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Biochemical mechanisms of bornyl caffeate induced cytotoxicity in rat pheochromocytoma PC12 cells

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ABSTRACT

The chemopreventive and antineoplastic activities of caffeic acid derivatives are highly dependent on the chemical structures and cancer cell types. The objective of the present study was to investigate the cytotoxicity of bornyl caffeate and the underlying molecular mechanisms in rat pheochromocytoma PC12 cells. Our initial studies demonstrated that bornyl caffeate exhibited potent cytotoxicity in PC12 cells in a concentration- and time-dependent manner. By examining the cell morphology on a fluorescence microscope and detecting the cell surface phosphoserine with Annexin V-FITC, we proposed that bornyl caffeate could induce apoptosis in PC12 cells. We tested this hypothesis by investigating the effects of bornyl caffeate on several apoptosis-related biomarkers. These experiments showed that bornyl caffeate induced the up-regulation of Bax and down-regulation of Bcl-xl, the disruption of mitochondrial membrane potential, the activation of caspase 3 and the cleavage of PARP. Mechanistic studies further revealed that bornyl caffeate caused the depletion of glutathione (GSH), generation of superoxide ion and progressive activation of p38 mitogen-activate protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) in a concentration-dependent manner. In particular, GSH depletion appeared to be the most important mechanism underlying the cytotoxicity of bornyl caffeate. The preservation of the intracellular GSH contents with N-acetyl-L-cysteine (NAC), GSH and vitamin C abolished the effect of bornyl caffeate on the activation of p38 MAPK and JNK, preserved the integrity of mitochondrial membrane and ultimately rescued the cells from drug-induced cell death. These results suggest that bornyl caffeate induces apoptosis in PC12 cells via stimulating the depletion of GSH, the generation of reactive oxygen species (ROS) and the dissipation of mitochondrial transmembrane potential.

1. Introduction

Adrenal pheochromocytomas and extra-adrenal sympathetic paragangliomas are rare neuroendocrine tumors secreting high amounts of catecholamines noradrenaline, adrenaline and dopamine [1,2]. Current therapeutic strategies include the pharmacological inhibition of catecholamines' actions and the laparoscopic removal of tumors [3]. However, prognosis remains an ongoing challenge when metastasis of pheochromocytoma occurs. Deregulated apoptosis is well-known to enhance the resistance of the tumor cells to chemotherapy, radiation and immunotherapy [4]. Thus, the therapeutic restoration of normal apoptotic pathway in tumors allows the effective elimination of the primary tumor cells and the prevention of tumor metastasis [4,5]. For the initial stage screening of drugs, rat dopaminergic PC12 cells are widely used as an *in vitro* cell model of pheochromocytoma for investigating apoptosis and neuronal differentiation [6].

Most of the existing anticancer drugs mainly target three major mechanisms as follows: (1) inhibition of oncogenic lesions and induction of apoptosis in tumors; (2) elimination of the lesions that suppress apoptosis in tumor cells; (3) disruption of the survival signals in tumor cells. To target Mechanism-1, anticancer drugs largely interfere with the basic machinery of DNA synthesis and cell division. For example, overexpression of c-myc proteins promotes apoptosis in the cells when cell growth is arrested at various points in the cell cycle [7]. Deregulated proliferation of tumor cells can also be modulated by inhibitors of receptor tyrosine kinases (RTKs), Ras, downstream signaling kinases such as the mitogen-activate protein kinase (MAPK), Akt pathway and CDKs [8]. To target Mechanism-2, tumors tolerate serious stress due to the malfunctions of the p53 pathway in most cancers. Thus, therapeutic strategies are under evaluation to reactivate p53 function and subsequently induce apoptosis in a variety of tumor cells [9].

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Moreover, some proteins (e.g., Bcl-2) directly mediate the apoptotic pathways to support the development of cancers and the acquisition of resistance to conventional cancer therapies. Interestingly, the anticancer drugs targeting Bcl-2-related proteins induce apoptosis in tumor cells regardless of the p53 status [10]. To target Mechanism-3, Brc-Abl inhibitor STI571 induces apoptosis by inhibiting the components of the signaling cascades in chronic myelogenous leukaemia [11], whereas inhibitors of VEGF expression and VEGF receptors Flt-1 and KDR signaling pathways kill tumor via inhibiting angiogenesis [11,12]. Therefore, there is no doubt that induction of apoptosis is the core of different drug discovery strategies against various cancers.

Natural products constitute a rich resource of alternative antineoplastic and chemopreventive drug candidates [13–15]. Based on the anticancer mechanisms, these natural products are classified into two major groups: (1) target-specific cytotoxic anticancer drugs. These compounds such as camptothecin and vinblastine kill cancer cells essentially by the anticancer principles of the conventional medicines [16]; (2) chemopreventive drugs. Most of natural products regulate the survival and growth of cancer cells via the chemopreventive mechanisms. The cytotoxicity of these chemopreventive agents is mostly weak to moderate compared with the existing cytotoxic anticancer drugs [13,14]. One of the chemopreventive mechanisms is the induction of apoptosis via the activation of p38 MAPK and c-jun N-terminal kinase (JNK) [17,18]. JNK, p38 MAPK, and extracellular signal-regulated kinase (ERK) 1/2 are three major serine/threonine kinases in the regulation of cell proliferation, differentiation and apoptosis [19]. For example, upon the activation by stress stimuli, p38 MAPK and JNK directly regulate programmed cell death. The activation of p38 MAPK and JNK often causes the disruption of mitochondrial membrane potential (MMP) and the depletion of intracellular reduced glutathione (GSH) [20–22]. Importantly, the extent of the intracellular GSH depletion dictates the progression of cell death in response to different apoptotic stimuli [23].

Caffeic acid derivatives exert a wide range of biological and pharmacological activities such as antioxidant, anti-inflammatory, chemopreventive, anticancer and antibacterial properties in a structure-dependent and cell-type-specific manner [24–26]. Several previous studies have shown that caffeic acid amide and ester analogues (e.g., caffeic acid phenethyl ester) induce apoptosis in various tumor cells or virally transformed cells [27–31]. Along this line, bornyl caffeate structurally bearing two anti-inflammatory and anti-bacterial natural products caffeic acid and borneol was initially identified in several plants such as *Piper philippinum*, *Coreopsis mutica var. mutica* and *Verbesinatur bacenina Kunth* [32–34]. Bornyl caffeate was recently evaluated as the potent inhibitor of human neutrophil elastase [35], HIV integrase [36] and trypanosome cysteine protease [33]. We have recently demonstrated that bornyl caffeate induced apoptosis in a variety of cancer cell lines [37]. In particular, the effect of bornyl caffeate on the intracellular GSH levels appeared to be cell-type specific. Presumably, a sophisticated intracellular system determines whether caffeic acid derivatives serve as the scavengers of free radicals or the eliminators of endogenous antioxidants such as GSH [38–40]. In the present study, we further investigated the apoptotic mechanisms underlying the cytotoxicity of bornyl caffeate in PC12 cells with a research focus on GSH depletion, ROS formation and mitochondrial dysfunction.

2. Materials and methods

2.1. Chemicals and reagents

The antibodies against phospho-p38, p38, phospho-JNK, JNK were purchased from Cell Signaling Technology (Boston, MA, USA). The antibodies against Bax, Bcl-xl, cleaved caspase 3 and PARP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody against GAPDH and goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). Bornyl caffeate was prepared by exhaustive esterification of caffeic acid with borneol as described previously [37,41]. Other chemicals were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA) unless otherwise indicated.

2.2. Cell culture and drug treatment

Rat pheochromocytoma PC12 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA), and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum (HS) and 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 37 °C in a humidified 5% CO_2 atmosphere. For drug treatment, the cells at 70–80% confluence were treated with bornyl caffeate in complete growth medium, whereas the control cells were treated with dimethyl sulfoxide (DMSO) under the same conditions.

2.3. Cell viability assay

The cell viability was evaluated by a standard colorimetric assay as previously described [42]. Briefly, PC12 cells (1×10^4/100 μl) were seeded in a 96-well microplate and were treated with bornyl caffeate as indicated. At the end of drug treatment, the cell monolayers were incubated with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) solution (0.5 mg/ml) in phosphate-buffered saline (PBS) for 4 h. The formation of purple formazan was measured in relation to the absorbance at 570 nm on a microplate reader (Bio-Rad, Hercules, CA, USA). The cell viability was presented as percentage relative to the vehicle-treated controls.

2.4. Lactate dehydrogenase (LDH) release assay

LDH activity in the cell culture medium was measured by a colorimetric assay using the cytotoxicity cell death kit from Sigma–Aldrich (St. Louis, MO, USA) according to the manufacturer's instructions. In brief, PC12 cells were seeded at the density of 1×10^4 cells/100 μl in a 96-well microplate and treated with bornyl caffeate, the supernatants were collected and assayed for LDH activity based on the absorbance at 490 nm on a Bio-Rad microplate reader (Hercules, CA, USA). The results were presented as percentage relative to the vehicle-treated controls.

2.5. Morphological observation of nuclear changes

At the end of drug treatment, the cells were stained with the dye Hoechst 33258. The cell morphology was examined on a Zeiss fluorescence microscope from Carl Zeiss (Göttingen, Germany). The apoptotic cells were characterized by chromatin condensation and nuclear fragmentation.

2.6. Flow cytometric analysis of apoptotic cells

After the treatment with bornyl caffeate for 12 h, the cells (1×10^6) were freshly harvested and washed with PBS. The cells were then incubated for 10 min with Annexin V-FITC and PI at room temperature in the darkness. The stained cells were subsequently analyzed on a FACS Calibur flow cytometer from BD Biosciences (San Jose, CA, USA).