

Proinsulin C-peptide activates cAMP response element-binding proteins through the p38 mitogen-activated protein kinase pathway in mouse lung capillary endothelial cells

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Proinsulin C-peptide has been reported to have some biological activities and to be possibly involved in the development of diabetic microangiopathy. In the present study, we examined the effects of C-peptide on the mitogen-activated protein kinase pathway in LEII mouse lung capillary endothelial cells. Stimulation of the cells with C-peptide increased both p38 mitogen-activated protein kinase (p38MAPK) and extracellular signal-regulated kinase (ERK1/2) activities and activity-related site-specific phosphorylation of the respective kinases in a concentration-dependent manner, but failed to activate c-Jun N-terminal kinase. Stimulation of the cells with C-peptide also induced site-specific phosphorylation of cAMP response element (CRE)-binding protein (CREB)/activating transcription factor 1 (ATF1), and

thereby binding of these transcription factors to CRE. Among three CREB kinases tested, phosphorylation of mitogen-activated protein kinase-activated protein kinase 2 (MAPKAP-K2) was induced after stimulation with C-peptide. The phosphorylation of CREB, ATF1 and MAPKAP-K2 were inhibited by SB203580, a p38MAPK inhibitor, but not by PD98059, an ERK kinase inhibitor. These results indicate that C-peptide activates p38MAPK followed by MAPKAP-K2 to enhance DNA–CREB/ATF1 interactions.

Key words: activating transcription factor (ATF), extracellular signal-regulated kinase (ERK), insulin, c-Jun N-terminal kinase (JNK), MAPK-activated protein kinase 2 (MAPKAP-K2).

INTRODUCTION

Insulin and C-peptide, a connecting segment of proinsulin, are secreted simultaneously from pancreatic β -cells into the circulation. In contrast to insulin, C-peptide had been considered biologically inactive, but increasing evidence strongly suggests that C-peptide has hormonal activities of its own [1], possibly through a stereospecific receptor protein [2,3].

Administration of C-peptide to patients or animals with Type I diabetes has been shown to ameliorate symptoms of diabetic complications, including renal [4–6] and microvascular [7–9] dysfunctions. Short-term infusion of C-peptide also improves the autonomic nerve functions, mainly parasympathetic components, in patients with polyneuropathy and Type I diabetes mellitus [10]. Administration of C-peptide to rats antagonizes sympathetically mediated stress responses in an atropine-dependent manner [11]. These findings imply that the effects of C-peptide on renal and vascular functions may be, at least in part, attributed to alteration of the balance between sympathetic and parasympathetic nerve activities.

In addition to these indirect effects, C-peptide has been shown to induce dilation of isolated skeletal arterioles in the presence of insulin [12], suggesting a direct action of C-peptide on the vascular system. This is also supported by the findings that C-peptide inhibits leucocyte–endothelium interactions, increases mRNA expression of endothelial nitric oxide synthase in the lung, and enhances NO production in aortic tissue [13].

We previously demonstrated activation by C-peptide of p44/42 mitogen-activated protein kinase (MAPK), also known as an

extracellular signal-regulated kinase (ERK), in Swiss3T3 fibroblasts [14]. The ERK pathway has been shown to be involved in endothelial nitric oxide synthase gene expression [15,16]. Thus, we assumed that C-peptide might stimulate the MAPK pathways and activate transcription factors in lung capillary endothelial cells.

To test this hypothesis, we determined the effects of C-peptide on phosphorylation and activation of ERK and two stress-activated protein kinases, p38MAPK and c-Jun N-terminal kinase (JNK), in LEII mouse lung capillary endothelial cells, and compared them with the effects of insulin. Furthermore, the effects of C-peptide on transcription factors downstream of the MAPK-signalling pathways were also examined.

EXPERIMENTAL

Materials

Human C-peptide was generously provided by Eli Lilly Co. (Indianapolis, IN, U.S.A.). Reverse sequence (retro)- or all-D-amino acid human C-peptides were obtained from BioSynthesis (Lewisville, TX, U.S.A.). Rat C-peptides 1 and 2 were bought from Yanaihara Institute (Shizuoka, Japan). Bovine insulin was purchased from Sigma (St. Louis, MO, U.S.A.). Following antibodies were purchased from Cell Signalling Technology (Beverly, MA, U.S.A.): phospho-specific ERK1/2 (p44/42MAPK, Thr-202/Tyr-204), ERK1/2, phospho-specific p38MAPK (Thr-180/Tyr-182), p38MAPK, phospho-specific JNK (Thr-183/Tyr-185), JNK, phospho-specific cAMP response element (CRE)-binding protein (CREB; Ser-133), phospho-specific activating transcrip-

Abbreviations used: ATF, activating transcription factor; CRE, cAMP response element; CREB, CRE-binding protein; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKAP-K, MAPK-activated protein kinase; MEK, ERK kinase; MSK, mitogen- and stress-activated protein kinase; NF κ B, nuclear factor κ B; p38MAPK, p38 mitogen-activated protein kinase; RSK, ribosomal S6 kinase.

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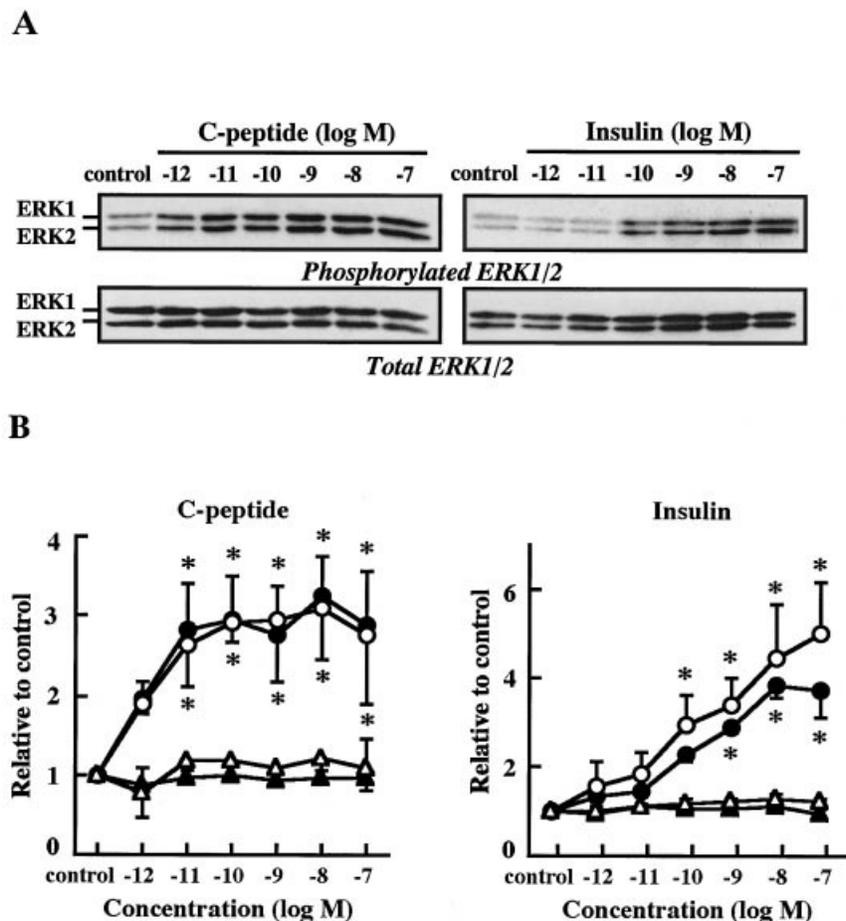


Figure 1 Effects of C-peptide and insulin on phosphorylation of ERK1/2 (p44/42MAPK) in LEII cells

LEII mouse lung capillary endothelial cells were stimulated with increasing concentrations of human C-peptide (left-hand panels) or bovine insulin (right-hand panels) for 3 min. Cell lysates were prepared as described in the Experimental section, and were subjected to analysis using antibodies against ERK1/2 and phospho-specific ERK1/2. (A) Representative results of Western blot analysis are shown. (B) The amounts of ERK1 (\blacktriangle), ERK2 (\triangle) and phosphorylated ERK1 (\bullet) and ERK2 (\circ) are expressed relative to those of non-stimulated control cells. Data are means \pm S.E.M. for three independent experiments. * $P < 0.05$ versus controls.

tion factor (ATF) 2 (Thr-71), phospho-specific c-Jun (Ser-73), c-Jun, phospho-specific MAPK-activated protein kinase 2 (MAPKAP-K2) (Thr-334), phospho-specific mitogen- and stress-activated protein kinase (MSK) 1 (Ser-360) and phospho-specific p90 ribosomal S6 kinase (RSK; Thr-360/Ser-364). Antibody to phospho-specific JNK (Thr-183/Tyr-185) was also bought from Promega (Madison, WI, U.S.A.). Antibodies to CREB, ATF2 and MAPKAP-K2 were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Antibodies to CREB and phospho-specific CREB also reacted with ATF1 and phosphorylated ATF1 (Ser-63), respectively, since the phosphorylated epitope of ATF1 is identical to that of CREB [17]. Antibodies to MSK1 and p90RSK were bought from Upstate Biotechnology (Lake Placid, NY, U.S.A.).

Cell culture and treatments

LEII mouse lung capillary endothelial cells [18] were cultured on 100 mm dishes in Dulbecco's modified Eagle's medium (Gibco-BRL, Gaithersburg, MD, U.S.A.) supplemented with 10% fetal bovine serum in 5% CO₂ in humidified air at 37 °C. After cells were grown to confluence, they were cultured in a

serum-free medium for 16–24 h to render them quiescent. The cells were then treated with C-peptide, insulin or PMA (100 nM; Sigma), and controls with vehicle (PBS) at 37 °C. Subsequently, the cells were washed twice with PBS and lysed with 1 ml of ice-cold lysis buffer [50 mM Hepes (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10 mM sodium pyrophosphate, 2 mM NaVO₃, protease inhibitor mixture (Complete™; Boehringer Mannheim, Mannheim, Germany) and 1% Nonidet P-40]. The lysate was kept on ice for 30 min and centrifuged at 15000 g for 20 min at 4 °C. The resulting supernatant was stored at –80 °C. Proteins were determined by the method of Lowry et al. [19] using BSA as a standard.

Treatment of cells with 50 μ M PD98059 [an ERK kinase (MEK) inhibitor; Biomol Research Laboratories, Plymouth Meeting, PA, U.S.A.] or 20 μ M SB203580 (a p38MAPK inhibitor, Wako Pure Chemical Co., Osaka, Japan) for 2 h. The treated cells were then stimulated by C-peptide for 3 min.

Western-blot analyses

Aliquots of the supernatant (40 μ g of protein) were separated by SDS/PAGE and transferred on to PVDF membranes

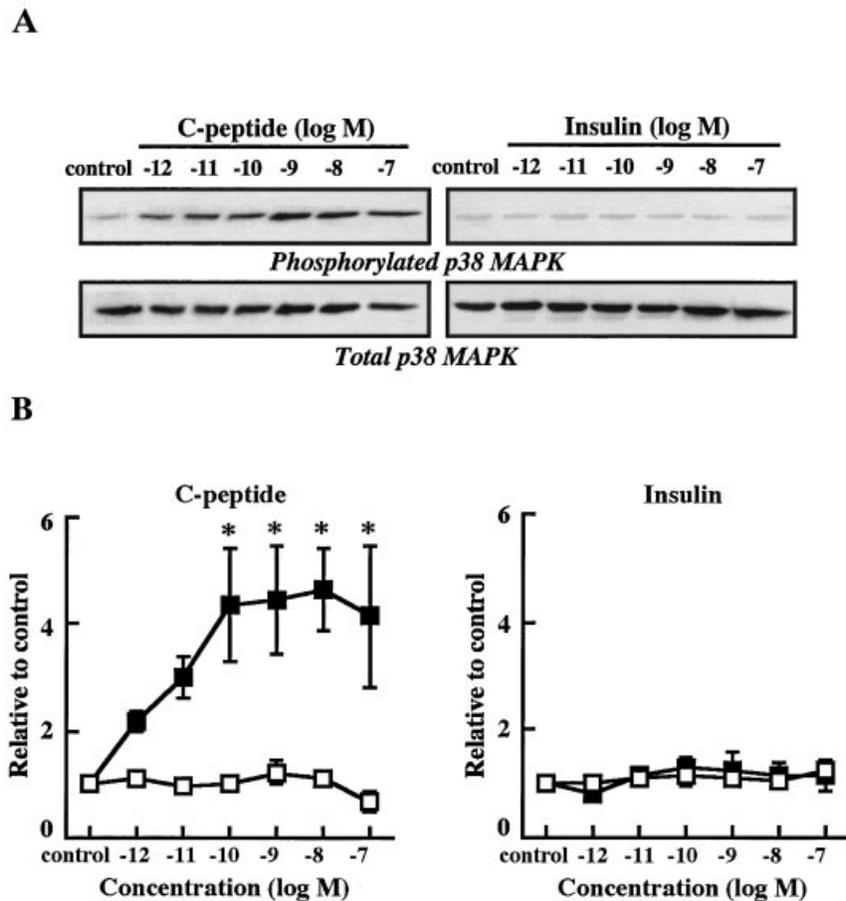


Figure 2 Effects of C-peptide and insulin on phosphorylation of p38MAPK in LEII cells

LEII cells were stimulated as in the legend for Figure 1, and cell lysates were analysed by using antibodies against phospho-specific p38MAPK and p38MAPK (total protein). (A) Representative results of Western blot analysis are shown. (B) The amounts of phosphorylated and total p38MAPK (■ and □ respectively) are expressed relative to those of non-stimulated control cells. Data are means \pm S.E.M. for three independent experiments. * $P < 0.05$ versus controls.

(Immobilon™; Millipore, Bedford, MA, U.S.A.). The membranes were incubated in a blocking buffer [20 mM Tris/HCl (pH 7.5)/150 mM NaCl] containing 0.1% Tween 20 and 5% skimmed milk, and then in the buffer containing an antibody. The bound antibody was visualized using horseradish peroxidase-linked goat anti-rabbit immunoglobulin (Zymed Laboratories, South San Francisco, CA, U.S.A.) and an enhanced chemiluminescence system (Amersham, Little Chalfont, Bucks., U.K.). The intensity of chemiluminescence for the corresponding bands was analysed by using NIH Image, a public-domain image-processing and analysis program.

MAPK assays

MAPK activities were measured using ERK1/2 (p44/42MAPK), p38MAPK and SAPK/JNK assay kits (Cell Signalling Technology), according to the manufacturer's recommended protocols. Briefly, LEII cells were treated with either PBS or 1 nM human C-peptide for 3 min, washed with ice-cold PBS and lysed in a buffer [20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM β -glycerophosphate, 2.5 mM sodium pyrophosphate, 1 mM NaVO_3 , protease inhibitor mixture (Complete™) and 1% Triton X-100]. After centrifugation at 15000 g for 10 min at 4 °C, the supernatant was subjected to

immunoprecipitation with the beads conjugated with either phospho-specific ERK1/2 (Tyr-204) monoclonal antibody, phospho-specific p38MAPK (Thr-180/Tyr-182) monoclonal antibody or c-Jun fusion protein. Each immunoprecipitate was washed twice with ice-cold buffer for cell lysis, twice with ice-cold kinase buffer [25 mM Tris/HCl (pH 7.5), 5 mM β -glycerophosphate, 2 mM dithiothreitol, 0.1 mM NaVO_3 and 10 mM MgCl_2], and finally resuspended in the kinase buffer containing ATP and a corresponding substrate for respective kinases. The reaction mixtures were incubated for 30 min at 30 °C, and then boiled in SDS-gel loading buffer. The samples were subjected to SDS/PAGE followed by Western blot analyses using either antibody to phospho-specific Elk-1 (Ser-383) for ERK, phospho-specific ATF2 (Thr-71) for p38MAPK or phospho-specific c-Jun (Ser-63) for JNK. MAPK activities were quantified by scanning the X-ray film as described above.

Gel mobility-shift assays

Nuclear extract was prepared as follows: LEII cells were treated with either PBS or 1 nM human C-peptide for 3 min, washed with ice-cold PBS and suspended in 800 μ l of a buffer consisting of 20 mM Hepes (pH 7.9), 2 mM MgCl_2 , 1 mM EDTA, 1 mM EGTA, 1 mM NaVO_3 , 20 mM NaF, 1.25 mM dithio-

threitol and protease inhibitor mixture (Complete™). Nuclei were then pelleted by centrifugation at 1000 *g* for 30 s, and the supernatant was carefully removed. The nuclei were resuspended in 400 μ l of the buffer containing 0.2% Nonidet P-40 and immediately centrifuged at 10000 *g* for 1 min. The resulting pellet was incubated with the buffer containing 420 mM KCl and 20% glycerol for 30 min at 4 °C, and the sample was centrifuged and the nuclear proteins in the supernatant were transferred to a fresh vial and stored at -80 °C.

A double-stranded oligonucleotide probe containing the consensus CRE-binding site (Santa Cruz Biotechnology) was labelled with [γ -³²P]ATP by polynucleotide kinase (Promega). Nuclear extract (15 μ g) was incubated with 10 fmol of probe in 20 μ l of binding buffer [10 mM Hepes (pH 7.9), 30 mM NaCl, 1 mM EDTA, 5 mM MgCl₂, 5% glycerol, 0.1% Nonidet P-40, 1 mg/ml BSA and 1 μ g of poly(dI-dC)] for 30 min at room temperature. For competition assays, a 100-fold molar excess of unlabelled consensus oligonucleotide probe or mutant oligonucleotide probe with an AC-to-TG substitution in the CRE-binding motif (Santa Cruz Biotechnology) was added to nuclear extracts for 20 min before addition of the labelled probe. The supershift assay was performed by incubating nuclear extract with 1 μ g of antibody against CREB/ATF1, ATF2 or nuclear factor κ B (NF κ B) p65 (Santa Cruz Biotechnology) for 10 min at room temperature before addition of the labelled probe. After incubation, samples were subjected to 4% PAGE in 25 mM Tris/HCl (pH 8.5), 190 mM glycine and 1 mM EDTA. The gel was subsequently dried and the DNA-protein complexes were detected by autoradiography.

Statistical analysis

Data were expressed as means \pm S.E.M. and analysed by ANOVA followed by Fisher's protected least-squares difference or Student's *t* test. A *P* value of less than 0.05 was considered to be statistically significant.

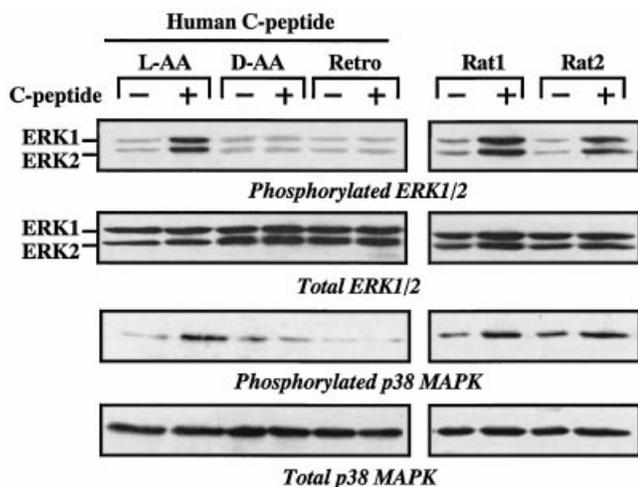


Figure 3 Effects of various types of C-peptide on phosphorylation of ERK1/2 and p38MAPK in LEII cells

LEII cells were treated with PBS (-) or 1 nM human C-peptide (L-AA), all D-amino acids human C-peptide (D-AA), retro-sequenced human C-peptide (retro), rat C-peptide 1 (Rat1) or rat C-peptide 2 (Rat2) for 3 min. Cell lysates were subjected to Western blot analysis of p38MAPK and ERK1/2. Representative results from three independent experiments are shown.

RESULTS

C-peptide activates ERK1/2 and p38MAPK, but not JNK in LEII cells

Both proinsulin C-peptide and insulin activate ERK1/2 in Swiss3T3 cells [14]. To examine whether this is also the case in LEII mouse lung capillary endothelial cells, the cells were incubated with the two peptides and phosphorylation of ERK1/2 at Thr-202 and Tyr-204 necessary for kinase activation were determined. As shown in Figure 1, C-peptide at 10 pM and higher concentrations induced a significant increase of ERK1/2 phosphorylation, without noticeable effects on the total amount of ERK1/2. Similarly, insulin caused phosphorylation of ERK1/2 in LEII cells dose-dependently from 100 pM (Figure 1). The effects of C-peptide and insulin on site-specific phosphorylation of p38MAPK (Thr-183/Tyr-185) were also determined in LEII cells. As shown in Figure 2, C-peptide increased p38MAPK phosphorylation in a concentration-dependent fashion, without changes in the total amount of p38MAPK. In contrast, insulin did not elicit p38MAPK phosphorylation at all (Figure 2). We also determined whether C-peptide and insulin activated JNK in

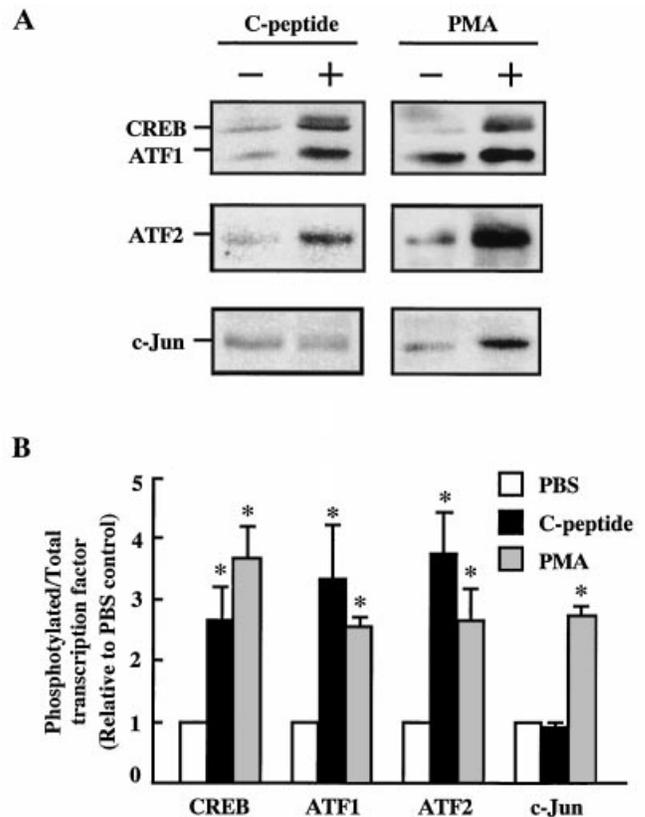


Figure 4 Effects of C-peptide on phosphorylation of transcription factors in LEII cells

LEII cells were stimulated with PBS (-, white columns), human C-peptide (1 nM; +, black columns) for 3 min, or PMA (100 nM; +, grey columns) for 10 min. The cell lysates were analysed by using antibodies against phospho-specific CREB/ATF1, ATF2 and c-Jun, and antibodies to respective nuclear factors to determine total amounts of protein. (A) Representative results of Western blot analysis using phospho-specific antibodies are shown. (B) The ratio of phosphorylated nuclear factor to total amount of the protein was expressed relative to that in PBS control cells. Data are means \pm S.E.M. for three independent experiments. **P* < 0.05 versus PBS controls.

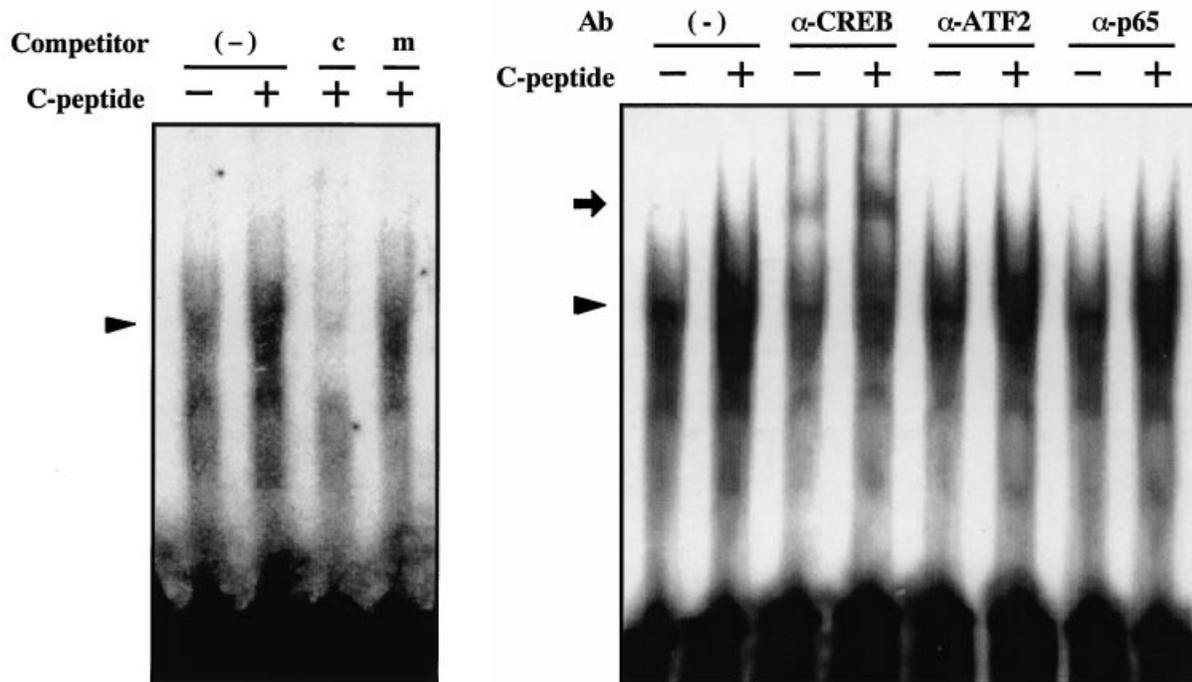


Figure 5 Effects of C-peptide on the interaction of nuclear factors and an oligonucleotide containing the CRE consensus sequence

Left-hand panel: LEII cells were treated with PBS (–) and human C-peptide (1 nM, +) for 3 min. Nuclear extract was incubated with ^{32}P -labelled oligonucleotide containing the CRE consensus sequence, and then the mixture was fractionated by 4% PAGE. Protein–oligonucleotide complex is indicated by an arrowhead. For competition assay, nuclear extract was first incubated with either 100-fold excess amounts of unlabelled consensus (c) or mutant (m) oligonucleotide, and subsequently with the labelled probe. Right-hand panel: the supershift assay was performed by incubating nuclear extract with antibody (Ab) against CREB/ATF1, ATF2 or NF κ Bp65 for 10 min before addition of the labelled probe. The supershifted band is indicated by an arrow.

LEII cells, but no clear signal for phosphorylated JNK was detected even after using two different antibody sources under our experimental conditions (results not shown).

To confirm that the C-peptide-induced phosphorylation of ERK1/2 and p38MAPK activates these enzymes, kinase activities of ERK1/2 and p38MAPK were measured. The ERK1/2 and p38MAPK activities increased 3.1 ± 0.1 -fold ($n = 4$) and 2.9 ± 0.3 -fold ($n = 5$), respectively, after a 3 min stimulation with C-peptide (1 nM) compared with those treated with PBS ($P < 0.05$). The kinase activity of JNK remained unchanged even after stimulation with C-peptide [1.1 ± 0.1 -fold ($n = 4$)], while it increased 2.7 ± 0.8 -fold ($n = 4$) after treatment with PMA.

The ability of some other forms of C-peptide to activate ERK1/2 and p38MAPK was also examined. Similarly to human C-peptide, rat C-peptides 1 and 2 induced phosphorylation of both enzymes, while neither retro-sequenced nor all-D-amino acid human C-peptide induced phosphorylation (Figure 3).

C-peptide activates CREB/ATF1 transcription factors through the p38MAPK-mediated pathway

Transcription factors are important targets of the MAPK superfamily. For example, both ERK1/2 and p38MAPK stimulate ATF2 [20,21]. CREB and ATF1 have been identified as substrates for RSK-B [22], MSK1 [23] and MAPKAP-K2 [24,25], all of which are direct effectors of p38MAPK. CREB also serves as a substrate for RSK, a downstream target of the ERK pathway [25,26]. Thus, to determine whether C-peptide activates the CREB/ATF family proteins, phosphorylation of CREB (Ser-

133), ATF1 (Ser-63) and ATF2 (Thr-71) was examined. As shown in Figure 4, stimulation of LEII cells with C-peptide significantly increased phosphorylation of CREB. C-peptide also elicited phosphorylation of ATF1 and ATF2, but not of c-Jun, a substrate of JNK. Activation of protein kinase C by transient PMA treatment caused phosphorylation of both c-Jun and CREB/ATF family proteins.

To confirm that the C-peptide-induced phosphorylation of the CREB/ATF family proteins actually promotes the binding of these proteins to DNA, gel mobility-shift assays were performed by using an oligonucleotide probe containing the consensus nucleotide sequence of CRE. Nuclear extract from the cells stimulated with C-peptide enhanced formation of the oligonucleotide–protein complex, which disappeared in the presence of unlabelled consensus oligonucleotide, but not of a mutant oligonucleotide (Figure 5, left-hand panel). Moreover, the mobility of the complex was retarded by addition of an antibody to CREB/ATF1, but not of those against ATF2 (related) and NF κ Bp65 (unrelated; Figure 5, right-hand panel).

Next, the involvement of ERK1/2 and/or p38MAPK in the C-peptide-induced phosphorylation of CREB/ATF1 was determined. As shown in Figure 6, pre-treatment of the cells with SB203580, a p38MAPK inhibitor, completely prevented the C-peptide-induced phosphorylation of CREB/ATF1 and activation of p38MAPK, while it did not affect ERK1/2 phosphorylation. In contrast, pre-treatment of the cells with PD98059, a MEK inhibitor, had no effect on the C-peptide-induced phosphorylation of CREB/ATF1, while it inhibited the ERK1/2 phosphorylation without affecting p38MAPK activity.

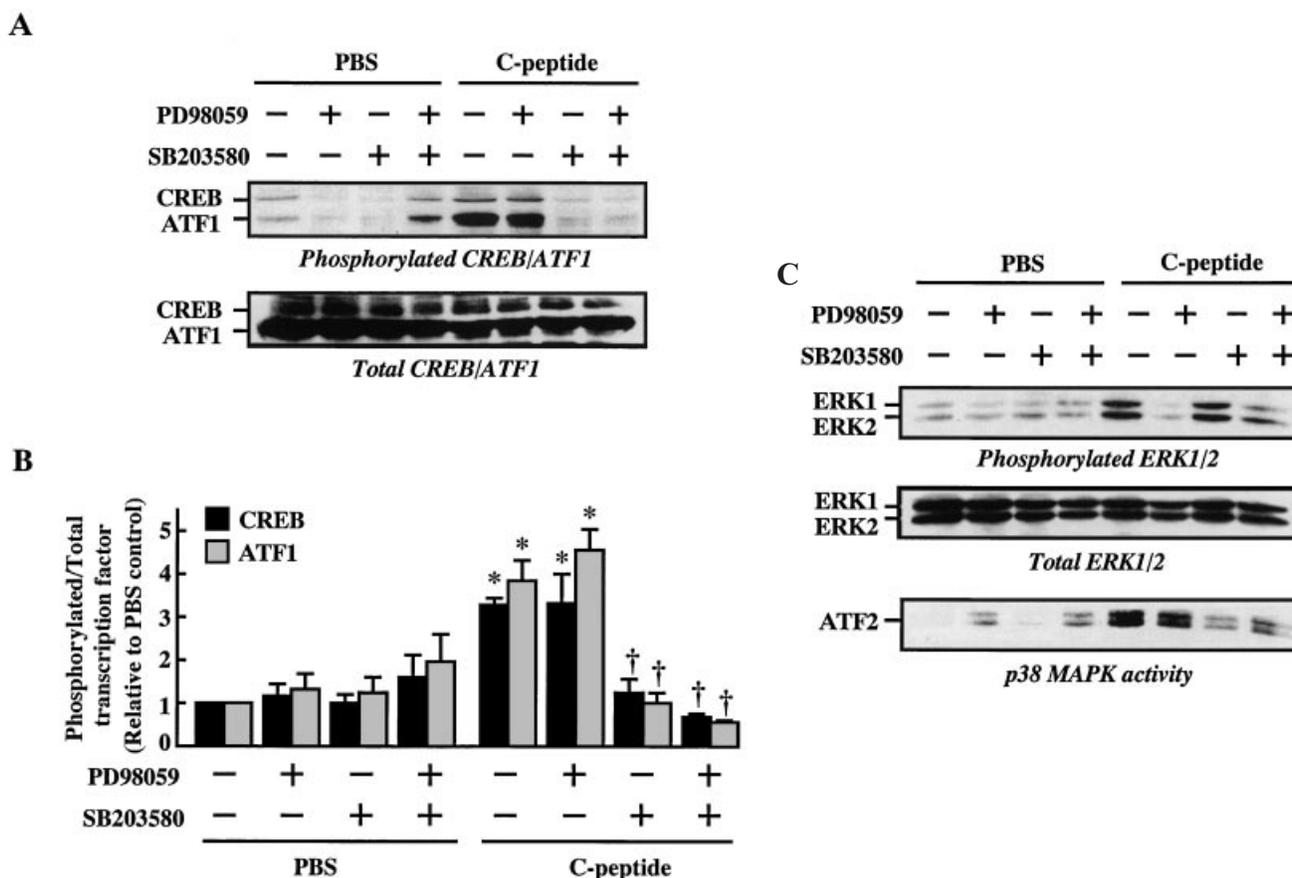


Figure 6 Effects of inhibition of MEK and/or p38MAPK on C-peptide-induced phosphorylation of CREB/ATF1 in LEII cells

LEII cells were treated with PD98059 (a MEK inhibitor, 50 μ M) and/or SB203580 (a p38MAPK inhibitor, 10 μ M) for 2 h, and stimulated with PBS or C-peptide (1 nM) for 3 min. The cell lysates were analysed by using antibodies against phospho-specific and total CREB/ATF1 and ERK1/2, and also used for measurement of p38MAPK activity. (A) Representative results of Western blot of CREB/ATF1 are shown. (B) The ratio of phosphorylated CREB and ATF1 to total amount of the proteins are expressed relative to those of PBS control cells without inhibitor. Data are means \pm S.E.M. for three independent experiments. * $P < 0.05$ versus PBS controls. † $P < 0.05$ versus without inhibitor treatment. (C) Representative results of a Western blot of ERK1/2 and p38MAPK activity (phosphorylation of ATF2) are shown.

Since p38MAPK has been shown not to directly phosphorylate CREB and ATF1, activation of MSK1, MAPKAP-K2 and RSK, which may be responsible for the phosphorylation [23–26], were examined by detecting activity-related site-specific phosphorylation of these kinases. As shown in Figure 7, stimulation with C-peptide increased phosphorylation of MAPKAP-K2 and RSK, but failed to induce phosphorylation of MSK1. The phosphorylation of MAPKAP-K2 was largely inhibited by SB203580, but not by PD98059 (Figure 7). In contrast, the phosphorylation of RSK was prevented by PD98059, but not by SB203580.

DISCUSSION

In the present study, we demonstrated that proinsulin C-peptide induced rapid phosphorylation and activation of p38MAPK in LEII mouse lung capillary endothelial cells. Stimulation of the cells with C-peptide also elicited phosphorylation and activation of ERK1/2 (p44/42MAPK), confirming our previous findings in Swiss3T3 and 3T3-F442A cells [14]. However, C-peptide failed to induce phosphorylation and activation of JNK, another member of the MAPK family, and c-Jun.

Both p38MAPK and ERK1/2 phosphorylation increased dose-dependently and reached maximal levels at 100 pM

C-peptide. These effective concentrations of C-peptide are comparable with the K_{ass} value (2.0–3.3 nM) obtained by an *in vitro* binding study [2] and with circulating concentrations (100 pM–1 nM) of C-peptide in healthy subjects [27–29]. Thus, it might be that C-peptide fully interacts with its putative receptor(s) and activates the p38MAPK and ERK pathways under normal physiological conditions. Since the MAPK pathways are known to regulate various cellular processes, thereby controlling cell survival and adaptation [20], our results imply that deficiency of C-peptide may cause some vascular dysfunction in the case of Type I diabetes mellitus.

At present, information about a putative receptor for C-peptide is limited. Rigler et al. [2] have detected the binding of C-peptide to cell membranes from fibroblast and endothelial cells, which was displaced by unlabelled native C-peptide (L-form), but not by all D-amino acid or scrambled C-peptide, using the fluorescence correlation spectroscopy method [2]. In line with their results, we found that native C-peptide, but neither D-form nor retro-sequenced C-peptide, induced phosphorylation of both p38MAPK and ERK1/2, suggesting the presence of a stereospecific receptor for C-peptide in capillary endothelial cells. Moreover, rat C-peptides 1 and 2, which have nine amino acid substitutions compared with human C-peptide, were also effective

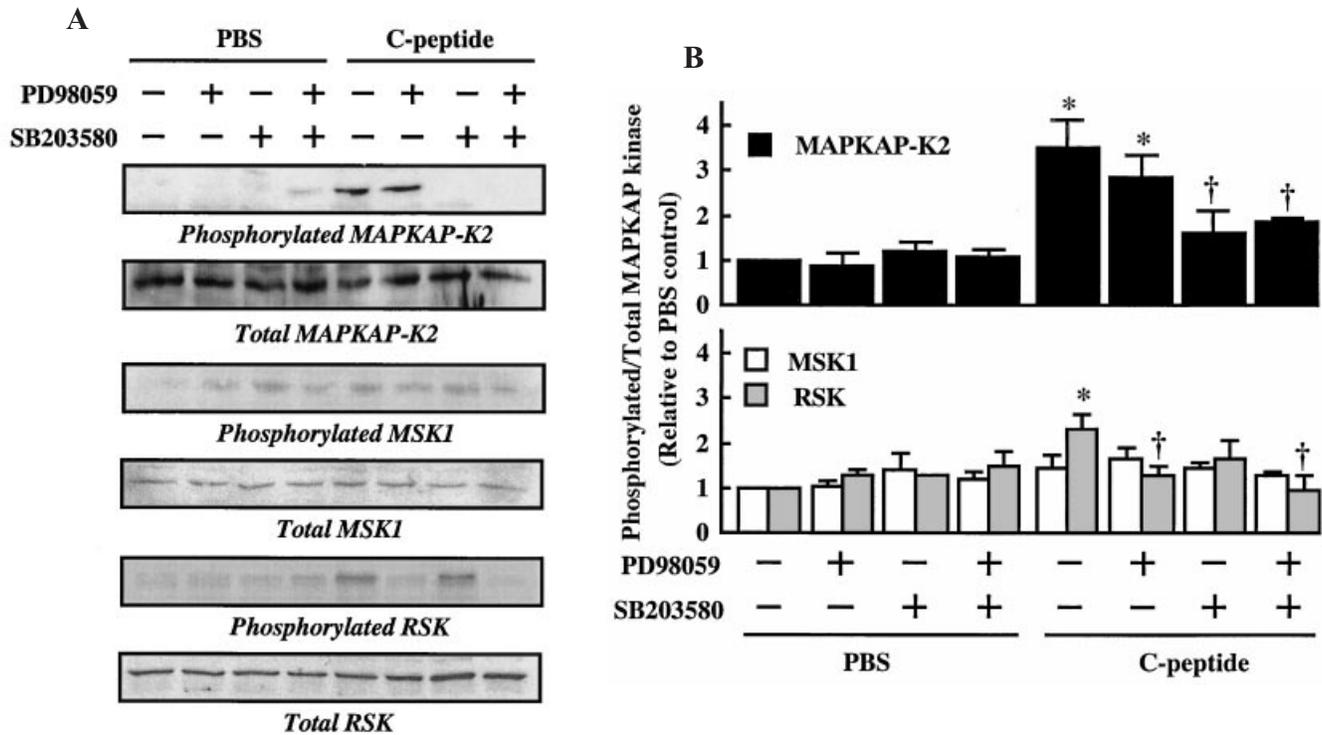


Figure 7 Effects of inhibition of MEK and/or p38MAPK on C-peptide-induced phosphorylation of three CREB kinases in LEII cells

LEII cells were treated with PD98059 and/or SB203580 for 2 h, and stimulated with PBS or C-peptide (1 nM) for 3 min. The cell lysates were analysed by using antibodies against phospho-specific and total MAPKAP-K2, MSK1 and RSK. Left-hand panel: representative results of the Western blot are shown. Right-hand panel: the ratios of phosphorylated to total amount of respective kinases are expressed relative to those of PBS control cells without inhibitor. Data are means \pm S.E.M. for three independent experiments. * $P < 0.05$ versus PBS controls. † $P < 0.05$ versus without inhibitor treatment.

in MAPK phosphorylation, indicating that these substitutions may not alter the C-peptide structure essential for its interaction with the receptor.

C-peptide is known to have some insulinomimetic effects. For example, administration of C-peptide to patients or animals with Type I diabetes increases glucose uptake in skeletal muscle [8,30,31]. More recently, Li et al. [32] reported that C-peptide enhances glycogen synthesis in L6 myoblasts, possibly through a crosstalk mechanism with the insulin-signalling pathway at the level of the insulin receptor [32]. In addition, insulin activates p38MAPK in 3T3-L1 adipocytes [33,34] and isolated skeletal muscle [35], and the ERK pathway in 3T3-L1 cells [14,33,36]. However, in LEII cells insulin showed no stimulatory effect on p38MAPK activation, while it activated the ERK pathway. These results indicate that the activation of p38MAPK by C-peptide is based on some insulin-independent signalling pathway.

In the present study, we also demonstrated that C-peptide induced the rapid phosphorylation of CREB and ATF1 and their association with the oligonucleotide containing the CRE. As the phosphorylation and DNA binding of CREB and ATF1 are usually followed by activation of CRE-dependent transcription [23,37], it would be expected that C-peptide promotes CRE-dependent gene expression in capillary endothelial cells. CREB is known to be phosphorylated by various protein kinases, including protein kinase A, Ca²⁺- and calmodulin-dependent protein kinase IV (CaMK IV), RSK, RSK-2, MAPKAP-K2, MSK1 and protein kinase B (Akt) [20–26,37–39]. The C-peptide-induced phosphorylation of CREB and ATF1 were completely inhibited by a p38MAPK inhibitor, but not by a MEK inhibitor,

suggesting that some kinases activated by p38MAPK, such as MAPKAP-K2 or MSK1, may be responsible for the phosphorylation. In LEII cells, C-peptide stimulated phosphorylation of MAPKAP-K2, but not of MSK1, which was selectively inhibited by a p38MAPK inhibitor. These results suggest that MAPKAP-K2 is involved, at least in part, in the C-peptide-induced phosphorylation and activation of CREB under control of p38MAPK. RSK is unlikely to be involved in the C-peptide-induced CREB phosphorylation, since C-peptide-induced phosphorylation of RSK was prevented by a MEK inhibitor, but not by a p38MAPK inhibitor.

ATF2 is a member of the CREB/ATF family and binds to CRE as a homodimer or heterodimer with c-Jun [40]. Transcriptional activity of ATF2 is controlled by phosphorylation at Thr-69 and Thr-71 by stress-activated kinases, including p38MAPK [20,21,40]. In the present study, C-peptide also induced phosphorylation of ATF2, but it did not elicit its binding to CRE (Figure 5). These results suggest a minor contribution of ATF2 to CRE-dependent transcription in LEII cells.

In summary, we have demonstrated that proinsulin C-peptide induces site-specific phosphorylation and activation of p38MAPK and ERK1/2, but not of JNK, in LEII lung endothelial cells. Since insulin induces phosphorylation of ERK1/2, but not of p38MAPK, in LEII cells, C-peptide may utilize different signalling pathways from those of insulin. C-peptide-induced p38MAPK activation causes phosphorylation of CREB and ATF1, and their binding to CRE, inducing activity-related phosphorylation of MAPKAP-K2, a CREB kinase. Collectively, C-peptide activates CREB/ATF1 through the p38MAPK path-

way and thereby may promote CRE-dependent gene expression in capillary endothelial cells.

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