Renoprotective effects of C-peptide in the Dahl salt-sensitive rat

R. Taylor Sawyer, Elizabeth R. Flynn, Zachary M. Hutchens, Jr., Jan M. Williams, Michael R. Garrett, and Christine Marie-Bilkan

1Department of Physiology and Biophysics, University of Mississippi Medical Center, Jackson, Mississippi; 2Department of Pharmacology and Toxicology, University of Mississippi Medical Center, Jackson, Missouri; and 3Center for Excellence in Cardiovascular-Renal Research, University of Mississippi Medical Center, Jackson, Mississippi

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Sawyer RT, Flynn ER, Hutchens ZM Jr, Williams JM, Garrett MR, Marie-Bilkan C. Renoprotective effects of C-peptide in the Dahl salt-sensitive rat. Am J Physiol Renal Physiol 303: F893–F899, 2012—Previous studies have demonstrated that renoprotective effects of C-peptide in experimental models of diabetes-induced renal disease may be mediated via lowering blood glucose. The present study examined the renoprotective effects of C-peptide in a model of nondiabetic renal disease, the Dahl salt-sensitive (SS/jr) rat. SS/jr rats were placed on a 2% NaCl diet for 2 wk (HS2, resulting in mild to moderate renal injury) or 4 wk (HS4, resulting in advanced renal injury) and then received either vehicle (veh) or C-peptide (Cpep) for additional 4 wk. Urine albumin (UAE) and protein (UPE) excretion rates were measured at baseline (i.e., before initiation of veh or Cpep treatment) and 4 wk later (i.e., at the time of death). Glomerular permeability, indexes of glomerulosclerosis and tubulointerstitial fibrosis, the presence of inflammatory cells, and protein expression of transforming growth factor-β (TGF-β) and podocin were measured at the time of death. In HS2 + veh rats, UAE and UPE increased by 74 and 92%, respectively, from baseline and the time of death. While HS2 + Cpep attenuated this increase in UAE and UPE, HS4 + Cpep had no effect on these parameters. Similarly, HS2 + Cpep reduced glomerular permeability, tubulointerstitial fibrosis, renal inflammation, TGF-β, and podocin protein expression, while HS4 + Cpep had no effect. These studies indicate that C-peptide is renoprotective in nondiabetic experimental models with mild to moderate renal injury.

MATERIALS AND METHODS

Study design. Male SS/jr rats were obtained from our breeding colony (M. R. Garrett). The animals were raised on a low (0.3%) NaCl diet (TD7034; Harlan, Madison, WI) until 6 wk of age, at which time they were placed on a high (2%) NaCl diet (TD94217; Harlan) and given free access to tap water. After either 2- or 4-wk pretreatment with 2% NaCl, animals were randomly divided to receive either vehicle or C-peptide for additional 4 wk, during which all animals continued to receive a 2% NaCl diet. Thus our experiment had four treatment groups: 2-wk pretreatment with 2% NaCl followed by vehicle (HS2 + veh; n = 5), 2-wk pretreatment with 2% NaCl followed by C-peptide (HS2 + Cpep; n = 5), 4-wk pretreatment with 2% NaCl followed by vehicle (HS4 + veh; n = 5), and 4-wk pretreatment with 2% NaCl followed by C-peptide (HS4 + Cpep; n = 6). The reason for selecting shorter (2 wk) and longer (4 wk) pretreatment periods with 2% NaCl was to allow for development of milder (2 wk) and more severe (4 wk) renal injury.

The vehicle (0.9% saline) and rat C-peptide (50 pmol·kg⁻¹·min⁻¹; American Peptide, Sunnyvale, CA) were continuously administered for 4 wk via osmotic minipumps (type 2004; Alzet, Cupertino, CA) that were implanted subcutaneously in the nape of the neck. Animals were placed in metabolic cages for a 24-h period to measure food and water intake and urine collection for determination of UAE and urinary protein excretion (UPE). These measurements were performed 1 day before the osmotic minipumps delivering vehicle or C-peptide were implanted (baseline) as well as 3 days before the animals were killed. One day before death, catheters were implanted in the femoral artery to measure blood pressure and in the femoral vein to measure glomerular filtration rate (GFR) as described below. The animals were allowed to recover overnight, and mean arterial pressure (MAP) and GFR measurements were taken the next day. After data collection, the rats were killed and the kidneys were removed and weighed. Parts of the kidneys were then either snap frozen (for protein analysis), fixed in 10% buffered formalin (for histology and immunohistochemistry), or used in the measurement of the glomerular permeability.

All experiments were performed in compliance with the guidelines recommended by the National Institutes of Health and approved by the University of Mississippi Medical Center, 2500 North State St., Jackson, MS 39216 (e-mail: cmari@umc.edu).

* R. T. Sawyer and E. R. Flynn contributed equally to this work.

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F893
Table 1. Metabolic and renal parameters in SS/jr rats pretreated with 2% NaCl for 2 wk

<table>
<thead>
<tr>
<th>Parameters</th>
<th>HS2 + Veh (n = 5)</th>
<th>HS2 + Cpep (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>325 ± 22</td>
<td>340 ± 24</td>
</tr>
<tr>
<td>Kidney/body weight, g/kg</td>
<td>5.03 ± 0.4</td>
<td>5.01 ± 0.4</td>
</tr>
<tr>
<td>Blood glucose, mg/dl</td>
<td>93.8 ± 8.5</td>
<td>93.0 ± 8.2</td>
</tr>
<tr>
<td>Plasma insulin, ng/ml</td>
<td>3.21 ± 0.40</td>
<td>4.04 ± 0.50</td>
</tr>
<tr>
<td>Plasma C-peptide, nM</td>
<td>1.48 ± 0.06</td>
<td>1.87 ± 0.06*</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>187 ± 17</td>
<td>187 ± 15</td>
</tr>
<tr>
<td>GFR, ml/min</td>
<td>2.09 ± 0.30</td>
<td>2.03 ± 0.26</td>
</tr>
<tr>
<td>GFR, ml·min⁻¹·g kidney wt⁻¹</td>
<td>1.33 ± 0.38</td>
<td>1.27 ± 0.39</td>
</tr>
</tbody>
</table>

Values are means ± SE. SS/jr, Dahl salt-sensitive rats; MAP, mean arterial pressure; GFR, glomerular filtration rate; Cpep, C-peptide; HS2, 2% NaCl diet for 2 wk; Veh, vehicle. *P < 0.05 vs. HS2 + veh.

Western blotting. Homogenized, denatured protein samples were separated through SDS-PAGE precast gels (Bio-Rad, Hercules, CA) and then transferred to a nitrocellulose membrane. The membranes were then blocked first with 5% nonfat milk and then incubated overnight at 4°C with antiserum against transforming growth factor-β (TGF-β; 1:500 rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA) or podocin (1:2,000 rabbit polyclonal; Abcam, Cambridge, MA). The membranes were then incubated with anti-rabbit antibodies conjugated to horseradish peroxidase, and proteins were visualized by enhanced chemiluminescence (Thermo Scientific, Rockford, IL). All the membranes were stripped using a stripping buffer (Thermo Scientific) and reprobed with an antibody against β-actin (1:1,100 mouse monoclonal; Cell Signaling, Danvers, MA). The densities of the specific bands were quantified using Scion Image beta (version 4.02) software and then normalized to the amount of protein loaded in each well using the densitometric analysis of β-actin from the same membrane.

Measurement of the reflection coefficient of albumin in glomeruli. The reflection coefficient of albumin (dorab) in isolated glomeruli was measured using a modification of the Savin technique (23). Briefly, high molecular weight FITC-labeled dextran was infused through the femoral vein, and after 5 min the glomeruli were isolated using the sieving method in Hanks’ buffer solution containing 6% BSA. The FITC-labeled dextran in the glomeruli was visualized using the 80-μl fast-exchange perfusion chamber mounted on the stage of an inverted microscope (TS100; Nikon, Melville, NY) and imaged with the InCyt IM1 imaging system (Intracellular Imaging, Cincinnati, OH) using an excitation filter of 475 nm and an emission filter of 530 nm. The dorab was determined by measuring the changes in fluorescence in each glomerulus after the concentration of BSA was lowered in the bath from 6 to 4%. The dorab was calculated as the measured percentage change of fluorescent intensity divided by the expected percentage change in glomerular volume relative to the 33% decrease in oncotic pressure. A minimum of 10 glomeruli was studied from each rat.

Statistical analysis. All values are expressed as means ± SE. The data, except for the analysis of UAE and UPE, were analyzed using Student’s t-test (Prism 4, Graph Pad Software, San Diego, CA). The UAE and UPE data were analyzed using two-way ANOVA (SigmaStat software version 3.5). P values <0.05 were considered statistically significant.

RESULTS

Metabolic parameters. No differences in body weight, kidney/body weight ratio, blood glucose, or plasma insulin were observed between vehicle and C-peptide treated rats after either a 2-wk (Table 1) or 4-wk (Table 2) pretreatment with 2% NaCl. As expected, plasma C-peptide levels were increased by 26% in the HS2 + Cpep compared with HS2 + veh (Table 1). Similarly, plasma C-peptide levels were increased by 17% in HS4 + Cpep compared with HS4 + veh (Table 2).
Renal function, albuminuria, and proteinuria. No differences in MAP or GFR were observed between HS2 + veh and HS2 + Cpep (Table 1) or HS4 + veh and HS4 + Cpep (Table 2).

UAE increased by 71% between baseline and the time of death in HS2 + veh, and this increase was prevented in HS2 + Cpep animals (Fig. 1A, left). Similarly, UAE increased by 93% between baseline and the time of death in HS4 + veh; however, HS4 + Cpep had no effect on the development of albuminuria (Fig. 1A, right).

UPE increased by 75% between baseline and the time of death in HS2 + veh, and this increase was prevented in HS2 + Cpep animals (Fig. 1B, left). Similarly, UAE increased by 61% between baseline and the time of death in HS4 + veh; however, HS4 + Cpep was unable to prevent this (Fig. 1B, right).

Integrity of the glomerular filtration barrier. Since we found that HS2 + Cpep, but not HS4 + Cpep, reduced UAE and UPE, we only assessed the parameters of the integrity of the glomerular filtration barrier after the 2-wk pretreatment period with 2% NaCl. Specifically, we assessed the permeability of the glomerular filtration barrier by measuring the reflection coefficient of albumin (dσ Alb) as well as protein expression of podocin, an integral component of the slit diaphragm (7). The HS2 + Cpep animals had a 14% increase in dσ Alb compared with HS2 + veh (Fig. 2A), suggesting that C-peptide improves the glomerular filtration barrier. In addition, HS2 + Cpep increased podocin protein expression by 47% compared with HS2 + veh, suggesting that C-peptide protects the integrity of the slit diaphragm.

Renal pathology. Two weeks of pretreatment with 2% NaCl were not associated with significant glomerular injury; however, prominent segmental glomerulosclerosis was present in rats following a 4-wk pretreatment with 2% NaCl (Fig. 3A, left). Neither HS2 + Cpep or HS4 + Cpep had an effect on the index of glomerulosclerosis (Fig. 3A, right).

Mild tubulointerstitial fibrosis, characterized by accumulation of extracellular matrix (blue color in the Masson’s trichrome-stained images, Fig. 2B, left), the presence of inflammatory cells, and tubular dilatation, was observed in HS2 + veh animals. The index of TIFI decreased by 41% in HS2 + Cpep compared with HS2 + veh (Fig. 3B, right). Tubulointerstitial fibrosis was even more apparent in HS4 + veh (Fig. 2B, left). However, unlike HS2 + Cpep, HS4 + Cpep was unable to reduce TIFI (Fig. 3B, right).

Renal inflammation. Renal inflammation, as measured by the presence of CD68-positive cells, was evident in HS2 + veh animals (Fig. 4, left) and HS2 + Cpep reduced the abundance of CD68-positive cells by 15% (Fig. 4, right). The presence of CD68-positive cells was even more apparent in HS4 + veh animals (Fig. 4, left); however, HS4 + Cpep was unable to reduce CD68-positive cell abundance (Fig. 4, right).

To further assess the contribution of C-peptide in reducing renal inflammation in rats after the 2-wk pretreatment with 2% NaCl, we measured TGF-β protein expression. HS2 + Cpep reduced TGF-β protein expression by 27% compared with HS2 + veh (Fig. 5).

DISCUSSION

C-peptide has recently emerged as a biologically active peptide and a potential therapeutic treatment for diabetic end-organ complications, such as diabetic nephropathy (9–10, 21, 24). However, there is still some controversy related to the mechanisms by which C-peptide exerts its renoprotective effects. Previous studies (11, 18, 22) have reported that one of the mechanisms by which C-peptide protects the target organ in the setting of diabetes may be via lowering blood glucose levels. Indeed, findings (14) from our own laboratory have indicated that chronic (4 wk) administration of C-peptide to STZ-treated rat reduces albuminuria, glomerular hyperfiltra-
C-peptide in renal pathophysiology, we examined its effects in the form of renal injury. To better understand the effects of C-peptide without the confounding effect of glucose lowering, we found no evidence of glomerular injury based on histological evaluations, we cannot exclude the possibility of molecular changes evident by light microscopy.

In addition to attenuating UAE and UPE, we found that treatment with C-peptide reduced tubulointerstitial fibrosis associated with a 2-wk pretreatment with 2% NaCl in the SS/jr rat. Furthermore, C-peptide reduced the abundance of CD68-positive cells (indicating the presence of inflammatory cells) and TGF-β protein expression in these animals, both of which contribute to the development and progression of tubulointerstitial fibrosis (15, 31). Studies (12, 19, 31) in experimental models of diabetic and nondiabetic renal disease have shown that attenuating the abundance of inflammatory cells and TGF-β protein expression is renoprotective. While C-peptide has previously been shown to exert antifibrotic effects in the diabetic kidney via attenuating TGF-β signaling (4, 6), this is, renal disease (9–10, 21, 24) as well as in patients with microalbuminuria associated with type 1 diabetes (10) in which C-peptide is renoprotective. Interestingly, C-peptide was unable to reduce UAE and UPE in the more severe form of renal injury (i.e., 4-wk pretreatment with 2% NaCl), suggesting that C-peptide may be ineffective in attenuating albuminuria and proteinuria in the presence of more advanced/severe renal injury. It is conceivable that the lack of an effect of C-peptide in these animals may be dose related; however, a pilot study performed in our laboratory using a higher dose of C-peptide (80 pmol·kg⁻¹·min⁻¹) did not result in additional renoprotection (data not shown).

Unlike in experimental models of diabetic renal disease where C-peptide reduces glomerular hyperfiltration (16, 18, 21, 24, 26), we did not see any effects of C-peptide on GFR in either the mild-moderate or the more severe form of renal injury. Similarly, C-peptide had no effects on MAP in any of the treatment groups. Thus it appears that C-peptide only affects GFR in the setting of glomerular hyperfiltration, as is the case in early diabetic renal injury. Since we observed no effects of C-peptide on GFR or MAP, we sought to explore other mechanisms that could explain the reduction in UAE and UPE in animals exposed to the 2-wk pretreatment with 2% NaCl. Our studies (23) show that treatment with C-peptide is associated with an improvement of the reflection coefficient of albumin, indicating reduced leakiness/permeability of the glomerular filtration barrier. Furthermore, the permeability of the glomerular filtration barrier is also dependent on its structural integrity. Specifically, alterations in the expression of the integral elements of the slit diaphragm, such as podocin, are associated with a leaky glomerular filtration barrier and thus increase in UAE and UPE (7, 27–28). We found that treatment with C-peptide prevents the decrease in podocin protein expression. These observations are consistent with the concept that one of the mechanisms by which C-peptide attenuates albuminuria and proteinuria is via preserving the integrity of the glomerular filtration barrier. It should be noted that while we found no evidence of glomerular injury based on histological evaluations, we cannot exclude the possibility of molecular changes in the glomerulus initiated in response to high salt. Indeed, this concept is supported by the fact that changes in podocin protein expression were evident despite the absence of histological changes and that C-peptide preserved glomerular integrity via increasing podocin expression without structural changes evident by light microscopy.

In addition to attenuating UAE and UPE, we found that treatment with C-peptide reduced tubulointerstitial fibrosis associated with a 2-wk pretreatment with 2% NaCl in the SS/jr rat. Furthermore, C-peptide reduced the abundance of CD68-positive cells (indicating the presence of inflammatory cells) and TGF-β protein expression in these animals, both of which contribute to the development and progression of tubulointerstitial fibrosis (15, 31). Studies (12, 19, 31) in experimental models of diabetic and nondiabetic renal disease have shown that attenuating the abundance of inflammatory cells and TGF-β protein expression is renoprotective. While C-peptide has previously been shown to exert antifibrotic effects in the diabetic kidney via attenuating TGF-β signaling (4, 6), this is,
to the best of our knowledge, the first report on the anti-inflammatory effect of C-peptide in the kidney. C-peptide has, however, been shown to exert anti-inflammatory effects in sepsis and myocardial ischemia-reperfusion injury (2). Several studies (1) have suggested that increased protein in the proximal tubules, as a result of increased leakage of proteins across the glomerular filtration barrier, may lead to development of tubulointerstitial fibrosis directly or via increasing the expression of proinflammatory cytokines. Since C-peptide reduced delivery of proteins in the proximal tubules, via reducing glomerular permeability, and reduced renal inflammation, it is conceivable that C-peptide reduced tubulointerstitial fibrosis via these mechanisms. In contrast to the effects of C-peptide on reducing tubulointerstitial fibrosis in rats pretreated with 2% NaCl for 2 wk, C-peptide had no effect on tubulointerstitial fibrosis in rats pretreated with 2% NaCl for 4 wk. These observations are consistent with the lack of an effect of C-peptide on UAE and UPE observed in rats with advanced renal injury.

Fig. 3. Effects of C-peptide on renal pathology in SS/jr rats fed a high salt diet. A: periodic acid-Schiff-stained sections and index of glomerulosclerosis (GSI) expressed in arbitrary units (AU). B: Masson’s trichrome-stained sections and index of tubulointerstitial fibrosis (TIFI) expressed in AU. Values are expressed as means ± SE. HS2, 2-wk pretreatment with 2% NaCl; HS4, 4-wk pretreatment with 2% NaCl.
While our study found that C-peptide attenuated albuminuria, proteinuria, tubulointerstitial fibrosis, TGF-β, and abundance of inflammatory cells in SS/jr rats, we observed no effect of C-peptide on glomerulosclerosis. This lack of an effect of C-peptide may not be so surprising given the absence of significant structural changes in the glomerulus in the early stages of the disease (i.e., 2-wk pretreatment with 2% NaCl). Studies (20–21) in experimental models of diabetic renal disease have consistently shown that C-peptide reduces glomerular hypertrophy, but no studies to date have reported the effects of C-peptide on glomerulosclerosis in experimental models of diabetic renal disease. In contrast to the 2-wk pretreatment with 2% NaCl, the SS/jr rat exposed to a 4-wk pretreatment with 2% NaCl did show moderate glomerulosclerosis. However, similar to other parameters of advanced renal injury, C-peptide was unable to reduce glomerulosclerosis.

To date, studies have exclusively focused on examining the renoprotective effects of C-peptide in the setting of diabetes. We believe that the major strength of the present study is the fact that it demonstrates renoprotective effects of C-peptide in a nondiabetic experimental model. One of the limitations of the present study is clearly the lack of mechanistic insight into the actions of C-peptide in the kidney. Unlike the diabetic kidney, we demonstrate that C-peptide exerts its actions through mechanisms other than glucose regulation in the nondiabetic kidney. However, future studies are clearly needed to examine the precise mechanisms of action of C-peptide in the kidney under either diabetic or nondiabetic conditions.

In conclusion, the present study shows that C-peptide exerts renoprotective effects in an experimental model of mild-moderate renal injury independent of blood glucose control. Although these beneficial effects of C-peptide appear to be limited to renal injury of mild-moderate severity, these observations provide evidence for a biologically active role of C-peptide in the prevention and treatment of chronic kidney disease.

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Fig. 4. Effects of C-peptide on CD68-positive cell abundance in SS/jr rats pretreated with 2% NaCl for 2 wk. CD68 immunolocalization (brown staining indicated by arrowheads; original magnification = ×400) and quantitative analysis of CD68-positive cell abundance. Values are expressed as means ± SE.

Fig. 5. Effects of C-peptide on transforming growth factor (TGF)-β protein expression in SS/jr rats pretreated with 2% NaCl for 2 wk. Top: representative immunoblot of TGF-β protein expression. Bottom: densitometric scans of the 50-kDa band in ROD expressed as a ratio of renal TGF-β/β-actin. Values are expressed as means ± SE.
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: R.T.S., E.R.F., Z.M.H.J., and J.M.W. performed experiments; E.R.F. and J.M.W. analyzed data; J.M.W., M.R.G., and C.M.-B. edited and revised manuscript; M.R.G. and C.M.-B. conception and design of research; C.M.-B. interpreted results of experiments; C.M.-B. prepared figures; C.M.-B. drafted manuscript; C.M.-B. approved final version of manuscript.

REFERENCES