C-peptide induces a concentration-dependent dilation of skeletal muscle arterioles only in presence of insulin

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Jensen, Michael E., and Edward J. Messina. C-peptide induces a concentration-dependent dilation of skeletal muscle arterioles only in presence of insulin. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H1223–H1228, 1999.—In this study we tested the hypothesis that C-peptide alone or in conjunction with insulin may cause a dilation of skeletal muscle arterioles. First-order arterioles (88 µm) isolated from rat cremaster muscles were pressurized (65 mmHg), equilibrated in a Krebs bicarbonate-buffered solution (pH 7.4), gassed with 10% O2 (balance 5% CO2, 85% N2), and studied in a no-flow state. C-peptide administered at concentrations of 0.3, 1, 3, 10, 100, 300, and 3,000 ng/ml evoked arteriolar dilation that was not concentration dependent. In contrast, the administration of the four lower physiological concentrations of C-peptide to arterioles exposed to a nondilating concentration of insulin evoked a significant concentration-dependent increase in arteriolar diameter from 8.6 to 42.3% above control. The arteriolar dilation to C-peptide in the presence of insulin was completely inhibited by administration of Nω-nitro-L-arginine (10–4 M). Responses to ACh and adenosine were not enhanced when these drugs were administered in the presence of insulin. These results indicate that C-peptide has the capacity to evoke arteriolar dilation in skeletal muscle via a nitric oxide-mediated mechanism that appears to be enhanced by an interaction with insulin. Furthermore, the effects of insulin appear to be specific for C-peptide and are not the result of a general enhancement of endothelium-dependent or endothelium-independent dilation.

microcirculation; vasodilation; rats; insulin-dependent diabetes mellitus; arterioles; nitric oxide; Nω-nitro-L-arginine; vascular smooth muscle; endothelium

SINCE THE DISCOVERY of insulin, metabolic control of diabetic patients has greatly improved. However, microvascular complications and associated neuropathy, retinopathy, and reduced skeletal muscle perfusion are still common among many diabetic patients despite well-controlled glucose levels (20).

Insulin is first formed as proinsulin with connecting peptide (C-peptide) serving as the link between the α- and β-chains of the molecule (10). Before 1975, it was thought that C-peptide’s primary and only function was to contribute to the secondary and tertiary structure of the hormone (10, 15). Thus C-peptide was thought to be without biological activity even though it is released in equimolar amounts with insulin into the circulation (10). In one of the first studies to suggest a biological action for rat C-peptide in vivo, it was found that administration of pharmacological amounts of C-peptide had no effect on fasting plasma glucose concentrations but that C-peptide could reduce the increase in plasma insulin levels induced by glucose as well as prolong the hypoglycemia evoked by insulin (23). These studies support the idea that C-peptide alone has no biological activity in vivo but that it may exert some effects through an interaction with insulin.

Recent studies, however, suggest that C-peptide may possess biological activity on its own. In vitro, in human skeletal muscle specimens from healthy subjects, C-peptide was found to stimulate glucose transport in a dose-dependent manner, suggesting that it may be involved in the regulation of carbohydrate metabolism (26). Low rates of C-peptide infusion in type 1 diabetic patients (insulin-dependent diabetes mellitus; IDDM) decreased glomerular filtration by 7%, increased effective plasma flow by 3%, and increased whole body glucose utilization by 25% (9). In another study, the effects of C-peptide on forearm blood flow, capillary diffusion capacity, and substrate exchange in normal subjects and IDDM patients were studied during rhythmic forearm exercise on a hand ergometer. Before the administration of C-peptide, forearm blood flow, capillary diffusion capacity, and glucose uptake were lower in the IDDM patients than in the normal subjects during exercise (8). Administration of C-peptide during exercise increased forearm blood flow by 27%, capillary diffusion capacity by 52%, and glucose uptake (8). It was also found that 7% of the administered C-peptide was cleared on a single passage through forearm tissues. The authors concluded that C-peptide replacement in type 1 diabetic patients leads to normalization of blood flow and capillary diffusion capacity during exercise and that skeletal muscle is a major site of C-peptide removal (8). This study was the first to demonstrate that C-peptide could increase skeletal muscle blood flow, albeit during exercise and only in type 1 diabetic patients (8).

Diabetic patients, specifically IDDM patients, produce neither insulin nor C-peptide and receive only exogenous insulin. As a result, it has been suggested that microvascular complications arising in diabetic patients despite replacement therapy with insulin may be due to the absence of endogenous and/or exogenously coadministered C-peptide (21).

On this basis, it seems possible that both insulin and C-peptide may play a role in microvascular regulation in healthy humans, and perhaps replacement therapy with C-peptide and insulin might provide a better control over metabolic and vascular complications associated with diabetes mellitus. Consequently, the present investigation was designed to test the hypothesis that C-peptide alone and/or in conjunction with insulin could be an important vasoactive agent with the poten-
tial for contributing to the control of blood flow in skeletal muscle. Toward this end, we studied the effects of C-peptide alone and in combination with insulin on isolated skeletal muscle arterioles. The results of these studies could have important implications with regard to the vascular complications associated with diabetes.

METHODS

Preparation. Six- to eight-week-old male Wistar rats were anesthetized with pentobarbital sodium (50 mg/kg) administered subcutaneously. Both cremaster skeletal muscles were exposed and cleared of adhering fascia so as to separate the muscle tissue from the scrotal sac. An incision was then made on the ventral surface of each muscle to expose and remove the testis and epididymis. Each cremaster muscle was excised by a transverse incision at its base, as close to the abdominal wall as possible. After excision, the muscles were immediately immersed in a cooled (0–4°C) MOPS-buffered physiological salt solution (MPSS). First-order arterioles were dissected free from surrounding skeletal muscle, and a 2.0- to 3.0-mm length of the dissected arteriole was removed and transferred to a special microvessel chamber (Living Systems Instrumentation, Burlington, VT) containing a Krebs bicarbonate-buffered physiological salt solution (PSS) at room temperature. Arterioles were prepared and studied as previously described (17). Briefly, the arteriole was cannulated at one end with the inlet glass micropipette and gently flushed with PSS to remove any blood from the lumen of the vessel. The free end of the arteriole was then cannulated with the outlet glass micropipette, and the transmural pressure slowly increased by way of the inlet micropipette to 65 mmHg. The transmural pressure was increased and maintained for the duration of the experiment with a pressure-servo syringe reservoir system (Living Systems Instrumentation). Temperature within the vessel chamber was thermostatically maintained at 34°C by passing PSS through a heating coil (Radnoti Glass Technology, Monrovia, CA). The total volume of PSS bathing the arteriole was maintained at 100 ml, and all drugs were added to the reservoir with final concentrations being reported. When originally cannulated, the arteriolar diameters ranged from 183 to 215 µm. After arterioles were cannulated, they were then allowed to equilibrate for 1 h, during which they developed myogenic tone and achieved resting control diameters averaging 83 µm. After equilibration, the arterioles were tested for their capacity to dilate to ACh (10⁻⁷ M) and to constrict to phenylephrine (PE; 10⁻⁷ M). Vessels that either failed to develop tone during equilibration or failed to respond to these vasoactive agents were considered injured by the procedure and deemed unsuitable for inclusion in this study.

Perfusates. The PSS used to both flush during cannulation and suffice the arteriole in the chamber was equilibrated with 10% O₂-5% CO₂ and the balance of nitrogen, with a pH of 7.40, and contained (in mM) 110.0 NaCl, 5.0 KCl, 2.5 CaCl₂, 1.0 MgSO₄, 1.0 KH₂PO₄, 10.0 glucose, 24.0 NaHCO₃, and 0.02 EDTA. The MPSS (pH 7.40) was made up of (in mM) 145.0 NaCl, 5.0 KCl, 2.0 CaCl₂, 1.0 MgSO₄, 1.0 NaH₂PO₄, 5.0 glucose, 2.0 pyruvate, 0.02 EDTA, and 3.0 MOPS. All salts and chemicals were analytic grade and obtained from J. T. Baker Chemical (Phillipsburg, NJ).

Drugs. Porcine insulin, ACh, and PE were purchased from Sigma Chemical (St. Louis, MO). Porcine insulin was used for these studies because of its availability, cost, and the fact that insulin from various species interacts with porcine insulin sera to more or less the same degree (10). Adenosine and N⁵-nitro-L-arginine (L-NNA) were purchased from Aldrich Chemical (Milwaukee, WI). ACh and PE were dissolved in PSS, whereas insulin was first dissolved in normal saline with further dilutions being made in PSS. Biosynthetic human C-peptide was purchased from Sigma Chemical and donated by Eli Lilly (Indianapolis, IN). The purity of the C-peptide and insulin preparations was >99%, as determined by HPLC analysis. C-peptide was dissolved in 0.5 M acetic acid, with further dilutions being made with distilled water. The vehicle for C-peptide was without any effect (data not shown).

Analytic procedures and statistical methods. Arteriolar luminal diameters were measured using an electronic image-shearing device (Video Caliper, Microrculation Research Institute, Texas A&M University, College Station, TX). Changes in intraluminal diameter and transmural pressure of the perfusion fluid were continuously measured and recorded with a computer data-acquisition system (Biopac Systems, Goleta, CA). Maximum responses are reported, and the data were analyzed by two-way ANOVA for repeated measures and multiple comparisons procedures with ANOVA method or by two-way ANOVA and Student-Newman-Keuls method for multiple comparisons. Data are presented as means ± SE. The value n represents the number of animals studied, and significant differences were established at the P < 0.05 level.

Experimental protocol. Arterioles were randomly assigned to one of five experimental groups. Preparations for all groups were the same, but the drugs used varied. Group 1 was used to generate a concentration-response (0.3, 1, 3, 10, 100, 300, and 1,000 ng/ml) curve for C-peptide in the absence of insulin. The concentrations of C-peptide chosen for the other protocols in this study (0.3, 1, 3, and 10 ng/ml) are equivalent to basal levels found in peripheral blood in humans (0.5–3.0 ng/ml) (4, 5) and rats (0.4–1.1 ng/ml) (6). The highest amounts used (10 ng/ml) represent C-peptide concentrations in peripheral blood after nutritional stimulation in humans (1, 5, 19). Vessels in group 2 were incubated in a nonvasodilator concentration of insulin (1 µU/ml) for 30 min and then subjected to incremental cumulative concentrations of C-peptide (0.3, 1, 3, and 10 ng/ml) and after a 45-min incubation with L-NNA (10⁻⁴ M). In group 3, vessels were treated with increasing concentrations of ACh (10⁻¹², 10⁻¹¹, 10⁻¹⁰, 10⁻⁹, 10⁻⁸, and 10⁻⁷ M) before and after a 30-min incubation in a nonvasodilator concentration of insulin (1 µU/ml). Vessels in group 4 were treated with increasing concentrations of adenosine (10⁻⁹, 10⁻⁸, 10⁻⁷, 10⁻⁶, and 10⁻⁵ M) before and after a 30-min incubation with a nonvasodilator concentration of insulin (1 µU/ml). The 30-min period of insulin incubation was utilized to demonstrate the failure of insulin to produce vasodilation at the concentration used; as in a previous study (2), we demonstrated that it took 10–15 min for insulin to evoke a maximum vasodilator response.

RESULTS

Effects of C-peptide on skeletal muscle arterioles. In this group, arterioles averaged 78.4 µm in diameter. As can be seen in Fig. 1, the response to C-peptide was highly variable in all seven experiments, and a clear concentration-response curve was not obtainable. Furthermore, the responses to C-peptide at each concentration were not significantly different from zero or from each other. Statistically, the changes in diameter were not significantly different from zero because of the inconsistency and variability of the responses across animals. However, in a single vessel, whatever the response was, it was consistent and was of a magnitude to be different...
from zero in some cases, particularly at the higher doses. The average increase in diameter, as a percentage of the control diameter, was 0, 4, 3, 6, 17, 5, and 34% for 0.3, 1, 3, 10, 100, 300, and 1,000 ng/ml, respectively. Depending on the concentration of C-peptide as well as the sensitivity of the individual arteriole, changes in arteriolar diameter were apparent within 10 min of administration, with the maximum response obtained within 15 min. Every vessel was allowed at least 20 min to reach a maximum sustained response.

Effects of insulin on arteriolar dilation to C-peptide. In this group of arterioles, control diameters averaged 88 µm. In the eight experiments, cumulative concentration-response curves were generated in response to 0.3, 1, 3, and 10 ng/ml C-peptide after incubation of the arteriole with a nondilating concentration of insulin (1 µU/ml) for 30 min. The duration of incubation served as a control to demonstrate that 1 µU/ml insulin produced no vasodilation, confirming our previous finding (2). Arterioles significantly dilated to all concentrations of C-peptide after insulin administration (Fig. 2). Increases in arteriolar diameter, as a percentage of control diameter, to C-peptide (0.3, 1, 3, and 10 ng/ml) averaged 0, 4, 3, and 6% before treatment with insulin and 9, 18, 33, and 42% after insulin treatment. As a result, insulin pretreatment contributed to C-peptide evoking significant concentration-dependent responses.

Effects of L-NNA on arteriolar dilation to C-peptide. In seven experiments, administration of L-NNA completely inhibited the insulin-induced arteriolar dilator responses to C-peptide (Fig. 3).

Effects of insulin on arteriolar dilation to ACh. In six experiments, cumulative concentration-response curves were generated for ACh before and after pretreatment with insulin. In this group, arterioles averaged 89.7 µm in diameter. Before insulin, arteriolar diameters increased by 0, 4, 2, 4, 21, and 59% in response to ACh concentrations of $10^{-12}$, $10^{-11}$, $10^{-10}$, $10^{-9}$, $10^{-8}$, and $10^{-7}$ M, respectively. After the addition of insulin, arteriolar diameters increased by 2, 7, 7, 8, 30, and 64% in response to the same concentrations of ACh. Therefore, insulin administration had no significant effect on arteriolar dilation to ACh (Fig. 4).

Effects of insulin on arteriolar dilation to adenosine. In seven experiments, cumulative concentration-response curves were generated for adenosine before and after pretreatment with insulin. In this group, arterioles averaged 82.6 µm in diameter. Before insulin, arteriolar diameters increased by 0, 2, 12, 20, and 32% in response to adenosine concentrations of $10^{-9}$, $10^{-8}$, $10^{-7}$, $10^{-6}$, and $10^{-5}$ M, respectively. After the addition of insulin, arteriolar diameters increased by 0, 1, 14, 20,
and 33% in response to the same concentrations of adenosine. Insulin thus failed to potentiate arteriolar dilations in response to increasing concentrations of adenosine (Fig. 5).

**DISCUSSION**

In the present study, we have shown that physiological concentrations of C-peptide evoked dilation of isolated skeletal muscle arterioles that were not consistent or concentration dependent. In contrast, physiological concentrations of C-peptide did evoke significant concentration-dependent dilation of skeletal muscle arterioles in the presence of a nonvasodilating concentration of insulin. The responses to C-peptide, in the presence of insulin, were completely inhibited by L-NNA, indicating a role for nitric oxide. The insulin and C-peptide interaction also appears to be specific because insulin failed to augment the arteriolar dilation in response to either ACh or adenosine.

A direct comparison of our findings with others is not entirely possible because our study deals with isolated skeletal muscle arterioles and because only recently has C-peptide become the focus of vascular studies. Therefore, very few studies are available for analysis. The first report suggesting a vasodilator role for C-peptide in skeletal muscle found that C-peptide did not influence resting blood flow in either IDDM patients or normal subjects (8). However, during forearm exercise, C-peptide was able to increase blood flow in IDDM patients but not in normal subjects (8). The effects of C-peptide in the IDDM patients apparently contributed to a normalization of forearm blood flow only during exercise, even though blood flow was lower in the IDDM patients both at rest and during exercise, as compared with the normal subjects (8). The reason for this is unknown at this time. In contrast to C-peptide's lack of an effect on skeletal muscle blood flow under resting conditions in normal human subjects, infusion of pharmacological amounts of rat 1 and human C-peptide to isolated perfused rat hindquarters was reported to increase capillary filtration coefficients and permeability-surface area product and to lower vascular resistance slightly (12). This later study, as well as our own, indicates that C-peptide is capable of skeletal muscle dilation in normal animals.

A few studies have reported on the vascular effects of C-peptide in vascular compartments other than skeletal muscle. The first study to examine the effects of C-peptide, in the renal circulation of IDDM patients, indicated that administration in low amounts decreased glomerular filtration rate (GFR) by 7%, whereas effective renal plasma flow increased by 3% (9). In isolated rat kidney, perfused at 8°C, C-peptide had no effect on GFR and total vascular resistance (12). In contrast, C-peptide was found to decrease diabetes-induced increases in blood flow, in rats with streptozocin-induced diabetes in the anterior uvea, retina, and sciatic nerve (6). In patients with type 1 diabetes, C-peptide increased capillary blood cell velocity in the cutaneous circulation without effecting laser Doppler flow, whereas in normal subjects, C-peptide was without effect (3). Thus, on the basis of these few reports, it would seem that C-peptide is most effective, with regard to vascular effects, in animals and humans with diabetes. In these animals and humans in which there are less than normal amounts of insulin and C-peptide, the administration of C-peptide is effective. The above findings suggest that in diabetic patients, who lack endogenous C-peptide, replacement of this peptide may exert beneficial effects by restoring vascular and circulatory deficiencies back to normal, as found in healthy subjects.

In our studies, C-peptide was inconsistent in evoking concentration-dependent arteriolar dilation in the absence of insulin. It should be pointed out that in our experiments we tested human C-peptide on isolated rat skeletal muscle arterioles. Some of the variability in...
arterial dilation may be attributed to the use of non-species-specific C-peptide. However, with regard to C-peptide, there is a 70% homology between rat and human material, and there are no reported differences in activity (10). Of the published studies dealing with the vascular actions of C-peptide, few have attempted the analysis of dose-response or concentration-response effects. In one study, C-peptide also failed to elicit a dose-response relationship with regard to decreasing GFR in IDDM patients (9). However, C-peptide appears to be able to evoke a dose-response effect with regard to increasing glucose uptake in vivo in type 1 diabetic patients (9) and in vitro in human skeletal muscle strips (26). Dose-response effects have also been reported with regard to C-peptide stimulation of rat renal tubular Na\(^{+}-\)K\(^{+}\)-ATPase activity (13).

Our findings suggest that, in the absence of insulin, C-peptide is a weak and variable dilator of skeletal muscle arterioles. However, in the presence of insulin, vasodilation to C-peptide is concentration dependent and endothelium dependent. Furthermore, the complete inhibition of the arteriolar dilation to C-peptide by \(-\)-NNA indicates that the response is nitric oxide mediated. Of particular interest is the necessity for arteriolar exposure to a nonvasodilating concentration of insulin for C-peptide to produce a consistent concentration-dependent response. Previous studies have suggested that there may exist an interaction between C-peptide and insulin in the regulation of glucose utilization (24). Our study, perhaps for the first time, demonstrates that an interaction between C-peptide and insulin exists with regard to skeletal muscle arteriole dilation. That this interaction was not observed for either ACh or adenosine further suggests that this interaction may be specific and of some biological importance. Although in our study we used a subphysiological concentration of insulin and found no interaction with ACh, a recently published report indicates that hyperinsulinemia potentiated the forearm vasodilation of intrabrachially administered ACh in normotensive subjects and hypertensive patients (18). In rats with streptozotocin-induced diabetes, \(^{125}\)I-labeled albumin permeation in ocular tissue, nerves, and aorta was 25% higher than in controls (6). Neither insulin nor C-peptide alone was capable of correcting this abnormality (6). However, C-peptide in combination with insulin significantly reduced the \(^{125}\)I-labeled albumin permeation in all of the tissues (6). These authors concluded that there is a “synergism” between insulin and C-peptide in reversing diabetes-induced vascular dysfunction (6). Our findings suggest that a strong C-peptide-insulin interaction also exists in normal arterioles that contributes to an enhanced vasodilation.

It has been well established that C-peptide possesses an important biological role in facilitating the formation of the correct secondary and tertiary structures of insulin. Despite this role, C-peptide was previously considered to be a biologically inert substance, since it lacked insulin-like effects on isolated rat adipose tissues (25). However, it seems unlikely that C-peptide is released along with insulin for the sole purpose of disposal as a biosynthetic by-product because it has an extremely long half-life (14) in part due to the fact that a major portion of its uptake and catabolism occurs in the kidney (14). Our findings and the results of other studies support this idea, especially since recent studies have uncovered a number of other potential regulatory effects of C-peptide. In short-term studies, C-peptide increases whole body glucose utilization in both resting and exercising type 1 diabetic patients (8). In exercising patients, glucose utilization is increased by as much as 25% (9, 27). C-peptide also increases glucose transport and glycogen content in both healthy and IDDM patient skeletal muscle (27) and lowers blood glucose, HBA\(_{1C}\), and fructosamine (6, 7), further supporting a role in glucose homeostasis and metabolism. Only a few studies have been carried out in rats using C-peptide. In normal rats, C-peptide appears to significantly diminish glucose-induced increases in plasma insulin, yet it enhances the hypoglycemic effect of exogenously administered insulin in diabetic rats (23). These findings suggest that C-peptide may act to regulate insulin release as well as interact competitively with insulin in regulating plasma glucose levels.

Several recent studies involving the effects of C-peptide on glucose utilization in streptozotocin-induced diabetic rats suggest a positive interaction between C-peptide and insulin; namely, infusion of C-peptide in the presence of insulin increases the rate of metabolic clearance for glucose in diabetic rats by >75% (24). These results confirm a positive interaction between C-peptide and insulin on glucose utilization in diabetic rats. Furthermore, it was suggested that this interaction is compatible with the idea that C-peptide enhances insulin sensitivity but not insulin responsiveness, since C-peptide clearly produces a marked effect with both low- and high-concentration insulin infusions (24). In the present study, C-peptide, in the presence of insulin, was able to produce a significant concentration-dependent vasodilation in arterioles from normal non-diabetic rats. Because this concentration-dependent dilation failed to occur in the absence of insulin, it is suggested that insulin plays a permissive role in C-peptide-mediated arteriolar dilation. Although the nature of the interaction between C-peptide and insulin is not known, several experiments have proposed that C-peptide interacts with a receptor in a manner that differs from most peptides. Generally, peptides act only by binding to stereospecific receptors (6). In the case of C-peptide, its effects are believed to be mediated by nonchiral interactions rather than through stereospecific receptors (6, 16). Indeed, the sequence of amino acids in the peptide chain appears to be critical for C-peptide activity but is independent of the direction of the sequence and its chirality (6). This would still imply that the actions of C-peptide are mediated via the insulin receptor. However, certain studies on glucose transport have suggested that the actions of C-peptide are not mediated via the insulin receptor, since it fails to displace radiolabeled \(^{125}\)I-labeled insulin binding from partially purified insulin receptors (27). In a previous report, C-peptide was able to stimulate Na\(^{+}-\)
K\textsuperscript{+}-ATPase in a concentration-dependent manner in rat proximal renal tubule segments (13). Because experi-
mental diabetes is accompanied by reduced Na\textsuperscript{+}-K\textsuperscript{+}-
ATPase activity in the kidney (22) and skeletal muscle (11), it is possible that the nature of C-peptide's effects on
blood flow and vascular smooth muscle relies on its interaction with Na\textsuperscript{+}-K\textsuperscript{+}-ATPase. However, our data
support the notion that C-peptide arteriolar dilation is mediated by nitric oxide, most likely by activation of
soluble guanylate cyclase.

In summary, we have shown that C-peptide can dilate isolated arterioles from normal rats, that its
dilator action is more consistent and concentration dependent when administered in the presence of insu-
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C-peptide plays an important biological role by interacting with insulin, the absence of which is seen in the
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ministration of C-peptide.

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