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*J Immunol* 2008; 181:6406-6416; doi: 10.4049/jimmunol.181.9.6406
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Cannabinoids Inhibit HIV-1 Gp120-Mediated Insults in Brain Microvascular Endothelial Cells

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HIV-1 infection has significant effect on the immune system as well as on the nervous system. Breakdown of the blood-brain barrier (BBB) is frequently observed in patients with HIV-associated dementia (HAD) despite lack of productive infection of human brain microvascular endothelial cells (HBMEC). Cellular products and viral proteins secreted by HIV-1 infected cells, such as the HIV-1 Gp120 envelope glycoprotein, play important roles in BBB impairment and HIV-associated dementia development. HBMEC are a major component of the BBB. Using cocultures of HBMEC and human astrocytes as a model system for human BBB as well as in vivo model, we show for the first time that cannabinoid agonists inhibited HIV-1 Gp120-induced calcium influx mediated by substance P and significantly decreased the permeability of HBMEC as well as prevented tight junction protein down-regulation of ZO-1, claudin-5, and JAM-1 in HBMEC. Furthermore, cannabinoid agonists inhibited the transmigration of human monocytes across the BBB and blocked the BBB permeability in vivo. These results demonstrate that cannabinoid agonists are able to restore the integrity of HBMEC and the BBB following insults by HIV-1 Gp120. These studies may lead to better strategies for treatment modalities targeted to the BBB following HIV-1 infection of the brain based on cannabinoid pharmacotherapies. The Journal of Immunology, 2008, 181: 6406–6416.

The blood-brain barrier (BBB), a regulated interface between the peripheral circulation and the CNS, is comprised of the cerebral microvascular endothelium, which, together with neurons, astrocytes, pericytes, and the extracellular matrix, constitutes a “neurovascular unit” (1–3).

Most forms of brain damage are associated with BBB disruption, resulting in secondary damage to neural cells. The interendothelial space of the cerebral microvasculature is characterized by the presence of a junctional complex that includes adherens junctions (AJs), tight junctions (TJ), and gap junctions (3–7). Whereas gap junctions mediate intercellular communication, both AJs and TJ act to restrict the permeability across the endothelium. AJs are ubiquitous in the vasculature and mediate the adhesion of endothelial cells to each other, contact inhibition during vascular growth and remodeling, initiation of cell polarity, and the regulation of paracellular permeability (2). The primary component of AJs is VE-cadherin. The TJ are the main components that confer low paracellular permeability and high electrical resistance (2). Electrical resistance in vivo across the barrier usually is >1000 ohm × cm² due to the TJ (8, 9).

The zonula occludens (ZO) proteins are involved in the coordination and clustering of protein complexes to the cell membrane and in the establishment of specialized domains within the membrane (10–13). ZO-1 links transmembrane proteins of the TJ to the actin cytoskeleton (10–13). The primary cytoskeletal protein, actin, has known binding sites on all ZO proteins and on claudins and occludin (10). Actin filaments serve both structural and dynamic roles in the cell. ZO-1 binds to actin filaments and to the C terminus of occludins and claudins (10), which couples the structural and dynamic properties of perijunctional actin to the paracellular barrier (10). Interestingly, ZO-3 expression is absent in endothelial cells (10–13).

The HIV invades the CNS early after viral exposure and causes progressive cognitive, behavior, and motor impairments years later with the onset of immune deficiency (for review, see Refs. 14 and 15). One of the major mediators of neuroAIDS is the transmigration of HIV-infected leukocytes across the BBB into the CNS. Infected peripheral immune-competent cells, in particular macrophages, appear to infiltrate the CNS and provoke a neuropathological response involving all cell types in the brain. The course of HIV-1 disease is strongly influenced by viral and host factors, such as the viral strain and the response of the host’s immune system (14, 15). In addition, HIV-1-dependent disease processes in the periphery have a substantial effect on the pathological changes in the CNS.

HIV infection alters BBB structure and function (14, 15), suggesting that altered BBB function is an early event in HIV-associated dementia (HAD), likely through the viral products activating brain endothelial cells such as HIV-1 Gp120, resulting in changes to the BBB by which substances pass through it. Alterations in the BBB can result from events occurring on either side of the BBB, with a net effect of changing the ability of the BBB to...
properly regulate the substances entering it. Indeed, HIV-infected patients showed changes in BBB integrity over a 2-year period although there was no sign of neurological disease (16). Furthermore, BBB leakage was greater in patients with AIDS than among asymptomatic HIV-seropositive individuals (17, 18). Reduction in staining for the TJ ZO-1 protein was also observed in basal ganglia and subcortical white matter in patients who died with HIV along with accumulation of HIV-infected brain macrophages (18, 19). Thus, TJ play an important role in the integrity and maintenance of human brain microvascular endothelial cells (HBMEC) and the BBB.

The HIV-1 envelope protein Gp120, in particular, has been proposed as an etiologic agent of neuronal loss as well as for development of the HAD complex (20–23). There is a massive shedding of the envelope Ags of HIV-1, such as Gp120, as detected by immunoelectron microscopic studies (20, 21). Gp120, shed from HIV-1 and HIV-1-infected cells, was shown to be present in the blood of HIV-1-infected patients (20, 21). Gp120 has also been detected in the cerebrospinal fluid of HIV-1-infected patients (20, 22) and is neurotoxic both in vitro and in vivo. The role of Gp120 in contributing to HAD has been highlighted by the observation that free Gp120 protein was found in the brains of AIDS patients (20, 22). HIV-1 Gp120 protein was reported to induce endothelial cell apoptosis and to down-regulate TJ proteins in monolayer cultures of HBMEC. These effects may lead to alteration of both the functional and the molecular properties of the BBB, which could increase trafficking of HIV and HIV-1 infected cells into the CNS and contribute to the pathogenesis of HAD. HIV-1 Gp120 from both CCR5 and CXCR4-tropic viruses were shown in a monolayer of HBMEC to diminish integrity and to increase permeability and monocyte migration through CCR5/CXCR4 receptor-mediated protein kinase C activation and [Ca²⁺]i release (24, 25). In addition, Gp120 was shown to alter TJ protein expression and brain endothelial cell permeability by a mechanism involving substance P (SP) (26–30), although the molecular mechanisms of these Gp120-mediated effects are not well defined. It is widely reported that SP and its receptor (neurokinin-1R) are potent modulators of neuroimmunoregulation and HIV/AIDS infection. SP was shown to induce and mediate inflammation, angiogenesis, infections, intestinal mucosal immunity, and stress. SP is able to activate several immune cells, such as CD4⁺ and CD8⁺ T lymphocytes, mast cells, NK cells, and macrophages.

Endocannabinoids are involved in neuroprotection through a number of biochemical pathways (31). Neuroprotective effects have been described for the 2-arachidonoylglycerol (2-AG) endocannabinoid in several neurotoxicity models (32–34). The identification of cannabinoid receptors (CB1 and CB2) and their endogenous lipid ligands has triggered an exponential growth of studies exploring the endocannabinoid system and its regulatory functions in health and disease. Cannabinoids (active ingredients in marijuana) have been shown to be useful in the treatment of some cancer patients and to reduce pain and improve the quality of life in patients with AIDS (31). However, whether cannabinoid agonists can initiate protective activities by shielding the BBB and protecting HBMEC following Gp120-mediated damage and loss of integrity is not known.

The endocannabinoids 2-AG and anandamide are endogenous cannabinoids as well as members of the eicosanoid class of cannabinoids, which are arachidonic acid derivatives. Anandamide is a natural endocannabinoid that undergoes rapid enzymatic degradation by the enzyme fatty acid amide hydrolase (termed FAAH). Inhibition of FAAH enzyme causes accumulation of anandamide in sites of its natural production (31–34).

The study of permeability across the BBB should use a reproducible in vitro model that recapitulates the functional and structural properties of the BBB in situ. During brain development, brain microvascular endothelial cells (BMEC) gradually acquire the ability to form a highly selective barrier. Astrocytes play an inductive role in this process (35). BMEC are joined together by intercellular TJ that are responsible for the acquisition of selective permeability. This property is specific to brain TJ and probably depends on the higher density of proteins such as occludins and ZO-1 in the BBB TJ. Cocultures of BMEC with astrocytes established a tight permeability barrier across the BMEC monolayer as was demonstrated by a significant increase in transendothelial electrical resistance (TER) of ~1000  ohm × cm² as compared with TEER of ~350  ohm × cm² for HBMEC monolayers, as well as showed significant reduction in the paracellular permeability for [³H]inulin and [¹⁴C]sucrose, as compared with BMEC alone (35–39).

The BBB model consists of HBMEC and astrocytes cocultured on opposite sides of gelatin-coated tissue culture inserts that permit astrocyte processes to penetrate the insert and establish contact with the HBMEC as described previously (35–39). In this model, HBMEC differentiate and express BBB markers as a result of contact with astrocytes, such as the glucose transporter GLUT-1 and γ-glutamyl transpeptidase, and have enhanced expression of TJ proteins. The dynamic in vitro model of the BBB mimics both functionally and anatomically the brain microvascular endothelial cells, creating quasi-physiological conditions for the coculturing of human endothelial cells and astrocytes.

In this study, using in vitro and in vivo BBB models, we demonstrate that CB1-based cannabinoid agonists and the FAAH inhibitor URB597 prevented the down-regulation of the TJ ZO-1 and claudin-5 expression, as well as inhibited Gp120-mediated damage of brain endothelium. These data support a potential therapeutic role for cannabinoid agonists as protective agents that preserve BBB integrity and may improve the pathogenesis of HIV-associated neurodegenerative diseases such as HAD.

Materials and Methods

Reagents and materials

The following reagents were used: mAbs for Von Willebrand factor (Santa Cruz, Biotechnology); actin (Chemicon); Gp120 MN protein (Protein Science); anti-CB1, anti-CB2, anti-GAPDH, and NK-1R Abs (Santa Cruz Biotechnology); anti-ZO-1 Ab and anti-claudin-5 (Chemicon); tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-mouse IgG (Dako-Cytomation); Titanium One-Step RT-PCR kit (BD Biosciences); BD Advantage 2 PCR kit (BD Biosciences); and Substance P ELISA kit (Cayman Chemical Company).

HBMEC

HBMEC were purchased from Cell Systems. The cells were grown to confluence in 100-mm plastic dishes (Corning) using CSC complete medium (Cell Systems) containing 10% FBS with a seeding density of 7.5 × 10⁴/cm². After 24 h incubation at 37°C in an incubator containing humidified atmosphere and 5% CO₂. The HBMEC used in our study formed tubular-like networks on matrigel and had the ability to uptake acetylated low-density lipoprotein, indicating that these cells maintained the signature properties of brain endothelial cells. The cells also produced von Willebrand factor and GLUT-1 and γ-glutamyl transpeptidase endothelial-specific markers (40–42). Cells were used only up to passage 6. HBMEC were recharacterized regularly to confirm that the cells retained their CNS properties. The cells were restained with Abs for Von Willebrand factor, a marker for endothelial cells. These cells were cultured regularly for astrocyte contamination by staining with anti-glia fibrillary acidic protein and found to be free of such cells.

Cocultures of HBMEC and human astrocytes

Human astrocytes were obtained from Science Cell Research Laboratories and were cultured based on the protocol provided by the company. Astrocytes were characterized to be >95% immunoreactive for glial fibrillary acidic protein. Astrocytes were used from passages 3–7 for the experiments as described below. The astrocyte-endothelial cocultures were established
as a BBB model system using the Transwell coculture system (Corning Costar) as described (36). Briefly, HBMEC and astrocytes were cocultured on opposite sides of poly-L-lysine-coated tissue culture inserts with 8-μm pores (Corning) that permit astrocyte processes to penetrate the insert and establish contact with the endothelial cells as described previously (36). In pores (Corning) that permit astrocyte processes to penetrate the insert and establish contact with the endothelial cells as described previously (36). In pores (Corning) that permit astrocyte processes to penetrate the insert and establish contact with the endothelial cells as described previously (36). In pores (Corning) that permit astrocyte processes to penetrate the insert and establish contact with the endothelial cells as described previously (36).

To study the effect of HIV-1 Gp120 and TNF-α on the fluid phase permeability of HBMEC, A. HBMEC were cultured to confluence on Transwell polycarbonate membranes and were incubated with HIV-1 Gp120 (10 ng/ml) or with TNF-α (10 ng/ml) for 6 and 24 h, as indicated. After 4 h of exposure to Lucifer Yellow (LY), the amount of LY in the lower chamber was measured. Control cells, untreated for each time point, were also used in measuring LY. The results are the mean ± SD of three experiments. **, p < 0.01. B. Monocyte transmigration across HBMEC monolayers. Cells were untreated (−) or treated (+) with Gp120 (10 ng/ml). For treatment with Abs, mAbs against Gp120 (α-Gp120; used at 20 μg/ml), CXCR4 Ab (α-CXCR4), or isotype control Abs (α-Control) were used (at a concentration of 30 μg/ml). Abs were added to the HBMEC monolayers for 2 h before fresh isolated monocytes were added to the upper chamber. Monocytes transmigrated across the HBMEC were counted (n = 4 per condition). The results are the mean ± SD of three experiments. **, p < 0.01.

**FIGURE 1.** The effect of HIV-1 Gp120 and TNF-α on the fluid phase permeability of HBMEC. A. HBMEC were cultured to confluence on Transwell polycarbonate membranes and were incubated with HIV-1 Gp120 (10 ng/ml) or with TNF-α (10 ng/ml) for 6 and 24 h, as indicated. After 4 h of exposure to Lucifer Yellow (LY), the amount of LY in the lower chamber was measured. Control cells, untreated for each time point, were also used in measuring LY. The results are the mean ± SD of three experiments. **, p < 0.01. B. Monocyte transmigration across HBMEC monolayers. Cells were untreated (−) or treated (+) with Gp120 (10 ng/ml). For treatment with Abs, mAbs against Gp120 (α-Gp120; used at 20 μg/ml), CXCR4 Ab (α-CXCR4), or isotype control Abs (α-Control) were used (at a concentration of 30 μg/ml). Abs were added to the HBMEC monolayers for 2 h before fresh isolated monocytes were added to the upper chamber. Monocytes transmigrated across the HBMEC were counted (n = 4 per condition). The results are the mean ± SD of three experiments. **, p < 0.01.

**FIGURE 2.** CB1 cannabinoid receptor expression analysis in HBMEC. A. RNA expression by RT-PCR analysis. Total RNA was extracted from HBMEC. Using the Titanium One-Step RT-PCR kit (BD Biosciences), the primer sequences for CB1 were 5′-GCTTGCGGTGGCCAAGCTTC-3′ (sense) and 5′-GACGCACGCCGATCAATGG-3′ (antisense). The size of the cDNA fragment obtained by RT-PCR was 278 bp for CB1. Lane M, m.w. standards. Lane 1, CB1 cannabinoid receptor; B. Protein expression analysis by Western blot (WB) assay of HBMEC. Total cell lysates were obtained from HBMEC that were either untreated or treated for 4 h with the cannabinoid agonists THC (10 nM) or the endogenous cannabinoid agonist 2-AG (10 nM). In some lanes, HBMEC were pretreated with the CB1 specific inhibitor AM251 (4 h at 10 nM). Cell lysates were analyzed by Western blotting with specific Abs against CB1 (Santa Cruz Biotechnology). For equal loading, we used GAPDH as control. C. Protein expression analysis of CB1 and CB2 by Western blot assay in HBMEC. Total cell lysates obtained from untreated HBMEC or HBMEC treated with exogenous cannabinoid agonist THC (4 h; 10 nM) or CP55940 (1 h; 1 ng/ml) or with the endogenous cannabinoid agonist 2-AG (1 h at 10 nM), as indicated.

**Table I. Effect of Gp120 on SP secretion**

<table>
<thead>
<tr>
<th>Treatment (pg/ml)</th>
<th>SP Secretion (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-HBMEC</td>
<td>163 ± 25</td>
</tr>
<tr>
<td>HBMEC plus Gp120</td>
<td>255 ± 17</td>
</tr>
<tr>
<td>HBMEC plus Gp120 plus control Ab</td>
<td>244 ± 22</td>
</tr>
<tr>
<td>HBMEC plus Gp120 plus Gp120 Ab</td>
<td>183 ± 18</td>
</tr>
<tr>
<td>HBMEC plus Gp120 plus spantide (SP antagonist)</td>
<td>166 ± 32</td>
</tr>
</tbody>
</table>

* HBMEC (2.5 × 10⁵ cells) were cultured in the presence of Gp120 (10 ng/ml for 6 h) alone or Gp120 with control Ab (20 μg/ml), Gp120 Ab (20 μg/ml), or the SP antagonist spantide (1 ng/ml). The secretion of SP in the supernatant was measured using a Substance P ELISA kit (Cayman Chemical). The results are the means of determinations from three experiments (each in quadruple) ± SD.

**SP treatment**

Confluent cultured endothelial cells were treated with SP (1 ng/ml) or control vehicle (0.9% NaCl solution, normal saline) dissolved in growth medium for the indicated times. Cells were then washed and harvested for immunoblotting or immunoprecipitation assays or fixed with 3.7% paraformaldehyde for immunocytochemical study. SP concentration was measured by the Substance P ELISA kit (Cayman Chemical).

**Gp120 activation of HBMEC**

HIV-1 Gp120MN protein was used in all experiments at a concentration of 10 ng/ml or as indicated. As a control, we used heat-inactivated Gp120 (10 ng/ml) and/or Gp120 Ab as indicated.

**Cell preparation for electrophoresis and Western blot analysis**

HBMEC were lysed using lysis buffer (20 mM HEPES, 0.42 NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 2% Nonidet P-40,
FIGURE 3. The effects of cannabinoids on calcium ion flux alterations following SP treatment of HBMEC. A, A calcium ion flux alteration assay was performed following SP treatment in HBMEC. a, The calcium ion concentration in the HBMEC control; b, the calcium ion concentration of HBMEC following treatment with SP (5 min at 5 ng/ml); c, the calcium ion concentration of HBMEC treated with CP55940 (1 h at 1 ng/ml); d, the calcium ion concentration of HBMEC treated with CP55940 (1 h; 1 ng/ml) followed by SP treatment (5 min at 5 ng/ml). B, Measurement of Ca\textsuperscript{2+} concentration ratio in HBMEC samples.

RT-PCR analysis of CB1 mRNA

Total RNA was extracted from HBMEC with TRIzol (Invitrogen) and CB1 primers individually. One microgram of RNA was reverse-transcribed and followed by PCR using a Titanium One-Step RT-PCR kit (BD Biosciences). The primer sequences for CB1 were 5'-GCCTGGCGGTGGCA GACCTCC-3' (sense) and 5'-GACGACGGCGATACAATGG-3' (antisense); PCR products were electrophoretically separated on 1.2% agarose gels and visualized by ethidium bromide staining. The size of the cDNA fragments obtained by RT-PCR was 278 bp for CB1.

For genotyping of CB1\textsuperscript{-/-} and CB2\textsuperscript{-/-} mice, DNA was extracted from the tails followed by PCR analysis using an Advantage 2 PCR kit (BD Biosciences).

Calcium ion concentration assay

HBMEC were treated with the calcium-sensitive dye fluo-3-acetoxymethyl ester (fluo-3-AM) for 1 h. Cells were then washed twice with PBS and analyzed using confocal microscopy analysis to detect the free cytosolic calcium concentration at 488 nm. SP (1 ng/ml), calcium ionophore A23187 (10\textsuperscript{-5} M), and EDTA (5 × 10\textsuperscript{-3} M) were applied individually and calcium ion concentration alterations in cultured cells were determined.

Immunostaining and quantitation of tight junctions

HBMEC were fixed with 3.7% paraformaldehyde in PBS containing 0.2% Triton X-100 (T-PBS) for 20 min at room temperature. The fixed cells were washed three times with T-PBS for 10 min each, then soaked in 10% nonfat milk-T-PBS blocking solution at room temperature for 30 min and the supernatant was analyzed by 10% SDS-PAGE. The proteins on the gel were transferred to polyvinylidene difluoride membranes (NEF Life Science Products, Boston, MA) for 4 h. The polyvinylidene difluoride membrane was soaked in 5% nonfat milk-Tween 20-TBS (t-TBS) blocking solution containing anti-CB1 Ab (Santa Cruz Biotechnology) or anti-ZO-1, anti-claudin-5, anti-junctional adhesion molecule (JAM)-1, or GAPDH Abs (Chemicon) as the primary Ab. Actin (Chemicon) was also used for comparison of protein content in the different groups. The membranes were then washed with t-TBS for 5 min four times and incubated with a t-TBS solution containing goat anti-mouse IgG or anti-rabbit IgG (Amersham Pharmacia) as the secondary Ab for 2 h. Target proteins were detected using ECL (ECL kit, PerkinElmer Life Sciences) and autoradiographed with x-ray film (Fuji Film).

Restriction of paracellular transport of small ions was determined by analysis of TEER. For each filter, the electrical resistance system with current-passing and voltage-measuring electrodes was used. TEER (ohm cm\textsuperscript{2}) was calculated from the display electrical resistance on the readout screen by subtraction of the electrical resistance of a blank filter and a correction for filter surface area. The resulting TEER values represented the resistance ("tightness") of the endothelial cell monolayers. The relative ratio between the TEER value for the HBMEC samples and the TEER value of control untreated HBMEC was calculated for each sample.

In vivo permeability of the BBB by d-mannitol

BBB "opening" in mice was induced by 1.6 M d-Mannitol infusion as described (43). This "opening" of the BBB was shown to be mediated by SP (44, 45). Damage to the BBB was judged by extravasation of Evans blue (EB) dye (43, 46). Fifteen minutes after 1.6 M d-Mannitol infusion, EB dye was injected i.v. (20 mg/kg body weight). After 1 h, animals were anesthetized with an i.p. injection of 100 mg/kg body weight ketamine hydrochloride and were transcardially perfused with normal saline to clear blood vessels of the dye. The brains were then removed, the hemispheres separated, EB was extracted, and its fluorescence was measured using a fluorospectrophotometer at a wavelength of 620 nm. Calculations were based on external standards in the solvent (100–500 ng/ml). Eluted EB dye was expressed as grams per milligram of brain tissue.

Statistical analysis

The values for the permeability assay, endothelial migration assay, and transendothelial electrical resistance are presented as the mean ± SD. The one-way ANOVA test was used to compare the means of three groups of data. If the one-way ANOVA test indicated an overall significant difference (p < 0.05), Tukey’s multiple comparison test was used to determine significant differences in the mean between any two groups. The independent t test was used to analyze data from the Western blot assay. Differences were considered to be significant at p < 0.05.
Results

Effects of HIV-1 Gp120 on HBMEC permeability

To analyze the effects of HIV-1 Gp120 on HBMEC permeability, the fluid phase permeability changes of HBMEC were measured by the colorimetric method. An increase in transcytosis was observed after the HIV-1 Gp120 treatment of HBMEC (Fig. 1A). The increase in transcytosis was similar to that obtained upon treatment of HBMEC with TNF-α/H9251, a well-characterized and potent cytokine known to induce transcytosis (Fig. 1A) (47). Heat-inactivated Gp120 had no significant effect on the permeability of HBMEC (data not shown).

Next, to investigate the involvement of HIV-1 Gp120 in the transendothelial migration of monocytes, cells were treated for 6 h with Gp120 (10 ng/ml) or treated with heat-inactivated Gp120 as controls, as described previously (25). Freshly isolated monocytes were isolated as described (25) and were added to the upper compartment of Transwell tissue culture inserts containing HBMEC monolayers and allowed to migrate for 2 h (37°C in 5% CO2). The migrated monocytes in the lower chamber were stained with a macrophage marker (CD68) and counted as described (25). Each treatment condition was performed in triplicate. As shown in Fig. 1B, treatment of HBMEC with Gp120 significantly enhanced monocyte transmigration. Furthermore, the Gp120-induced monocyte migration was inhibited by specific Abs for Gp120 (H11011/70%) and by specific Abs for CXCR4, whereas control isotype Ab had no inhibitory effects. No effects on migration were observed with the heat-inactivated Gp120 protein (data not shown). Polymyxin B also did not affect the Gp120-induced transmigration of monocytes, indicating that the Gp120 effect was not due to a contamination with LPS (data not shown). Migration in response to MCP-1 (10 ng/ml) served as a positive control (data not shown) (48).

Gp120 enhances SP expression in HBMEC

Because HIV-1 Gp120 was reported to induce SP secretion (26–28), we then examined the expression of SP in HBMEC. When HBMEC were cultured in the presence of HIV-1 Gp120, these cells secreted high levels of SP (Table I). Secretion of SP was blocked by Gp120 Ab or by the SP antagonist spantide.
The effects of HIV-1 Gp120 and SP on Ca\(^{2+}\) influx in HBMEC

Cannabinoid agonist CP55940 inhibited Gp120-mediated calcium influx in HBMEC

Expression of cannabinoid receptors CB1 and CB2 in HBMEC

CB1 mRNA expression was found in HBMEC by RT-PCR analysis (Fig. 2A). Western blot analysis showed positive expression of CB1 protein in HBMEC and this expression was up-regulated in the presence of the cannabinoid receptor ligands Δ⁹-tetrahydrocannabinol (THC) and 2-AG or the potent synthetic cannabinoid receptor agonist CP55940 (Fig. 2, B and C). However, in the presence of the CB1-specific inhibitor AM251, the expression of CB1 was significantly decreased in the THC-treated cells but not in 2-AG-treated cells (Fig. 2B). AM251 is a specific inhibitor for CB1-based cannabinoid agonists (such as THC), but not CB2-based cannabinoids (such as 2-AG). The increased expression levels of CB1 within 1 h strongly suggest that upon binding of a cannabinoid agonist to CB1 receptor, the cannabinoid agonist prevents CB1 degradation. The down-regulation of CB1 by HIV-1 Gp120 was on the level of protein (translation level) and not on the level of mRNA (transcription level) (data not shown). In addition, we examined the expression of CB2 in HBMEC. CB2 expression was detected by RT-PCR analysis (data not shown) and was up-regulated by the 2-AG and CP55940 agonists, but not by THC, which has low binding affinity to CB2 (Fig. 2C).

Cannabinoid agonist CP55940 inhibited Gp120-mediated calcium influx in HBMEC

The effects of HIV-1 Gp120 and SP on Ca\(^{2+}\) influx in HBMEC monolayer cultures was reported recently (25). Gp120 mediated its effects on Ca\(^{2+}\) influx through SP secretion. SP significantly increased the calcium ion concentration in the cocultures of the HBMEC/astrocyte model, which was blocked by the cannabinoid agonist CP55940 (Fig. 3).

Effects of cannabinoids on HIV-1 Gp120 mediated down-regulation of ZO-1 and claudin-5 expression in HBMEC

The TJ proteins ZO-1 and claudin-5 are central molecules in maintaining the integrity of the TJ (2, 3). To examine the changes in TJ protein expression in HBMEC following treatment with HIV-1 Gp120, cocultures of HBMEC were immunostained with specific Abs against ZO-1 and claudin-5. The localization of ZO-1 (Fig. 4A) and claudin-5 (Fig. 4B) at junctional regions between endothelial cells is consistent with the expression of high-resistance TJ, a hallmark of microvascular endothelial cells of the brain that define the BBB. The staining patterns of ZO-1 and claudin-5 in HBMEC demonstrate the integrity of the HBMEC system in significantly retaining the localization of these TJ-associated proteins. Upon exposure to Gp120, the expression of both ZO-1 and claudin-5 at interendothelial junctional regions was markedly altered in cultured HBMEC (see color Fig. 4). When the time of Gp120 exposure was extended to 8 h, the effects were more significant for both ZO-1 and claudin-5 (data not shown). For immunostaining and quantitation of TJ, HBMEC were fixed and immunostained with specific Abs against ZO-1, claudin-5, or control isotype Abs. TJ proteins were visualized using fluorescence microscopy (Leica). The relative staining along intercellular borders (presumed sites of junctional contacts) was determined by quantitative fluorescence analysis. The numbers of cells expressing the labeled markers along the entire lateral cell membrane were then determined by counting. At

FIGURE 5. Inhibition of permeability alteration of brain microvascular endothelial cells following HIV-1 Gp120 treatment in a human BBB model by cannabinoids. A–C, HIV-1 Gp120 (A and C; 10 ng/ml for 6 h) and SP (B; 1 ng/ml for 5 min) induced alterations in the permeability of HBMEC, as indicated by TEER. HBMEC cultures were treated with cannabinoid agonists such as CP55940 and ACEA (10⁻⁶ M for 1 h) in the presence (+) or absence (−) of cannabinoid antagonist CB1-specific inhibitor AM251 (1 h at 10 nM) or with SP inhibitor (1 h at 1 ng/ml) or URB597 (1 ng/ml for 1 h), as indicated. For treatments of cannabinoids plus Gp120, the cultures of HBMEC were treated with Gp120 (10 ng/ml for 6 h) plus cannabinoid compounds (as indicated above). The relative ratio of TEER in Gp120-treated samples as compared with untreated control HBMEC was measured for each sample; n = 8. The p values with an asterisk (*, p < 0.05; **, p < 0.01) shows significant differences from specific groups. This is a representative experiment of eight. The value of TEER with medium alone was 937.7 ± 9.4. The results are expressed as mean percent of controls ± SD. D, Inhibition of SP secretion by cannabinoids in a human BBB model following Gp120 treatment. Cannabinoid compounds (ACEA and CP55940), in the presence (+) or absence (−) of CB1-specific inhibitor AM251, were added to HBMEC in BBB cocultures as indicated. Bars represent the mean ± SD; n = 3. The p values with asterisk (**, p < 0.01) show significant differences from the indicated specific groups. This is a representative experiment of three.
least 25 different fields derived from five independent experiments were counted for each marker and time point.

Next, we examined the potential of cannabinoid agonists to prevent Gp120-mediated damage of TJ in HBMEC. Addition of the cannabinoid agonist CP55940 prevented the down-regulation of ZO-1 and claudin-5 in HBMEC following treatment with Gp120, leading to continuous uniform and linear TJ structures that preserved the integrity of the TJ in the HBMEC (Fig. 4, A and B). Approximately 70% of the expression levels of ZO-1 and 90% of the expression levels of claudin-5 were observed in the presence of CP55940 cannabinoid agonist, as compared with control samples (Fig. 4C).

Cannabinoids prevent decrease in ZO-1, JAM-1 and claudin-5 expression in HBMEC following exposure to HIV-1 Gp120

Because the reduced intensity of immunostaining of ZO-1 and claudin-5 at interendothelial junctions could be derived from alterations in subcellular distribution and/or the expression level of these proteins, Western blotting of whole cell lysates was performed to assess changes in TJ protein content. Western blot analysis using specific Abs for ZO-1, claudin-5, JAM-1, and actin was performed to quantitate the down-regulation of TJ. The intensity of immunoblotted bands of ZO-1 and claudin-5 in cell lysates was compared with that of actin. HIV-1 Gp120 treatment of HBMEC resulted in a decrease in claudin-5, JAM-1, and ZO-1 content (Fig. 4D), whereas addition of the cannabinoid agonist CP55940 prevented the HIV-1 Gp120-induced down-regulation of ZO-1, JAM-1, and claudin-5 (Fig. 4D). The effects of Gp120 on down-regulation of TJ expression was on the level of translation (protein level) and not on the level of mRNA (transcription level) (data not shown). Thus, CP55940 has protective effects on HBMEC by preserving TJ expression in these cells.

Effects of cannabinoid agonists on the inhibition of permeability changes induced by Gp120 in the human BBB model system using TEER assay

The permeability of the BBB coculture model system was analyzed by measuring TEER (49). The TEER values represented the resistance (“tightness”) of the endothelial cell. Both Gp120 and SP induced significant changes in HBMEC permeability (Fig. 5, A–C). The CB1-specific agonist arachidonyl-2′-chloroethylamide (ACEA) and the cannabinoid ligand CP55940 inhibited the permeability changes of HBMEC induced by Gp120 and restored the integrity of the HBMEC as compared with the untreated HBMEC (Fig. 5A). Furthermore, the SP inhibitor, alone or together with ACEA, blocked the SP-mediated damage of HBMEC (Fig. 5B). Next, we analyzed the effects of endocannabinoids on HBMEC permeability. Treatment with the FAAH inhibitor URB597, which...
blocks anandamide’s metabolic degradation and results in accumulation of anandamide levels, was also able to inhibit HIV-1 Gp120-mediated permeability changes of HBMEC (Fig. 5C). Finally, HIV-1 Gp120 induced SP secretion up to ~350 pg/ml, which was inhibited by both cannabinoid agonists, ACEA and CP55940 (Fig. 5D). These HIV-1 Gp120-mediated effects on SP secretion were modulated via the CB1 receptor (Fig. 5D), because the CB1-specific inhibitor AM251 prevented these protective effects of cannabinoids.

Analysis of endothelial cell integrity and ZO-1 localization following HIV-1 Gp120 treatment in a BBB coculture model and its modulation by cannabinoids

Endothelial integrity is regulated by the cytoskeleton and TJ. The linkage between actin and ZO forms the strong micro-framework of the endothelial cells. To examine the changes in ZO-1 TJ protein expression in HBMEC following treatment with HIV-1 Gp120, HBMEC were immunostained with specific Abs against ZO-1. As shown in color Fig. 6A, ZO-1 expression was detected in TJ in HBMEC but was significantly decreased in HBMEC treated with HIV-1 Gp120. Treatment with HIV-1 Gp120 resulted in discontinuous uniform and linear structures of TJ, and the TJ were completely damaged, forming unconnected tight junctional structures. However, addition of CP55940 prevented the down-regulation of ZO-1 expression in HBMEC following treatment with HIV-1 Gp120, leading to continuous uniform and linear TJ structures that preserved the integrity of the TJ in HBMEC. Interestingly, significant colocalization of ZO-1 and CB1 in the TJ in HBMEC was induced following treatment with CP55940 and was quantitated by confocal analysis in the Gp120 plus CP55940-treated cells, as compared with untreated cells and cells treated with Gp120 (Fig. 6B).

Cannabinoids increased the association of CB1 with ZO-1 in HBMEC

Because ZO-1 and CB1 were colocalized in the presence of cannabinoid agonists (Fig. 6), we then analyzed the association of CB1 with ZO-1. Endogenous association of ZO-1 with CB1 was observed in HBMEC in the presence of the cannabinoid agonist CP55940 (Fig. 6C). The association of ZO-1 with CB1 was mediated via the NH2 terminus of ZO-1 but not the C terminus of ZO-1 (Fig. 6D). Treatment of HBMEC led to down-regulation of ZO-1 and CB1 expression. However, in the presence of cannabinoid agonist, both CB1 and ZO-1 expression were preserved.

Inhibition of the transmigration of human monocytes across the BBB by cannabinoid agonists

To further investigate the protective effects of cannabinoid agonists on HBMEC permeability, we first examined the transmigration of normal human monocytes across the HBMEC/astrocyte cultures following exposure to HIV-1 Gp120 in the presence or absence of cannabinoid agonist CP55940 (which binds to both CB1 and CB2) or the CB1-specific agonist ACEA. Normal human monocytes were isolated as described (25). As shown in Fig. 7A, Gp120 induced the migration of monocytes that was inhibited by ACEA and CP55940, suggesting that CB1-based cannabinoid compounds may mediate their protective effects on brain microvascular endothelium and prevent monocyte transmigration following exposure to HIV-1 Gp120.

Cannabinoid agonists inhibited BBB permeability in vivo

We next used an in vivo model to examine the potential of cannabinoids in preventing BBB permeability changes. We used a known method of high osmotic solution (1.6M D-mannitol) to increase the permeability of the BBB in mice using Evans blue dye as a macromolecular marker (43, 46). This opening of the BBB was shown to be mediated by SP (43, 46). BBB “opening” in mice was induced by 1.6 M D-mannitol infusion as described (43, 46). Damage to the BBB was judged by EB dye (43, 46). Fifteen minutes after 1.6 M D-mannitol infusion, EB dye was injected i.v. (20 mg/kg body weight). After 1 h, animals were anesthetized and transcardially perfused with normal saline to clear blood vessels of the dye. The brains were then removed, the hemispheres separated,
and EB was extracted and measured using a fluorospectrophotometer at a wavelength of 620 nm. Calculations were based on external standards in the solvent (100–500 ng/ml). Eluted EB dye was expressed as grams per milligram of brain tissue. As shown in Fig. 7B, the high osmotic solution induced permeability changes in the BBB. These permeability changes were correlated with the expression levels of ZO-1 and claudin-5 and to the damaged TJ in the BBB of these animals. Both SP inhibitor and cannabinoid agonist ACEA significantly reduced the BBB permeability changes in vivo and inhibited the opening of the BBB in mice (Fig. 7B).

Next, frozen brain sections from untreated mice and mice treated with D-mannitol (to induce lesions) were fixed, permeabilized, and stained with specific Abs against ZO-1 and claudin-5. ZO-1 expression in the normal brain endothelium was more abundant and continuous in control mice, whereas down-regulation of both ZO-1 and claudin-5 were observed in the lesioned areas of treated mice (Fig. 7C).

Taken together, these results demonstrate that cannabinoid agonists prevented down-regulation of ZO-1, JAM-1, and claudin-5 expression in HBMEC following HIV-1 Gp120-mediated insult, resulting in inhibition of the permeability changes and sustaining the integrity of BMEC and the BBB, using cocultures of HBMEC/astrocytes and in vivo mice.

Discussion
To date, there is little known about how the BBB could be modulated to help prevent the development of HAD. In addition, there is no effective therapy to stop or prevent damage of the BBB during the HAD disease cascade. The search for interventions to protect against destruction of the BBB might help treat diseases that feature BBB damage. Using a human BBB model involving cocultures of HBMEC and human astrocytes as well as an in vivo mouse model, our results and those of others (30) showed that the secreted HIV-1 protein Gp120, which plays a major role in HAD, induced SP secretion and increased BBB permeability. More specifically, both Gp120 and SP decreased ZO-1 and claudin-5 protein expression, leading to increased BBB permeability (Figs. 4 and 5). These permeability changes were prevented by the cannabinoid agonist CP55940 and the CB1-specific agonist ACEA (Figs. 4–6). These cannabinoids prevented the down-regulation of ZO-1 and claudin-5 protein expression in HBMEC following Gp120 or SP-induced damage in HBMEC. Thus, cannabinoids are neuroprotective compounds that prevent the degradation of CB1 and the down-regulation of ZO-1 and claudin-5 TJ protein expression in HBMEC following insult, resulting in preservation of endothelial cell structure and function.

The endocannabinoid 2-AG was shown to have neuron-protective properties in animal models of ischemic brain injury (33). Endocannabinoids are involved in neuroprotection through numerous biochemical pathways. The endocannabinoid 2-AG was released in mouse brain after closed head injury, and treatment with exogenous 2-AG exerts neuroprotection via the central cannabinoid receptor CB1 (33). This process involves inhibition of inflammatory signals that were mediated by activation of the transcription factor NF-κB. 2-AG decreased BBB permeability and inhibited the acute expression of the main proinflammatory cytokines TNF-α, IL-1β, and IL-6. It also augmented the levels of endogenous antioxidants (33). In this study, we found that CP55940 and ACEA protected HBMEC against HIV-1 Gp120-related toxicity through a different mechanism. Addition of cannabinoid agonists such as ACEA and CP55940 inhibited SP secretion (Table I) and prevented the degradation of ZO-1 and claudin-5 in HBMEC following treatment with either HIV-1 Gp120 (Fig. 4) or SP, leading to continuous uniform and linear TJ structures that preserved the integrity of the TJ in the HBMEC (Fig. 4). The down-regulation of ZO-1 and claudin-5 was mediated by SP directly. However, the possibility that this down-regulation is also mediated by proinflammatory cytokines induced by SP, such as TNF-α, IL-6 and IL-1β, needs to be investigated in future studies. The current finding suggests that the mechanism by which
cannabinoids exert their effects on the BBB may involve inhibition of the early induction of SP by Gp120 through preservation of the TJ in HBMEC. These protective effects of cannabinoids are mediated through CB1 receptors, but not CB2 receptors, because AM251 (the specific inhibitor for CB1), but not AM630 (CB2-specific inhibitor) (data not shown), specifically blocked these protective effects on the HBMEC.

Circulating HIV-1 Gp120 might bind to anti-HIV-1 envelope Abs synthesized during early infection or may act on the BBB by altering the permeability of the brain microvascular endothelium, thus promoting the entry of HIV-1 into the CNS. This process might account for the early CNS involvement before overt neurological findings. Other proposed mechanisms, such as recruitment of HIV-1-infected monocytes mediated by increased adhesion molecule expression on the brain vessels, might be involved in the later stages of infection and amplify the CNS invasion (50). SP, a potent modulator of neuroimmunoregulation, and its receptor, NK-1R, may be involved in the modulation of HIV-1 infection, both in vivo and in vitro (26–29, 51) HIV-1-positive children have higher plasma levels of SP compared with HIV-1-negative children (51). SP was also found to play a critical role in the HIV-1 Gp120-induced increase in permeability of the rat brain microvascular endothelium, and this effect of SP was abrogated by the SP antagonist (spantide) and/or by anti-SP polyclonal Ab (52). In addition, significant SP immunoreactivity was observed in HIV-1 Gp120 transgenic mouse brain vessels, suggesting that SP is involved in HIV-1 Gp120-induced changes in the vascular component of the BBB (52). In the present study, we demonstrate that HIV-1 Gp120 caused SP secretion in HBMEC (Table I) and that both HIV-1 Gp120 and SP resulted in significant permeability changes in these cells, using a human BBB culture model that enables selective study of the physiology, pharmacology, and pathophysiology of the BBB. Thus, HIV-1 Gp120, through induction of SP, plays a key role in the disruption of the BBB and facilitates the transmigration of monocytes and leukocytes into the brain (Fig. 7A). The increased transmigration was shown to be directly correlated with increased BBB disruption. In addition, using an in vivo mouse model, we showed that cannabinoid agonist inhibited permeability changes induced by D-mannitol through SP induction (Fig. 7B).

The TJ play a central role in sealing the intercellular space in adjacent endothelial cellular sheets (2, 3). Through these “barrier” and “fence” functions of TJ, endothelial cellular sheets establish various compositionally distinct fluid compartments. Therefore, TJ are considered to be fundamental structures in multicellular organisms (4). HIV-1 Gp120 was shown to compromise BBB integrity and enhance monocyte migration across the BBB (49). HIV-1 Gp120 was reported to cause disruption and down-regulation of ZO-1, ZO-2, occludin, and claudin-5 (50). Based on our results, both HIV-1 Gp120 and SP induced changes in ZO-1 and claudin-5 TJ expression and the TJ were damaged, resulting in unconnected tight junctional structures (Fig. 4).

Specific association of ZO-1 with CB1 through the NH2 terminus of ZO-1 was observed in the presence of cannabinoid agonists (Fig. 6). As shown in Fig. 6A, Gp120 induced the down-regulation of ZO-1 and CB1 expression in HBMEC. However, in the presence of cannabinoid ligands, CB1 binds to its cognate cannabinoid agonist, which prevents CB1 degradation. This “activated” CB1 is then able to bind ZO-1 and form a complex of ZO-1/CB1 in HBMEC, strongly suggesting that CB1, through its interaction with ZO-1, protects ZO-1 from HIV-1 Gp120-mediated degradation. In conclusion, the present study describes the mechanisms of HIV-1 Gp120-induced permeability changes in HBMEC, through SP, by down-regulation of ZO-1 and claudin-5 protein expression. Furthermore, we show that cannabinoid agonists are protective agents that prevent this down-regulation and thus preserve the expression of ZO-1 and claudin-5 in the BBB, resulting in sustained BBB integrity. In addition, using an in vivo mouse model of BBB opening by D-mannitol through SP modulation (44, 45), we observed significant down-regulation of both ZO-1 and claudin-5 in the lesion in vivo (Fig. 7C). Furthermore, we showed that the cannabinoid agonist ACEA as well as an SP inhibitor (Spantide) alone or in combination, significantly inhibited BBB permeability in vivo and prevented Gp120-induced down-regulation of TJ expression in HBMEC (Fig. 7B). These results provide important information on the therapeutic potential of cannabinoids and/or SP inhibitors to inhibit the transmigration of HIV-1-infected cells across HBMEC and to prevent damage of HBMEC following insult by HIV-1 secreted proteins such as HIV-1 Gp120 (Fig. 8). These studies should provide insights into preventive and/or therapeutics to sustain BBB integrity in patients who developed HAD, based on SP inhibitors and/or cannabinoid pharmacotherapies.

Acknowledgments
We thank Chiuang-Pei Wang for typing assistance and Janet Delahanty and Makara Men for editing the manuscript. We thank Xin Li for technical assistance. We thank Dr. Rick Rogers (Lab Imaging, Harvard Medical School, Boston, MA) for help in immunostaining studies of tight junctions.

Disclosures
The authors have no financial conflict of interest.

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