Prevention of Vascular and Neural Dysfunction in Diabetic Rats by C-Peptide

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C-peptide, a cleavage product from the processing of proinsulin to insulin, has been considered to possess little if any biological activity other than its participation in insulin synthesis. Injection of human C-peptide prevented or attenuated vascular and neural (electrophysiological) dysfunction and impaired Na^+- and K^+-dependent adenosine triphosphate activity in tissues of diabetic rats. Nonpolar amino acids in the midportion of the peptide were required for these biological effects. Synthetic reverse sequence (retro) and all-D-amino acid (enantio) C-peptides were equipotent to native C-peptide, which indicates that the effects of C-peptide on diabetic vascular and neural dysfunction were mediated by nonchiral interactions instead of stereospecific receptors or binding sites.

Human insulin is synthesized in pancreatic beta cells as part of a larger proinsulin molecule consisting of an A chain and a B chain linked by a 31–amino acid connecting peptide (C-peptide, molecular weight = 3020) and two pairs of dibasic amino acids. C-peptide is postulated to promote alignment of the A and B chains for the formation of disulfide bonds between them (*1*). C-peptide is subsequently cleaved from proinsulin but remains in the secretory granule and is cosecreted with insulin in response to glucose stimulation. Plasma levels of C-peptide and insulin are markedly decreased in humans and animals with insulin-dependent diabetes. C-peptide is considered to possess little if any biological activity other than its role in insulin synthesis (*1*, *2*); however, there are several reports indicating biological activity (*3*). Among the biochemical and physiological abnormalities (in noninsulin-requiring tissues) associated with diabetes, vascular dysfunction, which is manifested by changes in blood flow and increased albumin permeation, and impaired nerve conduction with decreased Na^+- and K^+-dependent adenosine triphosphatase (Na^+,K^+-ATPase) activity are well documented in diabetic humans and animals. We describe the beneficial effects of human C-peptide on these abnormalities in animal models of diabetes and the structural determinants of C-peptide efficacy.

Biosynthetic human C-peptide (*4*) (130 nmol per kilogram of body weight) was injected subcutaneously twice daily for 5 weeks in control rats and in rats with streptozotocin-induced diabetes (*5*). Before injection, rat plasma C-peptide levels were 0.45 to 0.9 nM in control rats and below 0.4 nM in diabetics. Peak human plasma C-peptide levels were about 9 nM at 10 and 30 min after injection and were undetectable at 3 hours. Vascular and neural function were assessed 3 to 6 hours after injection of C-peptide. C-peptide markedly attenuated diabetes-induced increases in blood flow (6) in the anterior uvea, retina, and sciatic nerve (Fig. 1) and also prevented increased ^{125}I-labeled albumin permeation (6) in these tissues and in the aorta (Fig. 1) of diabetic rats. C-peptide did not affect vascular function in these tissues in control rats or in other tissues not affected by diabetes. C-peptide also prevented the decreased caudal motor nerve conduction velocity (MNCV) (6) in diabetic rats but had no effect on MNCV in control rats (Table 1). C-peptide had no effect on sorbitol levels in the sciatic nerve or retina (Table 1) or in other tissues examined. Thus, the beneficial effects of C-peptide were not mediated by normalization of the increased sorbitol pathway metabolism that has been implicated in the pathogenesis of diabetic complications in animal models of diabetes (*7*). C-peptide had no effect on food consumption, body weight, or plasma glucose levels in control or diabetic rats (Table 1).

Because vascular dysfunction in diabetic humans and animals is not rapidly reversible by intensive insulin treatment or islet transplants (*8*), the capacity of C-peptide (alone or in combination with a low dose of insulin) to reverse diabetes-induced vascular dysfunction was assessed. After 8 weeks of untreated diabetes, rats were assigned to groups receiving (i) a single daily injection of NPH (natural protamine Hagadorn) insulin (10 U per kilogram of body weight), (ii) twice-daily injections of C-peptide as in the prevention experiment, (iii) NPH insulin plus C-peptide, or (iv) no treatment for an additional 4 weeks. Insulin with or without C-peptide did not reduce plasma glucose or glycated hemoglobin levels but increased body weight gain ~30% ($P < 0.005$) versus that of untreated and C-peptide–treated diabetics. In untreated diabetics, ^{125}I-albumin permeation in ocular tissues, nerves, and the aorta was ~25% higher than in the prevention study (which is consistent with longer duration of diabetes) and was unaltered by insulin or C-peptide treatment for the last 4 weeks; in contrast, insulin plus C-peptide decreased ^{125}I-albumin permeation 63% in the aorta, 67% in the anterior uvea, and 73% in the retina and nerves ($P \leq 0.005$ for all tissues). These

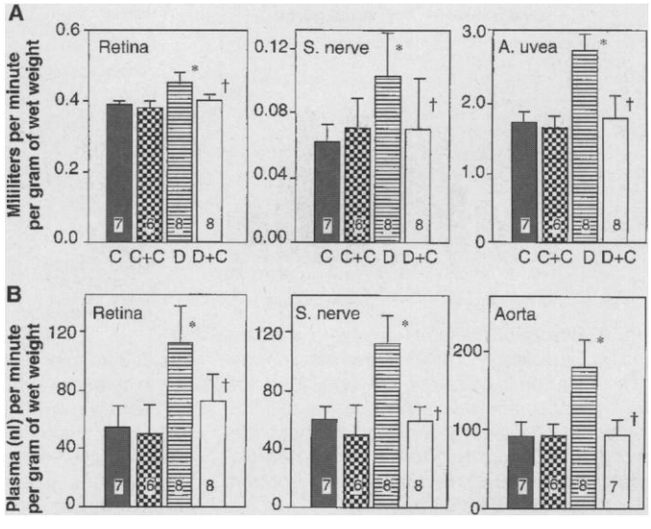

Fig. 1. Effects of human C-peptide injection on blood flow in the retina, sciatic nerve, and anterior uvea (**A**) and on vascular ^{125}I-albumin permeation in the retina, sciatic nerve, and aorta (**B**) in rats with diabetes of 5 weeks duration. The number of rats is shown at the bottom of each bar. Significantly different from controls (*12*): *$P < 0.001$. Significantly different from untreated diabetics: †$P < 0.001$.

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findings attest to a robust synergism between insulin and C-peptide in reversing diabetes-induced vascular dysfunction. Increased blood flow early after the onset of diabetes (observed in the 5-week prevention study) is a transient change (in contrast to increased albumin permeation) and was not evident after 12 weeks of untreated diabetes in the intervention study (neither insulin nor C-peptide affected blood flow).

The structural features of C-peptide critical for mediating its effects on diabetes-induced vascular dysfunction were investigated in a skin chamber granulation tissue model (9). In this model, exposure of granulation tissue (in nondiabetic rats) to buffer containing 11 to 30 mM D-glucose (but not L-glucose or 3-O-methylglucose) increased blood flow and vascular albumin permeation as observed in diabetic rats. The small amount of glucose added to the chamber had no detectable effect on systemic plasma glucose levels. Increases in blood flow and vascular ^{125}I-albumin permeation that were induced by 30 mM glucose were prevented by coadministration of 100 nM human C-peptide or 100 nM rat C-peptide 1 (*10*) with 30 mM glucose (Fig. 2). In contrast, 2 and 10 nM rat C-peptide 1, but not human C-peptide, reduced 30 mM glucose-induced vascular dysfunction about 50% (Fig. 2). In vessels exposed to 5 mM glucose, neither blood flow nor albumin permeation was affected by 10 nM human or rat C-peptide, but 100 nM concentrations of both peptides increased albumin permeation slightly, and 100 nM human C-peptide increased blood flow slightly (Fig. 2).

Decreased Na^+,K^+-ATPase activity is a characteristic abnormality in many tissues of diabetic animals and is prevented by interventions that also normalize associated vascular and neural dysfunction (*11*). Human C-peptide prevented decreased Na^+,K^+-ATPase activity (6) in the sciatic nerve of diabetic rats (Table 1) and in granulation tissue exposed to 30 mM glucose {11.6 ± 4.1 nmol of adenosine diphosphate (ADP) per milligram of protein per minute for 5 mM glucose (n = 6), 7.6 ± 1.1 for 30 mM glucose [n = 6, $P < 0.04$ (*12*) versus 5 mM], and 10.8 ± 2.3 for 30 mM glucose plus C-peptide (n = 7)}. The finding that elevated glucose levels caused diabetes-like vascular dysfunction and impaired Na^+,K^+-ATPase activity in granulation tissue in nondiabetic rats (i) indicates that vascular dysfunction induced by elevated glucose levels is not prevented by normal plasma levels of insulin and C-peptide and (ii) suggests that supraphysiological C-peptide levels are required to prevent vascular dysfunction induced by hyperglycemia.

The efficacy of human C-peptide in attenuation of vascular dysfunction in hyperglycemic and diabetic rats is not surprising in view of substantial homology between rat C-peptide 1 and human C-peptide. The longest homologous sequence occurs in the midportion of the peptides from C11 through C16 (Fig. 3). The slightly greater efficacy of rat versus human C-peptide (at concentrations of 2 to 10 nM) may result from differences in amino acid sequence. To examine the importance of the primary structure of human C-peptide in mediating its vascular effects, a reverse sequence (retro) human C-peptide was synthesized (*10*). When coadministered at a concentration of 100 nM with 30 mM glucose, the retro C-peptide was almost as effective as native C-peptide (Fig. 3). A scrambled C-peptide was then synthesized (Fig. 3) (*10*) in which the amino acid composition was identical to that of the native peptide, but the sequence was randomized. This peptide was inactive (Fig. 3). The biological activity of the retro C-peptide suggested that C-peptide activity was not dependent on chiral interactions with stereospecific receptors or binding sites. We then synthesized all–D–amino acid (enantio) human C-peptide (*10*). This peptide was equipotent to native C-peptide at concentrations from 50 to 500 nM (the data at 100 nM concentration in Fig. 3), although at concentrations up to 1 nM it was not recognized by a polyclonal antibody to native

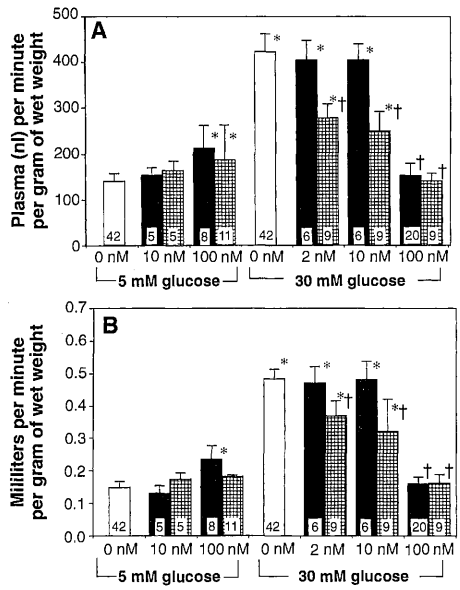

Fig. 2. Effects of human C-peptide (solid bars) and rat C-peptide 1 (cross-hatched bars) on vascular ^{125}I-albumin permeation (**A**) and blood flow (**B**) in skin chamber granulation tissue vessels. The number of chambers is shown at the bottom of each bar. Significantly different from 5 mM glucose (*12*): *$P < 0.05$. Significantly different from 30 mM glucose: †$P < 0.05$.

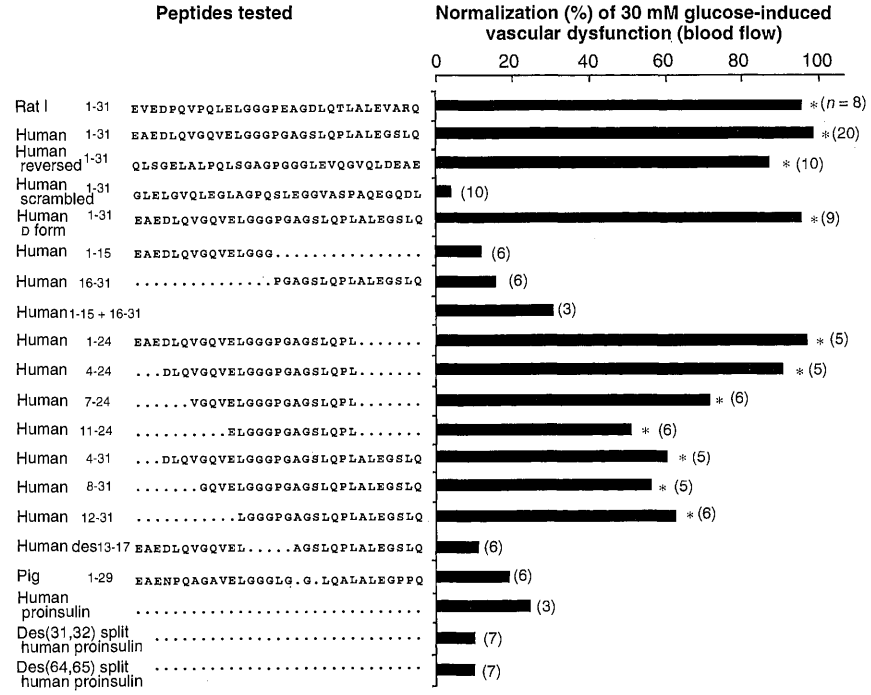

Fig. 3. Summary of effects of C-peptides and fragments on 30 mM glucose-induced vascular dysfunction (blood flow) in the skin chamber granulation tissue model. All peptides were coadministered at a concentration of 100 nM with 30 mM glucose. Efficacy is expressed as an average percent of the effect of 100 nM human C-peptide. Because the reductions (by the peptides) in 30 mM glucose-induced increases in ^{125}I-albumin permeation and blood flow were the same, only the blood flow data are shown. The number in parentheses to the right of each bar is the number of chambers assessed. Scheffe's interval test was used to assess differences (*12*). Significantly different for 30 mM glucose: *$P < 0.05$.

all–L–amino acid C-peptide in a radioimmunoassay sensitive to 10 pM of native C-peptide (*13*). Thus, the sequence of amino acids in C-peptide is critical for biological activity but not the direction of peptide bonds or chirality, confirming that the action of C-peptide is not mediated by stereospecific receptors or binding sites.

Next, the activity of various fragments and synthetic analogs of human C-peptide was assessed (*10*). All of the peptides were coadministered at a concentration of 100 nM with 30 mM glucose. Intact human proinsulin and both split forms (*4*) des(31, 32) and des(64,65) were inactive (Fig. 3). Human C-peptide containing the 31 and 32 arginines and the 64 lysine and 65 arginine residues of proinsulin possessed full activity (*13*). C-peptide fragments lacking the first three and the last seven amino acids (human 4 to 24 in Fig. 3) retained almost full activity. Removal of amino acids C4 to C6 and C7 to C10 resulted in progressive loss of activity (70% for C7 to C24 and 50% for C11 to C24); C4 to C31, C8 to C31, and C12 to C31 all possessed about 60% activity ($P < 0.05$ versus 30 mM glucose alone and versus 30 nM glucose plus C1 to C31 for all five fragments). Human C1 to C15, C16 to C31, and human des(13–17) lacked significant biological activity. Thus, amino acid residues in the midportion of human C-peptide are critical for prevention of glucose-induced vascular dysfunction. Pig C-peptide (which contains only 29 amino acid residues and in which C16 Pro is replaced by Leu, and C17 Gly and C18 Ala are deleted) also lacked biological activity. The amino acid composition of the midportion of mammalian C-peptides is substantially conserved and contains a high proportion of nonpolar amino acids flanking a C16 proline (*1*). In human C-peptide, the sequence from C13 to C19 is GGGPGAG (which is virtually symmetrical for nonpolar amino acids on either side of C16 proline), whereas in rat C-peptide 1 the sequence is GGGPEAG.

Circular dichroism spectra revealed a low signal intensity for both human and rat peptides. The signal intensity was higher for rat than for human C-peptide, which is indicative of a more stable structure for the rat peptide (Fig. 4A). The spectra were nearly identical for the native and retro human C-peptides. Predictions of secondary structure based on LINUS (*14*) indicated the presence of an α helix of approximately three amino acid residues for native human and eight amino acid residues for rat C-peptide. This was consistent with the circular dichroism spectra. Interpretation by RASMOL (*15*) of supersecondary structures predicted by LINUS, with the use of the algorithm by Kabsch and Sander (*16*) for pattern recognition of hydrogen-bonded and geometrical features in protein secondary structure, indicated the presence of hydrogen bonds in the midportion of both human and rat C-peptides. This supports the presence of a turn-like structure in this region of both peptides (Fig. 4B) (*17*). A turn-like structure in the midportion of C-peptides also is supported by predictions of Snell and Smyth (*1*) and Steiner (*1*) [based on the criteria of Chou and Fasman, Lewis *et al.*, and Crawford *et al.* (*18*)] and by the hydrodynamic data of Markussen and Schiff (*1*). Thus, a nonpolar turn-like structure in the midportion of C-peptide is important for its biological activity. Although the occurrence of multiple Gly residues is rare in turn-like structures, the GGGPGAG motif is reminiscent of the beta turn observed in fibrinopeptide A that contains three Gly residues (*19*). Whether the predicted turn-like structure

Table 1. Effects of human C-peptide on body weight and metabolic parameters in rats with streptozotocin-induced diabetes of 5 weeks duration.

	Control	Control + C-peptide	Diabetes	Diabetes + C-peptide
Number of rats	7	6	8	8
Body weight (g)				
Initial	216 ± 17*	217 ± 21	224 ± 23	212 ± 21
Final	359 ± 34	357 ± 41	333 ± 26‖	307 ± 21‖
Food consumption (g/day)	19.4 ± 1.4	20.3 ± 3.4	30.0 ± 6.8	31.1 ± 7.3
Plasma glucose (mM)	8.7 ± 1.0	7.6 ± 2.9	30.0 ± 6.2‖	26.6 ± 4.1‖
MNCV (m/s)†	38.2 ± 0.9	36.2 ± 3.3	33.4 ± 1.3‖	37.1 ± 0.8¶
Sorbitol‡				
Retina	86 ± 19	139 ± 62	756 ± 284‖	913 ± 243‖
Sciatic nerve	125 ± 18	163 ± 56	1556 ± 338‖	1587 ± 306‖
Na^+,K^+-ATPase§				
Sciatic nerve	23.7 ± 5.7	—	13.7 ± 2.7‖	20.8 ± 4.8¶

*Mean ± SD. †Caudal MNCV was measured as meters per second as described in (*6*). ‡Sorbitol levels (nmol/g wet weight) were determined as their butylboronate derivatives by gas chromatography mass spectrometry (*22*). §Na^+,K^+-ATPase (nanomoles of ADP per milligram of protein per minute) was measured as described in (*6*). ‖ Significantly different from controls (*12*): $P < 0.005$. ¶ Significantly different from untreated diabetics: $P < 0.005$.

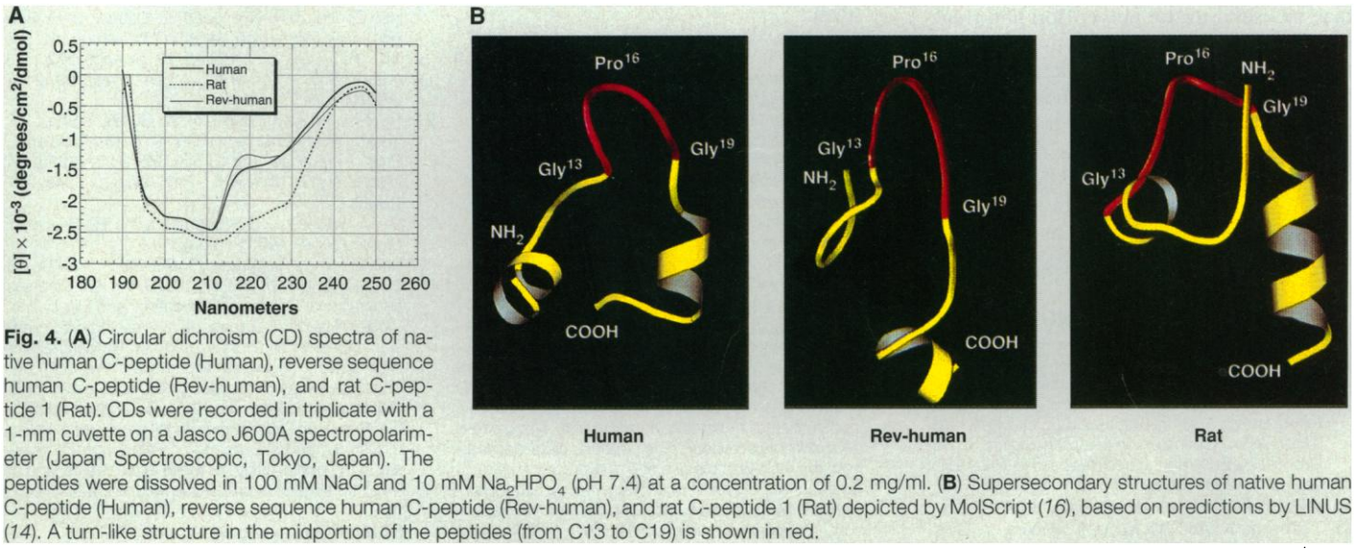

Fig. 4. (**A**) Circular dichroism (CD) spectra of native human C-peptide (Human), reverse sequence human C-peptide (Rev-human), and rat C-peptide 1 (Rat). CDs were recorded in triplicate with a 1-mm cuvette on a Jasco J600A spectropolarimeter (Japan Spectroscopic, Tokyo, Japan). The peptides were dissolved in 100 mM NaCl and 10 mM Na_2HPO_4 (pH 7.4) at a concentration of 0.2 mg/ml. (**B**) Supersecondary structures of native human C-peptide (Human), reverse sequence human C-peptide (Rev-human), and rat C-peptide 1 (Rat) depicted by MolScript (*16*), based on predictions by LINUS (*14*). A turn-like structure in the midportion of the peptides (from C13 to C19) is shown in red.

would be stable remains to be established.

Although the mechanisms that mediate vascular and neural dysfunction induced by diabetes are unclear, several lines of evidence support an important role for hyperglycemia-induced cytosolic reductive stress, a "hypoxia-like" increased cytosolic ratio of free NADH/NAD^+ (NAD, nicotinamide adenine dinucleotide), in initiating the metabolic imbalances (*20*) that cause vascular and neuroelectrophysiological dysfunction (*7*). In animal models of diabetes, this redox change results largely from increased oxidation of sorbitol to fructose coupled to reduction of NAD^+ to NADH by sorbitol dehydrogenase in the second step of the sorbitol pathway. Because C-peptide had no effect on tissue sorbitol levels (Table 1) and does not prevent reductive stress (*13*), we hypothesize that is may inhibit, or compensate for, a cascade of metabolic imbalances caused by hyperglycemia-induced reductive stress (*7*, *20*). The finding that the retro- and D-enantiomers are equipotent to the native peptide is analogous to the properties of retro- and D-enantiomers of amphipathic antimicrobial peptides such as cecropins, mangainins, and dermaseptins (*21*). Evidence that the effects of these antimicrobial peptides are mediated by nonchiral interactions with membrane lipids, resulting in formation of ion channels and inhibition of phospholipase A_2 activity (*21*), raises the possibility that C-peptide effects may be mediated by corresponding mechanisms resulting in normalization of enzyme activities altered by diabetes.

C-peptide prevents diabetes- and hyperglycemia-induced vascular and neural dysfunction in animal models of diabetes by nonchiral mechanisms rather than by stereospecific receptors or binding sites. To the extent that C-peptide treatment may be useful in the prevention and treatment of diabetic complications in humans, it should not be considered an alternative to insulin or other blood glucose–lowering agents. Instead, it could be used in combination with them to reduce the need for virtual normalization of blood glucose levels (and the attendant restrictions in lifestyle and increased risks of hypoglycemia and obesity).

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5. Animals were cared for in accordance with National Institutes of Health guidelines on laboratory animal welfare. Diabetes was induced in male Sprague-Dawley rats (body weight, 300 g) by intravenous injection of 50 mg of streptozotocin (Zanosar, Upjohn). Control rats received only buffer.
6. Regional blood flows were assessed by the reference sample microsphere method by injection of 11.3-μm ^{46}Sc microspheres in anesthetized rats as described by Tilton *et al.* (*20*). Vascular albumin permeation (^{125}I-albumin permeation) was quantified by use of ^{125}I- and ^{131}I-albumin (*20*). Caudal MNCV was measured in anesthetized rats (*11*). Na^+,K^+-ATPase activity was measured in crude homogenates by a modification of the coupled enzyme method (*11*).
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10. With the exception of scrambled human C-peptide, des(13–17) human C-peptide, and all-D-amino acid C-peptide (which are described below), synthetic peptides were from the Lilly Research Laboratories in Indianapolis, IN, and were prepared by solid-phase synthesis on an Applied Biosystems (ABI) Model 430A peptide synthesizer with the use of the Boc protecting strategy. Each residue was double-coupled with the use of either dicyclohexylcarbodiimide-initiated symmetric anhydride or 1-hydroxybenzotriazole activation. The peptides were cleaved from the resin with the use of 9:1 hydrogen fluoride/m-cresol at 0°C for 1 hour, followed by extraction with 0.1 M ammonium bicarbonate and lyophilization. The peptides were purified by rpHPLC on a Vydac C18 column using acetonitrile gradient elution with either 0.1 M ammonium acetate or 0.1 M ammonium bicarbonate buffers and analyzed by electrospray ionization mass spectrometry (ESI-MS). Native pig C-peptide (Lilly Research Laboratories) was isolated and purified from a trypsin digestion of des(62,63) split porcine proinsulin [R. E. Chance, In *DIABETES: Proceeding of the Seventh Congress of the International Diabetes Federation*, R. R. Rodriquez and J. Vallance-Owen, Eds. (Excerpta Medica, Amsterdam, 1971), pp. 292–305]. Scrambled human C-peptide, human C-peptide des(13–17), and all-D-amino acid C-peptide were synthesized by the Protein and Nucleic Acid Chemistry Laboratory at Washington University on an ABI model 431A peptide synthesizer. Amino acid activation was performed with the use of HBTU [2-(1H-benzotriazol-1-yl)1,1,3,3,tetramethyluronium hexafluorophosphate]. The alpha amino group of the amino acids was FMOC (9-fluorenylmethoxycarbonyl) protected, and the side chain groups were protected by tertiarybutyl (Asp, Ser, and Glu), and trityl (Gln). FMOC deprotection was performed with 20% piperidine in *N*-methylpyrrolidone. The peptide was simultaneously deprotected and cleaved from the resin with triflouroacetic acid/phenol/thioanisole/water/ethanedithiol (85:5:4:4:2) for 2 hours. Chromatographic analysis and purification of the peptide were performed as described [J. Gorka, D. W. McCourt, B. D. Schwartz, *Pept. Res.* **2**, 376 (1989)]. Electrospray mass spectrometry of the purified peptide was performed on a Vestec model 201 electrospray mass spectrometer.
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12. All results are expressed as mean ± 1 SD. Unless otherwise stated, overall group comparisons for each parameter were performed by the Van der Waerden test [E. L. Lehman, *Nonparametrics: Statistical Methods Based on Ranks* (Holden-Day, San Francisco, CA, 1975)]. When this test was significant at $P < 0.05$, individual pairwise group differences were assessed by the general linear model procedure with the use of SAS (SAS Institute, Cary, NC). Schéffe's interval test [H. Schéffe, *The Analysis of Variance* (Wiley, New York, 1959)] was also performed by SAS.
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