EXPERIMENTAL STUDY

C-peptide increases the expression of vasopressin-activated calcium-mobilizing receptor gene through a G protein-dependent pathway

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Abstract

Objective: Although an increasing number of reports suggest that physiological concentrations of C-peptide protect against the development of diabetic nephropathy, possibly through the modulation of Na–K pump activity, the intracellular pathways controlled by C-peptide are still unrecognized. C-peptide and vasopressin share similar intracellular effects including the activation of calcium influx and endothelial nitric oxide synthase. Both hormones stimulate also the activity of Na–K pump activity. Whether the activity of C-peptide is mediated by the recently identified vasopressin-activated calcium-mobilizing receptor (VACM-1) has never been previously investigated.

Design and methods: To clarify this issue, we evaluated the effect of C-peptide on VACM-1 RNA (measured by semiquantitative RT-PCR) and protein expression (measured by immunoblotting) in human skin fibroblasts (where a specific binding of C-peptide was demonstrated) and in human mesangial cells, the cellular target of diabetic nephropathy.

Results: C-peptide-induced activation of VACM-1 was demonstrated in fibroblasts from six healthy individuals (0.51 ± 0.1 vs 1.48 ± 0.4 , arbitrary units $\pm$ S.E., $P = 0.025$). This finding was paralleled by an increased VACM-1 protein expression (5.64 ± 1.0 vs 8.47 ± 1.2 , arbitrary units $\pm$ S.E., $P = 0.043$). Similar results were confirmed in three independent cultures of human mesangial cells. VACM-1 activation in fibroblasts was insensitive to phosphatidylinositol-3-kinase inhibitor LY294002, but was inhibited by pertussis toxin, suggesting that activation of VACM-1 could be mediated by a G protein-coupled receptor.

Conclusions: This study demonstrates for the first time that C-peptide activates VACM-1, possibly through a G protein-coupled receptor. Further studies are needed to clarify whether VACM-1 is involved in the protective effect of C-peptide against the development of diabetic nephropathy.

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Introduction

Although for a long time thought to have no biological function, C-peptide – the product, along with insulin, of the cleavage of proinsulin (1, 2) – is now considered a peptide hormone, independent of insulin, possibly acting through a G protein-coupled membrane receptor (3–5). Recent evidence suggests that C-peptide might have a protective role in the development of diabetic complications (6–9). In particular, the finding that pancreas transplantation induces a reversal of diabetic nephropathy (10), along with the recent evidence that type 1 diabetic patients with kidney and islet transplants have a better renal prognosis than patients transplanted with kidney alone (11), has suggested the hypothesis that C-peptide might protect the diabetic kidney from the development of nephropathy (12, 13). In line with these findings, we found a decreased C-peptide/creatinine ratio in the plasma of type 1 diabetic

patients with nephropathy when compared with normoalbuminuric patients of similar age and duration of diabetes (14). How C-peptide can possibly exert its beneficial effect is presently unknown. In animal models Na–K pump activity is decreased by hyperglycemia and C-peptide reverses this dysfunction (6) by modulating a not yet completely understood intracellular pathway (5). Activation of the Na–K pump by C-peptide was also demonstrated in man (15), but whether the protection by C-peptide against diabetic complications is mediated by this mechanism remains unclear. Of interest, recent findings indicate that C-peptide increases calcium influx and stimulates endothelial nitric oxide synthase (16, 17), possibly through the activation of the mitogen-activated protein (MAP) kinase (18).

Among the modulators of the Na–K pump activity, vasopressin has an established agonist role (19, 20) and it was also shown to increase nitric synthase

expression levels (21). A new receptor for vasopressin, the vasopressin-activated calcium-mobilizing receptor (VACM-1) has been recently demonstrated. This receptor was first identified in the rabbit (22) and its presence was then extended also to humans (23). Human VACM-1 gene is localized on chromosome 11q22-23 (23) and is a member of the cullin gene family expressed in vascular endothelial cells and medullary collecting tubule cells (24). Although the intracellular functions of VACM-1 are not yet completely understood, a contribution of this gene to the regulation of cell signaling (25) and in particular of the MAP kinase cascade, an intracellular pathway involved in the control of Na-K pump activity (26), has been recently demonstrated (27).

As the full comprehension of the intracellular activity of C-peptide could be of great importance toward the understanding of the pathogenesis of diabetic complications, the aim of the present study was therefore to clarify whether VACM-1 expression is modulated by C-peptide.

Materials and methods

The study was approved by the Ethics Committee of the San Raffaele Scientific Institute, Milan, Italy and informed consent was obtained from all participants. Forearm skin biopsies were obtained from six healthy non-diabetic individuals.

Synthetic human C-peptide was prepared by Primm SRL, Milan, Italy. The sequence of human C-peptide has been previously published (28). The purity of synthetic C-peptide was higher than 95% by HPLC.

Culture of fibroblasts

A skin specimen of approximately 2 mm³ was taken by excision under local anesthesia from an avascular area of the anterior aspect of the forearm. Cells were grown to confluence in 60 mm culture dishes in minimum Eagle's medium (MEM) supplemented with 10% fetal calf serum. After four passages, fibroblasts were harvested and frozen in liquid nitrogen in several aliquots. Cells were then thawed and grown as previously described (29). The experiments were performed in subconfluent cells at the sixth and seventh subculture.

Experimental procedure

Human skin fibroblasts were thawed and grown to confluence in two 90 mm culture dishes in the same medium as described above. When at confluence, cells were split and seeded (50 000 cell per dish) in dishes containing MEM supplemented with 10% human serum with low C-peptide concentration (see below). The medium was changed every 7 days until the cells reached subconfluence (2 weeks). At this point the medium was changed once again and to the dishes

was added the same medium either containing or not containing 1.0 nmol/l C-peptide.

Human serum with low C-peptide concentration

An additive effect of C-peptide cannot be appreciated using serum from normal animals or healthy subjects (3, 4) because full saturation of the receptor specific for C-peptide will have occurred already in these conditions. To perform the above described experiments we therefore used a pool of sera from type 1 diabetic patients containing virtually no C-peptide.

A blood sample (20 ml) was obtained from ten patients affected by type 1 diabetes of more than 10 years' duration, who were informed of the experimental procedure and gave their consent. The blood sample was taken from fasting patients only if blood glucose was lower than 6.5 mmol/l according to a capillary blood glucose monitoring device. One hundred milliliters of serum were finally pooled, and laboratory analysis gave the following results: glucose: 6.0 mmol/l, C-peptide: 0.036 nmol/l, insulin: 120 pmol/l. The serum pool was then frozen at -80 °C.

RNA extraction

Total RNA was extracted from cultured skin fibroblasts using RNAfast reagent (Molecular System, San Diego, CA, USA) and frozen at -80 °C. cDNA was retrotranscribed using the SuperScript II Reverse Transcriptase (Gibco, Gaithersburg, MD, USA).

Semiquantitative RT-PCR

RT-PCR primers were designed along the sequence of VACM-1 (GenBank AC NM 003478): forward primer (position 321-341), 5'-GCACGAGTACTGAGCCATCA-3' and reverse primer (position 684-704) 5'TGCCTTCT-CAAAATTGTCCC-3', amplified portion of the sequence 321-704; size 384 bp).

In the case of β -actin: forward primer 5'-TGACGGG-GTCAACCCACACTGTGCCCATCTA-3' and reverse primer 5'-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3' were used for amplification of a 646 bp fragment of human β -actin cDNA located between nucleotides 2198 and 3065 of the reported human gene sequence (GenBank AC E00829).

PCR conditions were optimized for Mg²⁺ concentration, primer concentration, primer annealing temperature and number of cycles for VACM-1 and β -actin cDNA amplification. VACM-1 cDNA fragments were amplified through 32, 36 and 40 cycles, and β -actin fragments through 22, 26 and 30 cycles, using the following amplification profile: 40 s at 94 °C; 30 s at 59 °C (VACM-1)/61 °C (β -actin); and 50 s at 72 °C.

After amplification, PCR products were separated by electrophoresis on a 1% agarose gel and were allowed

to react with ethidium bromide for band visualization. The optical density of bands was quantified by Scion Image densitometry (National Institute of Health, Bethesda, MD, USA). Finally, expression of VACM-1 was normalized by β -actin values. The effect of C-peptide on VACM-1 expression was confirmed in fibroblasts from the same individuals as described above grown to confluence with MEM supplemented with 10% fetal calf serum and then made quiescent by substitution of the culture medium with MEM supplemented with 0.3% fetal calf serum.

Immunoblotting

Total proteins of human skin fibroblasts or human mesangial cells treated either with or without C-peptide for 24 h were extracted in Laemmli buffer (Tris-HCl 62.5 mmol/l, pH 6.8, 20% glycerol, 2% SDS, 5% β -mercaptoethanol) and their concentration was finally measured according to Lowry *et al.* (30).

Thirty-five micrograms of total proteins were electrophoresed on 7% SDS-PAGE gels and blotted onto nitrocellulose (Schleicher & Schuell, Dassel, Germany). Blots were then stained with Ponceau S.

Membranes were blocked for 1 h in TBS (Tris (10 mmol/l), NaCl (150 mmol/l)), 0.1% Tween-20, 5% non-fat dry milk, pH 7.4 at 25 °C, incubated for 12 h with 200 μ g/ml of a polyclonal rabbit anti-VACM-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:200 or with a polyclonal rabbit anti- β -actin antibody (Abcam, Cambridge, MA, USA) diluted 1:1000 in TBS-5% milk at 4 °C, washed four times with TBS-0.1% Tween-20, then incubated

with a peroxidase-labeled goat anti-rabbit IgG secondary antibody diluted 1:1000 (DAKO, Glostrup, Denmark) in TBS-5% milk, and finally washed with TBS-0.1% Tween-20. The resulting bands were finally visualized using enhanced chemiluminescence (SuperSignal; Pierce, Rockford, IL, USA).

Effect of inhibitors

To clarify which class of receptors are involved in the C-peptide-induced activation of VACM-1, the effect of C-peptide on VACM-1 activity was evaluated either in the presence of LY294002, a strong inhibitor of phosphatidylinositol-3-kinase, one of the intracellular mediators of insulin action (31–33), or in the presence of pertussis toxin, the specific inhibitor of G protein-coupled receptors (34, 35). Three cultures of human skin fibroblasts were grown as described above and then treated for 24 h with or without 1.0 nmol/l C-peptide in the presence or absence of either 50 μ mol/l LY294002 or 1 μ g/ml pertussis toxin.

Titration curve and time course

Two cultures of human skin fibroblasts were grown as described above and then treated for 24 h with 0.01, 0.1, 1.0 and 10 nmol/l C-peptide to define the titration curve of C-peptide on VACM-1 RNA expression. The time course of the effect of C-peptide on VACM-1 RNA expression was also evaluated by exposing two cultures of human skin fibroblasts to 1.0 nmol/l C-peptide for different incubation times (0, 2 and 24 h and 7 days of incubation).

Figure 1 (A) VACM-1 RNA expression in human skin fibroblasts. Semiquantitative RT-PCR of VACM-1 and β -actin performed in one cell culture at three different incubation times (2 and 24 h and 7 days) in the presence (+ C-peptide) or absence (– C-peptide) of 1.0 nmol/l C-peptide. For each incubation time three different numbers of cycles of PCR were tested: 32 (lane 1), 36 (lane 2) and 40 (lane 3) for VACM-1 and 22 (lane 1), 26 (lane 2) and 30 (lane 3) for β -actin. (B) VACM-1 protein expression in human skin fibroblasts. Immunoblotting of VACM-1 and β -actin performed in two independent cell cultures incubated for 24 h in the absence (– C-pep) or presence (+ C-pep) of 1.0 nmol/l C-peptide.

Culture of human mesangial cells

Human mesangial cells were obtained from renal tissue using the technique of Striker *et al.* (36) with minor modifications. Human kidneys not suitable for transplantation or fragments of cortical tissue excised during nephrectomies were obtained from the surgical department of our Institute. Glomeruli were isolated from the renal cortex by serial sieving, digested with collagenase (type IV, 750 U/ml) and seeded in culture dishes. Glomerular cores were plated out in RPMI supplemented with 20% fetal calf serum, 5 µg/ml insulin, 5 µg/ml transferrin, 20 ng/ml selenium, 100 U/ml penicillin, 100 µg/ml streptomycin. Mesangial cell outgrowths appeared after 7–10 days in culture. Non-adherent glomeruli were removed by washing and the adherent cells were further cultured in the same medium. Cells were characterized by morphology (stellate or fusiform), and by the use of specific antibodies

(stained for smooth muscle actin and Thy-1, and not stained for cytokeratin and factor VIII), thus excluding contamination of epithelial and endothelial cells. Mesangial cells in culture were treated as previously described for human skin fibroblasts; incubation with C-peptide was stopped after 24 h. Three independent mesangial cell lines (between the sixth and eighth sub-culture) were used for these experiments.

Results

Human skin fibroblasts

RNA expression of VACM-1 was evaluated in human skin fibroblasts by semiquantitative RT-PCR and, as shown in Fig. 1A and Fig. 2A, C-peptide activation of VACM-1 was demonstrated in six individuals (0.51 ± 0.1 vs 1.48 ± 0.4 , arbitrary units $\pm$ S.E., $P = 0.025$).

To verify whether the change of RNA expression of VACM-1 was paralleled by a change of expression of its protein, we evaluated protein expression of VACM-1 by immunoblotting. As a result (Fig. 1B and Fig. 2B), VACM-1 protein expression was also increased by the addition of C-peptide (5.64 ± 1.0 vs 8.47 ± 1.2 , arbitrary units $\pm$ S.E., $P = 0.043$) and, as shown in Fig. 2C, levels of RNA and protein expression of VACM-1 were significantly correlated to one another.

Figure 2 (A) RNA expression of VACM-1 in the absence (circles) or presence (triangles) of 1.0 nmol/l C-peptide for 24 h ($*P = 0.025$). Expression levels of VACM-1 are corrected for expression levels of β -actin. (B) Protein expression of VACM-1 in the absence (circles) or presence (triangles) of 1.0 nmol/l C-peptide for 24 h ($**P = 0.043$). (C) Correlation between RNA and protein expression of VACM-1 in the absence (circles) or presence (triangles) of 1.0 nmol/l C-peptide for 24 h ($P = 0.01$).

Figure 3 (A) Titration curve of the effect of C-peptide on the activation of VACM-1 RNA expression in two human skin fibroblasts cultures. Bars show mean and S.E. (B) Time course of the effect of C-peptide on the activation of VACM-1 RNA expression in two human skin fibroblasts cultures. Results are expressed as percentage of activation where the control (time zero, no C-peptide) is considered to be equal to 100%.

Titration curve and time course

As shown in Fig. 3A and B, incubation of human skin fibroblasts with 1.0 nmol/l C-peptide for 24 h was shown to induce the larger increase of VACM-1 RNA expression and was therefore used as the standard incubation procedure to test the effect of C-peptide on VACM-1 gene and protein expression.

Effect of inhibitors of phosphatidylinositol-3-kinase and G proteins on VACM-1 expression

As shown in Fig. 4A and B, C-peptide was confirmed to activate VACM-1 expression (0.84 ± 0.2 vs 1.38 ± 0.1 , arbitrary units $\pm$ S.E., $P < 0.05$, $n = 3$). This phenomenon was not affected by the addition of 50 μ mol/l phosphatidylinositol-3-kinase inhibitor LY294002 (0.71 ± 0.03 vs 1.12 ± 0.07 , $P < 0.05$, $n = 3$) and was instead blunted by the addition of 1 μ g/ml pertussis toxin, the specific inhibitor of G proteins (0.89 ± 0.09 vs 0.70 ± 0.1 , $P = \text{NS}$, $n = 3$).

Human mesangial cells

The effect of C-peptide on VACM-1 gene and protein expression was then verified in human mesangial cells. As a result, in three independent mesangial cell cultures, VACM-1 RNA expression (0.57 ± 0.2 vs 1.13 ± 0.3 , arbitrary units $\pm$ S.E., $P = 0.024$, Fig. 5A) and expression of VACM-1 protein evaluated by immunoblotting (7.10 ± 1.5 vs 15.49 ± 3.22 , arbitrary units $\pm$ S.E., $P = 0.006$, Fig. 5B) were significantly increased by the addition of C-peptide (1.0 nmol/l for 24 h), in line with the results previously shown for human skin fibroblasts.

Discussion

The results of the present study demonstrate for the first time that C-peptide modulates VACM-1 gene expression in human skin fibroblasts and mesangial cells.

Prompted by the evidence that C-peptide (6, 15) and vasopressin (19, 20) are both established stimuli for the Na–K pump we focused our attention on VACM-1, showing by semiquantitative RT-PCR and immunoblotting that C-peptide increases both VACM-1 gene and protein expression.

The human skin fibroblast is one of the cells where a specific C-peptide binding has been suggested (4) and the evidence that C-peptide increases VACM-1 gene and protein expression in the six cultures of fibroblasts considered for the study strongly indicates this receptor as a possible mediator of C-peptide activity. On the other hand, the finding that also in the human mesangial cell C-peptide exerts a similar effect on VACM-1 expression is in line with the possibility that the C-peptide receptor might be present also in these cells.

Figure 4 (A) Effect of inhibitors on VACM-1 RNA expression in human skin fibroblasts. Semiquantitative RT-PCR of VACM-1 and β -actin performed in one cell culture after 24 h of incubation with (+C-pep) or without (– C-pep) 1.0 nmol/l C-peptide and in the presence or absence of either 50 μ mol/l phosphatidylinositol-3-kinase inhibitor LY294002 or 1 μ g/ml G protein inhibitor pertussis toxin. (B) Mean RNA expression of VACM-1 of three independent cultures of human skin fibroblasts treated for 24 h with or without 1.0 nmol/l C-peptide in the presence or absence of either 50 μ mol/l LY294002 or 1 μ g/ml pertussis toxin. * $P < 0.05$ when compared with the same cells grown without C-peptide, ** $P = \text{NS}$ when compared with the same cells grown without C-peptide.

Interestingly, the evidence that the major intracellular effects of C-peptide, i.e. increased calcium influx (16) and activation of the endothelial nitric oxide synthase (17) – paralleled by the *in vivo* finding that C-peptide in type 1 diabetic patients leads to a redistribution in skin microvascular blood flow levels (37) – are similar to the ones induced by vasopressin (21, 38) supports indirectly the hypothesis that at least part of C-peptide activity might be mediated by VACM-1 activation.

Despite several arguments confirming the existence of a specific C-peptide receptor (4), its responsible gene has not yet been identified, thus hampering the

Figure 5 (A) VACM-1 RNA expression in human mesangial cells. Semiquantitative RT-PCR of VACM-1 and β -actin performed in three independent cell cultures after 24 h of incubation in the presence (+C-pep) or absence (–C-pep) of 1.0 nmol/l C-peptide. (B) VACM-1 protein expression in human mesangial cells. Immunoblotting of VACM-1 and β -actin performed in three independent cell cultures incubated for 24 h in the absence (–C-pep) or presence (+C-pep) of 1.0 nmol/l C-peptide.

possibility of definitely clarifying which cells express this receptor and at the same time of defining its role in mediating C-peptide effect. Our findings, however, by demonstrating that pertussis toxin, but not LY294002, inhibits the C-peptide-induced activation of VACM-1 expression are in line with previous studies suggesting that C-peptide activity might be mediated by a G protein-coupled receptor (3, 4).

Mesangial cells represent the established cellular target of diabetic nephropathy (39). The evidence that C-peptide activates VACM-1 expression also in these cells might suggest that this pathway could be directly involved in the beneficial effect of C-peptide against the development of diabetic nephropathy. The evaluation in human mesangial cell of the effect of VACM-1 activation on Na–K pump activity and in parallel on the production of matrix components (such as collagens and fibronectin), whose overproduction usually goes along with the development of diabetic nephropathy, could be of great help toward the comprehension of the cellular mechanisms underlying the pathogenesis of this complication.

In vitro growing cells tend to de-differentiate and the original phenotype is progressively lost (40). To avoid this problem we studied fibroblasts and mesangial cells at an early culture passage (between the sixth

and seventh subculture), in line with previous reports (36).

Finally, the recent report from the Diabetes Control and Complication Trial that patients with sustained C-peptide secretion levels have a lower incidence of diabetic complications (41) can be explained by a protective effect induced by the parallel remnant secretion of insulin but also, as suggested by the authors as well, by an independent beneficial effect of C-peptide by itself.

In conclusion, this study demonstrates that in both human skin fibroblasts and mesangial cells C-peptide exerts at least some of its effects through the modulation of the expression of the VACM-1 gene. VACM-1 activation was maintained after phosphatidylinositol-3-kinase inhibition and blunted by pertussis toxin, the specific inhibitor of G protein-coupled receptor, in line with the hypothesis that this intracellular pathway mediates C-peptide activity. Because of the small number of cell cultures used in these experiments, the evidence that semiquantitative RT-PCR allows only an approximate quantification of mRNA and the lack of controls done using an inactive form of C-peptide, our results have to be considered preliminary and further investigations will now be needed to confirm this hypothesis and to clarify whether VACM-1 activation is involved in the protective effect of C-peptide against the development of diabetic nephropathy.

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