Curcumin, a Potential Inhibitor of Up-regulation of TNF-alpha and IL-6 Induced by Palmitate in 3T3-L1 Adipocytes through NF-kappaB and JNK Pathway[1]

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Objective To investigate the attenuating effect of curcumin, an anti-inflammatory compound derived from dietary spice turmeric (*Curcuma longa*) on the pro-inflammatory insulin-resistant state in 3T3-L1 adipocytes. **Methods** Glucose uptake rate was determined with the [^{3}H] 2-deoxyglucose uptake method. Expressions of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were measured by quantitative RT-PCR analysis and ELISA. Nuclear transcription factor kappaB p65 (NF-κB p65) and mitogen-activated protein kinase (MAPKs) were detected by Western blot assay. **Results** The basal glucose uptake was not altered, and curcumin increased the insulin-stimulated glucose uptake in 3T3-L1 cells. Curcumin suppressed the transcription and secretion of TNF-α and IL-6 induced by palmitate in a concentration-dependent manner. Palmitate induced nuclear translocation of NF-kB. The activities of Jun NH2-terminal kinase (JNK), extracellular signal–regulated kinase1/2 (ERK1/2) and p38MAPK decreased in the presence of curcumin. Moreover, pretreatment with SP600125 (inhibitor of JNK) instead of PD98059 or SB203580 (inhibitor of ERK1/2 or p38MAPK, respectively) decreased the up-regulation of TNF-α induced by palmitate. **Conclusion** Curcumin reverses palmitate-induced insulin resistance state in 3T3-L1 adipocytes through the NF-kB and JNK pathway.

Key words: Curcumin; Insulin resistance; Inflammation; Adipocyte; Free fatty acids

INTRODUCTION

Although type 2 diabetes is closely associated with obesity, the mechanisms by which obesity leads to type 2 diabetes remain unclear. Insulin resistance is a common pathogenesis of obesity and type 2 diabetes because obesity leads to hyperlipidemia. High level of free fatty acids (FFAs) in plasma and tissue reduces insulin sensitivity, impairs insulin signaling and induces insulin resistance[1–8].

Pro-inflammatory cytokines play a critical role in the development of insulin resistance[9] and their levels are elevated in insulin-resistant states like obesity and type 2 diabetes[10-13]. *In vivo* and *in vitro* studies demonstrated that activation of pro-inflammatory pathways is mechanically linked to insulin resistance, and that the NF-κB pathway plays a critical role in lipid-induced insulin resistance. Moreover, data suggest that FFA-derived metabolic products can activate JNK, NF-κB, and protein kinase θ (PKCθ)[14-17], all of which can phosphorylate insulin receptor substrate-1 (IRS-1) on serine residues. Consequently, IRS-1 activation through tyrosine phosphorylation is impaired, leading to a reduction in insulin receptor-mediated signaling and subsequent insulin resistance.

Curcumin derived from the rhizome of the herb *Curcuma longa* has been used for centuries in Asia as a dietary spice, and may be of therapeutic benefits to several diseases[18-21]. Curcumin has been used traditionally as an antidiabetic agent[22-24]. Its potential antidiabetic effect is determined, based on murine animal models. Previous studies have confirmed that oral curcumin treatment improves hyperglycemia in KK-Ay mice and streptozotocin-treated rats[25-29]. Curcumin also exerts potential anti-inflammatory effects by inhibiting pro-inflammatory cytokines and chemokines, adhesion molecules, cyclooxygenase-2, tissue factor and inducible nitric oxide synthase in diverse cell types (pancreatic cells, chodrocytes, and

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hepatic cells)[30-31]. These suppressive effects are due to the inhibition of the NF-kB pathway and other pro-inflammatory signaling pathways including MAPKs[33-36]. Although these pro-inflammatory signaling pathways might be involved in the pathogenesis of type 2 diabetes, there is no evidence that curcumin is an anti-inflammatory agent against obesity-induced insulin resistance. The present study was designed to study the underlying mechanism of curcumin to reduce pro-inflammatory cytokines in 3T3-L1 adipocytes with FFA- induced insulin resistance. Our specific aim was to investigate whether the effect of curcumin on adipokins is dependent on the NF-κB and MAPKs pathways. The results of this study may provide potential evidence for the treatment of obesity and type 2 diabetes with curcumin.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's medium and fetal bovine serum (FBS) were purchased from GIBCO (BRL, USA). Curcumin, palmitate, SP600125, PD98059 and SB203580 were purchased from Sigma-Aldrich (St. Louis, MO). [^{3}H] 2-deoxyglucose was obtained from PerkinElmer Life and Analytical Sciences. Rabbit antibody to SAPK/JNK (Thr183/ Thr185), phospho-SAPK/JNK (Thr183/Thr185), p38MAPK, phospho-p38MAPK, p44/ 42MAPK (Thr202/Tyr204), phospho-p44/42MAPK (Thr202/ Tyr204) were purchased from Cell Signaling (Beverly, MA). NF-κB p65 antibody and HRP-conjugated anti-rabbit antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). ECL plus Western blot detection system was purchased from Amersham Biosciences (GE Healthcare, UK). TNF-α, IL-6 ELISA kit were purchased from Jingmei (Jingmei Biotech, China).

Cell Culture and Treatment

3T3-L1 preadipocytes (American Type Culture Collection, Manassas, VA) were grown in DMEM containing 10% fetal bovine serum (FBS) and fed every 2 days. Two days after confluence (day 0), the medium was switched to DMEM supplemented with 10% FBS, 5 μg/mL insulin, 0.5 mmol/L 3-isobutyl-1-methylxanthine and 1 μmol/L dexamethasone. On day 2, the medium was changed to DMEM containing 10% FBS and 5 μg/mL insulin. Beginning on day 4, the medium was changed to DMEM containing only 10% FBS, and cells were given fresh medium every 2 days. Unless indicated otherwise, adipocytes were used 10-12 days after differentiation. After DMEM supplemented with 0.5% FFA-free bovine albumin, 3T3-L1 adipocytes were treated with 0.25 mmol/L palmitate in the presence of 0.5% FFA-free BSA with or without the indicated doses of curcumin (final concentration: 5 μmol/L,10 μmol/L, 20 μmol/L) for 24 h.

[^{3}H] 2-Deoxyglucose Uptake Assay

3T3-L1 preadipocytes (5×10^5/well) were differentiated to adipocytes in a 24-well plate. After serum-starvation in 0.2% BSA DMEM overnight, the cells were incubated in 0.2% BSA DMEM containing 0.25 mmol/L PA or (and) 5 μmol/L, 10 μmol/ L or 20 μmol /L curcumin for 24 or 48 h. The cells were then incubated in 1 mL Krebs/ Ringer phosphate (KRP)/HEPES (131.2 mmol/L NaCl, 4.71 mmol/L KCl, 2.47 mmol/L $CaCl_2$, 1.24 mmol/L $MgSO_4$, 2.48 mmol/L Na_3PO_4, 10 mmol/L HEPES, pH 7.4) with or without 100 nmol/L insulin for 30 min at 37 ℃, after washed three times in KRP/HEPES buffer. Finally the cells were incubated in 1 mL KRP/HEPES containing 0.5 μCi/mL 2-deoxy-D-[^{3}H] glucose for 10 min at 37 ℃. The cells were washed three times with ice-cold PBS and solubilized in 1 mL 0.1 mol/L NaOH for 2 h. Radioactivity was determined by liquid scintillation spectrometry. Non-specific deoxyglucose uptake was measured in the presence of 20 μmol/L cytochalasin B, and specific glucose uptake was detected from the subtracted total uptake. Three replicate wells were set up and each experiment was performed in triplicate.

Cell Lysates and Western Blot

Cells were washed with phosphate-buffered saline and lysis buffer [1% Triton X-100, 50 mmol/L KCl, 25 mmol/L HEPES, pH 7.8, 10 μg/mL leupeptin, 20 μg/mL aprotinin, 125 μmol/L dithiothreitol (DTT), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mmol/L sodium orthovanadate] and then added to the cells. The lysate was centrifuged at 12 000 rpm for 10 min. Cytosolic and nuclear fractions were separated (first buffer: 10 mmol/L HEPES pH 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA, 1.5 mmol/L $MgCl_2$, 0.1% NP40, 1 mmol/L DTT; second buffer: 20 mmol/L HEPES pH 7.9, 420 mmol/L NaCl, 0.1 mmol/L EDTA, 1.5 mmol/L $MgCl_2$, 25% glycerol, 1 mmol/L DTT, 0.5 mmol/L PMSF). Protein concentrations were measured with a BCA protein assay kit (Pierce, USA). The lysate was boiled in a SDS loading buffer and applied on SDS-PAGE. Following gel transference, polyvinylidene difluoride (PVDF) membranes were blocked with 1% BSA in phosphate-buffered saline-Tween 20 for 1 h. Membranes were probed

with primary antibodies overnight at 4 ℃ followed by incubation for 1 h with secondary HRP-conjugated antibodies diluted at 1:5000 in a blocking solution. Proteins were visualized by ECL substrate. Primary antibodies (JNK, Phospho-SAPK/JNK, p44/42 MAPK, Phospho-p44/42 MAPK, p38 MAPK, and Phospho-p38 MAPK) were diluted at 1:1000 according to the manufacturer's protocol. NF-κB antibody was diluted at 1:200.

RNA Isolation and Quantitative RT-PCR Analysis

RNA was isolated from the cells with TRIzol according to its manufacturer's protocol and reverse transcribed to cDNA using a RNA PCR kit (Takara, Dalian, China) according to its manufacturer's protocol. Primer and probe sequences are as follows, from the 5' to the 3'end: TNF-α forward ACCTTTCCAGATTCTTCCCTGAG, reverse CCCGGCCTTCCAAATAAATACATT, probe ACAGCCTTCCTCACAGAGCCAGCC; IL-6: forward GAGGATACCACTCCCAACAGACC, reverse AAGTGCATCATCGTTGTTCATACA, probe CAGAATTGCCATTGCACAACTCTTTTCTCA; β-actin: forward CTTCTTTGCAGCTCCTTCGTTG, reverse ATGGAGGGGAATACAGCCCG, probe CCACACCCGCCACCAGTTCGCC. RT-PCR was performed on an ABI Prism 7500 fast sequence detection system (Applied Biosystems), with the TaqMan fluorogenic detection system. Twenty-five microliters of reaction volume were used per well, and all samples were run in triplicate. The expression of target genes was normalized to β-actin RNA measured simultaneously.

Enzyme-linked Immunosorbent Assays

To measure the TNF-α and IL-6 secretion from 3T3-L1 cells, the medium was collected and quantified using a commercial ELISA according to its manufacturer's protocols.

Statistical Analysis

All results are expressed as $\bar{x} \pm s$. Statistical and graphical analysis was performed with SPSS version 10.0. The significance of differences between groups was determined by Student's t test. $P<0.05$ was considered statistically significant.

RESULTS

Curcumin Elevated Insulin-induced Glucose Uptake in 3T3-L1 Adipocytes with Insulin Resistance

3T3-L1 adipocytes were used as a cellular model to analyze the insulin signaling pathway, and treated with palmitate (0.25 mmol/L, 24 h) to induce insulin resistance. Insulin-induced glucose uptake was measured to determine insulin sensitivity. The results showed that insulin-induced glucose uptake was increasingly higher than that in the normal control group, but was inhibited by as much as 67% after incubation with palmitate for 24 h. However, intervention with curcumin reversed the situation completely, and curcumin increased insulin-stimulated 2-deoxyglucose glucose uptake in 3T3-L1 adipocytes in a dose-dependent manner (Fig. 1).

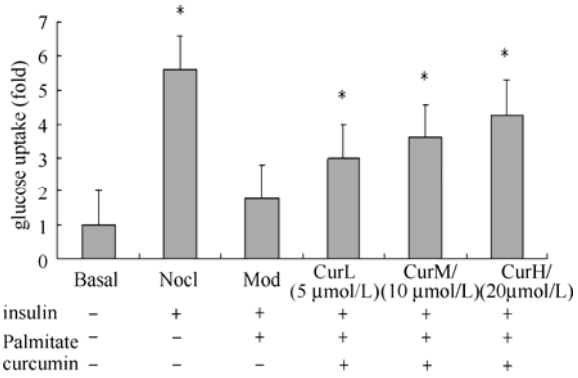

FIG. 1. Effects of curcumin on insulin-stimulated 2-deoxyglucose glucose uptake in 3T3-L1 adipocytes. 3T3-L1 proadipocytes were cultured and differentiated to adipocytes. 3T3-L1 adipocytes were then exposed to 0.25 mmol/L palmitate for 24 h. Thereafter, [^{3}H] 2DG uptake was determined as described under "Materials and Methods". 2DG uptake in the absence (-) or presence (+) of 100 nmol/L insulin with or without curcumin (5, 10, 20 μmol/L). Data represent the mean of at least three independent experiments. $^*P<0.05$ *vs*. the group treated with palmitate plus insulin.

Inhibitory Effects of Curcumin on Expressions of TNF-α and IL-6 mRNAs Induced by Palmitate in 3T3-L1 Adipocytes

3T3-L1 adipocytes were stimulated with palmitate for 24 h in the absence or presence of curcumin pretreatment. Real-time-PCR was used to examine whether curcumin inhibits the up-regulation of TNF-α mRNA induced by palmitate. The results showed that TNF-α mRNA and IL-6 mRNA levels increased in palmitate-treated 3T3-L1 cells, but significantly declined after curcumin treatment (Figs. 2A and B). In addition, conditioned medium was harvested from palmitate-treated adipocytes with or without

curcumin for ELISA. In the experiments, similar results were obtained. Curcumin actually blocked the palmitate-induced accumulation of TNF-α and IL-6 in the culture medium (Fig. 2C and D).

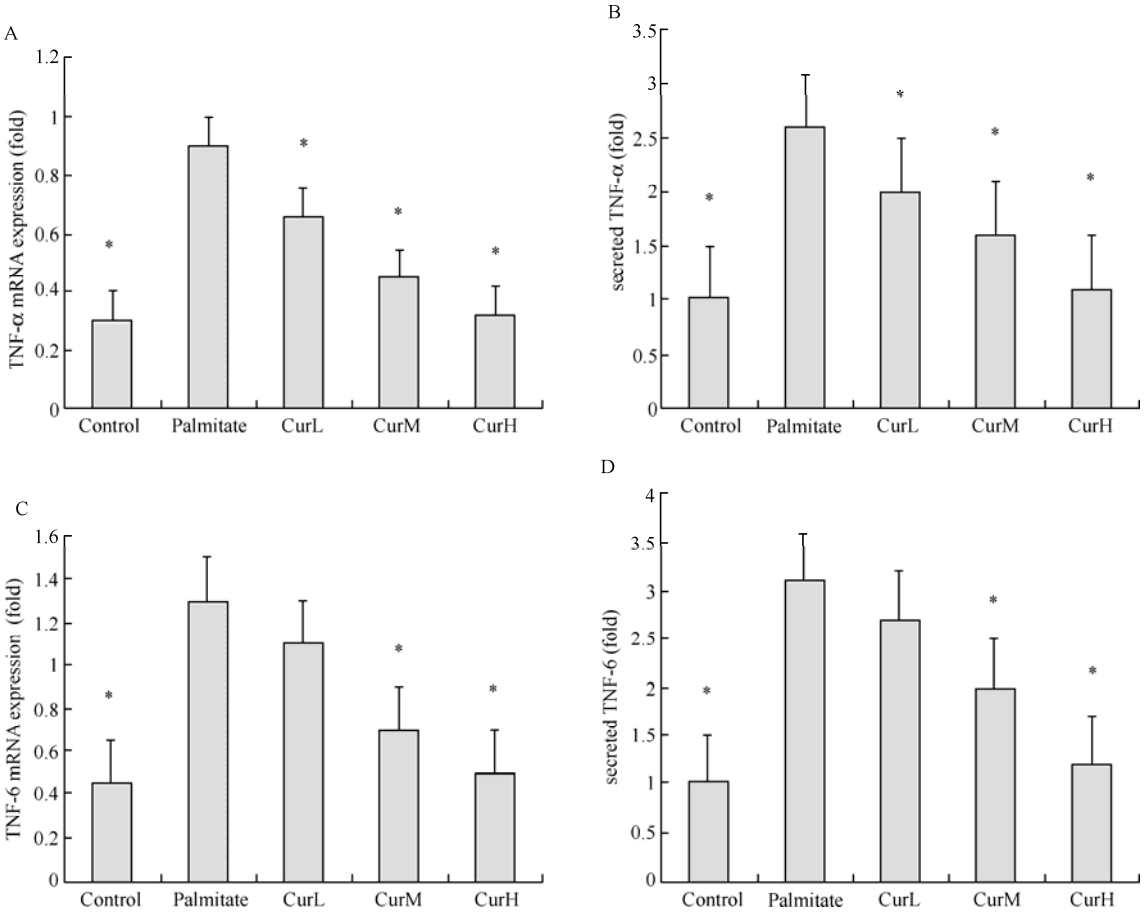

FIG. 2. Inhibitory effects of curcumin on the expressions of TNF-α and IL-6 mRNAs in 3T3-L1 adipocytes. Cells were starved for 6 h, and treated with curcumin (5, 10, 20 μmol/L) for 1 h prior to treatment with 0.25 mmol/L palmitate for 24 h. Conditioned medium was then collected. (A) Expression of TNF-α mRNA which was normalized to β-actin; (B) TNF-α accumulation in the media; (C) Expression of IL-6 mRNA, which was normalized to β-actin; (D) IL-6 concentration. Relative levels of TNF-α and IL-6 released from the control were assessed in three independent experiments. $^{*}P<0.05$ *vs*. the group treated with palmitate alone.

Inhibitory Effects of Curcumin on Protein Expression of Nuclear NF-κB p65 Induced by Palmitate in 3T3-L1 Adipocytes

Owing to a critical role of NF-kB in the development of insulin resistance, the level of p65 which is a functionally active subunit of NF-kB was measured in this study. The effect of curcumin on NF-κB p65 protein level in the whole-cell extracts and nuclear extracts with NF-κB p65 antibody was detected by Western blot assay. In 3T3-L1 adipocytes, palmitate (0.5 mmol/L, 24 h) induced the protein expression of nuclear NF-κB p65, and curcumin was found to be able to reduce the expression of NF-κB p65 protein in a dose-dependent manner ($P<0.05$). However, the whole NF-κB p65 protein level was unchanged during this study (Fig. 3). These results indicate that curcumin could inhibit palmitate-induced translocation of p65 to nuclei.

Inhibitory Effects of Curcumin on Palmitate-induced Activities of MAPKs in 3T3-L1 Adipocytes

Whether curcumin inhibits MAPKs phosphorylation in 3T3-L1 adipocytes was investigated in this study. After incubation with different concentrations of curcumin for 1 h before

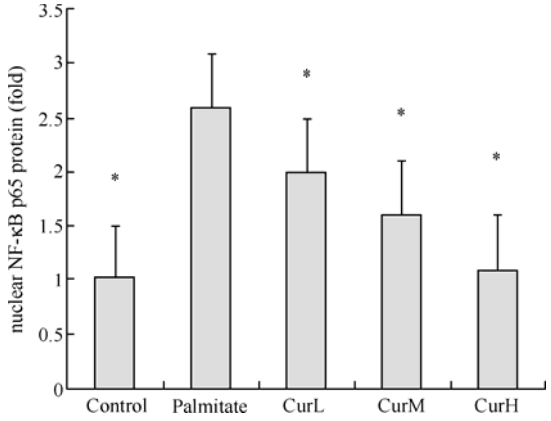

FIG. 3. Effect of curcumin on the whole NF-κB p65 and nucleic NF-κB p65 protein expression in 3T3-L1 adipocytes. Cells in a serum-poor medium were starved for 6 h, then pretreated with curcumin at 5, 10, 20 μmol/L for 1 h, and then with 0.25 mmol/L palmitate for 24 h. Whole NF-κB p65 and nucleic NF-κB p65 protein expression levels were determined in the whole cell lysate and nucleic cell lysate by Western blot. Each experiment was performed in triplicate. $^{*}P<0.05$ vs. the group treated with palmitate alone.

palmitate-treatment, activities of JNK, ERK and p38MAPK in 3T3-L1 adipocytes were determined. Curcumin inhibited the activities of JNK, ERK, and p38MAPK in a dose-dependent manner. Curcumin at the concentration of 20 μmol/L significantly inhibited JNK phosphorylation but did not alter the overall expression of these proteins (Fig. 4A- C).

Inhibitory Effects of the JNK Inhibitor SP600125 on the Expression of TNF-α Induced by Palmitate in 3T3-L1 Adipocytes

Whether palmitate-induced expressions of TNF-α and IL-6 depend on the activation of MAPK pathways was examined. Incubation with a JNK inhibitor (SP600125) markedly inhibited the effect of pamitate on the expression of TNF-α mRNA (Fig. 5A). Curcumin alone at the concentration of 20 mmol/L inhibited TNF-α and JNK (Fig. 5B). ERK or p38MAPK inhibited by PD98059 or SB203580 did not affect TNF-α (data not shown).

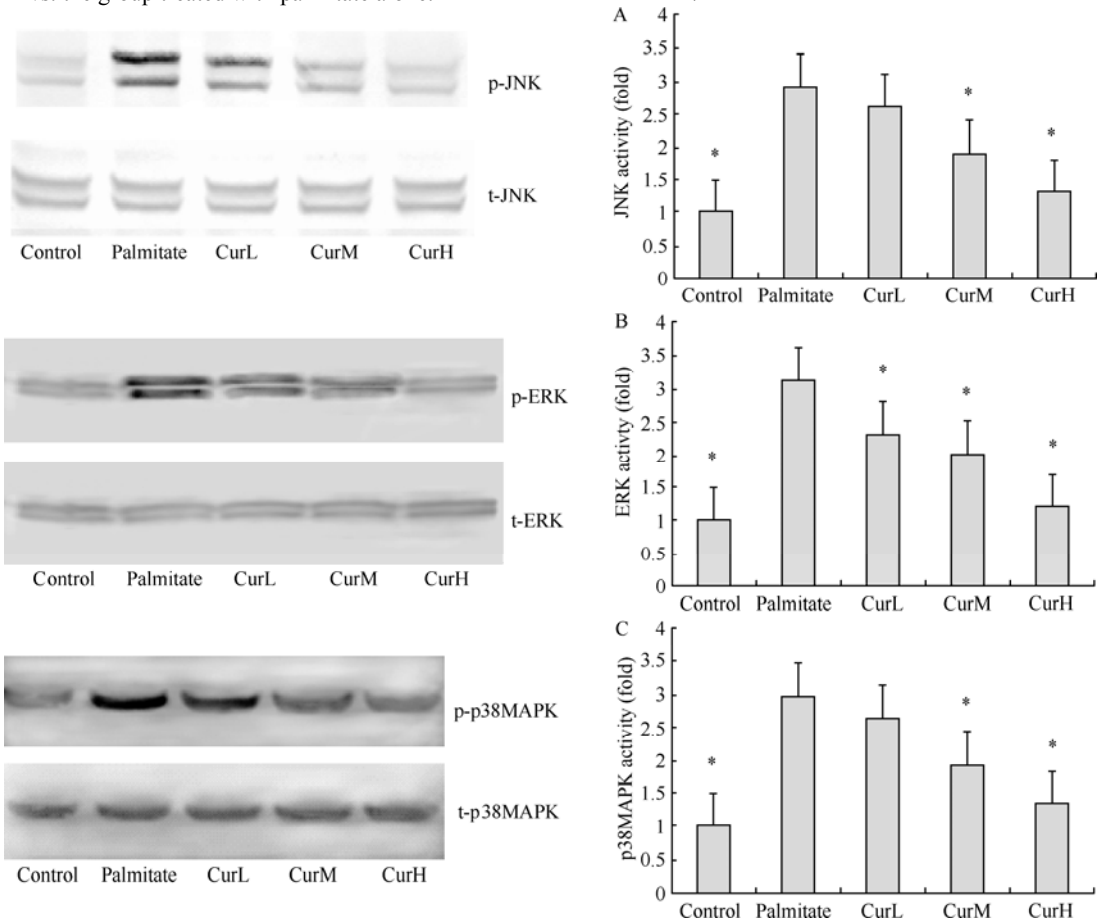

FIG. 4. Effects of curcumin on activities of JNK (A), ERK (B), and p38MAPK (C), as well as expression of these proteins (D) in 3T3-L1 adipocytes. Cells were starved for 6 h, and treated with curcumin (5, 10, 20 μmol/L) for 1 h prior to treatment with 0.25 mmol/L palmitate. The cell lysates were resolved by SDS-PAGE and analyzed using antibodies against total and phosphorylated MAPKs. Representative blots are shown from three independent experiments. $^{*}P<0.05$ *vs*. the group treated with palmitate alone.

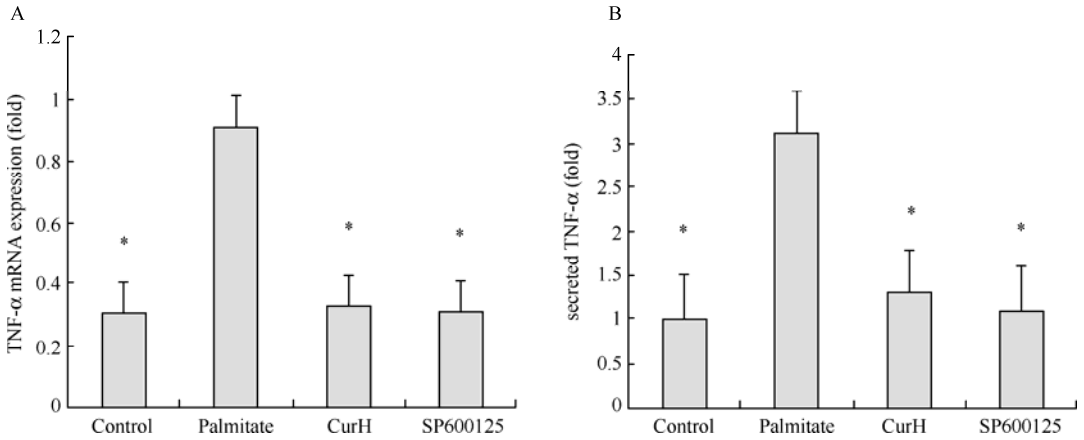

FIG. 5. Effects of SP600125 on the expression of TNF-α mRNA in 3T3-L1 adipocytes (A) and TNF-α accumulation in the media (B). Cells were starved for 6 h, and treated with curcumin (20 μmol/L) and SP600125 (20 μmol/L) for 1 h prior to treatment with 0.25 mmol/L palmitate. Each experiment was performed in triplicate. *P<0.05 *vs*. the group treated with palmitate alone.

DISCUSSION

Obesity-induced insulin resistance is the principal etiological factor for type 2 diabetes. Several lines of evidence suggest that a low-grade inflammation in subjects with obesity and type 2 diabetes augments the severity of insulin resistance[10-13, 34-35]. Given the increasing prevalence of obesity and type 2 diabetes, regulation of inflammatory responses may be useful in preventing or ameliorating insulin resistance. It would be advantageous to identify potential therapeutic nutrients/functional foods to improve insulin sensitivity. Interestingly, our results suggest that curcumin, which has been used for centuries in traditional oriental medicine to treat mainly inflammatory disorders, exerts insulin-sensitizing effects by attenuating the inflammatory profile in 3T3-L1 adipocytes.

Pro-inflammatory cytokines are closely associated with insulin resistance. Obesity results in overproduction of insulin-desensitizing cytokines including TNF-α and TNF-α, which in turn contributes to insulin resistance. It was reported that TNF-α induces perturbations in insulin signaling cascades of insulin-sensitive tissues, such as liver, muscle, and adipose tissue[36-38]. Because adipocytes are a major source of IL-6[39-40], IL-6 may act locally and systemically to induce insulin resistance, and becomes a target for insulin-mediated glucose disposal[41]. It has also been shown that IL-6 impairs both of insulin action and signaling pathway. Cytokines inhibit the transcriptional activity and protein expression of several molecules, like IRS-1 and glucose transporter 4 (GLUT4) related to insulin signaling and action[42]. The anti-inflammatory properties of curcumin have been verified in experimental studies[27-33]. Consistent with the anti-inflammatory hypothesis, our study showed that curcumin could attenuate the pro-inflammatory phenotype of palmitate-stimulated 3T3-L1 adipocytes, and that pretreatment with curcumin could antagonize the up-regulation of palmitate-stimulated TNF-α and IL-6.

It was recently reported that saturated fatty acid palmitate activates NF-κB activity and induces TNF-α and IL-6 expression in 3T3-L1 adipocytes[43]. It was also reported that treatment with a mixture of saturated and unsaturated free fatty acid impaires insulin signaling at multiple sites, decreases insulin-stimulated GLUT4 translocation and glucose transport, and activates the stress/inflammatory JNK pathway in 3T3-L1 adipocytes[44]. Obesity-induced insulin resistance might be prevented by knocking out various components of the inflammatory response [TNF-α, IkB kinase (IKK-β), or JNK] or giving pharmacological anti-inflammatory treatment[45-47]. Our study found important changes in components of the NF-κB expression complex and MAPKs activation, suggesting that curcumin exerts its anti-inflammatory effect by inhibiting NF-κB because curcumin downregulates the expression of nuclear NF-κB p65 and some cytokines are regulated by NF-κB[48-49]. Interaction between curcumin and JNK in 3T3-L1 adipocytes was also explored in our study. JNK is a central mediator of FFA effects and TNF-α is a downstream target of JNK. Our data show that curcumin could significantly inhibit the activation of JNK which can be blocked by SP600125. The TNF-α level was suppressed and p38MAPK inhibition did not affect the levels of TNF-α, suggesting that additional mechanisms may be involved in affecting gene expression. The relative role of curcumin and JNK in mediating FFA-induced insulin resistance may be different from that of ERK or p38MAPK. Curcumin promoted insulin-stimulated

glucose transport in pretreated 3T3-L1 adipocytes, which was most evident in insulin-resistant palmiteta-treated adipocytes.

In conclusion, curcumin exerts its anti-inflammatory effect on palmitate-induced insulin resistance in 3T3-L1 adipocytes and obesity-induced insulin resistance may be reduced by nutrient-based anti-inflammatory strategies. Further study is required to identify the other underlying mechanisms of insulin resistance.

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