Thyroid hormone ameliorates diabetic nephropathy in a mouse model of type II diabetes

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Abstract

Conventional therapies for diabetic patients, such as strict glycemic control, do not completely stop the progression of diabetic nephropathy. Serum-free tri-iodothyronine (T_3) levels were lower in patients with type II diabetes. The purpose of this study was to test a hypothesis that treatment with T_3 would improve diabetic nephropathy in db/db mice, a model of type II diabetes. Male db/db mice (16 weeks) were treated with T_3 for 4 weeks. Urinary excretions of albumin and blood glucose levels were measured. Kidneys were collected for histological examination and molecular assays of transforming growth factor- $\beta 1$ (TGF- $\beta 1$) expression and phosphatidylinositol 3-kinase (PI3K). T_3 attenuated albuminuria in db/db mice, suggesting an improved kidney function. T_3 significantly decreased accumulation of

collagenous components in cortical interstitium (interstitial fibrosis) and expansion of mesangial matrix in glomeruli (glomerulosclerosis) and prevented the loss of glomeruli in db/db mice. Therefore, T_3 improved the renal structural damage seen in diabetic mice. Notably, diabetic nephropathy was accompanied by a significant decrease in PI3K activity and an increase in TGF- $\beta 1$ expression in kidneys. T_3 restored renal PI3K activity, attenuated hyperglycemia, and decreased renal TGF- $\beta 1$ expression in db/db mice. These effects of T_3 were abolished by simultaneous treatment with PI3K inhibitor (LY294002). These data suggest that T_3 prevented progressive kidney damage and remodeling in db/db mice by improving insulin signaling (e.g. PI3K activity).

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Introduction

Diabetic nephropathy is now the most common cause of end-stage renal disease (ESRD), which significantly contributes to the high mortality in type II diabetic patients (Ruggenenti & Remuzzi 1998). It is projected that 30–40% of patients with type I diabetes and 5–10% of patients with type II diabetes eventually develop ESRD (Ruggenenti & Remuzzi 1998, Lin & Sun 2010a). Conventional therapies such as strict glycemic control and antihypertensive treatment do not completely stop the progression of diabetic nephropathy in diabetic patients (Ruggenenti & Remuzzi 1998).

The db/db mouse is a useful animal model of type II diabetes with a point mutation in leptin receptor gene, which develops hyperglycemia, insulin resistance in peripheral tissues, and obesity by 8 weeks of age (Breyer *et al.* 2005). The db/db mice exhibit renal glomerular mesangial matrix expansion, increases in renal collagen and serum creatinine, albuminuria, and a decrease in glomerular filtration rate (GFR), which are comparable to advanced human diabetic nephropathy with renal insufficiency (Sharma *et al.* 2003).

Glomerular mesangial matrix expansion has been considered the essential characteristic of diabetic nephropathy in human (Sharma *et al.* 2003).

Hypothyroidism is accompanied by a decrease in GFR and renal blood flow (Iglesias & Diez 2009). The low level of tri-iodothyronine (T_3) is associated with a survival disadvantage of chronic kidney disease (Iglesias & Diez 2009). Type II diabetic patients with subclinical hypothyroidism are associated with an increased risk of diabetic nephropathy (Chen *et al.* 2007). In contrast, hyperthyroidism increases GFR and renal blood flow (Iglesias & Diez 2009).

Clinical studies indicated that serum-free T_3 level was about 47% lower in patients with type II diabetes compared with non-diabetic patients (Islam *et al.* 2008). There were no significant differences in serum levels of free thyroxine (T_4) and TSH between the control and the study subjects (Islam *et al.* 2008). The serum level of T_4 was lower in db/db mice and the tissue level of T_3 was lower in liver and brain due to impaired deiodination in these tissues of db/db mice (Kaplan & Young 1987). The purpose of this study was to test our hypothesis that chronic administration of T_3 would attenuate diabetic nephropathy in db/db mice.

Materials and Methods

Animals

BKS.Cg- $+Lepr^{db}/+Lepr^{db}/OlaHsd$ mice (db/db mice) and BKS.Cg- $m+/+Lepr^{db}/OlaHsd$ (lean mice) (all males, 12 weeks) were purchased from Harlan (Indianapolis, IN, USA). All mice were housed at room temperature ($25 \pm 1^\circ\text{C}$) and were provided with Purina laboratory chow (No. 5001) and tap water which was made available *ad libitum*. This study was approved by the Institutional Animal Care and Use Committee at the University of Oklahoma Health Sciences Center.

Experimental protocol

The kidneys used for this study were from the same groups of animals used in our previous publication on the effects of T_3 on diabetes in db/db mice (Lin & Sun 2010b). Briefly, three groups of db/db and three groups of lean mice were used (all 12 weeks, five mice/group). Following a 4-week control period, three groups of each strain (16 weeks) received i.p. injections of vehicles (35% DMSO and PBS), T_3 (7 ng/g b.w. in PBS, Sigma), and LY294002 (3 $\mu\text{g/g}$ dissolved in 35% DMSO, Sigma) followed by T_3 respectively. The same doses of T_3 and LY294002 were given twice daily (0900 and 1700 h) for 18 days (14 ng/g per day for T_3 and 6 $\mu\text{g/g}$ per day for LY294002). LY294002 was given 20 min before injection of T_3 . Animals were further treated with the increased dose of T_3 (28 ng/g per day) and LY294002 (9 $\mu\text{g/g}$ per day) for another 10 days. A group treated with LY294002 alone was not included because the db/db mice died within 2–3 days after treatments, probably due to severe hyperglycemia. At 4 weeks prior to the treatment and during week 4 of the treatment, 24-h urine samples were collected for measurements of urinary albumin.

At the end of week 4 of the treatments with T_3 , animals were killed and blood was collected in EDTA for measuring blood glucose and plasma T_3 . Blood glucose was measured using a *Reli On Ultima* glucose reader (Solartek Products, Inc., Alameda, CA, USA). Plasma T_3 was measured using a T_3 ELISA kit (Alpha Diagnostic International, San Antonio, TX, USA). Animals were then perfused transcardially using heparinized saline. Following perfusion, kidneys were isolated and weighed. The left kidney from each mouse was fixed with 4% paraformaldehyde in PBS for 24 h. The kidneys were set in a plane perpendicular to the long axis and were embedded in paraffin for histological and immunohistochemical analysis.

Measurements of albumin

Urinary albumin excretion was measured with a mouse-specific microalbuminuria ELISA kit (Albuwell M; Exocell, Philadelphia, PA, USA) according to the manufacturer's instruction.

Morphological investigations

A series of cross sections of the left kidney (3–5 μm) were cut at the vascular poles. Tissue sections (four to five specimens per group) were stained with periodic acid Schiff (PAS), Masson's trichrome, or H&E.

For sections stained with Masson's trichrome (5 μm thickness), images of cortex from three consecutive sections for each animal were collected at equal exposure conditions under Nikon Eclipse Ti microscopy (magnification $\times 100$). The fraction area for collagenous components in cortex was obtained with NIS-Elements BR 3.0 (Nikon, Melville, NY, USA). Images of sections for each animal were also collected at equal exposure conditions under the microscopy at the magnification of 400.

For sections stained with PAS (3 μm), images of 20 glomeruli for each animal were collected at equal exposure conditions and at the magnification of 400 under a microscopy (Nikon Eclipse Ti). Mesangial matrix area was defined by

Figure 1 Treatments with T_3 decreased albuminuria in db/db mice. T_3 decreased urinary albumin excretion in db/db mice (A). T_3 increased kidney mass in lean and db/db mice (B). Data = mean $\pm$ S.E.M. $n=5$. * $P<0.05$, *** $P<0.001$ versus lean-DMSO-PBS group; +++ $P<0.001$ versus db/db-DMSO-PBS; ### $P<0.001$ versus db/db-DMSO- T_3 .

PAS-positive and nuclei-free area in the mesangium. The glomerular area was defined by tracing along the borders of the capillary loop. Relative mesangial area (defined as fraction area of mesangial matrix area over glomerular area) was obtained using Image J (NIH freeware, Bethesda, MD, USA).

For sections stained with H&E (5 μ m), the number of glomeruli was quantified using Nikon Eclipse Ti microscopy at a magnification of 100. Briefly, the kidneys were sectioned in a plane perpendicular to the long axis, beginning at the vascular poles. The kidneys were cut at 250 μ m intervals. The number of glomeruli was quantified in three randomly chosen fields in each section and averaged based on five consecutive sections for each animal. The area (mm²) of each field was measured. Images of cortex for each animal were also collected at equal exposure conditions at a magnification of 400.

Phosphatidylinositol 3-kinase activity

Lysates of mouse kidney were immunoprecipitated with antibody against phosphatidylinositol 3-kinase (PI3K; Millipore, Temecula, CA, USA). PI3K activity in the immunocomplexes was determined using a PI3K ELISA kit (Echelon Biosciences, Salt Lake City, UT, USA).

Western blotting

Lysates of mouse kidney under reduced conditions were directly subjected to SDS-PAGE followed by western blotting with an antibody against transforming growth factor- β 1 (TGF- β 1; Santa Cruz Biotechnology, Santa Cruz, CA, USA, TGF- β 1 and its precursor) and then with an antibody against β -actin (Santa Cruz) after stripping the blot.

Statistical analysis

Data were analyzed using a two-way ANOVA (strain and treatments). The Newman–Keuls procedure was used to assess differences between means. Data were expressed as mean $\pm$ S.E.M. A value with $P < 0.05$ was considered significant.

Results

T₃ decreased urinary albumin excretion in diabetic mice

At the age of 12 weeks, the db/db mice had a significantly higher level of urinary albumin than that of the lean mice (Fig. 1A). No significant difference of urinary albumin excretion was found between any two groups of db/db mice or lean mice at the age of 12 weeks (Fig. 1A). Albuminuria of the db/db mice at the age of 20 weeks was significantly increased compared with that at the age of 12 weeks (Fig. 1A). Treatment with T₃ significantly decreased albuminuria in db/db mice (Fig. 1A). Treatment with PI3K inhibitor (LY294002) abolished the beneficial effect of T₃ on albuminuria in db/db mice (Fig. 1A), suggesting that this effect may be mediated by PI3K.

There was a significant decrease in kidney mass in the db/db-DMSO-PBS group (control) versus the lean-DMSO-PBS group at the age of 20 weeks (Fig. 1B). Treatment with T₃ significantly increased the kidney mass in lean mice (Fig. 1B). Body weight was significantly greater in the db/db-DMSO-PBS group than in the lean groups (Lin & Sun 2010b). Treatment with T₃ did not affect body weights in either lean or db/db mice (Lin & Sun 2010b).

Figure 2 Treatment with T₃ attenuated collagen deposition in renal cortex of db/db mice. Representative photomicrographs of Masson's trichrome-stained kidney sections with original magnification $\times 400$ (A). Arrows indicate trichrome-positive collagenous components in cortical interstitium (blue). Kidney sections were cut at their vascular poles. Quantitative analysis of collagenous components in renal cortex (B). Images of cortex from three consecutive sections for each animal, collected at equal exposure conditions under Nikon Eclipse Ti microscopy ($\times 100$), were used in the analysis. Data = mean $\pm$ S.E.M. $n = 4-5$. ** $P < 0.01$ versus the lean-DMSO-PBS; + $P < 0.05$ versus the db/db-DMSO-PBS group; ## $P < 0.01$ versus the db/db-DMSO-T₃ group.

T_3 attenuated collagen accumulation in kidney cortex in diabetic mice

The Masson trichrome staining showed that the 20-week-old db/db mice had significantly higher levels of collagenous components in cortical interstitium compared with those of the lean mice (Fig. 2A and B). Treatments with T_3 significantly decreased collagenous components in db/db mice (Fig. 2A and B). PI3K inhibitor abolished the beneficial effect of T_3 (Fig. 2A and B). These data indicated that T_3 prevented interstitial fibrosis in db/db mice and that PI3K was important in mediating this beneficial effect in db/db mice.

T_3 decreased glomerular mesangial matrix expansion in diabetic mice

The db/db mice showed significant glomerular mesangial matrix expansion compared with the lean mice (Fig. 3A and B), indicating glomerular remodeling. Treatments with T_3 abolished glomerulosclerosis in db/db mice (Fig. 3A and B). PI3K inhibitor partially blocked the effect of T_3 on the mesangial matrix expansion.

T_3 attenuated kidney damage in diabetic mice

The db/db mice showed renal tubule atrophy, some lost typical structures of cortical tubules (Fig. 4A), suggesting renal remodeling in diabetic mice. T_3 restored tubular structures (Fig. 4A). The number of glomeruli was significantly decreased in the db/db-DMSO-PBS group compared with the lean-DMSO-PBS groups (Fig. 4B), suggesting a loss of glomeruli at the age of 20 weeks. The glomeruli were replaced

by massive cellular infiltrates in the diabetic mice. T_3 prevented the loss of glomeruli in the db/db mice (Fig. 4B). These beneficial effects of T_3 may be mediated by PI3K because they could be abolished by the PI3K inhibitor (Fig. 4B).

T_3 increased PI3K activities in kidneys and attenuated hyperglycemia in diabetic mice

The PI3K activity (PIP₃ products) was significantly lower in kidneys of the db/db-DMSO-PBS mice than that in the lean mice (Fig. 5). T_3 significantly increased the PI3K activity in kidneys of both db/db and lean mice (Fig. 5). The PI3K inhibitor abolished the stimulating effects of T_3 on PI3K activities.

The blood glucose level was significantly higher in the db/db-DMSO-PBS mice than in the lean mice (Lin & Sun 2010b). T_3 (14 ng/g per day) significantly attenuated the blood glucose level in the db/db mice but not in the lean mice. An increase in the dose of T_3 (28 ng/g per day) further attenuated hyperglycemia (Lin & Sun 2010b). The PI3K inhibitor abolished the anti-hyperglycemic effects of T_3 (Lin & Sun 2010b).

The basal level of plasma T_3 in db/db mice (61.7 ± 5.2 ng/dl) was significantly lower than that of the lean mice (86.5 ± 7.2 ng/dl). Daily administration of T_3 (28 ng/g per day) for 7 days increased the plasma levels of T_3 by 2.7-folds in db/db mice (Lin & Sun 2010b). As we reported recently (Lin & Sun 2010b), the basal level of plasma insulin was not significantly different between the lean and the db/db mice, although the latter had severe hyperglycemia. Treatment with T_3 significantly increased the plasma level of insulin (Lin & Sun 2010b).

Figure 3 Treatment with T_3 prevented mesangial matrix expansion in db/db mice. Representative photomicrographs of PAS-stained kidney sections with original magnification of $\times 400$ (A). Arrows indicate PAS-positive mesangial material (pink). Quantitative measurements of mesangial matrix expansion (B). Relative mesangial matrix area is expressed as PAS-positive mesangial matrix per total glomerular tuft cross-sectional area. Kidney sections were cut at their vascular poles. An average value was obtained from analyses of 20 glomeruli per mouse. Data = mean $\pm$ S.E.M. $n=4-5$. *** $P<0.001$ versus the lean-DMSO-PBS group; +++ $P<0.001$ versus the db/db-DMSO-PBS group; ### $P<0.001$ versus the db/db-DMSO-T₃ group.

Figure 4 Treatments with T₃ prevented cellular infiltration in cortical tubules and glomeruli and restored the number of glomeruli in kidney in db/db mice. Representative photomicrographs of H&E-stained kidney cortical sections showing typical structures of cortical glomeruli and tubules with original magnification of $\times 400$ (A). Arrows indicate cellular infiltration. The average number of glomeruli was obtained from three groups of identical 5 μ m cross sections separated by the 250 μ m intervals (B). Data = mean $\pm$ S.E.M. $n=4-5$. *** $P<0.001$ versus the lean-DMSO-PBS group; +++ $P<0.001$ versus the db/db-DMSO-PBS group; ### $P<0.001$ versus the db/db-DMSO-T₃ group.

T₃ decreased TGF- β 1 expression in kidneys of diabetic mice

The basal level of TGF- β 1 (precursor) expression was barely detectable in lean mice (Fig. 6). TGF- β 1 expression was significantly increased in the db/db-DMSO-PBS mice compared with the lean mice (Fig. 6). T₃ significantly decreased TGF- β 1 expression (Fig. 6). Notably, inhibition of PI3K not only abolished the effect of T₃ on TGF- β 1 expression but also significantly increased the TGF- β 1 level above that of the db/db-DMSO-PBS group.

Discussion

Renal fibrosis, including tubulointerstitial and glomerular fibrosis, is an important feature of diabetic nephropathy (Brosius 2008). Interstitial fibrosis seems to contribute to the deterioration of renal function, especially during the late stage of diabetic nephropathy (Brosius 2008). Diabetic glomerular

fibrosis is mainly due to accumulation of extracellular matrix proteins such as collagen and fibronectin in the mesangial interstitial space, which results in progressive diabetic nephropathy by reducing the surface area of glomerular capillaries for filtration (Steffes *et al.* 1989, Brosius 2008). This study showed that there was renal structural remodeling as evidenced by a loss of typical structures of renal tubules and glomeruli in db/db mice (Figs 2 and 4). The loss of glomeruli in db/db mice is surprising as it has not been observed in human and other experimental models of type II diabetes. The loss of glomeruli and cellular infiltration in the late stage of diabetes may be due to hyperglycemia-related vascular damages (Lehmann & Schleicher 2000, Koya *et al.* 2003). It is known that long-term hyperglycemia causes vascular oxidative stress resulting in vascular damage and cellular infiltration (Suzuki & Miyata 1999, Lehmann & Schleicher 2000, Koya *et al.* 2003), which may be eventually replaced by fibrosis (Lehmann & Schleicher 2000). Interestingly, treatments with T₃ prevented accumulation of collagenous components in cortical interstitium and glomerular mesangial matrix expansion in diabetic mice (Figs 2 and 3).

TGF- β has been shown to be linked to renal fibrosis in diabetic nephropathy in animals and humans (Lehmann & Schleicher 2000, Zhu *et al.* 2007, Chiarelli *et al.* 2009). Suppression of TGF- β 1 inhibited hyperglycemia-induced collagen synthesis and prevented glomerular fibrosis and renal insufficiency in db/db mice (Lehmann & Schleicher 2000, Ziyadeh *et al.* 2000). This study revealed that T₃ significantly attenuated the increase in TGF- β 1 expression in kidneys of db/db mice (Fig. 6). This effect may be mediated by an increase in PI3K activity because inhibition of PI3K abolished

Figure 5 Treatment with T₃ enhanced the activities of PI3K in kidneys of db/db mice. PI3K activity in kidneys was measured with a kit after tissue lysates were immunoprecipitated with antibody against PI3K. Data were normalized to the amount of protein used in the immunoprecipitation and the time for enzyme reaction. Data = mean $\pm$ S.E.M. $n=5$. *** $P<0.001$ versus the lean-DMSO-PBS group; ^^^ $P<0.001$ versus the lean-DMSO-T₃ group; +++ $P<0.001$ versus the db/db-DMSO-PBS group; ### $P<0.001$ versus the db/db-DMSO-T₃ group.

Figure 6 Treatment with T₃ decreased TGF-β1 expression in kidneys of db/db mice. Representative western blotting of TGF-β1 and β-actin (A). Quantification of TGF-β1 expression (B). TGF-β1 was normalized with β-actin, which was then calculated as fold changes of the control (lean-DMSO-PBS) group. Data = mean ± S.E.M. *n* = 5. ***P* < 0.01 versus the lean-DMSO-PBS group; +*P* < 0.05, +++*P* < 0.001 versus the db/db-DMSO-PBS; ###*P* < 0.001 versus the db/db-DMSO-T₃ group.

the attenuating effect of T₃ on the up-regulation of TGF-β1 in diabetic kidneys (Fig. 6). This finding further suggested that PI3K may be involved in the up-regulation of TGF-β1 in diabetic nephropathy. Further studies are required to determine whether PI3K regulates TGF-β1 via its direct action on TGF-β1 or through its effect on glycemia in diabetic kidneys. Therefore, the beneficial effects of T₃ on diabetic nephropathy may be mediated, at least in part, by its attenuating effect on TGF-β1 expression. These findings also support a notion that thyroid hormone is an important regulator of renal structure in diabetic mice.

Although renal hypertrophy is found in the early stage of diabetes (Sharma *et al.* 2003), this study showed that kidney mass did not remain increased in diabetic mice at the age of 20 weeks (Fig. 1B). This finding is consistent with a report that renal hypertrophy disappears in db/db mice at the age of 21 and 25 weeks (Koya *et al.* 2000). The decrease in kidney mass is probably due to the loss of glomeruli and tubules and structural remodeling at a late stage of diabetes.

The db/db mice showed progressive renal functional damage as evidenced by a significant increase in urinary excretion of albumin (Fig. 1A), which is consistent with the

published data (Ziyadeh *et al.* 2000, Cohen *et al.* 2001). Notably, treatments with T₃ markedly ameliorated renal functional damage. Plasma T₃ levels were significantly decreased in db/db mice (Lin & Sun 2010b). It was reported that female db/db mice aged 7 weeks have lower T₄ levels in serum and lower T₃ levels in the liver and brain (Kaplan & Young 1987). An epidemiological study indicated that type II diabetic patients with subclinical hypothyroidism are associated with an increased risk of diabetic nephropathy (Chen *et al.* 2007). The GFR in hypothyroid patients is approximately one-third lower than that of the euthyroid individuals (Singer 2001). Suher *et al.* (2005) showed that subjects with hypothyroidism are associated with increased urinary albumin excretion rate and decreased creatinine clearance. In corroboration with our findings, T₃ was reported to have beneficial effects on renal function in a diabetic patient with end-stage renal failure and severe hypothyroidism (van Welsem & Lobatto 2007). Renal function was improved by T₃ in 32 patients with hypothyroidism (den Hollander *et al.* 2005). Therefore, treatments with thyroid hormone may be an effective therapeutic approach for delaying progressive renal damage or even renal failure in diabetic patients with hypothyroidism or subclinical hypothyroidism.

Given that hyperglycemia may be involved in the development of diabetic nephropathy (Brosius 2008) and that T₃ decreased blood glucose levels in db/db mice (Fig. 5A and B), the anti-hyperglycemic effect of T₃ may contribute to its protective effects on diabetic nephropathy. On the other hand, strict glycemic control does not completely stop the progression of diabetic nephropathy in diabetic patients (Ruggenenti & Remuzzi 1998). It is noted that T₃ restored the decreased activity of PI3K in diabetic kidneys (Fig. 5C). The present data suggest that T₃ ameliorated diabetic nephropathy in db/db mice, probably by increasing PI3K activity because inhibition of PI3K activity abolished the beneficial effect of T₃. Therefore, the beneficial effects of T₃ on diabetic nephropathy may be mediated by the increased insulin synthesis and release, enhanced insulin signaling, and improved insulin resistance by T₃ (Lin & Sun 2010b). The limitation of this study is that it cannot determine the relative importance of the increased renal PI3K activity and the attenuated hyperglycemia in the beneficial effect of T₃ on diabetic nephropathy. A further study is needed to address this important question by controlling glucose levels in all groups.

The PI3K/AKT signaling was activated in renal cortex of db/db mice in the early phase of diabetes (at ages of 6–8 weeks) likely due to an increase in insulin receptor activities (Feliers *et al.* 2001). Activation of the PI3K/AKT signaling has been considered an important factor for diabetic renal hypertrophy, which contributes to diabetic nephropathy (Price 2007). On the other hand, the PI3K/AKT signaling was found to be lower in glomeruli of 12-week-old db/db mice, which has been linked to the death of podocytes of db/db mice (Tejada *et al.* 2008). Most recent data indicated that the down-regulation of PI3K/AKT signaling contributes

to renal tubular apoptosis in kidneys of streptozotocin-induced diabetic mice and apoptosis in renal proximal tubular cells (Rane *et al.* 2010). Apoptosis has been considered as an initiator of diabetic nephropathy (Kumar *et al.* 2004). Therefore, the PI3K/AKT signaling may play different roles at different stages of diabetic nephropathy.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Z S conceived and designed the experiment and wrote the paper. Y L designed and performed the experiments, analyzed the data, and wrote the paper.

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