Uric Acid Promotes Diabetic Retinopathy Progression through Transforming Growth Factor β 2(TGF- β 2) Signaling and Beneficial Effects of Fenofibrate

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Research Article

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Abstract

Purpose: To elucidate the role of TGF- β 2 and Fenofibrate in DR concomitant with HUA.

Methods: *In vitro*, human retinal endothelial cells (HRECs) were incubated with high glucose(HG), HUA and HG mixed HUA respectively. The apoptosis rate was evaluated by TUNEL assay and Capase-3 activity. TGF- β 2 and VEGF was detected by Western blot and real-time PCR. The therapeutic effect of Fenofibrate was revealed by detecting the level of peroxisome proliferator activated receptor α (PPAR α) through Western blot and real-time PCR.

Results: The level of cellular apoptosis, TGF- β and VEGF was conducted to increase most significantly in presence of HG mixed HUA stimulated HRECs. HG mixed HUA led to PPAR α suppression which resulted in activated TGF- β 2 expression, and this effect could be inhibited by Fenofibrate.

Conclusion: TGF- β 2 might be a pivotal mediator for the progression of DR with uric acid stress and Fenofibrate could be an important pathway to ameliorate diabetic complication.

Introduction

Our previous study has revealed that high uric acid might be a pathogenetic mediator of diabetic retinopathy(DR) by upregulating cell apoptosis and promoting inflammatory response of the retina. We also identified *in vitro* and *in vivo* the activation of Notch signaling pathway, which involved in high-uric acid in high-glucose stress in DR(1).

Previous findings have shown that the Notch signaling pathway can cross-talk and integrate with other signaling pathways in a series of pathological processes ranging from epithelial to mesenchymal transition (EMT), fibrotic diseases and cancer metastasis. Accumulating evidence indicates that Transforming growth factor β (TGF- β) is the major pathologic contributor to ocular fibrotic diseases, such as proliferative vitreoretinopathy (PVR) and proliferative diabetic retinopathy (PDR)(2, 3). And TGF- β 2, one isoform of TGF- β superfamily, has been found to significantly increase in the vitreous body of diabetes mellitus patients, which could activate tissue inhibitor of metalloproteinases (TIMP)-1 and is associated with neovascularization of PDR patients(4). LA. Khuu et al(5)revealed a significant increase in TGF- β 2 level in the aqueous humor of non-proliferative DR (NPDR) patients compared to the health subjects. Recently, the crosstalk between Notch and TGF- β 2 signaling has been shown to be a key regulator for EMT in retinal pigment epithelial (RPE) cells(2). However, the cellular processes mediated by TGF- β 2 behind the process of endothelial dysfunction in the presence of high-glucose with high-uric acid conditions has not fully been elucidated. Additionally, in DR vascular system, ligands of TGF- β superfamily were activated ubiquitously to promote retinal endothelial cells proliferation and angiogenesis and showed its pro-angiogenic effects(6). Thus, the exact function of the TGF- β 2 increment in diabetic retinal microangiopathy need be further elucidated.

Fenofibrate, acting as peroxisome proliferator activated receptor (PPAR α) agonist, is primarily administered for reducing total cholesterol, triglycerides and LDL. And now, fenofibrate has been elucidated to have beneficial effects of reducing the progression of diabetic retinopathy(7). For instance, fenofibrate was confirmed to activate PPAR α and negatively mediate metabolic injury induced by high glucose in vitro in DR(8). Australia also approved the use of fenofibrate for its specific indication of slowing the progression of existing diabetic retinopathy in type 2 diabetes patients(9). Furthermore, fenofibrate could reduce uric acid concentrations, even after gout patients who have already been given allopurinol(10). A large clinical study demonstrated that fenofibrate run-in could lower plasma uric acid by an average of 20% and reduce almost half in first gout attack(11).

Based on the reasoning above, we hypothesized that fenofibrate may suppress the promoting role of high uric acid on DR and TGF- β 2 signaling might involve endothelial cell and retinal vessels dysfunction under DR combined with high-uric acid stressful condition.

Materials And Methods

Cell culture

HRECs (Human retinal endothelial cell, Applied Cell Biology Research Institute), in EGM2-MV culture medium (Lonza Group AG) mixed with 10% FBS (Gibco), 100 U/ml penicillin and 2 mM glutamine, were added normal level of glucose (5 mmol/l glucose), high level of glucose (HG, 30 mmol/l glucose), high level of uric acid (HUA, 12 mg/dL), HG mixed HUA for 48 h, respectively, at 37 °C under 5% CO₂.

RNA isolation and RT-PCR

RNAs, isolated using TRIzol, were transcribed into cDNA reversely (PrimeScriptTM RT reagent kit), and then RT-PCR was performed (SYBR Premix Ex Taq II). Above kits were purchased from Takara. 2- $\Delta\Delta$ CT method was to analyze fold changes.

Western blot

The protein lysates were separated in SDS-PAGE for further transfer onto PVDF membranes (Millipore). The membranes were cultured in 5% non-fat milk prepared with TBS for 1-2 h. Next, the membranes were incubated with primary antibodies overnight at 4 °C, following HRP-labeled secondary antibodies (Sigma) for 2 h at room temperature. The primary antibodies were purchased from Abcam.

Caspase-3 activity

Diluting buffer was prepared according to the instructions of CaspACETM Assay System kit for drawing Standard curve. Prepared pancreatic cancer cells were added with lysis buffer (100 μ l/5 $\times$ 10⁶ cells). After

centrifugation at 18000g for 12 min at 4 °C, the supernatant was added to the prepared reaction system added with detection buffer beforehand. After the addition of Ac-DEVD-pNA (2 mmol/L), the incubation was conducted at 37 °C for 60-120 min. D405 was analyzed when obvious color change occurred.

TUNEL assay

Apoptotic cells were detected using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) system (Promega) according to the manufacturer's instructions. Representative images were observed and photographed by confocal microscope.

Statistical analysis

The data was performed with GraphPad Prism 6 software. Normally distributed data were expressed as mean $\pm$ SD. Differences between means were analyzed by using unpaired t test and one-way ANOVA test. The Bonferroni correction was applied for multiple comparison analysis. $p < 0.05$ was interpreted as statistically significant.

Results

Activated TGF- β 2 signaling is associated with the apoptosis in HRECs cultured in high-glucose and high uric acid

Apoptosis of endothelial cells is a major pathological change in DR(12), Coincidentally, uric acid could exacerbate vascular endothelial cell apoptosis in middle cerebral artery occlusion rat model (13), so we speculated that high-glucose mixed high-uric acid might be involved with apoptosis in HRECs. Firstly, we observed the level of cellular apoptosis in the TUNEL analysis. As shown in Fig. 1A, treated with high-glucose, high-uric and high-glucose mixed high-uric acid could cause the increase in cellular apoptosis, and the increment of apoptosis was the most significant in cells cultured with high-glucose mixed high-uric acid. We also analyzed caspase-3 activity presented with apoptotic level. Caspase-3 activity was higher either in high glucose or in high uric acid stimulation compared with control group, and was highest in the high-glucose mixed high-uric acid group (Fig. 1B). There was evidence that TGF- β can ameliorate the apoptosis in corneal epithelial cells cultured in high glucose(14). Next, to investigate the link between TGF- β 2, a polypeptide member of TGF- β superfamily, and HRECs apoptosis in high glucose mixed high uric acid, we incubated HRECs with high glucose, high uric acid and high glucose mixed high uric acid separately and analyzed each expression of TGF- β 2 and smad2/3, mediators of intracellular signaling, at protein and mRNA levels. TGF- β 2/ smad2/3 expression in HRECs treated with high glucose or high uric acid was much higher than the control group, and remained highest in the group of high glucose mixed high uric acid at protein level (Fig. 1C). The same results were observed at mRNA level

(Fig. 1D). These data supported the hypothesis that TGF- β 2 signaling is associated with the excess of cells apoptosis caused by uric acid in DR.

High-glucose concomitant high uric acid upregulates levels of VEGF in HRECs

Several studies have postulated VEGF as one of the pivotal factors in the progression of DR(15), hence, we analyzed the expression of VEGF family (VEGF A/ VEGF R1/ VEGF R2) in HRECs exposed to normal glucose, high glucose, high uric acid or high glucose mixed high uric acid. We found that VEGF A/ VEGF R1/ VEGF R2 expression in HRECs treated with high glucose or high uric acid was much higher than that of the control group, and remained highest in the group of high glucose plus high uric acid at protein level (Fig. 2A). The same results were observed at mRNA level (Fig. 2B). These results indicated that uric acid may promote DR via upregulating levels of VEGF family (VEGF A/ VEGF R1/ VEGF R2).

The inhibition of TGF- β 2 signaling suppresses cellular apoptosis and VEGF expression in HRECs exposed to High-glucose concomitant high uric acid

To furtherly analyze the role of TGF- β 2 activation in mediating the progression of DR combined with high uric acid, we used TGF- β 2 neutralization antibody to block TGF- β 2 signaling. The results showed that both the level of cellular apoptosis and VEGFA/ VEGFR1/ VEGFR2 expression were down regulated by TGF- β 2 inhibition (Fig. 3). Therefore, these results confirmed that uric acid may promote DR through TGF- β 2-dependent upregulation of VEGF.

Fenofibrate inhibits TGF- β 2 expression through PPAR α activation in HRECs

Fenofibrate, a widely administered lipid-lowering drug, was uncovered to be beneficial to reduce the progression of DR(16). Therefore, we investigated whether the role of fenofibrate as the PPAR α agonist could reduce the effect of uric acid in the development of DR by suppressing the TGF- β 2 signaling. As shown in Fig. 4, we found that high glucose mixed high uric acid could decrease expression of PPAR α and this effect was abolished by fenofibrate. Furthermore, fenofibrate could also reversed the effect of high glucose plus high uric acid on TGF- β 2/ smad2/3 expression. These results demonstrate that fenofibrate could decrease TGF- β 2 expression through PPAR α activation in HRECs.

Discussion

In this study, we demonstrated evidence that activation of TGF- β 2 signaling was closely associated with the effect of UA on DR progression. Furthermore, treated with high-glucose and high-uric acid, TGF- β 2 signaling could increase cell apoptosis and levels of VEGF A/VEGF R1/VEGFR2 in HRECs. Notably, fenofibrate could suppress TGF- β 2 signaling expression, subsequently reverse the increase in cell apoptosis in HRECs via PPAR α activation.

The pathogenesis of DR has been characterized in the context of high glucose, high blood pressure, and serum triglyceride levels, although several evidences has demonstrated that high serum uric acid (SUA) concentration is involved with the progression of DR(17-19), however, the molecular features resulting from high SUA on DR are poorly understood. Recently, several research has shown that TGF- β plays a pivotal role in the pathogenesis of microvascular complications in DR (20, 21). Our previous study has reported that TGF- β 2 has played a growth-suppressive role in pancreatic cancer cells after radiation(22). Furthermore, it has also been identified that TGF- β 2 could serve as a negative regulator of IL-6 signaling and suppresses the anti-apoptosis effect of IL-6(23). In prostate cancer, findings suggested that the growth characteristics of tumor cell behavior is reflected by active secretion of uric acid, which is positively correlated to the effects of members of TGF- β superfamily(24). In our study, we have shown that the enhanced expression level of TGF- β 2 signaling, cultured with high-glucose and high uric acid, may contribute to the induction of apoptosis in HRECs. Our data are in accordance with a previous study partially (25) , as demonstrating that metabolic disorders and hyperuricemia rise upon tendon cells death and influence the synthesis of pleiotropic cytokines as TGF- β and IL-6.

The early histological damage hallmark of DR are thickening of retinal capillary basement membranes and increased apoptosis of retinal pericytes and endothelial cells. DR is a consequential pathological processes from the breakdown of blood-retinal barrier, the leakage of retinal vasculature, occlusion of retinal vessels to retinal ischemia and hypoxia.(21, 26). Our observation presented that under high glucose concomitant high uric acid stress, the level of cells apoptosis and excessive VEGF expression showed a most significant increase in comparison with the result of cultured with high-glucose or high-uric acid respectively. One clinical study supported our assumption in which DR patients showed showed higher UA and VEGF concentrations in vitreous body of DR patients compared to the controls(18). Several previous studies have established the pathogenic potentiality of VEGF for DR(15, 27). Several postulations estimating the pro- and anti-angiogenic VEGF homeostasis in diabetic angiogenic disorders indicated that although anti-VEGF injections could improve vascular damage in DR patients, while repeated anti-VEGF injections in DR rat model resulted in detrimental effect to the retina by increasing retinal ganglion cells apoptosis and necrosis(28, 29). In addition, the in vivo study demonstrated that levels of both VEGFR1 and VEGFR2 were significantly higher in DR patients aqueous humor than those control eyes(30). We also observed a concomitant increased trend of the level of VEGFA along with a consistent increased level of VEGF R1,VEGFR2 treated with high glucose or high uric acid respectively. This study further demonstrated that under high-glucose mixed high- uric acid stress, the increment of VEGF family expression was the most statistically significant. Although details of the functional interaction of high uric acid on the progression of DR are enigmatic, our data suggested that the increase of VEGF is closely related to the effect of uric acid on adverse prognosis for DR.

Recently, in regard to the fenofibrate therapy, plasma uric acid concentrations showed a significantly effective reduction(31). Moreover, as an agonist for PPAR α , fenofibrate was supposed to inhibit the renal organic anion transporter URAT1 to exert uric acid-lowering effect, which was independent of lipid-profile changes(32). In addition to its role in modulating uric acid, action of fenofibrate has been confirmed to play the role of vasodilation, ameliorating inflammation and cell apoptosis that may be beneficial in diabetic retinopathy(16, 33). But not all the effects of fenofibrate to reduce the progression of DR are dependent on PPAR α and the exact mechanism remains to be confirmed.

A recent study focused on the gonadal function of diabetic rats found that PPAR γ , another member of the nuclear receptor family, can regulate expression of TGF- β 1 to improve testicular function(34). The current study also demonstrated a new founding that exposure to high glucose mixed high uric acid could attenuate PPAR α expression. Furthermore, fenofibrate was shown to activate PPAR α expression and reduce HRECs apoptosis in vitro via TGF- β 2 signaling. Thus, our results suggested that the inhibitory role of fenofibrate could mediate the influence of high uric acid on the progression of DR through the TGF- β 2-dependent signaling.

In summary, the present findings demonstrated that high uric acid can activate apoptosis and upregulate the level of VEGF in vitro DR model, likely through TGF- β 2 signaling pathway, which promoted the aggravation of DR damage. Fenofibrate was supposed to activate PPAR α and alleviate high uric acid-induced cells apoptosis via TGF- β 2 suppression in HRECs. Our findings may provide new insights into the effect of uric acid on DR and fenofibrate supplementation might be an adjunctive therapy for DR patients combined with hyperuricemia.

Declarations

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Competing interests:

The submission is not under review at any other publication, in whole or in part, and all the authors listed have approved the enclosed manuscript. All the authors declare no financial conflict of interest and have fulfilled the criteria of authorship for the manuscript.

Availability of data and material

The data sets supporting the results of this article are included within the article.

Code availability

Not applicable

Authors' contributions:

Dandan Zhu performed the experiments, analyzed the data and wrote the manuscript. Chi Fang explained the data and wrote the manuscript. Zhu Mei performed the Western blot experiments.

Consent to participate

Not applicable

Consent for publication

Written informed consent for publication was obtained from all participants.

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Figures

Figure 1

Activated TGF- β 2 signaling is associated with the apoptosis in HRECs exposed to High-glucose and high uric acid Increased expression of TGF- β and cellular apoptosis in human retinal endothelial cells. A Left, TUNEL for observing apoptotic human retinal endothelial cells. right, apoptotic human retinal endothelial cell rate. B caspase-3 activity. *** $p < 0.001$. ** $p < 0.01$. * $p < 0.05$ C-D. Western blot (C) and RT-qPCR (D) for

detecting TGF-β2/SMAD2/3 expression respectively at protein and mRNA levels in human retinal endothelial cells in different groups. N, normal glucose. HG, high glucose, HUA, high uric acid, HG+HUA, high glucose plus high uric acid. ***p < 0.001. **p < 0.01

Figure 2

High-glucose and high uric acid upregulates levels of VEGF in HRECs Increased expression of VEGF in human retinal endothelial cells. A-B. Western blot (A) and RT-qPCR (B) for detecting VEGF A/VEGF R1/VEGF R2 expression respectively at protein and mRNA levels in human retinal endothelial cells in different groups. N, normal glucose. HG, high glucose, HUA, high uric acid, HG+HUA, high glucose plus high uric acid. ***p < 0.001. **p < 0.01

Figure 3

The inhibition of TGF- β 2 signaling suppress cellular apoptosis and VEGF expression in HRECs exposed to High-glucose plus high uric acid HRECs were treated with TGF- β 2 neutralization antibody. A Upper, TUNEL for observing apoptotic human retinal endothelial cells. lower, apoptotic human retinal endothelial cell rate. B caspase-3 activity. *** $p < 0.001$. ** $p < 0.01$. C-D Western blot (C) and RT-qPCR (D) for detecting p-SMAD2/3/VEGF A/VEGF R1/VEGF R2 expression respectively at protein and mRNA levels in HRECs in different groups. N, normal glucose. HG+HUA, high glucose plus high uric acid. HG+HUA+ neutralization, high glucose plus high uric acid with TGF- β 2 neutralization antibody. *** $p < 0.001$. ** $p < 0.01$

Figure 4

Fenofibrate inhibits TGF- β expression through PPAR α activation in HRECs. Western blot (A) and RT-qPCR (B) for detecting PPAR α / TGF- β 2/SMAD2/3 expression respectively at protein and mRNA levels in HRECs in different groups. N, normal glucose. HG+HUA, high glucose plus high uric acid. HG+HUA+ fenofibrate, high glucose plus high uric acid with fenofibrate. ***p < 0.001. **p < 0.01.