NFKB plays a critical role mediating COX2 expression in renal medullary interstitial cells (RMICs). The trans-activating ability of NFKB can be modified by another nuclear factor C/EBPβ that can physically bind to NFKB and regulate its activity. Because the COX2 promoter also contains a C/EBPβ site adjacent to the NFKB site, the present study examined whether these two transcription factors cooperate to induce COX2 expression following hypertonic stress. Hypertonicity markedly induced COX2 expression in cultured medullary interstitial cells by immunoblot analysis. The toxicity-induced COX2 expression was suppressed by mutant IκB (IκBm) that blocks NFκB activation, demonstrating that toxicity-induced COX2 expression depends on NFκB activation. However, mutation of the NFKB site in the COX2 promoter failed to abolish toxicity-induced COX2 reporter activity. IκB kinase-1 (IKK1) significantly induced COX2-luciferase activity by 2.3-fold (n = 10, p < 0.01); mutation of the NFKB site also failed to abolish IKK1-stimulated COX2 reporter activity (86 ± 3.1% of wild type, p > 0.05, n = 4). Interestingly, mutation of the C/EBPβ site of the COX2 gene significantly reduced both IKK1 and hypertonicity-induced COX2 reporter activity (p < 0.01). To further examine the potential role of C/EBPβ in toxicity-induced COX2 expression, a dominant negative C/EBPβ-p20 was transduced into RMICs. C/EBPβ-p20 markedly suppressed hypertonic (550 mOsm) induction of COX2 (immunoblot) to a similar extent as IκBm. No additional suppression was observed when both NFKB and C/EBPβ were simultaneously blocked by IκBm and C/EBPβ-p20. Interestingly, IKK-induced COX2 expression was not only blocked by IκBm, but also completely abolished by C/EBPβ-p20. Further studies demonstrated physical association of C/EBPβ to NFKB p65 by coimmunoprecipitation. Importantly, this interaction between C/EBPβ and NFKB was greatly enhanced following hypertonic stress. These studies indicate C/EBPβ is required for the transcriptional activation of COX2 by NFKB, suggesting a dominant role for the C/EBPβ pathway in regulating induction of RMIC COX2 by hypertonicity.

Cyclooxygenase (COX) is a key enzyme in the conversion of arachidonic acid to prostaglandin H, which is further catalyzed to five major bioactive prostaglandins (e.g. PGE2, PGI2, PGF2α, PGD2, and TXA2) through their distinct synthases. Two isoforms of COX have been identified, designated COX1 and COX2 (1, 2). COX1 is constitutively expressed in most tissues detected and is thought to carry out housekeeping functions, such as cytoprotection of the gastric mucosa, regulation of renal blood flow, and control of platelet aggregation. In contrast, COX2 mRNA and protein are normally undetectable in most tissues, but can be rapidly induced by a variety of stimuli, including various cytokines, growth factors, oncogenes, endotoxins, and chemicals (2). Accumulating evidence suggests that COX2-mediated prostaglandins play important roles in regulating cellular homeostasis, inflammation, and tumorigenesis (2–5).

The kidney is one of the few organs where constitutive COX2 expression is detected. Renal medullary interstitial cells (RMICs) are a major site of COX2 expression in the kidney (6–8). Recent studies indicate that the hypertensive environment in renal medulla is an important factor contributing to COX2 expression (7, 9). Expression of COX2 plays an important role promoting renal medullary interstitial cells to survive otherwise lethal changes in environmental tonicity (7, 10), which is critical to the regulation of urinary concentrating ability. The mechanism by which renal medullary interstitial cell COX2 expression is regulated following hypertonic stress has only been partially characterized (7, 9). Studies suggest that in RMICs, hypertensive stress activates nuclear factor NFKB, and this is critical for induction of COX2 expression in renal medullary interstitial cells (7). NFKB has also been reported to be an important signaling pathway promoting COX2 expression by such stimuli as hypoxia and tumor necrosis factor, etc. (11–16). NFKB binding sites have been identified in the promoter region of the COX2 gene (17, 18), making it likely that binding of the NFKB protein to the NFKB cis-acting element is responsible for increased COX2 expression. However, recent studies indicate that the mechanism underlying NFKB-associated COX2 expression is more complex. Interactions between NFKB and other nuclear factors such as C/EBP, SP1, and PPAR have been reported (19–21). Cross-talk among these transcriptional factors can be critical for their transcriptional activity (22–24). The present studies examined the mechanism by which NFKB activates COX2 gene expression in cultured renal medullary interstitial cells.

**MATERIALS AND METHODS**

**Cell Culture**—Rabbit medullary interstitial cells were cultured as described previously (6). Briefly, female New Zealand White rabbits were anesthetized (44 mg/kg ketamine and 10 mg/kg xylazine, i.m.). The left kidney was removed, and the medulla was dissected and minced with a razor blade under sterile conditions in 5 ml of sterile precipitation assay; C/EBP, CCAAT/enhancer-binding protein; IKK, IκB kinase; Ad, adenosovirus; GFP, green fluorescent protein.

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‡ The abbreviations used are: COX, cyclooxygenase; RMIC, renal medullary interstitial cells; IL, interleukin; ChIP, chromatin immunoprecipitation assay; C/EBP, CCAAT/enhancer-binding protein; IKK, IκB kinase; Ad, adenosovirus; GFP, green fluorescent protein.
RPMI 1640 plus 10% (v/v) fetal bovine serum (Hyclone, Logan, Utah). This homogenate was injected subcutaneously in the abdominal wall using a 14-gauge needle. Twenty days postsurgery, subcutaneous nodules appeared. The rabbits were re-anesthetized and sacrificed by decapitation, and the nodules removed under sterile conditions. Nodules were minced into 1-mm fragments and explanted in 75-cm² tissue culture plates. Cells were cultured in RPMI 1640 tissue culture medium supplemented with 10% (v/v) fetal bovine serum, and streptomycin and penicillin. Cultures were incubated at 37 °C in 5% O₂, 5% CO₂. Tissue culture medium was changed every 48–72 h. Mouse RMICs were prepared as reported (7). C57BL/6J mice were sacrificed, and kidneys were minced into 1-mm fragments and explanted in 75-cm² tissue culture plates. Cells were cultured in RPMI 1640 tissue culture medium containing 10% fetal bovine serum. Cells were studied in their third to fourth passages.

These cells exhibited characteristic abundant oil red O-positive lipid droplets, a characteristic of type I RMICs (25).

**Immunoblotting**—Immunoblots were performed on whole cell lysates from cultured RMICs. The protein concentration was determined using the bicinchoninic acid protein assay (Sigma). Thirty micrograms of protein extract were loaded in each lane of a 10% SDS-PAGE minigel and run at 120 V. Protein was transferred to a nitrocellulose membrane at 22 V overnight at 4 °C. The membrane was washed three times with TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20) and then incubated in blocking buffer (Tris 20 mM, pH 7.5, NaCl 150 mM, 5% Carnation nonfat dry milk, pH 7.5) for 1 h at room temperature. The membrane was then incubated with an antihuman COX2 antibody (1:1,000, 160,108, Cayman), anti-C/EBPβ (1:100 sc-150, Santa Cruz Biotechnology) or anti-p-C/EBPβ (1:20,000, 3084, Cell Signaling Technology) antibody in blocking buffer overnight at 4 °C. Following washing (3×), the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (1:20,000, Jackson Immuno-Research Laboratories) for 1 h at room temperature, followed by three 15-min washes. Antibody labeling was visualized by addition of the chemiluminescence reagent (Renaissance, PerkinElmer Life Sciences), and the bands were exposed to Kodak X-AR 5 films.

**Immunoprecipitation**—Immunoprecipitations were performed on nuclear lysates from cultured RMICs. Lysates containing 1 mg/ml of rabbit IgG and 20 μl of 25% protein A-agarose beads were washed three times with IP buffer and were resuspended in 1 ml of IP buffer (Tris 20 mM, pH 7.5, NaCl 150 mM, EDTA 1 mM, EGTA 1 mM, Triton 100-1). The beads were washed three times with IP buffer and were resuspended in 30 μl of 2× sample buffer. Samples were boiled for 2 min, and 20 μl of precipitated proteins were added to each lane of a SDS-PAGE gel. Ad-IkBαm, Ad-IKKα, Ad-C/EBPβ-p20, and Ad-GFP—Adenoviral vectors, encoding a dominant negative IκBα and a constitutively active IκBα kinase 1 (IKK1) or a dominant negative C/EBPβ-p20, were used to modulate NFκB and C/EBP activity, respectively, in cultured renal medullary interstitial cells. The trans-dominant inhibitor of NFκB, IκBmut (avian IκBαS36/40A) was provided by Dr. Timothy Blackwell (7). Ad-IκBβm-p20 was provided by Dr. Linda Sealy. Constitutively active IKK1 (IKK1) cDNA was kindly provided by Dr. Frank Mercurio (Sigma, St. Louis, MO). Ad-C/EBPβ-p20 and IκBα were used at 1 μg/ml for IKK1 adenovirus construction (7). The IKK1 was made constitutively active by Ser-Glu mutations in Ser176 and Ser180 residues (26). An adenovirus expressing green fluorescent protein was constructed as described (27) for a control adenovirus. For infection of RMICs, 200 μl of virus (multiplicity of infection, 100) was added to each culture dish, and GFP adenovirus was used to adjust for equal loading. After a 2-h incubation, the virus was removed, and fresh Dulbecco’s modified En-}

**RESULTS**

**Mutation of the NFκB site of the COX2 Reporter Fails to Suppress Induction of the COX2 Reporter by Hypertonic Stress**—Our previous studies demonstrate that hypertonicity activates NFκB, and blocking NFκB by a mutant IκB dramatically suppresses hypertonic induction of COX2, suggesting that NFκB mediates hypertonicity-induced COX2 expression (7). Two NFκB binding sites have been identified in the human COX2 promoter (−446 to −437 and −223 to −214) (31). To examine whether hypertonicity-induced COX2 expression is mediated via binding of NFκB protein to the NFκB element of the COX2 gene, a COX2 luciferase transcription reporter system with mutant NFκB element was used. Hypertonic stress in RMICs significantly increased COX2 reporter activity in both 891-bp COX2 luciferase reporter construct (Fig. 1) and 327-bp COX2 reporter construct (Fig. 2)-transfected cells. Surprisingly, mutation of NFκB sites in the COX2 promoter luciferase reporters failed to abolish hypertonic stress-induced COX2 reporter activity in either COX2 reporter constructs. In contrast, mutation of the C/EBPβ binding site completely blocked hypertonic activation of COX2 reporter activity (Fig. 2).
**Blocking of C/EBPβ Suppresses Hypertonic Induction of COX2 Protein Expression.**—To further examine the involvement of C/EBPβ in COX2 expression following hypertonic stress, a dominant negative isoform of C/EBPβ, C/EBPβ-p20 (p20) was used to block C/EBPβ activity (32, 33). As shown in Fig. 3, induction of COX2 expression by hypertonic stress was suppressed by IκB mutant that blocked NFκB activation, consistent with our previous findings (7). These studies now find that a dominant negative C/EBPβ-p20 also dramatically reduced the ability of hypertonicity to induce COX2 expression. More importantly, combined treatment with C/EBPβ-p20 and IκBm did not further reduce COX2 expression, suggesting these two factors participate in the same signaling pathway.

C/EBPβ-p20 Suppresses IKK-induced COX2 Protein Expression in RMICs—To further test the hypothesis that NFκB and C/EBPβ participate in the same signaling pathway, we examined the effect of inhibiting C/EBPβ in NFκB-induced COX2 expression. NFκB was activated by adenoviral transduction with IκB kinase 1 (IKK1). As expected, IKK1, which phosphorylates IκB and activates NFκB, dramatically induced COX2 expression. However, IKK1-induced COX2 expression was blocked not only by an inactive IκBm, but also by blocking C/EBPβ with C/EBPβ-p20 adenovirus (Fig. 4).

**Mutation of the COX2 Promoter C/EBPβ Binding Site Suppresses IKK-activated COX2 Reporter Activity.—**To further investigate whether C/EBPβ is involved in the transcription mechanisms underlying NFκB-induced COX2 expression in cultured RMICs, the effect of IKK on the COX2 luciferase reporter system was examined. IKK1 increased COX2 reporter activity by 3-fold (p < 0.01, Fig. 5). However unexpectedly, mutation of the NFκB site failed to completely abolish IKK1-induced COX2 reporter activity. In contrast, mutation of C/EBPβ site completely abolished IKK-induced COX2 reporter activity.

**Hypertonic Stress Enhances Interaction of C/EBPβ and p65 in Cultured Renal Medullary Interstitial Cells.—**To further examine whether C/EBPβ is associated with NFκB, we examined whether physical interaction between NFκB and C/EBPβ could be detected by coimmunoprecipitation. Nuclear protein extract
Hypertonic Stress Increases Binding of C/EBPβ and NFκB p65 to the Endogenous COX2 Promoter—To examine whether hypertonic stress can enhance the binding of C/EBPβ and NFκB to the endogenous COX2 promoter, a chromatin precipitation assay was conducted. An expected PCR product (417 bp) was obtained. Nucleotide sequencing confirmed that the PCR product was identical to the mouse COX2 promoter from −568 to −151. As shown in Fig. 7, hypertonic stress enhanced the binding of both NFκB p65 and C/EBPβ to the COX2 promoter in a time-dependent manner, with maximal binding at 1 h following hypertonic stress. This binding of p65 and C/EBPβ to the COX2 promoter was specific, because transcription factor Sp1 antibody failed to pull-down the COX2 gene detected using the same PCR primers (data not shown).

C/EBPβ Site Is Required for NFκB to Bind to the COX2 Promoter—To further determine whether the C/EBPβ site in the COX2 promoter is involved in NFκB binding to the COX2 promoter, human COX2 promoter constructs with or without C/EBPβ site mutation were transfected into cultured mouse interstitial cells. The binding ability of NFκB to the COX2 promoter constructs was determined by a modified ChIP assay. Because the transfected constructs were from the human COX2 promoter and the host cells were from mouse, this allowed us to specifically amplify the transfected human COX2 promoter using PCR primers specific for human COX2, to examine the effect of mutation of transcription factor binding elements on NFκB binding. An expected PCR product (241 bp) was obtained from cells transfected with the human COX2 promoter, but not cells transfected with control vector. As shown in Fig. 8, hypertonic stress increased binding of p65 to the wild-type COX2 promoter. This hypertonic stress-associated binding of p65 was not abolished in cells transfected with a NFκB binding site mutant construct, but was abolished by mutation of both the NFκB and C/EBPβ binding sites. These results were consistent with functional studies using the luciferase reporter assay (Fig.
C/EBPβ in NFκB-mediated COX2 Expression

**DISCUSSION**

COX2 is an inducible form of cyclooxygenase, and its expression levels regulate endogenous prostaglandin synthesis. Numerous studies have indicated that COX2-derived prostaglandins play an important role in modulating organ development, cardiovascular homeostasis, and inflammatory reaction. Conversely, aberrant expression of COX2 is associated with tumorigenesis. Elucidating the mechanism by which COX2 expression is regulated will be crucial in understanding these COX2-regulated physiological and/or pathophysiological processes. COX2 expression is regulated at multiple levels, including transcriptional and post-transcriptional levels. Several putative cis-acting elements have been identified in the 5′-upstream region flanking the COX2 gene, including AP2, STAT1, STAT3, NFκB, SP1, NF-IL6 (C/EBP), and CRE sites (17, 18). Several transcription factors, including NFκB, C/EBP, CREB, AP-1, and PPARγ, have been reported to regulate COX2 expression (28, 35–40). However, the signal transduction pathways leading to activation of these transcription factors are extremely diverse and depend on the cell types studied. The present studies demonstrate a novel transcriptional mechanism underlying NFκB regulation of COX2 expression. In medullary interstitial cells, activation of COX2 by the NFκB pathway relies on an intact C/EBPβ element, rather than the NFκB element alone. These studies demonstrate positive interaction between NFκB and C/EBPβ binding sites on the COX2 gene.

The present mechanisms facilitating survival in the hypertonic conditions is an important characteristic of the cells residing in the renal medulla. The importance of COX activity in maintaining viability of renal medullary cells has long been recognized, based on observations that COX2-inhibiting NSAIDs may cause severe renal medullary injury including papillary necrosis (41). Recent studies show that hypertonicity induces COX2 and that this plays an important role in promoting survival of renal medullary interstitial cells residing in this otherwise lethal hypertonic environment (7, 10, 34, 42). Our previous studies indicate that hypertonicity-induced COX2 expression in RMCs is mediated by NFκB. These studies showed that water deprivation not only increased renal medullary COX2 expression, but also increased renal NFκB activity (7). Blocking NFκB activation using an IκB mutant dramatically suppressed hypertonic induction of COX2 expression in cultured renal medullary interstitial cells (7). Although NFκB activation is also reported to promote COX2 expression by other stimuli (11–16), the promoter-based mechanisms have not been fully characterized, partially because the presence of the putative NFκB site in the COX2 gene has led to the assumption that this site is the target of NFκB.

The present study unexpectedly found that mutation of NFκB site in the COX2 gene failed to block COX2 expression by hypertonic stress, suggesting that the NFκB element in the COX2 gene promoter is not critical. In contrast, mutation of the C/EBPβ binding site, which is located adjacent to the NFκB site, abolished induction of COX2 expression by hypertonicity. The involvement of C/EBPβ in hypertonic trans-activation of COX2 expression is also supported by studies showing increased binding of C/EBPβ as well as NFκB p65 to the endogenous COX2 promoter. The C/EBPβ pathway does not appear to be separate from the NFκB pathway, because the additive effect of C/EBPβ blockade and NFκB blocking was not observed. Moreover, mutation of C/EBPβ site not only abolished hypertonicity-induced COX2 expression, but also abolished IKK-induced COX2 expression, whereas mutation of the NFκB site of the COX2 gene failed to abolish IKK-induced COX2 expression, suggesting that the NFκB cis-acting site is not critical for IKK-induced COX2 expression. Rather the C/EBPβ site appears to be integral to the mechanism of NFκB activation, leading to COX2 expression.

C/EBPβ belongs to the basic leucine zipper C/EBP family that is comprised of six members, C/EBPα, β, γ, ε, δ, and ξ. C/EBPβ is closely related to C/EBPα and C/EBPδ, but is distantly related to C/EBPγ, C/EBPε, and C/EBPξ (43, 44). Several truncated forms of C/EBPβ have been reported (45). The low molecular weight form of C/EBPβ (C/EBPβ-p20) has been shown to function as a dominant negative form of C/EBP (46). Other studies demonstrate that C/EBP family members are capable of interacting with members of NFκB (Rel) family members (22–24). Overlapping or adjacent NFκB/C/EBPβ binding sites are located within the promoter regions of IL-6, IL-8, IL-12, angiotensinogen, serum amyloid A, and COX2 genes (24, 47, 48), indicating a close relationship between NFκB and C/EBP in transcriptional regulation of these proteins (19). Adams et al. (49) reported that nuclear Rel/C/EBPβ heteromer is important in PGG-glucan-induced Rel-A/C/EBPβ-related transcription. A p65/C/EBPβ complex, activated following lipopolysaccharide liver, is a potent activator of serum amyloid-A expression, promoting transcription from either NFκB or C/EBP elements within the promoter (24). The present studies now show that the C/EBPβ site of the COX2 promoter is more critical for activation of COX2 expression than the NFκB site, because mutation of the C/EBPβ site significantly blocked IKK-induced COX2 reporter activity, whereas mutation of the NFκB site failed to block IKK-associated COX2 expression. The in vivo DNA binding studies show that the C/EBPβ site on the COX2 promoter plays an important role in mediating p65 binding to the COX2 promoter (Fig. 8). Based on these observations, it may be hypothesized that activated Rel protein(s) may interact with C/EBP(s) in renal medullary interstitial cells. This protein complex may be recruited to COX2 promoter DNA through interaction at the C/EBPβ site of the COX2 gene, thereby enhancing transcription of COX2 expression. This hypothesis is further supported by immunoprecipitation studies demonstrating increasing physical association between Rel A (p65) and C/EBPβ following hypertonic stress.

Although the cis-acting site for the β isoform of C/EBP has been identified in the COX2 promoter, other C/EBP family members could also bind to the C/EBPβ site and trans-activate COX2 gene expression (39). Overexpression of murine C/EBPβ and C/EBPδ produced a dose-dependent increase in basal and IL-1-stimulated COX2 luciferase reporter activity. C/EBPβ

![Image](image75x649 to 288x738)
caused a greater enhancement of basal and IL-1-stimulated COX2 promoter activity than C/EBPβ, suggesting that C/EBPβ is a stronger trans-activator. Overexpression of C/EBPβ-p20, a dominant negative C/EBP inhibitor, which retains the C-terminal DNA binding domain and the leucine zipper region but lacks the N-terminal trans-activating domain of C/EBPβ (50), not only blocks C/EBPβ-induced COX2 expression, but can also block C/EBPβ-induced COX2 expression (51). Nevertheless, in the present study, C/EBPα and -β do not seem to be involved, because immunoblotting failed to detect C/EBPα and -β expression in cultured renal medullary interstitial cells. It has been reported that C/EBPβ phosphorylation (Thr-235) is associated with ERK/Ras-induced activation of C/EBPβ (52, 53). However, Thr-235 phosphorylation of C/EBPβ does not seem to be critical in mediating interaction with p65 and promoting COX2 transcription following hypertonic stress, because hypertonicity did not change C/EBPβ phosphorylation (Fig. 6C). The mechanism by which hypertonicity enhanced interaction of C/EBPβ and NFκB remains to be explored.

In summary, the present study indicates that C/EBPβ is required for the transcriptional activation of COX2 by NFκB following hypertonic stress, suggesting a dominant role for the C/EBPβ pathway in regulating induction of RMIC COX2 by hypertonicity.

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