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Inhibitory effect of ERK1/2 and AP-1 by hyperoside isolated from *Acanthopanax sessiliflorus*

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ABSTRACT

Consumption of fruits and vegetables is correlated with a lower incidence of cancer. Here, we identified hyperoside as an active compound from *Acanthopanax sessiliflorus*, and investigated the effect of hyperoside on UVB-induced transactivation of activator protein 1 (AP-1) and on the mitogen-activated protein kinase signalling pathway in JB6 P + cells. Hyperoside inhibited UVB-induced AP-1 transactivation. It inhibited the UVB-induced phosphorylation of $p90^{RSK}$. Kinase assays revealed that hyperoside significantly inhibited ERK1/2 activity. Furthermore, hyperoside bound to ERK1/2 to suppress its activity. In addition, phosphorylation of cAMP response element binding protein (CREB) and signal transducers and activators of transcription (STAT) 3 were suppressed by hyperoside. Overall, these results indicate that hyperoside may be a promising chemopreventive agent that acts by suppressing the transactivation of AP-1 and the phosphorylation of $p90^{RSK}$, CREB, and STAT3 through the binding and inhibition of ERK1/2.

1. Introduction

Transcription factors control the transfer of genetic information from DNA to mRNA, thereby regulating protein levels; their functions, in turn, are commonly regulated by protein phosphorylation and dephosphorylation via direct or indirect mechanisms (Whitmarsh & Davis, 2000). The transcription factor activator protein-1 (AP-1) plays a critical role in inflammation and various types of cancer, including skin, breast, and cervical cancer (Eferl & Wagner, 2003). In transgenic mice, AP-1 must be transactivated as a prerequisite for its tumour promotion activity (Young et al., 1999). Diverse transcriptional complexes are formed by homodimerisation and heterodimerisation of cAMP response element binding protein (CREB), cAMP response element modulator (CREM), and activating transcription factor 1 (ATF-1) (Siu & Jin, 2007). In some cancers, CREB expression is elevated (Abramovitch et al., 2004), and members of the signal transducers and activators of transcription (STAT) family of proteins have been known to act as both signal messengers and transcription factors. In particular, STAT3 is involved in a wide variety of human cancers, including skin cancer (Ahsan, Aziz, & Ahmad, 2005).

Diverse stimuli, including UV radiation, activate extracellular signal-regulated kinase (ERK), which then translocates to the nucleus where it phosphorylates its substrates, including ribosomal S6 kinase (RSK), Elk-1, c-Jun, and c-Myc (Roberts & Der, 2007). This in turn elicits the induction of early gene transcription, causing cell proliferation or differentiation (Eferl and Wagner, 2003). Thus, upregulation of the ERK signalling cascade is important in oncogenesis and cell transformation (Sebolt-Leopold & Herrera, 2004). The critical role of ERK2 has not been clarified because of embryonic lethality in ERK2 null knockout mice; however, in a conditional knockout system, it was shown that ERK2 is involved in memory and learning (Satoh et al., 2007). ERK1 knockout mice were viable, fertile, and of normal size, but had a twofold reduction in the number of mature thymocytes (Pages et al., 1999). Recently, we showed that mitogen-activated kinase (MEK) and ERK signalling represent a major signalling pathway in anchorage-independent cell growth and that this growth is significantly inhibited by naturally occurring phytochemicals suppressing MEK-ERK signalling (Kang et al., 2007; Lee et al., 2008). We suggested that ERK signalling may be a promising target for new chemopreventive and anticancer agents, as blocking ERK-mediated signalling would

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likely suppress downstream signalling, such as RSK translocation and AP-1, CREB, and STAT activation.

Several epidemiological studies have shown that the topical application of naturally occurring phytochemicals can inhibit carcinogenesis (Ross & Kasum, 2002; Surh, 2003). Furthermore, the roots and stems of *Acanthopanax sessiliflorus* have been shown to elicit biological functions, including anti-platelet aggregation, anti-pancreatic lipase, and anti-inflmmatory effects (Yang et al., 2009; Yoshizumi et al., 2006; Zhou, 1985). In our previous studies, natural phytochemicals could inhibit UVB-induced skin cancer in mice, and we have suggested that this effect may be attributable to the action of small-molecule inhibitors (Jung et al., 2008). One dietary flavonoid present in numerous plant parts, including mature saskatoon fruits and almond, is querecetin-3-*O*-galactoside (hyperoside), which has been shown to inhibit hydrogen peroxide-induced apoptosis in pheochromocytoma PC12 cells and lung fibroblasts, owing to its antioxidant capacity (Liu, Tao, Zhang, Lu, & Wei, 2005). However, the possibility of using hyperoside as a functional food material and its molecular mechanism are still unknown.

In the present study, we isolated hyperoside from *A. sessiliflorus*, which is a member of the Araliaceae family and is found in eastern Asia and far western Russia (Fujikawa et al., 2002; Sun et al., 2007; Yi et al., 2002), and identified it as an active compound of *A. sessiliflorus* fruit. We investigated its effect on UVB-induced signalling pathways in mouse epidermal JB6 P^+ cells and showed that hyperoside inhibits ERK1/2 kinase activity, which consequently inhibits UVB-induced transactivation of AP-1 and phosphorylation of $p90^{RSK}$, CREB, and STAT3. These findings suggest that hyperoside may be a potent chemopreventive agent, acting to inhibit UVB-induced ERK1/2 and AP-1 activity.

2. Materials and methods

2.1. Extraction and identification

The fully matured *A. sessiliflorus* fruit was harvested and collected from Jeongseon Agricultural Technology & Extension Center in Gangwon, South Korea in October, 2006. The air-dried whole *A. sessiliflorus* fruits (1.5 kg) were percolated with MeOH and H_2O (8:2) at 25 °C for 1 week. The residue obtained after the removal of the solvent (400 g) was diluted with H_2O (500 ml) and extracted using ethylacetate (500 ml) in triplicate. Accordingly, the EtOAc fraction (12.7 g) was concentrated *in vacuo* and the residues were dissolved in methanol to analyze by LC–ESI-MS (liquid chromatography–electron spray ionisation-mass spectrometry. Mass spectra were obtained via either direct infusion or liquid chromatographic introduction into a Varian 500-MS instrument (Varian Inc., CA, USA), which consists of a 212-LC binary gradient solvent delivery pump, a ProStar 335 photodiode array detector, a ProStar 410 autosamper and an iontrap mass spectrometer. The chromatographic separation of the compounds was achieved using the Chromosep SS C18 column [150 × 2.0 mm i.d., 5 μm (Varian, CA, USA)] at a flow rate of 0.2 ml/min. Mobile phases A and B were high-purity water and acetonitrile, respectively, both containing 0.1% formic acid. Gradient elution was conducted as follows: 0–55 min for 5–70% B with a linear gradient, followed by 55–60 min of 100% B. Therefore, the chemical structure of hyperoside was identified in a comparison of our researchable flavonoid MS/MS library and mass spectral results of previous study data with the literature values (Chen, Song, Guo, Liu, & Liu, 2002) .

2.2. Materials

Hyperoside (95%) was purchased from Sigma–Aldrich (St. Louis, MO). Eagle's minimum essential medium (MEM), gentamicin, and L-glutamine were obtained from Gibco–BRL (Carlsbad, CA). Foetal bovine serum (FBS) was purchased from Gemini Bio-Products (Calabasas, CA). Antibodies, to detect phosphorylated p38 (Tyr180/Tyr182), total p38, phosphorylated JNK (Thr183/Tyr185), total JNK, phosphorylated p90RSK (Thr359/Ser363) and total p90RSK, were purchased from Cell Signalling Biotechnology (Beverly, MA). Antibodies against phosphorylated ERK1/2 (Thr202/Tyr204), and total ERK were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The active ERK protein was obtained from Upstate Biotechnology (Lake Placid, NY). ATP and the chemiluminescence detection kit were purchased from Amersham Pharmacia Biotech (Piscataway, NJ), and the protein assay kit was obtained from Bio-Rad Laboratories (Hercules, CA). G418 and the luciferase assay substrate were purchased from Promega (Madison, WI).

2.3. Cell culture

The JB6 P + mouse epidermal cell line was cultured in monolayers at 37 °C in a 5% CO_2 incubator in 5% FBS–MEM, 2 mM L-glutamine, and 25 g/ml of gentamicin. The JB6 P + mouse epidermal cell line was stably transfected with the AP-1 luciferase reporter plasmid and maintained in 5% FBS–MEM containing 200 μg/ml of G418.

2.4. Luciferase assay for AP-1 transactivation

Confluent monolayers of JB6 P + cells stably transfected with the AP-1 luciferase plasmid were harvested, and 8×10^3 viable cells suspended in 10 ml of 5% FBS/MEM were added to each well of a 96-well plate. Plates were incubated at 37 °C in a 5% CO_2 incubator. When cells reached 80–90% confluence, they were starved by culturing in 0.1% FBS–MEM for a further 24 h. The cells were treated with hyperoside (20, 40, and 80 μM) 1 h before exposure to UVB (0.05 J/cm^2) and were then incubated for 6 h. Cells were disrupted with 100 μl of lysis buffer [0.1 M potassium phosphate buffer (pH 7.8), 1% Triton X-100, 1 mM dithiothreitol (DTT), and 2 mM ethylenediamine tetraacetic acid (EDTA)] and luciferase activity was measured using a luminometer (Luminoskan Ascent; Thermo Electron, Helsinki, Finland).

2.5. Western blot assays

For the Western blots, cells (1.5×10^6) were cultured in a 10 cm dish for 48 h, and then starved in 0.1% FBS–MEM for 24 h to eliminate FBS activation of MAPKs. The cells were then treated with hyperoside (20, 40, and 80 μM) for 1 h and irradiated with UVB (0.05 J/cm^2). The protein concentration was determined using a dye-binding protein assay kit (Bio-Rad Laboratories), as described in the manufacturer's manual. Lysate protein (40 μg) was subjected to 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech). After transfer, the membrane was incubated with the specific primary antibody at 4 °C overnight. Protein bands were visualised by a chemiluminescence detection kit (Amersham Pharmacia Biotech) after hybridisation with a horseradish peroxidase (HRP)-conjugated secondary antibody.

2.6. In vitro ERK 1 and 2 kinase assay

ERK kinase activity was assayed in accordance with instructions provided by Upstate Biotechnology (Billerica, MA). Twenty nanograms of ERK 1 or 2 protein were added to the mixture containing myelin basic protein, 5 × assay buffer, and diluted [γ-32P]ATP solution, with hyperoside or without hyperoside. Reactions were