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Proinsulin C-peptide induces c-Jun N-terminal kinase 1 expression in LEII mouse lung capillary endothelial cells

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Abstract

To characterize the roles of C-peptide in vascular homeostatic processes, we examined the genes regulated by C-peptide in LEII mouse lung microvascular endothelial cells. Treatment of the cells with C-peptide increased the expression of c-Jun N-terminal kinase 1 (JNK1) mRNA dose-dependently, accompanied by an increase in JNK1 protein content. Prior treatment of the cells with PD98059, an ERK kinase inhibitor or SB203580, a p38MAPK inhibitor, abrogated the C-peptide-elicited JNK1 mRNA expression. These results indicate that C-peptide increases JNK1 protein levels, possibly through ERK- and p38MAPK-dependent activation of JNK gene transcription.

Key words: C-peptide, diabetes mellitus, JNK

C-peptide, a connecting segment of proinsulin, is secreted from pancreatic β -cells into the circulation along with insulin. In contrast to insulin, C-peptide had been considered biologically inert, but increasing evidence strongly suggests that C-peptide has its own hormonal activity¹⁷⁾. For instance, the administration of C-peptide has been shown to diminish leakage of albumin and fluorescein across the blood-retinal barrier^{8,9)}, indicating the ameliorating effects of C-peptide on diabetes-induced microvascular dysfunction^{3,6,8,9)}.

Although no receptor for C-peptide has been identified, it was shown that specific binding sites for C-peptide are present on the surface of human renal tubular cells, endothelial cells, and fibroblasts using fluorescence correlation spectroscopy¹⁴⁾. In addition, it was shown that C-peptide activates protein kinase C (PKC) and subsequently mitogen-activated protein kinase (MAPK), which is also known as extracellular signal-regulated kinase (ERK), in the aforementioned cells^{10-12,18)}, and the ERK pathway has been shown

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to be involved in eNOS gene expression in the aortic endothelium^{12,16}. Moreover, stimulation of LEII mouse lung capillary endothelial cells with C-peptide led to considerable activation of a different type of MAPK in endothelial cells, namely p38MAPK, and through that to the activation of transcription factors such as cyclic AMP response element-binding protein (CREB) and activating transcription factor 1 (ATF1)¹¹. However, it is still unclear which genes are regulated by C-peptide in microvascular endothelial cells.

To further characterize the roles of C-peptide in vascular homeostasis, we examined C-peptide-regulated genes by macro array analysis using Atlas Rat Toxicology 1.2 Array II nylon membrane (Clontech, Palo Alto, CA, USA). In brief, total RNA was extracted from LEII cells treated with C-peptide or PBS for 1 hr using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and poly (A)⁺ RNA was prepared from pooled RNA from three independently treated-plates after it had been subjected to DNase treatment. The membrane was hybridized with ³²P-cDNA probes overnight at 68°C and analyzed with an image analyzer (BAS-2500, Fuji film, Tokyo, Japan) and Array Gauge software (Fuji film). Based on the differences between the treatments, four genes of interest were selected, tumor necrosis factor receptor 1 (TNFR1), Fas antigen, N-cadherin, and c-Jun N-terminal kinase 1 (JNK1).

The expression of these genes was examined further by RT-PCR. In brief, the primers used were mouse TNFR1 (GenBank accession number: NM_011609, a primer for the amplification of the 512-840nt region of the coding region), forward: 5'-CTG CAG GGAGTG TGA AAA GG-3', reverse: 5'-CCT CAT TTT TCT TGC AGT GG-3'; mouse Fas antigen (M83649, 248-556nt), forward: 5'-GAG GAC TGC AAA ATG AAT GG, reverse: 5'-GCG ATT TCT GGG ACT TTG TT-3'; mouse N-cadherin (AB008811, 1055-1435nt), forward: 5'-CAT CAA TGG CAA TCA AGT GG-3', reverse: 5'-GCT GTG GCT GTG TTT GAA AG-3'; mouse JNK1 (NM_016700, 1135-1433nt), forward-

5'-CTC TCC AGC ACC CAT ACA TC-3', reverse: 5'-CTG TAT CCG AGG CCA AAG TC-3'; and mouse GAPDH (M32599, 52-345nt), forward: 5'-GAAGGTCGGTGTGAACGGATT-3', reverse: 5'-AAGACACCAGTAGACTCCACGA-3'. The PCR conditions involved initial denaturation at 94°C for 5 min, 25 cycles (JNK1 and Fas antigen) or 27 cycles (TNFR1, N-cadherin and GAPDH) of denaturation at 94°C for 30 sec; annealing at 57°C

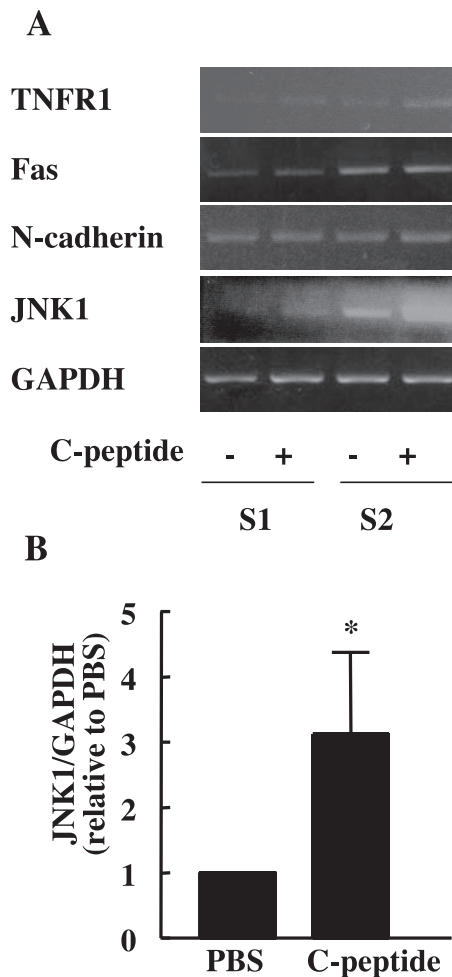


Fig. 1. C-peptide selectively up-regulates JNK1 gene expression in LEII cells. LEII cells were stimulated with either PBS (-) or C-peptide (+, 1 nM) for 1 hr. Then, their RNA was isolated, and RT-PCR was performed for the TNFR1, Fas, N-cadherin, JNK1 and GAPDH genes. Representative results of two independent samples (S1, S2) are shown in (A). The mRNA expression of JNK1 was quantified by real time RT-PCR in (B). Data are shown as means \pm SEM (n = 11) of values relative to the expression of the control cells and were analyzed using the Student's *t*-test. *, *p* < 0.0001 vs. PBS

(TNFR1 and N-cadherin), 59°C (JNK1), or 60°C (Fas antigen, GAPDH) for 30 sec; and extension at 72°C for 1 min, and final extension at 72°C for 7 min. The PCR products were analyzed in 1.5% agarose gel stained with ethidium bromide and verified by direct DNA sequencing. As a result, treatment with C-peptide clearly increased JNK1 mRNA expression in two independent samples, but failed to augment the expression of TNFR1, Fas antigen or N-cadherin mRNA (Fig. 1A).

The induction of a significant increase in JNK1 expression by C-peptide treatment was further confirmed by real-time RT-PCR (Fig. 1B), which was performed as follows: the RT products and cloned cDNA for JNK1 and GAPDH were amplified with specific primers and SYBR Green I Master mix (Roche Diagnostics, Mannheim, Germany) and detected with a fluorescence thermal cycler (Light Cycler System, Roche). The amplification program involved an initial denaturation step at 95°C for 30 sec; followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 62°C for 5 sec, and extension at 72°C for 10 sec; and finally heating to 95°C.

In addition, the cell lysate was extracted from

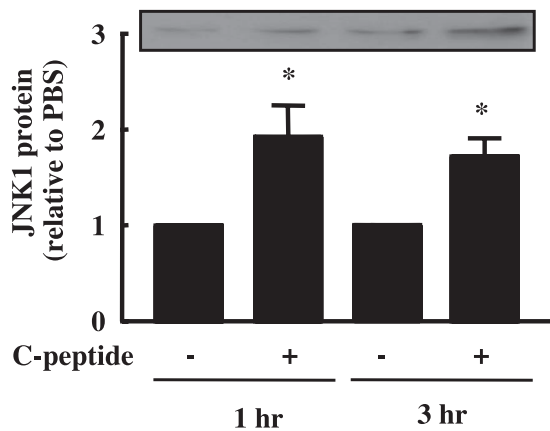


Fig. 2. C-peptide increases the amount of JNK1 protein in LEII cells. LEII cells were stimulated with either PBS (-) or C-peptide (+, 1 nM) for 1 hr and 3 hr, and the expression of JNK1 protein was analyzed. Representative blots and quantitative changes in JNK1 protein levels are shown. Data are shown as means \pm SEM ($n = 4$) of the intensities of the bands, expressed as fold increase above the value for PBS treatment, and were analyzed using the Student's *t*-test. *, $p < 0.01$ vs. the corresponding PBS treatment.

LEII cells treated with C-peptide or PBS for 1 hr or 3 hr using ice-cold lysis buffer containing 5 mM EDTA, 10 mM Na pyrophosphate, 2 mM NaVO₃, protease inhibitor mixture (Complete™, Boehringer Mannheim, GmbH, Germany) and 1% Nonidet P-40 and was centrifuged at 15,000 \times g for 20 min at 4°C. Aliquots of the supernatant (40 μ g of protein) were analyzed for JNK1 protein by the Western Blot method using JNK antibody (1:1000, Cell Signaling Technology, Beverly, MA), and the intensity of the protein bands produced was analyzed using NIH Image, a public domain image processing and analysis program. As shown in Fig. 2, treatment of the cells with C-peptide significantly increased JNK1 protein expression. The increase in JNK1 mRNA was dependent on the C-peptide concentration, and it was induced

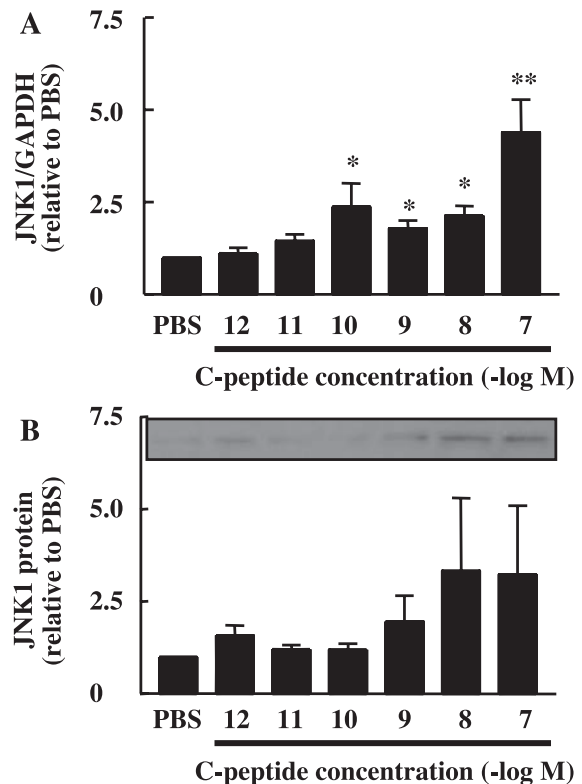


Fig. 3. C-peptide dose-dependently increases JNK1 mRNA expression. LEII cells were treated with either PBS or increasing concentrations of C-peptide for 1 hr. The mRNA expression (A) and protein amount (B) of JNK1 were measured by real time RT-PCR and Western blotting. Data are shown as means \pm SEM ($n = 3$) and were analyzed by ANOVA followed by Fischer's post hoc test. *, $p < 0.05$ vs. PBS; **, $p < 0.0001$ vs. PBS

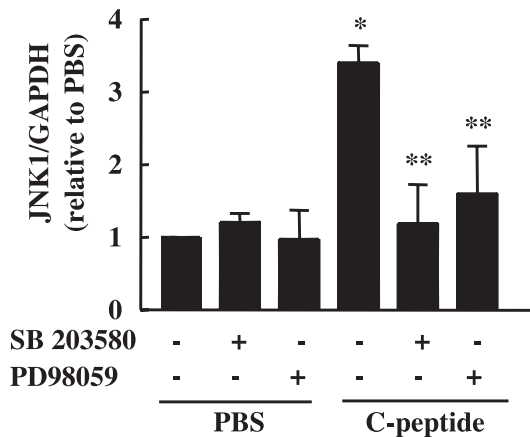


Fig. 4. ERK and p38MAPK are involved in C-peptide-induced JNK1 mRNA expression. LEII cells were treated with either PD98059 or SB203580 for 2 hr and then stimulated with either PBS or C-peptide (1 nM) for 1 hr. The mRNA expression of JNK1 was quantified by real time RT-PCR. Data are shown as means \pm SEM (n = 5) of values relative to the expression of PBS control cells without inhibitor treatment and were analyzed by ANOVA followed by Fischer's post hoc test. *, $p < 0.001$ vs. PBS without inhibitor; **, $p < 0.005$ vs. C-peptide without inhibitor

at concentrations as low as 0.1 nM (Fig. 3A), while the concentration of JNK1 protein tended to increase at 1nM and above (Fig. 3B).

To examine the involvement of MAPK pathways in C-peptide-induced JNK1 expression, the cells were treated with either PD98059 (an ERK kinase inhibitor, 50 μ M, Biomol Research Laboratories, Plymouth Meeting, PA, USA) or SB203580 (a p38MAPK inhibitor, 20 μ M, Calbiochem, La Jolla, CA, USA) for 2 h before C-peptide treatment. As shown in Fig. 4, the increased expression of JNK1 mRNA induced by C-peptide was abrogated by both PD98059 and SB203580. Transcription of the mouse JNK1 gene is positively controlled by activator protein 2 (AP-2), a transcription factor⁴. C-peptide has been shown to stimulate a PKC/I κ B/NF- κ B signaling pathway in fibroblasts, leading to COX-2 gene transcription¹³, which is also regulated by PKC/p38MAPK/AP-2 signaling⁵. Moreover, ERK is involved in AP-2-dependent transcription of matrix metalloproteinase 2 and G protein-coupled receptor kinase 3^{7,15}. Collectively, the present results suggest that C-peptide increases JNK1

protein levels, possibly through JNK gene transcription via ERK- and p38MAPK-dependent activation of AP-2.

The physiological and patho-physiological relevance of *de novo* induction of JNK1 by C-peptide is currently unknown. It has been reported that C-peptide activates JNK kinase activity as well as ERK in human renal tubular cells¹⁹, but JNK activation was not seen in LEII cells¹¹. As JNK1 is known to regulate cell growth, motility, apoptosis, and inflammatory responses^{1,2}, the up-regulation of JNK1 protein expression by C-peptide might play a role in the maintenance of the integrity of microvascular endothelium cells.

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