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Phloretamide, an apple phenolic compound, activates the Nrf2/ARE pathway in human hepatocytes

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ABSTRACT

The aim of this study was to evaluate the effect of phloretamide (PA), an apple constituent, on the activation of the Nrf2 transcription factor and the expression of its target genes: glutathione S-transferases (*GSTs*), NAD(P)H:quinone oxidoreductase-1 (*NQO1*) and heme oxygenase-1 (*HO-1*) in normal human THLE-2 hepatocytes and the hepatoma HepG2 cell line. PA did not show significant cytotoxicity towards THLE-2 cells but such an effect was observed in HepG2 cells (IC_{50} ~200 μM).

The treatment of cells with PA resulted in the translocation of Nrf2 from cytosol to nucleus in both cell lines, but increased the level of its transcript and protein only in THLE-2 cells. In this cell line an increased level of *GSTA*, *GSTP*, *GSTT*, *NQO1* mRNA was also observed. Increased expression of *GSTs* was confirmed by enhancement of their protein levels. The increase in p53 protein content observed in THLE-2 may be associated with its stabilization induced by the enhancement of NQO1 level. PA did not affect *Nrf2*, *GSTs*, *NQO1* or *HO-1* expression in HepG2 cells.

These results suggest that PA has rather chemopreventive than chemiotherapeutic potential and acts similarly as apple dihydrochalcones through the induction of detoxification/antioxidative enzymes.

1. Introduction

Human hepatocellular carcinoma (HCC) constitutes about 85% of primary liver cancers recorded in cancer data banks. Globally around 440 000 new cases of HCC occur annually, accounting for around 5.5% of all human cancers. Due to the high prevalence of liver tumors strategies aiming at the prevention or blockade of hepatocarcinogenesis are highly desirable. A number of non-nutrient compounds from plants and fruits have been reported to possess anticancer activity (Sivalokanathan et al., 2006). Chemopreventive effects exerted by plant compounds are often attributed to their enhancement of carcinogen detoxification and antioxidant defense systems. These processes are mostly mediated by such detoxifying/antioxidant enzymes as glutathione S-transferase (GST) isoenzymes, NAD(P)H:quinone oxidoreductase-1 (NQO1) and heme oxygenase-1 (HO-1). The induction of these enzymes is attributed to the NF-E2-related factor-2 (Nrf2) which binds to an upstream regulatory sequence – the antioxidant-response element (ARE) and activates the expression of various genes encoding cytoprotective proteins. Several naturally occurring polyphenols demonstrate the ability to activate the Nrf2/ARE pathway (Suhr et al., 2008). Recently it was shown that phloretin, a dihydrochalcone present in apples in a glucosidic form (phloridzin), modulated the metabolism of a potent hepatocarcinogen, aflatoxin B1, in alpha mouse liver 12 cells (AML_{12}). The increased conjugation of its ultimate carcinogenic metabolite (AFB_1-8,9-epoxide) with glutathione as result of GST induction was partially associated with the activation of Nrf2/ARE pathway (Gao et al., 2012). Moreover, it was also shown that the consumption of the cloudy apple juice rich in polyphenols by healthy volunteers reduced genotoxicity, enhanced antigenotoxicity and modulated GSTT2 gene expression in some individuals (Petermann et al., 2009). These effects might be at least partly attributed to the activity of phloretin (Veeriah et al., 2008). Further studies (Soyalan et al., 2011) demonstrated that apple juice, in which phloretin derivatives represent ~30% of total polyphenols, modulate the expression of ARE-dependent genes in rat liver and colon.

Abbreviations: ARE, antioxidant-response element; DAC, decitabine; DMEM, Dulbecco's Modified Eagle's Medium; HO-1, heme oxygenase-1; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; HCC, hepatocellular carcinoma; GST, glutathione S-transferases; NQO1, NADPH:quinone oxidoreductase 1; Nrf2, nuclear factor erythroid-2-related factor 2; PA, phloretamide; ROS, reactive oxygen species.

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There is evidence that fresh apples contain also phloretamide (PA), the derivative of phloretic acid which is the product of phloretin metabolism (Duke, 2000). PA was initially indentified in xylem sap of *Malus domestica* (Rybicka, 1986). Further studies have shown that this compound may act as a plant growth hormone and interact with mistletoe lectin 1 (Meyer et al., 2007).

In order to evaluate the potential hepatoprotective activity of PA, we studied its effect on the Nfr2/ARE pathway activation and expression profile of Nrf2 target genes – *GSTA*, *GSTP*, *GSTM*, *GSTT*, *NQO1* and *HO-1* in human normal THLE-2 hepatocytes and a hepatoma HepG2 cell line. Moreover, the effect of PA on the possible cross-talk between Nrf2 activation and p53 expression as well as *GSTP1* hypermethylation in HepG2 cells was analyzed. The methylation of the promoter region of this gene may contribute to the pathogenesis of liver cancer. The results showing significant differences between normal and cancer cells suggest that PA has chemopreventive potential and acts similarly as apple dihydrochalcones through the induction of detoxification/antioxidative enzymes.

2. Materials and methods

2.1. Chemicals

Phloretamide (PA, Fig. 1) was synthesized as described – US Patent No.: US 7,923,578 B2. Decitabine, antibiotics solution (10^4 U penicillin, 10 mg streptomycin, 25 μg amphotericin B), dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM), epidermal growth factor (EGF), RIPA buffer and Tris were obtained from Sigma–Aldrich (St. Louis, MO, USA). HepG2 cells were provided by Prof. Zofia Mazerska from the Department of Drug Technology and Biochemistry, Gdańsk University of Technology, Poland. The THLE-2 cell line (ATCC-CRL-2706) was purchased from the American Type Culture Collection (Rockville, MD, USA). PA was dissolved in DMSO (100 mM stock solution) and stored at −20 °C. Beta-naphtoflavone was dissolved in DMSO and was used as a positive control of phase II enzymes induction. Decitabine was dissolved in PBS buffer and stored as 1 mM solution. TrueStart Hot Start Taq DNA Polymerase from Fermentas (Burlington, Canada) was used in methylation-specific PCR reactions. All primers used in PCR reactions were obtained from oligo.pl (Warsaw, Poland). Protease inhibitor cocktail was obtained from Roche Diagnostics GmbH (Penzberg, Germany). Primary and secondary antibodies against GSTA, GSTP, GSTM, GSTT, NQO1, HO-1, p53, Nrf2, Keap-1 and beta-actin were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rainbow colored protein molecular weight marker was purchased from Amersham Pharmacia Biotechnology (Piscataway, NJ, USA). All other chemicals were commercial products of the highest purity available.

2.2. Cell culture

HepG2 cells were maintained in DMEM containing 10% fetal bovine serum and antibiotics while THLE-2 cells were cultured in BEGM supplemented with Bullet Kit (ATCC) and additionally 10% fetal bovine serum, 5 ng/ml EGF, 70 ng/ml phosphoethanolamine. The cells were grown in a humidified incubator at 37 °C in the atmosphere of 5% CO_2. To assess the effect of PA on the measured parameters, 5×10^5 cells were seeded per 100 mm ø culture dish. After 24 h of initial incubation the cells were treated with 2, 10, 20 μM PA or 0.1% vehicle control. The incubation was continued for subsequent 72 h and cells were harvested.

2.3. Cell viability assay

The effect of PA on cell viability was assessed with the MTT assay, according to standard protocols. Briefly, 10^4 HepG2 or THLE-2 cells were seeded per well in a 96-well plate. After 24 h of preincubation in DMEM containing 5% FBS or BEGM supplemented with Bullet Kit (ATCC) and additionally 10% fetal bovine serum, 5 ng/ml EGF, 70 ng/ml phosphoethanolamine, PA was added to culture medium in various concentrations and cells were incubated for subsequent 72 h. DMSO concentration did not exceed 0.1%. After 72 h, cells were washed twice with PBS buffer and fresh medium containing MTT salt (0.5 mg/ml) was added. After 4 h incubation, formazan crystals were dissolved in acidic isopropanol and absorbance was measured at 540 and 690 nm. All the experiments were repeated three times, with at least three measurements per assay.

In all the subsequent experiments, non-toxic concentrations of PA were used, ranging from 2 to 20 μM. DMSO concentration did not exceed 0.1%.

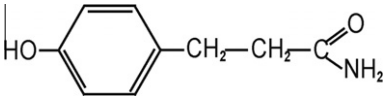

Fig. 1. Chemical structure of phloretamide (3-(*p*-hydroxyphenyl)-propionic acid amide).

2.4. Cell fractionation

The cytosol and nuclear extracts from HepG2 and THLE-2 were prepared using the Nuclear/Cytosol Fractionation Kit (BioVision Research, CA USA). Whole cell lysates were prepared from HepG2 and THLE-2 using RIPA buffer.

2.5. Preparation of DNA and RNA

Extraction of DNA and total RNA from cells was performed using GeneMatrix Universal DNA/RNA/Protein Purification Kit (EurX, Poland) according to the manufacturer's instructions.

2.6. Real-time PCR

Total RNA was subjected to reverse transcription using RevertAid Kit (Fermentas, Burlington, Canada) followed by quantitative real-time PCR. For real-time analyses the Maxima SYBR Green Kit (Fermentas) and a BioRad Chromo4 were used. The protocol started with 5 min. enzyme activation at 95 °C, followed by 40 cycles of 95 °C for 15 s, 54 °C for 20 s and 72 °C for 40 s and final elongation at 72 °C for 5 min. Product size verification was undertaken using melting curve analysis. The level of expression of TBP and PBGD reference genes was used for data normalization. The Pfaffl relative method (Pfaffl, 2001) was used for fold-change quantification. Primer sequences are listed in Table 1.

2.7. Nrf2 activation measurement

Nrf2 activation was assessed by an enzymatic immunoassay using the Nrf2 Transcription Factor ELISA Assay Kit (Active Motif, Rixensart, Belgium) according to the manufacturer's instructions. Nuclear extracts were incubated in the oligonucleotide-coated wells where the oligonucleotide sequence contains the ARE consensus binding site (5′-GTCACAGTGACTCAGCAGAATCTG-3′). Subsequently, wells were washed and incubated with the antibody against Nrf2. The addition of an HRP-conjugated secondary antibody provided a sensitive colorimetric readout at 450 nm.

2.8. Western blot

For the determination of the level of GSTA, GSTP, GSTM, GSTT, NQO1, HO-1, p53, Nrf2 and Keap-1 proteins the immunoblot assay was used. Protein content in the samples was determined with the Lowry method (1951). All the experiments were repeated three times. Whole cell lysates, nuclear extracts or cytosolic proteins (50–100 μg) were separated on 12% or 10% SDS–PAGE slab gels and proteins were transferred to nitrocellulose membranes (Laemmli, 1970; Towbin et al., 1979). After blocking with 10% skimmed milk, proteins were probed with rabbit anti-human GSTA, goat anti-rat GSTM, rabbit anti-human GSTP, human anti-mouse GSTT, goat anti-human NQO1, goat anti-mouse HO-1, mouse anti-human p53, goat anti-rat Keap-1, rabbit anti-mouse Nrf2, rabbit anti-human β-actin antibodies. The β-actin protein was used as an internal control. As the secondary antibodies in the staining reaction, the alkaline phosphatase-labeled anti-goat IgG, anti-mouse IgG or anti-rabbit IgG were used. The amount of the immunoreactive product in each lane was determined using the Quantity One software (BioRad Laboratories, Hercules, CA, USA). Values were calculated as relative absorbance units (RQ) per mg protein.

2.9. DNA methylation analysis

The methylation status of GSTP1 was evaluated using methylation-specific polymerase chain reaction (MSP) (Hermann et al., 1996) The EZ DNA Conversion Kit from ZymoResearch (Orange, CA, USA) was used for the bisulfite conversion of DNA samples. Primers and reaction conditions used for MSP were previously published (King-Batoon et al., 2008). DNA isolated from lymphocytes of healthy blood donors was used as the negative control and a completely methylated human DNA from New England Biolabs (Ipswich, MA, USA) as the positive control in MSP reactions. Amplification products were resolved on 2.5% agarose gels and visualized in UV after ethidium bromide staining.

2.10. Statistical analysis

The statistical analysis was performed by one-way ANOVA. The statistical significance between the experimental groups and their respective controls was assessed by Tukey's post hoc test, with $p < 0.05$.