mTOR Plays a Critical Role in p53-induced Oxidative Kidney Cell Injury in HIVAN


Department of Medicine, Feinstein Institute for Medical Research, Hofstra North Shore LIJ Medical School, Department of Pathology, New York Medical College, New York, USA, and University of Bristol, Bristol, UK

Address for correspondence:
Pravin C. Singhal, M.D.
Division of Kidney Diseases and Hypertension
100 Community Drive
Great Neck, NY 11021
Tel. 516-465-3010
516-465-3011
psinghal@nshs.edu

Running head: mTOR and p53 in HIVAN

Copyright © 2013 by the American Physiological Society.
Abstract

Oxidative stress has been implicated to contribute to HIV-induced kidney cell injury; however, the role of p53, a modulator of oxidative stress, has not been evaluated in the development of HIV-associated nephropathy (HIVAN). We hypothesized that mammalian target of rapamycin (mTOR) may be critical for the induction of p53 mediated oxidative kidney cell injury in HIVAN. To test our hypothesis, we evaluated the effect of an mTOR inhibitor, rapamycin, on kidney cell p53 expression, downstream signaling, and kidney cell injury both in in vivo and in vitro studies. Inhibition of the mTOR pathway resulted in Tg26 mice down regulation of renal tissue p53 expression, associated downstream signaling, and decreased number of sclerosed glomeruli, tubular microcysts, apoptosed, and 8-OHdG +ve cells in Tg26 mice. mTOR inhibition not only attenuated kidney cell expression of p66ShcA and phospho-p66ShcA but also reactivated redox-sensitive stress response program in the form of enhanced expression of MnSOD and catalase. In in vitro studies, the mTOR inhibitor also provided protection against HIV-induced podocyte apoptosis. Moreover, mTOR inhibition down regulated HIV-induced podocyte (HP/HIV) p53 expression. Since HP/HIV silenced for mTOR displayed lack of expression of p53 as well as attenuated podocyte apoptosis, this suggests that mTOR is critical for kidney cell p53 activation and associated oxidative kidney cell injury in HIV milieu.
HIV-associated nephropathy is the manifestation of specific genetic (Apol1), environmental (HIV infection), and host factors (2,10,17,34,45). On that account, HIVAN patients are being treated with highly active anti-retroviral therapy (HAART). Since the advent of HAART, there has been significant decrease in the number of HIVAN patients (2). Nonetheless, in a recent report, 50% of HIVAN patients progressed to end stage kidney disease (ESKD) despite being on HAART (4). Therefore, treatment of HIVAN still poses a challenge.

Patients with HIV infection display an imbalance in oxidative stress response in the form of lower levels of antioxidants- ascorbic acid, tocopherols, and selenium (7,12, 13), and higher levels of malondialdehyde and hydroperoxides (14, 40). Induction of oxidative stress had contributed to HIV disease pathogenesis in multiple ways: enhanced viral replication (31, 42), increased inflammatory immune response (15), and accelerated loss of immune function (44). The role of oxidative stress in the development of HIVAN is also being increasingly recognized (20, 38, 39).

We have previously reported the role of p66ShcA pathway in the deactivation of redox-sensitive redox program by HIV in both podocytes and tubular cells (38, 39). p53 has been shown to be upstream of p66ShcA signaling and known to activate the promoter of p66ShcA (47): P53-null mice displayed attenuated p66ShcA expression and diminished ROS generation (47). Interestingly, down regulation of p66ShcA in this model was not associated with enhanced longevity; on the contrary, these mice died prematurely because of enhanced carcinogenesis (47). Unlike p53-null mice, p66ShcA knockout mice not
only displayed attenuated ROS generation but also had a longer life span (30, 32). It has been suggested that p53 only induced ROS generation signaling through p66ShcA (47); whereas, its downstream signaling pertaining to tumor suppression was not mediated through p66ShcA pathway. We asked whether activation of p53 in HIVAN was also associated with activation of p66ShcA pathway.

The mTOR pathway plays an important role in the development and progression of HIVAN (25). We have previously demonstrated that kidney cells displayed enhanced expression of phospho-mTOR both in HIVAN mice and HIVAN patients (25, 35). In *in vivo* studies, inhibition of mTOR pathway slowed down the progression of HIVAN; whereas in *in vitro* studies inhibition of mTOR pathway prevented HIV-induced tubular cell growth. Recently, we demonstrated the role of oxidative stress in the development HIV-induced kidney cell injury (20, 38, 39). We now hypothesize that mTOR pathway may be modulating HIV-induced kidney cell injury by down regulating p53-mediated oxidative stress.

P53 plays a central role in responding to DNA damage and inhibits mTOR pathway in stressed cells (18). P53 inhibits mTOR by activation of AMPK and subsequent regulation of the TSC1/TSC2 complex (21). Inactivation of either TSC1/TSC2 complex or AMPK inhibits the impact of p53 on mTOR pathway (21). Through inhibition of mTOR, p53 could regulate inhibition of translation and activation of autophagy; thus, communication between p53 and mTOR pathway may be contributing to normal cell growth and proliferation. Conversely, HIV
promoted lymphocyte apoptosis through mTOR-mediated activation of p53 pathway (8, 9).

In the present study, we evaluated the effect of inhibition of the mTOR pathway on renal tissue p53 expression and associated down stream signaling in HIVAN (Tg26) mice. In addition, we studied the contributory role of mTOR-mediated p53-induced kidney cell injury.
Methods and Materials

HIV transgenic (Tg26) mice

We have used age and sex matched FVB/N (control) and Tg26 (with FVB/N background) mice. Breeding pairs of FVBN mice were obtained from Jackson Laboratories (Bar Harbor, ME). Breeding pairs to develop Tg26 colonies were kindly gifted by Prof. Paul E. Klotman M.D., President and CEO, Baylor College of Medicine, Houston, TX). Tg26 transgenic animals have the proviral transgene, pNL4-3: d1443, which encodes all the HIV-1 genes except $gag$ and $pol$ rendering the mice noninfectious.

The Ethics Review Committee for Animal Experimentation of Long Island Jewish Medical Center approved the experimental protocol.

Experimental studies

Three week old Tg26 and FVBN (control) mice ($n=6$) were administered either normal saline (Tg26) or rapamycin (5 mg/Kg every other day, intraperitoneal; Tg26/Rapa) for eight weeks and then were anesthetized and sacrificed. Both kidneys were excised; one was processed for histological studies while the other was used for RNA and protein extraction. Three-micrometer sections were prepared and stained with hematoxylin-eosin and Periodic-Acid Schiff (PAS). Renal sections were coded and examined under light microscopy. Twenty random fields (20X)/mouse were examined to score percentage of the involved glomeruli and tubules. Glomerular lesions were classified as segmental glomerulosclerosis (SGS), global glomerulosclerosis (GGS), and collapsing glomerulosclerosis (CGS). Severity of tubular injury was scored in the form of
tubular dilatation (% of dilated tubules/section) and size of microcysts (1+ to 4+)
by two investigators who were unaware of the experimental conditions.

**Preparation of podocytes**
Human podocytes were prepared and transduced with HIV (NL4-3) as described previously (16).

**TUNEL Assay**
TUNEL assay was performed using Apoptosis Detection Tacs TdT Kit (R&D System, Minneapolis, MN) as described previously (16).

**Immunohistochemical studies**
Renal cortical sections from control and HIV-1 transgenic (Tg26) mice were de-paraffinised and immunolabeled for 8-OHdG, phospho-p66ShcA and TUNEL as described previously (20, 38, 39).

**Silencing of mTOR/p53**
HIV/HPs (HIV-transduced podocytes) were transfected with 25 nM mTOR/p53-siRNA (Cell Signaling) with Siport Neofax transfection reagent and suspended in optiMEM media for 24 hrs. Control and transfected cells were used under control and experimental conditions.

**Western blotting studies**
Control and experimental cells or renal tissues were lysed and Western blots were prepared and probed for p53, p66ShcA (total), phospho-p66ShcA, p21, p27, GADD45, and Fas. Protein transfer on nitrocellulose blots were checked by Ponceau Red dye. Equal protein loading was confirmed by stripping and probing the same blots for actin.
**Reverse Transcription PCR Analysis**

Control (HP; transduced with empty vector) and experimental (HIV/HP) cells and renal tissues from Tg26 and Tg26/Rapa mice were used to quantify mRNA expression of p53, p21, p27, GADD45, MnSOD, and catalasae. RNA was extracted using TRIzol (Invitrogen corp.). For cDNA synthesis, 2 μg of the total RNA was preincubated with 2 nmol of random hexamer (Invitrogen Corp) at 65°C for 5 min. Subsequently, 8μl of the reverse-transcription (RT) reaction mixture containing cloned AMV RT, 0.5 mmol each of the mixed nucleotides, 0.01 mol dithiothreitol, and 1000 U/mL RNase out (Invitrogen Corp) was incubated at 42°C for 50 min. For a negative control, a reaction mixture without RNA or reverse transcription (RT) was used. Samples were subsequently incubated at 85°C for 5 min to inactivate the RT.

Quantitative PCR was carried out in an ABI Prism 7900HT sequence detection system using the primer sequences (mouse) as shown below:
<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Reference Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase</td>
<td>F GCTGAAGTTGAACAGAGATG</td>
<td>NM_009804.2</td>
</tr>
<tr>
<td></td>
<td>R GTCATCACGCTGAGTCTG</td>
<td></td>
</tr>
<tr>
<td>MnSOD</td>
<td>F GACCTGCCTACGACTATG</td>
<td>NM_013671.3</td>
</tr>
<tr>
<td></td>
<td>R GACCTTGCTCCTTATGGAAGC</td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td>F GCAACTATGGCTTCCACCTG</td>
<td>NM_011640.3</td>
</tr>
<tr>
<td></td>
<td>R CTCCTGACATGTCGACT</td>
<td></td>
</tr>
<tr>
<td>P21</td>
<td>F GTGATTGCAGAGCCGTCAATG</td>
<td>NM_00111099.1</td>
</tr>
<tr>
<td></td>
<td>R TCTCTTGACAGAAGCCATC</td>
<td></td>
</tr>
<tr>
<td>P27</td>
<td>F AGGAGAGCGAGATGTGGTAGC</td>
<td>NM_009875.4</td>
</tr>
<tr>
<td></td>
<td>R CAGAGTTGCTGAGACCCAA</td>
<td></td>
</tr>
<tr>
<td>Fas L</td>
<td>F CAGCTCTCCACCTCGAGAGG</td>
<td>NM_010177.4</td>
</tr>
<tr>
<td></td>
<td>R AGATCCCTCAAAAATGATCGAGA</td>
<td></td>
</tr>
<tr>
<td>GADD45</td>
<td>F AATATGACTTTGGAGGAATTC</td>
<td>NM_007836.1</td>
</tr>
<tr>
<td></td>
<td>R ATTCGGATGCCATCACGCGTTC</td>
<td></td>
</tr>
<tr>
<td>p53 (Human)</td>
<td>F GTTCCGAGAGATGGATGAGG</td>
<td>Accession:X02469</td>
</tr>
<tr>
<td></td>
<td>R TCTGAGTCCCTTGATGTGAG</td>
<td>M60950</td>
</tr>
<tr>
<td>MnSOD (Human)</td>
<td>F AGTTCAATGGTGAGTTGATGAT</td>
<td>NG_008729.1</td>
</tr>
<tr>
<td></td>
<td>R CAAATCCCACAGTGGATTA</td>
<td>BC_012423.1</td>
</tr>
</tbody>
</table>

SYBR green was used as a detector and ROX as a stabilizing dye. Results (means ± S.D.) represent number of cell samples or animals as described in the legend. The data were analyzed using the Comparative $C_T$ method ($\Delta\Delta^C_T$ method). Differences in $C_T$ are used to quantify relative amount of PCR target contained within each well. The data were expressed as relative mRNA expression in reference to control, normalized to quantity of RNA input by performing measurements on an endogenous reference gene, GAPDH.

**Statistical analysis**

For comparison of mean values between two groups, the unpaired t test was used. To compare values between multiple groups, analysis of variance
(ANOVA) and a Bonferroni multiple range test were used to calculate the p-value. Statistical significance was defined as $P<0.05$. All values are displayed as means ± SD.
Results

**Tg26 mice display kidney cell oxidative damage, apoptosis and HIVAN phenotype**

Renal cortical sections of control and Tg26 mice were immunolabeled for 8-hydroxy deoxy guanosine (8-OHdG) and TUNEL assay. Representative microphotographs are shown in Fig. 1. Tg26 mice displayed enhanced number of 8-OHdG +ve cells (Fig. 1B) as well as increased number of apoptosed kidney cells/field (Fig. 1D) when compared to control mice (Figs 1A and 1C). Renal cortical sections of control and Tg26 mice were also stained with Periodic Acid Schiff Reagent (PAS) and evaluated for renal histology. Representative microphotographs of renal cortical sections of a control and a Tg26 mouse are shown in Figs. 1E and 1F, respectively. The Tg26 mouse displayed a sclerosed glomerulus and a tubular microcyst.

**Renal tissues of Tg26 mice displayed enhanced expression of p53 and down stream signaling molecules**

Cellular lysates of renal tissues of control and Tg26 mice were electrophoresed and probed for p53, p21, p27, GADD45, and Fas. The same blots were reprobed for actin. Representative gels displaying renal tissues from two different mice are shown in Fig. 2. Cumulative data are shown in bar graphs (n=3). Renal tissues of Tg26 mice displayed enhanced expression of p53 (Figs 2A and 2B). Similarly, renal tissues of Tg26 mice displayed enhanced expression of p21 (Figs 2C and 2D), p27 (Figs. 2E and 2F), GADD45 (Figs. 2G and 2H) and Fas (Figs. 2I and
2J) when compared to respective control mice. These findings indicate that there is an activation of p53-mediated down stream signaling in renal tissues of HIVAN mice.

**mTOR inhibition attenuates HIV-induced kidney cell expression of p66shcA in HIVAN mice**

We previously reported the role of p66ShcA pathway in HIV-induced oxidative kidney cell injury both *in vitro* and *in vivo* studies (20, 39). To determine the role of mTOR in HIV-induced kidney cell injury, renal cortical sections of control, Tg26, and Tg26/Rapa were immunolabeled for p66ShcA (total), phospho-p66ShcA. Representative microphotographs are shown in Fig. 3A. Tubular cells in Tg26 mice displayed enhanced expression of both p66ShcA and phospho-p66ShcA; however, renal cortical sections of control and Tg26/Rapa (rapamycin) reveal minimal (background) expression of phospho-p66ShcA. To quantify renal tissue p66ShcA (both total and phosphorylated fractions), preparations of renal tissue lysates from three different animals were electrophoresed and probed for total p66ShcA (Fig. 3B) and phospho-p66ShcA (Fig. 3C). The same blots were reprobed for actin (since HIV infection as well as mTOR inhibition altered expression of total p66ShcA, we have used actin to indicate the protein loading). Representative gels are shown in Figs. 3B and 3C. Cumulative data are shown in bar graphs. These findings indicated that mTOR inhibition had the potential to inhibit p66ShcA expression in HIVAN mice.
mTOR inhibition attenuates oxidative DNA damage in kidney cells in HIVAN mice

If mTOR could inhibit oxidative stress pathway in HIVAN mice, we asked whether mTOR also provided protection against HIV-induced oxidative kidney cell damage (38). To determine the effect of mTOR inhibition on oxidative DNA damage, renal cortical sections of control, Tg26, and Tg26/Rapa were immunolabeled for 8-OHdG. Representative microphotographs are shown in Fig. 4A. Cumulative data in the form of 8-OHdG +ve cells/tubule are shown in Fig 4B. These findings indicate that mTOR inhibition provides protection against HIV-induced oxidative kidney cell DNA damage.

mTOR inhibition attenuated kidney cell apoptosis in HIVAN mice

If mTOR inhibition was associated with diminished oxidative kidney cell DNA damage in HIVAN mice, we expected a decreased number of apoptosed kidney cells in Tg26/Rapa mice. Renal cortical sections of control, Tg26, and Tg26/Rapa were stained for TUNEL +ve cells. Representative microphotographs are shown in Fig. 5A. Cumulative data of TUNEL +ve cells/field are shown in Fig. 5B (n=3). Renal cortical sections of Tg26 mice displayed increased number of TUNEL +ve cells/field; however, mTOR inhibition decreased number of TUNEL +ve cells/field in Tg26/Rapa mice.

mTOR inhibition attenuates renal lesions in HIVAN mice
Renal cortical sections of control, Tg26, and Tg26/Rapa were evaluated for the severity of renal lesions. Representative microphotographs are shown in Fig. 6A. Cumulative data in terms of % glomeruli showing sclerosis and collapsing phenotype are shown in Fig. 6B. Control mice did not display any glomerular or tubular lesions. Tg26 mice displayed both sclerosed (segmental glomerular sclerosis, SGS), collapsed (collapsing glomerular sclerosis, CGS) and globally sclerosed (global glomerular sclerosis (GGS) glomeruli. Tg26 mice also showed tubular dilatation with microcyst formation; whereas, rapamycin-receiving Tg26 mice displayed only a few sclerosed glomeruli (SGS and CGS) and none with global sclerosis (GGS). Similarly, rapamycin-receiving mice displayed only occasional dilated tubules and significant decrease in microcyst size.

**mTOR inhibition attenuates renal tissue expression of p53 and associated down stream signaling in HIVAN mice**

To determine the effect of mTOR inhibition on p53 pathway, renal tissue lysates of control, Tg26, and Tg26/rapa were electrophoresed and probed for p53 and reprobed for actin. Representative gels from two different mice are displayed in Fig. 7A. Cumulative data in bar graphs are also shown (n=3). Renal tissues of Tg26/rapa mice displayed attenuated p53 expression when compared with Tg26 mice.

To determine the effect of mTOR inhibition on p53-mediated down stream signaling, protein blots of renal tissues of control, Tg26, Tg26/Rapa were probed for p21, p27 and Fas proteins. The same blots were reprobed for actin.
Representative gels from two different mice are shown in Figs. 7B, 7C and 7D, respectively. Cumulative data in bar graphs (n=3) are shown in Figs 7B, 7C, and 7D. Renal tissues of Tg26 mice displayed enhanced expression of p21, p27 and Fas; whereas, mTOR inhibition attenuated expression of p21, p27 and Fas in Tg26/Rapa mice.

To determine the effect mTOR inhibition on renal tissue transcription of p53 and associated down stream molecules, total RNAs were extracted from renal tissues of control, Tg26, and Tg26/Rapa mice (n=3) and probed for p53, p21, p27, GADD45. As shown in Fig. 8A, renal tissues of Tg26 mice displayed enhanced p53 mRNA expression; whereas, mTOR inhibition attenuated this effect in Tg26/Rapa mice. Similarly, mTOR inhibition attenuated expression of p21 (Fig. 8B), p27 (Fig. 8C), and GADD45 (Fig. 8D) in Tg26/Rapa mice.

These findings indicate that mTOR not only inhibits renal tissue transcription and protein expression of p53, but also down regulates p53-mediated down stream signaling in HIVAN mice.

**mTOR inhibition enhances renal tissue expression of antioxidants and down regulates expression of proapoptotic molecules**

Since mTOR inhibition down regulated renal tissue p66ShcA phosphorylation in HIVAN mice, we expected that it would also reactivate redox-sensitive stress response program in HIVAN mice. To quantify the transcription of antioxidants, total RNA was extracted from renal tissues from control, Tg26, and Tg26/Rapa
mice and probed for MnSOD and catalase by real time PCR studies. As expected renal tissues from Tg26 mice displayed attenuation of mRNA expressions of MnSOD (Fig. 9A) and catalase (Fig. 9B). On the other hand, mTOR inhibition enhanced the expression of both MnSOD and catalase in HIVAN mice. These findings indicate that mTOR inhibition has the potential to reactivate redox-sensitive stress response program in HIVAN mice.

To determine the effect of mTOR inhibition on HIV-induced expression of pro-apoptotic molecules, total RNA was extracted from renal tissues of control, Tg26, and Tg26/Rapa mice and probed for FasL (Fas ligand) by real time PCR studies. As shown in Fig. 9C, renal tissues from Tg26 mice displayed enhanced transcription of FasL, whereas, mTOR inhibition attenuated FasL expression in Tg26/Rapa mice.

**mTOR is critical for podocyte p53 expression**

To silence mTOR in human podocytes (HP), HPs were transfected with either scrambled siRNA (SCR) or mTOR-siRNA. Protein blots of control, HP/SCR and HP/mTOR-siRNA were probed for mTOR. The same blot was reprobed for actin. Representative gel and densitometric analysis are shown in Fig. 10A.

To determine whether mTOR is critical for podocyte p53 expression in HIV milieu, protein blots of control, HIV-transduced HP (HP/HIV), mTOR-siRNA-transfected HIV/HP, scrambled siRNA- transfected HIV/HP were probed for p53 and reprobed for actin. Cumulative data are shown in bar graphs (n=3). HP/ HIV
displayed enhanced expression of p53, whereas, HP/HIV lacking mTOR
displayed attenuated expression of p53 (Fig. 10B). These findings indicate that
mTOR expression is critical for HIV-induced podocyte p53 expression.

To determine the effect of mTOR inhibition on podocyte transcription of p53 in
HIV milieu, HP and HP/HIV were incubated in media with or without rapamycin (1
ng/ml) for 48 hours. Total RNA was extracted and probed for p53 (n=3). As
shown in Fig. 10C, HIV enhanced p53 mRNA expression in podocytes; however,
rapamycin attenuated this effect.

To determine the effect of mTOR inhibition on podocyte transcription of HIV-
mediated down regulation of MnSOD, podocytes-treated with above mentioned-
conditions underwent RNA extraction followed by probing for MnSOD. As
expected HIV down regulated podocyte expression of MnSOD; however,
rapamycin inhibited this effect (Fig.10D).

**mTOR inhibition attenuates HIV-induced podocyte apoptosis**

HPs were grown on coverslips and then transduced with either empty vector or
HIV in the presence or absence of rapamycin (1 ng/ml). After 48 hours, cells
were assayed for apoptosis by TUNEL assay (n=3). HP/HIV showed a greater
number of TUNEL +ve cells (P<0.01) when compared with the HP. However,
proapoptotic effect of HIV was inhibited (P<0.05) by rapamycin (HP, 1.5 ± 0.6;
HP/HIV, 8.2 ± 1.1; HP/Rapa, 2.1 ± 0.8; HP/HIV/Rapa, 3.4 ± 1.2 %TUNEL +ve
cells/field, n=6).
To determine whether HIV-induced podocyte apoptosis was mediated through p53, HP/HIV were transfected with either p53-siRNA (HP/HIV-p53siRNA) or scrambled siRNA (HP/HIV-SCR). Control, HP/HIV, HP/HIV-p53siRNA, and HP/HIV-SCR were evaluated for % apoptosed cells by TUNEL assay. HP/HIV displayed greater % (P<0.01) of apoptosed cells when compared to control (Control, 2.8 ± 0.5% vs. HP/HIV, 22.8 ± 3.5% apoptosed cells/field; n=3). On the other hand, HP/HIV lacking p53 displayed decreased % (P<0.01) of apoptosed cells when compared to HP/HIV (HP/HIV, 22.8 ± 3.5% vs. HP/HIV-p53siRNA, 9.0 ± 0.8% apoptosed cells, n=3). These findings indicate that HIV induces podocyte apoptosis partially through p53 expression.
Discussion

In the present study, renal tissues of HIVAN mice displayed enhanced p53 expression and associated down stream signaling. The latter was associated with enhanced kidney cell oxidative kidney cell damage, apoptosis, glomerulosclerosis and microcyst formation. On the other hand, mTOR inhibition in HIVAN not only down regulated renal tissue p53 expression and down stream signaling, but also provided protection against p53 mediated oxidative kidney cell injury. Additionally, kidney cells in HIVAN mice displayed enhanced phosphorylation of p66ShcA; however, the mTOR inhibition not only down regulated phosphorylation of p66ShcA but also reactivated redox-sensitive response program in the form of enhanced expression of MnSOD and catalase.

In *in vitro* studies, HIV promoted podocyte apoptosis but mTOR inhibition attenuated this effect of HIV. Similarly, podocytes lacking p53 displayed resistance to apoptosis in HIV milieu. Since HIV/HP after silencing for mTOR also displayed attenuated expression of p53, there seems to be a causal relationship between mTOR and kidney cell p53 expression and associated oxidative kidney cell injury in HIV milieu.

mTOR signaling contributes towards maintenance of normal physiological as well as development of pathological states. For example, it is essential for growth and development but also contributes to the development of diabetes, cardiac hypertrophy, malignancies, neurodegenerative syndromes, and aging (43, 51, 53). In experimental animal models, inhibition of mTOR signaling increases
lifespan (5, 49). Caloric restriction, an intervention that extends life span, has also been implicated to work through mTOR pathway (41). The mTOR modulation late in life also increases mouse life span. Since oxidative stress is the major player in aging it is likely that mTOR inhibition might have served as anti-oxidative therapy. In the present study too, mTOR inhibition mitigated HIV-induced oxidative kidney cell injury.

P53 has been reported to cause inhibition of mTOR activation, whereas, mTOR has been reported to activate p53-mediated downstream signaling (8, 9). Interestingly, mTOR pathway has been reported to contribute to the pathogenesis of diabetic and HIV-associated nephropathy (25, 26). Moreover, the effector molecules of these entities- glucose and HIV- have also been reported to activate both kidney cell mTOR and p53 pathways (23, 35, 36, 46). However, there is no data on cross talk between mTOR and p53 in the development of these diseases. Since both glucose and HIV have been reported to enhance ROS generation by kidney cells, it is likely that ROS induced kidney cell DNA damage might have stimulated up regulation of p53 pathway (38). Additionally, both glucose and HIV have been demonstrated to activate the renin angiotensin system, which has been reported to induce oxidative damage in a variety of cells including kidney cells (3, 11, 38). However, the present study suggests a direct relationship between mTOR and p53 pathways. On that account, inhibition of mTOR pathway also down regulated not only the expression of p53 but also attenuated the downstream signaling and the outcome of the p53 activation.
p53 has been reported to be the modulator of oxidative stress - it down regulates low oxidative stress and exacerbates higher levels of stresses (22). For example, at low levels of oxidative stress, p53 activates antioxidant genes such as sestrin and glutathione peroxidase (1, 6, 16). Additionally, p53 induces the expression of TP53-induced glycolysis and apoptosis regulator, which slows glycolysis and promotes the production of NADPH to decrease ROS levels (50). Moreover, p53 suppresses the expression of phosphoglycerate mutase (PGM), which diminishes mitochondrial oxidative respiration and thus, attenuates ROS generation (24). Oxidative DNA damage stimulates p53, which further enhances expression of p21 and p27 to provide time to repair damaged DNA in injured cells (11). On the other hand, at higher levels of oxidative stress, p53 induces pro-oxidative genes including p53-inducible gene 3 (PIG3) and proline oxidase (27, 28). Additionally, it causes apoptosis by enhancing expression of BAX and PUMA (19). These roles of p53 suggest that severity of oxidative stress determines whether p53 will contribute or provide protection against cellular injury. In the present study, p53 contributed to HIV-induced oxidative stress by enhancing phosphorylation of p66ShcA and deactivating the redox-sensitive stress response in the form of attenuation of MnSOD and catalase expression in HIVAN mice.

We previously reported that HIV deactivated redox-sensitive stress response program in kidney cells in vitro and in vivo studies (20, 39). In these studies HIV stimulated kidney cell phosphorylation of p66ShcA and associated down stream phosphorylation of Foxo3A. The activation of the latter pathway
was associated with attenuated generation of MnSOD and catalase (39). It was suggested that attenuated generation of free radical scavengers in HIV milieu causes accumulation of ROS and associated oxidative stress kidney cell injury in HIVAN. In our current study also, kidney cells displayed enhanced oxidative DNA damage and thus confirming the occurrence of ongoing of HIV-induced ROS-mediated kidney cell injury in HIVAN. On the other hand, mTOR inhibition not only enhanced renal tissue expression of MnSOD and catalase in HIVAN mice but also decreased oxidative kidney cell DNA damage. These findings indicate that mTOR inhibition has potential to reverse HIV-induced deactivation of redox-sensitive stress response program in HIVAN mice.

Several chemotherapeutic agents have been reported to induce p53-mediated apoptosis through the activation of FasR-FasL pathway (29, 48, 52). These studies suggested that presence of p53 was critical for expression of FasR in these cells (29). These investigators identified a p53-responsive element within the first intron of the FasR gene, and three putative elements within the promoter. The intronic element conferred transcriptional activation by p53 and cooperated with p53-responsive elements in the promoter of the FasR gene. In the present study the activation of p53 pathway was associated with renal tissue expression of FasR. The latter seems to be involved in the induction of kidney cell apoptosis in HIVAN mice.
FasL pathway has been reported to contribute to kidney cell apoptosis in HIVAN (37). In these studies the activation of FasL was attributed to HIV-induced kidney cell NF-κB activation (37). We also demonstrated that HIV enhanced podocyte NF-κB activation (33). Interestingly, mTOR inhibition attenuated HIV-induced kidney cell NF-κB activation (33). In the present study, renal tissues of HIVAN mice also displayed upregulation of FasL. The mTOR, not only inhibited renal tissue expression of FasL in HIVAN mice but also decreased number of apoptosed number of cells. It was likely that this effect of mTOR on inhibition of renal tissue FasL expression was mediated through modulation of NF-κB activation in HIV milieu.

We have summarized a proposed schematic mechanism delineating the role of mTOR in p53 mediated oxidative kidney cell injury in Fig. 11. Since we have previously reported the role of HIV in the activation of mTOR pathway in kidney cell both in vivo (25) and in vitro (35), we did not emphasize this aspect in the present manuscript. Also, we have previously reported the role of HIV in the activation of p66ShcA pathway and deactivation of redox-sensitive response program both in podocytes (20) and tubular cells (39).

We conclude that mTOR inhibition provides protection against p53-induced oxidative damage in HIVAN. The current study provides mechanistical insight into the pathogenesis of HIVAN and also provides a basis for the therapeutic strategies for treatment of HIVAN.
Acknowledgement

This work was supported by grants RO1DK084910 and RO1 DK083931 (PCS) from National Institutes of Health, Bethesda, MD.
References


contributes to early cellular response to DNA damage. Oncogene. 29:1431-50, 2010


41. Speakman JR, Mitchell SE. Caloric restriction. Mol Aspects Med. 32:159-221, 2011


Figure Legends

1. Tg26 mice display kidney cell oxidative damage, apoptosis and HIVAN phenotype

Renal cortical sections of control and Tg26 mice were stained with PAS and immunolabeled for 8-hydroxy deoxy guanosine (8-OHdG) and TUNEL. Representative microphotographs are shown.

A. Renal cortical section from a control mouse displaying 8-OHdG +ve cells (darkly stained nuclei)

B. Renal cortical section from a Tg26 mouse displayed enhanced number of 8-OHdG +ve cells.

C. Renal cortical section from a control mouse stained for TUNEL.

D. Renal cortical section from a Tg26 mice displaying TUNEL +ve cells (indicated by arrows).

E. Renal cortical section from a control mouse displaying a normal glomerulus and tubules.

F. Renal cortical section from a Tg26 mouse displaying a sclerosed glomerulus (indicated by an arrow) and a microcyst (indicated by an arrowhead).

2. Renal tissues of Tg26 mice displayed enhanced expression of p53 and down stream signaling molecules
Cellular lysates of renal tissues of control and Tg26 mice were electrophoresed and probed for p53, p21, p27, GADD45, and Fas. The same blots were reprobed for actin.

A. Representative gels displaying renal tissue expression of p53 from two control (FVBN) and two Tg26 mice.

B. Densitometric data in the form of a bar diagram (n=3). **P<0.01 compared to FVBN.

C. Representative gels displaying renal tissue expression of p21 from two control (FVBN) and two Tg26 mice.

D. Densitometric data in bar graphs (n=3). *P<0.05 compared to FVBN

E. Representative gels displaying renal tissue expression of p27 from two control (FVBN) and two Tg26 mice.

F. Cumulative data in the form of a bar diagram (n=3). **P<0.01 vs. FVBN.

G. Representative gels displaying renal tissue expression of GADD45 from two control (FVBN) and two Tg26 mice.

H. Cumulative data are shown in bar graphs (n=3). *P<0.05 vs. FVBN

I. Representative gels displaying renal tissue expression of Fas from two control (FVBN) and two Tg26 mice.

J. Densitometric analysis is shown in bar graphs (n=3). **P<0.01 vs. FVBN

3. mTOR inhibition attenuates HIV-induced kidney cell expression of p66shcA in HIVAN mice
Renal cortical sections of control, Tg26, and Tg26/Rapa were immunolabeled for total p66ShcA and phospho-p66ShcA.

A. Representative microphotographs of renal cortical sections from a control, Tg26, and Tg26/Rapa mice. Control and Tg26/Rapa mice displayed mild staining for p66ShcA in both glomerular and tubular cells; whereas, Tg26 mice displayed moderate staining in tubules. Both control and Tg26/Rapa displayed minimal staining (background) of kidney cells for phospho-p66ShcA. On the other hand tubular cells displayed expression of phospho-p66ShcA (Darkly staining cells) in a Tg26 mouse.

B. Protein blots from control, Tg26, and Tg26/Rapa were probed for p66ShcA (n=3). The same blots were reprobed for actin. Representative gels are shown (from 3 different lysates). The upper lane displays renal tissue expression of p66ShcA (total) by control and experimental mice. The lower panel displays actin expression by renal tissues under similar conditions. Bar diagram displays densitometric analysis (n=3).

C. Protein blots from control, Tg26, and Tg26/Rapa were probed for phospho-p66ShcA$^{\text{Ser36}}$ (n=3). The same blots were reprobed for actin. Representative gels are shown. The upper lane displays renal tissue expression of phospho-p66ShcA$^{\text{Ser36}}$ by control and experimental mice. The lower panel displays actin expression by renal tissues under similar conditions. Bar diagram displays densitometric analysis (n=3).
mTOR inhibition attenuates oxidative DNA damage in kidney cells in HIVAN mice

Renal cortical sections of control, Tg26, and Tg26/Rapa were immunolabeled for 8-OHdG.

A. Representative microphotographs displaying 8-OHdG +ve cells (darkly stained nuclei) in cortical sections of control (FVBN), Tg26 and Tg26/Rapa are shown.

B. Cumulative data (n=3) of 8-OHdG +ve cells/tubule are shown.

mTOR inhibition attenuated kidney cell apoptosis in HIVAN mice

A. Representative microphotographs displaying TUNEL +ve cells (dark blue stained nuclei) in cortical sections of control (FVBN), Tg26 and Tg26/Rapa are shown.

B. Cumulative data (n=3) of TUNEL +ve cells/field are shown.

mTOR inhibition attenuates renal lesions in HIVAN mice

Renal cortical sections from control, Tg26 and Tg26/Rapa were stained with PAS and scored for severity of renal lesions.

A. Representative microphotographs from a control, Tg26, and Tg26/Rapa mice are shown. Control mouse showed normal glomeruli and tubules. Tg26 mouse displayed a collapsed glomerulus (indicated by a black arrow) and microcysts (orange arrows). Tg26/Rapa mouse displayed a
glomerulus with segmental sclerosis (indicated by an arrow) and dilated tubules (indicated by orange arrows).

B. Cumulative data (n=3) showing % glomeruli showing sclerosed and collapsed phenotype.

7. **mTOR inhibition attenuates renal tissue expression of p53 and associated down stream signaling in HIVAN mice**

A. Protein blots of renal tissues from controls, Tg26, and Tg26/rapa were probed for p53 and reprobed for actin. Representative gels from two different mice are displayed. The upper lane displays renal tissue p53 expression by control and experimental mice. The lower lane displays their actin content under similar conditions. Cumulative densitometric data are shown in bar graphs (n=3).

B. Western blots of renal tissue lysates from controls, Tg26, and Tg26/rapa were probed for p21 and reprobed for actin. Representative gels from two different mice are displayed. The upper lane displays renal tissue p21 expression by control and experimental mice. The lower lane displays their actin content under similar conditions. Cumulative densitometric data are shown in bar graphs (n=3).

C. Preparations of renal tissue lysates from controls, Tg26, and Tg26/rapa were electrophoresed and probed for p27. The same blots were reprobed for actin. Representative gels from two different mice are displayed. The upper lane displays renal tissue p27 expression by control and experimental mice. The lower
D. Proteins were extracted from renal tissues from controls, Tg26, and Tg26/rapa and Western blots were probed for Fas. The same blots were reprobed for actin. Representative gels from two different mice are displayed. The upper lane displays renal tissue Fas expression by control and experimental mice. The lower lane displays their actin content under similar conditions. Cumulative densitometric data are shown in bar graphs (n=3). Renal tissues of Tg26 mice displayed three fold higher Fas expression.

8. mTOR inhibition attenuate renal tissue transcription of p53 and downstream molecules

Total RNAs were extracted from renal tissues of control, Tg26, and Tg26/Rapa mice and probed for p53, p21, and p27 (n=3). Results (cumulative data) are shown only in Tg26 and Tg26/Rapa mice (as fold changes) when compared to respective FVBN (control).

A. Renal tissue mRNA expression of p53.
B. Renal tissue mRNA expression of p21.
C. Renal tissue mRNA expression of p27.
D. Renal tissue mRNA expression of GADD45.

9. mTOR inhibition enhances antioxidant expression and down regulated expression of pro-apoptotic molecules
Total RNAs were extracted from renal tissues of control, Tg26, and Tg26/Rapa mice and probed for MnSOD, catalase, and FasL (n=3). Results (cumulative data) are shown only in Tg26 and Tg26/Rapa mice (as fold changes) when compared to respective FVBN (control).

A. Renal tissue mRNA expression of MnSOD

B. Renal tissue mRNA expression of catalase.

C. Renal tissue mRNA expression of FasL.

10. mTOR is critical for podocyte p53 expression

A. Human podocytes (HP) were transfected with either scrambled siRNA (SCR) or mTOR-siRNA. Protein blots of control, HP/SCR and HP/mTOR-siRNA were probed for mTOR. The same blot was reprobed for actin. A representative gel and densitometric analysis are displayed.

B. Protein blots of control, HIV-transduced HP (HP/HIV), mTOR-siRNA-transfected HIV/HP, scrambled siRNA- transfected HIV/HP were probed for p53 (n=3). The same blots were reprobed for actin. Cumulative data are shown as bar graphs (n=3).

C. HP and HP/HIV were incubated in media for 48 hours. Total RNA was extracted and probed for p53 (n=3). Cumulative data are shown as bar graphs.
D. HP and HP/HIV were incubated in media for 48 hours. Total RNA was extracted and probed for MnSOD (n=3). Cumulative data are shown as bar graphs.


HIV enhances ROS generation by multiple ways, including kidney cell activation of RAS (renin angiotension syste), phosphphorylation of p66ShcA; the later phosphorylates Akt. HIV can also phosphorylate AkT through the activation of kinases. Akt-induced phosphorylation of Foxo3A deactivates redox-sensitive stress program in the form of attenuated generation of SOD and Cat (catalase) leading to accumulation of ROS. Phosphorylation of Akt enhances activation of mTOR, which enhances expression of p53 and associated activation p66ShcA. ROS-induced DNA damage also activates p53 pathway, which may lead to the expression of inhibitors of cell cycle (p21, p27, and GADD45) and proapoptotic molecules such as Fas.
Fig. 1

A  8-OHdG  
B  
C  TUNEL  
D  
E  PAS  
F  
Fig. 2

A | B
---|---
p53/Actin | Tg26
FVBN

C | D
---|---
p21/Actin | Tg26
FVBN

E | F
---|---
p27/Actin | Tg26
FVBN

G | H
---|---
GADD45/Actin | Tg26
FVBN

I | J
---|---
Fas/Actin | Tg26
FVBN
Fig. 3

A

Phospho-p66ShcA

FVBN

p66ShcA (Total)

Tg26

Tg26 + Rapa

B

p66Shc/Actin

0.5

P<0.01

Tg26

Phospho-p66Shc/Actin

0.1

P<0.01

FVBN

C

Phospho-p66Shc/Actin

1.5

P<0.01

Tg26 + Rapa

0.5

0
Fig. 4

A

B

# 8-OHdG +ve cells/tubule

Tg26/Rapa

Tg26

FVBN

P<0.01

P<0.01

0

2

4

6

8

10

12

14

8-OHdG
Fig. 5.

A

FVB/N                         Tg26                      Tg26/Rapa

TUNEL +ve cells/field

B

![Bar graph showing TUNEL +ve cells/field for FVB/N, Tg26, and Tg26/Rapa with P<0.01 for each comparison.](image)

Fig. 5.
Fig. 6

A

B
Fig. 7

A

B

C

p21

Actin

p53

Actin

p27

Actin

p21/actin

P<0.05

P<0.001

P<0.01

Fas/Actin

P<0.01

P<0.01

P<0.01

FVBN

Tg26

Tg26/Rapa

0

2.0

4.0

6.0

8.0

0

2.0

4.0

6.0

8.0

0

2.0

4.0

6.0

8.0

0

2.0

4.0

6.0

8.0

0

2.0

4.0

6.0

8.0

0

2.0
**Fig. 8**

A. Tg26 vs. Tg26/Rapa: p53 mRNA (fold change) 

B. Tg26 vs. Tg26/Rapa: p27 mRNA (fold change) 

C. Tg26 vs. Tg26/Rapa: GADD45 mRNA (fold change) 

D. Tg26 vs. Tg26/Rapa: P<0.01
Fig. 9

A. MnSOD mRNA (fold change)

B. Catalase mRNA (fold change)

C. FasL mRNA (fold change)
**Fig. 10**

**A**

- **p53**/Actin (fold change): 3.0, 2.0, 1.0, 0.0
- P<0.05

**B**

- MnSOD mRNA (fold change): 2.0, 1.5, 1.0, 0.5
- P<0.01

**C**

- mTOR/Actin (fold change): 6.0, 4.0, 2.0, 0.0
- P<0.01

**D**

- P53/mTOR siRNA
- HP/HIV, HP/HIV/Rapa
- P<0.01