

Caffeic Acid Phenethyl Ester Inhibits PDGF-Induced Proliferation of Vascular Smooth Muscle Cells via Activation of p38 MAPK, HIF-1 α , and Heme Oxygenase-1

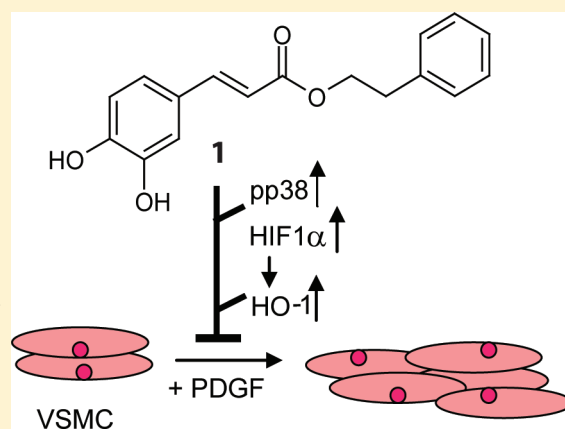
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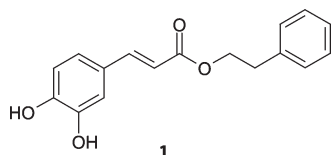
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S Supporting Information

ABSTRACT: Hyperproliferation of vascular smooth muscle cells (VSMCs) is critically involved in the onset of atherosclerosis and restenosis. Although caffeic acid phenethyl ester (CAPE, **1**), one of the main constituents of honeybee propolis, has been shown to exert a beneficial effect in models of vascular injury in vivo, detailed mechanistic investigations in vascular cells are scarce. This study has examined the antiproliferative activity of **1** in platelet-derived growth factor (PDGF)-stimulated primary rat aortic VSMCs and aimed to shed light on underlying molecular mechanisms. Compound **1** inhibited the proliferation of VSMCs upon exposure to PDGF in a dose-dependent manner by interfering with cell cycle progression from the G0/1- to the S-phase. Enhanced phosphorylation of p38 mitogen-activated protein kinase (MAPK) as well as stabilization of hypoxia-inducible factor (HIF)-1 α and subsequent induction of heme oxygenase-1 (HO-1) could be identified as molecular events contributing to the observed growth arrest in PDGF-activated VSMCs upon exposure to **1**.



trans-Caffeic acid phenethyl ester (CAPE, **1**) is a phenolic antioxidant that has been identified as one of the major components of honeybee propolis.¹ The biological activities of propolis and **1** are diverse and include anti-inflammatory, anticarcinogenic, and antioxidant activities.^{2–5} In the context of vasculoproliferative disorders, **1** has been shown to prevent neointima thickening after balloon angioplasty in rats and rabbits in vivo.^{6,7} Its underlying molecular mode of action is not yet completely resolved. Inhibition of nuclear factor (NF)- κ B as well as of the mitogenic kinases MEK and AKT have been proposed,^{6,7} however, without clear causal evidence. This study therefore examined (i) whether **1** affects proliferation of primary rat aortic vascular smooth muscle cells (VSMCs) after stimulation with platelet-derived growth factor (PDGF), a potent physiological mitogen for VSMCs,⁸ and (ii) what signaling pathways are involved.



RESULTS AND DISCUSSION

Treatment of quiescent VSMCs with different concentrations of **1** (1–10 μ M) caused a dose-dependent inhibition of PDGF

(20 ng/mL)-triggered proliferation (Figure 1A). Compound **1** blocked PDGF-induced DNA synthesis completely at a concentration of 5 μ M (Figure 1B). First indications of cell death in cultivated VSMCs were observed only at doses higher than 25 μ M of **1** after an incubation time of 48 h, as evident by morphological changes, detachment of the cells, and a decline of cell viability (assessed by trypan blue exclusion) to 65% (data not shown). Staining nuclei with propidium iodide and subsequent flow-cytometric cell cycle analysis consistently showed that cells that are treated with 5 μ M **1** are retained in the G0/1-phase of the cell cycle (Figure 1C). Hyperphosphorylation of the retinoblastoma protein, indicative of progression through S-phase, was abolished in cells treated with **1**, providing evidence for the G1 arrest at the protein level (Supporting Information, Figure S1). These data are in apparent contrast to a recent report showing arrest of coronary smooth muscle cells⁷ in S-phase upon treatment with **1**. The reason for this discrepancy may lie in different experimental parameters (e.g., cell type, species, age of donors, media composition, starvation, stimulus, etc.).

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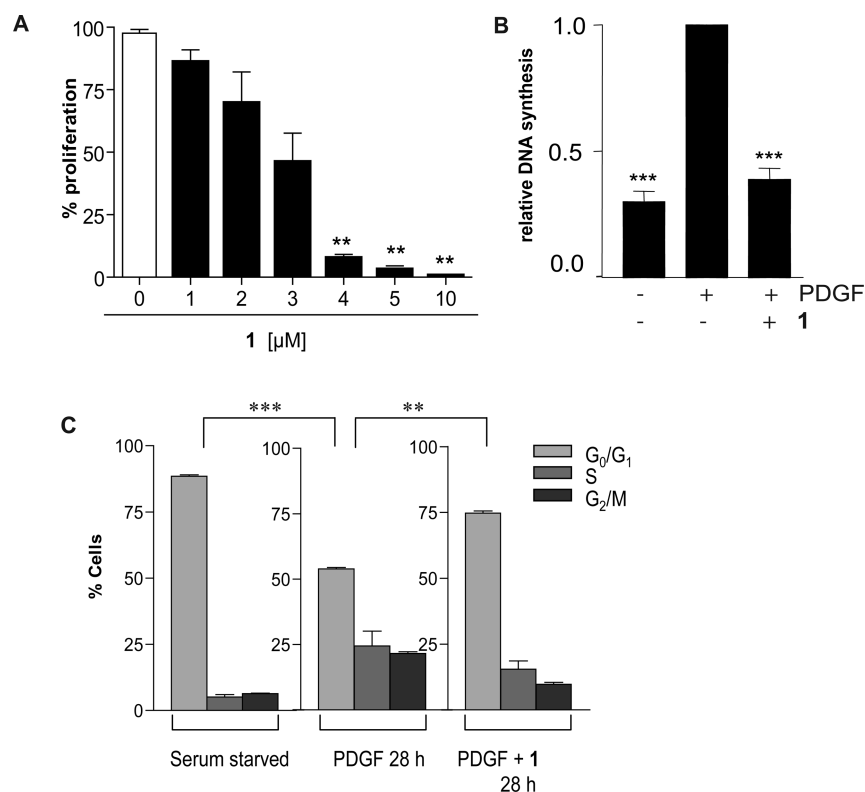


Figure 1. PDGF-induced proliferation of VSMCs is inhibited by **1**. (A) Quiescent VSMCs were treated with the indicated concentrations of **1** (CAPE) or vehicle before they were stimulated with PDGF (20 ng/mL) at $t(0)$ for 24 h. Then, cells were counted, and the increase in cell number in the vehicle-treated cells was set to 100%. (B) Quiescent VSMCs were treated with **1** (CAPE, 5 μ M) for 30 min before they were stimulated with PDGF (20 ng/mL) for 24 h, and incorporation of ³H-thymidine was determined. (C) Quiescent VSMCs were treated with 5 μ M CAPE for 30 min and then stimulated with PDGF (20 ng/mL) for 28 h before cell cycle analysis was performed as described in the Experimental Section ($n = 3$, ** $p < 0.01$, *** $p < 0.001$; ANOVA, Dunnett's post test).

Compound **1** is a known inhibitor of the transcription factor NF- κ B.⁹ NF- κ B regulates cell cycle progression and proliferation of a number of cell types.¹⁰ Since it is controversial as to whether PDGF triggers activation of NF- κ B in VSMCs,^{11,12} we investigated whether PDGF can lead to degradation of the inhibitor of NF- κ B ($I\kappa$ B), one of the first steps in the canonical pathway of NF- κ B activation. Whereas tumor necrosis factor (TNF)- α , a known activator of NF- κ B, triggers complete degradation of $I\kappa$ B between 15 and 45 min after exposure, PDGF had no effect on the levels of $I\kappa$ B (Supporting Information, Figure S2A) over time. It cannot be entirely excluded that PDGF activates NF- κ B via an alternative pathway, independent of $I\kappa$ B degradation. However, a NF- κ B-dependent luciferase reporter construct failed to be activated by PDGF in VSMCs (data not shown). Moreover, 5 μ M of **1** that effectively blocked VSMC proliferation did not reduce the TNF- α -induced NF- κ B binding activity in VSMCs, as seen in a gel shift assay (Supporting Information, Figure S2B). From these data it may be concluded that (i) PDGF does not induce NF- κ B activity in PDGF-induced VSMCs, (ii) concentrations of **1** as low as 5 μ M are not able to block NF- κ B activation, and, thus, (iii) inhibition of NF- κ B cannot explain the observed antimetogenic activity of **1** in PDGF-stimulated VSMCs.

Upon stimulation of quiescent VSMCs with PDGF, several growth-related signaling pathways are activated, such as the mitogen-activated protein kinases (MAPK, i.e., p38, ERK1/2, JNK1/2), the AKT kinase pathway or signal transducer and

activator of transcription (STAT)3 (Figure 2A), which are pivotal to translate the proliferative stimulus into cell division.¹³ In contrast to the data of Ho et al.,⁷ none of these early signaling nodes were inhibited by **1** in VSMCs employed in this study. Compound **1** even caused a highly reproducible enhanced and prolonged activation of p38 MAPK at a concentration of 5 μ M (Figure 2B). Moreover, the p38 kinase inhibitor SB203580 (10 μ M) significantly reduced the growth inhibitory effect of **1**, indicating that p38 kinase activity contributes to its antiproliferative effect (Figures 2C).

Compound **1** is known to induce heme oxygenase-1 (HO-1) in other cell types,¹⁴ and HO-1 has consistently been reported to lead to cell-cycle arrest in VSMCs.^{15,16} Therefore, it was tested as to whether **1** is capable of inducing HO-1 expression in VSMCs and whether this can account for the observed cell cycle arrest. Compound **1** as well as the positive control, cobalt protoporphyrin (CoPP, 10 μ M), markedly increased HO-1 expression in VSMCs (Figure 3A). Inhibition of HO-1 induction by transfection with a specific siRNA significantly diminished the growth arrest elicited by **1** (Figure 3B), demonstrating that HO-1 induction plays a role in the antiproliferative response of this compound. Several transcription factors are reported to mediate expression of HO-1, including NF-E2-related factor-2 (Nrf2) and hypoxia-inducible factor (HIF)-1 α .^{17,18} Compound **1** has moreover been reported to inhibit HIF prolylhydroxylase and thereby to prevent proteasomal degradation of HIF-1 α that normally ensures low levels of HIF-1 α under nonhypoxic conditions.¹⁹

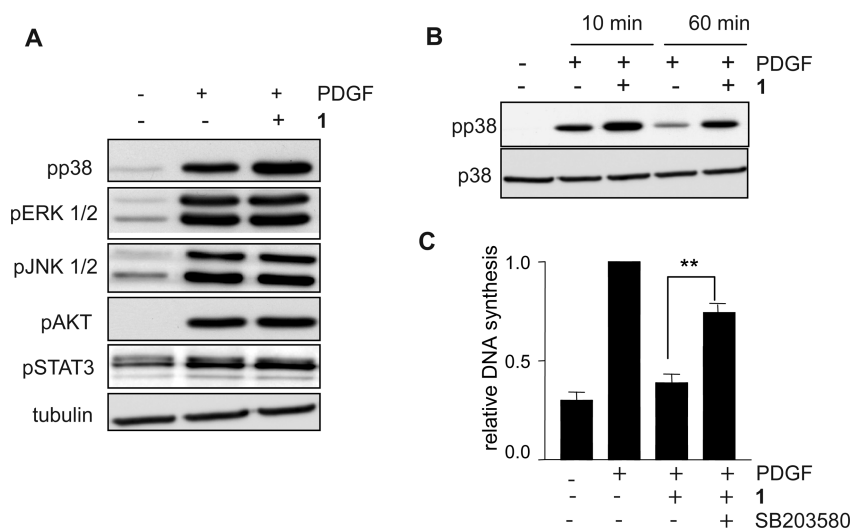


Figure 2. Compound **1** does not interfere with early signaling steps, but enhances p38 phosphorylation upon PDGF stimulation in VSMCs. (A) Quiescent VSMCs were treated with vehicle or **1** (CAPE, 5 μ M) for 30 min before they were stimulated with PDGF (20 ng/mL) for 10 min. Total cell lysates were subjected to Western blot analysis for the indicated proteins. (B) Quiescent VSMCs were treated with vehicle or **1** (CAPE, 5 μ M) for 30 min and then stimulated with PDGF for 10 and 60 min, respectively. Total cell lysates were subjected to immunoblot analysis for levels of phosphorylated (pp38) and total p38. Representative blots of three independent experiments with consistent results are depicted. (C) Quiescent VSMCs were treated with vehicle, **1** (CAPE, 5 μ M), a p38 inhibitor (SB203580; 10 μ M), and PDGF (20 ng/mL) as indicated for 24 h before their relative DNA synthesis was determined by assessing incorporated 3H-thymidine ($n = 3$, ** $p < 0.01$ Student's t -test).

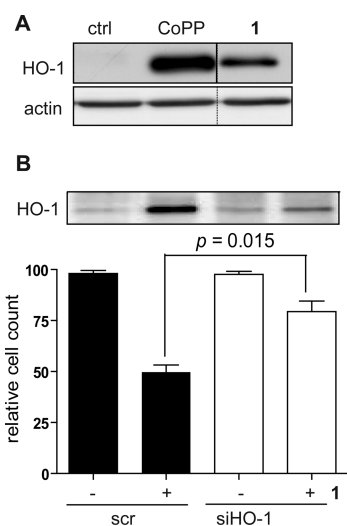


Figure 3. HO-1 induction contributes to growth arrest elicited by **1** in VSMCs. (A) VSMCs were treated with vehicle (ctrl), **1** (CAPE, 5 μ M), and CoPP (10 μ M) for 16 h, respectively. Total cell lysates were subjected to immunoblot analysis for HO-1 and actin levels. Representative blots of three independent experiments are shown. (B) VSMCs were transfected with siRNA, scrambled (scr) or specific for HO-1 (siHO-1), starved, treated with **1** (CAPE), and stimulated with PDGF. After 24 h cells were counted ($n = 3$, Student's t -test). A representative immunoblot showing the degree of HO-1 downregulation is depicted.

Furthermore, the catechol **1** is able to reduce basal ROS levels and the intracellular boost of reactive oxygen species upon PDGF stimulation (up to 23 h after addition of the stimulus) (Figure 4A). Interestingly, antioxidants, such as quercetin, were recently reported to lead to a stabilization of HIF-1 α and to growth arrest under normoxic conditions.²⁰ Therefore, HIF-1 α occurred as a likely candidate to be targeted by **1** in our setting. Indeed **1** led to an accumulation of HIF-1 α in proliferating VSMCs (Figure 4B) 4 h

after stimulation with PDGF. Moreover, HO-1 induction by **1** was dose-dependently reduced by co-incubation with the known HIF-1 α inhibitor YC-1²¹ (Figure 4C). These data indicate that **1** leads to stabilization of HIF-1 α under normoxic conditions, which contributes to HO-1 induction, in turn resulting in growth arrest of VSMCs. Enhanced stabilization of HIF-1 α may also play a role in the reported beneficial effects of **1** in various ischemia/reperfusion injury models. The observed sustained activation of p38 could not be connected causally to stabilization of HIF-1 α since co-treatment with a p38 inhibitor did not change HIF-1 α stabilization by **1** (data not shown). However, p38MAPK is reported to contribute to HIF-1 α transcriptional activation and to be involved in HO-1 expression.^{22,23} Although 100 μ M of YC-1 was able to completely abrogate HO-1 induction of **1**, it cannot be ruled out that other transcription factors besides HIF-1 α are activated as well. An obvious candidate is Nrf2, which has already been reported to be activated by **1** and to account for HO-1 induction in various cell types including VSMC.^{24,25} Moreover, p38-mediated phosphorylation is involved in full activation and nuclear translocation of Nrf2.²⁶ Data obtained after knockdown of Nrf2 levels, however, did not suggest a prominent role of Nrf2 in the HO-1 induction by **1** in PDGF-stimulated VSMCs (data not shown).

Overall, **1** inhibits PDGF-induced proliferation of rat VSMCs in a dose-dependent manner without affecting early PDGF-induced signaling steps or interfering with the NF- κ B signaling cascade. This is the first report to show involvement of enhanced phosphorylation of p38 MAPK, stabilization of HIF-1 α , and subsequent induction of HO-1 in the antiproliferative activity of **1** in VSMCs in culture and possibly also in vivo.

EXPERIMENTAL SECTION

Reagents. *trans*-CAPE (**1**) was obtained from Calbiochem (purity $\geq 97\%$ (w/w; HPLC)). It was dissolved in DMSO to obtain a stock solution of 100 mM, which was stored at -20 $^{\circ}$ C. When stored in this

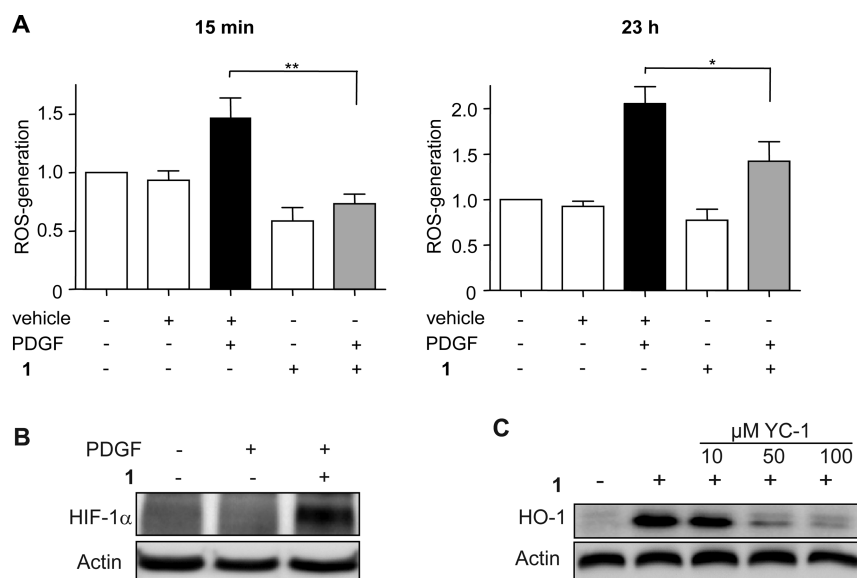


Figure 4. Compound **1** leads to stabilization of HIF-1 α , which contributes to HO-1 induction. (A) Quiescent VSMCs were pretreated with **1** (CAPE, 5 μ M) or vehicle as indicated for 30 min and stimulated with PDGF (20 ng/mL). After the given times, total ROS levels were determined by flow cytometric analysis of oxidized H₂DCF (n = 3, *p < 0.05; **p < 0.01, ANOVA). (B) Quiescent cells were pretreated with **1** (CAPE, 5 μ M) or vehicle and stimulated with PDGF. After 4 h, total cell lysates were prepared and subjected to immunoblot analysis for HIF1 α and actin. (C) VSMCs were treated with YC-1 as indicated for 15 min and then treated with **1** (CAPE, 5 μ M) or vehicle for 16 h. Total cell lysates were prepared and subjected to immunoblot analysis for HO-1 and actin. Representative blots of at least two independent experiments are depicted.

manner, **1** was stable up to 10 weeks and supported multiple freeze–thaw cycles. Due to photolability, the exposure of **1** to light was kept at a minimum (see Supporting Information, Figure S3). Antibodies for p(hospho)-p38, pAKT, pERK, pJNK, and pSTAT3 as well as pRb were from Cell Signaling, anti-HO-1 antibody was from Calbiochem, anti-HIF-1 α was obtained from Abcam, and antiactin or antitubulin antibodies were purchased from Santa Cruz. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody was from Dianova. Serotec provided HRP-conjugated goat anti-mouse antibody. Recombinant PDGF-BB was obtained from Bachem. Sigma-Aldrich provided propidium iodide and YC-1. Cobalt(III)-protoporphyrin was from Alexis, and the p38 inhibitor SB 203580 was from Calbiochem. TNF- α was from R&D Systems. Scrambled siRNA as well as oligofectamine were from Invitrogen, and siRNA specific for HO-1 came from Dharmacon.

Cell Culture. Rat primary aortic vascular smooth muscle cells were isolated from male Sprague–Dawley rat thoracic aortas by enzymatic digestion and cultivated as described previously.²⁷

Western Blotting. VSMCs at 70 to 95% confluence in 60 mm dishes were rendered quiescent by incubation with serum-free DMEM for 24 h. After treatment and stimulation with PDGF (20 ng/mL) as indicated, cells were harvested on ice, and Western blot was performed as described previously.²⁷

Cell Cycle Analysis. Performed as described.²⁷

Methyl-³H-thymidine Incorporation. Cells were starved for 48 h to render them quiescent. After the indicated treatment, cells were stimulated with 20 ng/mL PDGF-BB for 18 h. Then cells were pulse labeled (2 μ Ci/mL methyl-³H-thymidine) for 6 h, and the amount of incorporated ³H-thymidine was assessed: cells were washed (PBS) and incubated with ice-cold 5% trichloroacetic acid for 15 min at 4 °C. After washing with 70% ethanol wells were dried and cells dissolved in 0.5 mL of 0.4 M NaOH for 15 min at RT. In scintillation vials, 0.4 mL of the NaOH solution was added to 0.2 mL of 1 N HCl. After adding 5 mL of Liquescent (Roth), the radioactivity in the vials was counted in an LS 6500 Beckman Coulter.

siRNA-Mediated Knockdown of HO-1. This was performed basically as reported²⁸ using commercially available siRNAs (Invitrogen/

Dharmacon) and lipofectamine (Invitrogen), according to the manufacturer's instructions.

Electrophoretic Mobility Shift Assay (EMSA). VSMCs were grown in six-well plates. After preincubation with vehicle or **1** for 30 min at the indicated concentrations, cells were treated with 10 ng/mL TNF- α . Nuclear extracts and EMSA experiments were performed as described previously.²⁹

Determination of Reactive Oxygen Species. Intracellular ROS were determined using dihydro-dichloro-fluorescein-diacetate (H₂DCF-DA, Molecular Probes, Invitrogen) and flow cytometric analysis as described previously.²⁸

Statistical Analysis. At least three independent experiments were performed for statistical evaluation. Data are presented as means \pm standard error of the mean (SE). If not stated otherwise, statistical differences were analyzed with GraphPad Prism using the indicated statistical test. p-Values of <0.05 were considered significant.

■ ASSOCIATED CONTENT

S Supporting Information. Immunoblots showing reduced phosphorylation of retinoblastoma protein in the presence of **1**, data addressing the role of NF- κ B in PDGF-induced proliferation and its inhibition by **1**, as well as an exemplary purity and stability check of **1** are available online and freely accessible at <http://pubs.acs.org>.

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Author Contributions

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DEDICATION

Dedicated to Dr. Koji Nakanishi, Columbia University, for his pioneering work on bioactive natural products.

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