Regulation of IκB Kinase ε Expression by the Androgen Receptor and the Nuclear Factor-κB Transcription Factor in Prostate Cancer

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Abstract

Although several genes have been associated with prostate cancer progression, it is clear that we are far from understanding all the molecular events implicated in the initiation and progression of the disease to a hormone-refractory state. The androgen receptor is a central player in the initiation and proliferation of prostate cancer and its response to hormone therapy. Nuclear factor-κB has important proliferative and antiapoptotic activities that could contribute to the development and progression of cancer cells as well as resistance to therapy. In this study, we report that IκB kinase ε (IKKe), which is controlled by nuclear factor-κB in human chondrocytes, is expressed in human prostate cancer cells. We show that IKKe gene expression is stimulated by tumor necrosis factor-α treatment in LNCaP cells and is inhibited by transfection of a dominant-negative form of IκBα, which prevents the nuclear translocation of p65. Furthermore, we found that tumor necrosis factor-α–induced IKKe expression is inhibited by an androgen analogue (R1881) in androgen-sensitive prostate cancer cells and that this inhibition correlates with the modulation of IκBα expression by R1881. We also noted constitutive IKKe expression in androgen-independent PC-3 and DU145 cells. To our knowledge, this is the first report of an IκB kinase family member whose expression is modulated by androgen and deregulated in androgen receptor–negative cells. (Mol Cancer Res 2007;5(1):87–94)

Introduction

Prostate cancer is the most common malignant disease among men in the Western world. The mainstay for prostate cancer control is radical surgery or radiotherapy for tumors confined to the prostate, whereas hormone therapy is commonly used alone or in combination with other treatments in advanced or high-risk prostate cancer. Eventually, prostate cancer stops responding to hormone therapy, yielding aggressive malignancies described as androgen independent or hormone refractory (1-3). Most men with hormone-refractory prostate cancer will die from their disease within 1 to 2 years (4). Understanding the biological mechanisms involved in prostate inflammation, androgen-independent growth, tumor progression, and metastasis has emerged as fundamental issues in prostate cancer research.

It is known that members of the Rel/nuclear factor-κB (NF-κB) family play an important role in the development and progression of several human malignancies. NF-κB gene products have also been shown to have important proliferative and antiapoptotic activities that could contribute to the development, progression, and resistance to therapy of tumor cells (5, 6). Previous studies have observed high activity and nuclear translocation of NF-κB in prostate cancer cells (7-13) and found that NF-κB nuclear localization was strongly predictive of recurrence in patients following radical prostatectomy (14, 15). Prominent constitutive activation of NF-κB was also observed in the PC-3 and DU145 prostate cancer cell lines lacking androgen receptor expression, whereas only low NF-κB activity was seen in the LNCaP androgen-sensitive cell line (16). Androgen receptor, which is a member of the steroid hormone receptor family of ligand-activated nuclear transcription factors, is central to the initiation and growth of prostate cancer and to its response to hormone therapy (17). The DNA-binding activity of NF-κB in CL2 cells, hormone repressor (HR) derivative of LNCaP cells, was found to be higher than in the parental cell line (9). These data suggest an antagonistic effect between androgen receptor and NF-κB activity and an inverse correlation between androgen receptor expression and constitutive NF-κB activity in prostate cancer cells. In fact, some suggest that constitutive activation of NF-κB may play a role in the progression of prostate cancer and contribute to prostate cancer cell survival following androgen withdrawal (7-13). In this regard, we and others have recently found that NF-κB nuclear localization/activity in primary prostate cancer tissues correlates with poor patient outcome and bone...
metastasis (10-18). Furthermore, we have found an increased nuclear NF-\(\kappa\)B localization in lymphocytes and malignant cells in prostate cancer metastases containing pelvic lymph nodes (18). We also observed nuclear localization of both canonical and noncanonical NF-\(\kappa\)B subunits in prostate cancer tissues, which suggests that different NF-\(\kappa\)B pathways may be activated in prostate cancer progression (19).

The classic NF-\(\kappa\)B transcription factor is a heterodimer composed of p50 and p65 (20). In unstimulated cells, NF-\(\kappa\)B is sequestered in the cytoplasm through an interaction with I\(\kappa\)B\(\alpha\). On stimulation of cells by specific stimuli, such as tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), I\(\kappa\)B\(\alpha\) is phosphorylated on Ser\(^{32}\) and Ser\(^{36}\) by the cytoplasmic I\(\kappa\)B kinase (IKK) complex, which consists of the IKK\(\alpha\) and IKK\(\beta\) kinases and the NF-\(\kappa\)B essential modulator/IKK\(\gamma\) regulatory protein (reviewed in ref. 21). Degradation of I\(\kappa\)B\(\alpha\) via the ubiquitin-proteasome pathway (22, 23) allows a rapid but transient translocation of NF-\(\kappa\)B to the nucleus, where it binds to \&\(\gamma\)B consensus sites and interacts with coactivators to promote transcription (23, 24).

Recently, two noncanonical homologues of IKKs [i.e., IKK\(\epsilon\) and TANK-binding kinase-1 (TBK-1)] have been identified as NF-\(\kappa\)B activators (25). Both kinases can phosphorylate I\(\kappa\)B\(\alpha\) but only on Ser\(^{36}\). Neither IKK\(\epsilon\) nor TBK-1 can phosphorylate I\(\kappa\)B\(\alpha\) on Ser\(^{32}\), a phosphorylation site necessary for the degradation of I\(\kappa\)B\(\alpha\) by the ubiquitination pathway (26, 27).

NF-\(\kappa\)B-dependent gene expression is impaired in embryonic fibroblasts from TBK-1-deficient mice, which die as a result of apoptotic liver degeneration (28).

TBK-1 is ubiquitously expressed whereas IKK\(\epsilon\) is constitutively expressed in lymphoid cells and human fibroblast-like synoviocytes from rheumatoid arthritis patients, although inducible in other cell types (29, 30). It has been shown that the NF-\(\kappa\)B p65 subunit is involved in the transcriptional regulation of the IKK\(\epsilon\) promoter in human chondrocytes (31). IKK\(\epsilon\) mRNA synthesis can be induced in many cell types in response to inflammatory cytokines (TNF-\(\alpha\) and interleukin-\(1\beta\)) and lipopolysaccharides, indicating that proinflammatory agent-mediated stimuli can modulate its expression (32, 33).

Despite the importance of IKK\(\epsilon\) in the NF-\(\kappa\)B pathway, little is known about IKK\(\epsilon\) expression in prostate cancer. In this study, we looked at IKK\(\epsilon\) expression in prostate cancer cell lines and how its expression varies in response to TNF-\(\alpha\) and androgen.

Results

TNF-\(\alpha\) Induction of IKK\(\epsilon\) Expression in the LNCaP Cell Line

As NF-\(\kappa\)B p65 subunit is involved in the transcriptional regulation of IKK\(\epsilon\) promoter in human chondrocytes and IKK\(\epsilon\) could be responsible for the activation of NF-\(\kappa\)B, a component of prostate cancer progression, we investigated IKK\(\epsilon\) expression in relation to NF-\(\kappa\)B nuclear translocation in the androgen-sensitive LNCaP cells. Under standard culture conditions, no endogenous IKK\(\epsilon\) expression was detectable by Western blot analysis in the cytosolic fraction of LNCaP cells (Fig. 1A). Similarly, no NF-\(\kappa\)B p65 subunit expression was detectable in the nuclear fraction. The addition of TNF-\(\alpha\) rapidly induced p65 nuclear translocation (Fig. 1B). Although increased endogenous IKK\(\epsilon\) expression was visible for at least 24 h, it was first detectable in the cytosolic fraction only after 4 h of TNF-\(\alpha\) treatment (Fig. 1A). Hence, p65 translocation occurs before IKK\(\epsilon\) expression. In addition, IKK\(\epsilon\) expression did not increase p65 translocation (Fig. 1B). Interestingly, TBK-1 expression was constitutive and was not modulated by TNF-\(\alpha\) stimulation.

Correlation between I\(\kappa\)B\(\alpha\) and IKK\(\epsilon\) mRNA Expression

Following stimulation of nearly confluent LNCaP cells with TNF-\(\alpha\), IKK\(\epsilon\) mRNA expression was quantified by real-time PCR. Similarly to what was observed by Western blot, we found that IKK\(\epsilon\) expression was dramatically increased by TNF-\(\alpha\) treatment, 6-fold after 2 h and 8-fold after 4 h of TNF-\(\alpha\) treatment, compared with mock-treated cells (Fig. 2A). High IKK\(\epsilon\) mRNA levels were still detected at 8 h after TNF-\(\alpha\) treatment. We also measured I\(\kappa\)B\(\alpha\) mRNA expression in parallel as a means to follow the NF-\(\kappa\)B transcriptional activity. A TNF-\(\alpha\) treatment induced a rapid translocation of NF-\(\kappa\)B (Fig. 1A) concomitant with an increase in I\(\kappa\)B\(\alpha\) mRNA transcription (Fig. 2B). I\(\kappa\)B\(\alpha\) mRNA seems to increase at the same time as IKK\(\epsilon\) mRNA after TNF-\(\alpha\) treatment, although I\(\kappa\)B\(\alpha\) gene activation was less important compared with mock-treated cells (5-fold after 2 h). The increase in the expression of this gene was also maintained 8 h after TNF-\(\alpha\) treatment.

Implication of Androgen Receptor in the Regulation of

IKK\(\epsilon\) Expression in LNCaP Cell Line

To test the implication of NF-\(\kappa\)B in IKK\(\epsilon\) gene activation in the LNCaP cell line, we transiently transfected the pCMV-I\(\kappa\)Bdn construct in these cells. The I\(\kappa\)Bdn is a dominant-negative construct of the NF-\(\kappa\)B inhibitor, which cannot be phosphorylated and thereby inhibits the activation of NF-\(\kappa\)B. As a control for this experiment, we used the pCMV-Neo plasmid. Transfection of I\(\kappa\)Bdn dramatically blocked p65 nuclear translocation after TNF-\(\alpha\) treatment (Fig. 3A). Eight hours (Fig. 3B) or 24 h (data not shown) after stimulation, IKK\(\epsilon\) expression was observed in the control cells, whereas no IKK\(\epsilon\) protein could be detected in the LNCaP pCMV-I\(\kappa\)Bdn cells after TNF-\(\alpha\) treatment (Fig. 3B). Degradation of endogenous I\(\kappa\)B\(\alpha\) was shown as a control of TNF-\(\alpha\) stimulation (Fig. 3B). The inhibition of NF-\(\kappa\)B nuclear translocation had no effect on TBK-1 expression.

Implication of Androgen Receptor in the Regulation of

IKK\(\epsilon\) Expression in Prostate Cancer Cell Lines

Given these observations suggesting that the NF-\(\kappa\)B p65 subunit is involved in TNF-\(\alpha\)–induced IKK\(\epsilon\) expression in androgen-sensitive LNCaP cell line and because of several studies have suggested an antagonistic relationship between androgen receptor and NF-\(\kappa\)B, we chose to examine the effect of androgen receptor stimulation by androgen on IKK\(\epsilon\) expression. Other studies have suggested that androgen receptor stimulation by androgen can maintain I\(\kappa\)B\(\alpha\) levels and consequently block NF-\(\kappa\)B activity (34, 35). To determine if androgen receptor stimulation by androgen could control I\(\kappa\)B\(\alpha\) expression levels, we used cycloheximide to block translation in LNCaP cells. Figure 4 shows that inhibition of
translation prevents an androgen analogue-induced (R1881) increase in IκB-α protein (Fig. 4, lanes 4 and 5). To determine whether androgen modulation of IκB-α could affect the induction of IKK-ε by TNF-α, we pretreated our cells for 2 h with R1881 before TNF-α stimulation. As LNCaP cells do not constitutively express IKK-ε, TNF-α stimulation was required to observe the effects of androgen on IKK-ε protein levels. We analyzed IKK-ε and IκB-α protein levels 8 h following TNF-α stimulation (Fig. 4). R1881 treatment alone did not induce IKK-ε expression (Fig. 4, lane 4) as opposed to a clear induction by TNF-α (Fig. 4, lane 2). A decrease in IKK-ε up-regulation by TNF-α, which correlated with an increase in IκB-α protein, was observed when cells were pretreated with R1881 for 2 h before TNF-α stimulation (Fig. 4, lane 2 versus Fig. 4, lane 6). Interestingly, 8 h following TNF-α + R1881 costimulation, IKK-ε levels were equivalent to that observed after TNF-α treatment alone (Fig. 4, lane 8 versus Fig. 4, lane 2) despite a modulation of IκB-α levels.

IKK-ε Expression in Hormone-Resistant Versus Hormone-Sensitive Prostate Cancer Cells

Next, we determined the level of IKK-ε expression in PC-3 and DU145 androgen-independent cells compared with 22Rv1 and LNCaP androgen-sensitive cells. We observed high constitutive IKK-ε expression in PC-3 and DU145 cells that was not affected by TNF-α treatment (Fig. 5A). Moreover, IKK-ε expression in unstimulated DU145 and PC-3 cells was higher than in TNF-α-stimulated LNCaP and 22Rv1 cells. TNF-α treatment also induced a weak IκB-ε expression in the 22Rv1 cell line, which was only detectable after long exposures (Fig. 5B). Differences observed between IKK-ε expression in androgen-sensitive and androgen-independent cell lines were not correlated with variations in NF-κB activity (Fig. 5C). In fact, PC-3 and 22Rv1 NF-κB activity was only weakly affected by TNF-α treatment (1.4- and 1.3-fold, respectively) compared with DU145 and LNCaP cells (3- and 6.8-fold, respectively). After TNF-α stimulation, NF-κB activity in LNCaP cells dramatically increased and was higher than in all other cell lines studied. Comparable NF-κB activities were measured for DU145 and LNCaP cells in unstimulated conditions (Fig. 5C). Although NF-κB activity was stimulated by TNF-α in DU145 cells, no increase in IKK-ε expression levels was observed. Finally, TBK-1 expression was not affected by TNF-α treatment in any of the cell lines studied. However, TBK-1 expression level differed among the cell lines and did not correlate with IKK-ε expression or NF-κB activity levels.

Discussion

In the present study, we report for the first time NF-κB regulation of IKK-ε expression in androgen-dependent prostate cancer cell lines. It is known that IKK-ε is predominantly expressed in cells and tissues of the immune system, such as peripheral blood leukocytes, thymus and spleen (26-32), and murine embryonic fibroblasts (33). Recently, constitutive IKK-ε expression was also detected in human chondrocytes from cartilage and in the C28/I2 human chondrocyte cell line (31). Our data show differential expression of IKK-ε in the prostate cancer cell lines tested, and expression levels seem to be linked to the androgen receptor status of the cells. In androgen-sensitive cell lines, such as LNCaP and 22Rv1, IKK-ε expression can be induced in response to TNF-α stimulation. In androgen-independent PC-3 and DU145 cell lines, IKK-ε is constitutively expressed. These observations contrast with results presented in a recent report that showed an equal and constitutive IKK-ε expression in PC-3 and LNCaP cells (36). TBK-1 has been reported to be ubiquitously expressed (26-32). Although we observed some variations in TBK-1 levels between prostate cancer cell lines tested, neither TNF-α nor R1881 treatment had an effect on its expression.

Using LNCaP cells, we found that IKK-ε mRNA and protein synthesis correlate with p65 nuclear translocation and IκB-α mRNA synthesis in response to TNF-α treatment. These results suggest a role for NF-κB in the regulation of IKK-ε expression, in contrast to previous findings in murine embryonic fibroblasts.
study, which showed that the interaction between NF-κB p65 protein and −833/−847 κB sites on the IKKe promoter occurs and this interaction leads to the activation of the IKKe gene (31). Because NF-κB is thought to be constitutively activated in androgen-independent prostate cancer cells (7-9) and IKKe is a target gene of NF-κB, it is tempting to speculate that constitutive expression of IKKe in PC-3 and DU145 cells is the consequence of elevated NF-κB activity. However, this hypothesis is too simplistic as we observed equivalent NF-κB activity in LNCaP and DU145 cells in unstimulated conditions (Fig. 5C), although DU145 cells constitutively express IKKe whereas this protein is not detectable in LNCaP cells under these conditions. Likewise, the small difference in NF-κB activity observed between PC-3 and LNCaP cells could not completely explain the difference in IKKe expression in these cell lines.

These observations lead us to investigate a role for the androgen receptor in the modulation of IKKe expression. Cross-modulation, transcriptional interference, and physical interaction between androgen receptor and NF-κB have been described (40, 41). This interaction could explain the downregulation in IKKe expression after androgen receptor stimulation by the androgen analogue R1881 in LNCaP cells. One study showed that direct protein-protein inhibition of NF-κB by androgen receptor does not occur in the nucleus and incubation of LNCaP cells with dihydrotestosterone before NF-κB activation inhibited NF-κB-DNA complex formation (35). These authors suggested that NF-κB may be sequestered

Moreover, IKKe is thought to have a significant kinase activity when expressed and isolated from unstimulated cells (27-38). In addition, another study showed that expression of IKKe is sufficient to induce phosphorylation, nuclear translocation, and DNA binding of IRF-3 and IRF-7 (39). Moreover, a recent study showed a correlation between IKKe expression and the phosphorylation of p65 on Ser536 in several non-prostate cancer cell lines as opposed to prostate cancer cell lines where this correlation was not observed (36). Consequently, we were expecting to observe NF-κB activity during IKKe expression. In this study, we failed to find any p65 nuclear accumulation following an increase in IKKe synthesis after TNF-α treatment of LNCaP cells. In fact, we observed a decrease in nuclear p65 levels 6 h after TNF-α stimulation, whereas IKKe protein levels increased. These results suggest that the TBK-1/IKKe complex does not activate p65 translocation and canonical NF-κB activity in prostate cancer cells.

The role of NF-κB protein in the induction of IKKe expression was verified by transfecting a dominant-negative form of IκBα, the inhibitor of p65/p50 NF-κB dimer, in LNCaP cells. pCMV-IκBαdn inhibited the induction of p65 nuclear translocation by TNF-α treatment and blocked IKKe expression. These observations correlate well with a previous

Figure 2. Effect of TNF-α on IκBα and IKKe mRNA expression in LNCaP cells. Cells were grown to 80% confluency and treated with TNF-α (10 ng/mL) or mock treated for 8 h. Each mRNA sample (2 μg) was used to synthesize cDNAs, which were used for quantitative real-time PCR analysis. Each sample was tested in duplicate. C1 values were determined and used to calculate relative mRNA expression (sample/actinB ratio). Fold induction was calculated relative to untreated cells for each gene. Columns, mean of two independent experiments; bars, SE.

Figure 3. Effect of p65 nuclear translocation inhibition on IKKe expression. Cells were grown to 80% confluency and transfected with the pCMV-IκBαdn vector or the pCMV-Neo vector as a control. Thirty-six hours later, cells were treated with TNF-α (10 ng/mL) for either 30 min or 8 h. Equal amounts of nuclear and cytosolic proteins were migrated by SDS-PAGE, transferred onto nitrocellulose membranes, and probed with appropriate antibodies. A. Membrane containing nuclear extracts was immunoblotted with anti-p65 antibody. Equal loading was tested with an anti-α-tubulin antibody. B. Membrane containing cytosolic extracts was successively immunoblotted (after stripping) with anti-IKKε/TBK-1 and anti-p65. Anti-IκBα antibody recognized endogenous IκBα but failed to detect IκBαdn. Equal loading was tested with an anti-α-actin antibody.
The study of this phenomenon and the interactions between kinase members in androgen-independent prostate cancer cells present, for the first time, the deregulated expression of an IκBα response to androgen stimulation (Fig. 6). Moreover, we show that androgens also modulate IKKβ expression in hormone-independent cells and that this is potentially mediated by IκBα degradation. These observations favor a role for the androgen receptor on IκBα protein expression rather than on IκBα degradation. Further studies need to be conducted to clarify the androgen-dependent control of androgen receptor on IKKβ activity.

In summary, we have identified and characterized NF-κB as a regulator of the IKKβ gene in human prostate cancer cells. We have also shown that androgens modulate IKKβ regulation in androgen receptor–positive hormone-sensitive prostate cancer cells and that this is potentially mediated by IκBα synthesis in response to androgen stimulation (Fig. 6). Moreover, we present, for the first time, the deregulated expression of an IκB kinase member in androgen-independent prostate cancer cells.

The study of this phenomenon and the interactions between NF-κB, androgen receptor, and IKKβ will certainly be critical in improving our understanding of the development of hormone-independent cells and metastatic prostate disease.

**Materials and Methods**

**Cell Lines and Cell Culture**

Androgen-independent PC-3 and DU145 cells and androgen-sensitive 22Rv1 and LNCaP cells were purchased from the American Type Culture Collection (ATCC CRL-1435, ATCC HTB-81, ATCC CRL-2505, and ATCC CRL-1740, respectively; Manassas, VA). Cells were routinely grown in RPMI 1640 (Wisent, Inc., St.-Bruno, Quebec, Canada) supplemented with 100 μg/mL gentamicin, 0.25 μg/mL amphotericin B (Invitrogen, Paisley, United Kingdom), and 10% FCS. Human recombinant TNF-α was purchased from Roche Applied Science (Indianapolis, IN). Androgen analogue R1881 (methyltrienolone) was obtained from Perkin-Elmer (Wellesley, MA), and cycloheximide was from Supelco (Bellefonte, PA).

**Transfection and Luciferase Assays**

Transfection of LNCaP cells with the plasmid pCMV-IκBαdn (40 μg/plate), obtained from Clontech (Mountain View, CA), was done in 150-mm tissue culture plates when cells reached 80% to 90% confluence using LipofectAMINE reagent (Invitrogen) according to the manufacturer’s instructions.

For luciferase assays, prostate cancer cells were plated on 48-well plates (3 × 10^4 per well) and incubated with RPMI 1640 containing 10% FCS for 24 h. Transfections were done

![Image](image-url)
using LipofectAMINE reagent. NF-κB activity was measured using the 3×B-conA-Firefly plasmid (400 ng/well of 3×B-conA-Firefly; ref. 16). The total amount of plasmid DNA was adjusted to 500 ng/well by addition of pCMV-Renilla plasmid (Promega, Madison, WI), which codes for the Renilla luciferase gene under the control of the cytomegalovirus promoter. After 24 h, medium was replaced with RPMI 1640 plus 10% FCS containing TNF-α (10 ng/mL). Cells were collected after 24 h of incubation using the lysis buffer provided in the luciferase kit (Promega). Luciferase activities were measured using the Dual-Luciferase Assay System (Promega) with the aid of a multiplate luminometer (BMG Labtechnologies, Inc., Durham, NC). Luciferase activities were normalized using the Renilla activity of the samples as measured by the multiplate luminometer. All transfection experiments were carried out in duplicate and repeated at least thrice.

Protein Extraction

After cell treatments, media were aspirated, cells were scraped and washed twice with cold PBS, and pellets were frozen at −80°C. Ice-cold buffer I (10 mmol/L HEPES, 50 mmol/L NaCl, 10 mmol/L EDTA, 5 mmol/L MgCl2) with freshly added protease and phosphatase inhibitors (10 μg/mL aprotonin, 2 μg/mL leupeptin, 2 μg/mL pepstatin, 10 μmol/L phenylmethylsulfonyl fluoride, 200 μmol/L NaF, 50 μmol/L Na3VO4) was added and cells were incubated on ice for 30 min. Cell membranes were lysed by incubating with 1% NP40 for 10 min. Cytosolic fractions were collected after centrifugation (3,000 × g for 5 min at 4°C). Ice-cold buffer II (10 mmol/L HEPES, 400 mmol/L NaCl, 0.1 mmol/L EDTA, 0.5 mmol/L DTT) with freshly added protease and phosphatase inhibitors was then added to the nuclear aggregates and incubated on ice for 1 h. Nuclear protein fractions were collected after centrifugation (14,000 × g for 15 min at 4°C). Each fraction was immediately stored at −80°C. Whole-cell extracts were obtained after 30 min of incubation in lysis buffer (1% NP40, 10% glycerol, 50 mmol/L Tris, 2 mmol/L EDTA, 5 mmol/L NaF, 150 mmol/L NaCl) and 30 min of centrifugation (14,000 × g at 4°C). Whole-cell extracts were immediately stored at −80°C.

Protein concentration was measured by Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA) according to the manufacturer’s instructions.

Western Blot Analysis

For Western blot analysis, an appropriate amount of protein from whole-cell extracts or cell fractions (10-30 μg) was resolved on 7.5% to 10% polyacrylamide gels and then transferred onto a nitrocellulose membrane. Membranes were blocked using 5% nonfat dry milk in TBS-Tween 0.05% buffer [20 mmol/L Tris, 140 mmol/L NaCl (pH 8.0)] overnight at 4°C and probed using appropriate primary antibody in blocking buffer for 1 h at room temperature. Membranes were then incubated with appropriate secondary antibody conjugated with horseradish peroxidase (Amersham Life Sciences, Inc., Arlington Heights, IL) in blocking buffer for 1 h at room temperature and developed with enhanced chemiluminescence substrate (Amersham Life Sciences).

Anti-IKKα antibodies (polyclonal rabbit antibody IMG-5571) and TBK-1 antibodies (clone 72B587) were purchased from Ingemex (San Diego, CA) and anti-androgen receptor (clone AR441) was purchased from NeoMarkers (Fremont, CA). Antibodies against p65 (clone F-6) and IκBα (clone C-21) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). To ensure equal protein loading, membranes were
probed with anti-α-tubulin (clone TU-02; Santa Cruz Biotechnology) and anti-actin (clone AC-15) antibodies (Abcam, Cambridge, United Kingdom).

Quantitative Real-time PCR

LNCaP cells were plated at a density of 5 × 10^5/mL and treated with TNF-α (10 ng/mL) or water. After 8 h, medium was removed and RNA was extracted with Trizol reagent according to the manufacturer’s instructions (Invitrogen). The concentration of RNA samples was determined using a Beckman (Mississauga, Ontario, Canada) DU-600 spectrophotometer. RNA (2 μg) was used to synthesize cDNA using the SuperScript First-Strand Synthesis System (random hexamer method) according to the manufacturer’s instructions (Invitrogen, Burlington, Ontario, Canada). The QuantiTect SYBR Green PCR kit was used as recommended (Qiagen, Mississauga, Ontario, Canada). Real-time PCRs were done on a Rotor-Gene RG-300 (Corbett Research, Sydney, New South Wales, Australia). Optimal threshold and reaction efficiency were determined using the Rotor-Gene software. Melt curves were done twice and real-time measurements were done in duplicate for each gene in each experiment. Primer sequences used were as follows: IsxBz, 5'-CTGGCTTTCTCCAACCTTCC-3' (forward) and 5'-GTCTCGAGCTGATGAC-3' (backward); actinB, 5'-ACCCCTGGATCTCCATCCAC-3' (forward) and 5'-GTATCCGGCTCGGAAG-3' (backward); and IKKe, 5'-CTCCTCTTTGAAGACTGATA-3' (forward) and 5'-GGCGGATGTATGTTATGC-3' (backward).

Primers for each target gene were designed with the help of the Primer3 software (43).

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