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ORIGINAL ARTICLE

PI3K/Akt mediates expression of TNF-α mRNA and activation of NF-κB in calyculin A-treated primary osteoblasts

L Qiu[1], L Zhang[1], L Zhu[1], D Yang[1], Z Li[1], K Qin[2], X Mi[3]

Departments of [1]Endodontics and [2]Orthodontics, School of Stomatology, China Medical University, Shenyang, China; [3]Department of Pathology, Basic Medical College, China Medical University, Shenyang, China

OBJECTIVE: The effect of calyculin A (CA), a serine/threonine protein phosphatase inhibitor, on tumor necrosis factor-α (TNF-α) in primary osteoblasts was investigated to determine whether protein phosphatases could affect primary osteoblasts and if so which signaling pathways would be involved.

MATERIALS AND METHODS: Primary osteoblasts were prepared from newborn rat calvaria. Cells were treated with 1 nM CA for different time periods. The expressions of TNF-α and GAPDH mRNA were determined by RT-PCR. Cell extracts were subjected to SDS-PAGE and the activation of Akt and NF-κB were analyzed by western blotting.

RESULTS: Calyculin A-treatment markedly increased the expression of TNF-α mRNA and enhanced the phosphorylation level of Akt (Ser473) in these cells. Pretreatment with the PI3K inhibitor LY294002 suppressed the increase in TNF-α mRNA expression and the phosphorylation of Akt in response to CA. Western blot analysis showed that CA stimulated the phosphorylation and nuclear translocation of NF-κB in primary osteoblasts, and these responses were blocked by pretreatment with LY294002.

CONCLUSION: Calyculin A elicits activation of PI3K/Akt pathway which leads to expression of TNF-α mRNA and activation of NF-κB. This NF-κB activation involves both phosphorylation and nuclear translocation of NF-κB.
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Keywords: PI3K/Akt; NF-κB; TNF-α; calyculin A; primary osteoblasts

Introduction

Protein phosphatases can dephosphorylate many different substrates *in vitro* and *in vivo* (Honkanen and Golden, 2002). They are involved in various aspects of cell regulation, and play important roles in metabolism (Yoshida *et al*, 2003; Haneji, 2005; Morimoto *et al*, 2005; Moorhead *et al*, 2007). Intracellular phosphatases play a central role in signal transduction pathways related to inflammation (Shanley *et al*, 2001). Inhibition of protein phosphatases increase the level of phosphorylated cellular proteins, leading to the production of pro-inflammatory cytokines and the induction of systemic inflammatory responses (Honkanen and Golden, 2002). Inhibition of MAPK phosphatase can trigger a cascade of signaling events including phosphorylation of p38 and JNK pathway that ultimately lead to the production of a variety of proinflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin (IL)-1α, and IL-6 (Wang *et al*, 2007).

Calyculin A (CA) is an inhibitor of protein phosphatases type 1 and type 2A, which dephosphorylate serine and threonine residues in eukaryotic cells (Suganuma *et al*, 1990). CA was reported to stimulate the production of TNF-α in monocytes, macrophages and cultured osteoblasts (Sung *et al*, 1992; Boehringer *et al*, 1999; Qiu *et al*, 2007). Most investigations have focused on cell lines, little is known whether CA might have an effect on TNF-α expression *in situ*. The biological features of primary osteoblasts taken directly from fresh tissue do not change much from their original tissue. The study of mechanisms in primary cells will be important for bridging basic to clinical research. Therefore, it is important to prepare and use primary osteoblasts in experiments to examine the more precise biological effects of CA.

Phosphatidylinositol 3-kinase (PI3K) is a lipid-modifying enzyme that catalyzes the phosphorylation of phosphoinositides. These lipid products act as secondary messengers to activate downstream protein kinases, including Akt/protein kinase B (Okamura *et al*, 2005). PI3K/Akt has been shown to have a role in regulating TNF-α (Giri *et al*, 2004). Recent reports suggest a role for PI3K/Akt in the activation of NF-κB. The PI3K/Akt pathway was shown to positively regulate NF-κB in HepG2 cells via phosphorylation and increased

Correspondence: L. Qiu, Department of Endodontics, School of Stomatology, China Medical University, 117 North Nanjing Street, Heping District, Shenyang 11002, China. Tel: 86 24 22891701, Fax: 86 24 22892645, E-mail: drqlh@yahoo.com
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transactivation of p65NF-κB (Thomas *et al*, 2002). Activation of PI3K/Akt has been implicated in playing a pivotal role in activation of NF-κB (Reddy *et al*, 2000).

In this study, we examined the effect of PI3K on the expression of TNF-α mRNA in CA-treated primary osteoblasts prepared from newborn rat calvaria. We also investigated the relationship between PI3K and NF-κB in CA-treated primary osteoblasts. Our results indicate that PI3K/Akt regulates the expression of TNF-α mRNA and activation of NF-κB in CA-treated primary osteoblasts.

Materials and methods

Materials

Alpha-modified Eagle's minimal essential medium (α-MEM), fetal bovine serum (FBS), and prestained molecular weight markers were purchased from GIBCO (Gaithersburg, MD, USA). CA was purchased from Sigma (St. Louis, MO, USA), prepared in dimethyl sulfoxide, and protected from light. Reagents for RT-PCR were purchased from Invitrogen (Carlsbad, CA, USA). LY294002 was purchased from Biomol (Philadelphia, PA, USA). The mouse monoclonal antibody against Akt and the rabbit polyclonal antibody against phospho-Akt (Ser473), p65NF-κB, and phospho-p65NF-κB (Ser536) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other materials used were of the highest grade commercially available.

Cell culture

To isolate primary osteoblasts, calvaria were obtained from newborn (2-or 3-day old) Wistar rat. The calvaria were digested in 0.25% trypsin for 15 min, followed in 1 mg ml^{-1} collagenase type II solution (Worthington Biochemical, Lakewood, NJ, USA) for 90 min at 37°C with constant agitation. After centrifugation, the supernatant was discarded and the remaining bone fragments were agitated by pipetting with α-MEM containing 10% FBS, 50 μg ml^{-1} ascorbic acid, and 2 mM β-glycerophosphate to wash cells off the bone surface. The suspension was plated onto plastic dishes to collect primary osteoblasts and cultured overnight in the medium (as above) at 37°C in a humidified incubator supplied with 5% CO_2 and 95% air. The media was changed every 3 days and serial subcultures were prepared after the cells had reached confluence. High alkaline phosphatase activity was confirmed for the cultured cells, which is indicative of a mature osteoblast phenotype. For experiments, cells were plated in plastic dishes at a density of 7500 cells cm^{-2} and incubated for the desired periods.

RNA preparation and RT-PCR

After the appropriate treatment, total cellular RNA was extracted from confluent cells using the Trizol reagent (Invitrogen) following the manufacturer's protocol. The extracted RNA was quantified by absorbance at 260 nm and the cDNA was synthesized according to the manufacturer's protocol (Invitrogen). PCR was performed on the cDNA using the following sense and antisense primers: Rat TNF-α (forward) 5′-ATGGCATGGATCTCAAAGAC-3′. Rat TNF-α (reverse) 5′-CGGACTCCGTGATGTCTAAG-3′; Rat GAPDH (forward) 5′-GACCCCTTCATTGACCTCAAC-3′.Rat GAPDH (reverse) 5′-CTTCTCCATGGTGGTGAAGA-3′.

After denaturation at 94°C for 10 min, PCR amplification was performed under the following condition: 94°C for 50 s, 55°C for 50 s, and 68°C for 60 s, for the indicated number of cycles in each experiment. The reaction was terminated after 5 min elongation step at 68°C. PCR products were analyzed on 2% agarose gels containing 400 ng ml^{-1} ethidium bromide.

SDS-PAGE and Western blot analysis

After appropriate periods of cultivation, the cells were washed twice with ice cold PBS and collected into lysis buffer containing 1 mM DTT, 1 mM PMSF, 1 μg ml^{-1} leupeptin, 2 μg ml^{-1} aprotinin, 5 mM EGTA, and protein phosphatase inhibitor cocktails (Sigma-Aldrich, St Louis, MO, USA) in PBS. The cells were suspended gently and incubated on ice for 30 min, allowed to swell. Nuclear and cytosolic fractions were obtained from the cells as previously described (Yoshida *et al*, 2005). Protein concentrations of the samples were determined by Bradford assay (Bio-Rad, Hercules, CA, USA). The supernatants were denatured in sample buffer and heated in boiling water for 5 min.

Twelve micrograms of each sample and prestained molecular weight markers were separated by SDS-PAGE and transferred electrophoretically to PVDF membranes (Bio-Rad). The membranes were blocked for 2 h in 5% skim milk in PBS containing 0.1% Tween-20 (PBS-Tween). The membranes were incubated overnight at 4°C in primary antibody diluted 1:300 to 1:1000 in the blocking solution. The membranes were then washed 4 times for 30 min with PBS-Tween and subsequently incubated for 2 h at ambient temperature with second antibodies (diluted at 1:5000). Membranes were exposed to the enhanced-chemiluminescence reagents (Amersham Pharmacia Biotech, Uppsala, Sweden) and then to the X-ray film. The membranes were reprobed by stripping the antibodies off the membranes with 2% SDS and 0.35% 2-mercaptoethanol in 62.5 mM Tris–HCl (pH 6.8) for 30 min at 50°C, followed by blocking and re-incubation with another antibody according to the protocol above.

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Statistical analysis

All data were expressed as mean ± SD. Comparisons were made using the unpaired Student *t*-test. Differences in the data were considered significant when $P < 0.01$.

Results

Calyculin A increased TNF-α mRNA expression in primary osteoblasts

The expression of TNF-α mRNA in primary osteoblasts was examined by RT-PCR. Amplified bands of approximately 324 bp, corresponding to TNF-α mRNA, were detected in the cells treated with 1 nM CA following

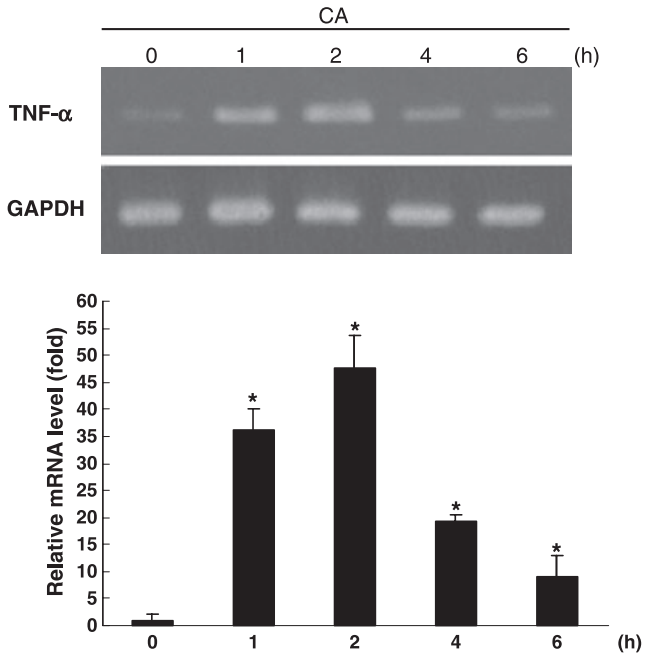

Figure 1 Calyculin A (CA) increased tumor necrosis factor-α (TNF-α) mRNA expression in primary osteoblasts. Primary osteoblasts were treated with 1 nM CA for different time periods as indicated. Total RNAs were isolated and expression of TNF-α and GAPDH mRNA were analyzed by semiquantitative RT-PCR. The density ratio (TNF-α/GAPDH) is also shown. Data represents the means of three independent experiments. $*P < 0.01$, compared with control group

amplification of cDNA for 35 cycles. CA stimulated expression of TNF-α mRNA in primary osteoblasts in a time-dependent manner (Figure 1). The level of TNF-α mRNA was low in the unstimulated cells and it increased to a maximum at 2 h in the CA-treated cells. A band of approximately 219 bp, representing GAPDH mRNA, was amplified from the same cells after 28 cycles (Figure 1). The GAPDH mRNA was constitutively expressed and its level was not affected by CA-treatment. The ratio of the TNF-α and GAPDH mRNA was also shown in Figure 1. Densitometric analysis revealed a 47-fold increase in the level of TNF-α mRNA expression in cells treated with 1 nM CA for 2 h compared with control cells.

Expression of TNF-α mRNA was increased via the PI3K/Akt pathway

It is well known that the PI3K/Akt pathway plays an important role in TNF-α production (Giri *et al*, 2004). To examine the role of PI3K in the CA-treated primary osteoblasts, PI3K activity was blocked with LY294002, a highly selective inhibitor of PI3K (Cross *et al*, 1995). Following 1 h of pretreatment with LY294002, nearly confluent cells were treated with 1 nM CA for 2 h. LY294002 had little effect on TNF-α mRNA expression by itself, but it significantly suppressed CA-stimulated expression of TNF-α mRNA in primary osteoblasts (Figure 2a). Western blot analysis showed that the anti-phospho-Akt (Ser473) antibody recognized a major band with an estimated molecular weight of 60 kDa

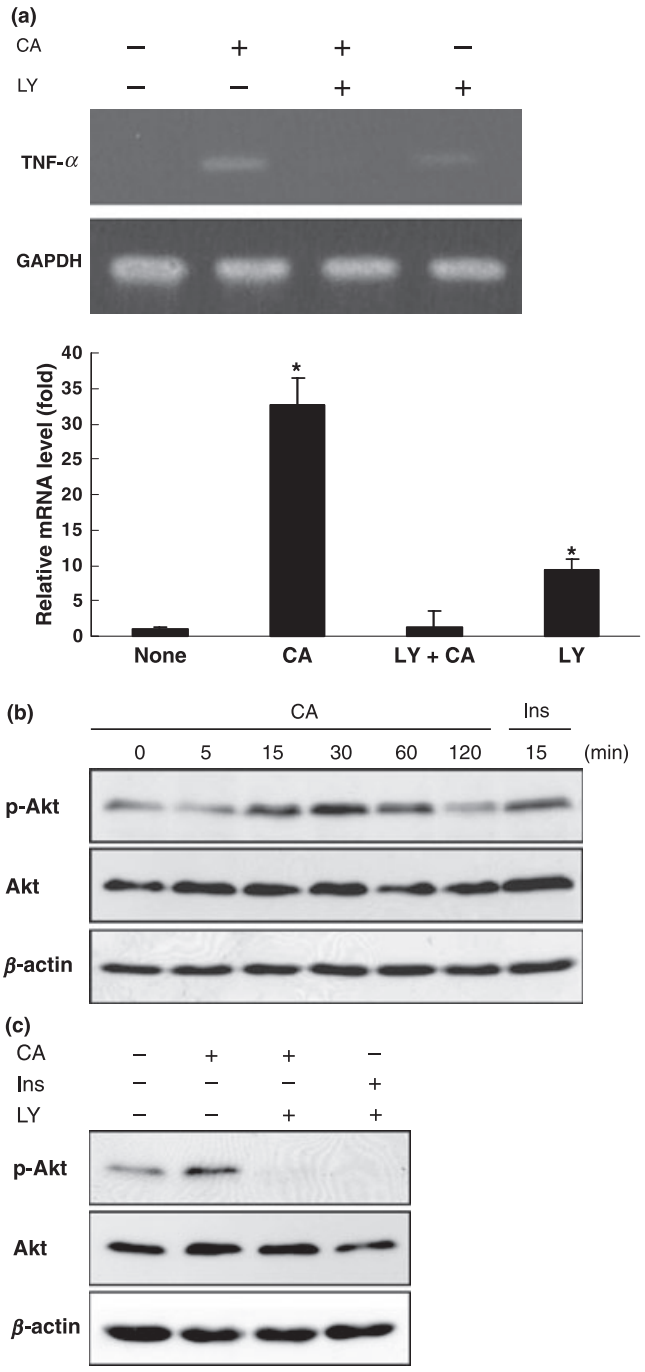

Figure 2 Expression of TNF-α mRNA was increased *via* the PI3K/Akt pathway. (**a**) Primary osteoblasts were pretreated with 10 μM LY294002 (LY) for 1 h, followed by co-incubation with 1 nM CA for 2 h. Total RNAs were isolated and TNF-α and GAPDH mRNA expressions were determined by RT-PCR. The density ratio (TNF-α/GAPDH) is also shown. Data represents the means of three independent experiments. $*P < 0.01$, compared with control group. (**b**) The cells were treated with 1 nM CA for the indicated time period, or with 10 μg ml^{-1} insulin for 15 min. The extracts were subjected to SDS-PAGE and western blotting with antibodies against phospho-Akt (Ser473, p-Akt), Akt, and β-actin. (**c**) The cells were pretreated with 10 μM LY for 1 h, then incubated with 1 nM CA for 30 min or with 10 μg ml^{-1} insulin for 15 min. The extracts were subjected to SDS-PAGE followed by western blot analysis with antibodies against phospho-Akt (Ser473, p-Akt), Akt, and β-actin

even in the unstimulated cells (Figure 2b, upper panel) and the band intensity increased up to 30 min after CA-treatment. However, the anti-phospho-Akt (Thr308) antibody did not react with any proteins prepared from CA-treated cells (data not shown). Because insulin induces the phosphorylation of Akt (Inoki *et al*, 2002), primary osteoblasts were also treated with 10 μg ml^{-1} insulin as a positive control. Insulin induced the phosphorylation of Akt in these cells (Figure 2b, upper panel). The amount of total Akt protein was not changed by CA treatment (Figure 2b, middle panel), and equal loading of protein was confirmed by Western blotting for β-actin (Figure 2b, bottom panel). PI3K activity is important in this process because pretreatment with LY294002 completely abolished the CA-stimulated Akt phosphorylation at Ser473 (Figure 2c).

The phosphorylation of NF-κB is stimulated by CA and inhibited by LY294002 in primary osteoblasts

NF-κB is one of the most important transcription factors in many cells (Wang and Baldwin, 1998). To examine the involvement of NF-κB in CA-treated primary osteoblasts, the cells were treated with 1 nM CA for different time periods. Because TNF-α induces the phosphorylation of NF-κB (Kurokouchi *et al*, 2001), the primary osteoblasts were also treated with 10 ng ml^{-1} TNF-α as a positive control. Western blot analysis demonstrated that the anti-phospho-Ser536 p65NF-κB antibody recognized a band of approximated 65 kDa in the CA-treated primary osteoblasts (Figure 3a, upper panel). The increase in phospho-Ser536 p65NF-κB reached a maximal level at 1 h. The bound antibody was stripped off the membrane and the membrane was re-incubated with antibodies against p65NF-κB and anti-β-actin, respectively. The staining intensities of these antibodies were not affected by CA-treatment (Figure 3a).

Activation of PI3K/Akt differentially regulates downstream effectors including NF-κB in signaling cascades from different types of cells (Reddy *et al*, 2000; Blum *et al*, 2001). To clarify the interaction of PI3K/Akt and NF-κB in CA-treated primary osteoblasts, the cells were pretreated with LY294002 for 1 h, followed by treatment with 1 nM CA for 1 h. The phosphorylation level of NF-κB was detected by Western blotting. CA treatment alone increased the phosphorylation of NF-κB, whereas LY294002 pretreatment inhibited the CA-stimulated phosphorylation of NF-κB (Figure 3b).

The nuclear translocation of NF-κB was stimulated by CA and inhibited by LY294002 in primary osteoblasts

To examine the intracellular localization of NF-κB in the CA-treated primary osteoblasts, cytosolic (C) and nuclear (N) fractions were prepared from the cells. The staining of p65NF-κB was strong in the cytosolic fraction, but weak in the nuclear fraction, of the unstimulated cells (Figure 4). Increased staining of p65NF-κB occurred in the nuclear fraction after the cells were treated with 1 nM CA for 1 h (Figure 4). However, the intensity of p65NF-κB staining decreased in the nuclear fraction when cells were pretreated with

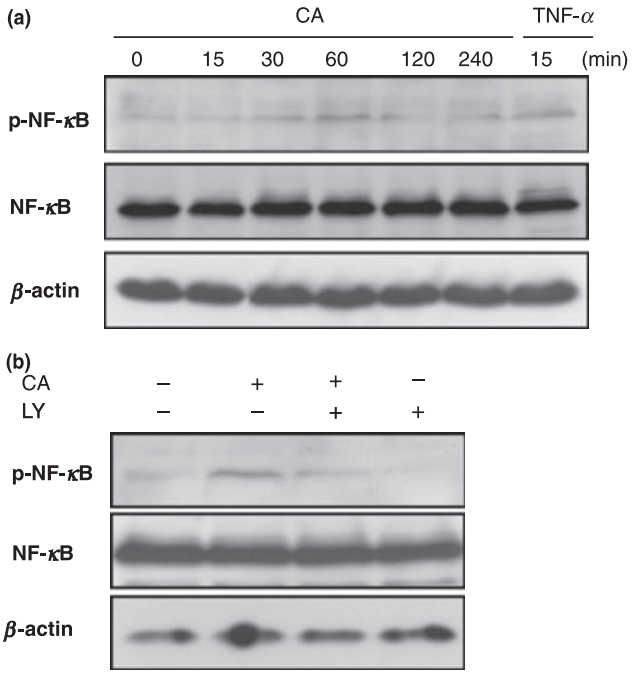

Figure 3 The phosphorylation of NF-κB is stimulated by Calyculin A (CA) and inhibited by LY294002 in primary osteoblasts. (**a**) Primary osteoblasts were treated with 1 nM CA for the indicated periods, or with 10 ng ml^{-1} tumor necrosis factor-α (TNF-α) for 15 min. The extracts were subjected to SDS-PAGE and analyzed for phospho-p65NF-κB (Ser536, p-NF-κB). The antibody was stripped off the membrane and the blots were re-probed with the anti-p65NF-κB antibody (NF-κB) and the anti-β-actin (β-actin) antibody, respectively. (**b**) Primary osteoblasts were pretreated with 10 μM LY294002 (LY) for 1 h, then incubated with 1 nM CA for 1 h. The extracts were subjected to SDS-PAGE and Western blot analysis using the anti-phospho-Ser536 p65NF-κB antibody (p-NF-κB). The antibody was stripped off the membrane and the blot was re-probed with anti-p65NF-κB (p65NF-κB) antibody and anti-β-actin (β-actin) antibody, respectively

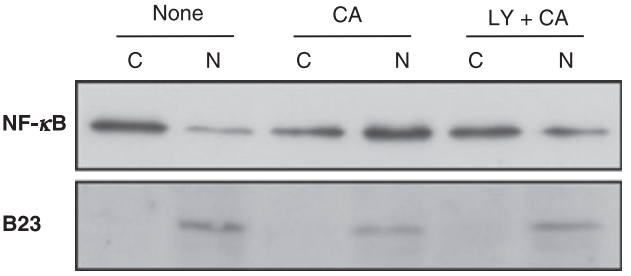

Figure 4 The nuclear translocation of NF-κB was stimulated by Calyculin A (CA) and inhibited by LY294002 in primary osteoblasts. Primary osteoblasts were either treated with 1 nM CA for 1 h, or pretreated with 10 μM LY294002 (LY) for 1 h followed by co-incubation with 1 nM CA for 1 h. Cytosolic (C) and nuclear (N) cell fractions were prepared. Proteins from each fraction were subjected to SDS-PAGE and Western blot analysis using anti-p65NF-κB antibody. The blots were re-probed with an anti-B23 antibody

LY294002, indicating that LY294002 pretreatment suppressed the CA-induced translocation of NF-κB. The nuclear fraction was positively identified by labeling

of the phosphoprotein B23, which is a major protein of the nucleolus and located in nucleus (Ulanet *et al*, 2003). The Western blots were stripped and re-probed with an anti-B23 antibody to determine the purity of the cell fractions. The anti-B23 antibody did not interact with any protein in the cytosolic fraction; however, it interacted with a nuclear protein in both the untreated and CA-treated cells, validating the high purity of the nuclear preparation.

Discussion

Tumor necrosis factor-α is an important inflammatory cytokine in the osteoblast microenvironment. Inappropriate production of TNF-α can increase the release of soluble factors such as RANKL from osteoblasts, which in turn stimulate osteoclasts to absorb bone (Zou *et al*, 2001; Nanes, 2003). Thus, the identification of mechanisms regulating TNF-α production is important for understanding bone formation. In this study, osteoblasts were isolated from newborn rat calvaria and found to increase the TNF-α mRNA expression in response to CA treatment.

Some signaling pathways correlate with expression of TNF-α mRNA (Bhat *et al*, 1998; Lee *et al*, 2006). However, it is not clear which pathways are related to expression of TNF-α mRNA in primary osteoblasts. Our results show that the PI3K signaling pathway is involved in the CA-stimulated TNF-α mRNA expression. CA penetrates the cell membrane and inhibits the intracellular protein phosphatases, which are counterbalanced by kinase activity (Tanaka *et al*, 2007). It may be that inhibition of phosphatase activity by CA leads to an alteration in the balance between kinase and phosphatase activity, so that there is an increase in the activity of kinase, such as PI3K. In many cases, Akt functions directly downstream of PI3K (Franke *et al*, 2003; Koyasu, 2003; Kim *et al*, 2005). Following PI3K activation, Akt is recruited to the plasma membrane and activated via dual phosphorylation of Ser473 or Thr308 (Martelli *et al*, 2006). Our results show that Akt is phosphorylated following treatment with CA. However, CA did not directly induce the phosphorylation of Akt. As pretreatment of LY294002 completely inhibited the phosphorylation of Akt and the expression of TNF-α mRNA in primary osteoblasts, it is possible that CA activates PI3K, which subsequently phosphorylates Akt.

In addition to a role for PI3K in the expression of TNF-α mRNA, the PI3K pathway also positively regulated NF-κB activation in CA-stimulated primary osteoblasts. CA stimulated the phosphorylation and nuclear translocation of NF-κB, and LY294002 pretreatment abolished this activation. In another study, over-activation of the p110 subunit of PI3K led to a marked induction of p65NF-κB gene activity (Sizemore *et al*, 1999). Phosphorylation of Akt was also necessary to mediate phosphorylation of p65NF-κB in IL-1-treated HepG2 cells (Thomas *et al*, 2002). In this study, CA treatment led to an increase of Akt phosphorylation at 30 min and activation of NF-κB at 1 h. Furthermore, the signaling pathway upstream of PI3K to downstream of NF-κB was blocked by LY294002 treatment. Therefore, it is inferred that PI3K is the upstream activator of NF-κB in CA-treated primary osteoblasts.

It has been reported that CA and okadaic acid, another protein phosphatase inhibitor, stimulated the phosphorylation of p65NF-κB in human osteoblastic osteosarcoma MG63 cells after treatment for 1 or 2 h (Ozaki *et al*, 2006; Tanaka *et al*, 2007). Our data also show that CA stimulated the phosphorylation of NF-κB after 1 h of treatment. However, both studies show that the phosphorylation of p65NF-κB stimulated by phosphatases inhibitors occurred more slowly than that induced by TNF-α. TNF-α stimulated the rapid phosphorylation of NF-κB by specific receptors (Kurokouchi *et al*, 2001). The different signal transduction pathways would operate via specific receptors.

IκB-α may be phosphorylated and ubiquitinated, thus allowing phosphorylation and translocation of NF-κB to the nucleus. It has been reported that CA caused the degradation of IκB, and subsequently stimulated the phosphorylation of p65NF-κB in macrophages and human MG63 cells (Grossman *et al*, 2002; Tanaka *et al*, 2007). However, CA had no effect on the degradation of IκB-α in primary osteoblasts (data not shown). Our data showed that CA stimulated the phosphorylation and nuclear translocation of NF-κB through the PI3K pathway in primary osteoblasts. Therefore, we can postulate that CA not only stimulated activation of NF-κB through IκB–mediated effects, as shown by others, but that it may also impose an additional level of regulation through the PI3K pathway. Although PI3K/Akt and NF-κB may represent the major pathway in the CA-treated primary osteoblasts, the signal cascades are rather complicated. More detailed experiments are needed to understand the relationship between these molecules in this system.

In summary, the current study demonstrates that intracellular phosphatase activity plays an important role in modulating TNF-α gene expression in osteoblasts. CA activates the PI3K/Akt pathway in primary osteoblasts, which stimulates the expression of TNF-α mRNA.

This study also demonstrated that CA-stimulated the phosphorylation and nuclear translocation of NF-κB were controlled through the PI3K pathway. It is most likely that PI3K/Akt signaling acts as an upstream stimulator of NF-κB in CA-treated primary osteoblasts.

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Author contributions

L. Qiu was involved in conception and design, financial support and manuscript writing; L. Zhang was responsible for

conception and design. L. Zhu along with D. Yang and Z. Li were involved in performing experiments. K. Qin took part in conception and design and was involved in financial support. X. Mi take care of data and analysis.

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