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ORIGINAL ARTICLE

Protective potential of klotho protein on diabetic retinopathy: Evidence from clinical and *in vitro* studies

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ABSTRACT

Aims/Introduction: The purpose of the present study was to observe the relationship between serum α-klotho (KL) protein level and diabetic retinopathy (DR), and to further examine the effects of KL protein on apoptosis induced by palmitic acid (PA) in human retinal endothelial cells.

Materials and Methods: A total of 17 healthy people and 60 type 2 diabetes patients were included. According to the results from fundus fluorescein angiography, the diabetes patients were divided into three subgroups: without DR, non-proliferative DR and proliferative DR. Serum KL level was measured by enzyme-linked immunosorbent assay. *In vitro*, human retinal endothelial cells were exposed to PA with or without KL protein. Apoptosis rates were analyzed by flow cytometry analysis. Apoptotic-related protein expressions were detected by western blotting analysis.

Results: Serum KL level was lower in diabetes patients than that in healthy participants ($P = 0.007$), and was gradually decreased among the without DR, non-proliferative DR and proliferative DR subgroups ($P = 0.045$). A logistic regression analysis showed that after adjusting for the other confounding factors, serum KL level was independently and negatively related with DR ($P = 0.049$). Furthermore, the increased apoptosis rates induced by PA were inhibited with the addition of KL protein. Consistently, KL protein reversed the expression levels of the increased pro-apoptotic protein Bax and the decreased anti-apoptotic protein Bcl-2 induced by PA. However, the anti-apoptotic effect of KL protein was attenuated by LY294002 through the phosphatidylinositol 3 kinase-serine/threonine kinase pathway.

Conclusions: The data suggested that KL protein was probably a potential protective factor against retinopathy in type 2 diabetes patients.

INTRODUCTION

Diabetic retinopathy (DR) is one of the most common microvascular complications of diabetes. The number of persons with visual impairment as a result of DR is increasing worldwide[1–3]. Studies showed that the retinal vascular endothelial cells apoptosis have an important role in the pathogenesis of DR[4]. Thus, it will be important to identify and intervene in the various pathological factors that cause endothelial cells apoptosis.

Although hyperglycemia is the main initiator of DR, dyslipidemia is also an important risk factor. In a meta-analysis and systematic review, the results showed that lipid-lowering agents presented a protective effect on retinopathy progression in diabetes patients[5]. Basic studies showed that dyslipidemia was implicated in the pathological processes of DR and prediabetic retinopathy[6–8]. Palmitic acid (PA), a saturated free fatty acid, could induce retinal endothelial cell damage and participates in the progression of DR[6,7].

The α-klotho (KL) gene was first described in 1997 as an anti-aging gene and codes for a single-pass transmembrane protein[9]. At the cellular level, the extracellular domain of KL protein can be cleaved by proteolytic enzymes and released into the circulation as soluble KL[10]. Studies have shown that KL protein plays vasculoprotective roles by reducing oxidative stress and apoptosis of vascular endothelial cells, and enhancing

endothelial function[11]. Studies found that hyperlipidemia could downregulate KL gene expression[12], and KL gene deficiency promoted the development of high-fat diet-induced arterial stiffening and hypertension[13]. The data suggested that KL protein was likely to play a pivotal role in vascular protection through the antagonism of lipotoxicity.

Previous studies showed that the KL gene had a critical function for retinal health[14,15]. Therefore, based on the corresponding theoretical basis, we aimed to observe the relationship between serum KL level and DR in type 2 diabetes patients. Furthermore, *in vitro*, the effect of recombinant KL protein on apoptosis induced by PA was observed in human retinal endothelial cells (HRECs).

METHODS

Participants
The participants included hospitalized patients with type 2 diabetes in November 2015 to November 2016, and the non-diabetic healthy population who carried out physical examinations in the same period at the First Affiliated Hospital of Chongqing Medical University in Chongqing, China. Diagnosis of diabetes was based on the criteria from the World Health Organization (1999 version). Exclusion criteria: (i) patients with acute diabetic complications; (ii) patients with type 1 diabetes mellitus or other types of diabetes; (iii) patients with acute and chronic infection symptoms; (iv) patients with severe liver and kidney dysfunctions, severe cardiovascular diseases, malignant tumors and so on; (v) patients who smoked more than one cigarette per day, and who drank excessive alcohol (20 g/day or 140 g/week in men and 10 g/day or 70 g/week in women); and (vi) patients who had received eye surgery and had other eye diseases. Eventually, 60 patients with type 2 diabetes and 17 healthy individuals were included. According to the 2002 international clinical standard on the severity of DR[16], all diabetes patients underwent fundus fluorescein angiography and were divided into the following groups: without DR (NDR), non-proliferative DR (NPDR) and proliferative DR (PDR). The healthy population underwent fundus photography and confirmed that there were no abnormities in the fundus.

Informed consent was obtained from all participants before the survey, and this study was approved by the Research Ethics Committee of the First Affiliated Hospital of Chongqing Medical University.

Measurements of anthropometric and laboratory parameters
In all participants, the sex, age, height, weight, systolic blood pressure (SBP), diastolic blood pressure (DBP), total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-c), high-density lipoprotein cholesterol (HDL-c), serum creatinine (Scr), blood urea nitrogen (BUN), uric acid (UA) and fasting plasma glucose (FPG) were collected. The information about duration of diabetes, glycated hemoglobin and percentage of diabetic nephropathy (DN) were also collected. DN was diagnosed according to the albuminuria levels:

normoalbuminuria (<30 mg/24 h), microalbuminuria (30–300 mg/24 h) and macroalbuminuria (>300 mg/24 h)[17]. The serum lipid profiles including TC, TG, HDL-c and LDL-c were measured by an enzymatic assay (Wako Diagnostics, Tokyo, Japan). The FPG was tested by hexokinase assays (Olympus Diagnostics, Tokyo, Japan). The Scr, BUN and UA were tested by enzymatic methods (Roche Diagnostics, Mannheim, Germany).

Measurement of serum KL level
Fasting venous blood samples were collected in coagulation-promoting tubes and were centrifuged at 1620 g for 10 min. The serum samples were extracted and stored at −80°C. Serum KL level was measured by enzyme-linked immunosorbent assay. The assay kit was purchased from Immuno-Biological Laboratories (Takasaki, Japan), and the detection range was from 93.75 to 6,000 pg/mL.

Cell culture
Human retinal endothelial cells were purchased from TongPai Biotechnology Co., Ltd (Shanghai, China).

Cells were grown in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco), 100 μg/mL streptomycin (HyClone, Logan, UT, USA) and 100 U/mL penicillin (Hyclone) under a humidified atmosphere containing 5% CO_2 at 37°C. Cells with passages 3–15 were used. When reaching approximately 80% of confluence in 60-mm culture dishes, the cells were treated with PA (Sigma-Aldrich, St. Louis, MO, USA) after pre-treatment with or without recombinant human KL protein (R&D Systems, Minneapolis, MN, USA) and the phosphatidylinositol 3 kinase-serine/threonine kinase (PI3K/Akt) inhibitor, LY294002 (Sigma-Aldrich). Namely, the cells were randomly divided into a normal control (Ctr) group, PA group, PA + KL group and PA + KL + LY294002 group.

Cell viability assay
To determine the pro-apoptotic effect of PA on HRECs, cells were first intervened with different concentrations of PA for 24 h, and the viability was measured with a cell counting kit-8 (CCK-8; Beyotime Institute of Biotechnology, Shanghai, China) following the manufacturer's instructions. Cells were seeded in a 96-wells plate and intervened with PA (0, 200, 400, 800 μmol/L) for 24 h. Then cells were incubated with the mixed liquor (10 μL CCK-8 reagent + 100 μL RPMI-1640 medium) at 37°C for 1 h. And the optical density value was measured at 450 nm.

Detection of apoptosis by flow cytometry analysis
Apoptosis was carried out using an apoptosis assay kit (Sungene Biotech, Tianjin, China). The apoptotic cells were measured using annexin V-FITC assay conjugated to propidium iodide by flow cytometry with BD FACSVantage SE cytometer (BD Biosciences, San Jose, CA, USA).

Western blot analysis

Total protein was extracted. The cells were washed with ice-cold phosphate-buffered saline and collected in radioimmuno-precipitation assay lysis buffer (Beyotime Institute of Biotechnology). The protein concentrations were measured with BCA method (Boster Biological Technology, Wuhan, China). The Bcl-2, Bax, Akt and p-Akt antibodies were ordered (Cell Signaling Technology, Beverly, MA, USA). Phosphospecific protein level was compared with its total protein level, and others were normalized to β-actin.

Statistical analysis

Statistical analysis was carried out using SPSS 21.0 (SPSS Inc., Chicago, IL, USA).

Clinical data are presented as the mean ± standard deviation for normally distributed variables, and the median (interquartile range [IQR]) for abnormal distributions. Unpaired *t*-test and Mann–Whitney *U*-test were used for comparisons of normally and abnormally distributed continuous variables between the two groups, respectively. Multiple and pairwise comparisons were determined by analysis of variance and Student–Newman–Keuls tests for normally distributed data, and Kruskal–Wallis and stepwise–step-down tests for abnormal distributions. Categorical variables are presented as the percentage (%). The χ^2-test was used to compare categorical variables. A logistic regression analysis was used for multivariate analysis to analyze the association between serum KL protein and DR. Data from cells are shown as the mean ± standard error of the mean. *P*-values (two-tailed) <0.05 are regarded as significant differences.

RESULTS

Baseline characteristics

The anthropometric and biochemical characteristics of participants are shown in Table 1. The study included 77 participants, consisting of 47 men and 30 women. Compared with the healthy population (NC group), the SBP, DBP and FPG in diabetes patients (diabetes group) significantly increased (all *P* < 0.05), whereas the HDL-c decreased (*P* < 0.05). There were no significant differences in sex, age, body mass index, TC, LDL-c, TG, Scr, BUN and UA between the two groups (all *P* > 0.05).

Additionally, the subgroup analyses were carried out among the NDR, NPDR and PDR groups. The results found that the Scr, BUN, UA levels and duration of diabetes among the subgroups were gradually increased (all *P* < 0.05). Compared with the NDR group, the Scr and UA levels increased in the NPDR and PDR group (all *P* < 0.05); and the BUN and duration of diabetes obviously increased, whereas the glycated hemoglobin level reduced in the PDR group (all *P* < 0.05). There were no significant differences in sex, age, BMI, SBP, DBP, HDL-c, LDL-c, TC, TG, FPG and percentage of DN among the three groups (all *P* > 0.05).

Comparison of serum KL level

As shown in Figure 1a, the serum KL level was lower in the diabetes group (566.25 pg/mL, IQR 471.57–705.43 pg/mL) than that in the NC group (768.98 pg/mL, IQR 544.41–1095.52 pg/mL; *P* = 0.007). Furthermore, subgroup analyses showed that the serum KL levels in the NDR, NPDR and PDR groups were 699.16 pg/mL (IQR 518.91–829.74 pg/mL), 583.40 pg/mL (IQR 471.24–700.70 pg/mL) and 495.88 pg/mL (IQR 407.85–571.11 pg/mL), respectively, and were gradually decreased (*P* = 0.045). Compared with the NDR group, the serum KL levels were significantly reduced in the PDR group (*P* = 0.013); however, there was no significant difference between NDR and NPDR groups (*P* = 0.425), and NPDR and PDR groups (*P* = 0.123; Figure 1b). Additionally, although there was no obvious statistical difference between the two groups (*P* = 0.173), the serum KL level was lower in the NDR group than that in the NC group.

Association analysis between serum KL level and DR

Diabetic retinopathy was served as the dependent variable, and the sex, age, BMI, SBP, DBP, HDL-c, LDL-c, TC and TG, FPG, duration of diabetes, DN state and serum KL level as the independent variables. A logistic regression analysis was carried out to analyze the association between serum KL protein and DR in diabetes patients. The results showed that after adjusting for the above-mentioned confounding factors, the serum KL level was independently related with DR (B −0.005; SE 0.002; *P* = 0.049; OR 0.995; 95% CI 0.990–1.000).

Effects of PA on HRECs viability

Human retinal endothelial cells were treated with PA in different concentrations (0, 200, 400, 800 μmol/L) for 24 h, and the cell viability was detected by the CCK-8 method. The results showed that the cell activity was decreased with the increase of PA concentrations and was shown in a concentration-dependent manner (Figure 2). Furthermore, the cell viability was significantly decreased in the concentration of 800 μmol/L, which suggested that there might be an obvious cytotoxic effect. Then we selected 400 μmol/L, 24 h as the intervention concentration and time of PA for the subsequent experiments.

Effects of KL protein on PA-induced apoptosis in HRECs

To determine the effects of KL protein on cell apoptosis induced by PA, HRECs were pretreated with 400 pmol/L recombinant human KL protein for 1 h (the selection of concentration and intervention time based on previous relevant reports[18,19]), and then were treated with PA for 24 h. The results showed that after pretreatment with KL protein, the increased apoptosis rate induced by PA was significantly decreased (*P* < 0.05; Figure 3a, b), and the Bcl-2 expression was upregulated and Bax expression was downregulated (*P* < 0.05; Figure 3c,d).

The PI3K/Akt pathway plays an important role in regulating cell survival. Akt is a pivotal effector and could be activated

Table 1 | Comparison of clinical and biochemical data of subjects

Variable	NC (n = 17)	DM (n = 60)	P	DM			P
				NDR (n = 27)	NPDR (n = 17)	PDR (n = 16)	
Sex, males (%)	52.9%	63.3%	0.574	55.6%	64.7%	68.7%	0.524
Age (years)	57.0 ± 5.1	57.8 ± 5.6	0.595	58.0 ± 4.7	57.4 ± 5.5	57.9 ± 7.1	0.925
BMI (kg/m^2)	23.00 ± 3.51	24.15 ± 3.20	0.228	24.00 ± 2.85	23.85 ± 3.46	24.95 ± 3.68	0.623
SBP (mmHg)	120.0 ± 10.8	138.9 ± 20.1	0.001	135.5 ± 17.0	140.6 ± 23.5	142.9 ± 21.3	0.478
DBP (mmHg)	75.9 ± 8.6	83.0 ± 12.5	0.046	81.7 ± 12.2	82.2 ± 15.0	86.2 ± 10.4	0.505
HDL-c (mmol/L)	1.52 ± 0.34	1.12 ± 0.28	<0.001	1.13 ± 0.32	1.05 ± 0.18	1.18 ± 0.28	0.468
LDL-c (mmol/L)	2.92 ± 0.44	2.81 ± 0.95	0.626	2.82 ± 0.92	2.65 ± 1.08	3.01 ± 0.87	0.622
TC (mmol/L)	4.90 ± 0.61	4.30 ± 1.21	0.053	4.36 ± 1.12	3.91 ± 1.37	4.77 ± 1.06	0.176
TG (mmol/L)	1.37 (1.10–2.25)	1.38 (0.97–2.35)	0.837	1.42 (0.89–2.13)	1.33 (0.92–2.51)	1.41 (1.15–2.65)	0.759
FPG (mmol/L)	5.28 ± 0.30	8.57 ± 3.04	<0.001	8.21 ± 2.78	9.19 ± 3.51	8.50 ± 3.02	0.588
Scr (μmol/L)	74.24 ± 12.57	74.53 ± 22.69	0.959	59.41 ± 11.76	80.47 ± 21.06*	93.75 ± 21.59*·**	<0.001
BUN (mmol/L)	5.73 ± 1.07	6.97 ± 2.91	0.090	5.56 ± 1.41	6.98 ± 2.73	9.33 ± 3.52*·**	<0.001
UA (μmol/L)	342.29 ± 70.50	326.43 ± 84.17	0.481	278.67 ± 66.13	360.47 ± 76.85*	370.88 ± 80.39*	<0.001
Duration (years)				6.0 (2.0–10.0)	9.0 (6.5–12.0)	10.0 (7.3–18.5) *	0.032
HbA1c (%)				9.80 ± 2.00	10.16 ± 2.30	7.94 ± 1.59*·**	0.004
DN (%)				40.7%	52.9%	56.2%	0.556

Data are presented as the mean ± standard deviation for normally distributed variables, and the median (interquartile ranges) for abnormal distributions. Unpaired t-test and Mann–Whitney U-test were used for comparisons of normally and abnormally distributed continuous variables between two groups, respectively. Multiple and pairwise comparisons were determined by analysis of variance and Student–Newman–Keuls tests for normally distributed data, and Kruskal–Wallis and stepwise–step-down tests for abnormal distributions. Categorical variables were presented as the percentage (%). The χ^2-test was used to compare categorical variables. Statistical differences were defined by P-values (two-tailed) <0.05. *P < 0.05 versus without diabetic retinopathy (NDR). **P < 0.05, proliferative diabetic retinopathy versus non-proliferative diabetic retinopathy. BMI, body mass index; BUN, blood urea nitrogen; DBP, diastolic blood pressure; DM, diabetes mellitus; DN, diabetic nephropathy; FPG, fasting plasma glucose; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; SBP, systolic blood pressure; Scr, serum creatinine; TC, total cholesterol; TG, triglyceride; UA, uric acid.
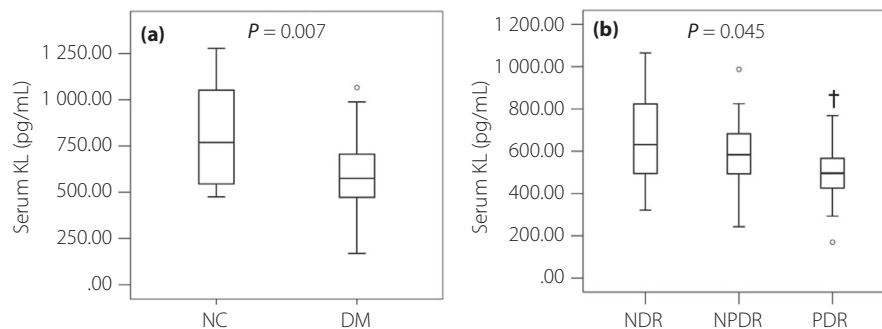

Figure 1 | Serum klotho (KL) levels in diabetes and diabetic retinopathy (DR) patients. (a) Comparison of serum KL levels between healthy control participants (NC group; n = 17) and diabetes patients (DM group; n = 60). (b) Comparison of serum KL levels among diabetes patients without diabetic retinopathy (NDR; n = 27), diabetes patients with non-proliferative diabetic retinopathy (NPDR; n = 17) and diabetes patients with proliferative diabetic retinopathy (PDR; n = 16). Data are represented as box plots. A Mann–Whitney U-test was used for comparisons of continuous variables between two groups. Kruskal–Wallis and stepwise–step-down tests were used for multiple and pairwise comparisons. Statistical differences were defined by P-values (two-tailed) <0.05. †P < 0.05 versus NDR group.

through phosphorylation by the products of PI3K. In the present study, the expression of p-Akt was decreased after exposure of HRECs to PA for 24 h; however, its level was restored to the control level after pretreating with KL protein (Figure 4a,b). Furthermore, to test whether the PI3K/Akt signaling pathway was involved in the inhibitory effects of KL protein on PA-induced cell damage, the inhibitor of PI3K, LY294002, was applied. HRECs were pretreated with LY294002 (10 μmol/L) for 1 h and 400 pmol/L KL for another 1 h, and then treated with PA for 24 h. The results showed that LY294002
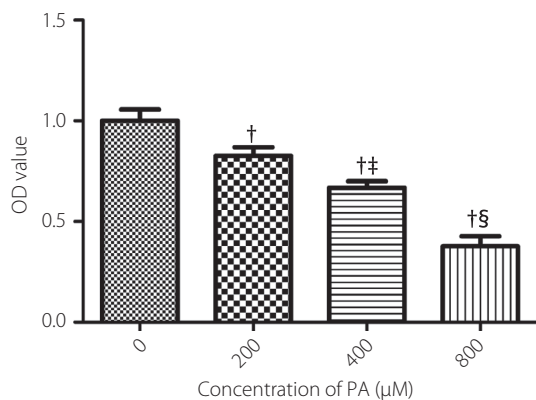
Figure 2 | Effects of different concentrations of palmitic acid (PA) on cell viability. human retinal endothelial cells were treated with different concentrations of PA (0, 200, 400, 800 µmol/L). After the treatments for 24 h, cell viability was assessed by cell counting kit-8 assay. Data are represented as the mean ± standard error of the mean ($n = 3$). Analysis of variance and Student–Newman–Keuls tests were carried out for multiple and pairwise comparisons. Statistical differences were defined by *P*-values (two-tailed) <0.05. [†]*P* < 0.05 versus 0 µmol/L; [‡]*P* < 0.05 versus 200 µmol/L; [§]*P* < 0.05 versus 400 µmol/L. OD, optical density.

intervention significantly weakened the inhibitory effects of KL protein on cell apoptosis induced by PA (Figure 4c,d).

DISCUSSION

The present data showed that the serum KL level was decreased in DR patients, and it was independently and negatively correlated with DR. In addition, KL protein could ameliorate the apoptosis induced by PA in HRECs, and the mechanisms might be involved in the activation of PI3K/Akt signaling pathway.

A variety of pathophysiological factors *in vivo* could affect KL gene expression. Studies have shown that hyperglycemia, oxidative stress, inflammation and angiotensin II significantly inhibited KL gene expression[20–23]. The present results showed that the serum KL protein level was evidently reduced in diabetes patients, which was consistent with previous studies[24–28].

The reduced serum KL level was probably an independent risk factor for DR. In the study of Słomiński *et al.*[29], the findings suggested that the KL gene polymorphism protected against the development of retinopathy in patients with type 1 diabetes, which might be involved in the increased KL protein levels and/or activity and/or trafficking of the protein that improved the inflammatory status and delayed endothelial dysfunction. The present results showed that serum KL protein
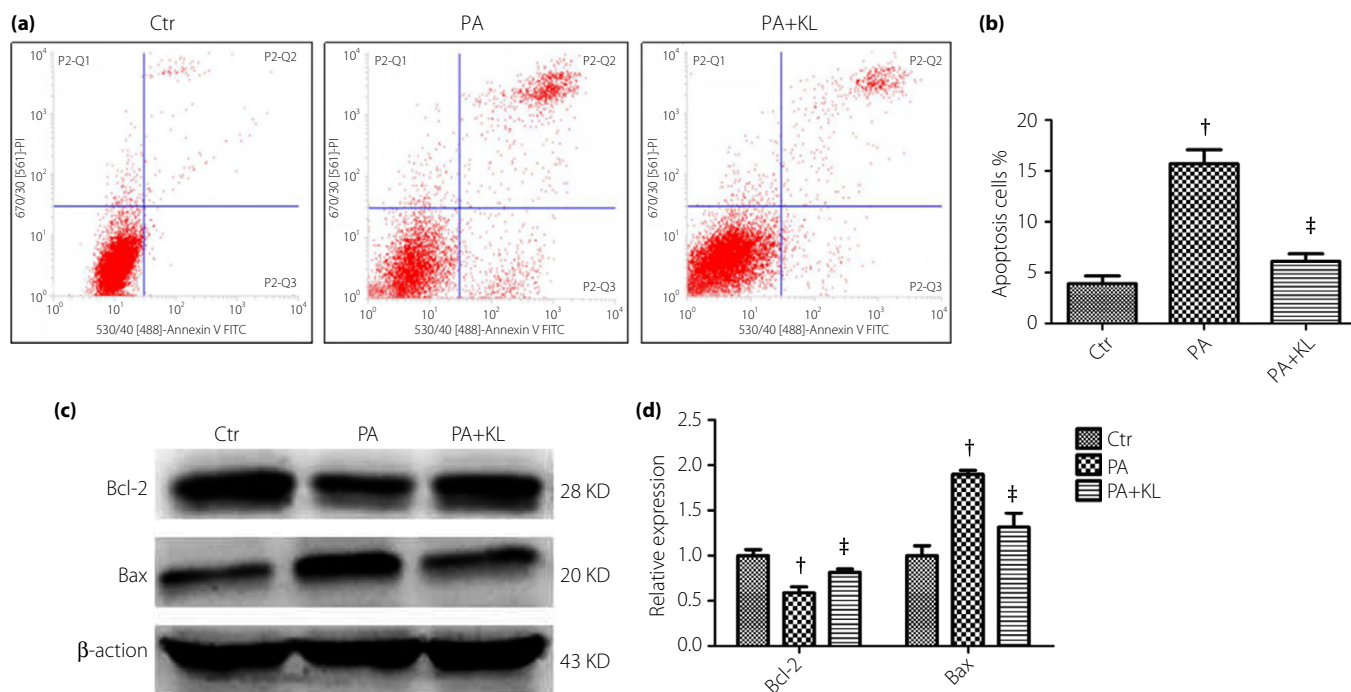

Figure 3 | Effects of klotho (KL) protein on palmitic acid (PA)-induced apoptosis in human retinal endothelial cells. Cells were pretreated with recombinant human KL protein (400 pmol/L) for 1 h and then treated with PA (400 µmol/L) for 24 h. (a,b) The cell apoptosis rate was detected using flow cytometry analysis. (c,d) Levels of Bcl-2 and Bax were detected by western blot analysis. Data are represented as the mean ± standard error of the mean ($n = 3$). Analysis of variance and Student–Newman–Keuls tests were carried out for multiple and pairwise comparisons. Statistical differences were defined by *P*-values (two-tailed) <0.05. [†]*P* < 0.05 versus control (Ctr) group; [‡]*P* < 0.05 versus PA group.
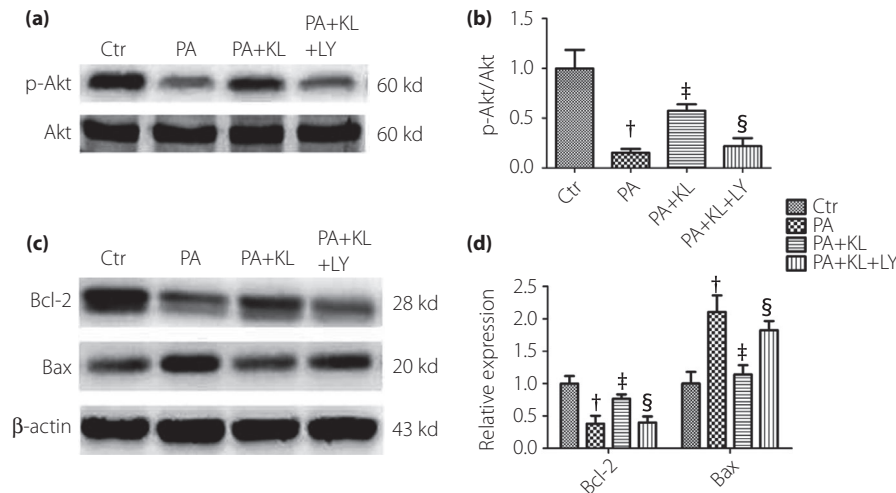
Figure 4 | Klotho (KL) protein inhibited palmitic acid (PA)-induced apoptosis by activating the phosphatidylinositol 3 kinase-serine/threonine kinase (PI3K/Akt) pathway in human retinal endothelial cells. Cells were pre-incubated with the PI3K inhibitor, LY294002 (10 µmol/L), for 1 h, followed by co-treatment with recombinant human KL protein (400 pmol/L) for 1 h and then with PA (400 µmol/L) for 24 h. (a,b) Level of p-Akt was detected by western blot analysis. (c,d) Levels of Bcl-2 and Bax were detected by western blot analysis. Data are represented as the mean ± standard error of the mean ($n = 3$). Analysis of variance and Student–Newman–Keuls tests were carried out for multiple and pairwise comparisons. Statistical differences were defined by P-values (two-tailed) <0.05. [†]$P < 0.05$ versus control (Ctr) group; [‡]$P < 0.05$ versus PA group; [§]$P < 0.05$ versus PA + KL group. LY, LY294002.

levels showed a downward trend with the increase of severity of retinopathy. However, it should be pointed out that serum KL protein was mainly from the kidney, and the renal and serum KL protein levels were significantly reduced in the DN state[28,30–32]. Consistent with previous results, the subgroup analyses were carried out according to the patients without DN (NDN group) or with DN (DN group) among diabetes patients, and the results showed that compared with the NDN group (682.60 pg/mL, IQR 526.52–829.74 pg/mL), the serum KL level in the DN group (474.18 pg/mL, IQR 381.78–604.76 pg/mL) was significantly decreased ($P < 0.001$). The DN state served as one of the important adjustment variables, and a logistic regression analysis was carried out to analyze the association between serum KL and DR. The results showed that the serum KL level was independently and negatively related with DR after adjusting for the DN status and other variables. In a brief summary, according to the above-mentioned results, we speculated that if the serum KL level was raised, there might be great improvement effects on DR. Consequently, we further observed the effects of KL protein in in vitro cell culture experiments.

The synthesis and decomposition function of adipose tissue is impaired in diabetes. In addition to hyperglycemia, dyslipidemia has also been found to be closely related with the DR progression. The present clinical data showed that the serum HDL-c level was significantly decreased in the diabetes patients compared with the healthy participants; however, there were no differences in TC, LDL-c and TG between the two groups, and furthermore, the blood lipid levels had no obvious differences in each subgroup according to the severity of retinopathy. We inferred that the use of lipid-lowering drugs in diabetes patients

might be the main reasons leading to no obvious differences. PA, one of the main sources of serum free fatty acid, was significantly increased in the diabetes patients and closely associated with DR[33]. In accordance with previous studies[6,7,34], the present results showed that PA could significantly induce retinal endothelial cell apoptosis. Fenofibrate, a peroxisome proliferator-activated receptor alpha agonist, could improve dyslipidemia. However, studies have confirmed that the improvement effects of fenofibrate on DR might not be due to its lipid-lowering effects, but mainly depended on its anti-inflammatory, anti-oxidant and anti-apoptosis functions[35]. This suggested that if there was a molecule against lipotoxicity-induced injury, it would have important values to delay the progression of retinopathy or other diabetic complications.

Previous studies showed that KL protein probably had an important role in vascular protection through the antagonism of lipotoxicity[12,13]. In clinical data, we found that the serum KL protein level was lower in the DR population and was an independent risk factor for DR. Therefore, we further observed the effects of KL protein through an in vitro cell experiment. The results showed that the administration of recombinant KL protein could evidently inhibit apoptosis induced by PA in HRECs, suggesting that KL protein might have a direct protective effect on DR.

The PI3K/Akt pathway plays an important role in regulating cell survival, and Akt is a pivotal effector. Studies reported that KL protein could activate the PI3K/Akt pathway to play protective roles. In the study of Zeldich et al.[19], administration of recombinant KL protein could inhibit the glutamate and amyloid-induced neuronal cell oxidative stress injury, whereas PI3K

inhibitors LY294002 significantly weakened the protective effect of KL protein. In the present study, our results showed that KL protein significantly reversed the decreased expression level of p-Akt induced by PA. Additionally, although KL protein attenuated apoptosis caused by PA in HRECs, the inhibitory effects of KL were obviously abolished by LY294002. This suggested that KL protein could inhibit HRECs damage induced by PA through activating the PI3K/Akt signaling pathway. Nevertheless, contrary to the study of Yamamoto *et al.*[36], the results showed that KL protein could activate the fork transcription factor and then enhance the resistance of cells to oxidative stress, but the mechanism was involved in inhibiting the insulin/insulin-like growth factor receptor 1/PI3K/Akt signaling pathway. Therefore, further experiments are necessary to elaborate the effects of KL protein on the Akt signaling pathway.

It should be pointed out that there were several limitations. Regarding clinical data, first, the sample size was relatively small in the present study. Second, the detailed history of medication, especially the use of angiotensin II receptor antagonist and insulin, should be obtained, and it would be better to carry out subgroup analyses. Regarding cell data, first, when exploring the mechanisms, we only discussed the PI3K/Akt signaling pathway, and other pathways were unclear. In addition to the PI3K inhibitor, the Akt inhibitor needed to be used in order to elaborate this signal pathway. Second, the concentration of KL protein was selected mainly according to the previous reports, and we examined the effects of KL protein only in *in vitro* experiments. Third, PA stimulation alone did not represent the state of diabetes. By all accounts, further large sample studies are required to exhaustively analyze the relationship between the serum KL level and DR. Further basic studies *in vivo* should be carried out to explore the protective roles and dose–effect of KL protein on DR.

In conclusion, the serum KL level was independently and negatively correlated with retinopathy in type 2 diabetes patients. Furthermore, KL treatment can inhibit apoptosis induced by PA in HRECs, and the potential mechanism might be involved in the activation of the PI3k/Akt signaling pathway. The data suggested that KL protein was likely to be a new and important target for DR.

ACKNOWLEDGMENTS

DISCLOSURE

The authors declare no conflict of interest.

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