Inhibition of Microsomal Triglyceride Transfer Protein Expression and Apolipoprotein B100 Secretion by the Citrus Flavonoid Naringenin and by Insulin Involves Activation of the Mitogen-Activated Protein Kinase Pathway in Hepatocytes

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Microsomal triglyceride transfer protein (MTP) is necessary for hepatocyte assembly and secretion of apolipoprotein (apo)B100-containing lipoproteins. The citrus flavonoid naringenin, like insulin, decreased MTP expression in HepG2 cells, resulting in inhibition of apoB100 secretion; however, the mechanism for naringenin is independent of insulin receptor substrate-1/2. Recently, it was reported that insulin decreased MTP expression in HepG2 cells via the mitogen-activated protein kinase (MAPK)/extracellular signal–regulated kinase (ERK) (MAPK\textsuperscript{erk}) pathway. We hypothesized that naringenin acts via a similar mechanism. Inhibition of MAPK kinase (MEK) 1/2 in HepG2 cells significantly attenuated the naringenin- and insulin-induced reduction in MTP expression. Both naringenin and insulin increased ERK1/2 phosphorylation, which was completely inhibited by MEK1/2 inhibition and enhanced by inhibition of MAPK\textsuperscript{p38}, a negative regulator of MAPK\textsuperscript{erk} activity. Inhibition of MEK1/2 significantly attenuated both the naringenin- and insulin-induced decrease in apoB100 secretion demonstrating a direct link between MAPK\textsuperscript{erk} activation and apoB100 secretion. Furthermore, both compounds increased MAPK\textsuperscript{p38} activation, and therefore inhibition of MAPK\textsuperscript{p38} amplified the naringenin- and insulin-induced decrease in apoB100 secretion. We conclude that MAPK\textsuperscript{erk} signaling in hepatocytes is critical for inhibition of apoB100 secretion by naringenin and insulin. Therefore, naringenin may prove useful for activating insulin-signaling pathways important for regulation of hepatocyte lipid homeostasis. Diabetes 54:1676–1683, 2005

It is well established that insulin resistance and type 2 diabetes result in overproduction of hepatic apolipoprotein (apo)B-containing lipoproteins, which confers an increased atherosclerotic risk (1,2). Microsomal triglyceride transfer protein (MTP) is rate-limiting for hepatic lipoprotein assembly. MTP catalyzes the transfer of lipids to newly synthesized apoB within the endoplasmic reticulum facilitating secretion of nascent lipoproteins (3). Recent evidence from the fructose-fed hamster model of insulin resistance has shown that an increased activity of MTP contributes to the increased secretion/overproduction of hepatic apoB100 lipoproteins (4). Furthermore, amelioration of hepatic insulin resistance in this model resulted in normalization of MTP expression and reduction of the overproduction of apoB100-containing lipoproteins (5). Under normal culture conditions, insulin negatively regulates the expression of MTP in hepatocytes (6). Therefore, other compounds that activate intracellular insulin-signaling pathways may also decrease MTP expression and potentially be used in patients to treat dyslipidemia associated with insulin resistance.

Previous studies in cell culture systems and animal models have shown that the grapefruit flavonoid naringenin dramatically decreases the secretion of hepatic lipoproteins (7–14). We reported that naringenin dose-dependently inhibited apoB secretion in HepG2 cells (11) resulting in increased intracellular apoB degradation via a proteasomal rapid kinetic pathway (8). Initial mechanistic studies revealed that naringenin inhibited the expression and activity of acyl CoA:cholesterol acyltransferase-2 and MTP, whereas apoB mRNA was unaffected (11). Subsequent experiments revealed that decreased acyl CoA: cholesterol acyltransferase activity and cholesteryl ester availability within the ER lumen were not requirements for the naringenin-induced inhibition of apoB secretion (8). In other studies, treatment of streptozotocin-induced diabetic rats with naringenin 7-O-β-D-glucoside (isolated from a Korean folk remedy) reduced blood glucose, triglycerides, and total cholesterol, indicating that naringenin may have insulin-like properties (15). Recently, we reported that naringenin increases the activity of phosphatidylinositol (PI) 3-kinase independent of insulin receptor sub-
strate (IRS-1)-1/2 (9). The increase in PI 3-kinase activity translated to an increase in cytosolic and nuclear sterol regulatory element–binding protein (SREBP)-1 protein levels and LDL receptor (LDL-r) mRNA, an effect that was completely blocked by inhibiting PI 3-kinase with the specific inhibitor wortmannin. These responses contributed to the decrease in apoB100 secretion from hepatocytes, thus supporting the concept that naringenin activates at least one insulin-signaling pathway (9). However, despite complete abrogation of the increase in SREBP-1 and LDL-r expression, wortmannin blocked the naringenin-induced decrease in apoB100 secretion by only 30%. Therefore, we hypothesized that naringenin might also activate other intracellular insulin-signaling cascades involved in regulation of apoB100 secretion.

Naringenin potently decreases the activity and expression of MTP, contributing to its ability to inhibit secretion of apoB100 from hepatocytes (8,10,11). Recently, insulin was shown to decrease MTP mRNA in hepatocytes (6) via activation of the mitogen-activated protein kinase (MAPK)/extracellular signal–regulated kinase (ERK) (MAPKerk) pathway (16). The MAPKerk pathway is involved in the intracellular regulation of gene transcription (17) and it is activated either via IRS-1/2 or the adapter protein Shc, two direct substrates of the insulin receptor (18). Major kinases in this pathway that are sequentially activated are Ras, Raf-1, MAPK kinase (MEK)-1/2, and ERK1/2 (19). In addition, MAPKp38 modulates the MAPKerk pathway, and activation of this kinase normally functions as a negative regulator of MEK1/2-ERK1/2 signaling, thus providing fine-tuning of this pathway (20).

Therefore, the objectives of the present study were to determine whether naringenin inhibits MTP expression via the MAPKerk pathway by using specific inhibitors of MEK1/2 and MAPKp38. Furthermore, we hypothesized that the MAPKerk-mediated naringenin- and insulin-induced decrease in MTP expression would attenuate hepatic secretion of apoB100. In this article, we show that naringenin, like insulin, decreases MTP mRNA abundance via the MAPKerk pathway, and this depends on the phosphorylation of ERK1/2. Furthermore, we demonstrate for the first time that activation of the MAPKerk pathway is responsible for part of the decrease in apoB100 secretion that is observed with both naringenin and insulin. Simultaneous inhibition of PI 3-kinase and MEK1/2 in HepG2 cells completely blocks the naringenin- and insulin-induced decrease in apoB100 secretion.

**RESULTS**

**Naringenin and insulin decrease MTP expression via activation of MEK1/2.** Insulin decreases MTP expression via activation of the MAPKerk pathway in HepG2 cells (16). Because we previously showed that naringenin induces a 32% decrease in MTP expression within 24 h (11), we determined whether naringenin mediates this effect via MAPKerk activation. Incubation of HepG2 cells with naringenin (100 μmol/l) or insulin (100 nmol/l) for 6 h significantly decreased the expression of MTP to 67 and 45% of control (Fig. 1). In contrast, UO124 (10 μmol/l), the inactive derivative of naringenin (100 μmol/l), partially restored the insulin-induced expression of MTP (P < 0.01 compared with naringenin alone) (Fig. 1A). Preincubation with the selective MEK1/2 inhibitor, U0126 (10 μmol/l), completely blocked the naringenin-inhibited decrease in MTP expression up to 77% of control (P < 0.01 compared with insulin alone), whereas the null isomer (U0124) did not alter the insulin effect.

Activation of the MAPKp38 side-pathway normally decreases the activation of the MAPKerk pathway and therefore acts as a dampener or fine-tunes the signal. When MAPKp38 was inhibited with the selective inhibitor SB203580 (10 μmol/l), the basal level of MTP expression was significantly decreased to 52% of control (Fig. 1B).

**Statistical analysis.** All values are presented as means ± SE of at least three experiments done in duplicate. Means were compared by t tests to determine statistical significance compared with control unless otherwise stated. P < 0.05 was considered significant.**
However, the addition of naringenin or insulin in the presence of the MAPKp38 inhibitor did not potentiate the decrease in MTP expression after 6 h (Fig. 1B). Also, SB202474 (10 μmol/l), the inactive derivative of SB203580, did not alter MTP expression on its own or in the presence of naringenin or insulin. Preincubation of cells with the PI 3-kinase inhibitor wortmannin did not affect MTP mRNA levels in the presence of naringenin or insulin (data not shown).

Naringenin and insulin increase the phosphorylation of ERK1/2 and MAPKp38. To confirm that naringenin and insulin were activating the MAPKerk/MAPKp38 pathway, we examined the time course of ERK1/2 or MAPKp38 phosphorylation by naringenin and insulin. HepG2 cells were preincubated with naringenin (100 μmol/l) or insulin (100 nmol/l) for 15, 30, or 60 min, and the phosphorylation of ERK1/2 or MAPKp38 relative to total ERK1/2 or MAPKp38 protein levels, respectively, were compared with control values. At all time points, there was basal phosphorylation of ERK1/2 or MAPKp38 in control cells (Fig. 2A and B). Naringenin had no effect at 15 min, whereas at 30 min, phosphorylation of ERK1/2 increased by 1.8-fold (P < 0.05). In contrast, the phosphorylation of ERK1/2 by insulin peaked earlier, reaching 2.9-fold over control by 15 min (P < 0.05), and levels were still significantly elevated 2.1-fold at 30 min (P < 0.05). Values for both compounds returned to baseline by 60 min (Fig. 2A). Naringenin significantly increased the phosphorylation of MAPKp38 2.3-fold at 15 min (P < 0.05) and stayed at similar levels up to 60 min. Similarly, the phosphorylation of MAPKp38 by insulin was enhanced 3.4-fold by 15 min (P < 0.05) and also remained elevated up to 60 min (Fig. 2B).

Naringenin and insulin alter the phosphorylation of ERK1/2 via activation of MEK1/2 and MAPKp38. ERK1/2 phosphorylation was determined in HepG2 cells preincubated for 30 min in the absence or presence of the MEK1/2 inhibitor UO126 (10 μmol/l) or the nonactive isoform UO124 (10 μmol/l), followed by a 30-min incubation with naringenin (100 μmol/l) or insulin (100 nmol/l) (Fig. 3A). ERK1/2 phosphorylation were abolished in the presence of UO126 confirming that this compound inhibits MEK1/2, whereas the inactive UO124 had no significant effect. Both naringenin (P < 0.05) and insulin (P < 0.01) significantly increased the phosphorylation of ERK1/2, which was completely inhibited in cells preincubated with UO126 (P < 0.01 for both). The nonactive isoform had no effect on the naringenin-induced increase in ERK1/2 phosphorylation; however, it reduced ERK1/2...
Naringenin and insulin decrease apoB100 secretion via activation of the MAPK<sub>erk</sub> pathway. As previously reported (9), incubation of HepG2 cells with naringenin (100 μmol/l) or insulin (100 nmol/l) for 24 h resulted in a significant decrease in the secretion of apoB100 to 31 and 56% of control, respectively (P < 0.01) (Figs. 4A and B).

Preincubation of cells with the MEK1/2 inhibitor (UO126 [10 μmol/l]) alone increased basal apoB100 secretion to 145% of control (Fig. 4A), consistent with inhibition of basal ERK1/2 phosphorylation (Fig. 3A). The UO126 compound blocked ~50% of the naringenin-induced inhibition of apoB100 secretion (P < 0.05 compared with naringenin alone), whereas UO126 plus insulin completely blocked the insulin-induced decrease in apoB100 secretion such that secretion reached 121% of control (P < 0.05 compared with insulin alone). Conversely, the inactive isoform, UO124 (10 μmol/l), had no significant effect under any experimental condition.

Incubation of HepG2 cells with the MAPK<sub>p38</sub> inhibitor, SB203580 (10 μmol/l), resulted in a significant decrease in secreted apoB100 to 59% of control (P < 0.01), and the inactive inhibitor slightly reduced apoB100 to 87% of control (not significant) (Fig. 4B). Preincubation with SB203580 caused apoB100 secretion to decrease a further 24% in the presence of naringenin and similarly enhanced the insulin-induced decrease by 25% (P < 0.05 compared with naringenin or insulin treatment alone, respectively). The inactive SB202474 did not significantly alter the naringenin- or insulin-mediated decrease in apoB100 secretion (Fig. 4B).

To determine if any of the treatments influenced apoB100 secretion through effects on apoB expression, we measured apoB mRNA abundance. HepG2 cells were treated with naringenin (100 μmol/l), insulin (100 nmol/l), UO126 (10 μmol/l), UO124 (10 μmol/l), SB203580 (10 μmol/l), SB202474 (10 μmol/l), or wortmannin (1 μmol/l) for 6 h. UO124 and SB202474 modestly increased apoB mRNA to 109 and 129% of control, respectively (P < 0.05), whereas other values were unchanged from control (data not shown).

**Simultaneous inhibition of PI 3-kinase and MEK1/2 completely inhibits the naringenin- and insulin-mediated decrease in apoB100 secretion.** Previously, we demonstrated that incubation of HepG2 cells with the PI 3-kinase inhibitor wortmannin prevented most of the insulin-mediated decrease in apoB100 secretion (9). However, the naringenin-induced reduction in apoB100 secretion was only partially blocked. Therefore, we investigated the effect of combined inhibition of MAPK<sub>erk</sub> and PI 3-kinase on apoB100 secretion over a 24-h period. Preincubation of HepG2 cells with wortmannin (1 μmol/l) resulted in a significant 30% increase in the basal levels of apoB100 secretion (P < 0.01) (Fig. 5). Preincubation with wortmannin followed by incubation with naringenin or insulin blunted the naringenin-induced inhibition of apoB100 secretion by 31%, whereas the insulin effect was completely blocked such that apoB100 levels reached 126% of control (P < 0.05 compared with insulin alone, but similar to wortmannin alone). Preincubation of HepG2 cells with both wortmannin (1 μmol/l) and UO126 (10 μmol/l) before the addition of naringenin completely attenuated the naringenin effect relative to control; however,
levels remained significantly below (50%) those observed for co-incubation with the two inhibitors alone. In comparison, the combined inhibition of PI 3-kinase and MEK1/2 in the presence of insulin resulted in complete ablation of the insulin effect and stimulated apoB100 secretion to 219% of control levels ($P < 0.05$), a value similar to that observed for co-incubation with the two inhibitors alone.

**DISCUSSION**

The prevalence of insulin resistance and obesity is increasing rapidly worldwide and represents an increased risk for the development of dyslipidemia and ultimately atherosclerosis (23). Often the insulin resistance is due to defective intracellular insulin signaling; therefore, treatments that can potentially overcome this defect may prove very useful (24–27). Previously, we had shown that the grapefruit flavonoid naringenin activates the insulin-stimulated PI 3-kinase pathway within hepatocytes and does so independent of IRS-1/2 phosphorylation (9). We now extend those findings and show for the first time that naringenin decreases the expression of MTP via activation of the insulin-sensitive MAPKerk pathway and specifically by phosphorylation of ERK1/2 within this pathway. Importantly, a significant proportion of the naringenin-mediated decrease in hepatic apoB100 secretion also depends on activation of the MAPKerk pathway and is modulated by MAPKp38 activation. Furthermore, simultaneous inhibition of PI 3-kinase and MEK1/2 demonstrated that both pathways contribute significantly to the naringenin-stimulated decrease in apoB100 secretion. Insulin was previously shown to decrease MTP expression via the MAPKerk pathway (16); however, our results clearly identify that part of the insulin-mediated decrease in apoB100 secretion is also via activation of the MAPKerk pathway, an effect modulated by MAPKp38 activation. These results are summarized in Fig. 6.

We had previously reported a significant 30% decrease in MTP mRNA expression after 24-h exposure of HepG2 cells to 200 μmol/l naringenin (11). In the present set of experiments, 100 μmol/l naringenin decreased MTP expression by 33% after 6 h, suggesting a rapid response to this flavonoid. Furthermore, insulin rapidly decreased MTP mRNA expression by 55% after 6 h. The naringenin- and insulin-induced decreases in MTP expression both require activation of MAPKerk. Our observations for insulin are consistent with a recent report demonstrating that insulin inhibits an MTP-luciferase reporter construct in HepG2 cells within 6 h (16). Furthermore, naringenin and insulin decreased apoB100 secretion by 69 and 44%, respectively, after 24 h, which also required MAPKerk signaling. Whether the rapid and significant changes we observe in MTP mRNA translate into a significant decrease in MTP protein activity and confer the decrease in apoB100 secretion after 24 h is not entirely clear. The half-life of the MTP protein in HepG2 cells is reported to be 4.4 days (6). Previously, we demonstrated that naringenin is a direct inhibitor of MTP activity, which we concluded contributed to the rapid decrease in apoB100 secretion observed with this flavonoid (8). Indeed, pulse-chase experiments revealed similar kinetics for apoB100 secretion in HepG2 cells exposed to naringenin or an MTP inhibitor (10). In contrast, insulin does not inhibit MTP activity (6). This
difference provides an explanation for why insulin induces greater ERK1/2 activation but decreases apoB100 secretion less than naringenin. Collectively, these results indicate that decreased MTP expression by naringenin and insulin may not mediate acute changes in apoB100 secretion. Nevertheless, our novel observation that MAPKerk and MAPKp38 mediate a significant proportion of the acute decrease in media apoB100 directly links signaling through these pathways to hepatic apoB100 secretion.

The identity of the MAPKerk-sensitive pathway, responsible for the acute regulation of apoB100 secretion, remains to be elucidated. Activation of MAPKerk and/or MAPKp38 may directly affect apoB stability and/or degradation. It has previously been reported that insulin and naringenin do not alter the levels of apoB mRNA, which was confirmed in this study (11,28). However, pulse-chase experiments revealed that naringenin increases intracellular degradation of newly synthesized apoB100 in HepG2 cells (8). Early experiments using primary rat hepatocytes suggested that phosphorylation of apoB100 could be directly increased by insulin stimulation resulting in decreased apoB100 secretion (29–32). It is possible that MAPKerk activation directly alters the phosphorylation of apoB100 and both naringenin and insulin decrease its secretion, in part through this mechanism. Whether apoB100 contains the docking domains potentially required for direct MAPKerk-mediated apoB100 phosphorylation (33) remains to be elucidated.

The downregulation of MTP expression via the MAPKerk pathway we identified with naringenin and insulin likely results in longer-term adaptive decreases in MTP protein and activity leading to reduced apoB100 secretion. In support of this concept, we observed a significant decrease in MTP protein after a 5-day exposure of HepG2 cells to naringenin (11). Furthermore, the fructose-fed insulin-resistant Syrian Golden hamster displays signifi-

There is evidence that SREBP-1a and -2 are downstream effectors of the MAPKerk cascade leading to transactivation of the LDL-r gene (36,37). Furthermore, increased LDL-r expression decreases hepatocyte apoB100 secretion (38). In contrast, we previously demonstrated in HepG2 cells that the naringenin- and insulin-induced stimulation of LDL-r expression was completely blocked by inhibitors of PI 3-kinase, demonstrating that LDL-r expression was entirely upregulated by this pathway (9). However, it is unlikely that inhibition of apoB secretion in vivo by insulin or naringenin occurs via LDL-r upregulation, since apoB secretion was inhibited similarly by both compounds in wild-type and LDL-r (−/−) mouse hepatocytes (9).

Emerging evidence suggests that aberrant MAPKp38 signaling occurs in insulin-resistant or type 2 diabetic skeletal myocytes, adipocytes, and hepatocytes (16,26,27,39–41). Stimulation of MAPKp38 normally inhibits MEK1/2, thereby decreasing signaling through this pathway, and we show here that both PI 3-kinase and MEK1/2 pathways contribute significantly to the decreased apoB100 secretion induced by naringenin or insulin. Our results suggest that regulation of apoB100 secretion by naringenin is partly mediated (50%) through activation of both PI 3-kinase and MAPKerk, whereas the insulin effect is entirely through activation of these two pathways. Furthermore, we show that even in the absence of naringenin or insulin, MAPKerk and MAPKp38 regulate hepatocyte apoB100 secretion. Basal phosphorylation of ERK1/2 inhibits apoB100 secretion, which is attenuated by basal activity of MAPKp38 (Figs. 2 and 4).

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but not as potently as insulin. In addition, the time to peak activation of ERK1/2 phosphorylation was longer for naringenin (30 min) compared with insulin (15 min). The variation in the time course of ERK1/2 activation suggests that the mechanism whereby naringenin activates the MAPK<sup>p38</sup> pathway differs from that of insulin. This concept is consistent with our previous observation that naringenin activates PI 3-kinase independent of IRS-1/2 phosphorylation, suggesting activation of insulin signaling independent of the insulin receptor (9). The small membrane-bound GTPase Ras is the classic upstream activator of the Raf-1/MEK/ERK MAPK<sup>erk</sup> cascade (33) and can be activated either dependent on or independent of insulin receptor phosphorylation (43) (Fig. 6). A recent report showed that insulin inhibits MTP expression in HepG2 cells via Raf-1 but does so independent of Ras (16). Therefore, it is possible that naringenin activates the MAPK<sup>erk</sup> pathway via Ras, which may account for the longer time required for ERK1/2 phosphorylation compared with insulin.

Collectively, we demonstrate here that naringenin, like insulin, inhibits the expression of MTP via MEK1/2 activation and ERK1/2 phosphorylation. Importantly, naringenin and insulin cause an acute decrease in the secretion of apoB100 from HepG2 cells mediated by MAPK<sup>erk</sup>. Furthermore, MAPK<sup>p38</sup> is directly linked to apoB100 secretion such that blocking its activity enhances the naringenin- and insulin-induced activation of MAPK<sup>erk</sup> and decreases apoB100 secretion (Fig. 6). The activation of these insulin-signaling pathways in hepatocytes indicates that naringenin and related compounds may provide insights into novel treatments for insulin resistance in humans, especially if activation of peripheral kinases in these signaling cascades occurs independently of the insulin receptor and IRS-1/2.

**ACKNOWLEDGMENTS**

This work was supported by an operating (T-4386) and program (PG-4854) grant from the Heart and Stroke Foundation of Ontario (to M.W.H.). E.M.A. is the recipient of a joint Astra Zeneca/Heart and Stroke Foundation of Canada research fellowship.

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