JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Naringenin and Hesperetin Induce Growth Arrest, Apoptosis, and Cytoplasmic Fat Deposit in Human Preadipocytes

KEIKO MORIKAWA,* MITSUKO NONAKA, HIROMI MOCHIZUKI, KIE HANDA, HARUKA HANADA, AND KAORI HIROTA

Division of Clinical Nutrition, Department of Nutrition, Sagami Women's University, 2-1-1 Bunkyo, Sagamihara, Kanagawa 228-8533, Japan

Citrus flavonoids are reported to be promising bioactive compounds against hyperlipidemia and lipid biosynthesis. However, the mechanism of the lipid lowering effect by flavonoids remains unknown. The present study examines the effect of some flavanones on the adipocytic conversion of the human preadipocyte cell line, AML-I. Among four structure-related flavanones including naringenin, naringenin-7-rhamnoglucoside (naringin), hesperetin, and hesperetin-7-rhamnoglucoside (hesperidin), the aglycones such as naringenin and hesperetin exhibited the growth arrest of AML-I cells. When the cells were examined by Annexin V−FITC staining method, it was noticed that growth arrest was induced by apoptotic cell death. In the study of apoptosis-related protein in the naringenin-treated cells, anti-apoptotic proteins such as p-Akt, NF-κB, and Bcl-2 were decreased, and pro-apoptotic protein Bad was accumulated by Western blot analysis. Interestingly, exposure of AML-I cells to naringenin or hesperetin during short-term cultures increased cytoplasmic lipid droplets by Sudan Black B staining. Furthermore, expression of fatty acid synthase (FAS) and peroxisome proliferator activated receptor (PPAR)-γ was enhanced in naringenin-treated cells. These data suggest that apoptosis by flavanones does not inhibit the adipocytic conversion of AML-I preadipocytes. The result also indicates that adipocyte may not be a direct target for the lipid-lowering activity of the flavanones.

KEYWORDS: Flavanone; apoptosis; adipogenesis; preadipocyte

INTRODUCTION

Naringenin and hesperetin are present in grapefruit and orange juice mainly as their glycosylated forms, naringin and hesperidin, respectively. These compounds are promising bioactive compounds against hyperlipidemia and lipid biosynthesis. The glycosylated forms have been reported to reduce the concentration of plasma triglyceride and cholesterol in hyperlipidemic rats (*1, 2*). Significant decrease in plasma and hepatic cholesterol levels is observed in rats fed a mixture of naringin and hesperetin (*3*). The aglycones, naringenin and hesperetin, also reduce apoB accumulation in the media of HepG2 cells in a dose-dependent manner (*4*) via reduced activity and expression of acyl CoA: cholesterol acyltransferase and microsomal triglyceride transfer protein (*5*). Additionally, naringenin inhibits lipogenesis by suppressing glucose conversion to lipids and lipolysis by affecting epinephrine-stimulated glycerol release in rat adipocytes (*6*). Hesperetin suppresses both epinephrine- and theophylline-induced lipolysis in rat adipocytes (*7*), too. These data suggest that the mechanism of the lipid-lowering effect of flavanones may depend on the inhibition of lipolysis or biosynthesis in vivo. However, it remains unknown whether flavanones may affect adipogenesis along with the maturation process of preadipocytes.

Recent studies have reported that dietary flavonoids including catechin, quercetin, kaempferol (*8*), genistein (*9*), and epigallocatechin gallate (EGCG) (*10, 11*) are able to induce apoptosis and inhibit adipogenesis in 3T3-L1 adipocytes. The antiadipogenic effect of genistein or EGCG is due to the inhibition of C/EBP activity (*9, 10*). Suppression of adipogenesis by quercetin depends on the arrest of 3T3-L1 cell maturation by inhibiting GPDH activity, which is a key enzyme of lipid synthesis (*11*). Naringenin is reported to inhibit phosphotidylinositide-3-kinase activity and glucose uptake in 3T3-L1 adipocytes (*12*). In this study, we examined the effect of flavanones on the proliferation and differentiation of AML-I preadipocyte to explore the mechanism of their lipid-lowering effect.

MATERIALS AND METHODS

Cell Line. A human preadipocyte cell line, AML-I, is derived from a lineage of human bone marrow stromal cells. We have shown that AML-I cells are able to differentiate into adipocytes and increase lipid droplets in the cytoplasm following adipogenic stimulation as described previously (*13*). Using the AML-I cell model of preadipocytes, we investigated the effect of flavanones on the proliferation and differentiation of preadipocytes into adipocytes. The cell line was maintained

* Author to whom correspondence should be addressed (telephone/fax 81-42-749-4909; e-mail morikawa_keiko@isc.sagami-wu.ac).

10.1021/jf801965n CCC: $40.75
Published on Web 11/04/2008

under standard culture condition in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2 in air and 1% penicillin−streptomycin in our laboratory. AML-I is free of any contamination including mycoplasma. The cells were subcultured before leading confluency, and medium was replaced every 4 days, because serum component contains the factors facilitating cell differentiation from preadipocytes to adipocytes when they are confluent. Subconfluent cells obtained on day 3 from the medium change were utilized for the experiment.

Reagents. The flavanones used in this study including naringenin, naringin, hesperidin, and hesperetin were purchased from Sigma (St. Louis, MO). Human antibodies for NF-κB (p65), α-tubulin, Bcl-2, Akt1, p-Akt, Bad, PPAR-γ, FAS, and second antibodies for horseradish peroxidase-conjugated mouse anti-rabbit IgG or goat anti-mouse IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) or Amersham Farmacia Biotech (Arlington Heights, IL), respectively.

Culture Medium for Induction of Proliferation, Apoptosis, and Differentiation. In the experiments to induce proliferation, apoptosis, and differentiation, the culture medium were switched to media containing 0.5 mmol/L methylisobuthylxanthine (M), 0.5 μmol/L dexamethasone (D), and 1 mg/mL insulin (I) in dimethyl sulfoxide (DMSO) (final concentration of DMSO was 0.1%) in RPMI-1640/10% FBS (MDI medium), unless otherwise mentioned. Flavanones were reconstituted as 100 mmol/L stock solutions in DMSO in a sterile condition and stored at −20 °C. They were dissolved in appropriate concentrations with MDI medium immediately before the experiment. Unless otherwise noted, "vehicle" refers to 0.1% DMSO in MDI medium.

Assay for Cell Proliferation and Viability. Subconfluent AML-I cells were seeded in six-well plates at a density of 2.5×10^5 cells/mL/well in 5 mL of MDI medium on day 1. Four flavanones or vehicle (0.1% DMSO) was added to culture medium (MDI medium) at the time of plating at the concentrations of 100, 250, and 500 μM. On days 2, 4, and 6 after plating, viable and dead cell numbers were measured by trypan blue dye exclusion in a Neubauer hemocytometer.

Assay for Apoptosis by Annexin V−FITC Staining. To determine whether the reduced viability was based on apoptosis or necrosis, the flavanone-treated cells were stained using an Annexin V−FITC apoptosis detection kit (Sigma) and analyzed qualitatively and quantitatively by fluorescent microscopy. Briefly, 1×10^6 cells were washed twice, resuspended in PBS, and stained with 5 μL of Annexin V−FITC and 2.5 μL of propidium iodide (PI) according to the manufacturer's instruction with minor modification. Cytocentrifuge preparation of each sample was made, and apoptotic cells were determined by fluorescent microscopy. At least 400 cells were observed in each slide, and the percentage of Annexin V−FITC positive cells was counted in each sample. It was regarded that cells under early apoptotic process and late apoptotic process were stained with the Annexin V−FITC alone and Annexin V−FITC plus PI, respectively. Live cells showed no staining by either PI or Annexin V−FITC. Necrotic cells were stained by PI alone. The number of apoptotic cells among 400 cells was counted and expressed as percentage in flavanone- or vehicle-treated cells, respectively. As AML-I cells enter into apoptosis at a very low rate after culture with vehicle in MDI medium (spontaneous apoptosis), the specific apoptotic effect of naringenin was estimated by the following formula.

$$\text{specific apoptosis (\%)} = \left(\frac{\text{apoptosis by flavanone} - \text{spontaneous apoptosis}}{100 - \text{spontaneous apoptosis}}\right) \times 100$$

Western Blot Analysis. Expression levels of both apoptosis- and differentiation-related proteins were examined by Western blot analysis. AML-I cells were plated at a density of 2.5×10^5 cells/mL in 200 mm Petri dishes in the MDI media with vehicle (0.1% DMSO) or flavanone (100 or 500 μM) for the indicated duration. Afterward, media were aspirated, and cells were washed with cold PBS (pH 7.4). Cell extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL), according to the manufacturer's protocol. For Western blotting, 50 μg of protein was loaded for each lane on the compact 10% gels (Atto Corp., Tokyo, Japan), blotted on Immune-Blot PVDF membrane (Bio-Rad Laboratories, Hercules, CA). Blots were incubated with the indicated primary antibodies and subsequent 1:25000 or 1:50000 horseraddish peroxidase-conjugated second antibodies. All of the primary antibodies were used at the final concentration of 2 μg/mL. Blots were then visualized using the ECL detection system as described by the manufacturer (Amersham Pharmacia Biotech). For quantification analysis, the sum of the density of bands corresponding to protein blotting with the antibody under study was calculated and normalized to the amount of α-tubulin. After normalization with α-tubulin, changes in the expression of the protein under study in treated samples were expressed relative to the basal levels of this protein in untreated sample. The intensities of the bands were evaluated using an ATTO imazing analyzer system (Atto Corp.).

Assay for Adipogenesis. After culture in the presence of 0.1% DMSO or flavanone at the concentration of 100 or 500 μM in MDI medium, a cytocentrifuge preparation of each sample was made and cells were stained for lipid by incubation with the Sudan Black B stain (0.18% Sudan Black B made in 6% ethanol containing PBS) at 37 °C for 60 min. Mature adipocytes were determined by the presence of cytoplasmic lipid droplets using light microscopy. Amounts of fat droplets varied among the cells, but they appeared to be plentiful in large adipocytes. Adipocyte maturation was assessed semiquantitatively by the amounts of lipid droplets using a scoring method for evaluating leukocyte alkaline phosphatase activity in the histochemical procedure (*14*) with minor modification. At least 400 cells were counted on each slide. The stage of adipocyte maturation was assessed by the amounts of cytoplasmic lipid droplets. Briefly, the cells were classified into three categories: stage I for the cells having fewer than five lipid droplets in the cytoplasm, stage II for the cells that had more but unevenly distributed droplets, and stage III for the cells that contained dense lipid droplets.

Statistics. The data were expressed as mean ± SD, and comparison of ANOVA was performed to determine significant difference in multiple comparison. Values of $p < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

In the present study, we show that aglycone flavanones were able to induce not only growth arrest and apoptosis but also lipogenesis in the human preadipocyte cell line, AML-I.

For time course and concentration−response experiments on the cell growth, AML-I cells were treated with four flavanones at concentrations of 100, 250, and 500 μM or vehicle (0.1% DMSO) in MDI medium. Cell number and viability were assessed on days 2, 4, and 6 after the initiation of culture by trypan blue dye exclusion test, and the viable and dead cell numbers were counted by hemocytometer. Proliferation of AML-I cells was enhanced time-dependently until day 6 of the culture in the presence of vehicle with MDI culture medium. In the presence of aglycone flavanones such as naringenin or hesperetin, cell proliferation was significantly suppressed after day 4 at concentrations between 250 and 500 μM, although it was not significant at 100 μM (**Figure 1**). Cell viability was almost unchanged in the presence of vehicle until day 6, but it was significantly decreased in the presence of naringenin or hesperetin at concentrations between 250 and 500 μM time-dependently. In contrast, Harmon et al. reported that naringenin affects neither mitotic clonal expansion and triglyceride accumulation nor PPAR-γ expression in the 3T3-L1 preadipocyte cell line (*15*). Those authors examined the effect of naringenin on cell proliferation at concentrations of 5, 50, and 100 μM during 48 h of culture in MDI medium. In our study, AML-I cell proliferation was significantly arrested in the presence of aglycons such as naringenin and hesperetin at concentrations between 250 and 500 μM, although not significant at 100 μM,

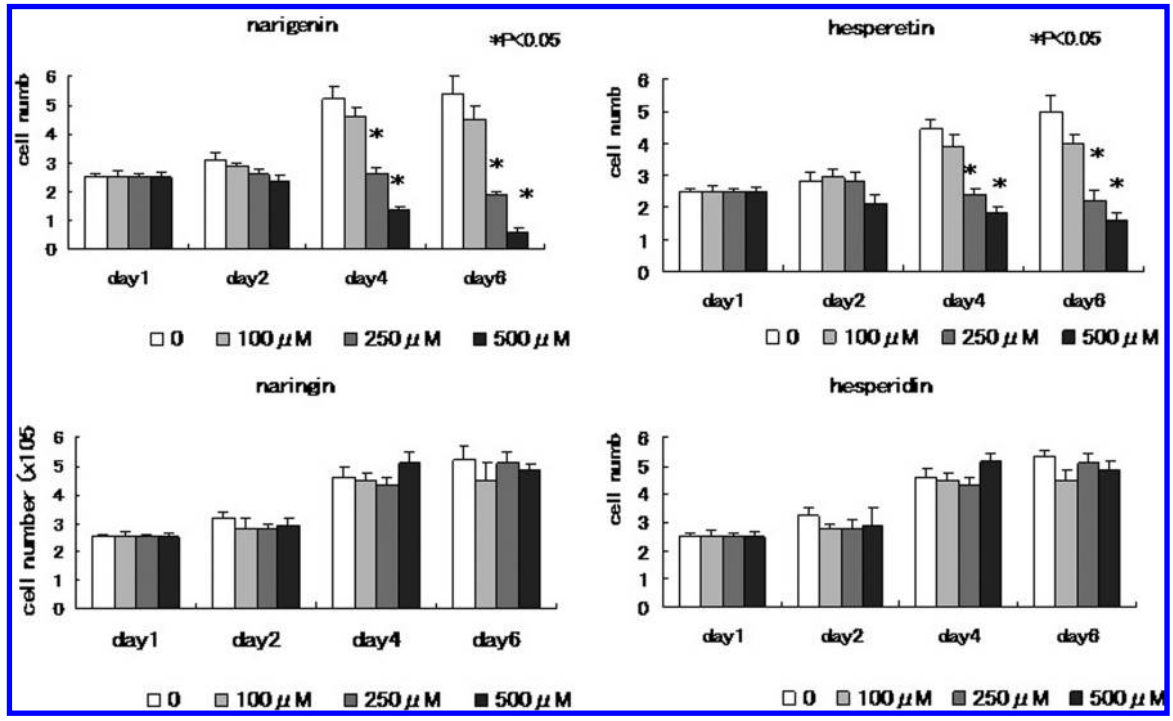

Figure 1. Effect of the flavanones on the growth of AML-I preadipocytes. AML-I preadipocytes were cultured in MDI medium in the presence of vehicle (0.1% DMSO) or four structure-related flavanones such as naringenin, naringin, hesperetin, and hesperidin. On days 2, 4, and 6, the cells were stained by trypan blue dye, and viable and dead cell numbers were counted by hemocytometer. Effects of naringenin, naringin, hesperetin, and hesperidin on the cell growth are shown in upper left, lower left, upper right, and lower right panels, respectively. The data shown are representative of three different experiments expressed as mean ± SD of triplicate cultures.

after 72 h of culture in MDI medium. The different results from Harmon et al. may depend on the difference of the time and concentration study examined and the discrete features of the preadipocyte cell line used. The doses tested in this study are comparable to those used by other researchers. Kuppusamy et al. observed antilipolytic action of hesperetin in rat adipocytes at concentrations of 125 and 250 μM (*7*). Szkudelska et al. examined the antilipogenic effect of naringenin in rat adipocytes at concentrations between 62.5 and 500 μM, and the effect was manifested at 250 μM and higher concentrations (*6*). These studies suggest that naringenin may influence some metabolic process in preadipocytes at higher concentrations.

Glycosylated forms such as naringin and hesperidin showed neither growth arrest nor cytotoxicity even at 500 μM during 5 days culture periods (**Figures 1** and **2**). These data suggest that flavanones containing the rhamnoglucoside moiety at position 7 were unable to induce growth arrest of AML-I cells. As for the structure−activity relationship, we already have reported that glycosylated forms of quercetin such as rutin and quercitrin showed poor growth arrest and apoptosis for AML-I cells compared with quercetin (*16*).

To determine whether flavanone-induced growth arrest was caused by apoptosis or necrosis, Annexin V−FITC apoptosis assay was performed in naringenin-, hesperetin- and vehicle-treated AML-I cells on day 3 of the culture (**Figures 3** and **4**). After counting of the apoptotic cells with fluorescent microscopy, the percent specific apoptosis was assessed using the formula shown under Materials and Methods. As shown in the lower parts of **Figures 3** and **4**, naringenin and hesperetin induced potent apoptotic activity at the concentration of 500 μM. These results suggest that growth arrest and cell death were induced by apoptotic activity of the flavanones.

Previously, we have shown that AML-I cells are able to differentiate into adipocytes and produce lipids following adipogenic stimulation using MDI medium (*13*). In this study, we examined the effect of naringenin and hesperetin on the lipogenesis of AML-I cells in MDI medium, respectively. On day 6 of the culture, the lipid droplet-containing cells were identified by Sudan Black B staining and with light microscopy. Unexpectedly, the cells treated with naringenin or hesperetin at the concentrations of 100 and 500 μM contained many more lipid droplets than those in vehicle as shown in **Figures 5** and **6** (upper parts). When the cell maturation was assessed semiquantitatively using our method described under Materials and Methods, stage II and III cells were significantly increased by naringenin or hesperetin treatment compared to the vehicle treatment, as shown in **Figures 5** and **6** (lower parts). As lipogenesis usually indicates the differentiation of preadipocyte into adipocyte, the increase of lipid droplets suggests that the flavanones may accelerate the adipocytic conversion of AML-I preadipocytes.

Next, we examined the expression of apoptosis-related proteins such as Bcl-2, Bad, AKT, p-AKT, and NF-κB in the naringenin-treated cells. Apoptosis-related proteins were obtained from the cell lysates of vehicle or naringenin (100 or 500 μM) treatment for 5, 12, or 24 h and analyzed by Western blotting. Expression of Akt did not change between naringenin- and vehicle-treated cells, but p-Akt was decreased in naringenin-treated cells compared with vehicle-treated cells. Expression levels of Bcl-2 and NF-κB were also reduced in the naringenin-treated cells in a time-dependent manner. In contrast, Bad was up-regulated in the naringenin-treated cells time dependently (**Figures 7** and **8**). Taken together, naringenin induced the accumulation of proapoptotic Bad, and the reduction of anti-apoptotic pAkt, Bcl-2, and NF-κB proteins in the cells. Apoptosis inhibitory protein, p-Akt, exerts its anti-apoptotic effect by binding to Bad. Although we did not examine the phosphorylated status of Bad, the inactivation of Akt and the

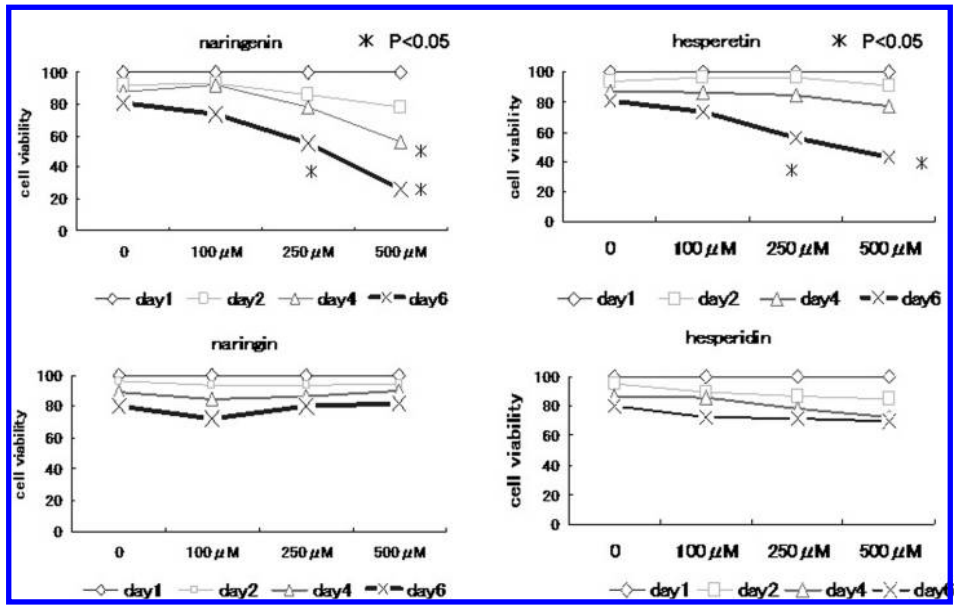

Figure 2. Effect of the flavanones on the viability of AML-I preadipocytes. AML-I preadipocytes were cultured in MDI medium in the presence of vehicle (0.1% DMSO) or four structure-related flavanones (naringenin, naringin, hesperetin, and hesperidin). On days 2, 4, and 6, the cell viability was assessed using trypan blue dye staining test. Effects of naringenin, naringin, hesperetin, and hesperidin on the cell viability are shown in upper left, lower left, upper right, and lower right panels, respectively. The data shown are representative of three different experiments expressed as mean ± SD of triplicate cultures.

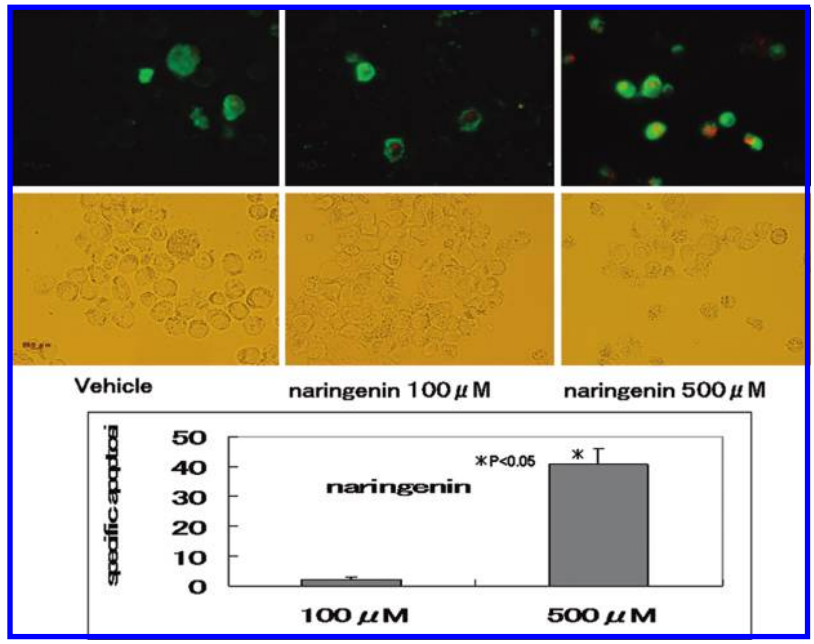

Figure 3. Apoptotic effect of naringenin. AML-I cells were cultured with vehicle (0.1% DMSO) or 100 or 500 μM naringenin, respectively. On day 3, cells were stained with Annexin V−FITC and propidium iodide and analyzed by fluorescence microscopy as shown in the upper panels. A representative experiment of four independent experiments is shown. Specific apoptosis (percent) was calculated with the formula shown under Materials and Methods and expressed in a graph shown in the lower panel. Experiments were repeated three times with similar results. Original magnification ×400 for all photographs. The asterisk indicates significant difference ($p < 0.05$) from vehicle-treated cells by ANOVA.

increase of Bad indicate the accumulation of unphosphorylated type of Bad in the cytosol. Another signal protein, NF-κB, is reported to play a survival role in oncogenesis because the inhibition of NF-κB in transformed cells can induce apoptosis (*17*). It has been reported that several biological activities of the flavonoids are mediated through the down-regulation of the NF-κB pathway (*17, 18*).

Then, we examined the expression of PPAR-γ and FAS proteins in the cells cultured in the presence or absence of naringenin (100 or 500 μM) with adipogenic stimulation (MDI medium) for 5 days by Western blot analysis. PPAR-γ is largely adipocyte-specific and is expressed at a low level in preadipocyte and at a maximal level in mature adipocyte (*19*). FAS is a key metabolic enzyme catalyzing the synthesis of long-chain fatty acids from 2-carbon precursors and plays a central role in de novo lipogenesis during the maturation of preadipocytes into adipocytes (*20*). As shown in **Figure 9**, both PPAR-γ and FAS were expressed in vehicle-treated cells when cultured with MDI medium. The presence of 500 μM naringenin in the MDI medium significantly increased the expression levels of these

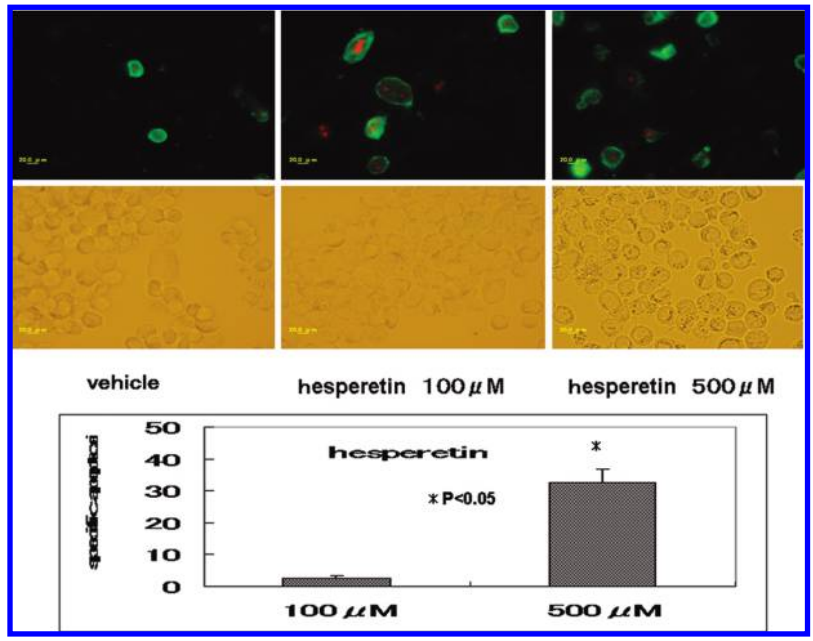

Figure 4. Apoptotic effect of hesperetin. AML-I cells were cultured with vehicle (0.1% DMSO) or 100 or 500 μM hesperetin, respectively. On day 3, cells were stained with Annexin V−FITC and propidium iodide and analyzed by fluorescence microscopy as shown in the upper panels. A representative experiment of four independent experiments is shown. Specific apoptosis (percent) was calculated with the formula shown under Materials and Methods and expressed in a graph shown in the lower panel. Experiments were repeated three times with similar results. Original magnification $\times$400 for all photographs. The asterisk indicates significant difference ($p < 0.05$) from vehicle-treated cells by ANOVA.

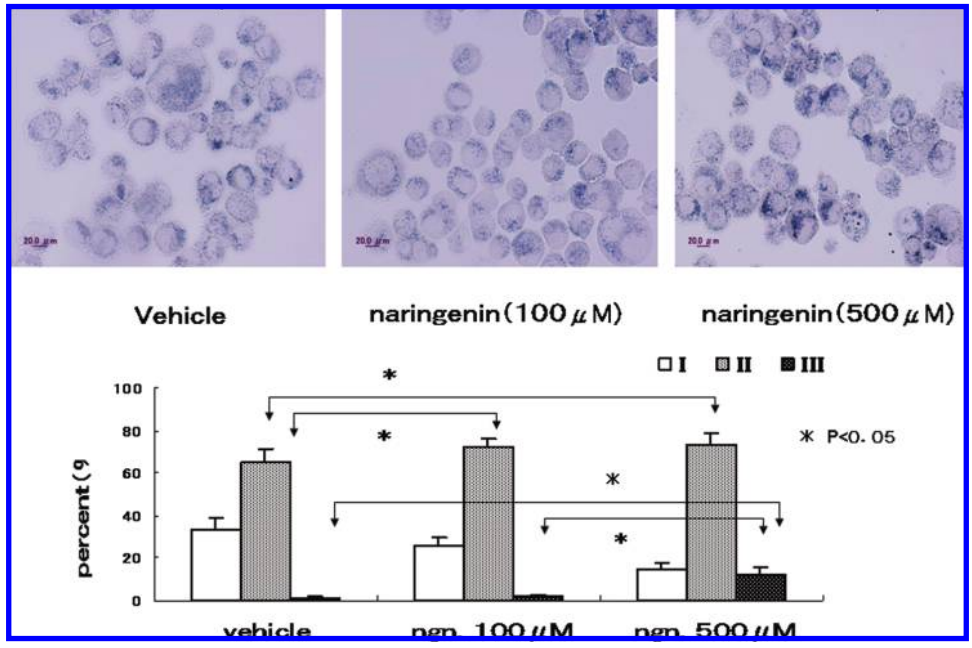

Figure 5. Effect of naringenin (ngn) on adipogenesis by AML-I cells. AML-I cells were cultured for 5 days in the presence of vehicle (0.1% DMSO) or naringenin at the concentrations of 100 and 500 μM in MDI medium, respectively. Afterward, cytocentrifuge preparation was made in each sample, and cytoplasmic fat drops were stained by Sudan Black B staining and observed under light microscopy. Four hundred cells in each sample were counted and classified semiquantitatively into I, II, and III cell stages as described under Materials and Methods. Values are expressed as percentage, and a representative experiment of three independent experiments is shown in a graph. Photographs were taken on day 6 of the culture (original magnification $\times$400). The asterisk indicates significant difference ($p < 0.05$) from control cells by Student t test.

proteins. These data suggested that adipogenic conversion of AML-I cells was more active in the presence of flavanone at the concentration capable of apoptosis.

The gene expression program leading to terminal differentiation is reported to initiate during and after the mitotic clonal expansion in 3T3-L1 preadipocytes (*19, 21*). Therefore, mitotic clonal expansion is thought to be a prerequisite for differentiation of 3T3-L1 preadipocytes into adipocytes (*21*). In contrast, several studies have reported that the cell proliferation process is not an obligatory step along the 3T3-L1 adipocyte maturation process (*22*). Similar observations have been reported in mouse embryo fibroblast (MEF), which is functionally similar to mesenchymal stem cells. Tang et al. showed that MEF undergoes mitotic clonal expansion and adipocyte differentiation at the same differentiation protocol as in 3T3-L1 preadipocytes (*21*). However, Kim et al. observed that the cell number and

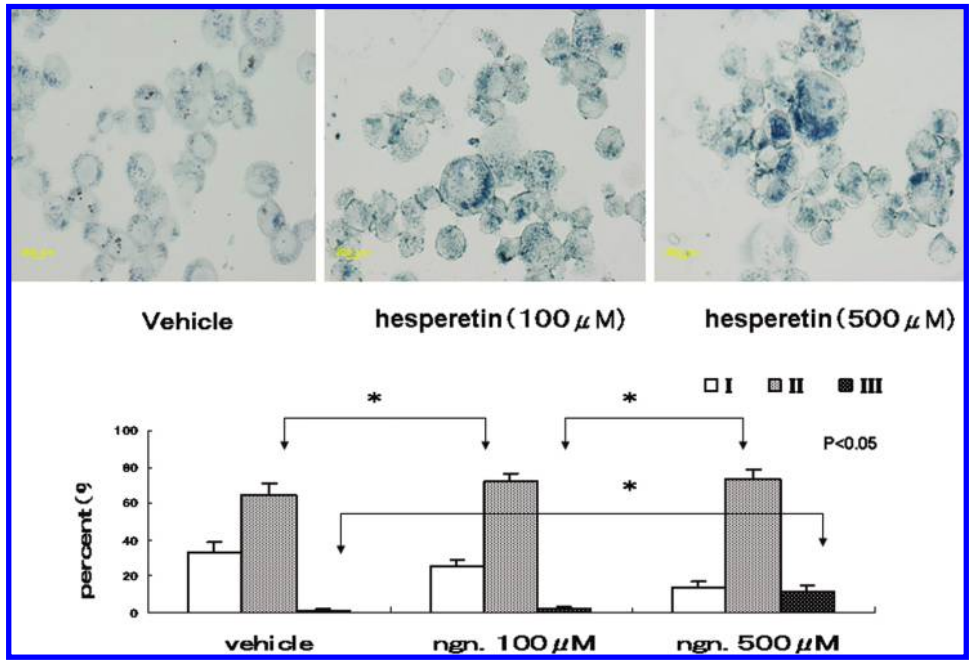

Figure 6. Effect of hesperetin (hpt) on adipogenesis by AML-I cells. AML-I cells were cultured for 5 days in the presence of vehicle (0.1% DMSO) or hesperetin at concentrations of 100 and 500 μM in MDI medium. Afterward, cytocentrifuge preparation was made in each sample, and cytoplasmic fat drops were stained by Sudan Black B staining and observed under light microscopy. Four hundred cells in each sample were counted and classified semiquantitatively into I, II, and III cell stages as described under Materials and Methods. Values are expressed as percentage, and a representative experiment of three independent experiments is shown in a graph. Photographs were taken on day 6 of the culture (original magnification $\times$400). The asterisk indicates significant difference ($p < 0.05$) from control cells by Student t test.

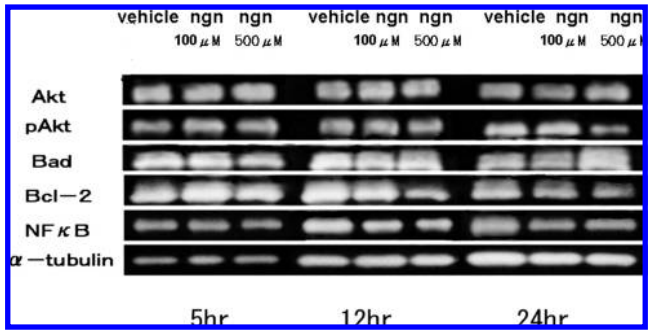

Figure 7. Expression of apoptosis-related proteins in the naringenin-treated cells. AML-I cells were treated with vehicle (0.1% DMSO) or 100 or 500 μM naringenin in MDI medium for 5, 12, or 24 h. Then, the cells were harvested and lysed for Western blot analysis as described under Materials and Methods. Blots were incubated with goat anti-human Akt, rabbit anti-human p-Akt, anti-human Bad, anti-human Bcl-2, and anti-human NF-κB antibodies, mouse anti-α-tubulin antibody, and the second antibodies. Data shown are representative of three independent experiments.

DNA synthesis do not increase during the early adipocyte differentiation phase in MEF (*23*). In this regard, there are conflicting data on the role of the mitogen-activated protein kinase (MAPK)-dependent signaling in adipocyte differentiation. Some have reported a requirement for rapid transient activation of extracellular signal-regulated kinase (ERK) for either clonal expansion or adipocyte differentiation, whereas others have described inhibition of differentiation by ERK activation (*23, 24*). It has been well-known that MAPK inhibitor, PD098059, blocks mitotic clonal expansion and accelerates adipocyte differentiation in confluent or postconfluent 3T3-L1 preadipocytes (*22*). Even in the absence of density-induced cell growth, inhibition of MAPK accelerates differentiation of subconfluent 3T3-L1 preadipocytes.

Whereas many studies have reported the inhibitory effect of flavonoids on the adipogenesis in 3T3-L1 preadipocytes (*8*−*11*), we have observed that flavonoids such as quercetin and EGCG are able to inhibit cell growth, induce apoptosis, and enhance adipogenesis in AML-I preadipocytes during adipogenic stimulation in MDI medium (*17*). As PPAR-γ is an absolute marker of differentiation, the increase of PPAR-γ and FAS proteins shown in naringein-treated cells in our study is proof of adipocytic conversion of preadipocytes.

Although the molecular basis of the mechanism of naringenin-induced effects in our study remains unknown, the molecular target for the flavanone is likely to be MAPKs. Inhibition of MAPKs by the flavanone may contribute to the activation of PPAR-γ, which leads to cell growth arrest, apoptosis, and further adipogenesis. It has been known that ligand activation of PPAR-γ inhibits cell growth and induces apoptosis in cancer cells (*25*). Activation of PPAR-γ by genistein at micromolecular concentrations increases adipogenesis and inhibits osteogenic differentiation by up-regulating the PPAR-γ transcriptional activity in KS483 cell line, which has the ability to differentiate into both osteoblasts and adipocytes (*26*). At high concentrations, genistein acts as a ligand of PPAR, leading to up-regulation of adipogenesis and down-regulation of osteogenesis, and at low concentrations, genistein acts as estrogen, stimulating osteogenesis and inhibiting adipogenesis in these cells (*26*).

Fink et al. reported that when mesenchymal stem cells are cultured under 1% oxygen atmosphere, an adipocyte-like phenotype with cytoplasmic lipid inclusions appears in these cells (*27*). However, hypoxia does not increase PPAR-γ and ADDI/SREBP1c proteins, early marker genes such as lipoprotein lipase, and mature adipocyte-specific genes such as leptin (*27*).

Orange juice and grapefruit juice contain high amounts of the flavanones, and the aglycones are formed from glycosylated forms in the intestine after ingestion. Although significantly

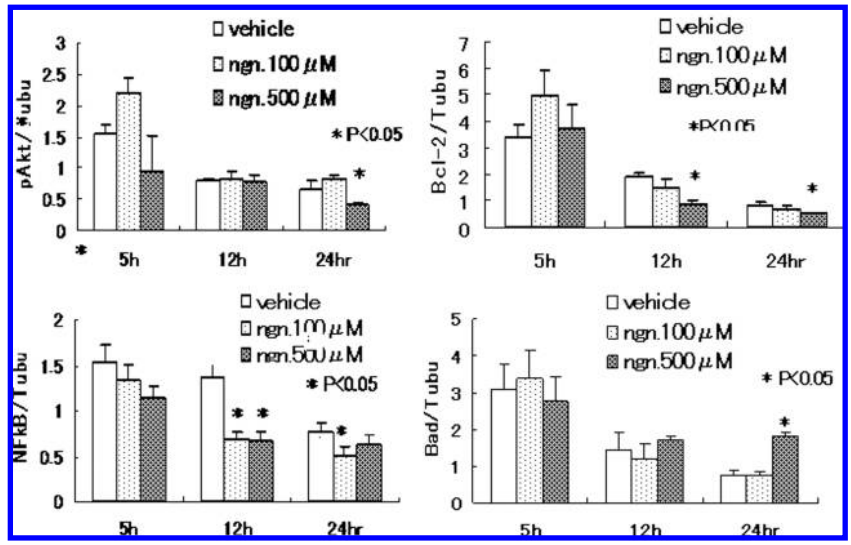

Figure 8. Mean ± SD of normalized densities of bands corresponding to pAKT, Bcl-2, NFkB, and Bad. The asterisk indicates significant difference ($p < 0.05$) from control cells by Student *t* test.

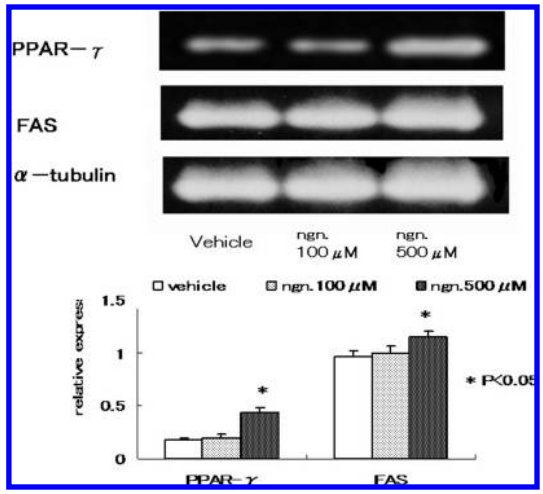

Figure 9. Effect of naringenin (ngn) on the expression of PPAR-γ and FAS proteins. AML-I cells were treated with vehicle (0.1% DMSO) or naringenin (100 or 500 μM) in the MDI medium for 5 days. Then, cells were harvested and lysed for Western blot analysis as described under Materials and Methods. Blots were incubated with mouse anti-human α-tubulin, mouse anti-human PPAR-γ, and goat anti-human FAS and the second antibodies. Experiments were repeated three times with similar results. The values of normalized densities of bands corresponding to FAS and PPAR-γ in the presence of vehicle or naringenin are shown in each graph (lower part). The asterisk indicates significant difference ($p < 0.05$) from control cells by Student *t* test.

larger flavanones can be taken up by individuals upon frequent consumption of flavanone-rich products, the plasma concentration of naringenin is reported to be rather low, from 70 to 112 nM after consumption of habitual diets (*28*). Pharmacokinetic study has demonstrated that both hesperetin and naringenin are rapidly absorbed and reach a peak at 4 and 3.5 h in the plasma after oral administration, respectively (*29*). Mean peaks of plasma concentrations for hesperetin and naringenin are reported to be 825.78 ng/mL (2731.8 nmol/L) and 2009.52 ng/mL (7386.6 nmol/L), respectively, when 135 mg of each compound is administered in humans (*29*). However, the plasma concentration of the flavanones can increase to even micromoles per liter after a single orange or grapefruit juice ingestion of 8 mL/kg of body weight (*30*). According to Erlund et al., the major factor affecting plasma levels is interindividual variation in the bioavailability of the flavanones, and it is remarkable (*30*). Furthermore, flavanones can accumulate in plasma after long-term intake, and they are distributed in tissues other than plasma. Distribution and accumulation of flavanones in other tissues cannot be ruled out because of the limit of detection of the analytical methods (*30*).

In any case, our results suggest that the lipid-lowering effect of flavanone in hyperlipidemia is unlikely to be caused by the inhibition of adipogenesis in preadipocytes.

ABBREVIATIONS USED

FAS, fatty acid synthase; PPAR, peroxisome proliferator activated receptor; EGCG, epigallocatechin gallate.

LITERATURE CITED

(1) Choi, G. S.; Lee, S.; Jeong, T. S.; Lee, M. K.; Lee, J. S.; Jung, U. J.; Kim, H. J.; Park, Y. B.; Bok, S. H.; Choi, M. S. Evaluation of hesperetin 7-*O*-lauryl ether as lipid-lowering agent in high-cholesterol-fed rats. *Bioorg. Med. Chem.* **2004**, *12*, 3599–3605.

(2) Kim, S. Y.; Kim, H. J.; Lee, M. K.; Jeon, S. M.; Do, G. M.; Kwon, E. Y.; Cho, Y. Y.; Kim, D. J.; Jeong, K. S.; Park, Y. B.; Ha, T. Y.; Choi, M. S. Naringin time-dependently lowers hepatic cholesterol biosynthesis and plasma cholesterol in rats fed high-fat nad high-cholesterol diet. *J. Med. Food* **2006**, *9*, 582–586.

(3) Bok, S. H.; Lee, S. H.; Park, Y. B.; Bae, K. H.; Son, K. H.; Jeong, T. S.; Choi, M. S. Plasma and hepatic cholesterol and hepatic activities of 3-hydroxy-3-methyl-glutaryl-CoA reductase and acyl CoA:cholesterol transferase are lower in rats fed citrus peel extract or a mixture of citrus bioflavonoids. *J. Nutr.* **1999**, *129*, 1182–1185.

(4) Borradaile, N. M.; Carroll, K. K.; Kurowska, E. M. Regulation of HepG2 cell apolipoprotein B metabolism by the citrus flavanones hesperetin and naringenin. *Lipids* **1999**, *34*, 591–598.

(5) Wilcox, L. J.; Borradaile, N. M.; Dreu, L. E.; Huff, M. W. Secretion of hepatocyte apoB is inhibited by the flavonoids, naringenin and hesperetin, via reduced activity and expression of ACAT2 and MTP. *J. Lipid Res.* **2001**, *42*, 725–734.

(6) Szkudelska, K.; Nogowski, L.; Nowicka, E.; Szkudelski, T. In vivo metabolic effects of naringenin in the ethanol consuming rat and the effect of naringenin on adipocytes in vitro. *J. Anim. Physiol. Anim. Nutr.* **2006**, *91*, 91–99.

(7) Kuppusamy, U. R.; Das, N. P. Antilipolytic action of hesperetin in rat adipocytes. *Planta Med.* **1993**, *59*, 508–512.

(8) Chien, P.-J.; Chen, Y.-C.; Lu, S.-C.; Sheu, F. Dietary flavonoids suppress adipogenesis in 3T3-L1 preadipocytes. *J. Food Drug Anal.* **2005**, *13*, 168–175.

(9) Harmon, A. W.; Patel, Y. M.; Harp, J. B. Genistein inhibits CCAAT/enhancer-binding protein β (C/EBP β) activity and 3T3-L1 adipogenesis by increasing C/EBP homologous protein expression. *Biochem. J.* **2002**, *367*, 203–208.

(10) Lin, J. I.; Della-Fera, A.; Baile, C. A. Green tea polyphenol epigallocatechin gallate inhibits adipogenesis and induces apoptosis in 3T3-L1 adipocytes. *Obesity Res.* **2005**, *13*, 982–990.

(11) Iwashita, K.; Yamaki, K.; Tsushida, T. Effect of flavonoids on the differentiation of 3T3-L1 adipocytes. *Food Sci. Technol. Res.* **2001**, *7*, 154–160.

(12) Harmon, A. W.; Patel, Y. M. Naringenin inhibits phosphoinositide 3-kinase activity and glucose uptake in 3T3-L1 adipocytes. *Biochem. Biophys. Res. Commun.* **2003**, *305*, 229–234.

(13) Torii, I.; Morikawa, S.; Nakano, A.; Morikawa, K. Establishment of a human preadipose cell line, HPB-AML-I:refractory to PPARγ-mediated adipogenic stimulation. *J. Cell Physiol.* **2003**, *197*, 42–52.

(14) Kaplow, L. S. A histochemical procedure for localizing and evaluating leukocyte alkaline phosphatase activity in smears of blood and marrow. *Blood* **1955**, *10*, 1023–1029.

(15) Harmon, A. W.; Harp, J. B. Differential effects of flavonoids on 3T3-L1 adipogenesis and lipolysis. *Am. J. Physiol. Cell Physiol.* **2001**, *280*, C807−C813.

(16) Morikawa, K.; Ikeda, C.; Nonaka, M.; Suzuki, I. Growth arrest and apoptosis induced by quercetin is not linked to adipogenic conversion of human preadipocytes. *Metabolism* **2007**, *56*, 1656–1665.

(17) Baldwin, A. S. Control of oncogenesis and cancer therapy resistance by the transcription factor NF-κB. *J. Clin. Invest.* **2001**, *107*, 241–246.

(18) Pellecchia, M.; Reed, J. C. Inhibition of anti-apoptotic Bcl-2 family proteins by natural polyphemols: new avenues for cancer chemoprevention and chemotherapy. *Curr. Pharmacol. Design* **2004**, *10*, 1387–98.

(19) Gregoire, F. M.; Smas, C. M.; Sul, H. S. Understanding adipocyte differentiation. *Physiol. Rev.* **1998**, *78*, 783–809.

(20) Weiss, G. H.; Rosen, O. M.; Rubin, C. S. Regulation of fatty acid synthetase concentration and activity during adipocyte differentiation. *J. Biol. Chem.* **1980**, *255*, 4751–4757.

(21) Tang, Q.-Q.; Otto, T. C.; Lane, M. D. Mitotic clonal expansion: a synchronous process required for adipogenesis. *Proc. Nat.l Acad. Sci. U.S.A.* **2003**, *100*, 44–49.

(22) Qiu, Z.; Wei, Y.; Chen, N.; Jiang, M.; Wu, J.; Liao, K. DNA synthesis and mitotic clonal expansion is not a required step for 3T3-L1 preadipocyte differentiation into adipocytes. *J. Biol. Chem.* **2001**, *276*, 11988–11995.

(23) Kim, K. A.; Kim, J. H.; Wang, Y.; Sul, H. S. Pref-1 (preadipocyte factor 1) activates the MEK/extracellular signal-regulated kinase pathway to inhibit adipocyte differentiation. *Mol. Cell. Biol.* **2007**, *27*, 2294–2308.

(24) Liao, Q. C.; Li, Y. L.; Quarles, L. D.; Xu, K. K.; Li, R.; Zhou, H. H.; Xiao, Z. S. Inhibition of adipocyte differentiation by phytoestrogen genistein through a potential downregulation of extracellylar signal-regulated kinases 1/2 activity. *J. Cell Biochem.* **2008**, *104*, 1853–1864.

(25) Rumi, M. A.; Sato, H.; Ishihara, S.; Ortega, C.; Kadowaki, Y.; Kinoshita, Y. Growth inhibition of esophageal squamous carcinoma cells by peroxisome proliferators-activated receptor-γ ligands. *J. Lab. Clin. Med.* **2002**, *140*, 17–26.

(26) Dang, Z.; Audinot, V.; Papapoulos, S. E.; Boutin, J. A.; Lowik, W. G. M. Peroxisome proliferators-activated receptorγ (PPARγ) as a molecular target for the soy phytoestrogen genistein. *J. Biol. Chem.* **2003**, *278*, 962–967.

(27) Fink, T.; Abildtrup, L.; Fogd, K.; Abdallah, B. M.; Kassem, M.; Ebbesen, P.; Zachar, V. Induction of adipocyte-like phenotype in human mesenchymal stem cells by hypoxia. *Stem Cells* **2004**, *22*, 1346–1355.

(28) Erlund, I.; Silaste, M. L.; Alfthan, G.; Rantala, M.; Kesaniemi, Y. A.; Aro, A. Plasma concentratins of the flavonids hesperetin, naringenin and quercetin in human subjects following their habitual diets, and diets high or low in fruit and vegetables. *Eur. J. Clin. Nutr.* **2002**, *56*, 891–898.

(29) Kanaze, F. I.; Bounartzi, M. I.; Georgarakis, M.; Niopas, I. Pharmacokinetics of the citrus flavanone aglycones hesperetin and naringenin after single oral administration in human subjects. *Eur. J. Clin. Nutr.* **2007**, *61*, 472–477.

(30) Erlund, I.; Meririnne, E.; Alfthan, G.; Aro, A. Plasma kinetics and urinary excretion of the flavanones naringenin and hesperetin in humans after ingestin of orange juice and grapefruit juice. *J. Nutr.* **2001**, *131*, 235–241.

Received for review June 27, 2008. Revised manuscript received September 9, 2008. Accepted September 29, 2008.

JF801965N