

Extracellular HIV-1 viral protein R affects astrocytic glyceraldehyde 3-phosphate dehydrogenase activity and neuronal survival

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Abstract Extracellular human immunodeficiency virus type 1 (HIV-1) viral protein R (Vpr) is a pleiotropic protein accomplishing several functions within the viral life cycle. While Vpr has been described extensively as an intracellular protein, very little is known about its role as an extracellular protein. In fact, HIV-1 Vpr has been detected in the blood, serum, and cerebrospinal fluid of HIV-1-infected patients, with concentrations increasingly higher in late-stage disease. To determine the role exogenous Vpr plays in HIV-associated central nervous system dysfunction, primary human fetal astrocytes were exposed to recombinant Vpr and a time- and dose-dependent decrease was demonstrated in two fundamental intracellular metabolites (adenosine-5'-triphosphate (ATP) and glutathione (GSH)). Additionally, exposure to exogenous Vpr led to increased caspase activity and secretion of proinflammatory cytokines IL-6 and IL-8 and chemoattractants, monocyte chemoattractant protein-1, and migration inhibition factor. Extracellular Vpr also dampened the glycolytic pathway through impairment of glyceraldehyde 3-phosphate dehydrogenase activity, causing a decline in the levels of ATP. The reduction in intracellular ATP increased reactive oxygen

species buildup, decreasing GSH concentrations, which affected several genes in the oxidative stress pathway. In addition, exposure of the SK-N-SH neuroblastoma cell line to conditioned medium from exogenous Vpr-treated astrocytes decreased synthesis of GSH, leading to their apoptosis. These observations point to a role that Vpr plays in altering astrocytic metabolism and indirectly affecting neuronal survival. We propose a model that may explain some of the neurological damage and therefore neurocognitive impairment observed during the course of HIV-1 disease.

Keywords Adenosine triphosphate · Astrocytic–neuronal network · Glutathione · Glyceraldehyde 3-phosphate dehydrogenase · HIV-1 viral protein R

Abbreviations

OS	Oxidative stress
ROS	Reactive oxygen species
GSH	Glutathione
GSSG	Oxidized glutathione
Vpr	Viral protein R
NAC	<i>N</i> -acetyl-L-cysteine
ANT	Adenine nucleotide translocator
MCP-1	Monocyte chemoattractant protein-1
MIF	Migration inhibitor factor
PAI-1	Plasminogen activator inhibitor-1
BSO	Buthionine sulfoximine
GST	Glutathione S-transferase
HA	Hemagglutinin
CPT	Camptothecin
qRT-PCR	Quantitative real-time PCR
HFA	Human fetal astrocytes
GFAP	Glial fibrillary acidic protein
SCARA3	Scavenger receptor class A member 3
rVpr	Recombinant Vpr
DGK	Diacylglycerol kinase
BBB	Blood–brain barrier

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Introduction

Oxidative stress (OS) is the condition wherein accumulation of reactive oxygen species (ROS) impairs intracellular protection mechanisms implicated in several pathological processes, including a number of neurodegenerative disorders (Baruchel and Wainberg 1992). Glutathione (GSH), a tripeptide composed of glycine, glutamate, and cysteine, plays a critical role in the removal of ROS. Within the central nervous system (CNS), astrocytes are the most critical cell type in regulating homeostasis and disposal of neurotoxic molecules (Shinya et al. 2004). By importing extracellular cysteine and glucose, astrocytes synthesize GSH and lactate (respectively) through aerobic glycolysis (Pellerin and Magistretti 1994); they then secrete GSH and lactate, which can be taken up by neurons, thus protecting them from OS. Exposure to HIV-1 increases ROS production in neuroglial cell cultures, leading to apoptosis (Pollicita et al. 2009), while excessive ROS accumulation within astrocytes may impair glutamate uptake (Sorg et al. 1997; Trotti et al. 1996), with subsequent extracellular glutamate accumulation and neuronal demise (Klegeris et al. 1997; Trotti et al. 1996). HIV-1-infected individuals are often at increased risk for OS due to impaired antioxidant defense mechanisms (Perl and Banki 2000; Treitinger et al. 2000) and decreased levels of GSH (Baruchel and Wainberg 1992). Imbalance between reduced GSH and oxidized glutathione (GSSG) triggers intracellular redox-sensitive pathways, ultimately causing DNA damage and correlating positively with CD4 T-cell count and disease progression (Herzenberg et al. 1997). Other HIV-1 proteins have been found to induce OS (Lassiter et al. 2009). Tat induces ROS accumulation through Nrf2 (Zhang et al. 2009) and inducible nitric oxide synthase signaling in astroglia (Liu et al. 2002), activation of macrophages and microglial cells, as well as T-cell infiltration in the CNS (Zhou et al. 2007), while gp120 sensitizes neurons to hydrogen peroxide-induced OS (Agrawal et al. 2010). HIV-1 viral protein R (Vpr) was found to induce OS through the hypoxia-inducible factor pathway in microglia (Deshmane et al. 2009), and we recently demonstrated Vpr as a causative agent of OS in an astrogloma cell line through depletion of adenosine-5'-triphosphate (ATP) and GSH reservoirs (Ferrucci et al. 2012). ROS-induced signaling leads to increased HIV-1 gene transcription (Perl and Banki 2000), which reseed viral infection.

Vpr has been shown to interact with the adenine nucleotide translocator (ANT) on the inner mitochondrial membrane (Jacotot et al. 2001, 2000), where amino acids Arg73 and Arg80 in Vpr are indispensable (Sabbah et al. 2006). Although these observations were determined using recombinant proteins or cell-free isolated mitochondria, there is no evidence that exogenous Vpr interacts directly with ANT, although recently Vpr was shown to transduce cells (Greiner et al. 2011; Sherman et al. 2002). Upon transduction, Vpr

may bind to ANT, thus hampering the passage of newly synthesized ATP molecules from the mitochondria, which in turn decreases the ATP-dependent concentration of GSH. Conversely, decreases in the levels of ATP and GSH could be the result of uncoupled signaling events, similar to the action of the chemotherapeutic agent doxorubicin. Through impairment of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the glycolytic pathway (Cui et al. 2006), decreased ATP induces excessive accumulation of ROS, thus reducing GSH storage. To test these hypotheses, the mode of action of Vpr was examined using a Vpr mutant protein with reduced binding affinity to ANT (Vpr R73,80A). Both parental and mutant Vpr caused reduction in the levels of ATP and GSH and equally affected GAPDH activity in human astrocytes, suggesting action through an ANT-independent signaling pathway. Additionally, increased secretion of proinflammatory cytokines (IL-6 and IL-8), monocyte chemotactic protein-1 (MCP-1), and migration inhibitor factor (MIF), and decreased levels of plasminogen activator inhibitor-1 (PAI-1) were observed. Removal of Vpr or Vpr R73,80A from the extracellular medium did not rescue astrocytic levels of either metabolite. Treatment of neurons with conditioned medium from Vpr- or Vpr R73,80A-exposed astrocytes reduced neuronal ability to synthesize GSH and led to neuronal apoptosis. These studies define a unique role for extracellular Vpr in altering astrocytic function, which impacts neuronal survival and might be associated with some of the pathological processes in during the course of HIV-1 disease.

Materials and methods

Cell maintenance *N*-acetyl-L-cysteine (NAC), buthionine sulfoximine (BSO), L-GSH, and doxorubicin hydrochloride were obtained from Sigma-Aldrich (St. Louis, MO, USA), while GSH beads for Vpr purification were from GE Healthcare (Waukesha, WI, USA). Whole cell lysates were prepared in 0.5× RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA), with the addition of a protease and phosphatase inhibitor cocktail (Calbiochem; EMD Millipore, Billerica, MA, USA); protein concentrations were calculated using the Bradford protein assay (Thermo Fisher Scientific). The pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA) was used to clone the 6 histidine (6His)-hemagglutinin (HA)-Vpr plasmid. Camptothecin (CPT) and the caspase inhibitor Ac-DEVD-CHO were purchased from BioVision (Milpitas, CA, USA) and Promega (Madison, WI, USA), respectively. Total cellular RNA was prepared as described by the manufacturer (USB Products; Affymetrix, Santa Clara, CA, USA). Primary human fetal astrocytes (HFAs) were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA) and maintained in astrocyte medium containing 1 % astrocyte

growth supplement, 2 % fetal bovine serum (FBS), and 1 % penicillin/streptomycin as described by the manufacturer. All experiments using HFAs were performed with cells passaged no more than twice as the astrocytic intracellular marker glial fibrillary acidic protein (GFAP) was gradually lost. The SK-N-SH neuroblastoma cell line (ATCC) was maintained in 1× Eagle's minimal essential medium supplemented with 10 % FBS, 1 mM sodium pyruvate, and 1.5 g/l sodium bicarbonate. All cells were maintained at 37 °C in 5 % CO₂ at 90 % relative humidity.

Plasmid construction and site-directed mutagenesis To clone Vpr, the noninfectious HIV-1 pNL4-3R⁺E⁻ molecular clone was used (obtained through the National Institutes of Health AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, provided by Dr. Nathaniel Landau; Connor et al. 1995; He et al. 1995). The 6His-HA-Vpr and glutathione S-transferase (GST)-6His-HA-Vpr plasmids were previously described (Ferrucci et al. 2012). The Vpr mutant plasmid (GST-6His-HA-Vpr R73,80A), wherein two arginine residues at positions 73 and 80 were changed to alanine, was obtained through two subsequent site-directed mutagenesis reactions from GST-6His-HA-Vpr plasmid using the QuickChange Site-Directed Mutagenesis procedure described by the manufacturer (Stratagene, La Jolla, CA, USA). Initially, the Vpr R73A variant (arginine to alanine change at position 73) was generated using the two reverse complement primers 5'-GCTGTTTATCCATTTTCGCAATTGGGTGTCGAC and 5'-GTCGACACCCAATTGCGGAAATG GATAAACAGC (the two nucleotides yielding the amino acid mutation are underlined). Parental plasmid DNA was digested by incubation with 10 U of Dpn I for 1 h at 37 °C, and the obtained PCR-amplified plasmid was transformed by heat shock into XL1-Blue-competent cells (Stratagene) to yield GST-6His-HA-Vpr R73A plasmid. This plasmid was then used to generate the double mutant GST-6His-HA-Vpr R73,80A with identical conditions as indicated above using the two reverse complement primers 5'-GGTGTGCGACATAGCGC AATAGGCGTTACTC and 5'-GAGTAACGCCTATTGCGCTATGTCGACACC (mutated nucleotides underlined). All plasmid DNAs were confirmed by sequencing (Genewiz, South Plainfield, NJ, USA).

Western immunoblot analyses Harvested cells were washed twice in 1× PBS, collected by low-speed centrifugation, and lysed by three freeze–thaw cycles. Lysed samples were cleared of nucleic acids and supernatant was collected, from which the protein concentration was assessed. Equal amounts of protein for all samples were resolved on a 10 % SDS-PAGE gel and transferred to a 0.45-μm Immobilon-P PVDF membrane, followed by blocking with 5 % (w/v) nonfat dry milk in 1× PBS (1 h at room temperature). Membranes were incubated overnight at 4 °C with

primary antibody; specific protein bands were detected using a horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Spotted antigen–antibody complexes were visualized using a chemiluminescent detection procedure (Pierce, Rockford, IL, USA). Densitometry was performed with an AlphaEase FC software package (Alpha Innotech, Randburg, South Africa). The following antibodies were used: GFAP, caspase-3 (Cell Signaling, Danvers, MA, USA); β-actin (Sigma-Aldrich); and GAPDH (GenScripts, Nanjing, Jiangsu, Japan).

Purification of recombinant Vpr Purification of Vpr or its mutant was performed as previously described (Ferrucci et al. 2012). Briefly, GST-6His-HA-Vpr or the Vpr R73,80A was induced by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside. Cultures were then pelleted by centrifugation and lysed in NETN lysis buffer, Tris pH 8.0 (20 mM), NaCl (100 mM), EDTA (1 mM), NP-40 (0.5 %), phenylmethylsulfonyl fluoride (1 mM), and protease inhibitor cocktail (1:100). The sonicated lysate was purified by affinity chromatography with GSH sepharose beads. The retained protein was washed and released from the column in elution buffer (30 mM reduced GSH in 50 mM Tris-HCl buffer, pH 8). Endotoxin was removed from eluted proteins with the ToxinEraser Endotoxin Removal procedure (GenScript). The GST tag was removed by cleavage with 10 U/mg thrombin for 2 h at room temperature. An additional affinity chromatography step was performed using a His SpinTrap nickel column (GE Healthcare) and protein dialyzed overnight at 4 °C in 1× PBS. All preparations employed in our study presented residual endotoxin concentration lower than the detectable levels (<0.001 EU/ml, as measured by the ToxinSensor Chromogenic LAL Endotoxin Assay procedure (GenScript)) and final protein concentration was measured at an absorbance of 280 nm.

ATP, GSH, and GAPDH activity assays For all experiments involving measurement of intracellular concentrations of ATP and GSH, we used the CellTiter-Glo Luminescent Cell Viability and GSH-Glo Glutathione Assay (Promega), respectively, as previously described (Ferrucci et al. 2012). Reduced GSH levels in SK-N-SH neuroblastoma cells were determined similarly with the following exceptions: cells were grown directly in single wells of a 96-well opaque plate, treated with conditioned medium for the indicated time, and lysed in 1× GSH-Glo reagent (Promega). For determination of the levels of reduced over oxidized glutathione (GSH/GSSG), a similar approach was followed using the GSH/GSSG-Glo Glutathione Assay (Promega) as described by the manufacturer. GSH/GSSG ratios were calculated from the formula $GSH = \frac{tGSH - GSSG}{2}$, then being $2 \cdot tGSH / GSSG - 1$.

To measure GAPDH intracellular activity, a fluorimetric assay procedure (KDalert GAPDH Assay; Ambion, Foster City, CA, USA) was used as described by the manufacturer. All untreated and treated cell populations were harvested and lysed in 100- μ l KDAlert lysis buffer. Ten microliter of lysate were dispensed in triplicate in single wells of a 96-well opaque plate with black walls. After addition of 90 μ l of master mix solution, the readings were taken using a FluoroMax-3 with a Spex Micromax 384 MicroPlate Reader (Horiba Scientific, Irvine, CA, USA) set at excitation and emission wavelengths of 560 and 590 nm, respectively. All readings were subtracted from the background value (lysis buffer alone). Graphs were plotted as percentage over untreated cells \pm standard errors.

Human cytokine array analyses Supernatant from untreated or Vpr-treated primary HFAs was collected at 48 h and used in the detection of differentially secreted cytokines utilizing the Proteome Profiler Array (R&D Systems, Minneapolis, MN, USA). Supernatants (500 μ l) from each of the two samples were incubated with a cytokine detection antibody cocktail and dispensed on top of each membrane. After an overnight incubation at 4 °C, cytokine–antibody complexes were detected with a streptavidin HRP-conjugated cocktail. Each cytokine, spotted in duplicate, was visualized by a chemiluminescent detection method (Pierce). To quantitate the brightness of each spot, densitometry was performed as described above, where each pair of cytokines was normalized to the untreated integrated density value, and the ratio of fold over untreated values was calculated. The average of each pair was plotted with its standard error; column bars with a positive or negative fold ratio represent cytokines up- or downregulated in extracellular Vpr-treated samples compared with untreated samples, respectively.

Caspase detection assay Caspase-3 and caspase-7 activities were measured using the luminescent Caspase-Glo 3/7 assay (Promega), based on a luminogenic substrate containing the tetrapeptide sequence DEVD, targeted by the two caspases. Primary HFAs were plated at a density of 2,000 cells in individual wells of a 96-well opaque tissue-culture plate and either left untreated or exposed to different compounds. As a positive control for induction of caspase-3 and caspase-7, HFAs were exposed for 48 h to 2 μ M CPT. HFAs were also treated alone or in combination with recombinant Vpr, anti-Vpr antibody (kind gift of Dr. Kopp, NIH, Bethesda, MD, USA), NAC, and the caspase inhibitor Ac-DEVD-CHO (Promega). NAC was applied to cells 24 h prior to harvesting. Forty-eight hours after exposure, cell culture medium was removed and HFAs were carefully washed in 1 \times PBS, lysed in situ, and incubated at room temperature in the dark for 20 min. Luminescent signals were recorded using a Glomax luminometer (Promega) and subjected to background correction (no cells). Augmented

caspase activity was detected as an enhanced luminescent signal. The data were plotted as fold over untreated cells.

Isolation of RNA and quantitative real-time PCR (qRT-PCR) microarray Total cellular RNA was isolated from primary HFAs (untreated or exposed to recombinant Vpr (rVpr) for 48 h) using the PrepEase RNA spin procedure as described by the manufacturer (USB, Affymetrix) and used to study the genes of the OS pathway that were differentially regulated. Five micrograms of total RNA was subjected to reverse transcription using the RT² First Strand procedure described by the manufacturer (SABiosciences, Frederick, MD, USA) to yield complementary DNA. qRT-PCR was performed on complementary DNA samples using RT SYBR Green/ROX qPCR Master Mix (SABioscience). The reaction mixtures (25 μ l) were dispensed in individual wells of a 96-well plate prespotted with primers of genes belonging to the OS and antioxidant defense pathway (SABioscience). Cycling conditions were set for 40 cycles, each consisting of 15 s at 95 °C and 1 min at 60 °C on an ABI 7300 Thermocycler (Applied Biosystems, Foster City, CA, USA). For each gene, a threshold cycle was calculated with the 7300 Thermocycler built-in software. Augmented mRNA levels in Vpr-treated samples were analyzed as fold over untreated (calibrator) and expressed in a linear scale. Using a software package (SABioscience), genes with the highest fold expression between untreated and treated were listed and plotted. For those genes with the highest fold values, protein levels were compared between untreated and treated samples by western immunoblotting as previously described.

In situ TUNEL assay To detect programmed cell death by terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay, the In Situ Cell Death Detection procedure was used as described by the manufacturer (Roche, Nutley, NJ, USA). Briefly, adherent SK-N-SH neuroblastoma cells were cultured on coverslips as described previously and exposed overnight to conditioned medium from astrocytes either left untreated or treated with rVpr. Cells were then washed twice with 1 \times PBS prior to fixation in 4 % paraformaldehyde for 20 min at room temperature. Permeabilization of plasma membranes was performed with 0.2 % Triton X-100 in 1 \times PBS for 5 min at room temperature. Cells were then blocked in 5 % goat serum, 1 % bovine serum albumin in 1 \times PBS for 1 h at room temperature and incubated with the TUNEL mixture (1:10 of Enzyme Solution to Label Solution, both Roche proprietary solutions) at 37 °C in the dark for 1 h. SK-N-SH neuroblastoma cells were subsequently incubated in 300 nM DAPI (4',6-diamidino-2-phenylindole) (Invitrogen)-containing 1 \times PBS for 2 min at room temperature to stain nuclei. Cells were washed three times with 1 \times PBS between each step.

Coverslips were then rinsed in ddH₂O, mounted on microscope slides with ProLong antifade reagent (Invitrogen), and immediately imaged with a deconvolution microscope (Olympus, Center Valley, PA, USA). For all cell types, a minimum of 10 fields at ×20 magnification were analyzed. The images reported are a representative field of the entire cell population. All fields of view were reported as two separate channels (DAPI and fluorescein isothiocyanate [FITC]) representing nuclei and TUNEL⁺ cells, respectively, until merged in a third image, wherein apoptotic cells displayed nuclei with an overlapped blue and green staining. As a positive control, SK-N-SH neuroblastoma cells were treated with 200 μM CPT to induce programmed cell death.

Statistical analysis The results were analyzed statistically by a two-tailed Student's *t* test. Differences between groups were considered significant if *p* values of <0.05 were obtained.

Results

Kinetic analysis of extracellular Vpr-induced decreases in the levels of ATP and GSH Since the first report of HIV-1 Vpr as an extracellular soluble protein (Levy et al. 1994), evidence has accumulated demonstrating the damaging effects this protein confers on both neurons and astrocytes (Cheng et al. 2007; Jones et al. 2007; Kitayama et al. 2008; Noorbakhsh et al. 2010; Regis et al. 2010; Sabbah and Roques 2005). HIV-1 Vpr has been detected in both the serum and cerebrospinal fluid (CSF) of HIV-1-infected patients and this protein has been shown to increase in quantity with disease progression (Levy et al. 1994, 1995). Additional studies have also shown that Vpr was found as a free extracellular protein in the serum of patients infected with HIV-1 (Hoshino et al. 2007; Jones et al. 2007). However, only the concentration of extracellular Vpr in the plasma of HIV-1-infected patients has been measured and was estimated to be 5–10 ng/ml (Hoshino et al. 2007). The concentration of extracellular Vpr has never been reported for CSF. We recently reported that treatment of the astroglioma U-87 MG cell line with extracellular recombinant HIV-1 Vpr protein-induced cellular deprivation of ATP and GSH, OS by increased accumulation of ROS, and a decreased ratio of reduced over oxidized glutathione upon (Ferrucci et al. 2012). In the same study, primary HFAs exposed to exogenous rVpr showed a significant decrease in the levels of ATP and GSH in a time- and rVpr dose-dependent manner, confirming results within the astroglioma U-87 MG cell line. To further examine the effects rVpr exerts on astrocytes, primary HFAs were exposed to different concentrations of rVpr, displaying a time- and dose-dependent decline in the GSH/GSSG ratio (reduced/oxidized glutathione), indicative of excessive oxidation (Table 1).

Table 1 Extracellular Vpr induces a time- and dose-dependent decline in GSH/GSSG ratios

Treatment	24 H	48 H
Untreated	53.4±3.8	55.6±2.5
Vpr 1 μg/ml	18.6±2.8*	7.0±2.4*
Vpr 2 μg/ml	12.6±1.4*	4.5±1.6*
Vpr 5 μg/ml	3.5±0.3**	1.6±0.2**

Although untreated HFAs did not show any significant variations after in vitro cultivation for 24 or 48 h, exposure to rVpr induced a dose- and time-dependent decrease in the levels of reduced, overoxidized glutathione (GSH/GSSG). Ratios at the beginning of the experiment (0 h) are 54.2±2.6

* *p* value <0.05

** *p* value <0.01

Extracellular Vpr activates caspase-3 and caspase-7

Previous studies reported activation of a caspase-induced cascade of events subsequent to Vpr exposure, leading to translocation of transcription factors to the nucleus, promoting DNA fragmentation and cell death in primary human astrocytes (Jones et al. 2007; Noorbakhsh et al. 2010). In a similar manner, HFAs exposed to exogenous Vpr were assessed for caspase-3 and caspase-7 activation through a luminescent assay (Fig. 1). HFAs treated with the apoptogenic drug CPT displayed almost a 2.5-fold increase in caspase-3 and caspase-7 activity (Fig. 1), while addition of a caspase inhibitor, acetylated synthetic tetrapeptide, inhibitor of caspase (Ac-DEVD-CHO), abrogated the observed increase. Exposure of

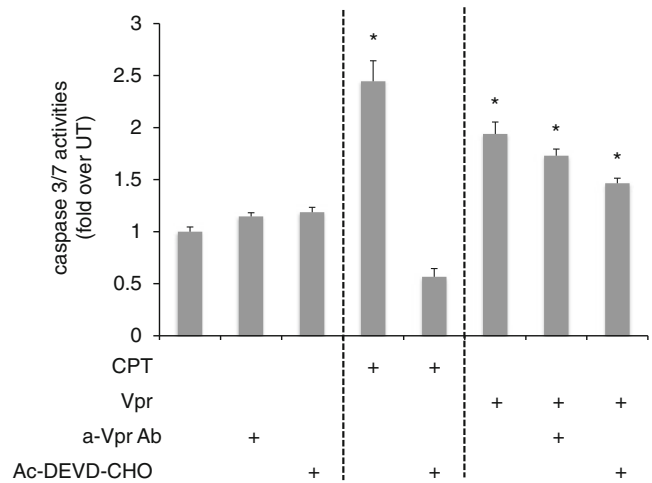


Fig. 1 Extracellular Vpr applied to HFAs induces activation of caspase-3 and caspase-7. HFAs were treated with different stimuli for 48 h and assayed for caspase activity using the Caspase-Glo 3/7 assay procedure. As a positive control, cells were treated for 48 h with CPT 2 μM, which inhibits DNA topoisomerase I in a caspase-dependent manner. Cells treated with rVpr (5 μg/ml) for 48 h similarly exhibited activation of caspase-3 and caspase-7. This effect was only partially recovered after treatment with anti-Vpr antibody or a caspase inhibitor (Ac-DEVD-CHO). **p* value <0.05 (Student's paired *t* test)

HFAs to rVpr promoted a twofold increase in caspase-3 and caspase-7, a result consistent with previous results obtained from examining mRNA levels (Noorbakhsh et al. 2010). Conversely, addition of Ac-DEVD-CHO or an anti-Vpr antibody to Vpr-treated HFAs only partially diminished the observed increases (Fig. 1, columns 7 and 8). Western immunoblotting analyses confirmed expression of cleaved caspase in rVpr-treated HFAs (data not shown). These results demonstrated that, within astrocytes exposed to rVpr, a series of intracellular events was initiated, commencing with decreased availability of ATP and GSH and culminating with caspase-dependent apoptosis.

Secretion of cytokines in extracellular Vpr-treated astrocytes HIV-1-induced neuropathogenesis is a concern among patients during the course of HIV-1 infection with an increasing number of individuals showing pathologies of neurodegeneration, such as excessive neuronal loss and abnormal astrocytic proliferation (Lipton 1994). Several studies have identified molecules playing a role in the observed effects including a number of viral proteins or virus-induced cellular cytokines. Given this, studies were performed to determine whether exposure of primary HFAs to rVpr induced secretion of cellular cytokines using a cytokine array containing many of the cytokines most commonly altered during the course of HIV-1 disease. Treatment of HFAs with rVpr-induced increased secretion of IL-6, IL-8, MCP-1, and MIF and decreased secretion of serpin E1, a serine proteinase inhibitor (Fig. 2a) also known as PAI-1. Semiquantitative densitometric analysis showed a significant increase in IL-6 (eightfold) as previously reported (Hoshino et al. 2010), as well as IL-8, MCP-1, and MIF (twofold), and a significant decrease in PAI-1 (threefold; Fig. 2b).

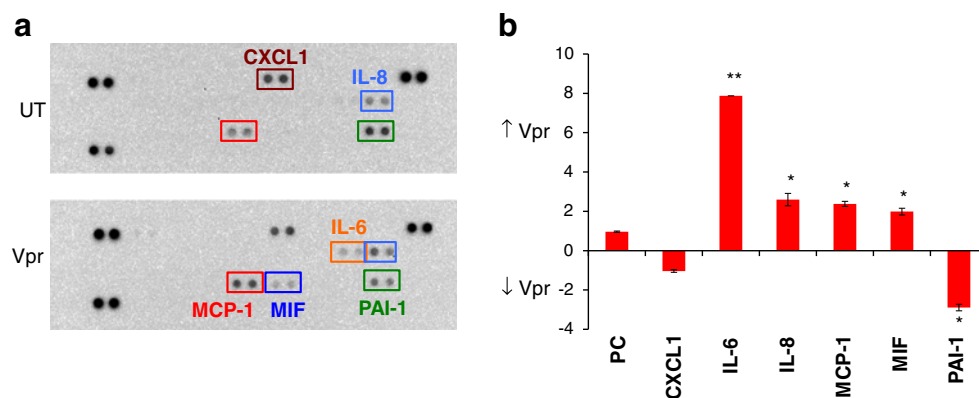


Fig. 2 Exogenous Vpr-treated primary HFAs display a different pattern of cytokine secretion. **a** Membranes spotted in duplicate with the most common cytokines were incubated with cell culture medium from either untreated (UT) or extracellular Vpr-treated HFAs (5 μ g/ml). Cytokines differentially expressed in the two cell supernatants are shown in different colored squares (orange IL-6, red MCP-1, blue MIF, light blue IL-8, green PAI-1, and maroon CXCL1). The doublet spots in the top left, top right, and bottom left corners are positive controls. **b** Densitometric analysis of the up- or downregulated cytokines present in the cell culture medium shows increased secretion of IL-6 (eightfold); increased secretion of IL-8, MCP-1, and MIF (twofold); and decreased secretion of PAI-1 (threefold). Levels of CXCL1 were unaltered. **p* value <0.05, ***p* value <0.01 (Student's paired *t* test)

Vpr-induced decreases in the levels of ATP and GSH is not through ANT binding We previously showed that exposure to extracellular Vpr affects both ATP and GSH concentrations (Ferrucci et al. 2012). Because ATP is involved in the synthesis of GSH from its precursor amino acid, we investigated whether the decreases in the two molecules were directly correlated. Previous reports showed binding between Vpr and the inner mitochondrial membrane protein ANT, responsible for the exchange of newly synthesized ATP molecules in the electron transport chain for cytoplasmic ADP (Pebay-Peyroula et al. 2003). A possible blockade of ADP/ATP exchange is detrimental intracellularly as shown by treatment with the ANT-binding bongkreikic acid (Halestrap et al. 1997). Indeed, a decline in the level of ATP could affect GSH concentrations, which is synthesized through two ATP-dependent reactions. For Vpr/ANT interaction to occur, two arginine residues at positions 73 and 80 of Vpr are of great importance (Jacotot et al. 2000; Sabbah et al. 2006). To test this hypothesis, a Vpr mutant protein (Vpr R73,80A) was constructed, wherein the ANT binding site was knocked out. HFAs were incubated in the absence or presence of rVpr or rVpr R73,80A, and the levels of both ATP and GSH were assayed (Fig. 3). Both the rVpr and rVpr R73,80A mutant-induced comparable decreases in the levels of ATP and GSH, without any statistical difference at 24 and 48 h after exposure (Fig. 3a and b, respectively). These observations suggested that a Vpr-induced mechanism other than binding to the ANT protein causes the pseudosynchronous declines in ATP and GSH. Additionally, we evaluated whether the Vpr-induced declines in ATP and GSH were reversible or not: HFAs were initially exposed to rVpr or rVpr R73,80A mutant for 48 h and cultivated for an additional 24 h with fresh medium. As shown in Fig. 3a and b, neither ATP nor GSH concentrations rebounded to sub-initial values upon removal of

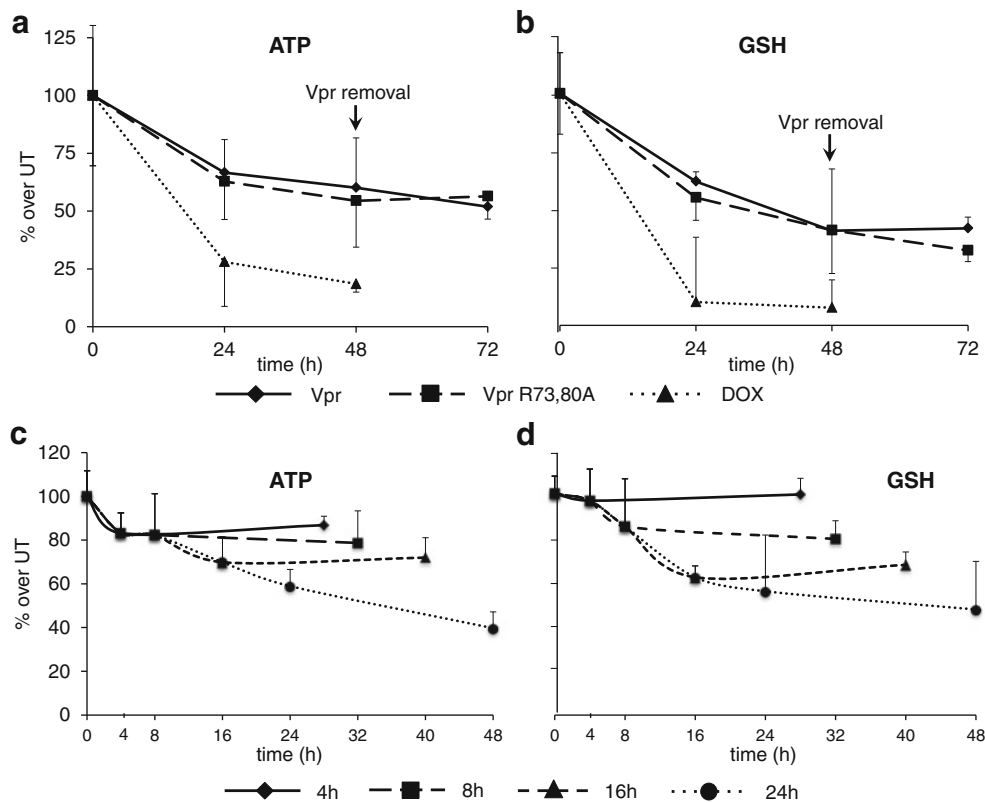


Fig. 3 Exposure of primary HFAs to extracellular Vpr or Vpr R73,80A induces irreversible decreases in both ATP and GSH metabolites through an ANT-independent pathway. **a** and **b** HFAs treated for 24 or 48 h with extracellular rVpr or rVpr R73,80A (with reduced binding affinity to ANT) were assayed for intracellular levels of ATP (**a**) and GSH (**b**). Upon removal of the protein in the extracellular medium and culture with fresh new medium for an additional 24 h, neither ATP nor GSH rebounded to initial values (doxorubicin was

used as a positive control). **c** and **d** A kinetic analysis denotes irreversible effects on the concentration of ATP and GSH induced by rVpr in HFAs. HFAs were incubated with rVpr (5 µg/ml) for 4, 8, 16, or 24 h, after which they were cultivated for an additional 24 h with fresh new medium. ATP levels dropped after exposure for 4 h (**c**) and to a greater extent after longer treatment periods, whereas the decrease in the level of GSH (**d**) was marginally delayed. Exposures for 24 h in the absence of Vpr did not lead to recovery of ATP or GSH levels

extracellular Vpr, which points to an irreversible effect. A kinetic analysis was then performed to determine whether the levels of ATP or GSH could be rescued after shorter exposure times. To address this point, HFAs were treated with rVpr for 4, 8, 16, or 24 h and subsequently cultivated for an additional 24 h with fresh Vpr-deficient medium. This experimental approach models removal of exogenous Vpr by antigen-presenting cells and allows analysis of the astrocytic ATP and GSH content at different exposure times. The concentration of ATP shows an initial steep drop for the first 4 h after exposure, which did not return to preexposure levels after removal of Vpr and further cultivation for 24 h with fresh medium (Fig. 3c). Conversely, the concentration of GSH was steady for the first 4 h after exposure and commenced to decline in the 4- to 8-h window (Fig. 3d). Addition of fresh medium for 24 h did not lead to recovery of either ATP or GSH for any exposure time examined (Fig. 3c and d).

Induction of OS by extracellular Vpr We have previously shown that exposure to extracellular Vpr induced decreases

in ATP and GSH levels and a shift in the intracellular GSH/GSSG ratio toward increased generation of ROS (Ferrucci et al. 2012), although the exact correlation remains elusive. Given this, the intracellular mechanisms involved in rVpr-induced OS were examined by identifying the genes within the antioxidant defense mechanism pathways that were differentially regulated. HFAs incubated in the absence or presence of extracellular rVpr was analyzed through a gene array containing the most relevant genes of the OS pathway. Six genes were significantly upregulated (*APOE*, *CCL5*, *DGKK*, *GPX5*, *NOS2*, and *PXDNL*), and 11 were significantly downregulated (*BNIP3*, *CYGB*, *DUOX2*, *DUSP1*, *FOXMI*, *GAPDH*, *HPRT1*, *MT3*, *MTL5*, *PTGS2*, and scavenger receptor class A member 3 (*SCARA3*); Table 2). Of immediate interest was the reduction in mRNA expression of a common housekeeping gene (*GAPDH*). All of the results reported herein were analyzed without considering *GAPDH* among the housekeeping genes in order not to skew the results. Four additional housekeeping genes (*B2M*, *HPRT1*, *RPL13A*, and *ACTB*)

Table 2 Genes up- and downregulated in extracellular Vpr-exposed HFAs

	Gene ID	Fold	Gene name
↑	<i>APOE</i>	7.9	Apolipoprotein E
	<i>CCL5</i>	4.2	RANTES
	<i>DGKK</i>	5.0	Diacylglycerol kinase kappa
	<i>GPX5</i>	5.1	GSH peroxidase 5
	<i>NOS2</i>	5.3	Nitric oxide synthase 2
	<i>PXDNL</i>	16.3	Peroxidasin homolog ligand
↓	<i>BNIP3</i>	−10.7	BCL2/adenovirus E1B 19 kDa interacting protein 3
	<i>CYGB</i>	−17.6	Cytoglobin
	<i>DUOX2</i>	−16.3	Dual oxidase 2
	<i>DUSP1</i>	−4.2	Dual specificity phosphatase 1
	<i>FOXMI</i>	−4.0	Forkhead box M1
	<i>MT3</i>	−120.1	Metallothionein 3
	<i>MTL5</i>	−4.4	Metallothionein-like 5
	<i>PTGS2</i>	−6.0	Prostaglandin-endoperoxidase synthase 2
	<i>SCARA3</i>	−7.2	Scavenge receptor class A, member 3
	<i>HPRT1</i>	−7.9	Hypoxanthine phosphoribosyltransferase 1
	<i>GAPDH</i>	−5.3	Glyceraldehyde 3-phosphate dehydrogenase

were used in the analysis as normalizing factors because their expression was found unvaried between untreated and Vpr-exposed HFAs.

Extracellular Vpr impairs the glycolytic pathway Recent studies underscored the role of *GAPDH* in apoptosis and OS, possibly due to its propensity for nuclear translocation (Dastoor and Dreyer 2001; Sukhanov et al. 2006; Thangima Zannat et al. 2011). Additionally, *GAPDH* activity was found to decline upon exposure to doxorubicin (a chemotherapeutic drug), directly correlating with a decline in ATP levels (Wolf and Baynes 2006). Decline in *GAPDH* activity, a key regulator enzyme in glycolysis, has been shown to be detrimental for all intracellular ATP-dependent metabolic processes. Western immunoblotting analysis of HFAs either untreated or treated with rVpr or with rVpr R73,80A showed only a minimal decrease in *GAPDH* expression 24 h after exposure (Fig. 4a). The decline was slightly more pronounced although not significant after 48 h of treatment, with about a 40 % reduction in protein expression determined by semiquantitative densitometric analysis (Fig. 4b). However, *GAPDH* activity was significantly decreased in HFAs treated with both rVpr or rVpr R73,80A (Fig. 4c and d). As a positive control, HFAs treated with two different concentrations of doxorubicin displayed about 20 and 75 % declines in *GAPDH* activity, respectively (Fig. 4c), as previously reported (Cui et al. 2006).

Vpr-induced effects on astrocytes can impact neuron function Astrocytes are key regulators in CNS homeostasis by tightly regulating brain homeostasis and supporting neurons architecturally and metabolically. One of the most

important metabolites secreted by astrocytes for neuronal homeostasis is GSH, which aids in maintaining a balanced neuronal redox state. Secreted GSH is initially cleaved into cysteine–glycine and subsequently into cysteine, which is then taken up by neurons to synthesize GSH intracellularly (Garg et al. 2008). Increased astrocytic oxidation in the form of decreased GSH release is translated to the neuronal compartment, because less cysteine is available for uptake from the extracellular environment, unless supplementation with a cysteine-containing compound has been provided. To demonstrate that conditioned medium from astrocytes treated with rVpr or rVpr R73,80A perturbs neuronal oxidation status, the concentration levels of GSH were measured in an SK-N-SH neuroblastoma cell line subjected to different treatments. SK-N-SH neuroblastoma cells exposed to rVpr- or rVpr R73,80A-conditioned medium for 6 h displayed a 50 % decrease in reduced GSH levels compared with untreated cells (Fig. 5a). These values could be partially rescued on supplementation with NAC (Fig. 5a), supporting the hypothesis that conditioned medium containing Vpr indirectly affects neuronal synthesis of GSH through less availability of cysteine, at least in this cell line model. An anti-Vpr antibody (a kind gift of Dr. Jeffrey Kopp, NIH, Kidney Disease Section) was added to all conditioned media containing Vpr to demonstrate that the observed effects were not directly driven by the presence of residual exogenous Vpr in the supernatant. As a positive control, SK-N-SH neuroblastoma cells were treated for 6 h with 100 μ M BSO, which irreversibly inhibits the first enzyme in GSH synthesis and may not be rescued by cotreatment with NAC (Fig. 5a). Additionally, to gain a better understanding of the downstream consequences

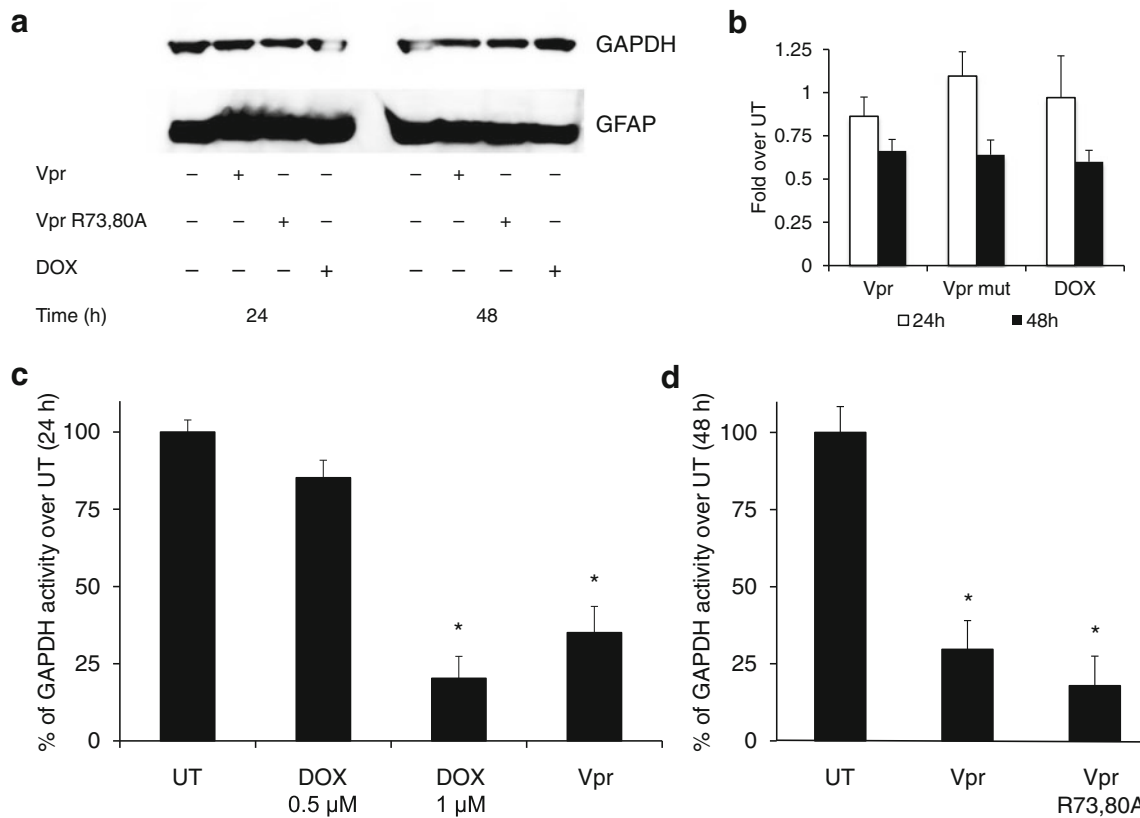


Fig. 4 Extracellular Vpr and Vpr R73,80A impair GAPDH activity. **a** Immunoblot assay shows small downregulation of GAPDH in both rVpr- and rVpr R73,80A-treated HFAs (5 μg/ml each) at 24 and 48 h after exposure. **b** Densitometric analysis showed virtually no difference in GAPDH expression at 24 h in rVpr-, rVpr R73,80A-, and doxorubicin (DOX, 1 μM)-treated HFAs, whereas a slight decrease (~0.5-fold)

was observed 48 h after exposure. **c** HFAs treated with DOX at two different concentrations (0.5 and 1 μM) or exogenous rVpr (5 μg/ml) showed decreased activity of GAPDH 24 h after exposure as measured by fluorimetric assay. **d** Both rVpr and rVpr R73,80A (5 μg/ml each) proteins induced decreased GAPDH activity 48 h after treatment. **p* value <0.05 (Student’s paired *t* test)

caused by rVpr exposure, SK-N-SH neuroblastoma cell survival was assessed through an in situ TUNEL assay. Untreated neuroblastoma cells showed minimal cell death after 6 h (Fig. 5b), while the apoptogenic drug CPT (10 μM) induced significant cell death (Fig. 5c). Similarly, cells exposed to conditioned medium treated with rVpr or rVpr R73,80A displayed increased apoptosis (Fig. 5d and e), as shown by the increased number of apoptotic nuclei (overlapping green and blue staining).

Discussion

HIV-1 infection of the CNS and its pathogenesis in this compartment continues to be an area of intense interest to the scientific community due to an increase in HIV-associated neurocognitive disorders (HAND) that has been noted for the past several years (Cysique et al. 2009; McArthur 2004; Nath et al. 2008). The CNS compartment is separated from the peripheral blood by the blood–brain barrier (BBB) which restricts access of the brain to pathogens, drugs, and cells of

the immune system (Strazza et al. 2011). Within the CNS, there are a number of cells that are infected by HIV-1 or dysregulated by the virus or components of the virus, including Vpr. Within the CNS, previous studies of extracellular Vpr have centered their attention on neurons, which are gradually lost throughout disease and especially during late stages resulting in a wide range of HAND. With respect to the effect of extracellular Vpr on neurons, studies have shown neurons exposed to rVpr are: (1) severely impaired, with inhibition of axonal outgrowth and death (Kitayama et al. 2008; Sabbah and Roques 2005) and (2) able to form porous channels at the plasma membrane (Piller et al. 1996), which caused aberrant inward sodium current in neurons (Piller et al. 1998; Potter et al. 2004) as well as rapid intracellular Ca²⁺ flux in astrocytes (Noorbakhsh et al. 2010). Abnormal concentrations of cytoplasmic Ca²⁺ could then lead to alteration in mitochondrial Ca²⁺ uptake, which, along with downregulation of GAPDH within the glycolytic pathway, could impair the efficiency of oxidative phosphorylation (Brookes et al. 2004), leading to a decline in the level of ATP. In fact, in studies using HFAs, a dose-dependent decrease was detected in the levels of ATP

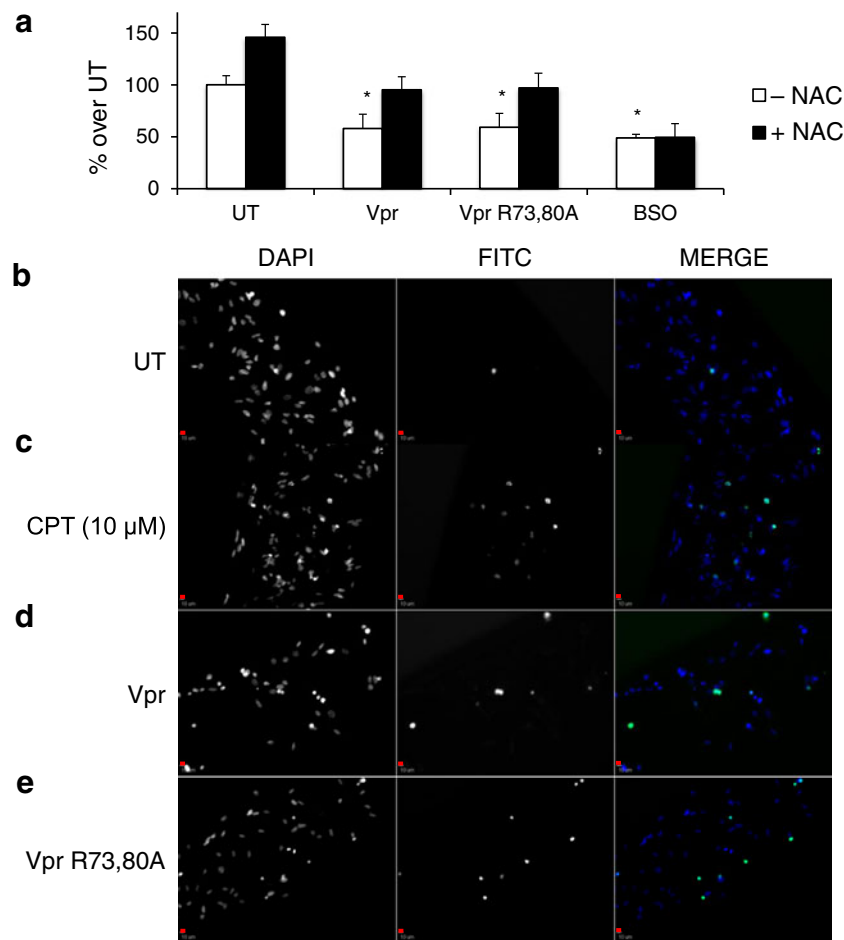


Fig. 5 SK-N-SH neuroblastoma cells exposed to Vpr- or Vpr R73,80A-treated HFA-conditioned medium display decreased level of GSH synthesis and increased apoptosis. **a** SK-N-SH neuroblastoma cells were exposed for 6 h to different media conditioned with HFAs and assayed for intracellular reduced GSH content. Conditioned medium from Vpr- or Vpr R73,80A-treated HFAs (for 48 h) induced about a 50 % decrease in neuronal GSH synthesis. These effects were recovered upon supplementation of NAC in the conditioned medium, while the oxidant BSO irreversibly inhibited GSH synthesis. Untreated (UT) SK-N-SH neuroblastoma cells (SK-N-SH cells exposed to conditioned medium from astrocytes that were not exposed to rVpr) also showed an

increase in GSH concentrations with NAC treatment (~50 %). Similarly, SK-N-SH neuroblastoma cells were exposed for 6 h to different HFA-conditioned media and subjected to the in situ TUNEL assay. Although untreated cells (UT) showed virtually a complete lack of apoptotic nuclei (**b**), SK-N-SH neuroblastoma cells exposed to the apoptogenic compound CPT (10 μM) (**c**), conditioned medium from Vpr- (**d**) or Vpr R73,80A (**e**)-treated HFAs (for 48 h) displayed an increase in the presence of apoptotic nuclei (FITC channels, *second column*), positively overlapping with DAPI staining (nuclei, *first column*), as shown in the merged figure (*third column*). All magnifications were $\times 10$. Scale bars 10 μm, **p* value <0.05 (Student's paired *t* test)

and GSH (Ferrucci et al. 2012; Table 1), and products of the aerobic respiration and antioxidant pathways, respectively.

To further investigate this association, herein, genes differentially regulated upon exposure to extracellular Vpr were examined with six genes upregulated and 11 downregulated (Table 2). Among the upregulated genes, *GPX5* is well known for reducing lipid peroxide and hydrogen peroxide to water (Chabory et al. 2010), although its role outside the epididymis of the mammalian male reproductive tract has not yet been explored. *DGKK* was upregulated about fivefold compared with untreated astrocytes (Table 2): upon activation of the G-protein-coupled receptor, diacylglycerol kinase (DGKs) phosphorylate 1,2-diacylglycerol into phosphatidic acid and activate

protein kinase C, thus leading to phosphorylation of several target proteins, such as kinases of the MAPK pathway. If confirmed at the protein level, upregulation of DGK could explain the observed increased phosphorylation of SAPK/JNK and p38 MAP kinases previously reported (Noorbakhsh et al. 2010). *CCL5* upregulation at the mRNA level (about fourfold) was not detected in the cytokine array (Fig. 2a), possibly underlying a post-translational sequestering effect or dampening of protein production. Vpr treatment also increased transcriptional expression of *ApoE*, essential in the catabolism of triglyceride-rich lipoproteins, recently associated with increased risk of Alzheimer's disease (Ma et al. 1994) and accelerated progression toward AIDS-defining illnesses (Cutler et al. 2004).

Most of the downregulated genes code for ROS-scavenging proteins, such as metallothionein 3 (*MT3*), cytoglobin (*CYGB*), *DUOX2*, and *SCARA3* (Table 2), confer cellular ability to detoxify ROS. Exogenous Vpr could possibly induce abundant ROS production by downregulating all these enzymes, thus promoting an oxidative intracellular status. Of particular interest was the downregulation (about fivefold at the mRNA level) of *GAPDH*, a common housekeeping gene. Our initially proposed mode of action by extracellular Vpr through binding to the ANT protein and blocking the passage of ATP for ADP between the mitochondrial matrix and the cytoplasm was challenged using a Vpr mutant protein with lesser ANT binding affinity (Fig. 3a and b). Instead these results suggested a pathway through impairment of one of the enzymes in the glycolytic pathway (*GAPDH*), which may compromise the conversion of glucose to pyruvate. *GAPDH* activity was severely impaired upon exposure to both parental and mutant Vpr (Fig. 4c and d) and only mildly underregulated at the mRNA and protein levels (Table 2 and Fig. 4a), which leads to reduced ATP synthesis owing to a lesser availability of substrate for the Krebs cycle and oxidative phosphorylation. These observations point to either direct binding of Vpr to *GAPDH* or an indirect mechanism through which Vpr affects *GAPDH* activity by removal of its substrate glyceraldehyde-3-phosphate (G3P). An additional mechanism could involve suppressing the reduction of NAD⁺ to NADH, which occurs simultaneously with the conversion of G3P to D-glycerate-1,3-bisphosphate. These results suggest that the intracellular cascade of events does not involve interaction between Vpr and ANT but rather a *GAPDH*-dependent mechanism, where impairment of the glycolytic pathway leads to ROS accumulation and decline in GSH levels (1 in Fig. 6).

Increased ROS accumulation could be either a direct consequence of prolonged ATP deprivation (Liu et al. 2005) or the result of a third-partner molecule causing the observed effects. A likely candidate may be an increase in intracellular calcium concentrations. Not only has Vpr been shown to induce Ca²⁺ entry (Acharjee et al. 2010; Noorbakhsh et al. 2010; Rom et al. 2009), possibly through cell membrane permeabilization (Macreadie et al. 1996; Piller et al. 1996, 1998), which can trigger the opening of mitochondrial permeability transition pores (Gincel et al. 2001), but also Ca²⁺ ions can freely diffuse through the mitochondria owing to their molecular size. Although under physiological conditions, Ca²⁺ is beneficial for cell survival as it stimulates the Krebs cycle and electron flow through the electron transport chain, thus increasing ATP levels, excessive concentrations of Ca²⁺ impair oxidative phosphorylation and increase ROS buildup (Brookes et al. 2004), thus perturbing mitochondrial integrity. Indeed, mitochondrial transition pores are closed and ANT is in its matrix conformational state, whereby the voltage-dependent anion channel (VDAC) does not allow passage of solutes

with a molecular weight greater than 1,500 Da (Halestrap 2009). Because there is no evidence that extracellular Vpr directly promotes opening of VDAC, which masks ANT from the cytoplasmic compartment, it is reasonable to hypothesize the existence of an intermediate molecule to promote pore opening. Overproduction of ROS then leads to a decrease in reduced GSH. Decreases in the intracellular levels of ATP and GSH explain mitochondrial membrane hyperpolarization (decreased $\Delta\psi$) with induction of pore opening, leading to programmed cell death through a caspase cascade (Fig. 1). Studies are under way to determine whether elimination of ROS (through supplementation of NAC or ATP-derived supplements) could restore *GAPDH* activity and functionality to counteract Vpr-induced downstream effects. Additionally, sustained ROS accumulation could trigger intracellular signaling and transcription of nuclear factor- κ B (NF- κ B)-dependent genes (2 in Fig. 6). The dual action of ROS on both caspase-dependent apoptosis and transcription of NF- κ B-dependent genes, including the HIV-1 long terminal repeat (LTR) promoter, is likely synchronized for the virus to take full advantage of the host cell machinery before its demise. Augmented viral production would then reseed infection in the CNS from persistently HIV-1-infected astrocytes.

The observed decrease in the levels of GSH could be either an immediate consequence of a decrease in ATP levels (although not through blockage of the ANT) or a consequence of an increased buildup of ROS within the intracellular environment. The interplay between ATP and GSH metabolites is particularly important, as different compounds might independently reduce the levels of ATP or GSH or both: for example, treatment with iodoacetate reduces ATP but not GSH (Verity et al. 1991). The role of *GAPDH* in OS has recently been evaluated and could be due to its nuclear translocation (Dastoor and Dreyer 2001), thus disabling its specific role in glycolysis and inducing apoptosis. A lower expression of *GAPDH* and/or a decreased activity due to an abundant translocation to the nucleus could explain the observed loss in ATP production. An additional study wherein bovine pulmonary artery endothelial cells were exposed to doxorubicin identified decreased activity of *GAPDH*, which correlated positively with ATP concentration (Cui et al. 2006). *GAPDH* could promote initiation of apoptosis through nuclear translocation (Sen et al. 2008), further depleting cytoplasmic storage levels of ATP in a vicious cycle. The results herein support a role for *GAPDH* as an intracellular sensor of oxidation.

We subsequently evaluated any alteration in the cytokine profile of Vpr-exposed astrocytes, because HIV-1 has been shown to induce secretion of proinflammatory and chemotactic cytokines. We demonstrated increased secretion of IL-6 (Fig. 2), as previously described in neuronal cells exposed to Vpr-treated, astrocytic-conditioned medium (Jones et al.

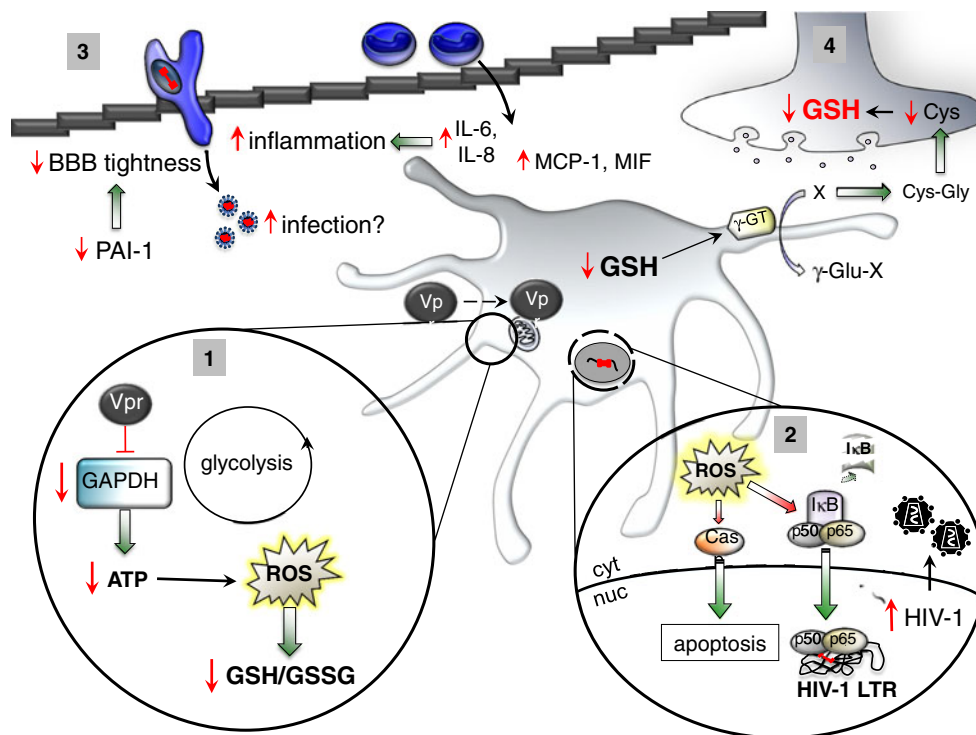


Fig. 6 Model of HIV-1 extracellular Vpr action on astrocytes. As exogenous Vpr gains access to the intracellular environment, it induces an initial decrease in ATP concentration through impairment of GAPDH. Prolonged ATP deprivation promotes increased ROS accumulation, which may no longer be detoxified by intracellular GSH, thus leading to a highly oxidized status (1). ROS buildup is potentially harmful because it induces transcription of oxidative-sensitive genes along with NF- κ B-dependent genes, including transcription driven from the HIV-1 LTR, which could enhance viral replication and spread from persistently infected astrocytes. ROS also induce caspase-dependent apoptosis (2). Additionally, exogenous Vpr induces secretion of

the proinflammatory cytokines IL-6 and IL-8 and the chemoattractants MCP-1 and MIF, which in turn could facilitate migration of monocytes from the periphery. Decreased levels of PAI-1 in the CNS could also reduce the integrity of the BBB, allowing passage of otherwise excluded molecules. Increased chemotaxis of HIV-1-infected monocytes may also reseed viral infection in the CNS (3). Finally, GSH synthesized within astrocytes may also be secreted and, on cleavage by gamma-glutamyl transpeptidase (γ -GT) into Cys-Gly, may be taken up by neurons as Cys, thus igniting GSH production within neurons. A reduction of astrocytic GSH is then reverberated on the neuronal compartment, leading to decreased synthesis of GSH and neuronal demise (4)

2007) or Vpr-treated monocyte-derived macrophages (Hoshino et al. 2010). IL-8 was also found to be increased as reported in a previous study in both T cells and macrophages infected with Vpr-containing HIV-1 virions (Muthumani et al. 2000). Two additional studies demonstrated increased IL-8 secretion in primary HFAs, either infected with an envelope glycoprotein G of vesicular stomatitis virus pseudotyped virus (Li et al. 2007) or by coinfection with hepatitis C virus (Vivithanaporn et al. 2010). Two other chemotactic cytokines were found to be elevated in the extracellular medium of Vpr-treated HFAs: MCP-1 and MIF. Previous studies showed MCP-1 was found increased in astrocytes cocultured with HIV-1-infected macrophages (Muratori et al. 2010), thus possibly contributing to increased recruitment of T lymphocytes and HIV-1-infected macrophages migrating through the BBB, which could reseed infection within the CNS. Interestingly, astrocytes treated with exogenous HIV-1 Tat secreted abundant MCP-1, which was also found in excess in the brains and CSF of patients with HIV-associated dementia (Maingat et al. 2011). Given that Vpr may be acting

through a similar mechanism as Tat. MIF is upregulated in several different inflammatory and infectious diseases: peripheral blood mononuclear cells from HIV-1-infected patients have been found to release excessive amounts of MIF, which in turn activates viral transcription and replication in HIV-1-infected CD4⁺ T cells (Regis et al. 2010). Additionally, activation of the toll-like receptor 4 has been shown to induce the secretion of MIF by mature dendritic cells (Popa et al. 2006), macrophages (Roger et al. 2003), and astrocytes (Krasowska-Zoladek et al. 2007). Recombinant Vpr-treated macrophages have been shown to induce IL-6 production through toll-like receptor 4/MyD88 signaling (Hoshino et al. 2010). If substantiated in vitro, these results not only would confirm a similar mechanism of activation between monocyte-derived macrophages and astrocytes by exogenous Vpr but also would explain the cause of increased secretions of MIF and IL-6. The observed alteration in cytokine secretion could be either a direct consequence of Vpr exposure or an indirect effect of an autocrine/paracrine process by action of the secreted cytokines or a synergistic effect of Vpr with any of the aforementioned

cytokines. Finally, decreased presence of PAI-1 in Vpr-exposed astrocytes could potentially cause deleterious effects in reducing the tightness of the BBB. PAI-1 is a member of the serine protease inhibitor 1 family, the primary inhibitor of tissue plasminogen activator, widely expressed both in the periphery and within the CNS, where astrocytes are the major source (Hino et al. 2001). A balanced production of tissue plasminogen activator and PAI-1 is vital in a highly regulated and selective environment such as the CNS, through tight junctions within endothelial cells at the lining of the BBB (Tran et al. 1998). All of this put together may point to extracellular Vpr contributing to increased BBB permeability as well as increased recruitment of cells, especially monocyte macrophage cells into the CNS through dysregulation of the astrocyte compartment (3 in Fig. 6).

Kinetic analysis of rVpr-treated HFAs indicated that exogenous Vpr inhibited astrocytic ability to synthesize both ATP and GSH within 24 h of exposure (Fig. 3). Short exposures to Vpr (4 h) were enough to compromise ATP pool levels (Fig. 3c), although GSH content seemed unvaried (Fig. 3d). However, the inability of HFAs to recover ATP values after a short 4-h exposure does not assure that Vpr-exposed astrocytes are similar to unexposed astrocytes. Fundamental functions such as removal of extracellular glutamate and neuronal support might be irreversibly compromised. Additionally, astrocytes preexposed to extracellular Vpr might be sensitized to future exposure because their ATP content has already declined and further treatment might lead to a larger and faster depletion. This difference in response to ATP and GSH at early time points after Vpr exposure could be explained by a delay in intracellular accumulation of ROS, whereas ATP decline is more rapid and caused by a direct impairment of GAPDH activity.

Lastly, augmented oxidation of GSH was shown to be detrimental for neuronal survival (4 in Fig. 6); indeed, GSH is secreted into the extracellular medium and catabolized into Cys-Gly, subsequently taken up by neurons as cysteine, which triggers GSH synthesis (Garg et al. 2008). Increased shift from GSH to GSSG glutathione within the astrocytes leads to diminished GSH secretion and uptake by the neuronal compartment (here modeled with the SK-N-SH neuroblastoma cell line), thus affecting their antioxidant pool (Fig. 5a) and inevitably leading to apoptosis (Fig. 5b–e and 4 in Fig. 6).

Given these and other studies, the importance of the role of astrocytes on support of the BBB and the neuronal population is once again highlighted. In the highly active antiretroviral therapy (HAART) era, where patients are living longer with more subdued chronic disease including HAND, understanding the interplay of astrocyte dysfunction in conjunction with other cells of the CNS, especially neurons, is increasingly important. In fact, these results provide yet another piece of evidence to suggest that HIV-1 may be inducing neuronal pathogenesis leading to HAND.

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