G Protein-Coupled Bile Acid Receptor TGR5 Activation Inhibits Kidney Disease in Obesity and Diabetes

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ABSTRACT

Obesity and diabetes mellitus are the leading causes of renal disease. In this study, we determined the regulation and role of the G protein-coupled bile acid receptor TGR5, previously shown to be regulated by high glucose and/or fatty acids, in obesity-related glomerulopathy (ORG) and diabetic nephropathy (DN). Treatment of diabetic db/db mice with the selective TGR5 agonist INT-777 decreased proteinuria, podocyte injury, mesangial expansion, fibrosis, and CD68 macrophage infiltration in the kidney. INT-777 also induced renal expression of master regulators of mitochondrial biogenesis, inhibitors of oxidative stress, and inducers of fatty acid β-oxidation, including sirtuin 1 (SIRT1), sirtuin 3 (SIRT3), and Nrf-1. Increased activity of SIRT3 was evidenced by normalization of the increased acetylation of mitochondrial superoxide dismutase 2 (SOD2) and isocitrate dehydrogenase 2 (IDH2) observed in untreated db/db mice. Accordingly, INT-777 decreased mitochondrial H2O2 generation and increased the activity of SOD2, which associated with decreased urinary levels of H2O2 and thiobarbituric acid reactive substances. Furthermore, INT-777 decreased renal lipid accumulation. INT-777 also prevented kidney disease in mice with diet-induced obesity. In human podocytes cultured with high glucose, INT-777 induced mitochondrial biogenesis, decreased oxidative stress, and increased fatty acid β-oxidation. Compared with normal kidney biopsy specimens, kidney specimens from patients with established ORG or DN expressed significantly less TGR5 mRNA, and levels inversely correlated with disease progression. Our results indicate that TGR5 activation induces mitochondrial biogenesis and prevents renal oxidative stress and lipid accumulation, establishing a role for TGR5 in inhibiting kidney disease in obesity and diabetes.

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Obesity and diabetes mellitus are the leading causes of renal disease.1–5 The pathogenesis of obesity and diabetes associated renal disease is multifactorial.6–14 In spite of all the beneficial interventions implemented in diabetic patients, including tight glucose and blood pressure control, renal disease still progresses in most patients.15,16 Additional treatments able to control pathogenic pathways involved in obesity and diabetic nephropathy (DN) are required.

Bile acids (BAs) act as signaling molecules that activate BA receptors which regulate BA homeostasis,
as well as glucose homeostasis, lipid homeostasis, and energy expenditure. Two major receptors for BA have been identified: the nuclear receptor farnesoid X receptor (FXR), and the membrane-bound, G protein-coupled receptor TGR5. Expression and function of TGR5 are distinct from expression and function of FXR, although in some cases they are complementary.\textsuperscript{17,18} While the effects of TGR5 in the muscle, brown adipose tissue, immune cells and enteroendocrine cells have been studied,\textsuperscript{19–26} the role of TGR5 in the kidney is not known.

TGR5 (GPBAR1 or GPR131) has been identified as a membrane BA-activated G protein-coupled receptor (GPCR, class A).\textsuperscript{19,20} TGR5 is a member of the rhodopsin-like subfamily of GPCRs (Class A), TGR5 mRNA is expressed in the gall bladder, kidney, brown adipose tissue, liver, intestine, and selected areas of the central nervous system. TGR5 is activated by several BAs, lithocholic acid being the most potent natural agonist with an EC\textsubscript{50} of 530 nM.\textsuperscript{17,18}

Upon ligand binding, TGR5 activation leads to the release of the Go\textsubscript{a} subunit and activation of adenylate cyclase. The increase in intracellular concentration of cyclic AMP activates protein kinase A (PKA), which phosphorylates cAMP-response element binding protein (CREB). Activated CREB transactivates its target genes by binding to cAMP-response elements (CREs) contained in their promoter.\textsuperscript{17,18}

In brown adipose tissue and muscle, BA-induced increases in cAMP lead to the activation of type 2 iodothyronine deiodinase (D2), which results in increased intracellular production of thyroid hormone. Activation of the thyroid receptor (TR) increases mitochondrial oxidative phosphorylation in muscle and uncoupling in brown adipose tissue, resulting in enhanced energy expenditure.\textsuperscript{24,27} TGR5 also increases PGC-1\textalpha expression, which is a master regulator of mitochondrial biogenesis and metabolism.\textsuperscript{24} In enteroendocrine cells, activation of TGR5 results in enhanced glucagon-like peptide 1 (GLP1) release, which induces insulin secretion and improves glucose homeostasis.\textsuperscript{17,22,23}

TGR5 activation also has immunomodulatory effects, in particular on macrophage function. TGR5 agonists inhibit NF-\kappaB, suppressing LPS-induced increases in IL-1\textalpha, IL-1\beta, IL-6, and TNF-\alpha production.\textsuperscript{19,25} By reducing the inflammatory response and lipid loading in macrophages, TGR5 activation inhibits atherosclerosis.\textsuperscript{26,28}

The purpose of the present study was to determine the expression and protective role of TGR5 in obesity-related glomerulopathy (ORG) and DN. Our studies indicate a novel role for TGR5 activation in inducing energy metabolism, mitochondrial biogenesis, and fatty acid oxidation in the kidney by activating AMPK, SIRT1, PGC-1\textalpha, ERR\alpha, and SIRT3, which lead to prevention of oxidative stress and lipid accumulation, thus firmly establishing an important role for TGR5 in preventing kidney disease in obesity and diabetes.

RESULTS

Treatment of Diabetic Mice with the TGR5-Specific Agonist INT-777 Prevents DN

TGR5 mRNA expression is decreased in diabetic mice (Figure 1A). To explore the role of TGR5 signaling in diabetic kidney disease, we treated db/db mice with TGR5 agonist INT-777. INT-777 decreased urinary albumin excretion (Figure 1B), glomerular mesangial expansion (Figure 1C), accumulation of extracellular matrix proteins (Figure 1D), prevented glomerular podocyte injury and loss (Figure 1, E–G), and decreased macrophage accumulation in the kidney (Figure 1H). Thus, INT-777 prevented all of the major phenotypic characteristics of DN. These beneficial effects are independent of any alterations in plasma glucose, cholesterol, or arterial systolic blood pressure, but were associated with a marked decrease and normalization of plasma triglycerides (Table 1).

Treatment of Diabetic Mice with INT-777 Increases Renal Mitochondrial Biogenesis, Decreases Oxidative Stress, and Increases Fatty Acid \(\beta\)-Oxidation

INT-777 induces significant increases in mRNA and/or protein abundance of (1) phospho-AMP kinase (p-AMPK) and phospho-ACC (Figure 2, A and B), (2) SIRT1 (Figure 2C), (3) PPAR\gamma coactivator-1\alpha (PGC-1\alpha) (Figure 2D), (4) estrogen related receptor-\alpha (ERR\alpha) (Figure 2E), (5) SIRT3 (Figure 2F), and (6) nuclear respiratory factor 1 (Nrf-1) (Figure 2G). These are important mediators of mitochondrial biogenesis, oxidative stress, and fatty acid \(\beta\)-oxidation. SIRT3 activity is increased following treatment with INT-777, as manifested by the decreased acetylation of SIRT3 targets SOD2 (Figure 2H) or IDH2 (Figure 2I).

INT-777 (1) decreases mitochondrial ROS production (Figure 3A), and (2) increases total superoxide dismutase (SOD) (Figure 3B) and mitochondrial SOD2 (Figure 3C) activity. This is associated with significant decreases in urinary thiobarbituric acid reactive substances (TBARS) (Figure 3D) and urinary \(\text{H}_2\text{O}_2\) excretion (Figure 3E), which are indicative of a decrease in overall renal oxidative stress.

INT-777 also (1) decreases DGAT1 (Figure 4A), an enzyme critical for triglyceride synthesis, (2) increases PPAR-\alpha (Figure 4B), a nuclear receptor critical for fatty acid oxidation, (3) increases uncoupling protein-2 (Figure 4C), and (4) increases carnitine palmitoyltransferase 1b (Figure 4D), which mediates fatty acid \(\beta\)-oxidation, resulting in decreased (5) renal neutral lipid accumulation (Figure 4E). These results are consistent with the capacity of INT-777 to reduce plasma triglycerides in diabetic mice (Table 1).

Treatment of Mice with Diet-Induced Obesity with INT-777 Prevents Obesity-Associated Nephropathy

TGR5 mRNA expression is also decreased in the kidneys of DIO mice (Figure 5A). INT-777 treatment of DIO mice decreased urinary albumin (Figure 5B), podocyte injury (Figure 5C), extracellular matrix proteins fibronectin and type IV collagen accumulation (Figure 5, D and E), and profibrotic growth factors TGF-\beta, CTGF, and PAI-1 (Figure 5F), CD68 macrophages...
Figure 1. TGR5 specific agonist INT-777 prevents DN in db/db mice. (A) TGR5 mRNA expression is decreased in the kidneys of mice with diabetes. (B) Urine albumin excretion is decreased with the treatment of INT-777 in db/db mice. (C) Periodic acid Schiff staining indicates marked mesangial expansion in db/db mice and treatment with INT-777 results in a significant decrease in mesangial expansion. (D) Fibronectin immunofluorescence microscopy indicates marked accumulation of glomerular matrix in db/db mice and treatment with INT-777 results in a significant decrease in...
(Figure 5G), proinflammatory cytokine and receptors MCP-1, TLR2, and TLR4 (Figure 5H). INT-777 also decreased neutral lipid accumulation as determined by label-free coherent anti-Stokes Raman (CARS) microscopy (Figure 5I). INT-777 induced significant increases in p-AMPK (Figure 5J), PGC-1α (Figure 5K), and SIRT3 protein abundance as determined both by Western blotting (Figure 5K) and immunofluorescence microscopy (Figure 5L). Increased SIRT3 activity resulted in increased MnSOD protein abundance (Figure 5M). Therefore, similar to the actions of INT-777 in diabetic mice, INT-777 prevents the phenotypic manifestations of obesity-related renal disease in mice with DIO.

In spite of similar caloric intake, average daily caloric intake low fat 12.8±0.8 versus low fat + INT-777 12.6±0.1, P=NS, high fat 21.7±1.3 versus high fat + INT-777 18.1±1.0, P=NS (Figure 5N), INT-777 prevented weight gain in DIO mice (Table 2). INT-777 also induced significant decreases in plasma triglyceride and cholesterol concentration (Table 2). The prevention of weight gain has been seen in previous studies as well21 and may be related to increased mitochondrial biogenesis in muscle and browning of adipose tissue.

Endogenous TGR5 Modulates Renal Expression of PGC-1α, ERRα, and SIRT3

To determine if TGR5 per se modulates renal expression of PGC-1α, ERRα, and SIRT3, we analyzed the kidneys of TGR5 knockout (KO) mice. TGR5 KO mice have significant decreases in PGC-1α, ERRα, and SIRT3 protein expression (Figure 6, A-C). INT-777 did not induce upregulation of PGC-1α, ERRα, or SIRT3 in TGR5 KO mice (Figure 6, A-C). These results are consistent with specific TGR5-mediated signaling induced by INT-777. In addition, the regulation of these signaling processes in the kidney is specific to the bile acid activated G protein–coupled receptor TGR5 as the BA-activated nuclear receptor FXR KO mice have no alterations in PGC-1α or SIRT3 protein (Figure 6D).

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<tr>
<th>Table 1. Metabolic parameters in diabetic mice</th>
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<td>Percentage of body weight change</td>
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<td>Plasma TG (mg/dl)</td>
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<tr>
<td>Plasma TC (mg/dl)</td>
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<td>Systolic blood pressure (mmHg)</td>
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Data are means±SEM (n=6 mice in each group).

aP<0.05 versus db/m.
bP<0.05 versus db/db.

TGR5 Signaling Acts on Both Glomerular and Tubular Fractions Isolated from Diabetic Mice

TGR5 mRNA is expressed in isolated glomeruli and tubules with a significantly higher level in the tubules (Figure 7A). To determine whether TGR5 acts in glomeruli versus tubules, we further checked the regulation of TGR5 targets in glomerular and tubular fractions isolated from db/db mice treated with INT-777. In glomeruli, we observed increased mRNA expression of PGC1α and SIRT3 (Figure 7B). A similar increase in PGC1α mRNA and protein (Figure 7C), as well as SIRT3 protein level, was found in the isolated tubule fraction (Figure 7D).

TGR5 Signaling Modulates Response of Podocytes and Proximal Tubular Cells to High Glucose

To further confirm the glomerular and tubular actions of TGR5 signaling and to determine a direct effect of TGR5 activation in the kidney independent of any systemic actions, we studied cultured human podocytes and human proximal tubule cells with the INT-777 treatment, respectively. TGR5 protein is expressed in human podocytes (Figure 7E) and INT-777 induced a rapid increase of cAMP at levels comparable to those observed when using forskolin stimulation (Figure 7F). INT-777 prevented high glucose-induced mitochondrial apoptosis as determined by a decrease in activated caspase 3/7 (Figure 7G) and mitochondrial damage as determined by Mito Green staining (Figure 7H). INT-777 increased mitochondrial oxygen consumption rate (Figure 7I) and suppressed mitochondrial ROS production as determined by Mito Sox staining (Figure 7J). INT-777 also induced increases in mitochondrial p-AMPK (Figure 7K) and PGC-1α protein (Figure 7L). INT-777 prevented podocyte lipid accumulation as determined by label-free CARS imaging (Figure 7M), which was mediated by a significant decrease in sterol regulatory element-binding protein 1 (SREBP-1) (Figure 7N) that would decrease fatty acid biosynthesis. (E) Synaptopodin immunofluorescence microscopy indicates significant decrease in the percentage of immunostaining area in glomerular tufts in db/db mice, which is indicative of decreased podocyte density, and treatment with INT-777 results in preservation of synaptopodin positive area. (F) Nephrin immunofluorescence microscopy shows that the treatment with INT-777 restores the decreased percentage of podocyte marker nephrin positive area in glomerular tuft in db/db kidneys. (G) p57 immunohistochemistry indicates significant decrease in podocyte number in db/db mice, and treatment with INT-777 prevents the loss of podocyte number. (H) CD68 immunofluorescence microscopy indicates the decreased macrophage accumulation by the treatment with INT-777 in db/db kidneys.
acid synthesis, and an increase in PPAR-α, long-chain acyl-CoA dehydrogenase (LCAD), and carnitine palmitoyl transferase-1 (CPT-1) that would increase fatty acid oxidation (Figure 7O). Indeed, INT-777 treatment induced an increase in the oxygen consumption rate when bovine serum albumin-palmitate was used as a substrate (Figure 7P), which is indicative of increased fatty acid oxidation activity.

In cultured human proximal tubule cells, there is also rapid cAMP response to INT-777 (Figure 7Q). Increased
TGR5 signaling led to the increased SIRT3 activity as shown by the reduction of acetylated SOD2 (Figure 7R).

**TGR5 Signaling in Human Kidney Disease in Obesity and Diabetes**

TGR5 is also expressed in human kidney, with much higher TGR5 mRNA expression in the tubules compared with glomeruli (Figure 8A). TGR5 protein is also expressed in the glomeruli and tubules as determined by immunohistochemistry (Figure 8B) and immunofluorescence microscopy (Figure 8C), using two different anti-human TGR5 antibodies. In spite of a significant decrease in TGR5 mRNA in the kidney biopsies of DN and ORG subjects (Figure 8D), we were unable to determine major and consistent differences in TGR5 protein level in glomeruli or tubules. There are several possible explanations for the discordant results in human diabetic tissue, with TGR5 mRNA being reduced while TGR5 protein appears unaffected by diabetes. These reasons include a biologic difference (e.g., prolonged protein half-life in diabetes compared with normal) and a methodologic difference (e.g., reduced dynamic range of immunohistochemistry compared with RT-PCR). Further, pathologic processes in humans are complex, and protein expression patterns may reflect underlying diseases, aging, co-morbidities, and the effects of medications. In addition, our inability to detect a change in glomerular or tubular TGR5 protein in spite of a decrease in TGR5 mRNA may be due to the specificity of the currently available antibodies.

However, the decrease in TGR5 mRNA is significantly correlated with (1) level of proteinuria (Figure 8E), (2) glomerulosclerosis (Figure 8F), (3) initial eGFR (Figure 8G), and (4) follow-up eGFR and progression of kidney disease (Figure 8H). Definition of slow versus rapid progression of eGFR decline in relationship of initial TGR5 mRNA level is based on initial and follow-up eGFR. In the whole patient cohort, the median rate of GFR decline was 0.6 ml/min/month (Supplemental Table 1). To study the association of TGR5 mRNA with longitudinal eGFR decline, we analyzed TGR5 expression after classifying the patients into two groups: (A) slow progressors: eGFR decline below 0.6 ml/min/month versus (B) fast progressors: eGFR decline above 0.6 ml/min/month. Median time for starting dialysis was 18 months in the fast progressors group versus 60 months for the slow progressors group.

On biopsy day the group’s demographic data were different only in HbA1c levels and age. The faster eGFR decline group showed significantly lower expression of TGR5 (Figure 8H). The decrease in TGR5 mRNA also correlated with decreases in (5) the glomerular podocyte markers nephrin and podocin (Figure 8, I and J).

In addition, the TGR5 targets PGC-1α, SIRT3, and ERRα that we identified in the mouse kidney, as well as human podocytes and proximal tubular cells, are also regulated in the human diabetic kidney, with significant decreases in PGC-1α (Figure 8K), SIRT3 (Figure 8L), and ERRα mRNA (Figure 8M). In fact the PGC-1α mRNA levels both correlate with

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**Figure 3.** Treatment of diabetic mice with INT-777 decreases oxidative stress. (A) Mitochondrial ROS generation is increased in mitochondria isolated from db/db mice and treatment with INT-777 decreased mitochondrial ROS generation. (B) Total SOD activity is decreased in the kidneys of db/db mice and treatment with INT-777 increases total SOD activity. (C) Mitochondrial SOD activity is decreased in mitochondria isolated from db/db mice and treatment with INT-777 increases mitochondrial SOD activity. (D) Urinary TBARS excretion, a marker of oxidative stress, is increased in db/db mice and treatment with INT-777 decreases urinary TBARS excretion. (E) Urinary H2O2 excretion, a marker of oxidative stress, is increased in db/db mice and treatment with INT-777 decreases urinary H2O2 excretion.
DISCUSSION

Our data demonstrate decreased TGR5 mRNA expression, with an inverse correlation between TGR5 expression level and progression of kidney disease defined by decrease in eGFR, glomerulosclerosis, proteinuria, and direct correlation with expression of the podocyte markers nephrin and podocin. Studies in cultured human podocytes and glomerular endothelial cells indicate that high glucose and/or fatty acids, two metabolic perturbations common in diabetes and obesity, induce downregulation of TGR5, thus providing a potential cause and mechanism for decreased TGR5 expression in obesity and in diabetes.

While these studies indicate the relevance of TGR5 to human disease, they are only correlative and do not provide a direct cause and effect relationship. We therefore utilized two well-studied mouse models of obesity and diabetes to determine whether TGR5 agonists would have any utility in prevention of DN and ORG. As in human disease, we found that TGR5 mRNA expression is also decreased in mouse models of diabetes and obesity. To determine the role of TGR5 in these conditions, we used the specific TGR5 agonist INT-777 (6a-ethyl-23(S)-methyl-cholic acid), a semisynthetic cholic acid derivative with systemic bioavailability. INT-777, due to its relatively low intestinal absorption, can effectively activate TGR5 in enteroendocrine cells, triggering GLP-1 secretion, and because of its systemic biodistribution can activate TGR5 in different districts, including the kidney.

INT-777 treatment in mice with DIO leads to enhanced mitochondrial function in muscle, brown adipose tissue, and enteroendocrine cells, resulting in increased energy expenditure and incretin secretion, and inducing a range of beneficial metabolic effects that include resistance to weight gain and hepatic steatosis, preservation of liver and pancreatic function, and maintenance of glucose homeostasis and insulin sensitivity. INT-777 also activates the TGR5-D2 signaling pathway, and robustly increases mitochondrial activity and oxidative phosphorylation. We found that INT-777 prevents diabetes-induced mitochondrial ROS generation, while it reverses the diabetes-induced decrease in superoxide dismutase activity. The net result is that INT-777 prevents diabetes-induced increases in urinary H₂O₂ and TBARS, well-established

In db/db mice, treatment with INT-777 decreases albuminuria, mesangial expansion, extracellular matrix protein accumulation, podocyte loss, and macrophage infiltration. TGR5 activates AMPK, SIRT1, PGC-1α, ERRα, SIRT3, and Nrf-1, indicating activation of mitochondrial biogenesis. This is consistent with several observations in animal models and humans linking reduced mitochondrial function and oxidative phosphorylation to the development of type 2 diabetes. INT-777 also activates the TGR5-D2 signaling pathway, and robustly increases mitochondrial activity and oxidative phosphorylation. We found that INT-777 prevents diabetes-induced mitochondrial ROS generation, while it reverses the diabetes-induced decrease in superoxide dismutase activity. The net result is that INT-777 prevents diabetes-induced increases in urinary H₂O₂ and TBARS, well-established

TGR5 mRNA expression (Figure 8N) and initial eGFR (Figure 8O).

Figure 4. Treatment of diabetic mice with INT-777 decreases lipid accumulation. (A) Representative images of oil red O staining, which stains for neutral lipids, show that neutral lipid accumulation in kidneys of db/db mice is prevented by the treatment with INT-777. The decrease in neutral lipid accumulation is associated with decrease in (B) DGAT1, mediator of triglyceride synthesis, and increases in (C) PPAR-α, (D) UCP-2, and (E) CPT1B, mediators of fatty acid synthesis.
Figure 5. TGR5 specific agonist INT-777 prevents nephropathy in diet-induced obesity mice. (A) TGR5 mRNA expression is decreased in the kidneys of mice with diet-induced obesity. (B) Urinary albumin excretion is increased in mice fed a high fat (HF) diet and treatment with INT-777 decreases albuminuria. (C) Nephrin immunofluorescence microscopy shows that the percentage of podocyte marker nephrin positive area in glomerular tuft, indicative of podocyte density, is restored by the treatment with INT-777 in db/db kidneys. (D) Fibronectin immunofluorescence microscopy indicates marked accumulation of glomerular matrix in HF mice and treatment with INT-777 results in a significant decrease in fibronectin immunostaining. (E) Type IV collagen immunofluorescence microscopy indicates that the marked accumulation of glomerular matrix in HF mice is significantly decreased by the treatment with INT-777. (F) Pro-fibrotic growth factors TGF-β, CTGF, and PAI-1 expression are increased in HF diet and treatment with INT-777 results in marked decreases in the expression of these profibrotic growth factors. (G) CD68 immunofluorescence microscopy indicates significant increase in immunostaining in HF mice, which is indicative of increased macrophage accumulation, and treatment with INT-777 results in decrease of CD68 immunostaining. (H) Proinflammatory cytokines MCP-1, TLR2, and TLR4 expression are increased in HF diet and treatment with INT-777 results in marked decreases in the expression of these proinflammatory cytokines. (I) Coherent anti-stokes Raman scattering (CARS) microscopy imaging indicates increased lipid accumulation in the kidneys of HF mice and treatment with INT-777 results in decreased lipid accumulation. (J and K) Representative Western blots show that INT-777 treatment increases expression of mediators of mitochondrial biogenesis, including p-AMPK (J), PGC-1α and SIRT3 (K). Increased SIRT3 expression with the INT-777 treatment is further manifested by immunofluorescence (L, green SIRT3, red F-actin). (M) Treatment of HF mice with INT-777 induced increased mitochondrial SOD protein. (N) Average caloric intake shows no difference in HF mice with INT-777 treatment from HF mice without treatment.
markers of oxidative stress. In addition, INT-777 also prevents lipid accumulation in the kidney by decreasing DGAT1, an enzyme involved in triglyceride synthesis, and increasing PPAR-α, an uncoupling protein-2, and CPT1β, an inducer of fatty acid oxidation and energy uncoupling.

Treatment of DIO mice with INT-777 decreased urinary albumin excretion, podocyte loss, accumulation of extracellular matrix proteins, expression of profibrotic growth factors TGFβ, CTGF, and PAI-1, accumulation of CD68 positive macrophages, and expression of proinflammatory cytokines MCP-1, TLR2, and TLR4. INT-777 also decreased accumulation of lipids and induced AMPK, PGC-1α, and SIRT3 expression. As AMPK activation has been found to markedly reduce glomerular TGF-β, collagen, and fibroinectin accumulation in several mouse models of diabetic kidney disease,32 our findings suggest an important role for the anti-inflammatory and anti-fibrotic effects induced by INT-777 treatment in chronic kidney disease.

The role of TGR5 in regulating PGC-1α, ERRα, and SIRT3, important elements in the control of mitochondrial biogenesis, antioxidant generation, and fatty acid β-oxidation, is illustrated by the observation that PGC-1α, ERRα, and SIRT3 mRNA expression are decreased in the kidneys of patients with ONG and DN. We have found that PGC-1α, ERRα, and SIRT3 protein expression are also reduced in the kidneys of TGR5 KO mice. Additionally, treatment with INT-777 in TGR5 KO mice does not induce PGC-1α, ERRα, and SIRT3, further indicating the key role of TGR5 in their upregulation and the specificity of INT-777 as a TGR5 agonist.

In previous studies we had demonstrated that agonists of FXR, the BA-activated nuclear receptor, can prevent diabetes and obesity-related kidney disease.47–49 In contrast, the effects of streptozotocin-induced hyperglycemia in the kidney were markedly accentuated in FXR KO mice.49 The effects of TGR5 in the kidney, however, are distinct from FXR, as in FXR KO mice, unlike in TGR5 KO mice, we did not see regulation of PGC-1α or SIRT3, thereby differentiating TGR5 from FXR actions in the kidney.

Because beneficial effects of TGR5 agonism could be mediated by its multiple extrarenal effects to regulate metabolism,19–26 we also performed studies in human podocytes cultured in the presence

Table 2. Metabolic parameters in diet-induced obesity mice

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<th>LF + INT-777</th>
<th>HF</th>
<th>HF + INT-777</th>
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<td>Final body weight (g)</td>
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<td>Percentage of body weight change</td>
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<td>Kidney weight (g)</td>
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<td>190±11</td>
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<td>Plasma TG (mg/dl)</td>
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<td>Plasma TC (mg/dl)</td>
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<td>Systolic blood pressure (mmHg)</td>
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<td>112±5</td>
<td>110±3</td>
<td>112±6</td>
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Data are means±SEM (n=6 mice in each group). LF, low-fat diet; HF, high-fat diet.

*P<0.05 versus LF.

**P<0.05 versus HF.
of high glucose and treated with INT-777. INT-777 reproduced most of the effects seen in vivo. INT-777 increased mitochondrial biogenesis and podocyte mitochondrial oxygen consumption rate and decreased mitochondrial ROS production. INT-777 activated AMPK and PGC-1α, and prevented high glucose induced podocyte lipid accumulation. The prevention of lipid accumulation was mediated by inhibition of fatty acid synthesis mediated by SREBP-1 and activation of fatty acid oxidation mediated PPAR-α, LCAD, and CPT-1β. Accordingly, in podocytes INT-777 induced fatty acid oxidation as determined by increased oxygen consumption rate, when cultured in the presence of palmitate.

Mitochondrial dysfunction has been proposed to play a critical role in the pathogenesis and complications of type 2 diabetes mellitus and obesity. A recent urine metabolomics-based study revealed evidence for suppression of mitochondrial activity in diabetic kidney disease, as well as decreased expression of PGC-1α mRNA. Our results are in agreement with these findings and furthermore indicate that TGR5 is an important regulator of PGC-1α in the kidney. It needs to be determined whether TGR5 activates PGC-1α via a PKA and p-CREB signaling pathway, via activation of AMPK which phosphorylates and activates PGC-1α, or via activation of SIRT1 which deacetylates and activates PGC-1α.

Previous studies have shown reduced PGC-1α levels in the diabetic kidney associated with reduced AMPK, reduced mitochondrial content, and reduced mitochondrial complex activity, and have demonstrated a beneficial effect of AMPK activation in preventing kidney disease in mouse models of obesity and diabetes. It is likely that these beneficial effects were mediated, at least in part, via activation of PGC-1α. PGC-1α is a master regulator of mitochondrial biogenesis. Our results in mice with diabetes and obesity and cultured human podocytes indicate that TGR5 stimulates the AMPK-SIRT1-PGC-1α axis, resulting in increased and even restored mitochondrial biogenesis.

TGR5 is also associated with activation of ERRα and SIRT3, a downstream target gene of PGC-1α, which have important effects in enhancing mitochondrial biogenesis, decreasing mitochondrial ROS generation and inducing fatty acid β-oxidation. Indeed, TGR5 activation in the kidney leads to decreased oxidative stress and lipid accumulation, effects also observed in cultured podocytes treated with INT-777.

In agreement with earlier studies, which have shown that TGR5 regulates energy metabolism and energy expenditure in the muscle, our studies also indicate that in spite of similar food intake, mice with high fat diet-induced obesity treated with INT-777 gain significantly less weight than their untreated counterparts. Our studies were not designed to measure energy expenditure in the kidney. However, the regulation of mitochondrial biogenesis in cultured podocytes, with increased mitochondrial respiration and fatty acid β-oxidation, suggests that TGR5 may also increase energy expenditure in the kidney.

In summary, our results indicate that TGR5 expression and activity is impaired in the kidneys of humans and rodents with obesity and diabetes. TGR5 activation induces mitochondrial biogenesis, while preventing renal oxidative stress and lipid accumulation, firmly establishing an important role for TGR5 activation in inhibiting kidney disease in obesity and diabetes.

**CONCISE METHODS**

**Animal Models: Diabetic Mice**

Eight-week-old male db/db and db/db mice (BLKS/J genetic background) were obtained from Jackson Laboratories (Bar Harbor, ME). They were maintained on a 12-h light/12-h dark cycle. They were fed for 12 weeks a regular chow diet or the TGR5 agonist 6-ethy-23(S)-methylcholic acid (6-EMCA, INT-777), 30 mg/kg body weight/day, admixed with chow. One week prior to the end of the study the mice were placed in metabolic cages for a 24-h urine collection. At the end of the study period, following anesthesia, blood was drawn from the aorta for chemistry and the kidneys were rapidly removed and processed for histology and immunofluorescence microscopy, RNA extraction for real-time PCR (QPCR), protein extraction for Western blotting, or mitochondria isolation.
Diet-Induced Obesity Mice

Eight-week-old C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME). They were maintained on a 12-h light/12-h dark cycle. They were fed for 12 weeks a low (10 kcal %) fat diet from Research Diets (D12450), or a high (60 kcal %) fat diet from Research Diets (D12492) supplemented with no addition or the TGR5 agonist 6α-ethyl-23(S)-methylcholic acid (6-EMCA, INT-777), 30 mg/kg body weight/day. At the end of the treatment period, mice were studied as above.

TGR5 KO Mice

TGR5 generalized KO mice and their littermates were a generous gift from Dr. Johan Auwerx and Dr. Kristina Schoonjans, Lausanne, Switzerland.21

FXR KO Mice

FXR generalized KO mice, on the C57BL/6J genetic background, originally generated by Frank Gonzalez,56 were obtained from The Jackson Laboratory (Bar Harbor, ME).

Animal studies and relative protocols adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee at the University of Colorado Denver.

Glomerular and Tubulo-Interstitial Cell Isolation from Mice

Mouse glomeruli were isolated from kidney cortex using the iron oxide injection method as described elsewhere.57 Those not collected for glomeruli in the purification process were saved as the tubular fraction. The

Figure 7. TGR5 signaling acts on both glomerular and tubular fractions. (A) mRNA level of TGR5 in isolated mouse glomeruli and tubules. (B) Treatment with INT-777 increases PGC1α and SIRT3 mRNA in isolated glomeruli from db/db mice. (C and D) Treatment with INT-777 increases PGC1α mRNA and protein level (C) and SIRT3 protein level (D) in the tubule fraction of db/db mice. (E) Both immunofluorescence microscopy and Western blot indicate the expression of TGR5 in cultured human podocytes. (F) INT-777 induces cAMP induction in cultured human podocytes. (G) Treatment with INT-777 prevents apoptosis, indicated by activated caspase 3/7, in human podocytes cultured with high glucose (HG) condition. (H) HG induces decrease in mitochondrial number as determined by Mito Green fluorescence staining, which is prevented by treatment with INT-777. (I) Treatment of HG podocytes with INT-777 increases oxygen consumption rate. (J) HG induces podocyte oxidative stress and ROS production as determined by MitoSox Red fluorescence staining, which is prevented by treatment with INT-777. (K) Treatment of podocytes with INT-777 increases p-AMPK protein. (L) HG induces decrease in PGC-1α protein, which is prevented by treatment with INT-777. (M) INT-777 prevents HG induced podocyte lipid accumulation as determined by CARS microscopy imaging. (N) HG induces increase in nuclear SREBP-1 protein, a transcriptional factor which is a master regulator of fatty acid and triglyceride synthesis, which is prevented by treatment with INT-777. (O) Treatment with INT-777 prevents HG induced decrease in PPAR-α, LCAD and CPT-1B, mediators of fatty acid β-oxidation. (P) Treatment of HG podocytes increases fatty acid β-oxidation rate. (Q) INT-777 induces cAMP induction in cultured human proximal tubule cells. (R) INT-777 increases SIRT3 activity in cultured human proximal tubule cells as shown by the reduction of acetylated SOD2.

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purity of glomerular and tubular preparation was verified under microscopy as well as podocin (glomerular) and tubular (NaPi-2a) markers.

**Human Kidney Samples**

Formalin-fixed paraffin-embedded (FFPE) tissue specimen renal biopsy material was obtained from the archives of the Columbia Renal Pathology Laboratory and from pathologic archives of the Department of Pathology at Rabin Medical Center. Kidney samples were obtained from leftover portions of diagnostic kidney biopsies of patients with DN (n=34) or ORG (n=33) and normal kidneys (n=17).

Control biopsies were renal biopsies that appeared normal by histologic, immunofluorescence, and electron microscopic examination. These controls were either obtained from renal biopsies performed for minimal isolated proteinuria or hematuria or tissue from uninvolved portions of a kidney at the time of nephrectomy for tumor or from candidate renal donors.58 DN kidney biopsies were from Rabin Medical Center.

ORG was defined morphologically as focal segmental glomerulosclerosis and glomerulomegaly occurring in obese patients with a body mass index higher than 30 kg/m. 59,60 Nine biopsy-proven ORG were from Rabin Medical Center archive and 24 were from Columbia University. Fluorescence for each cycle was quantitatively analyzed by an ABI Step One sequence detection system (PerkinElmer). In order to control for variation in the amount of DNA that was available for PCR in the different samples, gene expression of the target sequence was normalized in relation to the expression of an endogenous control, 18S rRNA or RPLPO (large ribosomal protein).

**Definition of Slow Versus Rapid Progression of eGFR Decline in Relationship of Initial TGR5 mRNA Level**

This is based on initial and follow-up eGFR. In the whole patient cohort, the median rate of GFR decline was 0.6 ml/min/month (Supplemental Table 1). To study the association of TGR5 mRNA with longitudinal eGFR decline, we analyzed TGR5 expression after classifying the patients into two groups: (A) slow progressors: eGFR decline below 0.6 ml/min/month versus (B) fast progressors: eGFR decline above 0.6 ml/min/month. Median time for starting dialysis was 18 months in the fast progressors group versus 60 months for the slow progressors group. On biopsy day the group’s demographic data were different only in HbA1c levels and age on the biopsy day. The faster eGFR decline group showed significantly lower expression of TGR5 (Figure 8H).

**Laser Capture Microdissection (LCM)**

LCM was performed using the PALM Micro Beam instrument (PALM, Carl Zeiss, Germany). FFPE blocks used were cut into sections (5 μm
thick) onto PALM membrane slides (PALM, Carl Zeiss), baked and deparaffinized with xylene, lightly stained with hematoxylin and eosin, and air-dried. All the glomeruli or the tissue surrounding the captured glomeruli were microdissected and captured on PALM adhesive cap tubes followed by total RNA extraction using the RNeasy FFPE Kit (Qiagen).

The study was approved by the Rabin Medical Center Institutional Ethics Committee and by the Columbia University Institutional Review Board.

Urine Chemistry
Urine albumin and creatinine concentrations were determined using kits from Exocell (Philadelphia, PA). Urinary H₂O₂ and TBARS level was measured using the kits from Thermo Fisher Scientific (Rockford, IL) and Bioassay Systems (Hayward, CA), respectively.

Quantitative Real-Time PCR
Quantitative real-time PCR was performed as previously described.47–49,62–67 Primer sequences are listed in Supplemental Table 2.

Western Blotting
Western blotting was performed as previously described.47–49,62–67 The antibodies against PGC-1α (EMD Millipore, Billerica, MA), p-AMPK, p-ACC, AMPK, SIRT1, SIRT3 (Cell Signaling Technology, Danvers, MA), ERRa (Epitomics, Burlingame, CA), SOD2 (Enzo Life Sciences, Farmingdale, NY), acetylated SOD2,68 and acetylated IDH2 (Genetel Laboratories, Madison, WI) were used for Western blotting.

Mitochondria Isolation
Freshly excised kidneys were homogenized with a small Dounce homogenizer in isolation buffer (50 ml 0.1 M Tris-MOPS, 5 ml 0.1 M
EGTA-Tris, 100 ml 1 M sucrose in 500 ml nanopure water, pH 7.4. The homogenate was centrifuged first at 800 g, 4°C for 10 min to remove tissue debris. The supernatant was centrifuged at 8000 g, 4°C for 10 min twice to obtain a mitochondrial pellet. The preparation was resuspended in a small amount of isolation buffer. The mitochondrial protein concentration was measured with a BCA protein assay kit (Pierce, Rockford, IL). The isolated mitochondria were used immediately for ROS measurements or were stored at −80°C until further analysis.

**Mitochondrial ROS Measurement**

H$_2$O$_2$ generation was used to measure the ROS in isolated mitochondria using Amplex Red (Life Technologies) as specified.\(^{69}\)

**SOD Activity Measurement**

The total SOD and mitochondrial SOD activities were measured by using SOD Assay kit from Cayman Chemical (Ann Arbor, MI).

**Histology Staining and Immunofluorescence Microscopy**

Sections (2 μm thick) cut from 10% formalin-fixed, paraffin-embedded kidney samples were used for periodic acid-Schiff (PAS) staining and immunohistochemistry for p57\(^{70}\) (performed by Kelly Hudkins and Charles Alpers, University of Washington, Seattle). Frozen sections were used for oil red staining of neutral lipid (cholesterol esters and triglycerides) deposits or for immunostaining for fibronectin and type IV collagen (Sigma-Aldrich), nephrin (a gift from Dr. Lawrence Holzman, University of Pennsylvania, Philadelphia, PA), synaptopodin (Sigma-Aldrich), and CD68 (AbD Serotec, Raleigh, NC), and imaged with a laser scanning confocal microscope (LSM 510; Carl Zeiss, Jena, Germany). TGR5 immunofluorescence was performed on human renal biopsy sections (University of Colorado Denver) with antibody from R&D Systems (Minneapolis, MN) (catalog number MAB4286).

**Immunohistochemical Staining**

De-identified renal biopsy sections (Columbia University, John Hopkins University, and Rabin Medical Center (Petah Tikva, Israel)) were used under IRB guidelines. Briefly, staining was performed on 5 μm formalin-fixed paraffin-embedded sections. Heat-induced antigen retrieval (pH 9) and peroxide block was performed in preparation for incubation with anti-TGR5 rabbit polyclonal antibody (1:200 for 2 h; Sigma-Aldrich, product number HPA062890) and detection with DAB.

**Quantification of Morphology**

All quantifications were performed in a masked manner. Using coronal sections of the kidney, 30 consecutive glomeruli per mouse, six mice per group were examined for evaluation of glomerular mesangial expansion. The index of the mesangial expansion was defined as ratio of mesangial area/glomerular tuft area. The mesangial area was determined by assessment of the PAS-positive and nucleus-free area in the mesangium using ScanScope image analyzer (Aperio Technologies, Vista, CA). ImageJ software was used to quantify the synaptopodin or nephrin immunofluorescence by measuring the percentage of the positive staining area in glomerular tuft area. p57 staining quantification was as described and the glomerular tuft area was used as a denominator to assess podocyte number.\(^{71}\)

**Podocyte Culture**

Human podocytes were maintained in RPMI 1640, 1% Insulin-Transferrin-Selenium, 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 33°C as previously described.\(^{72-77}\) Podocyte differentiation was induced by thermo-shifting the cells from 33°C to 37°C for 10 days. Podocytes were then treated for...
48 h with control (10 mM glucose + 20 mM mannitol as osmotic control), high glucose (30 mM), or high glucose plus INT-777 (10 μM). Mouse podocytes (line AI) were cultured similarly, with the addition of 30 units/ml of mouse interferon gamma.78

**Proximal Tubule Cell Culture**

Human proximal tubules were maintained in RPMI 1640, 1% Insulin-Transferrin-Selenium, 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 33°C. Cell differentiation was induced by thermo-shifting the cells from 33°C to 37°C for 3–5 days. Proximal tubules were then treated for 48 h with 10 mM normal glucose control, high glucose (30 mM), or high glucose plus INT-777 (10 μM).

**Mitochondrial Oxygen Consumption Rate**

Measurements of mitochondrial oxygen consumption rate were performed on Seahorse XF24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA). For these measurements 1.75 × 10^4 podocytes per well were seeded in 24-well microplates for 7 days. Baseline OCR was measured in control (vehicle) or INT-777 (10 μM or as indicated) treated cells. To determine fatty acid β-oxidation cells were treated with BAS-complexed palmitate (0.5 mM) followed by treatment with control or INT-777 (10 μM).

**Podocyte Staining for Mitochondrial ROS and Mitochondrial Number**

Human podocytes were cultured in 35 mm glass bottom culture dishes (MatTek Ashland, MA) for 10 days at 37°C. After culturing in the control or high glucose with or without INT-777 treatment, cells were incubated with (1) 200 nM Mitotracker deep green (Mito Green) (Life Technologies, Grand Island, NY) for 20 min to determine mitochondrial number or (2) 5 μM MitoSox Red (Life Technologies, Grand Island, NY) for 20 min to determine mitochondrial ROS at 37°C before taking images with a laser scanning confocal microscope LSM 510 (Carl Zeiss, Jena, Germany). Alternatively, human podocytes seeded in black-wall clear-bottom 96-well plates for 10 days were used for direct fluorescence measurement of Mito Green or MitoSox Red staining with Synergy 2 Microplate Readers (BioTek, Winooski, VT).

**CARS Microscopy**

We performed CARS microscopy for label-free imaging of lipid deposits in podocytes as previously described.79–81 Briefly, the CARS images were acquired using our custom CARS-TPAF (two-photon autofluorescence) multimodal platform Olympus FV-1000 microscope (Olympus America, Center Valley, PA) coupled to a picoEmerald™ system (APE; Angewandte Physik und Elektronik, Berlin, Germany) which is internally pumped by a picosecond laser (High-Q Laser, Rankweil, Austria; part of Newport Corporation, Irvine, CA) generating two beams, approximately 5 W at 532 nm and 1 W at 1064 nm. The repetition rate is 80 MHz with a pulse width of approximately 6 ps. The 532 nm output feeds an optical parametric oscillator (OPO) that generates an 816 nm laser beam with a maximum of approximately 1 W of power. The generated 816 nm laser beam (pump and probe) is combined with the 1064 nm laser beam (Stokes) and sent into the microscope to excite the sample for imaging. The pump/probe wavelength was chosen to excite the CH₂ Raman vibrational stretch at 2845 cm⁻¹ present in lipids. The resulting CARS signal is at approximately 662 nm. A 60× 1.2 NA objective (U Plan S Apo 60× IR W, Olympus America, Center Valley, PA) is used to focus the laser beams onto the sample. The laser powers measured at the objective are typically <70 mW for both the 816 nm and 1064 nm laser beams depending on the power necessary to image the sample. The pump/probe power is set at twice the Stokes power because this laser must serve both the pump and probe functions in generating a CARS signal. An external non-descanned detector in the epi direction is used to measure the TPAF signal from the sample through a 420–520 nm emission filter (hq470/100m-2p; Chroma Technology, Bellows Falls, VT). The CARS signal is measured in the forward direction with another non-descanned external detector through a 640–680 nm emission filter (hq660/40m-2p; Chroma Technology). The pixel dwell time is 10 μs and the image pixel resolution is 1600 by 1600. A Kalman averaging filter set to three scans/line time is used during image acquisition to improve the signal-to-noise ratio of the acquired images.

**Statistical Analysis**

Results are presented as the means±SEM for at least three independent experiments. Data were analyzed by ANOVA and Student–Newman–Keuls tests for multiple comparisons or by t test for unpaired data between two groups. Statistical significance was accepted at the P<0.05 level.

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**DISCLOSURES**

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**REFERENCES**


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