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Interactive role of human immunodeficiency virus type 1 (HIV-1) clade-specific Tat protein and cocaine in blood-brain barrier dysfunction: Implications for HIV-1–associated neurocognitive disorder

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In recent years, increasing interest has emerged to assess the human immunodeficiency virus type 1 (HIV-1) clade C viral pathogenesis due to its anticipated spread in the United States and other western countries. Previous studies suggest that clade C is less neuropathogenic than clade B; however, the underlying mechanism is poorly understood. Additionally, the interactive role of drugs of abuse such as cocaine on clade C–associated neuropathogenesis has not been reported. In the current study, we hypothesize that HIV-1 clade-specific Tat proteins exert differential effects on blood-brain barrier (BBB) integrity and cocaine further differentially aggravates the BBB dysfunction. We evaluated the effect of Tat B and Tat C and/or cocaine on the BBB integrity using an *in vitro* model constructed with primary human brain microvascular endothelial cells (HBMECs) and astrocytes. The BBB membrane integrity was measured by transendothelial electrical resistance (TEER) and paracellular permeability was measured by fluorescein isothiocyanate (FITC)-dextran transport assay and monocytes transmigration across the BBB. Results indicate that Tat B disrupts BBB integrity to a greater extent compared to Tat C and cocaine further differentially exacerbates the BBB dysfunction. This BBB dysfunction was associated with altered expression of tight junction proteins zona occuldens (ZO-1) and junctional adhesion molecule (JAM)-2. Thus, these results for the first time delineate the differential role of Tat B and Tat C and/or cocaine in BBB dysfunction, which may be correlated with the clade-specific differences observed in HIV-1–associated neurological disorders. *Journal of NeuroVirology* (2010) **16,** 294–305.

Keywords: blood-brain barrier; cocaine; HAND; HIV-1 clades

Introduction

The human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) epidemic remains a major global public health concern. During the later stages of disease, HIV-1–infected individuals suffer from a wide range of neurological abnormalities collectively known as HIV-1–associated neurocognitive disorder (HAND) (Antinori *et al,* 2007; Goodkin *et al,* 2001; Zheng and Gendelman, 1997; Mintz, 1994; McArthur *et al,* 1993; Robertson and Hall, 1992). HAND is characterized by neuroinflammation that occurs due to viral-host interactions, which results in increased permeability and enhanced transmigration of HIV-1–infected mononuclear phagocytes across the blood-brain barrier (BBB), leading to neuronal injury (Kanmogne *et al,*

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2007; Toborek *et al,* 2005; Avison *et al,* 2004; Kaul *et al,* 2001). Under physiological condition BBB restricts the entry of many blood-borne elements such as macromolecules and circulating leukocytes from the blood compartment to the brain (Lesniak and Brem, 2004). The selective permeability of BBB is attributed to distinct morphological and enzymatic properties of human brain microvascular endothelial cells (HBMECs), which enable them to form continuous tight junctions with minimal endocytic activity (Abbott, 2002).

HIV-1 proteins are known to play a pivotal role in causing BBB dysfunction. Several previous studies have shown that treatment with HIV-1 Tat and gp120 increases endothelial permeability by modulating the expression and distribution of tight junction proteins and further accelerates the HIV-1 neurotoxicity (Arese *et al,* 2001; Kim *et al,* 2003; Pu *et al,* 2003; Oshima *et al,* 2000). Our present understanding about the neuropathology of HIV-1 infection stems mainly from clade B subtype and very little is known about clade C subtype. Interestingly, isolated reports suggest very low neuropathogenesis in type C infections compared to B clade infections (Wadia *et al,* 2001; Satishchandra *et al,* 2000). Previous studies with HIV-1 Tat C proteins suggest that Tat C protein is functionally defective for its chemotactic (Ranga *et al,* 2004) and neurotoxic properties (Mishra *et al,* 2008) and its ability to activate the *N*-methyl-D-aspartate (NMDA) receptor on neurons (Li *et al,* 2008). Furthermore, *in vivo* studies in mice show less neurocognitive impairments with HIV clade C virus infection compared to clade B virus (Rao *et al,* 2008). However, the effect of HIV-1 clade C proteins on BBB integrity and HBMEC functions has not been studied yet.

The last decade has witnessed a great, entangled epidemic of drug abuse and HIV-1 infection (Dhillon *et al,* 2008; Aceijas *et al,* 2004; Hamers *et al,* 1997; Edlin *et al,* 1994). Drugs of abuse such as cocaine, methamphetamine, morphine, and others are known to increase the risk of acquiring HIV infection and further exacerbate the progression of HIV-associated neurological disorders (Nath *et al,* 2002; Fiala *et al,* 1996; Larrat and Zierler, 1993). Cocaine is one of the most widely abused drugs in the United States, and its use is spreading to other parts of the world including India where clade C infection predominates. Previous studies have shown that cocaine exacerbates HIV-1 neuroinvasion by increasing the expression of adhesion molecules and matrix metalloproteinases (MMPs) in HBMECs, thereby perturbing the BBB permeability and increasing transendothelial migration of activated immune cells (Gan *et al,* 1999; Zhang *et al,* 1998). However, the interactive role of cocaine on neuropathogenesis of clade C infection remains to be elucidated. Therefore, we hypothesize that HIV-1 B and C Tat exert differential effects on HBMECs leading to disruption of BBB integrity, and this effect is differentially exacerbated with cocaine treatment.

In order to elucidate the role of clade-specific viral protein in development of HAND, we herein report differential effects of HIV-1 B and HIV-1 C Tat protein on BBB integrity using an *in vitro* model system. Further, to delineate the role of clade-specific diversity in synergizing with cocaine, we treated *in vitro* BBB model with clade-specific Tat proteins in presence or absence of cocaine under identical culture conditions. We report greater disruption of BBB integrity and higher transendothelial migration of monocytes by clade B Tat protein compared to clade C Tat protein and cocaine further exacerbates the observed effects by B Tat protein. The molecular basis of this phenomenon was demonstrated by differential modulation of tight junction proteins, zona occuldens (ZO-1) and junctional adhesion molecule (JAM-2).

Results

Clade-specific differences on the BBB integrity by HIV-1 Tat protein and/or cocaine

The hallmark of HIV-1–associated neuropathogenesis is marked by loss of BBB integrity. We evaluated the effect of HIV-1 proteins (Tat B and Tat C), cocaine, or cocaine plus HIV-1 proteins on BBB permeability using an *in vitro* BBB model. The integrity of the BBB model was assessed by transendothelial electrical resistance (TEER) measurement in control and treated cultures. No difference in TEER values was observed at 0 time between the control and treated samples. After 24 h of treatment, TEER values decreased to 74.5% ± 1.2% ($P < .03$) with 100 ng/ml Tat B, 86.8% ± 2% (NS) with 100 ng/ml Tat C, and 76.4% ± 1.5% ($P < .05$) with 100 nM cocaine; similarly the combined treatment with Tat B plus cocaine significantly decreased TEER to 54.8% ± 2.7% ($P < .002$) compared to Tat C plus cocaine (70.7% ± 3%; $P < .02$) (Figure 1). Moreover, longer treatments of 48 h resulted in an additional decrease in TEER with Tat B (51.9% ± 2.5%; $P < .009$), Tat C (72% ± 2.2%; $P < .05$), and cocaine (57.6% ± 3.2%; $P < .04$), whereas combined treatment with Tat B plus cocaine showed significantly higher reduction in TEER values to 35.9% ± 4% ($P < .008$) compared to 63.6% ± 3.2% ($P < .03$) induced by Tat C plus cocaine treatment (Figure 1). Also, the observed difference in TEER values between Tat B– and Tat C–treated cultures was found to be significant at 48 h ($P < .01$). TEER readings were also significantly lower for cotreatment of Tat B and cocaine with respect Tat C and cocaine at 24 and 48 h.

To further confirm and complement the TEER measurement results, we assessed the paracellular transport in an *in vitro* BBB model using

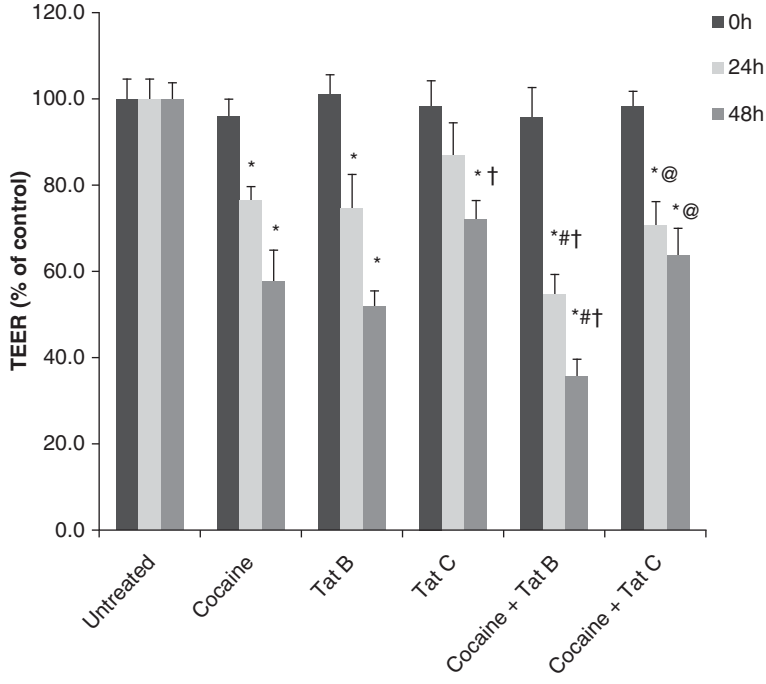

Figure 1 Effect of HIV-1 Tat proteins and/or cocaine on BBB membrane integrity. *In vitro* BBB model was established by growing primary HBMEC cultures in the upper side and HA cultures in the underside of 24-well tissue culture PET membrane inserts, pore size of 3 μm. After 5 days in culture, the BBB was then treated with 100 ng/ml Tat B, 100 ng/ml Tat C, and 100 nM cocaine, either alone or in combination, for 48 h. TEER (Ω/cm^2) was measured using a Millicell ERS system. Data are expressed as mean percent of controls ± SE of three independent experiments performed in replicates. Statistical significance was determined using Student's *t* test. *indicates statistical significance with respect to untreated control; #indicates statistical significance with respect to cocaine-treated cultures; †indicates statistical significance with respect to Tat B–treated cultures; and @indicates statistical significance between Tat B plus cocaine–treated and Tat C plus cocaine–treated cultures. We also evaluated effect of 100 nM Tat B and Tat C alone and in combination with 100 nM cocaine on BBB membrane integrity to account for the differences in molecular weight of Tat proteins and in ng/ml versus nM concentration. Results (not shown here) obtained were similar with that of 100 ng/ml Tat proteins.

FITC-dextran as a marker. Data presented in Figure 2 shows that individual treatment with Tat B, Tat C, and cocaine increased FITC-dextran transport by 25% ± 2% ($P < .009$), 10% ± 2% (NS), and 30% ± 3% ($P < .011$), respectively, compared to untreated control cultures. FITC-dextran transport by Tat B–treated cultures was significantly higher than Tat C–treated cultures ($P < .008$). The combined treatment of Tat B plus cocaine increased the FITC-dextran transport by 50% ± 1% ($P < .02$), which was significantly higher than individual treatment of Tat B ($P < .008$) or cocaine ($P < .02$). On the contrary, Tat C plus cocaine treatment demonstrated significantly less increase in FITC-dextran transport, 28% ± 2% ($P < .02$), compared to Tat B plus cocaine treatment. Also, the increase in FITC-dextran transport by combined Tat C plus cocaine treatment was not significantly different than the treatment of cocaine alone.

Differential alteration in transendothelial migration of monocytes across the BBB by HIV-1 Tat proteins and/or cocaine

Because cocaine or cocaine plus Tat proteins demonstrate synergistic effect on BBB membrane integrity as measured by TEER, we further evaluated whether the loss of BBB integrity was associated with increased transmigration of monocytes across the BBB. Our results (Figure 3) show an increase in monocytes migration with BBB cultures treated with Tat B, Tat C, and cocaine by 49% ± 5.3% ($P < .01$), 12% ± 4.8% (NS), and 67% ± 6.7% ($P < .01$), respectively. Tat B treatment showed significantly higher rate of monocyte transmigration compared to Tat C treatment ($P < .03$). Also, the combined treatment of Tat B plus cocaine caused significantly greater rate of monocytes migration, 156% ± 8.0% ($P < .003$), compared to Tat C plus cocaine treatment, 59% ± 4.0% ($P < .005$). The cotreatment results for Tat B plus cocaine were found to be significantly different compared to individual treatment of Tat B ($P < .0006$) and cocaine ($P < .02$). Additionally, the combined treatment of Tat C plus cocaine showed no significant difference in transmigration of monocytes compared to cocaine treatment alone. Thus, enhanced monocytes migration results may be correlated with diminished tightness of BBB membrane as shown in TEER studies (Figure 1).

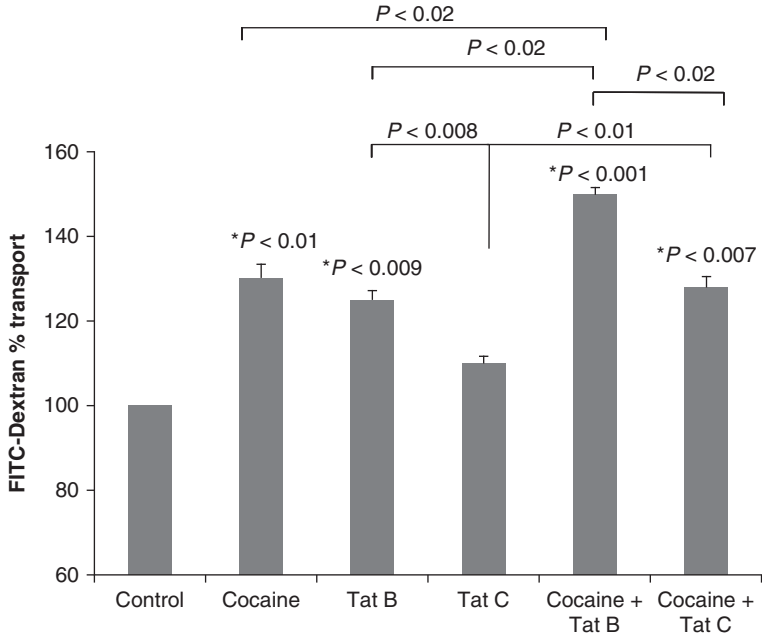

Figure 2 Effect of HIV-1 Tat proteins and/or cocaine on FITC-dextran transport in BBB model. FITC-dextran transport was measured in BBB model after 48 h of treatment with 100 ng/ml Tat B, 100 ng/ml Tat C, and 100 nM cocaine, either alone or in combination, followed by addition of FITC-dextran on the upper chamber of the insert. After 4 h of incubation, relative fluorescence units (RFUs) from the basal chambers of the inserts were measured. Results were expressed as % FITC-dextran transport with respect to the untreated control cultures and represented as mean ± SE of three independent experiments performed in replicates. Statistical significance was determined using Student's *t* test. *indicates statistical significance with respect to untreated control.

HIV-1 Tat B and Tat C and/or cocaine alter tight junction protein gene expression by primary HBMECs

Loss of BBB integrity is associated with alteration in expression of tight junction proteins such as ZO-1 and JAM-2 (Mahajan *et al,* 2008a, 2008b; Persidsky *et al,* 2006). Therefore, we analyzed the expression of tight junction proteins, ZO-1 (submembranous accessory protein) and JAM-2 (transmembranous protein) after treatment with Tat B, Tat C, and/or cocaine. Data presented in Figure 4a and b show the time kinetics of cocaine, Tat B, and Tat C effects on ZO-1 (Figure 4a) and JAM-2 (Figure 4b) gene expression by primary HBMECs at 12, 24, and 48 h of treatments. The results obtained indicate that primary HBMECs cultured with 100 nM cocaine significantly decreased ZO-1 gene expression at 12 h (transcript accumulation index [TAI] = 0.85 ± 0.050; $P < .05$), 24 h (TAI = 0.73 ± 0.03; $P < .03$), and 48 h (TAI = 0.69 ± 0.05; $P < .001$) compared to untreated control cultures. Tat B (100 ng/ml) treatment showed no significant effect on ZO-1 gene expression at 12 and 24 h, whereas a significant decrease was observed at 48 h (TAI = 0.85 ± 0.04; $P < .05$). However, Tat C did not show any significant effects on ZO-1 expression at 24 h (TAI = 0.98 ± 0.04) and 48 h (TAI = 0.95 ± 0.05). When cultures were simultaneously treated with Tat B plus cocaine, the ZO-1 gene expression was significantly down regulated at 12 h (TAI = 0.78 ± 0.03; $P < .05$), 24 h (TAI = 0.61 ± 0.02; $P < .02$), and 48 h (TAI = 0.55 ± 0.04; $P < .001$). On the other hand, Tat C and cocaine combined treatment showed modest decrease in expression of ZO-1 at 24 h (TAI = 0.78 ± 0.04; $P < .05$) and 48 h (TAI = 0.72 ± 0.03; $P < .05$). Also, Tat B treatment showed significant decrease in ZO-1 expression compared to Tat C treatment at 48 h ($P < .04$), whereas the combined treatment of Tat B plus cocaine was significantly different from individual treatment of Tat B, cocaine, and cotreatment of Tat C plus cocaine at 24 h ($P < .001$, $P < .04$, $P < .02$) and 48 h ($P < .001$, $P < .008$, $P < .01$), respectively.

Similarly, primary HBMEC cultures were treated with HIV-1 Tat proteins, cocaine, or cocaine plus Tat proteins and the effect on JAM-2 gene expression was studied. Our results (Figure 4b) show that cocaine 100 nM treatment significantly up-regulated JAM-2 gene expression at 24 h (TAI = 1.43 ± 0.13; $P < .05$) and 48 h (TAI=1.55 ± 0.1; $P < .01$) compared to untreated control cultures. Likewise, Tat B treatment increased the expression of JAM-2 24 h (TAI = 1.58 ± 0.15; $P < .02$) and 48 h (TAI = 1.7 ± 0.2; $P < .009$), whereas Tat C showed significant increase at only 48 h of treatment (TAI = 1.25 ± 0.12; $P < .05$). However, upon combined exposure of Tat B plus cocaine, significant increase in JAM-2 expression was then observed at 12 h (TAI = 1.7 ±

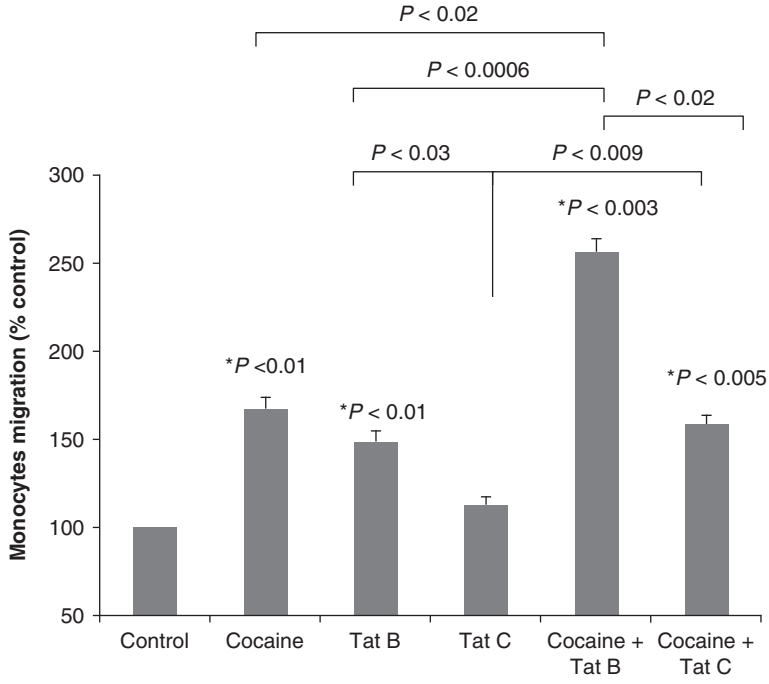

Figure 3 Monocytes migration across *in vitro* BBB model treated with HIV-1 Tat proteins and/or cocaine. The BBB layers were treated with Tat B (100 ng/ml), Tat C (100 ng/ml), cocaine (100 nM), or cocaine plus Tat proteins for 48 h prior to the transmigration assay. Monocytes (2×10^5 cells) were added per well into the upper chamber of the insert and the chambers were then incubated for 3 h at 37°C, 5% CO_2. After incubation time, cells were collected from the bottom chamber of the insert and counted using hemocytometer slide. The percentage of cells that transmigrated across the BBB with respect to the untreated control was calculated. Data represent mean ± SE of three independent experiments performed in triplicates. Statistical significance was determined using Student's *t* test. *indicates statistical significance with respect to untreated control.

0.13; $P < .04$), 24 h (TAI = 3.19 ± 0.3; $P < .005$), and 48 h (TAI = 3.40 ± 0.25; $P < .002$). On the other hand, Tat C plus cocaine combined treatment showed significant but less increase in JAM-2 expression at 24 h (TAI = 1.7 ± 0.2; $P < .05$) and 48 h (TAI = 1.80 ± 0.2; $P < .05$) compared to Tat B plus cocaine treatment. Further, significant differences were observed in JAM-2 expression between Tat B and Tat C treatment at 48 h ($P < .02$). Also, the combined treatment of Tat B plus cocaine were significantly different from cotreatment of Tat C plus cocaine at 24 h ($P < .05$) and 48 h ($P < .009$).

Based on the time kinetics results as presented in Figure 4a and b, the 24-h time point was selected to perform dose-response studies. Data presented in Figure 5a–c show the dose-response effects of Tat B (25–200 ng/ml), Tat C (25–200 ng/ml), and cocaine (10–1000 nM) treatment on ZO-1 and JAM-2 gene expression by primary HBMECs. Cocaine treatment showed dose-dependent decrease in ZO-1 gene expression at concentrations 100 nM (TAI = 0.7 ± 0.05; $P < .01$) and 1000 nM (TAI = 0.5 ± 0.04; $P < .003$) (Figure 5a). Tat B treatment also showed dose-dependent decrease at concentrations 100 ng/ml (TAI = 0.82 ± 0.05; $P < .04$) and 200 ng/ml (TAI = 0.7 ± 0.05; $P < .02$) (Figure 5b), whereas Tat C failed to significantly modulate the expression of ZO-1 in the concentration range of 25–200 ng/ml (Figure 5c). On the contrary, a dose-dependent increase in JAM-2 gene expression was observed by cocaine treatment at 10 nM (TAI = 1.2 ± 0.07; NS), 100 nM (TAI = 1.5 ± 0.1; $P < .02$), and 1000 nM (TAI = 2.2 ± 0.12; $P < .007$) (Figure 5a). Tat B treatment for 24 h also showed dose-dependent increase at 50 ng/ml (TAI = 1.1 ± 0.13; NS), 100 ng/ml (TAI = 1.7 ± 0.08; $P < .01$), and 200 ng/ml (TAI = 1.9 ± 0.08; $P < .005$) (Figure 5b), whereas Tat C showed significant increase only at the highest concentration tested, 200 ng/ml (TAI = 1.3 ± 0.09; $P < .05$) (Figure 5c).

HIV-1 Tat B and Tat C and/or cocaine modulate tight junction protein levels in primary HBMECs

Further to gene expression studies, we determined protein expression by Western blot analysis using specific antibody to quantitate ZO-1 protein levels in primary HBMECs treated with HIV-1 Tat proteins and/or cocaine. Primary HBMECs were incubated in the presence of 100 ng/ml Tat B, 100 ng/ml Tat C, and/or 100 nM cocaine for 48 h and then analyzed by immunoblotting. The results are expressed as percentage of ZO-1 protein levels with respect to control (Figure 6). The exposure of primary HBMECs to Tat B, Tat C, and cocaine decreased ZO-1 expression to 70% ± 4% ($P < .05$), 90% ± 5% (NS), and

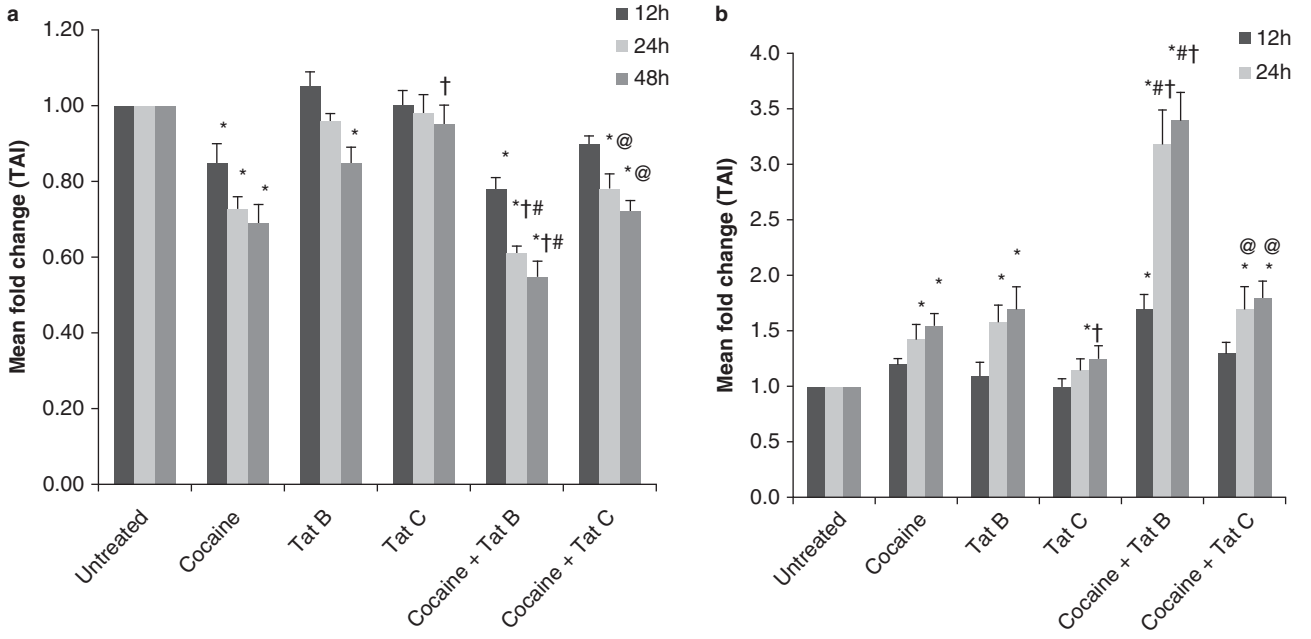

Figure 4 Time kinetics effect of HIV-1 Tat proteins and/or cocaine on ZO-1 and JAM-2 gene expression by primary HBMECs. Primary HBMECs (1×10^6 cells) were treated with clade-specific Tat proteins (100 ng/ml) and/or cocaine (100 nM) for 12, 24, and 48 h. RNA was extracted and reverse transcribed, followed by quantitative real-time PCR for ZO-1 (**a**) and JAM-2 (**b**) genes. Relative expression of mRNA species was calculated using the comparative C_T method. Data are mean ± SE of three independent experiments performed in replicates. Statistical significance was determined using Student's *t* test. *indicates statistical significance with respect to untreated control; #indicates statistical significance with respect to cocaine-treated cultures; †indicates statistical significance with respect to Tat B–treated cultures; and @indicates statistical significance between Tat B plus cocaine–treated and Tat C plus cocaine–treated cultures.

65% ± 3% ($P < .03$), respectively. The cultures treated with Tat B plus cocaine showed greater decrease to 56% ± 4.2% ($P < .01$), whereas a combination treatment of Tat C plus cocaine showed decrease to 70% ± 3% ($P < .02$) (Figure 6). The expression of ZO-1 protein by Tat B and Tat C treatment was found to be significantly different ($P < .05$). The combined effects of Tat B plus cocaine were significantly different from the combined effect of Tat C plus cocaine and are consistent with the results presented in Figures 1 to 3.

Discussion

The major group of HIV-1 strains that comprise the current global pandemic has diversified into at least 10 distinct subtypes or clades of which clade B infections are more common in the United States and western countries, whereas clade C infections are more prevalent in sub-Saharan Africa and Asia. HIV-1 infection eventually progresses to severe deficiency of various immunological functions and neurological abnormalities, especially during the later stages of the disease. Most of our present understanding of the pathophysiology and neuropathology of HIV-1 infection emanate mainly from B subtype. Previous reports suggest low incidence of neurocognitive impairments in clade C–infected patients compared to clade B–infected population (Wadia *et al,* 2001; Satishchandra *et al,* 2000; Heaton *et al,* 1995; White *et al,* 1995). However, more studies are ongoing to confirm whether clade B is more neuropathogenic than clade C.

The hallmark of HIV-associated neuropathogenesis is marked by loss of integrity of blood-brain barrier, which is a physiological dynamic barrier that results from the selectivity of the tight junctions between endothelial cells in central nervous system (CNS) vessels. At the interface, endothelial cells and associated astrocytes are stitched together by these tight junctions, which are comprised of smaller subunits of transmembrane proteins, such as occludin, claudins, junctional adhesion molecule, and others. Each of these transmembrane proteins is anchored into the endothelial cells by another protein complex that includes ZO-1 and associated proteins. ZO proteins are essential for targeting tight junction (TJ) structures, and they are linked to the actin cytoskeleton and related signal transducing mechanisms, critical for TJ function (Mehta and Malik, 2006; Luscinskas *et al,* 2002; Bazzoni *et al,* 2000). JAM proteins affect the passage of the cells when endothelial or mononuclear cells are activated. BBB dysfunction is commonly observed in HIV-infected patients, and it correlates with the cognitive impairment (Avison *et al,* 2004; Gendelman *et al,* 1997). The loss of BBB integrity enhances entry of

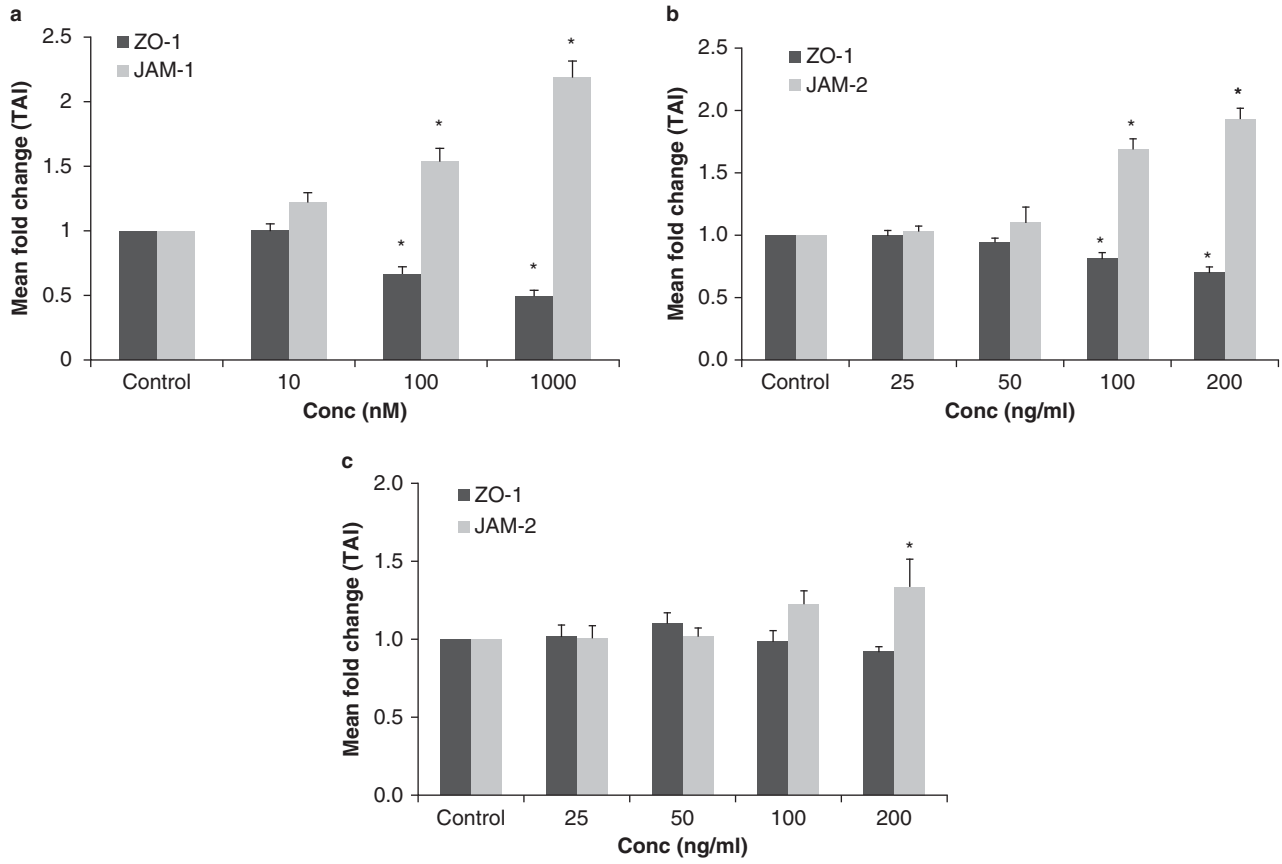

Figure 5 Dose-response analysis of HIV-1 Tat proteins and/or cocaine on ZO-1 and JAM-2 gene expression by primary HBMECs. Primary HBMECs (1×10^6 cells) were treated with clade-specific Tat proteins (25, 50, 100, 200 ng/ml) and/or cocaine (10, 100, 1000 nM) for 24 h. RNA was extracted and reverse transcribed, followed by quantitative real-time PCR for ZO-1 and JAM-2 genes for cultures treated with cocaine (**a**), Tat B (**b**), and Tat C (**c**). Heat-inactivated (HI) Tat B (**b**) and Tat C (**c**) served as controls. Relative expression of mRNA species was calculated using the comparative C_T method. Data are mean ± SE of three independent experiments performed in replicates. Statistical significance was calculated by Student's *t* test. *indicates statistical significance with respect to control.

toxins, free virus, and infected and/or activated monocytes and lymphocytes into the CNS (Kanmogne *et al,* 2007; Toborek *et al,* 2003). Therefore, the dysfunction of brain endothelium caused by HIV plays an important role in development of HAND (Toborek *et al,* 2005; Andras *et al,* 2003).

Furthermore, it is well established that parenteral drug abuse is a significant risk factor for contracting HIV infections and subsequent development of AIDS (Hahn *et al,* 1989; Curran *et al,* 1988; Des Jarlais and Friedman, 1988). Various investigators have shown that use of crack cocaine is more closely linked to HIV-1 clade B infection in the United States (Chaisson *et al,* 1989; Des Jarlais Friedman, 1988; Hubbard *et al,* 1988). Also, previous studies have shown that cocaine enhances the replication of HIV-1 clade B in *in vitro* cell culture system (Bagasra and Pomerantz, 1993; Peterson *et al,* 1991, 1992, 1993), suggesting a link between cocaine use and progression of HIV-1 clade B infections (Bayer *et al,* 1995; Chao *et al,* 1995; Heesch *et al,* 1995). Cocaine in combination with HIV-1 virus is also reported to up-regulate chemokine CCL2 and its receptor CCR2 in monocytes, leading to increased transmigration of monocytes across the BBB (Dhillon *et al,* 2008). However, the role of cocaine on neuropathogenesis of HIV-1 clade C infection has not been reported. Therefore, in the current study we report differential effects of HIV-1 Tat B and Tat C proteins and their interaction with cocaine on BBB integrity, tight junction protein expression, and transendothelial migration of monocytes across the BBB.

In current study, the treatment of BBB with HIV-1 Tat proteins (Tat B or Tat C), cocaine, or cocaine plus Tat proteins significantly increased the permeability of *in vitro* BBB, as indicated by decrease in TEER (Figure 1) and increased transport of FITC-dextran (Figure 2). Further, extent of increase in BBB permeability was higher with combined treatment of cocaine with Tat proteins compared to individual treatments. Previous study where HBMEC monolayer was treated with a combination of alcohol and HIV-1 gp120 has reported significant increase in permeability of the monolayer (Shiu *et al,*

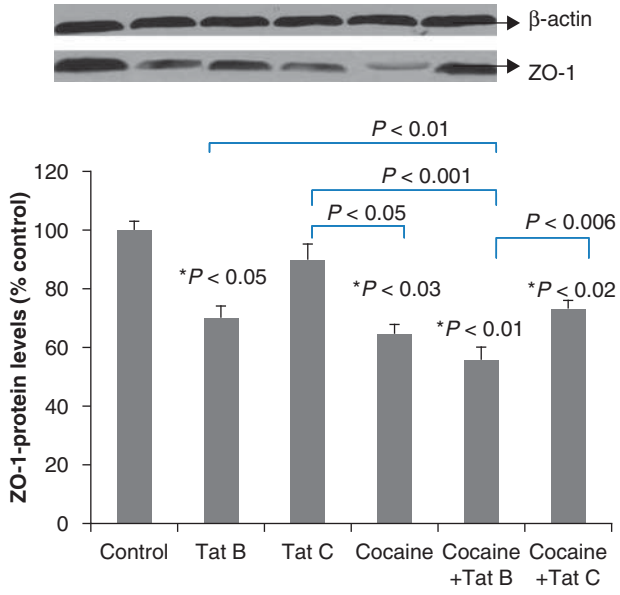

Figure 6 ZO-1 protein expression by primary HBMECs treated with HIV-1 Tat proteins and/or cocaine. Primary HBMECs (1×10^6) were treated with 100 ng/ml clade-specific Tat proteins and/or 100 nM cocaine separately for 48 h. The ZO-1 protein level in all the samples was quantitated by Western blot analysis. Data presented show a representative blot indicating modulation of ZO-1 protein expression and a bar graph representing the mean ± SE of % densitometric values of ZO-1 protein levels (% control) of three separate experiments performed. Statistical significance was determined using Student's *t* test. *indicates statistical significance with respect to untreated control.

2007). Additionally, we found that monocyte transmigration rate across the BBB model was higher with treatment of Tat B and cocaine alone and in combination compared to Tat C alone and in combination with cocaine (Figure 3). These observations suggest that HIV-1 Tat proteins, Tat B and Tat C, differentially modulate BBB integrity and addition of cocaine to HIV-1 proteins further aggravates the neuropathological condition with pronounced effects observed with Tat B plus cocaine than with Tat C plus cocaine. Furthermore, we found that Tat proteins and cocaine alone and in combination were not cytotoxic to HBMECs and HAs (data not shown). This confirms that the observed effects on BBB dysfunction were not directly attributed to the cytotoxic effects of Tat protein or cocaine on HBMECs and HAs.

Earlier studies from our laboratory on delineating the role of clade B and C in the development of neurological disorders have reported that Tat B and Tat C differentially modulate expression of neuropathogenic molecules, indoleamine-2,3-dioxygenase (IDO), and kynurenine by primary astrocytes (Samikkannu *et al,* 2009). We have also shown that Tat B and Tat C differentially induce the inflammatory cytokines by primary monocytes, leading to differential neuropathogenesis (Gandhi *et al,* 2009). Previous studies with cocaine have reported a dose-dependent increase in the transmigration of monocytes across the BBB (Gan *et al,* 1999). Also, methamphetamine and morphine in conjunction with HIV proteins (Tat and gp120) have been shown to impair BBB integrity and increase the transmigration of mononuclear cells across the BBB (Mahajan *et al,* 2008a, 2008b). We herein show differential effects of Tat B and Tat C in conjunction with cocaine on BBB integrity. Interestingly, our results indicate that Tat B plus cocaine induces higher BBB dysfunction than Tat C plus cocaine, suggesting that abuse of cocaine by HIV-1 clade B–infected subjects might increase the risk of developing HAND than clade C–infected subjects.

We further examined whether this disruption of BBB integrity is linked to altered expression of TJ proteins ZO-1 and JAM-2. Our results suggested that Tat B significantly down-regulated ZO-1 gene expression, whereas JAM-2 gene expression was significantly up-regulated in primary HBMECs (Figures 4a and b, 5a–c). On the other hand, Tat C showed relatively small alteration in ZO-1 and JAM-2 expression. Further, cocaine-associated exacerbation of HIV-1 Tat proteins response was significantly higher with clade B compared to clade C. Previous report on the treatment of HBMECs with cocaine has shown significant down-regulation in ZO-1 protein expression, which was associated with cytoskeletal rearrangement and formation of stress fibers (Dhillon *et al,* 2008). Likewise, treatment of HBMECs with methamphetamine and/or gp120 has been shown to down regulate ZO-1 expression and these effects are mediated by Rho-A activation (Mahajan *et al,* 2008b). Previous study with morphine and/or Tat has shown that JAM-2 expression is up-regulated and ZO-1 expression is down-regulated in brain endothelial cells and this modulation in tight junction protein expression is possibly mediated by activation of proinflammatory cytokines, intracellular Ca^{+2} release and activation of myosin light chain kinase (Mahajan *et al,* 2008a). As previously reported by us (Gandhi *et al,* 2009), Tat B and Tat C exert differential effects on expression of inflammatory cytokines by primary monocytes, with Tat B showing higher up-regulation of proinflammatory cytokines compared to Tat C. These differential effects of Tat B and Tat C on expression of proinflammatory molecules may play a role in modulating the expression of tight junction protein ZO-1 and JAM-2 and these effects are exacerbated by cocaine treatment. Our results on ZO-1 gene expression were further supported by protein expression studies in HBMECs by Western blot analysis (Figure 6). These results were in agreement with qRT-PCR and showed modulation of ZO-1 in a similar manner, wherein Tat B showed higher effects than Tat C and these effects were further exacerbated by cocaine treatment, with higher effect seen with Tat B plus cocaine compared to Tat C plus cocaine.

In summary, our results of clade-specific differences on BBB disruption support the clinical observation of greater neuropathogenic manifestations observed with HIV-1 clade B infections compared to HIV-1 clade C infections. Additionally, our study delineate the role of cocaine in exacerbating HAND, with higher effects seen in HIV-1 clade B than with HIV-1 clade C infections. The exact molecular mechanism underlying the differential effects of Tat B and Tat C in combination with cocaine on BBB integrity and differential neuropathogenesis is currently being investigated in our laboratory. Therefore, our results of differential effects of clade B and clade C in conjunction with cocaine on BBB integrity provide implications to clade-specific diversity in neurpathogenesis and open new avenues for developing therapeutic modalities for prevention of HAND.

Materials and methods

Cell culture and reagents

Primary cultures of human brain microvascular endothelial cells (HBMECs; catalog no. 1000) and human astrocytes (HAs; catalog no. 1800) were purchased from Sciencell Laboratories (Carlsbad, CA) and cultured as per supplier's instructions. Primary HBMECs were characterized by immunofluorescent method with antibodies to von Willebrand factor (vWF)/factor VIII and CD31 (platelet cell adhesion molecule [P-CAM]), by uptake of DiI-Ac-LDL (low-density lipoprotein), and by the formation of microtubular structure *in vitro.* HAs were characterized by immunofluorescent method with antibody to glial fibrillary acid protein (GFAP). Both the cultures were negative for HIV-1, hepatitis B virus (HBV), hepatitis C virus (HCV), mycoplasma, bacteria, yeast, and fungi. Primary HBMECs and HAs were obtained at passage 2 and used for all experiments between passages 2 and 8. Normal peripheral blood monocytes were isolated by a density gradient centrifugation process from HIV-1/HIV-2 and hepatitis B–seronegative donor leukopacks as described by us (Gandhi *et al,* 2009). HIV-1 clade B recombinant Tat protein was obtained from NIH AIDS Research and Reference Reagent Program and HIV-1 clade C recombinant Tat protein was commercially purchased from Diatheva (Fano, Italy). Cocaine was commercially purchased from Sigma Aldrich (St. Louis, MO). Tat and cocaine concentrations employed in the current study are used as described earlier for *in vitro* studies (Dhillon *et al,* 2008; Mishra *et al,* 2008; Ranga *et al,* 2004; Oshima *et al,* 2000; Gan *et al,* 1999; Zhang *et al,* 1998). Also, it is reported that localized Tat concentration is expected to be much higher than detected in sera from HIV-infected patients (as high as 40 ng/ml) (Xiao *et al,* 2000).

In vitro *BBB model*

The BBB model was established according to the procedure described earlier (Persidsky, 1999). The model consisted of two-compartment wells in a culture plate with the upper compartment separated from the lower by a cyclopore polyethylene terephthalate membrane (Collaborative Biochemical Products, Becton Dickinson, San Jose, CA) with a pore diameter of 3 μm. In a 24-well cell culture insert, 2×10^5 primary HBMECs were grown to confluency on the upper side whereas a confluent layer of primary HAs (2×10^5 cells/insert) was grown on the underside. Intactness of the BBB was determined by measuring the transendothelial electrical resistance (TEER) using Millicell ERS microelectrodes (Millipore, Billerica, MA). The electrical resistance of blank inserts with medium alone was subtracted from TEER readings obtained from inserts with confluent monolayers. The resulting TEER values represent the resistance of the endothelial cell monolayers. The BBB model was used for experiments at least 5 days after cell seeding. The BBB constructs were treated with HIV proteins (Tat B or Tat C, 100 ng/ml), cocaine (100 nM), or cocaine plus HIV proteins (Tat B or Tat C). TEER measurements were performed at 24 and 48 h after the treatment. The typical TEER values for untreated cultures were observed to be ~200 Ω/cm^2.

FITC-dextran transport

To assess the effect of clade-specific Tat protein and cocaine on the integrity of the *in vitro* BBB model, paracellular transport of flourescein isothiocyanate–labeled dextran (FITC-dextran; molecular weight 40000) was measured according to the procedure described earlier (Kanmogne *et al,* 2007). After the integrity of BBB was established by TEER measurement, the BBB monolayers were treated with HIV proteins (Tat B or Tat C, 100 ng/ml), cocaine (100 nM), or cocaine plus HIV proteins (Tat B or Tat C) and incubated for 48 h. After incubation, 100 μg/ml FITC-dextran (Sigma Aldrich, St. Louis, MO) was added to the upper chamber of the inserts and further incubated for 4 h. Samples were collected from the bottom chamber after 4 h and fluorescence intensity was measured at excitation wavelength 485 nm and emission wavelength 520 nm using Biotek Synergy HT multimode microplate reader instrument. FITC-dextran transport was expressed as percentage of FITC-dextran transported across the BBB into the lower compartment compared to untreated control cultures.

Monocyte transmigration assay

Monocyte transmigration assay was performed after treating BBB monolayers with HIV proteins (Tat B or Tat C, 100 ng/ml), cocaine (100 nM), or cocaine plus HIV proteins (Tat B or Tat C) for 48 h. Monocytes (2×10^5 cells) were added in the upper chamber of the insert and the plates were then incubated for 3 h at 37°C, 5% CO_2. After incubation, cells were collected from the bottom chamber of the insert and

counted using hemocytometer slide. Cell viability was assessed by trypan blue staining.

Quantitative real-time polymerase chain reaction (qRT-PCR)

In order to perform gene expression studies, 1×10^6 primary HBMECs were cultured in a 6-well tissue culture plate. For time kinetics experiments, HBMECs were cultured with HIV proteins (Tat B or Tat C, 100 ng/ml), cocaine (100 nM), or cocaine plus HIV proteins (Tat B or Tat C) for different time intervals, 12, 24, and 48 h. Based on the time kinetics data, dose-response experiments were performed with 25 to 200 ng/ml of Tat B and Tat C proteins, 10 to 1000 nM of cocaine alone, or in cocaine combination with Tat B/C for 24 h. Heat-inactivated Tat proteins were used as controls. Upon desired treatment period, cells were harvested, RNA from cell pellets was extracted using RNAeasy mini kit (Qiagen, GmbH, Germany), followed by cDNA synthesis using high-capacity reverse transcriptase cDNA kit (Applied Biosystems, Foster City, CA, USA) to perform qRT-PCR using Taqman gene expression assays (Applied Biosystems, Foster City, CA, USA) for ZO-1 (Hs01551876_m1), JAM-2 (Hs01022007_m1), and glutaldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1). GAPDH served as an internal control. Relative abundance of each mRNA species was assessed using brilliant Q-PCR master mix from Stratagene using M × 3000P instrument, which detects and plots the increase in fluorescence versus PCR cycle number to produce a continuous measure of PCR amplification. Relative mRNA species expression was quantitated and the mean fold change in expression of the target gene was calculated using the comparative C_T method as previously described by us (Gandhi *et al*, 2009). All data were controlled for quantity of RNA input by performing measurements on an endogenous reference gene, GAPDH. In addition, results on RNA from treated samples were normalized to results obtained on RNA from the control, untreated sample.

Western blot analysis

To assess protein expression, HBMEC cells were treated with HIV proteins (Tat B or Tat C, 100 ng/ml), cocaine (100 nM), or cocaine plus HIV proteins (Tat B or Tat C) for 48 h. After incubation, cells were harvested and lysed by lysis buffer with a complete cocktail of protease inhibitors (Pierce Chemical, Rockford, IL). Total protein content in these samples was determined using Bradford dye reagent (Pierce Chemical). Fifty microgram total protein was resolved on 5% Tris-HCl gel by polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and were probed with primary antibodies against ZO-1 (Cell Signaling Technology; 1:1000) and β-actin (Cell Signaling Technology; 1:1000), followed by secondary goat anti-rabbit immunoglobulin G (IgG) antibody (Cell Signaling Technology; 1:2500). Immunoreactive bands were visualized using a chemiluminescence's system according to the manufacturer's instructions (Pierce Chemical).

Statistics

Experiments were performed at least three times in replicates and the values obtained were averaged. Data are represented as mean ± SE. Comparisons between two groups were conducted using Student's paired *t* test. Differences were considered significant at $P \leq .05$.

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