A Novel Role of Proline Oxidase in HIV-1 Envelope Glycoprotein Induced Neuronal Autophagy

Jui Pandhare1,2,3*, Sabyasachi Dash1,2, Bobby Jones1,2,3, Fernando Villalta1,2,3, and Chandravanu Dash1,2,4

From The Center for AIDS Health Disparities Research1, School of Graduate Studies and Research2, Department of Microbiology and Immunology3, Department of Biochemistry and Cancer Biology4, Meharry Medical College. Nashville, Tennessee, USA.

Running title: Proline oxidase induces neuronal autophagy

*Author to whom correspondence should be addressed: 1005 Dr. DB Todd Jr. Blvd, Old Hospital Building, Room 5023, Nashville, TN 37208, Tel: 615-327-6940, Fax: 615-327-6929, Email: jpandhare@mmc.edu

Keywords: POX, HIV-1 gp120, ROS, autophagy

Background: Stress response autophagy is induced during HIV-1 glycoprotein “gp120” mediated neurotoxicity. However, the underlying mechanisms are poorly understood.

Results: HIV-1 gp120 induces Proline oxidase that elicits ROS mediated neuronal autophagy.

Conclusion: Protective autophagy during HIV-1 gp120 neurotoxicity is partly dependent on proline oxidase induced ROS.

Significance: This is the first report that demonstrates the functional role of proline oxidase in HIV-1 gp120 mediated neuronal autophagy.

SUMMARY

Proline oxidase (POX) catalytically converts proline to pyrroline-5-carboxylate (P5C). This catabolic conversion generates reactive oxygen species (ROS) that triggers cellular signaling cascades including autophagy and apoptosis. This study for the first time demonstrates a role of POX in HIV-1 envelope glycoprotein (gp120) induced neuronal autophagy. HIV-1 gp120 is a neurotoxic factor and involved in HIV-1 associated neurological disorders (HAND). However, the mechanism of gp120-mediated neurotoxicity remains unclear. Using SH-SY5Y neuroblastoma cells as a model; this study demonstrates that gp120 treatment induced POX expression and catalytic activity. Concurrently, gp120 also increased intracellular ROS levels. However, increased ROS had a minimal effect on neuronal apoptosis. Further investigation indicated that the immediate cellular response to increased ROS paralleled with induction of autophagy markers, beclin 1 and LC3-II. These data lead to the hypothesis that neuronal autophagy is activated as a cellular protective response to the toxic effects of gp120. A direct and functional role of POX in gp120 mediated neuronal autophagy was examined by inhibition and over-expression studies. Inhibition of POX activity by a competitive inhibitor “dehydroproline” decreased ROS levels concomitant with reduced neuronal autophagy. Conversely, overexpression of POX in neuronal cells increased ROS levels and activated ROS-dependent autophagy. Mechanistic studies suggest that gp120 induces POX by targeting p53. Luciferase reporter assays confirm that p53 drives POX transcription. Furthermore, data demonstrate that gp120 induces p53 via binding to the CXCR4 co-receptor. Collectively, these results demonstrate a novel role of POX as a stress response metabolic regulator in HIV-1 gp120 associated neuronal autophagy.
INTRODUCTION

Proline oxidase (POX) also known as proline dehydrogenase (PRODH), is a mitochondrial inner-membrane metabolic enzyme [1]. POX catalyzes the first step of proline catabolism by converting proline to delta-1-pyrroline-5-carboxylate (P5C) [2] (Fig. 1). Catalysis of proline by POX generates electrons that are generally donated into the electron transport chain (ETC) to generate ATP [3]. However, under cellular stress environment, these electrons can be channeled to generate ROS [4,5]. The significance of ROS in intracellular signaling is well documented including proliferation, gene activation, cell cycle arrest, autophagy, and apoptosis [6,7]. Thus, POX has been demonstrated to serve as a multifunctional stress responsive protein which on one hand can contribute to ATP production and on the other can mediate apoptosis through generation of ROS [8-10]. Furthermore, recent evidence suggests that under stress conditions POX generated ROS elicits cellular protective autophagic signaling pathways [11,12].

Several lines of evidence indicate a role of POX and proline metabolism in normal function and disease conditions in the brain. POX gene is widely expressed [13] and high affinity proline transporter molecules are also detected in the brain [14-16]. Studies show that mice lacking the POX gene show impairment of learning and memory [17]. Several reports have also suggested that proline metabolism is associated with schizophrenia [18-20]. P5C—the catalytic product of proline can also be converted to glutamate and gamma-aminobutyric acid (GABA), two neurotransmitters implicated in neurological disorders [21,22]. Even though these studies and others [23] emphasize a role of proline metabolism in neurological disorders, the impact of POX and proline metabolism in HIV-1 neurological disorders has not been elucidated.

The goal of this study is to examine a role of POX in HIV-1 associated neurological disorders (HAND). HAND affects 20-30% patients in the late stages of AIDS and is believed to be the most common cause of dementia worldwide among people aged 40 or less [24,25]. Even though antiretroviral therapy has reduced the severity of HAND [26], the incidence of this neurological disease continues to rise since HIV-1 patients are living longer [27]. However, the molecular details of HAND pathogenesis are not completely understood. The brain is a major target for HIV-1 infection [28]. The virus enters the brain within days-weeks of infection and progressive neuronal damage has been observed in infected patients [29-32]. Although neurons are refractory to HIV-1 infection, macrophages and microglia are the primary target cells that are infected by HIV-1 in the brain [30]. The neuronal damage in infected patients is partly driven by the neurotoxic effects of HIV-1 proteins produced from the infected cells in the brain [31,32].

The viral proteins with known neurotoxic effects are the HIV-1 envelope glycoprotein “gp120”, and the accessory viral proteins Tat, Nef, and Vpr [31-33]. However, accumulating evidence suggest that gp120 is one of the major drivers of the neuronal loss observed in HAND patients [34,35]. It is well documented that the neurotoxic effects of gp120 are predominantly induced by ROS mediated oxidative stress [36-38]. The cellular metabolic enzyme NADPH oxidase has been implicated as the biochemical mediator of gp120-mediated ROS generation [39]. However, the impact of gp120 on mitochondrial ROS mediated oxidative stress is yet to be elucidated. Given that POX is a well-established mitochondrial ROS-inducing redox enzyme, we hypothesized that POX-dependent ROS may contribute to HIV-1 gp120-induced neuronal oxidative stress. To test this, we used a neuronal model cell line and examined the effects of physiologically relevant concentrations of gp120 on POX expression and catalytic activity. We also examined the impact of gp120 on POX-mediated ROS production, neuronal autophagy and apoptosis. Our results demonstrate that gp120 induced expression and activity of POX in neuronal cells. Importantly, the enhanced POX activity resulted in higher levels of intracellular ROS production. Furthermore, POX-induced ROS elicited neuronal autophagy as a protective mechanism for the cellular stress of gp120. Our mechanistic studies suggest that gp120 regulates POX via CXCR4 mediated induction of p53. These results describe a novel mechanism for gp-120 mediated neuronal dysfunction and implicate POX as a potential biochemical modulator of HAND pathogenesis.
EXPERIMENTAL METHODS

Reagents – Recombinant HIV-1 gp120 IIIB, CXCR4 inhibitor-AMD3100 were obtained from the AIDS Research and Reference Reagent Program of NIH. 2',7'-dichloro-fluorescein diacetate, bafilomycin A1, N-acetylcysteine, dehydroproline, α-pifithrin, o-aminobenzaldehyde and anti-actin antibody were purchased from Sigma Chemicals (St. Louis, MO). Anti-LC3 was purchased from MBL, sequestosome 1 was purchased from Cell Signaling Technology and anti-p53 was from Santacruz Biotech. Anti-POX antibody was a gift from Dr. James Phang (NCI-Frederick).

Cell culture and HIV-1 gp120 treatment - Human neuroblastoma cells (SH-SY5Y) were purchased from American Type Culture Collection (Manassas, VA). The cells were maintained in a 1:1 mixture of DMEM and Ham’s F12 medium (Gibco, USA) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Gibco) containing 2 mM glutamine and 1% antibiotics (penicillin–streptomycin). Cells were maintained and cultured at 37°C in a humidified 5% CO2 atmosphere. For our studies we seeded appropriate number of SH-SY5Y cells and treated them with HIV-1 gp120 in a dose dependent manner from 50-400ng/mL. These concentrations were used since these concentrations have been suggested in literature to have physiological relevance [40,41].

Flow Cytometry - Neuronal apoptosis was determined by flow cytometry using the fluorescein isothiocyanate (FITC)–conjugated Annexin V apoptosis kit (Beckman Coulter, USA) according to the manufacturer’s instructions. Untreated and HIV-1 gp120 treated SH-SY5Y cells (1x10^6) were washed in 1x annexin binding buffer and incubated for 20 minutes with Annexin V-FITC at 4°C. After two washes with buffer, propidium iodide (100 μg/mL) was added. Thereafter, cells were analyzed immediately by flow cytometry using Becton Dickinson, San Jose, CA). Positive staining of the plasma membrane with annexin V and lack of concomitant staining of nuclei with PI was measured as apoptosis at an early stage. Whereas, positive staining of cells with both annexin V and PI was indicative of apoptosis at later stages.

Measurement of intracellular ROS - For measuring ROS levels in neuronal cells, 5x10^6 SH-SY5Y cells were seeded in 6 well plates in the growth medium before treatment. Cells were then treated with gp120, as specified, for an additional 24 h before analysis for ROS. For inhibition studies, cells were treated with 10 μM dehydroproline for 1 h prior to treatment with gp120. 2,7-Dichlorofluorescein diacetate (DCF-DA, from Sigma) was used as an indicator of the amount of intracellular ROS. On the day of the experiment, treatment medium was removed and cells were exposed to serum-free, phenol red-free medium containing 10 µM DCF-DA. Cells were exposed to the dye for 30 min in the dark to allow for equilibration. After two washes fluorescence was measured by flow cytometry. In parallel cells were solubilized with 0.5% SDS and 5 mM Tris HCl (pH 7.5). The fluorescent intensity of the lysate was determined using a spectrofluorometer (BioTek) with excitation and emission wavelengths of 485 nm and 530 nm, respectively. Samples were assayed in triplicate. Data are shown as arbitrary units of fluorescence ± SD.

Western blotting - SH-SY5Y cells (1x10^6) were treated with HIV-1 gp120 overnight. Cell lysates were prepared and quantified according to standard BCA protein assay (Pierce, USA). For lysis with separation of nuclear and cytoplasmic components, after treatment cells were lysed with Pierce NE-PER Nuclear and Cytoplasmic Extraction Reagents. Subcellular fractionation was performed as per the manufacturer’s instructions (Pierce, USA). Equal amounts of cell lysates were electrophoresed on SDS-polyacrylamide gels and transferred to nitrocellulose membranes using a semi-dry blotter (Bio-Rad). Membranes were blocked using Tris-buffered saline with 5% nonfat milk (pH 8.0; Sigma). Blots were then probed with the primary antibody in blocking buffer, and subsequently by a secondary antibody conjugated to horseradish peroxidase (1:2000). All blots were washed in Tris-buffered saline with Tween 20 (pH 8.0; Sigma) and developed using the enhanced chemiluminescence (ECL) procedure (Pierce, USA). Blots were routinely stripped by Restore Plus stripping buffer (Pierce) and reprobed with
anti-actin monoclonal antibody to serve as loading controls. Anti-rabbit or anti-mouse antibody (Santacruz, Piscataway, NJ) was used as secondary antibody.

**POX enzyme assay** – Untreated and treated SH-SY5Y cells after treatment were harvested in cold PBS, pelleted, and resuspended in cold sucrose buffer [250 mM sucrose, 3.5 mM Tris, and 1 mM EDTA (pH 7.4)]. Suspensions were then sonicated for 5 s at a setting of 25% (Branson Sonifier 450; Branson Ultrasonics Corp., Danbury, CT). Total protein was determined using the BCA protein assay (Pierce, USA). P5C formed due to POX mediated proline degradation was detected using a specific spectrophotometric method [5]. Briefly, P5C formed from the substrate proline was reacted with O-aminobenzaldehyde (OAB) and the resultant OAB-P5C complex was quantified. A 200 µl reaction mixture containing KPO₄ 0.1 M, pH 7.2, OAB 0.12 mg/ml, cytochrome c 0.012 mg/ml, proline 5mM and cell extract containing 50 µg protein was incubated for 45 min at 37 °C. The reaction was terminated by addition of 20 µl of OAB (10 mg/ml in 6N HCl). The samples were centrifuged and the absorbance of the supernatants was measured at 440 nm. All the reactions were performed in triplicates and proper protein controls were included for each measurement.

**Quantitative Real-time PCR** - Total RNA from treated and untreated cells was isolated using RNAeasy Kit (Qiagen, CA) according to the manufacturer’s instructions. The first-strand cDNA was synthesized using an iScript cDNA Synthesis Kit (Biorad, Richmond, CA). Then, the cDNAs were subjected to quantitative real-time PCR analysis. The sequences of the primers used to amplify beclin-1 in the current study were previously reported [42] and were as follows: beclin-1, (forward) 5’-AGC TGG AGC TGG ATG ATG AG-3’, and (reverse) 5’-CGA CCC AGC CTG AAG TTA TT-3’; GAPDH, (forward) 5’-GAA GGT GAA GGT CGG AGT C-3’ and (reverse) 5’-GAA GAT GGT GAT GGG ATC TT-3’. Real-time PCR was performed using iQ SYBR Green supermix in a C1000 Touch CFX96 Real time System (Biorad, Richmond, CA). All samples were analyzed in triplicates. The relative expression of the transcripts was normalized to the internal control gene GAPDH using the delta-delta Cq calculation method. For POX mRNA expression measurement, total RNA was isolated from untreated and gp120 treated SH-SY5Y cells. cDNA synthesis was carried out as described before and real time PCR was conducted using POX specific primers (Forward: 5’- CCACAGTGGACACAGTGCG, Reverse: 5’- GAAATGGGACAGCCTC). For copy number determination a standard curve was generated using the pcDNA-POX plasmid copies from 10⁰ through 10⁸.

**POX Over-expression** - SH-SY5Y cells were cultured in 6-well plates in the required growth medium and transfected with pcDNA control vector or POX expression vector a gift from Dr. James Phang (NCI-Frederick). Transfections were performed with Lipofectamine 2000 (Life Technologies, USA), according to the manufacturer’s directions. Over-expression of POX in the transfected cells was confirmed by western blot analysis as described before.

**Detection of Autophagosomes** - The visualization of autophagosomes was performed using GFP-LC3 expression vector (plasmid 24920: Addgene, Cambridge, MA). SH-SY5Y cells were cultured in 6-well plates and transfected with GFP-LC3 expression vector using Lipofectamine based methods as described earlier. The transfected cells were treated with appropriate concentration of HIV-1 gp120. The GFP-LC3-labeled autophagosomes were visualized by fluorescence microscopy.

**Luciferase based POX Promoter assay** - POX transcriptional activity was measured using the Luciferase Reporter Assay (Promega, Madison, WI, USA) according to the manufacturer's protocol. To determine the effect of HIV-1 gp120 on POX promoter activity, cells were transfected with the POX-Luciferase reporter construct (POX-Luc), a gift from Dr. James Phang (NCI-Frederick) and exposed to various concentration of HIV-1 gp120. The effect of p53 on POX promoter activity, was measured by co-transfection of POX-Luc construct along with equivalent amounts of p53 cDNA, or vector plasmid using Lipofectamine 2000 (Invitrogen, Gaithersburg, MD). Transfected cells were lysed and luciferase activity of the cell
extracts was measured using a plate reader (BioTek). Samples were assayed in triplicate.

**Statistical analysis** - Data were expressed as mean ± SD obtained from three independent experiments. Significance of differences between control and treated samples was determined by Student’s t test. Values of p < 0.05 were considered to be statistically significant.

**RESULTS**

**HIV-1 gp120 upregulates the mitochondrial redox enzyme POX.** Increased oxidative stress is a major driver of HIV-1 gp120 protein mediated neurotoxicity [36-38]. However the molecular and biochemical determinants leading to neuronal oxidative stress are not clearly defined. POX plays an important role in oxidative stress because of its ability to generate ROS [5,43] (Fig. 1). Therefore, we investigated whether POX is induced as an oxidative stress response enzyme by gp120. To test this we treated SH-SY5Y neuroblastoma cells with gp120 in a dose dependent manner. Cell lysates of gp120 treated cells were analyzed for POX expression by Western blot analysis. As described in Fig. 2A-B exposure of increasing concentrations of gp120 increased POX protein expression in a dose dependent manner. Densitometry analysis illustrated that a maximum induction of 4 fold POX expression was achieved in cells treated with 300 ng/mL of gp120 relative to untreated cells (Fig. 2B).

Next, we examined whether gp120 induced POX expression resulted in enhanced POX catalytic activity. This was tested by a spectrophotometric assay that detects the product of POX mediated proline degradation- “P5C” as an o-aminobenzaldehyde-P5C complex (Fig. 1) [5,43]. After treatment with gp120, cells were harvested and lysates were added to the reaction mixture and incubated for 45 min at 37°C. Data in Fig. 2C showed that gp120 treatment increased the amount of P5C produced in a dose-dependent manner. A maximum increase upto 3.5 fold in POX activity was obtained with 300 ng/mL of gp120 paralleling the maximum increase in POX protein expression at this concentration of gp120 (Fig. 2B). Collectively, data in Fig. 2 indicated that gp120 treatment induced POX expression and activity in neuronal cells.

To test that gp120 specifically targets POX, we treated cells with heat inactivated gp120 (200 ng/mL) and measured POX expression and activity. Western data in Fig 2D showed that exposure to heat inactivated gp120 failed to induce POX expression. Similarly, heat inactivated gp120 had minimal effect on POX activity (Fig. 2E).

**Upregulation of POX enhances intracellular ROS production.** POX is a mitochondrial metabolic enzyme and under cellular stress its catalytic activity is known to generate ROS [5,43] (Fig. 1). Therefore, we tested whether the increase in POX activity by gp120 enhanced ROS levels in neuronal cells. SH-SY5Y cells were treated with gp120 in a dose dependent manner. The levels of intracellular ROS were measured by the peroxide-sensitive fluorescent probe 2',7'-dichlorofluorescein (DCF). Significant increase in DCF fluorescence was observed in the cellular lysates of cells treated with 200 ng/mL of gp120 (Fig. 3A). A maximum increase of 3 fold in DCF fluorescence was observed with 400 ng/mL of HIV-1 gp120 (Fig. 3A). These data indicated a concentration dependent increase in intracellular ROS in gp120 treated neuronal cells.

Since intracellular ROS can be generated by several cellular processes [6,7,44], we examined whether gp120 induced upregulation of POX contributes to the increased ROS. To test this, SH-SY5Y cells were treated with gp120 (200 ng/mL) in the presence of proline-the substrate of POX, or dehydroproline (DHP)- a competitive inhibitor of POX. ROS was measured in the lysates of these cells by DCF fluorescence (Fig. 3B) and flow cytometry (Fig. 3C). As expected gp120 treated cells showed higher ROS levels compared to untreated cells (Fig. 3B-C). Addition of proline in the presence of gp120 further enhanced ROS levels, while cells treated with proline alone without gp120 had no effect on ROS levels. Conversely, data from cells treated with DHP illustrated that POX inhibition suppressed the increase in ROS in the gp120 treated cells (Fig. 3B-C). Both fluorimetry and flow cytometry measurements showed similar effects of proline and DHP on ROS. Collectively, these data strongly suggest that induction of POX contributes to the increased ROS levels in gp120 treated neuronal cells.
HIV-1 gp120-induced ROS shows minimal effect on neuronal apoptosis. Increased ROS plays important role in the induction of cellular apoptosis [45]. Therefore, we examined the effects of gp120-induced ROS (Fig. 3) on neuronal apoptosis. SH-SY5Y cells were exposed to different concentrations of gp120 and apoptosis was measured by flow cytometry. Annexin-V (AV) was used as an early apoptotic marker and propidium iodide (PI) as the late apoptotic marker [46]. Data presented in Fig. 4A demonstrated that treatment of cells with gp120 at 300 ng/mL for 24 h resulted in a moderate increase in AV staining. However, a two-fold increase in PI staining was only observed in cells treated with gp120 at 400 ng/mL (Fig. 4B). Interestingly, cells treated with 200 ng/mL or lower concentrations of gp120 showed minimal change in AV or PI staining compared to the untreated cells (Fig 4A and 4B). These results suggest that gp120 treatment for 24 h does not induce significant neuronal apoptosis. Furthermore, these observations implied that the increase in ROS production by gp120 in 24 h (Fig. 3) is most likely not sufficient to elicit apoptotic signal. To further understand the relationship between ROS and apoptosis, we measured ROS and apoptosis levels in cells treated with 200 ng/mL of gp120 as a function of time. As seen in Fig. 4C, gp120 increased ROS levels as a function of exposure time. Compared to the ROS levels at 24 h, the ROS levels increased approximately two fold by 48 h and three fold by 72 h in gp120 treated cells. Predictably, the ROS levels in untreated cells was also increased at 72 h, however this increase was significantly lower compared to the gp120 treated cells at 72 h (Fig. 4C). Interestingly, the significant increase in ROS by 200 ng/mL of gp120 treatment moderately increased neuronal apoptosis as a function of time (Fig. 4 D-E). After 24 h there was a minimal increase in AV (+) (Fig. 4D) or PI (+) (Fig. 4E) staining compared to the untreated cells. The AV (~3%) and PI (~7%) staining in treated cells increased modestly by 48 h-72 h. These data suggested that gp120 induced increase in ROS has limited effects on apoptosis as a function of time.

HIV-1 gp120 activates neuronal autophagy. Data in Fig 3A and Fig. 4D indicated that the neuronal cells treated with >200 ng/mL HIV-1 gp120 produced significantly higher levels of ROS. However, this increase in ROS induced minimal apoptosis (Fig. 4). Therefore, we investigated the fate of this ROS on neuronal cell function. It is well documented that ROS can function as a signaling molecule not only for apoptosis but for diverse cellular processes such as proliferation, gene activation, cell-cycle arrest and autophagy [6,7]. Autophagy is a self-defense mechanism against cellular stress/damage [47-50] and there is evidence that POX-dependent ROS induces stress response autophagy [11,51]. Therefore, we tested whether gp120 induced ROS elicited autophagy signals. We measured the expression of LC3-II in gp120 treated cells since the conversion of the cytosolic LC3-I (18 kDa) into the membrane-associated lipidated LC3-II form (16 kDa) serves as a marker for induction of autophagy [52]. Western blot analysis of lysates of SH-SY5Y cells treated with gp120 for 24 h showed significantly increased LC3-II conversion (Fig. 5A-B). The LC3-II conversion increased in a dose-dependent manner (Fig. 5A-B). At 200 ng/mL there was a two fold increase in LC3-II conversion that reached 4 fold at 400 ng/mL.

In parallel, we measured the mRNA levels of beclin-1- another autophagic marker required for the initiation of autophagosome formation [47,48]. In gp120 treated cells expression of beclin-1 increased in a concentration dependent manner (Fig. 5D), further supporting the induction of neuronal autophagy. We also analyzed the expression of autophagic marker sequestosome 1 (SQSTM1, also known as p62). p62 is a protein that is associated with autophagosomes and is degraded in the lysosomes after fusion of autophagosomes with lysosomes [53]. Therefore, reduction in p62 expression serves as an indicator of progression of autophagic process. Our western blot analysis revealed that the levels of SQSTM1/p62 were decreased in gp120 treated cells in a dose dependent manner (Fig. 5A and 5C). This decrease was significant in cells treated with 200-400 ng/mL of gp120 with the maximum decrease observed with 400 ng/mL. Collectively, the data in Fig. 5 illustrated that gp120 treatment induced neuronal autophagy.

To confirm that gp120 treatment induced neuronal autophagy, we measured the levels of LC3-II in gp120 treated SH-SY5Y cells in the presence of baflomycin A1 (BaF-A). BaF-A is an autophagic inhibitor that prevents the fusion of autophagosome and lysosome resulting in an accumulation of LC3-II [54]. Data in Fig. 6A
depicted that BaF-A treatment dramatically increased the levels of LC3-II in gp120 (200 ng/mL) exposed cells. To further prove the activation of autophagy by gp120, we examined the intracellular localization of LC3 in autophagic vesicles by transfecting a green fluorescent protein (GFP) tagged-LC3 plasmid into SH-SY5Y cells. Fluorescence microscopic analysis showed that in untreated cells GFP-LC3 was found mostly as diffused green fluorescence in the cytoplasm (Fig 6B). However, when these cells were treated with gp120, formation of green fluorescent puncta typical of autophagic activation were distinctly detected (Fig 6B). Notably, the number of puncta was significantly increased when gp120 treated cells were concomitantly exposed to BaF-A. These data strongly suggest that gp120 treatment induces neuronal autophagy as a cellular stress response.

**HIV-1 gp120 induced neuronal autophagy is dependent on ROS.** Next we examined whether gp120-induced neuronal autophagy is mediated by ROS. To test this, SH-SY5Y cells were treated with gp120 (200 ng/mL) in the presence of the ROS scavenger N-acetylcysteine (NAC) and formation of LC3-II was measured. Our data show that gp120 enhanced ROS production and NAC substantially suppressed gp120-induced ROS generation (Fig. 6C). Similarly, gp120 treatment increased expression of LC3-II (Fig 6D). In parallel, a significant decrease in lipidation of LC3-II was observed in the presence of NAC (Fig 6D). These data provide evidence that ROS is required for gp120-induced neuronal autophagy.

**POX-mediated ROS contributes to HIV-1 gp120-induced neuronal autophagy.** To determine whether upregulation of POX is the mediator of neuronal autophagy, we overexpressed POX in SH-SY5Y cells and measured the accumulation of LC3-II and beclin-1. Our data showed that overexpression of POX induced significant increase in the lipidation of LC3-II (Fig. 7A). In addition, neuronal cells over-expressing POX showed increased expression of beclin-1 (Fig. 7B) highlighting the contribution of POX-dependent initiation of autophagy. Moreover, this upregulation of autophagic markers was accompanied by an increase in ROS production in POX overexpressing cells (Fig. 7C). Interestingly, the addition of NAC to the cells overexpressing POX, suppressed the production of ROS (Fig. 7C) and beclin-1 (Fig. 7B). These data confirm the contribution of POX-induced ROS in the initiation of neuronal autophagy.

To further investigate that POX-induced ROS elicits neuronal autophagy, we examined the effects of POX inhibition on ROS generation and levels of LC3-II and beclin-1. As expected, treatment of SH-SY5Y cells with DHP (100 µM) minimally affected expression of LC3-II (Fig. 8D) and beclin-1 (Fig. 7E). However, gp120 (200 ng/mL) treatment significantly increased expression of these autphagic markers (Fig. 7D-E). Interestingly, in the presence of DHP, gp120 treated cells showed a decrease in beclin-1 and LC3-II expression (Fig. 7D-E). Moreover, a significant decrease in the generation of ROS was also observed in the cells treated with both DHP and gp120 (Fig. 7F). These data provide strong evidence of the contribution of POX in the generation of ROS and activation of neuronal autophagy.

**HIV-1 gp120 upregulates POX by regulating POX mRNA Transcription.** To understand the mechanism by which gp120 upregulates POX, we measured POX mRNA expression in gp120 treated SH-SY5Y cells using quantitative real time PCR (qPCR). Data in Fig. 8A indicated that the copy numbers of POX mRNA are significantly increased in cells treated with >200 ng/mL of gp120 relative to that of the untreated cells. To further demonstrate that gp120 regulates POX mRNA transcription we employed a luciferase reporter based promoter activation assay. In this assay we used a luciferase reporter that is driven by POX promoter whereas the control vector lacked any promoter sequence (Fig. 8B). We transfected SH-SY5Y cells with POX-Luc reporter construct or the empty vector. Then these cells were treated with gp120 in a dose dependent manner for 24 h and the POX promoter activity was measured in the cell lysates. Results in Fig. 8C showed that treatment with HIV-1 gp120 significantly stimulated the POX-promoter activity in a dose-dependent manner (Fig 2A). Interestingly, exposure to heat-inactivated gp120 failed to induce the POX-promoter activity (Fig. 8D), indicating the effect to be specifically mediated via HIV-1 gp120. Collectively, these studies strongly suggest that HIV-1 gp120 upregulates POX by regulating POX mRNA.
mRNA transcription specifically by enhancing POX promoter activity.

**HIV-1 gp120 mediated upregulation of POX is dependent on p53.** Next, we intended to identify the upstream regulators of POX gene targeted by gp120. Activation of the p53 and p53 target genes has been well documented during gp120-mediated neurotoxicity [55-57]. Furthermore, it is well established that POX is a downstream mediator in p53-induced cellular signaling [4,5,58]. Therefore, we investigated the effects of gp120 on p53 expression. To test this, the expression levels of p53 were measured by western blot after exposure to gp120 in SH-SY5Y cells. Data in Fig. 9 A-B showed that gp120 treatment resulted in a concentration dependent increase in p53 protein levels. Remarkably, this increase in p53 expression paralleled to the gp120-induced increase in POX expression (Fig. 2 B-C). POX being a p53 induced gene, we hypothesized that the induction of POX by gp120 in SH-SY5Y cells is mediated by p53. To test this, we repeated the POX promoter activation assay described in Fig. 9 In this experiment POX-Luc vector or the empty vector was transfected into SH-SY5Y cells in the presence or absence of a p53 overexpression construct. p53 overexpression in these cells was confirmed by western blot (data not shown). Concurrently, activation of POX promoter by p53 overexpression was measured by estimating luciferase activity in the cellular extract. Data in Fig. 9C illustrated that p53 significantly stimulated POX promoter activity. Specificity of p53 induced POX promoter activation was confirmed by α-pifithrin, which specifically inhibits p53 DNA-binding activity. Data showed that α-pifithrin abrogated activation of POX promoter by p53. These data strongly support the involvement of p53 in the gp120-induced POX expression.

Since nuclear p53 is known to induce autophagy inducing genes [59], next we tested the localization of p53 in gp120 treated cells. SHSY-5Y cells were treated with gp120 (200 ng/mL) followed by isolation of nuclear and cytoplasmic extracts and the expression of p53 was measured by western blotting (Fig 9D). Significantly higher levels of nuclear-p53 were observed in the gp120 treated cells as compared to untreated control. On the other hand, heat-inactivated gp120 failed to induce the nuclear localization of p53 indicating that gp120 specifically increases the nuclear localization of p53 that may in turn result in stimulation of POX.

**Involvement of CXCR4 in the up-regulation of p53 and POX by gp120.** Binding of HIV-1 gp120 to target cells require the CD4 primary receptor and either of the two chemokine co-receptors- CXCR4 or CCR5 [60]. Although neurons do not express the CD4 receptor they do express the HIV-1 co-receptors [56,61,62]. The binding of gp120 to CXCR4 is known to induce a number of signaling mechanisms [55-57,59,63,64]. Moreover, HIV-1 gp120 induced p53 expression has been reported to be regulated by CXCR4 [56,61,65,66]. Since SH-SY5Y cells used in our study have been shown to express CXCR4 [62], we investigated whether the gp120 induced p53 expression is mediated by CXCR4. To test this, we utilized a specific CXCR4 antagonist the bicyclam AMD3100 in our experiments [67-69]. SH-SY5Y cells were pretreated with AMD3100 (10 ng/ml) for 30 min prior to exposure to gp120. The effect of AMD3100 binding to CXCR4 on p53 expression was determined by western blotting. As expected gp120 induced p53 expression (Fig. 9 E-F). However, a significant reduction in the gp120-induced p53 increase was obtained in cells treated with the CXCR4 antagonist (Fig. 9 E-F), suggesting that the up-regulation of p53 is most likely mediated via binding of gp120 to CXCR4.

Therefore, we tested whether the engagement of CXCR4 with gp120 regulated POX promoter activity. We measured POX promoter activity in SH-SY5Y cells that were pretreated with AMD3100 in the presence and absence of gp120. AMD3100 treatment had minimal effect on POX promoter activity in the absence of gp120 (Fig. 9G). Interestingly, data also illustrated that the activation of POX promoter by gp120 was reduced in the presence of AMD3100 (Fig 9G). These data confirm that the induction of POX requires the engagement of gp120 with the CXCR4 receptor. Collectively, these observations provide mechanistic insight into the role of CXCR4/p53/POX axis in gp120 mediated neuronal autophagy.
DISCUSSION

HIV-1 infection in the brain leads to a range of neurological dysfunctions that are broadly termed as HAND [28]. Even though neurons are refractory to HIV-1 infection, neuronal apoptosis is one of the mechanisms by which HIV causes CNS dysfunction and injury [31]. Studies have showed that HIV-1 gene product “gp120” plays a critical role in neuronal apoptosis [31,34,35]. There is also evidence that gp120 induces apoptosis in other cells including lymphocytes [70] and cardiomyocytes [71]. Gp120-induced apoptosis has also been shown in hippocampal neurons [72]. Gp120 binds to the coreceptors CCR5 and CXCR4 expressed on neuronal cell membrane [61]. This binding has been proposed to play a critical role in eliciting neuronal apoptosis beginning with a series of signaling cascades initiated at the cell membrane by the binding of gp120. Many reports suggest that gp120 induced neurological dysfunctions and apoptosis involves ROS mediated oxidative stress [36]. Excess ROS destabilizes the cellular antioxidant defense system and induces neuronal apoptosis. However, biochemical mechanisms that increase HIV-1 gp120-induced ROS in neuronal cells are not clearly understood.

Intracellular ROS can be produced by a number of metabolic enzymes that are localized either in the plasma membrane or in the cytoplasm of cell [6]. For example, NADPH oxidase-a membrane associated enzyme has been implicated in HIV-1 gp120-induced ROS generation [39]. However, it is now increasingly recognized that mitochondrial ROS plays an important role in cellular oxidative stress [6,7,44]. Interestingly, a role of mitochondrial ROS in neuronal oxidative stress is yet to be clearly defined. Therefore, we considered the contribution of POX in this response because POX is a mitochondrial inner membrane enzyme that is well established to generate ROS [10,73]. This mitochondrial ROS generation by POX is dependent on its ability to generate electrons during proline catabolism (Fig. 1). Under normal metabolic settings the electrons generated during the conversion of proline to P5C by POX contribute to ATP production via the electron transport chain [3,43]. However, under cellular stress conditions, these electrons from the catalytic activity of POX can generate mitochondrial ROS [4,5]. Depending upon the type of cytotoxic stress, POX has been shown to be induced by p53, PPARγ ligands (in response to inflammatory stress) and oxidized low-density lipoproteins to generate superoxide radicals [5,11,58,74]. Therefore, induction of POX has been linked to ROS mediated apoptotic cell death [4]. Given that ROS plays an important role in HIV-1 gp120 mediated neurotoxicity (32), we hypothesized a role of POX-mediated ROS in neuronal oxidative stress.

Our data showed that treatment of physiologically relevant concentrations of soluble HIV-1 gp120 increased POX expression and catalytic activity in SH-SY5Y neuroblastoma cells (Fig. 2A-C). This induction seems to be specific given that heat inactivated gp120 failed to activate POX (Fig. D-E). This increase correlated with increased intracellular ROS production (Fig. 3) suggesting a functional role of POX in gp120 associated neurotoxicity. Since induction of POX mediates cellular apoptosis we envisioned that the increased ROS may cause neuronal cell death. Data in Fig. 4A-B illustrated that gp120 treatment for 24 h failed to minimally induce neuronal apoptosis even at the highest concentration of gp120 (400 ng/mL). Longer exposure of gp120 only moderately enhanced apoptosis (Fig. D-E). Surprisingly, treatment with 200 ng/mL gp120 for 24 h or longer significantly increased ROS production (Fig. 3). These data indicated that upon exposure of neuronal cells to gp120, POX-induced mitochondrial ROS may not be robust enough to induce apoptosis but play a role in alternative signaling mechanisms.

It is well documented that initiation of oxidative stress triggers pathways to counteract the foreseeing cellular damage [75]. Although ROS is a toxic byproduct of cellular metabolism, it also serves as stimulus for controlling pathways of cellular damage. For example increased ROS is known to enhance autophagy beyond basal levels during cell stress [76]. Autophagy is a highly regulated process that serves many functions in the cell, including maintaining cellular homeostasis, cell survival during stress or alternatively as a mechanism for cell death [47-50,77]. Whether there is a pro-survival autophagic response or one that eventually leads to cell death depends on the severity of the cellular stress. Autophagy is also an important process in the homeostatic maintenance of neuronal function [78]. For example, autophagy has also been reported to play a critical role in
various neurodegenerative disorders such as Alzheimer’s disease, Huntington’s disease, and Parkinson’s disease [77-80]. Recently, it has been reported that markers of autophagy are expressed in the postmortem brains of HIV-1 encephalitis patients [81]. Autophagy has also been shown to be an important mechanism in the HIV-1 envelope mediated CD4+ T cells death in uninfected bystander cells [82]. It has also been demonstrated that the neuronal cell line SK-N-SH when exposed to gp120 (200 ng/mL) resulted in the induction of autophagy [81]. Notably, SK-N-H cells are the parental cell line of the SH-SY5Y cells that are used in this study. Even though autophagy is shown to be involved in gp120 induced damage to the brain, the molecular mechanisms involved are not completely understood. Since we observed a 2-fold increase in ROS with 200 ng/mL of gp120 that did not cause apoptosis, we hypothesized that ROS may play a role in neuronal autophagy. Therefore we measured the levels of autophagy markers LC3-II and beclin-1. Our data illustrated that 24 h treatment of 200 ng/mL gp120 induced neuronal autophagic activity (Fig. 5). Even though these data support the previous reports, whether ROS generation plays a role in this effect remained unclear. To demonstrate that ROS is modulating the observed effect, we pre-treated the neuronal cells with ROS scavenger NAC prior to treatment with gp120. NAC pretreatment not only reduced ROS production but also decreased autophagic activity (Fig. 6). Thus, indicating that the increase of ROS induced by gp120 may be involved in promoting neuronal autophagy. In this context the contribution of POX becomes highly relevant because POX-dependent ROS has been shown to induce autophagy through a beclin-1 dependent pathway [45]. We also observed that inhibition of the POX catalytic activity by the competitive inhibitor DHP significantly reduced the levels of ROS and expression of autophagy markers in the presence of gp120 (Fig 7). Collectively, these data suggested the contribution of POX-dependent ROS in gp120-induced neuronal autophagy.

Interestingly, autophagy has also been shown to be an important mechanism in the HIV-1 envelope glycoprotein mediated CD4+ T cells death [82]. Moreover, prior to autophagic cell death, whether initially there is a prosurvival, protective induction of autophagy due to the ensuing oxidative stress because of HIV-1 gp120 has not been documented. To further validate a role of POX in the induction of autophagy, we overexpressed POX and measured its effect on autophagy markers in neuronal cells. POX overexpression markedly increased the levels of ROS and induced expression of the autophagy markers (Fig. 7). Moreover the addition of a ROS scavenger not only quenched the ROS generation induced by POX overexpression but also reduced the levels of the autophagy markers. Collectively, these data further suggested the contribution of POX-dependent ROS in gp120-induced neuronal autophagy.

Data in Fig. 8 and Fig. 9 suggested that gp120 induces the CXCR4/p53 axis to regulate POX expression in neuronal cells. HIV-1 gp120 binds to CD4 receptor and the co-receptors-CXCR4 or CCR5 [60]. Neurons lack the CD4 receptor, however express the HIV-1 co-receptors [56,61,62]. Furthermore, binding of gp120 to CXCR4 is known to induce a number of signaling mechanisms including p53 expression [56,61,65,66]. Since SH-SY5Y cells are known to express CXCR4 [62], we hypothesized a role of CXCR4/p53 axis in gp120 induced neuronal autophagy. The activation of POX leading to a proline-mediated ROS generation is known to be accompanied by the induction of p53 [4,5,58]. Previous studies have shown that proline oxidase is a p53-induced gene-6 (PIG6) [4,5,58]. Therefore we considered whether the gp120-induced upregulation of POX may be mediated by p53. We observed increased levels of p53 on exposure to gp120 (Fig. 9A-B) suggesting that p53 may regulate POX expression. The involvement of p53 was confirmed by the suppression of POX activation in presence of the inhibitor α-pifithrin, which specifically inhibits p53 DNA-binding activity. Accumulating evidence indicates that p53 can modulate autophagy depending on its subcellular localization [59], p53 is shown to stimulate autophagy when it is present in the nucleus and transactivates autophagy-stimulating genes [59]. This is important because we observed increased localization of p53 in the nucleus upon gp120 treatment (Fig. 9D) indicating that gp120 promotes nuclear translocation of p53, which may in turn result in activation of POX promoter. Moreover, in our study, gp120-induced increase in p53 expression and activation of POX promoter were suppressed by prior treatment of cells with the
CXCR4-antagonist AMD3100. We believe binding of gp120 to CXCR4 activates p53 resulting in its nuclear translocation which in turn results in the induction of POX.

Taken together, our data suggest an important role of POX in gp120-mediated neuronal function. Specifically, our results suggest that the oxidation of proline by POX enhances oxidative stress. POX-dependent ROS initially triggers autophagy and increased autophagy serves as a protective response to delay cell death in gp120 neurotoxicity. We believe that our study provides novel insights into the cellular mechanisms of HIV-1 gp120 mediated neurotoxicity. The observations in this study implicates that POX could serve as a potential target to inhibit HIV-1 associated neuronal dysfunction. Even though speculative, understanding the role of POX in neuronal autophagy and dysfunction may help us uncover novel targets for therapeutic intervention against HAND. However, this requires further studies in basic, translation and clinical fronts.

ACKNOWLEDGEMENTS: This work is partly supported by grants DA037779 to JP and DA024558, DA30896, DA033892, DA021471 to CD. BJ is supported by R25GM059994. We also acknowledge the RCMI Grant G12MD007586, the Vanderbilt CTSA grant UL1RR024975, the Meharry Translational Research Center (MeTRC) CTSA grant (U54 RR026140 from NCRR/NIH, the U54 grant MD007593 from NIMHD/NIH.
References:

1. Adams E, Frank L (1980) Metabolism of proline and the hydroxyprolines. Annu Rev Biochem 49: 1005-1061.
2. Phang JM (1985) The regulatory functions of proline and pyrroline-5-carboxylic acid. Curr Top Cell Regul 25: 91-132.
3. Yeh GC, Phang JM (1988) Stimulation of phosphoribosyl pyrophosphate and purine nucleotide production by pyrroline 5-carboxylate in human erythrocytes. J Biol Chem 263: 13083-13089.
4. Donald SP, Sun XY, Hu CA, Yu J, Mei JM, et al. (2001) Proline oxidase, encoded by p53-induced gene-6, catalyzes the generation of proline-dependent reactive oxygen species. Cancer Res 61: 1810-1815.
5. Pandhare J, Cooper SK, Phang JM (2006) Proline oxidase, a proapoptotic gene, is induced by troglitazone: evidence for both peroxisome proliferator-activated receptor gamma-dependent and -independent mechanisms. J Biol Chem 281: 2044-2052.
6. Holmstrom KM, Finkel T (2014) Cellular mechanisms and physiological consequences of redox-dependent signalling. Nat Rev Mol Cell Biol 15: 411-421.
7. Finkel T (2011) Signal transduction by reactive oxygen species. J Cell Biol 194: 7-15.
8. Phang JM, Donald SP, Pandhare J, Liu Y (2008) The metabolism of proline, a stress substrate, modulates carcinogenic pathways. Amino Acids 35: 681-690.
9. Phang JM, Liu W, Hancock C, Christian KJ (2012) The proline regulatory axis and cancer. Front Oncol 2: 60.
10. Phang JM, Liu W, Zabirnyk O (2010) Proline metabolism and microenvironmental stress. Annu Rev Nutr 30: 441-463.
11. Zabirnyk O, Liu W, Khalil S, Sharma A, Phang JM (2010) Oxidized low-density lipoproteins upregulate proline oxidase to initiate ROS-dependent autophagy. Carcinogenesis 31: 446-454.
12. Liu W, Phang JM (2012) Proline dehydrogenase (oxidase), a mitochondrial tumor suppressor, and autophagy under the hypoxia microenvironment. Autophagy 8: 1407-1409.
13. Gogos JA, Santha M, Takacs Z, Beck KD, Luine V, et al. (1999) The gene encoding proline dehydrogenase modulates sensorimotor gating in mice. Nat Genet 21: 434-439.
14. Fremeau RT, Jr., Caron MG, Blakely RD (1992) Molecular cloning and expression of a high affinity L-proline transporter expressed in putative glutamatergic pathways of rat brain. Neuron 8: 915-926.
15. Renick SE, Kleven DT, Chan J, Stenius K, Milner TA, et al. (1999) The mammalian brain high-affinity L-proline transporter is enriched preferentially in synaptic vesicles in a subpopulation of excitatory nerve terminals in rat forebrain. J Neurosci 19: 21-33.
16. Cohen SM, Nadler JV (1997) Proline-induced potentiation of glutamate transmission. Brain Res 761: 271-282.
17. Paterlini M, Zakharenko SS, Lai WS, Qin J, Zhang H, et al. (2005) Transcriptional and behavioral interaction between 22q11.2 orthologs modulates schizophrenia-related phenotypes in mice. Nat Neurosci 8: 1586-1594.
18. Liu H, Abecasis GR, Heath SC, Knowles A, Demars S, et al. (2002) Genetic variation in the 22q11 locus and susceptibility to schizophrenia. Proc Natl Acad Sci U S A 99: 16859-16864.
19. Liu H, Heath SC, Sobin C, Roos JL, Galke BL, et al. (2002) Genetic variation at the 22q11 PRODH2/DGCR6 locus presents an unusual pattern and increases susceptibility to schizophrenia. Proc Natl Acad Sci U S A 99: 3717-3722.
20. Jacquet H, Demily C, Houy E, Hecketsweiler B, Bou J, et al. (2005) Hyperprolinemia is a risk factor for schizoaffective disorder. Mol Psychiatry 10: 479-485.
21. Roussos P, Giakoumaki SG, Bitsios P (2009) A risk PRODH haplotype affects sensorimotor gating, memory, schizotypy, and anxiety in healthy male subjects. Biol Psychiatry 65: 1063-1070.
22. van Spronsen M, Hoogenraad CC (2010) Synapse pathology in psychiatric and neurologic disease. Curr Neurol Neurosci Rep 10: 207-214.
23. Wyse AT, Netto CA (2011) Behavioral and neurochemical effects of proline. Metab Brain Dis 26: 159-172.
24. Albright AV, Soldan SS, Gonzalez-Scarano F (2003) Pathogenesis of human immunodeficiency virus-induced neurological disease. J Neurovirol 9: 222-227.
25. Vivithanaporn P, Heo G, Gamble J, Krentz HB, Hoke A, et al. (2010) Neurologic disease burden in treated HIV/AIDS predicts survival: a population-based study. Neurology 75: 1150-1158.
26. Joska JA, Gouse H, Paul RH, Stein DJ, Flisher AJ (2010) Does highly active antiretroviral therapy improve neurocognitive function? A systematic review. J Neurovirol 16: 101-114.
27. Valcour V, Shikuma C, Shiramizu B, Watters M, Poff P, et al. (2004) Higher frequency of dementia in older HIV-1 individuals: the Hawaii Aging with HIV-1 Cohort. Neurology 63: 822-827.
28. McArthur JC, Steiner J, Sacktor N, Nath A (2010) Human immunodeficiency virus-associated neurocognitive disorders: Mind the gap. Ann Neurol 67: 699-714.
29. Clifford DB (2000) Human immunodeficiency virus-associated dementia. Arch Neurol 57: 321-324.
30. Rao VR, Ruiz AP, Prasad VR (2014) Viral and cellular factors underlying neuropathogenesis in HIV associated neurocognitive disorders (HAND). AIDS Res Ther 11: 13.
31. Kaul M, Garden GA, Lipton SA (2001) Pathways to neuronal injury and apoptosis in HIV-associated dementia. Nature 410: 988-994.
32. Nath A (2002) Human immunodeficiency virus (HIV) proteins in neuropathogenesis of HIV dementia. J Infect Dis 186 Suppl 2: S193-198.
33. Gonzalez-Scarano F, Martin-Garcia J (2005) The neuropathogenesis of AIDS. Nat Rev Immunol 5: 69-81.
34. Mocchetti I, Bachis A, Avdoshina V (2012) Neurotoxicity of human immunodeficiency virus-1: viral proteins and axonal transport. Neurotox Res 21: 79-89.
35. Acquas E, Bachis A, Nosheny RL, Cernak I, Mocchetti I (2004) Human immunodeficiency virus type 1 protein gp120 causes neuronal cell death in the rat brain by activating caspases. Neurotox Res 5: 605-615.
36. Agrawal L, Louboutin JP, Maruschich E, Reyes BA, Van Bockstaele EJ, et al. (2010) Dopaminergic neurotoxicity of HIV-1 gp120: reactive oxygen species as signaling intermediates. Brain Res 1306: 116-130.
37. Steiner J, Haughey N, Li W, Venkatesan A, Anderson C, et al. (2006) Oxidative stress and therapeutic approaches in HIV dementia. Antioxid Redox Signal 8: 2089-2100.
38. Hu S, Sheng WS, Lokensgard JR, Peterson PK, Rock RB (2009) Preferential sensitivity of human dopaminergic neurons to gp120-induced oxidative damage. J Neurovirol 15: 401-410.
39. Jana A, Pahan K (2004) Human immunodeficiency virus type 1 gp120 induces apoptosis in human primary neurons through redox-regulated activation of neutral sphingomyelinase. J Neurosci 24: 9531-9540.
40. Louboutin JP, Strayer DS (2012) Blood-brain barrier abnormalities caused by HIV-1 gp120: mechanistic and therapeutic implications. ScientificWorldJournal 2012: 482575.
41. Yang Y, Yao H, Lu Y, Wang C, Buch S (2010) Cocaine potentiates astrocyte toxicity mediated by human immunodeficiency virus (HIV-1) protein gp120. PLoS One 5: e13427.
42. Chen G, Ke Z, Xu M, Liao M, Wang X, et al. (2012) Autophagy is a protective response to ethanol neurotoxicity. Autophagy 8: 1577-1589.
43. Pandhare J, Donald SP, Cooper SK, Phang JM (2009) Regulation and function of proline oxidase under nutrient stress. J Cell Biochem 107: 759-768.
44. Finkel T (2012) Signal transduction by mitochondrial oxidants. J Biol Chem 287: 4434-4440.
45. Circu ML, Aw TY (2010) Reactive oxygen species, cellular redox systems, and apoptosis. Free Radic Biol Med 48: 749-762.
46. Vermes I, Haanen C, Reutelingsperger C (2000) Flow cytometry of apoptotic cell death. J Immunol Methods 243: 167-190.
47. Tanida I (2011) Autophagy basics. Microbiol Immunol 55: 1-11.
48. Tanida I (2011) Autophagosome formation and molecular mechanism of autophagy. Antioxid Redox Signal 14: 2201-2214.
49. Lee J, Giordano S, Zhang J (2012) Autophagy, mitochondria and oxidative stress: cross-talk and redox signalling. Biochem J 441: 523-540.
50. He C, Klionsky DJ (2009) Regulation mechanisms and signaling pathways of autophagy. Annu Rev Genet 43: 67-93.
51. Liu W, Glunde K, Bhujwalla ZM, Raman V, Sharma A, et al. (2012) Proline oxidase promotes tumor cell survival in hypoxic tumor microenvironments. Cancer Res 72: 3677-3686.
52. Kimura S, Fujita N, Noda T, Yoshimori T (2009) Monitoring autophagy in mammalian cultured cells through the dynamics of LC3. Methods Enzymol 452: 1-12.
53. Ichimura Y, Kominami E, Tanaka K, Komatsu M (2008) Selective turnover of p62/A170/SQSTM1 by autophagy. Autophagy 4: 1063-1066.
54. Vinod V, Padmakrishnan CJ, Vijayan B, Gopala S (2014) 'How can I halt thee?' The puzzles involved in autophagic inhibition. Pharmacol Res 82: 1-8.
55. Jayadev S, Yun B, Nguyen H, Yokoo H, Morrison RS, et al. (2007) The glial response to CNS HIV infection includes p53 activation and increased expression of p53 target genes. J Neuroimmune Pharmacol 2: 359-370.
56. Khan MZ, Shimizu S, Patel JP, Nelson A, Le MT, et al. (2005) Regulation of neuronal P53 activity by CXCR4. Mol Cell Neurosci 30: 58-66.
57. Garden GA, Morrison RS (2005) The multiple roles of p53 in the pathogenesis of HIV-associated dementia. Biochem Biophys Res Commun 331: 799-809.
58. Polyak K, Xia Y, Zweier JL, Kinzler KW, Vogelstein B (1997) A model for p53-induced apoptosis. Nature 389: 300-305.
59. Scherez-Shouval R, Weidberg H, Gonen C, Wilder S, Elazar Z, et al. (2010) p53-dependent regulation of autophagy protein LC3 supports cancer cell survival under prolonged starvation. Proc Natl Acad Sci U S A 107: 18511-18516.
60. Castedo M, Perfettini JL, Andreau K, Roumier T, Piacentini M, et al. (2003) Mitochondrial apoptosis induced by the HIV-1 envelope. Ann N Y Acad Sci 1010: 19-28.
61. Meucci O, Fatatis A, Simen AA, Bushell TJ, Gray PW, et al. (1998) Chemokines regulate hippocampal neuronal signaling and gp120 neurotoxicity. Proc Natl Acad Sci U S A 95: 14500-14505.
62. Clift IC, Bamidele AO, Rodriguez-Ramirez C, Kremer KN, Hedin KE (2014) beta-Arrestin1 and distinct CXCR4 structures are required for stromal derived factor-1 to downregulate CXCR4 cell-surface levels in neuroblastoma. Mol Pharmacol 85: 542-552.
63. Perfettini JL, Castedo M, Roumier T, Andreau K, Nardacci R, et al. (2005) Mechanisms of apoptosis induction by the HIV-1 envelope. Cell Death Differ 12 Suppl 1: 916-923.
64. Castedo M, Ferri KF, Blanco J, Roumier T, Larochette N, et al. (2001) Human immunodeficiency virus 1 envelope glycoprotein complex-induced apoptosis involves mammalian target of rapamycin/FKBP12-rapamycin-associated protein-mediated p53 phosphorylation. J Exp Med 194: 1097-1110.
65. Bardi G, Sengupta R, Khan MZ, Patel JP, Meucci O (2006) Human immunodeficiency virus gp120-induced apoptosis of human neuroblastoma cells in the absence of CXCR4 internalization. J Neurovirol 12: 211-218.
66. Khan MZ, Brandimarti R, Musser BJ, Resue DM, Fatatis A, et al. (2003) The chemokine receptor CXCR4 regulates cell-cycle proteins in neurons. J Neurovirol 9: 300-314.
67. De Clercq E, Yamamoto N, Pauwels R, Balzarini J, Witvrouw M, et al. (1994) Highly potent and selective inhibition of human immunodeficiency virus by the bicyclam derivative JM3100. Antimicrob Agents Chemother 38: 668-674.
68. Bridger GJ, Skerlj RT, Thornton D, Padmanabhan S, Martellucci SA, et al. (1995) Synthesis and structure-activity relationships of phenylenebis(methylene)-linked bis-tetraazamacrocycles that inhibit HIV replication. Effects of macrocyclic ring size and substituents on the aromatic linker. J Med Chem 38: 366-378.
69. Hendrix CW, Flexner C, MacFarland RT, Giandomenico C, Fuchs EJ, et al. (2000) Pharmacokinetics and safety of AMD-3100, a novel antagonist of the CXCR-4 chemokine receptor, in human volunteers. Antimicrob Agents Chemother 44: 1667-1673.
70. Wan ZT, Chen XL (2010) Mechanisms of HIV envelope-induced T lymphocyte apoptosis. Virol Sin 25: 307-315.
71. Twu C, Liu NQ, Popik W, Bukrinsky M, Sayre J, et al. (2002) Cardiomyocytes undergo apoptosis in human immunodeficiency virus cardiomyopathy through mitochondrion- and death receptor-controlled pathways. Proc Natl Acad Sci U S A 99: 14386-14391.
72. Thomas AG, Bodner A, Ghadge G, Roos RP, Slusher BS (2009) GCP II inhibition rescues neurons from gp120IIIB-induced neurotoxicity. J Neurovirol 15: 449-457.
73. Phang JM, Pandhare J, Liu Y (2008) The metabolism of proline as microenvironmental stress substrate. J Nutr 138: 2008S-2015S.
74. Maxwell SA, Davis GE (2000) Differential gene expression in p53-mediated apoptosis-resistant vs. apoptosis-sensitive tumor cell lines. Proc Natl Acad Sci U S A 97: 13009-13014.
75. Li L, Tan J, Miao Y, Lei P, Zhang Q (2015) ROS and Autophagy: Interactions and Molecular Regulatory Mechanisms. Cell Mol Neurobiol.
76. Azad MB, Chen Y, Gibson SB (2009) Regulation of autophagy by reactive oxygen species (ROS): implications for cancer progression and treatment. Antioxid Redox Signal 11: 777-790.
77. Lee JA (2012) Neuronal autophagy: a housekeeper or a fighter in neuronal cell survival? Exp Neurobiol 21: 1-8.
78. Xilouri M, Stefanis L (2010) Autophagy in the central nervous system: implications for neurodegenerative disorders. CNS Neurol Disord Drug Targets 9: 701-719.
79. Yu WH, Cuervo AM, Kumar A, Peterhoff CM, Schmidt SD, et al. (2005) Macroautophagy--a novel Beta-amyloid peptide-generating pathway activated in Alzheimer's disease. J Cell Biol 171: 87-98.
80. Cherra SJ, 3rd, Chu CT (2008) Autophagy in neuroprotection and neurodegeneration: A question of balance. Future Neurol 3: 309-323.
81. Zhou D, Masliah E, Spector SA (2011) Autophagy is increased in postmortem brains of persons with HIV-1-associated encephalitis. J Infect Dis 203: 1647-1657.
82. Espert L, Denizot M, Grimaldi M, Robert-Hebmann V, Gay B, et al. (2006) Autophagy is involved in T cell death after binding of HIV-1 envelope proteins to CXCR4. J Clin Invest 116: 2161-2172.
LEGENDS TO FIGURES

Fig. 1. Schematic diagram of proline metabolic pathway. POX is a mitochondrial inner membrane metabolic enzyme. POX metabolizes proline— an abundantly available substrate to generate electrons. Under normal metabolic settings the electrons generated during the conversion of proline to P5C contribute to ATP production via the electron transport chain. However, under cellular stress conditions, these electrons from the catalytic activity of POX are channeled to generate ROS.

Fig. 2. HIV-1 gp120 induces expression and activity of POX. (A) POX expression by western blot analysis. SH-SY5Y cells were treated with various concentrations of gp120 for 24 h. After treatment cells were harvested and cell lysates were prepared. The protein concentration of cell lysates was determined and equal amounts of cell lysates were electrophoresed on denaturing acrylamide gels and transferred onto nitrocellulose membrane by electroblotting. Western blots were performed using anti-POX antibody. Actin was used as a loading control (1:2000). Blots were developed using the enhanced chemiluminescence kit. (B) Densitometry of POX expression from n=3. (C) POX catalytic activity: POX activity in the cell lysates was measured using a specific spectrophotometric method as described in the methods section. P5C formed as a result of POX-mediated degradation of proline was reacted with O-aminobenzaldehyde (OAB) and the resultant OAB-P5C complex was quantified by measuring absorbance at 440 nm wavelength. (D-E) Effects of heat inactivated gp120 on POX expression and activity. For heat inactivation gp120 was incubated at 65 °C for 1 h. Then the heat inactivated gp120 was used to treat the SH-SY5Y cells for 24 h. POX expression (D) and activity (E) was measured in these cells and compared to cells treated with native gp120. The results are expressed as mean ± S.E. for three separate experiments conducted in triplicates. *p < 0.05 is for the comparison of gp120 treated cells vs untreated cells. **p < 0.05 is for the comparison of gp120 treated cells vs with heat inactivated gp120 treated cells.

Fig. 3. HIV-1 gp120 induced POX enhances intracellular ROS production. (A) SH-SY5Y cells were plated in 6-well plates and treated with various concentrations of gp120 for 24 h. Intracellular ROS production was measured using DCF assay as described under "Materials and Methods." Data are plotted relative to the ROS levels in the untreated cells from three separate experiments. *p < 0.05 is for the comparison of gp120 treated cells vs untreated cells. (B-C) POX-dependent ROS generation in gp120 treated SH-SY5Y cells. (B) SH-SY5Y cells treated with proline (5 mM) or POX inhibitor dehydroproline (DHP) (100 µM) for 1 hr, prior to treatment with gp120. After 24 h of treatment ROS was measured in the cellular lysates using DCF assay. The results are expressed as mean ± S.E. for three separate experiments. *p < 0.05 is for the comparison of gp120/proline or gp120/DHP treated cells vs cells treated with either proline or DHP alone. (C) Flow cytometry based ROS measurements. Cells were treated as described in (B) and DCF fluorescence in these cells was measured by flow cytometry.

Fig. 4. Measurement of HIV-1 gp120 induced neuronal apoptosis. SH-SY5Y cells were exposed to different concentrations of gp120 for 24h and apoptosis was measured by flow cytometry using Annexin-V (AV) as an early apoptosis marker and propidium iodide (PI) as the late apoptotic marker. After treatment cells were harvested and immediately stained with AV/PI and analyzed by flow cytometry. The percentage of (A) AV (+) and (B) PI (+) cells. (C-E) Time-dependent effects of HIV-1 gp120 treatment on ROS and apoptosis. SH-SY5Y were treated with gp120 (200 ng/mL) for various time periods as indicated followed by measurement of (C) ROS generation by DCF assay, (D) AV staining, and (E) PI staining. The results are expressed as mean ± S.E. for three separate experiments. *p < 0.05 is for the comparison of gp120 treated cells vs untreated cells.

Fig. 5. Activation of autophagy in HIV-1 gp120 treated SH-SY5Y cells. SH-SY5Y cells grown in 6 well culture dishes followed by treatment with HIV-1 gp120 for 24 h. (A) Western blot analysis was performed to measure the concentration-dependent effects of gp120 on the expression of LC3-II and SQSTM1 (p62). The expression of LC3-II and SQSTM1 was normalized to actin. Densitometry based quantification of (B)
LC3-II and (C) SQSTM1 (p62) expression. (D) The effect of gp120 on beclin-1 mRNA expression as measured by qPCR. After 24 h of treatment qPCR was performed using total RNA from treated cells and primers specific for beclin-1 as described in "Materials and Methods." Data are shown as relative beclin-1 mRNA levels normalized to GAPDH expression. *p < 0.05 is for the comparison of gp120 treated cells vs untreated cells.

Fig. 6. Effect of autophagy inhibitor and anti-oxidant on autophagy in HIV-1 gp120 treated SH-SY5Y cells. (A) SH-SY5Y were treated with BaF-A (5 nM) for 30 min prior to treatment with gp120 (200 ng/mL) for 24 h. Western blot analysis was performed to measure the expression of LC3-I/II. Actin was used as a loading control. (B) SH-SY5Y cells were transfected with a GFP-LC3 plasmid and then treated with 200ng/mL gp120 in the presence/absence of BaF-A (5 nM). The formation of GFP-LC3 puncta was examined under a fluorescence microscope. (C) SH-SY5Y cells were treated with 10 mM ROS scavenger, N-acetylcysteine (NAC), prior to treatment with 200ng/mL gp120. After 24 h ROS generation was measured by DCF assay. The results are expressed as mean ± S.E. for three separate experiments. *p < 0.05 is for the comparison of gp120 treated cells vs untreated cells. (D) Autophagy markers LC3-I and II were measured by Western blot using actin as a loading control.

Fig. 7. Contribution of POX-dependent ROS in activation of autophagy in HIV-1 gp120 treated SH-SY5Y cells. (A) SH-SY5Y cells were transfected with a POX expression vector or control vector as described in "Materials and Methods." The expression of POX and autophagy markers LC3-I and II were measured by Western blot using actin as a loading control. (B-C) SH-SY5Y cells were transfected with POX expression vector or control vector in the presence of NAC (10 mM). After 24 h. (B) qPCR analysis for beclin-1 expression was performed and the expression was normalized to GAPDH. (C) ROS generation was measured by DCF assay using flow cytometry as described previously. The results are expressed as mean ± S.E. for three separate experiments. (D-F) Effects of POX inhibitor DHP on neuronal autophagy. Prior to treatment with gp120 (200 ng/mL), SH-SY5Y cells were treated DHP (100 µM) for 1 hr. (D) After 24 h the expression of autophagy markers LC3-I and II were measured by Western blot using actin as a loading control. (E) beclin-1 expression by qPCR, and (F) ROS generation by DCF assay using flow cytometry. The results are expressed as mean ± S.E. for three separate experiments. *p < 0.05 is for the comparison of gp120 treated cells vs untreated cells.

Fig. 8. HIV-1 gp120 induces POX by regulating POX transcription. (A) SH-SY5Y cells grown in 6 well culture dishes followed by treatment with HIV-1 gp120 for 24 h. Genomic RNA was isolated from these cells and POX mRNA levels were measured by qPCR. Copy numbers were determined by using a standard curve that was generated using pcDNA-POX plasmid. Data is expressed as relative copy numbers to the untreated cells. (B) Schematic diagram of POX promoter based luciferase construct (POX-Luc). In this construct the POX promoter drives the luciferase gene. (C) We transfected SH-SY5Y cells with the POX-Luc reporter construct or the empty vector. Then these cells were treated with gp120 in a dose dependent manner for 24 h and the POX promoter activity was measured in the cell lysates. Gp120 treatment increased POX promoter driven luciferase activity. (D) Effects of heat inactivated gp120 on POX promoter activity. Data are representitive of three independent experiments conducted in triplicates. *p < 0.05 is for the comparison of gp120 treated cells vs untreated cells, **p < 0.05 is for the comparison of gp120 treated cells vs with heat inactivated gp120 treated cells.

Fig. 9. HIV-1 gp120 upregulates POX by binding to CXCR4 and up-regulating p53. (A) p53 expression by western blot analysis. SH-SY5Y cells were treated with various concentrations of gp120 for 24 h and equal amount of cell lysates were electrophoresed and probed by western blots using actin as the loading control. (B) Densitometry of p53 expression from n=3. *p < 0.05 is for the comparison of gp120 treated cells vs untreated cells. (C) Effects of p53 inhibition on POX promoter driven luciferase activity. We transfected SH-SY5Y cells with POX-Luc reporter construct or the empty vector along with an expression vector of p53. Luciferase activity in the cellular lysates were measured as described before. (D)
Nuclear localization of p53. p53 levels were determined in the cytoplasm and nucleus of SH-SY5Y cells by western blot in 200 ng/mL gp120 and heat inactivated gp120. (E) Effects of CXCR4 inhibitor-AMD3100 on p53 in gp120 treated cells. SH-SY5Y cells were pre-treated with AMD3100 (10 ng/mL) for 30 min. Thereafter, these cells were treated with 200 ng/mL of gp120 for 24 h. p53 levels in the cellular lysates were determined by western blot. (F) Effects of CXCR4 inhibitor AMD3100 on gp120-induced POX promoter activity. SH-SY5Y cells were pre-treated with AMD3100 (10 ng/mL) for 30 min. Thereafter, these cells were transfected with POX-Luc reporter construct or the empty vector and treated with 200 ng/mL of gp120 for 24 h. Luciferase activity in the cellular lysates were measured. Data are representative of three independent experiments conducted in triplicates.

Fig. 10. A model describing POX-induced ROS generation that functions as cellular a stress response sensor in HIV-1 induced neuronal autophagy. Our data suggest that HIV-1 gp120 induces expression and activity of the mitochondrial metabolic enzyme POX. Increased POX activity generates electrons and under gp120-mediated cellular stress, these electrons are channeled to generate ROS. The effect of this increased ROS is to induce neuronal protective signals specifically autophagy. Based on our data we propose that gp120 regulates POX by activating CXCR4 mediated induction of p53. Induction of p53 drives POX gene transcription leading to increased expression of POX. Collectively, our data underscore a functional role of POX in gp120 mediated neuronal autophagy.
Figure 1

ROS → e⁻ → ATP → POX → Proline → Pyrroline-5-Carboxylate (P5C)
Figure 2

(A) HIV-1 gp120 (ng/mL)

POX
β-actin

(B) POX Expression

0 50 100 200 300 400

HIV-1 gp120 (ng/mL)

(C) POX Activity

0 5 10 15 20

HIV-1 gp120 (ng/mL)

(D) POX Expression

Control HIV-1 gp120 Heat inactivated gp120

(E) POX Activity

Control HIV-1 gp120 Heat inactivated gp120
Figure 3

(A) Relative ROS

(B) Relative ROS

Control

HIV-1 gp120

(C) DCF Fluorescence

- Gp120
- Gp120+ PRO
- Gp120+ DHP
- Control

Downloaded from http://www.jbc.org/ by guest on March 24, 2020
Figure 4

(A) AV (%) vs. HIV-1 gp120 (ng/mL)

(B) PI (%) vs. HIV-1 gp120 (ng/mL)

(C) Relative ROS

(D) AV (%) vs. HIV-1 gp120 (200 ng/mL)

(E) PI (%) vs. HIV-1 gp120 (200 ng/mL)
Figure 5

(A) HIV-1 gp120 (ng/mL)  
| 0  | 400 | 200 | 300 | 400 |
|----|----|----|----|----|
| LC3-II | LC3-II | SQSTM1 (p62) | Actin |

(B) LC3-II (Fold change)  
| 0  | 100 | 200 | 300 | 400 |

(C) p62 (Fold change)  
| 0  | 100 | 200 | 300 | 400 |

(D) Beclin-1  
| 0  | 100 | 200 | 300 | 400 |
Figure 6

(A) Gp120 - + +
BaF-A - - +
LC3-I
LC3-II
Actin

(B) Control HIV-1 gp120 Gp120+BaF-A

(C) Relative ROS

(D) gp120 - - + +
NAC - + - +
LC3-I
LC3-II
Actin
Figure 8

(A) POX mRNA levels in cells treated with different concentrations of HIV-1 gp120 (ng/mL).

(B) Schematic representation of gene expression driven by Pox promoter and Luciferase reporter.

(C) POX-promoter driven Luciferase activity in cells treated with HIV-1 gp120.

(D) POX-promoter driven Luciferase activity in different treated conditions: Unreacted Control, Gp 20, Heat inactivated Gp 20.
Figure 9
A Novel Role of Proline Oxidase in HIV-1 Envelope Glycoprotein Induced Neuronal Autophagy
Jui Pandhare, Sabyasachi Dash, Bobby Jones, Fernando Villalta and Chandravanu Dash

J. Biol. Chem. published online September 1, 2015

Access the most updated version of this article at doi: 10.1074/jbc.M115.652776

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts