Inhibition of apoB secretion from HepG2 cells by insulin is amplified by naringenin, independent of the insulin receptor

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Abstract  Hepatic overproduction of apolipoprotein B (apoB)-containing lipoproteins is characteristic of the dyslipidemia associated with insulin resistance. Recently, we demonstrated that the flavonoid naringenin, like insulin, decreased apoB secretion from HepG2 cells by activation of both the phosphoinositide-3-kinase (PI3-K) pathway and the mitogen-activated protein kinase/extracellular-regulated kinase (MAPKerk) pathway. In the present study, we determined whether naringenin-induced signaling required the insulin receptor (IR) and sensitized the cell to the effects of insulin, and whether the kinetics of apoB assembly and secretion in cells exposed to naringenin were similar to those of insulin. Immunoblot analysis revealed that insulin stimulated maximal phosphorylation of IR and IR substrate-1 after 10 min, whereas naringenin did not affect either at any time point up to 60 min. The combination of naringenin and submaximal concentrations of insulin potentiated extracellular-regulated kinase 1/2 activation and enhanced upregulation of the LDL receptor, downregulation of microsomal triglyceride transfer protein expression, and inhibition of apoB-100 secretion. Multicompartmental modeling of apoB pulse-chase studies revealed that attenuation of secreted radiolabeled apoB in naringenin- or insulin-treated cells was similar under lipoprotein-deficient or oleate-stimulated conditions. Naringenin and insulin both stimulated intracellular apoB degradation via a kinetically defined rapid pathway. Therefore, naringenin, like insulin, inhibits apoB secretion through activation of both PI3-K and MAPKerk signaling, resulting in similar kinetics of apoB secretion. However, the mechanism for naringenin-induced signaling is independent of the IR. Naringenin represents a possible strategy for reduction of hepatic apoB secretion, particularly in the setting of insulin resistance.—Allister, E. M., E. E. Mulvihill, P. H. R. Barrett, J. Y. Edwards, L. P. Carter, and M. W. Huff. Inhibition of apoB secretion from HepG2 cells by insulin is amplified by naringenin, independent of the insulin receptor. J. Lipid Res. 2008. 49: 2218–2229.

Naringenin, like insulin, modulates hepatic VLDL apolipoprotein B-100 (apoB-100) production through activation of intracellular signaling cascades, namely the phosphoinositide-3-kinase (PI3-K) and mitogen-activated protein kinase/extracellular-regulated kinase (MAPKerk) cascades (1, 2). Activation of these two pathways results in upregulation of the LDL receptor (LDLr) and inhibition of microsomal triglyceride transfer protein (MTP), leading to the inhibition of apoB-100 secretion (1–4). Exposure of cells to insulin rapidly activates intracellular signaling by tyrosine phosphorylation of the insulin receptor (IR) and insulin receptor substrate-1 (IRS-1) within 10 min (5). However, the involvement of the IR and IRS-1 in mediating naringenin-induced cell signaling has not been clearly established. Preliminary experiments demonstrated that exposure of HepG2 cells to naringenin for 6 h did not increase IRS-1/2 tyrosine phosphorylation, suggesting that naringenin may activate these pathways independent of signaling through IRS-1/2 (2). Therefore, we hypothesized that rapid activation of MAPKerk signaling by naringenin is independent of IR and...
IRS phosphorylation. Because insulin and naringenin inhibit apoB-100 secretion through activation of the same signaling mechanisms, we postulated that naringenin has the ability to potentiate the effects of insulin in HepG2 cells and that naringenin sensitizes cells to insulin. Thus, examination of the molecular events in hepatocytes exposed to naringenin may provide insight into novel mechanisms for reduction of hepatic overproduction of apoB-100-containing lipoproteins, a characteristic of the dyslipidemia associated with insulin resistance (6–8).

Secretion of apoB-100-containing lipoproteins into the circulation is a complex process that requires the coordinated assembly of apoB, triglyceride (TG), free cholesterol, cholesteryl ester (CE), and phospholipids (PLs) within the endoplasmic reticulum (ER) (as reviewed in Ref. 9) (Fig. 1A). MTP is an absolute requirement for apoB secretion, inasmuch as it facilitates transfer of TG, CE, and PL to apoB as well as accretion of ER luminal TG for subsequent transfer to apoB (10). Assembly and secretion of apoB-100 involves a) APOB mRNA transcription and translation, b) translocation of apoB-100 across the ER membrane, and either c) association of apoB-100 with core and surface lipid via MTP and transport through the secretory pathway into plasma, or d) intracellular apoB-100 degradation. Although the proportion of apoB secreted from hepatocytes is predominantly regulated by an intracellular pool of secretion-coupled lipid, and by the transfer of these lipids to the nascent lipoprotein (11), each step may be subject to regulation. Pulse-chase experiments have provided a detailed examination of the kinetic movement of apoB-100 throughout the synthesis and secretory pathways and have revealed that in HepG2 cells, degradation of apoB-100 is mainly via a rapid proteasomal pathway, and to a lesser extent by ER luminal proteases or in post-ER compartments (3, 11). Previous pulse-chase studies in HepG2 cells analyzed by multicompartmental modeling have shown that naringenin inhibits apoB-100 secretion via increased intracellular degradation of apoB that is partially mediated through inhibition of MTP activity (12). Insulin has also been shown to inhibit the secretion of apoB-100 from hepatocytes (1, 2, 4, 13–16), mediated in part through activation of PI3-K activity (1, 2, 4) and inhibition of MTP.

Fig. 1. Multicompartmental modeling of apolipoprotein B-100 (apoB-100) synthesis and secretion from HepG2 cells. A schematic representation of the assembly and secretion of apoB-100-containing lipoproteins from hepatocytes (A). Secretion of apoB-100-containing lipoproteins requires the assembly of apoB, triglyceride (TG), free cholesterol, cholesteryl ester (CE), and phospholipid (PL) within the endoplasmic reticulum (ER). Microsomal triglyceride transfer protein (MTP) is an absolute requirement for apoB secretion, facilitating the transfer of TG, CE, and PL to apoB as well as accretion of ER luminal TG for subsequent transfer to apoB. Assembly and secretion of apoB-100 involves a) apoB mRNA transcription and translation, b) translocation of apoB-100 across the ER membrane, and either c) association of apoB-100 with core and surface lipid via MTP and transport through the secretory pathway into plasma, or d) intracellular degradation via the cytoplasmic proteasome or luminal proteases. A diagram of the multicompartmental kinetic model used for analysis of apoB secretion and intracellular degradation (B). Compartments 1 to 5 and 7 are within the HepG2 cell. Compartment 6 represents apoB in the culture media. Compartments 1 and 2 represent an intracellular pool of tracer and a delay compartment to allow for apoB synthesis after introduction of the tracer, respectively. Compartment 3 represents newly synthesized apoB. A parameter termed “Init” calculated by the model represents the amount of radioactive apoB-100 entering the system (initially compartment 3) required to achieve the tracer curve for total apoB (cell plus media) measured experimentally. ApoB-100 from compartment 3 may be transferred to compartment 4 and subsequently secreted. From compartment 4, apoB passes through a delay, compartment 5, before secretion into the media, compartment 6. ApoB may be degraded directly from compartment 3 by a rapid degradation pathway. A second, more slowly turning over pool of apoB destined for degradation is represented by compartment 7. The shaded compartments represent the compartments containing apoB radioactivity that were determined experimentally.
expression (1, 2). However, an extensive pulse-chase protocol coupled with multicompartmental modeling has not been used to characterize how insulin affects the kinetic movement of apoB-100 within hepatocytes.

The aim of this study is to compare the ability of naringenin and insulin to inhibit the secretion of apoB-100 in HepG2 cells and to determine whether naringenin activates rapid IR and IRS-1 phosphorylation. Furthermore, because naringenin and insulin both inhibit apoB-100 secretion via activation of the PI3-K and MAPK\(^{\text{ERK}}\) pathways, we hypothesize that exposure of cells to naringenin will potentiate or sensitize cells to the effects of insulin and that naringenin and insulin will induce similar kinetics for the attenuation of apoB-100 assembly and secretion from HepG2 cells.

**RESEARCH DESIGN AND METHODS**

**Cell culture and chemicals**

HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD) and grown as described previously (17). For experiments, HepG2 cells were plated in 6-well (35 mm) culture dishes from Falcon Scientific (VWR, Mississauga, ON) and cultured in MEM containing 5% human lipoprotein-deficient serum (LPDS) or serum-free media [0.5% insulin-free, fatty acid-free (FAF) BSA (Sigma, St. Louis, MO)]. Naringenin (Sigma) was solubilized in DMSO (concentration in cell cultures did not exceed 0.5% for any of the treatments). Bovine pancreatic insulin (Sigma) was solubilized in 0.1 N HCl.

**ApoB and extracellular-regulated kinase 1/2 immunoblotting**

ApoB secretion into the media was measured by immunoblot analysis as previously described (2). HepG2 cells grown in 6-well (35 mm) culture dishes were incubated for 24 h with either insulin (25 nM or 100 nM), naringenin (25 \(\mu\)M or 100 \(\mu\)M), or the combination of insulin and naringenin at various concentrations. Media apoB-100 was determined following 4.5% SDS-PAGE, transfer to PVDF membranes, and immunoblot analyses (2). Where indicated, HepG2 cells were preincubated for 30 min in the presence or absence of U0126 (10 \(\mu\)M) or U0124 (10 \(\mu\)M) (Calbiochem, San Diego, CA) followed by a further 23.5 h with either insulin, naringenin, or naringenin plus insulin. For extracellular-regulated kinase 1/2 (ERK1/2) phosphorylation experiments, HepG2 cells were grown in 6-well (35 mm) culture dishes and were incubated overnight in serum-free media to induce quiescence. A dose-response was established in cells incubated with increasing doses of naringenin for 30 min or insulin for 15 or 30 min. The time-course was determined in cells incubated with DMSO alone, naringenin (25 \(\mu\)M or 100 \(\mu\)M), insulin (25 nM or 100 nM), or the combination of naringenin and insulin at various concentrations for up to 60 min. For sensitizing experiments, cells were preincubated with naringenin (100 \(\mu\)M) for 30 min, followed by the addition of insulin (25 nM) for another 30 min. ERK1/2 phosphorylation and total ERK1/2 protein levels were measured following 12% SDS-PAGE, transfer to PVDF membranes, and immunoblot analyses using a phospho-specific ERK1/2 antibody and an ERK1/2 antibody (all from Cell Signaling Technology, Beverly, MA) as previously described (1). ERK1/2 phosphorylation was expressed as a ratio of the phosphorylated band to the protein band relative to control.

**Gene expression by quantitative real-time RT-PCR**

The expression of genes was determined in HepG2 cells grown in 6-well culture dishes and incubated in LPDS-MEM treatments for 6 h. Briefly, RNA was extracted using Trizol reagent (Invitrogen, Burlington, ON). Target gene RNA quantification was performed by quantitative real-time RT-PCR (qRT-PCR) on an ABI Prism (model 7900HT) Sequence Detection System (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Total RNA (10 \(\mu\)g) was reverse transcribed using the Applied Biosystems High-capacity cDNA archive kit according to the manufacturer’s protocol. cDNA (20–30 ng) was assayed in 20 \(\mu\)l reactions using the Taqman Assays-on-demand qRT-PCR protocol from Applied Biosystems. The primer probe sets for *MTP*, the *LDLr*, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), were obtained from Applied Biosystems (Hs00165177_m1, Hs00181192_m1, Hs99999905_m1). The standard curve method was used to determine mRNA abundance. Expression of *MTP* and expression of *LDLr* were normalized to *GAPDH* expression.

**Pulse-chase studies**

Secreted and cellular apoB-100, synthesized in the absence or presence of 0.1 mM oleic acid (OA) (Sigma) (complexed to FAF-BSA) was measured following precubation of cells for 24 h in the absence or presence of insulin (100 nM) or naringenin (100 \(\mu\)M). OA (0.1 mM) was added to the media for 20 min prior to the pulse. Cells were pulsed for 10 min with 100 \(\mu\)Ci/ml Tran \(^{35}\)S-label (1,000 Ci/mmol, L-[\(^{35}\)S]methionine and L-[\(^{35}\)S]cysteine; ICN, Costa Mesa, CA) and chased for a further 120 min (18). Media and cellular apoB-100 were immunoprecipitated using a polyclonal anti-human apoB antibody obtained from Boehringer Mannheim (Montreal, Canada) for the pulse-chase studies in LPDS media or from Midland Bioproducts Corporation (Boone, IA) for the pulse-chase studies with OA supplementation. ApoB-100 was resolved and quantitated as described previously (18).

**Multicompartmental modeling**

Data obtained from pulse-chase experiments, which included time points from 0 to 130 min postpulse, were analyzed by multicompartmental modeling using the SAAM II program (SAAM Institute, Seattle, WA) as previously described (12). We previously reported a model describing apoB synthesis, secretion, and degradation, which was developed using full-length apoB (apoB-100) radioactivity data obtained from pulse-chase experiments (18). Figure 1B shows the compartments and pathways between compartments included in the model. The model in-
ERK1/2 phosphorylation blocked the inhibitory effect of and 30 min, respectively (1). Furthermore, inhibition of resulting in maximal ERK1/2 phosphorylation at 15 min activated by insulin (100 nM) and naringenin (100 m), concen-
trations that decreased apoB-100 secretion by approximately 50% (2, 12). Control (DMSO-treated) HepG2 cells displayed no tyrosine phosphorylation of the immunoprecipitated β-subunit of the IR at any time point from 0 to 60 min (Fig. 2A). Insulin caused rapid tyrosine phosphorylation of the IR at 10 min, which remained activated up to 60 min. In contrast, naringenin did not cause tyrosine phosphorylation of the IR at any time point up to 60 min.

We observed basal tyrosine phosphorylation of IRS-1 in HepG2 cells, which was not stimulated in the presence of naringenin (100 μM) at 10, 20, 30, or 60 min (Fig. 2B). However, as expected, insulin (100 nM) maximally increased the tyrosine phosphorylation of IRS-1 at 10 min, which remained significantly elevated over the 60 min time-course. The role of IRS-2 was not pursued, inasmuch as its expression in HepG2 cells is very low compared with IRS-1 (data not shown).

Naringenin amplifies the ability of insulin to activate ERK1/2

Previously, we reported that in HepG2 cells, MAPKerk is activated by insulin (100 nM) and naringenin (100 μM), resulting in maximal ERK1/2 phosphorylation at 15 min and 30 min, respectively (1). Furthermore, inhibition of ERK1/2 phosphorylation blocked the inhibitory effect of both insulin and naringenin on apoB-100 secretion (1). In the present study, we tested the hypothesis that the addition of insulin to naringenin would enhance ERK1/2 phosphorylation. Initially, a dose-response for ERK1/2 activation by both insulin and naringenin was determined. HepG2 cells were incubated with increasing doses of naringenin for 30 min or insulin for 15 min, and the activation of ERK1/2 was determined. Naringenin dose-dependently increased ERK1/2 phosphorylation up to 2.8-fold at 100 μM, which did not increase further at 200 μM (Fig. 3A). Insulin induced a dose-dependent increase in ERK1/2 phosphorylation, reaching a maximum of 3-fold at 100 nM. Increasing the insulin concentration to 200 nM did not produce any further phosphorylation of ERK1/2 (Fig. 3B). Although quantitatively lower, the pattern for ratios of ERK1/2 phosphorylation to total ERK1/2 in cells incubated with insulin for 30 min was similar to that observed for 15 min of insulin exposure (data not shown).

This observation, together with our finding that naringenin and insulin activate the MAPKerk pathway via different mechanisms, suggested that the addition of naringenin to insulin would potentiate ERK1/2 phosphorylation. To test

**RESULTS**

Naringenin does not activate the IR or IRS-1 in cultured hepatocytes

Naringenin, like insulin, activates the MAPKerk pathway to inhibit MTP expression and apoB secretion (1); however, it is unknown whether naringenin does so via rapid activation of the IR and IRS-1. HepG2 cells were incubated with insulin (100 nM) and naringenin (100 μM), concentrations that decreased apoB-100 secretion by approximately 50% (2, 12). Control (DMSO-treated) HepG2 cells displayed no tyrosine phosphorylation of the immunoprecipitated β-subunit of the IR at any time point from 0 to 60 min (Fig. 2A). Insulin caused rapid tyrosine phosphorylation of the IR at 10 min, which remained activated up to 60 min. In contrast, naringenin did not cause tyrosine phosphorylation of the IR at any time point up to 60 min.

We observed basal tyrosine phosphorylation of IRS-1 in HepG2 cells, which was not stimulated in the presence of naringenin (100 μM) at 10, 20, 30, or 60 min (Fig. 2B). However, as expected, insulin (100 nM) maximally increased the tyrosine phosphorylation of IRS-1 at 10 min, which remained significantly elevated over the 60 min time-course. The role of IRS-2 was not pursued, inasmuch as its expression in HepG2 cells is very low compared with IRS-1 (data not shown).
this, HepG2 cells were initially incubated with naringenin (25 mM) plus insulin (25 nM), concentrations that induced submaximal ERK1/2 phosphorylation when used alone (Fig. 3A, B). Naringenin (25 mM) plus insulin (25 nM) resulted in a 1.5-fold and a 1.7-fold increase in ERK1/2 phosphorylation at 30 and 60 min, respectively (Fig. 4A). These increases were greater than the maximal increase for ERK1/2 phosphorylation observed for insulin (25 nM) alone or naringenin (25 mM) alone. Furthermore, the increase in ERK1/2 phosphorylation was sustained for up to 60 min, when the phospho-ERK1/2 response to either compound alone had returned to baseline. The maximal increase in ERK1/2 phosphorylation for naringenin (25 mM) plus insulin (25 nM) was similar to the maximal values observed for 100 mM naringenin (1.75-fold at 30 min) and for 100 nM insulin (1.7-fold at 15 min) when used alone (Fig. 4B). Incremental area under the curve for ERK1/2 phosphorylation from 0–60 min for naringenin (25 mM) plus insulin (25 nM) (27.2 ± 4.1) was significantly greater ($P < 0.05$) than either 25 mM naringenin (7.5 ± 3.7) or 25 mM insulin (12.2 ± 2.7) when used alone. A similar pattern of ERK1/2 phosphorylation was observed when naringenin (100 mM) was combined with insulin (25 nM), although the response was amplified further (Fig. 4B). With this combination, ERK1/2 phosphorylation increased 1.7-fold at 30 min and 2.4-fold at 60 min, which was similar to or greater than values observed for naringenin alone (100 mM) or insulin (100 nM) at any of the time points examined. Area under the curve for ERK1/2 phosphorylation from 0–60 min demonstrated that naringenin (100 mM) plus insulin (25 nM) (46.0 ± 2.2) was significantly greater ($P < 0.05$) than either naringenin alone at both 25 mM (7.5 ± 3.7) and 100 mM (21.7 ± 3.8) or insulin alone at both 25 mM (12.2 ± 2.7) and 100 mM (19.8 ± 8.3), suggesting that the effects are synergistic.

We next examined the potential for naringenin to sensitize HepG2 cells to insulin-stimulated ERK1/2 phosphorylation. HepG2 cells were preincubated for 30 min with 100 mM naringenin before adding 25 nM insulin. A time of 30 min was used because this was the time at which maximal ERK1/2 phosphorylation was observed for naringenin alone (Fig. 4B). ERK1/2 phosphorylation peaked 15 min following the addition of insulin, reaching values 2.6-fold greater than baseline (Fig. 4C). This was greater than peak values for insulin alone at 15 min (1.7-fold for 25 nM insulin, and 2.3-fold for 100 nM insulin) or naringenin alone at 30 min (1.8-fold at 100 mM). At 30 min following the addition of insulin (25 nM) to cells preincubated for 30 min with naringenin (100 mM), ERK1/2 phosphorylation was substantially greater than values for insulin or naringenin alone at the same time point. Area under the curve for ERK1/2 phosphorylation from 0–60 min for cells preincubated with naringenin (100 mM) for 30 min followed by the addition of insulin (25 nM) was significantly greater ($P < 0.05$) than values obtained over the same time-course by naringenin (100 mM) alone or insulin (25 nM) alone.

**Naringenin amplifies the ability of insulin to regulate the expression of MTP and the LDLr**

The expression of MTP and the LDLr in HepG2 cells is regulated by both naringenin and insulin through activation of the MAPKerk and PI3-K pathways (1, 2). To determine whether the ability of naringenin to amplify insulin-stimulated ERK1/2 phosphorylation extends to the regulation of MTP and LDLr expression, HepG2 cells were incubated for 6 h with naringenin (100 mM) alone or insulin (25 nM or 100 mM) alone, or were preincubated with naringenin (100 mM) followed by addition of insulin (25 nM). Insulin dose-dependently decreased MTP expression by 25% at 25 nM and 35% at 100 nM ($P < 0.05$ for both) relative to control. Preincubation with naringenin (100 mM) for 30 min followed by addition of insulin (25 nM) for a subsequent 5.5 h decreased MTP mRNA expression.

**Fig. 3.** Naringenin and insulin dose-dependently increase extracellular-regulated kinase 1/2 (ERK1/2) phosphorylation. HepG2 cells in serum-free media were treated with DMSO (control) and 12.5, 25, 50, 100, or 200 mM naringenin for 30 min (A) or DMSO and 12.5, 25, 50, 100, or 200 nM insulin for 15 min (B). Total and phosphorylated ERK1/2 were measured by immunoblot analysis and presented as a ratio of the phosphorylated form to total ERK1/2 protein (relative to DMSO control). Values are the mean ± SEM for six experiments. Values with different letters are significantly different at $P < 0.05$. 

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### Notes

- The Journal of Lipid Research is an academic journal that publishes research articles on lipid metabolism, its role in health and disease, and the development and evaluation of new therapeutic agents.
- The article discusses the effects of naringenin, a flavonoid commonly found in citrus fruits, on ERK1/2 phosphorylation and its potential role in regulating gene expression, specifically MTP and LDLr, in HepG2 cells.
- The studies were conducted under serum-free conditions with different concentrations of naringenin and insulin to determine dose-response effects.
- Key findings include the synergistic effect of naringenin and insulin on ERK1/2 phosphorylation, with significant amplification of insulin's effects upon preincubation with naringenin.
- Gene expression studies showed that naringenin, either alone or in combination with insulin, influenced MTP and LDLr expression, with potential implications for lipid metabolism and cardiovascular health.
Fig. 4. Addition of naringenin to insulin enhances and prolongs ERK1/2 activation in HepG2 cells. HepG2 cells in serum-free media were incubated with DMSO, naringenin (closed triangle), insulin (closed square), or insulin and naringenin added simultaneously (open diamond) from 0 to 60 min. Total and phosphorylated ERK1/2 were measured by immunoblotting. C: HepG2 cells in serum-free media were pretreated for 30 min with DMSO or naringenin (100 μM) followed by the addition of 25 nM insulin for a subsequent 30 min (closed diamond). Cells were also treated with insulin alone (at both 25 nM, open squares and 100 nM, closed squares) or 100 μM naringenin alone (closed triangles) from 0 to 60 min. Total and phosphorylated ERK1/2 were measured by immunoblot analysis and presented as a ratio of phosphorylated form to total ERK1/2 protein (relative to DMSO control). Mean incremental area under the curve (AUC) ± SEM are presented for four experiments. **P < 0.05 for simultaneous addition of naringenin (25 μM) plus insulin (25 nM) compared with naringenin alone (25 μM) and insulin alone (25 nM). ***P < 0.05 for simultaneous addition of 100 μM naringenin plus 25 nM insulin compared with naringenin alone (25 μM) and insulin alone (25 and 100 nM), and the simultaneous addition of naringenin (25 μM) and insulin (25 nM). †P < 0.05 for naringenin (100 μM) (0–60 min) plus insulin (25 nM) (30–60 min) compared with insulin alone (25 nM) and naringenin alone (100 μM).

Naringenin amplifies insulin’s ability to inhibit apoB secretion

The impact of combining naringenin and insulin on apoB secretion was determined in HepG2 cells incubated for 24 h with either compound alone or in combination (Fig. 5C). ApoB in the media was significantly decreased, by 68% (P < 0.05), by the combination of naringenin (25 μM) plus insulin (25 nM). This was a significantly greater reduction than for either naringenin alone at 25 μM (−9%) or insulin alone at 25 nM (−28%), suggesting a synergistic reduction of apoB secretion with this combination. This 68% decrease was greater than insulin alone at 100 nM (−41%) and similar to that observed for naringenin alone at 100 μM (−58%). Addition of 25 nM insulin to naringenin (100 μM) decreased apoB secretion by 68%, which was greater than that observed for naringenin alone (at both 25 μM and 100 μM) and insulin alone (at both 25 nM and 100 nM), and the same as the combination of naringenin (25 μM) plus insulin (25 nM). The combination of 100 μM naringenin plus 100 nM insulin also decreased apoB secretion by 68%, suggesting that maximal reduction by the combination was achieved by 25 μM naringenin plus 25 nM insulin.

ApoB secretion is inhibited by both naringenin alone (100 μM) (−58%; P < 0.05) and insulin alone (100 nM) (−42%; P < 0.05) in a MAPK<sup>erk</sup>-dependent manner (Fig. 5D). Addition of U0126, a specific MEK1/2 inhibitor, resulted in significant attenuation of the effects of both naringenin and insulin, whereas the inactive isof orm U0124 had no effect. These results confirm our previous observations (1). Figure 5D also demonstrates that the reduction of apoB secretion by the combination of naringenin plus insulin is dependent on MAPK<sup>erk</sup>. Addition of U0126 significantly blocked the majority of the effect on apoB secretion for all combinations, whereas U0124 had little or no effect. Therefore, inhibition of apoB secretion by 40% (P < 0.05), which was a greater reduction than that observed for 25 nM insulin alone or 100 μM naringenin alone (P < 0.05), and similar to that observed for 100 nM insulin alone (Fig. 5A). Preincubation with naringenin (100 μM) for 30 min followed by addition of insulin (100 nM) for a subsequent 5.5 h resulted in no further decrease in MTP mRNA expression. Incubation of cells with insulin for 6 h dose-dependently increased LDP expression 2.5-fold in the presence of 25 nM insulin and 3-fold with 100 nM insulin (P < 0.05 for both). Preincubation of cells with naringenin for 30 min prior to the addition of insulin (25 nM) for an additional 5.5 h increased LDLr expression 5-fold (P < 0.05), which was greater than that observed for 25 nM insulin alone (P < 0.05), 100 nM insulin alone, or 100 μM naringenin alone (P < 0.05) (Fig. 5B). Preincubation with naringenin (100 μM) for 30 min followed by addition of insulin (100 nM) for a subsequent 5.5 h resulted in no further increase in LDLr mRNA expression. These observations suggest that naringenin potentiates the effects of insulin on expression of MTP and expression of LDLr in HepG2 cells, both of which are determinants of net apoB-100 secretion.

Naringenin and insulin inhibit apoB secretion from HepG2 cells
by the combination of naringenin plus insulin is primarily regulated by activation of the MAPK_{erk} pathway.

**Naringenin and insulin both increase the degradation of apoB-100 through a kinetically defined, rapid pathway**

Activation of the PI3-K and MAPK_{erk} signaling pathways by naringenin or insulin leads to the inhibition of apoB-100 secretion from HepG2 cells (1, 2), suggesting that the kinetics of assembly and secretion of apoB-containing lipoproteins would be modulated through similar mechanisms. Although we have previously conducted detailed pulse-chase studies in HepG2 cells treated with naringenin, in which the kinetic parameters for the intracellular trafficking and secretion of apoB were quantitated using multi-compartmental modeling (12), similar quantitative studies have not been reported for insulin. Therefore, pulse-chase

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**Fig. 5.** Naringenin sensitizes HepG2 cells to the effects of insulin on mRNA expression of both MTP and the LDLr and apoB-100 secretion. Cells were pretreated with or without naringenin (100 μM) for 30 min before being treated with 25 nM or 100 nM insulin for a combined time of 6 h (cross-hatched bars). Insulin alone (25 nM or 100 nM, striped bars) for 6 h and naringenin alone (100 μM, solid bars) are also shown. MTP (A) or LDLr (B) expression was determined by quantitative real-time RT-PCR and normalized to GAPDH. Values are the mean ± SEM for five experiments. C: HepG2 cells were incubated in LPDS media for 24 h in the presence of DMSO, naringenin alone, insulin alone, or the combination of naringenin plus insulin at the indicated concentrations. Media was collected and apoB-100 measured by immunoblotting. D: HepG2 cells in LPDS media were preincubated for 30 min with DMSO, the MEK1/2 inhibitor UO126 (10 μM), or its inactive isoform UO124 (10 μM) followed by a 23.5 h incubation with naringenin alone, insulin alone, or the combination of naringenin plus insulin at the indicated concentrations. Media apoB-100 was measured by immunoblotting. Values are the mean ± SEM for five experiments. Values with different letters are significantly different at \( P < 0.05. \)
studies were conducted in HepG2 cells incubated with naringenin (100 μM) and insulin (100 nM) under basal (LPDS) conditions or following OA treatment, which stimulates apoB secretion. These concentrations of naringenin and insulin inhibit the accumulation of apoB-100 in the media of HepG2 cells by approximately 50% over 24 h [(1) and Fig. 5C]. Prior to the start of each pulse-chase, cells were preincubated for 24 h with or without naringenin or insulin in LPDS media alone. In some experiments, OA (0.1 mM) was added to the media for 20 min prior to the pulse. Media and cell lysates were collected at 10 time points throughout the pulse and the chase up to 2 h.

The data points shown in Fig. 6A–D represent radioactivity in full-length apoB-100 measured experimentally. The curves in each graph are fits to the experimental data obtained from analyses using the multicompartmental model shown in Fig. 1B. Under basal conditions, naringenin and insulin significantly decreased the amount of intracellular and secreted apoB-100 (Fig. 6A, C). Even under oleate-stimulated conditions, in which apoB-100 secretion is substantially increased in control cells, naringenin and insulin significantly decreased the intracellular accumulation and secretion of newly synthesized apoB-100 (Fig. 6B, D). Inspection of the curves indicated that under both conditions, naringenin and insulin decrease the peak of radioactivity in apoB-100 during the pulse and early chase period, prior to the appearance of any radiolabeled apoB-100 in the media. Although this is normally interpreted as a decrease in the synthesis of a protein, it has been well established that within hepatocytes, apoB-100 degradation can occur cotranslationally as well as post-translationally (3, 19). Cotranslational degradation is observed experimentally as a decrease in radiolabeled full-length apoB-100 appearing in the cell. Detailed pulse-chase protocols for apoB-100 cannot distinguish between apoB-100 synthesis and cotranslational degradation. In previous studies, we demonstrated, using the same protocol and multicompartmental modeling analyses, that in HepG2 cells incubated with lactacystin or ALLN to inhibit proteasomal degradation, peak intracellular apoB-100 radioactivity was substantially higher than in control cells (12). This increased peak radioactivity was interpreted as inhibition of cotranslational degradation, permitting increased availability of apoB-100 for particle formation. In the present studies peak intracellular apoB-100 radioactivity in insulin- or naringenin-treated cells was substantially lower than that of control cells (Fig. 6A, B). Taken together with our observations and those of others that neither naringenin nor insulin affects apoB mRNA (1, 14, 20–22), our results are consistent with the interpretation that both insulin and naringenin stimulate cotranslational degradation of apoB. However, an effect of insulin or naringenin on apoB-100 synthesis cannot be completely
was increased similarly. This degradation of apoB-100 is
proportionally. Compared with control cells, the Init parameter
increased 65% (P < 0.05) with the addition of OA, and was significantly decreased by the
addition of either naringenin (−56%) or insulin (−49%). In
cells cultured under basal conditions, the percent of apoB
secreted was significantly reduced by both naringenin (−65%) and insulin (−53%), whereas total degradation
was increased similarly. This degradation of apoB-100 is
independent of the Init parameter and therefore is indepen-
dent of cotranslational apoB degradation. The
decreased secretion for both treatments was reflected in a
decrease in the rate constant for secretion, k(4,3), and
due primarily to increases in the rate constant
for rapid degradation, k(0,3), although increases in the slow
degradation pathway, k(7,3), which represents less than
5% of total degradation, were observed. However, the
changes in k(7,3) did not reach statistical significance.
The addition of OA prior to and during the pulse
and chase period increased the percent of radiolabeled apoB-
100 secreted over 2-fold in control cells compared with
LPDS media, resulting in a proportional reduction in
apoB-100 degradation. The addition of OA exerts its stimu-
lation of secretion by decreasing the rapid component of
intracellular degradation, k(0,3). In OA-treated cells, the
percent of apoB secreted was also significantly decreased
by both naringenin (−77%) and insulin (−71%), whereas
the total degradation of apoB-100 increased proportion-
ally. The decreased secretion for both treatments was
reflected by a decrease in the rate constant for secretion,
k(4,3), and was due to significant increases in the rate con-
stant for rapid degradation, k(0,3), although nonsignif-
cicant increases in the slow degradation pathway, k(7,3),
were observed. Previously, we reported that degradation
from the rapidly turning over pool was largely proteasomal,
inasmuch as it was selectively inhibited by both ALLN and
lactacystin (12). This suggests that the increased rapid de-

### Table 1

Naringenin and insulin similarly stimulate the degradation and inhibit the secretion of apoB-100
from HepG2 cells

<table>
<thead>
<tr>
<th>LPDS</th>
<th>Control</th>
<th>Naringenin</th>
<th>Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Init (arbitrary units)</td>
<td>52.25 ± 7.61</td>
<td>18.98 ± 2.88</td>
<td>25.94 ± 8.18</td>
</tr>
<tr>
<td>Total degraded (%)</td>
<td>93.45 ± 1.25</td>
<td>97.67 ± 0.34</td>
<td>96.89 ± 0.47</td>
</tr>
<tr>
<td>Total secreted (%)</td>
<td>6.57 ± 1.25</td>
<td>2.33 ± 0.34</td>
<td>3.11 ± 0.47</td>
</tr>
<tr>
<td>k(4,3) (pools/min)</td>
<td>0.008 ± 0.001</td>
<td>0.004 ± 0.001</td>
<td>0.004 ± 0.001</td>
</tr>
<tr>
<td>k(0,3) (pools/min)</td>
<td>0.117 ± 0.022</td>
<td>0.152 ± 0.014</td>
<td>0.122 ± 0.006</td>
</tr>
<tr>
<td>k(7,3) (pools/min)</td>
<td>0.005 ± 0.003</td>
<td>0.007 ± 0.003</td>
<td>0.010 ± 0.003</td>
</tr>
<tr>
<td>Portion degraded via rapid pathway (%)</td>
<td>91.4 ± 1.6</td>
<td>93.9 ± 1.4</td>
<td>89.3 ± 2.4</td>
</tr>
<tr>
<td>Olate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Init (arbitrary units)</td>
<td>86.50 ± 12.8</td>
<td>30.08 ± 8.48</td>
<td>43.64 ± 9.31</td>
</tr>
<tr>
<td>Total degraded (%)</td>
<td>84.81 ± 4.05</td>
<td>96.53 ± 8.76</td>
<td>95.66 ± 11.1</td>
</tr>
<tr>
<td>Total secreted (%)</td>
<td>15.19 ± 4.05</td>
<td>3.47 ± 8.76</td>
<td>4.34 ± 11.1</td>
</tr>
<tr>
<td>k(4,3) (pools/min)</td>
<td>0.008 ± 0.002</td>
<td>0.0026 ± 0.0004</td>
<td>0.0030 ± 0.0007</td>
</tr>
<tr>
<td>k(0,3) (pools/min)</td>
<td>0.055 ± 0.014</td>
<td>0.081 ± 0.010</td>
<td>0.070 ± 0.009</td>
</tr>
<tr>
<td>k(7,3) (pools/min)</td>
<td>0.0007 ± 0.0002</td>
<td>0.0011 ± 0.0002</td>
<td>0.0008 ± 0.0001</td>
</tr>
<tr>
<td>Portion degraded via rapid pathway (%)</td>
<td>83.78 ± 4.94</td>
<td>95.29 ± 0.85</td>
<td>94.51 ± 1.69</td>
</tr>
</tbody>
</table>

Apolipoprotein B-100 (apoB-100) pulse-chase data were analyzed by multicompartamental modeling using
SAAM II. The percent of newly synthesized apoB-100 secreted and degraded was determined using the kinetic
model. Values are mean ± SEM for lipoprotein-deficient serum (LPDS) (n = 4) and oleate (n = 5).

* Means with different letters indicate statistical difference of at least P < 0.05.

* The Init parameter is calculated by the model and represents the amount of radioactive apoB-100 enter-
ing the system (initially compartment 3) required to achieve the tracer curve for total apoB (cell plus media) mea-
sured experimentally.

* Calculated using the formula [k(0,3) + k(7,3)] / k(4,3) + k(0,3) + k(7,3)] × 100.

* Calculated using the formula [k(4,3) / k(4,3) + k(0,3) + k(7,3)] × 100.

* k(4,3) is the rate constant for apoB-100 transfer from compartment 3 to compartment 4, or the rate constant
  for secretion, k(0,3) is the rate constant for apoB-100 degradation directly from compartment 3, or the rate
  constant for rapid degradation, k(7,3) is the rate constant for apoB-100 transfer from compartment 3 to com-
  partment 7, or the rate constant for slow degradation.

* The percent of apoB-100 degraded directly from compartment 3 is calculated using the formula [k(0,3) / k
  (4,3) + k(0,3) + k(7,3)] × 100.
activation of PI3-K and MAPKerk, which is linked to the
Nevertheless, the mechanism for the naringenin-induced
fails to phosphorylate IRS-1 in HepG2 cells at 6 h (2).
that naringenin does not activate the downstream effector
naringenin activation of both pathways occurs indepen-
dent of insulin. However, we show for the first time that
initiation of MAPKerk signaling involves farnesylation (unpub-
lished observations), sup-
porting the concept that initiation of MAPKerk signaling
by naringenin occurs through a mechanism distinct from
insulin. However, the identity of the cellular receptor and
upstream mediators responsible for the activation of Ras
by naringenin remains to be elucidated.

DISCUSSION

A major metabolic abnormality associated with insulin
resistance is dyslipidemia characterized by the overproduc-
tion of hepatic VLDL-apoB-100, which contributes to the
increased risk of cardiovascular disease in this population
(6–8). The failure of insulin to suppress hepatic de novo
lipogenesis, increased free fatty acid (FFA) flux, TG synthe-
sis, and decreased fatty acid oxidation collectively lead to
increased VLDL overproduction. Although the mecha-

isms regulating the assembly and secretion of hepatic
apoB-100-containing lipoproteins have been described
(3, 9, 11, 23), potential treatments to correct this imbal-
ance in VLDL production have not been identified. We
have previously shown that naringenin, like insulin, inhib-
its apoB-100 secretion via activation of two signaling path-
ways, namely PI3-K and MAPKerk (1, 2). In this study, we
show that in contrast to insulin, the ability of naringenin
to decrease apoB secretion in HepG2 cells does not
involve signaling through the IR. Naringenin activates
MAPKerk and PI3-K through a mechanism that does not
require phosphorylation of the IR or IRS-1. Furthermore,
we demonstrate that naringenin is able to sensitize HepG2
cells to insulin and/or potentiate the effects of insulin on
ERK1/2 signaling, the mRNA expression of both MTP and
the LDLr, and apoB secretion. Even though naringenin
and insulin utilize different mechanisms to initiate intra-
cellular signaling, they inhibit the assembly and secretion
of apoB-100 with similar kinetics.

The rapid increase in ERK1/2 phosphorylation [Fig. 4
and (1)] and PI3-K activity (2) by naringenin parallels
that of insulin. However, we show for the first time that
naringenin activation of both pathways occurs independent
of IR phosphorylation. Furthermore, we demonstrate that
naringenin does not activate the downstream effector
of IR activation, IRS-1, within 1 h of exposure. This is
consistent with our previous observations that naringenin
fails to phosphorylate IRS-1 in HepG2 cells at 6 h (2).
Nevertheless, the mechanism for the naringenin-induced
activation of PI3-K and MAPKerk, which is linked to the
regulation of apoB-100 secretion, is unknown. The activa-
tion of the MAPKerk pathway in hepatocytes by insulin
has been shown to require activation of Raf-1, the most
upstream member of the MAPKerk signaling cascade,
whereas the activation of Ras proteins, another important
upstream mediator of MAPKerk signaling, is not required
(24). Ras activation requires farnesylation (25). In prelimi-
nary experiments in HepG2 cells, we found that the phos-
phorylation of ERK1/2 by naringenin, but not insulin,
involves farnesylation (unpublished observations), sup-
porting the concept that initiation of MAPKerk signaling
by naringenin occurs through a mechanism distinct from
insulin. However, the identity of the cellular receptor and
upstream mediators responsible for the activation of Ras
by naringenin remains to be elucidated.

In a previous report, using multicompartamental anal-
ysis of pulse-chase experiments, we demonstrated that
naringenin inhibits apoB-100 secretion and stimulates
apoB-100 degradation, in both the absence of lipoproteins
and the presence of OA (12). However, this is the first
report of multicompartamental analyses of pulse-chase data
to define the ability of insulin to modulate the kinetics of
apoB-100 assembly and secretion. Analysis revealed that
naringenin and insulin modulated the intracellular kinet-
icsofapoB-100inaverysimilarmanner,inboththe
absence and presence of OA. Both compounds decrease
the availability of apoB for particle formation, with very
similar kinetics. Furthermore, insulin, like naringenin,
increases intracellular degradation of newly formed apoB-
100, and this degradation is from a kinetically defined,
rapidly turning over cellular apoB pool. The decrease in
apoB availability could be due to decreased synthesis or
enhanced cotranslational degradation of apoB; however,
pulse-chase studies cannot distinguish between the effects
of naringenin or insulin on synthesis versus cotranslational
degradation. Although it has been reported that insulin
inhibits hepatocyte apoB synthesis in a cell-free translation
system (16), this has not been a consistent finding in other
studies. In rat hepatocytes, insulin was shown to inhibit
the maturation phase of VLDL assembly by preventing
lipid transfer to pre-VLDL particles, resulting in preferen-
tial degradation of apoB-100 from these pre-VLDL par-
ticles (26). Furthermore, Chirieac et al. (27) examined
apoB-100 secretion by pulse-chase in primary mouse
hepatocytes and concluded that blocking the effect of in-
sulin with wortmannin increased the amount of apoB-100
available for secretion. It was not possible to distinguish
degradation decreased synthesis from increased cotranslational de-
gradation. The availability of apoB-100 is greatly enhanced
by proteasomal inhibitors, suggesting that this parameter
largely reflects cotranslational degradation (12). How-
erever, an effect of insulin or naringenin on apoB-100 synthesis
cannot be completely ruled out. Nevertheless, the kinetics
for apoB-100 availability are similar in cells exposed to
either insulin or naringenin.

The naringenin- and insulin-induced inhibition of se-
cretion results in enhanced degradation of apoB-100,
mainly via a kinetically defined rapid degradation path-
way. Previously, we demonstrated that this rapid pathway
is proteasomal, inasmuch as inhibitors of the cytosolic
proteasome selectively blocked this degradation pathway
(12). Increased proteasomal degradation occurs when
apoB is inadequately lipidated during assembly (as re-
viewed in Ref. 9). This is consistent with our findings that
a) naringenin and an exogenous inhibitor of MTP selec-
tively decrease the accumulation of newly synthesized TG
within the ER lumen (28), b) an MTP inhibitor enhances
the rapid apoB-100 degradation pathway (28), and c)
naringenin and insulin both decrease the expression of
MTP via activation of MAPKerk (1). The slow degradation
pathway for apoB, which accounts for less than 5% of total
apoB-100 degradation, was affected by naringenin, and, to
a lesser extent, by insulin. The nature of the slow degrad-
ation pathway has not been fully characterized, but may reflect
ER luminal degradation (3) or lysosomal degradation mediated by the LDL receptor (25). In isolated LDLr−/− mouse hepatocytes, modeling of pulse-chase experiments demonstrated that the slow apoB-100 degradation pathway was significantly decreased compared with wild-type hepatocytes (29). Our finding that both naringenin and insulin induce minor increases in the slow apoB-100 degradation pathway is consistent with the increase in LDLr expression resulting from the activation of the PI3-K and MAPKerk pathways by both naringenin and insulin (2).

Insulin resistance is often characterized by an elevation of circulating FFAs, and it is proposed that these FFAs exacerbate the insulin signaling defects, increase TG synthesis and apoB-100 secretion, and cause pancreatic β-cell toxicity, ultimately leading to decreased insulin secretion and type 2 diabetes (30). It is well established that OA increases the secretion of apoB from hepatocytes, because it increases the availability of substrate for the synthesis of TG, thereby providing more neutral lipid for apoB-containing lipoprotein formation, thus protecting apoB from cotranslational degradation or degradation of full-length apoB-100 (as reviewed in Ref. 20). Consistent with this observation, multicompartmental analysis of apoB-100 pulse-chase kinetic experiments revealed that OA treatment resulted in considerably more apoB-100 availability and significantly less apoB degradation, resulting in more apoB-100 secretion from HepG2 cells, compared with LPDS media (12). The kinetics of intracellular apoB-100 trafficking in naringenin- and insulin-treated cells exposed to OA are very similar. Naringenin and insulin substantially decrease apoB-100 secretion by greater than 70%, indicating that exposure of cells to either treatment completely overcomes the stimulatory effects of OA.

The ability of naringenin to activate intracellular signaling pathways independent of the IR and IRS-1 allows it to act as an insulin sensitizer. Initially, we established a dose-dependent relationship for the activation of ERK1/2 by naringenin or insulin. Naringenin- and insulin-induced ERK1/2 phosphorylation in HepG2 cells reached a maximal increase by 30 min at 100 μM of naringenin and by 15 min at 100 nM of insulin, a normal physiologic in vitro insulin concentration. One quarter of this insulin concentration (25 nM) increased ERK1/2 phosphorylation to a maximum of 1.5-fold at 15 min. Coincubation of insulin at 25 nM with naringenin (25 or 100 μM) significantly increased the peak of ERK1/2 activation, compared with either insulin or naringenin used alone. Furthermore, the time of ERK1/2 activation was substantially prolonged. Preincubation of HepG2 cells with naringenin (100 μM) for 30 min prior to the addition of 25 nM insulin for an additional 30 min increased ERK1/2 phosphorylation to levels similar to those achieved by 100 nM insulin alone. In cells exposed to 25 nM or 100 nM insulin alone for 60 min, ERK1/2 phosphorylation had returned to baseline levels, suggesting that preexposure to naringenin sensitizes cells to insulin, resulting in enhanced and prolonged cellular signaling.

The ability of naringenin to sensitize HepG2 cells to stimulation by insulin extended to the expression of MTP and the LDLr, both of which are important determinants of hepatocyte apoB-100 secretion (23, 29). We previously reported that expression of both genes is regulated in hepatocytes by naringenin and insulin through activation of the MAPKerk and PI3-K pathways (1, 2). We show that there is a dose-dependent decrease in MTP expression and increase in LDLr expression when cells are incubated with 25 or 100 nM insulin. Preincubation of cells with 100 μM naringenin prior to addition of 25 nM insulin reduced MTP expression and increased LDLr expression to levels similar to those achieved by 4-fold higher concentrations of insulin alone (100 nM). The combination of naringenin (25 μM) plus insulin (25 nM), at one-quarter of their IC50 concentrations, induced a significantly greater reduction in apoB-100 secretion than for either naringenin alone at 25 μM or insulin alone at 25 nM, suggesting a synergistic reduction of apoB secretion with this combination. Furthermore, this combination resulted in similar reductions in apoB-100, compared with either compound alone, at four times the concentration. Importantly, these observations solidify the concept that naringenin sensitizes HepG2 cells to a low dose of insulin. In insulin-resistant subjects, a given concentration of insulin fails to elicit a normal biological response. Therefore, naringenin may be able to restore sensitivity to insulin by priming the signaling pathways via a mechanism that does not involve activation of the IR.

In summary, naringenin inhibits the assembly and secretion of apoB-100-containing lipoproteins from HepG2 cells in a manner very similar to insulin. This is supported by detailed kinetic analysis of pulse-chase experiments, which revealed that the kinetics of apoB-100 secretion were similar for naringenin and insulin. One of the mechanisms involved includes activation by naringenin of PI3-K and MAPKerk signaling, two pathways that are also activated by insulin, to mediate the decrease in apoB-100 secretion. Of significance, we demonstrate that activation of these two cell signaling pathways by naringenin, in contrast to insulin, does not involve rapid activation of the IR or IRS-1. The exact mechanism of how naringenin activates PI3K and MAPKerk signaling has not been fully elucidated. However, the difference in activation of these two pathways probably underlies the ability of naringenin to either sensitize cells to insulin or potentiate the effect of insulin in mediating the inhibition of apoB-100 secretion. It will be imperative to test whether naringenin can ameliorate the hepatic VLDL-apoB-100 overproduction characteristic of insulin resistance.

REFERENCES


