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Gallate, the component of HIF-inducing catechins, inhibits HIF prolyl hydroxylase ☆,☆☆

Fuyo Tsukiyama [a], Yumi Nakai [b], Masataka Yoshida [a], Takahiro Tokuhara [a], Kiichi Hirota [c], Akiko Sakai [d], Hideyuki Hayashi [b,*], Takahiro Katsumata [a]

[a] *Department of Thoracic and Cardiovascular Surgery, Osaka Medical College, Daigakumachi, Takatsuki 569-8686, Japan*
[b] *Department of Biochemistry, Osaka Medical College, Daigakumachi, Takatsuki 569-8686, Japan*
[c] *Department of Anesthesia, Kyoto University Hospital, Kyoto University, Sakyo-ku, Kyoto 606-8507, Japan*
[d] *Department of Chemistry, Osaka Medical College, Daigakumachi, Takatsuki 569-8686, Japan*

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Abstract

Catechins have recently been reported to increase the cellular content of the hypoxia-inducible factor (HIF)-1α within mammalian cells. These catechins have a gallate moiety as a common structure. We now report that *n*-propyl gallate (nPG) also increases the HIF-1α protein in the rat heart-derived H9c2 cells. The increase was dose-dependent and reached a maximum at 2–4 h after the addition of nPG to the cells. nPG did not change the HIF-1α mRNA level, showing that the increase is a posttranscriptional event. Although nPG did not inhibit the HIF prolyl hydroxylase, gallate, the hydrolysis product of nPG, inhibited the enzyme completely at submillimolar concentrations. Model building studies on the human HIF prolyl hydroxylase 2 showed that the two phenolate oxygen atoms of gallate form a chelate with the active site Fe^{2+}, while the carboxyl group of gallate forms a strong ionic/hydrogen bonding interaction with Arg383, explaining why nPG, which has an esterified carboxyl group, is unable to inhibit the hydroxylase. Together with the observation that gallate was detected in the H9c2 cells treated with nPG, these results suggest that nPG incorporated into the cells is hydrolyzed and the released gallate inhibits the HIF prolyl hydroxylase, thereby reducing the HIF degradation rate and increasing the HIF-1α content.

Keywords: Gallic acid; *n*-Propyl gallate; Hypoxia inducible factor; Prolyl hydroxylase; Chelation; Ferrous ion; Ubiquitin

Gallic acid (3,4,5-trihydroxybenzoic acid) is a kind of polyphenol and is naturally found either as a free compound or combined with other compounds. These compounds include polyphenols such as catechins, which are flavonoid compounds that are found in green tea (tea tannin). Conversely, catechins predominantly exist as conjugates with gallic acid, such as (−)-epigallocatechin gallate (EGCG), (−)-epicatechin gallate (ECG), and (−)-gallocatechin gallate (GCG) [1]. These compounds have been shown to have antioxidant, anti-carcinogenic, and anti-inflammatory activities [2]. In addition to these actions, Zhou et al. recently reported that EGCG increases the content of the hypoxia inducible factor (HIF)-1α in T47D human breast tumor cells [1]. However, those catechins that are not conjugates with gallate have a low activity of inducing HIF-1α. Therefore, the action of the catechins on the HIF-1α expression is considered to be ascribed to the gallate moiety.

HIF-1α is induced under hypoxia and malnutrition conditions, and stimulates the expression of a variety of proteins including the vascular endothelial growth factor (VEGF), a factor that induces vascular proliferation in tissues [3–6]. Therefore, gallate conjugates may be useful for the treatment of ischemic heart disease and vascular diseases such as arteriosclerosis obliterans. Therefore, the

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☆☆ *Abbreviations:* EGCG, (−)-epigallocatechin gallate; HIF, hypoxia inducible factor; nPG, *n*-propyl gallate; PHD, prolyl hydroxylase.
* Corresponding author. Fax: +81 72 684 6516.
E-mail address: hayashi@art.osaka-med.ac.jp (H. Hayashi).

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mechanism by which gallate conjugates increase the content of HIF-1α is important for developing drugs using gallate conjugates as the lead compound.

Thomas and Kim recently reported that EGCG inhibits the degradation of HIF-1α via the ubiquitin pathway [7]. However, further studies are required to elucidate the precise inhibition mechanism, although they suggested the possibility that catechins may inhibit the HIF-1α hydroxylation, which is the required reaction for its ubiquitination.

In this study, we have chosen a simple gallate ester, *n*-propyl gallate (nPG), which one of us has found to induce HIF-1α in cultured cells and mice (Kimura and Hirota et al., submitted for publication), as a model compound for the gallate conjugates, and investigated the action of nPG on heart muscle cells and analyzed the mechanism by which nPG increases the HIF-1α content in the cells.

Materials and methods

Chemicals. *n*-Propyl gallate and gallic acid were purchased from Sigma. All other chemicals were of the highest grade commercially available.

Cell culture. Rat heart-derived H9c2 cells were purchased from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5.5 mM glucose supplemented with 10% fetal bovine serum [6].

Treatment with n-propyl gallate. *n*-Propyl gallate (nPG) was dissolved in DMSO to produce a 100 mM solution and then diluted with DMEM to a final concentration of 10, 50, 100, 200, and 400 μM. The cells were transferred to the above media containing nPG and then incubated for 1, 2, 4, 8, and 18 h.

Immunoblotting analysis. The cells were lysed using 200 μl of lysis buffer (50 mM Tris–HCl, 5 mM EDTA, 0.1% SDS, 1% Nonidet P-40, 150 mM NaCl, 1 mM Na_3VO_4, and 2 mM dithiothreitol). The lysis buffer contained one tablet of Protease Inhibitor Cocktail (complete mini, EDTA-free; Roche Applied Science, Mannheim, Germany) per 3 ml. After a 20-min continuous rocking at 4 °C, the cell extracts were centrifuged at 16,000*g* for 10 min at 4 °C to remove the cellular debris and nuclei. The proteins were quantified in the supernatant by the bicinchoninic acid method [8]. Samples with equal amounts of protein were subjected to 7.5% SDS–PAGE. The proteins were then transferred onto an Immobilon-P membrane (Millipore, Bedford, MD, USA). The membrane was blocked with 5% nonfat dried milk for 60 min and incubated overnight with the primary antibody that recognizes the rat HIF-1α (1:1000 dilution; Novus Biological, Littleton, Co, USA). After washing, the membranes were incubated for 1 h with the horseradish peroxidase-conjugated secondary antibody (Amersham Bioscience, Piscataway, NJ, USA), and the proteins were then visualized with enhanced chemiluminescence Western blotting detection reagents (Amersham Bioscience).

RT-PCR analysis of the HIF-1α mRNA. The total RNA was extracted from the cells using the RNeasy mini kit (Qiagen, Hilden, Germany). The RT-PCR was performed using the One-Step RT-PCR Kit (Qiagen), with 18 S rRNA as the endogenous control. The employed primers were as follows: HIF-1α forward primer 5′-CAGCAGACCCAGTTACAGAA-3′, HIF-1α reverse primer 5′-TCAGTTAACTTGATCCAAAG CTCT-3′, 18 S rRNA forward rimer 5′-ATCCTGCCAGTAGCATATGC-3′, and 18 S rRNA reverse primer 5′-ACCCGGGTTGGTTTTGATCTG-3′. The reaction conditions for the PCR were: pre-denaturation for 15 min at 95 °C, 30 cycles of amplification consisting denaturation for 40 s at 94 °C, annealing for 40 s at 58 °C, and extension for 90 s at 72 °C, with a final additional extension step for 10 min at 72 °C. The PCR products were run on 2% agarose gels.

HIF prolyl hydroxylase activity assay. The activity assay is conducted by measuring the amount of the product [1-^{14}C]-succinate formed from the substrate [5-^{14}C]-2-oxoglutarate, the latter being removed by precipitating as a dinitrophenylhydrazone after the enzymatic reaction [12]. The cells were washed twice in ice-cold PBS and then lysed in lysis buffer (see above). After 20 min at 4 °C with continuous rocking, the extracts were centrifuged at 3000*g* for 10 min at 4 °C to remove the cellular debris and nuclei. The cell extracts (0.2 mg protein/ml, 10 μl) were incubated in the 25 μl (final volume) reaction buffer containing 0.5 mM dithiothreitol, 50 μM ammonium ferrous sulfate, 1 mM ascorbate, 2 mg/ml bovine serum albumin, 0.4 mg/ml catalase, 0.3 mM [5-^{14}C]-2-oxoglutarate (1700 Bq), 40 mM Tris–HCl at pH 7.5, and 100 μM Pro-564 peptide [9]. The samples were then incubated at 37 °C for 10 min. A solution (25 μl) containing 20 mM nonlabeled succinate and 20 mM 2-oxoglutarate, serving as carriers for the radioactivity, and 25 μl of 0.16 M 2,4-dinitrophenylhydrazine in 30% $HClO_4$ were added. The precipitation was allowed to proceed for 30 min at room temperature. Mass precipitation was attained by adding 50 μl of 1 M 2-oxoglutarate and a further incubation for 30 min. The samples were then centrifuged at 3000*g* for 5 min. The supernatant (100 μl) was transferred into liquid scintillation vials, and the radioactivity was counted.

HPLC analysis. The cell suspension was mixed with an equal volume of chilled methanol, and the mixture was filtered through a membrane cartridge (pore size, 0.45 μm). The filtrate was injected onto an analytical TSKgel ODS-120T column (4.6 × 250 mm, 5-μm particle size; Tosoh Co., Tokyo, Japan). Gallate and nPG, which have oxidizable polyphenol structures, were detected electrochemically using ECD-300 (Eicom, Kyoto, Japan), the potential being set to 450 mV relative to the Ag/AgCl reference electrode. The mobile phase was a mixture of 0.025% phosphoric acid as solvent A and acetonitrile as solvent B. The column was first equilibrated with solvent A. After injection of the sample, the composition of solvent B was increased to 20% in 5 min, then to 50% in 4 min, kept at 50% for 4 min, and then returned to 0%. The flow rate was 1.0 ml/min. The metabolites were identified by co-chromatography or by comparison of their HPLC retention times with those of the authentic compounds.

Model building. Modeling of the human HIF prolyl hydroxylase 2 complexed with gallate was carried out on MOE (version 2005.6, Chemical Computing Group, Montreál, Canada) according to the manufacturer's instructions. The crystal structure of the human HIF prolyl hydroxylase 2 complexed with the inhibitor {[(4-hydroxy-8-iodoisoquinolin-3-yl)carbonyl]amino}acetic acid (PDB: 2G19) was used as the starting model. The structure of the inhibitor was changed to either gallate or 2-oxoglutarate, and the energies of the two structures were minimized using the force field MMFF94x while fixing all the atoms except for the ligands and the residues interacting with them, i.e., Arg383, Tyr303, Tyr310, Tyr329, Ile327 and Leu343.

Results

nPG increases the HIF content in the heart muscle cells

Cells were incubated with various concentrations (0, 10, 50, 100, 200, and 400 μM) of nPG for 2 h. The HIF-1α content in the cells, as evaluated by immunoblotting, increased in a dose-dependent manner (Fig. 1A). The time course of the change in the HIF-1α content was studied by fixing the nPG concentration to 200 μM and changing the incubation time (0, 1, 2, 4, 8, and 18 h). The HIF-1α content increased with time and reached a plateau from 2 to 4 h. After 4 h, the HIF-1α content decreased with time (Fig. 1B).

nPG does not change the HIF-1α mRNA content

In order to know whether the increase in the HIF-1α content is a transcriptional or a posttranscriptional event,

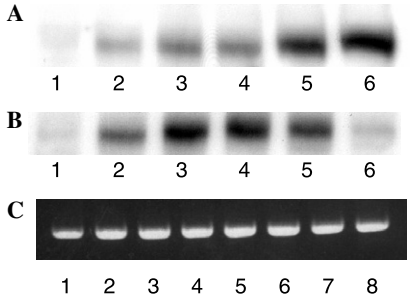

Fig. 1. (A) Immunoblotting of HIF-1α in H9c2 cells treated with various concentrations of *n*-propyl gallate (nPG). The H9c2 cells were incubated with nPG for 2 h, and the cell extracts were subjected to SDS–PAGE and immunoblotting using anti-rat HIF-1α antibody. nPG concentrations: lane 1, without nPG; lane 2, 10 μM; lane 3, 50 μM; lane 4, 100 μM; lane 5, 200 μM; lane 6, 400 μM. (B) Time course of the change in the HIF-1α content in H9c2 cells treated with nPG. The H9c2 cells were incubated with 200 μM nPG, and aliquots of the medium containing the cells were withdrawn for the immunoblotting analysis. Time after the addition of nPG: lane 1, 0 h; lane 2, 1 h; lane 3, 2 h; lane 4, 4 h; lane 5, 8 h; lane 6, 18 h. (C) HIF-1α mRNA levels of H9c2 cells. The H9c2 cells were treated with none (lane 1), 100 μM desferroxamine for 2 h (lane 2; positive control), 50 μM nPG for 0 h (lane 3), 1 h (lane 4), 2 h (lane 5), 4 h (lane 6), 8 h (lane 7), and 18 h (lane 8). The total RNA was extracted from the cells and RT-PCR was performed, then the product was run on 2% agarose gel electrophoresis and visualized with ethidium bromide.

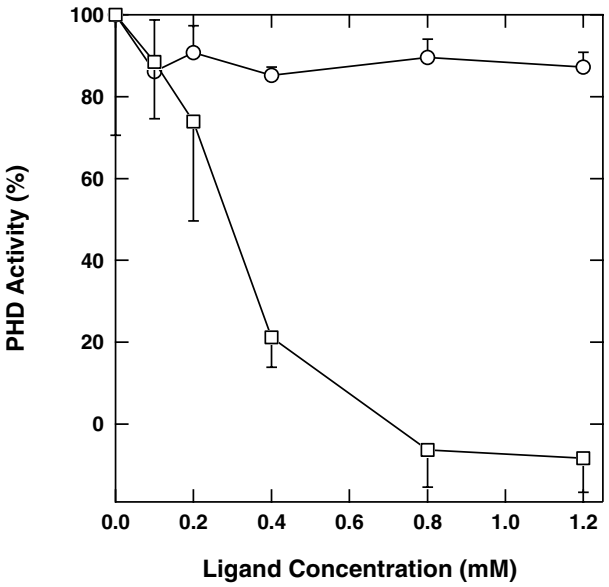

Fig. 2. Effect of *n*-propyl gallate (○) and gallate (□) on the HIF prolyl hydroxylase (PHD) activity. The PHD activity in the H9c2 cell extract was measured in the presence of various concentrations of *n*-propyl gallate and gallate. Error bars indicate S.D.

we analyzed the content of the HIF-1α mRNA. The amount of the PCR-amplified cDNA for the HIF-1α mRNA was essentially constant irrespective of the nPG concentration or the incubation period (Fig. 1C). This strongly suggests that the action of nPG to increase the HIF-1α content is mediated through posttranscriptional mechanisms.

Gallate, not nPG, inhibits the HIF prolyl hydroxylase activity

The effects of nPG and gallate, the possible hydrolyzed (deesterified) metabolite of nPG in the cells, on the activity of the HIF prolyl hydroxylase (PHD) were studied. nPG did not inhibit the PHD activity up to 1.2 mM (Fig. 2). On the other hand, gallate inhibited the PHD activity in a dose-dependent manner (Fig. 2). When the cell extract incubated with gallate was passed through a PD-10 desalting column, more than 95% of the activity was restored in the protein fraction (data not shown), indicating that the inhibition of PHD by gallate is reversible.

Modeling PHD complexed with nPG or 2-oxoglutarate

In order to explain the differential action of nPG and gallate on the PHD activity, a model building study was carried out on the human PHD2, the main prolyl hydroxylase involved in the HIF-1α degradation, and the structure of which has been recently elucidated [10]. In the structure, the active site of PHD2 contains a Fe^{2+} ion coordinated by two histidine residues, an aspartyl residue, and a water molecule [10]. The other two ligands are provided by the isoquinoline inhibitor {[(4-hydroxy-8-iodoisoquinolin-3-yl)carbonyl]amino}acetic acid (compound **A**), making up a six-coordinated structure. The carboxyl group of compound **A** forms an ionic/hydrogen bonding interaction with Arg383. The distance, as judged by the number of atoms, between the two oxygen atoms bound to Fe^{2+} and the carboxyl group of compound **A** is equal to that between the two hydroxyl groups and the carboxyl group of gallate and that between the α-keto/α-carboxyl groups and the γ-carboxyl group of 2-oxoglutarate. Therefore, gallate and 2-oxoglutarate were placed in the active site of PHD2 in such a way that the oxygen atoms of the two hydroxyl groups (gallate) or α-keto/α-carboxyl groups (2-oxoglutarate) coordinate to Fe^{2+} and the carboxyl group interacts with Arg383. Energy minimization was carried out for the ligand and the residues interacting with it. The obtained structures are shown in Fig. 3. As expected, the coordination to Fe^{2+} and the ionic/hydrogen bonding interaction of the carboxyl group to Arg383 are maintained during the energy minimization. The conformation of 2-oxoglutarate was the same as that observed for 2-oxoglutarate bound to the factor inhibiting HIF (FIH) [11], except for that the 2-oxoglutarate molecule is rotated by 90° between the two structures. This is probably due to the difference in the groups that interact with the γ-carboxyl group of 2-oxoglutarate; the γ-carboxyl group interacts with Arg383 in PHD2 whereas it interacts with Lys214 and Tyr145 in FIH. Importantly, the carbon skeleton and the groups and atoms interacting with Fe^{2+} and Arg383 are almost superimposable on the structure of nPG (Fig. 3). There is, however, a slight difference in the position of the two molecules; 2-oxoglutarate is shifted by ~1.1 Å to the right in Fig. 3. This shift of 2-oxoglutarate

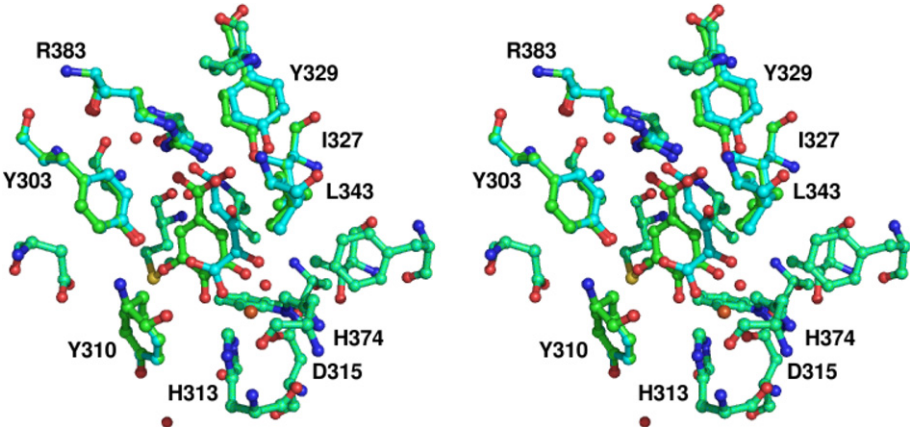

Fig. 3. Model for the active site of human PHD2 complexed with gallate (green) or 2-oxoglutarate (cyan). The models were created on the basis of the atomic coordinates of 2G19, and energy minimization was carried out for the ligands and the surrounding residues. Fe^{2+} is shown in orange. Val376, which locates in front of gallate and 2-oxoglutarate in this figure and forms a part of the pocket for accepting the ligands, is not shown for clarity. Drawn with PyMOL.

is induced by the shorter bond length (1.78 Å) between the α-carboxyl oxygen and Fe^{2+} compared to that (2.54 Å) between the α-keto oxygen and Fe^{2+}. The difference in the bond length is considered to be caused by the difference in the charge of the oxygen atoms; the carboxyl group has a formal charge of −1, whereas the keto group has no formal charge. In the case of gallate, the two phenolate oxygens are at roughly equal distances from Fe^{2+} (2.04 and 2.62 Å), reflecting the equal formal charge (−1) of the two oxygen atoms.

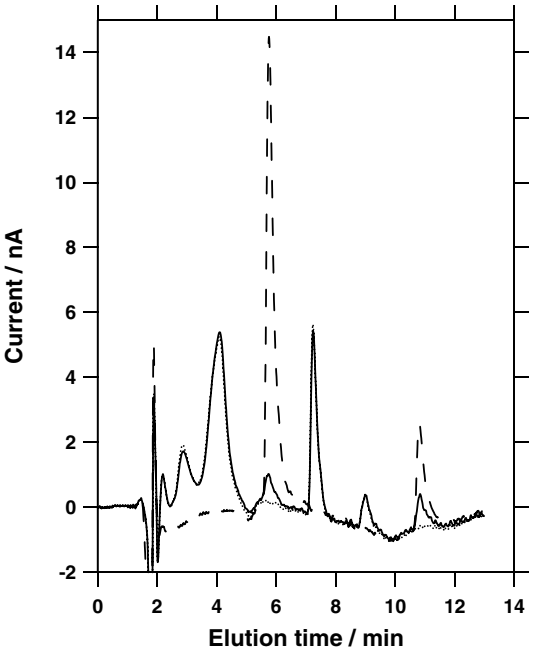

Fig. 4. HPLC analysis of gallate and nPG. Solid line: extract of the H9c2 cells incubated with 400 μM nPG for 2 h. Dotted line: extract of the H9c2 cells incubated without nPG. Dashed line: standard gallate and nPG (each 1 μM). Thirty-five microliters of the sample and standard solutions was injected. Gallate and nPG emerges at 6 and 11 min, respectively.

Detection of gallate in the cells treated with nPG

Cells were incubated with 400 μM nPG for 30 min, 1 and 2 h. The cells were collected and washed with PBS, then nPG and its derivatives were extracted with methanol, subjected to HPLC, and then electrochemically detected (Fig. 4 shows the data at 2 h). The amount of nPG found in the cells was 1590 (30 min), 1600 (1 h), and 1050 pmol/mg protein (2 h), whereas that of gallate was 68, 89, and 116 pmol/mg protein.

Discussion

We have shown that nPG increase the content of HIF-1α in human heart muscle cells (Figs. 1A and B). During the induction, the HIF-1α mRNA content was not changed (Fig. 1C), indicating that the increase in the HIF-1α protein content is due to the inhibition of the protein degradation rather than an increase in the protein synthesis.

The degradation of HIF-1α has been recently studied in detail, and much of the degradation mechanism has been elucidated ([12,13] for review, see Ref. [14]). HIF-1α is hydroxylated at Pro402 and Pro564 by the action of HIF prolyl-4-hydroxylase (PHD), a non-heme-iron-containing monooxygenease. The von Hippel–Lindau protein (pVHL) binds to the 4-hydroxylproline residue of the hydroxylated HIF-1α. pVHL assembles with cullin-2, elongin B/C, and the RING finger protein RBX1/ROC1 to form a heterooligomeric protein complex, which functions as a ubiquitin ligase. The polyubiquitinated HIF-1α is then degraded by the 26 S proteasome. Under hypoxia, the O_2-dependent hydroxylation of the proline residues is attenuated, and the HIF-1α protein escapes degradation and the HIF-1α content is increased. Combining this mechanism and the well-known fact that polyphenols are strong chelators for Fe ions, we can expect that nPG may exert its effect of increasing the HIF content by inhibiting the PHD activity through chelating

the active site Fe^{2+} ion. Therefore, we measured the PHD activity in the cell extract in the presence and absence of nPG. Contrary to our expectation, no inhibition of PHD was observed in the presence of nPG (Fig. 2). However, gallate, which is the hydrolyzed product of nPG but retains the polyphenol structure, showed a clear inhibition of PHD, with a half-maximum inhibition concentration of about 0.3 mM (Fig. 2). The inhibition was reversible, indicating that gallate reversibly binds to the active site of PHD.

In order to obtain insights into the inhibition mechanism by gallate, and not by nPG, a model building study based on the X-ray crystallographic structure of the human PHD [10] was carried out. The obtained structures showed that gallate binds to the active site of PHD with its phenolate oxygen atoms chelating Fe^{2+} and the carboxyl group binding to Arg383. The modeled co-substrate 2-oxoglutarate (2OG) has a conformation essentially superimposable on gallate, with its α-keto and α-carboxyl oxygen atoms chelating Fe^{2+} and the γ-carboxyl group binding to Arg383. This shows that gallate binds to the PHD by mimicking the structure of 2OG in the PHD active site. This model also indicates that if the carboxyl group of gallate is modified, as is the case with nPG, it would not be able to interact with Arg383. This clearly explains why nPG cannot inhibit the PHD activity. Apparently, the propyl group attached to the carboxyl group of gallate interferes with the binding of nPG to PHD. The HPLC analysis of the extract of the cells treated with nPG indicated that about 4–11% of nPG taken up by the cells is converted to gallate. Although it is difficult to know the exact concentration of gallate in the cytoplasm, the data strongly suggest that nPG, which has no inhibitory action on PHD, is deesterified to form gallate, and the liberated gallate inhibits PHD.

It has been reported that the local administration of the HIF-1α gene to the region of cardiac infarction and obstructed arteries promotes vascular neogenesis [15,16]. When tissues are under ischemic, malnutrition, or hypoxic conditions, various factors and enzymes interact with each other and promote vasculogenesis. HIF-1α is a factor that activates the array of these signal transduction reactions [14]. Contrary to the administration of genes for individual factors such as VEGF, gene therapy using the "master" gene HIF-1α activates various responses that are related to vasculogenesis, and, as the result, blood vessels induced by HIF-1α [17,18] have been reported to have a more mature structure than those induced by the single vasculogenic growth factors [19–21]. As indicated by Bruick et al. [22], developing drugs that selectively inhibit PHD will be useful for the treatment of vascular diseases. In the present study, we have shown that gallate directly inhibits PHD, and suggested that the actions of gallate-conjugated catechins to increase the HIF-1α content are mediated by the gallate released within the cells by hydrolysis. Altering the propyl group to other groups may lead to compounds with an increased cell membrane permeability or hydrolysis rate, which are expected to have a more potent activity of increasing HIF-1α than nPG. Additionally, as nPG is known to undergo additional metabolism other than simple hydrolysis in rat hepatocytes [23], controlling the metabolism of the gallate derivatives within the cells may further enhance the activity of these compounds.

References

[1] Y.D. Zhou, Y.P. Kim, X.C. Li, S.R. Baerson, A.K. Agarwal, T.W. Hodges, D. Ferreira, D.G. Nagle, Hypoxia-inducible factor-1 activation by (−)-epicatechin gallate: potential adverse effects of cancer chemoprevention with high-dose green tea extracts, J. Nat. Prod. 67 (2004) 2063–2069.
[2] Green Tea, Altern. Med. Rev. 5 (2000) 372–375.
[3] I. Baumgartner, A. Pieczek, O. Manor, R. Blair, M. Kearney, K. Walsh, J.M. Isner, Constitutive expression of phVEGF165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia, Circulation 97 (1998) 1114–1123.
[4] J.M. Isner, Myocardial gene therapy, Nature 415 (2002) 234–239.
[5] D.W. Losordo, P.R. Vale, J.F. Symes, C.H. Dunnington, D.D. Esakof, M. Maysky, A.B. Ashare, K. Lathi, J.M. Isner, Gene therapy for myocardial angiogenesis: initial clinical results with direct myocardial injection of phVEGF165 as sole therapy for myocardial ischemia, Circulation 98 (1998) 2800–2804.
[6] G. Wu, A.P. Mannam, J. Wu, S. Kirbis, J.L. Shie, C. Chen, R.J. Laham, F.W. Sellke, J. Li, Hypoxia induces myocyte-dependent COX-2 regulation in endothelial cells: role of VEGF, Am. J. Physiol. Heart Circ. Physiol. 285 (2003) H2420–H2429.
[7] R. Thomas, M.H. Kim, Epigallocatechin gallate inhibits HIF-1alpha degradation in prostate cancer cells, Biochem. Biophys. Res. Commun. 334 (2005) 543–548.
[8] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Measurement of protein using bicinchoninic acid, Anal. Biochem. 150 (1985) 76–85.
[9] G. Kaule, V. Gunzler, Assay for 2-oxoglutarate decarboxylating enzymes based on the determination of [1-^{14}C]succinate: application to prolyl 4-hydroxylase, Anal. Biochem. 184 (1990) 291–297.
[10] M.A. McDonough, V. Li, E. Flashman, R. Chowdhury, C. Mohr, B.M. Lienard, J. Zondlo, N.J. Oldham, I.J. Clifton, J. Lewis, L.A. McNeill, R.J. Kurzeja, K.S. Hewitson, E. Yang, S. Jordan, R.S. Syed, C.J. Schofield, Cellular oxygen sensing: crystal structure of hypoxia-inducible factor prolyl hydroxylase (PHD2), Proc. Natl. Acad. Sci. USA 103 (2006) 9814–9819.
[11] J.M. Elkins, K.S. Hewitson, L.A. McNeill, J.F. Seibel, I. Schlemminger, C.W. Pugh, P.J. Ratcliffe, C.J. Schofield, Structure of factor-inhibiting hypoxia-inducible factor (HIF) reveals mechanism of oxidative modification of HIF-1 alpha, J. Biol. Chem. 278 (2003) 1802–1806.
[12] G. D'Angelo, E. Duplan, N. Boyer, P. Vigne, C. Frelin, Hypoxia up-regulates prolyl hydroxylase activity: a feedback mechanism that limits HIF-1 responses during reoxygenation, J. Biol. Chem. 278 (2003) 38183–38187.
[13] K.B. Sandau, J. Zhou, T. Kietzmann, B. Brune, Regulation of the hypoxia-inducible factor 1alpha by the inflammatory mediators nitric oxide and tumor necrosis factor-alpha in contrast to desferroxamine and phenylarsine oxide, J. Biol. Chem. 276 (2001) 39805–39811.
[14] W.G. Kaelin Jr., The von Hippel–Lindau protein, HIF hydroxylation, and oxygen sensing, Biochem. Biophys. Res. Commun. 338 (2005) 627–638.
[15] K.A. Vincent, K.G. Shyu, Y. Luo, M. Magner, R.A. Tio, C. Jiang, M.A. Goldberg, G.Y. Akita, R.J. Gregory, J.M. Isner, Angiogenesis is induced in a rabbit model of hindlimb ischemia by naked DNA encoding an HIF-1alpha/VP16 hybrid transcription factor, Circulation 102 (2000) 2255–2261.

[16] K.G. Shyu, M.T. Wang, B.W. Wang, C.C. Chang, J.G. Leu, P. Kuan, H. Chang, Intramyocardial injection of naked DNA encoding HIF-1alpha/VP16 hybrid to enhance angiogenesis in an acute myocardial infarction model in the rat, Cardiovasc. Res. 54 (2002) 576–583.
[17] D.A. Elson, G. Thurston, L.E. Huang, D.G. Ginzinger, D.M. McDonald, R.S. Johnson, J.M. Arbeit, Induction of hypervascularity without leakage or inflammation in transgenic mice overexpressing hypoxia-inducible factor-1alpha, Genes Dev. 15 (2001) 2520–2532.
[18] R.K. Bruick, S.L. McKnight, Building better vasculature, Genes Dev. 15 (2001) 2497–2502.
[19] M. Detmar, L.F. Brown, M.P. Schon, B.M. Elicker, P. Velasco, L. Richard, D. Fukumura, W. Monsky, K.P. Claffey, R.K. Jain, Increased microvascular density and enhanced leukocyte rolling and adhesion in the skin of VEGF transgenic mice, J. Invest. Dermatol. 111 (1998) 1–6.
[20] F. Larcher, R. Murillas, M. Bolontrade, C.J. Conti, J.L. Jorcano, VEGF/VPF overexpression in skin of transgenic mice induces angiogenesis, vascular hyperpermeability and accelerated tumor development, Oncogene 17 (1998) 303–311.
[21] G. Thurston, C. Suri, K. Smith, J. McClain, T.N. Sato, G.D. Yancopoulos, D.M. McDonald, Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1, Science 286 (1999) 2511–2514.
[22] R.K. Bruick, S.L. McKnight, A conserved family of prolyl-4-hydroxylases that modify HIF, Science 294 (2001) 1337–1340.
[23] Y. Nakagawa, K. Nakajima, S. Tayama, P. Moldeus, Metabolism and cytotoxicity of propyl gallate in isolated rat hepatocytes: effects of a thiol reductant and an esterase inhibitor, Mol. Pharmacol. 47 (1995) 1021–1027.