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Introduction

Understanding molecular mechanisms regulating angiogenesis and systemic adaptive responses to reduced oxygen availability may lead to novel therapies for ischemic disorders. Tissues use many strategies, including induction of angiogenesis and alterations in metabolism, to survive under hypoxic conditions. Hypoxia-inducible factor 1 (HIF-1) activates the transcription of genes whose protein products mediate adaptive responses to hypoxia/ischemia, including erythropoietin (EPO), glucose transporter 1 (GLUT1), nitric oxide synthase 2 (NOS2), and vascular endothelial growth factor (VEGF) [1-4]. Ischemic heart disease is a major cause of mortality that is treated by pharmacologic agents, balloon angioplasty, and coronary artery-bypass graft surgery. Novel therapeutic strategies aiming to stimulate neovascularization and to induce expression of cytoprotective factors are now under development.

HIF-1 is a heterodimer consisting of a constitutively-expressed HIF-1β subunit and an O_2-regulated HIF-1α subunit [5]. Under normoxic conditions, members of the PHD/EGLN family hydroxylate the HIF-1α subunit on two conserved prolyl residues in an O_2-, Fe^{2+}-, and 2-oxoglutarate-dependent manner [6-8]. Hydroxylated HIF-1α molecules are polyubiquitinated and, hence, marked for proteasomal destruction, by an ubiquitin ligase that contains the pVHL tumor suppressor protein. Under conditions of low O_2 or low Fe^{2+}, or in the absence of pVHL, HIF-1α accumulates in its active form and activates the transcription of genes involved in adaptation to hypoxia. Concurrently with prolyl hydroxylation, hydroxylation of a conserved asparaginyl residue by FIH-1 regulates HIF-1 transcriptional activation by modulating

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recruitment of the coactivators CBP and p300 also in an O_2-, Fe^{2+}-, and 2-oxoglutarate-dependent manner [9-11]. Thus, hydroxylation of prolyl and asparaginyl residues in HIF-1α regulates its protein stability and transactivation function in an O_2-dependent manner.

Physiological stimuli other than hypoxia can also induce HIF-1 activation and the transcription of hypoxia-inducible genes. Signaling via the HER2/neu or IGF-1 receptor tyrosine kinase induces HIF-1 expression by an oxygen-independent mechanism [12, 13]. In addition to growth factors, nitric oxide, prostaglandin E_2, thrombin, angiotensin II, 5-hydroxytryptamine, and acetylcholine induce HIF-1 activation under non-hypoxic conditions [14, 15]. Taking account of the evidence that activation of HIF-1 is therapeutically beneficial in diseases characterized by acute or chronic ischemia [16, 17], low molecular weight compounds that induce HIF-1 may be candidates for the treatment of ischemic diseases.

HIF-1 activity is also affected by cellular redox conditions, which are maintained by the thioredoxin-redox factor 1 cascade [18, 19]. We screened redox-acting low molecular weight compounds to find an inducer of HIF-1 activity. In this report we demonstrate that n-propyl gallate (PG) strongly induces HIF-1 activity by inhibiting the activity of both prolyl and asparaginyl hydroxylases in various cell types, including epithelial, endothelial, and smooth muscle cells. Moreover, we demonstrate that PG is active in the kidney after systemic administration as determined by transcription profiling and production of erythropoietin, indicating that HIF-1 activity can be manipulated *in vivo* with an orally active low molecular weight compound.

BJ Biochemical Journal Immediate Publication. Published on 30 Nov 2007 as manuscript BJ20070824

Materials and Methods

Cell Cultures and Reagents

HEK293 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Hep3B and Caco-2 cells were maintained in MEM with Earl's salts supplemented with 10% FBS, essential amino acids and pyruvate, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. HT-29 and SW48 were grown in RPMI 1640 supplemented with 10% FBS. T-84 cells were grown in RPMI 1640 containing 10% FBS and Ham's F12:DMEM (1:1) containing 2 mM glutamine. Human umbilical vein endothelial cells (HUVECs) and human pulmonary arterial smooth muscle cells (HASMCs) were obtained from Kurabo (Osaka, Japan). Gallate, 3,4,5-Trihydroxybenzoic acid (GA), n-propyl gallate, 3,4,5-Trihydroxybenzoic acid propyl ester (PG), 3,4,5-Trihydroxybenzoic acid methyl ester (Methyl gallate), 3,4,5-Trihydroxybenzoic acid octyl ester (Octyl gallate), (-)-epigallocatechin-3-gallate, (-)-*cis*-2-(3,4,5-Trihydroxyphenyl)-3,4-dihydro-1(2H)-benzopyran-3,5,7-triol 3-gallate (EGCG), the iron chelator desferrioxamine (DFX), ascorbate and α-tocopherol were obtained from Sigma (St. Louis, MO). Cycloheximide (CHX), wortmannin, LY294002, genistein, PD98059, rapamycin, *N*-acetylcysteine (NAC) and dithiothreitol (DTT) were obtained from Calbiochem (San Diego, CA).

Plasmid Constructs

BJ Biochemical Journal Immediate Publication. Published on 30 Nov 2007 as manuscript BJ20070824

Reporter plasmid p2.1, which contains a 68-bp hypoxia response element (HRE) from the human enolase 1 (*ENO1*) gene inserted upstream of an SV40 promoter and *Photinus pyralis* (firefly) luciferase coding sequences, and its HRE-mutant p2.4 were described previously [20]. The reporter pVEGF-Kpn I contains nucleotides -2274 to +379 of the *VEGF* gene inserted into luciferase reporter pGL2-Basic (Promega, Madison, WI) [21]. The expression vector pGAL4/HIF-1α(531-826) and the reporter pG5E1bLuc, which contains 5 copies of a GAL4 binding site upstream of a TATA sequence and firefly luciferase coding sequences, were described previously [22]. The pCS2-Venus expression vector which encodes Venus, the F64L/M153T/V163A/S175G mutant of green fluorescent protein, was kindly provided by Dr. Atsushi Miyawaki [23]. The expression vector encoding a Venus-HIF-1α fusion protein was made from pCS2-Venus and HIF-1α cDNA by inserting HIF-1α sequences 3' to Venus coding sequences and designated pVenus-HIF-1α. The expression plasmid pCH-NLS-HIF1α(548-603)-LacZ was described previously [24]. The FLAG-tagged HIF-1α expression pcDNA3-FLAG-HIF-1α was described previously [15].

Immunoblot Assays

Whole cell lysates were prepared using ice-cold lysis buffer (0.1% SDS, 1% NP40, 5 mM EDTA, 150 mM NaCl, 50 mM Tris-Cl (pH 8.0), 1 mM sodium orthovanadate, and Complete protease inhibitor [Roche Diagnostics, Basel, Switzerland]) following a protocol described previously [15]. 100 μg aliquots were fractionated by 7.5% SDS-PAGE and subjected to immunoblot assay using mouse monoclonal antibody against HIF-1α (BD Biosciences, San Jose, CA) or

HIF-1β (H1β234; Novus Biologicals, Littleton, CO) at 1:1000 dilution and HRP-conjugated sheep antibodies against mouse IgG (GE Healthcare Bio-Science Corp., Piscataway, NJ) at 1:1000 dilution. Signal was developed by using ECL reagents (GE Healthcare Bio-Science Corp.).

Reverse Transcriptase-Polymerase Chain Reaction

The RT-PCR protocol is described elsewhere [25]. Cells were harvested and RNA was isolated with TRIzol (Invitrogen Corp., Carlsbad, CA). 1 μg of total RNA was subjected to first strand cDNA synthesis using random hexamers (SuperScript II RT kit, Invitrogen Corp.). cDNAs were amplified with TaqGold polymerase (Roche) in a thermal cycler with the specific primers (sequences provided on request). For each primer pair, PCR was optimized for cycle number to obtain linearity between the amount of input RT product and output PCR product. Thermocycling conditions were 30 s at 94°C, 60 s at 57°C, and 30 s at 72°C for 25 (VEGF and intestinal trefoil factor [ITF]), 27 (EPO), 25 (HIF1A), or 20 (18S rRNA) cycles preceded by 10 min at 94°C. PCR products were fractionated by 3% Nusieve agarose gel electrophoresis, stained with ethidium bromide, and visualized with UV.

Reporter Gene Assay

Reporter gene assays were performed in HEK293 cells [15, 26, 27]. $5x10^4$ cells were plated per well on the day before transfection. In each transfection, 200 ng of reporter gene, and 50 ng of the control plasmid pRL-SV40 (Promega), containing an SV40 promoter upstream of *Renilla*

BJ Biochemical Journal Immediate Publication. Published on 30 Nov 2007 as manuscript BJ20070824

reniformis (sea pansy) luciferase coding sequences, were pre-mixed with Fugene 6 transfection reagent (Roche). Cells were incubated with the reagents for 6 h, and then exposed to 20% or 1% O_2 for another 18 h. The cells were harvested and the ratio of firefly to sea pansy luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega). For each experiment, at least two independent transfections were performed in triplicate and representative data are shown. Results shown represent mean ± S.D. of three independent transfections. β-galactosidase (β-gal) activity was determined using a commercial assay system (Boehringer Ingelheim GmbH, Ingelheim, Germany). To normalize β-gal activity of each sample, pGL-Control plasmid was co-transfected with a β-gal-coding plasmid. Net β-gal count of each sample was divided by its luciferase count and normalized mean count ± S.D. of three independent transfections is shown as relative activity.

Confocal Microscopic Analysis

Chamber slides were mounted in 90% glycerol with 1 mg/ml p-phenylenediamine and examined with an MRC-1024 confocal microscope (Bio-Rad Laboratories, Hercules, CA).

In Vitro HIF-1α-VHL and HIF-1α-p300 Interaction Assays

Plasmids used in assays were described previously [10, 15]. Glutathione S-transferase (GST)-HIF-1α(429-608) and GST- HIF-1α(531-826) fusion proteins were expressed in *E. coli* as described [10, 15]. Biotinylated lysine-labeled proteins were generated in reticulocyte lysates with the TNT T7-coupled transcription/translation system using Transcend™

BJ Biochemical Journal Immediate Publication. Published on 30 Nov 2007 as manuscript BJ20070824

Biotinylated tRNA (Promega) based on T7-promoter driven plasmids vectors coding VHL and CH domain of p300 [15]. 25 μg aliquots of HEK293 cell lysate were preincubated with PG or DFX for 30 min at 30 °C, 2.5 μg of GST-HIF-1α(429-608) was added and incubated for 30 min at 30 °C. A 5 μl aliquot of *in vitro*-translated biotinylated VHL protein was mixed with 4 μg of GST fusion protein in a final volume of 200 μL of binding buffer (Dulbecco's PBS [pH 7.4], 0.1% Tween-20) and incubated for 2 h at 4 °C with rotation followed by addition of 10 μL of glutathione-Sepharose 4B beads (GE Healthcare Bio-Science Corp.) and incubation at 4 °C for 1 h. The beads were pelleted, washed 3 times in binding buffer, pelleted, resuspended in Laemmli sample buffer, and analyzed by SDS-PAGE. Proteins were transferred to PVDF membrane and visualized using streptavidin-labeled horseradish peroxidase and ECL reagent (GE Healthcare).

Gene silencing using short interfering RNA (siRNA)

Caco2 cells were transfected by 100 nM siRNA using HiPerFect Transfection Reagent (Qiagen Inc.) following a protocol provided by the manufacturer.

Transepithelial electrical resistance measurements

Measurement of transepithelial electrical resistance in Caco-2 cell monolayer was performing using a Millicell electric resistance system (ERS) ohmmeter (Millipore Corporation, Bedford, MA) as a barrier function assay. This device can measure electric resistance of epithelial cells in culture using a separate pair of Ag-AgCl electrolodes and a resistance meter. Fluid

BJ Biochemical Journal Immediate Publication. Published on 30 Nov 2007 as manuscript BJ20070824

resistance was subtracted and net resistance was calculated as ohm-square centimeter. Results are presented as the mean ± S.D. of three independent well.

Oral administration of PG and EPO ELISA

PG (100 mg/kg) was administered to male 57BL/6 mice daily for a week by oral gavage. 24 h after last administration of nPG, mice were anesthetized and blood was collected via heart. All samples were allowed to clot for 2 h at room temperature before centrifuging for 20 min at 1000 x *g*, and the serum was transferred to a 1.5-ml tube for EPO analysis using a commercial ELISA kit specific for mouse EPO (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. A kidney was harvested for the assessment of EPO mRNA by semiquantitive real time RT-PCR. Results are presented as the mean ± S.D. of 5 independent mice (ELISA study). Statistical analysis was performed using Student's *t* test and a P value <0.05 was considered significant.

Statistical analysis

Data are presented as ± S.D. Statistics were analysed with Student's *t*-test or, when appropriate, one-way analysis of variance followed by Dunnett's test.

BJ Biochemical Journal Immediate Publication. Published on 30 Nov 2007 as manuscript BJ20070824

Results

PG induces the accumulation of HIF-1α protein under non-hypoxic conditions

To examine the effect of PG on HIF-1 activity, HEK293 and Hep3B cells were exposed to 100 μM PG under non-hypoxic conditions (20% O_2) for 4 h. In both cell lines, 100 μM PG promoted the accumulation of HIF-1α protein (Fig. 1A, upper panels), similar to the effect of DFX, a known inducer of HIF-1 [28]. Expression of HIF-1β was not affected by PG treatment (Fig. 1A, lower panels). PG also induced HIF-1α accumulation in primary cultures of HUVECs (Fig. 1B, left panels), and HASMCs (Fig. 1B, right panels) without affecting the expression of HIF-1β.

Next, we investigated the dose-dependency of the PG effect on HIF-1α protein expression in HEK293 cells. PG promoted the accumulation of HIF-1α at doses from 25 to 400 μM in a dose-dependent manner (Fig. 1C, top, left panel). The effect of 100 μM PG peaked at 1 h, was sustained through 4 h and then gradually returned to the baseline levels by 24 h (Fig. 1C, top right panel). The time-course of HIF-1α accumulation induced by PG is quite different from that induced by DFX (Fig. 1C, bottom, right panel).

PG activates HIF-1-dependent gene expression

100 μM PG induced expression of VEGF mRNA and EPO mRNA in HEK293 cells without affecting the expression of HIF-1α mRNA (Fig. 2A), suggesting that PG directly affects HIF-1α protein accumulation, which leads to increased HIF-1 transcriptional activity. To test this

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hypothesis, HEK293 cells were transfected with the reporter p2.1; which contains a HIF-1-dependent HRE, or p2.4, which contains a mutation in the HIF-1 binding site [20]. PG induced HRE-dependent gene expression in a dose-dependent manner that was comparable to DFX (Fig. 2B). Expression of a dominant negative form of HIF-1α reduced p2.1 reporter gene expression (Fig. 2C). Transcription of the mutated reporter p2.4 was not significantly activated by PG (Fig. 2D). These results demonstrate that reporter gene activation in PG-treated cells was HRE- and HIF-1-dependent. PG also induced dose-dependent transcription of a luciferase reporter gene containing the *VEGF* promoter encompassing nucleotides -2274 to +379 relative to the transcription start site (Fig. 2E). Thus, PG treatment induces expression from both a native promoter and an isolated HRE.

PG induces nuclear localization and prolongs the half-life of HIF-1α protein

We next investigated the subcellular localization of HIF-1α. The fluorescent protein Venus was present in both the nucleus and cytoplasm (Fig. 3A, panel a). The Venus-HIF-1α fusion protein was localized mainly in the cytoplasm of untreated, non-hypoxic cells (panel b). Within 4 h, PG treatment induced translocation of Venus-HIF-1α from the cytoplasm into the nucleus (panel c), similar to effect of DFX (panel d). Next, we examined intracellular localization of HIF-1α using FLAG-tagged HIF-1α overexpressed in HEK293 cells. When FLAG-tagged HIF-1α is overexpressed in HEK293 cells, neither PG nor DFX induces further HIF-1α protein accumulation. But PG or DFX treatment facilitated nuclear translocation of overexpressed HIF-1α (Fig.3B). Althought these results indicated that PG as well as DFX induced at least

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GFP- or FLAG-tagged HIF-1α overexpressed by a promoter derived from virus in HEK293 cells, further analysis is still to be performed.

To determine whether PG treatment affected HIF-1α protein half-life, HEK293 cells were treated with 100 μM PG (Fig. 3B, lanes 4-6) or 100 μM DFX (lanes 7-9) for 4 h to induce HIF-1α expression, and then CHX was added to block ongoing protein synthesis. In the presence of CHX, the half-life of HIF-1α was > 60 min in PG-treated cells, which was longer than the half-life of HIF-1α in cells treated with DFX, which is known to block O_2-dependent degradation of HIF-1α. Taken together, our results indicate that PG induces the accumulation of HIF-1α protein by stabilizing HIF-1α protein and facilitates nuclear translocation of HIF-1α.

Effect of other gallates on HIF-1α accumulation

To explore the molecular mechanism by which PG induces HIF-1α, we tested GA and EGCG, which are compounds that are structurally related to PG. As shown in Fig. 4A, PG induced the accumulation of HIF-1α (lanes 2 and 3) but neither GA (lanes 4 and 5) nor EGCG (lanes 6 and 7) promoted the accumulation at any dose tested in HEK293 cells. Consistent with these results, the reporter gene assay demonstrated that only PG induced HRE-dependent gene expression (Fig. 4B). To explore the mechanisms behind nPG-induced HIF-1 activation, we examined trihydroxy compounds with similar structure with PG. The effects of methyl gallate, ethyl gallate, and octyl gallate were examined in addition to GA and PG (fig. 4C). 100 μM octyl gallate (lane 6) induced HIF-1α accumulation stronger than 100 μM PG (lane 5). In contrast,

BJ Biochemical Journal Immediate Publication. Published on 30 Nov 2007 as manuscript BJ20070824

100 μM methyl gallate (lane 3) and ethyl gallate (lane 4) had only weaker effect compared to 100 μM PG.

Next we performed experiments focused on HIF-1α hydroxylases. Both classes of HIF-α hydroxylases, PHDs and FIH, are members of the family of 2-oxoglutarate dioxygenases. The enzymes require Fe(II) and ascorbate as cofactors and 2-oxoglutarate and dioxygenase serve as prime substrates for the reaction. We, therefore, investigated the effect of 2-OG, ascorbate, and Fe(II) on PG-induced HIF-1α accumulation. As shown in figure fig. 4D, 15 mM 2-OG inhibited PG-induced HIF-1α accumulation. 100 μM Fe (II) also suppressed the accumulation but the effect was weaker than that of 15 mM 2-OG. In contrast, 100 μM ascorbate did not affect the PG-induced accumulation.

Gallates are reported to have potent antioxidative effects *in vitro* and *in vivo* [29, 30]. To test whether PG induces the accumulation of HIF-1α via its antioxidant activity, we treated HEK293 cells with other reagents that have antioxidant activity. Neither DTT, NAC, ascorbate, nor α-tocopherol induced HIF-1α protein accumulation (data not shown). Expression of the potent intracellular redox regulator thioredoxin or Cu/Zn superoxide dismutase did not induce HIF-1α accumulation in HEK293 cells (data not shown). Moreover, neither the PI3K inhibitor LY294002, the tyrosine kinase inhibitor genistein, the MEK inhibitor PD98059, the p38 MAPK inhibitor SB203580, nor the mTOR inhibitor rapamycin inhibited PG- or DFX-induced HIF-1α accumulation in HEK293 cells (data not shown).

BJ Biochemical Journal Immediate Publication. Published on 30 Nov 2007 as manuscript BJ20070824

PG inhibits HIF-1α hydroxylases

We examined whether prolyl or asparaginyl hydroxylase activity is affected by PG using *in vitro* pulldown assays. Incubation of a GST-HIF-1α (429-608) fusion protein with lysate from untreated cells demonstrated prolyl hydroxylation of HIF-1α as determined by its interaction with VHL (Fig. 5A, lane 2), which is hydroxylation-dependent. Lysate from cells treated with PG or DFX did not promote the interaction of GST-HIF-1α (429-608) with VHL (lanes 3-5). We also examined the effect of GA using two different protocols. In one protocol, cell lysates were prepared from untreated and GA-treated cells (Fig. 5B, left panel). In the other protocol, lysate was directly incubated with GA or vehicle (Fig. 5B, left panel, lane 4). Lysate from GA-treated cells promoted the interaction of GST-HIF-1α (429-608) with VHL (Fig. 5B, right panel, lane 8), similar to the effect of lysate from untreated cells.

Next, we examined asparaginyl hydroxylase activity in PG-treated HEK293 cell lysate. A GST-HIF-1α (531-826) fusion protein encompassing asparagine 803 was incubated with lysate and its interaction with the CH1 domain of p300 was tested. As shown in Fig. 5C, PG treatment promoted the interaction between HIF-1α and p300, which was similar to the effect of DFX treatment.

Next, we investigated the impact of PG on HIF-1α transcriptional activity. There are two independent transactivation domains (TADs) present in HIF-1α, which are designated as the amino-terminal (amino acids 531-575) and carboxyl-terminal (amino acids 786-826) TADs (TAD-N and TAD-C, respectively) [22]. A fusion protein consisting of the GAL4 DNA-binding domain fused to HIF-1α residues 531-826, which contains both of the TADs, is

BJ Biochemical Journal Immediate Publication. Published on 30 Nov 2007 as manuscript BJ20070824

expressed at similar levels under hypoxic and non-hypoxic conditions and thus can be used to examine the transcriptional activity of HIF-1α independent of its protein expression [10, 15, 22]. PG treatment increased transactivation mediated by GAL4-HIF-1α(531-826) in a dose-dependent manner, similar to the effect of DFX (Fig. 5D). These results demonstrate that PG promotes HIF-1α accumulation and HIF-1α transcriptional activity by inhibiting prolyl and asparaginyl hydroxylase activity, respectively.

PG treatment enhances cellular barrier function

Epithelial cells provide a barrier against external antigens and bacteria. Mucosal organs, including stomach and intestine, are dependent upon an extensive underlying vasculature, and therefore are susceptible to hypoxic-ischemic tissue damage. We exposed intestinal epithelial cell lines to PG under non-hypoxic conditions and examined HIF-1 activity and epithelial barrier function. Caco-2, HT-29, SW48, and T89 cells, which were derived from colorectal carcinomas, were exposed to PG at concentrations ranging from 50 to 400 μM. Compared to vehicle-treated cells, PG induced HIF-1α protein expression in a dose-dependent manner in all of the cell lines tested (Fig. 6A). We examined whether PG affects the expression of two genes that are regulated by HIF-1, using the RT-PCR technique to quantify mRNA expression in Caco-2 cells. PG induced the expression of ITF and VEGF mRNA to levels that were similar to those induced by hypoxia (Fig. 6B). In contrast, HIF-1α mRNA expression was not affected by exposure of the cells to PG or hypoxia. Pre-treatment of cells with a short interfering RNA (siRNA) that targets HIF-1α mRNA for degradation, blocked the induction of ITF mRNA in

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response to PG (Fig. 6C).

To determine whether PG altered mucosal barrier function, cultured intestinal epithelial cells were treated with vehicle, PG, or GA and the electrical resistance of the cells, which is a measure of barrier function, was examined using the Millicell-ERS ohmmeter system. Transepithelial resistance was increased 24 to 36 hours after PG treatment, whereas vehicle or GA had no significant effect (Fig. 6D).

PG induces EPO expression in vivo

Preclinical data suggest that a HIF-1 activator might be useful for the treatment of anemia and ischemic diseases [16, 31]. HIF prolyl and asparaginyl hydroxylation can be inhibited with small organic molecules [32, 33], leading to increased HIF activity. To explore whether PG can induce HIF-1 and its downstream gene expression *in vivo*, we administrated PG to mice to and examined serum levels of the erythropoietic cytokine EPO, which is the product of a HIF-1 target gene. Daily administration of PG to mice by oral gavage increased circulating EPO levels (Fig. 6E).

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Discussion

The results reported above demonstrate that treatment of several different cell types with PG induces HIF-1α protein accumulation and HIF-1 transcriptional activation, resulting in HIF-1-regulated gene expression. PG treatment inhibited the interaction between HIF-1α and VHL, increased the half-life of HIF-1α protein, induced nuclear translocation of overexpressed HIF-1α, promoted the interaction between HIF-1α and p300, and stimulated HIF-1α transactivation function. We demonstrated that PI3K, tyrosine kinase, MAPK, and mTOR inhibitors do not have any significant effects on PG-induced HIF-1α accumulation. In contrast, MAPK, PI3K, and certain tyrosine kinases play significant roles in growth factor-, or PGE_2-induced HIF-1α accumulation, in which increased HIF-1α translation is induced [12, 13, 34, 35]. The evidence indicates that PG is targeting a general mechanism in the HIF-1 signaling pathway, similar to hypoxia. In fact, PG suppresses prolyl HIF-1α hydroxylase activity, which determines the stability of HIF-1α protein [8]. Because O_2 is a required substrate for prolyl hydroxylase activity, these enzymes provide a direct link between reduced O_2 availability and adaptive responses to hypoxia that are mediated by HIF-1. In addition, the asparaginyl hydroxylase FIH-1 governs the interaction between HIF-1 and the transcriptional coactivator p300 [10, 11, 36]. PG also enhances the transcriptional activity of HIF-1α by suppressing the asparaginyl hydroxylase activity. Thus, PG inhibits the intracellular oxygen sensing system by blocking both prolyl and asparaginyl hydroxylase activity under non-hypoxic conditions.

BJ Biochemical Journal Immediate Publication. Published on 30 Nov 2007 as manuscript BJ20070824

Precedent studies reported that green tea catechins such as EGCG induced HIF-1 activation in T47D human breast carcinoma and PC3 human prostate cancer cells [37, 38]. In contrast, we did not observe an effect of EGCG on HIF-1 activity in HEK293 cells, Hep3B cells, HUVECs, or HASMCs. The varying effectiveness of different gallates as inducers of HIF-1α activity may be reflect differences in their ability to enter different cell types. The cell membrane permeability of PG is approximately 10-fold higher than that of GA [39]. The results shown in Fig. 5B are consistent with this hypothesis, as GA inhibited prolyl hydroxylase activity when added directly to lysates but not when added to intact cells.

Because gallates have iron chelating activity, the effect of PG may be attributable to this property. However, GA and EGCG, which have similar iron-chelating activity, did not induce HIF-1α accumulation (Fig.1A and 1B). Cell lysate from cultured HEK293 cells treated by PG but not GA did not suppressed the interaction between HIF-1α and VHL (Fig. 5B). 100 μM, which is equimoler to PG, $FeSO_4$ reversed only partially the effect of PG (Fig. 4D). In addition, it is reported that PG has a only weak binding constant for Fe^{2+} [29]. Finally, we indicated that as little as 25 μM PG significantly induced HIF-1α protein accumulation in HEK293 cells. We also observed that in human umbilical vein endothelial cells PG as low as 4 μM was enough to induce the accumulation of HIF-1α (data not shown). It is thus unlikely that PG activates HIF-1 by chelating Fe^{2+}. Gallates including PG also have potent antioxidant activity [30]. The finding that treatment with NAC, DTT, or α-tocopherol did not affect HIF-1 activity also suggests that the effect of PG cannot explained by its antioxidant activity. In Fig.4D, we

BJ Biochemical Journal Immediate Publication. Published on 30 Nov 2007 as manuscript BJ20070824

demonstrated that 15 mM 2-OG effectively inhibited the interaction between HIF-1α and VHL. We also previously suggested that GA binds to the PHDs by mimicking the structure of 2-OG in the PHD active site and demonstrated that PG added into culture media pass through plasma membrane and is hydrolyzed to GA [39]. We conclude that PG added to culture media enters cells and the hydrolyzed product GA directly inhibits prolyl and asparaginyl hydroxylases of HIF-1α resulting HIF-1α protein stabilization and transcriptional activation.

PG is approved as a food preservative that is added to prevent oxidation and regarded as safe. The implications of a dietary compound affecting the activity of a protein as pivotal as HIF-1 in the process of angiogenesis cannot be overlooked. Increased stabilization of HIF-1α might lead to increased angiogenesis through the coordinated induction of HIF-1 target genes encoding the angiogenic growth factors VEGF, platelet-derived growth factor B, and placental growth factor [40, 41]. Several studies have demonstrated that prolonged expression and activation of HIF-1α alone is sufficient to induce mature, physiologically functional, blood vessels and that even transient stabilization of HIF-1α might lead to changes in cellular metabolism, such as increased glucose uptake and glycolysis, that would allow cells to survive acute hypoxic insults [40]. For example, DFX administration has been shown to reduce infarct size in a rat stroke model [42]. In this study, we showed that PG induces ITF, which protects mucous epithelia from a range of insults, contributes to mucosal repair [43], and maintains barrier function of epithelial cells (Fig. 6D). In addition, oral administration of PG increased the levels of EPO mRNA in the kidney and circulating EPO protein in the blood (Fig. 6E). Taking account of the

Biochemical Journal Immediate Publication. Published on 30 Nov 2007 as manuscript BJ20070824

evidence that EPO confers a powerful protective effect to various organs and tissues [44, 45], PG may be useful as a lead compound for the development of novel drugs that protect against ischemic injury. Finally, our findings may have implications for people with diets that are unusually high in PG, such as those consuming 'health drugs' that contain this compound. Additional studies are required to further investigate the protective or pathological consequences of PG intake.

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