Molecular Mechanisms Involved in the Inhibition of MDA-MB-435 Breast Cancer Cells by Phenolic Acids from the Red Flesh Peach BY00P6653

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Keywords: MDA-MB-435, MAPK pathway, Prunus persica, caspase, chlorogenic acid, ERK1/2 phosphorylation, apoptosis

Abstract
A wide variety of fruits and vegetables extracts have been shown to protect against cancer cell growth in vitro. Increasing evidence suggests that phenolics compounds found in fruits and vegetables may have anticancer properties. However, the molecular mechanisms involved in the anti-proliferative activity exerted by these natural compounds are poorly understood.

Treatment of the estrogen-negative receptor MDA-MB-435 breast cancer cells with F1, containing mainly chlorogenic acid resulted in a dose-dependent reduction in cell viability with an IC50 = 150 mg chlorogenic acid equiv/L. Concomitantly, F1 treatments led to a time and dose-dependent ERK1/2 phosphorylation, and to a lesser extent c-Jun activation. The early and sustained activation of ERK1/2 was associated with up-regulation of the pro-apoptotic protein Bax. MEK1/2 inhibitor suppressed Bax activation and cytochrome c release from the mitochondria.

In conclusion, our studies employing the MEK-MAK inhibitor revealed that prolonged ERK activation by F1 mediated the apoptosis machinery and that MEK-MAK blockage modified the cytotoxicity exerted by F1 through mitochondria permeabilization, indicating that prolonged MEK-MAK activation may be linked to cell death.

INTRODUCTION
Breast cancer is the second leading cause of cancer death in women in the US. It was estimated that in 2007, 178,480 new cases and 40,460 deaths may occur (American Cancer Society, 2008). In recent years, much emphasis has been placed on functional properties of various fruits and vegetables and their contribution to human health. Peaches contain a variety of phytochemicals, such as phenolic acids, carotenoids and flavonoids, responsible for functional properties, which have been linked to the reduction or prevention of different types of cancer, including breast cancer (Dechsupa et al., 2007; Granado-Serrano et al., 2007; Hou, 2003; Miura et al., 2007; Tomas-Barberan et al., 2001).

Increasing interest in functional foods has motivated plant breeders to enhance functional properties of peaches to contain higher antioxidant activity and higher phenolic content (Cevallos-Casals et al., 2006; Vizzotto et al., 2007). Among these, the red flesh peach selection BY00P6653 was identified as having a potential for chemoprevention due to its higher toxicity to cancerous versus non cancerous breast cells (Vizzotto, 2005). Based on this preliminary study, our aim was to elucidate the molecular mechanisms behind the inhibitory effect of a fraction of phenolic acids extracted from the BY00P6653 peach against the estrogen independent MDA-MB-435 breast cancer cells. This study will provide insights on the chemopreventive or therapeutic effect of these natural compounds
MATERIALS AND METHODS

The red-fleshed peach selection, BY00P6653, obtained from the breeding program at USDA-ARS (SE Fruit & Nut Research Lab, Byron, GA) was used for this study. The phenolic acids fraction (F1) were isolated following the method reported by (Oszmianski et al., 1988). For the cell culture study, we used the estrogen-negative human breast cancer line MDA-MB-435, obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured according to ATCC specifications. The cell viability was measured by using a MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] assay (Mosmann, 1983). The role of the caspase activation on cell viability was assessed by using ZVAD-FMK (BD Bioscience, San Jose, CA), a broad-spectrum caspase inhibitor that prevents apoptotic cell death mediated by caspase activation. For western-blot analysis, the cells lysates were separated on SDS-polyacrylamide gel electrophoresis gels and transferred to PVDF membranes. Primary and secondary antibodies were used as recommended by the manufacturer: p-c-Jun, Bax or β-actin mouse monoclonal (Santa Cruz Biotechnology); ERK1/2, p-ERK1/2, cytochrome c rabbit monoclonal antibodies and the MEK1/2 inhibitor (U0126) (Cell Signaling Technology, Inc. Danvers, MA). Quantitative data represent mean values ± SD or SE (n ≥ 3). Data were analyzed by one-way analysis of variance (ANOVA) using SPSS version 15.0 (SPSS Inc., Chicago, IL). Post-hoc Tukey pairwise comparisons were used to separate means that were significantly (P < 0.05) different from each other.

RESULTS AND DISCUSSION

Cell Viability

The cell viability of MDA-MB-435 breast cancer cells was decreased in a dose-dependent manner (Fig. 1). Phenolic acids present in F1 were identified as hydroxycinnamic acid derivatives, mainly chlorogenic acid and caffeic acid (Vizzotto, 2005). This is in agreement with reports indicating the antiproliferative action of phenolic acids from BY00P6653 peach on breast cancer cells (Gonthier et al., 2003; Kampa et al., 2004).

Molecular Mechanism behind the Anticancer Activity of Phenolic Acids (F1) from Peach BY00P6653

1. Role of MAPK Pathway. The mitogen-activated protein kinases (MAPKs) are the family of kinases that transduce signals from the cell membrane to the nucleus in response to a wide range of stimuli, including stress (Wada and Penninger, 2004). We investigated the role of F1 on the activation of MAPK signaling pathway by western-blot analysis. Our results showed how F1 activated the extracellular signal-regulated kinase (ERK1/2) in a dose and time-dependent manner (Fig. 2). However, none of the other members of this family were activated: the c–Jun NH2-terminal kinase (JNK) or the p38-MAPK (data not shown). A late and weak activation of the transcription factor c-Jun was also observed. The c-Jun phosphorylation may occur downstream of ERK phosphorylation since the ERK inhibitor blocked c-Jun phosphorylation (data not shown). The MAPKs cell signaling pathway is frequently altered in a variety of human cancers; therefore, modulation of MAPK pathway by dietary compounds may provide novel strategies for the prevention and treatment of cancer (Sebolt-Leopold, 2000). The ERK is generally activated in response to growth stimuli, whereas JNK and p38 are known to be simultaneously activated in response to a variety of cellular and environmental stresses that trigger apoptosis. However, the activation of ERK cascade promotes contrary cellular responses upon cellular context (Marshall, 1995; Nguyen et al., 2003).

2. Role of Caspase Activation. Apoptosis might occur by activation of a caspase dependent pathway or an alternative pathway that may not involve caspase activation. Our results showed that pan-caspase inhibitor ZVAD-FMK had no effect on the inhibition of
cell viability of cells treated with F1 (Fig. 3) \((p > 0.05)\), indicating that caspase activation plays a minor or no role in the induction of apoptosis of MDA-MB-435 cells by F1.

3. Role of Mitochondria Pathway. It has been reported that the sustained ERK phosphorylation may target the mitochondrial pathway through Bax and Bak activation and mitochondria permeabilization that lead to cytochrome c release (Nguyen et al., 2003). Our results show that F1 activates the pro-apoptotic mitochondrial pathway through Bax activation, which leads to cytochrome c release. This effect was substantially suppressed by MEK1/2 inhibitor, which is upstream of ERK1/2 phosphorylation (Fig. 4). The use of agents that potentially may attack the mitochondria is interesting in cancer treatment, since the induction of apoptosis by this mechanism may involve the release of at least five apoptogenic proteins from the intermembrane mitochondrial space to the cytosol (Mohamad et al., 2005).

CONCLUSIONS
The anticancer activity exerted by F1 against MDA-MB-435 breast cancer cells is primarily mediated by sustained ERK activation which activates the mitochondrial pathway through Bax activation leading to cytochrome c release. These results may be relevant because phenolic acids present in peaches may act through a pathway that activate a cascade of reactions that would direct cancer cells to apoptosis.

Literature Cited
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Vizzotto, M. 2005. Inhibition of invasive breast cancer cells by selected peach and plum antioxidants. Dissertation, Texas A&M University, College Station.

Figures

Fig. 1. A representative evaluation of the concentration-dependent impact of F1 on the viability of human MDA-MB-435 breast cancer cells. MDA-MB-435 cells were incubated with various concentrations of F1 for 24 h and cell viability measured by MTT assay. Values are mean ± SD, n = 4.
Fig. 2. Peach BY00P6653, F1 activates MEK/ERK signaling pathway. Upper panel, MDA-MB-435 cells were treated for with 150 mg chlorogenic acid equiv./L of F1. Control (C), cells were cultured with serum free medium for 36 h. Positive control (+C) cells received medium containing 10% FBS 6 h before harvesting. Lower panel, MDA-MB-435 cells were treated with different doses of F1, control solvent (CS) received same volume of vehicle (DMSO). Cell lysates were subjected to western blot analysis using anti-ERK MAPK, phosphorylated ERK or c-Jun. In both panels, total β-actin were detected to show the relatively same amount of protein load.

Fig. 3. Effect of caspase inhibitor on the inhibition of cell viability. MDA-MB-435 cells were incubated with the vehicle (DMSO) or 50 μM of Z-VAD-FMK for 1 h and then cultured in the absence or presence of 150 mg/L of F1 without or with caspase inhibitor for 24h. Alfa-tocopheryl succinate (VES) was used as positive control for caspase-induction apoptosis. Cell viability was then assessed by MTT assay. Data are shown as mean ± standard error of the mean, n=4. Different letters indicate significance at the p < 0.05 level.
Fig. 4. MEK1/2 inhibitor suppresses Bax activation and cytochrome c release from mitochondria. MDA-MB-435 cells were treated with 0, 25, 50, 100, 150 and 200 mg chlorogenic acid equiv./L of peach BY00P6653 F1 for 36 hours. 10 μM of U0126 was used for MEK1/2 inhibition. Cells treated with 20 mg/L of alfa-tocopheryl succinate for 24h (VES) were used as positive control. Cells were harvested and lysed for Western blot analysis. Blots were incubated with mouse anti-Bax and rabbit anti-cytochrome c. β-actin was included as a loading control. The data represent a typical experiment conducted three times with similar results.