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ORIGINAL ARTICLE

Antioxidant activity of caffeoyl-prolyl-histidine amide and its effects on PDGF-induced proliferation of vascular smooth muscle cells

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Abstract Caffeic acid (CA) is one of the antioxidants found in plants, which protects vascular cells against vascular injuries from oxidative stress. In our previous study, caffeoyl-prolyl-histidine amide (CA-L-Pro-L-His-NH_2; CA-PH; a CA derivative) was synthesized, which exhibited a strong antioxidant activity with sufficient stability. In this study, we investigated the role of CA-PH in vascular smooth muscle cells (VSMCs) and confirmed the enhanced antioxidant activity of CA-PH compared with that of CA. In in vitro tube assays, CA-PH showed a higher free-radical-scavenging activity and lipid-peroxidation-inhibition activity than those of CA. In VSMCs, CA-PH significantly reduced hydrogen peroxide-induced ROS generation and increased the expression of heme oxygenase-1. Moreover, CA-PH effectively inhibited the platelet-derived growth factor-induced cellular proliferation of VSMCs, which was confirmed by a decrease in the expression of the proliferating cell nuclear antigen and the phosphorylation of Akt.

Keywords Caffeoyl-prolyl-histidine amide · Antioxidant · Vascular smooth muscle cells · Heme oxygenase-1

Introduction

Oxidative stress, including the production of intracellular reactive oxygen species (ROS), has been implicated in vascular injury leading to atherosclerosis, hypertension, restenosis, and vasospasm (Irani 2000; Kreuzer et al. 2003; Madamanchi et al. 2005). ROS participate in the regulation of vascular activities such as vascular proliferation, migration, apoptosis, inflammation, modification of the extracellular matrix, and pro-coagulant activity (Clempus and Griendling 2006; Gennaro et al. 2004). For instance, the platelet-derived growth factor (PDGF), a mitogen which is thought to be involved in atherogenesis, stimulates the production of hydrogen peroxide (H_2O_2) in vascular smooth muscle cells (VSMCs) and promotes growth and migration of the cells (Jackson et al. 1993; Lewis et al. 2001).

Antioxidant treatment is considered as a potential therapy to reduce the risk of cardiovascular diseases, since VSMC growth is redox-sensitive and oxidative stress stimulates the proliferation of these cells. It is well established that polyphenolic compounds isolated from herbal extracts can play significant roles in the prevention of atherosclerosis. As caffeic acid (CA) is one of the most abundant phenolic antioxidants present in plant foods, numerous studies have shown the protective effects of CA and its derivatives in cardiovascular diseases (Li et al. 2005; Roos et al. 2011; Suzuki et al. 2002).

Peptide conjugation to bioactive molecules is a simple and very efficient approach to modulate the inherent biological activities of those molecules by altering their

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chemical and physical properties (Kwak et al. 2012). This approach has negligible safety concerns, as peptides are generally nontoxic and are considered to be inert to the immune system. Caffeoyl-prolyl-histidine amide (CA-L-Pro-L-His-NH_2; CA-PH) is a strong antioxidant screened among a small set of library of caffeoyl dipeptide derivatives in the previous studies (Seo et al. 2010). However, underlying antioxidant mode of action of CA-PH remains unclear, especially in cell systems. This study was designed to demonstrate the enhanced antioxidative activity of CA-PH compared with caffeic acid (CA), both in an in vitro tube test and in cell systems. We investigated the antioxidant mechanism of CA-PH and examined the vascular protective role of CA-PH in rat aortic VSMCs after stimulation with PDGF.

Materials and methods

Reagents

Fmoc-Rink amide linker coupled to aminomethyl polystyrene (Rink amide AM; 100–200 mesh, 0.82 mmol/g) resin, filtered reactors (5 ml, Libra tube RT-5 M), benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP), hydroxybenzotriazole (HOBt), Fmoc-L-Pro-OH, and Fmoc-L-His(Trt)-OH were purchased from BeadTech (Seoul, Korea). Fmoc-D-Pro-OH, Fmoc-Hyp-OH, Fmoc-dehydroPro-OH, and Fmoc-D-His(Trt)-OH were purchased from Novabiochem (Darmstadt, Germany). Diisopropylethylamine (DIPEA) was bought from Alfa Aesar (Massachusetts, USA). Caffeic acid (CA), ninhydrin, linoleic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and recombinant rat PDGF-BB were bought from Sigma (St. Louis, USA). Ammonium thiocyanate (NH_4SCN), ferrous chloride ($FeCl_2$), and polyoxyethylenesorbitan monolaurate (Tween 20) were bought from Aldrich (St. Louis, USA). *N*-Methyl-2-pyrrolidone (NMP), piperidine, dichloromethane (DCM), diethyl ether, ethanol, and methanol were bought from Dae-Jung Chemicals (Korea). Trifluoroacetic acid (TFA) was purchased from Acros Organics (NJ, USA).

Synthesis of CA-PH and its derivatives

CA-PH and its derivatives were manually synthesized by SPPS via the Fmoc strategy on Rink amide AM resin (0.82 mmol/g). Each coupling reaction step was monitored by ninhydrin color test. Fmoc-amino acid (2 eq.), BOP (2 eq.), and HOBt (2 eq.) were dissolved in NMP and the mixture was added to the resin in the presence of DIPEA (4 eq.). Each coupling reaction was continued for 1.5 h at room temperature, and Fmoc deprotection was carried out via incubation in 20 % piperidine/NMP for 30 min. After peptide coupling and Fmoc deprotection, CA (2 eq.), BOP (2 eq.), and HOBt (2 eq.) in NMP were added to the dipeptide-anchored resin in the presence of DIPEA (4 eq.). CA coupling was continued for 3 h at room temperature, and CA-PH and its derivatives were separated from the resin by a cleavage cocktail (30 % TFA, 0.5 % DODT, 1 % anisole, and 0.5 % TIPS in DCM) for 1 h. The resin was filtered, and the filtrate was concentrated in vacuum, and then precipitated with cold diethyl ether. The resulting CA-PH and its derivatives were characterized using a QUATTRO Triple Quadrupole Tandem mass spectrometer (Micromass & Waters, Milford, USA) at the National Instrumentation Center for Environmental Management (NICEM, Seoul National University), and their purities were analyzed by high-performance liquid chromatography (HPLC; Thermo Scientific Spectra System AS3000) using an Aapptec SPIRIT PEPTIDE C18 reversed-phase column (120 Å, 5 μm, 4.6 × 250 mm) and the following conditions: gradient elution with A (0.1 % TFA in water) and B (0.1 % TFA in acetonitrile); from 10 to 90 % over 30 min; flow rate, 1.0 ml/min; detection, UV 260 nm. All CA-PH derivatives were purified on a semi-preparative RP-HPLC column (Waters μBondapak™ C18 reversed-phase column, 125 Å, 10 μm, 7.8 × 300 mm) using an A–B gradient (A: 0.1 % TFA in water; B: 0.1 % TFA in acetonitrile; from 10 to 90 % for 30 min; flow rate, 4.0 ml/min) and freeze-dried. The CA-PH and its derivatives used for NMR experiments were in 3 mM in methanol-*d*. The NMR spectra were acquired by a Bruker AVANCE-600 spectrometer (Bruker, Germany) operating at 600 MHz at 297 K. The fraction of s-*cis* proline conformer was determined by integrating well-resolved peaks in the one-dimensional ^{1}H -NMR spectra.

Analysis of free-radical-scavenging activity

DPPH free-radical-scavenging activity was determined by measuring the decrease in the absorbance of the DPPH solution after adding an antioxidant using a UV/visible spectrophotometer (Optizen 2120 UV, Mecasys Co. Ltd., Korea). Methanolic DPPH solution (0.1 mM, 1,480 μl) was mixed with 20 μl of CA-PH (25, 12.5 μM) or CA (25, 12.5 μM). The absorbance at 516 nm was monitored for 10 min. The percentage of DPPH radical was calculated using the following equation: DPPH radical (%) $= \mathrm{Abs}_{516\ \mathrm{nm}}(t = t')/\mathrm{Abs}_{516\ \mathrm{nm}}(t = 0) \times 100$. The control was a mixture of 0.1 mM methanolic DPPH solution (1,480 μl) and 20 μl of methanol. Each experiment was repeated three times in triplicate. A linoleic acid emulsion (25 mM) was prepared by mixing 0.142 g of linoleic acid, 0.142 g of Tween 20, and 50 ml of 0.1 M sodium phosphate buffer (pH 7.0). For the oxidation test, a reaction mixture was prepared with 0.5 ml of water, 2.5 ml

of the linoleic acid emulsion, 2.0 ml of 0.1 M sodium phosphate buffer, and 0.5 ml of CA or CA-PH (9, 45, and 90 μM). The reaction mixture was kept at 50 °C under dark conditions for 24 h. The control contained methanol instead of the antioxidant. To evaluate antioxidant activity, we used a modified ferric thiocyanate method. Twenty-five microliters of the reaction mixture was mixed with 1.175 ml of 75 % ethanol, 25 μl of 20 mM $FeCl_2$ in 3.5 % HCl, and 25 μl of 30 % NH_4SCN. The development of color from the reaction between Fe^{3+} and SCN^- reached a maximum in 3 min, and the absorbance at 500 nm was measured on a UV/visible spectrophotometer. The percentage of lipid-peroxidation inhibition was calculated by the following equation: inhibition of lipid oxidation (%) = $[\{Abs_{500\ nm}(t = 0) - Abs_{500\ nm}(t = t')\}/Abs_{500\ nm}(t = 0)] \times 100$. Each experiment was repeated three times in triplicate.

Isolation and culture of VSMCs from rat aorta

Rat VSMCs were isolated from the thoracic aortas of male Sprague–Dawley rats (ORIENT-Charles River Technology, Seoul, Korea) weighing 200–250 g, and were maintained in Dulbecco's Modified Eagle's Medium (Invitrogen, Grand Island, NY) containing 10 % fetal bovine serum (FBS), as previously described (Lee et al. 2006). For the experiments, cells from passages 3–8 were used, synchronized by serum deprivation for 24 h, and treated with 100 μM CA-PH or CA for 24 h before stimulation with PDGF-BB.

Measurement of ROS production in VSMCs

Intracellular ROS production was detected by flow cytometry using dichlorofluorescein diacetate (DCF-DA). After treating with or without CA and CA-PH for 24 h in VSMCs, 5-(and-6-)chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA; Molecular Probes Inc., Eugene, OR, USA) was added, and ROS generation was stimulated with 100 μM H_2O_2 and 10 ng/ml of PDGF for 30 min. VSMCs were washed with ice-cold PBS and detached from plates using trypsin. The cells were harvested by centrifugation (1,000 rpm, 5 min) and washed again with PBS. The cells were excited with a 488 nm argon ion laser in a flow cytometer (FACSCalibur; BD science, Franklin Lakes, NJ, USA), and the DCF emission was recorded at 530 nm. Data were collected from at least 10,000 cells.

Analysis of the proliferation of VSMCs

Cell proliferation was determined using a nonisotopic enzyme immunoassay for BrdU incorporation, according to the manufacturer's instructions. VSMCs were grown to subconfluence in 96-well plates and synchronized in the presence or absence of CA-PH or CA and PDGF (10 ng/ml) for 48 h. After incubation, the cells were labeled with BrdU for 2 h. The medium was removed, and the cells were fixed with fixing solution at room temperature for 30 min. The fixing solution was aspirated and an anti-BrdU antibody was added for 1 h. After washing, 100 μM of peroxidase-conjugated goat anti-mouse IgG was added for 30 min. After repeated washing, 100 μM of substrate was added, the plate was incubated in the dark for 2 h, and 100 μM of stop solution was added. Absorbance was read on a spectrophotometer microplate reader set at a dual wavelength of 450/550 nm. The background absorbance of cells that received no primary antibody was subtracted from the readings.

Immunoblot analysis

After CA and CA-PH treatment, VSMCs were collected and washed with PBS. After centrifugation, cell lysis was carried out by vigorous shaking for 15 min in Radio-Immunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS, 50 mM Tris–HCl (pH 7.4), 50 mM glycerophosphate, 20 mM NaF, 20 mM EGTA, 1 mM DTT, 1 mM Na_3VO_4, and protease inhibitors). After centrifugation at 14,000 rpm for 15 min, the supernatant was separated and stored at −80 °C until use. Protein concentration in supernatant fractions was determined using the Bradford assay. After addition of sample-loading buffer, protein samples were electrophoresed on a 10 % SDS polyacrylamide gel at 100 V for 2 h. Proteins were transferred onto polyvinylidene difluoride membranes at 20 V for 30 min. The blots were blocked with fresh blocking buffer (0.1 % Tween 20 in Tris-buffered saline (TBST), pH 7.4, containing 5 % non-fat dried milk) for 1 h at room temperature. Dilutions (1:1,000) of primary antibodies were prepared in TBST containing 5 % non-fat dried milk. After three washes with TBST, the blots were incubated with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) in TBST containing 5 % non-fat dried milk. The blots were washed again three times in TBST, and transferred proteins were incubated with ECL substrate solution (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for 1 min, according to the manufacturer's instructions. This was followed by visualization on an X-ray film.

Statistical analyses

Statistical analyses were performed using SPSS 10.0 software (SPSS Inc, Chicago, IL, USA) and $P < 0.05$ was considered statistically significant. Data are presented as the average ± SD.

Results

Synthesis of CA-PH and its derivatives

CA-PH and its derivatives were synthesized by SPPS using the Fmoc strategy on a Rink amide AM resin (Fig. 1). The resin was treated with 30 % TFA/0.5 % DODT/1 % anisole/0.5 % TIPS in DCM for 1 h at room temperature to separate the product. CA-PH and its derivatives were obtained as a white power with >95 % purity via semi-preparative RP-HPLC, and characterized by ESI–MS (Calculated: 414.4 $[M + H]^+$, observed: 414.7) and NMR spectroscopy.

In vitro radical-scavenging activity of CA-PH

As shown in Fig. 2a, the initial radical-scavenging activity of CA-PH was faster than that of CA, and CA-PH continuously scavenged DPPH radicals for 10 min, whereas CA immediately reacted with DPPH free radicals within 1 min. Moreover, 25 μM of CA-PH and CA scavenged DPPH radicals by 84 and 50 % in 10 min, respectively, whereas 12.5 μM of CA-PH and CA reduced DPPH radicals by 57 and 24 % in 10 min, respectively. A lipid-peroxidation test (Fig. 2b) showed that CA-PH inhibited lipid peroxidation by as much as 80 % at 9 μM, while CA showed inferior inhibition by 18 and 45 % at 9 and 90 μM, respectively. CA-PH derivatives were prepared, which contained L-proline (P), D-proline (p), hydroxyproline (Hyp), dehydroproline (dehydroP), L-histidine (H), and D-histidine (h), to study the relationships between the structure and antioxidant activity. All CA-PH derivatives exhibited enhanced antioxidative activities compared with CA in the lipid-peroxidation inhibition test (Fig. 2c). CA-Ph and CA-pH displayed slightly reduced antioxidative activities (by 9–10 %) compared with CA-PH. CA-HypH and CA-Hyph showed decreased antioxidant activities (by 15–20 %) compared with CA-PH. CA-ph, CA-dehydroPH, and CA-dehydroPh exhibited similar antioxidative activities as those of CA-PH.

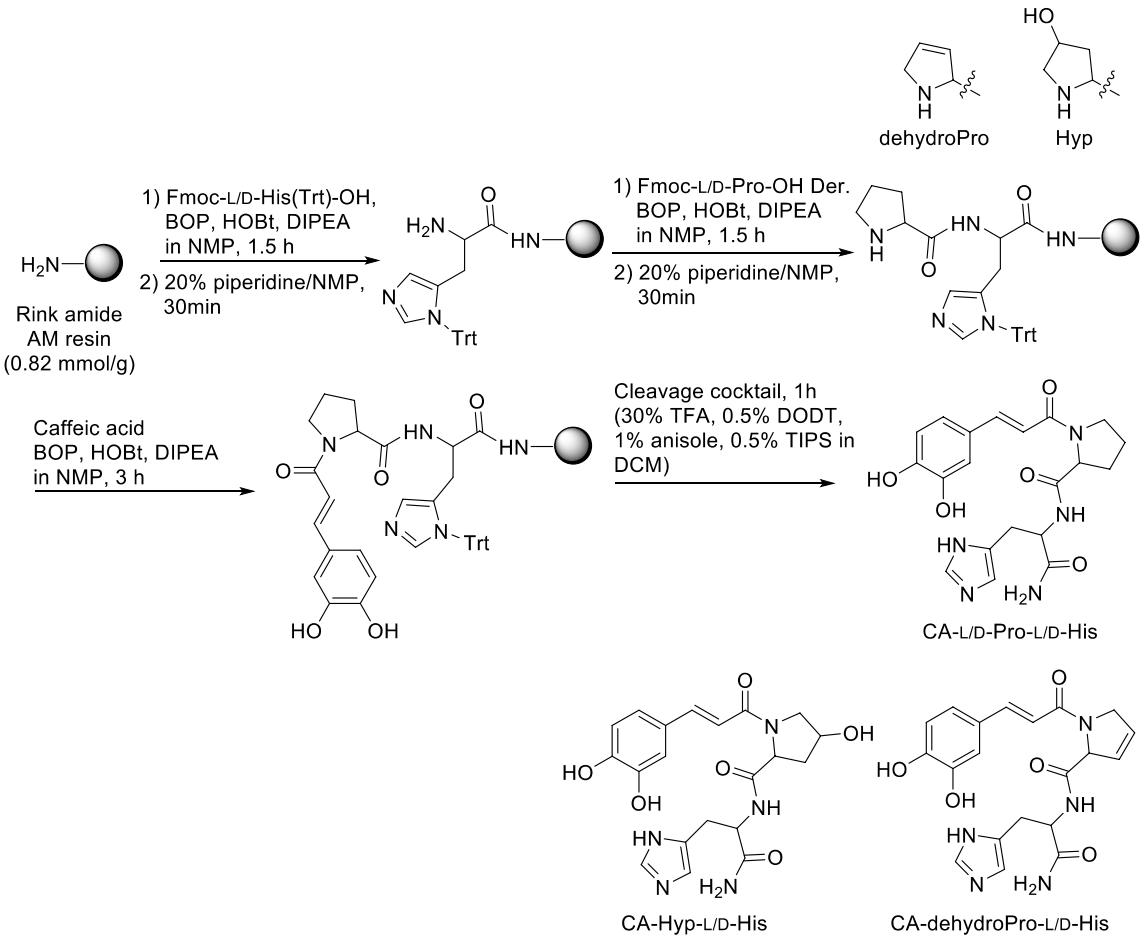

Fig. 1 Solid-phase synthesis of CA-Pro-His derivatives

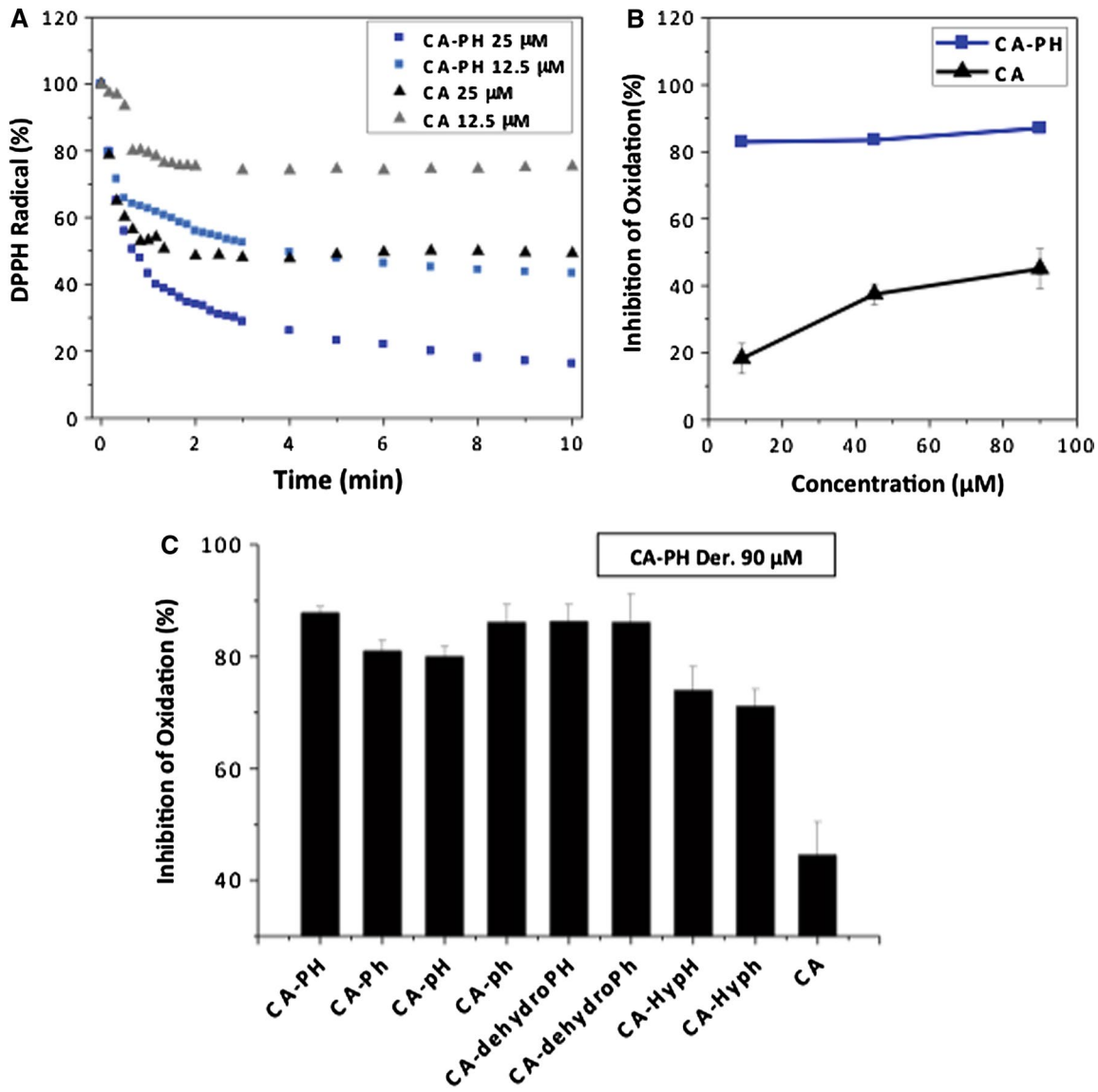

Fig. 2 In vitro radical-scavenging activity of CA-PH. **a** DPPH radicals left during 10 min treatment with 12.5 and 25 μM of CA-PH or CA. **b** Inhibition of lipid oxidation by CA-PH and CA at different concentrations. **c** Inhibition of lipid oxidation by CA-PH derivatives and CA at 90 μM. The data were obtained in triplicate and expressed as average ± SD

Antioxidative effects of CA-PH in VSMCs

To investigate the effects of CA-PH on VSMCs isolated from rat aorta, VSMCs were treated with CA-PH for 24 h before H_2O_2 treatment, and then analyzed for ROS generation using the fluorescent dye H_2DCF-DA, which bound to ROS. The presence of ROS labeled with H_2DCF-DA was quantified by a flow cytometry. The H_2O_2 treatment increased intracellular ROS generation in VSMCs, which was significantly inhibited by CA-PH (Fig. 3a). Moreover, CA slightly, but not significantly, inhibited the increase in ROS production. These results suggest that CA-PH can exert antioxidative activities against H_2O_2-induced oxidative stress in VSMCs, but that CA has a marginal effect regarding the inhibition of ROS generation compared with CA-PH. To test whether CA-PH could induce HO-1, which is an antioxidant enzyme, VSMCs were treated with 100 μM CA-PH for 24 h, and then assessed for HO-1 expression by the western blotting using a specific antibody against HO-1. CA-PH, but not CA, up-regulated HO-1 in VSMCs by up to twofold compared with control cells (Fig. 3b), suggesting that CA-PH plays a role as an antioxidant by inducing HO-1 expression in VSMCs.

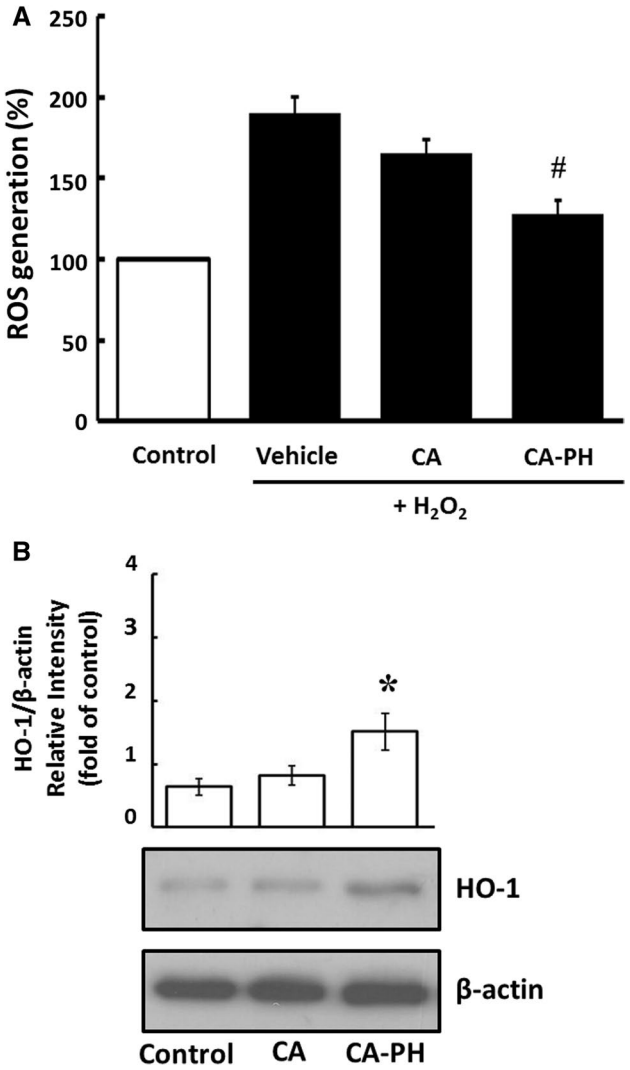

Fig. 3 Effects of CA-PH on ROS generation and HO-1 expression in VSMCs. VSMCs were treated with 100 μM CA and CA-PH for 24 h, and 100 μM H_2O_2 for 30 min, followed by the assessment of ROS generation by DCF-DA staining (**a**) and HO-1 expression was analyzed by western blotting, as described in the "Materials and methods" (**b**). VSMCs were treated with 100 μM of CA or CA-PH for 24 h, and then HO-1 expression was assessed. The data are expressed as the average ± SD. $^{\#}P < 0.05$ compared with H_2O_2-, vehicle-treated cells, $^{*}P < 0.05$ compared with control cells. The data are from three independent experiments

Inhibition of PDGF-induced VSMC proliferation by CA-PH

To elucidate the role of CA-PH in VSMCs, VSMCs were treated with 100 μM CA-PH for 24 h, and then stimulated with PDGF to induce cellular proliferation. These cells were assessed for cellular proliferation via a BrdU-incorporation assay. CA-PH effectively inhibited the proliferation of VSMCs in both the absence and presence of PDGF, which was accompanied by an antioxidative activity—i.e., the decrease of PDGF-induced ROS generation (Fig. 4a). The anti-proliferative effect of CA-PH was confirmed by analyzing the expression of PCNA and the phosphorylation of Akt induced by PDGF. CA-PH markedly reduced PDGF-induced PCNA expression and Akt phosphorylation to basal levels (Fig. 4b). Although not significant, CA yielded a slight inhibition of both cell proliferation and ROS generation (Fig. 4a, b). These results indicate that CA-PH can exert a more pronounced anti-proliferative effect than CA can against PDGF-induced oxidative stress in VSMCs, which might result from the enhanced antioxidative activity of CA-PH in VSMCs compared with that of CA.

Discussion

We examined the antioxidant activity of CA-PH compared with that of CA and found that CA-PH effectively scavenged free radicals and inhibited the PDGF-induced intracellular ROS generation and proliferation of VSMCs. We hypothesized that the bond-dissociation energy (BDE) of the O–H of CA would be reduced by the interaction with His imidazole in the CA-PH molecule. As shown in Fig. 2a, the initial DPPH free-radical scavenging activity of CA-PH was much faster than that of CA, which was observed more clearly at the concentration of 12.5 μM. Interestingly, CA-PH continuously quenched DPPH free radicals for 10 min, whereas CA immediately reacted with DPPH radicals within 1 min. Figure 2a indicates that CA-PH (25 μM) scavenged DPPH free radicals by 84 % in 10 min, whereas the same concentration of CA reduced DPPH free radicals by 50 % in 10 min. Even at the lower concentration of 12.5 μM CA-PH, DPPH radicals were reduced by 57 % in 10 min, although CA and CA-PH both have one catechol moiety. This could be explained by the fact that the nearby imidazolium can reactivate the antioxidant activity of CA by donating a hydrogen radical to the hydroxyl radical (PhO·) which is formed after CA-PH reacts with ROS. Subsequently, the resulting His imidazole radical cation might stabilize the semi-quinone radicals of CA after a second hydrogen atom abstraction (Fig. 5). The lipid-peroxidation test (Fig. 2b) showed that CA-PH exhibited a far superior antioxidative activity compared with CA at the experimental concentrations from 9 to 90 μM.

To study the relationships between the structure and antioxidant activity, CA-PH and its derivatives were prepared. It was hypothesized that the L or D form of the amino acids might change the distance and conformation between Pro and His, thereby affecting antioxidant activity. We expected a dramatic reduction of antioxidative activity in both CA-Ph and CA-pH, which have an alternative combination of L or D amino acids that can yield a longer distance

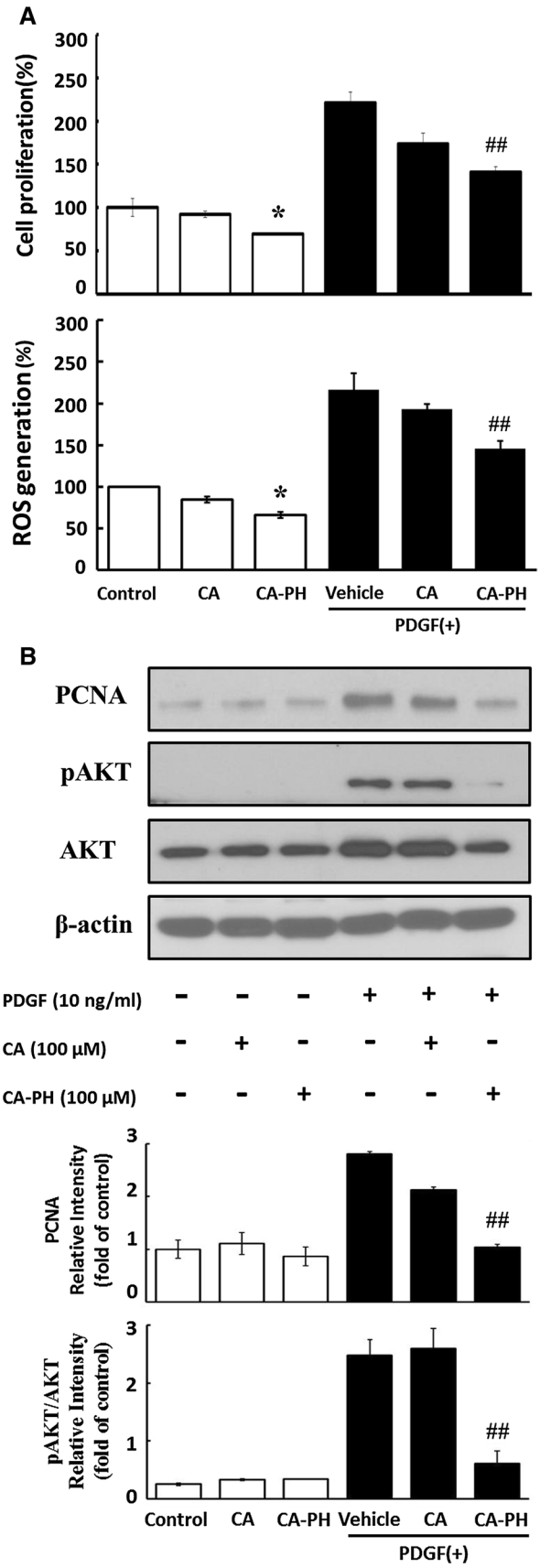

Fig. 4 Effects of CA-PH on the proliferation of VSMCs. **a** BrdU assay; **b** western blotting. VSMCs were starved and treated first with 100 μM of CA and CA-PH for 24 h, and then with 10 ng/ml PDGF. The cells were harvested and subjected to a BrdU assay and ROS generation (**a**), as well as western blotting to assess PCNA, pAkt, and Akt expression, as described in the "Materials and methods" (**b**). The data are expressed as the average ± SD. $^{*}P < 0.05$ compared with control cells, $^{\#\#}P < 0.001$ compared with PDGF-, vehicle-treated cells. The data are from three independent experiments

between Pro and His. However, CA-Ph and CA-pH exhibited a small decrease in lipid-oxidation-inhibitory activity. CA-HypH and CA-Hyph showed inferior antioxidative activity (by 15–20 %) compared with CA, which was probably due to increased hydrophilicity and a less optimized structure (Fig. 2c). In addition, the identification of the structures of those CA-PH derivatives by ^{1}H-NMR showed that they had a similar s-*cis* Pro conformation ratio. The only difference was that the CH = proton of CA-Hyph appeared at a relatively less downfield position (6.61 ppm) compared with other derivatives (6.70 ppm) (Table 1). This might be related to the inferior antioxidant activity of CA-Hyph observed in the lipid-peroxidation test.

We found that CA-PH effectively inhibited the intracellular ROS generation induced by H_2O_2 or PDGF in VSMCs. In addition, CA-PH increased the expression of HO-1, an intracellular antioxidant protein, and significantly inhibited the cellular proliferation induced by PDGF in VSMCs. In vascular cells, oxidative stress has been regarded as one of the risk factors for the development of cardiovascular diseases such as atherosclerosis and restenosis. In VSMCs, excessive ROS result in proliferation and migration of VSMCs, which are related to their proatherogenic changes (Elahi et al. 2009). Several studies have attempted to reduce oxidative stress in VSMCs, such as the regulation of HO-1 expression. HO-1 has been reported to protect vascular cells against vascular diseases including atherosclerosis (Juan et al. 2001; Yet et al. 2003). CA-PH exerted antioxidative effects in the oxidative state of VSMCs, as shown by the inhibition of ROS generation and up-regulation of HO-1. These effects were more pronounced in CA-PH than in CA in VSMCs—only CA-PH (and not CA) up-regulated HO-1—which suggests CA-PH to be a potent antioxidant in vascular cells. With excellent radical-scavenging and HO-1 inducing activity within cells, CA-PH can be a potential candidate for therapeutic reagent in treating vascular diseases.

PDGF regulates the tyrosine phosphorylation of various signaling proteins via intracellular production of H_2O_2 (Jackson et al. 1993). Recent findings also suggest that PDGF can trigger intracellular oxidative stress (Kreuzer et al. 2003; Sundaresan et al. 1995), which supports the

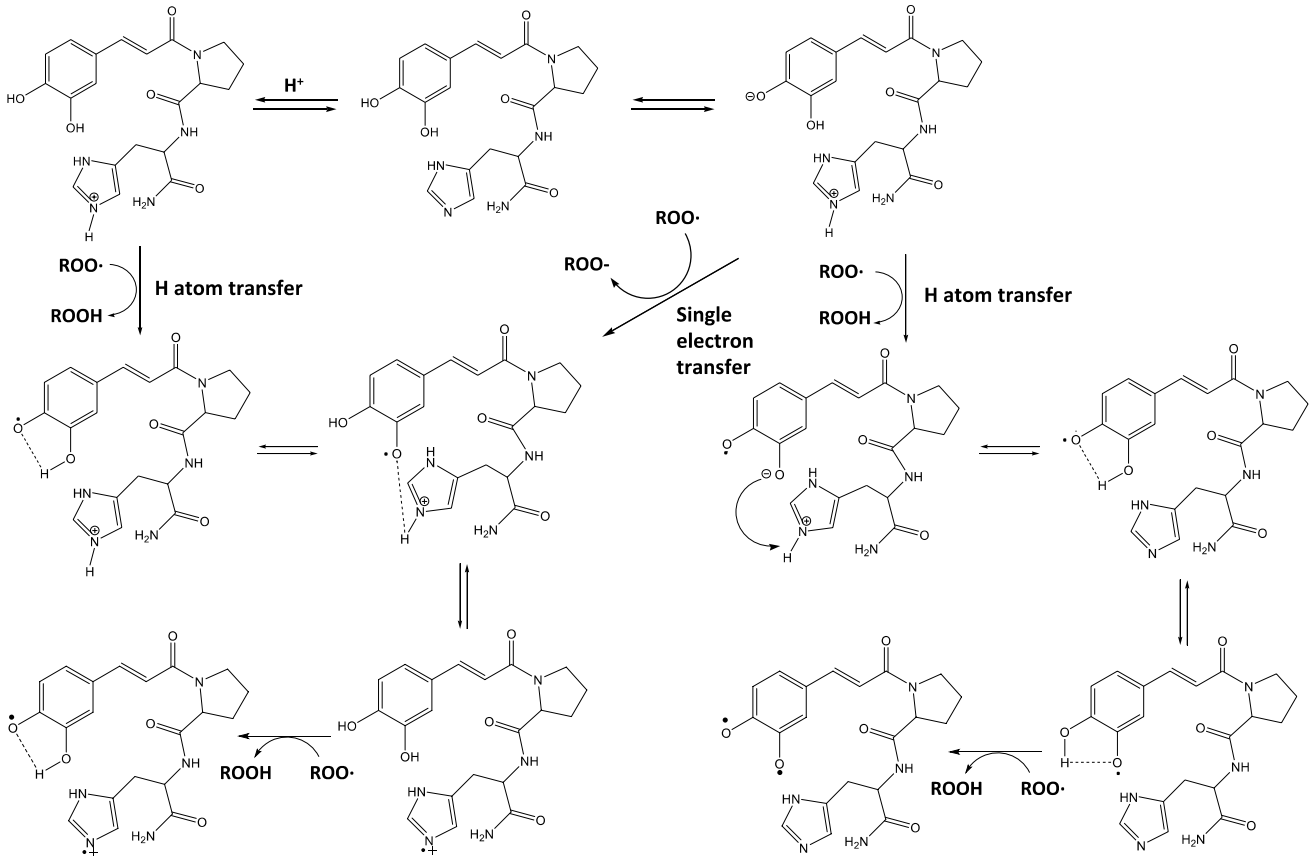

Fig. 5 Proposed antioxidant mechanism of CA-Pro-His with peroxy radicals based on H-atom transfer and single electron transfer mechanisms. The nearby imidazolium might be able to reactivate the antioxidant activity of CA by donating hydrogen radical to the hydroxyphenoxyl radical (PhO·) that is formed when CA-PH reacts with radicals

Table 1 H-NMR chemical shift (ppm, TMS) of CA-PH derivatives in s-*cis* pro conformation

	His $C^{\varepsilon 1}$-H	CA CH=
CA-PH	8.80	6.70
CA-Ph	8.80	6.69
CA-pH	8.80	6.68
CA-ph	8.81	6.71
CA-dehydroPH	8.80	6.71
CA-dehydroPh	8.81	6.71
CA-HypH	8.82	6.68
CA-Hyph	8.77	6.61

Samples were dissolved in methanol-*d*

possibility that ROS act as a key mediator in the regulation of PDGF-induced VSMC proliferation. In cultured VSMCs, we observed that PDGF potently induced intracellular ROS production, and that CA-PH effectively eliminated ROS production and impeded the cellular proliferation induced by PDGF. This suggests that the reduction of oxidative stress by CA-PH can lead to the inhibition of the PDGF-induced proliferation of VSMCs. Several receptor tyrosine kinases, including the receptors for PDGF, activate PI3 K/Akt, which is an important step in VSMC proliferation, cell-cycle progression, and cell survival (Higaki and Shimokado 1999). In this study, CA-PH inhibited the PDGF-stimulated phosphorylation of Akt and PCNA expression (Fig. 4b), confirming anti-proliferative effect of CA-PH in VSMCs. These results imply that CA-PH acts not only as a radical scavenger, but also as an inhibitor of VSMC abnormal proliferation by eliminating intracellular ROS and increasing HO-1 expression in VSMCs.

The differences of the DPPH free-radical-scavenging activity and the lipid-peroxidation-inhibition activity between CA-PH and CA led us to conclude that the exceptionally enhanced antioxidant activity of CA-PH comes from its unique structure. As Pro exists mainly in the *s-cis* conformation, His imidazole efficiently interacts with the catechol moiety of CA. It is likely that imidazolium reduces the phenoxy radicals, which are formed when CA-PH reacts with free radicals, and reactivates its antioxidant activity by forming a stabilized structure. Various CA-PH derivatives were additionally prepared to compare their

antioxidant activities according to their structures. These derivatives existed mostly in the *s-cis* Pro conformation, similar to CA-PH, and showed similar antioxidant activity compared with CA-PH. In addition to the in vitro tube test, CA-PH effectively eliminated intracellular ROS, induced HO-1 expression in VSMCs, and significantly impeded the proliferation of VSMCs. These effects were more effective with CA-PH than with CA, which might be the result of the enhanced antioxidant activity of CA-PH.

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Conflict of interest We have no conflict of interests.

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