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

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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.
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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.
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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,

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