Protective Effect of Baicalin on the TNF-α-Mediated Development of Insulin Resistance in Differentiated 3T3-L1 Cells

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Abstract – Adipose tissue-derived chronic inflammation contributes to development of insulin resistance in obesity, leading to type 2 diabetes and cardiovascular disease. Baicalin, a flavonoid, has antioxidant, anti-inflammatory, antihyperglycemic, anti-adipogenic, and antiobesity effects. However, whether baicalin attenuates adipose tissue-derived development of insulin resistance remains still unclear. This study was to investigate effect of baicalin on the inflammatory changes involved in the development of insulin resistance in adipose tissue. RAW 264.7 cells and differentiated 3T3-L1 adipocytes were pretreated with various concentrations of baicalin in complete media for 1 h and then cultured in the presence or absence of LPS or TNF-α. Our results demonstrated that baicalin remarkably inhibited LPS-induced production of TNF-α, IL-6, and NO by RAW 264.7 cells in a dose-dependent manner. Baicalin also inhibited TNF-α-induced production of IL-6 and PGE_2 in differentiated 3T3-L1 cells in a dose-dependent manner, while upregulated TNF-α-suppressed expression of adiponectin and PPAR-γ mRNA and IRS-1 protein. These findings suggest that baicalin may prevent the adipose tissue-derived development of insulin resistance in obesity.

Keywords – Baicalin, Adipocyte, Insulin resistance, IL-6; PGE_2, Adiponectin, PPAR-γ, IRS-1

Introduction

Obesity is strongly correlated with low grade chronic inflammation and consequently the development of insulin resistance in peripheral tissues such as skeletal muscle and the liver, leading to type 2 diabetes mellitus and cardiovascular disease (Berg and Scherer, 2005; Bastard *et al*., 2006; Winkler and Cseh, 2009). Obesity-related insulin resistance is characterized by increase in circulating levels of adipose-derived inflammatory factors that interfere with insulin action in peripheral tissues (Wellen and Hotamisligil, 2003; Xu *et al*., 2003). Adipose tissue including adipocytes and macrophages, is a highly active endocrine organ secreting several adipose-derived factors, such as TNF-α, IL-6, adiponectin, leptin, and resistin, which crucially contribute to an important link between chronic inflammation and insulin resistance (Trayhurn and Wood, 2004; Tilg and Moschen, 2006; Galic *et al*., 2010). Adipose macrophages that secrete inflammatory mediators such as TNF-α and IL-6, are further exacerbate the chronic inflammatory state by promoting insulin resistance in adipocytes (Permana *et al*., 2006; Gonzales and Orlando, 2008).

TNF-α and IL-6 are key inflammatory mediators strongly linked to development of insulin resistance in obesity. (De Taeye *et al*., 2007; Kim *et al*., 2009). TNF-α induces abnormal insulin signaling in adipocytes by loss of insulin receptor substrate (IRS)-1 expression (Stephens *et al*., 1997). IL-6 mediates hepatic insulin resistance in obesity that is characterized by impaired insulin receptor signaling in liver (Senn *et al*., 2002; Klover *et al*., 2005). The reduced gene expression of adiponectin, an anti-inflammatory adipokine, in adipose tissue is associated with insulin resistance, type 2 diabetes, obesity, or cardiovascular disease (Villarreal-Molina and Antuna-Puente, 2012; Fantuzzi, 2013). Peroxisome proliferator-activated receptor (PPAR)-γ is a negative regulator of obesity-related inflammatory responses and a key activator of insulin sensitivity (Yamauchi *et al*., 2002; Sharma and Staels, 2007). The abnormalities in adipose tissue-derived factors will be potential targets for the development of insulin resistance.

Baicalin, one of the major flavonoids in *Scutellaria baicalensis*, is well known to possess antioxidant and anti-inflammatory effects *in vivo* and *in vitro* (Krakauer *et al*., 2001; Lixuan *et al*., 2010). It has also been reported

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that baicalin had antihyperglycemic effects in diabetic rats and the possible mechanisms include increasing the hepatic glycogen content and glycolysis, and reducing the serum levels of TNF-α (Li *et al*., 2011). Long-term baicalin administration ameliorates metabolic disorders and hepatic steatosis in rats given a high-fat diet (Guo *et al*., 2009). Baicalin had anti-adipogenic and antiobesity effects via modulation of proadipogenic and antiadipogenic regulators of the adipogenesis pathway (Lee *et al*., 2009). However, whether baicalin attenuates adipose tissue-derived development of insulin resistance remains still unclear. In the present study, we observed effect of baicalin on the TNF-α-induced inflammatory changes involved in development of 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). Anti-insulin receptor substrate (IRS)-1 and anti-ß-Actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Dexamethasone, insulin, isobutylmethyxanthine, and recombinant TNF-α and IL-6 were purchased from Sigma (St. Louis, MO) except where indicated differently. Baicalin isolated in Radix of *Scutellaria baicalensis* was kindly provided by Dae Keun Kim professor in college of pharmacy, Woosuk University (Jeonlabuk Do, Korea) and dissolved in dimethyl sulfoxide (DMSO) for *in vitro* experiment.

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 baicalin 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 baicalin in complete media for 1 h and then cultured for 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 and cell supernatants stored at −70 ºC for IL-6 and PGE_2 assays.

Cytokine assay – The concentrations of cytokines such as TNF-α and IL-6 in the supernatants obtained 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 obtained 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 obtained from differentiated 3T3-L1 adipocytes was determined 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.

Western blot analysis – Following preincubation with baicalin and treatment of TNF-α 10 ng/ml or insulin, the differentiated 3T3-L1 adipocytes were washed with ice-cold PBS and then scraped from the plate in 500 μl of lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na_2-EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM PMSF). After 30 min at 4 ºC, the lysates were centrifuged ($15,000 \times g$ for 15 min) and stored at −80 ºC until use. Proteins were separated on SDS-PAGE and transferred to polyvinylidene fluoride (PVDF). Nonspecific binding was blocked with TBS-T (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% non-fat milk for 1 h. Primary antibodies were diluted and incubated with membranes overnight at 4 ºC with agitation. After washing three times with TBS-T, secondary antibodies were incubated for 1 h. After 5 additional washes with TBS-T, the bands were visualized with chemiluminescence according to the manufacturer's instructions.

Total RNA isolation and RT-PCR – Following preincubation with baicalin for 1 h, the differentiated 3T3-L1 cells were incubated for 4 h in the presence or absence of TNF-α 10 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'-CATAGCTACCTGGAGTACATG A-3' and antisense 5'-CATTCATATTGTCAGTTCTTCG-3'); adiponectin (sense 5'-GCAGAGATGGCACTCCTGG A-3' and antisense 5'- -3'); PPAR-γ (sense 5'-GCTCTAGA CGTGACAATCTGTCTGAGGTCTGTCAT-3' and antisense 5'-CGGGATCCGTTGTCGGTTTCAGAAATGCCTTGCA GTG-3'); GAPDH (sense 5'-GCCAAGGTCATCCATGA CAAC-3' and antisense 5'-AGTGTAGCCCAAGATGCC CTT-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

Baicalin inhibited LPS-induced production of proinflammatory cytokines and NO by RAW 264.7 cells – TNF-α is a potential mediator that links obesity-derived chronic inflammation with the development of insulin resistance (De Taeye *et al*., 2007). TNF-α is highly expressed in adipose macrophages in obese animals and human subjects and mediates impaired insulin sensitivity
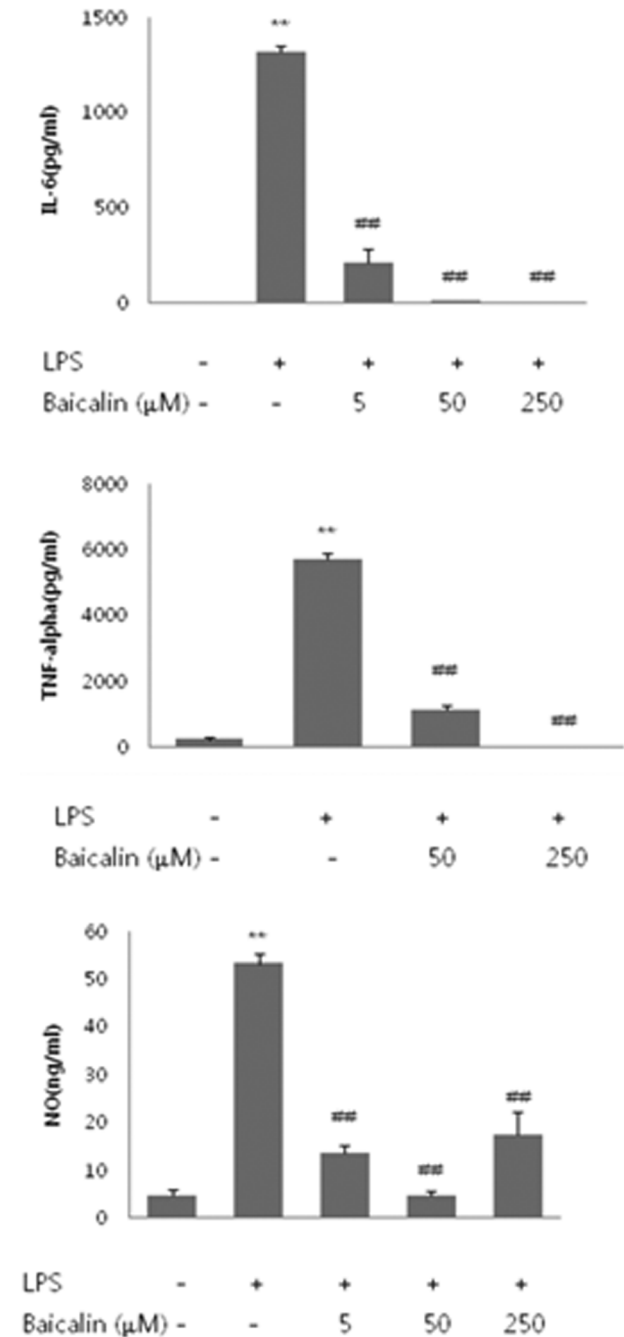

Fig. 1. Baicalin inhibited LPS-induced production of proinflammatory cytokines and NO in RAW 264.7 cells. RAW 264.7 cells were preincubated with various concentration of baicalin for 1 h and then cultured for 6 h for TNF-α production or 24 h for IL-6 and NO in the presence or absence of LPS. Concentration 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. [##] ($p < 0.01$): Significantly different from the value in positive controls.

in obesity (Stephens *et al*., 1997). Adipose macrophages that secrete inflammatory mediators, such as TNF-α and

IL-6, are further exacerbate the chronic inflammatory state by promoting insulin resistance in adipocytes (Permana *et al*., 2006; Kim *et al*., 2009). Gonzales and Orlando (2008) showed that TNF-α increased expression of IL-1β, IL-6, and COX-2 expression in differentiated adipocytes. Inducible NO synthetase was shown to be a key inflammatory mediator in obesity (Perreault and Marette, 2001). Herein, we examined whether baicalin attenuates the macrophage-derived production of IL-6, TNF-α, and NO. RAW 264.7 cells were pretreated with various concentrations of baicalin in complete media for 1 h and then cultured in the presence or absence of LPS 1 μg/ml at 37 °C in a humidified atmosphere containing 5% CO_2. Concentration of proinflammatory cytokines and NO in the cell supernatants was measured using ELISA. These results demonstrated that baicalin remarkably inhibited LPS-induced production of TNF-α, IL-6, and NO by RAW 264.7 cells in a dose dependent manner (Fig. 1). These results consist with the report that baicalin inhibited LPS-induced activation of macrophages and production of proinflammatory cytokines and NO in RAW264.7 cells and peritoneal macrophages (Liu *et al*., 2008). Therefore, our observations indicate that baicalin may protect from development of insulin resistance in adipocytes via inhibiting production of inflammatory mediators by adipose macrophages.

Baicalin attenuated TNF-α-induced production of IL-6 in differentiated 3T3-L1 cells – IL-6 is a key inflammatory mediator strongly linked to development of obesity and insulin resistance (Kim *et al*., 2009). IL-6 especially mediates hepatic insulin resistance that is characterized by impaired insulin receptor signaling in liver through STAT3-SOCS3 pathway (Senn *et al*., 2003). Circulating IL-6 produced by adipocytes plays an important role in obesity-associated hepatic insulin resistance at the cellular level (Klover *et al*., 2005). Our previous study demonstrated that gene expression of IL-6 with no mitogen was greatly enhanced in differentiated 3T3-L1 cells compared with RAW 264.7 cells (Chae and Shin, 2012), suggesting that low grade chronic production of IL-6 in adipocytes may contribute to the development of insulin resistance in obesity. Many studies demonstrated that baicalin had anti-inflammatory, anti-adipogenic and antiobesity effect (Krakauer *et al*., 2001; Lee *et al*., 2009; Lixuan *et al*., 2010). However, effect of baicalin on the production of IL-6 by adipocytes remains still unknown. In the present study, effect of baicalin on the TNF-α-induced production of IL-6 in differentiated 3T3-L1 adipocytes was examined. TNF-α was used to induce expression of IL-6 in differentiated adipocytes (Gonzales
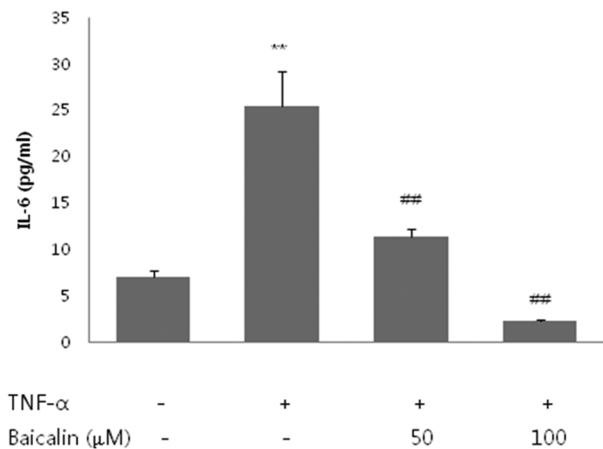

Fig. 2. Baicalin inhibited TNF-α-induced production of IL-6 in differentiated 3T3-L1 cells. Differentiated 3T3-L1 adipocytes were preincubated for 1 h with 50 and 100 μM baicalin and then cultured for 24 h in the presence of TNF-α for IL-6 production. Each value represents the mean $\pm$ S.E. Other legends and methods are the same as in Fig. 1. [**] (p < 0.01): Significantly different from the value in negative control. [##] (p < 0.01): Significantly different from the value in positive controls.

and Orlando, 2008). The differentiated adipocytes were pretreated with 50 and 100 μM baicalin 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. As shown in Fig. 2, these results demonstrated that baicalin remarkably suppressed TNF-α-induced production of IL-6 in the differentiated 3T3-L1 adipocytes, suggesting that baicalin may prevent the development of IL-6-mediated insulin resistance including hepatic insulin resistance.

Baicalin inhibited TNF-α-induced production of PGE$_2$ in differentiated 3T3-L1 cells – COX-2-mediated low-grade inflammation have been reported to contribute to systemic insulin resistance (Hsieh *et al*., 2009; Liu *et al*., 2009). TNF-α has been shown to increase expression of COX-2 in the differentiated adipocytes (Gonzales and Orlando, 2008). Herein, to examine effect of baicalin on the production of PGE$_2$ in adipocytes, differentiated 3T3-L1 cells were pretreated with 50 and 100 μM baicalin 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. These observations showed that baicalin significantly inhibited TNF-α-increased production of PGE$_2$ in the differentiated 3T3-L1 cells in a dose-dependent manner (Fig. 3). PGE$_2$ also aggravated IL-6-dependent insulin resistance in hepatocytes (Henkel *et al*. 2009). Therefore, these observations suggest that baicalin may attenuate PGE$_2$-mediated and IL-6-dependent development of insulin resistance in obesity via inhibition of
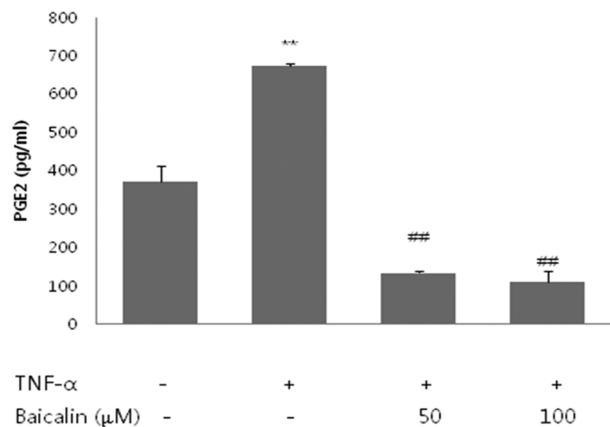
Fig. 3. Baicalin inhibited TNF-α-induced production of PGE_2 in differentiated 3T3-L1 cells. Each value represents the mean $\pm$ S.E. Other legends and methods are the same as in Fig. 2. [**] (p < 0.01): Significantly different from the value in negative control. [##] (p < 0.01): Significantly different from the value in positive controls.

production of PGE_2 in adipocytes.

Baicalin upregulated TNF-α-suppressed gene expression of adiponectin in differentiated 3T3-L1 cells – Adiponectin is an adipokine that promotes adipocyte differentiation, down-regulates tissue inflammation, and augments insulin sensitivity in adipocytes (Villarreal-Molina and Antuna-Puente, 2012; Fantuzzi, 2013). The reduced gene expression of adiponectin in adipose tissue is associated with insulin resistance, type 2 diabetes, obesity, or cardiovascular disease (Lee *et al*., 2013). TNF-α decreases adiponectin expression in differentiated adipocytes (Ruan and Lodish, 2003). In the present study, to investigate whether baicalin induces adiponectin expression in adipocytes, the differentiated 3T3-L1 adipocytes were pretreated with 50 or 100 μM baicalin 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 $^{\circ}$C in a humidified atmosphere containing 5% CO_2. These results were observed that baicalin upregulated TNF-α-suppressed gene expression of adiponectin in differentiated 3T3-L1 adipocytes (Fig. 4), suggesting that baicalin may lead to inhibition of inflammation and improvement of impaired insulin sensitivity in obesity via upregulation of adiponectin gene expression in adipocytes. It has been reported that adiponectin inhibits production of IL-6 in adipocytes (Ajuwon and Spurlock, 2005) and that adiponectin improves IL-6-mediated impairment of hepatic insulin signaling (Sun *et al*., 2011). Our results (Fig. 2) demonstrated that baicalin inhibited TNF-α-induced production of IL-6 in differentiated 3T3-L1 cells. Therefore, these results also suggest that baicalin may lead to improvement of impaired insulin sensitivity
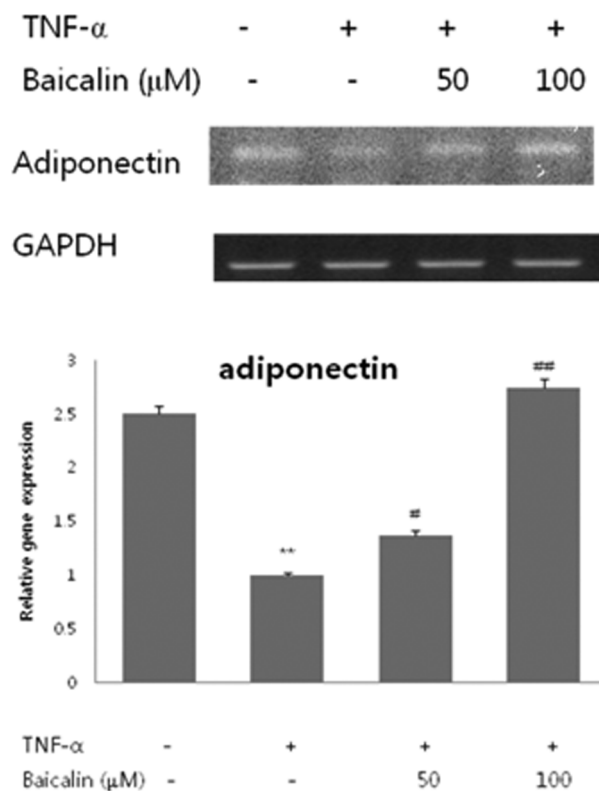

Fig. 4. Baicalin upregulated TNF-α-suppressed gene expression of adiponectin in differentiated 3T3-L1 cells. Differentiated 3T3-L1 adipocytes were pre-treated with 50 and 100 μM baicalin in complete media for 1 h and then cultured for 4 h the presence or absence of TNF-α 10 ng/ml. The cells were then harvested and total RNA was isolated from the cells. Expression of adiponectin mRNA was determined by RT-PCR. Each value represents the mean $\pm$ 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.

induced by adipocytes via upregulation of adiponectin gene expression and consequently inhibition of IL-6 production in adipocytes.

Baicalin upregulated TNF-α-suppressed gene expression of PPAR-γ in differentiated 3T3-L1 cells – PPAR-γ activation results in improvement of impaired insulin sensitivity through potentially beneficial effects on the expression and secretion of various factors involved in insulin resistance, including adiponectin, IL-6, TNF-α, and resistin, etc (Maeda *et al*., 2001; Yamauchi *et al*., 2002; Sharma and Staels, 2007). Baicalin has been reported to suppress NF-κB-mediated inflammation in aged rat kidney (Lim *et al*., 2012) and attenuate carbon tetrachloride-induced liver injury by activating PPARγ (Qiao *et al*., 2010). However, whether baicalin induces PPAR-γ expression in adipocytes remains unknown. In the present study, the differentiated 3T3-L1 adipocytes were pretreated with 50 or 100 μM baicalin in complete
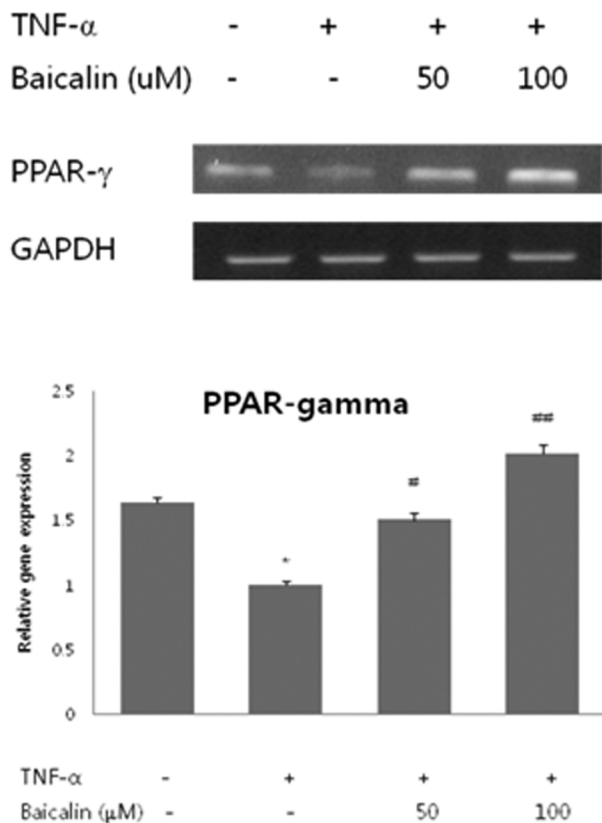
Fig. 5. Baicalin upregulated TNF-α-suppressed gene expression of PPAR-γ in differentiated 3T3-L1 cells. Differentiated 3T3-L1 adipocytes were pre-treated with 50 and 100 μM baicalin in complete media for 1 h and then cultured for 4 h the presence or absence of TNF-α 10 ng/ml. The cells were then harvested and total RNA was isolated from the cells. Expression of PPAR-γ mRNA was determined by RT-PCR. Each value represents the mean ± S.E. Other legends and methods are the same as in Fig. 4. * (p < 0.05): Significantly different from the value in negative control. # (p < 0.05) and ## (p < 0.01): Significantly different from the value in positive controls.
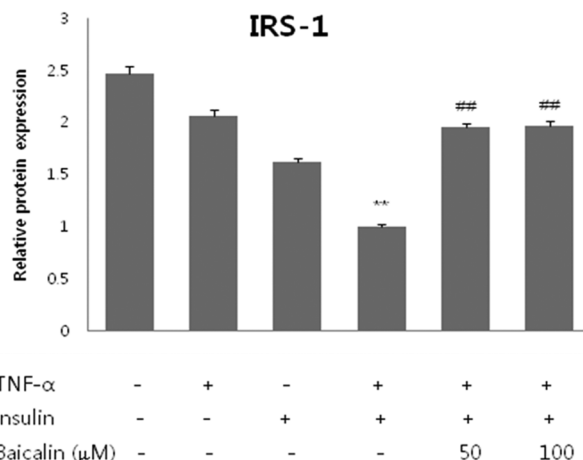

Fig. 6. Effect of baicalin on the TNF-α-suppressed expression of IRS-1 in differentiated 3T3-L1 cells. Differentiated 3T3-L1 adipocytes were pre-treated with 50 and 100 μM baicalin in complete media for 1 h and then cultured for 1 h in the presence or absence of TNF-α at 37 °C in a humidified atmosphere containing 5% CO_2. After 1 h incubation, the cells were stimulated with 100 nM human insulin for 3 min at 37 °C. The cells were then harvested and total cellular protein was isolated from the cells for western blot analysis. ** (p < 0.01): Significantly different from the value in negative control. ## (p < 0.01): Significantly different from the value in positive controls.

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. 5, these results demonstrated that baicalin upregulated TNF-α-suppressed gene expression of PPAR-γ in differentiated 3T3-L1 cells in a dose-dependent manner, indicating that baicalin may enhance inflammation-suppressed gene expression of PPAR-γ in adipocytes. PPAR-γ has also been reported to induce adiponectin gene expression in adipocytes (Astapova and Leff, 2012). Therefore, these findings suggest that baicalin-induced activation of PPAR-γ and consequently adiponectin induction in adipocytes may result in improvement of impaired insulin sensitivity in obesity.

Baicalin upregulated TNF-α-suppressed expression of IRS-1 in differentiated 3T3-L1 cells – IRS-1 plays an

important role in the metabolic actions of insulin mainly in skeletal muscle and adipose tissue (Bruning *et al*., 1997). It has been reported that baicalin had antihyperglycemic effects in diabetic rats and the possible mechanisms include increasing the hepatic glycogen content and glycolysis, and reducing the serum levels of TNF-α (Li *et al*., 2011). However, whether anti-diabetic effect of baicalin is associated with up-regulation of IRS-1 expression remains unclear. TNF-α impairs insulin signaling in adipocytes by loss of IRS-1 expression (Stephens *et al*., 1997). This study was examined whether baicalin reverses TNF-α-downregulated expression of IRS-1 in adipocytes. The differentiated 3T3-L1 adipocytes were pretreated with 50 and 100 μM baicalin in complete media for 1 h and cultured in the presence or absence of TNF-α 10 ng/ml at 37 °C, 5% CO_2 incubation for 1 h. Thereafter, the cells were stimulated with 100 nM human insulin for last 3 min at 37 °C. The cells were harvested and total proteins were isolated from the cells for western blot analysis. These results showed that insulin-dependent expression of IRS-1 protein was remarkably suppressed by TNF-α treatment in differentiated 3T3-L1 adipocytes, while was reversed by baicalin (Fig. 6). Therefore, these results suggest that baicalin may ameliorate impaired insulin sensitivity in obesity through upregulation of insulin-dependent expression of IRS-1 in adipocytes.

In conclusion, these results demonstrated that baicalin remarkably inhibited LPS-induced production of IL-6, TNF-α, and NO by RAW 264.7 cells in a dose-dependent manner. Baicalin 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 and IRS-1 protein. These findings suggest that baicalin may ameliorate adipose tissue-derived development of insulin resistance in obesity.

Acknowledgements

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