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 refractor (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...

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metastasis (10-18). Furthermore, we have found an increased nuclear NF-κB 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-κB subunits in prostate cancer tissues, which suggests that different NF-κB pathways may be activated in prostate cancer progression (19).

The classic NF-κB transcription factor is a heterodimer composed of p50 and p65 (20). In unstimulated cells, NF-κB is sequestered in the cytoplasm through an interaction with IκBα. On stimulation of cells by specific stimuli, such as tumor necrosis factor-α (TNF-α), IκBα is phosphorylated on Ser32 and Ser36 by the cytoplasmic IκB kinase (IKK) complex, which consists of the IKKα and IKKβ kinases and the NF-κB essential modulator/IKKγ regulatory protein (reviewed in ref. 21). Degradation of IκBα via the ubiquitin-proteasome pathway (22, 23) allows a rapid but transient translocation of NF-κB to the nucleus, where it binds to κB consensus sites and interacts with coactivators to promote transcription (23, 24). Recently, two noncanonical homologues of IKKs [i.e., IKKε and TANK-binding kinase-1 (TBK-1)] have been identified as NF-κB activators (25). Both kinases can phosphorylate IκBα but only on Ser36. Neither IKKε nor TBK-1 can phosphorylate IκBα on Ser32, a phosphorylation site necessary for the degradation of IκBα by the ubiquitination pathway (26, 27). NF-κB-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 IKKε 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-κB p65 subunit is involved in the transcriptional regulation of the IKKε promoter in human chondrocytes (31). IKKε mRNA synthesis can be induced in many cell types in response to inflammatory cytokines (TNF-α and interleukin-1β) and lipopolysaccharides, indicating that proinflammatory agent-mediated stimuli can modulate its expression (32, 33).

Despite the importance of IKKε in the NF-κB pathway, little is known about IKKε expression in prostate cancer. In this study, we looked at IKKε expression in prostate cancer cell lines and how its expression varies in response to TNF-α and androgen.

**Results**

**TNF-α Induction of IKKε Expression in the LNCaP Cell Line**

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

**Correlation between IκBε and IKKε mRNA Expression**

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

**Implication of NF-κB p65 Subunit in the Activation of IKKε Expression in LNCaP Cell Line**

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

**Implication of Androgen Receptor in the Regulation of IKKε Expression in Prostate Cancer Cell Lines**

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

IKKα Expression in Hormone-Resistant Versus Hormone-Sensitive Prostate Cancer Cells

Next, we determined the level of IKKα expression in PC-3 and DU145 androgen-independent cells compared with 22Rv1 and LNCaP androgen-sensitive cells. We observed high constitutive IKKα expression in PC-3 and DU145 cells that was not affected by TNF-α treatment (Fig. 5A). Moreover, IKKα 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 IKKα expression in the 22Rv1 cell line, which was only detectable after long exposures (Fig. 5B). Differences observed between IKKα 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).

Discussion

In the present study, we report for the first time NF-κB regulation of IKKα expression in androgen-dependent prostate cancer cell lines. It is known that IKKα 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 IKKα 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 IKKα 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, IKKα expression can be induced in response to TNF-α stimulation. In androgen-independent PC-3 and DU145 cell lines, IKKα is constitutively expressed. These observations contrast with results presented in a recent report that showed an equal and constitutive IKKα 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 IKKα mRNA and protein synthesis correlate with p65 nuclear translocation and IκBα mRNA synthesis in response to TNF-α treatment. These results suggest a role for NF-κB in the regulation of IKKα expression, in contrast to previous findings in murine embryonic fibroblasts.
nuclear translocation by TNF-α-expression. These observations correlate well with a previous LNCaP cells. pCMV-Iα form of Iα expression was verified by transfecting a dominant-negative increase. These results suggest that the TBK-1/IKK complex does not activate p65 translocation and canonical NF-kB activity during IKKα expression. Cells were grown to 80% confluency and transfected with expression and isolated from unstimulated cells (37-38). In addition, another study showed that expression of IKKα 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 IKKα 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 activation and nuclear translocation during IKKα expression. In this study, we failed to find any p65 nuclear accumulation following an increase in IKKα 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 IKKα protein levels increased. These results suggest that the TBK-1/IKKα 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 IKKα 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 IKKα expression. These observations correlate well with a previous study, which showed that the interaction between NF-κB p65 protein and −833/−847 κB sites on the IKKα promoter occurs and this interaction leads to the activation of the IKKα gene (31). Because NF-κB is thought to be constitutively activated in androgen-independent prostate cancer cells (7-9) and IKKα is a target gene of NF-κB, it is tempting to speculate that constitutive expression of IKKα 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 IKKα 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 IKKα expression in these cell lines.

These observations lead us to investigate a role for the androgen receptor in the modulation of IKKα expression. Cross-modulation, transcriptional interference, and physical interaction between androgen receptor and NF-κB have been described (40, 41). This interaction could explain the down-regulation in IKKα 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
The study of this phenomenon and the interactions between kinase members in androgen-independent prostate cancer cells is presented, for the first time, the deregulated expression of IkBα in response to androgen stimulation (Fig. 6). Moreover, we have shown that androgens modulate IKKα, a regulator of the IKKβ/IKKε complex, and androgen receptor–positive hormone-sensitive prostate cancer cells. These observations favor a role for the androgen receptor on IkBα degradation.

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

Transfection and Luciferase Assays

Transfection of LNCaP cells with the plasmid pCMV-IκBα-Badn (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

Figure 4. Inhibition of IKKε expression by androgen receptor (AR) stimulation. Western blot analysis of IKKε/TBK-1, androgen receptor, and IkBα protein levels after treatments by R1881 and TNF-α. Cells were grown to 80% confluency and pretreated 4 h with cycloheximide (50 μg/mL). R1881 (10 nmol/L) was added to the medium 2 h before TNF-α stimulation (T2) or at the same time (T0). Cells were treated with TNF-α (10 ng/mL) for 8 h. Equal amounts of proteins from whole-cell extracts were resolved by SDS-PAGE, transferred onto nitrocellulose membranes, and probed with appropriate antibodies. Equal loading was tested with an anti-actin antibody.

Figure 5. IKKε expression in androgen-independent prostate cancer cells. A. IKKε expression in prostate cancer cell lines was measured following treatment with TNF-α (10 ng/mL) for 8 h. Western blot analysis of IKKε and TBK-1 in total extracts. Equal amounts of protein from the four different cell lines were loaded on 7.5% SDS-PAGE. B. Longer exposure of A. C. NF-κB transcriptional activity was measured by luciferase assay following treatment with TNF-α (10 ng/mL) for 8 h. Cells were grown to 80% confluency and cotransfected with pCMV-Renilla and 3-β-conA-Firefly. Thirty-six hours later, cells were treated during 8 h with TNF-α (10 ng/mL) and subsequently assayed for luciferase activity. Transfection efficiency was normalized to that of Renilla luciferase. Fold induction of NF-κB activity.
using LipofectAMINE reagent. NF-κB activity was measured using the 3xB-conA-Firefly plasmid (400 ng/well of 3xB-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 aprotinin, 2 μg/mL leupeptin, 2 μg/mL pepstatin, 10 μmol/L phenylmethylsulfonyl fluoride, 200 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 72BS87) were purchased from Imgenex (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

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**Figure 6.** Schematic representation of the proposed regulation of IKKe expression in prostate cancer cell lines. TNF-α-mediated stimulation of TNF-receptor 1 (TNF-R1) induces the phosphorylation of IκBα via the IKKe-IKKα-NF-κB essential modulator (NEMO) complex. IκBα degradation by the proteasome releases p65/p50 NF-κB complex that translocates to the nucleus and induces the expression of several genes, such as IKKe and the NF-κB inhibitor IκBα. Androgen stimulation of prostate cells leads to the activation of the androgen receptor and its nuclear translocation. Activated androgen receptor induces IκBα synthesis, repression of NF-κB activity, and IKKe down-regulation.
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 $5 \times 10^{5}$ cells/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-Gen software. Melt curves for each primer exhibited a single peak, indicating specific amplification, which was also confirmed by agarose gel. $C_\text{t}$ values were determined using the Rotor-Gen software at the optimal threshold previously determined for each primer. Relative mRNA/actinB ratios were calculated using the method described by Pfaff et al. (42). Fold induction was calculated relative to the mock-treated control for each gene. Experiments were done twice and real-time measurements were done in duplicate for each gene in each primer. Primer sequences used were as follows: IskB, 5'-CTGGCTTCTTCTCACAATCTCA-3' (forward) and 5'-GTCTCGAGCTAGCATGCA3'- (backward); actinB, 5'-ACTCTTTCCAGCTCTCC-3' (forward) and 5'-GTATCTGGCTCAGAGGAGG-3' (backward); and IKKe, 5'-CTGGCTTGCTAATGACAGTGA-3' (forward) and 5'-GGGCGGTATGTATATGCCTT-3' (backward).

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

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