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 Pathway1

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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 [3H] 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 resistance9 and their levels are elevated in insulin-resistant states like obesity and type 2 diabetes10-13. In vivo and in vitro studies demonstrated that activation of pro-inflammatory pathways is mechanically linked to insulin resistance, and that the NF-kB pathway plays a critical role in lipid-induced insulin resistance. Moreover, data suggest that FFA-derived metabolic products can activate JNK, NF-kB, 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 diseases18-21. Curcumin has been used traditionally as an antidiabetic agent22-24. 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 rats25-29. 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...
hepatic cells\(^\text{[30-31]}\). These suppressive effects are due to the inhibition of the NF-κB pathway and other pro-inflammatory signaling pathways including MAPKs\(^\text{[33-36]}\). 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). \(\text{[3H]}\) 2-deoxyglucose was obtained from PerkinElmer Life and Analytical Sciences. Rabbit antibody to SAPK/JNK (Thr183/Thr185), phosho-SAPK/JNK (Thr183/Thr185), p38MAPK, phosho-p38MAPK, p44/42MAPK (Thr202/Tyr204), phosho-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 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.

**\(\text{[3H]}\) 2-Deoxyglucose Uptake Assay**

3T3-L1 preadipocytes (5 x 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 NaPO\(_4\), 10 mmol/L HEPES, pH 7.4) with or without 100 nmol/L insulin for 30 min at 37 °C, 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-[\(\text{3H}\)] glucose for 10 min at 37 °C. 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 °C 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 CCCGGGCTTCCTCAAATAAAATACATT, probe ACAGCCTTCTCACAGGCACCC; IL-6: forward GAGGATACCACTCCCAACAGACC, reverse AAGTGCATCATGTTGTTCATACA, probe CAGAATTGCCATTGCACAACTCTTTTCTCA; β-actin: forward CCTCCTTGAGCTCCCTTGGTG, reverse ATGGAGGGGAATACAGCCCG, probe CCACACCCGCCACCCAGTTCGCC. 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 $\overline{X} \pm s$. 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, [3H]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.](image)

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 inhibits inflammation in insulin resistance. 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).

**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-κB in the development of insulin resistance, the level of p65 which is a functionally active subunit of NF-κB 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.

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 palmitate on the expression of TNF-α mRNA (Fig. 5A). Curcumin alone at the concentration of 20 mmol/L inhibited TNF-α (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 palmitate-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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REFERENCES


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