO) production, and increased heme oxygenase-1 (HO-1) expression as well as the phosphorylation level of endothelial NO synthase (eNOS). The wound healing promoted by these compounds was inhibited by pretreatment with the NOS inhibitor L-NMMA and HO-1 inhibitor ZnPPIX, respectively. ZnPPIX inhibited the phosphorylation of eNOS as well. XA- and 4HD-dependent wound healing was also blocked by hemoglobin, a carbon monoxide (CO) absorbent. A CO-releasing molecule facilitated the wound healing, which was suppressed by pretreatment with L-NMMA. These results suggest that XA and 4HD stimulate wound healing by increasing HO-1 expression with subsequent CO production, which activates eNOS followed by NO generation. Our findings indicate that the two chalcones in *Angelica keiskei* may have a preventative effect against atherosclerosis.

Key words: *Angelica keiskei*, anti-oxidation, chalcones, endothelial cells, wound healing

Introduction

The two chalcones xanthoangelol (XA) and 4-hydroxyderricin (4HD) are major functional polyphenolic compounds in the edible herb *Angelica keiskei*, which is native to Japan. These two compounds are known to have various beneficial effects. They exhibit antihypertensive, anticancer, and diuretic effects (Enoki *et al*., 2007; Kimura and Baba, 2003; Kimura, 2005; Motani *et al*., 2008). The compounds also have a suppressive effect on blood glucose level (Shimizu *et al*., 1999; Matsuura *et al*., 2001), an antiulcer effect and an inhibitory effect on gastric secretion (Murakami *et al*., 1990), a beneficial effect on lipid metabolism

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(Ogawa *et al*., 2007), and an antibacterial effect (Inamori *et al*., 1991). However, their effect on preventing atherosclerosis is not clear. Accumulating evidence demonstrates that other polyphenolic compounds, such as resveratrol in red wine and epigallocatechin gallate in green tea, are effective in preventing atherosclerosis (Iijima *et al*., 2000; Iijima *et al*., 2002; Miura *et al*., 2001; Lorenz *et al*., 2004). Especially, resveratrol is believed to play a role in the unexpectedly low rates of heart disease and associated mortality in France, where a typical diet including dairy product and meat has a high fat content. Resveratrol has a number of beneficial effects, including protection of the endothelium from oxidized low-density lipoprotein (Ou *et al*., 2006), and anticancer and neuroprotective effects (Brisdelli *et al*., 2009). Epigallocatechin gallate was demonstrated to promote nitric oxide (NO) generation followed by vasodilation (Lorenz *et al*., 2004) and to prevent atherosclerosis in a mouse model (Miura *et al*., 2001).

Heart disease and cerebrovascular disease are major causes of death and account for approximately 25% of mortality in Japan. These diseases are caused by atherosclerosis, which is induced by lifestyle-related disease called metabolic syndrome such as obesity, diabetes, and hypertension. Metabolic syndrome is linked to the increase in reactive oxygen species (ROS) levels, which lead to endothelial injury. Monocytes and T-lymphocytes adhere to the injuries, eventually creating atheromas which result in vascular occlusion. Endothelial cells (ECs) adjacent to the wound repair the wounded area through the migration and/or proliferation of these cells. Since the delay in wound healing causes thrombus formation and vascular hyperpermeability leading to atherosclerosis, endothelial wound repair is a fundamental step in the prevention of atherosclerosis.

A number of endogenous molecules are involved in endothelial wound healing. NO generated by endothelial NO synthase (eNOS) acts as an endothelium-derived relaxing factor. NO also contributes to the proliferation and migration of ECs, and elicits cytoprotective and atheroprotective effects in the vasculature (Cooke, 2003; Gewaltig, 2002; Kibbe *et al*., 1999). Kuhlencordt *et al*. (2001) demonstrated that endothelial NO suppresses atherosclerosis and that atherosclerosis progresses rapidly in eNOS-knockout mice. Thus, regulating NO production is considered pivotal for endothelial wound healing. Heme oxygenase-1 (HO-1) is an enzyme that catabolizes heme and biliverdin into carbon monoxide (CO) and free iron. CO generated by HO-1 was indicated to phosphorylate eNOS and prevent hypertension via NO production (Wegiel *et al*., 2010; Zuckerbraun *et al*., 2006; Rodella *et al*., 2008).

In the present study, we aimed to evaluate the effect of XA and 4HD on endothelial wound healing, and to investigate the molecular mechanism of this effect, focusing on NO, eNOS, and HO-1.

Materials and Methods

1. Materials

Mixture of chalcones was perchased from Japan Bio Science Laboratory CO.,Ltd. and XA and 4HD were purified from the mixture in our laboratory. Hydroxytyrosol (HT) was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Lysophospatidic acid (LPA), Carbon monoxide-releasing molecules (CORM), N^G-methyl-$_L$-arginine acetate salt (L-NMMA) and hemoglobin were purchased from Sigma-Aldrich (Missouri, USA). Zinc (II) Protoporphyrin IX (ZnPPIX) was perchased from Merck Millipore (Darmstadt, Germany).

2. Cell culture

ECs were isolated from porcine pulmonary arteries and grown in Dulbecco's modified Eagle's medium (DMEM) at 37℃ in a humidified 5% CO_2 incubator. The DMEM containing low glucose was supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. ECs were analyzed between passages six to ten.

3. Wound-healing assay

ECs were seeded onto 3.5-cm plates at a density of 3×10^5 cells/plate and incubated for 7 h with DMEM containing 10% FBS. Cell culture medium was then replaced with DMEM containing 1% FBS and the ECs were incubated for another 16 h. Then the ECs were treated with test substances (4HD, XA, and CORM) and positive control (LPA). In wound-healing assays with inhibitors, L-NMMA, ZnPPIX or hemoglobin was applied 1 h before the addition of test substances and positive control. A wound was then created by scratching the monolayer of confluent, serum-starved ECs with a 200-μl pipette tip. The scratch area was imaged using an inverted microscope attached to a digital camera (Leica DM IBRE) immediately (0 h) and at 8 and 24 h after wound creation. The plate was incubated at 37℃ from wound creation to the 8- and

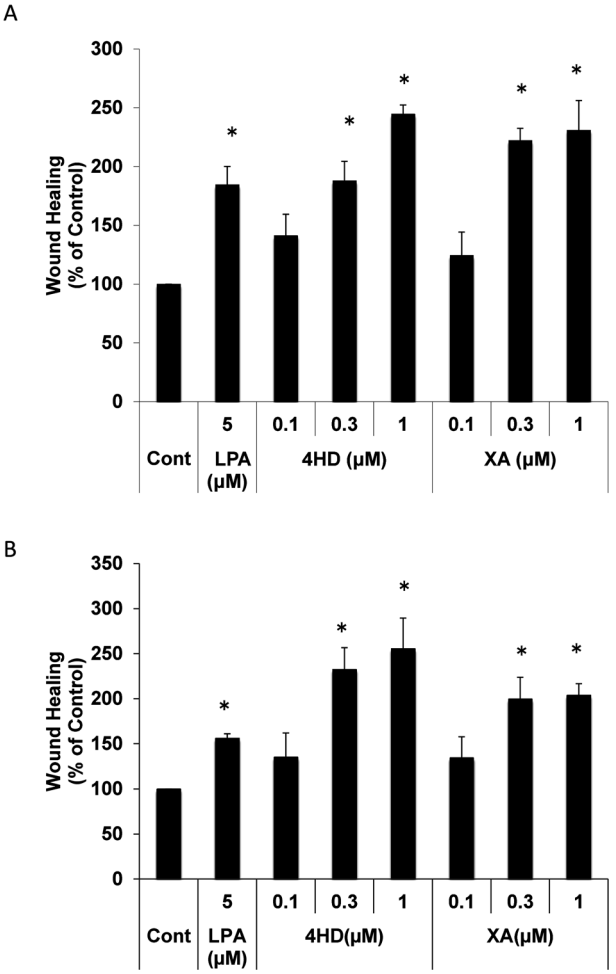

Fig. 1. 4HD and XA promote endothelial wound healing. Wound healing was determined 8 h (A) and 24 h (B) after wound creation in the presence and absence of the treatment of LPA, 4HD, and XA. LPA was used as a positive control. *$p<0.05$ vs. Cont.

24-h. Three microscopic fields were selected at random from the microscopic images at each time, and the number of cells in the fields was counted. Wound healing (%) was calculated as the value for each test substance or positive control versus the control valued at each time.

4. NO production assay

NO production was measured by fluorometric detection using DAF-2 DA, a cell-permeable and NO-sensitive fluorescent dye. ECs were seeded onto a 96-well plate (at 6×10^3 cells/well) and incubated with 10 μM DAF-2 DA in a dark condition for 45 min at 37℃. After washing the ECs with Hank's balanced salt solution (HBSS), NO production was stimulated by the addition of HBSS containing 4HD, XA or HT. Fluorescence intensities were measured using a spectrofluorophotometer (Powerscan HT, Dainippon Pharmaceutical, Tokyo, Japan) at excitation and emission wavelengths of 485 and 535 nm, respectively, after 1, 3, 6, and 12 h of treatment.

5. Western blot analysis

ECs were seeded onto 3.5-cm plates at a density of 2×10^5 cells/plate, and incubated for 7 h with DMEM containing 10% FBS. Cell culture medium was then replaced with DMEM containing 1% FBS and the cells were incubated for 16 h. After required treatment, ECs were washed twice with ice-cold PBS and suspended in a lysis buffer containing 50% glycerol, 100 mM NaF, 10 mM sodium pyrophosphate, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/mL antipain, 10 μg/mL leupeptin, and 10 μg/mL aprotinin. Lysates were separated by centrifugation at 17,900 g for 15 min at 4℃ and used for the experiment. Equal amounts of proteins per sample were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were then blocked with 5% non-fat dried milk or 5% BSA/TBS-T at room temperature and subsequently incubated overnight with antibodies at 4℃. Thereafter, blots were incubated with secondary antibodies at room temperature. Bands were detected using a Western Lightning™ Chemiluminescence Kit (Life Technologies Corporation, Santa Cruz, CA, USA) in accordance with the manufacturer's instructions. The intensity of each band was quantified using a LAS-4000 imaging mini analyzer (Fujifilm, Tokyo, Japan).

6. Statistical analysis

Data are expressed as mean values (±S.D.) for at least three independent experiments. Differences between treatments were analyzed using Student's t-test. P values less than 0.05 were considered statistically significant.

Results

1. 4HD and XA promote endothelial wound healing. We evaluated the effect of 4HD and XA on endothelial wound healing at 8 and 24 h after wound creation. The wound healing at both 8 h and 24 h was maximally promoted by 4HD and XA at a concentration of 1 μM (Fig. 1). 4HD tended to be more effective than XA in repairing the wound.

2. 4HD and XA increase endothelial NO production.

We evaluated the effect of 4HD and XA on endothelial NO production to investigate the mechanism for their promotion of wound healing. 4HD and XA time-dependently increased endothelial NO production at all tested concentrations (0.1, 0.3, and 1.0 μM) respectively, except for 0.1 μM XA (Fig. 2). The NO production level of 4HD tended to be more effective than XA. We used 4HD and XA at 1 μM for subsequent experiments, because the highest NO production was obtained at 1 μM.

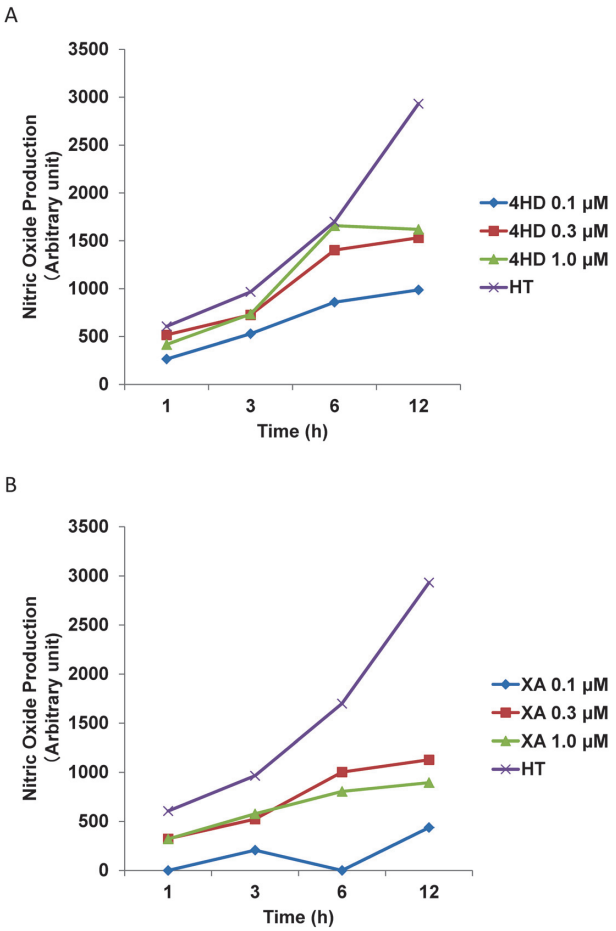

Fig. 2. 4HD and XA increase endothelial NO production. NO was quantified at 1, 3, 6, and 12 h after addition of 4HD (A) or XA (B). HT was used as the positive control.

3. 4HD and XA activates eNOS.

We evaluated the phosphorylation level of eNOS in 4HD- and XA-treated ECs to investigate whether the two chalcones activate eNOS. We targeted phosphorylation of eNOS at serine 1177 (Ser1177) because Ser1177 is one of the activation sites (Kukreja and Xi, 2007; Mount *et al*., 2007) and phosphorylated by various kinases including Akt, protein kinase A, and AMP-activated kinase (Fulton *et al*., 1999). Phosphorylated eNOS (Ser1177) level increased time-dependently from 3 h after addition of 4HD and from 6 h after addition of XA (Fig. 3). This effect of prolonged chalcone treatment on eNOS activation is consistent with the result of NO production shown in Fig. 2, suggesting that 4HD- and XA-induced NO production is linked to eNOS activation.

4. NO mediates 4HD- and XA-stimulated endothelial wound healing.

We investigated the effect of the NO inhibitor L-NMMA on 4HD- and XA-dependent wound healing. Pretreatment of ECs with 1 mM L-NMMA suppressed the wound healing promoted by 4HD and XA (Fig. 4), indicating that NO production mediates chalcone-enhanced endothelial wound healing.

5. 4HD and XA increase endothelial HO-1 protein expression.

We investigated HO-1 protein expression levels in 4HD- and XA-treated ECs to evaluate the role of HO-1 in chalcone-enhanced endothelial wound healing. HO-1 protein expression level increased time-depend-

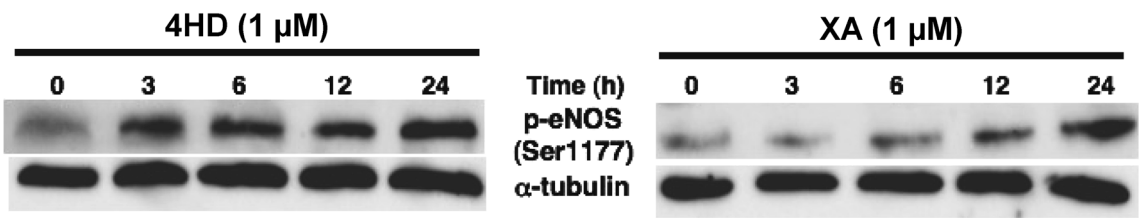

Fig. 3. 4HD and XA activate eNOS. Phosphorylated eNOS (p-eNOS) (Ser1177) levels in ECs were determined by western blotting after treatment with 4HD or XA for 0, 3, 6, 12, and 24 h. α-Tubulin was used as the internal standard.

ently from 3 h after addition of 4HD and from 6 h after addition of XA (Fig. 5), suggesting that HO-1 is likely to be involved in the promotion of wound healing by these chalcones.

6. HO-1 is involved in 4HD- and XA-dependent endothelial wound healing and eNOS phosphorylation.

We examined the effect of the HO-1 inhibitor ZnPPIX on 4HD- and XA-induced wound healing. Pretreatment of ECs with ZnPPIX inhibited wound healing (Fig. 6A). We also evaluated phosphorylated eNOS levels in 4HD- and XA-treated ECs after pretreatment with ZnPPIX. The inhibitor suppressed chalcone-induced eNOS phosphorylation (Fig. 6B). These data suggest that HO-1 is critically implicated in 4HD- and XA-stimulated endothelial wound healing by activating eNOS.

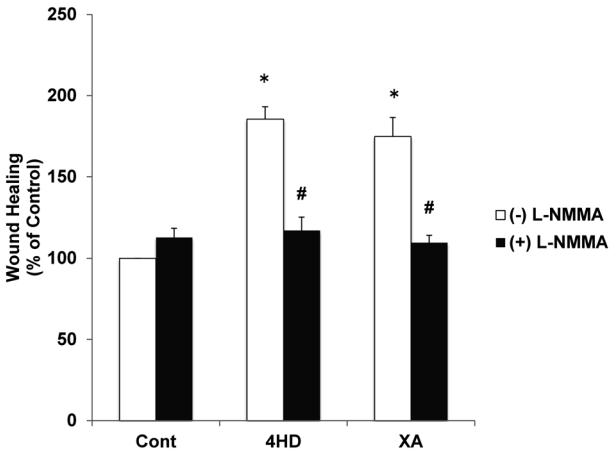

Fig. 4. NO mediates 4HD- and XA-promoted endothelial wound healing. Wound healing was determined 8 h after wound creation for LPA, 4HD, and XA-treated cells with and without pretreatment with 1 mM L-NMMA. $^{*}p<0.05$ vs. Cont(−); $^{\#}p<0.05$ vs. 4HD(−) or XA(−).

7. HO-1-dependent generation of CO activates eNOS.

We investigated the effect of CORM, a CO-releasing molecule, on wound healing to know whether HO-1-induced generation of CO plays an important role in wound healing. CORM promoted wound healing in a dose-dependent manner at 8 and 24 h after wound creation (Fig. 7). The eNOS inhibitor L-NMMA suppressed CORM-dependent wound healing (Fig. 8). hemoglobin (Hb), a CO absorbent, inhibited 4HD- and XA-related wound healing (Fig. 9). These data suggest that HO-1-dependent production of CO critically contributes to the wound healing induced by these chalcones through eNOS activation.

Discussion

In the present study, we investigated the effect of the chalcones 4HD and XA on endothelial wound healing. We found that both chalcones promote the wound healing of cultured ECs by increasing the expression of HO-1, which generates CO followed by the activation of eNOS with subsequent NO production.

The dysfunction of ECs induced by various stresses including oxidative stress is critically implicated in the initiation and progression of atherosclerosis. Therefore, the compounds that promote endothelial wound healing are strong candidates for anti-atherogenic factors. We have already found more than ten such candidates. They include resveratrol and its dimer ε-viniferin in red wine (Zghonda *et al*., 2011), HT in olive oil (Zrelli *et al*., 2011), carnosic acid in rosemary, and caffeoylquinic acid and chlorogenic acid in coffee. All of them are polyphenolic compounds and generate NO by activating eNOS, which is consistent with the present data of the chalcones. Interestingly, resveratrol, ε-viniferin, HT, carnosic acid raised HO-1 expression as 4HD and XA whereas caffeoylquinic acid and chlorogenic acid did not, suggesting that eNOS activation mechanism may be different, de-

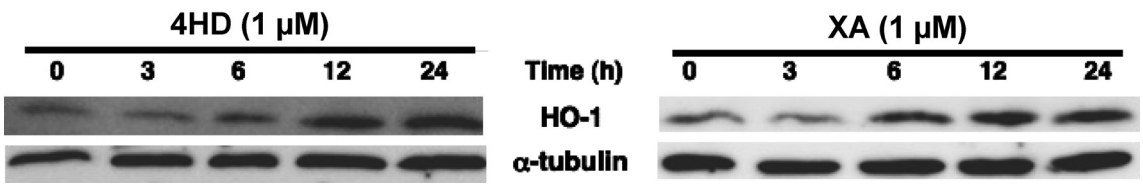

Fig. 5. 4HD and XA increase endothelial HO-1 protein expression. HO-1 expression levels in ECs were determined by western blotting analysis after treatment with 4HD or XA for 0, 3, 6, 12, and 24 h. α-Tubulin was used as the internal standard.

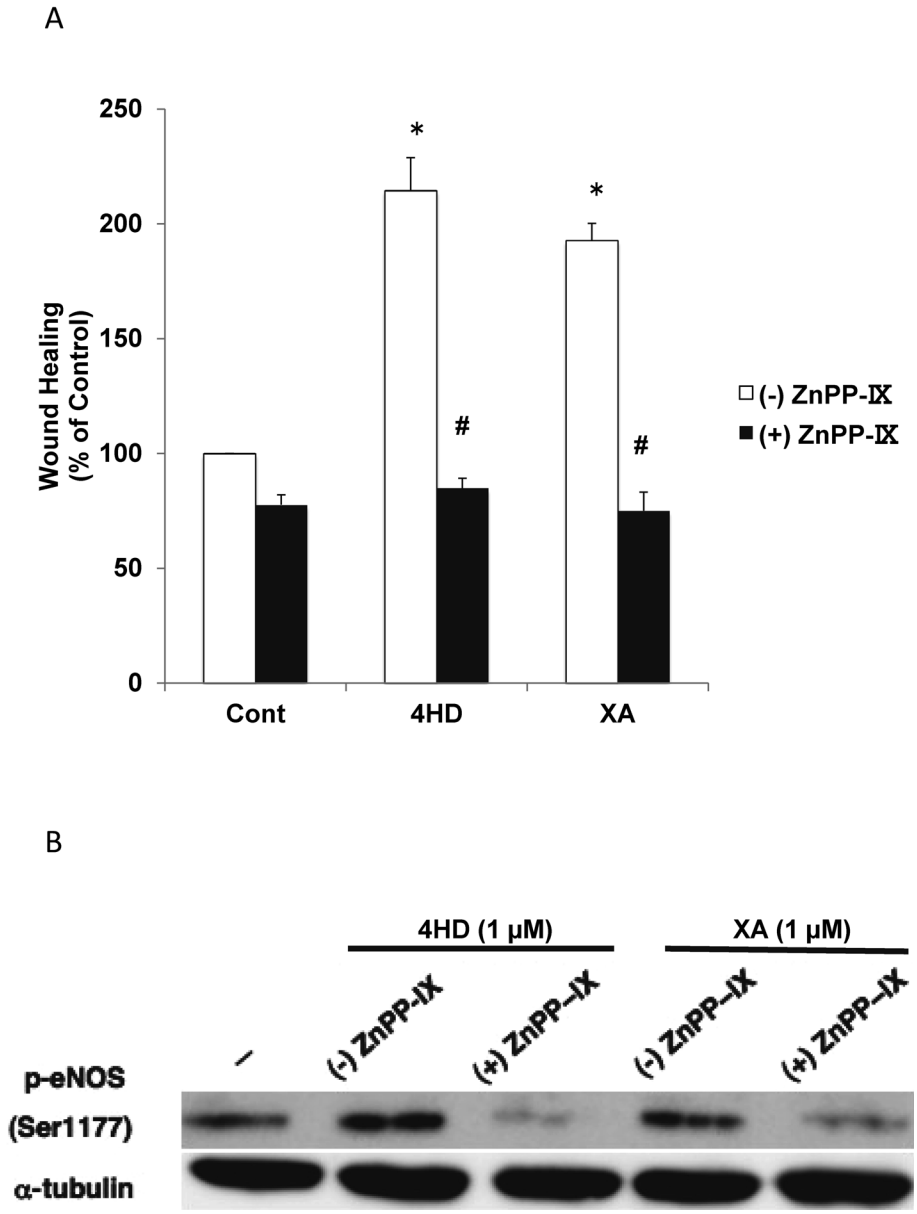

Fig. 6. HO-1 is involved in 4HD- and XA-dependent endothelial wound healing and eNOS phosphorylation. (A) Wound healing was determined 8 h after wound creation for 4HD- and XA-treated ECs with and without pretreatment with 1 μM ZnPPIX. $^{*}p<0.05$ vs. Cont(−), $^{\#}p<0.05$ vs. 4HD(−) or XA(−). (B) p-eNOS (Ser1177) levels in ECs were determined by western blotting after treatment with 4HD or XA for 5 h with and without pretreatment with 1 μM ZnPPIX.

pending on the polyphenolic compound (data not shown).

Our data demonstrate that 4HD- and XA-dependent increase in HO-1 expression and phosphorylation level of eNOS with subsequent NO production is sustained. In contrast, although acetylcholine induces vasorelaxation by activating eNOS followed by NO production via the PI3 kinase/Akt signaling pathway, this effect is short-lived (Zecchin *et al*., 2007; Seto *et al*., 2010). We consider that natural polyphenolic compounds including 4HD and XA obtained from normal dietary may be more effective in sustaining endothelial wound healing and NO generation than endogenous substances such as acetylcholine. In agreement with

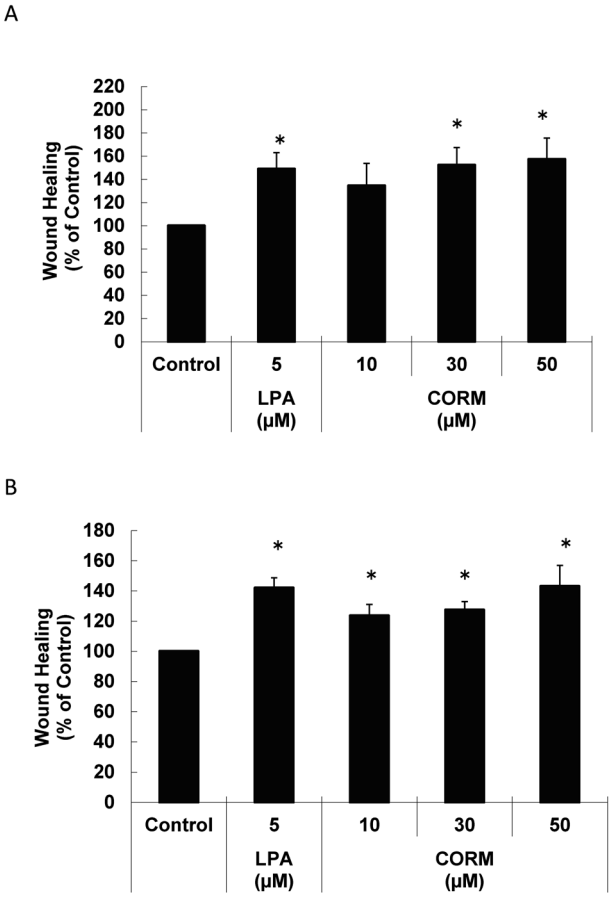

Fig. 7. CORM promotes endothelial wound healing. Wound healing was determined 8 h (A) and 24 h (B) after wound creation for LPA- and CORM-treated cells. LPA was used as a positive control. $^{*}p<0.05$ vs. Cont.

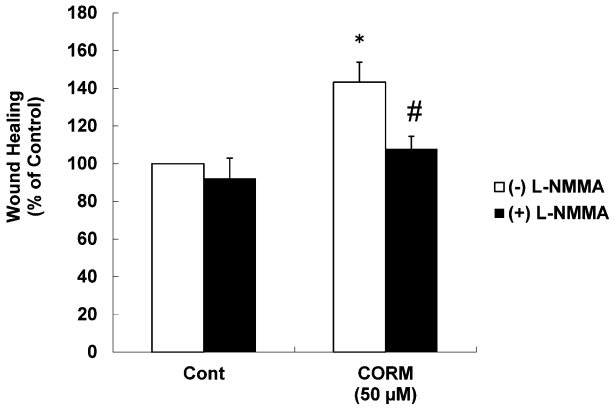

Fig. 8. L-NMMA suppresses CORM-dependent wound healing. Wound healing was determined 8 h after wound creation for CORM (50 μM)-treated ECs with and without pretreatment with 1 mM L-NMMA. $^{*}p<0.05$ vs. Cont(−), $^{\#}p<0.05$ vs CORM(−).

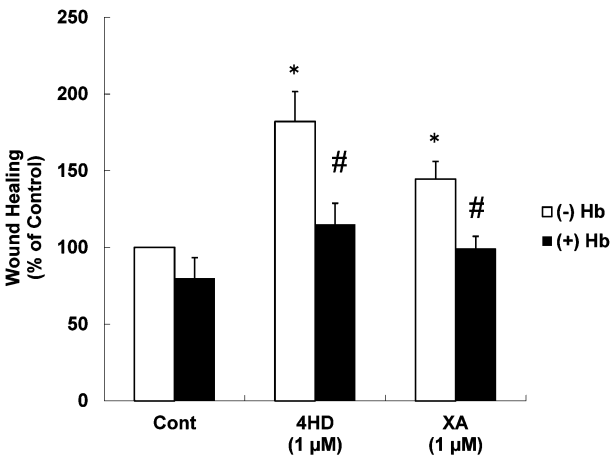

Fig. 9. Hemoglobin inhibits 4HD- and XA-dependent wound healing. Wound healing was determined 8 h after wound creation for 4HD- and XA-treated ECs with and without pretreatment with 10 μM Hb. $^{*}p<0.05$ vs. Cont(−), $^{\#}p<0.05$ vs. 4HD(−) or XA(−).

our thought, there are many reports indicating that natural polyphenolic compounds including 4HD and XA reduce blood pressure (Ogawa *et al*., 2005; Zghonda *et al*., 20112).

Conclusions

We investigated two chalcones, 4HD and XA, for their potential to promote wound healing. These chalcones are present in the edible herb *Angelica keiskei*. The prevention of various diseases through diet has received much attention recently. Prompt healing of endothelial injury is important for the prevention of atherosclerosis. 4HD and XA are known to have some beneficial health effects, but their efficacy against atherosclerosis is still unclear. Therefore, the present study aimed to elucidate the effects of 4HD and XA on *in vitro* endothelial wound healing and understand the molecular mechanism of their effects.

We found that the chalcones 4HD and XA promote wound healing by increasing HO-1 expression with subsequent CO production, which activates eNOS followed by NO generation. Our findings suggest that these two chalcones in *Angelica keiskei* may have a preventative effect against atherosclerosis.

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