Published in final edited form as:

AIDS. 2010 May 15; 24(8): 1107–1119. doi:10.1097/QAD.0b013e328337b0ab.

HIV-1 viral protein r induces ERK and caspase-8 dependent apoptosis in renal tubular epithelial cells

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Abstract

Objective—HIV-associated nephropathy is the most common cause of end stage renal disease in persons with HIV/AIDS and is characterized by focal glomerulosclerosis and dysregulated renal tubular epithelial cell (RTEC) proliferation and apoptosis. HIV-1 viral protein r (Vpr) has been implicated in HIV-induced RTEC apoptosis but the mechanisms of Vpr-induced RTEC apoptosis are unknown. The aim of this study was therefore to determine the mechanisms of Vpr-induced apoptosis in RTEC.

Methods—Apoptosis and caspase activation were analyzed in human RTEC cells (HK2) after transduction with Vpr-expressing and control lentiviral vectors. Bax and BID were inhibited with lentiviral shRNA, and ERK activation was blocked with the MEK1,2 inhibitor, U0126.

Results—Vpr induced apoptosis as indicated by caspase 3/7 activation, PARP-1 cleavage and mitochondrial injury. Vpr activated both caspases-8 and 9. Inhibition of Bax reduced Vpr-induced apoptosis, as reported in other cell types. Additionally, Vpr induced cleavage of BID to tBID and suppression of BID expression prevented Vpr-induced apoptosis. Since sustained ERK activation can activate caspase-8 in some cell types, we studied the role of ERK in Vpr-induced caspase-8 activation. Vpr induced sustained ERK activation in HK2 cells and incubation with U0126 reduced Vpr-induced caspase-8 activation, BID cleavage and apoptosis. We detected phosphorylated ERK in RTEC in HIVAN biopsy specimens by immunohistochemistry.

Conclusions—These studies delineate a novel pathway of Vpr-induced apoptosis in RTEC, which is mediated by sustained ERK activation, resulting in caspase 8-mediated cleavage of BID to tBID, thereby facilitating Bax-mediated mitochondrial injury and apoptosis.

Keywords

vpr; apoptosis; caspases; AIDS-Associated Nephropathy; Extracellular Signal-Regulated MAP Kinases; kidney

Introduction

HIV-associated nephropathy (HIVAN) is the most common cause of end stage renal disease in HIV seropositive patients and results from direct infection of renal epithelial cells [1,2]. In addition to its role in glomerular pathogenesis [3,4], recent studies have demonstrated that HIV-1 *vpr* (viral protein r) induces dysregulation of cytokinesis and apoptosis in renal

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tubular epithelial cells (RTEC) *in vitro*, which correlate with RTEC hypertrophy and apoptosis in HIVAN biopsy specimens [5].

Vpr induces apoptosis in many cell types, however, the mechanisms by which it induces cell death vary and are often cell-type specific (reviewed in [6]), with both caspase-8 and caspase-9 mediated mechanisms having been implicated [7–9]. In some non-renal cell types, Vpr induces activation of cellular DNA damage responses leading to G2/M arrest and Bax-dependent apoptosis [10–14]; in others, Vpr directly injures mitochondria [15].

Mitogen activated protein kinases (MAPK) are important mediators of HIV-induced renal pathogenesis [15,16]. While ERK activation is classically associated with promoting cellular proliferation [17], it can also induce cell cycle arrest and/or apoptosis, particularly in the presence of cellular stressors such as DNA damage [18,19]. Sustained activation of ERK can induce apoptosis in neurons via a caspase-8 dependent pathway that is independent of Fas or FADD [18,19].

Here, we report that transduction of a human RTEC cell line (HK2) with Vpr induced mitochondrial damage and apoptosis that was dependent upon activation of caspase-8 and caspase-9. Knock-down of BID and/or Bax expression using lentiviral shRNA vectors suppressed Vpr-induced apoptosis. We then demonstrate that Vpr-induced apoptosis was associated with prolonged ERK activation, and that inhibition of ERK activation with the specific MEK inhibitor U0126 reduced Vpr-induced apoptosis, caspase-8 and -9 activation, and BID cleavage to tBID. Importantly, activated ERK was detected in RTEC in HIVAN patient biopsy specimens, strongly suggesting that these findings are relevant to HIVAN pathogenesis *in vivo*.

Methods

Cell culture

HEK 293T cells were cultured in DMEM with 10% FBS, penicillin/streptomycin and L-glutamine. Adult human proximal tubule epithelial cells (HK-2) (described in [20]) were cultured in serum-free keratinocyte media with growth supplement (GIBCO).

Generation of pseudotyped virus, transfection and infection

Lentiviral vectors were pseudotyped with vesicular stomatitis virus glycoprotein (VSV-g) and expressed *vpr* (VSV-HR-vpr-IRES-EGFP, abbreviated HR-Vpr), empty vector (VSV-HR-IRES-EGFP, abbreviated HR). HK2 cells were transduced according to previously published methods [2]. Lentiviral shRNA vectors included VIRHD/E/siLuc [21] (abbreviated shLuc, gift of Dr. Luca Gusella, Mt. Sinai School of Medicine), shBID and shBax (OpenBiosystems, Cat# RHS4430-99157271 and Cat# RHS4430-99140545).

For mitochondrial assays using JC-1, we used VSV-pseudotyped lentiviral vectors encoding hexahistidine (His) and hemagglutinin (HA)-tagged Vpr (pHR-His-HA-VPR-IRES-GFP, abbreviated pHA-Vpr) and His-HA-tagged pHA-Vpr(Q65R) (inactive mutant) in which the start codon for GFP was mutated and a premature stop codon was added to GFP using the QuickChange site directed mutagenesis kit (Stratagene). pHA-Vpr and pHA-Vpr(Q65R) were gifts of Vincente Planelles, University of Utah [22].

Caspase and ERK inhibition

Caspase-8 and -9 were inhibited using Z-IETD-FMK and Z-LEHD-FMK, respectively (R&D Systems) at 60μM. ERK activation was inhibited by incubation with U0126 (Cell Signaling) at 10μM.

Cell cycle analysis

Cells were collected by trypsinization, fixed in 50% ethanol, stained with propidium iodide (Invitrogen) and treated with 0.01ug/ul percent RNase A (Puregement, Gentra Systems). Cell cycle parameters were measured using the FACScaliber flow cytometer equipped with CellQuest Software in the Mount Sinai Flow Cytometry Shared Research Facility and analyzed using FlowJo software version 6.4.7. Cell fragments were excluded from analysis on the forward scatter/side scatter plot. Gates from control transductions were applied to other experimental groups. Each experiment was conducted at least three separate times and representative plots are provided.

Real-time PCR

RNA was extracted using the RNeasy Mini Kit (Qiagen). cDNA was made using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). qPCR was performed using QuantiTect SYBR Green PCR Kit (Qiagen) at the Mount Sinai Quantitative PCR Shared Research Facility according to the protocol: 95C 15min, 40 cycles of 94C 15sec, 58C 30sec, 72C 30sec. Primers were to Bax (sense: 5'-TTTGCTTCAGGGTTTCATCC, anti-sense: 5'-CAGTTGAAGTTGCCGTCAGA) with cyclophilin as a housekeeping gene (sense: 5'-AGGGTGGTGACTTTACACGC, antisense: 5'-ATCCAGCCATTTCAGTCTTGG). The threshold cycle (Ct) for cyclophilin was subtracted from the Ct for each transcript (ΔCt). The fold-change difference in expression for each gene was defined as $2^{(\Delta Ct_{\text{experiment}} - \Delta Ct_{\text{control}})}$. Unpaired t-tests with a 2-sided P value of <0.05 were used to compare the expression of each gene.

Western blots

Whole-cell extracts were run on a 4–20% Tris-glycine gel (Invitrogen) and transferred to PVDF membranes (Millipore). Primary antibodies were to beta actin (Santa Cruz), PARP-1, phospho-ERK1,2, total ERK1,2, BID, JNK, and phospho-JNK (Cell Signaling). Secondary antibodies were horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse (Jackson ImmunoResearch), and detection was by chemiluminescence (ECL; Amersham Biosciences).

Mitochondrial assays

To assess the integrity of mitochondrial membrane polarization (MMP), cells were incubated with Mitotracker RedCMXRos (Invitrogen) for 30min, trypsinized and analyzed by flow cytometry. MMP was also assessed by incubating cells with 5μg/mL of JC-1 (Anaspec) prior to flow cytometric analysis of red and green fluorescence.

Caspase activation assays

Activation of Caspase 3/7, 8 and 9 was measured using Caspase Glo Assay kits (Promega). Luciferase activity was measured using a Wallac 1420 Manager Plate Read (Perkin Elmer). CaspaseGlo data is represented as the average of five experiments conducted in parallel.

Renal biopsy specimens and immunohistochemistry

Human biopsy material was collected previously from HIV infected patients after informed consent under a protocol approved by the Mount Sinai Institutional Review Board. Kidney tissue was obtained from 8 patients with HIVAN. Kidney biopsies from 6 HIV-seronegative patients with minimal change disease (MCD) were collected at Columbia University under a protocol approved by its Institutional Review Board. Immunostaining of paraffin-embedded renal biopsy specimens for phospho-ERK1,2 was conducted as described previously [15].

Statistical analysis

Flow cytometry data were analyzed using a chi-square test. Statistical significance was defined as $P < 0.05$. qPCR and caspase activation data were analyzed using the Student's t-test for populations with unequal variance and statistical significance was defined as 2-sided $P < 0.05$.

Results

Vpr induces apoptosis in human kidney cells

Previous studies in our lab demonstrated that Vpr induces apoptosis in human renal tubular epithelial cells (HK2) as determined by annexin V/propidium iodide (PI) staining [5]. We examined cellular DNA content by staining HK2 with PI after HR-Vpr transduction to characterize cell cycle regulation and determine the relative proportion of cells that became hypodiploid (an indicator of apoptosis) compared to cells transduced with empty HR control vector. Five days after transduction, analysis of propidium stained cells revealed that Vpr induced a marked increase in the proportion of hypodiploid cells (33.8%) compared to control-transduced cells (6.3%) (Fig. 1A). Furthermore, Vpr-transduction led to a dramatic increase in the percentage of hyperploid ($>4N$) cells (32.2%) as compared to control (5.6%, $p < 0.0005$).

In non-renal cell types, Vpr-induced apoptosis is accompanied by caspase activation and PARP-1 cleavage [10]. We therefore studied whether Vpr induces activation of caspase 3/7 and PARP-1 cleavage. HR-Vpr- and HR control-transduced HK2 cells were assayed for caspase 3/7 activity five days post transduction. Caspase 3/7 activity was 60 percent greater at five days post transduction with Vpr than control (Fig. 1B, $p < 0.001$). Whole cell lysates of HK2 cells were harvested three and five days after transduction with Vpr or control vector and PARP-1 cleavage was analyzed by western blotting. Increased levels of the PARP-1 89 and 24kD cleavage fragments were detected in HR-Vpr-transduced cells three days post transduction and levels increased further at five days, relative to HR control-transduced cells (Fig. 1C). Therefore, Vpr transduction of HK2 cells leads to apoptosis as measured by propidium staining, caspase 3/7 activity and PARP-1 cleavage.

Vpr transduction reduces mitochondrial oxidative capacity in human kidney cells

To further characterize whether Vpr expression induces mitochondrial injury in HK2 cells, Vpr- and control-transduced cells were assayed by flow cytometry five days post transduction after incubation with Mitotracker RedCMXRos, a fluorescent dye whose accumulation in mitochondria is dependent upon intact mitochondrial membrane polarization (MMP). Control-transduced cells displayed a single peak, indicating that cells were homogenous with respect to their content of oxidizing mitochondria (Fig. 1D). However, the HR-Vpr-transduced cells exhibited two peaks, one consisting of HK2 cells with less Mitotracker accumulation than control and another population with levels of Mitotracker accumulation that were similar to or higher than control (Fig. 1D). When the same cells were subsequently stained with propidium iodide, the percentage of hypodiploid Vpr-transduced cells was similar to the percentage of cells with reduced Mitotracker RedCMXRos staining (Fig. 1D), suggesting that the cells with reduced content of normal mitochondria were apoptotic. While these data do not explain why some Vpr-transduced cells had increased Mitotracker RedCMXRos staining, since Vpr is known to induce cellular hypertrophy in HK2 cells [5], it is likely that this population consists of cells with a higher number of mitochondria that have not yet undergone apoptosis.

To confirm whether reduced Mitotracker RedCMXRos staining indicated Vpr-induced loss of MMP, we transduced cells with lentiviral vectors expressing either wild-type Vpr (HA-

Vpr- Δ GFP) or mutated Vpr (HA-Vpr(Q65R)- Δ GFP) which is inactive due to mutation of its DCAF1 binding site [22]. GFP was mutated in these vectors to allow use of JC-1, a mitochondrial dye which shifts from red to green fluorescence when there is loss of MMP. Flow cytometric analysis of JC-1-stained HK-2 cells five days after transduction revealed that HA-Vpr- Δ GFP induced loss of MMP in 33.3% of cells, whereas MMP was reduced in only 6% of cells transduced with HA-Vpr(Q65R)- Δ GFP (Fig 1E).

Vpr induces caspase-8 and caspase-9 activity in human kidney cells

Vpr has been shown to induce apoptosis by intrinsic and extrinsic pathways [7]. We therefore studied activation of caspase-8 and caspase-9 in Vpr-transduced HK2 cells. Five days after transduction with HR-Vpr or HR control vector, we found that caspases-8 and 9 were both activated by Vpr as compared to control (Fig. 2A and B).

To determine whether activation of caspase-8 and 9 contributed to Vpr-induced apoptosis, the caspases were inhibited using Z-IETD-FMK and Z-LEHD-FMK, respectively. Inhibition of caspase-8 reduced apoptosis from 35% to 15.5% in HR-Vpr-transduced cells ($p < 0.0001$). Inhibition of caspase-9 reduced Vpr-induced apoptosis from 35% to 19.2% ($p < 0.0001$). Co-incubation with caspase-8 and -9 inhibitors decreased apoptosis from 35% to 12.2% ($p < 0.0001$, Fig. 2C). These results confirmed that caspase-8 and 9 activation are important in Vpr-induced apoptosis in HK2 cells, however, their effects were not additive. There was no evidence that apoptosis was preferentially prevented in hyperploid or G2/M phase cells. Caspase inhibition also reduced death in cells transduced with the HR control vector.

Bax and BID mediate Vpr-induced cell death

Bax mediates Vpr-induced apoptosis in HeLa cells [11]. To investigate the role of Bax in Vpr-induced apoptosis in HK2 cells, we knocked down Bax expression using a lentiviral shRNA vector (shBax) prior to transduction with HR-Vpr or HR control vector. Transduction of HK2 cells with shBax reduced Bax mRNA expression by 89% in control-transduced cells and 83% in HR-Vpr-transduced cells compared to control shRNA (shLuc) (Fig. 3A). shBax transduction decreased the percentage of cells undergoing Vpr-induced apoptosis from 49% to 11.7% five days after transduction (Fig. 3B, $p < 0.0005$). Compared to cells transduced with HR-Vpr and shLuc, in cells transduced with Vpr and shBax, the decrease in cell death was associated with a concomitant increase in cells in G0/G1 (20.3% to 37.6% $p < 0.0005$) and G2/M (14.3% to 30.5%, $p < 0.0005$). Knock-down of Bax also reduced death in control-transduced cells with concomitant small increases in each phase of the cell cycle.

In caspase-8 mediated apoptosis, caspase-8 cleaves BID to tBID and tBID subsequently promotes the proapoptotic activity of Bax. We therefore studied whether Vpr induces BID cleavage in HK2 cells. Whole cell extracts from Vpr- and control-transduced cells were collected three and five days post transduction and analyzed by western blotting using anti-BID antibody. tBID became detectable at three days and levels increased at five days post transduction with Vpr (Fig. 3C).

To determine whether BID is necessary for Vpr-induced apoptosis, BID expression was knocked down using lentiviral shRNA (shBID). Transduction of HK2 cells with shBID reduced BID protein levels (Fig. 3D). HK2 cells were transduced with shBID two days before transduction with HR-Vpr. Five days after transduction with HR-Vpr cells were stained with propidium and analyzed by flow cytometry. shBID decreased the percentage of cells that became hypoploid after Vpr transduction from 35.2% to 9.8% ($p < 0.0005$). This decrease in cell death was associated with a corresponding increase in hyperploid cells from

16.6% to 34% (Fig. 3E, $p < 0.0005$) compared to cells transduced with shLuc control shRNA vector. Together, these experiments implicate BID in Vpr-induced cell death in HK2 cells.

To determine whether the effects of BID and Bax are additive, we simultaneously transduced HK2 cells with shBID and shBax three days before transduction with HR-Vpr and analyzed propidium stained cells five days later. Co-transduction with shBID and shBax reduced the Vpr-induced hypodiploid fraction from 21.3% to 6.7% ($p < 0.0005$), and increased the hyperploid fraction from 14.4% to 27.6% (Fig. 3F, $p < 0.0005$). Transduction of HK2 cells with shBID, shBax, or both vectors suppressed Vpr-induced cell death to levels one-third those of HR-Vpr with shLuc control (Fig. 3G). There was no evidence that the effects of shBID and shBax on cell death were additive.

Caspase 8 activation by Vpr is due to prolonged ERK activation

MAPK pathways have been implicated as mediators of HIV renal pathogenesis [15] and prolonged ERK activation can induce caspase 8 dependent apoptosis [18]. JNK has a role in Vpr-induced apoptosis in monocytic cells [16]. We therefore studied whether Vpr induces activation of JNK and/or ERK in HK2 cells. Western blotting of total cell lysate collected after transduction with HR-Vpr and HR-control revealed no evidence of JNK activation (Fig. 4A), but there was a striking increase in ERK phosphorylation one and five days after Vpr transduction relative to control (Fig. 4B).

To determine whether ERK activation was necessary for activation of caspase-8, MEK1 and -2 (activators of ERK) were inhibited using the specific inhibitor U0126. Inhibition of ERK phosphorylation was confirmed by western blotting of protein lysate from HR-Vpr and HR-control transduced cells after treatment with U0126 (Fig. 4C). Incubation with U0126 for the third and fourth days or all five days after transduction with Vpr inhibited cleavage of BID to tBID (Fig. 4C). These data demonstrate that prolonged inhibition of ERK activation diminished BID cleavage in Vpr transduced cells.

We then studied whether treating Vpr-transduced HK2 cells with U0126 could prevent Vpr-induced apoptosis. Cells were transduced with HR-Vpr and subsequently incubated with U0126 for either all five days prior to analysis or for the final two days prior to analysis. Flow cytometric analysis of PI-stained cells revealed that MEK inhibition with U0126 for the final 2 days prior to analysis reduced the hypoploid fraction in Vpr-transduced cells from 28.1% to 22.1% (Fig. 4C, $p < 0.0005$). Incubation of U0126 for all five days after HR-Vpr transduction further decreased accumulation of hypodiploid cells to 16.1% (Fig. 4D, $p < 0.0005$).

MEK inhibition with U0126 also had pronounced effects upon cell cycle dynamics in Vpr-transduced cells. Incubation with U0126 for the final two days prior to analysis eliminated the >8N peak (Fig. 4D(v)) and incubation with U0126 for all five days following Vpr transduction decreased the percentage of hyperploid cells from 38.2% to 12.8% (Fig. 4D(vi), $p < 0.0005$) and induced a shift of hyperploid cells to the G2/M peak. Interestingly, MEK inhibition with U0126 had no effect upon the percentage of G0/G1 cells, suggesting that U0126 did not exert its effects by preventing cells from transitioning from G0/G1 to S phase but prevented Vpr-transduced cells from progressing from G2 through mitosis.

Since we had found that MEK inhibition with U0126 decreased Vpr-induced cell death, we studied the effect of U0126 upon caspase-8 and -9 activation five days after transduction with Vpr. Incubation with U0126 reduced Vpr induced activation of caspases-8 and 9 to levels found in control transduced cells (Fig. 4E and F, $p < 0.0001$). These results demonstrate that ERK activation is required for Vpr-induced caspase-dependent apoptosis in HK2 cells.

ERK is activated in RTEC in patients with HIVAN

To determine whether ERK is activated in RTEC in HIVAN specimens *in vivo*, we performed immunohistochemistry to detect phospho-ERK in HIVAN biopsy samples and biopsy samples from patients with minimal change disease (MCD), a form of proteinuric renal disease in which, in contrast to HIVAN, patients rarely develop tubulointerstitial injury or progressive renal failure. We detected phospho-ERK in the RTEC in HIVAN biopsies but not MCD (Fig. 5A). These results confirm that the ERK activation that we observed in Vpr transduced HK2 cells *in vitro* recapitulates findings in patients with HIVAN *in vivo*.

Discussion

In developed countries, widespread availability of antiretroviral therapy has led to dramatic reductions in morbidity and mortality due to opportunistic infections. Renal disease has become the fourth leading diagnosis contributing to death among Americans with HIV/AIDS [23] and HIVAN is the leading cause of ESRD in HIV seropositive patients [24]. The mechanisms by which expression of viral genes in RTEC leads to tubulointerstitial disease, characterized by dysregulated RTEC apoptosis and proliferation, remain poorly characterized. Previous studies have described HIV-1 Vpr as a principal HIV gene responsible for inducing RTEC hypertrophy and apoptosis [5]. Here, we define the mechanisms of Vpr-induced apoptosis in human RTEC.

Vpr can induce apoptosis in many cell types but the mechanisms by which it does so differ depending upon cell type and context [6]. Most previous research has focused upon the ability of Vpr to injure mitochondria either by direct binding to the adenine nucleotide transporter on the outer mitochondrial membrane, or via upregulation of Bax with resultant loss of mitochondrial membrane polarization and release of proapoptotic factors, culminating in caspase-9 activation and apoptosis [25,26].

When we found that Vpr induced apoptosis in RTEC, we initially hypothesized that the mechanism of apoptosis in these cells was via Bax-dependent mitochondrial injury, leading to activation of caspase-9 as reported in HeLa cells by Andersen *et al* [11]. As we predicted, Vpr induced caspase-9 activation and we also found that Bax expression was important for Vpr-induced apoptosis. To our surprise, however, we also found that Vpr activated caspase-8 and that inhibition of caspase-8 was as effective in preventing cell death as caspase-9 inhibition. Moreover, caspase-9 inhibition did not reduce cell death more than caspase-8 inhibition alone, suggesting that caspase-9 might be acting downstream of caspase-8. While our data demonstrating a role for caspase-8 in Vpr-induced apoptosis were unexpected, Vpr has been reported to induce caspase-8 mediated apoptosis in human neurons and CD8 cells [27,28].

Since active caspase-8 can cleave BID to tBID, which can then facilitate Bax-induced mitochondrial injury, we tested whether suppression of BID expression could prevent Vpr-induced apoptosis. Transduction of HK2 cells with shRNA directed against BID prevented apoptosis as effectively as Bax shRNA, and co-suppression of Bax and BID did not further reduce apoptosis, suggesting that BID is upstream of Bax-induced apoptosis in Vpr-transduced HK2 cells. Activation of caspase-8 is classically associated with receptor mediated (extrinsic) apoptosis [29,30]. In preliminary studies, we were unable to inhibit Vpr-induced apoptosis using blocking antibodies directed against Fas, the TNF- α receptor, or the TRAIL receptor (data not shown). Conaldi *et al.* also reported that HIV-1 induced apoptosis in human RTEC is not prevented by Fas blocking antibodies [31].

Previous studies have described the role of the HIV-1 Nef protein in ERK activation in podocytes. This study is the first to focus on ERK activation in RTEC in HIVAN and is also

the first to demonstrate a role for Vpr-induced activation of ERK as a stimulus for apoptosis. While other studies have reported a role for JNK activation in Vpr-induced apoptosis in non-renal cell types [16], we found no evidence of JNK activation in Vpr-transduced HK2 cells. As expression of HIV-1 Nef can also activate ERK signaling in podocytes, the combined effects of Nef and Vpr on ERK signaling in RTEC require future study.

Our studies demonstrate that ERK activation is necessary for caspase-8 activation in Vpr-transduced RTEC and that inhibition of ERK kinases MEK1 and MEK2 with the highly specific inhibitor U0126 decreases caspase-8 activation and cleavage of BID to tBID. We therefore propose a model where prolonged ERK activation results in caspase-8 activation, leading to cleavage of BID to tBID, which facilitates Bax-induced mitochondrial permeabilization, caspase-9 activation, and cell death. This model is depicted in Fig. 5B, and is similar to the pathway described for neuronal apoptosis in response to oxidative stress [32] and in human embryonic kidney (HEK) 293 cells subjected to prolonged ERK activation [18]. The mechanism by which Vpr induces ERK activation in RTEC is unknown.

Though ERK activation is commonly associated with promotion of cellular proliferation, ERK signaling can also induce cell cycle arrest in several cell types [33], [34]. Furthermore, prolonged ERK activation facilitates glutamate and oxidative stress-induced toxicity in neuronal cell lines and primary culture [19], and apoptosis in 293T cells [18]. ERK has also been implicated *in vivo* in a neuronal ischemic injury model [35]. Importantly, ERK activation occurs in response to various DNA damage stimuli [36], and mediates cell-cycle arrest and apoptosis after certain forms of DNA damage, effects that were inhibited by U0126 [37]. Recent studies have demonstrated that strong ERK activation causes cell cycle arrest via phosphorylation of Cdc25A [38]. Interestingly, the phosphorylation of Cdc25A induced by ERK activation is similar to Cdc25A phosphorylation by Chk1, which occurs in response to activation of DNA damage response pathways. Since Vpr has been shown to induce ATR-dependent Chk1 activation in some cell types [39], further research is needed to determine whether Vpr-induces ATR/Chk1 dependent cell cycle arrest in human RTEC. Moreover, Vpr has been recently demonstrated to activate DNA damage response in RTEC as detected by accumulation of γ -H2AX foci [40]. Since some DNA damaging agents including cisplatin, can induce ERK-dependent RTEC apoptosis *in vitro* and *in vivo* [41], future studies should further characterize Vpr-induced DNA damage response in RTEC and the mechanism by which it may lead to ERK activation.

In conclusion, these studies have identified a novel mechanism for Vpr induced apoptosis in human RTEC. We have demonstrated that Vpr induces ERK dependent activation of caspase-8, leading to cleavage of BID to tBID, and ultimately, caspase-9 activation and apoptosis. Moreover, ERK is activated in RTEC in biopsy samples from patients with HIVAN, a disease characterized by RTEC injury and apoptosis, but not in minimal change disease, a renal disease characterized by proteinuria without tubular injury or progressive renal failure.

Acknowledgments

Support: This research was funded by the NIH/NIDDK (grant numbers R01 DK078510 and P01 DK56492) and the Howard Hughes Medical Institute Medical Student Research Fellowship.

Alexandra Snyder conducted the experiments, and in conjunction with Michael J. Ross, wrote the text and created the figures. Jeremy S Leventhal performed studies analyzing BID knock-down at the protein level. Paul Rosenstiel assisted in analysis of ERK activation and Kevin Barley performed studies of JNK activation. Zygimantas C Alsauskas performed studies on effects of Vpr on MMP. Pengfei Gong, and Justin JK Chan assisted in cell culture and generation of lentiviral vectors. John C He performed immunohistochemistry of patient biopsy specimens. Mary E Klotman, Michael J Ross* and Paul E Klotman provided guidance, supervision, and funding for this project.

This work was generously supported by NIH grants R01DK078510 (Ross) and P01DK056492 (Klotman) and an HHMI Medical Student Research Fellowship.

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Figure 1.

Vpr induces apoptosis and diminishes mitochondrial membrane polarization (MMP) in human RTEC. A) Transduction of HK2 cells with HR-Vpr increased the hypodiploid fraction from 6.3% to 33.8% of cells at 5 days post infection (dpi) relative to cells transduced with empty control lentiviral vector (HR) as determined by propidium iodide staining ($p < 0.0005$). B) Caspase 3/7 activity increased significantly at 5 days post infection in HR-Vpr transduced cells relative to control-transduced cells ($*p < 0.001$). C) Transduction with HR-Vpr increased PARP-1 cleavage at 3 dpi and 5 dpi relative to control transduction. D) (i) 13.7% of control-transduced cells developed low MMP as compared to 44.5% of Vpr-transduced cells ($p < 0.0005$). (ii) Overlay of flow cytometry data from HR-Vpr-transduced cells (arrow) and HR control-transduced cells (arrowhead) stained for actively oxidizing mitochondria with Mitotracker RedCMXRos. (iii) 8.5% of control-transduced cells became hypodiploid as compared to 37.0% of HR-Vpr-transduced cells ($p < 0.0005$). E) Transduction with HA-Vpr induced mitochondrial membrane depolarization in 33.3% of cells after 5 days whereas transduction with HA-Vpr-Q65R induced mitochondrial membrane depolarization in only 6% of cells as detected by staining with JC-1 ($p < 0.0001$).

Figure 2.

Vpr activates caspase-8 and 9. HR-Vpr transduction of HK2 cells results in increased caspase-8 (A) and caspase-9 (B) activation relative to control-transduced cells. * $p < 0.0001$

C) Inhibition of caspase-8 by Z-IETD-FMK reduced Vpr-induced apoptosis from 35% to 15.5% of HR-Vpr-transduced cells. Inhibition of caspase-9 with Z-LEHD-FMK reduced Vpr-induced apoptosis from 35% to 19.2% of HR-Vpr-transduced cells. Combined inhibition of caspase-8 and -9 decreased apoptosis to 12.2% of HR-Vpr-transduced cells. * $p < 0.0001$ for increase in percentage of hypodiploid cells in HR-Vpr versus HR control transduction and for decrease in hypodiploid cells in HR-Vpr-transduced cells after incubation with caspase inhibitors versus no inhibitor.

A **B**

Figure 3.

shRNA to Bax or BID decreases Vpr-induced cell death in a non-additive manner. A) Transduction with shBax 2 days prior to HR or HR-Vpr reduced Bax RNA levels by 89% in HR-transduced cells and by 83% in HR-Vpr-transduced cells compared to co-transduction with shLuc. * $p < 0.0001$ B) shBax decreased cell death from 49% to 11.7% of HR-Vpr-transduced cells at 5 dpi, with a corresponding increase in the percentage of G₀/G₁ and G₂/M cells. * $p < 0.0005$ decrease in hypodiploidy in (iv) compared to (iii). C) tBid was detected 3 and 5 dpi following HR-Vpr-transduction of HK2 cells infection. D) Transduction of HK2 cells with shBID reduced total BID protein levels compared to shLuc control at 5dpi. E) Transduction with shBID 2 days prior to HR or HR-Vpr decreased Vpr-induced cell death from 35.3% to 9.8% of cells with a corresponding increase in the hyperploid population from 16.6% to 34.0%. * $p < 0.0005$ for change in hypodiploid and hyperploid populations in (iii) compared to (i) and for (iv) compared to (iii). (F) Transduction of HK2 cells with shBID

and shBax 2 days prior to transduction with HR-Vpr reduced the percentage of hypodiploid cells from 21.3% to 6.7% ($p<0.0005$) with a corresponding increase in the hyperdiploid population from 14.4% to 27.6% ($p<0.0005$). G) Inhibition of Bax, BID, or both reduced the hypodiploid fraction to approximately one third of levels induced by transduction with HR-Vpr and shLuc. * $p<0.0005$

Figure 4.

MEK1,2 blockade inhibits Vpr-induced ERK activation, BID cleavage, cell death, and caspase-8 and caspase-9 activation A) Transduction with HR-Vpr did not increase levels of phospho-JNK (p-JNK) at 2 or 5 days post infection (dpi) compared to HR control. Lysate from MDA-MB-231 cells stimulated with TAT-EGFR was used as positive control. B) Transduction with HR-Vpr increased ERK phosphorylation, especially at 1 and 5 dpi. C) Incubation with U0126 for 2 or 5 days inhibited Vpr-induced ERK activation and cleavage of BID to tBID. D) Incubation with U0126 decreased the proportion of hyperploid cells, especially >8N cells (vertical arrows), increased the proportion of G2/M cells (arrowheads), and decreased cell death. The effects of U0126 were more pronounced with incubation for 5 days (vi) than for 2 days (v). * $p < 0.0005$ for changes in hypodiploid, G2/M and hyperploid fractions in (iv) compared to (i) and (v) and (vi) compared to (iv). Incubation of HK2 cells

with the MEK1,2 inhibitor U0126 reduced caspase-8 activation (E) and caspase-9 activation (F) following HR-Vpr transduction to levels similar to those found in cells transduced with the HR control vector. * $p < 0.0001$

Figure 5.

A) ERK is activated in RTEC in HIVAN biopsy samples. Phospho-ERK was detected in RTEC in HIVAN biopsies samples but not in biopsies from patients with minimal change disease. B) Schema depicting hypothesized pathway for Vpr-induced apoptosis in RTEC.