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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.
Infections, intestinal mucosal immunity, and stress. SP is able to was shown to induce and mediate inflammation, angiogenesis, in-
these Gp120-mediated effects are not well defined. It is widely
addition, Gp120 was shown to alter TJ protein expression and
protein kinase C activation and \( \text{Ca}^{2+} \)
monocyte migration through CCR5/CXCR4 receptor-mediated
of HBMEC to diminish integrity and to increase permeability and
increase trafficking of HIV and HIV-1 infected cells into the CNS
functional and the molecular properties of the BBB, which could
impaired neurodegenerative diseases such as HAD.

The HIV-1 envelope protein Gp120, in particular, has been pro-
posed as an etiologic agent of neuronal loss as well as for develop-
ment 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 cul-
tures 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 \([\text{Ca}^{2+}]_i\) 32 release (24, 25).
In addition, Gp120 was shown to alter TJ protein expression and
brain endothelial cell permeability by a mechanism involving sub-
stance 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 mod-
ulators of neuroimmunoregulation and HIV/AIDS infection. SP
was shown to induce and mediate inflammation, angiogenesis, in-
fecions, intestinal mucosal immunity, and stress. SP is able to
activate several immune cells, such as CD4+ and CD8+ T lympho-
cytes, 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) endo-
cannabinoid in several neurotoxicity models (32–34). The identi-
fication of cannabinoid receptors (CB1 and CB2) and their endog-
enous 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 mari-
juana) 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 ago-
nists 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 can-
nabinoids, which are arachidonic acid derivatives. Anandamide is
a natural endocannabinoid that undergoes rapid enzymatic degra-
dation 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 repro-
ducible in vitro model that recapitulates the functional and struc-
tural 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 estab-
lished a tight permeability barrier across the BMEC monolayer as
was demonstrated by a significant increase in transendothelial
electrical resistance (TEER) of \( \sim 1000 \text{ ohm cm}^2 \) as compared
with TEER of \( \sim 350 \text{ ohm cm}^2 \) for HBMEC monolayers, as well
as showed significant reduction in the paracellular permeability for
\([^3H]\text{inulin and }[^{14}C]\text{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 con-
tact with astrocytes, such as the glucose transporter GLUT-1 and
\( \gamma \)-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 dem-
strate that CB1-based cannabinoid agonists and the FAAH in-
hibitor 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-associ-
ated 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 Sci-
cence); anti-CB1, anti-CB2, anti-GAPDH, and NK-1R Abs (Santa Cruz
Biotechnology); anti-ZO-1 Ab and anti-claudin-5 (Chemicon); tetrameth-
ylrhodamine isothiocyanate (TRITC)-conjugated anti-mouse IgG (Dako-
Cytomation); Titanium One-Step RT-PCR kit (BD Biosciences); BD Ad-
vantage 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 me-
dium (Cell Systems) containing 10% FBS with a seeding density of 7.5 \times
(10^6)/cm^2 at 37°C in an incubator containing humidified atmosphere and 5%
\( \text{CO}_2 \). 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 en-
dothelial cells. The cells also produced von Willebrand factor and GLUT-1
transporter and \( \gamma \)-glutamyl transpeptidase endothelial-specific markers
(40 –42). Cells were used only up to passage 6. HBMEC were recharac-
terized 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 cultures were analyzed routinely for astrocyte con-
tamination by staining with anti-glial 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 fibrillar
acidic protein. Astrocytes were used from passages 3 –7 for the experiments
as described below. The astrocyte-endothelial cocultures were established

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as the glucose transporter GLUT-1 and this model, endothelial cells differentiate and express BBB markers, such as TJ proteins (36). Cocultures exhibit barrier function with 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.

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.

**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’-GCCTGGCGGTGCGACAGCTCC-3’ (sense) and 5’-GCAGCAAGGCTCAATATGG-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; 20 ng/ml) or with the endogenous cannabinoid agonist 2-AG (1 h at 10 nM), as indicated.

**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 at 1 ng/ml) followed by SP treatment (5 min at 5 ng/ml). B, Measurement of Ca$^{2+}$ concentration ratio in HBMEC samples.

### 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 three times with PBS and analyzed using confocal microscopy analysis to detect the free cytosolic calcium concentration at 488 nm. SP (1 μg/ml), calcium ionophore A23187 (10$^{-5}$ M), and EDTA (5 × 10$^{-5}$ 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-Triton X-100 (T-PBS) for 20 min at room temperature. The fixed cells were then washed with T-PBS for 5 min four times and incubated with a T-PBS 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 autographed with x-ray film (Fuji Film).

### 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’-GCCTGGCGGTGCA GACCTCC-3’ (sense) and 5’-GCAGCAGCCGAATGAA (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$^{-/-}$ and CB2$^{-/-}$ mice, DNA was extracted from the tails followed by PCR analysis using an Advantage 2 PCR kit (BD Biosciences).

25% glycerol, at 4°C. The mixture was centrifuged at 12,000 rpm at 4°C for 30 min and the supernatant was analyzed by 10% SDS-PAGE. The proteins on the gel were transferred to polyvinylidene difluoride membranes (NEN 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 control 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 autographed with x-ray film (Fuji Film).

#### 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 $p < 0.05$. 

### 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.
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. 1 A). 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. 1 A) (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 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, 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
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-6, and IL-1β. 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 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, 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 infection by HIV-1 secreted proteins such as HIV-1 Gp120 (Fig. 8). These studies should provide insights into preventive and/or therapeutic strategies 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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