

Inhibition of Net HepG2 Cell Apolipoprotein B Secretion by the Citrus Flavonoid Naringenin Involves Activation of Phosphatidylinositol 3-Kinase, Independent of Insulin Receptor Substrate-1 Phosphorylation

Nica M. Borradaile, Linda E. de Dreu, and Murray W. Huff

The flavonoid naringenin improves hyperlipidemia and hyperglycemia in streptozotocin-treated rats. In HepG2 human hepatoma cells, naringenin inhibits apolipoprotein B (apoB) secretion primarily by inhibiting microsomal triglyceride transfer protein and enhances LDL receptor (LDLr)-mediated apoB-containing lipoprotein uptake. Phosphatidylinositol 3-kinase (PI3K) activation by insulin increases sterol regulatory element-binding protein (SREBP)-1 and LDLr expression and inhibits apoB secretion in hepatocytes. Thus, we determined whether naringenin activates this pathway. Insulin and naringenin induced PI3K-dependent increases in cytosolic and nuclear SREBP-1 and LDLr expression. Similar PI3K-mediated increases in SREBP-1 were observed in McA-RH7777 rat hepatoma cells, which express predominantly SREBP-1c. Reductions in HepG2 cell media apoB with naringenin were partially attenuated by wortmannin, whereas the effect of insulin was completely blocked. Both treatments reduced apoB100 secretion in wild-type and LDLr^{-/-} mouse hepatocytes to the same extent. Insulin and naringenin increased HepG2 cell PI3K activity and decreased insulin receptor substrate (IRS)-2 levels. In sharp contrast to insulin, naringenin did not induce tyrosine phosphorylation of IRS-1. We conclude that naringenin increases LDLr expression in HepG2 cells via PI3K-mediated upregulation of SREBP-1, independent of IRS-1 phosphorylation. Although this pathway may not regulate apoB secretion in primary hepatocytes, PI3K activation by this novel mechanism may explain the insulin-like effects of naringenin in vivo. *Diabetes* 52:2554–2561, 2003

From the Departments of Medicine and Biochemistry and the Robarts Research Institute, University of Western Ontario, London, Ontario, Canada.

Address correspondence and reprint requests to Dr. Murray W. Huff, The Robarts Research Institute, 4-16, 100 Perth Dr., London, Ontario N6A 5K8, Canada. E-mail: mhuff@uwo.ca.

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apoB, apolipoprotein B; apoB-Lp, apolipoprotein B-containing lipoprotein; CE, cholesteryl ester; ER, endoplasmic reticulum; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IRS, insulin receptor substrate; LDLr, low-density lipoprotein receptor; LXR, liver X receptor; MTP, microsomal triglyceride transfer protein; nSREBP, nuclear sterol regulatory element-binding protein; PI3K, phosphatidylinositol 3-kinase; PVDF, polyvinylidene fluoride; SREBP, sterol regulatory element-binding protein; TG, triglyceride.

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Flavonoids are naturally occurring polyphenols found in all foods of plant origin. Because epidemiological studies support an inverse association between dietary flavonoid intake and cardiovascular disease (1,2), there is considerable interest in determining the mechanisms responsible for this reduced disease risk. The grapefruit flavonoid naringenin reduces plasma lipids and atherosclerosis in rodents (3–8). Furthermore, a single i.p. injection of naringenin 7-O- β -D-glucoside (isolated from a Korean folk remedy) reduced blood glucose, triglycerides (TGs), and total cholesterol in streptozotocin-induced diabetic rats (4), indicating that naringenin may have insulin-like properties.

We previously reported that the aglycone naringenin reduces accumulation of apolipoprotein B (apoB) in the media of cultured human hepatoma cells (HepG2) (9). ApoB scaffolds the assembly of VLDL in the liver by cotranslationally associating with phospholipids, cholesterol, cholesteryl esters (CEs), and TGs in the endoplasmic reticulum (ER) (10). Additional neutral lipid is recruited throughout the remainder of the secretory pathway. Microsomal triglyceride transfer protein (MTP) mediates the accumulation of these lipids within the ER lumen (11–13) and their transfer to apoB (14) and thus is an absolute requirement for apoB-containing lipoprotein (apoB-Lp) assembly and secretion. We recently reported that naringenin inhibits MTP activity, thereby reducing neutral lipid accumulation in the microsomal lumen and subsequent apoB lipidation and secretion (13,15). We concluded that this is likely the primary mechanism responsible for plasma lipid-lowering in rodents. However, hepatocyte LDL receptor (LDLr) expression can regulate net apoB secretion by mediating the uptake of newly secreted particles and by targeting nascent apoB-Lp to degradation during the assembly process (16). We recently showed that naringenin substantially increases the expression and activity of the LDLr in HepG2 cells (17), an effect also observed with insulin (18).

The regulation of LDLr expression by insulin is not completely understood. Activation of the insulin receptor stimulates tyrosine phosphorylation of insulin receptor substrate (IRS) proteins, including IRS-1 and -2, which bind and activate phosphatidylinositol 3-kinase (PI3K) (19). PI3K then activates phosphoinositide-dependent ki-

nases and protein kinase B (PKB)/cAkt. This pathway is important in mediating the metabolic effects of insulin. Activation of the PI3K/PKB/cAkt pathway by insulin increases the expression of sterol regulatory element-binding protein-1 (SREBP-1) in cultured rat hepatocytes (20,21). SREBPs belong to the basic helix-loop-helix-leucine zipper (bHLH-Zip) family of transcription factors. Unlike other members of this family, they are synthesized as inactive, ER membrane-bound precursors (22). Through a two-step proteolytic process, the NH₂-terminal bHLH-Zip domain is released and translocates to the nucleus. Mature, nuclear SREBP (nSREBP) activates transcription by binding sterol regulatory elements in promoters of target genes. SREBP-1a and -1c are transcribed from a single gene using alternate start sites, whereas SREBP-2 is encoded by a separate gene. SREBP-1a, the predominant SREBP-1 isoform expressed in cell lines, is a potent activator of genes involved in both cholesterol and fatty acid metabolism. SREBP-1c, predominant in primary cell cultures and intact tissues, preferentially activates genes involved in fatty acid metabolism (22). SREBP-1a and -1c activate several insulin-sensitive hepatic genes, including fatty acid synthase, acetyl CoA carboxylase, L-pyruvate kinase, glucokinase, and the LDLr (23–27). Expression of the LDLr is particularly sensitive to activation by SREBP-1a (26). Because both SREBP-1a and -1c are expressed in HepG2 cells (28), an increase in SREBP-1 expression should enhance LDLr expression and ultimately reduce media apoB accumulation. Thus, we hypothesized that naringenin increases LDLr expression via a PI3K-mediated increase in SREBP-1a expression and that this mechanism is shared by insulin.

In this report, we show that both naringenin and insulin increase precursor SREBP-1 and mature nSREBP-1 levels in a PI3K-dependent manner, leading to increased LDLr expression in HepG2 cells. We further show that this mechanism contributes to the ability of naringenin to reduce apoB accumulation in the media. Finally, in contrast to insulin, we demonstrate the novel activation of PI3K by naringenin, apparently independent of IRS-1 tyrosine phosphorylation.

RESEARCH DESIGN AND METHODS

Cell culture and chemicals. HepG2 and McA-RH7777 cells obtained from American Type Culture Collection (Rockville, MD) were cultured and used in experiments as described previously (13,17). Primary mouse hepatocytes were isolated from anesthetized adult male C57Bl/6 (Charles River, St. Constant, QC) and LDLr^{-/-} (The Jackson Laboratory, Bar Harbor, ME) mice fed ad libitum, as previously described (12) with the following modifications. Livers were successively perfused through the vena cava with Gibco Liver Perfusion Medium, Gibco Liver Digest Medium, and Gibco Hepatocyte Wash Medium (all from Invitrogen, Burlington, ON). Released hepatocytes were washed once with Hepatocyte Wash Medium and suspended in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) and 20 mU/ml insulin. Cells were plated on BD BioCoat Cellware mouse type IV collagen-coated six-well, 35-mm plates (VWR) at 6.5×10^5 cells/well. For experiments, hepatocytes were maintained in Dulbecco's modified Eagle's medium containing 10% lipoprotein-deficient FBS. Naringenin, wortmannin, and LY294002 (all from Sigma, St. Louis, MO) and BMS 197636 (a gift from Dr. J. Wetterau, Bristol-Myers Squibb, Princeton, NJ) were solubilized in DMSO (concentration in cell cultures did not exceed 0.5%). Bovine pancreatic insulin (Sigma) was solubilized in 0.01 N HCl.

mRNA abundance. HepG2 or McA-RH7777 cells were preincubated for 30 min with either wortmannin (1 μ M) or LY294002 (50 μ M), followed by 6 h with insulin (100 nmol/l) or naringenin (200 μ M). Total RNA was isolated using Trizol (Life Technologies, Mississauga, ON). Custom oligonucleotide probes corresponding to human LDLr (accession no. L29401) 5'-CT

CGCCACTTAGGCAGTGGAACTCGAAGGCCGAGCAGGGGCTACTGTCCCG GTTCCAC-3', rat SREBP-1 (accession no. AF286470) 5'-ACTCACAGGGTCT GCAAGGGGCTGCCTGCACGGCTGTGCC-3' and human GAPDH (accession no. M32599) 5'-CGGAAGGCCATGCCAGTGAGCTTCAATG-3'. mRNA was quantitated using an RNase protection assay described previously (29). Data are expressed as band intensity for the gene of interest compared with glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

SREBP-1 immunoblotting. HepG2 cells were preincubated for 30 min with either wortmannin (1 μ M) or LY294002 (50 μ M), followed by 6 h with either insulin (100 nmol/l) or naringenin (200 μ M). For measurement of cytoplasmic SREBP-1, cells were lysed in PBS containing 5 mmol/l EDTA, 1 mmol/l EGTA, 1% sodium deoxycholate, 1% IGEPAL, 2 mmol/l PMSF, 0.1 mmol/l leupeptin, 100 units/ml aprotinin, and 2 mg/ml ALLN. Lysates were incubated on ice (30 min), followed by centrifugation (10 min, 10,000g, 4°C) to obtain a postnuclear supernatant. For measurement of mature SREBP-1, nuclear extracts were prepared by lysing HepG2 cells in 500 μ l of 10 mmol/l Tris-HCl, 10 mmol/l NaCl, 3 mmol/l MgCl₂, 0.5% IGEPAL, and a mixture of protease inhibitors (2 mmol/l PMSF, 0.1 mmol/l leupeptin, 100 units/ml aprotinin, 2 mg/ml ALLN, 0.5 mg/ml benzamide, and 5 μ g/ml pepstatin) (21). Lysates from duplicate dishes were incubated on ice (30 min) and homogenized by 15 passes through a 25-gauge needle. Nuclei were pelleted by centrifugation for 10 min (500g, 4°C) and washed once with the same buffer. Nuclei were resuspended in hypertonic buffer (10 mmol/l HEPES, 0.42 mol/l NaCl, 1.5 mmol/l MgCl₂, 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mmol/l DTT, and protease inhibitors [described above]) and allowed to swell for 30 min at 4°C, with vortexing. Clear nuclear extracts were obtained by centrifugation (30 min, 100,000g, 4°C). Postnuclear (10 μ g of protein) and nuclear extracts (50 μ g of protein) were resolved by 6% SDS-PAGE and electrophoretically transferred onto polyvinylidene fluoride (PVDF) membranes. Membranes were blocked (16 h, 4°C) with 5% nonfat dry milk in PBS, 0.1% Tween-20. Human SREBP-1 was detected using monoclonal antibody 2A4 (NeoMarkers, Fremont, CA) and a peroxidase-conjugated anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) and was visualized using BM Chemiluminescence Blotting Substrate (Boehringer Mannheim). Bands were quantified using an imaging densitometer (GS-700; BioRad, Mississauga ON). Optical densities of SREBP-1 bands were linear over the range of protein loaded.

ApoB immunoblotting and immunoprecipitation. ApoB secretion into the media of HepG2 cells preincubated for 30 min in the absence or presence of wortmannin (1 μ M), followed by an additional 24 h with insulin (100 nmol/l), BMS 197636 (10 nmol/l), or naringenin (200 μ M), was measured by Western blotting, as described previously (13). ApoB secretion into the media of primary hepatocytes isolated from wild-type and LDLr null mice was measured by immunoprecipitation. Hepatocytes were incubated for 24 h with or without insulin (100 nmol/l) or naringenin (200 μ M) and 100 μ Ci/ml Tran [35S]label (1,000 Ci/mmol [35S]methionine and [35S]L-cysteine; ICN, Costa Mesa, CA). Media apoB (full-length apoB100, and apoB48) was immunoprecipitated using a polyclonal anti-human apoB antisera (Chemicon International, Temecula, CA) essentially as previously described (13). Immunoprecipitates were resolved by 4.5% SDS-PAGE, and bands corresponding to apoB100 and apoB48 were visualized and quantified using a phosphorimager (Molecular Dynamics).

PI3K activity. HepG2 cells grown to 80% confluence were incubated overnight in media containing 0.5% insulin-free, fatty acid-free BSA (Sigma) to induce quiescence. Immunoprecipitable PI3K activity was assessed using a protocol from Upstate Biotechnology (www.upstate.com), with modifications. Briefly, cells were stimulated for 10 min with either insulin (100 nmol/l) or naringenin (200 μ M). Cells were washed with ice-cold Buffer A (20 mmol/l Tris-HCl, 137 mmol/l NaCl, 1 mmol/l CaCl₂, 1 mmol/l MgCl₂, and 0.1 mmol/l sodium orthovanadate) and lysed, on ice, in 1 ml of Buffer A with 1% IGEPAL, 1 mmol/l PMSF, and 0.02 mmol/l leupeptin. Lysates were centrifuged briefly to pellet insoluble material. PI3K was immunoprecipitated with 5 μ l of anti-PI3K antisera (Upstate Biotechnology, Lake Placid, NY; 1 h, 4°C). The immunocomplex was captured by addition of 60 μ l of protein G PLUS-agarose bead slurry (Santa Cruz Biotechnology) and successively washed with Buffer A plus 1% IGEPAL, followed by 0.1 mmol/l Tris-HCl, 5 mmol/l LiCl, 0.1 mmol/l sodium orthovanadate, and TNE (10 mmol/l Tris-HCl, 150 mmol/l NaCl, 5 mmol/l EDTA, 0.1 mmol/l sodium orthovanadate). Washed immunoprecipitates were resuspended in 50 μ l of TNE and 10 μ l of 100 mmol/l MgCl₂. Wortmannin (1 μ M) and LY294002 (50 μ M) were added to some samples. Phosphatidylinositol (20 μ g) and [³²P]ATP (30 μ Ci, 3,000 Ci/mmol) were added to initiate the reaction, which proceeded for 10 min at 37°C. The reaction was stopped with 6 N HCl, and lipids were extracted with chloroform:methanol (1:1). Lipid products were separated by thin-layer chromatography in chloroform:methanol:water:ammonium hydroxide (60:47:11.3:2) and visualized using a phosphorimager. Spots corresponding to phosphatidylinositol

monophosphate were quantified using Image Quant software (Molecular Dynamics).

Detection of phosphorylated IRS-1 and -2. HepG2 cells were incubated for 6 h with either insulin (100 nmol/l) or naringenin (200 μ mol/l) and then lysed in 2 ml of buffer containing 50 mmol/l Tris-HCl, 150 mmol/l NaCl, 1 mmol/l EGTA, 1 mmol/l NaF, 0.25% sodium deoxycholate, 1% IGEPAL, 1 mmol/l sodium orthovanadate, 1 mmol/l PMSF, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, and 1 μ g/ml pepstatin. Cells were disrupted with five passes through an 18-gauge needle. Lysates were incubated on ice (30 min), followed by centrifugation (10 min, 10,000g, 4°C) to obtain a postnuclear supernatant. IRS-1 and IRS-2 (5–10 μ g of cell lysate protein) were resolved by 6% SDS-PAGE. PVDF membranes were blocked with 3% nonfat dry milk in PBS and incubated with polyclonal antibodies for IRS-1 or -2 (Upstate Biotechnology; 16 h, 4°C). Incubation with secondary antibody and visualization of IRS proteins were performed as described for apoB. For immunoprecipitation of IRS-1 and -2, 150–500 μ g of cell lysate was incubated with 4 μ g of anti-IRS-1 or -2 (16 h, 4°C). Immunocomplexes were captured with 100 μ l of protein G PLUS-agarose bead slurry (Santa Cruz Biotechnology) and washed three times with PBS. Immunoprecipitates were resolved by 6% SDS-PAGE. PVDF membranes were blocked with 1% blocking solution (Boehringer Mannheim; 1 h, 4°C), followed by incubation with monoclonal anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology; 16 h, 4°C). Incubation with secondary antibody and visualization of phosphorylated IRS-1 and -2 were performed as described for apoB.

TG and fatty acid synthesis. Cells were preincubated for 30 min with or without wortmannin (1 μ mol/l) or LY294002 (50 μ mol/l), followed by 6 h with [14 C]acetic acid (0.5 μ Ci, 57 mCi/mmol) and insulin (100 nmol/l) or naringenin (200 μ mol/l). Incorporation of [14 C]acetic acid (Amersham) into TGs was determined as described previously (30). For determining incorporation of [14 C]acetic acid into fatty acids, cellular lipids were extracted and saponified (30). Fatty acids were then extracted with hexane from the aqueous phase and quantified (31).

Statistics. All data are presented as mean \pm SE. Means were compared by *t* tests to determine statistical significance ($P < 0.05$).

RESULTS

Naringenin increases LDLr expression in HepG2 cells via a PI3K-mediated increase in SREBP-1. Insulin induces SREBP-1 expression via the PI3K pathway (20,21), and SREBP-1 mediates activation of the LDLr promoter by insulin (27). Because we previously showed that naringenin induces a fivefold increase in LDLr expression within 24 h (17), we determined whether naringenin mediates this effect via a PI3K-dependent increase in SREBP-1 expression. HepG2 cells preincubated for 30 min with or without the specific PI3K inhibitors wortmannin (1 μ mol/l) or LY294002 (50 μ mol/l) were further incubated for 6 h with or without insulin (100 nmol/l) or naringenin (200 μ mol/l). Previous reports indicate that insulin increases LDLr (18) and SREBP-1 (20,21) expression within this time frame. Both insulin and naringenin increased LDLr mRNA abundance 1.8-fold ($P < 0.05$; Fig. 1). These increases were completely blocked by preincubation with either wortmannin or LY294002. Similarly, insulin and naringenin increased cellular content of the 125-kDa precursor SREBP-1 by 1.7-fold and 2.2-fold, respectively ($P < 0.05$; Fig. 2A). These increases were, again, completely prevented by preincubation with PI3K inhibitors. This pattern was maintained for transcriptionally active, 68-kDa nuclear SREBP-1. Insulin and naringenin increased nSREBP-1 content 1.6-fold and 1.7-fold, respectively ($P < 0.05$; Fig. 2B), and these effects were completely prevented by preincubation with either wortmannin or LY294002. This observation extended to McA-RH7777 cells, a rat hepatoma line expressing predominantly SREBP-1c (32). In these cells, insulin and naringenin increased SREBP-1 mRNA by 1.7-fold and 1.6-fold, respectively ($P < 0.05$). As in HepG2

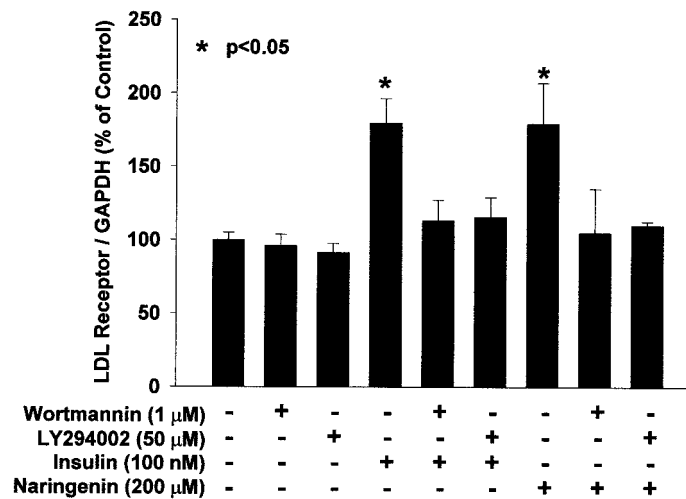


FIG. 1. PI3K-dependent upregulation of LDLr expression by insulin and naringenin. HepG2 cells were preincubated for 30 min in the absence (–) or presence (+) of the PI3K inhibitors wortmannin and LY294002, followed by an additional 6-h incubation in the absence (–) or presence (+) of insulin or naringenin. LDLr mRNA abundance was measured by RNase protection and normalized to the abundance of GAPDH mRNA before conversion to percentage of control. The control LDLr/GAPDH ratio is 0.395 ± 0.02 . Values are means \pm SE for at least three experiments.

cells, these effects were completely prevented by preincubation with LY294002 (data not shown).

Inhibition of net apoB secretion by naringenin in HepG2 cells is partially mediated by activation of PI3K. We next determined whether the PI3K-mediated increase in LDLr expression with naringenin contributed significantly to its effect on apoB secretion. HepG2 cells were preincubated for 30 min with or without wortmannin (1 μ mol/l), followed by 24 h with or without insulin (100 nmol/l); a specific MTP inhibitor, BMS 197636 (10 nmol/l, used as a negative control); or increasing concentrations of naringenin. A 24-h time point was chosen to allow for translation of LDLr message, accumulation of apoB in the media, and uptake of apoB-Lp from the media. The reduction in media apoB accumulation with insulin (–44%; $P < 0.01$) was nearly completely prevented (70%) by preincubation with wortmannin (Fig. 3A). In contrast, the reduction in media apoB with BMS 197636 (–69%; $P < 0.01$) was not affected by preincubation with wortmannin. The effect of PI3K inhibition on the ability of naringenin to reduce media apoB was intermediate compared with insulin and BMS 197636 (Fig. 3B). The reduction in media apoB with naringenin (–89%, 200 μ mol/l; $P < 0.05$) was partially prevented (21%, 200 μ mol/l; $P < 0.05$) by preincubation with wortmannin. A similar effect was observed with 75 μ mol/l naringenin. Wortmannin alone did not affect media apoB accumulation.

The contribution of the LDLr to the effect of naringenin on apoB secretion was evaluated in hepatocytes isolated from wild-type and LDLr null mice, which express predominantly SREBP-1c. Cells were incubated for 24 h with either insulin (100 nmol/l) or naringenin (200 μ mol/l) and [35 S]Trans label. Basal apoB100 accumulation in the media of LDLr $^{-/-}$ hepatocytes was significantly higher than that of wild-type cells (Table 1), as observed previously (16). However, both insulin and naringenin reduced apoB100 accumulation in the media of wild-type (–31% and –49%,

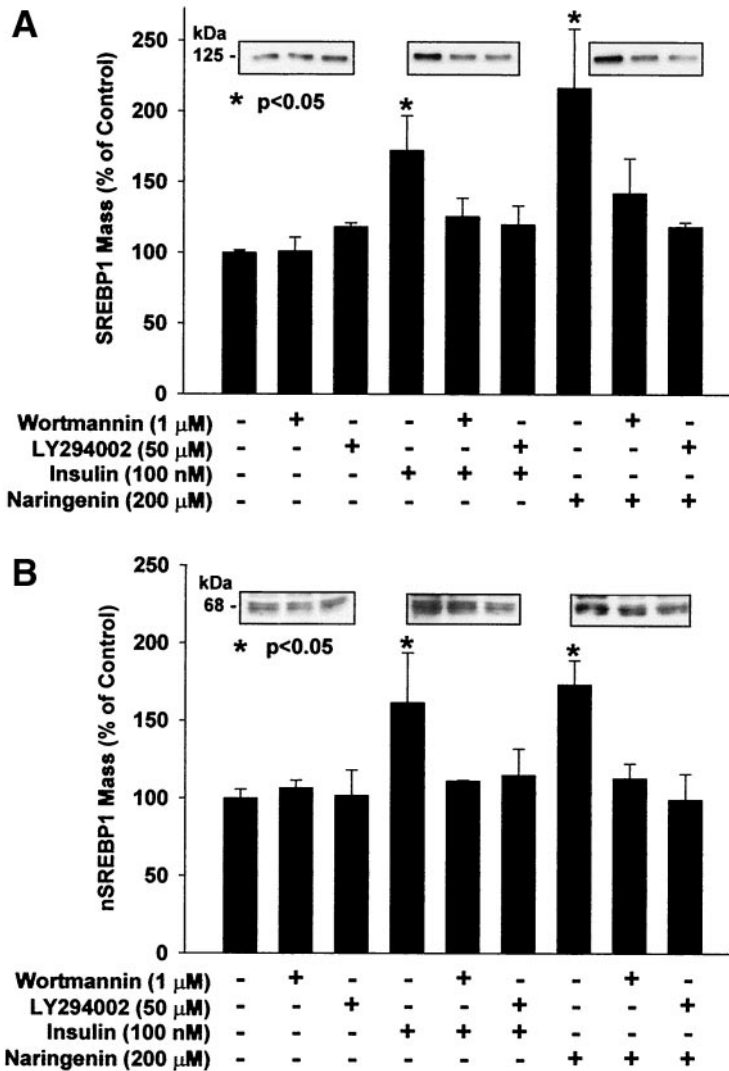


FIG. 2. PI3K-dependent upregulation of SREBP-1 expression by insulin and naringenin. HepG2 cells were preincubated for 30 min in the absence (–) or presence (+) of the PI3K inhibitors wortmannin and LY294002, followed by an additional 6-h incubation in the absence (–) or presence (+) of insulin or naringenin. Precursor (A) and mature (B) forms of SREBP-1 were detected in cytoplasmic and nuclear extracts, respectively, by immunoblotting. Insets are representative Western blots. Bands were quantified by densitometry. Control optical density values are 0.43 ± 0.007 and 0.63 ± 0.04 for precursor and nuclear proteins, respectively. Values are means \pm SE for at least three experiments.

respectively) and LDLr null hepatocytes (–20% and –40%, respectively) to approximately the same extent. Similar reductions were observed for apoB48.

Activation of PI3K by naringenin does not involve tyrosine phosphorylation of IRS-1. Insulin and naringenin increased total immunoprecipitable PI3K activity 1.5-fold and 1.6-fold, respectively ($P < 0.0001$), within 10 min (Fig. 4). This activity was inhibited by the addition of exogenous wortmannin and LY294002. To determine whether the naringenin-induced increase in PI3K activity involved activation of the insulin receptor, we examined the tyrosine phosphorylation of IRS proteins after 6-h incubations with insulin or naringenin. Insulin reduced the electrophoretic mobility of IRS-1, whereas naringenin had no effect (Fig. 5A, top). Furthermore, only IRS-1 immunoprecipitated from cells treated with insulin was detectable with anti-phosphotyrosine (4G10; Fig. 5A, bottom). In contrast, IRS-2 electrophoretic mobility was unchanged with either insulin or naringenin; rather, the quantity of protein was reduced with both treatments (Fig. 5B, top). However, as with IRS-1, IRS-2 was phosphorylated in the presence of insulin (Fig. 5B, bottom), although not to an extent sufficient to inhibit its electrophoretic mobility. Despite decreased IRS-2 content in naringenin-treated

cells, phosphorylated protein was detected at a level comparable to control cells (Fig. 5B, bottom).

Naringenin-induced increases in TG and fatty acid synthesis are not mediated by PI3K. We previously showed that naringenin increases TG synthesis by ~30% in HepG2 cells (13,15,17). Because SREBP-1 is the principal regulator of fatty acid synthesis in the liver (22) and we have shown here that naringenin increases both cytoplasmic and nuclear SREBP-1 (Fig. 2), we postulated that increased TG synthesis is secondary to PI3K-mediated increases in SREBP-1. HepG2 cells were preincubated for 30 min with or without PI3K inhibitors, followed by 6 h with [14 C]acetic acid with or without insulin or naringenin. Insulin increased the incorporation of radiolabel into both fatty acids and TG (35 and 28%, respectively; $P < 0.05$; Table 2). These increases were prevented by preincubation with PI3K inhibitors. In contrast, increases in fatty acid and TG synthesis with naringenin (21 and 30%, respectively; $P < 0.05$) were not completely prevented by PI3K inhibition. Preincubation with wortmannin did not prevent the increased TG synthesis observed with naringenin, whereas LY294002 partially prevented the effect. Neither PI3K inhibitor prevented the increase in fatty acid

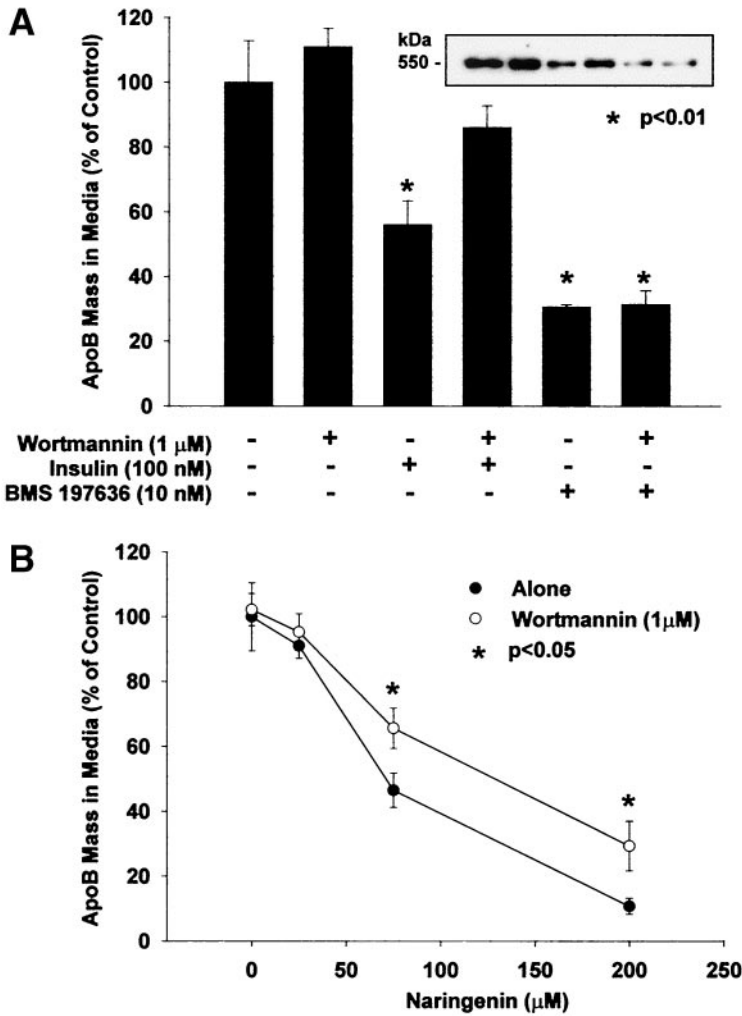


FIG. 3. PI3K-dependent inhibition of apoB secretion by insulin and naringenin. HepG2 cells were preincubated for 30 min in the absence (-) or presence (+) of the PI3K inhibitor wortmannin, followed by an additional 24-h incubation in the absence (-) or presence (+) of insulin or the specific MTP inhibitor BMS 197636 (A) or increasing concentrations of naringenin (B). ApoB in the media was detected by immunoblotting and quantified by densitometry. Control optical density values are 0.27 ± 0.035 and 0.33 ± 0.034 for A and B, respectively. The inset is a representative Western blot. Values are means \pm SE at least three experiments.

synthesis. The PI3K inhibitors alone did not affect lipid syntheses.

DISCUSSION

Current systemic pharmacological agents used for the treatment of type 2 diabetes improve glycemic control but have varying effects on the dyslipidemias commonly associated with this disease (33). Because coronary artery disease is the leading cause of death in patients with type 2 diabetes, the development of treatments that also improve known cardiovascular risk factors, such as hyperlipidemia, is important (33). Here we demonstrate for the first time the activation of PI3K in cultured hepatocytes by the grapefruit flavonoid naringenin, apparently indepen-

dent of IRS-1 phosphorylation. Activation of this pathway leads to increased expression of SREBP-1 and the LDLr, resulting in a net reduction in apoB secretion in HepG2 cells.

We previously described a fivefold induction of LDLr expression after 24 h of incubation with naringenin, resulting in a 1.8-fold increase in receptor activity (17). As shown in Fig. 1, naringenin increases LDLr mRNA to the same extent as insulin within 6 h. These increases were completely prevented by preincubation with PI3K inhibitors at concentrations previously shown to block insulin-induced effects in cultured hepatocytes (20,34-36). Furthermore, naringenin increased the cellular content of the 125-kDa precursor SREBP-1 within 6 h, in a PI3K-

TABLE 1
ApoB secretion from wild-type and LDLr null mouse hepatocytes in the presence of insulin and naringenin

Treatment	ApoB100 Phosphorimager volume		ApoB48 Phosphorimager volume	
	Wild-type	LDLr ^{-/-}	Wild-type	LDLr ^{-/-}
None	2,596 \pm 32 (100)	4,416 \pm 581 (100)	23,186 \pm 571 (100)	22,524 \pm 2,721 (100)
Insulin (100 nmol/l)	1,793 \pm 338 (69)	3,547 \pm 579 (80)	16,992 \pm 2,956 (73)	16,470 \pm 2,712 (73)
Naringenin (200 μ mol/l)	1,322 \pm 118* (51)	2,656 \pm 308* (60)	13,516 \pm 1,723* (58)	13,839 \pm 3,460 (61)

Data are means \pm SE for three experiments from independent hepatocyte isolations. Primary mouse hepatocytes were incubated for 24 h with either naringenin or insulin and [³⁵S]Tran-label. ApoB was immunoprecipitated, resolved by SDS-PAGE, and quantified as described in RESEARCH DESIGN AND METHODS. *P < 0.05 compared with untreated control cells. Numbers in brackets are percentages of control (untreated) values.

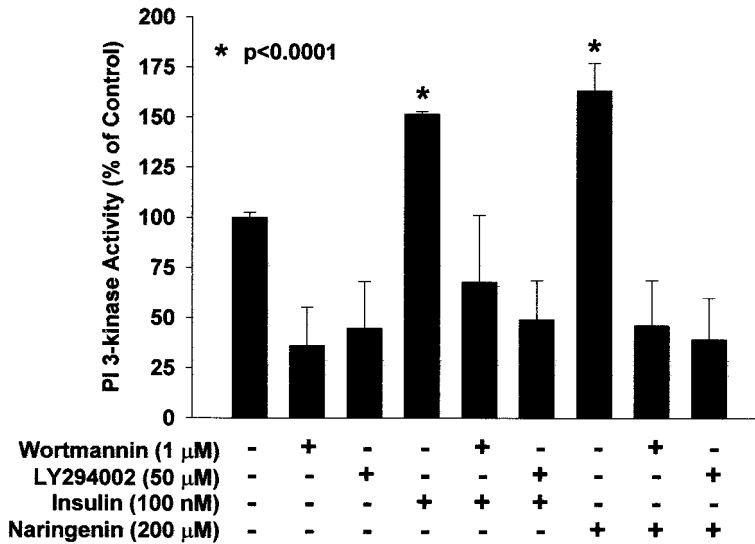


FIG. 4. PI3K activity is increased by insulin and naringenin. HepG2 cells were incubated for 10 min in the absence (-) or presence (+) of insulin or naringenin. Immunoprecipitable PI3K activity was assayed by the incorporation of [γ^{32} P]ATP into phosphatidylinositol monophosphate. The PI3K inhibitors wortmannin and LY294002 were added exogenously, at the time of the assay (absence or presence indicated by - and +, respectively). Labeled products formed were quantitated using a phosphorimager. The control volume value is $301,370 \pm 7,315$. Values are means \pm SE for three experiments.

dependent manner (Fig. 2A). The same pattern was observed for nuclear SREBP-1 (Fig. 2B), supporting the concept that newly synthesized precursor is rapidly cleaved and translocated to the nucleus, as previously described for insulin (21). The SREBP-1 antibody (2A4) that we used does not distinguish between isoforms. However, because SREBP-1a and -1c are expressed in a 2:1 ratio in HepG2 cells (28) and LDLr expression is controlled mainly by SREBP-1a (26), our data suggest that the PI3K-dependent effects of insulin and naringenin on the LDLr are mediated by SREBP-1a.

Insulin inhibits apoB secretion in cultured hepatocytes (37), and this effect involves PI3K activation (34–36). As shown in Fig. 3A, insulin reduced media apoB accumulation by 44% over 24 h, and this effect was nearly completely prevented by preincubation with wortmannin. For naringenin, however, we recently demonstrated that reduced apoB secretion is primarily due to inhibition of MTP-mediated apoB lipidation (13). In the present study, inhibition of apoB secretion by the specific MTP inhibitor BMS-197636 was unaffected by preincubation with wortmannin. Thus, we predicted that the remainder of the effect of naringenin on apoB secretion, not accounted for by MTP inhibition, would be sensitive to PI3K inhibition. Indeed, wortmannin prevented 21% of the reduction in media apoB accumulation observed with naringenin over 24 h (Fig. 3B). This PI3K-mediated portion of the effect could involve changes in several steps of the apoB synthesis and assembly process, including decreased apoB translation (38), increased apoB degradation (34), and decreased lipoprotein maturation (36). Recent evidence also suggests that the LDLr regulates both the uptake and the secretion of apoB from hepatocytes by endocytosing the secreted protein at the cell surface and by binding nascent apoB within the ER and shunting it to a degradation pathway (16). Because PI3K inhibition completely prevented the naringenin-induced increase in LDLr expression (Fig. 1), we conclude that PI3K-mediated upregulation of this receptor contributes to the observed reduction in media apoB accumulation in HepG2 cells. However, the role of LDLr in regulating apoB-Lp secretion in vivo remains controversial. A recent study comparing wild-type and LDLr null mice showed no difference in VLDL produc-

tion (39). Furthermore, given that SREBP-1c is predominant in intact liver (22), it is unlikely that LDLr expression is regulated via PI3K in vivo, suggesting that the contribution of the LDLr to the effects of naringenin on hepatic apoB-Lp production in vivo are relatively minor. In support of this, recent evidence suggests that the LDLr is not involved in the suppression of VLDL apoB secretion by insulin in primary mouse hepatocytes (40). Our data demonstrate that this also applies to naringenin. Similar reductions in apoB secretion from wild-type and LDLr null hepatocytes were observed after treatment with either insulin or naringenin. Nevertheless, we show that insulin and naringenin increase SREBP-1 expression, in a PI3K-dependent manner, in McA-RH7777 rat hepatoma cells

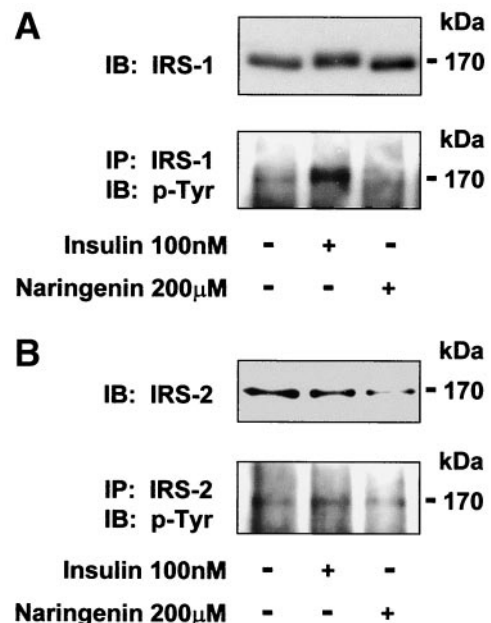


FIG. 5. IRS-1 is phosphorylated in the presence of insulin but not in the presence of naringenin. HepG2 cells were incubated for 6 h in the absence (-) or presence (+) of insulin or naringenin. Cell lysates were immunoblotted (IB; top panels) for IRS-1 (A) or IRS-2 (B) or immunoprecipitated (IP; bottom panels) with anti-IRS-1 or anti-IRS-2, followed by immunoblotting (IB) for phosphotyrosine. Experiments were done twice with identical results.

TABLE 2
Fatty acid and TG synthesis in the presence of insulin and naringenin

Pretreatment	Treatment	Fatty Acids (nmol [¹⁴ C]acetate/mg cell protein)	Triglycerides (nmol [¹⁴ C]acetate/mg cell protein)
None	None	3.58 ± 0.21	1.09 ± 0.02
Wortmannin (1 μmol/l)	None	3.97 ± 0.24	1.16 ± 0.14
LY294002 (50 μmol/l)	None	3.34 ± 0.34	1.09 ± 0.13
None	Insulin (100 nmol/l)	4.85 ± 0.44*	1.39 ± 0.08*
Wortmannin (1 μmol/l)	Insulin (100 nmol/l)	3.95 ± 0.09	1.20 ± 0.07
LY294002 (50 μmol/l)	Insulin (100 nmol/l)	3.37 ± 0.24	1.02 ± 0.07
None	Naringenin (200 μmol/l)	4.32 ± 0.19*	1.42 ± 0.08*
Wortmannin (1 μmol/l)	Naringenin (200 μmol/l)	5.16 ± 0.66*	1.47 ± 0.06*
LY294002 (50 μmol/l)	Naringenin (200 μmol/l)	3.95 ± 0.52	1.42 ± 0.10*

Data are means ± SE for a minimum of three experiments. HepG2 cells were pretreated for 30 min with PI3K inhibitors, followed by a 6-h incubation with either naringenin or insulin and [¹⁴C]acetate. Lipids were extracted and isolated by thin-layer chromatography as described in RESEARCH DESIGN AND METHODS. **P* < 0.05 compared with untreated control cells.

that, like primary hepatocytes, express predominantly SREBP-1c (32). Thus, naringenin, like insulin, may stimulate SREBP-1c-sensitive genes in vivo.

The rapid increase in PI3K activity with naringenin paralleled that of insulin (Fig. 4). However, in contrast to insulin, naringenin did not induce phosphorylation of IRS-1 (Fig. 5A), suggesting that PI3K is activated downstream of the insulin receptor. Like insulin, naringenin decreased IRS-2 levels (Fig. 5B). In hepatocytes, this negative feedback response to PI3K activation has been linked to both decreased expression mediated via an insulin response element in the IRS-2 promoter (41) and increased degradation via the proteasome (42). Because naringenin reduces IRS-2 levels, apparently without activating the insulin receptor, it may be useful for elucidating the involvement of PI3K in controlling IRS-2 content. Given that phosphorylated IRS-2 was detectable in naringenin-treated cells, despite reduced protein content (Fig. 5B), the activation of PI3K by naringenin via IRS-2 cannot be excluded. Naringenin may also activate PI3K directly or indirectly via small GTPases known to regulate this enzyme (43). Activity of the PI3K pathway can also be maintained downstream of the insulin receptor by reducing the activity of lipid phosphatases that regulate cellular levels of the phosphatidylinositol products of PI3K (19). However, it is unlikely that naringenin acts via this mechanism because increased PI3K activity was measured directly (Fig. 4).

Naringenin, like insulin (44), increases fatty acid and TG synthesis in HepG2 cells (Table 2). The effect of insulin is entirely mediated by PI3K and is consistent with the concept that insulin-induced activation of the PI3K pathway increases the expression of SREBP-1, the principle regulator of lipid synthesis in the liver (22). In contrast, the effects of naringenin are not completely prevented by PI3K inhibition, suggesting that the flavonoid activates lipogenesis through an alternative pathway. Recently, Joseph et al. (45) demonstrated that the liver X receptor (LXR) regulates the expression of fatty acid synthase both directly, via an LXR recognition sequence, and indirectly by increasing SREBP-1c expression. We recently found that naringenin has LXR agonist-like activity in cultured macrophages (unpublished data). Thus, further study will be required to determine whether the increased lipogenesis involves LXR activation by naringenin.

Flavonoids have been identified as the antidiabetic

components in a number of traditional ethnic remedies (4,46–48). However, the mechanisms whereby these molecules exert their hypoglycemic action have not been demonstrated. Here, we showed that naringenin increases LDLr expression via PI3K-mediated upregulation of SREBP-1. Although this mechanism contributes to the reduction in net apoB secretion from HepG2 cells, MTP inhibition is likely the primary mechanism whereby naringenin reduces plasma lipids in vivo (13,15). However, the novel mechanism whereby naringenin activates PI3K and upregulates SREBP-1, apparently independent of IRS-1 tyrosine phosphorylation, may account for the insulin-like effects of naringenin 7-*O*-β-glucoside in streptozotocin-treated rats (4). Whether this mechanism is common to all flavonoids that display antidiabetic activity warrants further investigation.

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