Transcriptional Regulation of Cyclooxygenase-2 Gene in Pancreatic β-cells

Fan Yang and David Bleich

Susan and Leslie Gonda (Goldschmeid) Diabetes & Genetic Research Center
Department of Diabetes, Endocrinology, & Metabolism
City of Hope National Medical Center
1500 East Duarte Road
Duarte, CA 91010

Corresponding Author:
David Bleich, MD
Department of Diabetes, Endocrinology, & Metabolism
City of Hope National Medical Center
1500 East Duarte Road
Duarte, CA 91010

TELE: 626-256-4673 ext 68251
FAX: 626-301-8212
EMAIL: dbleich@coh.org

Running Title: COX-2 gene expression in pancreatic β-cells
ABBREVIATIONS

AhR, aryl hydrocarbon receptor; C/EBP, CCAAT/enhancer-binding protein; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; CREB, cAMP response element-binding protein; IBMX, 3-isobutyl-1-methylxanthine; Luc, luciferase gene; NF-IL-6, nuclear factor interleukin-6; PGE₂, prostaglandin E₂; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.
SUMMARY

Prostaglandin E₂ (PGE₂) has been shown to negatively affect pancreatic β-cell function, and its inducible synthesis is mediated in part by cyclooxygenase-2 (COX-2). Regulation of basal and inducible COX-2 gene expression in pancreatic β-cells is not fully understood. In this report, we used pancreatic β-cells (RINm5F) to explore the molecular mechanisms regulating COX-2 promoter activity. Through deletion analysis of a –907/+70 base pair (bp) 5’ upstream region of the mouse COX-2 gene, we identified an inhibition domain (-804/-371) and an activation domain (-371/+70). The highest promoter activity was seen when the promoter was reduced to –371 bp. Several cis-acting elements were selected for site-directed mutations in the activation domain. We identified three sites that were essential for basal COX-2 promoter activity: 1. C/EBP (CCAAT/enhancer-binding protein), 2. AhR (aryl hydrocarbon receptor), and 3. CREB (cAMP response element binding protein). Single mutation of each individual site inhibited 70 to 80% of basal COX-2 promoter activity. Double mutation of the AhR and CREB binding sites showed synergy in repressing COX-2 promoter activity as did mutation of all three sites. We demonstrated that the transcription factors from RINm5F nuclear extracts specifically bound to oligonucleotides containing C/EBP, AhR, or CREB consensus sites. Forskolin, an activator of adenyl cyclase, increased COX-2 promoter activity via the CREB site. COX-2 promoter activity was also increased by TCDD, an AhR activator, through the AhR site. Both forskolin and TCDD increased COX-2 mRNA in a dose-dependent manner. We consider these three transcriptional regulators of COX-2 expression to be potential targets for the prevention of β-cell damage mediated by PGE₂.
INTRODUCTION

Cyclooxygenase (COX) is a key enzyme that catalyzes the conversion of arachidonic acid to prostaglandins (PGs). COX-1 and COX-2, two isozymes of COX, are encoded by two separate genes located on different chromosomes (1). COX-1 is constitutively expressed in most tissues and indeed has a GC-rich housekeeping promoter (2). In contrast, COX-2 is normally undetectable in most tissues, but can be rapidly induced by tumor promoters (3), growth factors (4), cytokines (5), viruses (6), and other stimuli. In some cases COX-2 induction is primarily due to mRNA stabilization, while in other instances COX-2 induction is predominantly dependent on transcriptional activation (7-10). Transcriptional activation of COX-2 in particular has been studied in depth because of its role in inflammation, immune responses, and carcinogenesis (11). The transcriptional activation of COX-2 is mediated by the binding of inducible transcriptional factors to cis-acting elements in the COX-2 promoter. The specific factors involved in COX-2 activation depend on both cell type and stimulus. For example, a consensus cyclic AMP response element (CRE) site and two nuclear factor interleukin-6 sites are essential for induced COX-2 expression in activated mast cells (12) and endotoxin treated macrophages (13), while NF-κB p65 transcription factor mediates the induction of COX-2 by hypoxia in vascular endothelial cells (14).

The COX-2 gene regulates diverse biological effects in mammalian tissues by increasing the synthesis of prostaglandin E2 (PGE₂). PGE₂ modulates downstream signaling pathways involved in cell adhesion, vasodilation, and cell proliferation (15-19). PGE₂ has long been known to impair β-cell function (20-22). Studies first conducted about thirty years ago demonstrated that PGE2 impaired glucose-stimulated insulin secretion (GSIS). Even though COX-2 is absent under basal conditions in many tissues, the pancreatic β-cell is somewhat unique because COX-2 is constitutively active without exogenous stimuli (22). Moreover, hyperglycemia appears to induce COX-2 mRNA in cultured human pancreatic islets (23). Upon
addition of interleukin-1β, COX-2 mRNA and protein typically increase 3-4 fold while end product PGE₂ increases >100-fold (24).

More recently, inhibition of COX-2 was shown to preserve β-cell function (25) and increased basal insulin secretion (Bleich et al., unpublished data). COX-2 levels are relatively low in healthy β-cells. When β-cells are placed in a stressful environment, COX-2 gene expression is up-regulated (22). Though COX-2 plays an important role in β-cell function and insulin secretion, no systematic study of COX-2 expression at the transcriptional level has been performed in pancreatic β-cells. One previous study showed that mutation of the NF-IL-6 binding motif reduced basal promoter activity by 50%, while IL-1β coordinately regulated increased NF-κB binding and decreased NF-IL-6 binding to the COX-2 promoter in HIT-T15 β-cells (26). Therefore, the molecular regulation of COX-2 gene in β-cells and pancreatic islet is incompletely understood.

In order to investigate the transcriptional regulation of COX-2 promoter in pancreatic β-cells, we used a 1-kilobase mouse COX-2 gene 5' flanking region (-907/+70 bp) linked to luciferase reporter gene in RINm5F β-cells. Through deletion studies, we found one activating domain (-371/+70 bp). We then identified three transcription factors that were required for basal COX-2 promoter activity: 1. cyclic AMP response-element binding protein (CREB) 2. Aryl hydrocarbon receptor (AhR) and 3. CCAAT/enhancer-binding protein (C/EBP). We identified these by mutating cis-acting elements in the activating domain. Importantly, the induction of COX-2 promoter activity by forskolin, an adenylyl cyclase activator, and TCDD, an AhR activator, was abolished by mutation of their respective promoter binding sites. We also determined that nuclear extracts from RINm5F cells specifically bound the DNA elements containing CREB, AhR, and C/EBP sites. To our knowledge, this is the first study to analyze in detail the COX-2 promoter in β-cells. These findings will be helpful for understanding or controlling β-cell function via modification of COX-2 gene expression.
EXPERIMENTAL PROCEDURES

Experimental Reagents—Forskolin, 3-isobutyl-1-methylxanthine (IBMX), RPMI-1640 medium with L-glutamine were obtained from Sigma Chemical Co., St. Louis, MO. Fetal bovine serum and antibiotics-antimycotics were obtained from Invitrogen, Carlsbad, CA. 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) was obtained from Cambridge Isotope Laboratories, Andover, MA. Forskolin, IBMX, and TCDD were dissolved in dimethyl sulfoxide (DMSO). SuperFect transfection reagent and one-step RT-PCR kit were obtained from Qiagen Inc., Valencia, CA. Dnase I Treatment and Removal reagents were obtained from Ambion, Inc., Austin, TX and TRI Reagent for total RNA isolation was purchased from the Molecular Research Center, Inc., Cincinnati, OH.

Cell Culture—RINm5F pancreatic β-cells were grown in RPMI 1640 (with L-glutamine) supplemented with 10% fetal bovine serum, 10 mM HEPES and 1% antibiotics-antimycotics at 37°C with 5% CO2. Cultures were fed with fresh medium two times per week.

Plasmid Construction—The various 5’ deleted fragments of mouse COX-2 promoter were derived from the plasmid pTIS10L (27) (a gift from Dr. Harvey Herschmann, UCLA School of Medicine) and amplified using PCR. The upstream and downstream PCR primers contained Kpn I and Xba I restriction sites respectively. The fragments were digested by Kpn I and Xba I and were inserted to the luciferase reporter vector pGL3 Basic (Promega, Inc. Madison, WI) that contained Firefly luciferase cDNA. The following primers were used: upstream primers, from –907, 5’GGGGTACCAGCAAAATTTTTTATCAAAACTGTGGTCTG; from -804, 5’GGGGTGACCCCCTTGGCCATAACATTTCTTGTAAACATGGA; from –568, 5’GGGGTACCGGGAGGGAAGCTGTGACGCTTTGAGCTTT; from –371, 5’GGGGTACCCGGAGGAAGCTGTGACGCTTTGAGCTTT; from –229, 5’GGGGTACCCGGAGGAAGCTGTGACGCTTTGAGCTTT.
from –149, 5’GGGGTACCCGCTGCGGTTCTTGCGCAACTCAGTGC; from –70, 5’GGGGTACCCAGAGTCACCACTACGTCACGTGGAGTCCG; from –40, 5’GGGGTACCCTTTACAGACTTAAAAGCAAGGTTC; downstream primer +70, 5’GAAGATCTCAGTGCTGAGATTCTT-CGTGAGCAGGTCC. Deletion constructs containing various promoter regions of mCOX-2 were named pCOX2 (-907/+70), pCOX2 (-804/+70), pCOX2 (-568/+70), pCOX2 (-371/+70), pCOX2 (-229/+70), pCOX2 (-149/+70), pCOX2 (-70/+70) and pCOX2 (-40/+70), relative to the transcription start site of the COX-2 gene. Restriction enzyme digestions and direct DNA sequencing (City of Hope Medical Center Core DNA Facility) were performed in order to confirm the proper sequence of all constructs.

Site-Directed Mutagenesis—Mutant COX-2 promoter constructs were made using QuikChange II Site-Directed Mutagenesis kit (Stratagene, Inc., La Jolla, CA) according to the manufacturer’s instruction. Construct pCOX2 (-371/+70) was used as a template for the other constructs. Mutant constructs were named by transcription factors. The consensus binding site of each transcription factor was mutated as shown in Table 1 and 2. The underlined nucleotides indicate the mutations. All the mutations were confirmed by direct DNA sequencing.

Transient Transfection and Luciferase Assays—The day before transfection, RINm5F cells were plated into six-well tissue culture plates at a density of 5 X 10^5 cells per well. Transfections were performed using SuperFect reagent (Qiagen), following the manufacturer’s protocol. Each transfection was performed using 1.5 µg of Firefly luciferase reporter construct DNA that contained various deletions and site-directed mutants of COX-2 promoter gene plus 0.1 µg of an internal control Renilla luciferase reporter plasmid pRL-TK (Promega Inc., Madison, WI). Two and one half hours after transfection, the medium was removed by aspiration and replaced with normal culture media containing 10% FBS and antibiotics. Following an overnight
recovery period, the transfected cells were cultured in medium with 0.2% BSA for 48 hours. All treatments were performed in 0.2% BSA medium 24 hours before cells were collected. Cell extracts were prepared for luciferase determination according to the protocol accompanying the Dual-Luciferase Reporter Assay System (Promega). Firefly and Renilla luciferase activities were measured with a TD-20/20 luminometer (Turner Designs, Inc., Sunnyvale, CA). Firefly luciferase activity was normalized to Renilla luciferase activity. Each experiment was performed in triplicate and repeated two or three times independently.

**RNA Preparation and Relative RT-PCR**—1 x 10⁶ RINm5F cells were seeded in 60 mm culture dishes. On the next day, the cells were switched to 0.2% BSA medium for 24 hours. Cells were changed to fresh 0.2% BSA medium and different concentrations of forskolin or TCDD were added. Fours hours later, the cells were collected and total RNA was isolated with TRI Reagent (Molecular Research Center). In order to remove contaminating DNA, RNA samples were treated with Dnase I, using Dnase Treatment & Removal Reagents (Ambion Inc.). RT-PCR assays were performed with the QIAGEN One-Step RT-PCR kit. The sense and antisense primers for rat COX-2 were 5'-TGGTGCCGGGTCTGATGATG and 5'-GCAATGCAGGTCTGATACTG. The level of 18S ribosomal RNA was used as an internal standard. The primers and competitors of 18S RNA were from QuantumRNA™ 18S Internal Standards kit (Ambion, Inc.). The amplification process was conducted for 36 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, and extension at 72 °C for 2 min. The RT-PCR products were fractionated on 1.2% agarose gels and photographed using an Alphalmager 2000 Documentation and Analysis System (Alpha Innotech Corp., San Leandro, CA).

**Nuclear Extracts**—3 X 10⁶ RINm5F cells were seeded in 100 mm dishes in triplicate. Next day, the cells were switched to 0.2% BSA medium for 48 hours to maintain the same
culturing condition as the transfected cells used for luciferase assays. Cells were washed once with PBS and scraped in PBS. Then, the cells were centrifuged for 3 min at 3000 RPM and the pellet suspended in 300 µl NP 40 lysis buffer (10 mM Tris, pH 7.5; 10 mM NaCl; 3 mM MgCl₂; 0.5% NP 40). After centrifugation, the pellet was treated with 50 µl low salt buffer (20 mM NaCl, 1 mM EDTA, 20% glycerol, 20 mM HEPEs, pH 7.9) and 50 µl high salt buffer (1 M NaCl, 1 mM EDTA, 20% glycerol, 20 mM HEPEs, pH 7.9) with vortexing in a cold room. The mixture was rotated at 4 °C for 30 min followed by the addition of 100 µl low salt buffer. After centrifugation at 15,000 RPM for 15 min, the remaining supernatant was the nuclear extract. Protein concentrations were measured using Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA).

Electrophoretic Mobility Shift Assays (EMSA)—The sense sequences of the oligonucleotides tested were as follows: AhR wild type, 5’CTCTCATTGCGTGGGTAAAGCCTGC; AhR mutant, CTCTCATTTTTTGGGTAAAGCCTGC; C/EBP wild type, TTGGTGGGGGTTGGGGAAAGCCTAAGC; C/EBP mutant, TTGGTGGGGTCTGGGGAAAGCCTAAGC; CREB wild type, GTCACCACTACGTCACGTGGAGTCCGC; CREB mutant, GTCA-CCACTATTGCACGTGGAGTCCGC. To prepare the double-stranded oligonucleotides, single-stranded forward and reverse oligonucleotides were annealed by heating to 95 °C and cooling slowly to room temperature in TEN buffer (10 mM Tris, 1 mM EDTA, 0.1 M NaCl, pH 8.0). The double-stranded oligonucleotides (4 pmol) were then digoxigenin-labeled at the 3’ end with enzyme and reagents supplied in DIG Gel Shift kit (Roche Applied Sciences, Indianapolis, IN). Binding reactions were conducted with 16 µg of nuclear protein and 0.4 ng of digoxigenin-labeled probe following the manufacturer’s protocol. Binding complexes were separated on a 6% DNA retardation gel running at 75 V for one and half hours, after which the gel was transferred to positive-charged nylon membrane by electro-blotting (30 min, 400 mA). The DNA
was fixed to membrane by UV cross-link, and chemiluminescent signals were recorded on X-ray film.

Statistics—Student’s *t*-test was used to evaluate statistical significance of differences between two groups. *P* < 0.05 was considered statistically significant.
RESULTS

Analysis of Basal COX-2 Promoter Activity in Pancreatic \( \beta \)-Cells—Deletion mutants of COX-2 promoter-driven luciferase reporter gene constructs and control pGL3 Basic vector were transiently transfected into RINm5F cells for 72 hours and relative luciferase activity (the ratio Firefly luciferase to Renilla luciferase) of each reporter gene construct was measured as shown in Fig. 1. The \(-371/+70\) bp region gave the highest level of COX-2 promoter activity. This finding is similar to the original report in COS-1 cells (27) where two COX-2 promoter fragments, pTIS10L\((-963/+70)\) and pTIS10S\((-371/+70)\) were tested. Here, \(-371/+70\) showed much higher promoter activity than \(-963/+70\). Our serial promoter deletions from \(-907\) bp to \(-371\) bp showed a five-fold increase in promoter activity, consistent with the notion that this region contains inhibitory domains. The deletions from \(-371\) bp to \(-40\) bp caused about 95% loss of luciferase activity. Luciferase activity of reporter construct pCOX2\((-40/+70)\) was almost at the level of the pGL3 Basic vector. These results indicated that the region between \(-371\) bp and \(-40\) bp contains activation domains for basal COX-2 expression in \( \beta \)-cells. Since a presumptive TATA element is located at \(-30\) bp, all reporter constructs of the COX-2 promoter contained this TATA element.

Identifying COX-2 Promoter Activation Elements—One inhibition domain \((-907/-371\) bp) and one activation domain \((-371/+70\) bp) were identified from the results in Fig. 1. In further studies we focused on the activation domain. To investigate the transcriptional activators in this domain, we mutated presumptive \textit{cis}-acting transcriptional regulatory elements by site-directed mutagenesis (Table 1). These sites were obtained by screening promoter region \(-371/+70\) with two computer programs called Matrix Search for Transcription Factor Binding Sites (MATCH) and Pattern Search for Transcription Factor Binding Sites (PATCH) (provided by Transfac\textsuperscript{\textregistered}Professional 7.4). If the binding sites of two transcription factors were close or
overlapping, we mutated them at the same time and essentially eliminated two DNA binding sites in one experiment. The factors that contained close sequences were NF-1/SP-1, and those that contained overlapping sequences were WT-1/GR, (NF-κB1/2)/C/EBP, and CREB/E-box. First, we picked five locations for site-directed mutations. We mutated these five locations separately and determined luciferase activities (Fig. 2). Compared with the wild type pCOX2(-371/+70), mutation of the NF-1/SP-1 site did not change the promoter activity, while mutation of the WT-1/GR site only decreased promoter activity by thirty percent. In contrast, mutation of the AhR site, the (NF-κB1/2)/C/EBP site, or the CREB/E-box site caused major decreases in COX-2 promoter activity by 70%, 74%, and 84%, respectively. Since the AhR site contained no overlapping DNA binding domains, we confirmed that AhR was essential for basal COX-2 expression in β-cells. Since the other two locations contain two transcriptional factors, we performed further studies to determine which factor was essential for COX-2 gene expression.

**CREB and C/EBP, but Not NF-κB 1/2 or E-box, Are Essential for Basal COX-2 Expression**—In Fig. 2., we mutated nucleotides overlapping the NF-κB1/2 and C/EBP binding region and the CREB and E-box binding region. Thus, we could not determine which two sites were responsible for COX-2 promoter activation. To solve this problem, we mutated non-overlapping binding sites in the NF-κB1/2 and C/EBP region and the CREB and E-box region and tested these new promoter constructs. As shown in Table 2, the mutation sites were carefully selected so that we could discriminate between individual overlapping transcription factor binding sites (e.g. NF-κB1/2 and C/EBP binding domains shared bases –93 to –90; therefore, bases –89 and –88 were mutated in the NF-κB1/2 site, while bases –95 and –94 were mutated in the C/EBP site). Using the wild type pCOX2(-371/+70) construct and the four mutant constructs shown in Table 2, we transiently transfected these reporter constructs into RINm5F cells and determined luciferase activities. As shown in Figure 3, the NF-κB1/2
mutation did not inhibit the reporter activity, while the C/EBP mutation strongly repressed reporter activity by 73% compared to the wild type. Similarly, the E-box mutation slightly decreased reporter gene activity, while the CREB mutation strongly inhibited the promoter activity by 83%. These results indicated that C/EBP and CREB sites were essential for basal COX-2 expression in β-cells.

**Synergistic Effect of Mutations in AhR, C/EBP and CREB Sites on COX-2 Promoter Activity**— Though single mutation of AhR, C/EBP, or CREB binding site decreased promoter activity by 70-80%, it was unknown whether these sites worked synergistically in regulating the COX-2 promoter. To this end, we designed luciferase reporter constructs containing double mutations of the AhR and C/EBP sites, AhR and CREB sites, C/EBP and CREB sites, and mutation of all three sites. The relative luciferase activities of three double mutant constructs and a triple mutant construct are shown in Figure 4. Double mutation of AhR and C/EBP sites, or CREB and C/EBP sites did not show synergy, while double mutations of AhR and CREB sites exhibited a further 1.5 to 2-fold reduction in reporter activities compared to single mutations. The triple mutation of AhR, C/EBP, and CREB strongly repressed luciferase activity 2-fold more than the respective single mutation. These results indicated that no synergy existed between AhR and C/EBP sites or CREB and C/EBP, whereas significant synergy was seen between AhR and CREB sites or among all three sites.

**Cyclic AMP Activator, Forskolin, Increases COX-2 Promoter Activity and COX-2 mRNA Level**—Based on the observation that mutation of the CREB site inhibited COX-2 promoter activity in β-cells (Fig. 3 and 4), we investigated whether increasing endogenous cyclic AMP (cAMP) by adding the cAMP activators, forskolin and IBMX, could also increase COX-2 promoter activity. Forskolin, an adenyl cyclase activator, and IBMX, a phosphodiesterase
inhibitor, were chosen for these studies. RINm5F cells were transfected with pCOX2(-371/+70) luciferase reporter construct and the next day, cells were starved in 0.2% BSA medium for 24 hours. The transfected cells were treated with forskolin, 4 and 10 µg/ml, and IBMX, 100 and 400 µM, for 24 hours. Forskolin increased luciferase activity 3-fold when compared to untreated cells (Fig. 5), while IBMX increased luciferase activity 2-fold (data not shown). When the CREB site was mutated, forskolin could not increase the mutant reporter luciferase activity (Fig. 5). We also tested other two constructs, pCOX2(-804/+70) and pCOX2(-907/+70), which contained longer promoter fragment than pCOX2(-371/+70). Even though the –804/+70 bp and –907/+70 bp fragments contained an inhibiting domain, the luciferase activities of pCOX2(-804/+70) and pCOX2(-907/+70) were still increased by the addition of forskolin (10 µg/ml) (Fig. 6). Since forskolin only increased the wild type COX-2 promoter activity, but not the CREB site mutant promoter activity, this indicated that cAMP activators increased the transcription of COX-2 gene via the CREB binding sites. So too, forskolin treatment increased COX-2 mRNA level of RINm5F cells in a dose-dependent manner (Fig. 7A). These experiments told us that the CREB site in the COX-2 promoter played an important role in both basal and induced expression of the COX-2 gene.

TCDD, a Ligand of AhR, Increases COX-2 Promoter Activity via AhR Binding Site in β-cells—AhR, a basic helix-loop-helix transcription factor, activates genes when bound by environmental contaminants like TCDD and other polychlorinated biphenyl compounds (28). Since the COX-2 promoter contained an AhR site, we investigated whether TCDD affected COX-2 promoter activity in β-cells. Wild type and AhR mutant constructs of luciferase reporter pCOX2(-371/+70) were transfected to RINm5F cells. After starvation with 0.2% BSA for 24 hours, the cells were treated with 1 nM and 10 nM TCDD for another 24 hours. Then, the relative luciferase activities were determined as shown in Fig. 8. TCDD increased luciferase
activity by ~1.5-fold in wild type pCOX2(-371/+70) promoter construct. Interestingly, TCDD did not increase AhR mutant luciferase reporter activity. Therefore, TCDD increased COX-2 promoter activity via the AhR site. TCDD also increased COX-2 mRNA in a dose-dependent manner using semi-quantitative RT-PCR, as shown in Figure 7B. These results indicated that AhR played a role in both basal and inducible COX-2 gene expression.

**Nuclear Factors from RINm5F Cells Specifically Bind the COX-2 Promoter Elements Containing AhR Site, CREB Site, or C/EBP Site**—We performed electrophoretic mobility shift assays (EMSA) to investigate whether the transcription factors from RINm5F cells could specifically bind COX-2 promoter elements containing AhR site, CREB site, or C/EBP site. As shown in figure 9A, a complex was formed between digoxigenin-labeled probe that contained AhR wild type site and nuclear extract protein from RINm5F cells. This binding was specific since complex formation was inhibited by 50-fold molar excess of unlabeled AhR wild type probe, but not by 50-fold molar excess of unlabeled AhR mutant probe. The formation of binding complex between digoxigenin-labeled probe that contained CREB wild type site and nuclear extract is shown in figure 9B. The specificity of binding was demonstrated by the inhibition of complex formation with 50-fold molar excess of unlabeled CREB wild type probe, but not by 50-fold molar excess of unlabeled CREB mutant probe. Figure 9C shows that the complex was formed between digoxigenin-labeled C/EBP wild type probe and nuclear extract protein. A 50-fold molar excess of unlabeled C/EBP wild type probe inhibited the formation of binding complex, whereas a 50-fold molar excess of unlabeled C/EBP mutant probe did not inhibit the complex formation. Therefore, the binding between C/EBP probe and nuclear extract protein was specific. These results indicated that nuclear factors from RINm5F cell nuclear extracts specifically bound the COX-2 promoter elements containing AhR site, CREB site, and C/EBP sites.
DISCUSSION

Up-regulation of COX-2 expression causes increased synthesis of PGE$_2$, resulting in impairment of pancreatic $\beta$-cell insulin secretion. The selective COX-2 inhibitor NS-398 has been demonstrated to partially restore GSIS in HIT cells and islets treated with IL-1$\beta$ for 24 hours (25) and to prevent low-dose streptozotocin-induced diabetes in mice (29). Immunostaining studies showed that COX-2 is expressed in islet-infiltrating macrophages, and that insulin and COX-2 expression disappeared concomitantly from $\beta$-cells when NOD mice progressed toward overt diabetes (30). These results suggested that COX-2 activation might play a pathogenic role in type 1 diabetes. Other studies showing that hyperglycemia induced both IL-1$\beta$ and COX-2 in human pancreatic $\beta$-cells (23,31) provided a link between type 2 diabetes, cytokine-mediated islet dysfunction, and PGE$_2$. Thus, it is critical to know how basal and induced COX-2 expression is regulated in $\beta$-cells. In this report, we identify an activating domain (-371/+70) through a series of promoter deletions using a 1-kb (-907/+70) mouse COX-2 promoter. Then, by site-directed mutation of putative transcription factor binding sites, we established that the CREB, AhR, and C/EBP binding sites are not only essential for basal COX-2 expression, but also important for induced COX-2 expression. We also studied the synergistic effect of these three binding sites.

In our studies, we observed the highest COX-2 activity when the promoter was deleted to $-371$ bp. This result is similar to a mouse COX-2 promoter study in NIH 3T3 cells (27) and a human promoter study in human endometrial stromal cells (7). Therefore, most of the COX-2 promoter activity may come from the proximal 5' flanking region to transcription start site (+1). In contrast to most other mammalian cell types, islets of Langerhans constitutively express COX-2 more so than COX-1 (22). To investigate the transcription factors responsible for basal COX-2 activity in $\beta$-cells, we use site-directed mutagenesis to mutate putative cis-acting elements as shown in Tables 1 and 2. We found that mutations in the AhR, CREB and C/EBP
binding sites greatly decreased 70 – 80% of promoter activity (Fig. 2 and 3). However, mutation of SP-1, NF-1, NF-κB 1 and 2 did not affect the basal expression of the COX-2 gene in β-cells. Mutation of WT-1 and GR sites decreased promoter activity by 30%, while mutation of E-box caused a 20% decrease in promoter activity. Therefore, AhR, CREB, and C/EBP sites were responsible for basal expression of COX-2 gene in β-cells.

Stimulation of adenyl cyclase leads to cAMP generation and subsequent PKA activation. Thereafter, PKA phosphorylates critical transcription factors like CREB, CREM and ATF1. These phosphorylated factors can then initiate transcription of target genes. CREB transcription factor has been reported to play a role in regulating basal COX-2 expression in colon carcinoma cells with high basal COX-2 expression (32). CREB has also been shown to be responsible for induced expression of COX-2 gene, such as the induction of COX-2 expression in activated mast cells (12), UVB induction of COX-2 expression in human keratinocytes (33), and shear stress-induced COX-2 promoter expression in osteoblastic MC3T3-E1 cells (34). In the murine COX-2 promoter, CREB site (-56/-52) and E-box site (-53/-48) are overlapped. In our study, when the non-overlapped bases of CREB site in the –371/+70 bp region were mutated, the promoter activity was decreased 70% of wild type (Fig. 3). In contrast, mutation of E-box only caused 20% decrease in promoter activity (Fig. 3). E-box sequences, CACGTG, are the binding sites for basic helix-loop-helix (bHLH) transcription factors, such as c-Myc. In mouse skin carcinoma cells, mutation of E-box dramatically decreased basal expression of COX-2 (35). Thus, the effect of E-box on the murine COX-2 promoter activity is tissue-specific. To investigate the effect of the CREB site on COX-2 promoter activity, we treated RINm5F cells with the cAMP activator, forskolin. Here, COX-2 promoter activity was increased two to three fold by treatment with forskolin (10 μg/ml) (Fig. 5 and 6). Mutation of the CREB site completely abolished the effect of forskolin on promoter activity (Fig. 5). These results suggested that forskolin increased COX-2 promoter activity via the CREB site. Forskolin also increased the
expression of COX-2 mRNA in RINm5F cells in a dose-dependent manner (Fig. 7A). COX-2 mRNA expression can be upregulated either by increasing the transcription rate (34-36) or by increasing mRNA stability via highly conserved AU-rich element in 3′-untranslated region (3′-UTR) (37). Thus, further studies will be needed to confirmed whether forskolin increased COX-2 mRNA by increased COX-2 transcription rate or through both increased transcription rate and increased RNA stability. We conclude that the CREB site in COX-2 promoter is not only important for basal expression, but also for induced expression through the cAMP/protein kinase A pathway in pancreatic β-cells.

The family of C/EBP transcription factors is composed of basic leucine-zipper DNA-binding proteins and has six members so far: C/EBPα, C/EBPβ, C/EBPδ, C/EBPγ, C/EBPε, and C/EBPζ (38). C/EBP family members work as pivotal regulators of cellular differentiation, terminal function, and response to inflammatory insult (38). It was identified that some members of C/EBP family could bind to NF-IL-6 site (35). By searching a computer program of Transcription Factor Binding Sites (PATCH) in the murine COX-2 5′ flanking region (–966/+70 bp), we found one C/EBP site (GTTGGG, -95/-90) which can bind both C/EBPα and C/EBPβ and is overlapped with NF-κB 1 and 2 (TGGGGA, -93/-88). NF-κB 1 (p105/p50) and NF-κB 2 (p100/p52) belong to the family of NF-κB transcription factors that play a critical role in cellular inflammatory responses, cell stress responses, cancer development and so on (39,40). When the overlapped bases of C/EBP and NF-κB 1 and 2 sites were mutated, the basal COX-2 promoter activity was decreased about 70% (Fig. 2). To identify which factor played the key role in this effect, we mutated the non-overlapped bases of these two sites (Table 2). Our results (Fig. 3) showed that mutation of the C/EBP site was responsible for the decreased basal COX-2 promoter activity, whereas mutation of the NF-κB 1 and 2 site did not affect basal COX-2 expression. EMSA demonstrated that transcription factors from RINm5F β-cells could specifically bind to the DNA element containing the C/EBP site (Fig 9C). Since both C/EBPα
and C/EBPβ can bind this putative C/EBP site, further experiments will be performed to determine which isoform regulates basal COX-2 expression in β-cells.

The aryl hydrocarbon receptor (AhR), a basic helix-loop-helix transcription factor, mediates carcinogenic and teratogenic effects of environmentally toxic chemicals, such as dioxin (41). AhR was originally characterized because of its high affinity binding of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (41). Under basal conditions cytosolic AhR is associated with heat shock protein 90 and the hepatitis B virus X-associated protein (42). Upon ligation with TCDD, AhR dissociates from its complex and migrates to the nucleus where it binds to the aryl hydrocarbon nuclear translocator (ARNT) (43). This heterodimeric complex then binds to its DNA binding site (i.e. the xenobiotic response element) and activates the target gene. It was reported that TCDD increased COX-2 mRNA expression in mouse hepatoma cells via AhR because anti-sense oligonucleotides to AhR mRNA inhibited the TCDD effect (44) and that TCDD increased COX-2 mRNA and protein expression in C3H/M2 mouse fibroblasts (45). In this study, we identified that the AhR site is required for maintenance of basal COX-2 expression in β-cells (Fig. 2) and TCDD increased the promoter activity via the AhR site (Fig. 8). TCDD increased COX-2 mRNA in a dose-dependent manner in RINm5F cells (Fig. 7B). We also demonstrated that transcription factors from RINm5F cell extracts specifically bound the DNA element containing AhR site by EMSA (Fig. 9A). These findings are the first to elucidate the effect of AhR transcription factor on COX-2 gene expression in pancreatic β-cells. The biological relevance of AhR-dependent gene activation in pancreatic β-cells has yet to be determined, but we speculate that agents like TCDD may modulate β-cell function through activation of COX-2.

In summary, we identified that AhR, CREB, and C/EBP transcription factors play a critical role in the basal expression and induced expression of COX-2 gene in pancreatic β-cells through deletion mutations and site-directed mutagenesis of putative binding sites in –907/+70
bp 5' flanking region of murine COX-2 gene. These three transcription factors work independently, but double mutations of AhR and CREB sites or triple mutations of all three sites still showed a synergistic effect. We also demonstrated that TCDD, an environment contaminant, increased COX-2 expression via AhR transcription factor activation. These findings will be helpful to better understanding the role of COX-2 in pancreatic β-cell function.
REFERENCES


FIGURE LEGENDS

Fig. 1. **Deletion analysis of the mouse COX-2 promoter in pancreatic β-cells.** The promoter activity of a series of 5’-deletions made in the COX-2 promoter-flanking region was analyzed by transient transfection into RINm5F cells. COX-2 promoter deletion mutant constructs were named according to the length of the regulatory region. Relative luciferase activities were expressed as the mean ± SD. Experiments were performed in triplicate and repeated three times independently. Serial deletion mutants demonstrated the significance of the −371 bp flanking region for basal promoter activity. Luc: luciferase gene; +1: transcription start site.

Fig. 2. **Identification of transcription factors responsible for COX-2 promoter activity.** The promoter activity of a series of site-specific mutants made in the COX-2 promoter-flanking region (-371/+70) was analyzed by transient transfection into RINm5F cells. The site-specific mutation is indicated by a black oval. Except for the AhR site, two transcription factors were mutated together for the other four sites as indicated in the figure. Results were expressed as the mean ± SD. The experiments were performed in triplicate and repeated twice independently. **P < 0.01 compared with the value from wild type reporter construct, pCOX2(-371/+70).

Fig. 3. **Quantification of wild type and mutant COX-2 promoter luciferase activity.** The promoter activity of a series of site-specific mutants made in the COX-2 promoter-flanking region (-371/+70) containing the cis-acting elements, C/EBP, NFκB 1 and 2, CREB, E-box, was analyzed by transient transfection into RINm5F cells. Mutant COX-2 promoter constructs were named by the mutated transcription factor. Relative luciferase activity was expressed as the
mean ± SD. The experiments were performed in triplicate and were repeated twice independently. **P < 0.001 compared with the wild type.

Fig. 4. Synergy between AhR, C/EBP, and CREB sites in the murine COX-2 promoter. The promoter activity of a series of site-specific mutants made in the COX-2 promoter-flanking region (-371/+70) was analyzed by transient transfection into RINm5F cells. The TATA box (TATA) and three cis-acting sites, AhR, C/EBP and CREB sites, are indicated. The site-specific single mutation, double mutations, or triple mutations are indicated by black ovals. Relative luciferase activities are expressed as the mean ± SD. Experiments were performed in triplicate and repeated twice independently. *P < 0.05 compared with the value from the single mutation (eg. Luciferase activities of AhR and CREB double mutation or triple mutation are significantly lower than their respective single mutation).

Fig. 5. Effect of forskolin on COX-2 promoter activity conferred by a 5′ flanking DNA fragment (-371/+70) and its CREB site mutant. RINm5F cells transfected cells with pCOX2(-371/+70) and its CREB site mutant were starved 24 hours in 0.2 % BSA medium before treatment with 4 ng/ml or 10 ng/ml forskolin in 0.2% BSA medium for another 24 hours. The data are expressed as the mean ± SD of two independent experiments in triplicate. Light gray bars denote wild type DNA fragment (-371/+70) and black bars denote CREB site mutant. **P < 0.01 compared with control.

Fig. 6. Effect of forskolin on COX-2 promoter activity with different length of 5′ flanking DNA fragments. Transfected cells with pCOX2(-371/+70), pCOX2(-804/+70), or pCOX2(-907/+70) were starved 24 hours in 0.2 % BSA medium before forskolin treatment (10 µg/ml) for another 24 hours in 0.2 % BSA medium. The relative luciferase activity is expressed as the
mean ± SD of two independent experiments in triplicate. Light gray bars and black bars denote without and with forskolin treatment, respectively. *P < 0.05, **P < 0.01 compared with control.

Fig. 7. **Effect of forskolin and TCDD on COX-2 mRNA expression in β-cells.** Relative RT-PCR was performed with total RNA isolated from RINm5F cells without or with forskolin (0, 5, 10 µg/ml) or TCDD (0, 1, 5, 10 nM) for 4 hours, using gene-specific primers. 18S RNA was used as an internal control.

Fig. 8. **Effect of TCDD on COX-2 promoter activity conferred by a 5' flanking DNA fragment (-371/+70) and AhR site mutant.** Transfected RINm5F cells with pCOX2(-371/+70) or its CREB site mutant were starved 24 hours in 0.2 % BSA medium before treatment with 1, 5, 10 nM TCDD for another 24 hours in 0.2 % BSA medium. The data are expressed as the mean ± SD of two independent experiments in triplicate. Light gray bars and black bars represent wild type DNA fragment (-371/+70) and AhR site mutant, respectively. *P < 0.05 compared with control.

Fig. 9. **Complex formation between nuclear protein extracts from RINm5F cells and AhR, CREB, or C/EBP DNA probe.** EMSA was performed as described in Experimental Procedures. Binding complex of nuclear factors with digoxigenin-labeled AhR DNA probe (A), CREB DNA probe (B), C/EBP DNA probe (C) are indicated by an arrow. Specificity of binding was determined by the addition of a 50-fold molar excess of unlabeled wild type (wt) probe or a 50-fold molar excess of unlabeled mutant probe (mu). Arrow: complex formation; NS: non-specific binding; Free: free probe.
Table 1  Mutations of cis-acting elements in the region –371/+70 bp of m-COX-2 promoter

<table>
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<th>TF Name</th>
<th>*Consensus Sequences</th>
<th>Mutations</th>
<th>Positions</th>
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<tbody>
<tr>
<td>SP-1</td>
<td>GGGCGG</td>
<td>TGCTTG</td>
<td>-239</td>
</tr>
<tr>
<td>NF-1</td>
<td>TGGCA</td>
<td>TTTCA</td>
<td>-222</td>
</tr>
<tr>
<td>AhR</td>
<td>GCGTG</td>
<td>TTTTG</td>
<td>-167</td>
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<tr>
<td>GR</td>
<td>CAGAG</td>
<td>CATAT</td>
<td>-120</td>
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<tr>
<td>WT-1</td>
<td>GAGGGGGAA</td>
<td>TAGTTGGA</td>
<td>-116</td>
</tr>
<tr>
<td>C/EBP</td>
<td>GTTGGG</td>
<td>GTTTGG</td>
<td>-95</td>
</tr>
<tr>
<td>NF-κB1/2</td>
<td>TGGGGA</td>
<td>TTTGTA</td>
<td>-93</td>
</tr>
<tr>
<td>CREB</td>
<td>CGTCA</td>
<td>CTAAA</td>
<td>-56</td>
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<tr>
<td>E-box</td>
<td>CACGTG</td>
<td>AAATTG</td>
<td>-53</td>
</tr>
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</table>

* Only sense sequences are shown.
Table 2  Mutations of *cis*-acting elements in the region –371/+70 bp of m-COX-2 promoter

<table>
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<th>*Consensus Sequences</th>
<th>Mutations</th>
<th>Positions</th>
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<td>C/EBP</td>
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<td>TCTGGG</td>
<td>-95</td>
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<tr>
<td>E-box</td>
<td>CACGTG</td>
<td>CATTTT</td>
<td>-53</td>
</tr>
</tbody>
</table>

* Only sense sequences are shown.
Luc
Luc
Luc
Luc
Luc
Luc
Luc
Luc

Relative Luciferase Activity

Fig. 1

Murine COX-2 gene 5’ flanking region
Murine COX-2 gene 5’ flanking region  Relative Luciferase Activity

Fig. 2
Fig. 3
AhR  C/EBP  CREB  TATA

m-COX-2 gene 5' flanking region

pCOX2
(-371/+70)

Luc

pGL3 basic

Relative Luciferase Activity

Fig. 4
Fig. 5

Relative Luciferase Activity

Forskolin (µg/ml)

wt

CREBmu

**

Fig. 5
Fig. 6
Fig. 7
Fig. 8

Relative luciferase activity vs. TCDD (nM)

wt
AhRmu

* Significant difference
Fig. 9
Fig. 9
Transcriptional regulation of cyclooxygenase-2 gene in pancreatic beta-cells

Fan Yang and David Bleich

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