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Regulation of adipocytokine secretion and adipocyte hypertrophy by polymethoxyflavonoids, nobiletin and tangeretin

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ABSTRACT

Aims: The polymethoxyflavonoids nobiletin and tangeretin possess several important biological properties such as neuroprotective, antimetastatic, anticancer, and anti-inflammatory properties. The present study was undertaken to examine whether nobiletin and tangeretin could modulate adipocytokine secretion and to evaluate the effects of these flavonoids on the hypertrophy of mature adipocytes.
Main methods: All experiments were performed on the murine preadipocyte cell line 3T3-L1. We studied the formation of intracellular lipid droplets in adipocytes and the apoptosis-inducing activity to evaluate the effects of polymethoxyflavonoids on adipocyte differentiation and hypertrophy, respectively. The secretion of adipocytokines was measured using ELISA.
Key findings: We demonstrated that the combined treatment of differentiation reagents with nobiletin or tangeretin differentiated 3T3-L1 preadipocytes into adipocytes possessing less intracellular triglyceride as compared to vehicle-treated differentiated 3T3-L1 adipocytes. Both flavonoids increased the secretion of an insulin-sensitizing factor, adiponectin, but concomitantly decreased the secretion of an insulin-resistance factor, MCP-1, in 3T3-L1 adipocytes. Furthermore, nobiletin was found to decrease the secretion of resistin, which serves as an insulin-resistance factor. In mature 3T3-L1 adipocytes, nobiletin induced apoptosis; tangeretin, in contrast, did not induce apoptosis, but suppressed further triglyceride accumulation.
Significance: Our results suggest that nobiletin and tangeretin are promising therapeutic candidates for the prevention and treatment of insulin resistance by modulating the adipocytokine secretion balance. We also demonstrated the different effects of nobiletin and tangeretin on mature adipocytes.

Introduction

Obesity is closely associated with insulin resistance, which causes various diseases including type 2 diabetes, hypertension, coronary heart disease, and cancer (Kopelman, 2000). Obesity-related insulin resistance is attributable to an increase in the number and size of adipocytes differentiated from fibroblastic preadipocytes at the cellular level in adipose tissues. Furthermore, such adipocyte hypertrophy subsequently affects the kinetics of the secretion of adipocytokines derived from adipocytes, which are involved in insulin resistance. In obese states, the plasma concentration of the most representative adipocytokine adiponectin is decreased (Maeda et al., 1996; Arita et al., 1999); adiponectin is considered as a physiologically active protein that improves insulin sensitivity (Yamauchi et al., 2001; Díez and Iglesias, 2003). On the other hand, monocyte chemoattractant protein (MCP)-1 or resistin, which is present in high amounts in obese states and in hypertrophic adipocytes, is implicated in the development of insulin resistance and atherosclerosis (Sartipy and Loskutoff, 2003; Haluzik and Haluzikova, 2006; Kim et al., 2006). These results suggest that the modulation of the balance between the secretion of adiponectin and insulin-resistant factors through the regulation of adipocyte hypertrophy is a useful strategy for preventing the development of obesity-related pathologies.

Recently, several flavonoids have been shown to possess anti-hypertrophic effects against adipocytes through the regulation of multiple actions. Hsu and Yen (2007) have reported that rutin, quercetin, and resveratrol inhibited adipogenesis in 3T3-L1 adipocytes. Furthermore, Hsu and Yen (2008) have reported that epigallocatechin-3-gallate, quercetin, and genistein show anti-obesity effects by inducing apoptosis in adipocytes.

Polymethoxyflavonoids (PMFs) belong to a class of flavones that are commonly found in citrus fruits. The 2 most common PMFs nobiletin and tangeretin exist mainly in the peel of oranges and

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tangerines (Horowitz and Gentili, 1977). It has been reported that PMFs play important roles in a number of biological functions. They have been reported to possess neuroprotective (Datla et al., 2001), antimetastatic (Sato et al., 2002), anticancer (Silalahi, 2002), and anti-inflammatory properties (Lin et al., 2003). However, the effects of PMFs on adipocyte hypertrophy and adipocytokine secretion remain to be fully elucidated.

In the present study, we examined the effects of nobiletin and tangeretin on the secretion of adiponectin and MCP-1 and hypertrophy of mature adipocytes. We demonstrated that combined treatment of differentiation reagents with nobiletin or tangeretin differentiated 3T3-L1 preadipocytes into adipocytes possessing less intracellular triglyceride as compared to vehicle-treated differentiated 3T3-L1 adipocytes. Both flavonoids increased the secretion of an insulin-sensitizing factor, adiponectin, but concomitantly decreased the secretion of the insulin-resistance factor MCP-1 in 3T3-L1 adipocytes. Furthermore, nobiletin was found to decrease the secretion of resistin, which served as an insulin-resistance factor. In mature 3T3-L1 adipocytes, nobiletin induced apoptosis; tangeretin, in contrast, did not induce apoptosis, but suppressed further triglyceride accumulation.

Materials and methods

Materials

Nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone) (Fig. 1A), and tangeretin (5,6,7,8,4′-pentamethoxyflavone) (Fig. 1B) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Pioglitazone was purchased from Sigma (St. Louis, MO). These compounds were soluble in dimethyl sulfoxide (DMSO), and the dissolved concentration of solvent in culture medium was less than 0.4%. The murine preadipocyte cell line 3T3-L1 was obtained from the American Type Culture Collection (Manassas, VA). All other reagents were of analytical grade.

Cell culture

3T3-L1 murine preadipocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Biowest, Nuaille, France) at 37 °C in a humidified atmosphere with 5% CO_2 until confluence. After 2 days from confluence, designated as day 0, the cells were switched to differentiation medium (DM) containing 1 μM insulin, 1 mM isobutylmethylxanthine (IBMX), and 1 μM dexamethasone in DMEM as described previously (Green and Meuth, 1974), for another 2 days. During this time, the cells were treated with DM in the presence of various concentrations of nobiletin or tangeretin to examine the effect on adipogenic differentiation. After 2 days (i.e., on day 2), the cell culture medium was replaced with a maintenance medium consisting of 10% FBS/DMEM; the cells were then maintained for another 6 days (i.e., till day 8), after which mature adipocytes with accumulated fat droplets had formed. On day 8, when the differentiation was complete, the cells were harvested or treated with nobiletin or tangeretin up to 4 days (till day 12) to examine the effect of flavonoids on mature 3T3-L1 adipocytes. All media contained 50 μg/mL of penicillin, 50 μg/mL of streptomycin, and 100 μg/mL of neomycin.

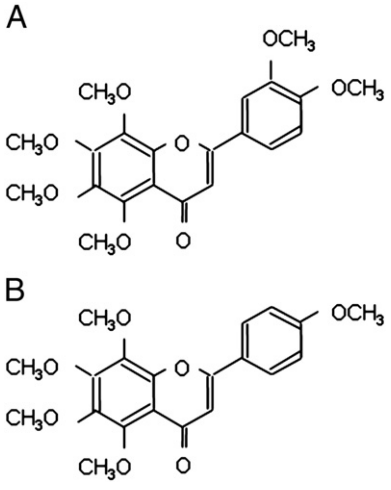

Fig. 1. Chemical structures of nobiletin [A] and tangeretin [B].

Oil red O staining

On day 8, the 3T3-L1 adipocytes plated onto collagen I-coated 24-well plate were washed twice with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS(−)), fixed with 4% paraformaldehyde diluted with PBS(−) for 60 min, and washed 3 times with distilled water. The cells were then stained with 0.5% Oil red O (Sigma) for 60 min, and the nuclei were stained with hematoxylin. After this staining procedure, the cells were washed 3 times with distilled water and were observed under an optical microscope.

Measurement of intracellular triglycerides

The 3T3-L1 adipocytes plated onto 60-mm dish till day 8, 10, and 12 were washed with PBS(−) and scraped on ice in 1 mL saline solution (25 mM Tris–HCl (pH 7.5) and 1 mM EDTA). The cells were homogenized by sonication of cell suspension and were assayed for total triglycerides by using the Triglyceride E-Test Wako (Wako Pure Chemicals), in accordance with the manufacturer's instructions. The protein concentration of the cellular fraction was measured by using a bicinchoninic acid (BCA) protein analysis kit (Pierce Biotechnology, Rockford, IL). The results were expressed as the ratio of the total triglycerides to cellular protein.

Analysis of adipocytokine secretion

The amount of adiponectin, MCP-1, and resistin in the harvested medium on day 8 was determined by the Quantikine® Mouse Adiponectin/Acrp30 Immunoassay (R&D Systems Inc., Minneapolis, MN), Quantikine® Mouse CCL2/JE/MCP-1 Immunoassay (R&D Systems), and Quantikine® Mouse Resistin Immunoassay (R&D Systems) respectively, in accordance with the manufacturers' instructions.

Apoptosis assay

Apoptosis was detected through a Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling (TUNEL) assay. In brief, day 8 3T3-L1 adipocytes plated onto collagen I-coated cover slips were washed twice with PBS(−), fixed with 4% paraformaldehyde diluted with PBS(−) for 15 min, and washed 3 times with PBS(−). The cells were permeabilized with methanol for 5 min, and then washed twice more with PBS(−). Thereafter, the cells were incubated for 1 h in a humidity box at 37 °C in 200 μL of a DNA-labeling solution containing 180 μL of labeling safe buffer (Takara Bio Inc., Otsu, Japan) and 20 μL of terminal deoxynucleotidyl transferase (TdT) enzyme (Takara Bio). After a brief wash with PBS(−), the cells were incubated with propidium iodide (PI) for 10 min at room temperature in the dark to stain their nuclei. The cells were then observed under a fluorescent microscope.

Western blot analysis

3T3-L1 adipocytes plated onto 35-mm dishes were harvested and lysed in RIPA buffer containing 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 20 mM