Original Paper

Inhibition of the Expression of TGF- β 1 and CTGF in Human Mesangial Cells by Exendin-4, a Glucagon-like Peptide-1 Receptor Agonist

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Key Words

Connective tissue growth factor (CTGF) • Exendin-4 • Human mesangial cells (HMCs) • Transforming growth factor- β 1 (TGF- β 1)

Abstract

Background: Despite the presence of glucagon-like peptide-1 receptor (GLP-1R) in kidney tissues, its direct effect on diabetic nephropathy remains unclear. The transforming growth factor- β 1 (TGF- β 1) and the connective tissue growth factor (CTGF) both induce extracellular matrix accumulation and persistent fibrosis in the glomerular mesangium of patients with diabetic nephropathy. **Objective:** Herein, we demonstrate that a GLP-1R agonist, exendin-4, exerts renoprotective effects through its influence on TGF- β 1 and CTGF in human mesangial cells (HMCs), cultured in a high glucose medium. **Method:** HMCs, cultured in a high glucose medium, were used for the current study. The direct effect of exendin-4 on TGF- β 1 and CTGF expression was confirmed in HMCs. MDL-12330A (a specific adenylate cyclase inhibitor) and PKI14-22 (a protein kinase A inhibitor) were used to examine the role of the cAMP signaling pathway in exendin's anti-fibrosis action. **Results:** The findings showed that exendin-4 inhibited the proliferation of HMCs, and upregulated the expression of TGF- β 1 and CTGF, induced by high glucose. The effect of exendin-4 is largely dependent on the activation of adenylate cyclase. **Conclusion:** This study provides new evidence that GLP-1 acts as an antifibrotic agent in HMCs.

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Introduction

About 30-40% of diabetic patients, worldwide develop diabetic nephropathy (DN). DN is histologically characterized by the accumulation of extracellular matrix (ECM) proteins, such as collagen, in the glomerular mesangium. The transforming growth factor- β_1 (TGF- β_1) is a major fibrogenic growth factor in the pathogenesis of glomerulosclerosis and interstitial fibrosis. It not only induces collagen and matrix synthesis directly, but also enhances the expression of connective tissue growth factor (CTGF), mRNA and proteins [1]. CTGF can induce proliferation, collagen synthesis, and chemotaxis in mesenchymal cells [2, 3]. It also promotes fibronectin (FN) synthesis in mesangial cells [4-6] and seems to mediate at least part of the TGF- β_1 -induced extracellular matrix accumulation and persistent fibrosis [7-9]. As a result, TGF- β_1 and CTGF are supposed to be useful molecular markers of the fibrotic response [2, 3, 10], which is an important function in the treatment of DN.

Glucagon-like peptide-1 (GLP-1) is an incretin hormone secreted by the small intestine in response to nutrient ingestion. It is almost immediately degraded by dipeptidyl peptidase-4 (DPP4). Although glycaemic control is the major physiological function of GLP-1, however, it plays much more important roles. GLP-1 acts through the GLP-1R, a G-coupled protein receptor, abundantly found in the gastrointestinal tract. The GLP-1R is also expressed in the nervous system, heart, vascular smooth muscles, proximal tubules, and glomerulus of the kidney [11-13]. Park et al. [14] found that treating normal people with a GLP-1 agonist upregulated the expression of GLP-1R, which was present in decreased concentrations in the glomerulus, and ameliorated renal lesions in *db/db* (diabetes) mice. The expression of TGF- β_1 was also down-regulated after incretin treatment in these studies. Activation of the GLP-1R can trigger generation of the second messenger cAMP, followed by the activation of the protein kinase A (PKA) [15].

Exendin-4 (Ex-4), a GLP-1R agonist, is a recently established modality for treatment of type 2 diabetes. It has been found that Ex-4 can suppress the expression of TGF- β_1 in DN patients [16]. The study by Chaykovska et al. have confirmed that the treatment of around five to six nephrectomized rats for four days with DPP4 inhibitor can increase the circulating levels of intact bioactive GLP-1 and can significantly reduce the expression of TGF- β_1 to baseline levels [17]. This data highlights the potential suppressive effects of GLP-1 on the expression of TGF- β_1 . To our knowledge, till date no research work has been performed to study the effects of GLP-1 on the expression of TGF- β_1 in HMCs.

This study aimed to examine the effect of GLP-1 on the expression and synthesis of TGF- β_1 and CTGF in HMCs, cultured in a high glucose media, using the long-acting GLP-1 agonist, Ex-4.

Patients and Methods

Cell line and reagents

For the current research work, primary HMCs and the mesangial cell medium were purchased from ScienCell Research Laboratories (Cat. No. 4200). The RT-PCR system was purchased from Takara Biotechnology (Dalian, China subsidiary of Japan TaKaRa Bio Inc.). Antibodies against CTGF and TGF- β_1 were purchased from Proteintech Group Biotechnology (Proteintech Group, Inc. China). D-glucose and other chemicals were of analytical reagent grade and were purchased locally from commercial suppliers.

Cell culture

Primary HMCs were seeded in 25 cm² tissue culture flasks in a mesangial cell medium. The culture medium was supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin, in an atmosphere containing 5% CO₂. The cell medium was changed every other day until the cells became confluent. HMCs at approximately 70-80% confluence were cultured in a serum-free 1640 medium for 24hrs to synchronize the cell growth.

Table 1. PCR primers and cycling condition

	Primer	Condition	Aplified Base pair
TGF- β_1	forward 5'-GCCCTGGACACCAACTATTGC-3'	95°C,30s→60°C,20s →72°C,50s 30 cycles	161bp
	reverse 5'-AGGCTCCAAATGTAGGGCAGG-3'		
CTGF	forward 5'-CTAAGACCTGTGGGATGGGC-3'	95°C,30s→57°C,20s →72°C,50s 30 cycles	379bp
	reverse 5'-CTC AAAGATGTCATTGTCCCC-3'.		
β -actin	forward 5'-GTGGGGCGCCCCAGGCACCA-3'	95°C,30s→57°C,20s →68°C,50s 30 cycles	539bp
	reverse 5'-CTCCTTAATGTCACGCACGATTTC-3'.		

Cell proliferation measurements

The cell proliferation was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl -tetrazolium bromide (MTT) assays in 96-well microplates. Growth medium (180 μ l) was added to every well and after 44 hrs, 20 μ l of 5 mg/ml MTT was added to each well. After four hours, the medium was replaced with 200 μ l of dimethyl sulfoxide (DMSO) and the 96-well microplates were shaken gently for 10mins. The optical density (OD) value of the cultured cells was then measured at 570 nm and these data was transformed into a variable, which represented the number of cells in each well by using a curve that correlated absorbance by the HMCs to the number of HMCs.

Experimental design

HMCs were thoroughly washed and were incubated with or without 0.03, 0.3, and 3 nmol/l Ex-4 for 1 hr, followed by incubation with the growth medium containing 30 mmol/l glucose (ScienCell 4201+D-glucose) for 24 hrs. It has been previously reported that the main effects of GLP-1 are mediated through the activation of adenylate cyclase and the production of cAMP [18]. Therefore, to inhibit the exenatide signal, HMCs were incubated with 5 μ mol/l MDL-12330A (Sigma-Aldrich), a specific adenylate cyclase inhibitor, and 10 μ mol/l PKI14-22 (Sigma-Aldrich), a protein kinase A (PKA) inhibitor, for 30 mins before adding Ex-4. Control HMCs (NG) were incubated in a medium containing 5.6 mmol/l glucose (ScienCell 4201). Total RNA was obtained from HMCs for further analysis.

RNA extraction and RT-PCR analyses for expression of TGF- β_1 mRNA and CTGF mRNA

The expression of mRNA was determined by RT-PCR, applying the reverse transcription kit (Promega,USA), using β -actin as an internal control. Primers and cycling conditions for PCR have been described in Table 1. The PCR products were subjected to 2% agarose gel electrophoresis and the resulting gel was analyzed with a R-200 Bioimaging system (Shanghai Fu Dan). Band intensity values for TGF- β_1 and CTGF mRNA were normalized to those of β -actin mRNA, and the resultant TGF- β_1 and CTGF: β -actin ratios were plotted to illustrate variations in the gene expression.

Western blot analyses of TGF- β_1 and CTGF

For protein extraction, each dish was treated for 60 mins with ice-cold lysis buffer. The lysates were then centrifuged at 10,000 $\times$ g for 5 min at 4°C. Subsequently, samples (50 μ g of protein/lane) were separated by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) (7%) and were transferred to polyvinylidene fluoride (PVDF) membranes, and incubated for 60 mins at room temperature (22°C) in TTBS (tris-buffered saline with 0.05% Tween-20 detergent at pH 7.4) plus 5% BSA. PVDF membranes were then incubated overnight at 4°C with CTGF, TGF- β_1 , and β -actin antibodies. After thoroughly washing the PVDF membranes the next morning, membranes were again incubated for 100 mins at room temperature with goat antirabbit IgG horseradish peroxidase conjugate. Proteins present in the membrane were then detected using an ECL (enhanced chemiluminescence) detection system and the band intensities were measured using ImageJ.

Fig. 1. Effect of Exendin-4 on overproliferation induced by HG. HMCs proliferation was measured with an MTT assay. Values are expressed as the means $\pm$ SD. HG induced a significant increase in HMCs proliferation compared with the control group (NG). HMCs proliferation was inhibited significantly in absence of Ex-4, especially at 0.3 nM and 3 nM. * $p < 0.05$ versus NG group; # $p < 0.05$, ## $p < 0.01$ versus HG group.

Statistical analyses

The effect of Ex-4 on TGF- β_1 and CTGF mRNA expression and protein production was assessed by ANOVA tests. Specific differences were tested with unpaired *t*-tests. Data were expressed as mean $\pm$ SD and $p < 0.05$ was considered to be statistically significant.

Results

Exendin-4 inhibits the HG (high glucose)-induced HMCs over-proliferation

We examined the antiproliferative effects of Ex-4 on HMCs, treated with high glucose concentration (30 mmol/l) and analyzed HMCs' proliferation with MTT assays. The proliferation of HMCs obtained its peak value in the HG group (Fig. 1). The addition of 0.03 nmol/L Ex-4 decreased the HMC proliferation significantly ($p < 0.05$, $n = 5$) while adding 0.3 nmol/L Ex-4 overinhibited the HMC proliferation ($p < 0.01$, $n = 5$). However, no additive effect was observed on HMC proliferation when the dose was increased to 3 nmol/L.

Exendin-4 decreases the HG induced upregulated mRNA levels of TGF- β_1 and CTGF

To examine the antifibrotic effects of Ex-4 on HG treated HMCs, we focused on TGF- β_1 and CTGF mRNA. HMCs were incubated with or without 0.03, 0.3 and 3 nmol/l Ex-4 for 1 h, followed by incubation with the growth medium containing 30 mmol/l glucose for 24 h and the reverse transcribed RNA was subjected to RT-PCR. HG concentrations upregulated mRNA levels of TGF- β_1 and CTGF in HMCs and Ex-4 inhibited the upregulated mRNA levels of TGF- β_1 and CTGF (Fig. 2). HMCs in the HG group showed a significantly higher mRNA level of TGF- β_1 and CTGF as compared to the NG group ($p < 0.01$, $n = 3$). Treatment of 0.03 nmol/L Ex-4 suppressed the upregulation of TGF- β_1 mRNA ($p < 0.05$, $n = 3$). Expression of TGF- β_1 was significantly inhibited following 0.3 nmol/L Ex-4 ($p < 0.05$, $n = 3$). However, no additive effect could be observed on expression of TGF- β_1 when the dose was increased to 3 nmol/L. The HG induced upregulation of CTGF mRNA was also attenuated by Ex-4 ($p < 0.01$, $n = 3$).

Exendin-4 reduces the synthesis of HG induced TGF- β_1 and CTGF proteins

To further confirm the antifibrotic effects of Ex-4 on HMCs, we investigated changes of TGF- β_1 and CTGF proteins by western blot analysis (Fig. 3). HG induced the overexpression of TGF- β_1 ($p < 0.05$, $n = 3$) and CTGF proteins ($p < 0.05$, $n = 3$). The overexpression of TGF- β_1 was attenuated by Ex-4 ($p < 0.05$, $n = 3$). The addition of 0.3 nM Ex-4 was more effective than the addition of 0.03 nM Ex-4 ($p < 0.05$, $n = 3$). However, no effect was seen in TGF- β_1 and CTGF protein when the dose was increased to 3 nmol/L. The addition of 0.03 nM Ex-4 showed no significant decrease in expression of CTGF protein in lysates while the overexpression of CTGF was attenuated by 0.3 nM and 3 nM Ex-4 ($p < 0.05$, $n = 3$). Obtained results confirm

Fig. 2. Ex-4 inhibits the upregulated mRNA levels of HG treated TGF- β_1 and CTGF. Representative RT-PCR results show HG upregulated mRNA levels of TGF- β_1 and CTGF in HMCs and Ex4 inhibits the upregulated mRNA levels of TGF- β_1 and CTGF induced by high glucose. ^{aa} $p < 0.01$ versus the control group; ^b $p < 0.05$ versus HG group; ^c $p < 0.05$ versus HG + 0.03 nM Ex4 group.

Fig. 3. The upregulated synthesis of TGF- β_1 and CTGF proteins induced by high glucose were inhibited by exendin-4. The band intensities were measured using ImageJ. Representative Western blot results show the upregulated synthesis of TGF- β_1 and CTGF proteins induced by high glucose were inhibited by Ex-4. ^a $p < 0.05$ versus the control group; ^b $p < 0.05$ versus HG group; ^c $p < 0.05$ versus HG + 0.03 nM Ex4 group.

that Ex-4 decreases the synthesis of the fibrotic cytokines, TGF- β_1 and CTGF, in HG treated HMCs.

Effects of the regulation of the cAMP signaling pathway on TGF- β_1 and CTGF

The addition of MDL-12330A and PKI14-22 did not affect the upregulated mRNA levels of HG treated TGF- β_1 and CTGF. HMCs in the MDL group and PKI14-22 group showed the same high expression of TGF- β_1 and CTGF mRNA with the HG group (Fig. 4). Treatment of 0.3nmol/L Ex-4 suppressed the upregulation of TGF- β_1 mRNA and CTGF mRNA ($p < 0.01$, $n = 3$). The inhibitory effect of Ex-4 on the expression levels of TGF- β_1 and CTGF was significantly reversed by MDL-12330A and PKI14-22 ($p < 0.01$; $n = 3$). These results suggest that the

Fig. 4. The addition of MDL-12330A or PKI14-22 significantly suppressed the inhibitory effect of exendin-4 on the expression of TGF- β_1 mRNA and CTGF mRNA. To inhibit the exenatide signal, HMCs were incubated with 5 μ mol/l MDL-12330A or 10 μ mol/l PKI14-22 for 30 min before adding exendin-4. Data of median fluorescence intensity relative to the control. Representative RT-PCR results showed the inhibitory effect of exendin-4 on the expression of TGF- β_1 mRNA and CTGF mRNA in HMCs was suppressed by MDL-12330A and PKI14-22 significantly. ^{bb}*p* < 0.01 versus HG group; ^{dd}*p* < 0.01 versus HG + 0.3 nM Ex4 group.

inhibitory effects of Ex-4 on the expression of TGF- β_1 and CTGF are largely dependent on the activation of adenylate cyclase. Obtained results also confirm that PKA is involved in the inhibitory effect of Ex-4.

Discussion

Expanding mesangial fibrosis is one of the main mechanisms that correlate closely with declining renal function. It is widely accepted that these processes are mediated by TGF- β_1 [19, 20] whose expression is elevated in renal tissues of DN patients [21]. Previous studies have confirmed that the treatment of diabetic mice (db/db) by neutralising an antibody to TGF- β_1 attenuated glomerular hypertrophy and prevented increased the expression of collagen and fibronectin, also the glomerular mesangial matrix expansion [22]. It has also been found that glomerular CTGF mRNA and protein levels increase in murine models of DN, initially in mesangial cells [23]. CTGF has many of the same profibrotic actions as TGF- β_1 and may act as a mediator of TGF- β_1 actions on mesenchymal cells [24]. Another published study has even reported that the level of FN increased significantly in both rabbit and human mesangial cells when cultured in a medium containing 30 mmol/l glucose [25]. Therefore, we added D-(+)-glucose to the mesangial cells medium of 30 mmol/l concentration. In our study, HG induced a significant increase in the proliferation of HMCs as compared with the control group and upregulated mRNA levels and synthesis of TGF- β_1 and CTGF in HMCs. Observations suggest that an increasing risk of renal interstitial fibrosis is induced by high glucose.

GLP-1 is a hormone secreted by the L-cells of the small intestine and stimulates glucose-dependent insulin response [26–28]. In addition, GLP-1 has other effects that may improve the pathophysiology of the diabetic state, such as suppression of glucagon secretion [27], inhibition of gastrointestinal secretion and motility [29], and inhibition of food intake [30]. Accordingly, enhancement of GLP-1 actions appears to have ideal profiles for the treatment of type 2 diabetes. However, a single administration of GLP-1 is not effective for the treatment for diabetes, because the protein gets rapidly degraded by DPP-4. Thus, GLP-1R agonists that are resistant to DPP-4, such as Ex-4, are currently being used for the treatment of type

2 diabetes. Ex-4 is a 39-amino acid peptide that was originally isolated from the salivary secretions of the Gila monster lizard [31]. It shares approximately 53% homology with the mammalian incretin GLP-1. Ex-4 binds to the mammalian receptor and activates it for synthesis of GLP-1, cloned from pancreatic β -cells. One of the major objectives of treatment of type 2 diabetes is to prevent DN, therefore, in this context several studies investigated the effects of Ex-4 on the nephropathic system [32, 33]. Considering the vital role of HMCs in DN, we examined the effect of Ex-4 on the HG treated HMCs. We found that 0.3 and 3 nmol/l Ex-4 inhibited the overproliferation of HG treated HMCs in a dose-dependent manner during treatment of humans [34, 35]. TGF- β_1 and CTGF secreted by HMCs have paracrine actions in renal interstitial fibrosis [36]. Our results show that addition of either 0.3 or 3 nmol/l Ex-4 decreases the mRNA and protein levels of TGF- β_1 and CTGF. HMC overproliferation and secretions of fibrotic cytokines are beneficial in interstitial fibrosis, leading to declining renal function in DN patients [37]. Treatment with Ex-4 could possibly improve the renal interstitial fibrosis in DN effectively.

GLP-1 receptor is a well-known G-protein-coupled receptor, the activation of which results in increased cAMP concentration due to combined activation of adenylate cyclase [38]. The study by Masayuki Arakawa suggested that Ex-4 markedly reduced the accumulation of monocytes/macrophages in the vascular walls, at least partly suppressing the inflammatory response in macrophages through the activation of the cAMP/PKA pathway [39]. We therefore investigated the involvement of the intracellular signaling cascade in the GLP-1 reno-protective effect. To explore the mechanism of Ex-4 induced suppression of TGF- β_1 and CTGF expression in HMCs, we preincubated HMCs with MDL-12330A, a specific adenyl cyclase inhibitor and PKI14-22, a specific PKA inhibitor. Our study results showed that the addition of MDL-12330A significantly suppressed the inhibitory effect of Ex-4 on TGF- β_1 and CTGF mRNA. These results suggest that the inhibitory effects of Ex-4 on TGF- β_1 mRNA and CTGF mRNA are largely dependent on the activation of adenylate cyclase. We also investigated the downstream pathway of cAMP using PKI14-22, a specific PKA inhibitor. Similar to MDL-12330A, the inhibitory effect of Ex-4 was significantly reversed by PKI14-22, suggesting the involvement of PKA in anti-matrix accumulation or anti-fibrosis effect of Ex-4.

In conclusion, our study suggests that appropriate concentrations of Ex-4 can significantly reduce the elevated expression levels of TGF- β_1 and CTGF mRNA in the HG induced HMCs, partly via the cAMP/PKA pathway. Thus, GLP-1 activation may improve conditions of renal interstitial fibrosis, whose treatment earlier was difficult in DN patients. These unique effects of GLP-1 activation may be helpful to design new therapies for renal impairment in type 2 diabetic patients.

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