Proinsulin C-peptide constricts glomerular afferent arterioles in diabetic mice. A potential renoprotective mechanism

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Nordquist L, Lai EY, Sjöquist M, Patzak A, Persson AEG. Proinsulin C-peptide constricts glomerular afferent arterioles in diabetic mice. A potential renoprotective mechanism. Am J Physiol Regul Integr Comp Physiol 294: R836–R841, 2008. First published December 12, 2007; doi:10.1152/ajpregu.00811.2007.—Objective: an increased glomerular filtration rate (GFR) has been postulated as a potential mechanism involved in the progression of diabetic nephropathy. Studies suggest that C-peptide exerts a renoprotective effect on diabetes. The peptide decreases hyperfiltration in patients with type 1 diabetes, as well as in diabetic animal models. In this study, we investigated whether C-peptide causes a change in arteriolar diameter. Research Design and Methods: C57-Bl mice were made diabetic by means of a single intravenous injection of alloxan 2 wk prior to the experiment. Age-matched normoglycemic mice served as controls. Afferent arterioles, intact with the glomeruli, were dissected and microperfused. The effect of luminal application of C-peptide, compared with scrambled C-peptide or vehicle, was investigated. The effect of the Rho-kinase inhibitor Y-27632 was also investigated. Results: C-peptide constricted afferent arterioles in diabetic mice by −27% compared with the control value. Normoglycemic arterioles administered C-peptide displayed a delayed and minute response (−4%). Scrambled C-peptide or vehicle administration, whether administered to hyperglycemic or normoglycemic mice, did not induce any effect. Addition of Y-27632 abolished the effect of C-peptide. Conclusion: C-peptide induces constriction of afferent arterioles in diabetic mice. This can reduce enhanced GFR and may be one of the mechanisms in the renoprotective action of C-peptide in diabetes. Diabetes; glomerular filtration rate; renal function

Despite improvements in treatment, diabetes still leads to a number of complications. The initial phase following the onset of diabetes is commonly associated with an increased glomerular filtration rate (GFR), an increase that has been postulated as a potential mechanism involved in the progression of diabetic nephropathy (33, 63). Approximately one-third of all type 1 diabetic patients develop diabetic nephropathy (28), and one-fifth are diagnosed with end-stage renal disease (10a, 36). During the past few years, studies have reported renoprotective effects from exogenously administered proinsulin connecting peptide, C-peptide, to C-peptide-deficient diabetic animals and patients. C-peptide is the 31-residue cleavage product of insulin synthesis, and in healthy mammals, this peptide is secreted from the islets of Langerhans together with insulin (19, 34). When, as in type 1 diabetes, insulin production is impaired, C-peptide will be affected to the same extent. C-peptide was long thought to be biologically inert (62), but has more recently become viewed as a bioactive molecule. Several studies have reported renoprotective effects of C-peptide in diabetic subjects and animal models (20, 23, 51). The peptide seems to acutely decrease hyperfiltration in patients with type 1 diabetes (23), and the replacement of C-peptide in type 1 diabetic patients during a period of 1–3 mo, is accompanied by improvements in renal function (14). In diabetic animal models, C-peptide has also been shown to reduce renal hypertrophy, proteinuria, and albuminuria (52, 58). Notably, peptides with the same amino-acid composition, but in randomized sequences, have not been shown to result in any effects (45, 53, 54). The mode of action by which C-peptide reduces hyperfiltration is unknown.

The renal glomerular afferent arterioles exert critical actions to control glomerular capillary pressure, thereby influencing GFR. Thus dilation of the afferent arterioles could cause glomerular hyperfiltration. Contraction, on the other hand, will reduce capillary pressure and GFR. Thus one possible mode of action for the C-peptide’s renoprotective function is via the afferent arterioles.

To gain further understanding of the mechanisms by which C-peptide mediates its action, it is of considerable importance to localize a site of effect and a mechanism of action. Our hypothesis was that C-peptide induces afferent arteriolar vasoconstriction. Such an action could underpin the potential renoprotective effects of this molecule. Therefore, in this study, we investigated whether intraluminally administered C-peptide causes a change in arteriolar diameter.

Rho and Rho-kinase are important Ca2+ sensitizing factors in the mediation of renal afferent vasoconstriction (30) and are also necessary for a MAPK-induced contractile response. Thus, to further elucidate the properties of C-peptide, the effect of Rho-kinase inhibitor Y-27632 on the effect of C-peptide was investigated, and the peptide was compared with three well-known vasoconstrictors: angiotensin II, norepinephrine, and KCl.

Research Design and Methods

Animals. The study was performed on arterioles isolated from C57-Bl mice weighing ~25 g and purchased from Mollegaard and Bomholtgaard (M&B, Ry, Denmark). The mice were allowed free access to water and standardized mouse chow (Ewos, Södertälje, Sweden). All experiments were approved by The Uppsala Ethical Committee for Animal Experiments, and were performed in accordance with national guidelines for the care and use of laboratory animals.

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Induction of diabetes. C57-B1 mice were made diabetic by means of a single intravenous injection of alloxan (75 mg/kg, Sigma Chemical, St. Louis, MO) 2 wk prior to the experiment. Animals were considered diabetic if the plasma glucose concentration rose to $\geq 16$ mM within 60 h after the injection and remained at this level at the time of the experiment. Age-matched normoglycemic mice served as controls. Blood samples were obtained from the cut tips of the tails and analyzed by glucose reagent strips (MediSense, Bedford, MA). Among the normoglycemic animals, plasma glucose concentrations varied between 5.5 and 7 mM. An animal was excluded from the study if it exhibited a weight loss $\geq 10\%$ of its body weight or if blood glucose exceeded 30 mM.

**Solutions and drugs.** DMEM was used during dissection and perfusion of the arterioles. Glucose was adjusted to 5.56 mM in the DMEM for the normoglycemic arterioles and to 17.51 mM in the DMEM for the hyperglycemic arterioles. These concentrations do not in themselves affect the afferent arterioles (29). BSA was added to the DMEM to make up a concentration of 0.1%, after which the pH was adjusted to 7.4. The BSA and angiotensin II were obtained from Sigma-Aldrich (Stockholm, Sweden), DMEM from Invitrogen (Stockholm, Sweden), the mouse C-peptide from Thermo Electron (Ulm, Germany), and the scrambled C-peptide from Dr Ekberg’s laboratory, Section of Clinical Physiology, Karolinska Institute (Stockholm, Sweden).

**Procedure.** Afferent arterioles from normoglycemic and alloxan-treated hyperglycemic mice were manually isolated and perfused in a procedure that conforms to that developed by Edwards (11) and modified by Patzak et al. (42): the mice were killed and their kidneys were immediately removed and sliced along the cortico-medullary axis. The arterioles were then prepared at 4°C in albumin-enriched DMEM. The afferent arterioles, intact with the glomeruli and arteriolar stems, were dissected and cut at the proximal end. The arteriole was then fixed on the stage of a confocal microscope by being sucked into a holding pipette (aperture of roughly 26 μm). A perfusion pipette of 5 μm in diameter was advanced into the lumen of the arteriole and the arteriole microperfused with DMEM solution (Fig. 1). The arterioles were all perfused at 37°C, with a pressure of 80 mmHg in the pressure head.

**Experimental protocol.** After 15 min of warm-up for stabilization, vessel reactivity to KCl was tested, and after a 5-min control period a 1-min control period was used to obtain baseline values. Thereafter, changes in luminal diameter were measured every 12 s during 60 s.

**Other vasoconstricting agents.** The constricting properties of three well known vasoconstrictors, angiotensin II (10 nM, n = 6 and 0.1 nM, n = 6), norepinephrine (10 μM, n = 6), and KCl (100 mM, n = 7), were estimated on renal afferent arterioles using a modified version of the C-peptide protocol. After 15 min of warm-up for stabilization, the afferent arterioles were perfused with C-peptide (5 nM) in DMEM (n = 7).

**Data collection and statistical analysis.** The experiments were recorded by a video system, off-line digitized and analyzed as described previously (41). Every 5 min during the C-peptide experiments and every 12 s during the constrictor experiments, in control as well as treatment periods, five measurements of the luminal diameter of the afferent arterioles were averaged. All statistical analyses were performed using GraphPad Prism software (GraphPad Software, San Diego, CA). Descriptive statistics are presented as mean values ± SE.

Multiple data sets between groups were analyzed with nonparametric Kruskal Wallis followed by Dunn’s post hoc test or ANOVA followed by Fisher’s post hoc test when appropriate. Multiple data sets within groups were analyzed with ANOVA followed by Dunnett’s post hoc test when appropriate. For all comparisons, P < 0.05 was considered to be statistically significant.

**RESULTS**

**Afferent arteriole diameter.** The initial diameters of the arterioles did not differ between the normoglycemic and hyperglycemic groups (Table 1). At a concentration of $5 \times 10^{-9}$ M, C-peptide administered to afferent arterioles from hyperglycemic mice reduced afferent arteriolar diameter within 10 min compared with baseline values ($-15.8 \pm 6.7\%$; $P < 0.05$; Fig. 2). After 30 min, C-peptide had decreased afferent arteriolar diameter by $-26.7 \pm 7.7\%$ in the hyperglycemic group compared with baseline values ($P < 0.001$) and to the diameter in the other groups ($P < 0.001$). At a concentration of $5 \times 10^{-10}$ M, C-peptide administered to afferent arterioles from hyperglycemic mice reduced afferent arteriolar diameter within 30 min compared with baseline values ($-7.0 \pm 2.4\%$; $P < 0.05$; Fig. 2). Normoglycemic arterioles administered C-peptide showed a minor but significant decrease in arteriolar diameter only after 30 min ($-4.1 \pm 1.4\%$; $P < 0.001$; Fig. 3) compared with baseline values. Scrambled C-peptide perfused through the arterioles, whether from hyperglycemic or normoglycemic mice, did not induce any effect ($P > 0.5$ and 0.7, respectively). Vehicle administration did not affect the diameter in hyperglycemic ($P > 0.2$) or normoglycemic groups ($P > 0.7$).

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**Fig. 1.** The setup of the renal afferent arteriole experiments. The microphotograph shows a glomerulus and its afferent arteriole held by 2 holding pipettes. The perfusion pipette is inserted into the holding pipette on the right hand side.
alone, did not affect vessel diameter (data not shown). Administration of Rho-kinase inhibitor Y-27632 was prevented by the inhibition of Rho-kinase with Y-27632/H11002.

Angiotensin II (10^(-7) M, n = 6, 10^-10 M, n = 6), norepinephrine (10^-6 M, n = 6), and KCl (100 mM, n = 7). At a high concentration, angiotensin II (10^(-7) M) achieved a maximal diameter decrease of 51.2 ± 16.7% in arteriole diameter within 24 s, whereas a lower concentration (10^-10 M) decreased the diameter by 9.7 ± 2.1% within 48 s. The infusion of norepinephrine (10^-6 M) achieved a maximal diameter decrease of 72.2 ± 7.3% within 24 s. The infusion of 100 mM KCl, finally, induced a close to 100% decrease in arteriolar diameter (-96.9 ± 2.9%) within 24 s.

The effect of Rho-kinase inhibition. The effect of C-peptide was prevented by the inhibition of Rho-kinase with Y-27632 (Fig. 5). Administration of Rho-kinase inhibitor Y-27632 alone, did not affect vessel diameter (data not shown).

**Fig. 2.** The effect of proinsulin C-peptide (5 nM, n = 7, ●), scrambled C-peptide (5 nM, n = 7, ○), and vehicle perfusion (n = 4, □) on the diameter of afferent glomerular arterioles from hyperglycemic C57-B1 mice. *P < 0.05 vs. all other groups, +P < 0.05 vs. the control value within the same group. Values are presented as means ± SE.

**Fig. 4.** The effect of norepinephrine (NE; 10 μM, n = 6, □), KCl (n = 7, 100 mM, §) and ANG II (n = 6, 100 pM, □) on the diameter of afferent glomerular arterioles from normoglycemic C57-B1 mice. *P < 0.05 vs. the control value within the same group. Values are presented as means ± SE.

**DISCUSSION**

In this study, we report that C-peptide induces a marked constriction in glomerular afferent arterioles from hyperglycemic, but not from normoglycemic, animals. Increases in GFR, largely due to an increased glomerular capillary hydraulic pressure, are apparent in early diabetes mellitus (39). Agents that lower glomerular capillary hydraulic pressure, slow the progression of renal injury in diabetes (5). Thus, if the C-peptide-induced constriction of the glomerular afferent arteriole occurs in vivo, this may explain the beneficial effects of C-peptide substitution in diabetes. Preliminary data from our lab, showing a reduction in stop-flow pressure, support these findings (L. Nordquist, R. Brown, A. Fasching, M. Sjöquist, and F. Palm, unpublished observations). However, C-peptide possesses no effects on renal blood flow, which may indicate a concomitant dilating effect on the efferent arteriole (20, 23, 52).

The fact that normoglycemic mice administered C-peptide initially did not show any difference in renal afferent arteriole diameter, is consistent with previous studies (20, 32). Although no receptor for C-peptide has yet been fully characterized or sequenced, studies have shown binding of C-peptide to cell membranes and inhibition of C-peptide effects by pertussis toxin, suggesting a G protein-coupled receptor (27, 40, 45). Although remaining receptor binding seems unlikely under these conditions, this could be explained by residual downstream effects from endogenous C-peptide in these arterioles in the beginning of the experiments, possibly combined with an

**Table 1.** Mean diameter of renal afferent arterioles from NG animals, HG animals, and from animals receiving either 5 nM or 0.5 nM C-peptide of 5 nM scramble C-peptide, or 5 nM C-peptide after incubating with 1 μM Rho-kinase inhibitor Y-27632

<table>
<thead>
<tr>
<th>Time, min</th>
<th>Control</th>
<th>Scrambled C-Peptide (5 nM)</th>
<th>C-Peptide (5 nM)</th>
<th>C-Peptide (0.5 nM)</th>
<th>Y-27632 &amp; C-Peptide (5 nM)</th>
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<tbody>
<tr>
<td>Afferent arteriole diameter in hyperglycemic animals, μm</td>
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<tr>
<td>0</td>
<td>10.3±1.2</td>
<td>10.6±0.6</td>
<td>10.2±1.2</td>
<td>12.3±0.9</td>
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<td>10</td>
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<td>10.7±0.7</td>
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<td>9.8±0.8</td>
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<td>20</td>
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<td>7.9±1.3</td>
<td>12.1±1.0</td>
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<tr>
<td>30</td>
<td>10.4±1.2</td>
<td>10.8±0.7</td>
<td>7.6±1.1</td>
<td>11.7±1.1</td>
<td>9.6±0.9</td>
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<tr>
<th>Time, min</th>
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<th>Scrambled C-Peptide (5 nM)</th>
<th>C-Peptide (5 nM)</th>
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<tbody>
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<td>Afferent arteriole diameter in normoglycemic animals, μm</td>
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<tr>
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<td>12.4±0.4</td>
<td>10.0±0.7</td>
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<tr>
<td>30</td>
<td>12.1±0.4</td>
<td>10.1±0.7</td>
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Values are means ± SE. n = 4 for normoglycemic animals (NG); n = 4 for hyperglycemic animals (HG); n = 9 and 7 for NG and HG receiving C-peptide, respectively; n = 7 for HG receiving 0.5 nM C-peptide; n = 5 each for NG and HG receiving 5 nM scrambled C-peptide; n = 7 for HG receiving 5 nM C-peptide after incubating with 1 μM Y-27632.
upregulation of C-peptide’s receptors, and thereby downstream signaling, in the diabetic state. Plasma C-peptide concentrations in the normoglycemic animals should be ~0.4–1 nM (1, 21, 50), whereas C-peptide concentrations in the hyperglycemic animals are likely ~0.2 nM (21, 50). Several receptors are known to be upregulated in the diabetic state, and although no receptor has been identified for C-peptide in the afferent arterioles specifically, studies have reported specific binding of C-peptide to the cell membrane of several human cell types (45). In addition, several studies suggest the involvement of a G protein-coupled receptor (4, 40, 59). However, for residual effects of C-peptide to be a probable explanation, either slow dissociation of C-peptide from its receptor or time consuming downstream events are required. Dissociation of C-peptide from its putative receptor appears to be in the same range as those observed for, e.g., insulin, galanin, or α-bungarotoxin (45), and although the in vivo effect of C-peptide has been shown to last at least 30 min after a single bolus injection (38), it remains unclear whether the second messenger cascades initiated by C-peptide have decay times extended enough for this model to be plausible. Consistent with previous studies, perfusion with scrambled C-peptide did not produce any effect (18, 21, 64).

Previous studies have showed normalizing effects of C-peptide on diabetes-impaired blood perfusion and blood cell velocity (15, 22). However, these studies investigated tissues where diabetes induces a decrease in blood perfusion and showed that administration of C-peptide increased blood perfusion toward normal values (13, 43, 44). In renal vessels, on the other hand, diabetes will instead cause an increased blood flow (8). Blood flow seems to be differentially affected in, e.g., kidney vs. peripheral tissues in C-peptide deficiency (8–10). It has also been suggested that C-peptide may interact with two or more receptor molecules (45). Thus, taken together, it is likely that C-peptide exerts tissue- and/or state-specific effects.

The G protein Rho and its downstream effector Rho-kinase affect cytoskeleton and expression of integrins and also play important roles in mediating vasoconstriction in the kidney. Thus they regulate glomerular blood flow and GFR, as well as the function and structure of renal cells such as tubular epithelial cells and mesangial cells. Inhibition of Rho-kinase in constricted renal afferent arterioles, as well as in other vessels, causes a relaxation of the artery (6, 47, 56), suggesting that Rho-kinase acts as a regulator of the sensitivity of the arterial contractile apparatus. Rho/Rho-kinase seems to act in the tonic phase of constriction and will inactivate myosin phosphatase. With the phosphatase inactive, the myosin regulatory subunit will remain phosphorylated, inducing Ca²⁺ sensitivity. Thus, although this phase is stimulated by Ca²⁺ influx and Rho/Rho-kinase is activated by Ca²⁺ (35, 49), Rho/Rho-kinase maintains vasoconstriction at only modestly elevated Ca²⁺ levels, a phenomenon referred to as Ca²⁺-sensitization (26). Except for Ca²⁺ influx or Ca²⁺ sensitization, Rho-kinase activity has also been shown to be stimulated entirely Ca²⁺ independently (12, 17, 56).

We show in this study that the Rho-kinase inhibitor Y-27632 prevents the constricting effects of C-peptide in vitro. These results are congruent with previous studies and may in addition suggest a Ca²⁺-sensitizing effect (37, 57) of C-peptide. A mechanism for the actions of C-peptide has long been sought for. The finding in this study that inhibition of Rho-kinase abolishes the vasoconstricting ability of C-peptide, indicates that C-peptide is dependent on this cascade, possibly by the activation of Rho-kinase. These results are in line with previous data by Zhong et al. (64), showing translocation and binding of RhoA by C-peptide in renal tubular cells. A C-peptide-induced activation of Rho-kinase could imply that C-peptide effects are mediated via Ca²⁺ sensitivity (55, 56).

However, the preventive effects of Y-27632 on C-peptide-induced vasoconstriction of isolated afferent arterioles, do not necessarily imply that C-peptide activates Rho-kinase. C-peptide is known to increase intracellular Ca²⁺ in human tubular cells (53), smooth muscle cells (31), and aortic endothelial cells (60), and it has been reported that C-peptide activates PKC via increased intracellular Ca²⁺ in opossum proximal tubular cells (2). C-peptide also activates MAPK through a PKC-dependent mechanism (64). Importantly, PKC and MAPK both mediate contraction (16, 48, 61). Additionally, the MAPK cascade has been linked to phosphorylation of the regulatory subunit of the myosin light chain in the same location as myosin light chain kinase (46). Inhibition of Rho-kinase will allow for greater myosin phosphatase activity, which in turn decreases the phosphorylation status of the regulatory subunit of the myosin light chain. Therefore, it is possible that C-peptide induces phosphorylation of the myosin light chain in a non-Rho-A-dependent manner, such as PKC or MAPK.

Inhibition of Rho-kinase abolishes not only the vasoconstricting effects of C-peptide, but also the effect of other vasoconstricting agents, such as angiotensin II and norepinephrine (7). Comparing the constricting behavior of C-peptide to that of these vasoconstrictors gives a clearer picture of the potency of C-peptide as a constrictor of the afferent arterioles. The administration of C-peptide to isolated afferent arterioles, produced an effect comparable in magnitude to that of angiotensin II (24, 25), one of the most powerful endogenous vasoconstricting agents. However, as seen in Fig. 5, the effect of C-peptide takes considerably longer to develop (10 min) than that of the other vasoconstrictors, which will induce constriction in a matter of seconds. In addition, the C-peptide-induced vasoconstriction does not stabilize during the experiment. It is therefore possible that a prolonged administration of C-peptide would provide a yet greater effect on the efferent arterioles. An explanation for this could be a sequential cas-
cade of events leading up to the vasoconstriction, e.g., through activation of its receptor with subsequent effects on e.g., protein synthesis.

Perspectives and Significance

The constrictive effect of C-peptide on afferent arterioles found in this study supports previous findings that C-peptide affects diabetes-induced glomerular hyperfiltration, and that C-peptide might be used as a preventive drug to bring about a more efficient treatment for diabetic patients. Since the reducing effect of C-peptide on diabetes-induced glomerular hyperfiltration is achieved without affecting blood flow (52), further studies should be undertaken, investigating the effect of C-peptide on the intricate interplay between the afferent and the efferent arteriole. Concomitantly, the effectiveness of C-peptide on the prevention and reversal of diabetic complications should be more thoroughly investigated in human subjects. To achieve this, long-term, large-scale studies should be conducted to evaluate safety and health benefits of long-term administration of C-peptide.

CONCLUSIONS

The findings of this study show that the administration of C-peptide has effects on the renal afferent arteriole that could influence GFR, thereby at least partly preventing hyperfiltration. This effect seems to be mediated through Rho-kinase, ERK, and/or PKC. Thus it is possible that C-peptide contributes to the maintenance of normal renal arteriolar tone in normoglycemic mice.

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GRANTS


DISCLOSURES

No potential conflicts, personal or financial, exist regarding this study.

REFERENCES
