

Original Paper

Exendin-4 Alleviates High Glucose-Induced Rat Mesangial Cell Dysfunction through the AMPK Pathway

Wen-Wei Xu^{ab} Mei-Ping Guan^{ab} Zong-Ji Zheng^a Fang Gao^a Yan-Mei Zeng^a
Yan Qin^a Yao-Ming Xue^a

^aDepartment of Endocrinology and Metabolism, Nanfang Hospital, Southern Medical University, Guangzhou, P. R. China; ^bThese authors contributed equally to this work

Key Words

AMPK • Cell proliferation • Fibronectin • ERK • mTOR • MMP-2 • TIMP-2

Abstract

Background/Aims: Glucagon-like peptide-1 (GLP-1), which counteracts insulin resistance in humans with type 2 diabetes, has been shown to ameliorate diabetic nephropathy in experimental models. However, the mechanisms through which GLP-1 modulates renal function remained illdefined. The present study investigated the putative mechanisms underlying effects of exendin-4, a GLP-1 analog, on mesangial cell proliferation and fibronectin. **Methods:** Rat mesangial cells (MCs) were treated with exendin-4 under high glucose conditions. AMP-activated protein kinase (AMPK) inhibitors (compound C) and agonists (AICAR) were used to analyze the role of this kinase. Cell proliferation was measured using a MTT assay. Fibronectin expression and AMPK-signaling pathway activity were assessed using ELISA and Western blotting, respectively. The production of matrix metalloproteinase (MMP)-2 and tissue inhibitors of metalloproteinases (TIMP)-2 was evaluated using quantitative real-time RT-PCR. **Results:** Exendin-4 inhibited cell proliferation and fibronectin secretion in high glucose-induced MCs. It also caused phosphorylation of AMPK and subsequently increased the ratio of MMP-2 to TIMP-2, which resulted in the degradation of fibronectin. Exendin-4 reversed extracellular signal-regulated kinase (ERK) phosphorylation and enhanced expression of mammalian target of rapamycin (mTOR) in MCs. Moreover, the activation of the AMPK pathway by exendin-4 was induced by AICAR, which was inhibited by compound C. **Conclusion:** Exendin-4 exerts an inhibitory effect on cell proliferation and fibronectin secretion in rat MCs, partly through AMPK activation. These results may explain some of the beneficial effects of exendin-4 on the kidney.

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Yao-Ming Xue, MD, PhD

Department of Endocrinology and Metabolism, Nanfang Hospital, Southern Medical University, 1838, Guangzhou Avenue North, Guangzhou 510515 (China)
Tel. +86-20-61641635, Fax +86-20-87282018, E-Mail yaomingxue999@126.com

Introduction

Diabetic nephropathy (DN) has been the leading cause of end-stage renal disease in developed countries, while the number of patients with DN is increasing because of the high morbidity of diabetes. Furthermore, DN contributes to an increase of cardiovascular risk and all-cause mortality rates [1]. Therefore, treatment of DN, which improves the prognosis of diabetic patients, warrants further investigation. MC proliferation, extracellular matrix (ECM) accumulation and glomerular basement membrane thickening play important roles in the pathogenesis of DN. Resident and nonresident renal cells stimulated by hyperglycemia produce cytokines, growth factors such as transforming growth factor (TGF)- β 1 and other mediators that are responsible for structural alterations such as mesangial expansion and increased deposition of ECM proteins [2]. MCs also play a role in the synthesis, as well as the degradation of the ECM, which is mediated by proteinases such as matrix metalloproteinases (MMPs), including type IV collagen, laminin, fibronectin (FN) and proteoglycans [3, 4].

MMPs are a large family of zinc-dependent endopeptidases that are collectively capable of degrading all components of the ECM [5]. ECM activity is also regulated by a family of endogenous inhibitors known as tissue inhibitors of metalloproteinases (TIMPs) [6]. Imbalance between MMPs and TIMPs has been implicated in the disruption of ECM homeostasis [7, 8]. The decreasing ratios of MMPs to TIMPs, especially MMP-2 to TIMP-2 and MMP-9 to TIMP-1 may result in augmentation of ECM protein deposition.

ERK1 and ERK2 are related protein-serine/threonine kinases that participate in the RAS/RAF/MEK/ERK signal transduction cascade, which is involved in the regulation of a large variety of processes, including cell cycle progression, cell migration, differentiation, proliferation and transcription [9, 10]. *In vitro* studies have confirmed that ERK is activated in high glucose-induced mesangial cells [11, 12]. The mTOR pathway has a well-established role in cell proliferation; it can be activated by ERK and plays a part in DN [13].

As a GLP-1 receptor agonist, exendin-4 has been clinically used in patients with type 2 diabetes. A previous study demonstrated that exendin-4 ameliorated renal injury without lowering blood glucose levels in a streptozotocin (STZ)-induced rat model, showing that exendin-4 exerted renoprotective effects in blood glucose-level independent pathways [14]. An *in vitro* study also revealed that exendin-4 inhibited MC multiplication and expression of transforming growth factor- β 1 and connective tissue growth factor, both of which induced ECM accumulation [15]. Exendin-4 induced phosphorylation of AMPK, which reduced weight gain and insulin resistance in mice with high-fat diets [16]. Additionally, phosphorylated AMPK was down-regulated in the kidneys of db/db mice [17, 18].

Therefore, the aim of this study was to investigate the effect of exendin-4 on cell proliferation and ECM expression in MCs and to define the mechanism involved with AMPK pathway activity.

Materials and Methods

Reagents

Exendin-4 was purchased from AnaSpec, Inc. AMPK inhibitor (compound C) and AMPK agonist (5-aminoimidazole-4-carboxamide-1 β -D-ribofuranoside, AICAR) were purchased from Merck Millipore and Cell Signaling Technology, Inc., respectively. 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Merck Millipore.

Cell culture and treatment

Rat mesangial cell lines (HBZY-1) were routinely maintained in complete DMEM culture medium (Hyclone, Thermo, San Jose, CA), supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μ g/ml streptomycin (both from Sigma-Aldrich). The cells were kept at 37 °C in a humidified 95% air, 5% CO₂ atmosphere incubator designated as culture at a steady-state condition. The medium was changed every 2 days. Passages 10-15 of the

Table 1. Sequences of primers and annealing temperatures

Gene	Primers	Product size (bp)	Accession number
MMP-2	Forward: 5'-ACGGGCCGTACAATCTTCAC-3' Reverse: 5'-AGCAGGAGACAGGTAACCA-3'	83	NM_031054.2
TIMP-2	Forward: 5'-TTCTTCACCCCTTCCAAGC-3' Reverse: 5'-AACAGCGGGAGGGCATAAC-3'	82	NM_021989.2
MMP-9	Forward: 5'-GCCTGTGTTGGTCAGAAGA-3' Reverse: 5'-TAAAAGGGCCGGTAAGGTGG-3'	134	NM_031055.1
TIMP-1	Forward: 5'-TGAGAAGGGCTACCAGAGCG-3' Reverse: 5'-ATCGAGACCCCAAGGTATTGC-3'	84	NM_053819.1
mTOR	Forward: 5'-GGCCACCGTGTGTGAAGAA-3' Reverse: 5'-GACCCTGCACTGAGATCCTG-3'	143	NM_019906.1
β-actin	Forward: 5'-GCGAGTACAACCTTCTTGCAG-3' Reverse: 5'-GCCTTGACATGCCGGA-3'	115	NM_031144.3

cells were used in this study. MCs at approximately 80% confluence were cultured in 1% FBS DMEN medium for 24 hours for synchronization, and then were exposed to low glucose (5.6mM), high glucose (30mM), with or without the additional application of exendin-4 (0.1, 1, 10 or 100 nM), Compound C (10μM) or AICAR (1mM).

Cell proliferation (MTT) assays

MCs were seeded in 96-well flat bottom plates at a density of 4×10^3 cells/well (200 μl/well). After synchronization, cells were treated with different concentrations of the indicated reagent for 24 hours. Medium was then replaced with 20 μl of 0.01 mol/L Phosphate Buffered Saline (PBS) containing MTT (5 mg/ml). After a 4 h incubation at 37 °C, MTT-containing PBS was removed, and 150 μl of DMSO (Sigma-Aldrich) was added to each well. After gentle mixing for 15 min, the reduced purple formazan crystals were dissolved, and the absorbance was read at 490 nm using an ELx800 microplate (Bio-tek, Vermont, USA).

ELISA

Cell culture supernatants from different treatment groups were harvested and centrifuged at 2000 g for 20min. After centrifugation, the supernatants were then assayed for TGF-β1 and FN using the ELISA kits (Boster Biological Engineering Co., Wuhan, China). The absorbance was read at 450 nm with an ELx800 microplate (Bio-tek, Vermont, USA).

Quantitative real-time PCR

Total RNA was extracted from MCs lysed in RNAiso Plus (Takara, Shiga, Japan). The RNA quality was determined using a the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., MA, USA). Reverse transcription of RNA was carried out according to the instructions of PrimeScript RT Master Mix (Takara, Shiga, Japan). Real-time PCR was conducted with 40 ng of cDNA using an ABI Prism 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA), and PCR amplifications were performed with the All-in-One qPCR Mix (GeneCopoeia, Guangzhou, China) using primers shown in Table 1. The cycling program consisted of 10 min at 95 °C, followed by 40 cycles of 95 °C for 10s, 60 °C for 20s, and 72 °C for 34s. The reactions were quantified according to the amplification cycles when the PCR products of interest were first detected (threshold cycle, Ct). Each reaction was performed in triplicate. The expression of the transcripts was normalized to the levels of β-actin in the samples.

Western blot analysis

Western blotting was used to measure the AMPK and ERK levels in MCs. The antibodies used were as follows: phospho-AMPKα (Thr172) and AMPKα (Cell Signaling Technology,

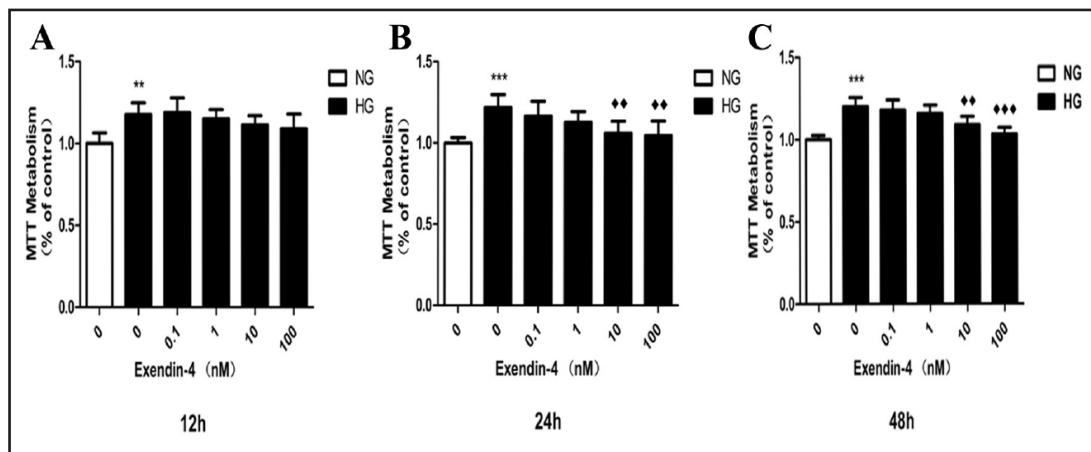


Fig. 1. MTT assay effects of exendin-4 on high glucose-induced MC proliferation. MCs were cultured in DMEM and 5.6 mM glucose (NG) or 30 mM glucose (HG) in the presence of exendin-4 concentrations of 0 (vehicle control), 0.1, 1, 10, and 100 nM for 12, 24 or 48 hours. Values are the mean \pm SD of six measurements. ** $P < 0.01$ and *** $P < 0.001$ vs. NG group; ◆◆ $P < 0.01$ and ◆◆◆ $P < 0.001$ vs. HG group.

Danvers, MA, USA); phospho-p44/42 MAPK (ERK1/2) and p44/42 MAPK (ERK1/2) (Cell Signaling Technology, Danvers, MA, USA). Briefly, the total protein of the MCs was extracted with RIPA Lysis Buffer (Beyotime Institute of Biotechnology, Beijing, China) according to the manufacturer's instructions, which was then subjected to 10% SDS-PAGE (Bio-Rad Laboratories, Hercules, CA, USA). The separated proteins were transferred to polyvinylidene fluoride membranes (Merck Millipore, MA, USA) by electrotransfer. The membranes were then blocked with Tris-buffered saline with 0.05% Tween (TBST) and 5% (wt/vol.) non-fat dry milk, and subsequently immunoblotted with primary antibodies, followed by IRDye 800CW-conjugated secondary antibody (LI-COR, Lincoln, NE). The infrared fluorescence image was obtained using the Odyssey infrared imaging system (LI-COR, Lincoln, NE), and the bands were quantified by Quantity One Version 4.4.0.

Statistical analysis

Data are presented as the mean \pm SD. Differences between the groups were examined for statistical significance by ANOVA with Bonferroni correction, using SPSS version 13.0. A value of $P < 0.05$ was considered statistically significant.

Results

Determination of the dose-dependent effect of exendin-4 on MC viability using MTT

MCs cultured in high glucose (30 mM) were used to mimic hyperglycemia in this study because MC proliferation is one of the features of early stage DN. Cell viability was examined by MTT assay to evaluate the effect of exendin-4 on mesangial cells. Cell proliferation was significantly increased in high glucose-cultured MCs relative to the normal glucose (5.6 mM) group. The level of cell proliferation in the high glucose (HG) group increased by $17.5 \pm 7.3\%$ ($P = 0.001$), $21.7 \pm 8.0\%$ ($P < 0.001$) and $19.9 \pm 5.7\%$ ($P < 0.001$) at 12, 24 and 48 h (Fig. 1), respectively. Compared to the high glucose group, the threshold concentration of exendin-4 that caused decreased MC viability was 10 nM ($106.0 \pm 7.4\%$ vs. $121.7 \pm 8.0\%$, $P < 0.01$) at 24 hours (Fig. 1B), with maximal effect at 100 nM ($104.4 \pm 9.0\%$ vs. $121.7 \pm 8.0\%$, $P < 0.01$). Similar results were observed in groups treated for 48 hours ($108.9 \pm 5.0\%$ vs. $119.9 \pm 5.7\%$, $P < 0.01$; $103.2 \pm 4.0\%$ vs. $119.9 \pm 5.7\%$, $P < 0.001$) (Fig. 1C). However, exendin-4 seems to have no significant effect on high glucose-induced MCs at 12 hours (Fig. 1A).

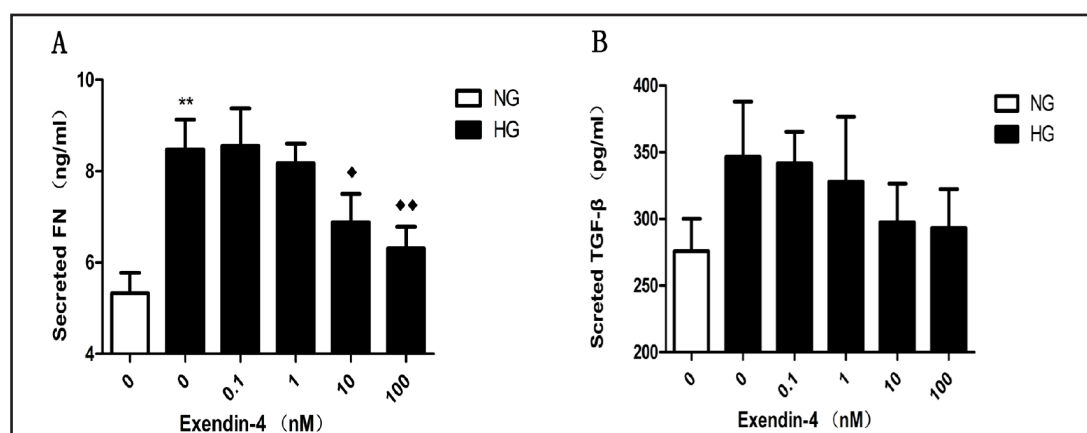


Fig. 2. Expression of FN and TGF-β1 in the cell culture supernatants. Supernatants were assayed by ELISA after treatment with different concentration of exendin-4 for 24 hours. Values are the mean \pm SD of three measurements. ** $P < 0.01$ vs. NG group; $\blacklozenge P < 0.05$ and $\blacklozenge\blacklozenge P < 0.01$ vs. HG group.

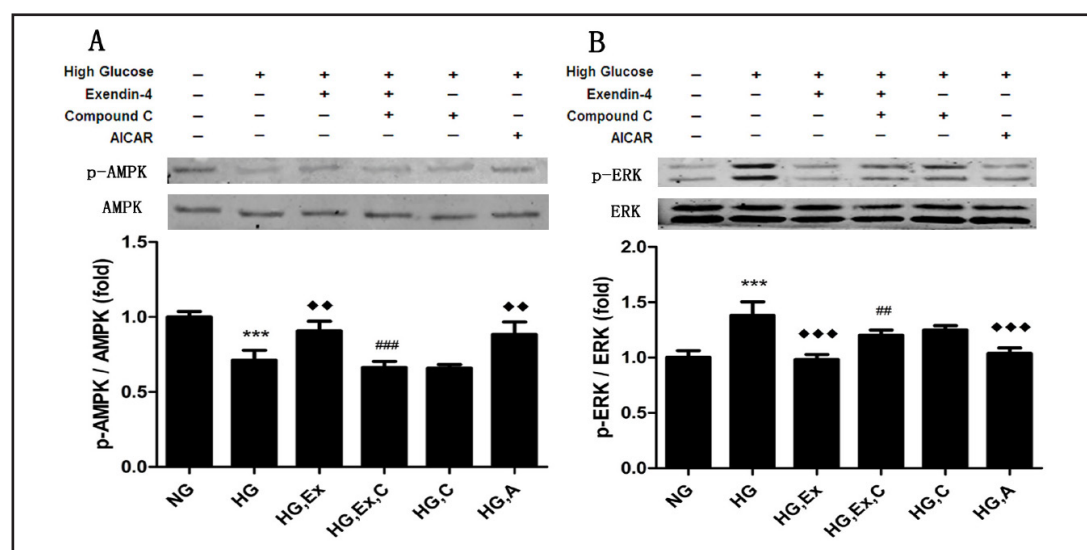


Fig. 3. Effects of exendin-4 on AMPK and ERK activities under high glucose conditions with or without the addition of compound C and AICAR. Ex: exendin-4; C: compound C; A: AICAR. MCs were incubated for 24 h in the presence or absence of exendin-4 (10 nM). In different groups, cells were treated with compound C (10 μ M) or AICAR (1 mM) for 1 h before glucose modulation. Values are the mean \pm SD of four measurements. *** $P < 0.01$ vs. NG group; $\blacklozenge\blacklozenge P < 0.01$ and $\blacklozenge\blacklozenge\blacklozenge P < 0.001$ vs. HG group; $\#\# P < 0.01$ and $\#\#\# P < 0.001$ vs. HG+Ex group.

Effect of exendin-4 on the secretion of FN in cell culture supernatants

The production of FN and TGF-β1 in cell culture supernatants is summarized in Fig. 2. The secretion of FN was higher from MCs in the high glucose group than in the normal glucose group, whereas expression was down-regulated after treatment with high concentrations (10 nM and 100 nM) of exendin-4 ($P < 0.05$ and $P < 0.01$ respectively) (Fig. 2A). Large doses of exendin-4 also reduced the secretion of TGF-β1, however, the change was not statistically significant (Fig. 2B).

Exendin-4 attenuated high glucose-induced phosphorylation of ERK through AMPK signaling

In MCs cultured with high glucose AMPK phosphorylation was inhibited, while AMPK activity was significantly increased by exendin-4 (Fig. 3A). Exendin-4 also reduced the high

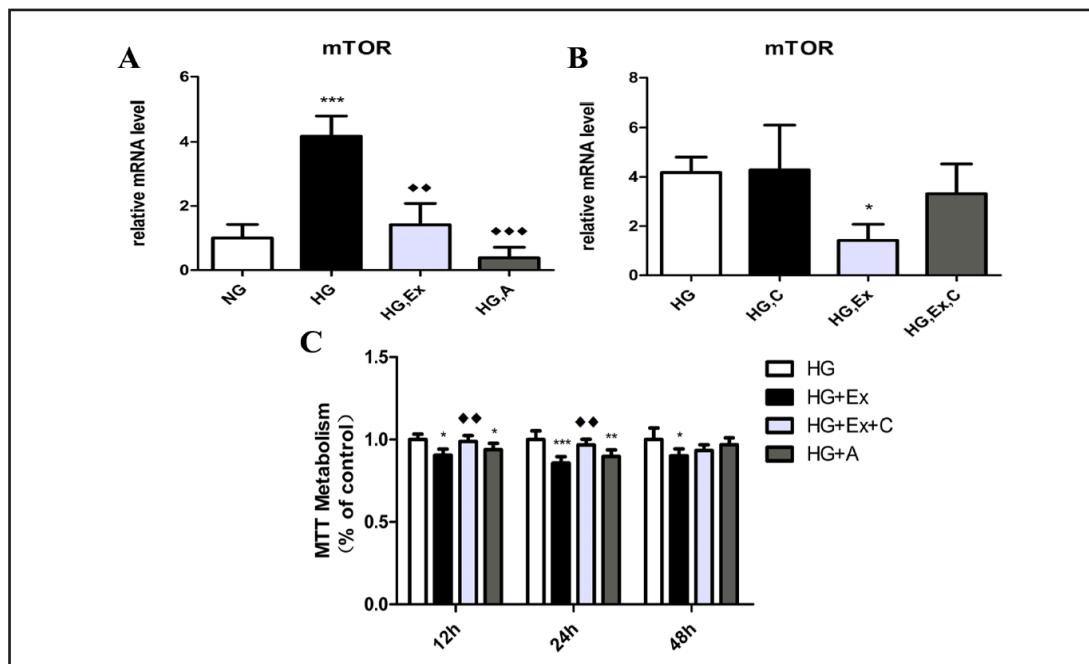


Fig. 4. Regulation of the AMPK pathway affects mTOR expression and cell viability. (A) MCs were incubated for 24 h with normal glucose or high glucose to measure the gene expression of mTOR. Exendin-4 (10 nM) or AICAR (1 mM) were used to assess the effect of AMPK on mTOR mRNA expression. N=3, ***P < 0.001 vs. NG group; ♦♦P < 0.01 and ♦♦♦P < 0.001 vs. HG group. (B) The effect of compound C (10 μ M) on mTOR mRNA expression was then examined. N=3, *P < 0.05 vs. HG group. (C) Cell viability was also determined in MCs induced by high glucose with the above reagents. N=6, *P < 0.05, **P < 0.01 and ***P < 0.001 vs. HG group; ♦♦P < 0.01 vs. HG+Ex group.

glucose-induced phosphorylation of ERK. However, the effects of exendin-4 were attenuated by compound C. AICAR, an AMPK agonist, had a similar effect to exendin-4, decreasing ERK activity (Fig. 3B).

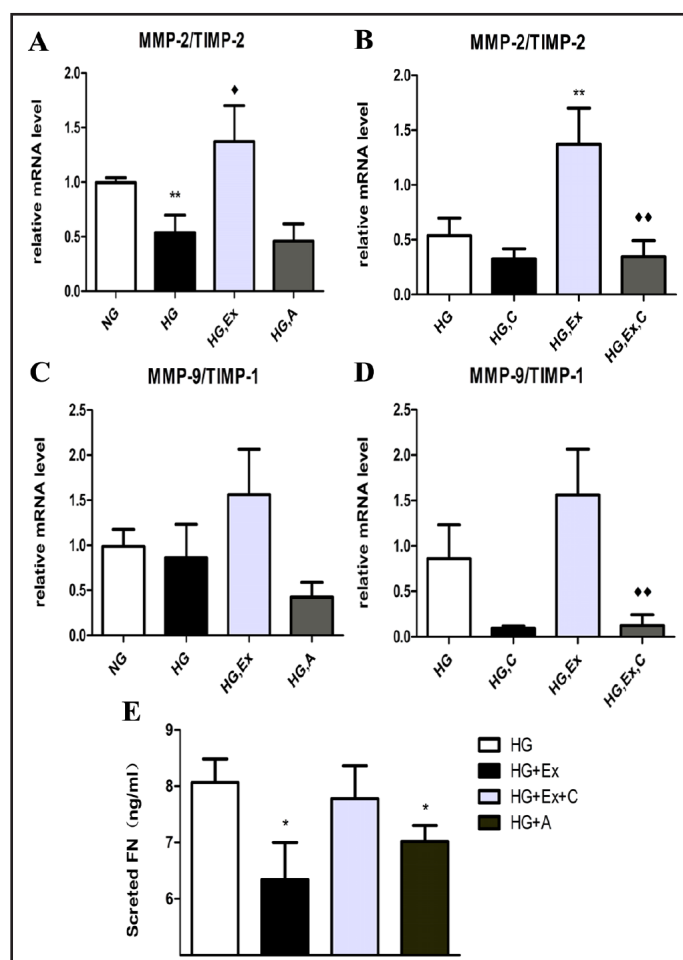
Effects of the regulation of the AMPK signaling pathway on cell proliferation and the expression of mTOR

The mRNA expression of mTOR was up-regulated in the high glucose group relative to the normal glucose group, whereas exendin-4, as well as AICAR, significantly inhibited high glucose-induced expression of mTOR (Fig. 4A). Down-regulation of mTOR mRNA levels by exendin-4 was attenuated by compound C, though the change was not significant (Fig. 4B). Similar to exendin-4, AICAR had an inhibitory effect on cell proliferation, which could be attenuated by compound C (Fig. 4C).

Effects of AMPK signaling pathway regulated MMP-2/TIMP-2 expression on FN secretion

We examined the mRNA expression levels of MMP-2, MMP-9, TIMP-2 and TIMP-9 in our study. The levels of these genes were inhibited by high glucose, but no significant changes were observed among the high glucose group and the high glucose groups treated with exendin-4 or AICAR (data not show). However, in the group treated with exendin-4, the mRNA expression levels of MMP-2/TIMP-2 were up-regulated (Fig. 5A). The changes of the mRNA levels of MMP-2/TIMP-2 and MMP-9/TIMP-1 by exendin-4 were reversed with the addition of compound C, which increased and decreased the mRNA expression of TIMP-2 and MMP-9, respectively in group co-treatment with high glucose and exendin-4 (Fig. 5B-D). Moreover, compound C attenuated the effect of exendin-4 on FN secretion in cell culture supernatants (Fig. 5E).

Fig. 5. Expression of MMP-2/TIMP-2, MMP-9/TIMP-1 and FN under AMPK pathway regulation. The ratios of MMP-2 to TIMP-2 and MMP-9 to TIMP-1 as well as the secretion of FN were evaluated under AMPK regulation. (A, C) N=3, **P < 0.01 vs. NG group; ◆P < 0.05 vs. HG group. (B, D) N=3, **P < 0.01 vs. HG group; ◆◆P < 0.01 vs. HG+Ex group. (E) N=3, *P < 0.05 vs. HG group.



Discussion

As an incretins, GLP-1 augments glucose-induced insulin release from pancreatic β -cells and suppresses glucagon secretion through its association with its high-affinity receptor, GLP-1R. GLP-1R is present in extrapancreatic tissues, such as kidneys, vascular tissues and the central nervous system. *In vivo* studies have shown some functional relevance for its presence in the kidney. However, endogenous GLP-1 is rapidly degraded by dipeptidyl peptidase (DPP)-4, which limits its application. Therefore, GLP-1R agonists that are resistant to DPP-4, such as exendin-4, are currently being used for treating type 2 diabetes. Additionally, these agonist most likely have a beneficial effect on renal function [19]. In STZ-induced diabetic rats, exendin-4 protected against the development and progression of DN by inhibiting the AGE-RAGE-mediated ADMA generation [20]. It also exerted renoprotective effects through its anti-inflammatory action via the GLP-1 receptor, without lowering blood glucose [14]. Correlative studies have shown that exendin-4 can enhance the phosphorylation of AMPK in the liver of DIO mice and in hepatocytes [17, 21], which was down-regulated in the kidneys of db/db mice [18]. Initially, AMPK was solely recognized as a highly conserved sensor of cellular energy that regulated Ca^{2+} and K^{+} channels in different cell types [22, 23]. However, further studies have demonstrated that AMPK also plays a role in the amelioration of epithelial cell damage caused by renal ischemia [24] and cell proliferation through the attenuation of MEK-ERK signaling [25] as well as mTOR activity [26, 27]. Besides, increased AMPK activity downregulates nuclear factor- κB (NF- κB) signaling which can activate proinflammatory mediators such as TNF- α and promote cell proliferation via SIRT1, peroxisome proliferator-activated receptor γ co-activator 1 α (PGC-1 α), Forkhead box O (FoxO) family and p53 [28, 29].

In experimental animal models, MC proliferation frequently, precedes and is linked to the increase of ECM in the mesangium and glomerulosclerosis. The proliferation of MCs, which are a part of the glomerular capillaries, can be initiated by basic fibroblast growth factor (bFGF). MC proliferation is maintained by an autocrine mechanism involving platelet-derived growth factor (PDGF). Subsequent development of sclerosis is mediated by TGF- β [30]. Reduction of MC proliferation in glomerular disease models by treating with a low-protein diet, heparin or antibodies to PDGF, has been shown to reduce ECM protein deposition and glomerulosclerotic changes [31]. Here, we observed that exendin-4 inhibited MC proliferation in a dose and time-dependent manner according to cell viability assays; in most time course studies, the effective inhibitory concentrations were 10 and 100nM. Exendin-4 also caused AMPK phosphorylation with down-regulation of phospho-ERK and mTOR. However, ERK can also be phosphorylated by the AMPK inhibitor compound C, which increased the expression of mTOR as well. Thus, AMPK activation by exendin-4 could be an upstream effect of ERK phosphorylation and mTOR activation. We also showed that compound C attenuated the inhibitory effect on cell proliferation by exendin-4, and the AMPK agonist AICAR had a similar effect to exendin-4, indicating that the AMPK pathway plays an important role in MC proliferation.

Furthermore, in our experiments the increased expression of FN induced by high glucose was alleviated by exendin-4. As one of the most important ECM proteins, glucose-induced FN expression in MCs results in the accelerated progression of glomerulosclerosis [32]. Therefore, the degradation of FN or other ECM proteins by any means seems to be beneficial in the treatment of DN. Previous studies have implied that MMPs play an important role in regulating physiological homeostasis and pathological disorders of the kidney through modulating the decomposition of ECM components, including FN [6]. As their name implies, the MMP gelatinases, also called MMP-2 and MMP-9, cleave denatured collagen (gelatin) as well as FN in basement membranes [4]. DN has been shown to be associated with decreased expression of MMP-2 and MMP-9 [33, 34]. The roles of MMPs in DN seem to vary within different clinical studies. Glomerular MMP-2 expression was decreased in patients with DN compared with normal controls [35]. However, another study showed that increased MMP-9 in plasma precedes the development of microalbuminuria [36].

TIMPs, a family of endogenous, specific inhibitors of MMPs, have varying specificities for different MMPs; for instance, TIMP-2 and TIMP-1 are the specific inhibitors of MMP-2 and MMP-9, respectively. Generally, TIMPs combine in a 1:1 stoichiometry with MMPs to cause an inhibitory effect [37]. A recent study suggested a link between an increase in TIMP-2 expression and the activity of STZ-induced diabetic rats [38]. In another study of 35 diabetic patients, TIMP-1 expression was elevated in serum and urine, and urinary TIMP-1 levels correlated with increased urine albumin. Nevertheless, circulating TIMP-1 and TIMP-2 are decreased in patients with DN when compared with patients with diabetes [39]. Due to the complexity of MMPs and TIMPs expression levels in DN, the ratios of MMPs to TIMPs may be a more suitable method to evaluate the balance between MMPs and TIMPs. In our study, exendin-4 increased the expression of MMP-2/TIMP-2 induced by high glucose, which could be reversed by compound C, exerting a similar change of FN expression in the culture supernatants. The above results indicate that the inhibitory effect of exendin-4 on FN secretion, which modulated ECM remodeling, may be regulated through the expression of MMP-2/TIMP-2 via AMPK pathway activation.

In conclusion, our study shows that exendin-4 exerts an inhibitory effect on mesangial cell proliferation under high glucose conditions by decreasing the phosphorylation of ERK and the expression of mTOR, which is partly mediated by AMPK activation. In addition, AMPK phosphorylation induced by exendin-4 modulates the deposition of fibronectin, most likely by regulating the ratio of MMP-2 to TIMP-2. These effects may explain some of the observed renoprotective properties of exendin-4, as well as its beneficial effects on diabetic nephropathy.

Conflict of Interest

The authors declare no conflicts of interest.

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