Inhibition of p66ShcA Longevity Gene Rescues Podocytes from HIV-1-induced Oxidative Stress and Apoptosis*

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Glomerular visceral epithelial cells (podocytes) play a critical role in the pathogenesis of human immunodeficiency virus (HIV)-associated nephropathy. A key question concerns the mechanism(s) by which the HIV-1 genome alters the phenotype of the highly specialized, terminally differentiated podocytes. Here, using an in vitro system of conditionally immortalized differentiated human podocytes (CIDHHPs), we document a pivotal role for the p66ShcA protein in HIV-1-induced reactive oxygen species generation and CIDHP apoptosis. CIDHP transfected with truncated HIV-1 construct (NL4-3) exhibit increased reactive oxygen species metabolism, DNA strand breaks, and a 5-fold increase in apoptosis, whereas the opposite was true for NL4-3/CIDHP co-transfected with mu-36p66ShcA (mu-36) dominant negative expression vector or isoform-specific p66-small interfering RNA. Phosphorylation at Ser-36 of the wild type p66ShcA protein, required for p66ShcA redox function and inhibition of the potent stress response regulator Foxo3a, was unchanged in mu-36/NL4-3/CIDHP but increased in NL4-3/CIDHP. Acute knockdown of Foxo3a by small interfering RNA induced a 50% increase in mu-36/NL4-3/CIDHP apoptosis, indicating that Foxo3a-dependent responses promote the survival phenotype in mu-36 cells. We conclude that inhibition of p66ShcA redox activity prevents generation of HIV-1 stress signals and activation of the CIDHP apoptosis program.

The podocyte, strategically positioned along the glomerular basement membrane, is a critical component of the glomerular filtration barrier, functioning in tandem with its associated slit diaphragm to limit passage of albumin and plasma proteins to the urinary space (1, 2). Compelling evidence (3–7) supports a key role for HIV-1 gene products in the podocyte injury that leads to a breach in the integrity of the glomerular filtration barrier and the massive proteinuria that characterizes HIVAN. The absence of podocyte regeneration after cell injury or apoptosis is a major limitation to the development of innovative therapeutic strategies to arrest or prevent HIVAN and other glomerular diseases. Accordingly, interventions that increase the resistance of this terminally differentiated cell population to death signals offer a novel approach to preserve the integrity and permselectivity of the glomerular filtration barrier.

Several lines of evidence support a dominant role for the p66ShcA protein in the intracellular pathways that convert oxidative stress to apoptosis (8, 9). The three overlapping Shc proteins, p66ShcA, p52ShcA, and p46ShcA, share a C-terminal Src homology 2 domain, central collagen homology region, and N-terminal phosphotyrosine binding domain. p46ShcA and p52ShcA are the product of alternative translation initiation sites within the same transcript, whereas p66ShcA is distinguished by a unique N-terminal region (collagen homology 2), generated by alternative splicing. p66ShcA has emerged as a genetic determinant of longevity in mammals (10) that controls mitochondrial metabolism and cellular responses to oxidative stress, aging, and apoptosis. The potent stress response regulator Foxo3A is a downstream target of p66ShcA redox signals that phosphorylate key regulatory sites, inhibiting transcription of Foxo3A stress-related gene products (11, 12). Because phosphorylation at a critical Ser-36 residue activates p66ShcA redox activity (13), mutation at this site should inhibit transactivation of reactive oxygen species (ROS)-dependent signals.

**The abbreviations used are: HIVAN, human immunodeficiency virus (HIV)-associated nephropathy; siRNA, small interfering RNA; CIDHP, conditionally immortalized differentiated human podocyte(s); GFP, green fluorescence protein; DPl, diphenylethionium; γH2AX, phospho-histone H2AX; DCFDA, 2′,7′-dichlorofluorescein (DCF) diacetate; SFM, serum-free media; EV, empty vector; PBMC, peripheral blood mononuclear cells; ROS, reactive oxygen species; ELSA, enzyme-linked immunosorbent assay; TUNEL, terminal dUTP nick-end labeling; PBS, phosphate-buffered saline; PKB, protein kinase B; HA, hemagglutinin.

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that target Foxo3A and genomic DNA, triggering activation of the apoptosis program. We have proposed a model in which inhibition of p66ShcA redox activity results in the activation of a Foxo3A-dependent stress program that shifts the phenotype of podocytes expressing HIV-1 genes away from apoptosis and toward cell survival.

In the present study conditionally immortalized differentiated human podocytes (CIDHPs) were genetically engineered to co-express a truncated HIV-1 construct (NL4-3-GFP) together with mutant-36p66ShcA (mu-36) or isoform-specific p66ShcA siRNA (p66-siRNA) to test the hypothesis that p66ShcA-deficient CIDHP will exhibit an oxidant-resistant phenotype and resistance to NL4-3-induced apoptosis signals. Our results document a pivotal role for p66ShcA redox activity in the NL4-3/CIDHP stress phenotype that is abrogated by co-transfection with mu-36 or p66Shc-siRNA, which in turn increases FOXO3a ability to promote the survival phenotype.

EXPERIMENTAL PROCEDURES

Previously, the lack of an in vitro podocyte culture system prevented a detailed analysis of the effects of HIV-1 gene expression on podocytes. However, with the establishment of conditionally immortalized human podocytes (CIDHP), it is possible to study HIV-1 genes responsible for cellular phenotype changes in vitro studies.

In the present study podocytes (CIDHP) were either a transduced or transfected HIV-1 construct under a natural long term repeat promoter in gag/pol-deleted NL4-3:EGFP construct. In both cases the HIV-1 genome integrates into the host cell genome and expresses its genes as it does under natural conditions. Therefore, this is the best possible way that HIV genes can be expressed by using an in vitro system, which imitates maximum closeness to physiological conditions (14, 15). Furthermore, the same plasmid construct had been used earlier to generate a Tg mouse model of HIVAN (16).

Preparation of Podocytes—Human podocytes were obtained from Dr. Moin A. Saleem (Children’s Renal Unit and Academic Renal Unit, University of Bristol, South Mead Hospital, Bristol, UK). Human podocytes were conditionally immortalized by introducing temperature-sensitive SV40-T antigen after transfer to the nonpermissive temperature (37 °C). The cell lysate was centrifuged, and 20 μl of supernatant was used to transfect the cell death ELISA was also performed using the Cell Death Detection ELISA Plus kit (Roche Applied Science) according to the manufacturer’s instructions. The assay is based on endogenous endonuclease cleavage of chromosomal DNA into mono- and oligonucleosomes, which are detected in cytoplasmic fraction using biotin-labeled anti-histone antibody in a 96-well ELISA format. In brief, HIV-1 vector-transduced podocytes were lysed in 200 μl of lysis buffer for 2 h at room temperature. The cell lysate was centrifuged, and 20 μl of supernatant was transferred on streptavidin-coated microplate wells. Subsequently, 80 μl of immuno-reagent was added followed by incubation for 2 h at room temperature under gentle shaking. The wells were washed thoroughly 4 times in washing buffer, and then 100 μl of ABTS substrate solution was added and incubated under gentle shaking for 10–20 min. The color development by peroxidase was measured by absorbance at A405-490 nm. PODocyte apoptosis was measured in both conditionally immortalized human podocytes and CIDHPs at the indicated time periods. To determine the role of oxidative stress, the cell death ELISA was also performed on HIV- and vector-transduced CIDHPs in the presence of NADPH oxidase inhibitor diphenyleneiodonium (DPI, 5 μM).

TUNEL Assay—Podocytes were grown on collagen-coated coverslips at nonpermissive temperature and transduced with pseudotyped replication-deficient HIV-1 and vector control
viruses for 48 h. TUNEL assay was performed using Apoptosis Detection Tacs TdT kit (R&D System, Minneapolis, MN). In brief, the cells were fixed in 3.7% formaldehyde in 1× PBS. The cells were permeabilized in Cytonin solution for 15 min at room temperature and then quenched in 3% H2O2 in methanol for 5 min. The cells were washed and treated with TdT-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 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.

Annexin V-phosphatidylethanolamine Staining—Podocytes were grown on collagen-coated flasks at non-permissive temperature (CIDHPs) and transduced with pseudotyped replication deficient HIV-1 and vector control viruses for 24 h. Subsequently, cells were harvested and stained with annexin V-phosphatidylethanolamine and 7-aminoactinomycin D as described in the kit (Annexin V-PE apoptosis detection kit, BD Biosciences Pharmingen). The Cells were analyzed by flow cytometry using a FACS-Vantage flow cytometer (BD Biosciences). Transduced cells were identified by the presence of annexin V staining in the absence of 7-aminoactinomycin D (7-AAD) staining, and necrotic cells were identified by the presence of 7-AAD staining. p < 0.05 was used to define statistical significance.

Immunofluorescence Detection of Oxidant Stress Associated with Podocyte HIV-1 Expression—The trafficking of 2,3,4- and 5,6-pentafluorodihydrotetramethyllrosamine (PF-HTMRos or Redox Sensor Red CC-1, Molecular Probes, Eugene, OR) was used to detect reactive oxygen intermediates in CIDHPs expressing HIV-1. Redox Sensor Red CC-1 is oxidized in the presence of O2· and H2O2. In brief, human podocytes transfected with plasmid constructs, including NL4-3 HIV (pNL4-3: ΔG/P-EGFP, NL4-3/DCIHP), empty vector, mu-36 p66shc (dominant negative, DN, mu-36p66ShcA) + NL4-3 HIV, mu-36 p66shcA, or NL4-3 + DPI, were loaded at 37 °C for 20 min with Redox Sensor Red CC-1 (1 μM) and a mitochondria-specific dye, MitoTracker Green FM (50 nM; Molecular Probes). Culture slides were washed and mounted with PBS and visualized with Nikon fluorescence microscope (Nikon Eclipse E800) equipped with triple filter cube and charge-coupled device (CCD) 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.
Immunofluorescence Detection of DNA Strand Breaks—Differentiated human podocytes (CIDHP) transfected with plasmid constructs including NL4-3 HIV, empty vector, or mu36-p66ShcA were plated on poly-D-lysine-coated Lab-Tek culture slides. Cells were fixed and permeabilized with a buffer containing 0.02% Triton X-100 and 4% formaldehyde in PBS. Fixed cells were washed 3 times in PBS and blocked in 1% bovine serum albumin for 30 min at 37 °C. Phospho-histone H2AX (H9253-H2AX) was detected by a mouse monoclonal antibody that recognizes phosphorylated serine within the amino acid sequence 134–142 of human histone H2A.X (UBI) and rhodamine-conjugated goat anti-mouse secondary antibody (Molecular Probes). Negative controls were performed in the presence of nonspecific isotype antibody in place of primary antibody. In all variables DNA was counterstained with 4',6-diamidino-2-phenylindole. Specific staining was visualized with an inverted Olympus 1X 70 fluorescence microscope equipped with a Cook Sensicom ER camera (Olympus America, Melville, NY). Final images were prepared with Adobe Photoshop to demonstrate subcellular localization of γH2AX. The percentage of localization of γH2AX was calculated from the entire volume of the fluorescent signal was calculated with Microsoft excel using equation \((\text{Ft} - \text{Fo})/\text{Fo} \times 100\) (20).

Western Blotting Studies—Empty vector (EV)/CIDHPs, EV/mu36-p66Shc/CIDHPs, NL4-3/CIDHPs, or mu36-p66Shc/NL4-3/CIDHPs were incubated in media for 48 h. At the end of the incubation period, cells were harvested, lysed in radioimmune precipitation assay buffer containing 50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% deoxycholate, 0.1% SDS, 1× protease inhibitor mixture (Calbiochem, Mixture Set I), 1 mM phenylmethylsulfonyl fluoride, and 0.2 mM sodium orthovanadate. Protein concentration was measured with the BCA Protein Assay kit (Pierce). Total protein extracts (20 μg/lane) were separated on a 15% PAGE pre-made gel (Bio-Rad) and transferred onto a nitrocellulose membrane using Bio-Rad miniblot apparatus. Nitrocellulose membranes were then processed further for immunostaining with primary antibodies against p66(ShcA) (recognizes all ShcA isoforms, Cell Signaling), mouse monoclonal anti-phospho-ShcA-Ser-36 (Calbiochem), anti-phospho-Foxo3a (Th-32), rabbit polyclonal antibody/pS9138/321Foxo3a/ps253/Foxo3a (Cell Signaling

Determination of ROS Kinetics in HIV-1-transduced Podocytes—The kinetics of ROS metabolism in HIV-1-expressing podocytes was determined by measuring the intensity of the fluorescent signal from the redox-sensitive fluorophore 2',7'-dichlorofluorescein diacetate (DCFDA) at multiple time points. DCFDA is converted by intracellular esterases to 2',7'-dichlorodihydrofluorescein, which in turn is oxidized by H2O2 to the fluorescent 2',7'-dichlorohydrofluorescein (DCF). Briefly, CIDHPs transduced with either pseudo-virus (NL4-3HIV or empty vector) or transfected with plasmid constructs (pNL4-3 HIV or empty vector) were plated in 24-well plates in phenol red-free Dulbecco’s modified Eagle’s medium (DMEM) for 48 h at 37 °C. Subsequently, cells were washed with phenol red-free DMEM and incubated in serum-free media (SFM) containing DCFDA (10 mM) for 30 min. Cells were washed, and DCF fluorescence was detected by a Fluorescence Multi-Well Plate Reader CytoFluor 4000 (PerSeptive Biosystems) set for excitation of 485 nm and emission of 530 nm. The intensity of the fluorescent signal was calculated with Microsoft excel using equation \((\text{Ft} - \text{Fo})/\text{Fo} \times 100\) (20).

FIGURE 2. NL4-3-induced ROS generation in CIDHP. Panel A, cells were plated in SFM and loaded with the redox-sensitive dyes Red CC-1 and the mitochondria-specific dye MitoTracker Green FM and CIDHP expressing EV, NL4-3, and NL4-3 plus the free radical scavenger 10 μM DPI in media. NL4-3/CIDHP show bright yellow/orange fluorescence because of the co-localization of Red CC-1 and MitoTracker Green FM in mitochondria, whereas in NL4-3/CIDHP + DPI, the fluorescent signal is attenuated. Panel B, DPI inhibits the kinetics of NL4-3-induced ROS generation. CIDHP were plated in SFM and loaded with the redox-sensitive probe DCFDA. The intensity of the DCF signal was determined at the indicated intervals. Data are presented as the means and represent three independent experiments. *, \(p < 0.01\) versus other variables.

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Technology, Beverly, MA), anti-phospho-S473/Akt (Cell Signaling Technology), and anti-AKT and anti-Foxo3A and subsequently with horseradish peroxidase-labeled appropriate secondary antibodies. The blots were developed using a chemiluminescence detection kit (Pierce) and exposed to x-ray film (Eastman Kodak Co.). Equal protein loading and protein transfer were confirmed by immunoblotting for determination of actin protein using a polyclonal /H9251 -Actin antibody (I-19, Santa Cruz, CA) on the same Western blots.

To explore whether HIV-1 infection also enhances phospho-p66ShcA in primary human cells, peripheral blood mononuclear cells (PBMC) were isolated from fresh blood. PBMC were incubated in media containing either buffer or X4 strain, HIV-1HT/92/599 for variable periods (6 and 24 h). Afterward, non-internalized virus was removed by incubation with 0.05% trypsin at 37 °C for 10 min followed by extensive washing. Viral stock of HIV-1HT/92/599 was prepared in phytohemagglutinin-activated primary lymphocytes cultured in the presence of interleukin-2. Proteins were isolated from control and HIV-1-infected PBMC. Western blots were prepared and probed for phospho-p66ShcA and actin as mentioned above.

RESULTS

To explore the role of HIV-1 genes in the phenotype expressed by podocytes in HIVAN, CIDHP were transfected with the truncated HIV-1 construct NL4-3 (lacking gag and pol genes) using the natural long term repeat promoter.

**NL4-3 Decreases Survival of CIDHP**—CIDHP were transduced or transfected with NL4-3:EGFP plasmid construct. Fig. 1A is a representative microphotograph showing GFP(/H11001 CIDHP. In the various experimental protocols the % of GFP(/H11001 cells ranged from 60 to 80%. As shown in Fig. 1B, NL4-3/CIDHP show a progressive decline in cell viability at 48, 72, and 96-h post-transduction, as judged by trypan blue exclusion. To test if NL4-3 activates the death program by apoptosis, two approaches were employed; annexin V binding (Fig. 1C) and TUNEL assay (Fig. 1D). A 5-fold increase in NL4-3/CIDHP apoptosis was detected by both annexin V binding and TUNEL assay. Taken together, NL4-3 is a potent stimulus for apoptosis in CIDHP.

**NL4-3-induced Apoptosis Is ROS-dependent**—ROS are known to induce multiple DNA lesions ranging from single base modifications to single strand DNA breaks and potentially lethal double strand DNA breaks (18, 19). HIV-1-in-
duced oxidative stress has been linked to apoptosis in macrophages and neurons (20, 21). To test if NL4-3 increases intracellular ROS production, CIDHP were transfected with EV or NL4-3 and maintained in media for 48 h. Cells were then loaded with Red CC-1 and the mitochondria-specific dye MitoTracker Green FM. As shown in Fig. 2A, NL4-3/CIDHP show bright yellow/orange fluorescence due to colocalization of oxidized Red CC-1 and MitoTracker Green FM in mitochondria, indicative of augmented ROS production. An identical analysis with NL4-3/CIDHP in the presence of the free radical scavenger DPI shows no increase in the intensity of the fluorescent signal.

To determine whether NL4-3 shifts the kinetics of ROS metabolism, CIDHP were loaded with the redox-sensitive fluoroprobe dichlorofluorescein (DCFDA), which in turn is oxidized to the highly fluorescent DCF, and the intensity of the fluorescent signal was measured at multiple time points (Fig. 2B). NL4-3/CIDHP kinetic curves show a marked increase in amplitude that was sustained over the 3-h interval. An identical analysis with NL4-3/CIDHP + DPI or EV/CIDHP shows suppressed rates of ROS metabolism.

*p66ShcA Redox Activity Is Indispensable for NL4-3-induced ROS Generation*—We next asked if p66ShcA is required for NL4-3-induced ROS production. To compete with endoge-
To determine whether NL4-3-induced ROS production is attenuated in CIDHP expressing mu-36, cells were loaded with the redox-sensitive fluoroprobe DCFDA, and the intensity of the 2',7'-dichlorohydrofluorescein signal was determined at multiple time points. As shown in Fig. 3C, NL4-3/CIDHP expressing mu-36 showed marked attenuation in the amplitude of ROS kinetic curves, whereas NL4-3/CIDHP show a sustained increase of this parameter.

**Knockdown of p66ShcA Inhibits NL4-3-induced ROS Generation**—As a second approach we used siRNA strategy to inhibit endogenous p66ShcA redox function. An isoform-specific p66-siRNA (23) was used to knockdown p66ShcA expression (Fig. 4A) in CIDHP. Nuclear lamin siRNA was used as control. p66-siRNA cells show minimal levels of p66ShcA protein, whereas in control cells p66ShcA expression was not affected by lamin siRNA. We next asked if p66-siRNA/CIDHP exhibit resistance to NL4-3-induced ROS generation. As shown in Fig. 4B, NL4-3/CIDHP show bright yellow orange fluorescence, indicative of augmented ROS production, whereas in NL4-3/CIDHP transfected with p66-siRNA, the fluorescent signal is attenuated. To quantify the intensity of fluorescent signal per cell, we repeated this analysis using Red CC-1 alone (Fig. 4C). As shown in panel D, p66-siRNA reduced the intensity of the fluorescent signal to control values. Taken together, p66ShcA redox function is indispensable for NL4-3 ROS generation.

**Inhibition of p66ShcA Attenuates NL4-3-induced DNA Damage and Apoptosis**—Cell survival and longevity are closely linked with the maintenance of genomic stability (24, 25). The DNA double helix is a target for ROS-dependent signals. We hypothesized that p66ShcA-deficient cells will be resistant to NL4-3-induced oxidative DNA damage. To evaluate foci of DNA breaks, immunolabeling of NL4-3/CIDHP, indicative that NL4-3-induced ROS production, whereas in NL4-3/CIDHP transfected with p66-siRNA, the fluorescent signal is attenuated. To quantify the intensity of fluorescent signal per cell, we repeated this analysis using Red CC-1 alone (Fig. 4C). As shown in panel D, p66-siRNA reduced the intensity of the fluorescent signal to control values. Taken together, p66ShcA redox function is indispensable for NL4-3 ROS generation.

**Inhibition of p66ShcA Prevents Phosphorylation at Thr-32 of the Foxo3A Protein**—The redox function of the p66ShcA protein is activated by ROS-dependent signals that phosphorylate a critical Ser-36 residue, located at the N-terminal region (26). We hypothesize that levels of phospho-Ser-36 will be increased in NL4-3/CIDHP, whereas phospho-Ser-36 is not.

**HIV-1 Induces Podocyte Apoptosis**

In a previous study, we have demonstrated that HIV-1 entry into podocytes induces ROS production (19). These observations are important because podocytes are the major site of nephrotic syndrome, and oxidative stress has been implicated in the pathogenesis of this disease (20). To determine whether HIV-1 entry into podocytes induces ROS production, we used the redox-sensitive fluoroprobe DCFDA. As shown in Fig. 5, the intensity of the DCFDA signal in HIV-1-exposed podocytes was significantly lower than in control cells, indicating that HIV-1-induced ROS production is attenuated in CIDHP expressing mu-36.

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expected to increase in NL4-3/CIDHP expressing the dominant negative mu-36. To test this hypothesis, cell lysates were probed with anti-ShcA/p66 (Ser(P)-36) mouse monoclonal antibody that recognizes the 66-kDa isoform of ShcA, phosphorylated at Ser-36 (22). As shown in Fig. 7A, NL4-3/CIDHP show up-regulation in phospho-Ser-36 levels, whereas NL4-3/CIDHP expressing mu-36 show no detectable alteration in phosphorylation status of Ser-36.

p66ShcA interacts with Foxo3A by facilitating ROS-dependent activation of Akt/PKB (27–29), which in turn phosphorylates and inactivates Foxo3A via an evolutionary conserved pathway (27, 28). To test if this signaling pathway is dormant in NL4-3-expressing mu-36, the phosphorylation status of Ser-473 of Akt/PKB protein was examined (28). Consistent with result of phospho-Ser-36, NL4-3/CIDHP show increased levels of phospho-Ser-473 of the Akt/PKB protein (Fig. 7A), but phospho-Ser-473 remained unchanged in NL4-3/CIDHP expressing mu-36.

The phosphorylation status of Foxo3A and its subcellular localization are critical for its transcriptional activity (27, 28). Phosphorylated Foxo3A is exported from the nucleus, whereas unphosphorylated Foxo3A is transcriptionally active in the nucleus. The sites of Akt/PKB-dependent Foxo3A phosphorylation have been mapped to three key regulatory residues, Thr-32, Ser-318/321, and Ser-253. As shown in Fig. 7A, phospho-Thr-32 levels were also increased in NL4-3/CIDHP, but no alteration in the phosphorylation status of Ser-318/321 or Ser-253 was detected (Fig. 7B). An identical analysis performed with NL4-3/CIDHP expressing mu-36 shows no change in the phosphorylation status of the three regulatory residues. To determine whether primary HIV-1 strain has the potential to promote phosphorylation in human immune cells, freshly isolated PBMC were infected with HIV-1 for 6 and 24 h and then evaluated for the expression of phospho-p66ShcA. As shown in Fig. 7C, HIV-1 stimulated phosphorylation of p66ShcA in PBMC.

Inhibition of p66ShcA Attenuates Nuclear Export of HA-Foxo3a—To evaluate Foxo3A subcellular localization, CIDHP were transfected with HA-Foxo3A and analyzed with an antibody directed against the HA epitope. In general, because of low endogenous levels of Foxo3A, the effects of p66ShcA inhibition were more evident after co-transfection with HA-Foxo3A expression plasmid. As shown in Fig. 8A, HA-Foxo3A is exported to the cytoplasmic compartment in NL4-3/CIDHP, indicated by the absence of purple fluorescence in the nuclear compartment, counterstained with 4′,6-diamidino-2-phenylindole (blue fluorescence). EV/CIDHP and NL4-3/CIDHP expressing mu-36 exhibit retention of HA-Foxo3A in the nucleus, indicated by the detection of purple staining against a blue background. A histogram of the % cells positive for cytoplasmic/nuclear versus cytoplasmic HA-Foxo3A (Fig. 8B) shows a marked reduction in cytoplasmic/nuclear HA-Foxo3A and increase in cytoplasmic HA-Foxo3A in NL4-3/CIDHP, whereas for EV/CIDHP and NL4-3/CIDHP expressing mu-36, the % of cells positive for cytoplasmic/nuclear HA-Foxo3A shows a 3- and 4-fold increase, respectively. Taken together, inhibition of p66ShcA redox function attenuated NL4-3-induced phosphorylation and nuclear export of HA-Foxo3A.
Knockdown of Foxo3A Increases NL4-3-induced Apoptosis in mu-36/CIDHP—To test if the survival phenotype in NL4-3/CIDHP expressing mu-36 is mediated by Foxo3A stress program, cells were transfected with Foxo3A-siRNA (Fig. 9A). As shown immediately below, nuclear lamin siRNA did not affect Foxo3A expression, whereas Foxo3A siRNA cells show knockdown of Foxo3A expression (Fig. 9B). Analysis of apoptosis by ELISA cell death assay detected a 50% increase in NL4-3/CIDHP cells expressing mu-36 and Foxo3A-siRNA (Fig. 9C). Taken together, Foxo3A is necessary for induction of the mu-36 oxidant-resistant pro-survival phenotype in NL4-3/CIDHP.

DISCUSSION

The present study documents a pivotal role for p66ShcA protein on the redox status of podocytes expressing HIV-1 genes. We have shown that inhibition of p66ShcA redox activity induces a strong oxidant-resistant phenotype in NL4-3/CIDHP that shifts the kinetics of intracellular ROS metabolism and promotes the survival phenotype by attenuating or preventing oxidative DNA damage and apoptosis. Finally, in p66ShcA-deficient CIDHP expressing NL4-3, we document a key role for Foxo3A in the orchestration of the stress response and survival phenotype.

Several lines of investigation indicate HIV-1-induced proteins expressed by the host in response to viral infection contribute to the protean manifestations of AIDS, including muscle wasting, insulin resistance, and cardiomyopathy (30). In this regard, HIV-1-specific RNA and proviral DNA have been detected in renal epithelium and podocytes from patients with HIVAN (5). Until recently, the lack of an in vitro podocyte cell culture system prevented a detailed analysis of HIV-1 gene expression in podocytes. The availability of conditionally immortalized human podocytes (14, 15) and the NL4-3 construct (16, 17) provide the experimental tools for such an analysis. Recently, a paradigm based on HIV-1-induced overproduction of ROS has been proposed to account for the activation transduction of NL4-3 redox signals that alter the stress phenotype of CIDHP via the activation of the apoptosis program.

To the best of our knowledge, this is the first report documenting Foxo3A-induced stress resistance in a mammalian cell line expressing HIV-1 genes. The Foxo family of forkhead transcription factors (Foxo1, Foxo3, Foxo4, and Foxo6 in mammals) participate in various cell functions, including apoptosis, cell cycle progression, and antioxidant defense (35). Growth factor signaling via phosphatidylinositol 3-kinase and its downstream effector Akt/PKB to Foxo3A has been found to be evolutionary conserved for Foxo3A phosphorylation and nuclear export, resulting in the inhibition of Foxo3A transcriptional activity (27, 28, 36). Akt/PKB kinase activity is mediated by phosphorylation of Ser-473 at the C terminus and Thr-308 in the catalytic domain (29). The sites of Foxo3A phosphorylation by Akt/PKB have been mapped to three key regulatory residues, Thr-32, Ser-318/321, Ser-253. Interestingly, only Thr-32 showed increased levels of phosphorylation, which was not detected in NL4-3/CIDHP expressing mu-36. This finding is in agreement with that observed at regulatory resides of the upstream molecules, Ser-36 of p66ShcA and Ser-473 of Akt/PKB, for which no change in the phosphorylation status was detected in NL4-3/CIDHP expressing mu-36. Conversely, NL4-3/CIDHP with intact p66ShcA redox function show increased levels of phosphorylation at Ser-36, Ser-473, and Thr-32. The absence of serum or growth factors in these protocols strongly suggests changes in the phosphorylation status of Akt/PKB, reflect cell redox status, and are not mediated by phosphatidylinositol 3-kinase. Taken together, in CIDHP expressing HIV-1 genes we have identified a pivotal role for p66ShcA redox function in the evolutionary conserved phosphatidylinositol 3-kinase/Akt/PKB signaling module, which inactivates Foxo3A.

The phosphorylation status and subcellular distribution of Foxo3A are critical determinants of its transcriptional activity (27, 28). In general, because of low levels of endogenous Foxo3A expression, the effects of p66ShcA inhibition were more evi-
dent after co-transfection with HA-Foxo3A expression plasmid (26) and indirect immunofluorescent staining with an antibody against the HA epitope. Consistent with the increased levels of phospho-Thr-32 in NL4-3/CIDHP, HA-Foxo3A was almost exclusively localized to cytoplasm, where it is known to be sequestered by 14-3-3 proteins (26). Conversely, EV/CIDHP and NL4-3/CIDHP, in which there was no detectable alteration in phosphorylation levels of Thr-32, show a significant fraction of HA-Foxo3A retained at the cytoplasmic/nuclear boundary. Taken together, our findings are in agreement with previous reports indicating the phosphorylation status of Foxo3A is a key determinant of subcellular localization (26).

A fundamental mechanism by which cells defend themselves against oxidative stress is the repair of damaged DNA (37). The growth arrest and DNA damage response gene (Gadd45) is a direct target of Foxo3A that promotes Foxo3A-dependent DNA repair (27, 28). We evaluated the functional significance of inhibiting cross-talk between p66ShcA and Foxo3A by examining oxidative DNA damage and apoptosis. Our data show unequivocally, inhibition of NL4-3-induced p66ShcA redox activity attenuates or prevents DNA strand breaks and apoptosis, implying inhibition of p66ShcA may rescue NL4-3/CIDHP by enhancing Foxo3A ability to maintain genomic integrity. We performed Foxo3A knockdown experiments to test if Foxo3A is required to defend mu-36/CIDHP from NL4-3 danger signals that activate apoptosis. Acute knockdown of Foxo3A induced a 50% apoptosis in NL4-3/CIDHP expressing mu-36, indicating a Foxo3A-dependent response operates in the rescue of these cells. Taken together, our findings are consistent with a growing body of evidence suggesting an important role for Foxo3A in stress resistance and the aging process in mammals (27).

The present study has certain limitations, including the necessity to maintain cells under SFM to eliminate the confounding effect of serum and contained growth factors on signaling pathways linked to cell survival and oxidant stress (14). Moreover, we must acknowledge the limitations of short term in vitro cell culture systems in simulating the chronic in vivo condition of HIVAN. Finally, although beyond the scope of the present manuscript, we have not explored the impact of inhibiting p66ShcA redox activity on the HIV-1-induced stress phenotype(s) of podocyte dedifferentiation and podocyte proliferation, integral components of HIV-1 collapsing glomerulopathy. These important questions must await future investigations, with HIV-1 transgenic mice crossed with p66ShcA knock out mice or genetically engineered mice with mice with podocyte-specific deletion of p66ShcA.

Taking into account the above limitations, we believe that our work may have important implications for preserving the interface between matrix and cellular components of the glomerular filtration barrier. ROS inflict irreversible injury at multiple sites in the kidney, including the glomerular basement membrane, where ROS target matrix components and alter the distribution of anionic charges, impairing permselectivity (38, 39). The linkage between α-dystroglycan of podocytes and the globular domains of laminin and agrin in the glomerular basement membrane are disrupted by oxidative stress, an alteration that predisposes to podocyte detachment. Accordingly, the application of gene-based strategy targeting p66ShcA may represent an exciting new avenue of therapeutic intervention in the kidney. Recent efforts aimed at developing targetable transduction systems have made significant progress toward addressing the problems of cell type-specific recognition and insertion mutagenesis (40). Whether strategies that incorporate siRNA to silence disease-causing genes (41) such as p66ShcA can be applied in vivo remains to be determined.

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