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Naringin-induced p21WAF1-mediated G_1-phase cell cycle arrest via activation of the Ras/Raf/ERK signaling pathway in vascular smooth muscle cells

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ABSTRACT

The flavonoid naringin has been shown to play a role in preventing the development of cardiovascular disease. However, the exact molecular mechanisms underlying the roles of integrated cell cycle regulation and MAPK signaling pathways in the regulation of naringin-induced inhibition of cell proliferation in vascular smooth muscle cells (VSMCs) remain to be identified. Naringin treatment resulted in significant growth inhibition and G_1-phase cell cycle arrest mediated by induction of p53-independent p21WAF1 expression; expression of cyclins and CDKs in VSMCs was also down-regulated. In addition, among the pathways examined, blockade of ERK function inhibited naringin-dependent p21WAF1 expression, reversed naringin-mediated inhibition of cell proliferation and decreased cell cycle proteins. Moreover, naringin treatment increased both Ras and Raf activations. Transfection of cells with dominant negative Ras (RasN17) and Raf (RafS621A) mutant genes suppressed naringin-induced ERK activity and p21WAF1 expression. Finally, naringin-induced reduction in cell proliferation and cell cycle protein was abolished in the presence of RasN17 and RafS621A mutant genes. The Ras/Raf/ERK pathway participates in p21WAF1 induction, leading to a decrease in cyclin D1/CDK4 and cyclin E/CDK2 complexes and in naringin-dependent inhibition of cell growth. These novel and unexpected findings provide a theoretical basis for preventive use of flavonoids to the atherosclerosis disease.

1. Introduction

Atherosclerosis and restenosis represent the culmination of a series of cellular and molecular events involving proliferative abnormalities of vascular smooth muscle cells (VSMCs) (Ross, 1993). Cell proliferation is governed primarily by regulation of the cell cycle (Pardee et al., 1978), which consists of four distinct sequential phases (G_0/G_1, S, G_2, and M). Transition through the G1-phase of the cell cycle and entry into the S-phase requires binding and activation of cyclin/cyclin-dependent kinase (CDK) complexes, predominantly cyclin D/CDK4 or CDK6 and cyclinE/CDK2 (Sherr, 1994). Cyclin-dependent kinase inhibitors (CKIs) are naturally occurring gene products that inhibit cyclin/CDK activity, leading to G1-phase arrest (Xiong et al., 1993; Tanner et al., 1998). p21WAF1 (p21) is a primary negative regulator of CDK in VSMCs (Tanner et al., 1998). Kinase activity of the cyclin/CDK complexes is negatively regulated by CDK inhibitors, such as p21WAF1 (Xiong et al., 1993; Tanner et al., 1998). These inhibitors block cell-cycle progression by binding and inactivating the cyclin/CDK complex in the G1-phase, leading to cell cycle arrest (Xiong et al., 1993; Tanner et al., 1998).

GTP binding to the Ras protein initiates a protein kinase cascade, which activates extracellular signal-regulated kinase 1/2 (ERK1/2) via the protein kinase, Raf-1 (Waskiewicz and Cooper, 1995). In general, ERK activation is associated with VSMCs survival and proliferation in response to activation by proliferative factors (Millette et al., 2006; Duff et al., 1995; Mehta and Griendling, 2007). However, *in vitro* ERK activation contributes to cell death in other cell lines (Rundén et al., 1998; Lee et al., 2005; Park et al., 2003).

Recently, interest in the therapeutic use of nutraceuticals, such as flavonoids, for the prevention and treatment of many chronic diseases has increased. Among the naturally occurring flavonoids, naringin, an active compound existing in citrus fruits, was shown to be an effective anti-cancer, anti-oxidative and anti-atherogenic agent in animal studies (Le Marchand et al., 2000; So et al., 1996; Kanno et al., 2005; Ng et al., 2000; Lee et al., 2001). Although many studies have analyzed the effects of naringin on growth inhibition in various cell lines (Le Marchand et al., 2000; So et al., 1996; Kanno et al., 2005), the pathway that integrates cell cycle regulation and the signaling pathways involved in inhibition of cell proliferation remain to be identified.

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The purpose of the present study was to examine the roles of MAPK signaling pathways and p21WAF1-mediated G1 cell-cycle arrest in the regulation of the naringin-induced inhibition of VSMCs growth.

2. Materials and methods

2.1. Materials

Naringin was purchased from Wako Pure Chemical Industries, LTD. (Osaka, Japan). Polyclonal antibodies to cyclin E, CDK2 and CDK4 were obtained from Santa Cruz (Santa Cruz, CA). Polyclonal antibodies to cyclin D1, p21WAF1, p53, p27, ERK, Raf, phospho-Raf, phospho-ERK, p38 MAP kinase, phospho-p38 MAP kinase, JNK and phospho-JNK were obtained from New England Biolabs. PD98059, SP600125 and SB203580 were obtained from Calbiochem (San Diego, CA). Anti-Ras antibody was obtained from Transduction Laboratories. The pCMV vectors encoding either dominant negative Ras (RasN17) or dominant negative Raf (RafS621A) were purchased from Clontech. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

2.2. Cell Cultures

VSMCs were isolated from Sprague-Dawley rats. These explants were grown in DMEM containing 10% FBS, 2 mM glutamine, 50 μg/ml gentamycin, and 50 μl/ml amphotericin-B at 37 °C in a humidified 5% CO_2 atmosphere. MAP kinase inhibitors (PD98059, SP600125 and SB203580) were present in the culture medium during the 12 h naringin-treatment period.

2.3. Cell viability assay

Subconfluent, exponentially growing VSMCs were incubated with naringin in 24-well plates for various lengths of time. Cell viability was determined using a modification of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described previously (Moon et al., 2004).

2.4. [^{3}H]thymidine incorporation

VSMCs, grown to near confluence in 24-well tissue culture plates, were made quiescent and treated with naringin. [^{3}H]thymidine-incorporation experiments were performed as described previously (Moon et al., 2004).

2.5. Cell cycle analysis (FACS)

Cells were harvested, fixed in 70% ethanol, and stored at −20 °C. Cells then were washed twice with ice-cold PBS and incubated with RNase and propidium iodide, a DNA-intercalating dye. Cell cycle phase analysis was performed using a Becton Dickinson Facstar flow cytometer equipped with Becton Dickinson cell-fit software.

2.6. Immunoprecipitation, immunoblotting, and immune complex kinase assays

Growth-arrested cells were treated with naringin for various time periods at 37 °C in the presence of 10% FBS. Cell lysates were prepared, and immunoprecipitation, immunoblotting and immune complex kinase assays were performed as described previously (Moon et al., 2004).

2.7. Creation of p21WAF1 promoter reporter constructs

The human p21WAF1 promoter construct, WWW-luc (p21WAF1P), was a gift from Dr Bert Vogelstein (El-Deiry et al., 1994; Datto et al., 1995). Preparation of p21WAF1P Δ2.3 has been described previously by Datto et al. (1995).

2.8. Transient transfection

Each plasmid was transfected into VSMCs using a Superfect reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions [19]. Luciferase activity was measured using a luciferase assay system (Promega, Madison, WI) according to the manufacturer's instructions. Firefly luciferase activity was standardized to β-galactosidase activity.

2.9. Affinity precipitation of the active form of Ras (Ras-GTP)

Cells seeded in 100 mm dishes at subconfluency (5×10^4 cells/cm^2) were grown and treated with naringin for various lengths of time. After washing with ice-cold PBS, cells were lysed by addition of 500 μl lysis buffer (25 mM HEPES, 10 mM EDTA, 1% Igepal CA630, complete protease inhibitor cocktail from Roche Diagnostics, 1 mM sodium orthovanadate, and 10% glycerol). The lysate was clarified by centrifugation for 15 min at 14,000*g*, and the protein concentration of the lysate was determined using a BCA assay (Pierce, Rockford, IL). Equal amounts of cell lysates (500 μg) were subjected to affinity precipitation with Ras-GTP using 10 μl of an agarose suspension conjugated with a GST fusion protein that corresponded to the human Ras-binding domain of c-Raf (GST-RBD) (Upstate Biotechnology, Lake Placid, NY). After 1 h incubation at 4 °C, the agarose was washed three times with lysis buffer and boiled with 30 μl SDS sample buffer. The product was resolved by 15% SDS–PAGE, followed by immunoblotting with anti-Ras antibody (Moon et al., 2004).

2.10. Statistical analysis

When appropriate, data were expressed as means ± SE. Data were analyzed by factorial ANOVA and Fisher's least significant difference test where appropriate. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Naringin reduces VSMCs proliferation

To investigate the inhibitory effect of naringin on proliferation of VSMCs, the cells were grown in medium containing 10% FBS in the absence and presence of naringin (0–150 μM) for 24 h. As shown in Fig. 1A and B, naringin significantly inhibited cell viability and DNA synthesis in a concentration-dependent manner, as evidenced by the results of the MTT assay and the [^{3}H]thymidine incorporation experiment. The vehicle (ethanol) had no effect on basal cell viability and thymidine incorporation (data not shown).

3.2. Naringin-induced G1-phase cell cycle arrest

Based on flow cytometric analysis (Fig. 1 C–G), treatment with 100 μM naringin (the IC_{50} concentration as assessed by MTT assay and [^{3}H] thymidine incorporation) caused a significant increase in the number of cells in the G1-phase. This result suggests that naringin inhibits VSMCs growth by causing cell cycle arrest in the G1-phase.

Next, the effect of naringin on cell cycle regulatory molecules that affect the G1- and S-phases of the cell cycle was examined. Naringin treatment of VSMCs for 24 h resulted in a dose-dependent decrease in expression of cyclin D1 and cyclin E, as well as reductions in CDK2 and CDK4 (Fig. 2A). These results suggest that naringin-induced G1-phase cell cycle arrest, followed by inhibition of cyclinD1/CDK4 and cyclinE/CDK2. The kinases associated with CDKs drive cell cycle progression through the transition checkpoints, as they activate cyclins—the essential regulatory components of the cyclin-CDK complexes (Pardee et al., 1978; Sherr, 1994). Therefore, the kinase activities associated with CDK2 and CDK4 were assessed in naringin-treated cells. Naringin treatment of VSMCs inhibited kinase activity of both CDK2- and CDK4-immunoprecipitates in a dose-dependent manner (Fig. 2B).

3.3. Naringin-induced cell cycle arrest is associated with up-regulation of p21WAF1

The effect of naringin on induction of p21WAF1, which regulates entry into the S-phase at the G1–S phase transition checkpoint, was examined (Xiong et al., 1993; Tanner et al., 1998). Immunoblot analysis revealed that naringin treatment of VSMCs resulted in significant, dose-dependent induction of p21WAF1 compared with untreated cells (Fig. 2C); p27 was not affected by naringin treatment. Moreover, under similar experimental conditions, expression of p53, a tumor suppressor protein, was unaffected. These results suggest that involvement of p27 and p53 in naringin-induced cell cycle arrest is unlikely (Fig. 2C).

Naringin treatment strongly induced p21WAF1 expression in VSMCs. Because induction of p21WAF1 increases interaction between p21WAF1 and CDK, which, in turn, decreases kinase activity (Xiong et al., 1993; Tanner et al., 1998), the effects of naringin on interactions between p21WAF1 and CDKs were examined. In