A Dual Agonist of Farnesoid X Receptor (FXR) and the G protein-coupled Receptor TGR5, INT-767, Slows Down Age-Related Kidney Disease in Mice

Xiaoxin X. Wang1, Yuhuan Luo1, Dong Wang1, Luciano Adorini2, Mark Pruzanski2, Evgenia Dobrinskikh1, Moshe Levi1

1Division of Renal Diseases and Hypertension, Department of Medicine, Denver VA Medical Center and University of Colorado AMC, Aurora, Colorado; 2Intercept Pharmaceuticals, New York, NY.

Running title: FXR-TGR5 reverses age-related kidney disease

Address inquiries to: Moshe Levi, MD, 12700 East 19th Avenue, MC C281, Aurora, Colorado 80045
E-Mail: Moshe.Levi@ucdenver.edu; Fax: 303-724-4868; Tel: 303-724-4825

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ABSTRACT

Even in healthy individuals, renal function gradually declines during aging. However, an observed variation in the rate of this decline has raised the possibility of slowing or delaying age-related kidney disease. One of the most successful interventional measures that slows down and delays age-related kidney disease is caloric restriction. We undertook the present studies to search for potential factors that are regulated by caloric restriction and act as caloric restriction mimetics. Based on our prior studies with the bile acid-activated nuclear hormone receptor farnesoid X receptor (FXR) and G protein coupled membrane receptor TGR5 that demonstrated beneficial effects of FXR and TGR5 activation in the kidney, we reasoned that FXR and TGR5 could be excellent candidates. We therefore determined the effects of aging and caloric restriction on the expression of FXR and TGR5 in the kidney. We found that FXR and TGR5 expression are decreased in the aging kidney, and that caloric restriction prevents these age-related decreases. Interestingly, in long-lived Ames dwarf mice renal FXR and TGR5 expression levels were also increased. A 2-month treatment of 22-month-old C57BL/6J mice with the FXR-TGR5 dual agonist INT-767 induced caloric restriction-like effects and reversed age-related increases in proteinuria, podocyte injury, fibronectin accumulation, TGF-β expression, and, most notably, age-related impairments in mitochondrial biogenesis and mitochondrial function. Furthermore, in podocytes cultured in serum obtained from old mice, INT-767 prevented the increases in the proinflammatory markers TNF-α, toll-like receptor 2 (TLR2), and TLR4. In summary, our results indicate that FXR and TGR5 may play an important role in modulation of age-related kidney disease.

INTRODUCTION

A gradual decline in renal function occurs even in healthy aging individuals (1-3). The fastest growing group of people in the United States with impaired kidney function is the oldest age group. The population older than 65 years in the United States is expected to double in the next 20 years. The number of elderly worldwide is expected to triple from 743 million in 2009 to 2 billion in 2050. This will result in a marked increase in the elderly population with chronic kidney disease (CKD). This increase may be further amplified by other age-related co-morbidities including hypertension and the metabolic syndrome that accelerate age-related decline in renal function (4-8). Hypertension, obesity and insulin resistance can induce mitochondrial dysfunction, endoplasmic reticulum stress, oxidative stress, inflammation, altered lipid metabolism, and stimulation of profibrotic growth factors in the kidney, which collectively contribute to age-related kidney disease (1).

However, there is variation in the rate of decline given gender, race, and burden of co-morbid conditions (9-13). Although greater
glomerular, vascular and interstitial sclerosis is evident on renal tissue examination of healthy kidney donors with increasing age (14), closer examination of processes leading to sclerosis suggests a role for possible modifiable metabolic and hormonal factors that can decrease the rate of sclerosis. In this regard, the Baltimore Longitudinal Study of Aging revealed that nearly a third of older healthy adults have little change in renal function over time (15). These findings bring into question the inevitability of age-related decline in renal function and raise the possibility of slowing or delaying the process.

Similar findings have also been reported in rodent models of aging. Korstanje and colleagues at the Jackson Labs studied 30 inbred strains of mice determining urine albumin/creatinine ratio (ACR) and renal pathology at 12 months, 18 months, and 24 months of age (14). They found that while some strains of mice are more resistant to development of age-related albuminuria, other strains of mice are more susceptible, suggesting a genetic role in age-related kidney disease.

One of the most successful interventional measures that have been shown to slow down and delay age-related kidney disease is caloric restriction (16-20). Studies in our lab have demonstrated that caloric restriction prevents age-related kidney disease in part by preventing the increased expression of the sterol regulatory element binding proteins SREBP-1 and SREBP-2 that are master regulators of fatty acid, triglyceride and cholesterol synthesis (16, 17). The prevention of the age-related increases in SREBP-1 and SREBP-2 were associated with decreased renal triglyceride and cholesterol accumulation, decreased renal expression of growth factors, connective tissue growth factor (CTGF) and vascular endothelial growth factor (VEGF), matrix metalloproteinase inhibitor, plasminogen activator inhibitor-1 (PAI-1), resulting in prevention of mesangial expansion, podocyte injury and proteinuria (16, 17).

We undertook the present studies to search for potential additional factors that may be regulated by caloric restriction and thus act as caloric restriction mimetics. Based on our earlier work with the bile acid activated nuclear hormone receptor farnesoid X receptor (FXR) and G protein coupled membrane receptor TGR5 (also known as GPBAR1 or GPR131), where we have shown highly beneficial effects of FXR and TGR5 in the kidney (21-24), we reasoned that FXR and TGR5 could be excellent candidates, and we therefore determined the effects of aging and caloric restriction on the expression of FXR and TGR5 in the kidney.

In the current study, we determined the effects of the dual FXR-TGR5 dual agonist INT-767 (6α-ethyl-3α, 7α, 23-trihydroxy-24-nor-5-β-cholan-23 sulfate sodium salt) in aging mice. INT-767 is the first compound described so far to potently and selectively activate both BA receptors. INT-767 is a semi-synthetic analogue of the endogenous FXR agonist chenodeoxycholic acid (CDCA), with one fewer carbon on the side chain and a sulfate ester, lacking the COOH group of CDCA. INT-767 has similar lipophilicity and detergenty to CDCA but with a much lower pKa (<1), similar to taurine-conjugated CDCA (25). INT-767 is 300 times more potent than CDCA in FXR activation and about 5 times more potent than lithocholic acid (LCA), the endogenous TGR agonist, in TGR activation (26). INT-767 shows efficient intestinal absorption and its biliary excretion is facilitated by 3-glucuronidation (25). Thus, INT-767 possesses a pharmacokinetic profile suitable for targeting both FXR and TGR5. Consistent with its dual agonist activities, INT-767 induces FXR dependent lipid uptake by adipocytes, with the beneficial effect of shuttling lipids from central hepatic to peripheral fat storage, and promotes TGR5-dependent glucagon-like peptide-1 secretion by enteroendocrine cells, a validated target in the treatment of type 2 diabetes. Moreover, INT-767 treatment markedly decreases cholesterol and triglyceride levels in diabetic db/db mice and in mice rendered diabetic by streptozotocin administration (26). INT-767 has also marked anti-inflammatory (27), anti-atherosclerotic (28), and anti-cholestatic effects (29).

Our results indicate that FXR and TGR5 expression are decreased in the aging kidney, and that caloric restriction prevents the age-related decreases in FXR and TGR5 expression. 2-month treatment of 22-month-old aged C57BL/6J mice with the FXR-TGR5 dual
agonist INT-767 has caloric restriction-like actions and reverses age-related increases in proteinuria and podocyte injury and most notably age-related mitochondrial dysfunction. In addition, in podocytes cultured in the presence of serum obtained from old mice, INT-767 prevents the increases in the inflammatory markers TNF-α, TLR2, and TLR4. Our results therefore indicate that FXR and TGR5 may play an important role in modulation of age-related kidney disease.

RESULTS
Caloric Restriction prevents age-related decreases in renal FXR and TGR5 expression-
We found that compared to 5 months old ad lib fed mice, 24 months old ad lib fed mice had significant decreases in FXR and TGR5 mRNA expression. These decreases were prevented in 24 months old mice with life-long caloric restriction (Figure 1A). The effects of aging and caloric restriction on FXR were also documented at the protein level by Western blotting (Figure 1B). We could not perform Western blotting for TGR5 because presently there are no specific antibodies available for mouse TGR5. Although a TGR5 antibody did reveal parallel changes in TGR5 protein, based on Western blots performed with tissues from TGR5 KO mice, we do not believe that the signals are specific for TGR5.

Treatment of 22 months old aging ad lib fed mice for 2 months with the FXR and TGR5 dual agonist reverses the age-related increase in urinary albumin excretion—We then treated 22-month-old ad lib fed mice with the dual FXR and TGR5 agonist INT-767 to determine if a 2-month treatment would be able to reverse the age-related increase in urinary albumin excretion. Our results indicate that treatment of 22-month-old ad lib fed mice with INT-767 induced a significant decrease in urinary albumin, like the beneficial effects achieved with life-long caloric restriction (Figure 1C). In addition, INT-767 prevented the age-related decrease in synaptopodin expression, as determined by immunofluorescence microscopy (Figure 1D). The beneficial effects of INT-767 were associated with prevention of the age-related increases in transforming growth factor-β (TGF-β), fibroblast specific protein-1 (FSP-1) and fibronectin mRNA (Figure 1E) and protein (Figure 1F) as determined by immunofluorescence microscopy.

Treatment of 22 months old aging ad lib fed mice for 2 months with the FXR and TGR5 dual agonist reverses the age-related decreases in mitochondrial biogenesis and function—Since mitochondria have been known to play a crucial role in the process of aging (30-33), we examined if the treatment with INT-767 could modulate the mitochondrial biogenesis and function in the aging kidney. We found that mitochondrial to nuclear DNA ratio, a hallmark of mitochondrial biogenesis, as well as the master regulator of mitochondrial biogenesis, nuclear respiratory factor 1 (NRF1), were significantly decreased in the kidneys of 24-month-old ad lib fed mice and reversed by the treatment with INT-767 (Figure 2A). We also found that the INT-767 treatment concomitantly increased the activated AMPK level, and reversed the age-related decreases in SIRT1 mRNA and the nuclear hormone receptor estrogen related receptor-alpha (ERR-α) mRNA expression (Figure 2B). Furthermore, treatment of 22-month-old ad lib fed mice with INT-767 for 2 months reversed the age-related decrease in PGC-1α mRNA and protein, and mitochondrial SIRT3 mRNA and protein (Figure 2C). The increase in SIRT3 was accompanied by increases in medium-chain Acyl-CoA dehydrogenase (MCAD) protein, an important mediator of mitochondrial fatty acid β-oxidation (Figure 2C). At the same time INT-767 also reversed the age-related increase in acetylated form of mitochondrial isocitrate dehydrogenase (acetyl-IDH2/IDH2), another target of SIRT3 activity (Figure 2D). This is ultimately associated with reversal of the decreased mitochondrial complex I and complex IV activity in aged kidneys by 2-month INT-767 treatment, achieving levels identical to life-long caloric restriction (Figure 2D).

INT-767 decreases inflammation in human podocytes conditioned with aging serum—To determine if INT-767 has direct effects in aging podocytes we treated human podocytes with serum from young (4-month old) or old (28-month old) mice. Aging serum increased the expression of TNF-α, TLR2 and TLR4 (Figure 3). Treatment with INT-767 prevented these
changes (Figure 3), indicating a direct effect of FXR and TGR5 activation on the cultured podocytes.

**FXR and TGR5 expression are increased in long-lived Ames dwarf mice**—Since caloric restriction is associated with prevention of age-related pathologies and life and health span extension, we studied the Ames dwarf mice, that exhibit delayed aging and extended longevity to determine if FXR and TGR5 expression were regulated like caloric restriction. We found that like caloric restriction FXR and TGR5 mRNA levels are increased in the kidneys of Ames dwarf mice (Supplemental Figure S1A). In addition, we found that the genes involved in the mitochondrial biogenesis and function, including NRF1, SIRT1, PGC1α, ERRα, SIRT3, COX4, and LCAD, were also increased in the kidneys of Ames dwarf mice, consistent with the findings with the INT-767 treatment of aging C57BL/6 mice (Supplemental Figure S1B).

**DISCUSSION**

Our studies have identified the nuclear hormone receptor FXR and the G protein coupled receptor TGR5 as two important modulators of age-related kidney disease that are regulated by life-long caloric restriction. FXR and TGR5 expression are decreased in the aging kidney and caloric restriction results in increases in both FXR and TGR5 expression in the kidney.

Remarkably only a 2-month treatment of 22-month-old mice with the dual FXR-TGR5 agonist INT-767 reverses the age-related increase in urinary albumin excretion and the significant decrease of the podocyte marker synaptopodin. These effects were comparable to those achieved with life-long caloric restriction which is known to protect against age-related comorbidities including loss of renal function (16, 17).

These improvements in renal function were associated with reversal of the age-related impairments in mitochondrial biogenesis and function. INT-767, like caloric restriction, increased the mitochondrial DNA content and complex I and complex IV activities. These improvements were associated with significant increases in phospho-AMPK, PGC-1α, and Sirtuin 3 protein expression in the kidney. Increased Sirtuin 3 expression was paralleled by increased protein expression of MCAD, an important mediator of mitochondrial fatty acid β-oxidation, and decreased acetyl-IDH2, an important mediator of mitochondrial oxidative phosphorylation. INT-767 also increased the expression of Sirtuin 1 mRNA as well as the mitochondrial transcription factor Nrf-1 and estrogen related receptor-α expression. Once again these effects were identical to those achieved by lifelong caloric restriction.

Interestingly the long-lived Ames mice display changes in the kidney like those induced by FXR-TGR5 activation and caloric restriction, including increases in FXR and TGR5 and the mitochondrial related Nrf-1, Sirtuin 1, PGC-1α, Sirtuin 3, ERR-α, and the Sirtuin 3 target LCAD, an important mediator of mitochondrial fatty acid β- oxidation.

In addition, in human podocytes cultured in the presence of serum from young versus old mice, INT-767 prevented increases in TNF-α, TLR2 and TLR4 induced by serum from aged mice, indicating a direct beneficial effect of FXR and TGR5 activation on cultured podocytes.

In summary, our results indicate that FXR and TGR5 are upregulated by caloric restriction and may act as caloric restriction mimetics. Indeed 2-month treatment of aged mice with the FXR-TGR5 dual agonist INT-767 could reverse age-related kidney disease, including age-related impairments in mitochondrial biogenesis and mitochondrial function. Our studies therefore indicate that FXR and TGR5 agonists may play an important role in prevention and/or reversal of age-related decline in renal function.

**EXPERIMENTAL PROCEDURES**

*Animal models*—1) Aging mice: 3 months old and 22 months old C57BL/6 mice fed ad lib and 22 months old C57BL/6 mice with caloric restriction were obtained through the NIA aging colony. The mice were continued to be fed ad lib with NIH31 diet or caloric restricted NIH31 diet as per the NIA instructions. A group of 22 months old ad lib fed mice were also fed with diet containing INT-767 (6α-ethyl-3α, 7α, 23-trihydroxy-24-nor-5-β-cholan-23 sulfate sodium...
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salt), the dual FXR-TGR5 agonist, 30 mg/kg body weight/day (26). 2) Ames Mice: Ames dwarf mice and their controls were acquired from Jackson Laboratories (Bar Harbor, ME) and studied at 21 months old.

Animal studies and relative protocols were approved by the Animal Care and Use Committees at the VA and University of Colorado AMC.

Urine chemistry-Urine albumin and creatinine concentrations were determined with kits (Exocell, Philadelphia, PA).

RNA extraction and quantitative real-time PCR and Western blotting-Quantitative real-time PCR was performed as previously described (21-24). Primer sequences are listed in Supplementary Table 1.

Western blotting-Cortical homogenate protein content was measured by BCA assay (Thermo Fisher Scientific, Waltham, MA). Equal amount of total protein was separated by SDS-PAGE gels and transferred onto PVDF membranes. The antibodies against p-AMPK/AMPK (catalogue numbers 4184 and 2795, Cell Signaling, Danvers, MA), PGC-1α (catalogue number AB3242, Millipore, Billerica, MA), and SIRT3 (catalogue number 5490, Cell Signaling) were used for Western blotting. After HRP-conjugated secondary antibodies, the immune complexes were detected by chemiluminescence captured on UVP Biospectrum 500 Imaging System (Upland, CA) and the densitometry was performed with ImageJ software. β-actin (catalogue number A5316, Sigma, St Louis, MO) was used as a loading control and all signals were normalized to β-actin signal.

Immunofluorescence microscopy-Frozen sections (4μm-thick) were used for immunostaining for synaptopodin (catalogue number S9442, Sigma) and fibronectin (catalogue number F3648, Sigma) and imaged with a laser scanning confocal microscope (LSM 780, Zeiss, Germany).

Mitochondrial complex activity assay-Mitochondrial fraction was isolated from the kidney as previously described (24) and used for the measurement of complex I (NADH dehydrogenase) and complex IV (cytochrome c oxidase) activity with kits from Abcam (Cambridge, UK).

Podocyte cell culture- Human podocytes obtained from Dr. Moin Saleem 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 (34-36). Podocyte differentiation is induced by thermo-shifting the cells from 33°C to 37°C for 7 days. The differentiated podocytes were then cultured in the presence of 5% of the 4-month old C57/BL6 mouse serum or 5% of the 28-month old mouse serum obtained from NIA to replace fetal bovine serum for 72 hours. In the last 24 hours, 10µM INT-767 was added to the treatment group.

Statistical analysis-Results are presented as the means ± SE for at least three independent experiments. Data were analyzed by ANOVA and Student-Newman-Keuls tests for multiple comparisons or by Student's t test for unpaired data between two groups. Statistical significance was accepted at the P < 0.05 level.

Conflict of Interest: Luciano Adorini and Mark Pruzanski are employees of Intercept. These studies were in part supported by a medical school IIS grant to Moshe Levi.

Author contributions: XXW and ML conceived and designed the whole study. XXW, YL, DW, ED performed the study and analyzed the data. XXW, LA, MP, and ML wrote the paper. All authors analyzed the results and approved the final version of the manuscript.
REFERENCES

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FOOTNOTES
These studies were supported by NIH R01 AG049493, NIH R01 DK098336, and Intercept ISS grant to Moshe Levi.

FIGURE LEGENDS

FIGURE 1. Short-term treatment with INT-767 reverses age-related kidney disease. A-B) Caloric Restriction prevents age-related decreases in renal FXR and TGR5 expression. C) INT-767 induces a significant decreased in urinary albumin, like life-long caloric restriction. D) INT-767 prevents the age-related decrease in synaptopodin expression, as determined by immunofluorescence microscopy. E) INT-767 prevents the age-related increases in transforming growth factor-β (TGF-β), fibroblast specific protein-1 (FSP-1) and fibronectin mRNA. F) Age-related increase in fibronectin protein expression as determined by immunofluorescence microscopy is prevented by INT-767 treatment. (N= 6 mice. * p< 0.05 vs. 5mo-AL; ** p< 0.05 vs. 24mo-AL).

FIGURE 2. INT-767 treatment increases mitochondrial biogenesis and function. A) INT-767 increases mitochondrial to nuclear DNA ratio and mRNA level of nuclear respiratory factor 1 (NRF1) in aging kidneys. B) INT-767 treatment increases the activated AMPK level, and prevents the age-related decreases in SIRT1 mRNA and the nuclear hormone receptor estrogen related receptor-alpha (ERR-α) mRNA. C) INT-767 prevents the age-related decrease in PGC-1α mRNA and protein, mitochondrial SIRT3 mRNA and protein and its target medium-chain Acyl-CoA dehydrogenase (MCAD) protein. D) INT-767 reverses the age-related increase in acetylated form of mitochondrial isocitrate dehydrogenase as shown in the Western blot. 2-month INT-767 treatment also reverses the decreased mitochondrial complex I and complex IV activity in aged kidneys. (N= 6 mice. * p< 0.05 vs. 5mo-AL; ** p< 0.05 vs. 24mo-AL).

FIGURE 3. INT-767 has direct effects in cultured human podocytes. The expression of TNF-α, TLR2 and TLR4 are increased in the aging serum-conditioned human podocytes but this effect is abolished by treatment with INT-767. (N= 3. * p< 0.05 vs. young).
Figure 1

A

FXR

mRNA relative level

TGR5

mRNA relative level

5mo-AL  24mo-AL  24mo-CR

B

FXR protein

5mo-AL  24mo-AL  24mo-CR

FXR

β-actin

Densitometry unit

5mo-AL  24mo-AL  24mo-CR
Figure 1

C

Albuminuria

Albumin/creatinine ratio (mg/g)

0 50 100 150

5mo-AL 24mo-AL 24mo-AL-INT 24mo-CR

D

Synaptopodin

AL-young AL-old

CR-old AL-old-INT
Figure 1

E11

TGF-β

Fibronectin

FSP-1

mRNA relative level

mRNA relative level

mRNA relative level
Figure 1

Fibronectin

5mo-AL  24mo-AL

24mo-AL-INT  24mo-CR
Figure 2

A

Mitochondria to nuclear DNA ratio

[Graph showing the ratio for different conditions: 5mo-AL, 24mo-AL, 24mo-AL-INT, 24mo-CR.]

Nrf-1

[mRNA relative level for the same conditions as above.]

* and ** indicate statistical significance.
Figure 2

B

- **pAMPK** and **AMPK** 60kDa bands from 5mo-AL, 24mo-AL, 24mo-AL-INT, and 24mo-CR.

- **pAMPK/AMPK** densitometry units for 5mo-AL, 24mo-AL, 24mo-AL-INT, and 24mo-CR.

- **Sirt1** mRNA relative levels for 5mo-AL, 24mo-AL, 24mo-AL-INT, and 24mo-CR.

- **ERRα** mRNA relative levels for 5mo-AL, 24mo-AL, 24mo-AL-INT, and 24mo-CR.

Statistical significance indicated by asterisks (*p < 0.05, **p < 0.01**).
Figure 2

C

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**PGC1α/β-actin**

* * *

**MCAD/β-actin**

* * *
Figure 2

C

PGC1α

Sirt3

mRNA relative level

5mo-AL 24mo-AL 24mo-AL-INT 24mo-CR

mRNA relative level

5mo-AL 24mo-AL 24mo-AL-INT 24mo-CR
Figure 2

Acetyl-IDH2/IDH2 protein

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Densitometry unit

Complex I Activity

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Complex IV Activity

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Figure 3

**TNF-α**

- Young: 0.8
- Old: 1.0
- Old-INT: 1.2

**TLR2**

- Young: 100
- Old: 150
- Old-INT: 200

**TLR4**

- Young: 0.0
- Old: 0.2
- Old-INT: 0.4
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