Effects of Pentoxifylline on Soluble Klotho Concentrations and Renal Tubular Cell Expression in Diabetic Kidney Disease

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OBJECTIVE

The effect of pentoxifylline on Klotho levels in patients with type 2 diabetes mellitus with chronic kidney disease (CKD) was assessed in a post hoc analysis of the Pentoxifylline for Renoprotection in Diabetic Nephropathy (PREDIAN) trial.

RESEARCH DESIGN AND METHODS

Circulating and urinary tumor necrosis factor-α (TNF-α) and Klotho were measured before and after 1 year of pentoxifylline. The effect on Klotho expression was assessed in cultured renal tubular cells.

RESULTS

Pentoxifylline administration resulted in decreased serum and urinary TNF-α, whereas serum and urinary Klotho increased significantly. Changes in urinary Klotho, urinary TNF-α, and phosphorus were associated with changes in serum Klotho; changes in estimated glomerular filtration rate, urinary TNF-α, and albuminuria were related to urinary Klotho variation. In renal tubular cells, pentoxifylline prevented the decrease in Klotho expression induced by inflammatory cytokines or albumin.

CONCLUSIONS

Pentoxifylline increased Klotho levels in patients with diabetes with stage 3–4 CKD and prevented reduced Klotho expression in vitro. This beneficial effect may be related to anti-inflammatory and antialbuminuric activity.

Klotho is a transmembrane protein predominantly expressed in kidney tubular cells with a soluble form that functions as a humoral factor, with beneficial biological effects including antiaging and nephroprotective functions, as observed by its role in diabetic kidney disease (DKD) (1–6). Renal Klotho expression is markedly diminished in DKD (2), whereas serum Klotho decreases in patients with type 2 diabetes mellitus (T2DM) as nephropathy progresses (3).

The Pentoxifylline for Renoprotection in Diabetic Nephropathy (PREDIAN) trial (7) showed that pentoxifylline administration in patients with T2DM with stage 3–4 chronic kidney disease (CKD) results in a slower decrease in estimated glomerular filtration rate (eGFR) and a greater reduction of albuminuria. In this post hoc analysis, we investigated the effects of pentoxifylline on soluble Klotho levels. In addition, pentoxifylline regulation of Klotho expression was explored in proximal tubule epithelial cells exposed to albumin and inflammatory injury.

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RESEARCH DESIGN AND METHODS

Details of the PREDIAN trial have been previously published (7). Participants were randomly assigned to a pentoxifylline or control group, and the current results are after 1-year of follow-up. Tumor necrosis factor-α (TNF-α) was measured by immunoenzymatic ELISA and soluble Klotho by a solid phase sandwich ELISA (Immuno-Biological Laboratories, Takasaki, Japan). Urinary Klotho and TNF-α were normalized to urinary creatinine concentrations. All measurements were performed while blinded to participant characteristics and group assignment.

Mouse cortical tubule cells were cultured with the addition of 10 mg/mL BSA, 100 ng/mL recombinant human-soluble TNF-like weak inducer of apoptosis (TWEAK), or 30 ng/mL murine TNF-α after prior dose-response studies (8). Pentoxifylline was added 1 h before stimulation. Western blot used rabbit polyclonal anti-Klotho (1:500; Calbiochem, La Jolla, CA) as the primary antibody. One microgram RNA was reverse-transcribed, and real-time PCR was performed using the $2^{-\Delta\Delta Ct}$ method. Expression levels are expressed as ratios to GAPDH.

RESULTS

Of the 169 patients in the PREDIAN trial, 166 (85 control group, 81 pentoxifylline group) who completed 1-year follow-up were included in this analysis. Baseline eGFR was 37.4 ± 12.1 mL/min/1.73 m^2, and albuminuria 1,100 mg/day (640–1,800 mg/day); 68.3% of patients had stage 3 CKD. Serum and urinary Klotho were 295.9 pg/mL (223.4–406.5 pg/mL) and 54.1 ng/g (41.2–70.5 ng/g), respectively, and serum and urinary TNF-α were 15.2 pg/mL (11.9–17.4 pg/mL) and 16.1 ng/g (10.0–20.1 ng/g), respectively. Serum and urinary Klotho were higher in stage 3 than in stage 4 CKD (302.9 pg/mL [240–429.5 pg/mL] and 60.0 ng/g [44.9–88.7 ng/g] vs. 282.6 pg/mL [210.2–371.2 pg/mL] and 41.4 ng/g [30.1–52.2 ng/g]; $P = 0.13$ and < 0.001, respectively). Correlation analysis showed that serum Klotho was negatively associated with phosphorus ($r = -0.21$; $P < 0.01$), whereas urinary Klotho was directly related to eGFR ($r = 0.59$; $P < 0.01$) and inversely correlated with diabetes duration ($r = -0.17$; $P < 0.05$) and serum phosphorus ($r = -0.35$; $P < 0.01$). No correlation was observed between serum and urinary Klotho or between serum and urinary TNF-α.

After 1 year, the control group showed a mean reduction of 9.0% in eGFR ($P < 0.01$) and a 4.4% increase in albuminuria ($P < 0.01$), whereas in patients receiving pentoxifylline, eGFR decreased by 3.9% ($P < 0.01$) and albuminuria by 12.5% ($P < 0.001$) ($P < 0.01$ and < 0.001 between groups, respectively). Serum and urinary TNF-α did not change in the control group, but urinary TNF-α significantly decreased in the pentoxifylline group (Fig. 1*A*). Serum Klotho increased in patients receiving pentoxifylline (mean increment 5.9% vs. baseline [95% CI 2.4–9.4%]; $P < 0.05$), with a mean difference of 27.5 pg/mL between groups (95% CI 22–31.1 pg/mL; $P < 0.01$). Urinary Klotho decreased by 3.9% (−5.1 to −2.7%; $P < 0.01$) in the control group, whereas it increased by 9.3% (4.6–13.9%; $P < 0.001$) in the pentoxifylline group (mean difference 7.7 ng/g [5.9–9.4 ng/g]; $P < 0.001$ between groups) (Fig. 1*B*). Changes in urinary Klotho, urinary TNF-α, and phosphorus were independently associated with variations in serum Klotho (adjusted $R^2 = 0.44$; $P < 0.001$), whereas changes in eGFR, albuminuria, and urinary TNF-α were independently associated with urinary Klotho variations (adjusted $R^2 = 0.60$; $P < 0.0001$).

The effects of pentoxifylline on Klotho expression were analyzed in renal tubular cells. After exposure to albumin to mimic DKD, Klotho expression significantly decreased, which was abrogated by pentoxifylline. Pretreatment with pentoxifylline resulted in a dose-dependent increased expression of Klotho protein and mRNA (Fig. 1*C* and *D*). To mimic the inflammatory milieu of injured kidneys, tubular cells were exposed to TNF-α and TWEAK, cytokines with a proven ability to downregulate Klotho expression (8). Decreased Klotho expression induced by these cytokines was completely prevented by pentoxifylline (Fig. 1*E* and *F*).

CONCLUSIONS

This study shows that in patients with T2DM with stage 3–4 CKD, treatment with pentoxifylline leads to a significant increase in serum and urinary Klotho concentrations, with clinical findings supported by experimental data showing that pentoxifylline is able to prevent the downregulation of Klotho protein and mRNA expression induced by albumin, TNF-α, and TWEAK in renal tubular cells. The soluble form of Klotho functions as a humoral factor that regulates various signaling pathways with beneficial biological effects. The kidney is the main source of soluble Klotho (9), and Klotho production is severely reduced in human CKD (10). An early decrease in renal Klotho expression has been reported as a characteristic finding of DKD (2). In the current study, urinary Klotho levels were higher in participants with stage 3 CKD than in more advanced CKD and showed a direct correlation with eGFR. However, no association was found between serum and urinary Klotho, indicating that urinary Klotho is not the result of glomerular filtration. This finding is in accordance with studies that reported that soluble Klotho is not filtered across the glomerular barrier and that urinary Klotho is tubular in origin (11).

Only two previous clinical studies in patients with T2DM have analyzed the effect of therapies on soluble Klotho, specifically the effect of renin-angiotensin system blockade. Karalliedde et al. (12) reported that valsartan increased serum Klotho by 73.7%, whereas Lim et al. (13) observed that losartan enhanced circulating Klotho by 23%. In the current study, serum and urinary Klotho increased significantly by 5.9 and 9.3%, respectively. Of note, participants in previous studies had a preserved renal function (mean eGFR 92.3 mL/min/1.73 m^2), whereas eGFR in the current study was 37.4 ± 12.1 mL/min/1.73 m^2, reflecting an important reduction in renal functional mass, which can explain the lower percent increases of soluble Klotho. Finally, the current clinical study is the first to our knowledge of a therapeutic intervention on urinary Klotho.

Although the intimate mechanisms need additional investigation, pentoxifylline may positively regulate Klotho on the basis of its anti-inflammatory properties. Patients receiving pentoxifylline exhibited a decrease in serum and urinary TNF-α without correlation between these parameters, indicating that TNF-α is produced within the kidneys. Of note, changes in urinary TNF-α were negatively and independently associated with the variation in serum and urinary Klotho concentrations. Furthermore, our in vitro experiments showed that pentoxifylline prevented the TWEAK and TNF-α–induced Klotho downregulation; the reduction of urinary albumin excretion in pentoxifylline-treated patients could contribute to this effect because albuminuria causes tubular inflammation and renal injury (14) and directly reduces Klotho levels in tubular cells in vivo and in culture (15), as confirmed in the current study.

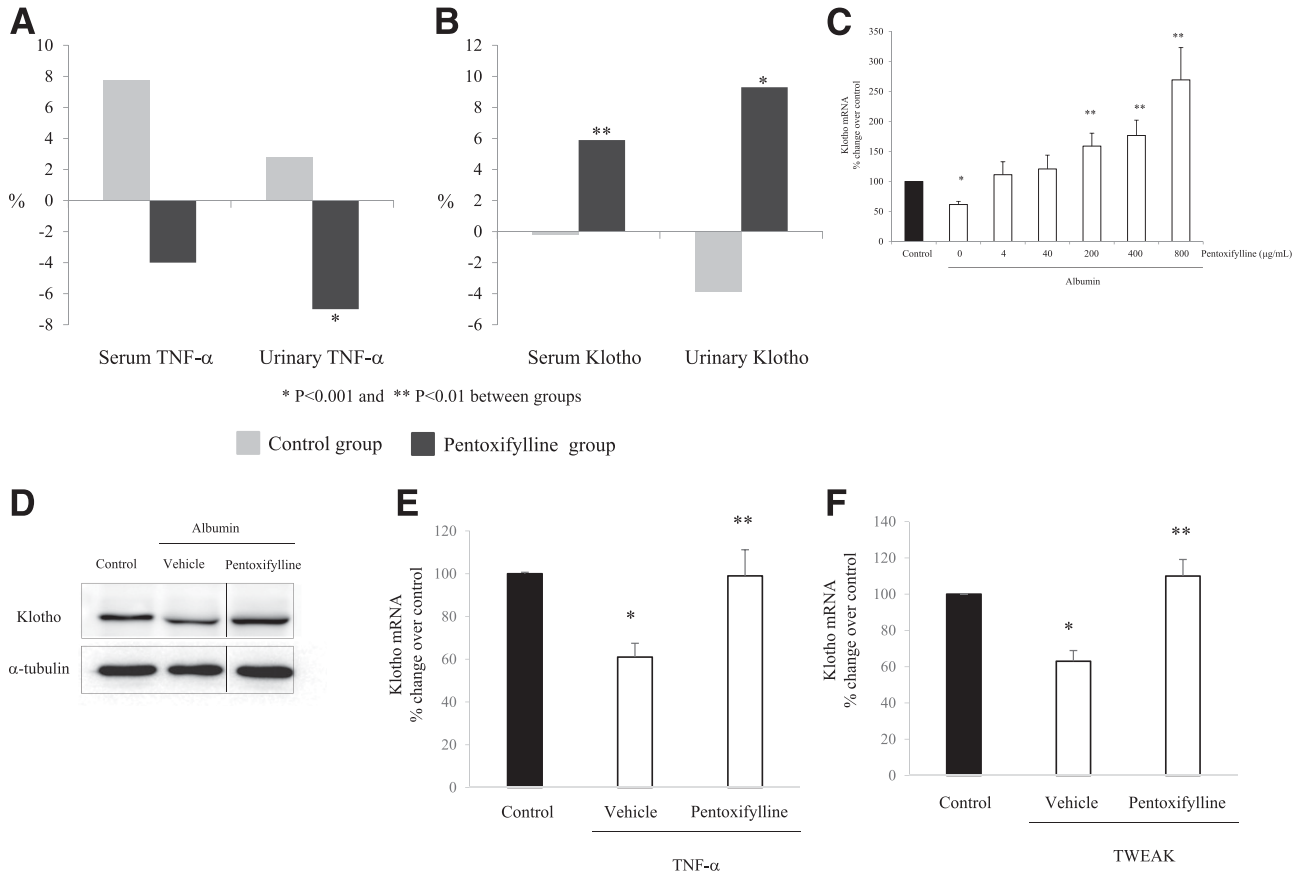

Figure 1—Clinical and in vitro effects of pentoxifylline. *A* and *B*: Mean percent changes of TNF-α and soluble Klotho with respect to baseline according to study group. *C–F*: Pentoxifylline prevents the decrease in Klotho expression in response to albumin and to the inflammatory cytokines TWEAK and TNF-α in vitro. *C*: Kidney tubular cells were stimulated with various concentrations of pentoxifylline for 1 h and then with 10 mg/mL albumin for 3 h. Klotho mRNA expression level was decreased after albumin stimulation alone but increased in cells pretreated with pentoxifylline. *$P < 0.002$ vs. control; **$P < 0.01$ vs. albumin alone. *D*: Cells were stimulated with 40 μg/mL pentoxifylline for 1 h and then with 10 mg/mL albumin for 3 h. Protein from whole-cell lysates was studied. A representative Western blot is shown. Noncontiguous lanes of the same gel have been spliced together. All the lanes were run on the same gel at the same time. This alteration is indicated in the figure by a dividing line between the lanes. The estimated size of the Klotho band is 131 kDa. *E* and *F*: Kidney tubular cells were stimulated with 40 μg/mL pentoxifylline for 1 h and with cytokines (30 ng/mL TNF-α and 100 ng/mL TWEAK) for 3 or 6 h. Inflammatory cytokines decrease Klotho mRNA levels, and this was prevented by pentoxifylline pretreatment. *$P < 0.005$ vs. control; **$P < 0.05$ vs. cytokine alone.

In conclusion, pentoxifylline administration to patients with T2DM with stage 3–4 CKD increased serum and urinary Klotho levels, which may be related to anti-inflammatory and antialbuminuric effects. Given the nephroprotective and antiaging effects of Klotho, further characterization of the Klotho-preserving effect of pentoxifylline may contribute to the design of novel therapeutic strategies aimed at preserving Klotho expression to improve kidney and survival outcomes in CKD.

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