Proinsulin C-Peptide Antagonizes the Profibrotic Effects of TGF-β1 via Up-Regulation of Retinoic Acid and HGF-Related Signaling Pathways

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Novel signaling roles for C-peptide have recently been discovered with evidence that it can ameliorate complications of type 1 diabetes. Here we sought to identify new pathways regulated by C-peptide of relevance to the pathophysiology of diabetic nephropathy. Microarray analysis was performed to identify genes regulated by either C-peptide and/or TGF-β1 in a human proximal tubular cell line, HK-2. Expression of retinoic acid receptor β (RARβ), hepatocyte growth factor (HGF), cellular retinoic acid-binding protein II (CRABPII), vimentin, E-cadherin, Snail, and β-catenin was assessed by immunoblotting. The cellular localization of vimentin and β-catenin was determined by immunocytochemistry. Changes in cell morphology were assessed by phase contrast microscopy. Gene expression profiling demonstrated differential expression of 953 and 1458 genes after C-peptide exposure for 18 h or 48 h, respectively. From these, members of the antifibrotic retinoic acid (RA) and HGF-signaling pathways were selected. Immunoblotting demonstrated that C-peptide increased RARβ, CRABPII, and HGF. We confirmed a role for RA in reversal of TGF-β1-induced changes associated with epithelial-mesenchymal transition, including expression changes in Snail, E-cadherin, vimentin, and redistribution of β-catenin. Importantly, these TGF-β1-induced changes were inhibited by C-peptide. Further, effects of TGF-β1 on Snail and E-cadherin expression were blocked by HGF, and inhibitory effects of C-peptide were removed by blockade of HGF activity. This study identifies a novel role for HGF as an effector of C-peptide, possibly via an RA-signaling pathway, highlighting C-peptide as a potential therapy for diabetic nephropathy. (Molecular Endocrinology 24: 822–831, 2010)

A very recent position statement from the American Diabetes Association set out standards of care and summarized recommendations for the treatment of microangiopathic complications in diabetes (1). In addition to tight glycemic control and appropriate screening, advice highlighted the need to: control hypertension and use renin-angiotensin system modifiers in nephropathy; employ laser photocoagulation in retinopathy; and to pay attention to foot care, relieving symptoms and considering revascularization in the management of neuropathy. These interventions are largely long established, but experience has shown that alone they cannot prevent the development of complications in millions of individuals with diabetes. Although undoubtedly pragmatic and helpful, these guidelines therefore reveal that major advances in the treatment of diabetic microangiopathy have unfortunately not been forthcoming in the last 15–20 yr.

C-peptide and insulin are released in equimolar amounts from pancreatic β-cells after cleavage of proinsulin (2). Although there was previously a consensus that C-peptide lacked biological activity, recent evidence demonstrates that this is incorrect and that C-peptide has multiple biological effects and may protect against development of microvascular complications in type 1 diabetes. Indeed C-peptide ad-

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ministration to either diabetic animals or patients with type 1 diabetes ameliorates neuropathic complications (3). Further, replacement of C-peptide in animal models of diabetes and in patients with type 1 diabetes also ameliorates a number of the structural and functional characteristics of diabetic nephropathy including attenuation of glomerular hyperfiltration, reduced microalbuminuria, decreased mesangial expansion, and increased endothelial nitric oxide synthase levels (4–6). We recently demonstrated that C-peptide alleviates TGF-β1-mediated phenotypic and morphological changes in proximal tubular cells (PTCs) associated with epithelial mesenchymal transformation (EMT) (7), which is key to the tubular atrophy and interstitial fibrosis that are central to the loss of renal function in diabetic nephropathy (8, 9).

These effects of C-peptide are robustly underpinned by multiple signaling events that have been described in various cell types. Thus, C-peptide activates ERK, phosphatidylinositol 3-kinase, and protein kinase C and evokes elevations of intracellular [Ca²⁺]. As a consequence, C-peptide activates several transcription factors including nuclear factor κ-light chain-enhancer of activated B cells, peroxisome proliferator-activated receptor-γ (10, 11), cAMP response element binding protein, and the activating transcription factor proteins (12). Alterations in transcription factor activity are generally accompanied by altered target gene expression and cell phenotypic changes, and we therefore investigated C-peptide-induced alterations in gene transcription in a human PTC line, HK-2, using microarray analysis. We were particularly interested to identify C-peptide-mediated regulation of components of pathways that may confer protection against the profibrotic effects of TGF-β in the kidney tubulointerstitium in diabetic nephropathy. Comprehensive expression profiling and functional cluster analyses revealed that C-peptide influenced the expression of hundreds of genes. From these, we selected candidate genes that have the potential to be central to the antifibrotic effects of C-peptide. In particular we focused on hepatocyte growth factor (HGF), which has recently been identified as a key antifibrotic cytokine, preventing tissue fibrosis after chronic injury. Indeed, several studies have demonstrated that HGF attenuates initiation of events that predispose to progressive loss of kidney function, whereas blockade of HGF signaling further exacerbates the extent and progression of renal fibrosis (13, 14). Furthermore, HGF mediates the recently reported antifibrotic effects of retinoic acid (RA) (15). Here we demonstrate that RA and HGF attenuate profibrotic effects of TGF-β1 in HK-2 cells. Further, we demonstrate that C-peptide increased the mRNA and protein levels of HGF and key components of the RA-signaling pathway. Importantly, we provide novel evidence that HGF is a downstream mediator of the protective effects of C-peptide on TGF-β1-induced EMT in PTCs.

Results

Identification of C-peptide-regulated genes by microarray analysis

Gene expression profiling of HK2 cells after 18 h exposure to C-peptide identified 953 genes regulated at greater than or equal to 2-fold (P < 0.05; 459 up and 494 down) compared with control, unstimulated cells. After 48 h C-peptide treatment, 1458 genes showed greater than or equal to 2-fold change in expression (P < 0.05; 712 up and 746 down). After exposure to TGF-β1 for 48 h, there were changes in expression of 1207 genes at greater than or equal to 2-fold (P < 0.05; 588 up and 619 down) compared with control conditions. Compared with TGF-β1 alone, cells treated with TGF-β1 and C-peptide displayed at least greater than or equal to 2-fold change in expression of 1026 genes (P < 0.05; 506 up and 520 down). Supplemental Microarray Data Files are published on The Endocrine Society’s Journals Online web site at http://mend.endojournals.org. All microarray data are available at National Center for Biotechnology Information Gene Expression Omnibus accession no. GSE 20247.

From the genes showing more than 2-fold up- or downregulation we searched for candidates potentially associated with propagation of, or protection from, TGF-β1-mediated tubulointerstitial fibrosis in diabetic nephropathy. We identified seven genes related to retinoid and HGF signaling (Table 1). These genes were of interest because retinoids have recently been identified as antifibrotic agents that antagonize the deleterious effects of TGF-β1 on kidney cells via HGF (16). These genes demonstrated either potentially favorable regulation by C-peptide, or deleterious regulation by TGF-β1, or deleterious regulation by TGF-β1 that was antagonized by C-peptide, or a mixture of these effects.

From these seven genes we selected three, i.e. CRABPII, RARβ, and HGF, and examined whether increased mRNA expression was reflected in altered protein expression. Immunoblotting demonstrated that C-peptide induced a concentration-dependent increase in RARβ [e.g. 5 nM increased expression to 186 ± 22% of control (Fig. 1A)]. CRABPII associates with retinoic acid and facilitates its binding to receptors (17). Immunoblot analysis confirmed that C-peptide induced a concentration-dependent increase in CRABPII (maximal 143 ± 14% of control at 5 nM) (Fig. 1B). Immunoblot analysis also demonstrated a concentration-dependent increase in HGF expression (Fig. 1C).

RA mediates antifibrotic actions through HGF (16), and thus a potential role for RA in the regulation of HGF was examined. Cells were treated with 9-cis-RRA to acti-
vate all members of the RAR and retinoid X receptor (RXR) families (17). 9-cis-RA induced a concentration-dependent increase in HGF expression to 113 ± 5%, 133 ± 6%, and 182 ± 5% of controls, at 10^-8, 10^-7, and 10^-6 M, respectively (Fig. 1D).

### E-cadherin expression is down-regulated by TGF-β1 but restored by 9-cis-RA

The ability of 9-cis-RA to mitigate the classical EMT changes in PTCs induced by TGF-β1 was examined. Cells incubated with TGF-β1 at low glucose for 48 h demonstrated reduced expression of E-cadherin (57 ± 5% of control). However, coapplication of TGF-β1 and 9-cis-RA (10^-8, 10^-7, or 10^-6 M) inhibited TGF-β1-mediated down-regulation of E-cadherin expression to 85 ± 6%, 82 ± 9%, and 90 ± 110% of controls, respectively (Fig. 2).

### C-peptide reverses TGF-β1-mediated up-regulation of the transcription factor Snail

The effect of TGF-β1 and C-peptide on Snail expression was assessed. At low glucose concentration, TGF-β1 increased Snail expression (188 ± 31% of control) (Fig. 3A). At high glucose concentration, expression of Snail was similar to that at the low glucose concentration. In addition, at high glucose, TGF-β1 increased Snail expression to 163 ± 23% of the low-glucose control (Fig. 3A). Coapplication of C-peptide and TGF-β1 abolished the effects of TGF-β1 on Snail expression at both low- and high-glucose concentrations (Fig. 3A). The effect of C-peptide was not reproduced by scrambled C-peptide (ScC-peptide) (data not shown).

TGF-β1 induction of Snail was also blocked by 9-cis-RA. When incubated in low glucose with TGF-β1 for 48 h, Snail expression increased to 265 ± 19% of the low-glucose control. However, coapplication of 9-cis-RA with TGF-β1 inhibited TGF-β1-mediated up-regulation of Snail expression (e.g., 165 ± 18% of control at 10^-8 M RA) (Fig. 3B).

### 9-cis-RA negates TGF-β1-induced changes in vimentin and β-catenin expression

Cells were incubated for 48 h in low-glucose media supplemented with either TGF-β1 (2 ng/ml), 9-cis RA (10^-8, 10^-7, 10^-6 M), or a combination of both. Under these conditions, TGF-β1 increased vimentin expression (171 ± 14% of low-glucose control). However, 9-cis-RA, at all concentrations tested, inhibited the TGF-β1-mediated up-regulation of vimentin expression (Fig. 4A). Incubation with 9-cis-RA alone had no effect on vimentin expression (Fig. 4A).

Typical of cells with an epithelial phenotype, HK2 cells under control conditions exhibited diffuse cytoplasmic vimentin staining, lacking discrete fibers (Fig. 4Bi). In response to TGF-β1, a network of vimentin filaments spanning the cell and establishing cell-to-cell contacts was observed (Fig. 4Bi). Application of 9-cis-RA (10^-5 M) blocked TGF-β1-induced vimentin fiber formation and restored the cellular distribution pattern to that under control conditions (Fig. 4Bii).

β-Catenin, found at the plasma membrane, is a central component of the E-cadherin cell adhesion complex. In EMT, down-regulation of E-cadherin allows the release and subsequent relocalization of β-catenin from the cell membrane (18). The present data show that in HK-2 cells, TGF-β1 failed to induce a change in the overall expression level of β-catenin (Fig. 4C). Typical of cells with an epithelial phenotype, β-catenin localized to the plasma membrane under control conditions (Fig. 4Bi). However, incubation with TGF-β1 resulted in dissociation of β-catenin with redistribution and accumulation in the cytoplasmic and nuclear compartments (Fig. 4Dii). This effect was prevented by coinubcation with C-peptide (Fig 4Diii). We then investigated the potential of 9-cis RA to influence TGF-β1-mediated changes in the cellular localization of β-catenin. Thus, cells were incubated in a low-

### Table 1. Changes in expression of RA and HGF-signaling components in response to C-peptide and/or TGF-β1

<table>
<thead>
<tr>
<th>Gene (Homo sapiens)</th>
<th>C-peptide&lt;sup&gt;a&lt;/sup&gt; vs. control (18 h)</th>
<th>C-peptide&lt;sup&gt;a&lt;/sup&gt; vs. control (48 h)</th>
<th>TGF-β1&lt;sup&gt;a&lt;/sup&gt; vs. control (48 h)</th>
<th>C-peptide ± TGF-β1&lt;sup&gt;a&lt;/sup&gt; vs. TGF-β1 (48 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRABPI, mRNA</td>
<td>-3.15</td>
<td>-3.0</td>
<td>&lt;±2</td>
<td>-3.1</td>
</tr>
<tr>
<td>CRABPII, mRNA</td>
<td>&lt;±2</td>
<td>&lt;±2</td>
<td>&lt;±2</td>
<td>2.9</td>
</tr>
<tr>
<td>RARβ transcript variant 1, mRNA</td>
<td>&lt;±2</td>
<td>76</td>
<td>4.8</td>
<td>6.1</td>
</tr>
<tr>
<td>Predicted: similar to RARγ-A (LOC654040), mRNA</td>
<td>2.2</td>
<td>&lt;±2</td>
<td>-14</td>
<td>7.6</td>
</tr>
<tr>
<td>RA induced 2</td>
<td>&lt;±2</td>
<td>&lt;±2</td>
<td>-2.3</td>
<td>2.9</td>
</tr>
<tr>
<td>HGF (hepapoietin A; scatter factor)</td>
<td>&lt;±2</td>
<td>32</td>
<td>-9</td>
<td>&lt;±2</td>
</tr>
<tr>
<td>HGF, transcript variant 3, mRNA</td>
<td>&lt;±2</td>
<td>&lt;±2</td>
<td>&lt;±2</td>
<td>4.8</td>
</tr>
</tbody>
</table>

<sup>a</sup>Numbers represent fold change under condition noted with superscript letter a compared with the second condition. Negative numbers indicate down-regulation by condition noted with superscript letter a. Where there was no change in gene expression, either up or down, between conditions or a fold change less than 2, this is indicated as less than ±2.
glucose media supplemented with either TGF-β1 (2 ng/ml), 9-cis RA (10^{-8}, 10^{-7}, 10^{-6} M) or a combination of both for 48 h. These conditions did not change the overall expression level of β-catenin (Fig. 4E) but 9-cis-RA prevented the TGF-β1-mediated change in its subcellular distribution (Fig. 4F, ii and iii, and data not shown).

**HGF mediates the inhibitory effects of C-peptide on TGF-β1-induced morphological and phenotypic changes**

HGF exerts strong antifibrotic effects (14), and we therefore assessed the effects of HGF on our previously reported TGF-β1-induced changes in HK-2 cell morphology (7). HK-2 cells were treated with TGF-β1 (2 ng/ml) either alone or in combination with HGF (40 ng/ml) for 48 h under low-glucose conditions. As previously observed (7), phase-contrast microscopy demonstrated that the typical epithelial cobblestone appearance of HK2 cells (Fig. 5Ai) was lost after TGF-β1 treatment with cells developing an elongated, fibroblast-like phenotype (Fig. 5Aii). Coapplication of HGF and TGF-β1 prevented these morphological changes (Fig. 5Aiii), whereas addition of HGF alone had no effect on cell morphology (data not shown). The transcription factor Snail is a strong repressor of E-cadherin in epithelial cell lines. Indeed, stable expression of Snail leads to loss of E-cadherin and induces EMT in epithelial cell lines (19). Because the loss of E-cadherin expression is pivotal to EMT and given the ability of HGF to prevent TGF-β1-mediated changes in cell morphology and the ability of C-peptide to enhance expression of HGF, we examined the potential role of HGF in the inhibitory effects of C-peptide on TGF-β1-induced changes in the expression of E-cadherin and Snail. Cells were incubated in low-glucose medium supplemented with various combinations of TGF-β1, HGF, C-peptide, and a concentration of an HGF-neutralizing antibody previously demonstrated to be effective in blocking the effects of HGF in cell culture (14). Consistent with earlier data (Fig. 2), TGF-β1 for 48 h reduced E-cadherin expression. As shown previously (7) C-peptide abolished this TGF-β1-mediated down-regulation of E-cadherin expression. However, in the presence of an HGF-neutralizing antibody, C-peptide failed to block the TGF-β1-mediated reduction in E-cadherin expression (Fig. 5B). E-cadherin expression was unaffected by HGF alone (Fig. 5B).

The expression of Snail was investigated in similar experiments. Thus, cells were incubated in low-glucose media with combinations of TGF-β1, HGF, C-peptide, and HGF-neutralizing antibody. Consistent with the earlier experiments (Fig. 2A), TGF-β1 increased the expression of Snail, and this was prevented by C-peptide (Fig. 5C). However, in the presence of the HGF-neutralizing antibody, C-peptide was unable to prevent the stimulatory effect of TGF-β1 on Snail expression (Fig. 5C). Snail expression was unaffected by HGF alone (Fig. 5C). Further-
more, the addition of HGF-neutralizing antibody alone had no significant effect on E-cadherin or Snail expression levels (Fig. 5, D and E, respectively).

**Discussion**

In diabetic nephropathy the profibrotic actions of TGF-β1 are key to the development of proximal tubular atrophy, matrix production and tubulointerstitial scarring that accompanies loss of renal excretory function (20). The reciprocal loss of tubular epithelial cells and accumulation of interstitial fibroblasts as a consequence of EMT promotes chronic renal fibrosis. EMT represents the process by which these epithelial cells transform to exhibit a mesenchymal phenotype (21, 22). Characteristic epithelial cell phenotypic changes associated with EMT include morphological alterations with reorganization of the actin cytoskeleton, de novo acquisition of mesenchymal cytoskeletal markers such as α-smooth muscle actin and vimentin, and the down-regulation of epithelial adhesion molecules such as E-cadherin and zona occludens protein ZO-1 (23, 24).

C-peptide has recently emerged as a bioactive peptide with the ability to activate multiple signaling pathways, including those regulated by transcription factors. These signaling functions solidly underpin a number of observed physiological effects of C-peptide and its protective actions against diabetic nephropathy and neuropathy (25, 26). We recently demonstrated the ability of C-pep-
tide to prevent PTC EMT by blocking a variety of TGF-β1-evoked cell-signaling events in these cells (7). These results suggested that C-peptide may be an important therapeutic candidate in the prevention of diabetic nephropathy. Accordingly, we now demonstrate that C-peptide inhibits TGF-β1-induced morphological and phenotypic changes in PTCs through the downstream actions of RA and HGF.

To further understand how C-peptide acts to alter kidney cell function and phenotype, we performed microarray analysis of genes regulated by C-peptide in the human PTC cell line HK-2. Abnormalities in TGF-β1 at both receptor and downstream signaling level have been discovered in a wide variety of disorders, including autoimmune diseases, malignancies, and chronic renal disease (27), and overwhelming evidence implicates TGF-β1 as the predominant factor mediating PTC phenotypic changes and fibrosis in diabetic nephropathy (28, 29). Production of TGF-β1 by PTCs in diabetes is stimulated in part by high-glucose and advanced-glycation end products (30).

Several hundred genes showed altered expression in response to exposure of cells to C-peptide, and, in addition, C-peptide had a salutary effect on changes in expression of some fibrosis-related genes mediated by TGF-β1. In particular, TGF-β1 tended to regulate key elements of the retinoid- and HGF-signaling pathways such that biological activity would be diminished, whereas C-peptide tended to do the opposite or indeed antagonize the effects of TGF-β1. Although not all of the gene changes under the different experimental conditions were in complete agreement, differences in timescale of regulation of the expression of different genes may mean the necessary window was missed. These findings emphasize the substantial biological activity of C-peptide and identify new pathways regulated by C-peptide that are of relevance to the pathophysiology of diabetic nephropathy. In the proximal tubule, TGF-β1 is a key mediator of EMT and modulates the expression of several epithelial cell recognition and organizational proteins, including the cadherins (31), catenins, and the actin cytoskeleton (32). The repression of some TGF-β1-induced gene transcriptional

FIG. 4. 9-cis-RA reverses TGF-β1-induced changes in vimentin and β-catenin expression and localization. HK-2 cells were grown in either low or high glucose for 48 h with TGFβ1 (2 ng/ml) alone or TGFβ1 (2 ng/ml) and the indicated concentrations of either 9-cis-RA or 5 nM C-peptide. The expression levels of vimentin (A) and β-catenin (C and E) were then determined by immunoblotting. The upper parts of panels A, C, and E are representative immunoblots showing expression of vimentin or β-catenin (upper blots) or the same blots stripped and reprobed for GAPDH as a loading control (lower blots). Band intensities were quantified by densitometry and the nonstimulated, low glucose control condition normalized to 100%, and all other conditions were compared with this. The lower part of the panel shows the mean ± SEM derived from densitometry of three blots. Each bar in the histogram represents the same condition in the blots above; solid pattern is low glucose and cross-hatched pattern is high glucose. Key significances are shown: **, P < 0.01; ***, P < 0.001, n = 3. The subcellular localization of vimentin (B) and β-catenin (D and F) in HK-2 cells was examined by immunocytochemistry and fluorescent microscopy. Cells were cultured in low glucose for 48 h in the absence (Bi, Di, Fi) or presence of TGFβ1 (2 ng/ml) either alone (Bii, Dii, Fii) or in combination with either 5 nM C-peptide (Diii) or 10−6 M 9-cis-RA (Bi, II). Images are representative of greater than or equal to three individual experiments (magnification ×40).
events by C-peptide illustrates its therapeutic potential to alleviate fibrosis in diabetic nephropathy, and this microarray analysis identifies a number of candidate genes toward which future therapies may be targeted.

Specifically, we identified a number of genes involved in RA actions that appeared to be beneficially regulated by C-peptide. Our interest in RA as a downstream target of C-peptide derived from its recently reported renoprotective effects in models of renal disease (16). Of the genes shown to be altered by C-peptide in the microarray analyses, we elected to verify that the protein products of selected genes were altered, namely CRABPII, RARβ, and HGF, all of which have demonstrated antifibrotic effects in the kidney. These experiments confirmed that C-peptide treatment resulted in changes at both the mRNA and protein levels and led us to further study the downstream role of RA and HGF in mediating the antifibrotic actions of C-peptide.

RA, an active metabolite of vitamin A, binds to receptors from one of two subfamilies, the RAR family and the RXR family (33). Upon ligand binding the receptors undergo nuclear translocation where they interact with specific cis-acting RA- and RXR-response elements in the promoter region of their target genes, thereby modulating gene transcription (34). As a critical modulator of renal tubulogenesis, the absence of RA signaling in double RAR/RXR knockout mice results in severe urogenital malformations (35). Although recent evidence has established a potent renoprotective role for RA in the kidney, little is known about the mechanisms through which its effects are mediated. However, it has been recently reported that RA reverses TGF-β1-mediated fibrosis in mesangial cells, with the downstream mediator of this response identified as HGF (16). The ability of HGF to prevent progressive loss of kidney function in chronic kidney disease (13) is underpinned by its ability to interrupt TGF-β1 signaling, most likely by suppression of TGF-β1/Smad-mediated gene activation (14, 36).

The results of our microarray studies therefore led us to assess the possibility that 9-cis-RA and HGF may mediate the C-peptide-induced inhibition of TGF-β1-dependent changes of EMT in PTCs. Taken together, the results provide novel evidence that: 1) C-peptide stimulates expression of HGF and several components of the RA-signaling pathway; 2) 9-cis-RA inhibits TGF-β1-induced changes in expression of the epithelial marker E-cadherin and its transcriptional repressor Snail in a manner similar to C-peptide; 3) 9-cis-RA blocks TGF-β1-induced up-regulation of vimentin expression while preventing the release of β-catenin from the cell periphery in a manner
similar to C-peptide; 4) 9-cis-RA stimulates HGF expression as does C-peptide, and finally; 5) the ability of C-peptide to reverse both TGF-β1-induced PTC morphology, and changes in expression of both E-cadherin and Snail are dependent on HGF signaling. These results strongly support a role for 9-cis-RA and HGF in antagonizing the fibrogenic action of TGF-β1 in HK-2 cells. Further they support the concept that the antifibrotic effects of C-peptide in EMT are driven by downstream enhancement of expression and activity of several elements of RA-signaling pathways, which, in turn, promote increased HGF activity, thus negating the pathological effects of TGF-β1. Although it is not possible from the current data to state definitively that enhanced RA signaling is absolutely obligate for increased C-peptide-evoked HGF expression, the effects of C-peptide and RA are completely congruent. We therefore postulate that C-peptide stimulation of key regulatory components of the RA pathway and then HGF, in conjunction with the observed inhibitory effects of both 9-cis-RA and HGF on TGF-β1-induced EMT, provides an integral connection between RA and HGF as downstream mediators of C-peptide.

An antagonistic action of C-peptide on TGF-β1-mediated effects in diabetic nephropathy most likely underpins the glomerular protection and antagonism of TGF-β1 observed in animal models of type 1 diabetes (37, 38). Therefore, these data identify downstream signaling pathways of C-peptide relevant to the protection against TGF-β1-induced EMT and further demonstrate biological activity and subsequent cellular effects elicited by C-peptide in cells of the proximal tubule. These observations position C-peptide as a potentially important therapeutic agent in diabetic nephropathy.

Materials and Methods

Materials

Human 31-amino acid C-peptide and a 31-amino acid scrambled (C-peptide) were provided by Dr. John Wahren (Karolinska Institute, Stockholm, Sweden). Tissue culture media and plastic ware were from Invitrogen Life Technologies (Paisley, UK). Immobilon P membranes were from Millipore (Watford, UK), enhanced chemiluminescence detection reagents from GE Healthcare (Little Chalfont, UK) and antifade Citifluor was from Agar Scientific (Essex, UK). Antibodies were from either Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), R&D Systems (Abingdon, UK), or Affinity Bioreagents (Cambridge, UK) as indicated. Recombinant human TGF-β1, 9-cis-RA, and other laboratory chemicals and reagents were from Sigma-Aldrich (Poole, UK).

Cell culture

HK-2 cells (passages 18–30) were maintained in DMEM/Ham’s F12 (DMEM/F12) (17.5 mM glucose), supplemented with 10% fetal calf serum, glutamine (2 mM), penicillin (100 IU/ml), and streptomycin (100 μg/ml). Cells were cultured at 37 C in a humidified atmosphere of 5% CO₂ in air. Before treatment, cells were cultured in DMEM/F12 low glucose (5 mM) (5 mM DMEM/F12) for 48 h. This medium was prepared as described previously (39) and was used to remove effects of the high-glucose concentration in normal culture medium.

For microarray analyses, cells were cultured in 5 mM DMEM/F12 without supplements (control) or with 5 nM C-peptide for either 18 h or 48 h. Alternatively, cells were treated with 2 ng/ml TGF-β1 either alone or in combination with C-peptide for 48 h. All treatments were performed in triplicate to yield 18 flasks, and RNA from each flask hybridized to a separate chip (n = 3 for each of six treatments). Flasks were subjected to identical media changes, and cells were cultured for identical periods in media without supplements. In all experiments, cells were serum starved overnight before agonist addition. Treatments were initiated such that 18- and 48-h incubation periods ended coincidentally, and all RNA was prepared at this point.

For assessment of the effects of TGF-β1 and C-peptide on protein expression by immunoblotting, cells were cultured in 5 mM DMEM/F12 for 48 h before treatment. Cells were then cultured in DMEM/F12 without supplements containing either low (5 mM) or high (25 mM) glucose for 48 h. These two different glucose concentrations were used to mimic previous experiments (7) such that we could examine effects of C-peptide under the equivalent of normo- and hyperglycemic conditions. Where required, cells were treated with either 2 ng/ml TGF-β1 with or without 5 nM C-peptide or alternatively with 5 nM ScC-peptide for 48 h. To assess the effect of C-peptide alone, cells were treated with either 1.25, 2.5, 5, or 10 nM C-peptide. For assessment of any antifibrotic effects of HGF, cells were cultured as above and treated with 2 ng/ml TGF-β1 with or without 40 ng/ml HGF for 48 h. To assess any role of HGF in mediating the effects of C-peptide, cells were treated as before with TGF-β1 (2 ng/ml) with or without C-peptide in the presence or absence of 1 μg/ml HGF neutralizing antibody. This antibody at this concentration has been shown by others to block HGF bioactivity (40). In all experiments, cells were serum starved overnight before ligand addition. The concentrations of TGF-β1 and HGF used have been demonstrated to have pro- and antifibrotic effects, respectively (7).

Microarray analyses

RNA was prepared by acid-guanidinium extraction (41) using a Genelute mammalian total RNA miniprep kit (Sigma-Aldrich) following the manufacturer’s instructions. RNA was reverse transcribed and directly hybridized to Illumina Human WG6 BeadChips for whole-genome expression by Geneservice (Cambridge, UK). The array intensity data were analyzed using Illumina BeadStudio software (version 3.1.3) (Illumina Cambridge, UK) for visualization and normalization. The quantile normalization method was used for all analyses, and average background correction was performed within the Beadstudio software. Both principal component analysis and statistical analysis (ANOVA) were undertaken using the Partek Genomics Suite software package (version 6.3) (Partek, MO). Before ANOVA, data were adjusted such that all values were greater than or equal to 0.0001 (to remove negative values) and all values were then log transformed (base 2). The ANOVA P values were adjusted using the Benjamini and Hochberg procedure to control for false discovery rates (42).
Immunoblotting

Cytosolic proteins were prepared and separated by SDS-PAGE and electoblotted onto Immobilon P membranes as described previously (44). Membranes were probed with polyclonal antibodies against human HGF, E-cadherin, Snail, β-catenin (R&D Systems), vimentin (Affinity Bioreagents, Cambridge, UK), cellular retinoic acid-binding protein II (CRABPII), and RARB (Santa Cruz Biotechnology) at dilutions of 1:100, 1:500, 1:100, 1:100, and 1:100, respectively. After three 10-min washes (PBS, 0.1% Tween 20), membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (diluted up to 1:40,000 in PBS, 0.05% Tween-20) for 60 min at 25°C followed by three 10-min washes (PBS, 0.1% Tween 20). Proteins were visualized using enhanced chemiluminescence and exposure to film. Blots were stripped and reprobed with anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:20,000; R&D Systems) to control for protein loading in subsequent densitometric analysis.

Immunocytochemistry

Cells at 80% confluence on coverslips were fixed with 4% paraformaldehyde. Nonspecific binding was blocked using 10% goat serum in PBS-Triton X-100 (0.01%) for 1 h at 25°C. After three 10-min washes with PBS, nuclei were stained using 4′,6-diamidino-2-phenylindole dihydrochloride (1 μM, 3 min). After three further 10-min washes, cells were incubated with TRITC-conjugated phalloidin diluted at 1:100 in PBS-Triton X-100 (0.01%) for 1 h at 25°C or incubated overnight at 4°C with primary antibody (1:100) diluted in PBS-Triton X-100 (0.01%). Cells were then washed with PBS and incubated with Alexa 488-conjugated secondary antibody for 1 h at 25°C in the dark. Secondary antibodies were diluted (1:400) in PBS Triton X-100 (0.01%). Cells were washed for three periods of 10 min and coverslips mounted in antifade Citifluor (glycerol/PBS solution) on glass slides. Immunofluorescence was visualized using an Axiovert 200 research inverted fluorescence microscope (Carl Zeiss Ltd., Welwyn Garden City, UK).

Analysis of autoradiograph data

Autoradiographs were quantified by densitometry (TotalLab 2003; Nonlinear Dynamics, Newcastle, UK). The nonstimulated, low-glucose (5 mM) control condition was normalized to 100%, and other experimental conditions were compared with this. Statistical analyses were performed by one-way ANOVA, and, where P < 0.05, compared using Tukey’s multiple-comparison posttest with P < 0.05 indicating statistical significance. Data are expressed as mean ± SEM, and n denotes the number of experiments.

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