Oscillating Fluid Flow Inhibits TNF-α-induced NF-κB Activation via an IκB Kinase Pathway in Osteoblast-like UMR106 Cells

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Fluid flow plays an important role in load-induced bone remodeling. However, the molecular mechanism of flow-induced signal transduction in osteoblasts remains unclear. In endothelial cells, fluid flow alters activation of NF-κB resulting in changes in expression of cell adhesion molecules. To test the hypothesis that fluid flow alters NF-κB activation and expression of cell adhesion molecules in osteoblastic cells, we examined the effect of oscillating fluid flow (OFF) on tumor necrosis factor (TNF)-α-induced NF-κB activation in rat osteoblast-like UMR106 cells. We found that OFF inhibits NF-κB-DNA binding activities, especially TNF-α-induced p65-p65 heterodimer NF-κB activation and TNF-α-induced intercellular adhesion molecule-1 mRNA expression. The inhibitory effects of OFF on both TNF-α-induced NF-κB activation and intercellular adhesion molecule-1 mRNA expression were shear stress-dependent and also increased with OFF exposure duration, indicating that OFF has potent effects on mechanotransduction pathways. OFF also inhibited TNF-α-induced IκBα degradation and TNF-α-induced IκB kinase (IKK) activity in a shear stress-dependent manner. These results demonstrate that IKK is an initial target molecule for OFF effects on osteoblastic cells. Thus, OFF inhibits TNF-α-induced IKK activation, leading to a decrease in phosphorylation and degradation of inhibitory IκBα, which in turn results in the decrease of TNF-α-induced NF-κB activation and potentially the transcription of target genes.

Skeletal systems are maintained by continuous bone remodeling. Mechanical loading, as well as a number of biochemical factors, regulates this bone remodeling. Mechanotransduction in bone has been proposed to involve a variety of biophysical signals including electrical potentials (streaming potentials and piezoelectric effects) and direct transduction of matrix strain. Recent studies suggest that shear stress is an important biophysical signal in bone cell mechanotransduction (1–3). Indeed, experiments designed to discriminate between flow and strain effects suggest that fluid flow-induced shear stress is a more potent stimulator of bone cells than substrate deformation (4, 5). As bone tissue is loaded in vivo, extracellular fluid in the canalicular network experiences a heterogeneous pressurization in response to the deformation of the mineralized bone matrix, resulting in generation of fluid flow along pressure gradients. When loading is removed, pressure gradients and flows are reversed. These fluid motions are dynamic and oscillatory in nature. Recently, Jacobs et al. (6) demonstrated that oscillating fluid flow (OFF), similar to what a bone cell might experience in vivo, mobilizes cytosolic calcium in osteoblastic cells. This was the first study to examine the effect of OFF on bone cells. Other studies have demonstrated that steady or pulsating fluid flow regulates many biochemical factors such as cytosolic calcium (4, 6), cAMP (1), prostaglandin E2 (2), inositol triphosphate (2), nitric oxide (7), cyclooxygenase-2 mRNA (8, 9), and c-Fos (9) in osteoblastic cells. However, the precise mechanism by which bone cells convert biophysical signals, such as fluid flow-induced shear stress, into these biochemical signals remains unclear.

A number of paracrine and autocrine factors have been identified that control bone remodeling. Tumor necrosis factor (TNF)-α, a cytokine synthesized in the bone microenvironment, has been shown to exert pleiotropic effects on osteoblasts and osteoblast-like cells (10, 11). It has also been shown that TNF-α increases the production of interleukin-6 and macrophage colony-stimulating factor in osteoblastic cells, thereby indirectly promoting differentiation of osteoclasts and enhancing bone resorption (12, 13). In addition, the production of TNF-α in pathological conditions such as estrogen deficiency and rheumatoid arthritis has been suggested to result in osteopenia and bone destruction adjacent to areas of inflammation (14–16).

We previously demonstrated that TNF-α induces the expression of intracellular adhesion molecule (ICAM)-1 through transcription factor NF-κB activation in osteoblasts, leading to the promotion of bone resorption (17, 18). The transcription factor NF-κB was first identified as a protein that binds to a specific DNA site in the intrinsic enhancer of the Igκ light chain gene. It is composed of homo- or heterodimers of members of the Rel family that control the expression of numerous genes involved in the immune and inflammatory responses, cell adhesion, and growth control. Moreover, NF-κB plays a role as a primary regulator of the stress response. NF-κB can be rapidly activated by many types of extracellular stimuli, including viral infection, bacterial products, oxidative stress, and physical stress (for reviews, see Refs. 19–21). Additionally in endothelial cells, fluid flow alters activation of NF-κB resulting in changes in expression of cell adhesion molecules (22–24). Therefore, we hypothesized that fluid flow-induced shear stress

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† The abbreviations used are: OFF, oscillating fluid flow; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor-α; ICAM-1, intercellular adhesion molecule-1; IKK, IκB kinase; PBS, phosphate-buffered saline; DTT, dithiothreitol.
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EXPERIMENTAL PROCEDURES

**Chemicals—**All reagents were obtained from Sigma except as otherwise noted.

**Fluid Flow Experiments—**UMR106 cells were grown on glass slides in minimal essential medium (Life Technologies, Inc., Rockville, MD) supplemented with 10% fetal bovine serum (HyClone, Logan, UT). Nearly confluent cells were incubated in minimal essential medium without fetal bovine serum for 24 h before experiments. OFF was generated as previously described with some modification (6). In brief, cells on glass slides were mounted in a parallel plate flow chamber attached to a custom designed fluid pump via rigid wall tubing. Cells were exposed to OFF, in the absence or presence of 1 ng/ml TNF-α, for various lengths of time at flow rates for 2 h. After exposure to OFF, cells adhering to glass slides were washed twice with 10 ml of phosphate-buffered saline (PBS) (Ca++ and Mg++ (PBS (−)); Life Technologies, Inc.) and were collected by trypsinization.

Cell viability was evaluated by a trypan blue dye exclusion test.

**Preparations of Nuclear and Cytosolic Protein Extracts—**Nuclear and cytosolic protein extracts were prepared as described previously with some modification (17). After being washed twice with 10 ml of PBS (−), the cells were incubated on ice for 5 min in 1 ml of PBS (−) (containing 2 mM EDTA (pH 8.0), harvested by scraping with a cell scraper, and then pelleted by centrifugation at 12,000 g for 15 s at 4 °C. The cell pellet was resuspended in 100 μl of Buffer A (25 mM m aprolin, 1 mM dithiothreitol (DTT), 10 mM HEPES-KOH (pH 7.9), 10 mM KCl, 10 μg of leupeptin, 1.5 mM MgCl₂, 100 μM pepstatin, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 0.5 mM NaVO₃, 1 mM sodium pyrophosphate, 5 μg/ml N-tosyl-L-phenylalanine chromethyl ketone, and 0.4% Nonidet P-40 (Fluka, Milwaukee, WI) and lysed by incubating on ice for 10 min and then centrifuged at 12,000 × g for 5 min. The supernatant was used as cytosolic protein extract. The pellet was washed with PBS (−), resuspended in 100 μl of Buffer C (1% Triton X-100, 20% glycerol, 20 mM HEPES-KOH (pH 7.9), 0.4 M KCl, 100 mM pepstatin, and 1 mM phenylmethylsulfonyl fluoride), and lysed by freezing and thawing. After centrifugation at 12,000 × g for 5 min at 4 °C, the supernatant was used as nuclear protein extract. Protein concentration was determined by a microassay kit (Bio-Rad, Hercules, CA) using bovine serum albumin as a standard. The nuclear and cytosolic protein extracts were aliquoted and stored at −80 °C until analysis.

**Electrophoretic Mobility Shift Assay—**Nuclear extracts (20 μg of protein) were used for electrophoretic mobility shift assay. The NF-κB consensus oligonucleotide was obtained from Promega (Madison, WI). The NF-κB oligonucleotide was labeled by T4 polynucleotide kinase in the presence of 20 μCi of [γ-32P]ATP (Amersham Pharmacia Biotech) and used as a probe. To identify the NF-κB subunits, supershift analysis was performed using antibodies directed against p50 and p65 (Santa Cruz Biotechnology, Santa Cruz, CA). The antibodies were added to the binding reaction mixture (40 mM HEPES-KOH (pH 7.9), 75 mM KCl, 0.5 mM EDTA (pH 8.0), 0.5 mM DTT, and 10% glycerol) before the addition of the labeled probe and incubated for 1 h at 4 °C. Samples were loaded on 4% polyacrylamide gels (29:1, acrylamide/bisacrylamide) containing 45 mM Tris-HCl (pH 8.0), 45 mM borax acid, and 1 mM EDTA (pH 8.0) for 3 h at 180 V. The gels were dried and autoradiographed to Kodak X-OMAT AR films at room temperature.

**Northern Blot Analysis—**Total RNA was extracted using an RNeasy kit (Qiagen, Valencia, CA). 15 μg of total RNA, as determined by a spectrophotometer, was fractionated in 1% agarose-formaldehyde gels and then centrifuged at 12,000 × g for 10 min at 55 °C. The heat-denatured probe for rat ICAM-1 cDNA was labeled with 50 μCi of [α-32P]dCTP (Amersham Pharmacia Biotech) using a random primed DNA labeling kit (Roche Molecular Biochemicals). The labeled cDNA probe was added to the solution, and the hybridization was performed for 20 h at 55 °C. After the hybridization, the membranes were washed with 50 mM sodium phosphate (pH 7.4) and 1% SDS twice for 5 min at 55 °C. Then the membranes were autoradiographed to films at −80 °C. The mRNA levels were normalized to GAPDH mRNA levels. Radioactivity of the band for the respective RNA was quantified by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**Western Blot Analysis—**The cytosolic extracts containing 60 μg of protein were mixed with 1 volume of SDS loading buffer containing 5% mercaptoethanol. After denaturing in boiling water for 10 min, the samples and molecular weight markers (Low or High; Bio-Rad) were separated on 10% or 6% SDS polyacrylamide gels and electoblotted onto membranes (Trans-Blot; Bio-Rad) using the Mini-Protein II system (Bio-Rad). The membranes were soaked for 30 min in TBST (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20) containing 3% skim milk. Then the membranes were incubated for 3 h with anti-IκBα, IκB kinase (IKKα or IKKβ antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:500 with TBST containing 3% skim milk. After three additional washes with TBST, the membranes were soaked in enhanced chemiluminescence detection reagents (ECL; Amersham Pharmacia Biotech) according to the manufacturer’s protocol. The membranes were exposed to film at −80 °C.

**IKK in Vitro Kinase Assay—**The cytosolic extracts containing 200 μg of protein were preincubated with 1 μg of IKKα antibody (Santa Cruz Biotechnology) for 1 h and then were incubated for 20 h, together with 20 μl of protein AG-agarose (Santa Cruz Biotechnology) at 4 °C. After washing four times with PBS (−), one-half of each of the immunocomplexes was subjected to IKK kinase assays. Kinase assays were performed as described previously with some modification (25). Briefly, each immunoprecipitate was resuspended in 25 μl of kinase buffer (10 mM ATP, 2 μCi of [γ-32P]ATP, 25 μg/ml aprotinin, 1 mM benzamidine, 1 mM DTT, 10 mM β-glycerophosphate, 20 mM HEPES-KOH (pH 7.9), 10 μg/ml leupeptin, 2 mM MgCl₂, 2 mM MnCl₂, 10 mM p-nitrophenyl phosphate, 2 μg/ml pepstatin, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 100 mM NaVO₃, and 25 μg of total RNA, as determined by a spectrophotometer, was fractionated in 1% agarose-formaldehyde gels and then centrifuged at 12,000 × g for 10 min at 55 °C. The heat-denatured probe for rat ICAM-1 cDNA was labeled with 50 μCi of [α-32P]dCTP (Amersham Pharmacia Biotech) using a random primed DNA labeling kit (Roche Molecular Biochemicals). The labeled cDNA probe was added to the solution, and the hybridization was performed for 20 h at 55 °C. After the hybridization, the membranes were washed with 50 mM sodium phosphate (pH 7.4) and 1% SDS twice for 5 min at 55 °C. Then the membranes were autoradiographed to films at −80 °C. The mRNA levels were normalized to GAPDH mRNA levels. Radioactivity of the band for the respective RNA was quantified by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**RESULTS**

**OFF Does Not Alter the Viability of UMR106 Cells Adhering to Glass Slides—**Trypan blue dye exclusion tests indicated that increasing flow rates up to 20 ml/min, inducing shear stresses up to 9.3 dyne/cm², tended to decrease the number of viable cells adhering to glass slides (Fig. 1). However, over 90% of cells were viable after exposure to OFF, which was not significantly different from untreated controls. Moreover, OFF did not affect cell viability relative to cells untreated with TNF-α at any flow rates examined. Thus, under our study conditions OFF was not cytotoxic.

**OFF Inhibits TNF-α-induced Activation of NF-κB—**We first examined the effect of OFF on NF-κB-DNA binding activity, in the absence or presence of TNF-α, by electrophoretic mobility shift assay using nuclear protein extracts obtained from UMR106 cells. A single NF-κB DNA complex was observed even in untreated control cells (Fig. 2A, lane 1). Exposure to OFF in the absence of TNF-α did not change this basal activation of the NF-κB DNA complex (lanes 2–4). In contrast, activation of two distinct complexes was observed in TNF-α-treated cells, namely a fast-migrating complex, which exhibited the same mobility and weak activity as the complex observed in untreated cells, and an additional slow-migrating complex,
which exhibited stronger activity (lane 5). Exposure to OFF decreased dramatically the TNF-α-induced activation of the slow-migrating NF-κB-DNA complex in a shear stress-dependent manner (lanes 6–8). However, the activity of the fast-migrating NF-κB-DNA complex was not altered by TNF-α or OFF. Time course studies (Fig. 2B) revealed that treatment with TNF-α resulted in a rapid (within 15 min) increase of the slow-migrating NF-κB-DNA complex, which continued throughout the 120-min treatment period. Once again, TNF-α did not affect the activation of the fast-migrating NF-κB-DNA complex. In contrast, exposure to OFF decreased markedly the TNF-α-induced activation of the slow-migrating NF-κB-DNA complex in a time-dependent manner, whereas it did not change the activation of the fast-migrating NF-κB-DNA complex.

We next characterized the NF-κB-DNA binding complexes in UMR106 cells utilizing supershift analysis with specific antibodies against p50 and p65, two of the more common members in the NF-κB/Rel family that form a dimer in rat osteoblastic cells (17). A weak single NF-κB-DNA complex in untreated cells (Fig. 2C, lane 1) was supershifted by anti-p50 antibody (lane 2) but not by anti-p65 antibody (lane 3), indicating that the complex represents a p50 homodimer NF-κB. On the other hand, anti-p50 antibody supershifted both fast- and slow-migrating complexes induced by TNF-α (lane 5), whereas anti-p65 antibody supershifted only the slow-migrating complex (lane 6). Thus, the slow- and fast-migrating NF-κB-DNA complexes represent a p50-p65 heterodimer and a p50 homodimer NF-κB, respectively.

OFF Inhibits a TNF-α-induced Increase in ICAM-1 mRNA Expression—ICAM-1 gene expression is mainly regulated by NF-κB in rat osteoblastic cells (17, 18). We therefore examined ICAM-1 mRNA expression to evaluate the effect of OFF on the expression of an endogenous NF-κB target gene. Basal ICAM-1 mRNA expression was detected even in the absence of TNF-α (Fig. 3A). This basal mRNA level was not altered by a 2-h exposure to OFF at any shear stress level examined. In contrast, treatment with TNF-α markedly increased ICAM-1 mRNA expression within 2 h. Exposure to OFF inhibited this TNF-α-induced ICAM-1 mRNA expression in a shear stress-dependent manner with the inhibition reaching statistical significance at 4.3 dynes/cm². Time course studies (Fig. 3B) revealed that TNF-α dramatically induced ICAM-1 mRNA expression during a 4-h exposure (lanes 1–5) while increasing...
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FIG. 3. Effects of OFF on ICAM-1 mRNA expression. The membranes were rehybridized with GAPDH cDNA probe. Representative autoradiographs are shown. After the radioactivity of the bands was measured by PhosphorImager, the ICAM-1 mRNA levels were normalized to GAPDH mRNA levels and then expressed as a percentage of untreated control level. A, the effect of OFF for 2 h on ICAM-1 mRNA expressions in the absence or presence of 1 ng/ml TNF-α at various shear stress levels as indicated in the figure. Values are expressed as mean ± S.E. (n = 3). *, statistically significant versus control; #, statistically significant versus TNF-α alone. B, the results of time course studies of ICAM-1 mRNA expression. Values are expressed as mean ± S.E. (n = 2).

FIG. 4. Effects of OFF on IκBα, IKKα, and IKKβ protein levels. 60 μg of cytosolic protein extracts were subjected to Western blot analysis. Cell treatments are indicated in the figure. Positions of molecular mass markers, carbonic anhydrase (35 kDa), ovalbumin (50 kDa), and bovine serum albumin (87 kDa), are indicated. These results are typical of two other experiments.

Effect of OFF on IκB Activation—To evaluate the mechanism by which OFF decreases TNF-α-induced IκBα degradation and NF-κB activation, we examined the activities of endogenous IKK utilizing IκBα as a substrate in an in vitro kinase assay. The weak IKK activation observed in untreated cells (Fig. 5A, lane 1) dramatically increased after a 30-min exposure to TNF-α (lane 2). Exposure to OFF at 9.3 dynes/cm² significantly inhibited this TNF-α-induced IκBα degradation. Protein levels of IKKα and IKKβ, two kinase members of the IKK family that regulate IκBα phosphorylation and thus degradation (21, 26–28), were not affected by exposure to TNF-α or OFF (Fig. 4, middle and lower panels, respectively).

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DISCUSSION

The present study demonstrates for the first time that fluid flow has an inhibitory effect on NF-κB-DNA binding activity in the nucleus, especially on the activation of the slow-migrating NF-κB-DNA complex induced by TNF-α. The results of super shift analysis clearly indicate that the slow-migrating NF-κB-DNA complex activated by TNF-α is the p50-p65 heterodimer NF-κB and the basal NF-κB-DNA complex activated constitutively in UMR106 cells is the p50 homodimer NF-κB. TNF-α-induced ICAM-1 mRNA expression was also inhibited by OFF as shown by Northern blot analysis. The inhibitory effects of OFF on both TNF-α-induced NF-κB activation and ICAM-1 protein was highly expressed in the cytosol in the absence of TNF-α (Fig. 4, upper panel, lane 1). Treatment with TNF-α markedly decreased IκBα protein levels within 60 min (lane 2), reflecting a degradation of IκBα. Exposure to OFF in the presence of TNF-α resulted in a substantial increase in IκBα protein levels in a shear stress-dependent manner (lanes 3–5). IκBα protein levels in cells exposed to TNF-α and OFF at 9.3 dynes/cm² were similar to untreated control levels. These results suggest that OFF-induced shear stress decreases TNF-α-induced IκBα degradation. Protein levels of IKKα and IKKβ, two kinase members of the IKK family that regulate IκBα phosphorylation and thus degradation (21, 26–28), were not affected by exposure to TNF-α or OFF (Fig. 4, middle and lower panels, respectively).
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mRNA expression were shear stress-dependent and also increased with OFF exposure duration. These results suggest that OFF has a potent biophysical effect on mechanotransduction pathways, especially at the transcriptional level in osteoblastic cells stimulated by TNF-α.

Theoretical models predict that the wall fluid shear stresses in the canaliculi of bone tissues are 6–30 dynes/cm² (3). The maximum flow rate used for our study was ± 20 ml/min, and frequency was 1 Hz. This fluid flow induces peak shear stresses of 9.3 dyne/cm² in our flow chamber. Thus, the fluid shear stress generated by our OFF system is within the range bone cells experience in vivo. Under the condition of our studies, fluid flow had no effect on cell viability or adhesion.

It has been demonstrated that the p50-p65 heterodimer NF-κB is capable of transactivating gene expression (29). Indeed, we previously have shown that TNF-α induces interleukin-6 and ICAM-1 gene expressions in rat osteoblastic cells via activation of the p50-p65 heterodimer NF-κB (17, 18). The shear stress and time course for OFF inhibition of TNF-α-induced ICAM-1 mRNA expression were similar to the shear stress and time course for the inhibition of TNF-α-induced NF-κB activation. Taken together with our previous findings, this suggests the possibility that the effect of OFF on TNF-α-induced ICAM-1 gene expression may be via an NF-κB-dependent pathway. However, a direct link between OFF, TNF-α, and ICAM-1 expression cannot be made without rigorous mutational analysis. In any case, our data clearly show an interaction of OFF with TNF-α that has a strong potential to alter bone cell activity.

In this study, neither OFF nor TNF-α affected the basal p50 homodimer NF-κB activation. Moreover, basal ICAM-1 mRNA expression, which was detected in the absence of TNF-α, was not altered by OFF. It has been reported that moderate levels of p50 homodimer NF-κB activation was conserved in other cells (30–32). The role for the p50 homodimer NF-κB activation in constitutive-type transcription is unclear, but it may provide low levels of transcriptional activity or it may serve as a transcriptional repressor protein (19, 33, 34).

In response to external stresses, mammalian cells rapidly translocate NF-κB to the nucleus. Once there, this protein binds to 10-base pair κB sites as a dimer within the DNA of specific genes, resulting in the regulation of transcription of these genes. The activity of NF-κB is tightly regulated by interactions with inhibitory IκB proteins in the cytoplasm, which block transport of NF-κB into the nucleus in the absence of activating signals. Most extracellular signals such as TNF-α activate NF-κB through a common pathway dependent on phosphorylation-induced degradation of IκB (for reviews, see Refs. 19, 20, and 35). In this study, we demonstrated that cytosolic IκBα protein levels were dramatically decreased by OFF and that OFF attenuated the inhibitory effect of TNF-α on IκBα.

Recent evidence suggests that IκBα degradation is regulated by phosphorylation via IκK (36). IκK is a protein complex the catalysis of which is generally carried out by a heterodimeric kinase consisting of IκKα and IκKβ subunits (21, 26–28).

Effect of OFF on IKK activity. 200 mg of cytosolic protein extracts were immunoprecipitated by anti-IKKα and, one-half of each immunocomplex was subjected to Western blot analysis using anti-IKKα and IKKβ antibodies. The radioactivities for IKK activities were measured by PhosphorImager and were expressed as a percentage of untreated control level. A, the effect of OFF at 9.3 dyne/cm² for 60 min on IKK activities in the absence or presence of 1 ng/ml TNF-α. Values are expressed as mean ± S.E. (n = 3). *, statistically significant versus control; #, statistically significant versus TNF-α alone. B, the effect of OFF on IKK activity at various shear stress levels as indicated in the figure. Values are expressed as mean ± S.E. (n = 2). C, the results of time course studies of IKK activation. Values are expressed as mean ± S.E. (n = 2).
NF-κB activation. Exposure to OFF inhibited TNF-α-induced activation of IKK in a shear stress-dependent manner. The shear stress-dependent pattern of OFF inhibition of TNF-α-induced IKK activation was similar to the shear stress-dependent pattern of the inhibition of TNF-α-induced NF-κB activation, ICAM-1 mRNA expression, and IkBα degradation, suggesting a link between these signaling pathways and OFF. Neither TNF-α nor OFF affected IkKα and IkKβ levels, suggesting that the effects we observed were not because of an effect on protein synthesis or degradation. Our results suggest that OFF is an initial target molecule for OFF effects on osteoblastic cells. OFF inhibits TNF-α-induced IKK activation, leading to a decrease in phosphorylation and degradation of inhibitory IkBα, which in turn results in the decrease of TNF-α-induced NF-κB activation and the transcription of target genes.

The precise mechanism by which OFF decreases TNF-α-induced IKK activity remains unclear. Recently it has been demonstrated that IKK itself is also phosphorylated and regulated by one or more upstream kinases (37). One possibility is that OFF affects dephosphorylation events by inducing conformational changes in these upstream kinases of IKK. Shear stress might lead directly to dephosphorylation by deactivating Ikara and inactivating them (39). On the other hand, it has been demonstrated that fluid flow rapidly activates mitogen-activated protein kinases, including extracellular signal-regulated kinase and c-Jun N-terminal kinase, both recognized stress-activated protein kinases (40–44), and focal adhesion kinase (45) in vascular endothelial cells. Therefore, another possibility is that OFF might activate upstream kinases, leading to phosphorylation of IKK itself and resulting in decreased IKK activity. Indeed, more recently it was discovered that phosphorylation of IKKα at C-terminal serines can also result in negative regulation of IKK activity by changing the conformation of the intrinsic kinase activator domain (46, 47). Another possibility is an indirect effect through which OFF-induced shear stress may modify distinct signaling factors that compete for or couple with TNF-α-induced IKK activation pathways.

An important point to consider is that our results were obtained with UMR106 cells, a rat osteogenic osteosarcoma cell line with osteoblastic characteristics. Although this cell line has provided many important insights into bone cell biology, especially as related to mechanotransduction, it could be argued that transcription factors in UMR106 cells are not the same as those in authentic osteoblasts. However, we believe that this is unlikely, because it has recently been shown that NF-κB is required for TNF-α activity in primary culture human osteoblasts (48).

In summary, our results suggest that OFF inhibits TNF-α-induced NF-κB activation in an osteoblastic cell line. Previous studies suggest that TNF-α increases accumulation of bone resorption stimulating cytokines by osteoblastic cells (12, 13) and may also stimulate osteoblastic differentiation, both of which may be mediated by NF-κB. (49) Thus, OFF may modulate bone turnover through its effect on bone cell activity.
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