INCREASING EVIDENCE SUPPORTS that oxidative stress is involved in mouse tubular cells both in vitro and in vivo.

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Salhan D, Pathak S, Husain M, Tandon P, Kumar D, Malhotra A, Meggs LG, Singhal PC. HIV gene expression deactivates redox-sensitive stress response program in mouse tubular cells both in vitro and in vivo. Am J Physiol Renal Physiol 302: F129–F140, 2012. First published October 12, 2011; doi:10.1152/ajprenal.00024.2011.—Human immunodeficiency virus (HIV)-1 has been reported to cause tubular cell injury both in vivo and in vitro. In the present study, we evaluated the role of oxidative stress in the induction of apoptosis in HIV gene expressing mouse tubular cells in vivo (Tg26, a transgenic mouse model of HIV-associated nephropathy) and in vitro (tubular cells were transduced with pNL4-3: ΔG/P-GFP, VSV.G pseudotyped virus) studies. Although Tg26 mice showed enhanced tubular cell reactive oxygen species (ROS) generation and apoptosis, renal tissue did not display a robust antioxidant response in the form of enhanced free radical scavenger (MnSOD/catalase) expression. Tg26 mice not only showed enhanced tubular cell expression of phospho-p66ShcA but also displayed nuclear Foxo3a translocation to the cytoplasm. These findings indicated deactivation of tubular cell Foxo3A-dependent redox-sensitive stress response program (RSSRP) in Tg26 mice. In in vitro studies, NL4-3 (pNL4-3: ΔG/P-GFP, VSV.G pseudotyped virus)-transduced mouse proximal tubular cells (NL4-3/MPTEC) displayed enhanced phosphorylation of p66ShcA. NL4-3/MPTECs also displayed greater (P < 0.01) ROS generation when compared with empty vector-transduced tubular cells; however, both diminution of p66ShcA and N-acetyl cysteine attenuated NL4-3-induced tubular cell ROS generation as well as apoptosis. In addition, both antioxidants and free radical scavengers partially inhibited HIV-induced tubular cell apoptosis. NL4-3/MPTEC displayed deactivation of RSSRP in the form of enhanced phosphorylation of Foxo3A and attenuated expression of superoxide dismutase (SOD) and catalase. Since both SOD and catalase were able to provide protection against HIV-1-induced tubular cell apoptosis, it suggests that HIV-1-induced proapoptotic effect may be a consequence of the deactivated RSSRP.

HIV gene expression deactivates redox-sensitive stress response program in mouse tubular cells both in vitro and in vivo

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INCREASING EVIDENCE SUPPORTS that oxidative stress is involved in the progression of human immunodeficiency virus (HIV) disease (32). First, glutathione and antioxidants including ascorbic acid, tocopherol, and selenium are frequently lower in patients with HIV-1 infection (1, 9–11, 15). Second, HIV-infected patients have higher levels of serum markers of oxidative stress-like malondialdehyde and hydroperoxides, and oxygen consumption rates, which increase the oxygen load (13, 24, 35, 39). Oxidative stress may contribute to HIV disease pathogenesis by enhancing viral replication (28, 40), increasing the inflammatory immune response (14), and accelerating the loss of immune function (41). Although oxidative stress has been considered an important mediator in the development and progression of renal disease in both human and experimental models of renal disease (38, 45, 47), the role of oxidative stress in the development and progression of HIV-associated nephropathy (HIVAN) is not clear (25). Recently, a paradigm based on HIV-1-induced overproduction of reactive oxygen species (ROS) has been proposed to account for the activation of apoptosis in peripheral blood mononuclear cells, neurons, endothelial cells, and podocytes (8, 17, 20, 21, 33, 43).

For a long time it was not clear whether the pathogenesis of HIVAN was due to HIV infection in the renal cell or due to an indirect effect of the systemically dysregulated immune system. Studies designed to address this issue showed that HIVAN can occur at any point during AIDS progression with no apparent correlation with either viral burden or CD4 T cell number (6, 46). Increasing evidence supports a role for HIV-1 infection of renal epithelium in the pathogenesis of HIVAN (25). Presence of conventional HIV-1 receptors such as CD4, CCR5, and CXCR4 in renal epithelial cells is controversial (7, 12). Recently, renal tubular cells have been shown to express DEC-205 that serves as a receptor for HIV-1 (18). However, HIV-1 entering through this receptor followed the endocytic pathway and did not replicate. To circumvent the problem of HIV-1 entry into tubular cells, we transduced tubular cells with an HIV-1 construct as described previously (20). Interestingly, in a recent study, human tubular cells displayed productive infection when they were cocultivated with HIV-infected lymphocytes (4).

HIV-1 transgenic mice (Tg26) develop renal lesions identical to those in HIVAN (23). The Tg26 transgenic animal has the proviral transgene, pNL4-3: d1443, which encodes all the HIV-1 genes except gag and pol and therefore the mice are noninfectious. These mice develop proteinuria at the age of 4 wk and undergo progressive renal failure (23). In the present study, we evaluated the occurrence of oxidative stress in Tg26 mice. In in vitro studies, we have transduced mouse tubular cells with the proviral transgene pNL4.3: dg/p-GFP, which was used for the development of Tg26 mice.

Microcystic dilatation of tubules is a unique tubular lesion, which has been reported only in patients with HIVAN (34). In addition, tubular lesions in patients of HIVAN display both proliferation and apoptosis (34). We and other investigators reported induction of apoptosis in renal proximal tubular epithelial cells in response to HIV-1 (22, 36). Recently, we highlighted the role of G2/M arrest in the development of HIV-1-induced tubular cell apoptosis (44). Despite all these reports, the involved mechanism in HIV-1-induced tubular cell...
apoptosis is far from clear. In the present study, we studied the role of oxidative stress in the induction of tubular cell apoptosis both in vivo and in vitro. In addition, we evaluated the involved molecular mechanism in oxidative stress-induced renal cell injury.

MATERIALS AND METHODS

HIV Transgenic Mice

We used age- and sex-matched FVB/N (control) and Tg26 (on FVB/N background) mice. Breeding pairs of FVB/N 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. The Tg26 transgenic animal has the proviral transgene pNL4-3; d1443, which encodes all the HIV-1 genes except gag and pol and therefore the mice are noninfectious (23). Mice were housed in groups of four in a laminar-flow facility (Small Animal Facility, Long Island Jewish Medical Center, New Hyde Park, NY). We are maintaining colonies of these animals in our animal facility. For genotyping of these animals, tail tips were clipped, DNA was isolated, and PCR was performed using following primers for Tg26: HIV-F 5'-ACATGAGCAGTGAGGGTGATGAG-3' and HIV-R 3'-CAAGAGACTCTGATGCGCAGGTG-5'. The Ethics Review Committee for Animal Experimentation of Long Island Jewish Medical Center approved the experimental protocol.

Proximal Tubular Cells

Mouse proximal tubular epithelial cells (MPTEC) were a gift from Dr. Poornima Upadhya (Long Island Jewish Medical Center, New Hyde Park, NY). Mouse tubular cells were characterized by their expression for cytokeratin-18, -19, and E-cadherin. Human tubular cells were obtained from American Tissue Culture Collection (Manassas, VA).

Production of Pseudotyped Retroviral Supernatant

Replication-defective viral supernatants were prepared as published previously (20). In brief, green fluorescence protein (GFP) reporter gene (from pEGFP-C1; Clontech, Palo Alto, CA) was substituted in place of gag/pol genes in HIV-1 proviral construct pNL4-3. This parental construct (pNL4-3; ΔG/P-GFP) was used to produce VSV.G pseudotyped viruses to provide pleiotropism and high-titer virus stocks. Infectious viral supernatants were produced by the transient transfection of 293T cells using Effectene (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The HIV-1 gagpol and VSV.G envelope genes were provided in trans using pCMV R8.91 and pMD.G plasmids, respectively (gifts of Dr. Didier Trono, Salk Institute, La Jolla, CA). As a negative control, virus was also produced from pHR-CMV-IRES2-GFP using pCMV Δgag/pol genes in HIV-1 proviral construct pNL4-3.

Reverse Transcription PCR Analysis

RNA was extracted from the kidneys of control and HIV-1 expressing tubular cells using TRIzol (Invitrogen, Carlsbad, CA). cDNA synthesis, 2 μg of the total RNA were preincubated with 2 nmol of random hexamer (Invitrogen) at 65°C for 5 min. Subsequently, 8 μl of the reverse-transcription (RT) reaction mixture containing Cloned AMV RT, 0.5 mmol each of the mixed nucleotides, 0.01 mol dithiothreitol, and 1,000 U/ml RNasin (Invitrogen) were incubated at 42°C for 50 min. For a negative control, a reaction mixture without RNA or RT was used. Samples were subsequently incubated at 85°C for 5 min to inactivate the RT. The following primers were used to probe for catalase and MnSOD: catalase forward GCTGAAGTGTACGGAGTG, reverse GTCATCAGCGTGAAGTGCTG; MnSOD forward GACCTGCTTACGACTATGG, reverse GACCTGCTTACGACTATGG.

Immunofluorescence Detection of Oxidant Stress Associated with Tubular Cell HIV-1 Expression

The trafficking of 2, 3, 4, and 5, 6-pentafluorodihydrotetramethylosamine (PF-HTMROS or Redox Sensor Red CC-1; Molecular Probes, Eugene, OR) was used to detect reactive oxygen intermediates in HIV-1 expressing tubular cells. Redox Sensor Red CC-1 is oxidized in the presence of O2 and H2O2. In brief, MPTECs transduced with NL4-3 or empty vector (EV) pseudotyped viruses were loaded at 37°C for 20 min with Redox Sensor Red CC-1 (0.5 μM) and a mitochondria-specific dye MitoTracker greenFM (100 nM; Molecular Probes). Culture slides were washed and mounted with PBS and visualized labeling buffer followed by PBS washing again. The cells were incubated in streptavidin-horseradish peroxidase solution for 10 min followed by washing in PBS and incubation in diaminobenzidine (DAB) substrate solution for 10 min. The cells were rinsed in H2O and counterstained with methyl green. The coverslips were mounted on the slide using aqueous mounting media and observed under light microscope.

In Vivo ROS Generation by Tubular Cells

Frozen kidney sections (8 μM) from HIV-1 transgenic mice were deparaffinized and antigen retrieval was done by microwave heating. The endogenous peroxidase was blocked with 0.3% hydrogen peroxide in methanol for 20 min at room temperature. Sections were washed in PBS thrice and incubated in blocking serum solution for 30 min at room temperature followed by incubation with primary antibodies against p66(ShcA) (recognizes all ShcA isoforms, 1:1,000, Cell Signaling Technology, Beverly, MA), pT32Foxo3A (Th-32) (1:100, Cell Signaling), catalase, or superoxide dismutase (SOD; Calbiochem, San Diego, CA) antibody for overnight at 4°C in a moist chamber. Each of the sections was washed thrice with PBS and incubated in secondary antibody at 1:250 dilutions at room temperature for 1 h. After being washed with PBS three times, sections were incubated in ABC reagent (Vector Laboratories, Burlingame, CA) for an h. Sections were washed thrice in PBS and then placed in DAB/hydrogen peroxide solution, counterstained with hematoxylin, dehydrated, and mounted with a xylene-based mounting media (Permount, Fisher Scientific, Fair Lawn, NJ). Appropriate positive and negative controls were used.

Immunohistochemical Staining

Renal cortical sections from control and HIV-1 transgenic (HIVAN) mice were deparaffinized and antigen retrieval was done by microwave heating. The endogenous peroxidase was blocked with 0.3% hydrogen peroxide in methanol for 20 min at room temperature. Sections were washed in PBS thrice and incubated in blocking serum solution for 30 min at room temperature followed by incubation with primary antibodies against p66(ShcA) (recognizes all ShcA isoforms, 1:1,000, Cell Signaling Technology, Beverly, MA), pT32Foxo3A (Th-32) (1:100, Cell Signaling), catalase, or superoxide dismutase (SOD; Calbiochem, San Diego, CA) antibody for overnight at 4°C in a moist chamber. Each of the sections was washed thrice with PBS and incubated in secondary antibody at 1:250 dilutions at room temperature for 1 h. After being washed with PBS three times, sections were incubated in ABC reagent (Vector Laboratories, Burlingame, CA) for an h. Sections were washed thrice in PBS and then placed in DAB/hydrogen peroxide solution, counterstained with hematoxylin, dehydrated, and mounted with a xylene-based mounting media (Permount, Fisher Scientific, Fair Lawn, NJ). Appropriate positive and negative controls were used.
with Nikon fluorescence microscope (Nikon Eclipse E800) equipped with triple filter cube and charge-coupled device camera (Nikon DXM1200). The staining was performed in quadruplicate for each group and 10 random fields were studied in replicate. Images were captured using Nikon ACT-1 (version 1.12) software and combined for publishing format using Adobe Photoshop 6.0 software.

**Annexin V-PE Staining**

EV/MPECs, NL4-3/MPTECs, mu-36p66ShcA/NL4-3/MPTECs, N-acetyl cysteine (NAC) + NL4-3/MPTECs were incubated in serum-free media (SFM) for 24 h. Subsequently, cells were harvested and stained with annexin V-PE and 7-AAD as described in the kit (Annexin V-PE apoptosis detection kit, BD Biosciences Pharmingen, San Diego, CA). The cells were analyzed by flow cytometry using a FACSc- Vantage flow cytometer (BD Biosciences). Transduced cells were identified by the presence of GFP fluorescence. Apoptotic cells were identified by the presence of annexin V staining in the absence of 7-amino-actinomycin D (7-AAD) staining, and necrotic cells were identified by the presence of 7-AAD staining.

**Silencing of p66ShcA, Akt, and Foxo3A**

MPTEc s were transfected either with 25 nM p66Shc (SHC-1 Silencer Select® Predesigned siRNA, cat. no. 4390771, Ambion, Austin, TX), 100 nM AKT siRNA (cat. no. SC-6211S Santa Cruz Biotechnology, Santa Cruz, CA), 100 nM Foxo3A-siRNA (cat. no. 040728-00, Ambion), or 100 nM scrambled siRNA (cat. no. SC-37007, Santa Cruz Biotechnology) with Siport Neofect transfection reagent and left in optiMEM media for 48 h. Control and transfected cells were used under control and experimental conditions.

**Immunodetection by Western Blot**

MPTEc s (control), NL4-3/MPTECs, and EV/MPTEc s were incubated in media for 48 h. At the end of the incubation period, cells were harvested and lysed in RIPA buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% deoxycholate, 0.1% SDS, 1 × protease inhibitor cocktail I (Calbiochem, EMD Biosciences, Gibbstown, NJ), 1 mM PMSF, and 0.2 mM sodium orthovanadate. Protein concentration was measured by the BCA Protein Assay kit (Pierce, Rockford, IL). Proteins were separated on 15% polyacrylamide gels (PAGE, Bio-Rad, Hercules, CA) and transferred onto a nitrocellulose membrane using Bio-Rad miniblot apparatus. Nitrocellulose membranes were then subjected to immunostaining with primary antibodies against p66ShcA (recognizes all ShcA isoforms, Cell Signaling, Danvers, MA), mouse monoclonal anti-phospho-ShcA-Ser-36 (EMD Biosciences), anti-phospho-FOXO3a (Th-32) rabbit polyclonal antibody (Cell Signaling), anti-SOD rabbit polyclonal antibody (Calbiochem), anti-catalase (Cell Signaling), and subsequently with horseradish peroxidase-labeled appropriated secondary antibodies. The blots were developed using a chemiluminescence detection kit (Pierce) and exposed to X-ray film (Eastman Kodak, Rochester, NY). Equal protein loading was confirmed by immunoblotting for actin protein using a polyclonal α-actin antibody (Santa Cruz Technology) on the same Western blots.

**Statistical Analysis**

For comparison of mean values between two groups, the unpaired t-test was used. To compare values between multiple groups, ANOVA was used to calculate a P value. Statistical significance was defined as P < 0.05. Results are presented and means ± SD.

**RESULTS**

**In Vivo Studies**

Renal tubular cells in Tg26 mice displayed enhanced ROS generation. To determine ROS generation by tubular cells, cryosections from HIV-1 transgenic (HIVAN) and age- and sex-matched control mice (FVB/N) were stained with redox-sensitive probe DHE and evaluated for ROS generation. Tubular cells from Tg26 mice showed enhanced ROS generation when compared with control mice. Representative micrographs of tubules from a control and an HIVAN mouse are shown in Fig. 1, A and B, respectively.

Renal tubular cells in Tg26 mice undergo enhanced apoptosis. To evaluate occurrence of tubular cell apoptosis, cryosections from control (FVB/N) and Tg26 mice were labeled for TUNEL and with a nuclear stain (DAPI). Renal cortical sections of Tg26 mice showed increased number of TUNEL-positive cells. Representative micrographs of tubular cells from a control and a Tg26 mouse are shown in Fig. 1, C and D, respectively.

As a second approach to occurrence of tubular cell apoptosis, renal cortical sections (paraffin) from control (FVB/N) and Tg26 mice were labeled for TUNEL. Increased number of tubular cells showed TUNEL-positive cells when compared with control mice. Representative microphotographs of renal sections from a control and a Tg26 mouse are shown in Fig. 1, E and F, respectively.

Tubular cells displayed enhanced phospho-p66ShcA expression in Tg26 mice. To determine the activation of p66ShcA pathway in Tg26 mice, renal sections from control (FVB/N) and Tg26 mice were immunolabeled for phospho-p66ShcA. Tubular cells of Tg26 mice showed increased cytoplasmic labeling for phospho-p66ShcA. Representative microphotographs of renal sections from a control and a Tg26 mouse are shown in Fig. 1, G and H, respectively.

To quantify renal tissue phospho-p66ShcA expression, proteins were extracted from renal tissues of FVB and Tg26 mice (n = 4) and immunoblots were probed for phospho-p66ShcA and total p66ShcA. Representative gels in duplicate are shown in Fig. 2A. Cumulative data of four sets of experiments are shown in the form of a bar diagram. Renal tissues of Tg26 mice displayed enhanced (P < 0.01) expression of phospho-p66ShcA when compared with FVB mice.

Cytoplasmic accumulation of phospho-Foxo3A in tubular cells of Tg26 mice. During deactivation of RSSRP, nuclear Foxo3A (active transcriptor of SOD and catalase) is phosphorylated (inactive) and exported to cytoplasm. To determine deactivation of stress response program in tubular cells, renal sections of control and Tg26 mice were immunolabeled for phospho-Foxo3A. Tubular cells from Tg26 mice showed enhanced tubular cell cytoplasmic accumulation of phospho-Foxo3A. Representative microphotographs from a control and Tg26 mouse are shown in Fig. 1, I and J, respectively.

To quantify renal tissue phospho-p66ShcA and phospho-Foxo3A expression, immunoblots were prepared from renal tissues of FVB and Tg26 mice (n = 3) and probed for phospho-p66ShcA, total p66ShcA, phospho-Foxo3A, and total Foxo3A. Representative gels are shown in Fig. 2, A and B. Cumulative data of three sets of experiments are shown in bar graphs. Renal tissues of Tg26 mice displayed enhanced expres-
sion of both phospho-p66ShcA (P < 0.01) and phospho-Foxo3A (P < 0.05) when compared with FVBN mice.

Absence of increment in tubular cell anti-oxidant response in Tg26 mice. To determine the tubular cell antioxidant response to HIV-1-induced oxidative stress, renal sections from control and Tg26 mice were immunolabeled for MnSOD expression. Tubular cells of Tg26 mice did not show any increase in SOD expression compared with control mice. Representative microphotographs of a control mouse and a Tg26 mouse are shown in Fig. 1, K and L, respectively.

Diminished renal tissue expression of MnSOD and catalase in Tg26 mice. To determine the involved mechanism of inadequate antioxidant response by renal cells in Tg26 mice, total RNA was extracted from two control and two Tg26 mice, followed by probing for MnSOD and catalase by RT-PCR. As shown in Fig. 3A, Tg26 mice showed attenuated mRNA expression of MnSOD and catalase.

To quantify renal tissue MnSOD and catalase, proteins were extracted from renal tissues of FVBN and Tg26 mice (n = 3), and Western blots were prepared and probed for MnSOD and catalase. Blots were stripped and reprobed for actin. Gels displaying expression of MnSOD and catalase are shown in Fig. 3, B and C, respectively. As shown Fig. 3B, renal tissues from Tg26 mice showed reduction (P < 0.05) in MnSOD expression compared with FVBN mice. Similarly, renal tissues from Tg26 mice displayed attenuation (P < 0.01) in catalase expression (Fig. 3C).
In Vitro Studies

Morphological effect of HIV-1 expression on MPTECs. To determine whether HIV-1 expression in tubular cell is associated with morphological phenotype, MPTECs were transduced with pseudotyped viruses containing EV or HIV-1 and then maintained in SFM for 48 h. Subsequently, cells were examined under fluorescence microscope for GFP expression as a marker of vector or HIV-1 expression. As shown in Fig. 4, more than 30% of cells showed GFP expression. Many NL4-3/MPTECs expressing GFP showed rounding, indicating ongoing cell injury.

HIV-1 promotes serine-36 phosphorylation of p66. Since HIV-1 is known to induce oxidative stress, we determined the effect of HIV-1 expression on serine-36 phosphorylation of p66 (ShcA) in MPTECs. As shown in Fig. 5A, NL4-3/MPTECs displayed enhanced serine-36 phosphorylation of p66 (ShcA) when compared with control and EV/MPTECs. To determine downstream activation of p66ShcA pathway, MPTECs treated under similar conditions were evaluated for phosphorylation of Akt. HIV-1 also promoted (P < 0.01) phosphorylation of Akt (Fig. 5B). These findings indicate that HIV-1 activated p66ShcA pathway in MPTECs.

To determine the role of specific HIV proteins contributing to HIV-induced p66ShcA phosphorylation, MPTECs were incubated in media containing either buffer, gp120 (50 ng/ml), Tat (50 ng/ml), Vpr (50 ng/ml), and mouse IgG (50 ng/ml) for 24 h. Subsequently, immunoblots were prepared and probed for phospho-p66ShcA and total p66ShcA. As shown in Fig. 5C, both gp120 and Tat enhanced (P < 0.01) tubular cell expression of phospho-p66ShcA, whereas Vpr and IgG did not alter tubular cell expression of phospho-p66ShcA.

Effect of HIV-1 expression on tubular cell ROS kinetics. To determine the effect of HIV-1 expression on tubular cell ROS kinetics, control, EV- or NL4-3-expressing tubular cells were incubated in SFM for 48 h at 37°C. Subsequently, cells were loaded with the redox-sensitive probe 2′, 7′-dichlorofluoro-...
rescein diacetate (DCFDA). The intensity of 2′, 7′-dichloro-
hydrofluorescein (DCF) fluorescent signal was determined
for 60 min at the indicated time periods. As shown in Fig. 6,
NL4-3/MPTECs showed greater ($P < 0.01$) generation of
ROS when compared with control and EV-expressing tubu-
lar cells.

$p66ShcA$ redox activity is critical for HIV-1-mediated ROS
generation. To determine whether $p66ShcA$ signaling is indis-
penable for HIV-mediated ROS generation, NL4-3/MPTECs
were transfected with mu-36 expression vector (to compete
with endogenous $p66ShcA$). A mutation at position 36
(serine to alanine) was constructed by standard methods and
both wild-type and mutant constructs were confirmed by
direct sequencing. Immunoblot analysis (Fig. 7A) shows
inhibition of the expression of phospho-$p66ShcA$ in mu-
36$p66ShcA$ NL4-/MPTECs when compared with NL4-3/
MPTECs, whereas NL4-3/MPTECs showed enhanced phos-
phorylation of $p66ShcA$ compared with EV/MPTECs.

![Fig. 4. Morphological effect of HIV-1 ex-
pression on mouse proximal tubular epithe-
pal cells (MPTECs). MPTECs were trans-
duced with either empty vector (EV/GFP/ 
MPTEC) or HIV-1 (NL4-3/GFP/MPTEC) 
and then maintained in serum-free media 
(SFM) for 48 h. Subsequently, cells were 
examined under fluorescence microscope for 
green fluorescence protein (GFP) expression 
as a marker of vector or HIV-1 expression.
Approximately 30% of cells showed GFP 
expression. Many NL4-3/MPTECs express-
ing GFP showed rounding, indicated by 
white arrows.](#)

![Fig. 5. HIV-1 promotes serine-36 phosphorylation of $p66$ (ShcA). A: control, EV/MPTECs, and NL4-3/MPTECs were incubated in SFM media for 24 h. At the end of the incubation period, proteins were extracted and Western blots from protein lysates were probed for phospho-$p66ShcA$ and total $p66ShcA$ ($n = 3$). Representative gel is shown. A bar diagram showing cumulative data of 3 experiments is also shown. *$P < 0.05$ compared with control, **$P < 0.01$ compared with control, ***$P < 0.05$ compared with EV. B: control, EV/MPTECs, and NL4-3/MPTECs were incubated in SFM media for 24 h. Subsequently, proteins were extracted and Western blots were prepared and probed for phospho-Akt and total Akt ($n = 3$). Representative gel is shown. A bar diagram showing cumulative data of 3 experiments is also shown. *$P < 0.05$ compared with other variables. C: MPTECs were incubated in media containing either buffer, gp120 (50 ng/ml), Tat (50 ng/ml), Vpr (50 ng/ml), and mouse IgG (50 ng/ml) for 24 h. At the end of the incubation period, immunoblots were probed for phospho-$p66ShcA$ and total $p66ShcA$. Representative gels are shown as insets. Cumulative data of 3 sets of experiments are shown in the form of a bar diagram. *$P < 0.01$ vs. control, Vpr, and IgG. **$P < 0.01$ vs. control, Vpr, and IgG.](#)
To determine the role of phospho-p66ShcA in ROS generation and site of ROS generation, EV/MPTECs, NL4-3/MPTECs, mu-36p66ShcA/MPTECs, and NL4-3/MPTECs/H11001 NAC were incubated in SFM for 48 h and then loaded with the redox-sensitive probes Red CC-1 and Mito Tracker green FM. As shown in Fig. 7, NL4-3/MPTECs (Fig. 7C) showed mitochondrial generation of ROS (yellow/orange fluorescence), whereas EV/MPTECs (Fig. 7B), mu-36p66ShcA/MPTECs (Fig. 7D), and NAC + NL4-3/MPTECs displayed minimal ROS generation.

To confirm the role of p66ShcA in HIV-1-infected tubular cell ROS kinetics, cells treated under above mentioned conditions were loaded with the redox-sensitive probe DCFDA. The intensity of DCF fluorescent signal was determined from 0–180 min at the indicated time periods. As shown in Fig. 8, HIV expression showed enhanced tubular cell generation of ROS; however, mu-36p66ShcA attenuated the effect of HIV. These findings indicated that p66ShcA is indispensable for the generation of ROS in tubular cells.

Role of oxidative stress in HIV-1-induced tubular cell apoptosis. We and other investigators previously reported proapoptotic effect of HIV-1 on tubular cells (33–35). To evaluate the role of oxidative stress in HIV-1-induced tubular cell apoptosis, EV/MPTECs and NL4-3/MPTECs were incubated in media containing either buffer, ascorbic acid (AA; 100 μM), DPI (5 μM), and NAC (50 μM) for 48 h. Subsequently, cells were prepared for apoptotic assay. As shown in Fig. 9A, NL4-3 enhanced (P < 0.001) tubular cell apoptosis when compared with control; however, AA, DPI, and NAC inhibited proapoptotic effect of HIV.

To determine the involved mechanism in antioxidant-mediated inhibition of apoptosis, EV/MPTECs and NL4-3/MPTECs were incubated in media containing either buffer SOD (10 μM) or catalase (2,000 U/ml) for 48 h followed by preparation of the cells for apoptosis assay. As shown in Fig. 9B, both SOD and catalase (P < 0.05) partially inhibited proapoptotic effect of HIV.

p66ShcA redox activity is critical for HIV-1-mediated apoptosis. To determine whether phosphorylation of p66ShcA and associated downsignaling are critical for HIV-1-induced tubular cell apoptosis, MPTECs were transfected with either p66-siRNA, Akt-siRNA, or scrambled (Scr)-siRNA. Twenty-four hours later, proteins were extracted and immunoblots were probed for either Shc proteins or Akt. Subsequently, blots were stripped and reprobed for actin. As shown in Fig. 10A, p66-siRNA downregulated expression of p66 but preservation of p52 and p46 in MPTECs. Similarly, Akt-siRNA downregulated MPTEC expression of Akt (Fig. 10B).

Control, EV/MPTECs, NL4-3/MPTECs, NL4-3/p66-SIRNA/MPTECs, NL4-3/Akt-siRNA/MPTECs, or NL4-3/Scr-siRNA/MPTECs were incubated in media containing either buffer, ascorbic acid (AA; 100 μM), DPI (5 μM), and NAC (50 μM) for 48 h. Subsequently, cells were prepared for apoptotic assay. As shown in Fig. 9B, both SOD and catalase (P < 0.05) partially inhibited proapoptotic effect of HIV.
MPTECs were incubated in SFM for 24 h, followed by staining with annexin V-PE/7-AAD and analysis by flow cytometry. As MPTECs were incubated in SFM for 24 h, followed by staining with DCFDA. The intensity of fluorescent signal was determined from 0 – 180 min at the indicated time periods. Results (means) are from 3 sets of experiments. **P < 0.01 compared with EV. NL4-3/p66ShcA, and NL4-3/MPTECs were incubated in SFM for 48 h. At the end of the incubation period, cells were loaded with DCFDA. The intensity of fluorescent signal was determined from 0 – 180 min at the indicated time periods. Results (means) are from 3 sets of experiments. **P < 0.01 compared with EV. NL4-3/p66ShcA, and NL4-3/MPTECs were incubated in SFM for 24 h, followed by staining with annexin V-PE/7-AAD and analysis by flow cytometry. As MPTECs were incubated in SFM for 24 h, followed by staining with DCFDA. The intensity of fluorescent signal was determined from 0 – 180 min at the indicated time periods. Results (means) are from 3 sets of experiments. **P < 0.01 compared with EV. NL4-3/p66ShcA, and NL4-3/MPTECs were incubated in SFM for 48 h. At the end of the incubation period, cells were analyzed for TUNEL assay. Results (means) are from 3 sets of experiments. *P < 0.05 compared with EV. NL4-3/MPTECs, NL4-3/NAC for the respective time points. **P < 0.001 compared with EV, NL4-3/p66ShcA, and NL4-3/NAC for the respective time points.

Figs. 8 and 9. Effect of HIV-1 on tubular cell apoptosis. A: EV/MPTECs and NL4-3/MPTECs were incubated in media containing either buffer, ascorbic acid (AA; 100 μM), DPI (5 μM), or NAC (50 μM) for 48 h. At the end of the incubation period, cells were analyzed for TUNEL assay. Results (means ± SD) are from 4 sets of experiments, each carried out in triplicate. *P < 0.01 compared with other variables. B: EV/MPTECs and NL4-3/MPTECs were incubated in media containing either buffer, superoxide dismutase (SOD; 10 μM) or catalase (2,000 U/ml) for 48 h. At the end of the incubation period, cells were analyzed for TUNEL assay. Results (means ± SD) are from 4 sets of experiments, each carried out in triplicate. *P < 0.01 compared with EV. **P < 0.005 compared with NL4-3- alone.

To establish a causal relationship between phosphorylation of p66ShcA and the phosphorylation of Foxo3A in NL4-3/MPTECs, immunoblots of EV/MPTECs, NL4-3/MPTECs, p66ShcA-siRNA/EV/MPTECs, and p66ShcA-siRNA/NL4-3/MPTECs were probed for phospho-Foxo3A and total Foxo3A. As shown in Fig. 12B, p66ShcA-siRNA/NL4-3/MPTEC displayed diminished (P < 0.01) expression of phospho-Foxo3A compared with NL4-3/MPTECs. These findings indicate that HIV-induced phosphorylation of p66ShcA is necessary for the phosphorylation of Foxo3A in NL4-3/MPTECs.

To establish a relationship between phosphorylation of Akt and the phosphorylation of Foxo3A, immunoblots of EV/MPTECs, NL4-3/MPTECs, Akt-siRNA/MPTECs, and Akt-siRNA/NL4-3/MPTECs were probed for phospho-Foxo3A and total Foxo3A. As shown in Fig. 13, Akt-siRNA/NL4-3/MPTEC displayed attenuated (P < 0.01) expression of phospho-Foxo3A compared with NL4-3/MPTECs. These findings suggest that HIV-induced phosphorylation of Akt is essential for the phosphorylation of Foxo3A in NL4-3/MPTECs.

Knockdown of FOXO3a by siRNA increases apoptosis in mu-36/NL4-3/MPTECs. To evaluate the role of the potent stress response regulator FOXO3a in the survival phenotype of mu-36/NL4-3/MPTECs, Foxo3a-siRNA was delivered by lipofectamine. Scramble siRNA was used as control. Foxo3a-siRNA cells showed downregulation of Foxo3a protein (Fig. 14A), whereas in control cells, Foxo3a expression was not affected by scramble siRNA. To test whether acute loss of

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**HIV-1 PROMOTES DEACTIVATION OF REDOX-SENSITIVE STRESS RESPONSE PROGRAM.** Since anti-oxidants could inhibit HIV-induced ROS generation as well as apoptosis, we suspected deactivation of RSSRP in NL4-3/MPTECs. To determine the effect of HIV-1 on RSSRP, control, EV/MPTECs, and NL4-3/MPTECs were evaluated for expression of phospho-Foxo3A. As shown in Fig. 11A, HIV enhanced phosphorylation of Foxo3A in tubular cells.

Since phosphorylation of Foxo3A facilitates translocation of nuclear Foxo3A (active transcription factor) to the cytoplasmic location (inactive status), we evaluated the effect of HIV-1 on tubular cell production of SOD and catalase. As shown in Fig. 11B, NL4-3/MPTECs displayed diminished SOD expression and no increase in catalase expression. These findings indicated NL4-3/MPTECs did not display robust antioxidant response despite ongoing proapoptotic oxidative stress.

**HIV-INDUCED PHOSPHORYLATION OF p66ShcA AND Akt IS ESSENTIAL FOR THE PHOSPHORYLATION OF Foxo3A.** To establish a causal relationship between phosphorylation of p66ShcA and the phosphorylation of Akt in NL4-3/MPTECs, immunoblots of EV/MPTECs, NL4-3/MPTECs, p66ShcA-siRNA/ EV/MPTECs, and p66ShcA-siRNA/NL4-3/MPTECs were probed for phospho-Akt and total Akt. As shown in Fig. 12A, p66ShcA-siRNA/NL4-3/MPTEC displayed diminished (P < 0.01) expression of phospho-Akt compared with NL4-3/MPTECs. These findings suggest that HIV-induced phosphorylation of p66ShcA is critical for the phosphorylation of Akt in NL4-3/MPTECs.
Foxo3a reverses rescue of NL4-3/MPTECs by mu-36, apoptosis was evaluated by FACS analysis. An increase \((P < 0.05)\) in apoptosis was detected in mu-36/NL4-3/MPTECs transfected with Foxo3a siRNA (Fig. 14, B and C), indicative Foxo3a-dependent responses in survival phenotype.

**DISCUSSION**

The present study demonstrated occurrence of enhanced tubular cell ROS generation and apoptosis in Tg26 mice. Despite an ongoing oxidative stress, tubular cells did not display an increase in their SOD expression. In addition, Tg26 mice showed diminished renal tissue mRNA as well as protein expression of MnSOD and catalase. Since HIV gene-expressing tubular cells (transduced with pNL4-3: \( \Delta G/P-GFP, VSV.G \) pseudotyped virus) not only showed enhanced expression of phospho-p66ShcA but also of phospho-Foxo3A, deactivation of RSSRP was suspected in tubular cells of Tg26 mice. In in vitro studies, HIV-1 not only stimulated tubular cell ROS formation, but also induced phosphorylation of p66ShcA and Akt is essential for the phosphorylation of Foxo3A, which is critical for HIV-1-mediated apoptosis. A: MPTECs were transfected with either p66-siRNA or scrambled (Scr)-siRNA. Immunoblots were probed for Shc proteins and actin. Top panel shows representative tubular cell expression of Shc proteins. Bottom panel shows expression of actin by MPTECs under similar conditions. B: MPTECs were transfected with Akt-siRNA or scrambled (Scr)-siRNA. Twenty-four hours later, cellular lysates were probed for Akt and actin. Top panel shows the effect of Akt-siRNA on tubular cell Akt expression. Bottom panel shows tubular cell actin expression under similar conditions. C and D: control MPTECs, EV/MPTECs, NL4-3/MPTECs, NL4-3/p66-siRNA/MPTECs, NL4-3/Akt-siRNA/MPTECs, or NL4-3/Scr-siRNA/MPTECs were incubated in SFM for 24 h, followed by staining with annexin V-PE/7-AAD and analysis by flow cytometry. Representative flow scans are shown. Cumulative data \((n = 3)\) are shown in the form of a bar diagram. *\(P < 0.01\) compared with control and EV. **\(P < 0.01\) compared with NL4-3. ***\(P < 0.05\) compared with NL4-3.

**Fig. 10.** p66ShcA redox activity is critical for HIV-1-mediated apoptosis. A: MPTECs were transfected with either p66-siRNA or scrambled (Scr)-siRNA. Immunoblots were probed for Shc proteins and actin. Top panel shows representative tubular cell expression of Shc proteins. Bottom panel shows expression of actin by MPTECs under similar conditions. B: MPTECs were transfected with Akt-siRNA or scrambled (Scr)-siRNA. Twenty-four hours later, cellular lysates were probed for Akt and actin. Top panel shows the effect of Akt-siRNA on tubular cell Akt expression. Bottom panel shows tubular cell actin expression under similar conditions. C and D: control MPTECs, EV/MPTECs, NL4-3/MPTECs, NL4-3/p66-siRNA/MPTECs, NL4-3/Akt-siRNA/MPTECs, or NL4-3/Scr-siRNA/MPTECs were incubated in SFM for 24 h, followed by staining with annexin V-PE/7-AAD and analysis by flow cytometry. Representative flow scans are shown. Cumulative data \((n = 3)\) are shown in the form of a bar diagram. *\(P < 0.01\) compared with control and EV. **\(P < 0.01\) compared with NL4-3. ***\(P < 0.05\) compared with NL4-3.
generation, but it also promoted induction of apoptosis. Since anti-oxidants inhibited HIV-1-induced tubular cell apoptosis, it suggests that HIV-1-mediated tubular cell apoptosis would be caused by ROS generation. Interestingly, transduction of mu-36p66ShcA not only prevented ROS generation by NL4-3/MPTECs but also provided protection against proapoptotic effect of HIV-1. Moreover, NL4-3/MPTECs displayed enhanced expression of phospho-p66ShcA, which was associated with enhanced phosphorylation of Akt and Foxo3A. In addition, p66ShcA-deficient MPTECs displayed resistance to HIV-induced phosphorylation of both Akt and Foxo3A3A; similarly, Akt-deficient MPTECs exhibited resistance to HIV-induced phosphorylation of Foxo3A. Furthermore, NL4-3/MPTECs displayed attenuated MnSOD expression and no increase in catalase expression. These findings indicated that HIV gene-induced deactivation of RSSRP facilitated the induction of apoptosis in tubular cells.

The mammalian adaptor protein ShcA has three isoforms p46, p52, and p66. They all share a common structure, but p66ShcA has an additional domain at its NH2 terminus. p52 and p46 are cytoplasmic signal transduction molecules involved in mitogenic signaling from activated tyrosine kinase receptors to Ras; on the other hand, p66ShcA regulates reactive ROS metabolism and apoptosis through its downstream signaling to FOXO pathway (30). Both p66ShcA knockout mice and cells have been demonstrated to have reduced levels of intracellular ROS and have been found to be resistant to apoptosis in response to different stimuli (5, 26, 27, 29). Although the exact mechanism of action of p66ShcA is not clear, it has been demonstrated that a portion of p66ShcA works as a redox enzyme in mitochondria and generates ROS and hence triggering the apoptosis (3, 5). Thus, mitochondrial ROS production is critical for the induction of cellular injury. This notion was also supported by increased p66ShcA gene expression in peripheral blood mononuclear cells of diabetic patients (27); moreover, p66ShcA KO mice showed resistance to develop diabetic glomerulopathy (26), cardiac stem cell aging, heart failure (3), and hyperglycemia-induced endothelial dysfunction (16).

Cellular survival against oxidative stress is dependent on its ability to generate anti-oxidant molecules such as MnSOD and catalase to metabolize ROS. This redox-sensitive stress response program (RSSRP) acts as a survival strategy for the stressed cells (30). Nevertheless, the net outcome (cell survival vs. death) determines whether this stress response was adequate or not. For an example, despite enhanced expression of MnSOD and catalase if cells undergo apoptosis, one would assume that stress response was there in the form of expression of MnSOD and catalase but was not robust enough to neutralize the ongoing oxidative stress. On the other hand, if cells show only enhanced expression of MnSOD and catalase without any increase in the number of apoptosed cells, one would assume that cellular response to oxidative stress was adequate to neutralize the exerted stress. In HIVAN mice, despite overt ROS generation, renal tissue did not show any increase in the expression of either MnSOD or catalase; moreover, there was evidence of ongoing oxidative stress-associated cellular injury in the form of tubular cell apoptosis. These findings indicated
that HIV-1 had compromised tubular cell RSSRP in Tg26 mice (in vivo studies). In in vitro studies, too, HIV-1-transduced tubular cells displayed attenuated expression of MnSOD expression and no increase in catalase expression despite ongoing oxidative stress. Since HIV-1-transduced tubular cells showed enhanced apoptosis, it would be suggestive whatever antioxidant reaction had been generated was not adequate to neutralize the ongoing oxidative stress. Interestingly, both MnSOD and catalase could inhibit HIV-1-induced tubular cell apoptosis in in vitro studies. These findings indicate that if HIV-1-transduced tubular cells would have optimal production of MnSOD and catalase, they might have been able to provide protection against ROS-mediated cellular injury.

What is the relevance of phosphorylation of p66ShcA in the manifestation of tubular cell apoptotic phenotype in HIVAN? ROS generation can induce phosphorylation of p66ShcA (24); on the other hand, p66ShcA stimulates mitochondrial ROS generation (3, 31). ROS production by p66ShcA appears to be a specialized function whereby electrons are subtracted from the mitochondrial electron transport chain to catalyze the partial reduction of molecular oxygen. In this context, p66ShcA can be regarded as an atypical signal transducer that converts proapoptotic signals into redox signals. Therefore, intracellular levels of ROS are decreased in p66ShcA−/− cells (16). Moreover, p66ShcA−/− mice have diminished levels of both systemic and intracellular oxidative stress (29). Pelicci and colleagues (27) demonstrated a novel role of WTP66ShcA as a genetic determinant of oxidative stress and longevity in mammals. Sv129p66ShcA−/− mice express a phenotype characterized by normal fertility and growth, increased resistance to ROS, and extended life span (27). In addition, the p66ShcA protein has been reported to play a critical role in the modulation of oxidative stress in diabetic milieu (29). In the present study, renal cells in both in vivo and in vitro studies not only showed increased expression of phospho-p66ShcA in HIV-1 milieu, but they also displayed enhanced ROS generation. Since NL4-3/MPTEC expressing mu36/p66ShcA not only displayed reduction in ROS generation but also resulted in diminished percentage of apoptosed cells, it appears that HIV-1-induced tubular cell oxidative stress and associated apoptosis were mediated via p66ShcA pathway.

The mammalian Forkhead homolog Foxo3a is a potent stress response regulator, linked to the transcription of genes involved in cell cycle progression, antioxidant defense, and DNA repair (2, 37). Foxo3a is a downstream target of p66ShcA that phosphorylates key regulatory sites including transcription of stress-related Foxo3a gene products (2, 30, 37). In the present study, HIV-1-induced tubular ROS generation invoked phosphorylation at a critical CH2 Ser-36 residue of the p66ShcA protein, a modification that serves to promote the intracellular generation of ROS (2, 37) and the recruitment of Akt/PKB, which directly phosphorylates and inactivates members of the Foxo family (19). Export of nuclear Foxo3a to cytoplasm is likely to compromise the production of anti-oxidants and thus may endanger cell survival strategy. In the present study, HIVAN mice not only showed enhanced phosphorylation of p66ShcA but also showed increased tubular cell accumulation of cytoplasmic phospho-Foxo3a (exported from the nuclear compartment); the latter occurrence provided an explanation for the inability of the part of HIV-1-transduced tubular cells to mount a robust antioxidant response, i.e., generation of MnSOD and catalase.

Recently in in vitro studies, Sun et al. (42) demonstrated that both high glucose and ANG II not only enhanced tubular cell expression of phospho-p66Shc but were also associated with increased number of apoptosed cells; on the other hand, over-expression of a dominant-negative Ser36 mutant p66Shc (p66ShcS36A) inhibited tubular cell p66ShcA expression as well as apoptosis. These findings are consistent with our observations.

We conclude that HIV gene expression in tubular cells stimulates ROS generation and activation of p66ShcA pathway. The latter deactivates tubular cell RSSRP and thus, leading to oxidative stress-induced tubular cell apoptosis. The present study provides an insight into the involved mechanism in HIV-1-mediated tubular cell apoptosis in HIVAN.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: D.S., S.P., and P.T. performed experiments; D.S., M.H., P.T., D.K., A.M., L.G.M., and P.C.S. approved final version of manuscript; M.H. and P.C.S. conception and design of research; D.K. analyzed data; A.M. and L.G.M. interpreted results of experiments; P.C.S. drafted manuscript; P.C.S. edited and revised manuscript.

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HIV-1 INDUCES TUBULAR CELL OXIDATIVE STRESS