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Caffeic acid phenethyl ester is a potent inhibitor of HIF prolyl hydroxylase: structural analysis and pharmacological implication

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Abstract

Caffeic acid phenethyl ester (CAPE) is an active component of propolis from honeybee. We investigated a potential molecular mechanism underlying a CAPE-mediated protective effect against ischemia/reperfusion (I/R) injury and analyzed the structure contributing to the CAPE effect. CAPE induced hypoxia-inducible factor-1 (HIF-1) α protein, concomitantly transactivating the HIF-1 target genes *vascular endothelial growth factor* and *heme oxygenase-1*, which play a protective role in I/R injury. CAPE delayed the degradation of HIF-1α protein in cells, which occurred by inhibition of HIF prolyl hydroxylase (HPH), the key enzyme for von Hippel–Lindau-dependent HIF-1α degradation. CAPE inhibition of HPH and induction of HIF-1α protein were neutralized by an elevated dose of iron. The catechol moiety, a chelating group, is essential for HPH inhibition, while hydrogenation of the double bond (–C=C–) in the Michael reaction acceptor markedly reduced potency. Removal of the phenethyl moiety of CAPE (substitution with the methyl moiety) severely deteriorated its inhibitory activity for HPH. Our data suggest that a beneficial effect of CAPE on I/R injury may be ascribed to the activation of HIF-1 pathway *via* inhibition of HPH and reveal that the chelating moiety of CAPE acted as a pharmacophore while the double bond and phenethyl moiety assisted in inhibiting HPH.

Keywords: Hypoxia-inducible factor-1; HIF prolyl hydroxylase; Caffeic acid phenethylester; Ischemic/reperfusion injury; Structure–activity relationship

1. Introduction

Caffeic acid phenethyl ester (CAPE), an active component of honeybee propolis extract, has been used as a traditional medicine for many years. The polyphenolic natural product has numerous biological activities, including antioxidant, anti-inflammatory, antiviral and immunomodulatory properties [1–4]. Clinically, it has been shown to inhibit the growth and metastasis of different types of tumor cells, to protect tissues from reperfusion injury in various ischemia/reperfusion (I/R) models and to inhibit pulmonary fibrosis [5–8]. At the molecular level, CAPE modulates the activities of the enzymes matrix metalloproteinase-9, focal adhesion kinase, inducible nitric oxide synthase, HIV integrase, lipoxygenase and cyclooxygenase-2 [9–14] and of the transcription factors Nrf2 and nuclear factor κB [15,16], which may play a role as molecular mechanisms underlying its clinical activities.

Hypoxia-inducible factor-1 (HIF-1) is a heterodimeric transcription factor that is composed of HIF-1α and aryl hydrocarbon receptor nuclear translocator (HIF-β) whose activity is mainly regulated by oxygen [17]. In normoxia, HIF-1α is continuously synthesized but rapidly ubiquitylated and subsequently degraded by the cellular proteasome. The posttranslational regulation of the protein is orchestrated by interaction with the E3 ubiquitin ligase von Hippel–Lindau (VHL) protein [18–21]. In hypoxia, this interaction is suppressed; HIF-1α, which is accompanied by its nuclear translocation, heterodimerization with HIF-1β and transcription of genes encoding proteins that function to increase angiogenesis and to promote cell survival and proliferation, is thereby stabilized [22]. The cellular-oxygen-sensing mechanism that determines HIF-1α/pVHL interaction consists of the oxygen-dependent enzymatic hydroxylation of two highly conserved proline residues [23,24]. To date, four human HIF prolyl hydroxylases (HPHs) have been cloned [25,26]. According to their catalytic mechanism, these HPHs belong to the family of oxygen-dependent, iron-dependent and 2-oxoglutarate-dependent dioxygenases. HPH-2 is ubiquitously expressed and exhibits the highest specific activity toward HIF-1 [25].

Abbreviations: CAPE, caffeic acid phenethyl ester; CNAPE, cinnamic acid phenethyl ester; DMC, dimethoxycinnamic acid phenethyl ester; DMDHC, dimethoxydihydrocinnamic acid phenethyl ester; DHC, dihydrocaffeic acid phenethyl ester; CAME, caffeic acid methyl ester; CAPEN, caffeic acid *n*-pentyl ester; CAHA, caffeic acid *n*-hexyl ester; HIF-1, hypoxia-inducible factor-1; HO-1, heme oxygenase-1; VEGF, vascular endothelial growth factor; HPH, HIF prolyl hydroxylase.

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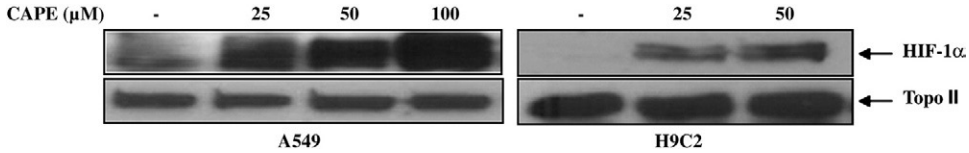

Fig. 1. CAPE up-regulates HIF-1α protein. Human lung carcinoma A549 cells and rat myocardial H9C2 cells were treated with CAPE for 4 h and lysed to obtain nuclear extracts. HIF-1α levels in the nuclear extracts were examined by Western blot analysis, as described under Materials and Methods.

Since normoxic induction of the HIF-1α protein, leading to activation of HIF-1, has shown a beneficial effect on ischemia and I/R injury of tissues [27–30], inhibition of HPH, leading to activation of HIF-1, has been thought as a feasible strategy to protect tissues from I/R injury. In fact, small molecules or small interfering RNAs (siRNAs) inhibiting HPH have been reported to elicit a protective effect on various I/R injury models [31–33], suggesting that potent HPH inhibitors that exhibit a low level of toxicity and side effects hold promise as new therapeutic options for diseases such as myocardial and peripheral ischemia and as chemopreventive agents that could be used to reduce the level of I/R injury following heart attack and stroke. In this study, we investigated a potential molecular mechanism underlying CAPE-mediated protection of I/R injury and analyzed its chemical structure for effects. Our data demonstrate that CAPE activated HIF-1 by inhibiting HPH and subsequently up-regulating the HIF-1 target genes *heme oxygenase-1* (*HO-1*) and *vascular endothelial growth factor* (*VEGF*), which are involved in HIF-1-mediated protection of I/R injury [27,34–36]. It was also revealed that the catechol moiety, a chelating group, acted as a pharmacophore for HPH inhibition and, moreover, that saturation of the double bond (–C=C–) in the Michael reaction acceptor or change in the phenethyl ester moiety to the methyl moiety markedly reduced its activity for HPH inhibition.

2. Materials and methods

2.1. Chemicals

CAPE was obtained from Alexis (San Diego, CA). Echinomycin (NSC-13502), 3,4-dimethoxycinnamic acid, 3,4-dimethoxyhydrocinnamic acid, 3,4-dihidroxyhydrocinnamic acid, cinnamic acid, sodium 2-ketoglutarate, sodium ascorbate, ferrous chloride and hexamethyl-phosphoramide were purchased from Sigma Chemical Co. (St. Louis, MO). Caffeic acid (3,4-dihydroxycinnamic acid), catechol and hexylchloride were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Reaction solvents were obtained from Junsei Chemical Co. (Tokyo, Japan). All other chemicals were reagent-grade commercially available products. The caffeic acid derivatives used in this report were prepared in our laboratory. The formation and isolation of caffeic acid derivatives were verified by thin-layer chromatography (TLC), infrared (IR), ^{1}H nuclear magnetic resonance (NMR) and element analysis (EA).

2.2. Synthesis of caffeic acid derivatives

For the synthesis of caffeic acid hexyl ester, 2.28 ml of 25% NaOH was added to a solution of caffeic acid (1.98 g) in 25 ml of hexamethylphosphoramide (HMPA). After stirring for 90 min, we added hexyl chloride (3.7 ml) dropwise into 15 ml of HMPA and stirred it for 2 days at room temperature. The reaction mixture was poured into ice

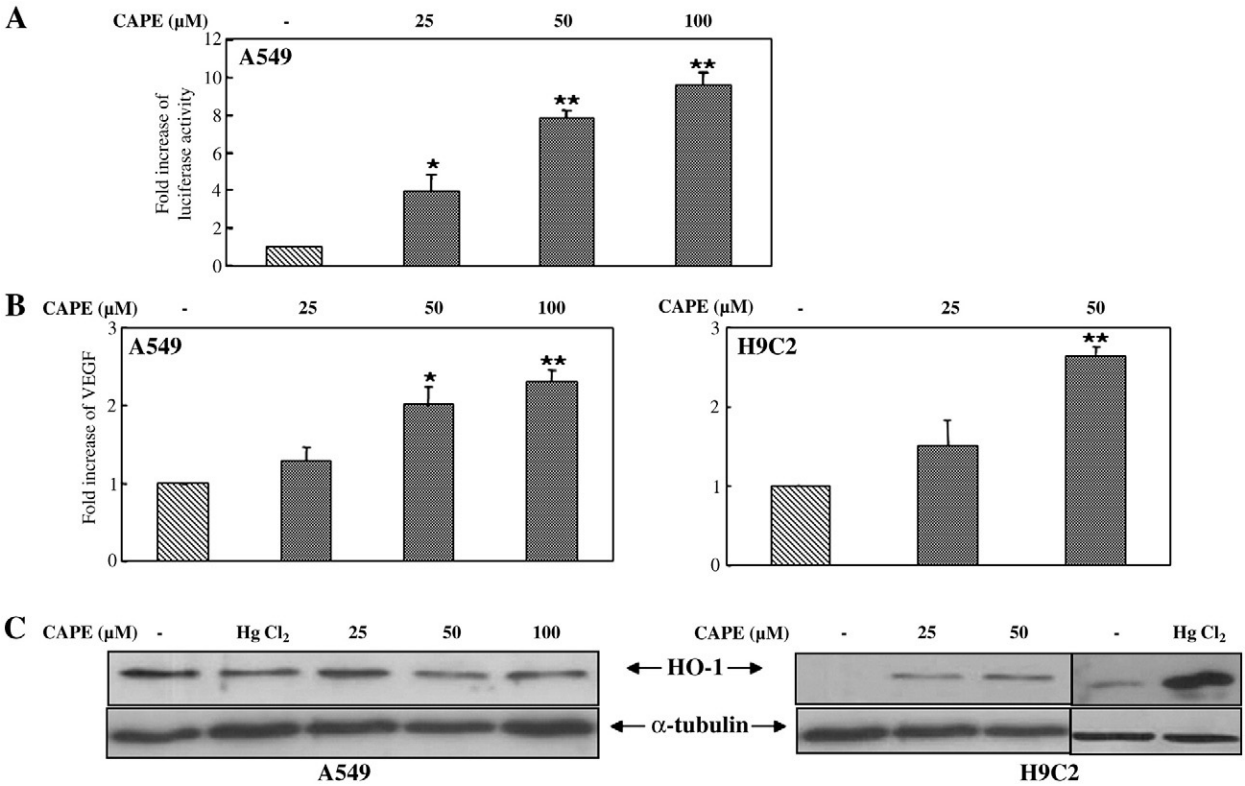

Fig. 2. CAPE activates HIF-1. (A) A549 cells were transfected with a HIF-1-responsive reporter gene plasmid and subsequently treated with CAPE for 10 h, and luciferase activity was measured and normalized to CMV *Renilla* luciferase activity. (B) Left: Human lung carcinoma A549 cells were treated with various concentrations of CAPE for 10 h, and VEGF in cell culture supernatants was analyzed as described under Materials and Methods. Right: The same experiment was performed in H9C2 cells. (C) Left: A549 cells were treated with various concentrations of CAPE or mercuric chloride ($HgCl_2$; 25 μM) for 4 h, and HO-1 levels were monitored in whole-cell lysates by Western blot analysis. Right: The same experiment was performed in H9C2 cells. The data in (A) and (B) are presented as mean±S.E. (n=3). *P<.05 and **P<.01 versus controls.

water (70 ml), which was extracted with diethyl ether. The ether extract was washed thoroughly with 1 N HCl, water and 10% $NaHCO_3$ solution, dried over anhydrous sodium sulfate and evaporated. Recrystallization in an appropriate ether/*n*-hexane ratio gave the final products. For the synthesis of dimethoxycinnamic acid phenethyl ester (DMC), dimethoxyhydrocinnamic acid phenethyl ester and cinnamic acid phenethyl ester (CNAPE), we added *N*,*N*′-carbonyldiimidazole (5.5 mmol; in portions), which was stirred at 0°C for 30 min, to a solution of dimethoxycinnamic acid, dimethoxyhydrocinnamic acid or cinnamic acid (5 mmol) in dimethylformamide (8 ml). The reaction mixture was added to a solution of phenethylalcohol (10 mmol) and triethylamine (20 mmol) in dimethylformamide (3 ml) and stirred for 1 h at room temperature. The reaction mixture was filtered, evaporated and subsequently dissolved with diethyl ether. The ether extract was washed with water and 10% $NaHCO_3$ solution, dried over anhydrous sodium sulfate and evaporated. Recrystallization in an appropriate ether/*n*-hexane ratio gave the final products. For the synthesis of caffeic acid methyl ester (CAME) and pentyl ester, Dowex 50WX8-200 (0.2 g) was added to caffeic acid (1 g) dissolved in absolute methanol or *n*-pentanol and heated under reflux for 1 day, and the reaction mixture was filtered and evaporated. The residue was dissolved with diethyl ether. The ether extract was washed with water and 10% $NaHCO_3$ solution, dried over anhydrous sodium sulfate and evaporated to afford the final products. For the synthesis of dihydrocaffeic acid phenethyl ester (DHC), phenethylalcohol (10 mmol) was added to a solution of dihydroxyhydrocinnamic acid (1 g) in tetrahydrofuran (20 ml) and Dowex 50WX8-200 (0.2 g) and heated under reflux for 1 day. The reaction mixture was filtered, evaporated and subsequently dissolved with diethyl ether. The ether extract was washed with water and 10% $NaHCO_3$ solution, dried over anhydrous sodium sulfate and evaporated. Recrystallization in an appropriate ether/*n*-hexane ratio gave the final products. The formation and isolation of caffeic acid derivatives were verified by TLC, IR, ^{1}H NMR and EA.

2.3. Cell culture and transient transfection

Human lung carcinoma A549 cells, rat heart myoblast H9C2 cells and human embryonic kidney 293 cells were grown in Dulbecco's modified Eagle's medium (Hyclone, South Logan, UT) supplemented with 10% fetal bovine serum (Hyclone) and penicillin/streptomycin (Hyclone). For transient transfection of plasmids, cells were plated in 6-cm dishes or six-well plates until 50–60% confluence on the day of transfection with Flag-VHL (5 μg; a gift from Dr. J. Issacs, Medical University of South Carolina) and HA-HIF-1α plasmid (5 μg; a gift from Dr. L. Neckers, National Cancer Institute), or with HIF-responsive luciferase (0.4 μg; a gift from Dr. G. Melillo, National Cancer Institute) and CMV *Renilla* luciferase plasmid (4 ng; Promega, Madison, WI). Fugene (Roche, Indianapolis, IN) was used as transfection reagent. At 1 day posttransfection, cells were treated with each reagent, as indicated in the figure legends. For transfection of siRNA, chemically synthesized double-stranded siRNAs

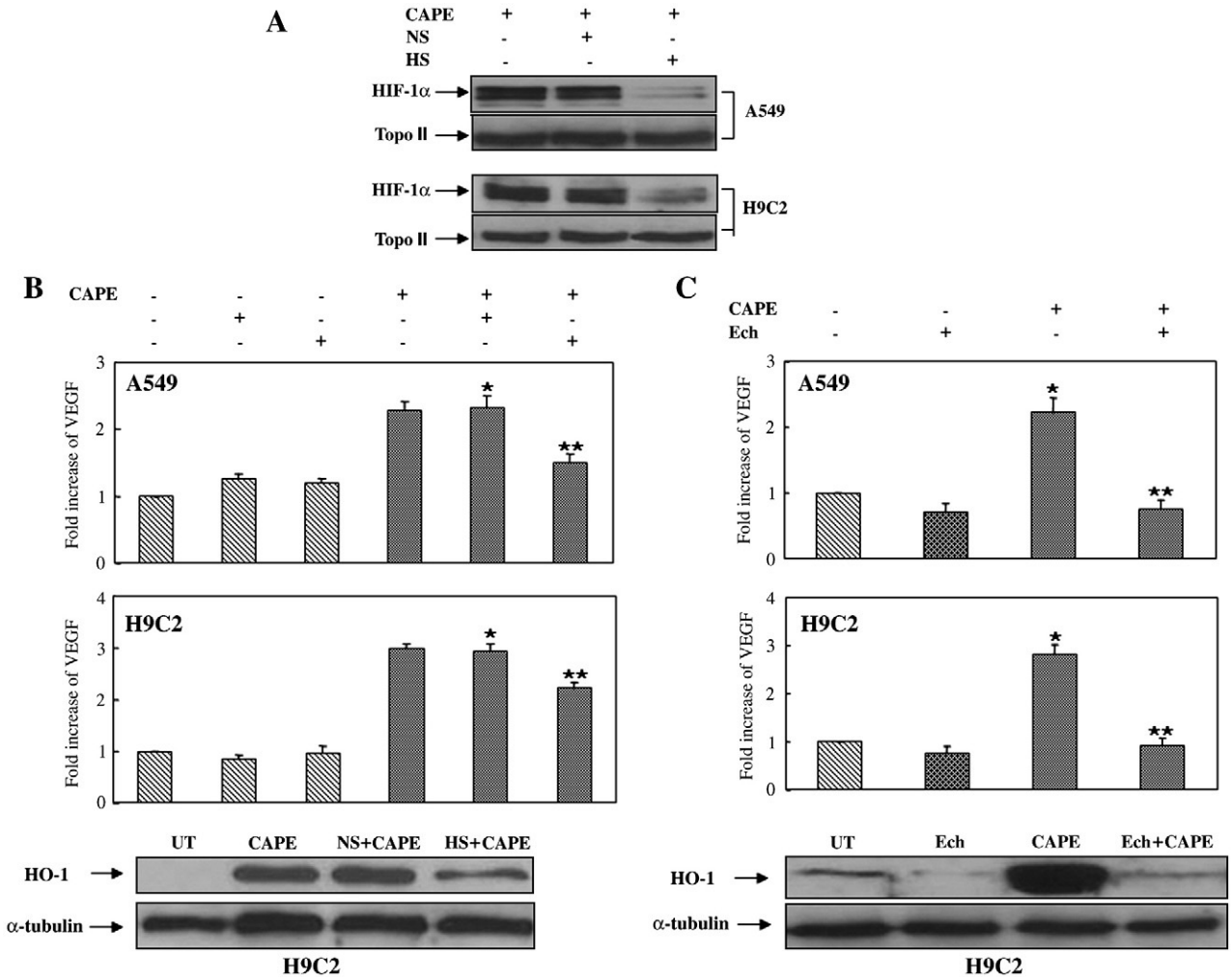

Fig. 3. CAPE mediates HO-1 induction, and VEGF is HIF-1 dependent. (A) Top: Chemically synthesized double-stranded siRNA specific for HIF-1α (HIF-1α siRNA; HS) was transfected (200 nmol/L) using a Dharmafect reagent. A nontargeting siRNA (NS) sequence was used as nonspecific control. At 1 day posttransfection, A549 cells were treated with CAPE (50 μM) and lysed to obtain nuclear extracts. HIF-1α levels in the nuclear extracts were examined by Western blot analysis. Bottom: The same experiment was performed in H9C2 cells. (B) Top: At 1 day posttransfection with HIF-1α siRNA (HS) or nontargeting siRNA (NS), A549 cells were treated with CAPE (50 μM) for 10 h, and VEGF in cell culture supernatants was analyzed. The data are presented as mean±S.E. (n=3). *(CAPE+NS); P<.01 versus **(CAPE+HS). Middle: The same experiment was performed in H9C2 cells. The data are presented as mean±S.E. (n=3). *(CAPE+NS); P<.05 versus **(CAPE+HS). Bottom: H9C2 cells, which had been transfected with HIF-1α siRNA (HS) or nontargeting siRNA (NS) for 1 day, were treated with CAPE (50 μM), and HO-1 levels were monitored in whole-cell lysates by Western blot analysis. (C) Top: A549 cells were treated with CAPE (50 μM) following incubation with echinomycin (Ech; 10 nM) for 30 min, and VEGF in cell culture supernatants was analyzed 10 h later. The data are presented as mean±S.E. (n=3). *(CAPE+Ech); P<.01 versus **(CAPE+Ech). Middle: The same experiment was performed in H9C2 cells. The data are presented as mean±S.E. (n=3). *(CAPE+Ech); P<.01 versus **(CAPE+Ech). Bottom: H9C2 cells were treated with CAPE (50 μM) following incubation with echinomycin (Ech; 10 nM) for 30 min, and HO-1 levels were monitored in whole-cell lysates by Western blot analysis.

specific for HIF-1α (HIF-1α siRNA) 5-AGAGGUGGAUAUGUGUGGGdTdT-3 and 5-CCCACACAUAUCCACCUCUdTdT-3 [37] were purchased from Dharmacon Research, Inc. (Chicago, IL). The siRNA was transfected (200 nmol/L) using a Dharmafect transfection reagent, in accordance with the manufacturer's instructions. A predesigned nontargeting siRNA sequence (Dharmacon Research, Inc.) was used as nonspecific control.

2.4. Immunoblot analysis and immunoprecipitation

Cells were lysed and nuclear or whole-cell extracts were prepared as described previously [38,39]. The protein concentration in supernatants was determined by the bicinchoninic acid method. Cellular extracts were electrophoretically separated using 7.5% or 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels. Proteins were transferred to nitrocellulose membranes (Protran, Schleicher and Schuell, Keene, NH), and HIF-1α was detected in nuclear extracts (30–40 μg) using a monoclonal anti-HIF-1α antibody purchased from Novus (Littleton, CO) and BD Biosciences Pharmingen (San Jose, CA). HO-1 protein was detected in whole-cell lysates (30–40 μg) using a polyclonal anti-HO-1 antibody (Stressgen, Inc., Victoria, BC, Canada). Peroxidase-conjugated anti-goat or anti-mouse secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used at a dilution of 1:2000. Signals were visualized using the SuperSignal chemiluminescence substrate (Pierce ImmunoPure, Rockford, IL). Experiments were performed in duplicate and normalized with antibodies to topoisomerase II (Santa Cruz Biotechnology) for HIF-1α and with antibodies to α-tubulin (Santa Cruz Biotechnology) for HO-1. For immunoprecipitation, 24 h after cotransfection of an HA-HIF-1α plasmid and a Flag-VHL plasmid in 293 cells, the transfected cells were treated as indicated in the figure legends and lysed using a lysis buffer (50 mM Tris–HCl pH 7.4, 0.75% NP-40, 150 mM NaCl, 1 mM EDTA, 0.3 μM aprotinin, 1 μM pepstatin and 1 mM phenylmethanesulphonylfluoride). The cell lysates (0.7 mg of protein) were incubated with 20 μl of anti-HA-antibody-bound beads (Covance, Berbeley, CA) or anti-Flag antibody, followed by addition of protein G agarose beads (Invitrogen, Carlsbad, CA). The beads were washed five times with the lysis buffer, resuspended in 1× SDS sample buffer and boiled for 3 min. Immunoprecipitated proteins were separated by 10% SDS-PAGE gels. Immunoblot analysis was performed as aforementioned.

2.5. In vitro VHL capture assay

Biotinylated wild-type or proline-hydroxylated peptides (corresponding to HIF residues 556–574) were synthesized (American Peptide Co., Sunnyvale, CA), dissolved in sterile water (500 μg/ml) and incubated with steptavidin beads (Pierce ImmunoPure) at 4°C for 2 h. The beads were washed twice with VHL binding buffer (20 mM Tris pH 8, 100 mM NaCl, 1 mM EDTA and 0.5% NP-40) and three times with reaction buffer (20 mM Tris pH 7.5, 5 mM KCl and 1.5 mM $MgCl_2$). For each condition, 2 μg of peptide/20 μl of beads was aliquoted into separate tubes, and the reaction buffer was added along with cofactors (100 μM 2-ketoglutaric acid, 100 μM L-ascorbic acid and 50 μM ferrous chloride). The beads and HPH cofactors were mixed at room temperature for 15 min in the reaction buffer. Prior to this incubation, any inhibitor or competing factor was added to the appropriate tubes. Separate *in-vitro*-translated (IVT) reactions (Promega) were sources for the HPH protein (the HPH-2 plasmid was kindly provided by S. McKnight, University of Texas Medical Center, Dallas, TX) and the Flag-VHL protein [40]. A 5-μl aliquot of IVT HPH-2 was added to the bead–peptide mixture for 1 h at 30°C. Subsequently, the beads were washed with VHL binding buffer, and 10 μl of Flag-VHL IVT was added to the beads overnight at 4°C. The beads were washed, SDS Laemmli buffer was added, the samples were boiled and subjected to SDS-PAGE, and resultant blots were probed for Flag.

2.6. VEGF analysis

Cells were treated as indicated in the figure legends. The medium was collected following 8 h of treatment. ELISA kits (R&D Systems, Minneapolis, MN) were used to assess secreted VEGF levels from an appropriate volume of medium. Each sample was

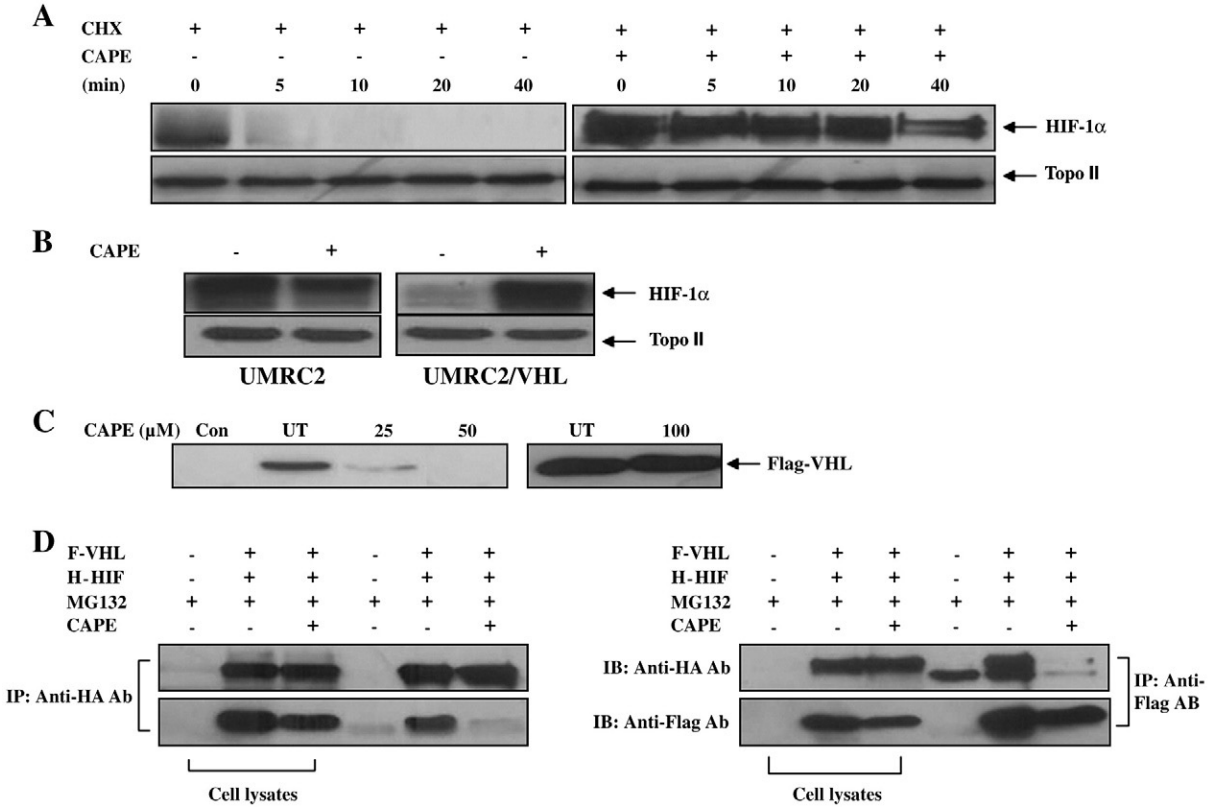

Fig. 4. CAPE activates HIF-1 by inhibiting HPH. (A) A549 cells were either left untreated or pretreated with CAPE (50 μM) for 4 h, followed by addition of cycloheximide (CHX; 200 μM) for the indicated times. Levels of HIF-1α in nuclear extracts were visualized by Western blot analysis. (B) Renal carcinoma cells that are deficient in VHL function (UMRC2) or a clonally selected line with VHL stably expressed (UMRC2/VHL) was treated with CAPE (50 μM) for 4 h, and HIF-1 protein was detected in nuclear extracts. (C) A VHL capture assay utilizing biotinylated HIF peptide was performed as described under Materials and Methods. Left: The assay was performed in the presence of cofactors (50 μM ferrous chloride, 100 μM sodium 2-ketoglutarate and 100 μM sodium ascorbate) and at the indicated concentrations of CAPE, and resultant blots were probed for Flag-VHL. The control lane (con) represents the assay in the absence of added cofactors, while the untreated (UT) lane contains all required cofactors. Right: The same assay was repeated by utilizing a chemically hydroxylated peptide and CAPE (100 μM). (D) 293 cells, cotransfected with HA-HIF-1α and Flag-VHL, were treated with CAPE in the presence of MG-132 and lysed 4 h later. HA-HIF-1α or Flag-VHL protein was immunoprecipitated by the addition of anti-HA-antibody-bound beads or anti-Flag antibody, as described under Materials and Methods. Immunoprecipitated proteins were solubilized in SDS sample buffer and separated by SDS-PAGE. Blots were probed with anti-Flag antibody or anti-HA antibody.

harvested for quantification of protein, which was used to normalize VEGF levels. An experiment for each condition was carried out in triplicate.

2.7. Data analysis

Results were expressed as mean±S.E. Statistical differences among the results of various groups were compared by Student's *t* test. $P<.05$ was considered significant.

3. Results

3.1. CAPE activates HIF-1

To explore a molecular mechanism for a CAPE-mediated protective effect of I/R injury, we examined whether CAPE could stimulate HIF-1, whose activation is reported to protect tissues from I/R injury. Human lung carcinoma A549 cells and rat myocardial H9C2 cells were treated with CAPE and lysed to obtain nuclear extracts. HIF-1α levels in the nuclear extracts were examined by Western blot analysis. As shown in Fig. 1, CAPE elevated the level of HIF-1α protein in a dose-dependent manner in both cell lines. To test whether HIF-1α protein elevated by CAPE could lead to activation of HIF-1, we transfected cells with a HIF-1-responsive reporter gene plasmid and subsequently treated them with CAPE for 10 h, and then we measured luciferase activity. As shown in Fig. 2A, consistent with Western blot results, CAPE increased luciferase activity in a dose-dependent manner. To ensure CAPE activation of HIF-1, we examined the expression of HIF-1 target genes following treatment with CAPE. Since *VEGF* and *HO-1* are HIF-1 target genes involved in HIF-1-mediated tissue protection from I/R injury, it was tested whether CAPE was able to increase VEGF secretion and the level of HO-1 protein in A549 and H9C2 cells. As shown in Fig. 2B and C, in H9C2 cells, CAPE elevated the levels of VEGF and HO-1 (right); however, in A549 cells, only VEGF induction was observed (Fig. 2B, left). HO-1 was not induced even by a typical and strong HO-1 inducer (mercuric chloride) in A549 cells (Fig. 2C, left), although the inducer increased HO-1 protein level effectively in H9C2 cells (Fig. 2C, right), suggesting that A549 cells have a defect in inducing HO-1. However, it does not seem that A549 cells have a general defect in inducing HO-1, since a number of stimulants, including hemin and tumor growth factor-β1, are reported to induce HO-1 in human alveolar epithelial cells [41–44]. For verification of the HIF-1 dependence of the CAPE induction of VEGF and HO-1, cells were transfected with HIF-1α siRNA or pretreated with a specific HIF-1 inhibitor (echinomycin) for 1 day [45], followed by treatment with CAPE, and then VEGF and HO-1 levels were measured. As shown in Fig. 3A, HIF-1α siRNA, but not nontargeting siRNA, reduced the level of HIF-1α protein in A549 cells (top) and H9C2 cells (bottom). Consistent with the result, HIF-1α siRNA significantly attenuated VEGF secretion in A549 cells (Fig. 3B,

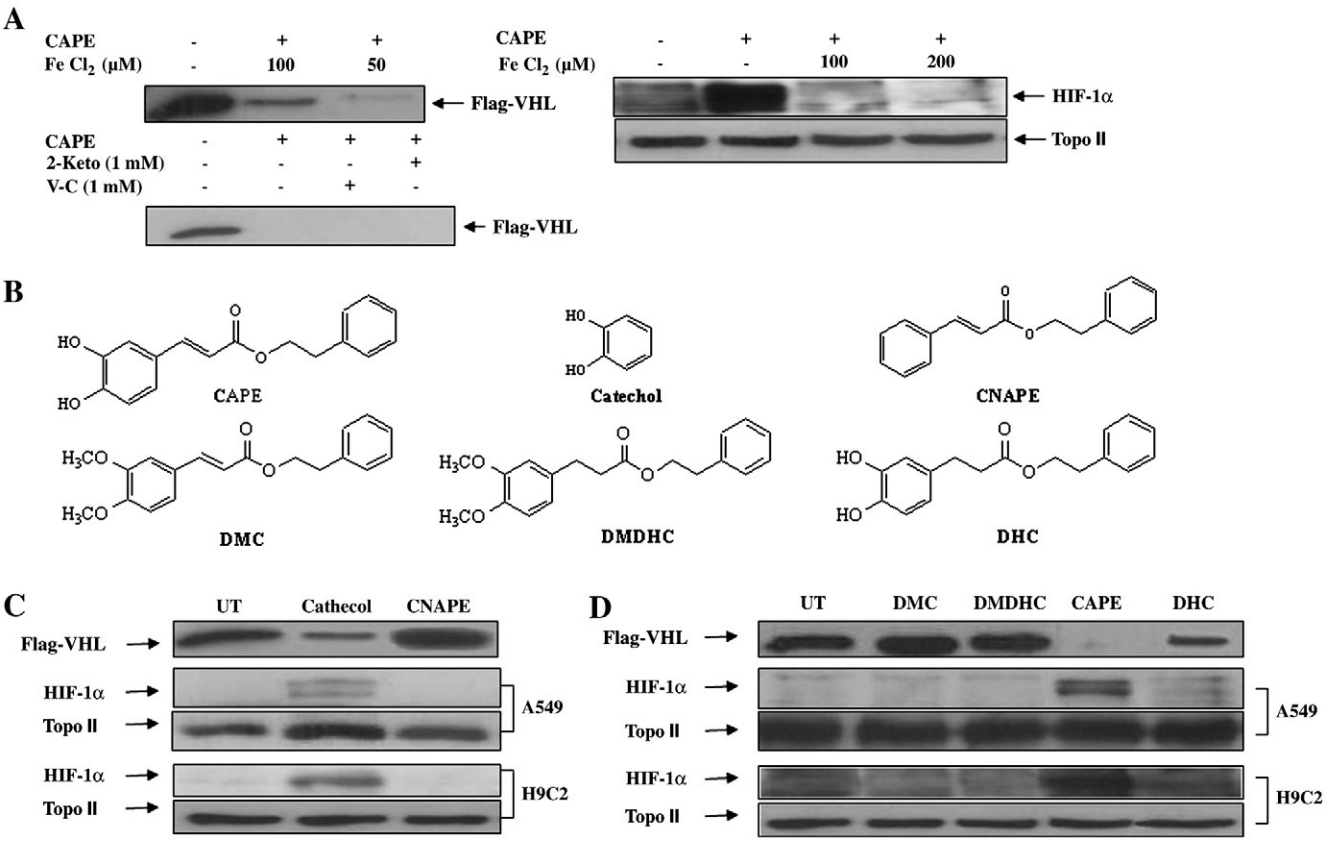

Fig. 5. The catechol moiety in CAPE is essential for inhibiting HPH and subsequently for activating HIF-1. (A) Left: A VHL capture assay was performed in the presence of CAPE (50 μM) and either escalating doses of iron, 2-ketoglutarate (2-Keto) or sodium ascorbate (V-C), and resultant blots were probed for Flag-VHL. Right: A549 cells were treated with CAPE (50 μM) for 4 h in the presence or in the absence of various ferrous chloride, and HIF-1α protein levels were monitored in nuclear extracts. (B) Chemical structures of CAPE, catechol, CNAPE, DMC, DMDHC and DHC. (C) Top: A VHL capture assay was performed in the presence of catechol (250 μM) or CNAPE (250 μM), and resultant blots were probed for Flag-VHL. Middle: A549 cells were treated with catechol (250 μM) or CNAPE (250 μM) and lysed to obtain nuclear extracts. HIF-1α levels in nuclear extracts were examined by Western blot analysis. Bottom: The same experiment as in (B) was performed in H9C2 cells. (D) Top: A VHL capture assay was performed in the presence of 50 μM CAPE, DMC, DMDHC or DHC, and resultant blots were probed for Flag-VHL. Middle: A549 cells were treated with 50 μM CAPE, DMC, DMDHC or DHC and lysed to obtain nuclear extracts. HIF-1α levels in nuclear extracts were examined by Western blot analysis. Bottom: The same experiment as in (B) was performed in H9C2 cells. (E) Top: 549 cells were transfected with a HIF-1-responsive reporter gene plasmid and subsequently treated with 50 μM CAPE, DMC, DMDHC or DHC, and luciferase activity was measured and normalized to CMV *Renilla* luciferase activity 10 h later. The data are presented as mean±S.E. ($n=3$). Second panel: A549 cells were treated with 50 μM CAPE, DMC, DMDHC or DHC, and VEGF in cell culture supernatants was analyzed 10 h later. The data are presented as mean±S.E. ($n=3$). Third panel: The same experiment was performed in H9C2 cells. The data are presented as mean±S.E. ($n=3$). Bottom: H9C2 cells were treated with 50 μM CAPE, DMC, DMDHC or DHC, and HO-1 levels were monitored in whole-cell lysates 4 h later.

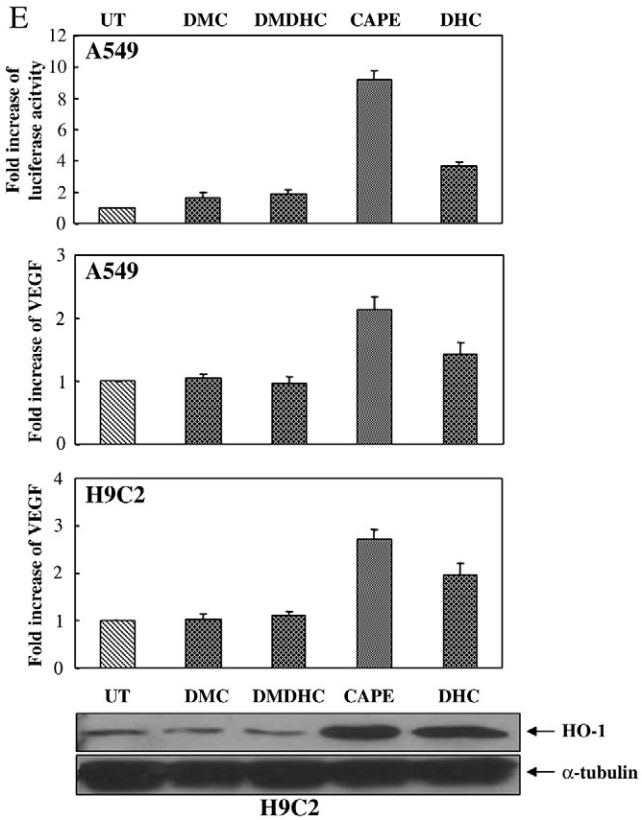

Fig. 5 (*continued*).

top) and H9C2 cells (Fig. 3B, middle), and HO-1 expression in H9C2 cells (Fig. 3B, bottom). Echinomycin completely abrogated the CAPE induction of VEGF and HO-1 in both cell lines (Fig. 3C, top, middle and bottom).

3.2. CAPE activates HIF-1 by inhibiting HPH

Since the α-subunit of HIF is tightly regulated at the post-translational level by protein degradation [19], we considered whether CAPE modulated HIF-1α stability. A549 cells were either left untreated or pretreated with CAPE for 4 h, followed by addition of the protein synthesis inhibitor cycloheximide for the indicated times. The disappearance rate of HIF-1α protein was then compared. As shown in Fig. 4A, HIF-1α protein was extremely labile, disappearing in 5 min in cells left untreated with CAPE. In marked contrast, a substantial amount of HIF-1α protein still remained in CAPE-pretreated cells 40 min after the addition of cycloheximide, suggesting that CAPE stabilized HIF-1α protein. The central molecular mechanism for regulating HIF-1α protein stability is VHL-dependent proteasomal HIF degradation, following hydroxylation of proline residues in HIF-1α by HPH [23,24]. We considered whether CAPE might impact upon the HIF-regulating pathway. To test this, we first examined the effects of CAPE on HIF-1α expression in either the parental VHL-deficient renal carcinoma cell line UMRC2 or UMRC2/VHL, which expresses a stably integrated construct encoding Flag-VHL. As shown in Fig. 4B, CAPE increased HIF-1α expression in UMRC2/VHL; however, when this experiment was repeated in the VHL-deficient parental line, CAPE was unable to induce HIF-1α expression. While these data support the involvement of VHL in CAPE-mediated HIF induction, it remains unclear how CAPE intervenes in VHL-dependent HIF-1α regulation. HPH is the key enzyme for VHL-dependent HIF degradation; thus, we examined whether CAPE affected HPH activity. To do this, we utilized an *in vitro* VHL capture assay with a biotinylated HIF peptide that contains a conserved proline residue subject to HPH-dependent hydroxylation. As shown in Fig. 4C (left), the association of VHL with the HIF peptide in the absence of exogenously added cofactors is undetectable (lane "con"). When the required cofactors for HPH are added, the association between the HIF peptide and VHL is markedly enhanced (lane "UT"). Strikingly, a 25 μM concentration of CAPE significantly reduced the association between HIF and VHL, and a 50 μM concentration completely abrogated the interaction between these proteins. Finally, we used a chemically synthesized hydroxylated peptide to verify whether CAPE impacts directly upon HPH activity and does not impair VHL protein. As shown in Fig. 4C (right), CAPE did not impair the ability of VHL to associate with hydroxylated HIF peptide up to 100 μM. In contrast, a HIF peptide in which the two proline residues were mutated to alanine failed to bind VHL under any circumstances (data not shown). Our data strongly support the premise that CAPE is a potent inhibitor of HPH. To test this notion in cells, we transfected an HA-HIF-1α plasmid and a Flag-VHL plasmid in 293 cells, followed by 4 h of treatment with a proteasome inhibitor MG-132 in the presence or in the absence of CAPE. After immunoprecipitation with anti-HA antibody or anti-Flag antibody, VHL or HIF-1α levels in immunocomplexes were monitored using anti-HA antibody or anti-Flag antibody. As shown in Fig. 4D, MG-132 increased the VHL or HIF-1α level in immunocomplexes and, consistent with the result of VHL capture assay, CAPE effectively prevented the coprecipitation of VHL-HIF-1α.

3.3. Structural analysis of CAPE for CAPE inhibition of HPH

We wished to explore how CAPE inhibited the enzyme. Since the enzyme requires cofactors to catalyze HIF-1α hydroxylation, we examined whether CAPE impaired the activity of the enzyme by affecting the availability of the required factors. *In vitro* VHL capture assay was performed in the presence of various concentrations of the factors. As shown in Fig. 5A, while a 10-fold increase in either 2-ketoglutarate or ascorbate did not at all affect the inhibitory effect of CAPE on the enzyme, an increase in iron concentration attenuated the CAPE effect, as represented by a partly restored VHL association (left). To test this further, we treated cells with CAPE in the presence of escalating doses of iron, and we monitored the HIF-1α level. Consistent with *in vitro* results, HIF-1α induction was abolished under iron-enriched conditions (right). This result suggests that CAPE inhibits HPH activity by reducing the availability of iron. Since the catechol moiety of CAPE possesses an iron-chelating activity, we examined whether the functional group was required for HPH inhibition. To do this, an *in vitro* VHL capture assay was performed with catechol and CNAPE, which has no side chain or 3,4-dihydroxy group in CAPE, respectively, as shown in Fig. 5B. As shown in Fig. 5C (top), catechol significantly attenuated VHL association at 250 μM, whereas CNAPE had no ability to inhibit HPH. Consistent with these results, catechol, but not CNAPE, induced HIF-1α protein in A549 cells (middle) and H9C2 cells (bottom). However, the potency of catechol-inhibiting HPH was much lower than that of CAPE, suggesting that the side chain of CAPE contributes to increasing potency. For further structural analysis, CAPE derivatives with variations in aromatic substituent and/or side chain, such as DMC, dimethoxydihydrocinnamic acid phenethyl ester (DMDHC) and DHC, were prepared (Fig. 5B), and their abilities to inhibit HPH and to induce HIF-1α protein were compared. As shown in Fig. 5D (top), DMC and DMDHC, which lack chelating activity, did not either attenuate VHL association in the *in vitro* assay (top) or induce HIF-1α protein in A549 cells (middle) and H9C2 cells (bottom), confirming the requirement for the catechol moiety. Moreover, DHC, where the Michael reaction

acceptor (–C=C–) is hydrogenated, showed a marked decrease in the potency of HPH inhibition and HIF-1α protein induction (Fig. 5D, top, middle and bottom). We also examined whether these results were correlated with the ability of CAPE derivatives to induce HIF-1-responsive luciferase and the HIF-1 target genes *VEGF* and *HO-1*. Cells were treated with the CAPE derivatives, and luciferase expression, VEGF secretion and HO-1 protein induction were monitored. As shown in Fig. 5E, consistent with the above results, DMC and DMDHC did not at all induce luciferase (top), VEGF (second and third panels) and HO-1 (bottom), and DHC showed a lower ability to induce luciferase and the target genes than did CAPE. Furthermore, it was examined whether the phenethyl moiety in CAPE played a role in the biological activities of the natural product. CAME, caffeic acid *n*-pentyl ester (CAPEN) and caffeic acid *n*-hexyl ester (CAHA) were prepared (Fig. 6A). CAME was designed to examine the structural role of the phenethyl moiety in CAPE. CAPEN and CAHA were introduced to see whether *n*-alkyl moieties (similar in molecular length to the phenethyl moiety) could replace the phenethyl moiety. The potency of the derivatives for HPH inhibition and HIF-1α protein induction was compared with that of CAPE. Substitution with the methyl moiety severely deteriorated the CAPE activity for HPH inhibition, indicating the importance of the phenethyl moiety. CAPEN, which was more potent than CAHA, was a little less potent than CAPE in inhibiting HPH (Fig. 6B). Consistent with the above results, as shown in Fig. 6C, the potency for HIF-1α protein induction was on the order of CAPE>CAPEN>CAHA>CAME. We also compared the potency of the derivatives for HIF-1 activation with the potency of the derivatives of CAPE. HIF-1-responsive luciferase induction and VEGF secretion were measured in A549 cells, followed by treatment with the derivatives or CAPE. As shown in Fig. 6D and E, the compounds increased luciferase induction and VEGF secretion, and their potency was on the same order as that for HPH inhibition.

4. Discussion

In this study, we investigated a potential molecular mechanism underlying a CAPE-mediated protective effect on I/R injury and the structure–activity relationship for CAPE inhibition of HPH and subsequent activation of HIF-1. Our data demonstrated that CAPE induced HO-1 and VEGF by activating HIF-1, which occurred by inhibition of HPH. It has also been revealed that the catechol moiety is essential for HPH inhibition, and that saturation of the double bond (–C=C–) and change in the length of the ester moiety impaired the ability to inhibit HPH.

Our data demonstrate that CAPE elevated the level of HIF-1α protein, which activated HIF-1, as shown in the luciferase experiment. Furthermore, CAPE induced the HIF-1 target genes *HO-1* and *VEGF*, at least partly, *via* HIF-1. HIF-1 dependence was demonstrated by data showing that expression of HO-1 protein and secretion of VEGF were significantly attenuated by a biological HIF-1 inhibitor (HIF-1α siRNA) or a chemical HIF-1 inhibitor (echinomycin). Since *HO-1* and *VEGF* are HIF-1 target genes involved in tissue protection from I/R injury [27,34–36], this result strongly suggests that CAPE-mediated HIF-1 activation is relevant to the CAPE effect that protects tissues from I/R injury.

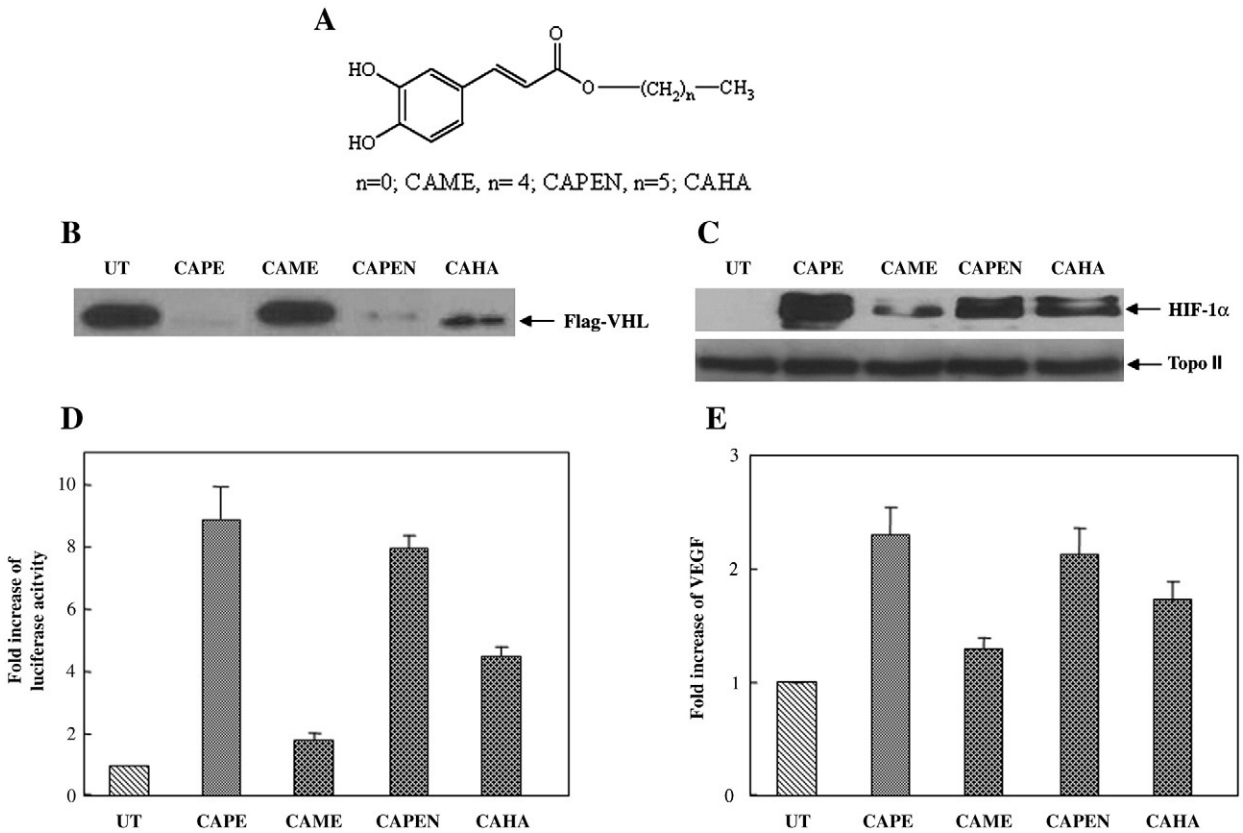

Fig. 6. Effects of the modification of the ester moiety in CAPE on HPH inhibition and HIF-1 activation. (A) Chemical structures of CAME, CAPEN and CAHA. (B) A VHL capture assay was performed in the presence of 25 μM CAPE, CAME, CAPEN or CAHA, and resultant blots were probed for Flag-VHL. (C) A549 cells were treated with 50 μM CAPE, CAME, CAPEN or CAHA and lysed to obtain nuclear extracts. HIF-1α levels in nuclear extracts were examined by Western blot analysis. (D) A549 cells were transfected with a HIF-1-responsive reporter gene plasmid and subsequently treated with 50 μM CAPE, CAME, CAPEN or CAHA, and luciferase activity was measured and normalized to CMV *Renilla* luciferase activity 10 h later. The data are presented as mean±S.E. (n=3). (E) A549 cells were treated with 50 μM CAPE, CAME, CAPEN or CAHA, and VEGF in cell culture supernatants was analyzed 10 h later as described under Materials and Methods. The data are presented as mean±S.E. (n=3).

Furthermore, we elucidated the molecular mechanism underlying CAPE-mediated HIF-1α protein elevation and subsequent activation of the HIF-1 pathway. Our data showing that CAPE delayed the degradation of HIF-1α protein and that CAPE mediated HIF-1α induction occurred only in cells with functional VHL, suggesting that the natural product stabilizes HIF-1α protein by preventing VHL-dependent HIF-1α degradation. This hypothesis can be validated by providing compelling evidence that CAPE inhibited HPH, thus interfering with hydroxylation of HIF-1α, a critical posttranslational modification for VHL-dependent degradation of HIF-1α. In the *in vitro* VHL capture assay where IVT HPH hydroxylates HIF peptide and, subsequently, the hydroxylated peptide associates with IVT VHL, we observed that addition of CAPE attenuated VHL association with HIF peptide, indicating a reduced hydroxylation of HIF peptide by CAPE-mediated HPH inhibition. This *in vitro* result was confirmed by demonstrating that CAPE decreased the level of VHL or HIF-1α precipitated together with HIF-1α or VHL in cells.

We suggest that HPH inhibition by CAPE occurred *via* a reduction in the availability of iron, an essential cofactor of the enzyme. This argument was supported by data showing that: (a) escalation of iron dose attenuated the CAPE effect on VHL association; (b) pretreatment with iron prevented HIF-1α protein induction by CAPE; and (c) dose changes in the other factors, ascorbate and 2-ketoglutarate, did not influence the CAPE effects on VHL association. In line with this finding, it was revealed that the catechol moiety (the chelating moiety of CAPE) was essential for HPH inhibition. The requirement for the catechol moiety was illustrated by showing that: (a) while catechol itself inhibited HPH, cinnamic phenethylester, a CAPE derivative without the catechol moiety, did not inhibit HPH at all; and (b) methylated CAPE derivatives with no chelating activity lost their ability to inhibit HPH and to induce HIF-1α protein, subsequently activating HIF-1 (HIF-1-dependent luciferase, HO-1 and VEGF induction). Although CAPE reportedly induces HO-1 *via* activation of Nrf2 [15], for which the Michael reaction acceptor functionality in CAPE is required, our data showing that CAPE induction of HO-1 was at least partly dependent on HIF-1 and that the catechol moiety in CAPE was essential for HIF-1 activation and HO-1 induction suggest that HIF-1 and Nrf2 are involved in CAPE-mediated HO-1 induction and that the structural requirement for CAPE for HO-1 induction is the catechol moiety rather than the Michael reaction acceptor at least in the cell line H9C2. Moreover, the aliphatic double bond (–C=C–) and the phenethyl ester moiety in CAPE seem to be important for the optimal interaction of CAPE with HPH. Hydrogenation of the double bond and substitution of the phenethyl moiety with the methyl moiety significantly reduced the potency to inhibit HPH. Our data demonstrated that substitution of the phenethyl moiety with *n*-alkyl moieties that are similar in molecular length (*n*-pentyl and *n*-hexyl) did not completely restore the activity for HPH inhibition. It is likely that the phenethyl moiety may interact with HPH with a little greater affinity than does *n*-alkyl moiety with a similar molecular length. Moreover, the potency of the *n*-alkyl derivatives (CAME, CAPEN and CAHA) for HIF-1α protein induction and HIF-1 activation (HIF-1-dependent luciferase induction and VEGF secretion) was on the same order as that for the inhibition of HPH, indicating that the *n*-alkyl derivatives also utilize the same pathway with CAPE for induction of HIF-1 target genes.

Although our molecular data suggest the possibility of the involvement of HPH–HIF-1 in CAPE-mediated tissue protection, it is uncertain whether this pathway is indeed implicated in the clinical beneficial effect of CAPE on I/R injury. The dose of CAPE, which is administered *via* parenteral routes, does not usually exceed 3 mg/kg, very likely achieving a plasma concentration of less than 25 μM [46,47]. Nevertheless, considering that the HPH–HIF-1 pathway is a promising molecular target for protective effect on I/R injury [31], our data providing the structural information of CAPE for inhibition of HPH may be useful for the drug design of a safe and potent therapeutic agent against I/R injury using the natural product CAPE as lead.

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References

[1] Son S, Lewis BA. Free radical scavenging and antioxidative activity of caffeic acid amide and ester analogues: structure–activity relationship. J Agric Food Chem 2002;50(3):468–72.
[2] Borrelli F, Maffia P, Pinto L, Ianaro A, Russo A, Capasso F, et al. Phytochemical compounds involved in the anti-inflammatory effect of propolis extract. Fitoterapia 2002;73(Suppl 1):S53–S63.
[3] Ho CC, Lin SS, Chou MY, Chen FL, Hu CC, Chen CS, et al. Effects of CAPE-like compounds on HIV replication *in vitro* and modulation of cytokines *in vivo*. J Antimicrob Chemother 2005;56(2):372–9.
[4] Park JH, Lee JK, Kim HS, Chung ST, Eom JH, Kim KA, et al. Immunomodulatory effect of caffeic acid phenethyl ester in Balb/c mice. Int Immunopharmacol 2004;4(3):429–36.
[5] Liao HF, Chen YY, Liu JJ, Hsu ML, Shieh HJ, Liao HJ, et al. Inhibitory effect of caffeic acid phenethyl ester on angiogenesis, tumor invasion, and metastasis. J Agric Food Chem 2003;51(27):7907–12.
[6] Calikoglu M, Tamer L, Sucu N, Coskun B, Ercan B, Gul A, et al. The effects of caffeic acid phenethyl ester on tissue damage in lung after hindlimb ischemia–reperfusion. Pharmacol Res 2003;48(4):397–403.
[7] Parlakpinar H, Sahna E, Acet A, Mizrak B, Polat A. Protective effect of caffeic acid phenethyl ester (CAPE) on myocardial ischemia–reperfusion-induced apoptotic cell death. Toxicology 2005;209(1):1–14.
[8] Ozyurt H, Sogut S, Yildirim Z, Kart L, Iraz M, Armutcu F, et al. Inhibitory effect of caffeic acid phenethyl ester on bleomycin-induced lung fibrosis in rats. Clin Chim Acta 2004;339(1–2):65–75.
[9] Hwang HJ, Park HJ, Chung HJ, Min HY, Park EJ, Hong JY, et al. Inhibitory effects of caffeic acid phenethyl ester on cancer cell metastasis mediated by the down-regulation of matrix metalloproteinase expression in human HT1080 fibrosarcoma cells. J Nutr Biochem 2006;17(5):356–62.
[10] Weyant MJ, Carothers AM, Bertagnolli ME, Bertagnolli MM. Colon cancer chemopreventive drugs modulate integrin-mediated signaling pathways. Clin Cancer Res 2000;6(3):949–56.
[11] Song YS, Park EH, Hur GM, Ryu YS, Lee YS, Lee JY, et al. Caffeic acid phenethyl ester inhibits nitric oxide synthase gene expression and enzyme activity. Cancer Lett 2002;175(1):53–61.
[12] Fesen MR, Kohn KW, Leteurtre F, Pommier Y. Inhibitors of human immunodeficiency virus integrase. Proc Natl Acad Sci USA 1993;90(6):2399–403.
[13] Sud'ina GF, Mirzoeva OK, Pushkareva MA, Korshunova GA, Sumbatyan NV, Varfolomeev SD. Caffeic acid phenethyl ester as a lipoxygenase inhibitor with antioxidant properties. FEBS Lett 1993;329(1–2):21–4.
[14] Michaluart P, Masferrer JL, Carothers AM, Subbaramaiah K, Zweifel BS, Koboldt C, et al. Inhibitory effects of caffeic acid phenethyl ester on the activity and expression of cyclooxygenase-2 in human oral epithelial cells and in a rat model of inflammation. Cancer Res 1999;59(10):2347–52.
[15] Balogun E, Hoque M, Gong P, Killeen E, Green CJ, Foresti R, et al. Curcumin activates the haem oxygenase-1 gene *via* regulation of Nrf2 and the antioxidant-responsive element. Biochem J 2003;371(Pt 3):887–95.
[16] Natarajan K, Singh S, Burke Jr TR, Grunberger D, Aggarwal BB. Caffeic acid phenethyl ester is a potent and specific inhibitor of activation of nuclear transcription factor NF-kappa B. Proc Natl Acad Sci USA 1996;93(17):9090–5.
[17] Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix–loop–helix–PAS heterodimer regulated by cellular O_2 tension. Proc Natl Acad Sci USA 1995;92(12):5510–4.
[18] Cockman ME, Masson N, Mole DR, Jaakkola P, Chang GW, Clifford SC, et al. Hypoxia inducible factor-alpha binding and ubiquitylation by the von Hippel–Lindau tumor suppressor protein. J Biol Chem 2000;275(33):25733–41.
[19] Huang LE, Gu J, Schau M, Bunn HF. Regulation of hypoxia-inducible factor 1alpha is mediated by an O_2-dependent degradation domain *via* the ubiquitin–proteasome pathway. Proc Natl Acad Sci USA 1998;95(14):7987–92.
[20] Salceda S, Caro J. Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin–proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. J Biol Chem 1997;272(36):22642–7.
[21] Tanimoto K, Makino Y, Pereira T, Poellinger L. Mechanism of regulation of the hypoxia-inducible factor-1 alpha by the von Hippel–Lindau tumor suppressor protein. EMBO J 2000;19(16):4298–309.

[22] Semenza GL. Hypoxia-inducible factor 1: master regulator of O_2 homeostasis. Curr Opin Genet Dev 1998;8(5):588–94.
[23] Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, et al. HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing. Science 2001;292(5516):464–8.
[24] Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, et al. Targeting of HIF-alpha to the von Hippel–Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. Science 2001;292(5516):468–72.
[25] Hirsila M, Koivunen P, Gunzler V, Kivirikko KI, Myllyharju J. Characterization of the human prolyl 4-hydroxylases that modify the hypoxia-inducible factor. J Biol Chem 2003;278(33):30772–80.
[26] Bruick RK, McKnight SL. A conserved family of prolyl-4-hydroxylases that modify HIF. Science 2001;294(5545):1337–40.
[27] Date T, Mochizuki S, Belanger AJ, Yamakawa M, Luo Z, Vincent KA, et al. Expression of constitutively stable hybrid hypoxia-inducible factor-1alpha protects cultured rat cardiomyocytes against simulated ischemia–reperfusion injury. Am J Physiol Cell Physiol 2005;288(2):C314–20.
[28] Chin BY, Jiang G, Wegiel B, Wang HJ, Macdonald T, Zhang XC, et al. Hypoxia-inducible factor 1alpha stabilization by carbon monoxide results in cytoprotective preconditioning. Proc Natl Acad Sci USA 2007;104(12):5109–14.
[29] Matsumoto M, Makino Y, Tanaka T, Tanaka H, Ishizaka N, Noiri E, et al. Induction of renoprotective gene expression by cobalt ameliorates ischemic injury of the kidney in rats. J Am Soc Nephrol 2003;14(7):1825–32.
[30] Cai Z, Zhong H, Bosch-Marce M, Fox-Talbot K, Wang L, Wei C, et al. Complete loss of ischaemic preconditioning-induced cardioprotection in mice with partial deficiency of HIF-1 alpha. Cardiovasc Res 2007;77(3):463–70.
[31] Natarajan R, Salloum FN, Fisher BJ, Kukreja RC, Fowler III AA. Hypoxia inducible factor-1 activation by prolyl 4-hydroxylase-2 gene silencing attenuates myocardial ischemia reperfusion injury. Circ Res 2006;98(1):133–40.
[32] Natarajan R, Salloum FN, Fisher BJ, Ownby ED, Kukreja RC, Fowler III AA. Activation of hypoxia-inducible factor-1 *via* prolyl-4 hydroxylase-2 gene silencing attenuates acute inflammatory responses in postischemic myocardium. Am J Physiol Heart Circ Physiol 2007;293(3):H1571–80.
[33] Nagle DG, Zhou YD. Natural product-derived small molecule activators of hypoxia-inducible factor-1 (HIF-1). Curr Pharm Des 2006;12(21):2673–88.
[34] Ockaili R, Natarajan R, Salloum F, Fisher BJ, Jones D, Fowler III AA, et al. HIF-1 activation attenuates postischemic myocardial injury: role for heme oxygenase-1 in modulating microvascular chemokine generation. Am J Physiol Heart Circ Physiol 2005;289(2):H542–8.
[35] Zhu Y, Zhang Y, Ojwang BA, Brantley Jr MA, Gidday JM. Long-term tolerance to retinal ischemia by repetitive hypoxic preconditioning: role of HIF-1alpha and heme oxygenase-1. Invest Ophthalmol Vis Sci 2007;48(4):1735–43.
[36] Mu D, Jiang X, Sheldon RA, Fox CK, Hamrick SE, Vexler ZS, et al. Regulation of hypoxia-inducible factor 1alpha and induction of vascular endothelial growth factor in a rat neonatal stroke model. Neurobiol Dis 2003;14(3):524–34.
[37] Gillespie DL, Whang K, Ragel BT, Flynn JR, Kelly DA, Jensen RL. Silencing of hypoxia inducible factor-1alpha by RNA interference attenuates human glioma cell growth *in vivo*. Clin Cancer Res 2007;13(8):2441–8.
[38] Andrews NC, Faller DV. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. Nucleic Acids Res 1991;19(9):2499.
[39] Jung YJ, Isaacs JS, Lee S, Trepel J, Neckers L. Microtubule disruption utilizes an NFkappa B-dependent pathway to stabilize HIF-1alpha protein. J Biol Chem 2003;278(9):7445–52.
[40] Isaacs JS, Jung YJ, Mimnaugh EG, Martinez A, Cuttitta F, Neckers LM. Hsp90 regulates a von Hippel Lindau-independent hypoxia-inducible factor-1 alpha-degradative pathway. J Biol Chem 2002;277(33):29936–44.
[41] Bundy RE, Hoare GS, Kite A, Beach J, Yacoub M, Marczin N. Redox regulation of p38 MAPK activation and expression of ICAM-1 and heme oxygenase-1 in human alveolar epithelial (A549) cells. Antioxid Redox Signal 2005;7(1–2):14–24.
[42] Ning W, Song R, Li C, Park E, Mohsenin A, Choi AM, et al. TGF-beta1 stimulates HO-1 *via* the p38 mitogen-activated protein kinase in A549 pulmonary epithelial cells. Am J Physiol Lung Cell Mol Physiol 2002;283(5):L1094–102.
[43] Lin CC, Chiang LL, Lin CH, Shih CH, Liao YT, Hsu MJ, et al. Transforming growth factor-beta1 stimulates heme oxygenase-1 expression *via* the PI3K/Akt and NF-kappaB pathways in human lung epithelial cells. Eur J Pharmacol 2007;560(2–3):101–9.
[44] Fukano Y, Oishi M, Chibana F, Numazawa S, Yoshida T. Analysis of the expression of heme oxygenase-1 gene in human alveolar epithelial cells exposed to cigarette smoke condensate. J Toxicol Sci 2006;31(2):99–109.
[45] Kong D, Park EJ, Stephen AG, Calvani M, Cardellina JH, Monks A, et al. Echinomycin, a small-molecule inhibitor of hypoxia-inducible factor-1 DNA-binding activity. Cancer Res 2005;65(19):9047–55.
[46] Ozyurt H, Ozyurt B, Koca K, Ozgocmen S. Caffeic acid phenethyl ester (CAPE) protects rat skeletal muscle against ischemia–reperfusion-induced oxidative stress. Vasc Pharmacol 2007;47(2–3):108–12.
[47] Ozyurt B, Iraz M, Koca K, Ozyurt H, Sahin S. Protective effects of caffeic acid phenethyl ester on skeletal muscle ischemia–reperfusion injury in rats. Mol Cell Biochem 2006;292(1–2):197–203.