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Proinsulin C-Peptide Stimulates a PKC/IκB/NF-κB Signaling Pathway to Activate COX-2 Gene Transcription in Swiss 3T3 Fibroblasts

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Proinsulin C-peptide causes multiple molecular and physiological effects, and improves renal and neuronal dysfunction in patients with diabetes. However, whether C-peptide controls the inhibitor κB (IκB)/NF-κB–dependent transcription of genes, including inflammatory genes is unknown. Here we showed that 1 nM C-peptide increased the expression of cyclooxygenase-2 (COX-2) mRNA and its protein in Swiss 3T3 fibroblasts. Consistently, C-peptide enhanced COX-2 gene promoter-activity, which was inhibited by GF109203X and Go6976, specific PKC inhibitors, and BAY11-7082, a specific nuclear factor-κB (NF-κB) inhibitor, accompanied by increased phosphorylation and degradation of IκB. These results suggest that C-peptide stimulates the transcription of inflammatory genes *via* activation of a PKC/IκB/NF-κB signaling pathway.

Key words: C-peptide, cyclooxygenase-2, diabetes, NF-κB–dependent transcription, PKC.

Abbreviations: COX-2, cyclooxygenase-2; IκB, inhibitor of NF-κB; NF-κB, nuclear factor-κB; iNOS, inducible nitric oxide synthase; IL-1, interleukin-1; PES-2, prostaglandin-endoperoxide synthase-2; PGE_2, prostaglandin E_2; *p*-APMSF, (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride.

C-peptide, the connecting segment of proinsulin, is secreted by pancreatic β-cells into the circulation together with insulin in equimolar quantities (*1*). Although C-peptide has been so far considered to be biologically inactive, recent studies have revealed that it exerts not only important physiological functions, but also has beneficial effects in patients with type 1 diabetes who lack this peptide, but not in healthy subjects (*2-4*). The cellular and molecular mechanisms underlying the actions of C-peptide are now beginning to emerge. C-peptide has been observed to probably bind to a membrane receptor coupled to a pertussis toxin–sensitive G-protein to elicit a transient increase in the intracellular Ca^{2+} concentration and to stimulate mitogen-activated protein kinase (MAPK)–dependent signaling pathways (*3-5*). Consequently, C-peptide stimulates Na^+,K^+-ATPase and endothelial nitric oxide synthase (eNOS) (*6*), the enzymatic activation of both of which has been reported not to be elicited in type 1 diabetes patients with nephropathy (*2*).

Type 1 diabetes mellitus is an autoimmune disease characterized by the selective destruction of insulin-secreting β-cells found in pancreatic islets of Langerhans. Islet-infiltrating macrophages and T lymphocytes secrete inflammatory cytokines such as IL-1, IFN-γ, and TNF-α (*7*). These cytokines inhibit glucose-stimulated insulin secretion and induce islet degeneration in isolated rat and human islets (*8-10*). The inhibitory and destructive effects of cytokines on β-cell function and islet viability have been demonstrated to be mediated in part through the expression of the inducible form of nitric-oxide synthase (iNOS) and the subsequent increased production of NO by β-cells (*11*). This has been further evidenced by more recent studies using selective inhibitors of iNOS (*12*, *13*) and iNOS-deficient mice (*14*), suggesting a primary role for NO as the mediator of cytokine-induced inhibition of glucose-induced insulin secretion and islet degeneration. Also, high glucose and diabetes induces cyclooxygenase-2 (COX-2) gene expression in human monocytes and islets (*15*, *16*), suggesting that COX-2 expression and PGE_2 formation by rat and human islets correlates with cytokine-induced islet damage. On the other hand, nuclear factor-κB (NF-κB) plays a pivotal role in the transcription activation not only of inflammatory cytokine genes, including IL-1β and TNF-α (*17*, *18*), but also of cytokine-inducible genes, such as the COX-2 and iNOS ones (*19*, *20*). In addition, prolonged treatment with IL-1β in combination with IFNγ and/or TNFα induces β cell death mostly through apoptosis in rodent and human islets (*8-10*). Furthermore, inhibition of cytokine-induced NF-κB activation prevents β cell apoptosis (*21*). These reported observations suggest the central role of NF-κB in cytokine-induced β cell apoptosis. But it remains unclear whether C-peptide controls NF-κB–dependent gene regulation. In the present paper

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we present the first evidence that C-peptide enhances expression of the COX-2 gene in a PKC/inhibitor of NF-κB (IκB)/NF-κB signaling–dependent manner in Swiss 3T3 fibroblasts.

MATERIALS AND METHODS

Chemicals and Materials—Mouse C-peptide, anti–COX-2 antibody, and anti–14-3-3β, anti–I-κBα and anti–phosphorylated I-κBα antibodies were obtained from Yanaihara Institute Inc., Cayman, and Santa Cruz Biotechnology, Inc., respectively. Plus reagent and Lipofectamine reagent, and a Dual-Luciferase® Reporter Assay system were purchased from Life Technologies, Inc. and Promega, respectively. Other chemicals and drugs were of reagent grade or of the highest quality available.

Cell Culture—Swiss 3T3 cells, a mouse embryonic fibroblast line, were cultured as reported previously (5).

Real Time RT-PCR Analysis—Swiss 3T3 cells were seeded on 35-mm dishes at a density of 1.0×10^5 cells/dish and then cultured for 24 h. For RT-PCR analysis of COX-2 expression, cells were incubated in serum-free medium with or without mouse C-peptide for the indicated times. Total RNA was isolated from cells using an RNeasy Mini Kit (Qiagen, Tokyo, Japan). Quantitative PCR was performed with an ABI Prism 7900 and SYBR Green Reagent (Applied Biosystems, Foster City, CA, USA). cDNA was synthesized from 0.5 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Absolute cDNA abundance was calculated using the standard curve obtained for rat genomic DNAs. The PCR conditions were as follows: 10 min at 95°C, then 50 cycles of 15 s at 94°C, 30 s at 60°C, 1 min at 72°C. The mRNA levels were normalized using mouse GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as the housekeeping gene. The primers for amplification were: *GAPDH*, 5′-CAA AAT GGT GAA GGT CGG TGT G-3′ (forward) and 5′-ATT TGA TGT TAG TGG GGT CTC G-3′ (reverse); and *COX-2*, 5′-GGA TGC ACG AGC AGC AGT TT-3′ (forward) and 5′-TTG ATG GAC GGG AAC AGG TT-3′ (reverse).

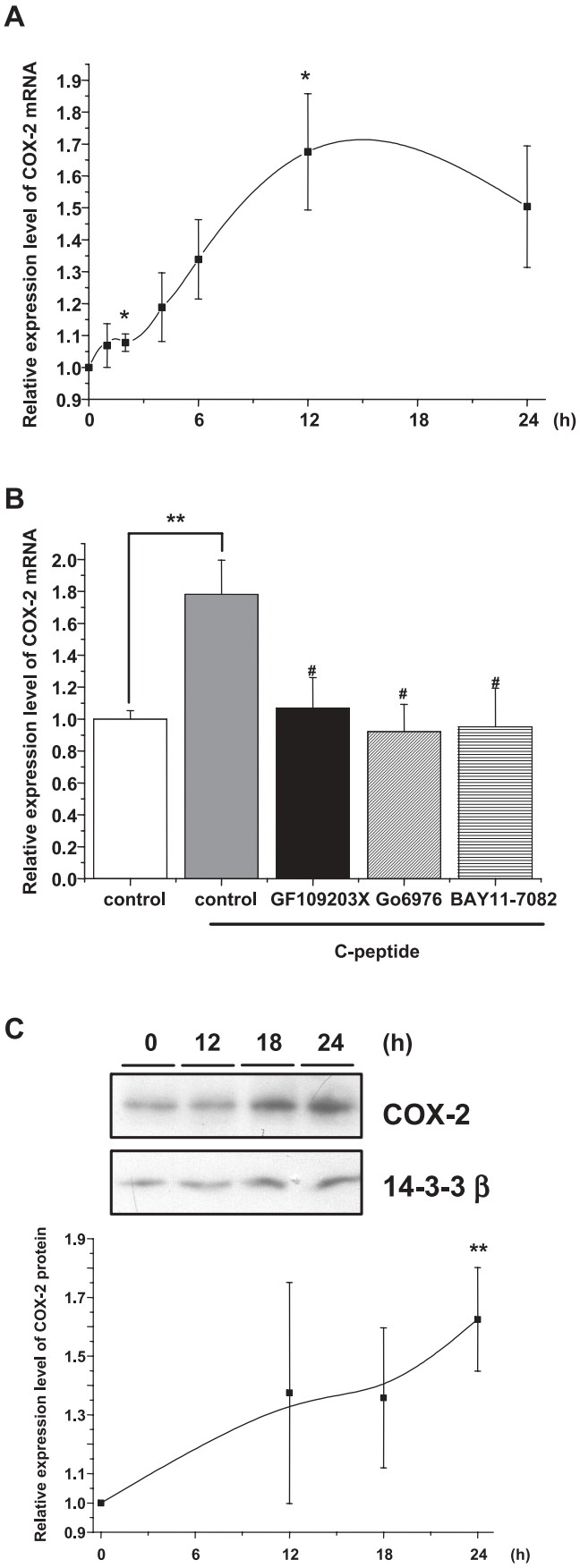
Western Blotting—Swiss 3T3 cells were seeded on 35-mm dishes at a density of 1.0×10^5 cells/dish and then cultured for 24 h. For Western blot analysis of COX-2 expression, cells were incubated in serum-free medium with or without mouse C-peptide for the indicated times. For Western blot analysis of the total IκB expression level and its phosphorylation, cells were incubated in Hank's buffer with or without mouse C-peptide for the indicated times. After incubation, cells were washed two times with PBS and then lysed with lysis buffer (1 mM EDTA, 1% SDS, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml chymostatin, 10 μg/ml antipain, 10 μg/ml phosphoramidon, 1 mM *p*-APMSF, 0.32 μM okadaic acid, 10 nM calyculin A, 10 mM NaF, 1 mM Na-orthovanadate, 50 μM bpV, 10 mM HEPES-HCl, pH 7.5). Cell lysates were boiled and centrifuged to obtain supernatants as cell extracts, which were subjected to Western blot analyses as described previously (*22*).

Plasmids—The fragment corresponding to nucleotide positions –327 to +59 from the transcription start site of the human PES-2 gene, of which the product catalyzes the rate-limiting step in the biosynthesis of prostaglandins (*23*), was generated by polymerase chain reaction (PCR) using 5′-GGG GGG TAC CAC TAC CCC CTC TGC TCC CAA ATT GGG GCA G-3′ (forward), 5′-GGG GGG CTA GCG CGC TGC TGA GGA GTT CCT GGA CGT GCT CC-3′ (reverse), and the human genome as a template. The fragment corresponding to nucleotide residues –220/+59 without an NF-κB–responsive element was generated by polymerase chain reaction (PCR) using 5′-GGG GGG TAC CAC TAC CCC CTC TGC TCC CAA ATT GGG GCA G-3′ (forward), 5′-GGG GGG CTA GCG CGC TGC TGA GGA GTT CCT GGA CGT GCT CC-3′ (reverse), and phPES(–327/+59) as a template. These PCR fragments were digested at *Kpn*I/*Nhe*I sites and then inserted into the *Kpn*I/*Nhe*I sites of pGL4.0 (Promega). Each construct was verified by DNA sequence analysis with a DNA sequencer, ABI 3700 (Applied Biosystems, Foster City, CA, USA).

Transfection and Reporter Gene Assay—Swiss 3T3 cells were seeded on 48-well plates at a density of 1.0×10^4 cells/well and then cultured for 24 h. Cells were co-transfected with 0.05 μg/well of pNF-κB-Luc, a firefly luciferase reporter construct containing five repeated NF-κB–responsive elements (*22*), phPES2(–327/+59)-Luc, a firefly luciferase reporter construct containing the human COX-2 gene promoter fragment with the NF-κB–responsive element, or phPES2 (–220/+59)-Luc without the NF-κB–responsive element, and 0.01 μg/well of a *Renilla* luciferase control vector (phRG-TK) using Plus reagent and Lipofectamine reagent according to the published method (*24*). Relative transcription activities were measured as described previously (*22*).

RESULTS AND DISCUSSION

COX-2 is the rate-limiting enzyme in the conversion of arachidonic acid (AA) to prostaglandin and is related to diseases, including inflammation and diabetes (*15*, *16*, *25*). Because C-peptide triggers multiple intracellular biochemical reactions sensitive to pertussis toxin in Swiss 3T3 fibroblasts, as reported previously (*6*), we first examined whether C-peptide affected the expression of COX-2 mRNA in Swiss 3T3 fibroblasts by quantitative PCR. C-peptide at 1 nM, which is close to the physiological range (1–3 nM) in human, increased the expression of COX-2 mRNA within 12 h after treatment (Fig. 1A). NF-κB has been reported to act as an intracellular signaling molecule downstream of PKC (*17*). Since Kitamura *et al.* have also reported that C-peptide stimulates PKC activity (*6*), we examined whether NF-κB was required for this stimulatory effect of C-peptide. The increase at 12 h after treatment was abolished by pretreatment with 1 μM GF109203X and Go6976, specific PKC inhibitors, or 1 μM BAY11-7082, a specific NF-κB inhibitor (Fig. 1B). Furthermore, treatment with 1 nM C-peptide augmented the expression of COX-2 mRNA to increase the expression of its protein at 24 h (Fig. 1C). Next, to determine whether this increase in the expression of COX-2 protein induced by C-peptide resulted from triggering of COX-2 gene transcription, the transcription activity of the COX-2 gene was analyzed by reporter gene assay (Fig. 2). Swiss 3T3 fibroblasts were transfected with phPES2 (–327/+59)-Luc, a human COX-2 reporter gene containing an NF-κB–responsive element, to assay the luciferase activity at 24 h after treatment with or without



1 nM C-peptide. As shown in Fig. 2A, in Swiss 3T3 fibroblasts, C-peptide increased the transcription activity of phPES2 (–327/+59)-Luc, which was inhibited by pretreatment with 1 μM GF109203X or Go6976, and 1 μM BAY11-7082 for 15 min, whereas C-peptide had no enhancing effects on the transcription activity of phPES2 (–220/+59)-Luc, in which the NF-κB–responsive element was not included (Fig. 2B), providing evidence that C-peptide triggers COX-2 gene transcription via a PKC/NF-κB–mediated signaling pathway.

Activation of NF-κB has been demonstrated to participate in induction of the gene expression of COX-2 (*19*). Therefore the present finding that C-peptide augments both COX-2 gene transcription and its protein expression raises the possibility that C-peptide stimulates NF-κB–dependent transcription. Expectedly, C-peptide increased NF-κB–dependent transcription (Fig. 3). Since the phosphorylation and subsequent degradation of IκBα are well known to serve as the mechanism underlying the activation of NF-κB–dependent transcription resulting from the nuclear translocation of NF-κB from the cytoplasm (*17–19*), the effects of C-peptide on the phosphorylation and degradation of IκBα were further examined by Western blotting. An increase in the level of phosphorylated IκBα was observed at 10 min after treatment with 1 nM C-peptide and the phosphorylation level reached the maximum level at 30 min after the treatment. After a further 30 min, the level of IκBα protein was observed to decrease (Fig. 4A), but to have returned to the initial level at 12 h after the treatment (Fig. 4B).

In the present study we obtained the first evidence that C-peptide activates the IκB/NF-κB system *via* a PKC-mediated signaling pathway to enhance COX-2 gene transcription, and thereby increases its protein expression in Swiss 3T3 fibroblasts, although the mechanism by which C-peptide produces a sustained increase in expression of COX-2 protein remains to be elucidated, suggesting the ability of C-peptide to stimulate NF-κB–dependent gene transcription *via* a PKC/IκB-mediated mechanism. NF-κB plays a central role in cytokine-induced β cell apoptosis as well as in the transcription activation of inflammatory cytokine genes (*17*, *18*) and cytokine-inducible genes, including COX-2 (*19*), in

Fig. 1. **Increasing effects of C-peptide on the mRNA and protein expression of COX-2 in Swiss 3T3 cells.** Cells were pre-incubated in serum-free DMEM containing 1 μM GF109203X or Go6976, specific PKC inhibitors, or 1 μM BAY11-7082, a specific NF-κB inhibitor, for 15 min, and then incubated in serum-free DMEM containing 1 nM mouse C-peptide for the indicated times. Preparation of total cellular RNA and cell extracts was conducted as described under "MATERIALS AND METHODS." Quantification of COX-2 mRNA expression was performed with PRISM7700 (A and B; $n = 4$). The expression of COX-2 mRNA is presented as the relative expression level normalized as to that of GAPDH mRNA. In C, proteins included in the cell extracts were separated on a 7% SDS-PAGE gel, followed by immunoblotting with anti–COX-2 and anti–14-3-3-β antibodies (upper panel). 14-3-3-β protein was used as a loading control. Each column represents the density of a specific single band corresponding to COX-2 protein normalized as to the expression of 14-3-3-β protein (lower panel). Values represent the means ± SEM for three independent experiments ($n = 4$). $^{*}p < 0.05$, $^{**}p < 0.01$ *versus* vehicle control; $^{\#}p < 0.05$ versus cells treated with C-peptide alone. Similar results were obtained in at least three independent experiments.

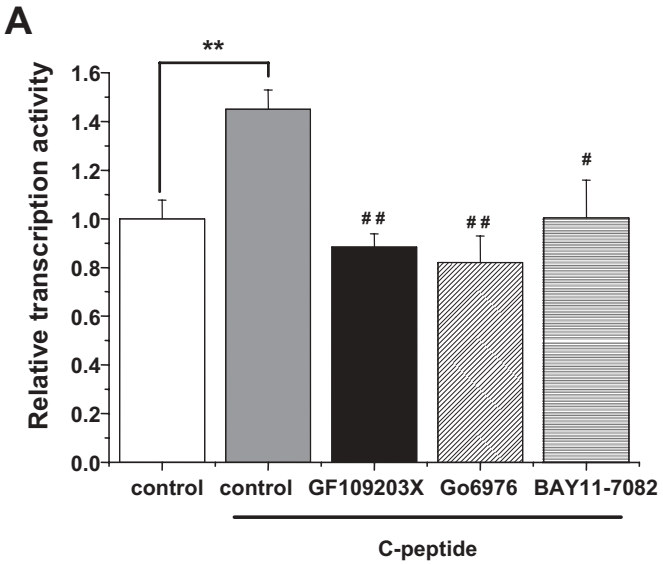


Fig. 2. **PKC-dependent stimulatory effect of C-peptide on COX-2 gene transcription in Swiss 3T3 cells.** Cells were co-transfected with phPES2 (–328/+59)-Luc (A) or phPES2 (–220/+59)-Luc (B), and a phRG-TK plasmid. After transfection, the cells were pre-incubated in serum-free DMEM containing 1 μM GF109203X, Go6976 or BAY11-7082 for 15 min, and then incubated in serum-free DMEM containing 1 nM mouse C-peptide for 24 h. Cells lysates were collected to assay the luciferase activity as described under "MATERIALS AND METHODS." Relative luciferase activity was calculated as the ratio of firefly luciferase activity to *R. reniformis* luciferase activity. Each column represents the mean ± SEM (n = 6). $^{**}p < 0.01$ *versus* vehicle control; $^{\#}p < 0.05$ *versus* cells treated with C-peptide alone. Similar results were obtained in at least three independent experiments.

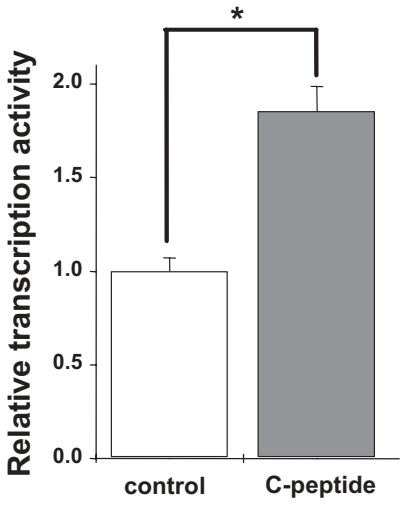


Fig. 3. **Stimulatory effect of C-peptide on NF-αB–dependent transcription in Swiss 3T3 cells.** Cells were co-transfected with pNF-κB-Luc and phRG-TK plasmids. After transfection, the cells were incubated in serum-free DMEM containing 1 nM mouse C-peptide for 24 h. Luciferase activity assay was performed as described in the legend to Fig. 2. Each column represents the mean ± SEM ($n = 6$). $^{*}p < 0.05$ *versus* vehicle control. Similar results were obtained in at least three independent experiments.

β cells. Pancreatic β-cells coexpress insulin and COX-2, and PGE_2 has been reported to inhibit glucose-induced insulin secretion in pancreatic β-cell line (*26*). C-peptide can also elicit a biochemical response in the pancreatic islet cells probably via activation of a membrane receptor coupled to

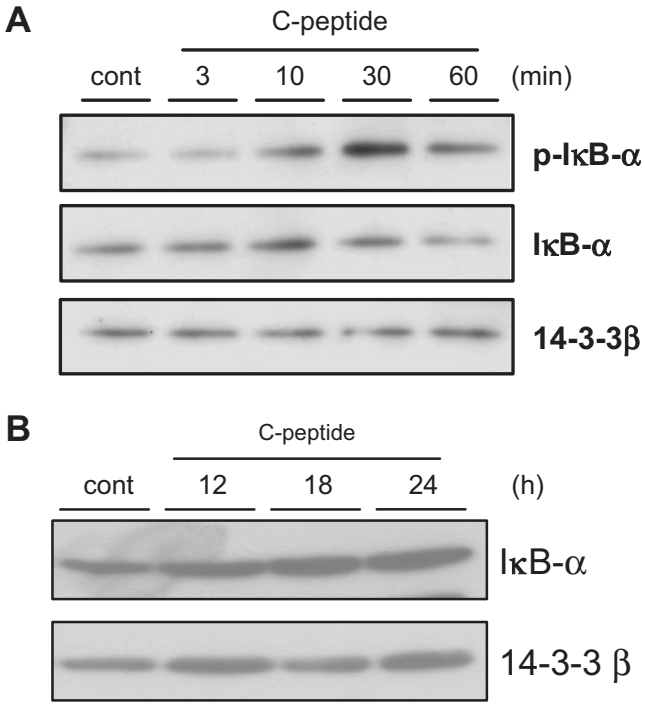


Fig. 4. **Stimulatory effects of C-peptide on phosphorylation and degradation of IκBα protein in Swiss 3T3 cells.** Cells were stimulated with 1 nM mouse C-peptide for the indicated times. Cell lysates were prepared as described under "MATERIALS AND METHODS," and fractionated on a 10% SDS-PAGE gel, followed by immunoblotting with anti–phospho-IκBα and anti–IκBα antibodies. 14-3-3-β protein was used as a loading control. Similar results were obtained in at least two independent experiments.

a pertussis toxin–sensitive G-protein (*2*). It is thus plausible that C-peptide may serve as an important modulator of insulin secretion in pancreatic β-cells in physiological circumstances, and be implicated in the progression towards diabetes, especially type 1 diabetes.

The most important finding in the present study is that C-peptide itself has the ability to stimulate NF-κB–dependent gene transcription *via* a PKC/IκB-mediated mechanism in Swiss 3T3 fibroblasts. It has been reported that in SH-SY5Y neuroblastoma cells, C-peptide alone little affects high-glucose–induced apoptosis, but exerts an anti-apoptotic effect on the apoptosis *via* activation and translocation of NF-κB in the presence of insulin (*27*). Together with the reported result, the finding in the present study suggests that activation of NF-κB–dependent gene transcription by C-peptide appears to be differentially controlled in a cell-type–dependent manner.

In summary, we found that proinsulin C-peptide activated a PKC/IκB/NF-κB signaling cascade, and thereby enhanced the transcription activity of the COX-2 gene to upregulate its protein expression in Swiss 3T3 fibroblasts. These results suggest that C-peptide may play crucial roles in the control of insulin secretion from pancreatic β-cells and in the pathogenesis of type 1 diabetes. Elucidation of the molecular and cellular mechanisms underlying C-peptide–induced modulation of inflammatory gene expression *via* NF-κB–dependent transcription may lead to the development of new therapies for diabetes.

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