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# Hesperidin Ameliorates TNF-α-Mediated Insulin Resistance in Differentiated 3T3-L1 Cells

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**Abstract** – Adipose inflammation is linked to the development of insulin resistance and type 2 diabetes. Hesperidin (HES) is a flavonoid with antioxidant, anti-inflammatory and anti-diabetic properties. However, whether HES improves inflammation-mediated insulin resistance in adipose tissues remains unclear. The purpose of this study was to investigate whether HES attenuates inflammation-mediated insulin resistance in adipose tissue. Herein, RAW 264.7 cells and differentiated 3T3-L1 adipocytes were pretreated with various concentrations of HES in complete media for 1 h and then cultured in the presence or absence of LPS or TNF-α. Our results demonstrated that HES remarkably inhibited LPS-induced production of IL-6, TNF-α, and NO by RAW 264.7 cells in a dose-dependent manner. Also, HES inhibited TNF-α-induced production of IL-6 and $PGE_2$ in differentiated 3T3-L1 cells, while upregulated TNF-α-suppressed expression of adiponectin and PPAR-γ mRNA. These findings suggest that HES may ameliorate inflammation-mediated insulin resistance in adipose tissue.
**Keywords** – Hesperidin; IL-6, $PGE_2$, Adiponectin, PPAR-γ, Insulin resistance, Adipocyte

## Introduction

Obesity is known as one of the most common risk factors for type 2 diabetes and cardiovascular disease (Bastard *et al*., 2006). Chronic adipose inflammation in obesity is associated with development of insulin resistance and metabolic syndrome leading to type 2 diabetes and cardiovascular disease (Xu *et al*., 2003). Adipose tissue is an immune organ that produces numerous immunomodulatory factors that link adipose inflammation to insulin resistance (Berg and Scherer, 2005). Macrophages in adipose tissue induce chronic inflammation and insulin resistance by secreting pro-inflammatory cytokines, COX-2, and inducible NOS (iNOS) (Weisberg *et al*., 2003; Dallaire *et al*., 2008; Hsich *et al*., 2009). TNF-α highly expressed in adipose macrophages in obese animals and human subjects, contributes to induction of insulin resistance and hepatic steatosis seen in obesity (Ruan and Lodish, 2003; De Taeye *et al*., 2007).

Hepatic insulin resistance is a major contributor to hyperglycemia in metabolic syndrome and type II diabetes (Meshkani and Adeli, 2009). IL-6 is preferentially released from adipose tissue, especially visceral fat, in obesity (Vozarova *et al*., 2001; Fontana *et al*., 2007). IL-6 plays a crucial role in linking obesity-derived chronic low grade inflammation and insulin resistance (Kim *et al*., 2009). Moreover, IL-6 has been reported to cause hepatic insulin resistance in mice (Klover *et al*., 2003). $PGE_2$ mediates development of insulin resistance as well as inflammatory responses and tissue injuries in obesity. $PGE_2$ release in adipose tissue and adipocytes has been reported to contribute to insulin resistance (Fain *et al*., 2004). COX-2 activation in fat inflammation has been shown to be associated with the development of insulin resistance and fatty liver in high fat-induced obese rats (Hsieh *et al*., 2009).

Adiponectin is a protective adipokine with anti-diabetic, anti-inflammatory, and anti-atherogenic properties on the insulin resistance in the metabolic syndrome and diabetes (Hosch *et al*., 2006; Buechler *et al*., 2011). Adiponectin was shown to inhibit LPS-induced NF-kappaB activation and IL-6 production in adipocytes (Ajuwon and Spurlock, 2005). Peroxisome proliferator-activated receptor (PPAR)-γ acts as a negative regulator of obesity-related inflammatory responses and a key activator of insulin sensitivity in adipocytes (Ricote and Glass, 2007). PPAR-γ plays a crucial role in the regulation of adipogenesis, lipid metabolism, and glucose homeostasis (Sharma and Staels, 2007; Staels, 2007).

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Hesperidin (HES) is a citrus flavonoid with antioxidant, immunomodulatory, and anti-inflammatory effects (Garg *et al*., 2001; Yeh *et al*., 2007; Jain and Parmar, 2010). Recently, HES was also shown to have antidiabetic and hypolipidemic effects in diabetic rats (Jung *et al*., 2006; Akiyama *et al*., 2009; Akiyama *et al*., 2010). However, whether HES improves inflammation-mediated insulin resistance in adipose tissues remains unclear. TNF-α treatment of differentiated adipocytes has been shown to induce inflammatory responses and insulin resistance in adipose (Ruan and Lodish, 2003). Accordingly, this study was examined whether HES improves the TNF-α-induced insulin resistance in differentiated 3T3-L1 cells.

## Experimental

**Materials** – RAW 264.7 cells and 3T3-L1 preadipocytes were purchased from the Korean Cell Bank (Seoul, Korea). Dexamethasone, insulin, isobutylmethyxanthine, recombinant TNF-α, IL-6, and IL-1β were purchased from Sigma (St. Louis, MO) except where indicated differently. Hesperidin (HES) was obtained from Dae Keun Kim professor in college of pharmacy, Woosuk University and dissolved in dimethyl sulfoxide (DMSO) for *in vitro* experiment.

**Extraction and isolation of hesperidin** – The fruit peels of *Citrus aurantium* were collected and air-dried in December 2009 at Wanju, Chonbuk, Korea. A voucher specimen was deposited in the herbarium of the college of pharmacy, Woosuk University (WSU-09-018). The $^{1}$H- and $^{13}$C-NMR spectra were determined on a JEOL JMN-EX 400 spectrometer. The TLC was carried out on pre-coated silica gel F254 plates (Merck, Darmstadt, Germany), and the silica gel for column chromatography was Kiesel gel 60 (230 - 400 mesh, Merck). The column used for LPLC was the Lobar A (Merck Lichroprep Si 60, 240 - 10 mm). The shade dried plant material (500 g) was extracted three times with MeOH at room temperature and filtered. The extracts were combined and evaporated in vacuo at 40 ºC. The resultant methanolic extract (95 g) was partitioned with ethyl acetate three times to afford an ethyl acetate-soluble fraction on drying (24 g). The ethyl acetate fraction (5 g) was chromatographed on silica gel column ($CH_2Cl_2$-MeOH, 10 : 1 → 1 : 1) to give six fractions (MC1-MC6). The fraction MC3 (1.2 g) was purified by Lobar-A column ($CH_2Cl_2$-MeOH, 5 : 1 1) to give hesperidin (HES; 210 mg) (Han *et al*., 2001).

**Cell culture** – RAW 264.7 cells were maintained in complete DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Sigma) and 1 × antibiotic/antimycotic (Invitrogen). The RAW 264.7 cells were pretreated with various concentrations of HES in complete media for 1 h and then cultured for 24 h for IL-6 and NO production and for 6 h for TNF-α in the presence or absence of LPS 1 μg/ml (Sigma Chemical Co., St., Louse, MO) at 37 ºC in a humidified atmosphere containing 5% $CO_2$. The cells were then harvested cell supernatants and stored at −70 ºC for proinflammatory cytokine and NO assays.

**Culture and differentiation of 3T3-L1 cells** – The 3T3-L1 preadipocytes were maintained in complete DMEM containing high glucose supplemented with 10% BCS (Sigma) and 1 × antibiotic/antimycotic. The confluent 3T3-L1 preadipocytes were maintained for 2 days in the complete DMEM with 1 μM insulin, 0.5 mM isobutylmethylxanthine and 0.1 μM dexamethasone and 10% FBS and then for 2 days in the culture medium with 1 μM insulin and 10% FBS. After an additional 3 - 6 days in culture medium, more than 90% of the cells had accumulated fat droplets. The differentiated 3T3-L1 adipocytes were pretreated with 50 and 100 μM HES in complete media for 1 h and then cultured for 4 h for IL-6 gene expression and 24 h for IL-6 and $PGE_2$ production in the presence or absence of recombinant TNF-α 10 ng/ml at 37 ºC in a humidified atmosphere containing 5% $CO_2$. The cells were then harvested for RT-PCR and cell supernatants stored at −70 ºC for IL-6 and $PGE_2$ assays.

**Cytokine assay** – The concentrations of cytokines in the supernatants from RAW 264.7 cells and differentiated 3T3-L1 cells were determined by using cytokine monoclonal antibodies (BD Biosciences Pharmingen, U.S.A.). All measurements were carried out in triplicate. The results were measured in picograms per milliliter at 450 nm using an ELISA microplate reader (Molecular Devices Co., Ltd., U.S.A.). The lower limit of sensitivity for each of the ELISA was equal to or smaller than 5 pg/ml.

**NO assay** – The concentrations of NO (nitric oxide) in the supernatants in RAW 264.7 cells harvested from the culture were assayed by adding 100 μl of freshly prepared Griess reagent to 100 μl of the sample in 96-well plates, and then reading the absorbance at 540 nm after 10 minutes using ELISA.

**$PGE_2$ immunoassay** – $PGE_2$ concentration in the supernatants of differentiated adipocytes was determined by using a monoclonal antibody enzyme immunoassay kit from Cayman Chemical, according to the manufacturer's instruction. Concentrations of $PGE_2$ were measured at 405 nm using ELISA.

**Total RNA isolation and RT-PCR** – Following preincubation with HES for 1 h, the RAW 264.7 cells, Hepa-1c1c7 cells, and differentiated 3T3-L1 cells were

incubated for indicated hours in the presence or absence of LPS 1 μg/ml, TNF-α 10 ng/ml, or IL-6 20 ng/ml at 37 °C and 5% $CO_2$. Total RNA was extracted from the cells using an RNA purification kit (QIAGEN) according to the manufacturer's instructions and quantitated spectrophotometrically at 260 nm. cDNA synthesis from total RNA (2 μg) was performed with QuantiTect® Reverse Transcription kit (QIAGEN). PCR was performed in a 20 μl final volume containing 2 μl of the first strand cDNA, 1 μM of sense and antisense primers (BIONEER, Kor.), and 10 μl of 400 nM of QuantiTect® SYBR Green PCR Master Mix (QIAGEN) using a MultiGene PCR (Labnet International Inc.). With a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as an internal control, was amplified by PCR at the same time. Amplification was performed for 15 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s in a thermocycler (GeneAmp 9600-R, Perkin-Elmer, Wellesley, MA). The primers were as follows: IL-6 (sense 5'-CATAGCTACCTGGAGTACATGA-3' and antisense 5'-CATTCATATTGTCAGTTCTTCG-3'); adiponectin (sense 5'-GCAGAGATGGCACTCCTGGA-3' and antisense 5'- -3'); PPAR-γ (sense 5'-GCTCTAGACGTGACAATCTGT CTGAGGTCTGTCAT-3' and antisense 5'-CGGGATCCG TTGTCGGTTTCAGAAATGCCTTGCAGTG-3'); GAPDH (sense 5'-GCCAAGGTCATCCATGACAAC-3' and antisense 5'-AGTGTAGCCCAAGATGCCCTT-3') was used as positive control. The amplified PCR products were analyzed by electrophoresis on a 1.2% agarose gels and visualized by ethidium bromide staining. Quantification of the band intensity on the Hyperfilm was performed using the public domain NIH image software.

**Statistical Analysis** – All data were expressed as means ± standard error (S.E.). Experiments were always run in triplicate and repeated at least twice. Analysis of variation and Student's *t*-test were used to determine statistical significance, and $p < 0.05$ was considered to be statistically significant.

## Results and Discussion

**Hesperidin inhibited LPS-induced production of proinflammatory cytokines and NO in RAW 264.7 cells** – An enhanced infiltration of macrophages in obese adipose tissue is characterized by low grade chronic inflammation (Weisberg *et al*., 2003). Production of proinflammatory cytokines by macrophages in obese tissue contributes to formation of insulin resistance (De Taeye *et al*., 2007). In the present study, RAW 264.7 cells were pretreated with various concentrations of HES in complete media for 1 h and then cultured for 6 h for TNF-α production or for 24 h for IL-6 and NO in the presence or absence of LPS 1 μg/ml at 37 °C in a humidified atmosphere containing 5% $CO_2$. Concentration of

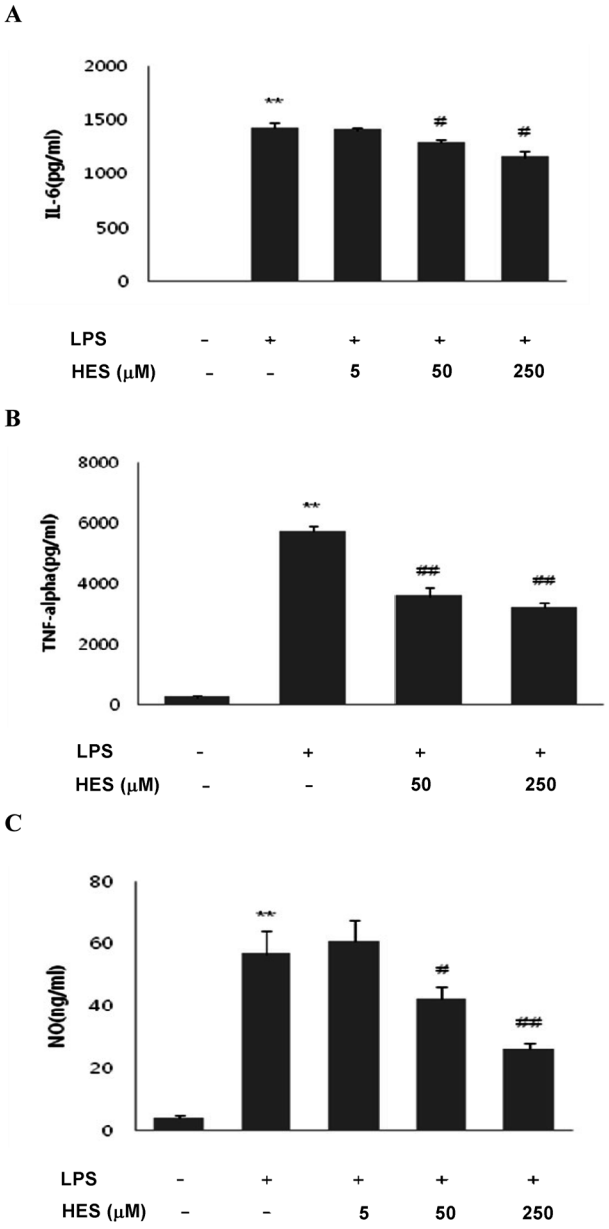


**Fig. 1.** Hesperidin inhibits LPS-induced production of proinflammatory cytokines and NO in RAW 264.7 cells.
RAW 264.7 cells were preincubated with various concentrations of hesperidin (HES) for 1 h and then cultured for 6 h for TNF-α production or 24 h for IL-6 and NO in the presence or absence of LPS. Concentration of cytokines and NO was measured using ELISA. Each value represents the mean ± S.E. $^{**}$ ($p < 0.01$): Significantly different from the value in negative control. $^{\#}$ ($p < 0.05$) and $^{\#\#}$ ($p < 0.01$): Significantly different from the value in positive controls.

proinflammatory cytokines and NO in the cell supernatants was measured using ELISA. These results demonstrated that HES remarkably inhibited LPS-induced production of IL-6, TNF-α, and NO by RAW 264.7 cells in a dose-dependent manner (Fig. 1). Expression of macrophage pro-inflammatory cytokines such as TNF-α and IL-6, and iNOS was been shown to contribute to induction of chronic inflammation and insulin resistance in adipose tissue (Perreault and Marette, 2001; Vozarova *et al*., 2001; Ruan and Lodish, 2003). Obese mice lacking iNOS were sensitized to the metabolic actions of rosiglitazone, a PPARγ agonist, that result in increased plasma adiponectin levels (Dallaire *et al*., 2008). Therefore, our observations indicate that HES may inhibit inflammatory responses for development of insulin resistance in adipose tissue via inhibition of production of TNF-α, IL-6, and NO by adipose macrophages.

**Hesperidin attenuated TNF-α-treated production of IL-6 in differentiated 3T3-L1 cells** – Circulating IL-6 produced by fibroblastic pre-adipocytes and differentiated adipocytes has been reported to play an important role in obesity-associated systemic chronic inflammation and insulin resistance, leading to type 2 diabetes mellitus (Pradhan *et al*., 2001; Kim *et al*., 2009). In addition, IL-6 mediates impaired insulin receptor signaling in liver (Senn *et al*., 2002; Klover *et al*., 2003; Klover *et al*., 2005). In the present study, comparison of differentiated 3T3-L1 adipocytes and RAW 264.7 cells on gene expression of IL-6 was carried out. As shown in Fig. 2A, our results demonstrated that IL-6 mRNA expression without stimulation of TNF-α was remarkably enhanced in differentiated 3T3-L1 adipocytes compared to those in RAW 264.7 cells. Our results support some evidences that adipose IL-6 may greatly contribute to increase in circulating IL-6 concentration under no stimulation in obesity. TNF-α, which is highly expressed in adipose macrophages in obese animals and human subjects, was implicated in the induction of insulin resistance in obesity and type 2 diabetes (Ruan and Lodish, 2003; De Taeye *et al*., 2007). Matsunaga *et al*. (2012) recently reported that exposure to TNF-α (10 ng/ml) for 24 h increased IL-6 secretion in 3T3-L1 adipocytes. Herein, we used TNF-α (10 ng/ml) as an inducer of IL-6 in differentiated 3T3-L1 cells. We examined effect of HES on the TNF-α-treated gene expression and protein production of IL-6 in differentiated 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were pretreated with 50 and 100 μM HES in complete media for 1 h and then cultured for 24 h for production of IL-6 in the presence or absence of TNF-α 10 ng/ml at 37 °C, 5% $CO_2$ incubation. Our results

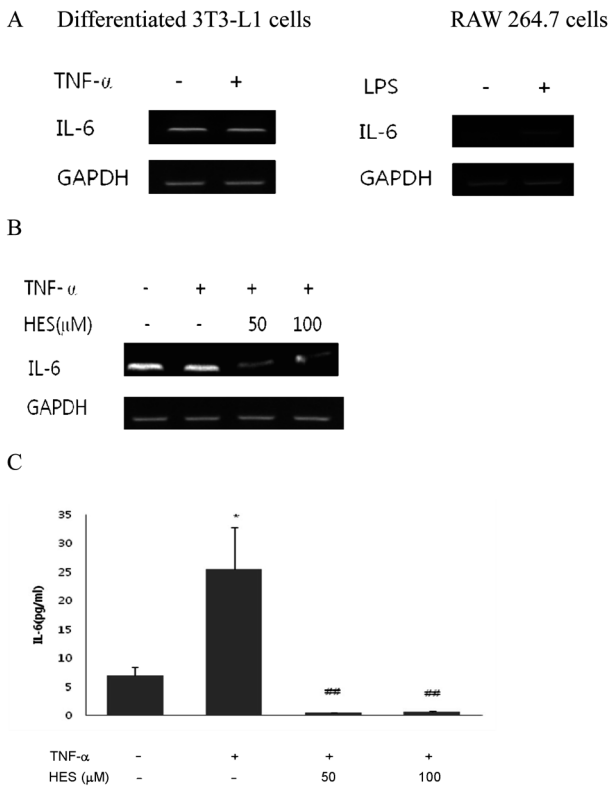


**Fig. 2.** Hesperidin inhibits TNF-α-induced production of IL-6 in differentiated 3T3-L1 cells.

IL-6 mRNA expression in differentiated 3T3-L1 adipocytes was compared to those in RAW 264.7 cells (A). Differentiated 3T3-L1 adipocytes were preincubated for 1 h with 50 and 100 μM HES and then cultured for 4 h in the presence of TNF-α for IL-6 mRNA expression (B) and for 24 h in the presence of TNF-α for IL-6 production (C). The cells were then harvested and total RNA was isolated from the cells. Gene expression was determined by RT-PCR. Each value represents the mean ± S.E. Other legends and methods are the same as in Fig. 1. $^{*}$ ($p < 0.05$): Significantly different from the value in negative control. $^{\#\#}$ ($p < 0.01$): Significantly different from the value in positive controls.

demonstrated that, while HES attenuated TNF-α-stimulated gene expression and protein production of IL-6 in differentiated 3T3-L1 cells (Fig. 2B and C). Moreover, these results showed that elevated gene expression and production of IL-6 in differentiated 3T3-L1 adipocytes even without TNF-α treatment were attenuated by HES. Liver is a target organ of IL-6 secreted by immune cells and adipose tissues. IL-6 was shown to induce impaired insulin sensitivity in hepatocytes (Senn *et al*., 2002; Senn *et al*., 2003). Therefore, our data indicate that HES may lead to prevent from development of IL-6-mediated-hepatic insulin resistance in obesity via inhibition of production of IL-6 by adipocytes.

**Hesperidin inhibited TNF-α-induced production of $PGE_2$ in differentiated 3T3-L1 cells** – $PGE_2$ release in

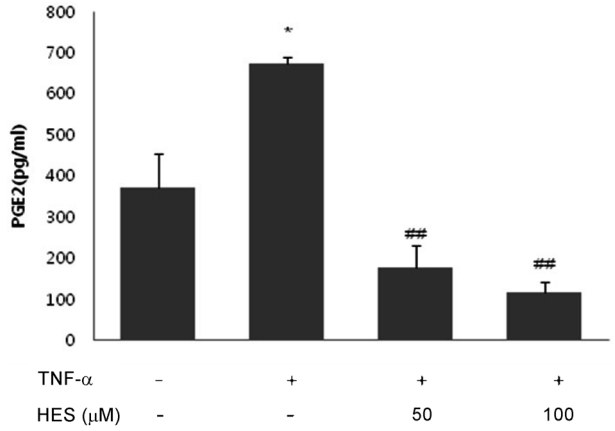


**Fig. 3.** Hesperidin inhibits TNF-α-induced production of $PGE_2$ in differentiated 3T3-L1 cells.

Differentiated 3T3-L1 adipocytes were preincubated for 1 h with 50 and 100 μM HES and then cultured for 4 h in the presence of TNF-α for 24 h in the presence of TNF-α for IL-6 production (C). Each value represents the mean ± S.E. Other legends and methods are the same as in Fig. 1. * ($p < 0.05$): Significantly different from the value in negative control. ## ($p < 0.01$): Significantly different from the value in positive controls.

adipose tissue and adipocytes has been also reported to contribute to insulin resistance (Hsieh *et al*., 2009). COX-2-mediated low-grade inflammation was shown to be important in the development of insulin resistance in fructose-fed rats (Liu *et al*., 2009). $PGE_2$ was also shown to play an important role in development of hepatic insulin resistance. Henkel *et al*. (2009) has reported that $PGE_2$ aggravated IL-6-dependent hepatic insulin resistance via increase in a serine phosphorylation of IRS-1 in hepatocytes. Therefore, adipose $PGE_2$ may be an important target for improvement of impaired insulin sensitivity in obesity. TNF-α treatment of differentiated adipocytes has been shown to increase IL-6 and COX-2 expression in a time dependent manner (Ruan and Lodish, 2003). We examined effect of HES on the TNF-α-induced production of $PGE_2$ in differentiated 3T3-L1 adipocytes. Herein, differentiated 3T3-L1 cells were pretreated with 50 and 100 μM HES in complete media for 1 h and then cultured for 24 h in the presence or absence of TNF-α 10 ng/ml at 37 °C, 5% $CO_2$ incubation. In the present study, we observed that HES significantly inhibited TNF-α-increased production of $PGE_2$ in the differentiated 3T3-L1 cells in a dose-dependent manner (Fig. 3). Therefore, these observations suggest that HES may attenuate $PGE_2$-mediated development of insulin resistance in obesity.

**Hesperidin upregulated TNF-α-suppressed expression of adiponectin mRNA in differentiated 3T3-L1 cells** – Adiponectin is a protective adipokine with anti-diabetic, anti-inflammatory, and anti-atherogenic properties in insulin

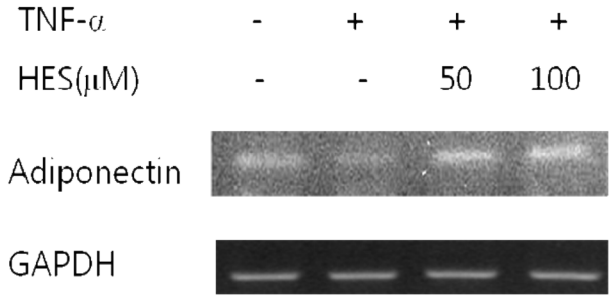


**Fig. 4.** Hesperidin upregulates TNF-α-suppressed expression of adiponectin mRNA in differentiated 3T3-L1 cells.

Differentiated 3T3-L1 adipocytes were pre-treated with 50 and 100 μM HES in complete media for 1 h and then cultured for 4 h the presence or absence of TNF-α 10 ng/ml. Other legends and methods are the same as in Fig. 1.

resistance in the metabolic syndrome and diabetes (Hosch *et al*., 2006; Buechler *et al*., 2011). TNF-α treatment in differentiated adipocytes has been shown to decrease adiponectin in a time dependent manner (Ruan and Lodish, 2003). In the present study, differentiated 3T3-L1 adipocytes were pretreated with 50 or 100 μM HES in complete media for 1 h and then cultured for 4 h in the presence or absence of TNF-α 10 ng/ml at 37 °C in a humidified atmosphere containing 5% $CO_2$. As shown in Fig. 4, these results demonstrated that HES upregulated TNF-α-suppressed expression of adiponectin in differentiated 3T3-L1 cells, suggesting that HES may protect from imflammation-related insulin resistance in obesity. Adiponectin also was shown to improve IL-6-mediated impairment of insulin signaling in HepG2 cells (Sun *et al*., 2011). Downregulation of serum adiponectin has been reported to be associated with enhanced IL-6 in human (Schultz *et al*., 2010). Adiponectin inhibited LPS-induced NF-kappaB activation and IL-6 production in adipocytes (Ajuwon and Spurlock, 2005). Moreover, adiponectin improved IL-6-mediated impairment of insulin signaling via downregulation of CRP gene expression and STAT3 phosphorylation in HepG2 cells (Zhang *et al*., 1996; Sun *et al*., 2011). Therefore, these findings suggest that HES may improve IL-6-mediated impairment of hepatic insulin sensitivity in obesity through enhancement of gene expression of adiponectin.

**Hesperidin upregulated TNF-α-suppressed expression of PPAR-γ mRNA in differentiated 3T3-L1 cells** – PPAR-γ is a negative regulator of obesity-related inflammatory responses and a key activator of insulin sensitivity in adipocytes (Ricote and Glass, 2007). PPAR-γ activation induced beneficial effects not only on glucose homeostasis and lipid metabolism but also on endothelial function and vessel wall inflammation (Sharma and Staels, 2007;

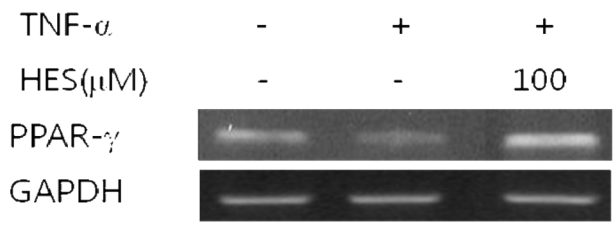


**Fig. 5.** Hesperidin upregulates TNF-α-suppressed expression of PPAR-γ mRNA in differentiated 3T3-L1 cells.
Differentiated 3T3-L1 adipocytes were pre-treated with 100 μM HES in complete media for 1 h and then cultured for 4 h the presence or absence of TNF-α 10 ng/ml. Other legends and methods are the same as in Fig. 1.

Staels, 2007). PPAR-γ activation was associated with potentially beneficial effects on the expression and secretion of a range of factors, including adiponectin, IL-6, TNF-α, and resistin, etc (Maeda *et al*., 2001; Ricote and Glass, 2007). In the present study, the differentiated 3T3-L1 adipocytes were pretreated with 50 or 100 μM HES in complete media for 1 h and then cultured for 4 h in the presence or absence of TNF-α 10 ng/ml at 37 ºC in a humidified atmosphere containing 5% $CO_2$. As shown in Fig. 5, these results demonstrated that HES upregulated TNF-α-suppressed expression of PPAR-γ mRNA in differentiated 3T3-L1 cells, indicating that HES may enhance inflammation-mediated suppressed expression of PPAR-γ mRNA in adipocytes. Furthermore, adiponectin gene expression has been reported to be induced by activation of PPAR-γ in 3T3-L1 adipocytes (Maeda *et al*., 2001). Therefore, these findings suggest that HES-increased expression of PPAR-γ gene in adipocytes may result in adiponectin expression in obesity.

In conclusion, these results demonstrated that HES remarkably inhibited LPS-induced production of IL-6, TNF-α, and NO by RAW 264.7 cells in a dose-dependent manner. HES also inhibited TNF-α-induced production of IL-6 and $PGE_2$ in differentiated 3T3-L1 cells, while upregulated TNF-α-suppressed expression of adiponectin and PPAR-γ mRNA. These findings suggest that HES may improve inflammation-mediated insulin resistance in obesity.

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