ARTICLE
Journal of Cellular Biochemistry 113:2560–2566 (2012)

Journal of Cellular Biochemistry

Inhibitory Effect of Paeoniflorin on the Inflammatory Vicious Cycle Between Adipocytes and Macrophages

Boren Jiang, Jie Qiao, Yuguo Yang, and Yingli Lu*

Laboratory of Endocrine and Metabolic Diseases, Department of Endocrine and Metabolic Diseases, Ninth People's Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai, China

ABSTRACT

Obesity is associated with a state of chronic, low-grade inflammation. It is considered that the paracrine loop involving free fatty acid (FFA) and tumor necrosis factor (TNF)α between adipocytes and macrophages establishes an inflammatory vicious cycle that augments the inflammatory changes and insulin resistance in obese adipose tissue. Paeoniflorin (PF), one of the major components of Paeony root, has been shown to have anti-inflammatory effects in vivo. We investigated the effect of PF on the production of FFA and TNFα in the interaction between adipocytes and macrophages. Coculture of 3T3-L1 adipocytes and RAW 264.7 macrophages markedly enhanced the production of TNFα and FFA compared with the control cultures, however, treatment with PF dose-dependently inhibited the production. We further examined the effects of PF on TNFα-stimulated adipocyte lipolysis and on FFA-induced macrophage TNFα expression. PF inhibited TNFα-stimulated adipocyte lipolysis in a dose-dependent manner, which was compatible with suppressed phosphorylation of TNFα-activated ERK1/2 and preserved downregulation of perilipin. Palmitate, one of the most important saturated FFAs, induced macrophage TNFα upexpression, but PF partially attenuated the effect. These results indicate that PF exhibits anti-inflammatory properties by inhibiting the vicious cycle between adipocytes and macrophages. PF may be useful for ameliorating the inflammatory changes in obese adipose tissue. J. Cell. Biochem. 113: 2560–2566, 2012. © 2009 Wiley Periodicals, Inc.

KEY WORDS: PAEONIFLORIN; MACROPHAGE; ADIPOCYTE; INFLAMMATION; OBESITY

Obesity is recognized as a risk factor for insulin resistance, cardiovascular diseases, and type 2 diabetes [Miranda et al., 2005]. Elevated FFA and chronic, low-grade inflammation are two mediators in the process [Fernandez-Real and Ricart, 2003; Lyon et al., 2003; Dandona et al., 2005]. Recent studies have demonstrated that obese adipose tissue is characterized by increased infiltration of macrophages [Weisberg et al., 2003; Xu et al., 2003]. It is considered that the paracrine loop involving adipocyte-derived free fatty acid (FFA) and macrophage-derived tumor necrosis factor (TNF)α establishes a vicious cycle that augments the inflammatory changes and insulin resistance in obese adipose tissue [Suganami et al., 2005]. TNFα, secreted from macrophages infiltrated into obese adipose tissue, induces the release of FFA from adipocytes via lipolysis; while FFA from adipocytes, plays a major role in the activation of macrophages and TNFα expression. Therefore, blocking the vicious cycle between adipocytes and macrophages is, therefore, now regarded as an important pharmacological target to prevent or treat obesity-associated diseases.

Paeoniflorin (PF) is one of the major components of Paeony root and it has been widely studied as antioxidant [Sun et al., 2007], antihyperglycemic agent [Hsu et al., 1997], cerebral and myocardial ischemia/reperfusion injury protector [Liu et al., 2006b; Nizamutdinova et al., 2008], endothelium-dependent vasodilator [Goto et al., 1996] and learning impairment attenuating agent [Ohta et al., 1994]. PF is effective for the treatment or control of chronic inflammatory conditions such as rheumatism [Yamahara et al., 1982; Zhang et al., 2008], dyslipidemia [Yang et al., 2004] and immunological liver injury [Liu et al., 2006a]. The antiinflammatory action of PF results from the disruption of the production of various inflammatory factors (e.g., cytokines such as TNFα, IL-6, cyclooxygenase-2) [Liu et al., 2006a; Zhang et al., 2008] and induction of lymphocytes apoptosis [Tsuboi et al., 2004]. Because obesity-induced inflammation entails an increase in the levels of proinflammatory mediators and the numbers of inflammatory cells, PF which elicits antiinflammatory responses, may have therapeutic potential for the treatment of enhanced inflammatory responses in obesity.

The aim of this study is to obtain in vitro evidences whether PF could modulate the production of proinflammatory mediators in the interaction between adipocytes and macrophages.

*Correspondence to: Dr. Yingli Lu, Professor of Medicine, Laboratory of Endocrine and Metabolic Diseases, Department of Endocrine and Metabolic Diseases, Ninth People's Hospital, School of Medicine, Shanghai Jiaotong University, 639 Zhizhaoju Road, Shanghai 200011, China. E-mail: emd9hospital@yahoo.com

Manuscript Received: 18 January 2009; Manuscript Accepted: 17 March 2009

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 24 April 2009

DOI 10.1002/jcb.22173 • © 2009 Wiley Periodicals, Inc.

MATERIALS AND METHODS

MATERIALS

PF was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), purity: ≥98% (HPLC). Recombinant murine TNFα, dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), palmitate, PD98059, SP600125, and BAY 11-7082 were obtained from Sigma–Aldrich (St. Louis, MO). Antibodies against phospho-ERK1/2, phospho-P_{38}, phospho-JNK phospho-IKKα/IKKβ and IκBα were purchased from Cell Signaling Technology Inc. (Danvers, MA); antibody against perilipin A was from Affinity Bioreagents (Golden, CO). 2-Deoxy-D-[1-^{3}H] glucose (2-DOG) was purchased from Amersham (Buckinghamshire, UK). Unless otherwise specified, all other reagents were purchased from Sigma.

CELL CULTURE

RAW264.7 macrophages and 3T3-L1 preadipocytes (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) containing 10% fetal bovine serum. Differentiation of 3T3-L1 preadipocytes to mature adipocytes was performed as described previously with some modifications [Tafuri, 1996]. Briefly, the fibroblasts were grown in DMEM containing 10% FCS, 25 mM glucose, 2 mM glutamine, 3.4 mg/ml $NaHCO_3$, 3.6 mg/ml HEPES, 64 μg/ml penicillin, 100 μg/ml streptomycin (growth medium) at 37°C in 5% CO_2 atmosphere. After confluence, the fibroblasts were maintained for another 2 days (defined as day 0). Differentiation was induced by treating the cells with standard differentiation inducers (DMEM containing 0.5 mM IBMX, 1 μM DEX, 0.17 μM INS, and 10% FCS) for 48 h (from day 0 to day 2). The cells were re-fed with DMEM supplemented with 0.17 μM INS and 10% FCS for the following 48 h (from day 2 to day 4), then the medium was replaced by growth medium, changed every 2 days. Eight days after induction of differentiation, the cells that accumulated large lipid droplets were used as hypertrophied 3T3-L1 adipocytes.

COCULTURE OF ADIPOCYTES AND MACROPHAGES

Differentiated 3T3-L1 adipocytes were cocultured with RAW 264.7 in the medium containing 2% fatty acid (FA)-free bovine serum albumin for 24 h in coculture systems. Adipocytes and macrophages were cocultured in a contact system as previously described [Suganami et al., 2005]. Briefly, differentiated 3T3-L1 adipocytes were incubated overnight in DMEM with 0.5% bovine serum albumin (BSA) in the absence of serum. RAW 264.7 cells (2×10^5 cells/ml) were plated onto dishes with serum-starved and hypertrophied 3T3-L1 cells by using transwell inserts with a 0.4-μm porous membrane (Corning), and the coculture was incubated in serum-free DMEM for 24 h. RAW264.7 and 3T3-L1 cells of equal numbers to those in the coculture were cultured separately as control cultures. PF was added to the coculture at various concentrations as shown in each figure. After 24 h of treatment, culture supernatants were collected and stored at −20°C until measurement.

MEASUREMENT OF TNFα, MCP-1, AND FFAS PRODUCTION

The concentrations of TNFα and MCP-1 in the culture supernatants were determined by enzyme-linked immunosorbent assay (R&D Systems), according to the manufacturer's protocol. The concentrations of FFAs in the medium were measured using an acyl-coenzyme A oxidase-based colorimetric assay kit (NEFA-C, WAKO Pure Chemicals).

LIPOLYSIS ASSAY

Differentiated 3T3-L1 adipocytes were incubated overnight in DMEM with 0.5% bovine serum albumin (BSA) in the absence of serum. The following morning, cells were treated as described in Results. Glycerol content of the incubation medium was transferred to another set of tubes and heated at 70°C for 10 min to inactivate any enzymes released by the cells. Samples (25 μl) were then assayed for glycerol using 175 μl of glycerol reagent (GPO Trinder Reagent A, Sigma) in a flat-bottom 96-well plate. After incubation for 5 min at 37°C, absorption was measured at 540 nm on a BioRad plate reader. Glycerol concentrations were calculated according to glycerol standard curve. Protein content was determined using the BCA protein assay (Pierce, Rockford, IL).

IMMUNOBLOT ANALYSIS

Cells were washed twice with ice-cold PBS and harvested in a lysis buffer (RIPA, 50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 5 μg/ml Aprotinin, 5 μg/ml Leupeptin, pH 7.4). After sonication and centrifugation, protein concentrations were determined in cell lysates using BCA protein assay kit. Equal amounts of protein from each sample were boiled in SDS-loading buffer for 5 min and loaded and separated by SDS–polyacrylamide gel electrophoresis. Gels were then blotted onto PVDF membranes (Amersham, Little Chalfont, UK) by Western blotting. Blots were blocked for 2 h at room temperature in Tris buffered saline, with 0.1% Tween 20 (TBS-T) and 5% non-fat dried milk and subsequently incubated overnight at 4°C in the presence of specific antibodies. Following washing steps in TBS-T and incubation with secondary antibodies conjugated to horseradish peroxidase, antigen-antibody complexes were detected by chemiluminescence and exposed to high-performance Kadak film. Films were scanned and specific bands were quantified using the Qualityone Image software.

INDUCTION OF INSULIN RESISTANCE AND GLUCOSE UPTAKE ASSAY

To induce insulin resistance, cells were chronically exposed to 10 ng/ml TNFα for 96 h [Iwata et al., 2001]. During the treatment period, fresh TNFα or PF was added every 48 h. Cells were washed three times with PBS, then incubated in Krebs-Ringer phosphate buffer (KRP, 1.32 mM NaCl, 4.71 mM KCl, 47 mM $CaCl_2$, 1.24 mM $MgSO_4$, 2.48 mM Na_3PO_4, 10 mM HEPES, pH 7.4) with or without 100 nM insulin at 37°C. After 30 min, 0.5 μCi/ml 2-DOG (final concentration) was added to the cells. After 10 min incubation, the medium was aspirated and the cells were washed three times with icecold KRP containing 10 mM glucose to terminate the reaction. The cells were lysed with 0.1 N NaOH and the radioactivity taken up by the cells was determined using a scintillation counter (Beckman

Instruments). Dpm value was corrected by protein content in each well which was measured with BCA protein assay kit.

REVERSE TRANSCRIPTION AND REAL-TIME POLYMERASE CHAIN REACTION

Total RNA from 3T3-L1 cells was isolated with TRIZOL Reagent and reverse transcription of 1 μg RNA was carried out with the SuperScript III Reverse Transcriptase following Invitrogen's protocol with Oligo dT primer. Quantitative PCR amplification and detection were performed with SYBR Premix Ex Taq (TaKaRa, Shiga, Japan), according to the manufacturer's protocol on ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA). Cycling parameters were 95°C for 10 s, then 40 cycles of 95°C for 5 s and 60°C for 34 s. As a reference gene, the mRNA level of 36B4 was determined in the real-time PCR assay for each RNA sample and was used to correct for experimental variations. Quantifications were performed in duplicate and the experiments were repeated independently three times. Melting curves were performed using Dissociation Curves software (Applied Biosystems) to ensure only a single product was amplified. The following primer sequences were used: TNFα 5′-CACAAGATGCTGGGACAGTGA-3′ (forward), 5′-TCCTTGATGGTGGTGCATGA-3' (reverse); 36B4, 5′-AAGCGCGT-CCTGGCATTGTCT-3' (forward), 5′-CCGCAGGGGCAGCAGTGGT-3' (reverse).

STATISTICAL ANALYSIS

Data were expressed as the mean ± SE. Statistical analysis was performed using ANOVA followed by Dunnett's *t*-test. A P value <0.05 (two-sided) was considered to be statistically significant.

RESULTS

INHIBITORY EFFECT OF PF ON INFLAMMATORY CHANGES BY COCULTURE OF ADIPOCYTES AND MACROPHAGES

To elucidate the effect of PF on the inflammatory interaction between adipocytes and macrophages, we treated the coculture of 3T3-L1 adipocytes and RAW 264.7 macrophages with PF. Our preliminary study had shown that the addition of PF at 1–100 mg/L concentrations for 24 h did not affect adipocytes and macrophages cell viability compared with vehicle (0.1% DMSO) alone (MTT assay, data not shown). Coculture of differentiated 3T3-L1 adipocytes and RAW264.7 cells showed an enhanced TNFα secretion over threefold that of the macrophages alone (Fig. 1A). PF dose-dependently suppressed the coculture-induced TNFα secretion. The MCP-1 secretion from RAW 264.7 macrophages or 3T3-L1 adipocytes was very low when separately cultured, however, the coculture of these cells in the contact system revealed a marked increase in MCP-1 level. PF treatment significantly decreased MCP-1 secretion level even at the lower concentration (12.5 mg/L; Fig. 1B). Similar effects of PF on FFA secretion were also observed. Coculture of these cells in the contact system revealed a marked increase in FFA level compared with that from adipocytes alone, but treatment of this coculture with PF significantly inhibited the coculture-induced FFA secretion (Fig. 1C). These results indicate that PF suppresses the coculture-induced production of proinflammatory factors.

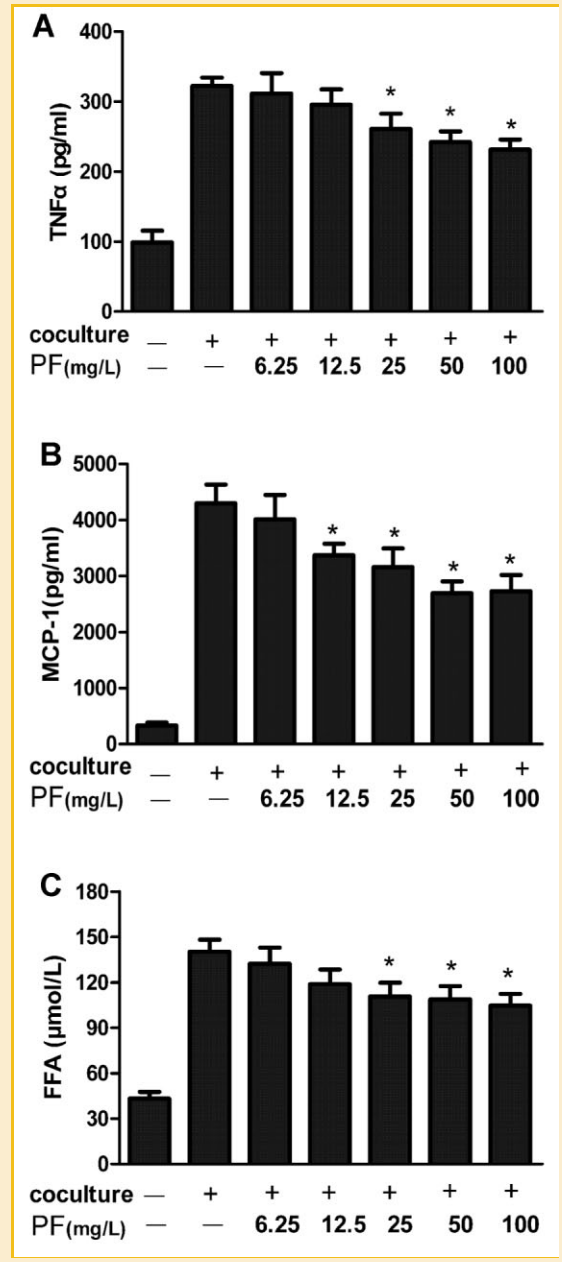

Fig. 1. Effect of PF on inflammatory changes induced by coculture of 3T3-L1 adipocytes and RAW 264.7 macrophages. Differentiated 3T3-L1 adipocytes were cocultured with RAW 264.7 macrophages (2×10^5 cells/well) for 24 h with or without various concentrations of PF. Released TNFα (A) and MCP-1 (B) levels in the coculture medium were measured by ELISA, and that from macrophages alone are used as a negative control. Secretion level of FFA (C) was also measured as described in Materials and Methods Section, and FFA from adipocytes alone was used as a negative control. The values are means ± SE of six tests. $^*P < 0.05$ compared with non-treated coculture.

EFFECT OF PF ON TNFα-STIMULATED LIPOLYSIS IN 3T3-L1 ADIPOCYTES

Now that the paracrine loop involving FFA and TNFα between adipocytes and macrophages establishes an inflammatory vicious cycle, we then investigated the inhibiting targets of PF on TNFα-FFA

inflammation cycle. We first explored the effect of PF on lipolysis in TNFα-treated adipocytes. Triacylglycerol hydrolysis proportionally releases glycerol and FFA from adipocytes, thus we assayed glycerol release in the culture medium as an index of lipolysis. 3T3-L1 adipocytes were stimulated with TNFα in the presence or absence of PF. As shown in Figure 2A, when 3T3-L1 adipocytes were pre-incubated with PF at indicated concentration, then treated with TNFα for 24 h, TNFα-stimulated glycerol release was blocked by PF in a concentration-dependent manner.

In 3T3-L1 adipocytes or differentiated human adipocytes, the activation of ERK1/2 appears to be a major pathway for the regulation of TNFα-stimulated lipolysis [Souza et al., 2003], and the phosphorylation of JNK and NF-κB may also participate in this process [Ryden et al., 2004; Laurencikiene et al., 2007]. To investigate the mechanisms by which PF inhibit TNFα-stimulated lipolysis, we performed immunoblot analysis to examine the phosphorylation of three mitogen-activated protein kinases (MAPKs) and IKK/NF-κB pathway, including ERK1/2, p38, JNK and IKK. As shown in Figure 2B, MAPK phosphorylation were promoted in TNFα-stimulated adipocytes, but treatment with 50 mg/L PF inhibited the phosphorylation of ERK1/2, JNK, P38 ($P < 0.05$). IKK phosphorylation and IκBα degradation were also promoted by TNFα, treatment with PF partially reversed the effects ($P < 0.05$). Perilipin coats the surface of intracellular lipid droplets in adipocytes, has a firmly established function to modulate lipolysis by limiting the lipase access to the triacylglycerol core stored within the lipid droplets [Souza et al., 1998; Londos et al., 1999]. PF also prevented the decrease in perilipin level in TNFα-treated adipocytes ($P < 0.05$; Fig. 2C). In the lipolysis assay, we found that PD98059 (ERK inhibitor), but not SP600125 (JNK inhibitor) or BAY11-7085 (NF-κB inhibitor) significantly suppressed TNFα-induced lipolysis in adipocytes. These results imply that PF exerts its antilipolytic action mainly through blocking TNFα-activated ERK1/2 and preventing the downregulation of perilipin.

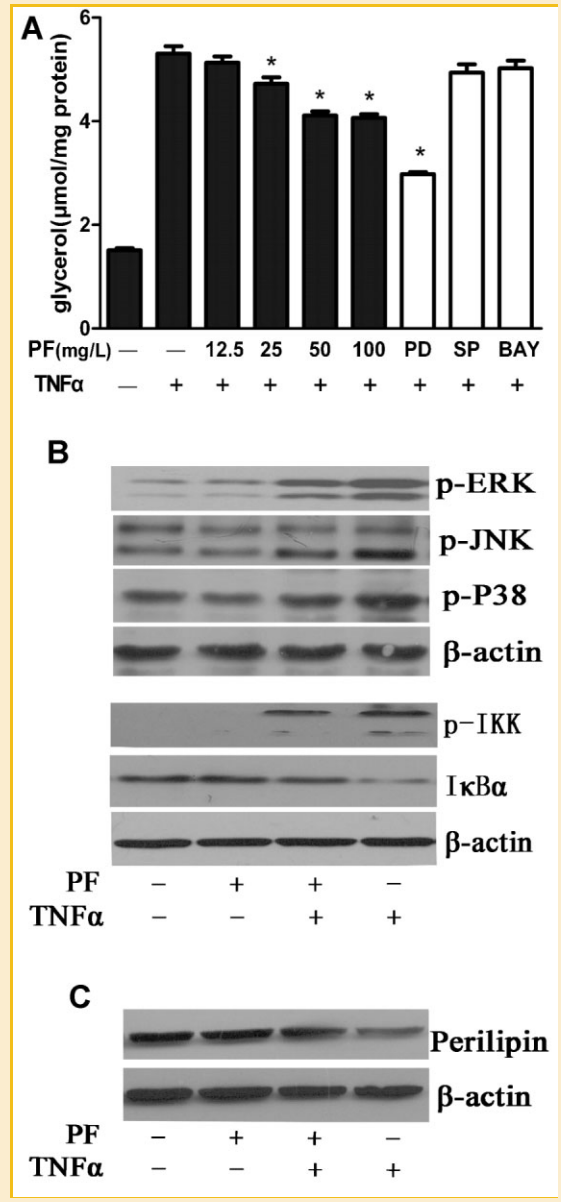

Fig. 2. PF attenuated adipocyte lipolysis stimulated by TNFα. A: 3T3-L1 adipocytes were preincubated with indicated concentrations of PF, 20 μM PD98059 (PD), 10 μM SP600125 (SP), 10 μM BAY11-7085 (BAY) for 2 h, and the cells were then treated for 24 h after the addition of 10ng/ml TNFα. Glycerol release in the culture media was assayed. Data are expressed as means ± SE of six tests. B: 3T3-L1 adipocytes were pretreated with 50 mg/L PF for 12 h. Cells were then stimulated with 10 ng/ml TNFα, PF, or both for another 5–15 min. Change of ERK, p38, JNK, IKK phosphorylation and IκBα degradation were analyzed by Western blotting. Results are representative of three individual experiments. C: Adipocytes were preincubated for 12 h with 50 mg/L PF, and then treated for 24 h in the presence of 10ng/ml TNFα, PF, or both. The cells were lysed and perilipin protein expression was analyzed by Western blot of whole cell lysates. Results are representative of three individual experiments. $^{*}P < 0.05$ versus 10 ng/ml TNFα alone.

EFFECT OF PF ON TNFα-INDUCED INSULIN RESISTANCE IN 3T3-L1 ADIPOCYTES

In addition to lipolysis acceleration, TNFα can induce adipocyte insulin resistance [Stephens et al., 1997; Qi and Pekala, 2000]. From Figure 3, treatment with 10 ng/ml TNFα for 96 h decreased the insulin-stimulated 2-DOG uptake by 45.7%. Co-treatment with PF antagonized the effect of TNFα and partially reversed TNFα-induced decrease in 2-DOG uptake in a dose-dependent manner. These results indicate that TNFα causes insulin resistance in 3T3-L1 adipocytes and PF antagonizes the effects.

EFFECT OF PF ON THE EXPRESSION OF TNFα INDUCED BY PALMITATE IN RAW 264.7 MACROPHAGES

From above, PF attenuated FFA release from adipocytes stimulated by TNFα. We then investigated whether PF could suppress FFA-induced TNFα expression in macrophages. RAW 264.7 macrophages were stimulated with palmitate, one of the most important saturated FFAs produced most abundantly in 3T3-L1 adipocytes, in the presence or absence of PF. From Figure 4, palmitate significantly enhanced TNFα mRNA expression in RAW264.7 cells and PF partially inhibited the effects. Moreover, ERK, JNK and NF-κB inhibitor significantly suppressed palmitate-induced TNFα mRNA upexpression in RAW264.7 macrophages ($P < 0.01$). These observations, taken together, indicate that ERK, JNK and NF-κB pathway are involved in palmitate-induced TNFα expression and PF may

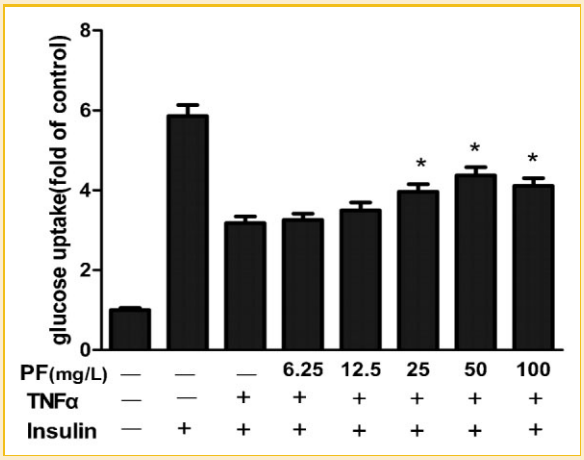

Fig. 3. Effect of TNFα and PF on 2-DOG uptake. Fully differentiated 3T3-L1 adipocytes were treated with TNFα (10 ng/ml) in the absence or presence of PF at the indicated concentration for 96 h, and then [^{3}H]2-DOG uptake was measured as described under Materials and Methods Section. Means ± SE for six independent experiments are shown. $^*P < 0.05$ versus 10ng/ml TNFα alone.

antagonize the effects of palmitate on RAW 264.7 cells through MAPK and IKK/NF-κB pathway.

DISCUSSION

It has been shown that paracrine interactions between adipocytes and nonadipocyte cells such as macrophages augment the inflammatory response of adipose tissue in obesity. For example, adipocyte-derived FFA and macrophage-derived TNFα constitute a vicious cycle [Weisberg et al., 2003; Suganami et al., 2005]. Our data also showed that the coculture of 3T3-L1 adipocytes and RAW 264.7 macrophages resulted in a marked upregulation of the expression of proinflammatory mediators, namely, TNFα, FFA, and MCP-1, which were consistent with a previous report [Suganami et al., 2005]. We originally found that PF significantly suppressed the coculture-induced upregulation of MCP-1, TNFα, and FFA, suggesting that PF might directly suppress chronic inflammatory responses in obese adipose tissue.

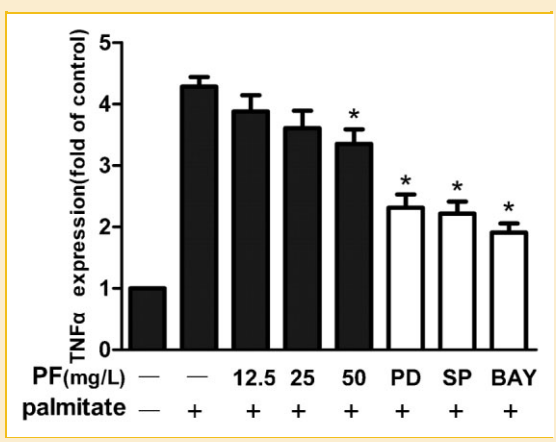

Fig. 4. Effect of PF on the expression of TNFα induced by palmitate in RAW 264.7 macrophages. RAW 264.7 cells were preincubated with indicated concentrations of PF, 20 μM PD98059 (PD), 10 μM SP600125 (SP), 10 μM BAY11-7085 (BAY) for 2 h, and the cells were then stimulated with palmitate (250 μM) for 24 h. TNFα expression was analyzed by real-time PCR. The values are means ± SE of four tests. $^*P < 0.05$ compared with palmitate alone.

Next we investigated the inhibiting targets of PF on TNFα-FFA inflammation cycle. TNFα, a representative proinflammatory cytokine, is implicated in the metabolic disturbances of chronic inflammation and pathogenesis of insulin resistance. Several laboratories have confirmed that TNFα can increase adipocyte lipolysis and release of FFA [Petruschke and Hauner, 1993; Doerrler et al., 1994; Green et al., 1994]. Besides, interstitial levels of TNFα and FFA in adipose tissue are positively correlated [Orban et al., 1999]. Therefore, TNFα plays an important role in elevated circulating FFA concentrations in obesity and type 2 diabetes. In the current study, we originally found that PF blocked adipocyte lipolysis stimulated by TNFα. Some previous researches have demonstrated that the ERK1/2 pathway in 3T3-L1 adipocytes and the JNK, IKK pathway in differentiated human adipocytes, participated in the regulation of TNFα-stimulated lipolysis [Zhang et al., 2002; Souza et al., 2003; Ryden et al., 2004]. Our results also showed that ERK inhibitor, but not JNK inhibitor or NF-κB inhibitor suppressed TNFα-induced lipolysis in 3T3-L1 adipocytes. Perilipin is a substrate-associating protein and an active participant in lipolytic modulation [Souza et al., 1998; Mottagui-Tabar et al., 2003]. Downregulated perilipin is thought to account for the increased lipolytic action of TNFα in differentiated adipocytes [Souza et al., 1998; Zhang et al., 2002]. In our study, PF not only attenuated the TNFα-stimulated ERK1/2 activation but also preserved the TNFα-decreased barrier function of perilipin. These two effects together predominantly account for the molecular basis of the antilipolytic action of PF in TNFα-stimulated 3T3-L1 adipocytes. In view of the different mechanisms of TNFα-induced lipolysis between 3T3-L1 adipocytes and human adipocytes, further researches are needed to study the effect of PF on human adipocytes. Additionally, NF-κB is a primary regulator of inflammatory responses. Activation of the NF-κB pathway is involved in the TNFα-induced proinflammatory cytokines production from adipocytes (e.g., MCP-1 and IL-6) [Suganami et al., 2007]. Therefore, the inhibition of NF-κB by PF may also play a role in the decreased inflammatory interaction between adipocytes and macrophages through reduced proinflammatory cytokines production from adipocytes.

In addition to lipolytic acceleration, TNFα can act to render adipocyte insulin resistance. We found that PF increased the insulin-stimulated 2-DOG uptake in TNFα-treated adipocytes, suggesting PF antagonizes the TNFα-induced insulin resistance. As we know, mechanisms of TNFα to render adipocyte insulin resistance are complex, which maybe related to the change of glucose transporter, insulin signaling inhibition, ceramide generation, etc. [Stephens et al., 1997; Qi and Pekala, 2000; Chavez and Summers, 2003]. Further studies are needed to elucidate the effects of PF. Yet from our

data, attenuation of TNFα-stimulated activation of JNK and IKK pathway by PF may play a part [Aguirre et al., 2000; Gao et al., 2002].

Finally we also found that PF inhibited FFA-stimulated TNFα expression in RAW264.7 macrophages. FFAs are important adipocyte-derived paracrine mediators of inflammation in macrophages. Saturated FFAs, such as palmitate, a major FFA released from 3T3-L1 adipocytes, can increase TNFα production and, thus, induce inflammatory changes in RAW264.7 [Lee et al., 2003]. The precise mechanisms by which FFAs enhance TNFα expression are currently unknown, but several lines of evidence demonstrate that elevated FFA concentrations lead to the induction of increased NF-κB binding activity in circulating mononuclear cells (MNCs) [Tripathy et al., 2003] and palmitate could increase TNFα expression through activation of MAP kinases and NF-κB in RAW 264.7 macrophages [Suganami et al., 2005; Suganami et al., 2007]. Thus, the effect of PF on MAP kinases and NF-κB signaling pathway in macrophages needs to be further elucidated. In addition, because of the differences among several macrophage cell lines and normal macrophages [Marantos et al., 2008], further studies are needed to verify the effects of PF.

Moreover, PF significantly decreased MCP-1 secretion level in the contact system (Fig. 1B). MCP-1 plays a crucial role in the augmentation of inflammatory responses in obesity by enhancing the migration of monocytes into adipose tissue and activation into macrophages [Kamei et al., 2006]. Our results imply PF may prevent the recruitment of monocytes into obese adipose tissue by reducing the production of MCP-1 by adipocytes and macrophages in vivo. Further studies are needed to clarify the effects of PF on the migration of monocytes and the macrophage infiltration in vivo and in vitro.

In summary, the results presented here showed that PF suppressed the production of proinflammatory mediators by adipocytes cocultured with macrophages. This effect of PF attributed not only to blocking TNFα-stimulated adipocytes lipolysis, but also to suppressing the production of TNFα in FFA-activated macrophages. PF may have a potential to improve chronic inflammatory conditions in obesity, and improve obesity-related insulin resistance.

REFERENCES

Aguirre V, Uchida T, Yenush L, Davis R, White MF. 2000. The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307). J Biol Chem 275:9047–9054.

Chavez JA, Summers SA. 2003. Characterizing the effects of saturated fatty acids on insulin signaling and ceramide and diacylglycerol accumulation in 3T3-L1 adipocytes and C2C12 myotubes. Arch Biochem Biophys 419:101–109.

Dandona P, Aljada A, Chaudhuri A, Mohanty P, Garg R. 2005. Metabolic syndrome: A comprehensive perspective based on interactions between obesity, diabetes, and inflammation. Circulation 111:1448–1454.

Doerrler W, Feingold KR, Grunfeld C. 1994. Cytokines induce catabolic effects in cultured adipocytes by multiple mechanisms. Cytokine 6:478–484.

Fernandez-Real JM, Ricart W. 2003. Insulin resistance and chronic cardiovascular inflammatory syndrome. Endocr Rev 24:278–301.

Gao Z, Hwang D, Bataille F, Lefevre M, York D, Quon MJ, Ye J. 2002. Serine phosphorylation of insulin receptor substrate 1 by inhibitor kappa B kinase complex. J Biol Chem 277:48115–48121.

Goto H, Shimada Y, Akechi Y, Kohta K, Hattori M, Terasawa K. 1996. Endothelium-dependent vasodilator effect of extract prepared from the roots of Paeonia lactiflora on isolated rat aorta. Planta Med 62:436–439.

Green A, Dobias SB, Walters DJ, Brasier AR. 1994. Tumor necrosis factor increases the rate of lipolysis in primary cultures of adipocytes without altering levels of hormone-sensitive lipase. Endocrinology 134:2581–2588.

Hsu FL, Lai CW, Cheng JT. 1997. Antihyperglycemic effects of paeoniflorin and 8-debenzoylpaeoniflorin, glucosides from the root of Paeonia lactiflora. Planta Med 63:323–325.

Iwata M, Haruta T, Usui I, Takata Y, Takano A, Uno T, Kawahara J, Ueno E, Sasaoka T, Ishibashi O, Kobayashi M. 2001. Pioglitazone ameliorates tumor necrosis factor-alpha-induced insulin resistance by a mechanism independent of adipogenic activity of peroxisome proliferator-activated receptor-gamma. Diabetes 50:1083–1092.

Kamei N, Tobe K, Suzuki R, Ohsugi M, Watanabe T, Kubota N, Ohtsuka-Kowatari N, Kumagai K, Sakamoto K, Kobayashi M, Yamauchi T, Ueki K, Oishi Y, Nishimura S, Manabe I, Hashimoto H, Ohnishi Y, Ogata H, Tokuyama K, Tsunoda M, Ide T, Murakami K, Nagai R, Kadowaki T. 2006. Overexpression of monocyte chemoattractant protein-1 in adipose tissues causes macrophage recruitment and insulin resistance. J Biol Chem 281:26602–26614.

Laurencikiene J, van Harmelen V, Arvidsson Nordstrom E, Dicker A, Blomqvist L, Naslund E, Langin D, Arner P, Ryden M. 2007. NF-kappaB is important for TNF-alpha-induced lipolysis in human adipocytes. J Lipid Res 48:1069–1077.

Lee JY, Plakidas A, Lee WH, Heikkinen A, Chanmugam P, Bray G, Hwang DH. 2003. Differential modulation of Toll-like receptors by fatty acids: Preferential inhibition by n-3 polyunsaturated fatty acids. J Lipid Res 44:479–486.

Liu DF, Wei W, Song LH. 2006a. Protective effect of paeoniflorin on immunological liver injury induced by bacillus Calmette-Guerin plus lipopolysaccharide: Modulation of tumour necrosis factor-alpha and interleukin-6 MRNA. Clin Exp Pharmacol Physiol 33:332–339.

Liu J, Jin DZ, Xiao L, Zhu XZ. 2006b. Paeoniflorin attenuates chronic cerebral hypoperfusion-induced learning dysfunction and brain damage in rats. Brain Res 1089:162–170.

Londos C, Brasaemle DL, Schultz CJ, Adler-Wailes DC, Levin DM, Kimmel AR, Rondinone CM. 1999. On the control of lipolysis in adipocytes. Ann NY Acad Sci 892:155–168.

Lyon CJ, Law RE, Hsueh WA. 2003. Minireview: Adiposity, inflammation, and atherogenesis. Endocrinology 144:2195–2200.

Marantos C, Mukaro V, Ferrante J, Hii C, Ferrante A. 2008. Inhibition of the lipopolysaccharide-induced stimulation of the members of the MAPK family in human monocytes/macrophages by 4-hydroxynonenal, a product of oxidized omega-6 fatty acids. Am J Pathol 173:1057–1066.

Miranda PJ, DeFronzo RA, Califf RM, Guyton JR. 2005. Metabolic syndrome: Definition, pathophysiology, and mechanisms. Am Heart J 149:33–45.

Mottagui-Tabar S, Ryden M, Lofgren P, Faulds G, Hoffstedt J, Brookes AJ, Andersson I, Arner P. 2003. Evidence for an important role of perilipin in the regulation of human adipocyte lipolysis. Diabetologia 46:789–797.

Nizamutdinova IT, Jin YC, Kim JS, Yean MH, Kang SS, Kim YS, Lee JH, Seo HG, Kim HJ, Chang KC. 2008. Paeonol and paeoniflorin, the main active principles of Paeonia albiflora, protect the heart from myocardial ischemia/reperfusion injury in rats. Planta Med 74:14–18.

Ohta H, Matsumoto K, Shimizu M, Watanabe H. 1994. Paeoniflorin attenuates learning impairment of aged rats in operant brightness discrimination task. Pharmacol Biochem Behav 49:213–217.

Orban Z, Remaley AT, Sampson M, Trajanoski Z, Chrousos GP. 1999. The differential effect of food intake and beta-adrenergic stimulation on adipose-derived hormones and cytokines in man. J Clin Endocrinol Metab 84:2126–2133.

Petruschke T, Hauner H. 1993. Tumor necrosis factor-alpha prevents the differentiation of human adipocyte precursor cells and causes delipidation of newly developed fat cells. J Clin Endocrinol Metab 76:742–747.

Qi C, Pekala PH. 2000. Tumor necrosis factor-alpha-induced insulin resistance in adipocytes. Proc Soc Exp Biol Med 223:128–135.

Ryden M, Arvidsson E, Blomqvist L, Perbeck L, Dicker A, Arner P. 2004. Targets for TNF-alpha-induced lipolysis in human adipocytes. Biochem Biophys Res Commun 318:168–175.

Souza SC, de Vargas LM, Yamamoto MT, Lien P, Franciosa MD, Moss LG, Greenberg AS. 1998. Overexpression of perilipin A and B blocks the ability of tumor necrosis factor alpha to increase lipolysis in 3T3-L1 adipocytes. J Biol Chem 273:24665–24669.

Souza SC, Palmer HJ, Kang YH, Yamamoto MT, Muliro KV, Paulson KE, Greenberg AS. 2003. TNF-alpha induction of lipolysis is mediated through activation of the extracellular signal related kinase pathway in 3T3-L1 adipocytes. J Cell Biochem 89:1077–1086.

Stephens JM, Lee J, Pilch PF. 1997. Tumor necrosis factor-alpha-induced insulin resistance in 3T3-L1 adipocytes is accompanied by a loss of insulin receptor substrate-1 and GLUT4 expression without a loss of insulin receptor-mediated signal transduction. J Biol Chem 272:971–976.

Suganami T, Nishida J, Ogawa Y. 2005. A paracrine loop between adipocytes and macrophages aggravates inflammatory changes: Role of free fatty acids and tumor necrosis factor alpha. Arterioscler Thromb Vasc Biol 25:2062–2068.

Suganami T, Tanimoto-Koyama K, Nishida J, Itoh M, Yuan X, Mizuarai S, Kotani H, Yamaoka S, Miyake K, Aoe S, Kamei Y, Ogawa Y. 2007. Role of the Toll-like receptor 4/NF-kappaB pathway in saturated fatty acid-induced inflammatory changes in the interaction between adipocytes and macrophages. Arterioscler Thromb Vasc Biol 27:84–91.

Sun R, Yi YP, Lv LL, Zhang ZP, Sun H, Liu GQ. 2007. Effects of paeoniflorin on pathological changes in global brain ischemia model rats. Zhongguo Zhong Yao Za Zhi 32:2518–2522.

Tafuri SR. 1996. Troglitazone enhances differentiation, basal glucose uptake, and Glut1 protein levels in 3T3-L1 adipocytes. Endocrinology 137:4706–4712.

Tripathy D, Mohanty P, Dhindsa S, Syed T, Ghanim H, Aljada A, Dandona P. 2003. Elevation of free fatty acids induces inflammation and impairs vascular reactivity in healthy subjects. Diabetes 52:2882–2887.

Tsuboi H, Hossain K, Akhand AA, Takeda K, Du J, Rifa'i M, Dai Y, Hayakawa A, Suzuki H, Nakashima I. 2004. Paeoniflorin induces apoptosis of lymphocytes through a redox-linked mechanism. J Cell Biochem 93:162–172.

Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr. 2003. Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest 112:1796–1808.

Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, Chen H. 2003. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J Clin Invest 112: 1821–1830.

Yamahara J, Yamada T, Kimura H, Sawada T, Fujimura H. 1982. Biologically active principles of crude drugs. II. Anti-allergic principles in ''Shoseiryu-To'' anti-inflammatory properties of paeoniflorin and its derivatives. J Pharmacobiodyn 5:921–929.

Yang HO, Ko WK, Kim JY, Ro HS. 2004. Paeoniflorin: An antihyperlipidemic agent from Paeonia lactiflora. Fitoterapia 75:45–49.

Zhang HH, Halbleib M, Ahmad F, Manganiello VC, Greenberg AS. 2002. Tumor necrosis factor-alpha stimulates lipolysis in differentiated human adipocytes through activation of extracellular signal-related kinase and elevation of intracellular cAMP. Diabetes 51:2929–2935.

Zhang LL, Wei W, Wang NP, Wang QT, Chen JY, Chen Y, Wu H, Hu XY. 2008. Paeoniflorin suppresses inflammatory mediator production and regulates G protein-coupled signaling in fibroblast-like synoviocytes of collagen induced arthritic rats. Inflamm Res 57:388–395.