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Pyrogallol inhibits the growth of lung cancer Calu-6 cells via caspase-dependent apoptosis☆

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ABSTRACT

Pyrogallol (PG) is a polyphenol compound and a known $O_2^{\bullet -}$ generator. We evaluated the effects of PG on the growth and apoptosis of human pulmonary adenocarcinoma Calu-6 cells. PG decreased the viability of Calu-6 cells in a dose- and time-dependent manner. The induction of apoptosis by PG was accompanied by the loss of mitochondrial membrane potential ($\Delta\Psi_m$), cytochrome *c* release from mitochondria and activation of caspase-3 and caspase-8. All tested caspase inhibitors, especially the pan-caspase inhibitor (Z-VAD), markedly rescued Calu-6 cells from PG-induced cell death. Rescue was accompanied by inhibition of caspase-3 activation and PARP cleavage. Treatment with Z-VAD also prevented the loss of mitochondrial membrane potential ($\Delta\Psi_m$). In conclusion, PG inhibits the growth of Calu-6 cells via caspase-dependent apoptosis.

1. Introduction

The mechanism of apoptosis mainly involves the mitochondrial and cell death receptor signaling pathways [1–3]. The key element in the mitochondrial pathway is the efflux of cytochrome *c* from mitochondria to the cytosol, where it subsequently forms a complex (apoptosome) with Apaf-1 and caspase-9, leading to activation of caspase-3 [4]. The cell death receptor pathway is characterized by the binding of cell death ligands and receptors, with subsequent activation of caspase-8 and caspase-3 [5,6]. Caspase-3 is an executioner caspase; its activation can directly dismantle cells by cleaving key proteins such as PARP.

Pyrogallol (PG) is a polyphenol compound and known superoxide anion ($O_2^{\bullet -}$) generator [7,8]. It has often been used to investigate the role of $O_2^{\bullet -}$ in biological systems. The superoxide anion ($O_2^{\bullet -}$) belongs to the reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$) and peroxynitrite ($ONOO^-$). ROS have recently been implicated in the regulation of many important cellular events, including transcription factor activation, gene expression, differentiation, and cell proliferation [9–11]. ROS are formed as by-products of mitochondrial respiration or certain oxidases such as nicotine adenine diphosphate (NADPH) oxidase, xanthine oxidase (XO), and a number of arachidonic acid oxygenases [12]. A change in the redox state of the tissue implies a change in the generation or metabolism of ROS. The principle metabolic pathways include superoxide dismutase (SOD), expressed as extracellular, cytoplasmic, and mitochondrial isoforms [13], which metabolize $O_2^{\bullet -}$ to H_2O_2. Further metabolism by peroxidases, including catalase and glutathione (GSH) peroxidase, yields O_2 and H_2O [14]. Cells possess antioxidant systems to control the redox state, which is important for their survival. Excessive production of ROS gives rise to the activation of events leading to death in several cell types [15–18]. Also, PG has been shown to induce the $O_2^{\bullet -}$-mediated death of several types of cell such as mesangial cells [19], human lymphoma cells [7], human glioma cells [20] and As4.1 juxtaglomerular cells [21]. The exact mechanisms involved in ROS-induced cell death are not fully understood.

Lung cancer is a major contributor to the variety cancer death in developed countries. Various novel therapeutic strategies are cur-

Abbreviations: PG, pyrogallol; ROS, reactive oxygen species; PARP, poly(ADP-ribose) polymerase; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; PI, propidium iodide; FITC, fluorescein isothiocyanate; DAPI, 4′-6-diamidino-2-phenylindole; Z-VAD-FMK, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketon; Z-DEVD-FMK, benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketon; Z-IETD-FMK, benzyloxycarbonyl-Ile-Glu-Thr-Asp-fluoromethylketon; Z-LEHD-FMK, benzyloxycarbonyl-Leu-Glu-His-Asp-fluoromethylketon; NADPH, nicotine adenine diphosphate; SOD, superoxide dismutase; XO, xanthine oxidase; GSH, glutathione.

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rently under consideration, as the clinical use of cytotoxic drugs is limited due to intrinsic or acquired resistance and toxicity [22]. Studies of the molecular mechanisms of cytotoxic drug action have shed light on the treatment of lung cancer, and novel agents that target specific intracellular pathways related to the distinctive properties of cancer cells continue to be developed. However, little is known about the relationship between PG and lung cancer cells.

In the present study we evaluated the effects of PG on the growth of human pulmonary adenocarcinoma Calu-6 cells and investigated its antiproliferative mechanism in relation to apoptosis.

2. Materials and methods

2.1. Cell culture

The human pulmonary adenocarcinoma Calu-6 cell line was obtained from the ATCC (HTB56) and maintained in a humidified incubator containing 5% CO_2 at 37 °C. Calu-6 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (GIBCO BRL, Grand Island, NY). Cells were routinely grown in 100-mm plastic tissue culture dishes (Nunc, Roskilde, Denmark) and harvested with a solution of trypsin–EDTA while in a logarithmic phase of growth. Cells were maintained in these culture conditions for all experiments.

2.2. Reagents

PG was purchased from the Sigma–Aldrich Chemical Company (St. Louis, MO). PG was dissolved in H_2O at 1×10^{-1} M as a stock solution. The pan-caspase inhibitor (Z-VAD-FMK; benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketon), caspase-3 inhibitor (Z-DEVD-FMK; benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketon), caspase-8 inhibitor (Z-IETD-FMK; benzyloxycarbonyl-Ile-Glu-Thr-Asp-fluoromethylketon) and caspase-9 inhibitor (Z-LEHD-FMK; benzyloxycarbonyl-Leu-Glu-His-Asp-fluoromethylketon) were obtained from R&D Systems, Inc. (Minneapolis, MN) and were dissolved in DMSO (Sigma) at 1×10^{-2} M as a stock solution. All stock solutions were wrapped in foil and kept at 4 or −20 °C.

2.3. Cell viability assay

The effect of PG on *in vitro* Calu-6 cell viability was determined by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye absorbance of living cells as previously described [23]. In brief, cells (5×10^4 cells per well) were seeded in 96-well microtiter plates in the presence of the designated doses of PG (Nunc, Roskilde, Denmark). After exposure to PG for 24, 48 or 72 h, 50 μl of MTT (Sigma) solution (2 mg/ml in PBS) were added to each well and the plates were incubated for an additional 3 or 4 h at 37 °C. MTT solution in the medium was aspirated off and 100 or 200 μl of DMSO were added to each well to solubilize the formazan crystals formed in viable cells. Optical density was measured at 570 nm using a microplate reader (Spectra MAX 340, Molecular Devices Co., Sunnyvale, CA). Each plate contained multiple wells at a given experimental condition and multiple control wells. This procedure was replicated for 2–4 plates per condition.

2.4. Sub-G1 analysis

Sub-G1 distribution was determined by propidium iodide (PI, Sigma–Aldrich; Ex/Em = 488 nm/617 nm) staining as previously described [23]. PI is a fluorescent biomolecule that can be used to stain DNA. In brief, 1×10^6 cells were incubated with the designated doses of PG with or without caspase inhibitor for the indicated amounts of time. Cells were then washed with phosphate-buffered saline (PBS) and fixed in 70% ethanol. Cells were washed again with PBS, then incubated with PI (10 μg/ml) with simultaneous RNase treatment at 37 °C for 30 min. Cell DNA content was measured using a FACStar flow cytometer (Becton Dickinson, San Jose, CA) and analyzed using lysis II and CellFIT software (Becton Dickinson) or ModFit software (Verity Software House, Inc., ME).

2.5. Morphological analysis of apoptosis by staining with 4′-6-diamidino-2-phenylindole (DAPI)

Cell morphology was evaluated by fluorescence microscopy following DAPI staining (Ex/Em = 364 nm/454 nm). Cells were cultured in eight-chamber glass slides (Lab-Tek, Nunc, Naperville, IL) containing 10% FBS in the absence or presence of 100 μM PG. After 72 h of incubation, slides were rinsed with PBS and fixed in 80% ethanol for at least 30 min. Cellular DNA was stained for 30 min with 1 μg/ml of DAPI dissolved in PBS. Slides were visualized on a Carl Zeiss fluorescence microscope (Germany). DAPI permeates the plasma membrane and yields blue chromatin. Viable cells display normal nuclear size and blue fluorescence, whereas apoptotic cells show condensed chromatin and fragmented nuclei.

2.6. Annexin V/PI staining

Apoptosis was determined by staining cells with annexin V-fluorescein isothiocyanate (FITC) (Ex/Em = 488 nm/519 nm) and PI labeling as described previously [24]. PI can be also used to differentiate necrotic, apoptotic and normal cells. This agent is membrane impermeant and generally excluded from viable cells. In brief, 1×10^6 cells were incubated with the designated doses of PG with or without caspase inhibitors for the indicated amounts of time. Cells were washed twice with cold PBS and resuspended in 500 μl of binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) at a concentration of 1×10^6 cells/ml. 5 μl of annexin V-FITC (PharMingen, San Diego, CA) and PI (1 μg/ml) were added and cells were analyzed using a FACStar flow cytometer (Becton Dickinson).

2.7. Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)

The mitochondrial membrane potential was monitored using the Rhodamine 123 fluorescent dye (Ex/Em = 485 nm/535 nm), which preferentially enters mitochondria based on their highly negative membrane potential ($\Delta\Psi_m$), as previously described [24]. Depolarization of mitochondrial membrane potential ($\Delta\Psi_m$) results in the loss of Rhodamine 123 and a decrease in intracellular fluorescence. In brief, 1×10^6 cells were incubated with the designated doses of PG with or without caspase inhibitors for the indicated amounts of time. Cells were washed twice with PBS and incubated with Rhodamine 123 (0.1 μg/ml; Sigma) at 37 °C for 30 min. PI (1 μg/ml) was subsequently added, and Rhodamine 123 and PI staining intensity were determined by flow cytometry.

2.8. Western blot analysis

The expression of apoptosis-related proteins was evaluated by Western blot analysis as described previously [23]. In brief, 1×10^6 cells were incubated with the designated doses of PG with or without a caspase inhibitor for 72 h. The cells were then washed in PBS and suspended in five volumes of lysis buffer (20 mM HEPES, pH 7.9, 20% glycerol, 200 mM KCl, 0.5 mM EDTA, 0.5% NP40, 0.5 mM DTT, 1% protease inhibitor cocktail (from Sigma)). Lysates were then collected and stored at −20 °C until further use. Supernatant protein