siveness. *Am J Physiol* 1993; **264**: C1367–C1387.
6. Blaustein MP: Endogenous ouabain: role in the pathogenesis of hypertension. *Kidney Int* 1996; **49**: 1748–1753.
7. Nelson LD, Unlap MT, Lewis JL, Bell PD: Renal arteriolar Na^+/Ca^{2+} exchange in salt-sensitive hypertension. *Am J Physiol* 1999; **276**: F567–F573.
8. Nelson LD, Mashburn NA, Bell PD: Altered sodium-calcium exchange in afferent arterioles of the spontaneously hypertensive rat. *Kidney Int* 1996; **50**: 1889–1896.
9. Unlap MT, Peti-Peterdi J, Bell PD: Cloning of mesangial cell Na^+/Ca^{2+} exchangers from Dahl/Rapp salt-sensitive/resistant rats. *Am J Physiol Renal Physiol* 2000; **279**: F177–F184.
10. Ashida T, Kuramochi M, Omae T: Increased sodium-calcium exchange in arterial smooth muscle of spontaneously hypertensive rats. *Hypertension* 1989; **13**: 890–895.
11. Ashida T, Kawano Y, Yoshimi H, Kuramochi M, Omae T: Effects of dietary salt on sodium-calcium exchange and ATP-driven calcium pump in arterial smooth muscle of Dahl rats. *J Hypertens* 1992; **10**: 1335–1341.
12. Kraev A, Chumakov I, Carafoli E: The organization of the human gene *NCX1* encoding the sodium-calcium exchanger. *Genomics* 1996; **37**: 105–112.
13. Kofuji P, Lederer WJ, Schulze DH: Mutually exclusive and cassette exons underlie alternatively spliced isoforms of the Na/Ca exchanger. *J Biol Chem* 1994; **269**: 5145–5149.
14. Barnes KV, Cheng G, Dawson MM, Menick DR: Cloning of cardiac, kidney, and brain promoters of the feline *NCX1* gene. *J Biol Chem* 1997; **272**: 11510–11517.
15. Lee SL, Yu AS, Lytton J: Tissue-specific expression of Na^+-Ca^{2+} exchanger isoforms. *J Biol Chem* 1994; **269**: 14849–14852.
16. Nicholas SB, Yang W, Lee SL, Zhu H, Philipson KD, Lytton J: Alternative promoters and cardiac muscle cell-specific expression of the Na^+/Ca^{2+} exchanger gene. *Am J Physiol* 1998; **274**: H217–H232.
17. Scheller T, Kraev A, Skinner S, Carafoli E: Cloning of the multipartite promoter of the sodium-calcium exchanger gene *NCX1* and characterization of its activity in vascular smooth muscle cells. *J Biol Chem* 1998; **273**: 7643–7649.
18. Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure: The sixth report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure. *Arch Intern Med* 1997; **157**: 2413–2446.
19. Kamide K, Tanaka C, Takiuchi S, *et al*: Six missense mutations of the epithelial sodium channel β and γ subunits in Japanese hypertensives. *Hypertens Res* 2004; 333–338.
20. Tanaka C, Kamide K, Takiuchi S, *et al*: An alternative fast and convenient genotyping method for the screening of angiotensin converting enzyme gene polymorphisms. *Hypertens Res* 2003; **26**: 301–306.
21. Inamoto N, Katsuya T, Kokubo Y, *et al*: Association of methylenetetrahydrofolate reductase gene polymorphism with carotid atherosclerosis depending on smoking status in a Japanese general population. *Stroke* 2003; **34**: 1628–1633.
22. Hwang EF, Williams I, Kovacs G, *et al*: Impaired ability of the Na^+/Ca^{2+} exchanger from the Dahl/Rapp salt-sensitive rat to regulate cytosolic calcium. *Am J Physiol Renal Physiol* 2003; **284**: F1023–F1031.
23. Meade TW, Imeson JD, Gordon D, Peart WS: The epidemiology of plasma renin. *Clin Sci (Lond)* 1983; **64**: 273–280.
24. Alderman MH, Madhavan S, Cohen H, Sealey JE, Laragh JH: Low urinary sodium is associated with greater risk of myocardial infarction among treated hypertensive men. *Hypertension* 1995; **25**: 1144–1152.
25. Furman I, Cook O, Kasir J, Low W, Rahamimoff H: The putative amino-terminal signal peptide of the cloned rat brain Na^+-Ca^{2+} exchanger gene (RBE-1) is not mandatory for functional expression. *J Biol Chem* 1995; **270**: 19120–19127.
26. Loo TW, Ho C, Clarke DM: Expression of a functionally active human renal sodium-calcium exchanger lacking a signal sequence. *J Biol Chem* 1995; **270**: 19345–19350.

FEBS 29203 FEBS Letters 579 (2005) 705–711

Chrysin suppresses lipopolysaccharide-induced cyclooxygenase-2 expression through the inhibition of nuclear factor for IL-6 (NF-IL6) DNA-binding activity

Kyung Jin Woo[a,b], Yong-Jin Jeong[b], Hiroyasu Inoue[c], Jong-Wook Park[a], Taeg Kyu Kwon[a,*]

[a] *Department of Immunology, School of Medicine, Keimyung University, 194 DongSan-Dong Jung-Gu, Taegu 700-712, South Korea*
[b] *Department of Food Science and Technology, Keimyung University, Taegu, South Korea*
[c] *Department of Food Science and Nutrition, Nara Women's University, Nara, Japan*

Received 13 September 2004; revised 4 December 2004; accepted 9 December 2004

Available online 4 January 2005

Edited by Frances Shannon

Abstract Chrysin is a natural, biologically active compound extracted from many plants, honey and propolis. It possesses potent anti-inflammation, anti-cancer and anti-oxidation properties. The mechanism by which chrysin suppresses COX-2 expression remains poorly understood. In the present report, we investigated the effect of chrysin on the expression of COX-2 in lipopolysaccharide (LPS)-activated Raw 264.7 cells. Chrysin significantly suppressed the LPS-induced COX-2 protein and mRNA expression in a dose-dependent manner. The ability of chrysin to suppress the expression of the COX-2 was investigated using luciferase reporters controlled by various *cis*-elements in COX-2 promoter region. Mutational analysis and electrophoretic mobility shift assay verified that nuclear factor for IL-6 was identified as responsible for the chrysin-mediated COX-2 downregulation. These results will provide new insights into the anti-inflammatory and anti-carcinogenic properties of chrysin.

Keywords: Chrysin; Cyclooxygenase-2; Nuclear factor for IL-6; Macrophage; Lipopolysaccharide

1. Introduction

Prostaglandins play important roles in many cellular responses including cell growth, ovulation, and immune functions, and inhibition of their synthesis is the target of most known nonsteroidal anti-inflammatory drugs [1,2]. Prostaglandins synthesis involve phospholipase catalyzed release of arachidonic acid from membranes phospholipids and its conversion by the two cyclooxygenase (Cox) enzymes to prostaglandins [2,3]. Cox-1 is constitutively expressed in tissues and is thought to be involved in homeostatic prostanoid biosynthesis [2–4]. COX-2 is thought to be the predominant isoform involved in the inflammatory responses [2–4]. Multiple lines of evidence suggest that COX-2 has a significant role in carcinogenesis. COX-2 is overexpressed in transformed cells as well as in various forms of cancer [5–7], because the targeted inhibition of COX-2 is a promising approach to inhibiting inflammation and carcinogenesis as well as to prevent cancer.

The flavonoids are a diverse family of chemicals commonly found in fruits and vegetables. Flavonoids are plant polyphenolic compounds, which comprise several classes including flavonols, flavanones, flavanols and flavans. Chrysin (5,7-dihydroxyflavone) is a natural flavonoid contained in many plant extracts, honey and propolis [8,9]. Recent studies have shown that chrysin inhibits activation of human immunodeficiency virus in models of latent infection [10]. Several studies in recent years have shown that chrysin has multiple biological activities, such as anti-inflammation, anti-cancer and anti-oxidation effects [11–14]. Recently, some flavonoids including chrysin exhibited weak peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist activities in an in vitro competitive-binding assay [15]. The PPAR-γ agonists have been considered to inhibit production of monocyte inflammatory cytokines and expression of inducible nitric oxide synthase and COX-2 [16]. However, the cellular and molecular mechanisms underlying chrysin-induced inhibition of COX-2 expression in macrophages are not clear.

In this study of chrysin anti-inflammatory properties, chrysin inhibited LPS-induced COX-2 expression in Raw 264.7 cells, apparently through blockading NF-IL6 binding to the COX-2 promoter, thereby inhibiting COX-2 expression.

2. Materials and methods

2.1. Cells and materials

All reagents were purchased from Sigma–Aldrich unless otherwise stated. Chrysin [(5,7-dihydroxyflavone) (Fig. 1)] was dissolved in dimethyl sulfoxide and freshly diluted in culture media for all in vitro experiments. The macrophage cell line Raw 264.7 was obtained from the American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 supplemented with 2 mM of L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum. The cells were subcultured twice weekly and grown on 6-well plates at 1×10^6cells/well, at 37 °C in fully humidified 5% CO_2 air. Anti-COX-2 and anti-NF-IL6 were purchased from Santa Cruz Biotechnology Inc. Anti-phospho-ERK, anti-phospho-JNK, and anti-phospho-p38 MAPK were purchased from New England Biolabs Inc. The inhibitors PD98059, SB203580, SP600125, genistein, and tyrphostin A 23 were purchased from Biomol.

2.2. Western blotting

Cellular lysates were prepared by suspending 1.5×10^6 cells in 100 μl of lysis buffer (137 mM NaCl, 15 mM EGTA, 0.1 mM sodium orthovanadate, 15 mM $MgCl_2$, 0.1% Triton X-100, 25 mM MOPS (4-morpholinepropane-sulfonic acid), 100 μM phenylmethylsulfonyl fluoride,

*Corresponding author. Fax: +82 53 250 7074.
E-mail address: kwontk@dsmc.or.kr (T.K. Kwon).

0014-5793/$30.00
doi:10.1016/j.febslet.2004.12.048

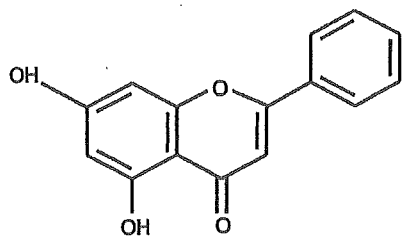

Fig. 1. Chemical structure of chrysin (5,7-dihydroxyflavone).

and 20 μM leupeptin, adjusted to pH 7.2), disrupted by sonication and extracted at 4 °C for 30 min. The proteins were electrotransferred to Immobilon-P membranes and detection of specific proteins was carried out with an ECL Western blotting kit according to the manufacturer's instructions.

2.3. RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated according to Chomczynski and Sacchi [17]. Single-strand cDNA was synthesized from 2 μg of total RNA using Moloney–Murine leukemia virus reverse transcriptase. The cDNA for COX-2 and actin were PCR amplified using the following specific primers: COX-2 (sense) 5′-CCGTGGTGAATGTATGAGCA-3′ and (antisense) 5′-CCTCGCTTCTGATCTGTCTT-3′. PCR amplification was carried out as follows: 1× (94 °C, 3 min); 30× (94 °C, 45 s; 59 °C, 45 s; and 72 °C, 1 min); and 1× (72 °C, 10 min). PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide.

2.4. DNA transfection and luciferase assay

Nuclear factor-κB (NF-κB) reporter constructs were purchased from Clontech. The human COX-2 promoter-containing plasmids have been described previously [18,19]. NF-κB reporter plasmids or COX-2 promoter plasmids were transfected into Raw 264.7 cells using the Lipofectamine reagent according to the manufacturer's instructions. To assess COX-2 promoter driving of the luciferase gene, cells were collected and disrupted by sonication in lysis buffer (25 mM tris-phosphate, pH 7.8, 2 mM EDTA, 1% Triton X-100, and 10% glycerol). After centrifugation, aliquots of the supernatants were analyzed with the luciferase assay according to the manufacturer's instructions.

2.5. Nuclear extract preparation and electrophoretic mobility shift assay (EMSA)

Preparation of nuclear extracts from control or drug-treated cells was carried out as described previously [20]. The sequences of the double-stranded oligonucleotides used to detect the DNA-binding activities of NF-κB, cAMP response element-binding protein (CREB), and NF-IL6 (CCAAT/enhancer-binding protein, C/EBP) are as follows: NF-κB, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′; CREB, 5′-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3′; and NF-IL6 (C/EBP), 5′-TGC AGA TTG CGC AAT CTG CA-3′. The reaction mixture for EMSA contained 20 mM Tris–HCl, pH 7.6, 1 mM dithiothreitol, 2 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1% NP-40, 1 μg of poly(dI-dC) and 5 μg nuclear proteins. Unlabeled wild type oligonucleotide was added into the reaction mixture and incubated for 10 min at room temperature. [^{32}P]-labeled probe DNA (300 000 cpm) was added, and the binding reaction was allowed to proceed for another 20 min. Mixtures were resolved on 8% polyacrylamide gels at 150 V for 4 h. Gels were dried and subjected to autoradiography.

3. Results

3.1. Chrysin inhibition of LPS-induced COX-2 mRNA and protein expression in RAW 264.7 cells

Unstimulated Raw 264.7 cells do not contain COX-2, whereas addition of 50 ng/ml LPS induced COX-2 synthesis in these cells (Fig. 2A). To investigate whether chrysin can inhibit LPS-induced COX-2 expression, Raw 264.7 cells were pretreated for 30 min with various concentrations of chrysin and subsequently treated with 50 ng/ml LPS. Cells pretreated

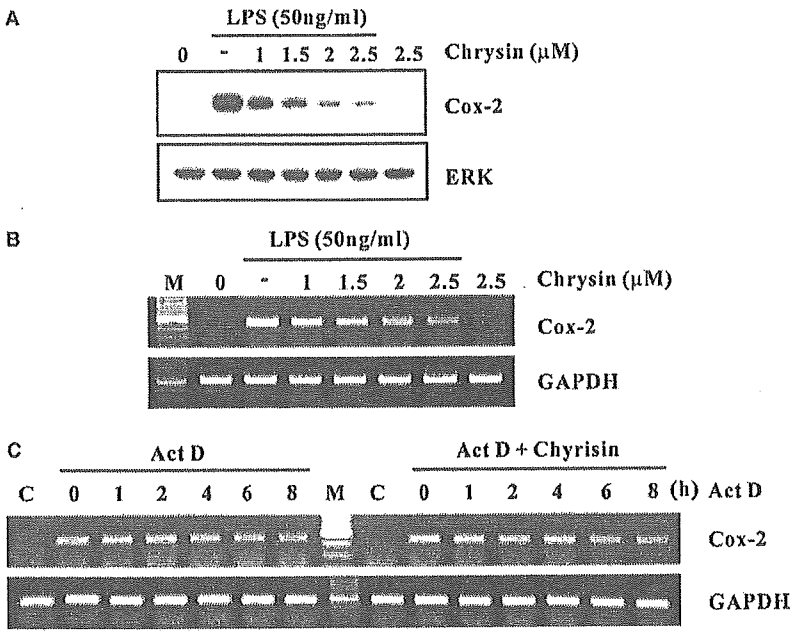

Fig. 2. Effect of chrysin on LPS-induced expression of COX-2 protein and mRNA. (A) Raw 264.7 cells were treated with indicated concentrations of chrysin in the presence of LPS (50 ng/ml) for 12 h. The cells were lysed, and the lysates were analyzed by immunoblotting using anti-COX-2. The blot was stripped of the bound antibody and reprobed with anti-ERK antibody to confirm equal loading. (B) Total RNA was prepared and RT-PCR analysis was performed as described in Section 2. (C) Raw 264.7 cells were treated with 50 ng/ml LPS for 8 h and then with or without chrysin (2.5 μM) in the presence of actinomycin D (5 μg/ml) for the indicated times. Total RNA was isolated and RT-PCR analysis was performed. A representative result is shown; two additional experiments yielded similar results.

with chrysin showed a dose-dependent inhibition of COX-2 protein expression following LPS stimulation. To assess the effect of chrysin on COX-2 mRNA expression, we measured mRNA levels using RT-PCR. The expression of COX-2 mRNA was hardly detectable in unstimulated cells. Raw 264.7 cells that were not treated with chrysin expressed high level of COX-2 mRNA when stimulated with 50 ng/ml LPS for 12 h. Addition of chrysin inhibited this LPS-stimulated COX-2 mRNA production in a dose-dependent manner (Fig. 2B). We also investigated whether chrysin stabilized COX-2 mRNA. Raw 264.7 cells were treated with vehicle or LPS for 8 h to induce COX-2 transcription, and then transcription was stopped with the addition of actinomycin D. RNA was isolated at different time points after treatment with actinomycin D and subjected to RT-PCR analysis. As shown in Fig. 2C, treatment with chrysin did not cause a significant change in the half-life of COX-2 mRNA.

3.2. *Effect of chrysin on phosphorylation state of MAPKs and tyrosine kinase activated by LPS*

To investigate whether the ERK, JNK, and p38 MAPK pathways are involved in LPS stimulation of Raw 264.7 cells, we examined whether selective MAPK inhibitors could affect LPS-stimulated COX-2 expression. SP600125 (a JNK inhibitor) profoundly inhibited LPS-mediated COX-2 expression, however, treatment with PD98059 (a MEK inhibitor) and SB203580 (a p38 MAP kinase inhibitor) slightly decreased COX-2 expression (Fig. 3A). To investigate whether the inhibition of COX-2 expression by chrysin is mediated through the modulation of the MAPK pathways, we examined the activation of the three MAPKs by detecting their dually phosphorylated forms in Western blots probed with specific anti-phosphokinase antibodies (Fig. 3B). LPS treatment induced a strong transient increase in phosphorylated MAPKs levels, which peaked at 30 min and declined thereafter. Overall, LPS treatment of macrophages stimulated these three MAPKs with similar kinetics. The phosphorylation of MAPKs was not suppressed by cotreatment with LPS and chrysin (Fig. 3B).

Genistein and tyrphostin A23, inhibitors of tyrosine kinase, were used to determine whether LPS-induced tyrosine phosphorylation participated in the signaling pathways for the COX-2 expression. Pretreatment of genistein and tyrphostin A23 inhibited COX-2 expression by Raw264.7 cells following stimulation with LPS (Fig. 3C). To investigate whether the tyrosine phosphorylation is involved in the chrysin-mediated inhibition of LPS-induced COX-2 expression,

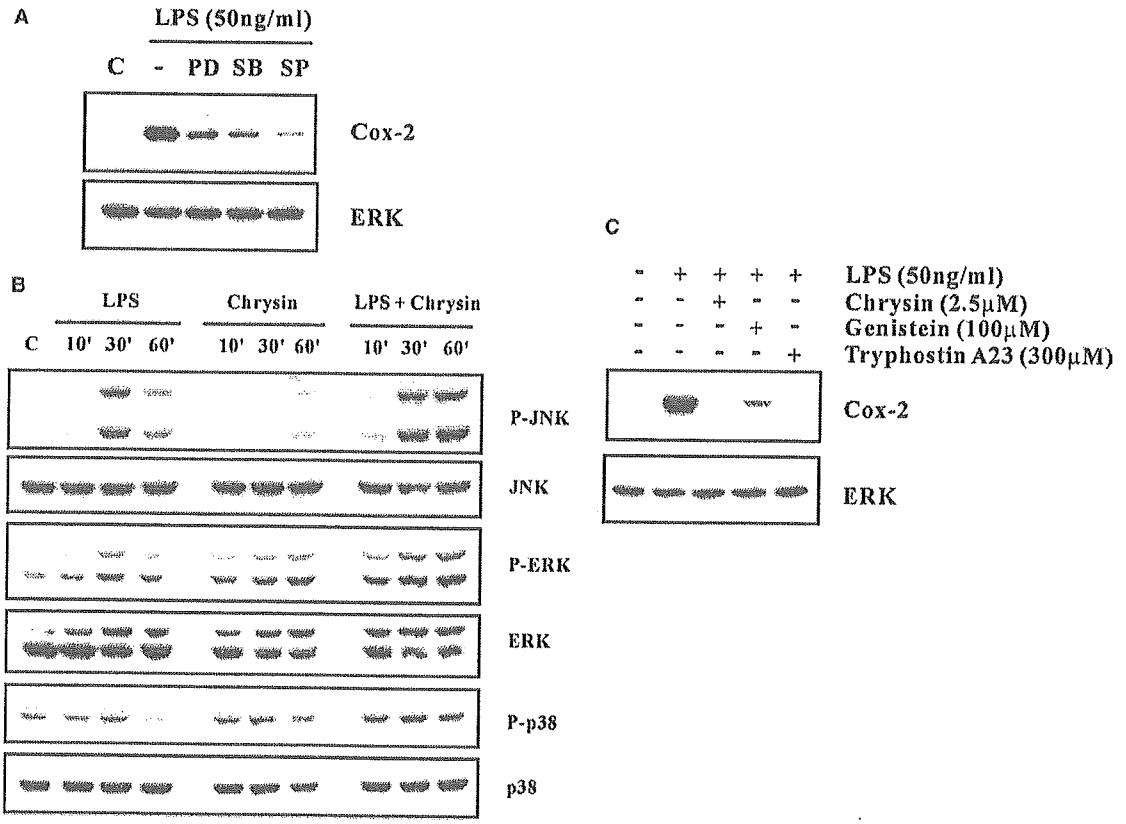

Fig. 3. Effect of chrysin on LPS-induced phosphorylation of MAPKs and tyrosine kinase in Raw 264.7 cells. (A) Raw 264.7 cells were pretreated with PD98059 (50 μM), SB203580 (10 μM) and SP600125 (20 μM) for 30 min followed by stimulation with LPS (50 ng/ml) for 12 h. COX-2 expression was determined by Western blot analysis. The blot was stripped of the bound antibody and reprobed with anti-ERK antibody to confirm equal loading. (B) Raw 264.7 cells were treated with vehicle, LPS (50 ng/ml), chrysin (2.5 μM) and LPS plus chrysin for indicated times. Equal amounts of lysates (40 μg) were subjected to electrophoresis and analyzed by immunoblotting using phosphorylation state-specific antibodies. To ascertain that the total level of each MAPK did not change, blots were stripped and reprobed with the antibodies raised against the corresponding phosphorylation-independent MAPK. The results presented are representative of three independent experiments. (C) Raw 264.7 cells were pretreated with genistein (100 μM), chrysin (2.5 μM) and tyrphostin A23 (300 μM) for 30 min followed by stimulation with LPS (50 ng/ml) for 12 h. COX-2 expression was determined by Western blot analysis. The blot was stripped of the bound antibody and reprobed with anti-ERK antibody to confirm equal loading.

the effect of chrysin on phosphotyrosine accumulation in stimulated cells exposed to LPS was studied. Chrysin did not affect in LPS-induced phosphotyrosine accumulations (negative data not shown).

3.3. Chrysin inhibits COX-2 promoter activity

To investigate the mechanism underlying the chrysin-mediated inhibition of LPS-induced COX-2 expression, we used phPES2 (−327/+59), a plasmid that expresses firefly luciferase under the control of the human COX-2 gene promoter (−327/+59). The COX-2 promoter region (−327/+59) contains three *cis*-acting elements, namely, an NF-κB binding site, an NF-IL6 (C/EBP) binding sites, and a CREB, all of which have been shown to be involved in the regulation of COX-2 gene transcription. To identify which *cis*-acting elements play a critical role in LPS-mediated COX-2 gene promoter (−327/+59) activity, mutants of the three *cis*-acting element were made and tested in transfection assay (Fig. 4A). A mutation in CREB or NF-IL6 significantly reduced the basal and LPS-induced promoter activities. Mutation at the NF-κB site also inhibits basal and LPS-induced promoter activities, but it show less inhibitory effect than that of CREB or NF-IL6. To examine the effect of chrysin on LPS-induced COX-2 promoter activities, a reporter assay was performed using phPES2 (−327/+57), phPES2 (CRM; mutant for CREB), phPES2 (ILM; mutant for NF-IL6) and phPES2 (KBM; mutant for NF-κB). As shown in Fig. 4B, chrysin inhibits twofold in LPS-induced COX-2 promoter activities in the phPES2 (−327/+57), phPES2 (CRM) and phPES2 (KBM) constructs transfected cells. However, chrysin-mediated COX-2 promoter inhibition measured by LPS-driven luciferase activity is significant less in the phPES2 (ILM) construct than in the constructs with the wild type, CREB-mutated promoter and NF-κB-mutated promoter. These data suggest that NF-IL6 is mainly involved in the chrysin-mediated inhibition of LPS-induced COX-2 promoter activity.

3.4. Inhibition of NF-IL6 binding activity by chrysin in LPS-induced Raw 264.7 cells

It is well known that NF-IL6 is an important transcription factor for the expression of COX-2 gene by LPS [21,22]. To determine whether these transcription factors are an important target for chrysin in Raw 264.7 cells, we performed an EMSA. Treatment of Raw 264.7 cells with 50 ng/ml LPS caused a significant increase in the DNA-binding activity of NF-IL6 within 4 h. To confirm that increasing bands were indeed NF-IL6 specific DNA–protein complexes, we tested binding of wild-type oligonucleotides against that of a mutant oligonucleotide lacking the NF-IL6 site. The wild-type competitor inhibited LPS-induced NF-IL6 binding activity,

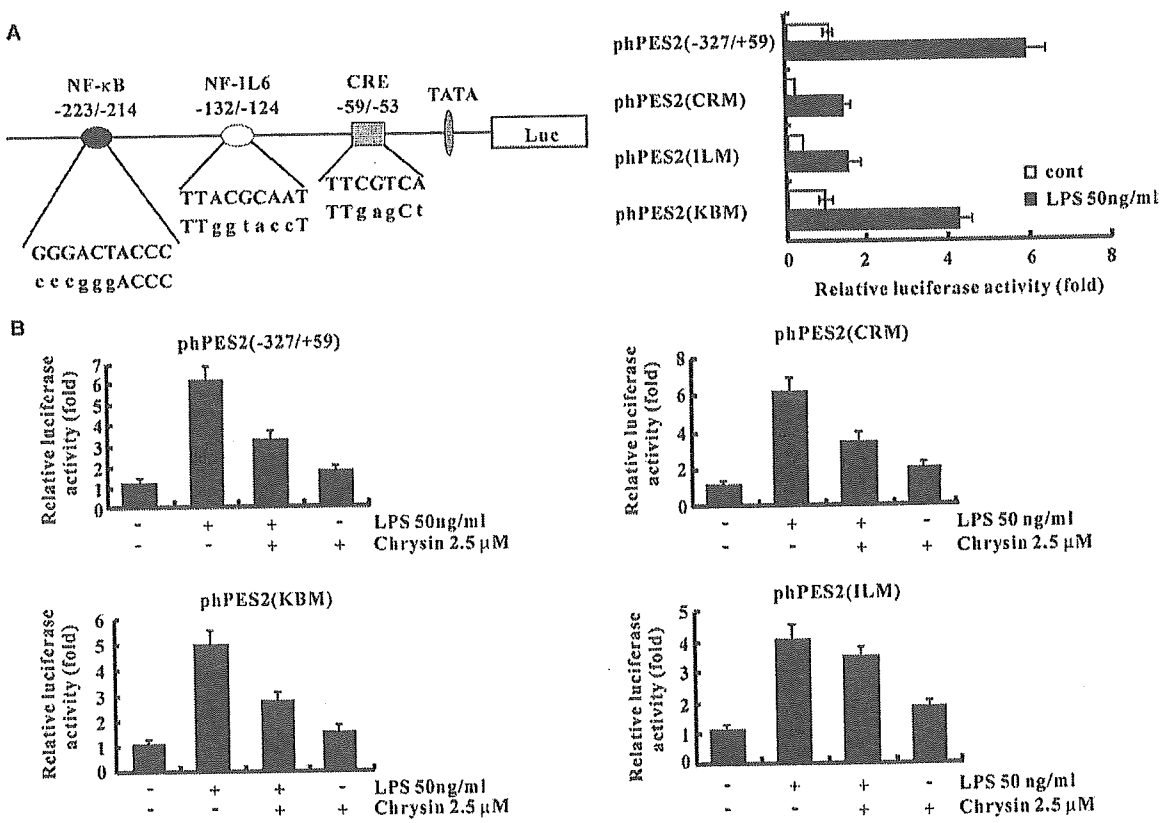

Fig. 4. Site-specific mutation of the COX-2 promoter region in response to chrysin. (A) The COX-2 gene promoter (−327/+59) was mutated at each putative transcriptional regulatory element. Lowercase letters in the lower sequence of each promoter indicate mutated bases, and the upper sequence shows wild-type bases. Wild type or mutant COX-2 promoters were transfected and treated with or without LPS (50 ng/ml) for 24 h and luciferase activity measured. Data represent the means ± S.D. of at least three independent experiments. (B) Wild type or mutant COX-2 promoters were transfected, and treated with or without chrysin in the presence of LPS (50 ng/ml). Data represent the means ± S.D. of at least three independent experiments.

whereas the mutant type competitor had no effect on the binding. The identity of the NF-IL6 protein in this complex was further confirmed by a super-shift assay using an antibody specific to NF-IL6 (Fig. 5A). LPS-induced NF-IL6 binding was markedly decreased at 2.5 μM chrysin treatment (Fig. 5A). The Western blot data suggest that NF-IL6 activity is not regulated NF-IL6 expression levels, because NF-IL6 protein expression of NF-IL6 remain unchanged regardless of whether the Raw 264.7 cells were treated with LPS and chrysin or not (Fig. 5B).

To determine whether chrysin inhibits activation of CREB and NF-κB through the inhibition of DNA binding of CREB and NF-κB, we examined the effect of chrysin on LPS-induced binding of CREB and NF-κB by EMSA. Chrysin did not affect the intensity of the CREB DNA complex induced by LPS or its migration in Raw 264.7 cells (negative data not shown). In the presence of chrysin, LPS-induced NF-κB binding was markedly increased in a dose-dependent manner (Fig. 6A). To determine the effect of chrysin on LPS-stimulated NF-κB-dependent reporter gene expression, we used a pNFκB-Luc plasmid, which was generated by inserting four spaced NF-κB binding sites into the pLuc-Promoter vector. Raw 264.7 cells were transiently transfected with the pNFκB-Luc plasmid and then stimulated with 50 ng/ml LPS either in the presence or absence of chrysin. Chrysin treatment significantly increased the LPS-induced increase in NF-κB-dependent luciferase enzyme expression (Fig. 6B). These results suggest that chrysin-mediated inhibition of LPS-induced COX-2 expression is associated with inhibition of NF-IL6 DNA-binding activity, but not in CREB and NF-κB DNA-binding activity.

4. Discussion

Chrysin is known to have anti-inflammatory and anti-cancer effects. Here, we demonstrate that chrysin inhibits COX-2 gene expression in LPS-stimulated cultured macrophages, and that this effect is mediated through the inhibition of NF-IL6 DNA-binding activity.

The regulation of COX-2 gene transcription is complex and varies according to the cell type and the stimulus applied. Studies have shown that CREB, NF-κB, and NF-IL6 (C/EBP) were commonly or individually involved in the regulation of the COX-2 gene [21–26]. NF-κB exerts as a positive regulator of several gene expressions involved in chronic inflammatory diseases [27]. NF-κB signaling has been implicated variously in the expression of COX-2 stimulated by several factors including LPS [28–30]. Our findings indicate that NF-κB binding activity and NF-κB luciferase activity are increased by treatment with chrysin (Fig. 6A and B). This renders NF-κB as an unlikely target for the pharmacological actions of chrysin. In addition, the mitogen-activated protein kinases play a critical role in the regulation of cell growth and differentiation and in the control of cellular responses to cytokines and stresses [31]. Studies using pharmacological inhibitors of MAPKs or their upstream protein kinase activators and dominant-negative mutant forms of protein kinases demonstrated the role of ERK, JNK, and p38 MAPK in transcriptional activation of COX-2 [32–35]. Moreover, MAP kinases are involved in the signaling pathway for LPS-induced COX-2 expression [22,36]. Hwang et al. [37] reported that blockade of ERK and p38 MAPK activities by PD98059 and SB203580, respectively, resulted in partial suppression of LPS-induced expression of COX-2 in Raw 264.7

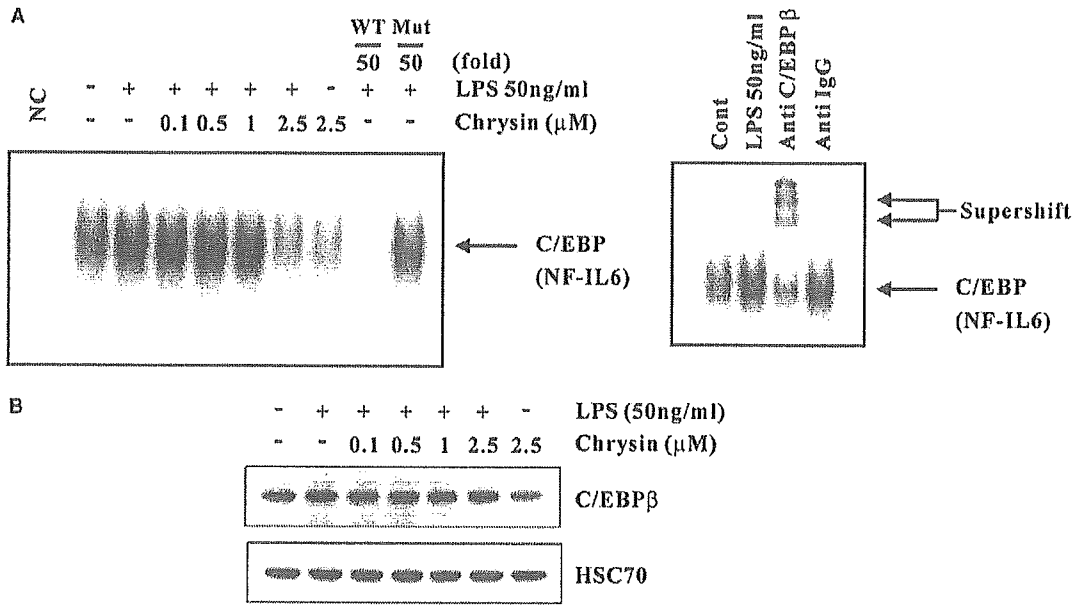

Fig. 5. Effect of chrysin on NF-IL6 DNA-binding activity in LPS-stimulated cells. (A) Raw 264.7 cells were pretreated with the indicated concentrations of chrysin for 30 min before incubation with LPS (50 ng/ml) for 4 h. EMSA analysis of the nuclear extracts was conducted using a [^{32}P]-labeled NF-IL6 oligonucleotide probe. Binding specificity was determined using the unlabeled wild-type probe or mutant-type containing the NF-IL6 binding sequence (50-fold in excess) to compete with the labeled oligonucleotide. To identify whether the bound proteins contain NF-IL6, super-shift analysis was performed using an antibody specific to NF-IL6. Super-shift with a rabbit IgG was used as a negative control. (B) Raw 264.7 cells were pretreated with the indicated concentrations of chrysin for 30 min before incubation with LPS (50 ng/ml) for 4 h. Expression levels of NF-IL6 were determined by Western blot analysis.

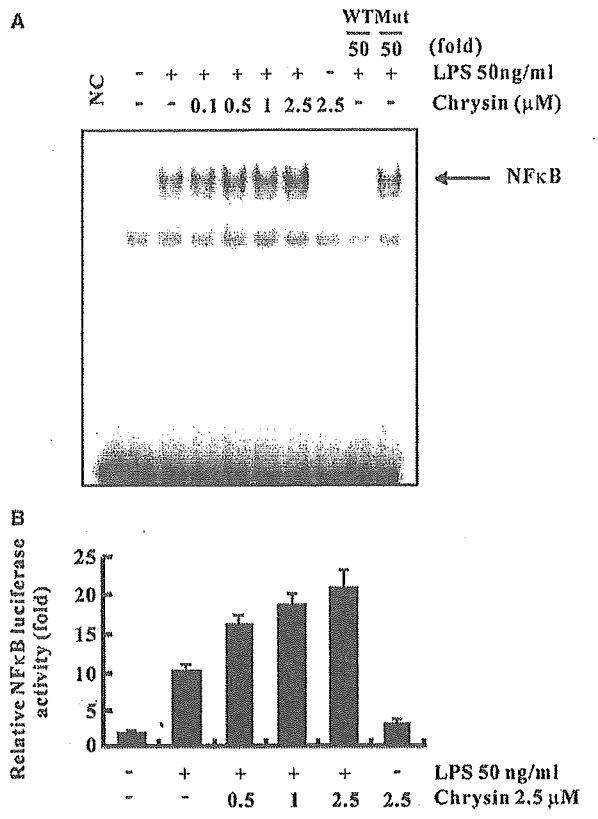

Fig. 6. Effect of chrysin on NF-κB DNA-binding activity and NF-κB promoter activity. (A) Raw 264.7 cells were pretreated with the indicated concentrations of chrysin for 30 min before incubation with LPS (50 ng/ml) for 4 h. EMSA analysis of the nuclear extracts was conducted using a [^{32}P]-labeled NF-κB oligonucleotide probe. Binding specificity was determined using the unlabeled wild-type probe or mutant-type containing the NF-κB binding sequence (25-fold in excess) to compete with the labeled oligonucleotide. (B) Cells were transiently transfected with a pNFκB-Luc plasmid containing four copies of the NF-κB binding site, treated with the indicated concentrations of chrysin and LPS for 24 h. The cells were lysed and luciferase activity was measured. Data represent the means ± S.D. of at least three independent experiments.

cells. To determine the specific signal transduction pathway that is involved in the chrysin-mediated inhibition of LPS-induced COX-2 expression, we examined the effects of chrysin on the activation of MAPKs induced by LPS, finding that LPS-induced MAPKs activations are not inhibited by chrysin. Therefore, the effect of chrysin on inhibition of LPS-induced COX-2 expression is probably not associated with MAP kinase activation.

NF-IL6, a member of the C/EBP family of transcription factors, is involved in inducing several acute-phase protein genes in response to immune and inflammatory stimulation. NF-IL6 also plays a major role in inducing the expression of COX-2 by cytokines (TNF-α and IL-1 β) and endotoxin [23,24,32]. Recently, Gorgoni et al. [38], reported that COX-2 induction by LPS was profoundly defective in C/EBP$^{-/-}$ macrophages, essentially due to impaired transcriptional induction via C/EBP promoter element. Here, we demonstrate that chrysin inhibits COX-2 gene expression in LPS-stimulated cultured macrophages, and we propose that the actions of chrysin are mediated by inhibition of NF-IL6 binding and transactivation. Several pieces of evidence support this proposal: (1) mutation of the NF-IL6 site inhibits the stimulatory effect of LPS, and the inhibitory effect of chrysin; (2) chrysin inhibited the DNA-binding activity of NF-IL6; and (3) chrysin had not inhibit in LPS-induced NF-κB binding and transactivation.

In summary, chrysin inhibits LPS-induced COX-2 expression in macrophages. These effects are mediated, at least in part, by inhibition of NF-IL6 activation. The fact that NF-IL6 is negatively regulated by chrysin is important because this transcription factor plays a critical role in the regulation of a variety of genes involved in inflammatory responses. In view of the facts that COX-2 plays an important role in mediating inflammatory responses and that chrysin may be an important determinant of clinical response in inflammatory diseases, further efforts to explore this therapeutic strategy appear warranted.

Acknowledgments: This work was supported by grants to T.K. Kwon from the MRC Program of the Korea Science & Engineering Foundation (No. R13-2002-028-03001-0).

References

[1] DeWitt, D.L. (1991) Prostaglandin endoperoxide synthase: regulation of enzyme expression. Biochim. Biophys. Acta 1083, 121–134.
[2] Smith, W.L., Garavito, R.M. and DeWitt, D.L. (1996) Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. J. Biol. Chem. 271, 33157–33160.
[3] Herschman, H.R. (1996) Prostaglandin synthase 2. Biochim. Biophys. Acta 1299, 125–140.
[4] Griswold, D.E. and Adams, J.L. (1996) Constitutive cyclooxygenase (COX-1) and inducible cyclooxygenase (COX-2): rationale for selective inhibition and progress to date. Med. Res. Rev. 16, 181–206.
[5] Chan, G., Boyle, J.O., Yang, E.K., Zhang, F., Sacks, P.G., Shah, J.P., Edelstein, D., Soslow, R.A., Koki, A.T., Woerner, B.M., Masferrer, J.L. and Dannenberg, A.J. (1999) Cyclooxygenase-2 expression is up-regulated in squamous cell carcinoma of the head and neck. Cancer Res. 59, 991–994.
[6] Kutchera, W., Jones, D.A., Matsunami, N., Groden, J., McIntyre, T.M., Zimmerman, G.A., White, R.L. and Prescott, S.M. (1996) Prostaglandin H synthase 2 is expressed abnormally in human colon cancer: evidence for a transcriptional effect. Proc. Natl. Acad. Sci. USA 93, 4816–4820.
[7] Subbaramaiah, K. and Dannenberg, A.J. (2003) Cyclooxygenase 2: a molecular target for cancer prevention and treatment. Trends Pharmacol. Sci. 24, 96–102.
[8] Williams, C.A., Harborne, J.B., Newman, M., Greenham, J. and Eagles, J. (1997) Chrysin and other leaf exudate flavonoids in the genus Pelargonium. Phytochemistry 46, 1349–1353.
[9] Rapta, P., Misik, V., Stasko, A. and Vrabel, I. (1995) Redox intermediates of flavonoids and caffeic acid esters from propolis: an EPR spectroscopy and cyclic voltammetry study. Free Radic. Biol. Med. 18, 901–908.
[10] Critchfield, J.W., Butera, S.T. and Folks, T.M. (1996) Inhibition of HIV activation in latently infected cells by flavonoid compounds. AIDS Res. Hum. Retroviruses 12, 39–46.
[11] Habtemariam, S. (1997) Flavonoids as inhibitors or enhancers of the cytotoxicity of tumor necrosis factor-alpha in L-929 tumor cells. J. Nat. Prod. 60, 775–778.
[12] Koganov, M.M., Dueva, O.V. and Tsorin, B.L. (1999) Activities of plant-derived phenols in a fibroblast cell culture model. J. Nat. Prod. 62, 481–483.
[13] Lapidot, T., Walker, M.D. and Kanner, J. (2002) Antioxidant and prooxidant effects of phenolics on pancreatic beta-cells in vitro. J. Agric. Food Chem. 50, 7220–7225.
[14] Cho, H., Yun, C.W., Park, W.K., Kong, J.Y., Kim, K.S., Park, Y., Lee, S. and Kim, B.K. (2004) Modulation of the activity of pro-inflammatory enzymes, COX-2 and iNOS, by chrysin derivatives. Pharmacol. Res. 49, 37–43.

[15] Liang, Y.C., Tsai, S.H., Tsai, D.C., Lin-Shiau, S.Y. and Lin, J.K. (2001) Suppression of inducible cyclooxygenase and nitric oxide synthase through activation of peroxisome proliferator-activated receptor-gamma by flavonoids in mouse macrophages. FEBS Lett. 496, 12–18.
[16] Jiang, C., Ting, A.T. and Seed, B. (1998) PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. Nature 391, 82–86.
[17] Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal. Biochem. 162, 156–159.
[18] Inoue, H., Yokoyama, C., Hara, S., Tone, Y. and Tanabe, T. (1995) Transcriptional regulation of human prostaglandin-endoperoxide synthase-2 gene by lipopolysaccharide and phorbol ester in vascular endothelial cells. Involvement of both nuclear factor for interleukin-6 expression site and cAMP response element. J. Biol. Chem. 270, 24965–24971.
[19] Inoue, H., Umesono, K., Nishimori, T., Hirata, Y. and Tanabe, T. (1999) Glucocorticoid-mediated suppression of the promoter activity of the cyclooxygenase-2 gene is modulated by expression of its receptor in vascular endothelial cells. Biochem. Biophys. Res. Commun. 254, 292–298.
[20] Baek, W.K., Park, J.W., Lim, J.H., Suh, S.I., Suh, M.H., Gabrielson, E. and Kwon, T.K. (2002) Molecular cloning and characterization of the human budding uninhibited by benomyl (BUB3) promoter. Gene 295, 117–123.
[21] Cho, Y.H., Lee, C.H. and Kim, S.G. (2003) Potentiation of lipopolysaccharide-inducible cyclooxygenase 2 expression by C2-ceramide via c-Jun N-terminal kinase-mediated activation of CCAAT/enhancer binding protein beta in macrophages. Mol. Pharmacol. 63, 512–523.
[22] Caivano, M. and Cohen, P. (2000) Role of mitogen-activated protein kinase cascades in mediating lipopolysaccharide-stimulated induction of cyclooxygenase-2 and IL-1 beta in RAW264 macrophages. J. Immunol. 164, 3018–3025.
[23] Yamamoto, K., Arakawa, T., Ueda, N. and Yamamoto, S. (1995) Transcriptional roles of nuclear factor kappa B and nuclear factor-interleukin-6 in the tumor necrosis factor alpha-dependent induction of cyclooxygenase-2 in MC3T3-E1 cells. J. Biol. Chem. 270, 31315–31320.
[24] Sorli, C.H., Zhang, H.J., Armstrong, M.B., Rajotte, R.V., Maclouf, J. and Robertson, R.P. (1998) Basal expression of cyclooxygenase-2 and nuclear factor-interleukin 6 are dominant and coordinately regulated by interleukin 1 in the pancreatic islet. Proc. Natl. Acad. Sci. USA 95, 1788–1793.
[25] Wardlaw, S.A., Zhang, N. and Belinsky, S.A. (2002) Transcriptional regulation of basal cyclooxygenase-2 expression in murine lung tumor-derived cell lines by CCAAT/enhancer-binding protein and activating transcription factor/cAMP response element-binding protein. Mol. Pharmacol. 62, 326–333.
[26] Tang, Q., Chen, W., Gonzales, M.S., Finch, J., Inoue, H. and Bowden, G.T. (2001) Role of cyclic AMP responsive element in the UVB induction of cyclooxygenase-2 transcription in human keratinocytes. Oncogene 20, 5164–5172.
[27] Barnes, P.J. and Karin, M. (1997) Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. N. Engl. J. Med. 336, 1066–1071.
[28] Rhee, S.H. and Hwang, D. (2000) Murine TOLL-like receptor 4 confers lipopolysaccharide responsiveness as determined by activation of NF kappa B and expression of the inducible cyclooxygenase. J. Biol. Chem. 275, 34035–34040.
[29] Chen, B.C., Chang, Y.S., Kang, J.C., Hsu, M.J., Sheu, J.R., Chen, T.L., Teng, C.M. and Lin, C.H. (2004) Peptidoglycan induces nuclear factor-kappaB activation and cyclooxygenase-2 expression via Ras, Raf-1, and ERK in RAW 264.7 macrophages. J. Biol. Chem. 279, 20889–20897.
[30] Liu, W., Reinmuth, N., Stoeltzing, O., Parikh, A.A., Tellez, C., Williams, S., Jung, Y.D., Fan, F., Takeda, A., Akagi, M., Bar-Eli, M., Gallick, G.E. and Ellis, L.M. (2003) Cyclooxygenase-2 is up-regulated by interleukin-1 beta in human colorectal cancer cells via multiple signaling pathways. Cancer Res. 63, 3632–3636.
[31] Johnson, G.L. and Lapadat, R. (2002) Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science 298, 1911–1912.
[32] Wadleigh, D.J., Reddy, S.T., Kopp, E., Ghosh, S. and Herschman, H.R. (2000) Transcriptional activation of the cyclooxygenase-2 gene in endotoxin-treated RAW 264.7 macrophages. J. Biol. Chem. 275, 6259–6266.
[33] Guan, Z., Buckman, S.Y., Pentland, A.P., Templeton, D.J. and Morrison, A.R. (1998) Induction of cyclooxygenase-2 by the activated MEKK1 → SEK1/MKK4 → p38 mitogen-activated protein kinase pathway. J. Biol. Chem. 273, 12901–12908.
[34] Guan, Z., Buckman, S.Y., Miller, B.W., Springer, L.D. and Morrison, A.R. (1998) Interleukin-1beta-induced cyclooxygenase-2 expression requires activation of both c-Jun NH2-terminal kinase and p38 MAPK signal pathways in rat renal mesangial cells. J. Biol. Chem. 273, 28670–28676.
[35] Yang, T., Huang, Y., Heasley, L.E., Berl, T., Schnermann, J.B. and Briggs, J.P. (2000) MAPK mediation of hypertonicity-stimulated cyclooxygenase-2 expression in renal medullary collecting duct cells. J. Biol. Chem. 275, 23281–23286.
[36] Luo, S.F., Wang, C.C., Chien, C.S., Hsiao, L.D. and Yang, C.M. (2003) Induction of cyclooxygenase-2 by lipopolysaccharide in canine tracheal smooth muscle cells: involvement of p42/p44 and p38 mitogen-activated protein kinases and nuclear factor-kappaB pathways. Cell Signal. 15, 497–509.
[37] Hwang, D., Jang, B.C., Yu, G. and Boudreau, M. (1997) Expression of mitogen-inducible cyclooxygenase induced by lipopolysaccharide: mediation through both mitogen-activated protein kinase and NF-kappaB signaling pathways in macrophages. Biochem. Pharmacol. 54, 87–96.
[38] Gorgoni, B., Caivano, M., Arizmendi, C. and Poli, V. (2001) The transcription factor C/EBPbeta is essential for inducible expression of the cox-2 gene in macrophages but not in fibroblasts. J. Biol. Chem. 276, 40769–40777.

[Cancer Biology & Therapy 4:2, 175-180, February 2005]; ©2005 Landes Bioscience

Research Paper

Selenomethionine Regulates Cyclooxygenase-2 (COX-2) Expression through Nuclear Factor-Kappa B (NF-κB) in Colon Cancer Cells

Durga P. Cherukuri[1]
Anne-Christine Goulet[1]
Hiroyasu Inoue[2]
Mark A. Nelson[1,*]

[1]Department of Pathology; Arizona Cancer Center; The University of Arizona College of Medicine; Tucson, Arizona USA

[2]Department of Pharmacology; National Cardiovascular Center Research Institute; Osaka, Japan

*Correspondence to: Mark A. Nelson, Ph.D. Associate Professor and Director of Research; P.O. Box 245042; 1501 N. Campbell Ave., #5208; Tucson, Arizona 85724 USA; Tel.: 520.626.1489; Fax: 520.626.8864; Email: mnelson@azcc.arizona.edu

Received 10/19/04; Accepted 12/08/04

Previously published online as a *Cancer Biology & Therapy* E-publication: http://www.landesbioscience.com/journals/cbt/abstract.php?id=1438

KEY WORDS

Selenomethionine, COX-2, NF-κB, colorectal cancer

ABBREVIATIONS

COX	cyclooxygenase
NF-κB	nuclear factor-kappaB
Se-Met	selenomethionine
EMSA	electrophoretic mobility shift assay
PG	prostaglandin
LPS	lipopolysachharide
CRE	cAMP response element
TCF-4	T-cell factor 4
NF-IL6	nuclear factor for interleukin-6 expression
PARP	poly (ADP-ribose) polymerase

ACKNOWLEDGEMENTS

This work was supported by grant CA097383 from NIH.

ABSTRACT

Previously, we showed that selenomethionine (Se-Met) inhibits growth of colon cancer cells via suppressing COX-2 expression at both mRNA and protein level. However, the molecular mechanism by which Se-Met suppresses COX-2 expression remains to be elucidated. To this end, we transiently transfected HCA-7 cells with different COX-2 promoter constructs followed by Se-Met treatment (90 μM) for 12 h. The results suggested the role of nuclear factor-kappa B (NF-κB) in transcriptional regulation of COX-2. We also observed complete inhibition of DNA binding activity of NF-κB in Se-Met (90 μM) treated HCA-7 cells as shown by electrophoretic mobility shift assay (EMSA). Supershift assays with anti-p65 antibody identified p65 subunit in the protein complex. We further demonstrate dose-dependent inhibition of nuclear translocation of NF-κB/p65 in Se-Met treated HCA-7 cells, which could explain the observed reduction in DNA binding of NF-κB/p65. These results suggest that Se-Met regulates COX-2 at transcriptional level by modulating the activity of NF-κB transcription factor.

INTRODUCTION

Colorectal cancer is one of the major leading cause of cancer-related deaths in the United States. Cyclooxygenases (COXs) exist in two isoforms—COX-1 and -2—that metabolize arachidonic acid to produce prostaglandins (PGs). Several studies have reported increased expression of COX-2 in colorectal cancer tissues, while COX-1 is constitutively expressed.[1-3] COX-2 is inducible in response to inflammatory cytokines, growth factors, oncogenes, and lipopolysachharide (LPS).[4,5] Direct evidence for the role of COX-2 in neoplastic transformation is shown by genetic studies. COX-2 knockout in $APC^{\Delta 716}$ mice showed significant reduction in the number and size of the polyps compared to the control mice.[6] In another study using transgenic mice it was shown that overexpression of COX-2 itself is sufficient to induce tumorigenesis by modulating the expression of pro- and anti-apoptotic proteins and these effects are mediated by PGs.[7] Elevated levels of PGs as a result of COX-2 overexpression in colon cancer cells play a key role in cell proliferation, apoptosis, angiogenesis, and tumor metastasis.[8-11] Taken together, these observations suggest that strategies directed at inhibition of COX-2 could be promising in the prevention/or treatment of colon cancer.[12]

Selenium is an essential trace element of human health and its cancer chemopreventive efficacy was demonstrated by epidemiological, cell culture, and human clinical trial studies.[13,14] However, the molecular mechanisms underlying the anti-cancer effects of Se are not well understood. We previously demonstrated that Se-Met (60 and 90 μM) inhibits the growth of colorectal cancer cells in a dose and time-dependent fashion.[15] We also showed inhibition of COX-2 expression by Se-Met that accounts for the observed reduction in prostaglandin E2 (PGE2) levels. Further, exogenously added PGE2 protected HCA-7 cells from the anti-proliferative effects of Se-Met, indicating the involvement of COX-2 dependent pathway in Se-Met mediated growth inhibition of HCA-7 cells. However, exactly how Se-Met regulates COX-2 expression in colon cancer cells remains to be determined.

COX-2 can be regulated at both transcriptional and post-transcriptional levels. Interleukin-1β and LPS induce COX-2 in colon cancer cells through increased mRNA synthesis.[4,5] In another study, it was shown that transfection of HCT116 colon cancer cells with a reporter gene containing 2.0 kb fragment of 5'-flanking region of COX-2 gene showed significant luciferase activity without any external stimulus compared to the normal epithelial cells.[16] Similar observations were also made in other colon cancer cell lines including HCA-7 and LS-174.[17] It can be drawn from these studies that some factors that are either constitutively expressed or induced with external stimuli could account for the

transcriptional regulation of COX-2. Further, regulation of COX-2 at post-transcriptional level through increased mRNA stability was also reported.[17,18] Binding sites for the regulatory elements including NF-κB, NF-IL6, CRE, PEA-3, SP-1, AP-2 and TCF-4 have been identified in the 5'-flanking region of the COX-2 gene.[19,20] COX-2 expression appears to be modulated by distinct mechanisms such as through NF-κB, cAMP response element (CRE), and T-cell factor-4 (TCF-4).[4,5,20-22] Based on our previous observations, we hypothesized that Se-Met regulates COX-2 at transcriptional level.

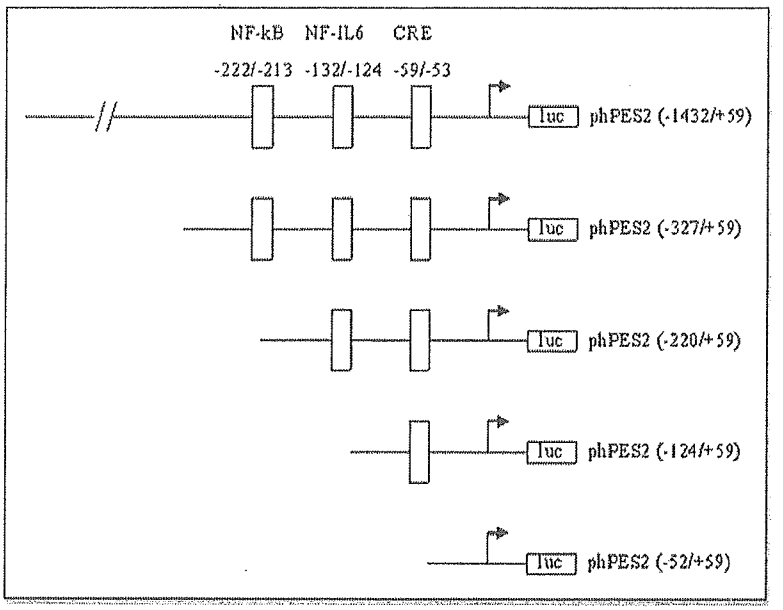

Figure 1. Schematic diagram of the human COX-2 promoter constructs. The positions of consensus binding sites for NF-κB, NF-IL6 and CRE in the 5'-flanking region of COX-2 relative to the transcription start site are indicated.

MATERIALS AND METHODS

Cell culture and reagents. HCA-7 colorectal cancer cells were maintained in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 1% pencillin/streptomycin at 37°C with 5% CO_2. Goat polyclonal anti-p65 antibody (sc-372) and mouse monoclonal anti-p50 antibody (sc-8414) were from Santa Cruz Biotechnology.

Transient transfection of HCA-7 cells with COX-2 promoter constructs and luciferase assay. HCA-7 cells were transiently transfected with various COX-2 promoter deletion constructs (-1432/+59, -327/+59, -220/+59, -124/+59, and -52/+59) as described previously.[17] 80,000 cells/well were plated in 24-well plates in DMEM medium and were allowed to grow for 24 h. After 24 h, cells were cotransfected with 0.5 μg of each COX-2 promoter construct and 10 ng of Renilla plasmid at 90% confluence using LipofectAMINE™ 2000 reagent (Invitrogen™). After 24 h of transfection, cells were treated with Se-Met at 90 μM for 12 h. Later the cells were washed twice with PBS, lysed with passive lysis buffer (Promega) and the cell lysates were stored at -80°C till further use. Firefly and renilla luciferase activities were measured using Dual luciferase reporter assay system (Promega) according to the protocol suggested by the manufacturer. Firefly luciferase values obtained with each COX-2 construct were normalized to the corresponding renilla luciferase values.

Se-Met treatment of HCA-7 cells. 8 x 10^4 cells were plated in 10 cm petri dish and were treated with seleno-L-methionine (Sigma) for 6 days as described previously.[15] Briefly, After 24 h of plating, cells were treated with 90 μM Se-Met (day 0). Fresh media with or without Se-Met was added on days 2, and 4 and the cells were collected on day 6 and stored at -80°C until next use.

Preparation of nuclear extracts. HCA-7 cells were gently lysed in sucrose buffer containing NP-40 (10 mM Tris pH 8.0, 3 mM CaCl2, 2 mM MgOAc, 0.1 mM EDTA, 0.32 M sucrose, 0.5% NP-40, 0.5 mM PMSF, and 1 mM DTT). The nuclei were spinned down by centrifugation at 500 g for 5 min at 4°C and the pellet was resuspended in a hypotonic low salt buffer (20 mM HEPES pH 7.9, 1.5 mM MgCl2, 20 mM KCl, 0.2 mM EDTA, 25% glycerol, 0.5 mM PMSF, and 0.5 mM DTT). To this suspension, equal volume of high salt buffer (20 mM HEPES pH 7.9, 1.5 mM MgCl2, 800 mM KCl, 0.2 mM EDTA, 25% glycerol, 1% NP-40, 0.5 mM PMSF, 0.5 mM DTT, and protease cocktail inhibitor) was added and incubated for 45 min on a rotator, and then centrifuged at 14000 g for 30 min at 4°C. Total nuclear protein was quantitated using BCA™ protein assay kit (Pierce).

Electrophoretic mobility shift assay (EMSA) and supershift analysis. 10 μg of nuclear proteins were incubated with 32p-labeled double stranded NF-κB consensus oligonucleotides (5'-AGT TGA GGG GAC TTT CCC AGG C-3') for 30 min at room temperature. Binding reaction was carried out in a final volume of 10 μl. Mutant NF-κB oligonucleotide with a single mutation in the NF-κB DNA binding site (5'-AGT TGA GGC GAC TTT CCC AGG C-3') was used as a control in this study. For competition experiments, nuclear extracts were incubated with an excess of unlabeled oligonucleotides for 30 min at room temperature followed by incubation with labeled oligonucleotides for another 30 min to confirm the specificity of NF-κB binding. After 1h incubation, the DNA: protein complexes were separated by electrophoresis through 5% nondenaturing polyacrylamide gel with a running buffer of 0.5x TBE. Gel was dried, and the DNA:protein complexes were visualized by autoradiography. In case of supershift analysis, nuclear protein extracts were incubated with goat anti-p65 antibody for 30 min, followed by incubation with labeled wild type NF-κB consensus oligonucleotides for another 30 min to identify NF-κB protein complex.

Western analysis. 50 μg of nuclear protein was separated on SDS-PAGE, and transferred to nylon membrane. The membrane was probed with goat polyclonal anti-p65 antibody (1:1000) overnight at 4°C. Later, the membrane was incubated with anti-goat IgG secondary antibody at 1:40,000 and the blots were subjected to autoradiography.

Statistical analysis. Statistical analysis was performed using student's t-test. Values at $P<0.05$ are considered significant.

RESULTS

NF-κB-mediated regulation of COX-2 by Se-Met. Our group previously demonstrated that Se-Met decreases COX-2 mRNA levels in dose-dependent fashion in HCA-7 colon cancer cells which could be due to either transcriptional regulation or decreased mRNA stability. We hypothesized transcriptional regulation of COX-2 by Se-Met. The 5'-flanking region of COX-2 gene has consensus binding sites for Tcf-4, NF-κB, NF-IL6 and CRE. In order to understand the regulation of COX-2 by Se-Met, we transiently transfected HCA-7 cells with human COX-2 promoter deletion constructs (Fig. 1) followed by Se-Met treatment at 90 μM for 12 h and the expression of each construct was determined by the luciferase activity. A time point of 12 h for Se-Met treatment was chosen based on our time-course experiments in which significant decrease in luciferase activity with 90 μM Se-Met was observed as early as 12 h compared to the untreated cells (data not shown).

As shown in Figure 2A, -220/+59 followed by -327/+59 promoter constructs showed highest luciferase activity compared to -1432/+59 construct, while -52/+59 construct which lacks the binding sites of all the regulatory elements exhibited >75% reduction in luciferase activity compared to -1432/+59.

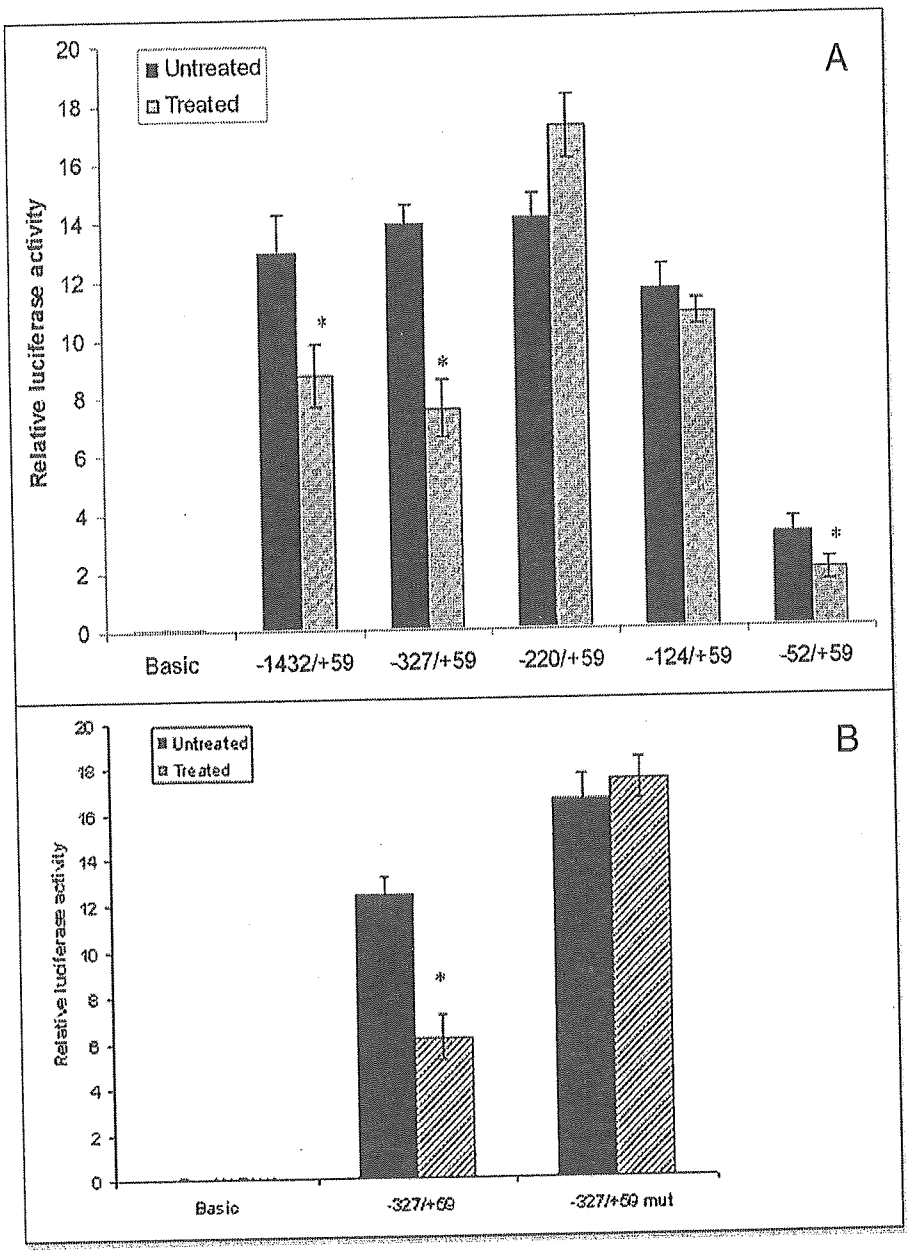

Figure 2. Transcriptional activity of COX-2 promoter constructs in HCA-7 cells treated with Se-Met at 90 μM. (A) HCA-7 cells were transfected with 0.5 μg of each COX-2 promoter construct for 24 h followed by Se-Met treatment for 12 h. Firefly and renilla luciferase activities were measured using Dual luciferase assay system (Promega). Firefly luciferase values were normalized to Renilla values. Mean ± S.E. of three independent experiments was plotted. (B) -327/+59 construct with a mutation in the NF-kB binding site was transfected into the HCA-7 cells for 24 h, followed by Se-Met treatment for 12 h. Data shown is derived from three independent experiments.

Deletion of NF-IL6 and CRE sites in -124/+59 and -52/+59 constructs resulted in a decrease of luciferase activity by 18% and 77%, respectively compared to -220/+59. These observations suggest that NF-IL6, and CRE element play an important role in regulating COX-2 transcription in HCA-7 cells. When transfected HCA-7 cells were treated with 90 μM Se-Met, -1432/+59 and -327/+59 constructs showed a significant ($P<0.05$) decrease in their luciferase activity by 33% and 46%, respectively compared to their untreated controls. No difference was seen in luciferase activity between control and Se-Met treatment with -220/+59 and -124/+59 COX-2 promoter constructs. These results indicate that Se-Met regulates COX-2 expression at transcriptional level possibly by targeting NF-κB.

To confirm the involvement of NF-κB transcription factor in Se-Met mediated COX-2 regulation, HCA-7 cells were transfected with -327/+59 construct carrying a mutation in NF-κB binding site. If Se-Met targets only NF-κB, no difference could be seen in luciferase activity between control and Se-Met treatment. The data in Figure 2B shows no decrease in luciferase activity with Se-Met treatment. Together, these observations suggest a role for NF-κB in the regulation of COX-2 by Se-Met.

Se-Met inhibits NF-κB DNA binding. Based on the observations from transfection experiments, we speculated that modulation of DNA binding of NF-κB by Se-Met could explain the observed decrease in luciferase activity in COX-2 promoter constructs that contains NF-κB binding sites in HCA-7 cells. We performed EMSA to examine binding of NF-κB using 32p-labeled consensus NF-κB double stranded oligonucleotides in both control and Se-Met 90 μM treated cells. NF-κB DNA binding was seen only with wild-type NF-κB oligonucleotides (Lane 2, Fig. 3), but not with mutant oligos (lane 5, Fig. 3). NF-κB binding was observed when labeled wild type-NF-κB oligonucleotides were incubated with nuclear extracts isolated from untreated HCA-7 cells (lane 2, Fig. 3), whereas this binding was completely inhibited in Se-Met treated HCA-7 cells (lane 3, Fig. 3). NF-κB binding was completely abrogated when the control nuclear extracts were incubated with an excess of nonlabeled wild type oligonucleotide (100x) for 30 min prior to the addition of labeled oligonucleotide (lane 4, Fig. 3), which clearly shows the specificity of the NF-κB binding. However, addition of nonlabeled mutant oligonucleotide had no effect on the binding (lane 6, Fig. 3).

NF-κB exists in the form of either homo- or heterodimer composed of five different subunits. To identify the subunits of NF-κB involved in this binding, supershift analysis was carried out by incubating the nuclear extracts with anti-p65- and anti-p50 antibodies. The binding complex was super-shifted only with p65 antibody but not with p50 (lanes 7 and 8, Fig. 3). Together these observations suggested that Se-Met treatment inhibits DNA binding of p65 subunit of NF-κB.

Decreased nuclear translocation of NF-κB/p65 with Se-Met treatment. In its inactive form, NF-κB is predominantly located in cytoplasm and bound to inhibitory IkB proteins. Degradation of these IkBs by phosphorylation at serine residues by upstream kinases results in release of NF-κB, which then translocates into the nucleus and activates its target genes. To determine whether inhibition of NF-κB DNA binding with Se-Met treatment is due to reduced nuclear accumulation of NF-κB/p65, western

analysis was carried out with nuclear extracts isolated from untreated and cells treated with Se-Met at 60 (IC_{50} dose) and 90 μM. Se-Met treatment resulted in dose-dependent reduction of NF-κB/p65 nuclear accumulation in HCA-7 cells (Fig. 4).

DISCUSSION

Dietary selenium supplementation with selenium-enriched yeast has been shown to be associated with marked reduction in the risks for developing prostate, lung, and colorectal cancer.[13] Currently, selenium alone or in combination with other chemopreventive agents is being evaluated in clinical trials against human colon and prostate cancers.[23,24] However, the molecular and cellular effects of selenium underlying its anti-cancer effects are not completely understood. Se-Met is an organic form of selenium present in a variety of natural foods and is being identified as a predominant form of Se in selenized yeast.[25] Previous in vitro studies have shown that Se-Met inhibits COX-2 expression in colon cancer cells.[15] However, whether COX-2 inhibition by Se-Met occurs at transcriptional or post-transcriptional level is not known. In the present study, we demonstrate that Se-Met significantly inhibits luciferase activity of COX-2 reporter constructs. This suggests that Se-Met inhibits COX-2 at the transcriptional level.

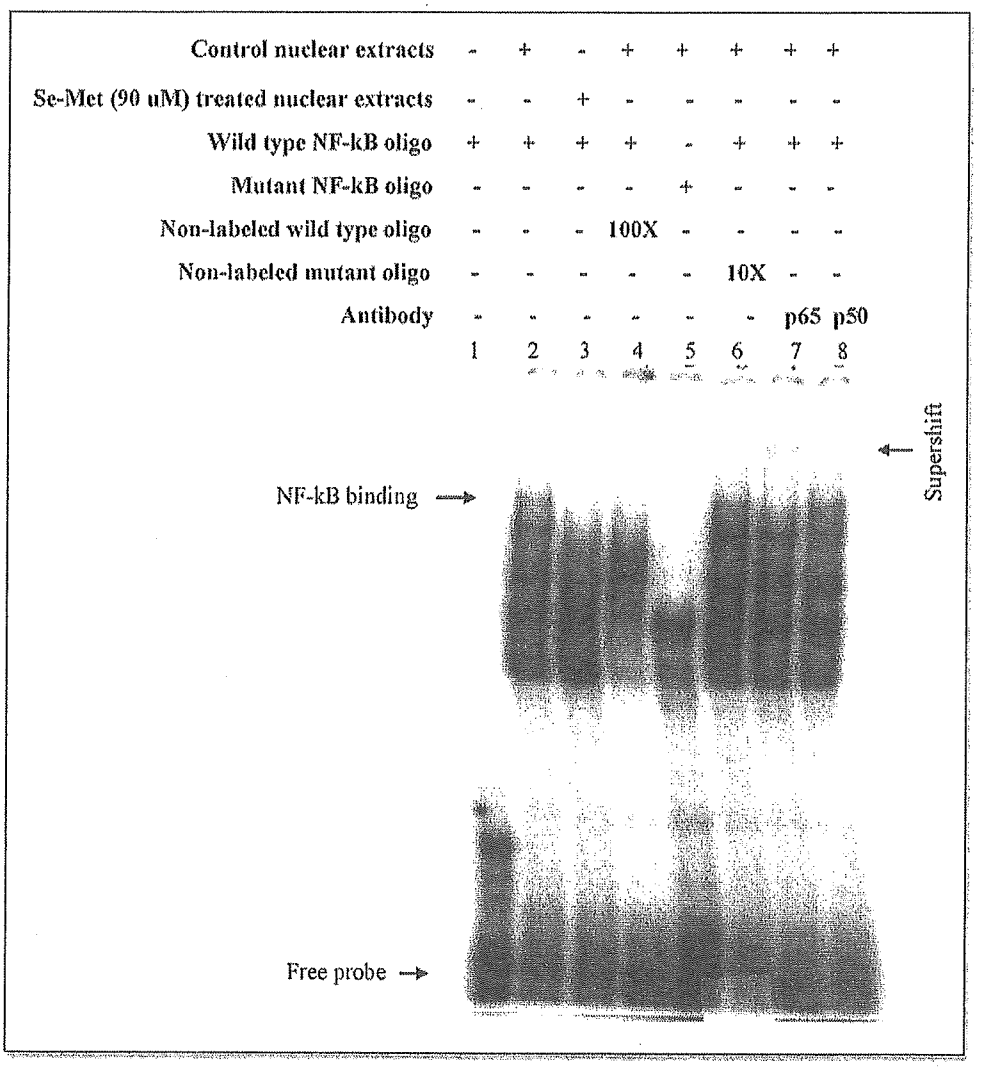

Figure 3. Effect of Se-Met on NF-kB DNA binding in HCA-7 cells. Cells were treated with 90 μM Se-Met for 6 days and nuclear extracts were prepared. EMSA was performed with 10 μg of nuclear proteins which were incubated with 32p-labeled double stranded NF-kB consensus oligonucleotide for 30 min. NF-kB binding complexes were analyzed by supershift analysis using anti-p65 and 50 antibodies. The data shown is a representative of the three experiments and similar results were observed in all the three experiments.

COX-2 promoter constructs using luciferase as a reporter gene were described in previous studies.[26,27] The COX-2 reporter construct -327/+59 showed slightly higher luciferase activity compared to that of -1432/+59 in HCA-7 cells. This observation suggests that -327/+59 region that contains consensus sequences for NF-κB site, NF-IL6 site, and CRE is sufficient for the induction of COX-2 transcription. Further, reduction in luciferase activity observed with the deletion of NF-IL6 site, and CRE indicate key role of these cis-acting elements in regulating COX-2 transcription. These data is consistent with the previous studies.[17,27] Post-transcriptional mechanisms also appears to be important for the sustained induction of COX-2.[17] Interestingly, Se-Met treatment at 90 μM significantly reduced the luciferase activity of those reporter constructs that contain binding sites for NF-κB. These data demonstrate that Se-Met inhibits COX-2 at transcriptional level by selectively targeting NF-κB transcription factor. These findings were further supported by using -327/+59 reporter construct with a mutation in NF-κB binding site in which Se-Met treatment did not alter the luciferase activity.

Electrophoretic mobility shift assays using double-stranded NF-κB consensus oligonucleotides showed that Se-Met completely inhibits NF-κB DNA binding in HCA-7 cells. This observation is consistent with previous studies using other selenium compounds in different cell types.[21,28-31] Competition experiments with an excess of nonlabeled wild type and mutant oligos showed binding specificity of NF-κB. Supershift analysis by incubating nuclear protein extracts with anti-p65 and p50 antibodies identified the presence of p65 protein but not p50, suggesting that Se-Met inhibits DNA binding of NF-κB/p65 protein. These results can be explained by the observed reduction in nuclear translocation of NF-κB/p65 by Se-Met

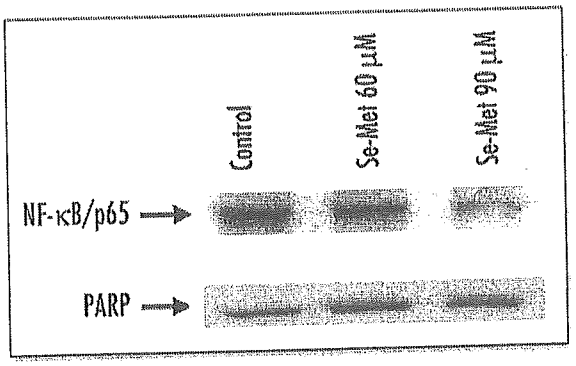

Figure 4. Se-Met inhibited nuclear translocation of NF-kB/p65 in HCA-7 colon cancer cells. Cells were treated with 60 & 90 μM Se-Met for 6 days, and the nuclear protein extracts were prepared. 50 μg of nuclear protein was separated on SDS-PAGE, transferred to nylon membrane, and probed with goat polyclonal anti-NFkB/p65 overnight. The experiment was repeated twice and similar results were observed. PARP was used as a loading control.

and this reduction is dose-dependent at 60 and 90 μM of Se dose. The molecular mechanisms by which Se-Met inhibits NF-κB activation requires further study. One possible mechanism could be inhibiting phosphorylation of IkB proteins by their upstream IKK kinases and thereby preventing their degradation as observed with some selenium compounds in prostate cancer cells.[30]

In this current study, we observed maximum inhibition of NF-κB by Se-Met at doses that are higher than the concentration seen in previous clinical trials.[13] However, our findings are supported by a recent study in which Se-Met maximally inhibited nuclear accumulation of NF-κB/p65, as measured by flow cytometric analysis, in peroxynitrite- and LPS-challenged leukocytes and polymorphonuclear cells at 100 μM dose.[32] Furthermore, it has been reported that prolonged periods of Se supplementation with 750–850 μg may not be associated with any adverse side effects.[33] Also in type II diabetic patients extra daily supplementation of Se at 960 μg (Se group) decreased the NF-κB/p65 DNA binding to the control levels (non-diabetic group) with no observed toxicity symptoms of Se.[31] Finally, it is worth noting that toxicity profile in high dose selenium supplementation conducted in men with biopsy-proven prostate cancer who were randomized to either 1600 or 3200 μg/day of selenized yeast suggest that doses greater than 400 μg/day could be given in controlled conditions for an extended period of time, without serious toxicity.[34]

In summary, our findings demonstrate that Se-Met inhibits COX-2 at transcriptional level by modulating NF-κB activation. These observations holds significance in light of a recent immunohistochemical study that showed elevated levels of NF-κB and further coexpression of NF-κB and COX-2 in moderately and well-differentiated colonic malignant epithelial cells, indicating the possibility of NF-κB mediated COX-2 induction in these cells.[35] In this context, Se-Met supplementation may be useful in suppression of COX-2 and its downstream effects by inhibiting NF-κB pathway in colorectal cancer.

References

1. Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN. Upregulation of cyclooxygenase-2 expression in human colorectal adenomas and adenocarcinomas. Gastroenterology 1994; 107:1183-8.
2. Khan KN, Masferrer JL, Woerner BM, Soslow R, Koki AT. Enhanced cyclooxygenase-2 expression in sporadic and familial adenomatous polyposis of the human colon. Scand J Gastroenterol 2001; 36:865-9.
3. Einspahr JG, Krouse RS, Yochim JM, Danenberg PV, Danenberg KD, Bhattacharya AK, Martinez ME, Alberts DS. Association between cyclooxygenase expression and colorectal adenoma characteristics. Cancer Res 2003; 63:3891-3.
4. Kojima M, Morisaki T, Izuhara K, Uchiyama A, Matsunari Y, Katano M. Lipopolysachharide increases cyclooxygenase-2 expression in a colon carcinoma cell line through nuclear factor-kB activation. Oncogene 2000; 19:1225-31.
5. Liu W, Reinmuth N, Stoelzing O, Parikh AA, Tellez C, Williams S, Jung YD, Fan F, Takeda A, Akagi M, Bar-Eli M, Gallick GE, Ellis LM. Cyclooxygenase-2 is upregulated by interleukin-1β in human colorectal cancer cells via multiple signaling pathway. Cancer Res 2003; 63:3632-6.
6. Oshima M, Dinchuk JE, Kargman S, Oshima H, Hancock B, Kwong E. Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). Cell 1996; 87:803-9.
7. Liu CH, Chang SH, Narko K, Trifan OC, Wu MT, Smith AE, Haudenschild C, Lane TF, Hla T. Overexpression of cyclooxygenase-2 is sufficient to induce tumorigenesis in transgenic mice. J Biol Chem 2001; 276:18563-9.
8. Tsujii M, Kawano S, Tsuji S, Sawaoka H, Hori M, DuBois RN. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. Cell 1998; 93:705-16.
9. Chen WS, Wei SJ, Liu JM, Hsiao M, Kou-Lin J, Yang WK. Tumor invasiveness and liver metastasis of colon cancer cells correlated with cyclooxygenase-2 (COX-2) expression and inhibited by a COX-2-selective inhibitor, Etodolac. Int J Cancer 2001; 91:894-9.
10. Sheng H, Shao J, Washington MK, DuBois RN. Prostaglandin E2 increases growth and motility of colorectal carcinoma cells. J Biol Chem 2001; 276:18075-81.
11. Pai R, Nakamura T, Moon WS, Tarnawski AS. Prostaglandins promote colon cancer cell invasion; signaling by cross-talk between two distinct growth factor receptors. FASEB J 2003; 17:1640-7.
12. Gupta RA, DuBois RN. Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. Nat Rev Cancer 2001; 1:11-21.
13. Clark LC, Combs GF Jr, Turnbull BW, Slate EH, Chalker DK, Chow J, Davis LS, Glover RA, Graham GF, Gross EG, Krongrad A, Lesher JL Jr, Park HK, Sanders BB Jr, Smith CL, Taylor JR. Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. A randomized controlled trial. Nutritional prevention of cancer study group. JAMA 1996; 276:1957-63.
14. Redman C, Scott JA, Baines AT, Basye JL, Clark LC, Calley C, Roe D, Payne CM, Nelson MA. Inhibitory effect of selenomethionine on the growth of three selected human tumor cell lines. Cancer Lett 1998; 125:103-10.
15. Baines A, Taylor-Parker M, Goulet AC, Renaud C, Gerner EW, Nelson MA. Selenomethionine inhibits growth and suppresses cyclooxygenase-2 (COX-2) protein expression in human colon cancer cell lines. Cancer Biol Ther 2002; 114:374-8.
16. Kutchera W, Jones DA, Matsunami N, Groden J, McIntyre TM, Zimmerman GA, White RL, Prescott SM. Prostaglandin H synthase 2 is expressed abnormally in human colon cancer: Evidence for a transcriptional effect. Proc Natl Acad Sci USA 1996; 93:4816-20.
17. Shao J, Sheng H, Inoue H, Morrow JD, DuBois RN. Regulation of constitutive cyclooxygenase-2 expression in colon carcinoma cells. J Biol Chem 2000; 275:33951-6.
18. Zhang Z, Sheng H, Shao J, Beauchamp RD, DuBois RN. Posttranscriptional regulation of cyclooxygenase-2 in rat intestinal epithelial cells. Neoplasia 2000; 2:523-30.
19. Tazawa R, Xu XM, Wu KK, Wang LH. Characterization of the genomic structure, chromosomal location and promoter of human prostaglandin H *synthase-2* gene. Biochem Biophys Res Commun 1994; 203:190-9.
20. Araki Y, Okamura S, Hussain SP, Nagashima M, He P, Shiseki M, Miura K, Harris CC.. Regulation of cyclooxygenase-2 expression by the Wnt and Ras pathways. Cancer Res 2003; 63:728-34.
21. Zamamiri-Davis F, Lu Y, Thompson JT, Prabhu KS, Reddy PV, Sordillo LM, Reddy CC.. Nuclear factor-kB mediates overexpression of cyclooxygenase-2 during activation of RAW 264.7 macrophages in selenium deficiency. Free Radical Biol Med 2002; 32:890-7.
22. Subbaramaiah K, Cole PA, Dannenberg AJ. Retinoids and carnosol suppress cyclooxygenase-2 transcription by CREB-binding protein/p300-dependent and -independent mechanisms. Cancer Res 2002; 62:2522-30.
23. Frank DH, Roe DJ, Sherry Chow HH, Guillen JM, Choquette K, Gracie D, Francis J, Fish A, Alberts DS. . Effects of a high-selenium yeast supplement on celecoxib plasma levels: A randomized phase II trial. Cancer Epidemiol Biomark Prev 2004; 13:299-303.
24. Pak RW, Lanteri VJ, Scheuch JR, Sawczuk IS. Review of vitamin E and selenium in the prevention of prostate cancer: Implications of the selenium and vitamin E chemoprevention trial. Integrated Cancer Ther 2002; 1:338-44.
25. Block E, Glass RS, Jacobsen NE, Johnson S, Kahakachchi C, Kaminski R, Skowronska A, Boakye HT, Tyson JF, Uden PC. Identification and synthesis of a novel selenium-sulfur amino acid found in selenized yeast: Rapid indirect detection NMR methods for characterizing low-level organoselenium compounds in complex matrices. J Agric Food Chem 2004; 52:3761-71.

26. Inoue H, Nanayama T, Hara S, Yokoyama C, Tanabe T. The cAMP response element plays an essential role in the expression of the human prostaglandin-endoperoxide *synthase-2* gene in differentiated in U937 monocytic cells. FEBS Lett 1994; 350:51-4.
27. Inoue H, Yokoyama C, Hara S, Tone Y, Tanabe T. Transcriptional regulation of human prostaglandin-endoperoxide *synthase-2* gene by lipopolysachharide and phorbol ester in vascular endothelial cells. Involvement of both nuclear factor for interleukin-6 expression site and cAMP response element. J Biol Chem 1995; 270:24965-71.
28. Kim IY, Stadtman TC. Inhibition of NF-κB DNA binding and nitric oxide induction in human T cells and lung adenocarcinoma cells by selenite treatment. Proc Natl Acad Sci USA 1997; 94:12904-7.
29. Prabhu KS, Zamamiri-Davis F, Stewart JB, Thompson JT, Sordillo LM, Reddy CC. Selenium deficiency increases the expression of inducible nitric oxide synthase in RAW 264.7 macrophages: Role of nuclear factor-kB in upregulation. Biochem J 2002; 366:203-9.
30. Gasparian AV, Yao YJ, Lu J, Yemelyanov AY, Lyakh LA, Slaga TJ, Budunova IV. Selenium compounds inhibit IkB kinase (IKK) and nuclear factor-kB (NF-κB) in prostate cancer cells. Mol Cancer Ther 2002; 1:1079-87.
31. Faure P, Ramon O, Favier A, Halimi S. Selenium supplementation decreases nuclear factor-kappa B activity in peripheral blood mononuclear cells from type 2 diabetic patients. Eur J Clin Invest 2004; 34:475-81.
32. Jozsef L, Filep JG. Selenium-containing compounds attenuate peroxynitrite-mediated NF-κB and AP-1 activation and *interleukin-8* gene and protein expression in human leukocytes. Free Radical Biol Med 2003; 35:1018-27.
33. Yang G, Yin S, Zhou R, Gu L, Yan B, Liu Y, Liu Y. Studies of safe maximal daily dietary Se-intake in a seleniferous area in China. Part II: Relation between Se-intake and the manifestation of clinical signs and certain biochemical alterations in blood and urine. J Trace Elem Electrolytes Health Dis 1989; 3:123-30.
34. Reid ME, Stratton MS, Lillico AJ, Fakih M, Natarajan R, Clark LC, Marshall JR. A report of high-dose selenium supplementation: response and toxicities. J Trace Elem Med Biol 2004;18:69-74.
35. Charalambous MP, Maihofner C, Bhambra U, Lightfoot T, Gooderham NJ. The colorectal cancer study group. Upregulation of cyclooxygenase-2 is accompanied by increased expression of nuclear factor-kB and IkB kinase- in human colorectal cancer epithelial cells. Br J Cancer 2003; 88:1598-604.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 16, Issue of April 22, pp. 16354–16359, 2005
Printed in U.S.A.

C/EBPβ and Its Binding Element Are Required for NFκB-induced COX2 Expression Following Hypertonic Stress*

Received for publication, September 28, 2004, and in revised form, February 7, 2005
Published, JBC Papers in Press, February 14, 2005, DOI 10.1074/jbc.M411134200

Jing Chen‡§, Min Zhao‡, Reena Rao‡, Hiroyasu Inoue¶, and Chuan-Ming Hao‡§‖

From the ‡Division of Nephrology, Department of Medicine, Vanderbilt University, Nashville, Tennessee 37232, the ¶Department of Pharmacology, National Cardiovascular Center Research Institute, 5-7-1 Fujishiro-dai, Suita, Osaka 565-8565, Japan, and the §Division of Nephrology, Huashan Hospital, Fudan University, Shanghai 200040, China

NFκB plays a critical role mediating COX2 expression in renal medullary interstitial cells (RMICs). The *trans*-activating ability of NFκB can be modified by another nuclear factor C/EBPβ that can physically bind to NFκB and regulate its activity. Because the COX2 promoter also contains a C/EBPβ site adjacent to the NFκB 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 tonicity-induced COX2 expression was suppressed by mutant IκB (IκBm) that blocks NFκB activation, demonstrating that tonicity-induced COX2 expression depends on NFκB activation. However, mutation of the NFκB site in the COX2 promoter failed to abolish tonicity-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 NFκB 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 tonicity-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 NFκB 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 NFκB p65 by coimmunoprecipitation. Importantly, this interaction between C/EBPβ and NFκB was greatly enhanced following hypertonic stress. These studies indicate C/EBPβ is required for the transcriptional activation of COX2 by NFκB, suggesting a dominant role for the C/EBPβ pathway in regulating induction of RMIC COX2 by hypertonicity.

Cyclooxygenase (COX)[1] 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 hypertonic 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, hypertonic stress activates nuclear factor NFκB, and this is critical for induction of COX2 expression in renal medullary interstitial cells (7). NFκB 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). NFκB binding sites have been identified in the promoter region of the *COX2* gene (17, 18), making it likely that binding of the NFκB protein to the NFκB *cis*-acting element is responsible for increased COX2 expression. However, recent studies indicate that the mechanism underlying NFκB-associated COX2 expression is more complex. Interactions between NFκB 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 NFκB 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

* This work was supported by NIDDK, National Institutes of Health Grant DK065024 (to C.-M. H.) and a Vanderbilt Discovery grant. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‖ To whom correspondence should be addressed: S3223 MCN, Vanderbilt University Medical Center, Nashville, TN 37232. Tel.: 615-343-9867; Fax: 615-343-4704; E-mail: chuanming.hao@vanderbilt.edu.

[1] 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, adenovirus; 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-cm^2 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 95% O_2, 5% CO_2. Tissue culture medium was changed every 48–72 h. Mouse RMICs were prepared as reported (7). C57BL/6J mice were sacrificed, and kidneys were rapidly removed and washed in Ringer's solution. The renal medulla was excised, minced, and placed in Ringer's solution containing collagenase (1 mg/ml) at 37 °C for 1 h with occasional agitation. The collagenase-treated tissue was then washed in Dulbecco's modified Eagle's medium (DMEM) three times and cultured in DMEM 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 (150 mM NaCl, 50 mM Tris, 0.05% Tween 20, and 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, 160106, Cayman), anti-C/EBPβ (1:400 sc-150, Santa Cruz Biotechnology), or anti-p-C/EBPβ (1:500, 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 ImmunoResearch 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 membrane was exposed to Kodak XAR-5 film.

Nuclear Protein Extraction and Immunoprecipitation—Cultured cells were washed with phosphate-buffered saline and lysed on ice for 15 min in hypotonic lysis buffer (10 mM HEPES, 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM NaF, 1 mM Na_3VO_3, and 0.08% Nonidet P-40) containing proteinase inhibitor mixture (1 tablet/10 ml, Complete Mini, Roche Applied Science). The cell lysate was centrifuged at 4 °C at 3,000 rpm for 5 min. The supernatant (cytoplasmic proteins) was stored at −80 °C. The pellet was washed with hypotonic lysis buffer two times and centrifuged at 13,000 rpm for 5 s. The supernatant was removed, and the pellet was resuspended in 50 μl of Dignin solution (20 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 420 mM NaCl, 50 mM β-glycerophosphate, 1 mM NaF, 1 mM Na_3VO_4, 1 mM dithiothreitol, 25% glycerol, pH 7.9) for 30 min and centrifuged for 10 min at 13,000 rpm. The supernatant nuclear protein was used for immunoprecipitation. 50 μg of nuclear protein extract was added to 500 μl of IP buffer (Tris 20 mM, pH 7.5, NaCl 150 mM, EDTA 1 mM, EGTA, 1 mM, Triton-100 1%). The nuclear protein was precleared by adding 0.2 μg of rabbit IgG and 20 μl of 25% protein A-agarose, incubated at 4 °C for 30 min, and centrifuged at 3,000 rpm. The supernatant was collected, and 0.4 μg of anti-C/EBPβ antibody was added and incubated at 4 °C for 2 h. 20 μl of 25% protein A-agarose beads were added and incubated at 4 °C overnight with mixing. The beads were washed three times with IP buffer and were resuspended in 30 μl of 2× sample buffer. The samples were boiled for 2 min, and 20 μl of precipitated proteins were added to each lane of an SDS-PAGE gel.

Ad-IκBmu, Ad-IKKα, Ad-CEBP-p20, and Ad-GFP—Adenoviral vectors, encoding a dominant negative IκB and a constitutively active IκB 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-C/EBPβ-p20 was provided by Dr. Linda Sealy. Constitutively active IKK1 (IKK1) cDNA was kindly provided by Dr. Frank Mercurio (Signal Pharmaceutical, San Diego, CA) and subcloned into pACCMV for IKK1 adenovirus construction (7). The IKK1 was made constitutively active by Ser-Glu mutations in Ser^{176} and Ser^{180} 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 Eagle's medium with 10% fetal bovine serum was added. Experiments were carried out 48–72 h after infection.

COX2 Reporter Studies—An 891-bp human COX2 luciferase reporter construct was generously provided by Dr. Lee-Ho Wang (17). A 327-bp human COX2 luciferase reporter construct, and its NFκB and C/EBPβ site mutants were provided by Dr. Hiroyasu Inoue (28). The NFκB and C/EBPβ site mutants have been shown to lack the ability to bind to NFκB and C/EBP, respectively (28, 29). Two NFκB sites in the 891-bp COX2 reporter construct were mutated via site-directed mutagenesis using primers: CGGCGGCGGGAGAGCTCATTCCCTGCGCCC (5′ sense), CAGGAGAGTGGCCACTACCCCCTCTGCT (3′ sense) (30) (QuikChange II Site-Directed Mutagenesis kits, Stratagene, La Jolla, CA). The firefly luciferase COX2 reporter plasmid and a plasmid containing *Renilla* luciferase driven by the TK promoter (Promega) were transfected into cells using SuperFect (Qiagen). Cells were lysed 48 h after transfection for luciferase activity measurement using the Dual Luciferase assay system (Promega). COX2 luciferase activity was adjusted by *Renilla* luciferase activity.

Chromatin Immunoprecipitation (ChIP) Assay—The ability of NFκB and C/EBPβ to bind to endogenous COX2 promoter was examined using the ChIP assay according to the manufacturer's protocol (Upstate Technologies, Lake Placid, NY). Briefly, cultured mouse renal medullary interstitial cells (7) were exposed to isotonic or hypertonic media for indicated periods of time. Cells were then cross-linked with 1% formaldehyde for 5 min. After washing with phosphate-buffered saline, cells were lysed with SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) containing proteinase inhibitors (Complete Mini, EDTA-free). The chromatin was sheared by sonication (strength, 20%; pulse, 12 s × three times). The cross-linked chromatin was quantified to determine the initial amount of DNA present in the different samples. 100 ng of DNA were used as input. The remaining chromatin fractions were precleared with salmon sperm DNA/protein A-agarose for 1 h and immunoprecipitated with antibodies (NFκB-p65 or CEBPβ, 200 μg/ml, Santa Cruz Biotechnology) overnight at 4 °C. The COX2 promoter DNA, bound to p65 and C/EBPβ, was analyzed by PCR using primers: sense, CGGAGGGTAGTTCCATGAAA; antisense, CAGGCTTTTACCCACGCAAA. PCR was performed at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, for 35 cycles.

To further examine whether the C/EBPβ site in the COX2 promoter plays an important role in mediating NFκB binding to the *COX2* gene, wild type or mutants of COX2 promoter constructs containing 327-bp human COX2 promoter sequences, were transfected into mouse interstitial cells using SuperFect (Qiagen). 24 h after transfection, cells were exposed to hypertonic stress for 1 h. The cells were cross-linked and precipitated as described in ChIP assay. The transfected human COX2 promoter bound to NFκB was detected by PCR using primers specific for the human *COX2* gene. PCR primers: sense, CCCCTCTGCTCCCAAATT; antisense, CGCTCACTGCAAGTCGTAT. The PCR was performed at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, for 35 cycles. Genomic DNA from a human cell line HEK293 cells was used as a positive control. Genomic DNA extracted from mouse renal medullary interstitial cells without transfection was used as a negative control.

RESULTS

Mutation of the NFκB site of the COX2 Promoter 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).

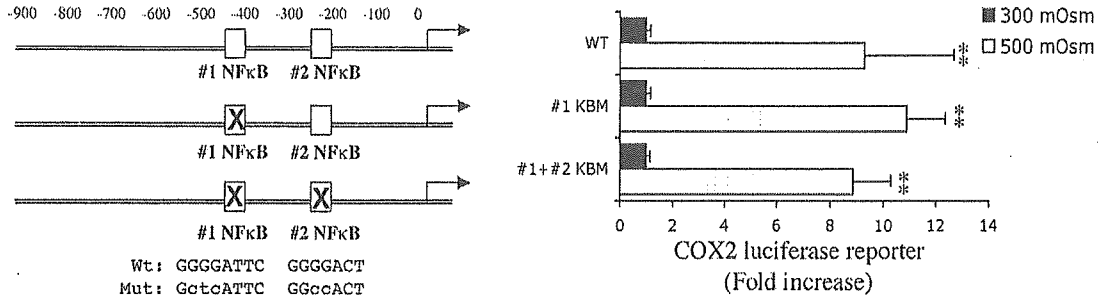

FIG. 1. **Effect of NFκB site mutation on hypertonicity-induced COX2 luciferase reporter activity in cultured renal medullary interstitial cells.** Cultured RMICs were co-transfected with wild-type or mutant COX2 promoter-driven firefly luciferase vector and TK-driven *Renilla* luciferase plasmid. Cells were exposed to isotonic (300 mOsm) or hypertonic (500 mOsm) medium. 24 h later, luciferase activities were determined as described under "Materials and Methods." **, $p < 0.01$ *versus* isotonic medium, $n = 6$. *KBM*, NFκB site mutation.

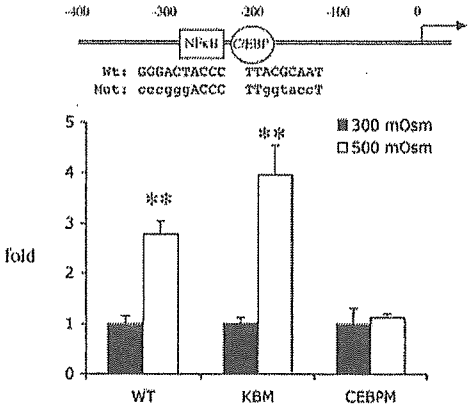

FIG. 2. **COX2 luciferase reporter activity following hypertonic stress.** Cultured RMICs were co-transfected with wild-type or mutant COX2 promoter-driven firefly luciferase vector and TK-driven *Renilla* luciferase plasmid. Cells were cultured in isotonic or hypertonic (500 mOsm) medium for 24 h. Luciferase activities were determined as described under "Materials and Methods." *WT*, wild type; *KBM*, NFκB site mutant; *CEBPM*, C/EBPβ site mutant. **, $p < 0.01$ *versus* 300 mOsm.

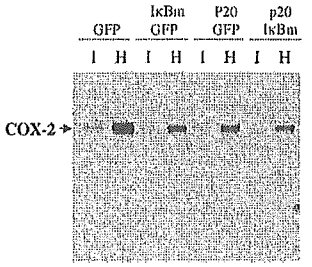

FIG. 3. **Effect of C/EBPβ-p20 on hypertonicity-induced COX2 expression.** Cultured RMICs were transduced with GFP, IκBm, C/EBP-p20, or p20 plus IκBm via adenoviral vectors. Cells were then exposed to isotonic (*I*, 300 mOsm) or hypertonic medium (*H*, 550 mOsm). 24 h later, cellular proteins were extracted and immunoblotted for COX2.

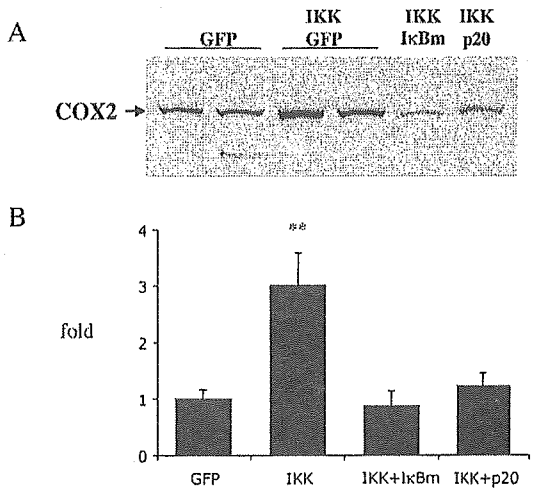

FIG. 4. **Effect of C/EBPβ-p20 on IKK1-induced COX2 expression.** Cultured RMICs were transduced with GFP, IKK1, IKK1 plus IκBm, or IKK plus C/EBP-p20 via adenoviral vectors. 24 h later, cellular proteins were extracted and immunoblotted for COX2. *A*, representative autoradiograph of immunoblot for COX2. *B*, densitometry analysis of immunoblot for COX2. **, $p < 0.01$; $n = 6$

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

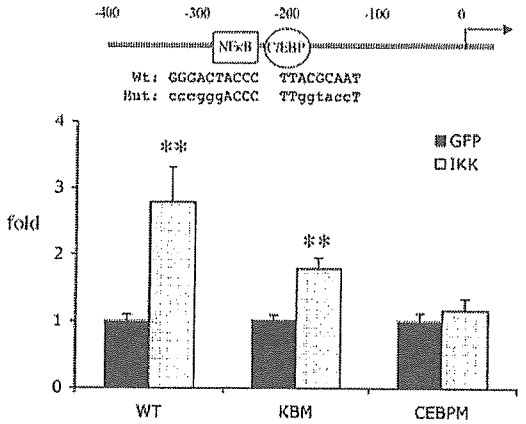

FIG. 5. **IKK-associated COX2 luciferase reporter activity.** Cultured RMICs were co-transfected with wild-type or mutant COX2 promoter-driven firefly luciferase vector and TK-driven *Renilla* luciferase plasmid. Cells were transduced with AdGFP or AdIKK. 24 h later, luciferase activities were determined as described under "Materials and Methods." *WT*, wild type; *KBM*, NFκB site mutant; *CEBPM*, C/EBPβ site mutant. **, $p < 0.01$ *versus* AdGFP

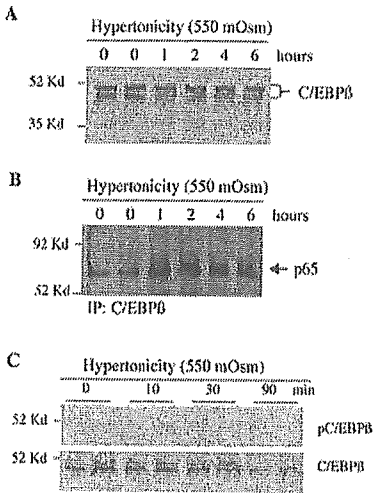

FIG. 6. **Effect of hypertonic stress on C/EBPβ expression (*A*), interaction between C/EBPβ and NFκB p65 (*B*), and C/EBPβ phosphorylation (*C*).** Renal medullary interstitial cells were cultured to confluent and exposed to hypertonic stress (550 mOsm) for indicated periods of time. *A*, whole cell protein extracts were separated on SDS-PAGE and blotted for C/EBPβ. *B*, nuclear protein extracts were immunoprecipitated by C/EBPβ antibody. C/EBPβ immunoprecipitated proteins were blotted for p65 as described under "Materials and Methods." *C*, whole cell protein extracts were blotted with anti-pC/EBP and C/EBP antibodies.

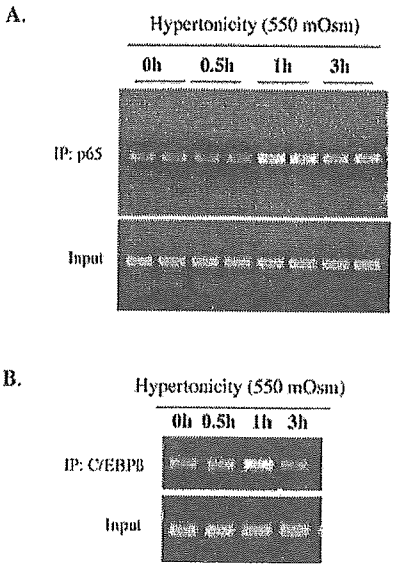

FIG. 7. **Effect of hypertonicity on binding of p65 (*A*) and C/EBPβ (*B*) to the COX2 promoter in renal medullary interstitial cells *in vivo*.** Cultured renal medullary interstitial cells were exposed to hypertonic medium for 0, 0.5, 1, and 3 h. Cells were fixed with formaldehyde. p65- or C/EBPβ-bound DNA was isolated via immunoprecipitation using anti-p65 or anti-C/EBPβ antibodies. 100 ng of genomic DNA was used as input. The p65- or C/EBPβ-bound COX2 promoter DNA was detected by PCR as described under "Materials and Methods."

was immunoprecipitated using anti-C/EBPβ antibody and separated with SDS-PAGE. As shown in Fig. 6, C/EBPβ antibody-immunoprecipitated proteins from cultured medullary interstitial cells include NFκB p65 immunoreactive protein, consistent with a physical association of p65 with C/EBPβ. The interaction between p65 and C/EBPβ appears to be specific, because no p65 was coprecipitated by PPARδ (data not shown), a transcription factor abundantly expressed in renal medullary interstitial cells (34). More importantly, this physical interaction was dramatically enhanced following hypertonic stress, despite the fact that hypertonic stress did not change C/EBPβ protein expression (Fig. 6). Only C/EBPβ but not C/EBPα, δ, and γ were detected in cultured renal medullary interstitial cells by immunoblot. Furthermore, none of these C/EBP isoforms was induced by hypertonic stress (data not shown). Hypertonicity did not change C/EBPβ phosphorylation (Thr-235) (Fig. 6*C*), suggesting that phosphorylation of the Thr-235 residue is not critical for hypertonic activation of C/EBPβ.

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/EBP 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.

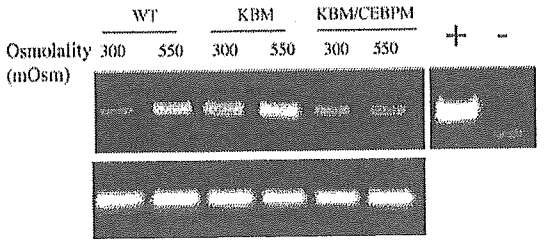

FIG. 8. **Effect of mutation of NFκB and C/EBPβ sites on hypertonicity-induced binding of p65 to the COX2 promoter.** Cultured mouse renal medullary interstitial cells were transfected with the human COX2 327-bp promoter construct with or without mutations of NFκB and C/EBP binding sites and exposed to hypertonic medium for 1 h. Cells were fixed with formaldehyde. p65-bound DNA was isolated via immunoprecipitation using anti-p65 antibody. The p65-bound-transfected COX2 promoter DNA was detected by PCR as described under "Materials and Methods." +, genomic DNA from human HEK293 cells was used as a positive control; −, DNA from mouse renal medullary interstitial cells without transfection was used as a negative control.

2), supporting a role for the C/EBPβ site in promoting NFκB-mediated, hypertonicity-induced 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/EBPβ), 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 presence of 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 RMICs 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/EBP pathway does not appear to be separate from the NFκB pathway, because the additive effect of C/EBP blockade and NFκB blocking was not observed. Moreover, mutation of C/EBP 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/EBP site appears to be integral to the mechanism of NFκB activation, leading to COX2 expression.

C/EBP belongs to the basic leucine zipper C/EBP family that is comprised of six members, C/EBPα, β, γ, ϵ, δ, and ζ. C/EBPβ is closely related to C/EBPα and C/EBPδ, but is distantly related to C/EBPγ, C/EBPϵ, and C/EBPζ (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/CEBP 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/CEPBβ heteromer is important in PGG-glucan-induced Rel-A/CEBPβ-related transcription. A p65/CEBPδ 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/EBP 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 coimmunoprecipitation 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/EBPδ

caused a greater enhancement of basal and IL-1-stimulated COX2 promoter activity than C/EBPβ, suggesting that C/EBPδ 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/EBPδ-induced COX2 expression (51). Nevertheless, in the present study, C/EBPα and -δ do not seem to be involved, because immunoblotting failed to detect C/EBPα and -δ 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. 6*C*). 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.

Acknowledgments—We thank Dr. Linda Sealy from Vanderbilt University for providing the C/EBPβ-p20 construct. We thank Dr. Matthew Breyer for support and critical reading (DK37097). We thank Reyadh Redha for assistance with RMIC preparation. We also thank Dr. Manakin B. Srichai for critical reading of the present manuscript. The IKK1 cDNA was a generous gift from F. Mercurio.

REFERENCES

1. Hla, T., Bishop-Bailey, D., Liu, C. H., Schaefers, H. J., and Trifan, O. C. (1999) *Int. J. Biochem. Cell Biol.* **31,** 551–557
2. Smith, W. L., and Langenbach, R. (2001) *J. Clin. Investig.* **107,** 1491–1495
3. FitzGerald, G. A., and Loll, P. (2001) *J. Clin. Investig.* **107,** 1335–1337
4. Subbaramaiah, K., and Dannenberg, A. J. (2003) *Trends Pharmacol. Sci.* **24,** 96–102
5. Breyer, M. D., Hao, C., and Qi, Z. (2001) *Curr. Opin. Crit. Care* **7,** 393–400
6. Guan, Y., Chang, M., Cho, W., Zhang, Y., Redha, R., Davis, L., Chang, S., DuBois, R. N., Hao, C. M., and Breyer, M. (1997) *Am. J. Physiol.* **273,** F18–F26
7. Hao, C. M., Yull, F., Blackwell, T., Komhoff, M., Davis, L. S., and Breyer, M. D. (2000) *J. Clin. Investig.* **106,** 973–982
8. Zhang, M. Z., Hao, C. M., Breyer, M. D., Harris, R. C., and McKanna, J. A. (2002) *Am. J. Physiol. Renal Physiol.* **283,** F509–516
9. Yang, T., Huang, Y., Heasley, L. E., Berl, T., Schnermann, J. B., and Briggs, J. P. (2000) *J. Biol. Chem.* **275,** 23281–23286
10. Moeckel, G. W., Zhang, L., Fogo, A. B., Hao, C. M., Pozzi, A., and Breyer, M. D. (2003) *J. Biol. Chem.* **278,** 19352–19357
11. Lo, C. J., Cryer, H. G., Fu, M., and Lo, F. R. (1998) *J. Trauma* **45,** 19–23; discussion 23–14
12. Nakao, S., Ogata, Y., Shimizu-Sasaki, E., Yamazaki, M., Furuyama, S., and Sugiya, H. (2000) *Mol. Cell Biochem.* **209,** 113–118
13. Paik, J. H., Ju, J. H., Lee, J. Y., Boudreau, M. D., and Hwang, D. H. (2000) *J. Biol. Chem.* **275,** 28173–28179
14. Roshak, A. K., Jackson, J. R., McGough, K., Chabot-Fletcher, M., Mochan, E., and Marshall, L. A. (1996) *J. Biol. Chem.* **271,** 31496–31501
15. Yan, X., Wu Xiao, C., Sun, M., Tsang, B. K., and Gibb, W. (2002) *Biol. Reprod.* **66,** 1667–1671
16. Tsai, S. H., Liang, Y. C., Chen, L., Ho, F. M., Hsieh, M. S., and Lin, J. K. (2002) *J. Cell. Biochem.* **84,** 750–758
17. Tazawa, R., Xu, X. M., Wu, K. K., and Wang, L. H. (1994) *Biochem. Biophys. Res. Commun.* **203,** 190–199
18. Okada, Y., Voznesensky, O., Herschman, H., Harrison, J., and Pilbeam, C. (2000) *J. Cell. Biochem.* **78,** 197–209
19. Perkins, N. D. (1997) *Int. J. Biochem. Cell Biol.* **29,** 1433–1448
20. Kleemann, R., Gervois, P. P., Verschuren, L., Staels, B., Princen, H. M., and Kooistra, T. (2003) *Blood* **101,** 545–551
21. Ikawa, H., Kameda, H., Kamitani, H., Baek, S. J., Nixon, J. B., Hsi, L. C., and Eling, T. E. (2001) *Exp. Cell Res.* **267,** 73–80
22. Stein, B., Cogswell, P. C., and Baldwin, A. S., Jr. (1993) *Mol. Cell. Biol.* **13,** 3964–3974
23. Diehl, J. A., and Hannink, M. (1994) *Mol. Cell. Biol.* **14,** 6635–6646
24. Ray, A., Hannink, M., and Ray, B. K. (1995) *J. Biol. Chem.* **270,** 7365–7374
25. Muirhead, E. E., Germain, G. S., Leach, B. E., Brooks, B., and Stephenson, P. (1973) *Prostaglandins* **3,** 581–594
26. Mercurio, F., Zhu, H., Murray, B. W., Shevchenko, A., Bennett, B. L., Li, J., Young, D. B., Barbosa, M., Mann, M., Manning, A., and Rao, A. (1997) *Science* **278,** 860–866
27. Hao, C. M., Komhoff, M., Guan, Y., Redha, R., and Breyer, M. D. (1999) *Am. J. Physiol.* **277,** F352–F359
28. Inoue, H., Yokoyama, C., Hara, S., Tone, Y., and Tanabe, T. (1995) *J. Biol. Chem.* **270,** 24965–24971
29. Inoue, H., and Tanabe, T. (1998) *Biochem. Biophys. Res. Commun.* **244,** 143–148
30. Schmedtje, J. F., Jr., Ji, Y. S., Liu, W. L., DuBois, R. N., and Runge, M. S. (1997) *J. Biol. Chem.* **272,** 601–608
31. Appleby, S. B., Ristimaki, A., Neilson, K., Narko, K., and Hla, T. (1994) *Biochem. J.* **302,** 723–727
32. Duong, D. T., Waltner-Law, M. E., Sears, R., Sealy, L., and Granner, D. K. (2002) *J. Biol. Chem.* **277,** 32234–32242
33. Jover, R., Bort, R., Gomez-Lechon, M. J., and Castell, J. V. (2002) *FASEB J.* **16,** 1799–1801
34. Hao, C. M., Redha, R., Morrow, J., and Breyer, M. D. (2002) *J. Biol. Chem.* **277,** 21341–21345
35. Wadleigh, D. J., Reddy, S. T., Kopp, E., Ghosh, S., and Herschman, H. R. (2000) *J. Biol. Chem.* **275,** 6259–6266
36. Xie, W., and Herschman, H. R. (1995) *J. Biol. Chem.* **270,** 27622–27628
37. Thomas, B., Thirion, S., Humbert, L., Tan, L., Goldring, M. B., Bereziat, G., and Berenbaum, F. (2002) *Biochem. J.* **362,** 367–373
38. Thomas, B., Berenbaum, F., Humbert, L., Bian, H., Bereziat, G., Crofford, L., and Olivier, J. L. (2000) *Eur. J. Biochem.* **267,** 6798–6809
39. Zhu, Y., Saunders, M. A., Yeh, H., Deng, W. G., and Wu, K. K. (2002) *J. Biol. Chem.* **277,** 6923–6928
40. Subbaramaiah, K., Lin, D. T., Hart, J. C., and Dannenberg, A. J. (2001) *J. Biol. Chem.* **276,** 12440–12448
41. Segasothy, M., Samad, S. A., Zulfigar, A., and Bennett, W. M. (1994) *Am. J. Kidney Dis.* **24,** 17–24
42. Rao, R., Hao, C. M., and Breyer, M. D. (2004) *J. Biol. Chem.* **279,** 3949–3955
43. Wedel, A., and Ziegler-Heitbrock, H. W. (1995) *Immunobiology* **193,** 171–185
44. Akira, S., and Kishimoto, T. (1997) *Adv. Immunol.* **65,** 1–46
45. Ossipow, V., Descombes, P., and Schibler, U. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 8219–8223
46. Liao, J., Piwien-Pilipuk, G., Ross, S. E., Hodge, C. L., Sealy, L., MacDougald, O. A., and Schwartz, J. (1999) *J. Biol. Chem.* **274,** 31597–31604
47. Ruocco, M. R., Chen, X., Ambrosino, C., Dragonetti, E., Liu, W., Mallardo, M., De Falco, G., Palmieri, C., Franzoso, G., Quinto, I., Venuta, S., and Scala, G. (1996) *J. Biol. Chem.* **271,** 22479–22486
48. Yoshimoto, T., Kojima, K., Funakoshi, T., Endo, Y., Fujita, T., and Nariuchi, H. (1996) *J. Immunol.* **156,** 1082–1088
49. Adams, D. S., Nathans, R., Pero, S. C., Sen, A., and Wakshull, E. (2000) *J. Cell. Biochem.* **77,** 221–233
50. Descombes, P., and Schibler, U. (1991) *Cell* **67,** 569–579
51. Harrison, J. R., Kelly, P. L., and Pilbeam, C. C. (2000) *J. Bone Miner Res.* **15,** 1138–1146
52. Hanlon, M., Sturgill, T. W., and Sealy, L. (2001) *J. Biol. Chem.* **276,** 38449–38456
53. Shuman, J. D., Sebastian, T., Kaldis, P., Copeland, T. D., Zhu, S., Smart, R. C., and Johnson, P. F. (2004) *Mol. Cell. Biol.* **24,** 7380–7391