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The citrus flavonoids hesperetin and naringenin block the lipolytic actions of TNF-α in mouse adipocytes

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ABSTRACT

Obese adipose tissue is characterized by an excessive production of inflammatory adipokines including tumor necrosis factor-α (TNF-α). TNF-α stimulates free fatty acid (FFA) secretion through adipocyte lipolysis, and increased plasma levels of FFA promote insulin resistance. In this report, we show that hesperetin and naringenin, two citrus flavonoids, inhibit TNF-α-stimulated FFA secretion from mouse adipocytes. These flavonoids block the TNF-α-induced activation of the NF-κB and ERK pathways. Moreover, hesperetin and naringenin prevent TNF-α from downregulating the transcription of two antilipolytic genes, perilipin and PDE3B. These effects are mediated through the inhibition of the ERK pathway. In contrast, the inhibition of the NF-κB pathway by hesperetin and naringenin suppresses the transcription of IL-6, which induces FFA secretion in an autocrine manner. Our results provide novel evidence that hesperetin and naringenin directly inhibit TNF-α-stimulated FFA secretion. These findings may be useful for ameliorating FFA-induced insulin resistance.

1. Introduction

Obese adipose tissue produces excess inflammatory adipokines, including tumor necrosis factor-α (TNF-α), which are involved in the development of insulin resistance and type 2 diabetes [1,2]. TNF-α plays a pivotal role in obesity-related insulin resistance, and its expression is increased in the obese adipose tissue of both rodents and humans [3–5]. TNF-α induces insulin resistance through its direct ability to attenuate insulin receptor signaling [6,7]. Additionally, several reports suggest that TNF-α indirectly promotes insulin resistance by increasing the circulating levels of free fatty acid (FFA) [8–10]. Interestingly, the interstitial levels of TNF-α and FFA in adipose tissue are positively correlated [11]. Chronically elevated plasma levels of FFA promote insulin resistance by impairing peripheral glucose utilization [10,12]. Thus, the reduction of excess FFA levels is important in the improvement of insulin resistance.

TNF-α promotes FFA secretion through adipocyte lipolysis. Although the mechanism by which TNF-α induces lipolysis has yet to be completely elucidated, studies have proposed that TNF-α downregulates the expression of antilipolytic genes, such as perilipin and phosphodiesterase-3B (PDE3B) [3,13,14]. Perilipin is located on the surface of intracellular triglyceride lipid droplets and regulates the access of hormone-sensitive lipase (HSL), which hydrolyzes triglycerides to FFA and glycerol [15–17]. PDE3B is the major hydrolytic enzyme of cAMP activated by insulin signaling [18,19]. Decreased intracellular levels of cAMP lead to reduced activity of protein kinase A (PKA), which phosphorylates and activates HSL [20]. Some intracellular signaling pathways mediate the effects of TNF-α on adipocyte lipolysis. Nuclear factor-κB (NF-κB) and extracellular signal-regulated kinase (ERK) are important for TNF-α-induced lipolysis, because they regulate the expression of lipolysis-related genes including perilipin and PDE3B [21–23]. Thus, the regulation of antilipolytic gene expression through the activation of intracellular signaling pathways may be the mechanism by which TNF-α induces lipolysis.

Flavonoids, which are found in fruits and vegetables, have beneficial effects on health. Hesperetin and naringenin are flavonoids that are abundant in citrus fruits, and they exert antioxidant, anti-inflammatory and antiproliferative effects [24–27]. A cohort study found that the intake of hesperetin and naringenin reduces the risk of chronic diseases such as cerebrovascular disease and asthma [28]. However, the effects of hesperetin and naringenin on adipocyte function, especially FFA secretion, remain largely unknown.

Abbreviations: TNF-α, tumor necrosis factor-α; FFA, free fatty acid; NF-κB, nuclear factor-κB; ERK, extracellular signal-regulated kinase; PDE3B, phosphodiesterase-3B; IL-6, interleukin-6; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

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In this report, we show that hesperetin and naringenin block TNF-α-stimulated FFA secretion by inhibiting the NF-κB and ERK pathways in mouse adipocytes. The inhibition of the ERK pathway prevents TNF-α from downregulating the transcription of two antilipolytic genes, perilipin and PDE3B. In contrast, the inhibition of the NF-κB pathway suppresses the transcription of IL-6, which also induces FFA secretion. Our results provide novel evidence that hesperetin and naringenin directly inhibit TNF-α-stimulated FFA secretion. These findings may be useful for developing treatments to ameliorate FFA-induced insulin resistance.

2. Materials and methods

2.1. Reagents

We purchased the following reagents: TNF-α, IL-6, control IgG and IL-6 neutralizing antibody (R&D Systems; Minneapolis, MN); hesperetin (Wako Pure Chemical Industries; Osaka, Japan); naringenin, isobutylmethylxanthine (IBMX) and dexamethasone (DEX) (Sigma; St. Louis, MO); insulin (Cell Science & Technology Institute; Sendai, Japan); Dulbecco's modified Eagle's medium (DMEM), bovine serum (BS) and fetal bovine serum (FBS) (Invitrogen; Carlsbad, CA); MG-132 (BIOMOL; Plymouth Meeting, PA); and U0126 (Cell Signaling Technology; Beverly, MA).

2.2. Cell culture

3T3-L1 cells (Health Science Research Resources Bank; Osaka, Japan) were maintained in DMEM supplemented with 10% BS. After the preadipocytes reached confluency, they were induced to differentiate into mature adipocytes by replacing the medium with 10% FBS-supplemented DMEM containing 0.5 mM IBMX, 0.25 μM DEX and 5 μg/mL insulin for 3 days. Then, the medium was replaced with 10% FBS-supplemented DMEM containing 5 μg/mL insulin, and it was changed every 3 days for the next 6 days. Primary adipose cells derived from mouse epididymal fat pads (Primary Cell; Hokkaido, Japan) were cultured according to the manufacturer's instructions.

2.3. Fatty acid assay

Differentiated adipocytes were incubated overnight in DMEM with 2.0% fatty acid-free bovine serum albumin (Sigma) in the absence of serum. The following morning, cells were treated with TNF-α or IL-6 for 24 h after a 30-min pretreatment with flavonoids, signaling inhibitors, or neutralizing antibodies. The levels of free fatty acids were determined by using an acyl-CoA oxidase-based colorimetric kit (NEFA-V2; Alfresa, Osaka, Japan).

2.4. Cell viability assay

Viability was assessed by using an MTT assay kit (BioAssay Systems; Hayward, CA), according to the manufacturer's instructions. This assay is based on the conversion of a tetrazolium salt, MTT, to formazan by living cells. In brief, cells were incubated with MTT reagent for 4 h at 37 °C, and the produced formazan was dissolved with solubilization solution. Absorbance was measured at 570 nm, and data from TNF-α-treated wells were expressed relative to control wells to obtain a cell survival index.

2.5. Real-time RT-PCR analysis

Total RNA was isolated (TRIzol reagent; Invitrogen), and the reverse transcription reaction was performed (Transcriptor First Strand cDNA Synthesis Kit; Roche, Basel, Switzerland), according to the manufacturers' instructions. In brief, the reverse transcription reaction was performed with oligo(dT) and random hexamers for 10 min at 25 °C, followed by 60 min at 50 °C. PCR amplification was performed by using LightCycler FastStart DNA Master Plus SYBR Green 1 (Roche). Each reaction contained 4 μL of 5× Master Mix, 1 μL of template cDNA and 500 nM primers in a final volume of 20 μL. Duplicate samples were incubated in the LightCycler for an initial denaturation at 94 °C for 30 s, followed by 40 PCR amplification cycles of 95 °C for 10 s, 60 °C for 5 s and 72 °C for 12 s. To confirm the amplification of specific transcripts, melting curve profiles were produced at the end of each PCR. The relative quantity of mRNAs was determined by using the comparative C_t method and normalized by using β-actin as an endogenous control. All samples were run in duplicate. The primers were as follows: perilipin forward, 5′-CTC TGG GAA GCA TCG AGA AG-3′; perilipin reverse, 5′-GCA TGG TGT GTC GAG AAA GA-3′; PDE3B forward, 5′-GGT GAT GGT GGT GAA GAA-3′; PDE3B reverse, 5′-AGT GAG GTG GTG CAT TAG-3′; IL-6 forward, 5′-AGT TGC CTT CTT GGG ACT GAT-3′; IL-6 reverse, 5′-TCC ACG ATT TCC CAG AGA AC-3′; β-actin forward, 5′-AGC CAT GTA CGT AGC CAT CC-3′; and β-actin reverse, 5′-TCC CTC TCA GCT GTG GTG GTG AA-3′.

2.6. Western blot analysis

Cells were lysed with RIPA buffer containing PMSF, proteasome inhibitor cocktail and sodium orthovanadate (Santa Cruz Biotechnology; Santa Cruz, CA). Whole cell extracts were separated on 10% SDS–PAGE gels and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories; Hercules, CA). The membranes were blocked with a TBS solution containing 0.1% Tween 20 (TBS-T) and 2% Blocking Agent (GE Healthcare UK Ltd; Buckinghamshire, UK). After three washes in TBS-T, the membranes were incubated with a 1:2000 dilution of a primary antibody. After another three washes in TBS-T, the membranes were incubated with a 1:10,000 dilution of the corresponding secondary antibody. Chemiluminescence reagent ECL Advance (GE Healthcare) was used to visualize the blots. Antibodies against IκB-α, phospho-ERK1/2 (Thr202/Tyr204) and β-actin were purchased from Cell Signaling Technology.

2.7. Statistical analysis

Values are given as means ± SD. Data were considered significant at $p < 0.01$ based on ANOVA with Student–Newman–Keuls (SNK) post-hoc test.

3. Results

3.1. Hesperetin and naringenin inhibit TNF-α-stimulated FFA secretion from mouse adipocytes

To determine if hesperetin and naringenin regulate TNF-α-induced lipolysis, we first measured FFA levels by performing a fatty acid assay. 3T3-L1 adipocytes were stimulated with TNF-α in the presence or absence of hesperetin or naringenin. Hesperetin and naringenin significantly inhibited TNF-α-stimulated FFA secretion (Fig. 1A). To further validate the inhibitory effects of these flavonoids on TNF-α-stimulated FFA secretion, we next performed the fatty acid assay with mouse primary adipocytes. As shown in Fig. 1B, similar results were obtained with the primary cells. There were no treatment-related changes in cell viability (Fig. 1C; 3T3-L1 adipocytes; Fig. 1D, primary cells).

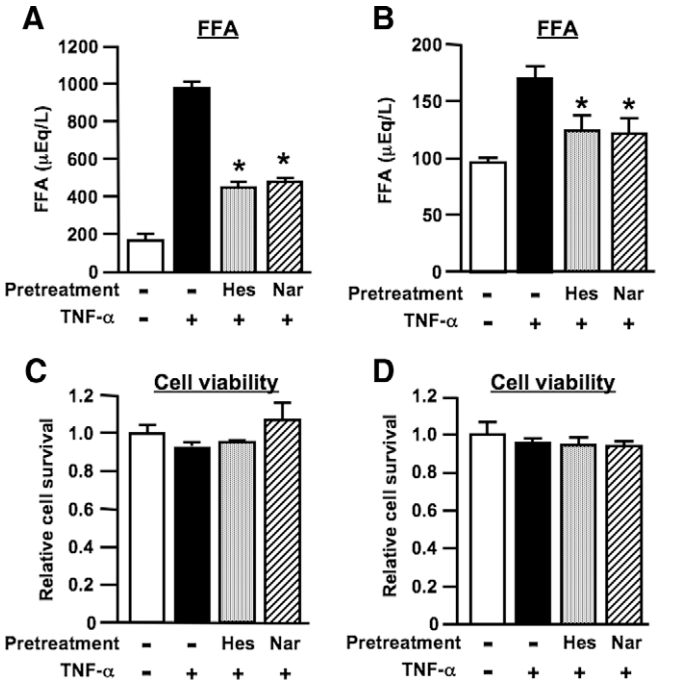

Fig. 1. Hesperetin and naringenin inhibit TNF-α-stimulated FFA secretion from mouse adipocytes. (A) 3T3-L1 adipocytes were pretreated with hesperetin (100 μM) or naringenin (100 μM) for 30 min and then stimulated with TNF-α (10 ng/mL) for 24 h. FFA levels in the culture medium were measured by fatty acid assay. (B) Mouse primary adipocytes were pretreated with hesperetin (100 μM) or naringenin (100 μM) for 30 min and then stimulated with TNF-α (50 ng/mL) for 24 h, followed by fatty acid assay. (C,D) Cell viability was determined by MTT assay and expressed as the ratio relative to the control (C, 3T3-L1 adipocytes; D, primary cells). Values are means ± SD of triplicate determinations. *$p < 0.01$ versus TNF-α-treated cells. Hes, hesperetin; Nar, naringenin.

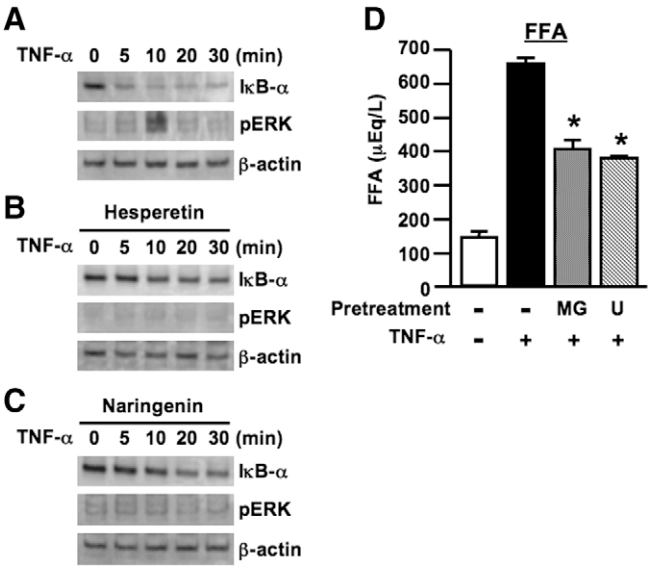

Fig. 2. Hesperetin and naringenin suppress TNF-α-stimulated FFA secretion by inhibiting the NF-κB and ERK pathways. (A–C) Cells were pretreated with hesperetin or naringenin for 30 min and then stimulated with TNF-α for 5–30 min. Total cell lysates were extracted, and IκB-α degradation and ERK phosphorylation were analyzed by Western blotting. β-Actin served as the loading control. Experiments were repeated three times. (D) Cells were pretreated with MG-132 (10 μM) or U0126 (10 μM) for 30 min, and then stimulated with TNF-α (10 ng/mL) for 24 h. FFA levels were measured by fatty acid assay. Values are means ± SD of triplicate determinations. *$p < 0.01$ versus TNF-α-treated cells. MG, MG-132; U, U0126.

3.2. Hesperetin and naringenin suppress TNF-α-stimulated FFA secretion by inhibiting the NF-κB and ERK pathways

To elucidate the mechanism by which hesperetin and naringenin inhibit TNF-α-stimulated FFA secretion, we sought to identify the intracellular signaling pathways that are regulated by hesperetin and naringenin. Because the NF-κB and ERK pathways are involved in TNF-α-induced lipolysis [21,22], we first determined whether hesperetin and naringenin directly influence the TNF-α-induced activation of the NF-kB and ERK pathways by assessing the inhibitor of κB-α (IκB-α) degradation and ERK phosphorylation. IκB-α forms a complex with the NF-κB transcription factor, preventing its translocation from the cytoplasm to the nucleus, the site of its transcriptional activity [29]. Thus, IκB-α degradation leads to the activation of NF-κB. As shown in Fig. 2A, IκB-α in adipocytes was degraded after a 5- to 30-min incubation with TNF-α, and ERK phosphorylation was increased after 10 min of incubation. Hesperetin and naringenin suppressed the IκB-α degradation and ERK phosphorylation (Fig. 2B and C). To confirm that the NF-κB and ERK pathways are involved in TNF-α-stimulated FFA secretion, we used MG-132 and U0126, which inhibit the NF-κB and ERK pathways, respectively. The inhibition of the NF-κB and ERK pathways suppressed TNF-α-stimulated FFA secretion in 3T3-L1 adipocytes (Fig. 2D). These data indicate that hesperetin and naringenin suppress TNF-α-induced adipocyte lipolysis by inhibiting the NF-κB and ERK pathways.

3.3. Hesperetin and naringenin prevent the TNF-α-mediated downregulation of perilipin and PDE3B

Because the downregulation of antilipolytic genes is a key step in TNF-α-induced lipolysis [13,14], we next determined whether hesperetin and naringenin influence the transcription of perilipin and PDE3B. Consistent with previous reports, TNF-α downregulated perilipin and PDE3B mRNA (Fig. 3A and B). However, hesperetin and naringenin prevented the TNF-α-induced decrease in perilipin and PDE3B mRNA. We next investigated whether the NF-κB and ERK pathways are involved in the TNF-α-mediated downregulation of perilipin and PDE3B by assessing the effects of MG-132 and U0126. Interestingly, the inhibition of the ERK pathway, but not the NF-κB pathway, blocked TNF-α-mediated downregulation of perilipin and PDE3B mRNA (Fig. 3C and D). These results indicate that hesperetin and naringenin prevent TNF-α-mediated downregulation of perilipin and PDE3B through the inhibition of the ERK pathway.

3.4. Hesperetin and naringenin inhibit TNF-α-stimulated FFA secretion through the downregulation of IL-6

Although we determined that the inhibition of the ERK pathway by hesperetin and naringenin is required for the recovery of antilipolytic gene expression, it was unclear how the inhibition of the NF-κB pathway by these flavonoids suppressed the TNF-α-stimulated FFA secretion. TNF-α is a powerful local regulator within adipose tissue and plays a pivotal role in the production of other adipokines [2,30]. Thus, it is possible that TNF-α produces other adipokines through the activation of the NF-κB pathway that lead to FFA secretion in an autocrine manner. One candidate adipokine is interleukin-6 (IL-6), which is also involved in insulin resistance and adipocyte lipolysis [31,32]. Therefore, we examined whether hesperetin and naringenin influence TNF-α-induced IL-6 synthesis. As shown in Fig. 4A, hesperetin and naringenin significantly downregulated the TNF-α-induced transcription of IL-6. We then used MG-132 and U0126 to determine if the NF-κB and ERK pathways are involved in TNF-α-induced IL-6 synthesis. Inhibition of the NF-κB pathway, but not the ERK pathway, blocked the TNF-α-induced transcription of IL-6 (Fig. 4B). These results suggest that hes-

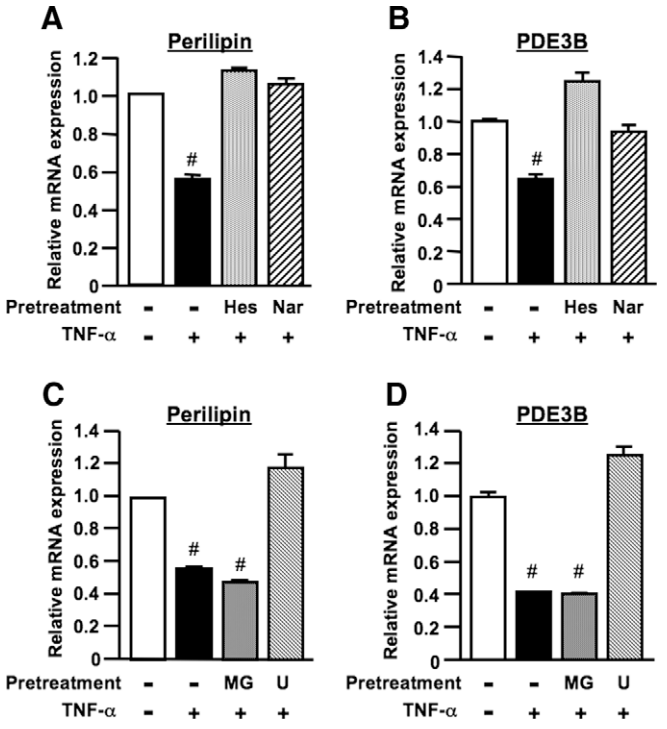

Fig. 3. Hesperetin and naringenin prevent TNF-α-mediated downregulation of perilipin and PDE3B. (A–D) Cells were pretreated with hesperetin, naringenin, MG-132 or U0126 for 30 min and then stimulated with TNF-α for 3 h. Total RNA was extracted to measure the mRNA levels of perilipin and PDE3B by real-time RT-PCR. Results are the means ± SD of three independent experiments. $^{\#}p < 0.01$ versus control cells. Hes, hesperetin; Nar, naringenin; MG, MG-132; U, U0126.

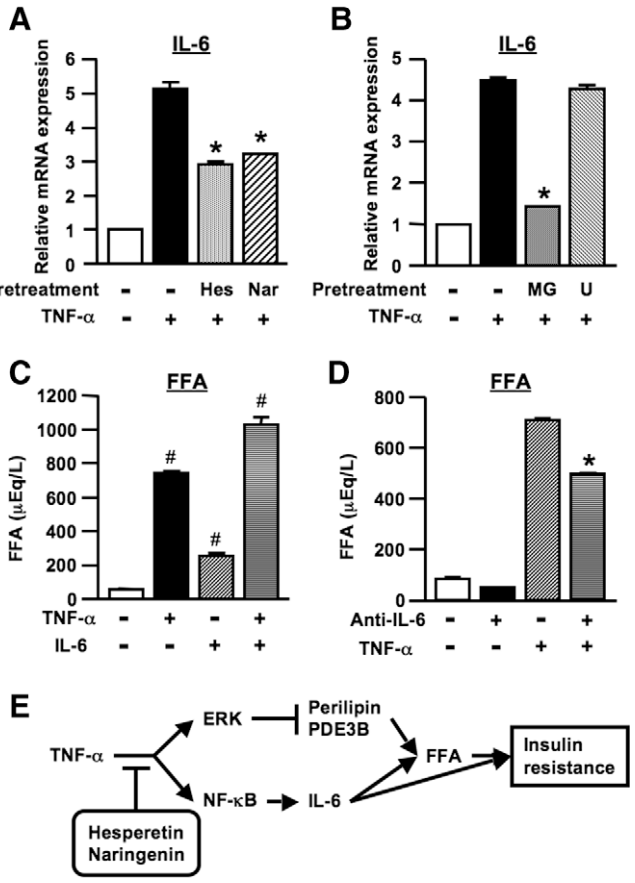

Fig. 4. Hesperetin and naringenin inhibit TNF-α-stimulated FFA secretion through the downregulation of IL-6. (A,B) Cells were pretreated with hesperetin, naringenin, MG-132 or U0126 for 30 min and then stimulated with TNF-α for 1 h. Total RNA was extracted to measure the mRNA levels of IL-6 by real-time RT-PCR. Results are the means ± SD of three independent experiments. $^{*}p < 0.01$ versus TNF-α-treated cells. (C) Cells were stimulated with TNF-α (10 ng/mL) and/or IL-6 (100 ng/mL) for 24 h. FFA levels were measured by fatty acid assay. Values are means ± SD of triplicate determinations. $^{\#}p < 0.01$ versus control cells. (D) Cells were pretreated with control IgG (10 μg/mL) or IL-6 neutralizing antibody (10 μg/mL) for 30 min and then stimulated with TNF-α for 24 h. FFA levels were measured by fatty acid assay. Values are means ± SD of triplicate determinations. $^{*}p < 0.01$ versus TNF-α-treated cells. (E) Schematic representation of the inhibition of TNF-α-induced lipolysis by hesperetin and naringenin. Hes, hesperetin; Nar, naringenin; MG, MG-132; U, U0126.

peretin and naringenin inhibit TNF-α-induced IL-6 synthesis through the inhibition of the NF-κB pathway. To confirm that IL-6 induces adipocyte lipolysis, we assessed the FFA secretion. As shown in Fig. 4C, IL-6 stimulated FFA secretion from 3T3-L1 adipocytes, although the effect was weaker than that of TNF-α. Cotreatment with TNF-α and IL-6 additively increased FFA secretion. To determine if the TNF-α-induced IL-6 production promotes adipocyte lipolysis in an autocrine manner, we assessed the effects of IL-6 neutralizing antibody on TNF-α-stimulated FFA secretion. Neutralization of endogenous IL-6 suppressed the TNF-α-stimulated FFA secretion (Fig. 4D). Collectively, these data indicate that the inhibition of the NF-κB pathway by hesperetin and naringenin suppresses TNF-α-stimulated FFA secretion through the downregulation of IL-6.

4. Discussion

In this report, we demonstrated that hesperetin and naringenin inhibit TNF-α-induced adipocyte lipolysis (Fig. 4E). We showed for the first time that these flavonoids inhibit both the NF-κB and ERK pathways, which in turn suppress TNF-α-stimulated FFA secretion. The inhibition of the ERK pathway is required for the recovery of perilipin and PDE3B mRNA. In contrast, the inhibition of NF-κB is involved in the downregulation of IL-6, which promotes FFA secretion in an autocrine manner. Thus, this study reveals a mechanism by which hesperetin and naringenin inhibit TNF-α-induced adipocyte lipolysis.

It is particularly interesting that hesperetin and naringenin regulate adipocyte function. Although previous studies showed that hesperetin and naringenin have various pharmacological properties including anti-inflammatory activity, most of these studies used non-fat cells, such as macrophages [26,27]. Despite recent studies demonstrating that adipocytes secrete bioactive factors involved in inflammation and metabolic disorders [2], the effects of hesperetin and naringenin on adipocyte function remain largely unknown. In this study, we demonstrated that hesperetin and naringenin inhibit TNF-α-induced adipocyte lipolysis through the regulation of ERK-dependent antilipolytic gene expression and NF-κB-dependent IL-6 synthesis. Thus, hesperetin and naringenin may prevent obesity-induced insulin resistance by inhibiting TNF-α-stimulated FFA secretion from adipocytes.

Understanding the primary target molecules of hesperetin and naringenin is key to the evaluation of these flavonoids as preventive or therapeutic agents for metabolic disorders. There is accumulating evidence that flavonoids, including hesperetin and naringenin, modulate the activity of a number of protein kinases that regulate various intracellular signaling cascades, such as phosphoinositide 3-kinase (PI3K), Akt/protein kinase B (Akt/PKB), tyrosine kinases, protein kinase C (PKC) and mitogen-activated protein kinase (MAPK). This modulatory action is mediated via the binding of the flavonoids to the ATP-binding sites on enzymes ([33] and references therein). In addition to protein kinases, the proteasome is also a target for flavonoids. Several studies have shown that flavonoids such as apigenin and quercetin inhibit the chymotryp-

sin-like activity of the proteasome [34,35]. In this study, we show that hesperetin and naringenin suppress TNF-α-induced ERK phosphorylation and IκB-α degradation. The inhibition of ERK phosphorylation by these flavonoids may result from modulating upstream regulatory kinases. In contrast, the inhibition of IκB-α degradation may result from inhibiting proteasome activity. Thus, hesperetin and naringenin are likely to have extensive target molecules and regulate cellular function.

Another important finding in our study is that hesperetin and naringenin regulate IL-6 synthesis in adipocytes. The expression of IL-6 and TNF-α is elevated in obesity [36]. In addition, IL-6 impairs insulin signaling and promotes insulin resistance [32,37]. Thus, the control of IL-6 expression is an important strategy for the improvement of insulin resistance. This study shows that hesperetin and naringenin inhibit TNF-α-induced IL-6 synthesis. This finding suggests that hesperetin and naringenin exert inhibitory effects on IL-6-mediated metabolic disorders.

5. Conclusion

This data suggest that hesperetin and naringenin inhibit TNF-α-induced adipocyte lipolysis and that they may be useful for ameliorating insulin resistance. Future studies will verify the ability of hesperetin and naringenin to inhibit lipolysis-related disorders *in vivo*.

Acknowledgments

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