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HIV-1 STIMULATES NUCLEAR ENTRY OF AMYLOID BETA VIA DYNAMIN DEPENDENT EEA1 AND TGF- β /SMAD SIGNALING

Ibolya E. András and Michal Toborek

Department of Biochemistry and Molecular Biology, University of Miami School of Medicine, Miami, FL

Abstract

Clinical evidence indicates increased amyloid deposition in HIV-1-infected brains, which contributes to neurocognitive dysfunction in infected patients. Here we show that HIV-1 exposure stimulates amyloid beta (A β) nuclear entry in human brain endothelial cells (HBMEC), the main component of the blood-brain barrier (BBB). Treatment with HIV-1 and/or A β resulted in concurrent increase in early endosomal antigen-1 (EEA1), Smad, and phosphorylated Smad (pSmad) in nuclear fraction of HBMEC. A series of inhibition and silencing studies indicated that Smad and EEA1 closely interact by influencing their own nuclear entry; the effect that was attenuated by dynasore, a blocker of GTP-ase activity of dynamin. Importantly, inhibition of dynamin, EEA1, or TGF- β /Smad effectively attenuated HIV-1-induced A β accumulation in the nuclei of HBMEC. The present study indicates that nuclear uptake of A β involves the dynamin-dependent EEA1 and TGF- β /Smad signaling pathways. These results identify potential novel targets to protect against HIV-1-associated dysregulation of amyloid processes at the BBB level.

Keywords

HIV-1; blood-brain barrier; amyloid beta

INTRODUCTION

Combination antiretroviral therapy (cART) changed HIV-1 infection from acute to chronic disease, dramatically increasing the number of infected individuals who are 50 or older [1]. Neurocognitive impairments and dementia associated with HIV-1 infection are more prevalent in these patients [2, 3]. In addition, abundant amyloid beta (A β) deposition is a hallmark of HIV-1 infected brains [3, 4]. Although amyloid pathology is different in HIV-1 and Alzheimer's disease (AD) [5], HIV-associated neurocognitive disorders (HAND) in the aged population correlate with the early beta-amyloidosis [1, 6]. Several mechanisms have

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Correspondence: Correspondence and requests for materials should be addressed to Michal Toborek and/or Ibolya E. András: 1011 NW 15th Street; Gautier Building, Room 528; Miami, FL 33136-1019, USA; Phone number: 305-243-0230. mtoborek@med.miami.edu and/or iandras@med.miami.

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been proposed to explain elevated levels of amyloid in HIV-1 brains. For example, it was demonstrated that HIV-1 can increase brain A β levels by increasing amyloid precursor protein [7], stimulating A β production [8, 9], or inhibiting its degradation [10]. In addition, cART was shown to contribute to elevated brain amyloid by decreasing microglial A β phagocytosis [11].

Important observations from our laboratories indicate that exposure to HIV-1 results in enhanced A β levels in human brain endothelial cells (HBMEC) and increased its transendothelial transfer [12, 13], suggesting that brain endothelial cells contribute to accumulation of A β in HIV-1-infected brains. These observations are consistent with the evidence that brain vascular dysfunction and the blood brain barrier (BBB) are playing important roles in amyloid pathology observed in AD [14]. Importantly, amyloid and HIV-1 potentiate their cerebrovascular toxicity by disrupting the integrity of the brain endothelium and stimulation of inflammatory responses [15, 16].

The present study is based on a novel observation that A β not only accumulates in the cytoplasm of HIV-1 exposed cells but also enters the nuclei. We indicate that this process involves a complex interplay between dynamin-dependent stimulation of the early endosomal antigen-1 (EEA1) and TGF- β /Smad signaling pathway.

MATERIALS AND METHODS

Cell cultures, HIV-1 infection, and treatment factors

Human brain microvascular endothelial cells (HBMEC) used in the present study represent a stable, well characterized, and differentiated human brain endothelial cell line [17]. The cells were cultured on collagen type I (BD Biosciences Pharmingen, San Jose, CA) coated dishes in EBM-2 medium (Clonetics, East Rutherford, NJ) supplemented with VEGF, IGF-1, EGF, basic FGF, hydrocortisone, ascorbate, gentamycin, and 0.5% fetal bovine serum (FBS) [17].

HIV-1 stock was generated using human embryonic kidney (HEK) 293T cells (American Type Culture Collection, Manassas, VA) transfected with pYK-JRCSF plasmid containing full-length proviral DNA as described earlier [18]. Throughout the study, HBMEC were exposed to HIV-1 particles for 24 h at the p24 level of 30 ng/ml. This type of exposure was shown to be highly effective in stimulation of cellular A β levels [12]. Plasma levels of p24 were reported to reach ~5–6 log₁₀ fg p24/ml in non-treated HIV-1-infected patients, which correspond to ~0.1–1 ng p24/ml [19]. HIV-1 infected monocytes from patients were shown to productively release live viruses in vitro [20] suggesting that HIV-1 may also directly affect the vascular endothelium and produce local p24 values that are higher than the average plasma levels.

A β (1–40) and A β (1–40) HiLyte were purchased from Anaspec (San Jose, CA). A β (1–40) was dissolved in sterile ultra-pure water (ELGA Purelab Classic, Lowell, MA). Freshly solubilized A β solutions without pre-aggregation were used for experiments. Such a form of A β was demonstrated to induce proinflammatory reactions in isolated rat brain microvessels [21]. A β (1–40) HiLyte was dissolved first in a basic buffer (0.1 M NH₄OH) than diluted further in PBS as suggested by the manufacturer. Cells were treated with A β (1–40) or A β

(1–40) HiLyte at the concentration of 1 μ M for 10 min or 1 h in complete medium. Similar treatment was shown to result in an uptake by the BBB [22] and stimulation of pro-inflammatory responses [15].

Inhibitors SB431542 (Tocris Bioscience, Bristol, UK), dynasore hydrate (Sigma Aldrich, St. Louis, MO) were dissolved in DMSO and further diluted in the cell culture media. Cells were pretreated with 10 μ M SB431542 for 2 h or 50 μ M dynasore for 30 min prior to A β exposure.

Cell fractionation

Cell fractionation was performed as described previously [23, 24] with minor modifications. Briefly, cells were solubilized in 1 ml of ice-cold MNE buffer (25 mM MES [morpholineethanesulfonic acid, pH 6.5]; 150 mM NaCl; 5 mM EDTA) with 1 mM sodium orthovanadate, protease inhibitors and 1% Triton X-100 on ice. The cell lysate was then homogenized with a loose-fitting Dounce homogenizer. The homogenate was centrifuged at 15000 $\times$ g for 15 min, 4 $^{\circ}$ C, and mixed with 1 ml of 90% sucrose in MNE buffer. The resulting 45% sucrose cell lysate was overlaid with 5 ml of 35% sucrose and 5 ml of 5% sucrose (w/v in MNE buffer), followed by centrifugation for 20 h at 35,000 rpm at 4 $^{\circ}$ C using a Beckman SW41 rotor. Twelve 1 ml fractions were collected from top to bottom and aliquots of each fraction were subjected to SDS-PAGE and immunoblotting to assess EEA1 and Smad2.

Immunoblotting

Homogenates of cultured HBMEC were prepared in lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 0.1 mg/ml phenylmethylsulfonyl fluoride, 0.5% Nonidet P40, 1 mM EDTA, 2.5 μ g/ml leupeptin, 1 μ g/ml pepstatin A, and 1 μ g/ml aprotinin). After centrifugation at 15,000 $\times$ g for 15 min, supernatants were collected and protein concentrations were determined using the Bradford protein assay. For isolation of cytoplasmic and nuclear fractions, NucBuster nuclear protein extraction kit (Invitrogen) was used following the manufacturer's protocol. Samples (8 μ g protein/lane for both cytoplasmic and nuclear fractions) were separated on SDS-polyacrylamide gel, blotted onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA), and incubated with the respective antibodies. Mouse monoclonal anti-EEA1 and mouse monoclonal anti-Nucleoporin p62 antibodies were from BD Transduction Laboratories (San Diego, CA). Rabbit monoclonal phospho-Smad2 and Smad2 antibodies were from Cell Signaling Technology (Boston, MA). Rabbit polyclonal anti-Rab5A and anti-GAPDH-HRP antibodies were from Santa Cruz Biotechnology. Secondary antibodies were purchased from Santa Cruz Biotechnology and Invitrogen (Carlsbad, CA). For visualization of detected proteins, immunoblots were analyzed using an ECL Western blot detection kit (Amersham Biosciences, Piscataway, NJ).

Confocal immunofluorescence microscopy

HIV-treated HBMEC cultured on collagen I coated chambered glass slides (BD Biosciences, San Jose, CA) were fixed with ethanol for 30 min at 4 $^{\circ}$ C. After washing with PBS and blocking with 3% bovine serum albumin in PBS for 30 min, samples were incubated

overnight at 4°C with the primary antibody. Then, the excess of primary antibody was removed, slides were washed with PBS, and incubated with Alexa Fluor⁵⁶⁸-conjugated secondary antibody for 2 h at room temperature. After washing with PBS, slides were mounted using ProLong Gold Antifade reagent containing 4',6-diamidino-2-phenylindole (DAPI, Invitrogen, Carlsbad, CA) to visualize the nuclei. Specimens were covered with coverslips and the immunofluorescent images were evaluated and captured using confocal microscopy.

The procedure was modified for nuclear and cytoplasmic A β measurements. HBMEC were exposed to HIV, followed by 10 min treatment with 1 μ M fluorescently labeled A β (A β HiLyte-Alexa Fluor⁴⁸⁸). The cells were then fixed, washed, and mounted as described above. Green fluorescence originating from A β HiLyte-Alexa Fluor⁴⁸⁸ was acquired directly using confocal microscopy (Olympus, Fluoview 1200, dry lens UPLFLN 40x NA: 0.75, room temperature) and did not require the use of primary or secondary antibody. Fields were chosen at random and acquisition and quantification were performed using the FV10-ASW3.1 software. For the nuclear A β measurements, mean fluorescence intensity was measured in nuclear areas outlined by the DAPI staining. For the cytoplasmic A β measurements, cells were outlined by differential interference contrast (DIC) technique and the corresponding fluorescence was measured excluding the DAPI positive nuclear areas.

EEA1 silencing

Human EEA1 siRNA (a pool of 3 target-specific 20–25 nt siRNAs) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cells were transfected with control siRNA or EEA1 siRNA-1 at a final concentration of 40 nM for 48 h using siPORT NeoFX transfection reagent from the Silencer siRNA Transfection II Kit (Ambion, Applied Biosystems, Foster City, CA) in OptiMEM I medium with GlutaMAX (Invitrogen, Carlsbad, CA).

Statistical Analysis

Data were analyzed using SigmaPlot for Windows 11.0 software (Systat Software Inc., San Jose, CA). One, two or three way ANOVA was used to compare responses among treatments. Treatment means were compared using All Pairwise Multiple Comparison Procedures and $p < 0.05$ was considered significant.

RESULTS

Exposure to HIV-1 increases nuclear localization of A β

To explore a hypothesis that brain endothelial cells participate in HIV-1-induced amyloid accumulation in the brains of infected individuals, HBMEC were exposed to HIV-1 particles (p24, 30 ng/ml), followed by a 10 min treatment with fluorescently labeled A β HiLyte (1 μ M). As illustrated in Figure 1, treatment with A β HiLyte alone resulted in weak A β fluorescence (Figure 1A). Importantly, a co-treatment with HIV-1 dramatically increased A β -specific fluorescence, which partly co-localized with DAPI positive staining, indicating a prominent nuclear localization (Figure 1B). Quantification of these results (Figure 1C) revealed that nuclear levels of A β increased more than 16 times in the presence of HIV-1.

While A β fluorescence was also elevated in the cytoplasm of exposed cells, this effect was less prominent compared to nuclei.

Exposure to HIV and/or A β alters cellular distribution of EEA1 and Smad2

Early endosomes are involved in intracellular movement of proteins; therefore, we evaluated if they have impact on HIV-1 induced nuclear entry of A β . As indicated in Figure 2A, early endosomal markers EEA1 and Rab5A are present in both endothelial cell nuclear and cytoplasmic fractions. Quantification of these results demonstrated that nuclear levels of EEA1 were markedly altered cells exposed for 10 min to HIV-1 alone and HIV plus A β . Similar effects were observed with A β treatment for 1 h but the changes were statistically significant only as the result of HIV-1 exposure. Because expression of Rab5A was only minimally affected by the employed treatment factors, we focused on EEA1 in the remaining experiments.

The impact of HIV-1 and/or A β exposure on cellular distribution of EEA1 was also explored using fractionation by sucrose density ultracentrifugation. In control samples, EEA1 was distributed in fractions 6–12 (Figure 2B). Treatment with HIV-1 particles caused a visible shift to the right, with EEA1 being detected mainly in fractions 7–12, with only a weak band present in fraction 6. Treatment with A β and a co-treatment with HIV-1 and A β also resulted in a shift to the heavier cellular fractions as the first EEA1 band became present in fraction 7.

Because early endosomal markers were shown to be involved in nuclear signaling via Smad [25], this signaling pathway was also included in our experiments. Treatment with HIV-1 plus A β resulted in significantly increased nuclear levels of both Smad2 and phosphorylated Smad2 (pSmad2) while exposure to HIV-1 alone increased only pSmad2 nuclear levels (Figure 2C). Exposure to A β and/or HIV-1 also induced changes in cellular Smad2 allocation as determined in cellular fractions obtained by sucrose density ultracentrifugation. Smad2 immunoreactivity in control cells was observed mainly in fractions 6–11. Exposure to A β and/or HIV resulted in a shift of Smad2 to heavier fractions, with Smad2 immunoreactivity becoming present only in fraction 7 (Figure 2D), mirroring the effects of A β and/or HIV on cellular EEA1 localization. This synchronized shift of both EEA1 (Figure 2B) and Smad2 (Figure 2D) within the cellular fractions suggests a coordinated movement within the cells and involvement of both proteins in cellular responses induced by HIV-1 and/or A β .

Immunofluorescent quantification of HIV-induced EEA1 and pSmad2 nuclear localization is depicted in Figure 3. In control cultures (Figure 3A), EEA1-positive immunoreactivity exhibited a vesicular cytoplasmic staining pattern that appeared to be more abundant in the perinuclear area. A weaker nuclear staining pattern with different sized dot-like structures was also observed. In addition, Smad2 immunoreactivity showed a punctated cytoplasmic and nuclear staining pattern. Consistent with the immunoblotting results, HIV exposure enhanced both EEA1 and pSmad2 nuclear immunoreactivity (Figure 3B). Quantification of these results is illustrated in Figures 3C and 3D, respectively. Images in the upper parts of the bar graphs represent the immunoreactivity intensity profiles for EEA1 (Figure 3C) and pSmad2 (Figure 3D) in a representative nuclear area.

Interplay between EEA1 and TGF- β /Smad signaling regulates their nuclear entry

In order to evaluate whether EEA1 can influence Smad signaling, EEA1 was silenced by a specific siRNA, followed by treatment with HIV-1 and/or A β . Figure 4A demonstrates the effectiveness of the procedure resulting in a marked decrease in nuclear and cytoplasmic levels of EEA1. While a combined exposure to HIV-1 plus A β elevated both pSmad2 and Smad2 in nuclear fractions, EEA1 silencing significantly attenuated these effects. In contrast, EEA1 silencing did not affect pSmad2 and Smad2 levels in the cytoplasm.

To determine if the TGF- β /Smad pathway is involved in HIV-1-induced EEA1 nuclear entry, cells were pretreated with 10 μ M SB431542, a specific TGF- β /Smad signaling inhibitor, for 2 h prior to exposure to HIV and/or A β (Figure 4B). SB431542 efficiently blocked Smad2 phosphorylation (positive control) in both nuclear and cytoplasmic fractions. Importantly, SB431542 markedly attenuated EEA1 levels in the nuclear fractions in all treatment groups. In contrast, EEA1 levels in the cytoplasmic fractions were barely affected by TGF- β /Smad signaling inhibition.

Dynamin provides a platform for HIV-1 and/or A β -induced EEA1 and Smad nuclear entry

EEA1 was reported to co-localize with dynamin in the perinuclear region of endothelial cells [26] and HIV-1 Tat-induced A β production was impaired in neurons transfected with dominant negative dynamin [9]. Therefore, we explored whether dynamin is involved in the HIV-1-induced nuclear EEA1 and Smad2 translocation. Control cells or cells exposed to HIV-1 for 24 h were pre-treated with dynamin inhibitor, dynasore (50 μ M), for 30 min prior to exposure to HIV-1 and/or A β . Pretreatment with dynasore reduced the intensity of the nuclear EEA1 levels in all treatment groups, with cytoplasmic EEA1 only minimally affected (Figure 5A). Quantification of these results demonstrated that the impact of dynasore on nuclear EEA1 levels was statistically significant in the HIV plus A β group.

Taking into consideration a strong inter-relationship between EEA1 and TGF- β /Smad signaling, we also evaluated the impact of dynamin on nuclear levels of pSmad and Smad2. Inhibition of dynamin attenuated HIV and/or A β -induced changes in pSmad2 levels in the nuclear fraction. Interestingly, blocking dynamin also markedly diminished pSmad levels in the cytoplasm (Figure 5B).

EEA1, TGF- β /Smad, and dynamin control in concert HIV-1-induced nuclear entry of A β

In the final set of experiments, the impact of EEA1, TGF- β /Smad, and dynamin on HIV-1-induced nuclear uptake of A β was evaluated. Using the previously established conditions, EEA1 was silenced by transfection with specific siRNA, TGF- β /Smad signaling was inhibited by SB431542 (10 μ M), and dynamin signaling by dynasore (50 μ M). Cells were also treated with HIV-1 and/or A β HiLyte, followed by visualization of A β HiLyte-specific fluorescence by confocal microscopy and quantification for nuclear localization. EEA1 silencing, pretreatment with SB431542, or dynamin inhibition significantly reduced the intensity of nuclear fluorescence of A β HiLyte in HIV-1-treated cells (Figure 6A, 6B, 6C, respectively). Because of its apparent location upstream from EEA1 or Smad2, we suggest that dynamin may act as a master regulator to control nuclear levels of A β via the EEA1 and TGF- β /Smad pathways.

The effects of EEA1 silencing, pretreatment with SB431542, and dynamin inhibition on HIV-1-induced A β HiLyte accumulation HBMEC were also measured by in cell fluorescence (Supplementary Figures 1A, 1B, and 1C). This method allows for quantitative measurement of the total A β levels without discrimination between nuclear and cytosolic fractions. As illustrated in Supplementary Figures 1A and 1B, EEA1 silencing and pretreatment with SB431542 protected against HIV-1-induced A β accumulation when measured in the whole cells. Dynamin inhibition appears to be effective only on nuclear A β uptake because did not change the overall cellular A β accumulation in the presence of HIV-1 (Supplementary Figure 1C).

DISCUSSION

Although A β accumulation has been linked primarily to the pathology of AD, a moderate brain accumulation of this peptide is also characteristic for normal aging. Moreover, emerging clinical data provide evidence that amyloid levels are increased in HIV brains [1, 5, 27]. There appears to be prevalence for amyloid deposition in the hippocampus and frontal lobe with deposits in pyramidal neurons, and along axonal tracks in HIV-infected individuals [3]. Prominent A β localization is also observed in the perivascular space in HIV brains [1, 27] indicating the importance of cerebral microvasculature in amyloid pathology. HIV-associated neurocognitive disorders (HAND) in older patients correlate with early beta-amyloidosis [1, 6]. Amyloid accumulation in the brain may also affect the rate of HIV infection [28].

The BBB plays an important role both in HIV-1 [29] and amyloid pathology [30], regulating both entry of the blood-borne amyloid into the brain and clearance from the brain [31]. At the same time, A β is toxic for the brain vasculature as demonstrated by a loss of occludin, claudin-5, and ZO-1 in A β -laden capillaries. Treatment with A β disrupted endothelial permeability, impaired glucose transport, and resulted in apoptosis [32]. The underlying mechanisms of A β toxicity in vascular system appear to be induction of reactive oxygen species, as A β cytotoxicity was reversed by administration of exogenous antioxidants and NOX-2 inhibitors. It was reported that HEK cells transfected with a construct of A β (1–43) exhibited nuclear A β accumulation [33]. A similar phenomenon was observed in CHO cells expressing A β in the endoplasmic reticulum [34]. However, to our knowledge, the present manuscript is the first report indicating enhanced exogenous A β accumulation in the nuclei of cells exposed to HIV-1.

The Smad signaling was linked to A β pathology [35]; therefore, this pathway was evaluated in the present study as a possible mediator of nuclear accumulation of A β . Exposure to HIV-1 and/or A β increased pSmad2 levels in the nuclear fractions, without affecting the total Smad2 levels in the cytoplasm. The mechanisms of these effects are not fully understood; however, HIV-1 gp120 [36] can signal via transforming growth factor- β 1 (TGF- β), providing an activation pathway for Smad2 stimulation. Indeed, Smad is typically activated by stimulation of the TGF- β family receptor complex, which comprises the activin-like kinase 5 (ALK5)/TGF- β type I receptor (T β RI) and the TGF- β type II receptor (T β RII) subunits. Binding of a ligand (e.g., TGF- β) to T β RII induces the assembly of both types of receptors into a complex, followed by transphosphorylation. Activated T β RI

phosphorylates receptor-activated Smads (Smad 2, 3, 5, and 8), which form a complex with Smad 4, translocate into the nucleus where they regulate the expression of various target genes. TGF- β /Smad pathway has been directly implicated in HIV-1 infection. For example, it was observed that HIV-1 and HIV-1 protein Tat increased TGF- β levels in the brain [37, 38]. In addition, TGF- β inhibited HIV-1 infection of mammary epithelial cells via downregulating the HIV-1 long terminal repeat (LTR) promoter; however, it increased infection of macrophages by HIV-1 by upregulation of the LTR promoter [39]. The p300-TGF- β /Smad binding was also shown to modulate HIV-1 gp120 induced tubular injury which may be relevant to HIV-associated nephropathy [36].

Important data from the present study indicate that inhibition of TGF- β /Smad protect against HIV-1-induced A β accumulation in the nuclei. These results are consistent with literature reports indicating that inhibition of this signaling pathway in mouse peripheral macrophages diminished A β deposits in the brain vessels [35]. Nevertheless, the role of Smad in A β pathology may be complex as it was also reported that AD brains are associated with deficiency of Smad [40, 41] and an aberrant localization of pSmad2/3 in the cytoplasm of the hippocampal neurons and in amyloid plaques [42]. In addition, inhibition of TGF- β /Smad increased vulnerability of hippocampal neurons to A β and enhanced neurodegeneration in an animal model [43]. The TGF- β /Smad signaling pathway was shown to be regulated by caveolin-1 in NIH-3T3 cells via interaction with the T β RI. While T β RI and Smad2 colocalize with caveolin-1 in caveolae, caveolin-1 can inhibit this pathway by blocking Smad2 phosphorylation [44]. In addition, it was demonstrated that HIV-1 gp41 has a caveolin-1 binding domain [45], suggesting that exposure of brain endothelial cells to HIV-1 may induce a direct binding of the virus to lipid rafts and/or caveolae, a hypothesis that is consistent with a possible raft-dependent endocytotic entry of HIV-1 to HBMEC [46]. These observations indicate a potential upstream gp41-caveolin-1-TGF- β RI signaling for the interactions of HIV-1 with brain endothelial cells.

Early endosomal proteins were suggested to regulate the Smad signaling pathway. For example, a dominant negative mutant of Rab5A caused amplification of Smad phosphorylation and its nuclear translocation, resulting in transcriptional activation of a Smad-dependent promoter in endothelial cells [25]. While expression of Rab5A was only slightly affected in the present study, exposure to HIV-1 and/or A β induced a prominent translocation of EEA1 into the nuclear fractions of HBMEC. This disconnection between these two early endosomal proteins was surprising because EEA1 was described as a Rab5 effector acting as an organelle-tethering molecule [47]. Nevertheless, their various nuclear entry in response to HIV-1 and/or A β treatment suggests a different role for EEA1 in the nuclear environment. Indeed, inhibition of Rab5A was shown to lead to increased Smad phosphorylation and nuclear translocation [25]. In contrast, the present study demonstrates that EEA1 silencing results in diminished Smad phosphorylation and nuclear entry with the concurrent attenuation of HIV-induced A β accumulation in HBMEC nuclei.

Dynamin-2 is a 100 kDa protein that co-localizes with several proteins, including EEA1, in the perinuclear region [26]. Dynamin has been implicated in vesicle fission and receptor endocytosis in endothelial cells [48–50] and may function as a signal transducing GTP-ase [51]. Importantly, it also plays a role in HIV-1 infection, as its inhibition blocked HIV-1

internalization, infection, and fusion with epithelial and lymphoid cells [52]. The results of the present study confirm the important role of dynamin in regulation of cellular processes. Pretreatment with dynasore affected nuclear localization of both EEA1 and Smad2/pSmad2 both in HIV-1 and/or A β treated and control cells, indicating the impact of dynamin on trafficking of intracellular proteins. Cytoplasmic levels of pSmad2 were also greatly affected by dynasore, further supporting a close interaction between dynamin and pSmad. Because dynamin appears to be upstream from Smad2 and EEA1, it may act as the master regulator of these signaling molecules and effectively control cellular uptake of A β . To support this statement, our important data indicate that inhibition of dynamin markedly attenuated HIV-1-induced nuclear uptake of A β . These results are in agreement with observations that transfection of HeLa cells with dominant negative dynamin diminished A β production [53]. In addition, dominant negative dynamin inhibited nuclear signaling of the intracellular domain (AICD) of the amyloid precursor protein (APP) [54]. Although A β has no overlapping sequence with AICD, these results support the role of dynamin in nuclear signaling.

CONCLUSIONS

The present study demonstrates the role of EEA1, TGF- β /Smad, and dynamin signaling in HIV-1-induced nuclear entry of A β . While EEA1 and Smad closely interact regulating each other nuclear translocation, dynamin appears to be a master regulator controlling EEA1 and Smad nuclear entry and, ultimately, A β nuclear accumulation (Figure 7).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

- HIV-1 induces nuclear accumulation of amyloid beta ($A\beta$) in brain endothelial cells.
- EEA-1 and TGF-B/Smad act in concert to regulate nuclear entry of $A\beta$.
- Dynamin appropriates the EEA-1 and TGF-B/Smad signaling.
- Dynamin serves as a master regulator of HIV-1-induced nuclear accumulation of $A\beta$.

Figure 1. Exposure to HIV-1 increases nuclear localization of Aβ

HBMEC were exposed to HIV-1 particles at p24 levels of 30 ng/ml for 24 h followed by co-treatment with 1 μ M fluorescently labeled Aβ HiLyte for 10 min. Aβ HiLyte (green fluorescence) was assessed by confocal microscopy in cells treated with Aβ alone (A) or co-treated with HIV-1 (B). DAPI staining was performed to visualize the nuclei (blue staining). Representative images from three experiments. Scale bar: 10 μ m. (C) Quantification of Aβ HiLyte nuclear and cytoplasmic levels. Values are mean $\pm$ SEM, n=13–17. ***Statistically significant at $p < 0.001$.

B

Figure 2. Exposure to HIV and/or A β alters cellular distribution of EEA1 and Smad2

HBMEC were exposed to HIV-1 particles at p24 levels of 30 ng/ml for 24 h followed by co-treatment with 1 μ M A β for 10 min or 1 h. (A) Early endosomal markers EEA1 and Rab5A were analyzed by immunoblotting in the cytoplasmic and nuclear fractions; n=4–12. (B) Cellular distribution of EEA1 by fractionation using sucrose density ultracentrifugation. (C) pSmad2 and Smad2 were analyzed by immunoblotting in the cytoplasmic and nuclear fractions after exposure to 1 μ M A β for 10 min; n=8–12. (D) Cellular distribution of Smad2 by fractionation using sucrose density ultracentrifugation. The images show representative blots and the bar graphs in A and C reflect quantitative densitometric data. Values are mean $\pm$ SEM. *Statistically significant at p<0.05. GAPDH and nucleoporin p62 (Np62) were used as internal loading controls for the cytoplasmic and nuclear fractions in A and C.

Figure 3. Exposure to HIV increases EEA1 and pSmad2 nuclear levels

HBMEC were exposed to vehicle (A; control cultures) or HIV-1 particles (B) at p24 levels of 30 ng/ml for 24 h. Expression of EEA1 (red fluorescence) and pSmad2 (green fluorescence) was assessed by confocal microscopy. DAPI staining was performed to visualize the nuclei (blue staining). Representative images from three experiments. Scale bar: 10 μ m. Quantification of EEA1 (C) and pSmad2 (D) levels in the nuclear area. Values are mean $\pm$ SEM, $n=15$. ***Statistically significant as compared to control at $p < 0.001$. Images in the upper parts of the bar graphs depict the immunoreactivity intensity profiles for EEA1 or pSmad2 in a representative nuclear area.

Figure 4. Interplay between EEA1 and TGF- β /Smad signaling regulates their nuclear entry
(A) HBMEC were exposed to HIV-1 and/or A β as in Figure 1 with or without EEA1 silencing. pSmad2 and Smad2 levels were analyzed by immunoblotting in nuclear and cytoplasmic fractions; n=8–12. **(B)** HBMEC were exposed to HIV-1 and/or A β as in Figure 1, with the selected cultures pretreated with TGF- β /Smad inhibitor SB431542 (SB, 10 μ M) for 2 h. Nuclear and cytoplasmic EEA1, pSmad2 and Smad2 levels were analyzed by immunoblotting; n=4–6. The images show representative blots from at least four experiments and the bar graphs reflect quantitative densitometric data. Values are mean $\pm$ SEM. Statistically significant at *p<0.05 or ***p<0.001. GAPDH and nucleoporin p62 (Np62) were detected as internal loading controls for the cytoplasmic and nuclear fractions, respectively.

Figure 5. Dynamin regulates HIV-induced EEA1 and pSmad2/Smad2 nuclear entry
 HBMEC were exposed to HIV-1 particles and/or A β as in Figure 1, with the selected cultures pretreated with dynamin inhibitor dynasore (Dy, 50 μ M) for 30 min. Nuclear and cytoplasmic EEA1 (A), pSmad2, and Smad2 (B) levels were analyzed by immunoblotting. The images show representative blots from at least four experiments and the bar graphs reflect quantitative densitometric data. Values are mean $\pm$ SEM, $n=4-6$. *Statistically significant at $p<0.05$. GAPDH and nucleoporin p62 (Np62) were detected as internal loading controls for the cytoplasmic and nuclear fractions, respectively.

Figure 6. EEA1, TGF- β /Smad, and dynamin are involved in HIV-1-induced nuclear entry of A β (A) EEA1 was silenced by transfection with specific siRNA, (B) TGF- β /Smad signaling was inhibited by SB431542 (10 μ M), and (C) dynamin signaling was blocked by dynasore (50 μ M). Such pretreated cultures were then exposed to HIV-1 particles and/or A β as in Figure 1, followed by the assessment of A β HiLyte fluorescence in nuclear areas by confocal microscopy as in Figure 1. At least 5 images for every experimental condition from different samples were randomly acquired. Values are mean $\pm$ SEM, n=250. ***Statistically significant at $p < 0.001$.

Figure 7. Schematic diagram of HIV-1-induced nuclear Aβ uptake in brain endothelial cells

Our data indicate that HIV-1-induced nuclear Aβ accumulation in brain endothelial cells is dynamin-dependent, involving EEA1 and the TGF-β/Smad signaling pathway. Dynamin colocalizes with EEA1 in the perinuclear region of endothelial cells, appears to be upstream from the EEA1- and TGF-β/Smad signaling routes and may act as a master regulator of these signaling pathways and control nuclear uptake of Aβ. These events may contribute to HIV-1-induced brain deposition of amyloid and ultimately to the development of cognitive dysfunction in HIV-1-infected brains. Abbreviations: Aβ, amyloid beta; EEA1, early endosomal antigen 1; TGFβ-R I, transforming growth factor beta receptor type I.