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The tetramethoxyflavone zapotin selectively activates protein kinase C epsilon, leading to its down-modulation accompanied by Bcl-2, c-Jun and c-Fos decrease

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ABSTRACT

Zapotin, a tetramethoxyflavone, is a natural compound with a wide spectrum of activities in neoplastic cells. Protein kinase C epsilon (PKCε) has been shown to be oncogenic, with the ability to increase cell migration, invasion and survival of tumor cells. Here we report that zapotin inhibits cell proliferation. In wild-type HeLa cells with basal endogenous expression of PKCε, the IC_{50} was found to be 17.9 ± 1.6 μM. In HeLa cells overexpressing doxycycline-inducible constitutively active PKCε (HeLaPKCεA/E), the IC_{50} was 7.6 ± 1.3 μM, suggesting that PKCε enhances the anti-proliferative effect of zapotin. Moreover, we found that zapotin selectively activated PKCε in comparison with other PKC family members, but attenuated doxycycline-induced PKCε expression. As a result of zapotin treatment for 6, 12 and 24 h, the doxycycline-induced levels of the two differently phosphorylated PKCε forms (87 kDa and 95 kDa) were decreased. Migration assays revealed that increasing concentrations of zapotin (from 3.5 to 15 μM) decreased migration of HeLaPKCεA/E cells. Furthermore, zapotin significantly increased the fraction of apoptotic cells in doxycycline-induced (HeLaPKCεA/E) cells after 24 h and decreased the levels of Bcl-2, c-Jun, c-Fos. This was accompanied by a degradation of PARP-1. In summary, activation of PKCε and down-modulation of the induced PKCε level by zapotin were associated with decreased migration and increased apoptosis. These observations are consistent with the previously reported chemopreventive and chemotherapeutic action of zapotin.

1. Introduction

Flavonoids belong to the family of polyphenolic compounds that are common components of human diet. Epidemiological studies have shown that consumption of flavonoids is associated with a low risk of cardiovascular diseases and cancer. Flavones, a group of flavonoids, are neuroprotective, cardioprotective and chemopreventive agents acting as antioxidants and modulators of protein kinases and lipid-dependent signaling pathways (Duraj et al., 2005). The flavone zapotin (5,6,2′,6′-tetramethoxyflavone) was first identified in the tropical fruit zapote blanco (*Casimiroa edulis*) (Murillo et al., 2007), later isolated from *Sargentia gregii* (Meyer et al., 1985) and extracted from the leaves of *Primula veris* (Budzianowski et al., 2005). In a previous study it was demonstrated that zapotin prevented colon carcinogenesis (Murillo et al., 2007). In human promyelocytic HL-60 leukemia cells, zapotin induced both, cell differentiation and apoptosis (Mata-Greenwood et al., 2001). Moreover, zapotin inhibited 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ornithine decarboxylase activity in human T24 bladder carcinoma cells and TPA-induced nuclear factor kappa B (NF-κB) activity in human HepG2 hepatocellular carcinoma cells (Maiti et al., 2007). However, any influence of zapotin on protein kinase pathways has not been reported so far. In our work, we focused on the effects of zapotin on protein kinase C (PKC) which is a family of ten isozymes comprising (i) the conventional PKCs α, βI, βII, γ, (ii), the novel δ, ε, θ, η, and (iii) the atypical λ/ι (mouse/human) and ζ. PKC isozymes play important roles in the activation of signal transduction pathways leading to synaptic transmissions, the activation of ion fluxes, secretion, proliferation, cell cycle control, ischemic preconditioning, differentiation and tumorigenesis. PKC has become of major interest as a target for therapeutic intervention in a range of different diseases such as allergy, asthma, rheumatoid arthritis, transplantation, AIDS, Alzheimer's disease, multiple sclerosis, hypertension, cardiac hypertrophy, ischemic insult, atherosclerosis, diabetes and cancer (Goekjian and Jirousek, 1999). The exact functions of the different PKC isozymes are not known at present. Here we investigated the influence of zapotin on the ten PKC isoenzymes and found that it selectively activates PKCε.

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2. Materials and methods

2.1. Isolation of zapotin

The leaves of *Primula veris L.* (cowslip) were collected from the field (A) and maintained by Dr. Maria Morozowska at the Department of Botany, Poznań University of Life Sciences, Poznań, Poland; in vitro cultures (B) were obtained by Dr. Maria Wesołowska at the Department of Pharmaceutical Botany and Plant Biotechnology, as described by Budzianowski et al. (2005). The dried leaves of each collection (A and B, each 80.0 g) were separately extracted with chloroform under reflux (7× 600 ml/h). The concentrated extracts (A: 8.0 g, B: 6.3 g) were separately chromatographed over cellulose CF-11 columns (Whatman, Maidstone, UK), eluted with a methanol–water mixture (ratio 7:3, v/v), and subsequently eluted from polyamide columns (Roth, Karlsruhe, Germany) with methanol to yield crude lipophilic flavone fractions (A: 3.4 g, B: 2.3 g). Portions of each of those fractions (A: 1.5 g, B: 1.8 g) were combined and separated on a silica gel column (Merck, Darmstadt, Germany) eluted with hexane, hexane–ethyl acetate mixtures (ratio 9:1, 8:2, 7:3, 6:4, v/v) and ethyl acetate to give fractions containing various mixtures of lipophilic flavones. The fractions containing zapotin were separated by preparative thin-layer chromatography on polyamide 6 (Macherey-Nagel, Düren, Germany) using acetic acid–water mixture (ratio 3:7, v/v) and on silica gel using hexane–ethyl acetate mixture (ratio 7:3, v/v) to give crude zapotin. This was purified by two-step column chromatography on Sephadex LH20 (Amersham-Pharmacia, Dübendorf, Switzerland) using methanol of HPLC grade and a mixture of redistilled ethanol and double distilled water (ratio 4:1, v/v) to yield a pure compound as white crystals from methanol (97 mg). For the experiments, a 40 mM stock solution in DMSO was used.

2.2. NMR identification of zapotin

The identity of the isolated zapotin sample was determined by ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra recorded for the solution in deuterated chloroform ($CDCl^{3}$) and compared to the spectral data previously published (Budzianowski et al., 2005). The purity of zapotin was determined to be 95% from the integrals observed in the ^{1}H NMR spectrum. Those integrals indicated 18 protons for zapotin and 2 protons for an impurity, which was visible as a broad singlet at 1.78 ppm and hence considered to correspond to water crystallization.

2.3. Cell culture

The HeLaPKCεA/E subline was derived from parental HeLa wild-type cells (HeLaWT; human epitheloid cervix carcinoma cells; ATCC No. CCL-2) by transfection with a pUHD 172-1-neo vector carrying a tetracycline/doxycycline-inducible Tet-on vector (Clontech, Palo Alto, CA) containing a constitutively active rat PKCε (PKCεA/E, Ala159 is replaced by Glu) (Garczarczyk et al., 2009). In this cell line the expression of PKCεA/E can be induced with 2 μg/ml doxycyline. This mutated form of PKCε is constitutively active without activators such as TPA. Cells were cultured in RPMI 1640 medium supplemented with 2 mM glutamine (Sigma Chemicals, Munich, Germany), 10% Tet-approved fetal bovine serum (Clontech, Mountain View, USA), 100 μg/ml geneticin and 100 μg/ml hygromycin (both from Roche Diagnostics, Mannheim, Germany). HeLaWT cells were cultured in RPMI 1640 medium with 10% fetal bovine serum and 50 μg/ml gentamicin (Sigma Chemicals, Munich, Germany).

2.4. Cell proliferation assay

Zapotin cytotoxicity was assessed as previously described (Rubis et al., 2008) using the MTT Proliferation Assay (Cell Proliferation Kit, Roche Diagnostics, Mannheim, Germany). Briefly, ~3000 HeLaWT or HeLaPKCεA/E cells/well were seeded in 96-well microplates and exposed to 1–25 μM of zapotin for 72 h. Cell viability was quantified using a Labsystems Multiscan RC spectrophotometer. IC_{50} values were calculated with CalcuSyn (Biosoft, Cambridge, UK) and standard deviation with Excel software. The final concentration of DMSO in the medium of controls and zapotin-treated cells was 0.1% and this did not show any effect on cell proliferation. The mean of 3 experiments, each in duplicate (+/− S.D.), is indicated in Fig. 2.

2.5. Western blot analysis

HeLaPKCεA/E cells were treated with 7.5, 15, 30 μM zapotin or 2 μg/ml doxycycline, or a combination of both compounds. Whole cell extracts were prepared using lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 1% Triton X-100, 100 mM PMSF, 25 μg/ml Na_3VO_4, 25 μg/ml NaF, 25 μg/ml leupeptin and 25 μg/ml aprotinin) as previously described (Garczarczyk et al., 2009). Protein concentration was measured with the Bradford assay (Sigma, Munich, Germany) and 30 μg (or 60 μg for phospho PKCε detection) of each extract was loaded onto SDS-PAGE ready gels (BioRad, Hercules, CA). Western blotting was performed by a standard procedure using PVDF membrane (Pierce Biotechnology, Rockford, USA). The following antibodies were used for detection: anti-PKCε, anti-pPKCε(Ser729), anti-PKCδ, anti-Bcl-2, anti-c-Jun, anti-c-Fos, anti-PARP-1, anti-NF-κB, anti-pNF-κB(Ser536), anti-actin, anti-GAPDH, anti-tubulin (all from Santa Cruz Biotechnology); 1 μg/ml of each primary antibody was used in the blotting solution. The proteins were visualized using SuperSignal® West Pico Chemiluminescent Substrate and a CL-X Posure™ film (Pierce Biotechnology, Rockford, USA). The optical density (Arbitrary Units) of the bands was measured using LabWorks software (UVP, Upland, CA). In Figs. 4 and 5, representatives of two experiments are shown.

To prepare the nuclear and cytosolic fractions, cells were homogenized in ice-cold lysis buffer (10 mM Hepes (pH 7.4), 1.5 mM $MgCl_2$, 10 mM KCl and 5 mg/ml aprotinin and 5 mg/ml leupeptin) for 5 min as described by Brodsky et al. (2010). The extract was centrifuged at 2400 *g* for 15 min, and the supernatants were centrifuged for 45 min at 14,000 *g*. The cytosolic extract was stored at 4 °C. The nuclear pellet was resuspended and incubation for 45 min in lysis buffer containing 20 mM Hepes (pH 7,4) 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA and 5 mg/ml aprotinin, and 5 mg/ml leupeptin. The nuclear lysates were centrifuged for 45 min at 14,000 *g* and the supernatant containing the soluble nuclear proteins was taken for further experiments. Protein concentrations were measured with the Bradford assay, and 40 μg protein from each extract was loaded onto 7–12% SDS-PAGE gels (BioRad, Hercules, CA). In Fig. 8, the scans of one representative experiment (out of three) are shown.

2.6. PKC activity

PKC assays were performed with 150 ng of each recombinant PKC isozyme (Proqinase, Freiburg, Germany) in 100 μl of 20 mM Tris–HCl pH 7.5, 20 mM $MgCl_2$, 1 mM $CaCl_2$, 50 μM substrate peptide (PKCα-19–31, RFARKGSLRQKNV; NeoMPS, Strasbourg, France), 10 μM phosphatidylserine, 1 μM TPA (Sigma, Munich, Germany), 40 μM ATP and 1 μCi γ-^{33}P-ATP (NEG602H, PerkinElmer, Waltham, MA). Zapotin was dissolved in DMSO. In untreated controls (set as 100%), the same volume of DMSO was added as in the zapotin-treated samples. After 10 min incubation at 30 °C, 50 μl of the reaction mix was transferred to a phosphocellulose disk (Whatman, Dassel, Germany), washed three times with 1.5% phosphoric acid and twice with distilled water. Subsequently, the disks were transferred into scintillation vials, and 3 ml of Ultima Gold (PerkinElmer, Waltham, MA) was added for determining the ^{33}P incorporation in a liquid scintillation counter. The data shown represents means of at least three independent experiments, each in triplicates (+/− S.D.).