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Lipid rafts and functional caveolae regulate HIV-induced amyloid beta accumulation in brain endothelial cells

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ABSTRACT

Amyloid beta (Aβ) levels are increased in HIV-1 infected brains due to not yet fully understood mechanisms. In the present study, we investigate the role of lipid rafts, functional caveolae, and caveolae-associated signaling in HIV-1-induced Aβ accumulation in HBMEC. Both silencing of caveolin-1 (cav-1) and disruption of lipid rafts by pretreatment with beta-methyl-cyclodextrin (MCD) protected against Aβ accumulation in HBMEC. Exposure to HIV-1 and Aβ activated caveolae-associated Ras and p38. While inhibition of Ras by farnesylthiosalicylic acid (FTS) effectively protected against HIV-1-induced accumulation of Aβ, blocking of p38 did not have such an effect. We also evaluated the role of caveolae in HIV-1-induced upregulation of the receptor for advanced glycation end products (RAGE), which regulates Aβ transfer from the blood stream into the central nervous system. HIV-1-induced RAGE expression was prevented by infecting HBMEC with cav-1 specific shRNA lentiviral particles or by pretreatment of cells with FTS. Overall, the present results indicate that Aβ accumulation in HBMEC is lipid raft and caveolae dependent and involves the caveolae-associated Ras signaling.

1. Introduction

Due to the success of antiretroviral therapy (ART), which changed the clinical picture of HIV-1 infection from acute to chronic disorder, there is a sharp increase in infected patients 50 years old and older [1]. In addition, the rate of new infections is also climbing in older people. This increase in age of the HIV-1 infected population constitutes a new challenge in the HIV/AIDS epidemic in the affluent countries. Indeed, older HIV-1 infected patients are more susceptible to neurocognitive impairments associated with the disease [2], and a significantly higher prevalence of dementia was observed in aged HIV-1-infected individuals as compared to younger patients [3]. Increased deposition of amyloid beta is also a characteristic in the HIV-1 infected brains [2,4]. HIV-associated neurocognitive disorders (HAND) in older patients appeared to correlate with early beta-amyloidosis characteristic for the Alzheimer's disease (AD) even if amyloid pathology is different in HIV-1 and AD [1]. Aβ accumulation in the brain may also affect the rate of HIV-1 infection probably via activated microglia and macrophages since these cell types have been found in the proximity to amyloid plaques [5].

The aim of the present study was to evaluate the role of lipid rafts and functional caveolae in Aβ accumulation in brain endothelial cells. This focus was prompted by the following factors: (a) the receptor for advanced glycation end products (RAGE), which may be involved in Aβ uptake by endothelial cells, is localized to caveolae [6,7]; (b) caveolin-1 was shown to play a role in activation of RAGE [8]; and (c) HIV-1 Tat can activate the caveolae-associated pathway in brain endothelial cells [9].

2. Materials and methods

2.1. Cell culture and treatment

Human brain microvascular endothelial cells (HBMEC) and human monocytic U937 cells were co-cultured as described earlier [10]. Cells were treated with freshly solubilized Aβ(1–40) (Anaspec, San Jose, CA) at the concentration of 1 μM for 10 min in complete medium.

HIV-1 stock was generated using human embryonic kidney (HEK) 293T cells (American Type Culture Collection, Manassas, VA) and used to infect U937 cells [10]. In the co-culture experiments, confluent HBMEC cultures growing on 6-well plates were exposed to U937 cells at a ratio of 1:1 and HIV-1 p24 at a level

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid beta; ART, antiretroviral therapy; BBB, blood-brain barrier; cav-1, caveolin-1; FTS, farnesylthiosalicylic acid; HAND, HIV-associated neurocognitive disorders; HBMEC, human brain microvascular endothelial cells; HEK, human embryonic kidney cells; MCD, beta-methyl-cyclodextrin; RAGE, receptor for advanced glycation end products.

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of 230 pg/ml. Non-infected U937 cells were used as controls. In the monoculture experiments confluent HBMEC cultured on collagen I coated chambered glass slides (BD Biosciences, San Jose, CA) were exposed to HIV-1 particles (initial p24 level of 30 ng/ml; final p24 level of 1 ng/ml).

2.2. Caveolin-1 silencing and shRNA lentiviral particles infection

Caveolin-1 (cav-1) silencing was performed as described by Lim et al. (2008) [11] using a mixture of siRNA corresponding to the nucleotides 69–87 of the human cav-1 mRNA sequence: 5′-CAUCUACAAGCCCAACAA C-dTdT-3′ (cav-1 siRNA-1) and to the nucleotides 223–241: 5′-CCAGAAGGGACACACAGUU-dTdT-3′ (cav-1 siRNA-2). Control siRNA was: 5′-AAAGAGCGACUUUACACAC-dTdT-3′.

HBMEC were infected with shRNA lentiviral particles containing target-specific constructs that encode shRNA designed to knock down cav-1 expression (Santa Cruz Biotechnology) using the manufacturer's instructions.

2.3. Purification of lipid rafts

Lipid raft membranes were prepared as described by Lim et al. [11] 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, centrifuged at 15000 × *g* for 15 min at 4 °C, followed by sucrose gradient ultracentrifugation. Aliquots were subjected to SDS–PAGE and immunoblotting to assess flotillin-1 and cav-1 as marker proteins for lipid rafts and caveolae, respectively.

2.4. Immunoblotting and confocal immunofluorescence microscopy

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 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 μg/ml aprotinin) and analyzed using a standard approach. Immunofluorescence was performed and evaluated as described earlier [10].

2.5. Ras and p38 activation assays

Ras Activation ELISA Kit (Millipore, Temecula, CA) was used to assess GTP-bound Ras. ELISA (Invitrogen, Camarillo, CA) was also used to quantitate phosphorylation of p38 at threonine 180 and tyrosine 182 as markers of p38 activation.

2.6. Statistical analysis

Data were analyzed using SigmaPlot 11.0 (Systat Software Inc., San Jose, CA). One way ANOVA was used to compare responses among treatments. Treatment means were compared using all pairwise multiple comparison procedures (Tukey Test) and $p < 0.05$ was considered significant.

3. Results

3.1. Exposure to HIV and/or Aβ alters cellular distribution of lipid rafts and caveolae

The cells were fractionated by sucrose density ultracentrifugation, followed by determination of flotillin- and cav-1 (markers of lipid rafts and caveolae, respectively) (Fig. 1A). Treatment with Aβ (1 μM) for 10 min resulted in a slight shift to the heavier cellular fractions. Treatment with HIV-1 particles caused an even more visible shift to the right. Interestingly, Aβ co-treatment of HBMEC exposed to HIV-1 resulted in a shift to the lighter fractions. A 2 h pretreatment with farnesylthiosalicylic acid (FTS, 20 μM), a specific Ras inhibitor, followed by co-exposure to Aβ and HIV-1 caused a flotillin-1 shift to heavier fractions.

Exposure to Aβ and/or HIV-1 also induced changes in cav-1 allocations to different cellular fractions (Fig. 1B). Treatment with Aβ and HIV-1 resulted in a shift of cav-1 to heavier fractions. In cells exposed to both Aβ and HIV-1, the overall changes were similar to those in HIV-1-treated HBMEC; however, they were more pronounced. Interestingly, pretreatment with FTS reversed the changes in cav-1 localization in Aβ and HIV-1 plus Aβ-treated samples.

Next, we determined the localization of Aβ in fractionated HBMEC cells (Fig. 1C). The most prominent Aβ immunoreactivity was detected in fractions 7 and 8; i.e., in the cellular fractions that correspond to lipid rafts and caveolae. Aβ immunoreactivity was increased by HIV-1 without changes in allocation of Aβ immunoreactivity among cellular fractions.

3.2. Disruption of lipid rafts and caveolae attenuates Aβ accumulation in HBMEC

Cholesterol depletion by methyl-beta-cyclodextrin (MCD; 5 mM for 30 min at 37 °C) was used to disrupt lipid rafts and evaluate whether intact lipid rafts are involved in HIV-1-induced accumulation Aβ. While treatment with Aβ and, in particular, Aβ plus HIV-1 resulted in a significant increase in Aβ levels (Fig. 2A), these effects were attenuated by MCD.

The protective effects of MCD on Aβ accumulation were confirmed in immunofluorescence microscopy studies. In these experiments, cells were exposed to Aβ(1–40) HiLyte Fluor 488 (producing green fluorescence) and co-stained for flotillin-1 immunoreactivity (red fluorescence). The regions of co-localizations of Aβ and flotillin are marked in yellow (Fig. 2B).

In order to explore the role of caveolae in Aβ accumulation in HBMEC, cav-1 was silenced, followed by treatment with Aβ (1 μM for 10 min) and/or HIV-1 particles (p24, 1 ng/ml). As shown in Fig. 2C, cav-1 silencing resulted in a marked decrease in Aβ levels in cells exposed to HIV-1 plus Aβ. To further confirm the role of cav-1 in Aβ accumulation, we established a cell line in which cav-1 was knocked-down by specific cav-1 shRNA. Aβ levels were markedly diminished in cav-1 shRNA cells treated with HIV-1 plus Aβ (Fig. 2D), providing additional evidence that functional caveolae contribute to Aβ accumulation in HBMEC.

3.3. Exposure to Aβ or HIV-1 activates caveolae-associated Ras and p38 signaling. Ras, but not p38, is involved in HIV-1-induced Aβ accumulation in HBMEC

In the next series of experiments, we evaluated the effects of HIV-1 and/or Aβ on activation of caveolae-associated Ras and p38 signaling in HBMEC using quantitative ELISA. Treatment with Aβ (1 μM) but not with HIV-1 particles (p24, 1 ng/ml) for 3 min resulted in an increase in GTP–Ras levels. However, GTP–Ras levels were increased in cells exposed to HIV-1 for 24 h. Additional exposure to Aβ for 3 min did not affect GTP–Ras levels as compared to cells treated with HIV-1 alone (Fig. 3A).

Exposure to 1 μM Aβ or HIV (p24, 1 ng/ml) for 10 min significantly increased phosphorylation of p38. In contrast, treatment with HIV for 24 h diminished levels of p-p38 as compared to control. The addition of Aβ had no effect on p-p38 levels in cells exposed to HIV-1 for 24 h (Fig. 3B).

Pre-incubation with FTS (20 μM; 2 h pretreatment) did not affect Aβ and/or HIV-1-stimulated p38 phosphorylation, indicating

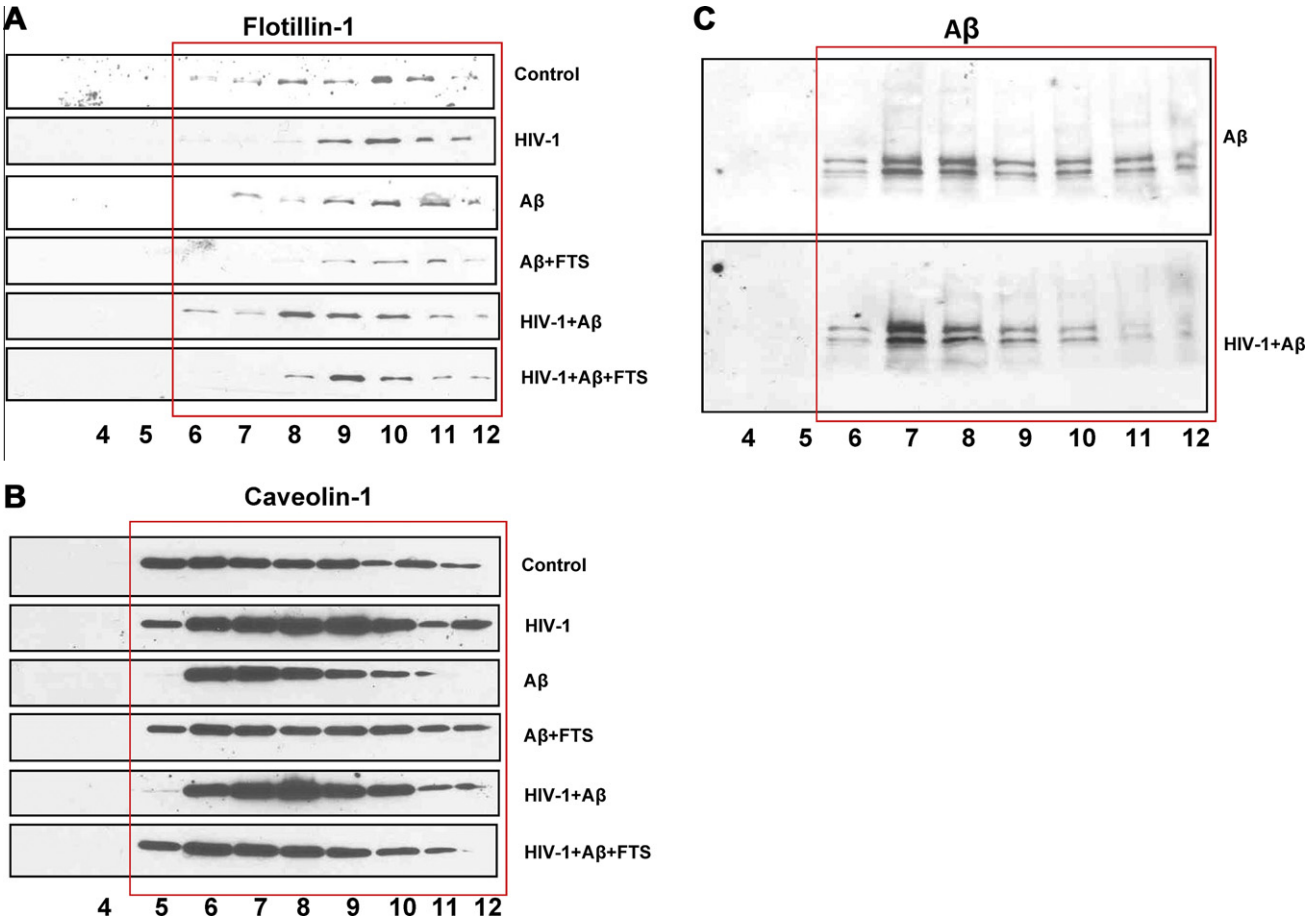

Fig. 1. Exposure to HIV and/or Aβ alters cellular distribution of lipid rafts and caveolae. HBMEC were exposed to HIV-1 particles for 24 h and/or treated with 1 μM Aβ(1–40) for 10 min. Selected cultures were pretreated with 20 μM FTS (a Ras inhibitor) for 2 h. Distribution of flotillin-1 (A), caveolin-1 (B) and Aβ (C) was determined by immunoblotting in cellular fractions obtained by sucrose density ultracentrifugation. The blots are representative images from three independent experiments.

that activation of p38 is not dependent on Ras activation (Fig. 3C). SB203580 (p38 inhibitor) served as a positive control (10 μM; 1 h pretreatment) in these experiments.

Consistent with the results shown in Fig. 2A, treatment with Aβ resulted in increased intracellular levels of Aβ and this effect was further potentiated in the presence of HIV-1 (Fig. 3D). Pretreatment with FTS markedly reduced the intensity of all Aβ-immunoreactive bands in HIV-1 plus Aβ-treated cells. In cultures exposed to Aβ without HIV-1 co-treatment, FTS had a tendency to decrease Aβ levels; however, these changes did not reach statistical significance. Independent of treatment conditions, pre-exposure to SB203580 did not affect Aβ in HBMEC.

3.4. Functional caveolae and Ras signaling regulate HIV-1 induced RAGE overexpression

A 24 h co-culture with HIV-1-infected U937 cells increased RAGE protein levels in HBMEC transfected with control shRNA as compared to the cells co-cultured with uninfected U937 cells. This effect was blocked in cells transfected with cav-1 shRNA (Fig. 4A). Pretreatment with FTS also attenuated RAGE levels in cultures exposed to HIV-1 plus Aβ (Fig. 4B).

4. Discussion

Lipid rafts and caveolae are important for the vascular effects of HIV. Adsorptive endocytosis by which HIV-1 can be taken up by endothelial cells involves these lipid microdomains. HIV-1 specific proteins, such as Tat and Nef, can also interact with caveolae/lipid rafts and thus exert their biological effects. Several receptors (e.g., VEGFR2 and RAGE) and signaling pathways (e.g., Ras and Rho) that are utilized by HIV-1 and HIV-1 proteins are localized to lipid rafts and caveolae in endothelial cells. Therefore, we focused on the role of these membrane domains in HIV-1-mediated alterations of Aβ metabolism at the BBB level.

Novel results of the present study indicate that exposure to HIV-1 viral particles can dramatically change lipid raft dynamics in HBMEC. Upon a 24 h exposure to HIV-1, lipid rafts shifted to heavier cellular fractions as assessed by determination of marker protein flotillin-1. Similar, although less dramatic, changes in lipid rafts were induced by treatment of HBMEC with Aβ. These findings are important because changes in lipid rafts may play a role in several brain pathologies, including neurodegenerative diseases, such as AD [12].

Specific forms of lipid rafts in vascular endothelial cells are caveolae characterized by transmembrane proteins called caveolins [13]. Cav-1 has been identified as the marker protein of caveolae, indispensible for the caveolae structure as cav-1 silencing disrupts the functional caveolae. Importantly, cav-1 was demonstrated to be up-regulated in the brains of old rats and in elderly human cerebral cortex, implying a correlation between Alzheimer's disease progression and cav-1 expression [14]. Our data indicate that treatment of HBMEC with HIV-1 and/or Aβ results in alterations of cellular distribution of caveolae as reflected by a shift of cav-1 to higher density cellular fractions.

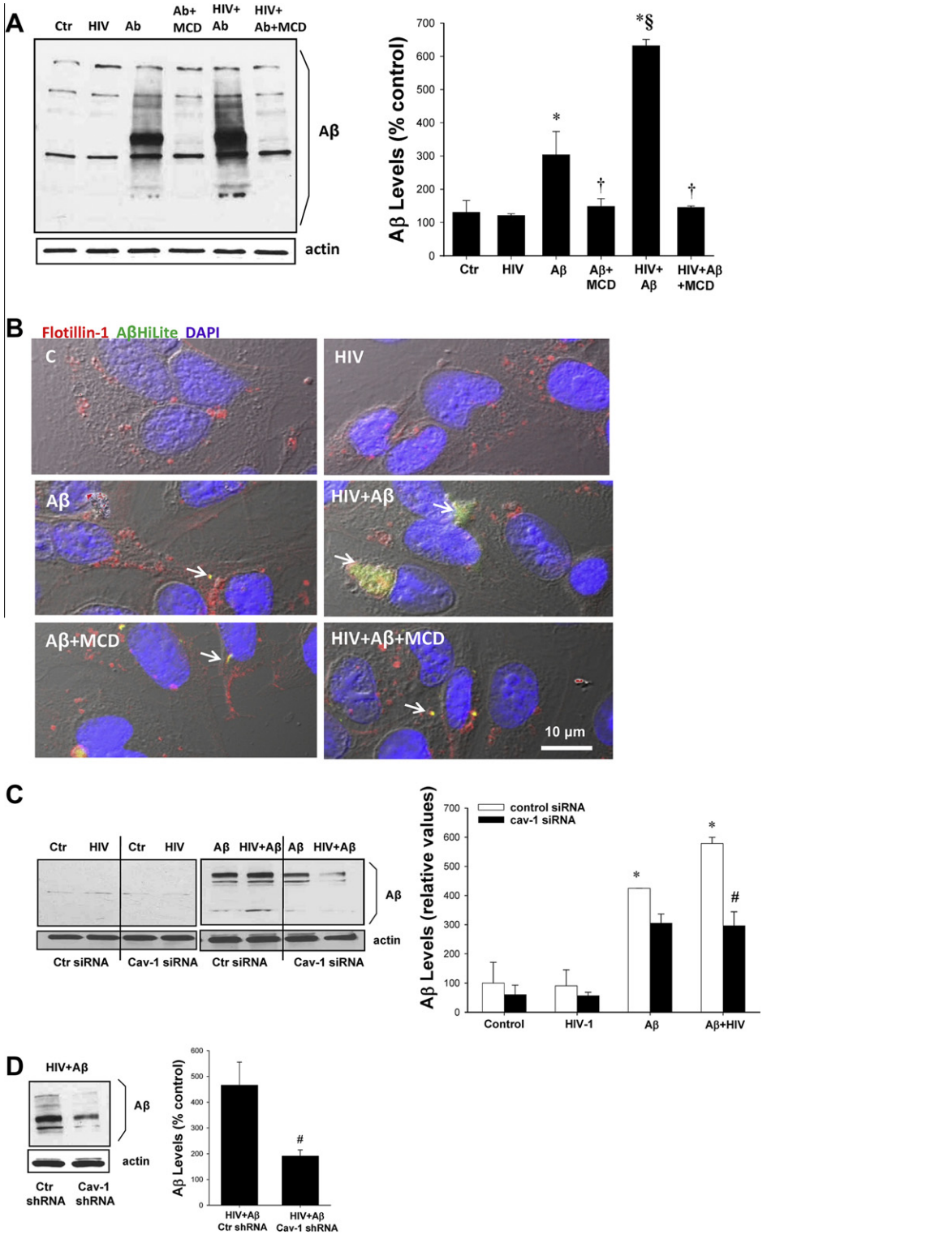

Fig. 2. Disruption of lipid rafts and caveolae attenuates Aβ accumulation in HBMEC. (A) HBMEC were exposed to HIV-1-infected or control U937 cells for 24 h and/or treated with 1 μM Aβ(1–40) for 10 min. Selected cultures were pretreated with 5 mM methyl-beta-cyclodextrin (MCD) for 30 min. Cellular levels of Aβ were analyzed by western blotting in whole cell lysates. (B) HBMEC were exposed to HIV-1 particles at p24 levels of 1 ng/ml for 24 h, followed by co-treatment with 1 μM fluorescently labeled Aβ(1–40) HiLyte for 24 h. In addition, selected cultures were pre-exposed to 5 mM MCD for 30 min before the Aβ(1–40) HiLyte exposure. Aβ(1–40) HiLyte (green fluorescence) and flotillin expression (red fluorescence) were assessed by fluorescence microscopy. DAPI staining was performed to visualize the nuclei (blue staining). The regions of co-localization of Aβ(1–40) HiLyte and flotillin are depicted in yellow (arrows). Representative images from three independent experiments. Scale bar: 10 μm. (C) HBMEC were transfected with control or caveolin-1 specific siRNA (Ctr siRNA or Cav-1 siRNA, respectively) and exposed to HIV-infected U937 cells and/or Aβ as in Fig. 2A. (D) Caveolin-1 shRNA cell line with knock down of caveolin-1 (cav-1) expression was established. Control (Ctr) or cav-1 shRNA cells were exposed to HIV-1 infected U937 cells for 24 h and Aβ (1 μM) for 10 min. Cellular levels of Aβ were determined by western blotting. Values are mean ± SEM (n = 4–8). *Statistically significant compared to vehicle-treated control. †Values in the group Aβ + MCD or HIV + Aβ + MCD are significantly different compared to the corresponding groups not exposed to MCD. §Values in the group HIV + Aβ are significantly different compared to the corresponding values in the Aβ or HIV group. #Values in the group HIV + Aβ + cav-1 siRNA or HIV + Aβ + cav-1 shRNA are significantly different compared to the corresponding values in the Aβ + HIV group.

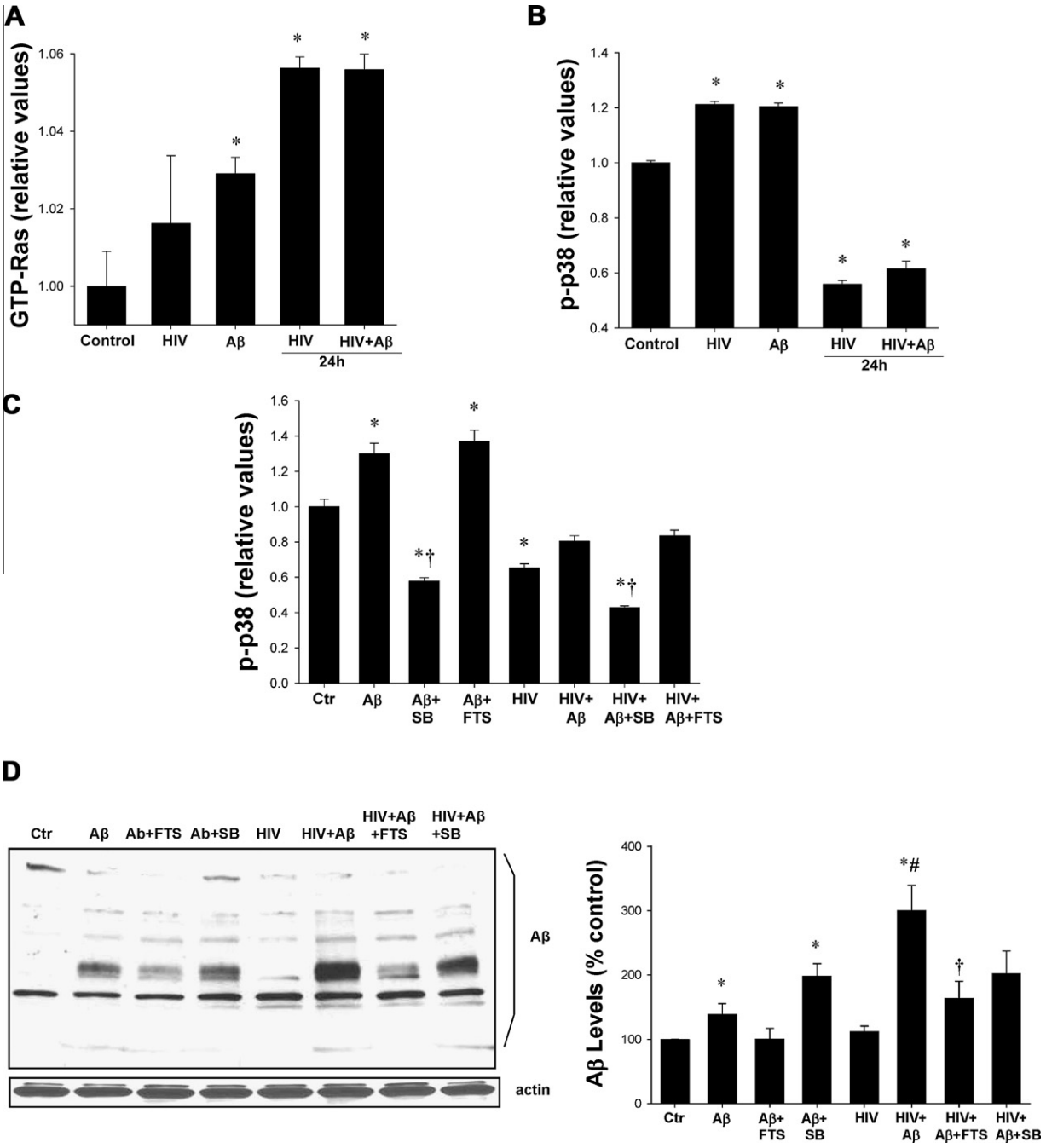

Fig. 3. Exposure to Aβ or HIV-1 activates caveolae-associated Ras and p38 signaling; Ras is involved in Aβ accumulation in HBMEC. (A) HBMEC were exposed to HIV-1 viral isolate (p24, 1 ng/ml) or to Aβ (1 μM) for 3 min. For co-exposure studies, HBMEC were treated with HIV viral isolate (p24, 1 ng/ml) for 24 h, followed by exposure to Aβ (1 μM) for 3 min. GTP–Ras level was assessed using the Ras activation ELISA assay and the values were normalized to cellular protein content. (B) HBMEC were exposed to HIV-1 viral isolate (p24, 1 ng/ml) or to Aβ (1 μM) for 10 min. For co-exposure studies, HBMEC were treated with HIV viral isolate (p24, 1 ng/ml) for 24 h, followed by exposure to Aβ (1 μM) for 10 min. Activation of p38 was measured by ELISA and normalized to protein levels. (C) HBMEC were pretreated with farnesylthiosalicylic acid (FTS, specific Ras inhibitor; 20 μM) for 2 h or with SB203580 (SB, 10 μM, specific inhibitor of p38; positive control) for 1 h prior to treatment with Aβ (1 μM) or HIV (p24, 1 ng/ml) for 10 min. Activation of p38 was assessed as in (B). (D) HBMEC were exposed to HIV-1 infected or uninfected U937 cells for 24 h, followed by co-exposure to 1 μM Aβ for 10 min. Selected cultures were pretreated with FTS or SB203580 as in (C). The blot (left panel) is the representative image from 4 independent experiments. The graph (right panel) represents the combined densitometric measurements of all Aβ-immunoreactive bands. Values are mean ± SEM, $n = 3–4$. *Statistically significant compared to vehicle-treated control. †Values in the groups treated with SB or FTS are significantly different compared to the corresponding groups not exposed to SB or FTS. #Values in the group HIV + Aβ are significantly different compared to the corresponding values in the Aβ or HIV group.

The mechanisms of HIV-1 and/or Aβ-induced redistribution of lipid rafts and caveolae are not fully understood. In cells treated with Aβ alone, they may involve alterations of the composition and/or solubility of these lipid domains, which are enriched in cholesterol, sphingolipids, and saturated fatty acids. Another possibility is the direct binding of Aβ to lipid rafts. Exposure to HIV-1 may also induce 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 [15]. In support of this hypothesis, it was demonstrated that HIV-1 gp41 has a cav-1 binding domain [16].

It is important to note that Aβ localizes in the same cellular fractions as those with the highest detectable levels of flotillin-1 and cav-1. This correlation suggests the dependency of Aβ uptake and cellular accumulation on lipid rafts or caveolae. Indeed, depletion

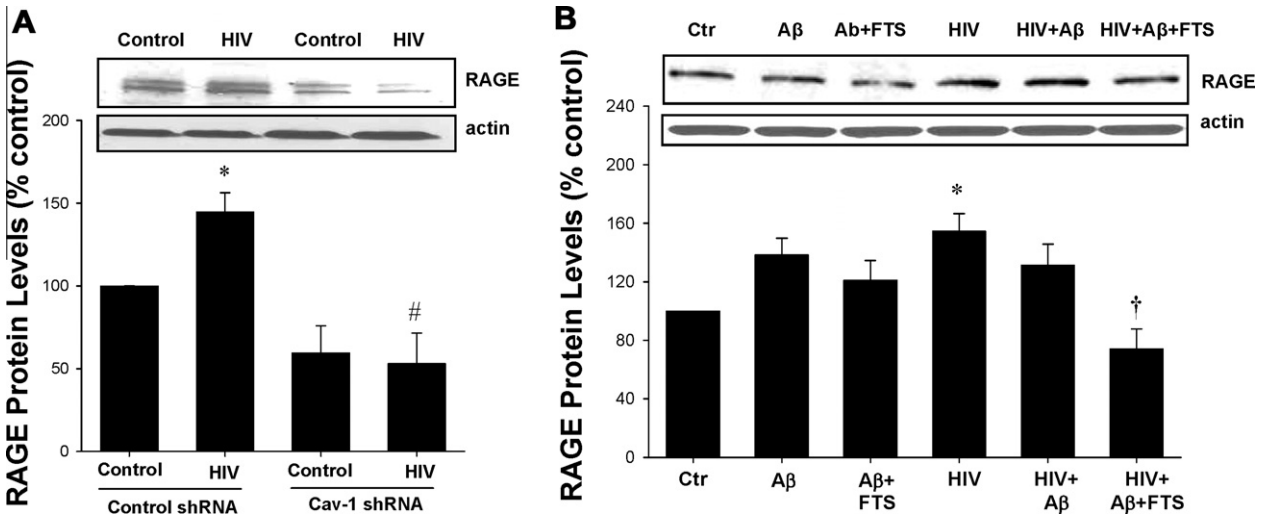

Fig. 4. Functional caveolae and Ras signaling regulate HIV-1-induced RAGE overexpression. (A) Cav-1 expression was silenced by infection of HBMEC with cav-1 shRNA. Then, control cells (infected with control shRNA) and cav-1 deficient cells were exposed to HIV-1 infected U937 cells for 24 h. RAGE protein levels were measured by immunoblotting and quantified by densitometry. The blot is the representative image from 10 to 11 independent experiments. The graph represents the quantified combined data from these experiments. (B) HBMEC were exposed to HIV-1 infected or uninfected U937 cells for 24 h and/or Aβ (1 μM) for 10 min. Selected cultures were pretreated with FTS (20 μM) for 2 h prior to Aβ treatment. RAGE protein levels were measured as in (A). The graph represents the combined densitometric measurements of all Aβ-immunoreactive bands. Values are mean ± SEM, $n = 8–10$. *Statistically significant compared to the respective vehicle-treated control. #Values in the group HIV + cav-1 shRNA are significantly different compared to the corresponding values in the HIV + control shRNA group. †Values in the group HIV + Aβ + FTS are significantly different compared to the corresponding values in the Aβ, HIV, or Aβ + HIV group.

of cholesterol from cell membranes by MCD clearly demonstrated that intact lipid rafts are needed for Aβ uptake as MCD pretreatment drastically diminished Aβ accumulation in control and HIV-1 exposed HBMEC. Similar dependency of Aβ uptake on lipid rafts was observed in neurons [17]. In addition, disruption of caveolae by silencing of cav-1 significantly protected against Aβ accumulation in HBMEC.

The presence of Aβ in similar cell fractions as flotillin-1 and cav-1 also suggests a possible interaction of Aβ with lipid rafts and/or caveolae. Therefore, we also investigated the role of caveolae-associated signaling mechanisms in HIV-1-induced Aβ accumulation in HBMEC. We focused on the Ras-MAPK and p38-MAPK signaling pathways because Ras is involved in HIV-1-Tat induced alterations of endothelial tight junctions [18] and p38 may participate in Aβ uptake by neurons [19]. In addition, it was shown that p38 has an important role in the brain pathology of HIV-1 infection [for review see [20]] and its activity can be induced by Aβ in brain endothelial cells [21].

Consistent with earlier reports [22,23], exposure to HIV-1 or Aβ activated both Ras and p38. While inhibition of Ras by FTS effectively protected against HIV-1 and/or Aβ-induced accumulation of Aβ, pretreatment with the p38 inhibitor SB203580 did not affect intracellular Aβ levels. This finding was unexpected in the light of literature reports showing the involvement of the p38 pathway in Aβ pathology [24].

Among potential mechanisms that can regulate Aβ levels in HBMEC and its transfer across the BBB, RAGE appears to play a critical role [25]. Our previous studies demonstrated that HIV-1 exposure results in RAGE overexpression, which then contributes to increased Aβ accumulation in HBMEC [10]. Importantly, RAGE is present in endothelial caveolae [6] and can be activated by cav-1 [8]. In agreement with these reports, our data indicate that HIV-1-induced upregulation of RAGE protein levels can be attenuated by cav-1 depletion from HBMEC. In addition, HIV-1-induced RAGE overexpression was prevented by pretreatment with FTS, further demonstrating the dependency of RAGE on caveolae-associated pathways.

In summary, the results of the present study provide evidence that HIV-1-induced accumulation of Aβ in HBMEC is lipid raft and caveolae dependent. In addition, these mechanisms involve Ras signaling via upregulation of RAGE expression. Overall, these results suggest that targeting Ras signaling and/or lipid rafts may be an effective strategy to protect against HIV-1-induced Aβ accumulation in the brain endothelium.

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