Transcriptional Roles of Nuclear Factor κB and Nuclear Factor-Interleukin-6 in the Tumor Necrosis Factor α-Dependent Induction of Cyclooxygenase-2 in MC3T3-E1 Cells

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When a mouse osteoblastic cell line MC3T3-E1 was cultured in the presence of tumor necrosis factor α (TNF-α), the release of prostaglandin E2 and the cyclooxygenase activity increased in a dose- and time-dependent manner. The increase of the enzyme activity was attributed mostly to the induction of cyclooxygenase-2 rather than cyclooxygenase-1 as judged by the inhibitory effect of NS398, Western blotting, and Northern blotting. In this system we attempted to elucidate the transcriptional regulation of the cyclooxygenase-2 gene. As examined by the luciferase assay, two positive regulatory regions (−186 to −131 and −512 to −385 base pairs) were found in the 5′-flanking promoter region of the mouse cyclooxygenase-2 gene in the TNF-α-stimulated cells. The former included putative NF-IL6 (C/EBPβ) and AP2 elements, and the latter contained the NF-κB motif. A DNA probe including the NF-IL6 and AP2 sites gave positive bands upon electrophoretic mobility shift assay using the nuclear extracts of MC3T3-E1 cells. The bands were supershifted by the addition of anti-NF-IL6 antibody but not by anti-AP2 antibody. A probe including the NF-κB site also gave positive bands, which were supershifted by anti-NF-κB p50 and p65 antibodies. Furthermore, when the motif of NF-IL6 or NF-κB or both was subjected to point mutation, the luciferase activity was markedly reduced. These data suggested a potential role of both NF-IL6 and NF-κB in the induction of cyclooxygenase-2 by TNF-α.

**Prostaglandin (PG)** endoperoxide synthase (EC 1.14.99.1) is the key enzyme in the biosynthetic pathway of PGs and thromboxanes from arachidonic acid. The enzyme is a bifunctional enzyme, and has the cyclooxygenase activity (from arachidonic acid to PGG2) and the hydroperoxidase activity (from PGG2 to PGH2). Two isozymes referred to as cyclooxygenase-1 and -2 in this paper are found in the mammalian tissues (1, 2). Cyclooxygenase-1 is generally considered as a constitutive enzyme, while cyclooxygenase-2 is rapidly and transiently induced by various cytokines, hormones, and tumor promoters (1, 2). In view of such an inducible nature the cyclooxygenase-2 has recently been a subject of active molecular biological investigations. Promoter regions of the cyclooxygenase-2 genes of mouse (3), rat (4), and human (5) have been cloned and sequenced. Regardless of the animal species these promoter regions contained a canonical TATA box and various putative transcriptional regulatory elements such as CRE, NF-IL6 (C/EBPβ), AP2, SP1, NF-κB, and GATA box. Among these elements, CRE (6, 7) and C/EBPβ (8) were shown to act as positive regulatory elements for the cyclooxygenase-2 transcription.

An osteogenic MC3T3-E1 cell line was established from newborn mouse calvaria. The cells differentiate into osteoblasts, and show calcification in vitro (9, 10). With this cell line we have been investigating the cyclooxygenase induction by epidermal growth factor (11, 12), transforming growth factor β (12), epinephrine (13), and PGs (14). More recently the cyclooxygenase-2 induction was demonstrated with the mouse cyclooxygenase-2 induction was demonstrated with the mouse cyclooxygenase-2 promoter system, where the induction of the enzyme activity increased in a dose- and time-dependent manner. The increase of the enzyme activity was attributed mostly to the induction of cyclooxygenase-2 rather than cyclooxygenase-1 as judged by the inhibitory effect of NS398, Western blotting, and Northern blotting. In this system we attempted to elucidate the transcriptional regulation of the cyclooxygenase-2 gene. As examined by the luciferase assay, two positive regulatory regions (−186 to −131 and −512 to −385 base pairs) were found in the 5′-flanking promoter region of the mouse cyclooxygenase-2 gene in the TNF-α-stimulated cells. The former included putative NF-IL6 (C/EBPβ) and AP2 elements, and the latter contained the NF-κB motif. A DNA probe including the NF-IL6 and AP2 sites gave positive bands upon electrophoretic mobility shift assay using the nuclear extracts of MC3T3-E1 cells. The bands were supershifted by the addition of anti-NF-IL6 antibody but not by anti-AP2 antibody. A probe including the NF-κB site also gave positive bands, which were supershifted by anti-NF-κB p50 and p65 antibodies. Furthermore, when the motif of NF-IL6 or NF-κB or both was subjected to point mutation, the luciferase activity was markedly reduced. These data suggested a potential role of both NF-IL6 and NF-κB in the induction of cyclooxygenase-2 by TNF-α.

**Experimental Procedures**

Materials—The materials used in this work were purchased as follows: [1-14C]arachidonic acid (2.1 GBq/mmol) and 35S-labeled anti-rabbit IgG whole antibody (74 TBq/mmol) from Amersharm International (Bucks, United Kingdom); Immobilon-P transfer membrane from Millipore (Bedford, MA); ATTO (220 TBq/mmol) from DuPont NEN; fetal bovine serum and newborn bovine serum from Irvine Scientific (Santa Ana, CA); penicillin G and phenylmethylsulfonyl fluoride from Sigma; α-modified Eagle’s minimum essential medium (α-MEM), recombinant mouse TNF-α, and lipofectAMINE from Life Technologies, Inc. (Gaithersburg, MD); ISETGEN from Nippon Gene (Tokyo, Japan); EMBL3 mouse genomic library from Clonetech (Palo Alto, CA); PicaGene luciferase assay system from Toyonk (Tokyo, Japan); poly(dI-dC)-poly(dI-dC) from Pharmacia (Uppsala, Sweden); anti-AP2, anti-C/EBPβ (NF-IL6), and anti-NF-κB p50 antibodies from Santa Cruz Bio-technology, Inc. (Santa Cruz, CA); anti-NF-κB p65 antibody from Serotec (Oxford, United Kingdom); and purified NF-κB p50 protein from Promega (Madison, WI). MC3T3-E1 cell line was kindly provided by Dr. H. Kodama of Ohu University, luciferase plasmid pX-P1 by Dr. T. Sakai of Kyoto Prefectural University, and expression vectors containing the genes of NF-IL6 and NF-κB p50 by Dr. S. Akira of Osaka University. NS398 was donated by Taisho Pharmaceutical Co. (Saitama, Japan).

Cell Culture—MC3T3-E1 cells (7.5 × 10^4 cells/dish) were plated in 150-mm plastic dishes with 30 ml of α-MEM containing 10% fetal bovine serum and 100 units/ml of penicillin G as described previously (11). The dishes were placed in a humidified 7% CO2, 95% air incubator.
at 37 °C. The cells were subcultured every 3 days.

Determination of PGE$_2$ Synthesis—MC3T3-E1 cells (6 x 10$^4$ cells/dish) were placed in 35-mm dishes with 2 ml of α-MEM containing 10% fetal bovine serum. Confluent cultures were usually obtained on the 4th day. Then, the medium was changed to α-MEM supplemented with 2% newborn bovine serum, and TNFα was added. At indicated time intervals, the culture medium was removed and subjected to radioimmunoassay for PGE$_2$ (11).

Cyclooxygenase Assay—MC3T3-E1 cells (9 x 10$^4$ cells/dish) were plated in 150-mm dishes with 30 ml of α-MEM containing 10% fetal bovine serum, and treated with TNFα. The cells were scraped from the dishes at various time intervals, suspended in 300 μl of 20 mM Tris-HCl (pH 7.4) containing 5 mM tryptoophan, and sonicated twice each for 3 s at 20 KHz. The sonicates were incubated with 10 μM [1-$^{14}$C]arachidonic acid (50,000 cpm in 5 μl of ethanol) for 2 min at 24 °C in a 100-μl assay mixture containing 100 mM Tris-HCl (pH 8.0), 2 μM hematin, and 5 mM tryptoophan. The reaction products were separated by TLC (15), and detected by a BAS2000 imaging analyzer (Fujix, Tokyo, Japan). Protein concentration was determined by the method of Lowry et al. (17) with bovine serum albumin as standard.

Western Blotting—A polyclonal anti-cyclooxygenase-2 was prepared with a peptide corresponding amino acids 579–594 of murine enzyme, and provided by Dr. Yoko Hayashi of this laboratory. The sonicates of MC3T3-E1 cells were subjected to 10% polyacrylamide gel electrophoresis in the presence of 0.1% SDS (10 μg of protein/lane). The protein bands were transferred to an Immobilon-P membrane, which was incubated with the polyclonal antibody against cyclooxygenase-2 and then with $^{35}$S-labeled anti-rabbit IgG whole antibody as the second antibody. Radioactivity of the immunocomplex was quantified by a BAS2000 imaging analyzer.

Northern Blotting—Total RNA was extracted from MC3T3-E1 cells using ISOGEN (a mixture of guanidine isothiocyanate and phenol) according to the manufacturer’s instruction. Northern blotting was performed as described previously (15, 18). The relative radioactivity of each band was estimated using a BAS2000.

Preparation of the 5′-Flanking Region of Mouse Cyclooxygenase-2 Gene and Construction of Luciferase Reporter Vectors—We screened a mouse genomic library constructed in EMBL3 to clone the 5′-flanking region of the mouse cyclooxygenase-2 gene by the method as described previously for 12-lipoxygenase (19). DNA fragments of mouse cyclooxygenase-2 promoter regions of various lengths or their point mutants were prepared from the clone by polymerase chain reaction, and were inserted into the plasmid pXP-1 as described previously for 12-lipoxygenase (20).

Transfection of Plasmids to MC3T3-E1 Cells and Luciferase Assay—MC3T3-E1 cells (1.4 x 10$^5$ cells/dish) were cultured for 2 days in 60-mm dishes with 5 ml of α-MEM containing 10% fetal bovine serum. For transfection the subconfluent cells were treated with plasmid DNA (2.3 μg) containing cyclooxygenase-2 promoter and luciferase reporter gene, standard plasmid DNA (1.2 μg) containing the β-galactosidase gene, and 2.3 ml of serum-free α-MEM for 4 h at 37 °C according to the manufacturer’s instructions. The medium was changed to 5 ml of α-MEM with 10% fetal bovine serum, and the transfected cells were further incubated for 3 days. Then the cells were stimulated with 20 ng/ml TNFα in the presence of α-MEM supplemented with 2% newborn bovine serum. After 12 h the cells were scraped from the dishes, and the luciferase activity was measured by a Lumat LB9501 luminescent perfusion spectrophotometer (Berthold, Germany) using a Pico Gene luciferase assay system according to the manufacturer’s instructions. The luciferase activities were normalized on the basis of β-galactosidase activities which were assayed as described previously (20).

Preparation of Nuclear Extracts—MC3T3-E1 cells (9 x 10$^4$ cells/dish) were placed in 150-mm dishes with 30 ml of α-MEM containing 10% fetal bovine serum, and stimulated with various concentrations of TNFα. After 1 h the cells were harvested, and nuclear extracts were prepared as described previously (20).

Electrophoretic Mobility Shift Assay—We synthesized the five oligonucleotides as shown in Fig. 4. The complementary oligonucleotides were annealed to the corresponding nucleotides as described by Berger and Kimmel (21), and the double-stranded oligonucleotides were purified electrophoretically on 15% polyacrylamide gel, end-labeled with [γ-$^{32}$P]ATP, and used as probes. The binding of the probes (10,000 cpm, about 10 fmol) to the nuclear extracts (2.3 μg protein) was performed in a 20-μl mixture containing 5 μg of poly(dI-dC)-poly(dI-dC), 15 mM Tris-HCl at pH 7.5, 1 mM EDTA, 10 mM KCl, 5 mM MgCl$_2$, 125 mM glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol. For the supershift experiment, antibody was added to the mixture. The mixture was incubated at 25 °C for 30 min, and applied to 4% polyacrylamide gel electrophoresis gels at a constant 150 V for 2 h. Distribution of the radioactivity on the dried gel was analyzed by a BAS2000 imaging analyzer.

RESULTS

Induction of Cyclooxygenase-2 by TNFα—When MC3T3-E1 cells were incubated with various concentrations of TNFα for 12 h, there was a dose-dependent increase in the amount of PGE$_2$ released into the medium (Fig. 1A). After the addition of 20 ng/ml TNFα, the PGE$_2$ release increased with a lag time of about 1 h, and reached a maximum at 9 h (Fig. 1B). As shown in Fig. 1, C and D, TNFα increased the cyclooxygenase activity dose and time dependently, and the enzyme activity continued to increase for 24 h to a specific activity of 0.8–2 nmol/2 min/mg of protein.

NS398, a specific inhibitor of cyclooxygenase-2 (22), inhibited the cyclooxygenase activity almost completely at 15 μM concentration (Fig. 1C and D), suggesting that most of the increased enzyme activity was attributable to cyclooxygenase-2 rather than cyclooxygenase-1. To confirm this finding we carried out Western blotting with an antibody specific for cyclooxygenase-2 (Fig. 2A). Without TNFα the cyclooxygenase-2 protein was undetectable, and the addition of 20 ng/ml TNFα increased the enzyme amount time dependently up to 9 h. There was a slight but reproducible decrease around 12 h, followed by an increase again.

Furthermore, as shown in Fig. 2B, the change in mRNA level was followed by Northern blot analysis. The addition of TNFα brought about a biphasic increase in the cyclooxygenase-2 mRNA. First, there was a rapid 10-fold increase reaching a maximum at 2 h, followed by a decrease at 3 h. Then, the mRNA increased again at 6–12 h. However, after the first transient peak the presence of 15 μM NS398 reduced the second peak. The cyclooxygenase-1 mRNA was not detectable under these experimental conditions.

These results presented in Figs. 1 and 2 demonstrated a rapid and marked induction of cyclooxygenase-2 by TNFα. The second peak of the cyclooxygenase-2 may be attributable to the enzyme which was induced by accumulating PGE$_2$ produced by the cyclooxygenase-2 at the first peak.
Functional Activity of Cyclooxygenase-2 Promoters—As illustrated at the top of Fig. 3, about 600 bp of the 5’-flanking region of mouse cyclooxygenase-2 gene contained various putative response elements; MEF-2 (a muscle-specific DNA binding protein, −490 to −481 bp), NFκB (−401 to −393 bp), SP1 (−239 to −234 bp), AP2 (−150 to −142 bp), NF-IL6 (−138 to −130 bp), CRE (−56 to −51 bp), and TATA box (−30 to −25 bp). The sequences of 5’-GGGATTCCC-3’ (−401 to −393 bp) and 5’-TTGCGCAAC-3’ (−138 to −130 bp) were identified as NFκB and NF-IL6 elements on the basis of their consensus sequences of 5’-GGGA(C/A)TN(T/C)CC-3’ and 5’-TTGCGCAAC-3’, respectively, as described previously (23).

For deletion analysis of the promoter region, we constructed luciferase vectors of various lengths covering the region from −821 to −43 bp (Fig. 3). Each vector was transfected to MC3T3-E1 cells by the lipofection method, and the luciferase activity of the cell lysate was measured at 12 h after the addition of 20 ng/ml TNFα. The addition of TNFα markedly stimulated the luciferase activity (closed column versus slashed column). The luciferase activity increased when two regions (−512 to −385 bp, −186 to −131 bp) were deleted, and these regions were presumed to have positive response elements. We noted NFκB consensus element (−401 to −393 bp), AP2 element (−150 to −142 bp), and NF-IL6 element (−138 to −130 bp).

Electrophoretic Mobility Shift Assay Targeting Positive Regulatory Elements—For further identification of these positive regulatory elements, we prepared five double-stranded oligonucleotide probes (g1, g2, ga, gβ, and gC). As shown in Fig. 4, g1 (−409 to −385 bp) contained NFκB and their vicinity, ga (−155 to −121 bp) contained AP2 and NF-IL6. g2, gβ, and gC were mutant probes for NFκB, AP2, and NF-IL6, respectively. We carried out electrophoretic mobility shift assay using the nuclear extracts of MC3T3-E1 cells preincubated with TNFα for 1 h. As shown in Fig. 5, when g1 probe was incubated with the nuclear extracts, a broad band of complex α was observed with increasing density depending on the amount of TNFα (lanes 3–6). The complex α was hardly detectable before the addition of TNFα (lane 2). Considering the subunit structure of NFκB (p50 and p65), the g1 probe was incubated with the purified NFκB p50, and a band appeared at the position of complex α (lane 7). The complex α was supershifted to complex β by the addition of anti-p50 antibody (lane 10), and was supershifted to complex γ by the addition of anti-p65 antibody (lane 11). The binding was not observed using the probe g2 with a mutation in NFκB motif (lanes 13 and 14).

When gα probe was incubated with the nuclear extracts, three complexes (a, b, and c) appeared with increasing density depending on the amount of TNFα (Figs. 6, lanes 3–6). These complexes were supershifted to bands d, e, and f by the addition of antibody against NF-IL6 (lane 8). The binding was scarcely observed by the use of the probe gC with a mutation in NF-IL6 (lanes 15–19). Moreover, the complex α was supershifted to complex e by the addition of anti-NFκBp50 (lane 9). The bands a, b, and c were not supershifted by anti-AP2 antibody (lane 7), and the binding profile was not affected by the use of the probe gB with a mutation in AP2 (lanes 10–14).

These data suggested that NFκB and NF-IL6 were increased by the addition of TNFα in a dose-dependent manner.

Possible Interaction of Both NFκB and NF-IL6 Binding Sites—For mutation analysis of the promoter region, we constructed luciferase vectors including mouse cyclooxygenase-2 promoter region (−512 to −123 bp) with site-specific mutations (Fig. 7). Each vector was transfected to MC3T3-E1 cells, and the luciferase activity was measured as described above. As shown in Fig. 7, the luciferase activity decreased by 63% in a mutant of NFκB, 82% in a mutant of NF-IL6, and only 19% in a mutant of AP2 site. By mutation of both NFκB and NF-IL6, the luciferase activity was lost to the level of PXP1. These data suggested that both NFκB and NF-IL6 in combination acted as transcription factors for the induction of cyclooxygenase-2.

Furthermore, expression vectors containing the genes of NF-IL6 and NFκB p50, respectively, were cotransfected to MC3T3-E1 cells together with a luciferase plasmid containing the promoter region of cyclooxygenase-2 (−512 to +123 bp). The luciferase activity increased depending on the amount of NF-IL6 DNA (data not shown). A similar experiment using the expression vector for NFκB p50 has so far been unsuccessful.

DISCUSSION

It was reported previously that TNFα increased the PGE2 production and the cyclooxygenase activity in mouse osteoblastic cell line MC3T3-E1 (24–26). We confirmed these findings (Fig. 1), and demonstrated that the increased enzyme activity was attributed mostly to the induction of cyclooxygenase-2 according to the inhibitory effect of NS398, Western blotting, and Northern blotting (Figs. 1 and 2). The mechanism of the biphasic increase of cyclooxygenase-2 protein and mRNA (Fig. 2) may be discussed as follows. First, TNFα activates phospholipase A2 (as reported in Ref. 27) and induces cyclooxygenase-2, resulting in an increased production of PGE2. Then, the produced PGE2 binds to a PGE receptor, and increases the cyclooxygenase-2 protein and mRNA again. Previously we reported the cyclooxygenase-2 induction by various PGS, including PGE2, which was a major arachidonate metabolite in this cell line (15).

Since the addition of TNFα brought about such a typical and prominent induction of cyclooxygenase-2 in MC3T3-E1 cells, we attempted to elucidate the transcriptional regulation of the cyclooxygenase-2 gene in this system. The luciferase assay (Figs. 3 and 7) and electrophoretic mobility shift assay (Figs. 5 and 6) revealed two positive regulatory elements on the 5’-flanking region of mouse cyclooxygenase-2 gene: NFκB motif
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NFκB motif (5'-GGGATTCCC-3') located at nucleotides 2401 to 2393 bp and NF-IL6 motif (also referred to as C/EBPb, 5'-TTGCG-CAAC-3') at 2138 to 2130 bp. NF-IL6 was originally identified as a nuclear factor binding to the IL-1 response element of the human IL-6 gene (28). By cDNA cloning NF-IL6 was found to be a member of the CCAAT enhancer-binding protein (C/EBP) family of basic-leucine zipper (bZIP) transcription factors (29). Subsequent studies suggested a regulatory role of NF-IL6 for the genes encoding many acute-phase proteins and cytokines (30). The NF-IL6 motif on the rat cyclooxygenase-2 gene (5'-TTATGCAAT-3' located at 2140 to 2132 bp) was previously reported to contribute to the induction of cyclooxygenase-2 by forskolin, follicle-stimulating hormone, and luteinizing hormone in granulosal cells (5). Recently it was reported that the cyclooxygenase-2 induction was mediated by an activating transcription factor/CRE element (5'-CGTCACGTG-3' at -56 to -48 bp) on the mouse cyclooxygenase-2 gene promoter (7) or by CRE element (5'-TTTCGTCA-3' at -59 to -53) in the human cyclooxygenase-2 gene (6). However, we could not demonstrate the involvement of the CRE motif in the TNFα-dependent cyclooxygenase-2 induction in MC3T3-E1 cells. The AP2 element (5'-CCGCTCGGG-3', -150 to -142 bp) close to the NF-IL6 was shown to be inactive (Figs. 3, 6, and 7).

NFκB was originally found as a binding protein to immunoglobulin κ light-chain enhancer (31), and it is now considered as a key transcription factor in the regulation of immune responses. Its activation is crucial for the induction of inflammatory genes, including cyclooxygenase-2. The CRE element, on the other hand, plays a role in the regulation of genes that are involved in cell proliferation and growth, such as the E2F family of transcription factors.
examined by electrophoretic mobility shift assay (Fig. 5), the nucleic acid of B cells, certain T cell lines and monocytes, and has a ubiquitously transcription factor. It is constitutively present in the nuclear extracts.

NF-IL6 (Fig. 7). Interaction of both NF-kB and NF-IL6 showed a lower luciferase activity than the homodimer of either p50 or p65 (36). As suggested by a finding that band a was supershifted to band e by anti-NF-kB p50 antibody in electrophoretic mobility shift assay with a probe including the NF-IL6 motif (lane 9 in Fig. 6).

Fig. 6. Electrophoretic mobility shift assay targeting AP2 and NF-IL6 sites. MC3T3-E1 cells were incubated for 1 h with various concentrations of TNFα. The nuclear extracts (2.3 μg of protein) from the cells stimulated with 0 ng/ml (lane 3), 0.2 ng/ml (lane 4), 2 ng/ml (lane 5), and 20 ng/ml (lanes 6–9, 11–14, and 16–19) TNFα were incubated with probe gA (wild type, lanes 3–9), gB (mutation for AP2, lanes 11–14), or gC (mutation for NF-IL6, lanes 16–19). The nuclear extracts before the addition of TNFα was also incubated with gA (lane 2). Anti-AP2 (lanes 7, 12, and 17), anti-NF-IL6 (lanes 8, 13, and 18), or anti-NF-kB p50 (lanes 9, 14, and 19) antibody was incubated together with the nuclear extracts. Lanes 1, 10, and 15 were for incubations without the nuclear extracts. Shifted bands indicated by a-g are described in the text.

Fig. 7. Mutations of NF-kB and NF-IL6 motifs. A mixture of each luciferase plasmid which is wild or mutated (cross indicates point mutation of wild plasmid) (2.3 μg) and β-galactosidase plasmid (1.2 μg) was transfected into MC3T3-E1 cells (1.4 × 10^5 cells/60-mm dish) by the lipofection method. The cells were cultured to confluency for 3 days, and incubated for 12 h with (closed column) or without (slashed column) 20 ng/ml TNFα. The luciferase activity was assayed, and normalized with the β-galactosidase activity. Data are means ± S.E. of triplicate determinations.

Individual binding sites for NF-IL6 and NF-kB are present in the promoter of the IL-6 gene, and the cooperation of these two factors plays an important role in transcription of IL-6 (37). A similar activation was reported for the IL-8 promoter, which also contains both NF-IL6 and NF-kB binding sites (37). The NF-kB p50 and NF-IL6 proteins directly interact in vitro, and the Rel homology domain and leucine-zipper motif, respectively, are important for this interaction (38).

TNFα enhanced the promoter activity of the cyclooxygenase-2 gene of MC3T3-E1 cells (Fig. 3), and increased the amounts of NF-kB and NF-IL6 bound to the DNA probes including the corresponding cis-elements (Figs. 5 and 6). The results were in parallel with the marked induction of cyclooxygenase-2 in the TNFα-stimulated cells (Figs. 1 and 2). It was shown that TNFα activated an endosomic acidic sphingomyelinase through the 55-kDa TNF receptor and then the produced ceramide triggered the activation of NF-kB (27). TNFα also induced NF-IL6 in the mouse lung, liver, and kidney (30). By analogy we presume that TNFα triggers the activation or induction of both NF-kB and NF-IL6, resulting in the induction of cyclooxygenase-2 in MC3T3-E1 cells. We should note that there are NF-kB and NF-IL6 sites in human (5) and rat (4) cyclooxygenase-2 genes: human, 5'-GGGATTCCC-3' (~447 to ~349 bp, NF-kB element) and 5'-TTAGGCAAT-3' (~324 to ~324 bp, NF-IL6 element); rat, 5'-GGGATTCCC-3' (~340 to ~336 bp, NF-kB element) and 5'-TTAGGCAAT-3' (~140 to ~132 bp, NF-IL6 element).

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