Effect of Cocaine on Human Immunodeficiency Virus–Mediated Pulmonary Endothelial and Smooth Muscle Dysfunction

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Human immunodeficiency virus (HIV)–associated pulmonary arterial hypertension (PAH) is a devastating, noninfectious complication of acquired immune deficiency syndrome, and the majority of HIV-PAH cases occur in individuals with a history of intravenous drug use (IVDU). However, although HIV-1 and IVDU have been associated with PAH independently or in combination, the pathogenesis of the disproportionate presence of HIV-PAH in association with IVDU has yet to be characterized. The objective of this study was to obtain a better understanding of the interactions between HIV-1 and cocaine to help uncover the mechanism(s) of the development of HIV-PAH. We observed that exposure of HIV-infected macrophages or HIV-Trans-Activator of Transcription (Tat)–treated pulmonary endothelial cells to cocaine enhanced the expression of platelet-derived growth factor (PDGF)-BB. Simultaneous treatment with Tat and cocaine, on the other hand, exacerbated both the disruption of tight junction proteins (TJPs), with enhanced permeability in pulmonary endothelial cells, and the proliferation of pulmonary smooth muscle cells (pSMCs) compared with either treatment alone. Histological examination of HIV plus IVDU human lung sections showed signs of early pulmonary arteriopathy, severe down-modulation of TJPs, and increased expression of PDGF-BB compared with the lung sections from individuals who are infected with HIV and without history of IVDU. Interestingly, blocking of PDGF receptor signaling with the receptor antagonist or small interfering RNA has been shown to inhibit the increase in proliferation of pSMCs on Tat and cocaine exposure. Our results, therefore, support an additive effect of cocaine to HIV infection in the development of pulmonary arteriopathy through enhancement of endothelial dysfunction and proliferation of pSMCs, while also suggesting PDGF–PDGF receptor axis as a potential target for use in clinical intervention.

Keywords: vascular remodeling; Trans-activator of transcription; platelet-derived growth factor; tight junction proteins

CLINICAL RELEVANCE

This article focuses on the yet-uncharacterized pathogenesis of the enhanced injury observed in the pulmonary vascular beds of intravenous drug users who are infected with human immunodeficiency virus (HIV). We report evidence for an additive effect of HIV infection and cocaine in (1) loss of endothelial integrity through down-regulation of tight junction proteins, and (2) proliferation of pulmonary smooth muscle cells. This work furthermore bolsters the case that platelet-derived growth factor BB and its receptor play a prominent role in viral protein- and cocaine-mediated smooth muscle hyperplasia, and thus offers potential targets for therapeutic intervention in HIV-associated pulmonary hypertension.

The complications associated with acquired immune deficiency syndrome in the antiretroviral therapy era have evolved from those of an infectious nature to ones stemming from consequences of prolonged survival. Prime examples of this shift are forms of vascular dysfunction arising in important end-organs, such as the heart, brain, and lungs (e.g., premature myocardial infarction [1–3], stroke [4, 5], and pulmonary arterial hypertension [PAH] [6, 7], respectively). Particularly severe, human immunodeficiency virus (HIV)–related PAH (HIV-PAH) is more likely to result in mortality than other complications of HIV infection, and serves as an independent predictor of death (8). The incidence of symptomatic HIV-PAH is unchanged since 1999, at 0.5% (9–11). However, reports of elevated pulmonary artery pressures by echocardiography (10, 12, 13) in as many as 35% of asymptomatic HIV-positive individuals (13) suggests that HIV-PAH is a more formidable problem than previously believed.

Although pulmonary vascular dysfunction arises independently of the route of infection with HIV, it is more common in individuals with a history of intravenous drug use (IVDU). A range of 59–70% of HIV-PAH cases are reported to be in individuals engaging in IVDU (14–16). Although the abuse of cocaine and other stimulants may be an independent risk factor in the development of pulmonary vascular injury (17–20), HIV-1 and cocaine interplay in the host might be involved in the exacerbation of pulmonary vasculature dysfunction, leading to the higher incidence of PAH in HIV-infected individuals with IVDU as a risk factor. Clearly, HIV and cocaine each have the capacity to orchestrate endothelial cell injury and dysfunction that may lead to abnormal smooth muscle cell (SMC) proliferation/migration and subsequent vascular remodeling. Others and we have previously reported

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down-regulation of endothelial tight junction proteins (TJPs) by either HIV-1 or cocaine that resulted in endothelial dysfunction with enhanced permeability (21–24). Furthermore, in our earlier studies, we demonstrated enhanced activation and virus production by HIV-infected macrophages on cocaine exposure (25), and, therefore, it is likely that the interplay of HIV-1 and cocaine in the host would accelerate the HIV-related pathogenesis. In light of the emerging realization of a possible relationship between HIV-infection and cocaine use in the development of pulmonary vascular dysfunction, we hypothesized that the use of cocaine by HIV-1 infected individuals can lead to synergistic or additive loss of endothelial integrity, followed by enhanced exposure of smooth muscles to viral proteins, cocaine, and other circulating mitogens, leading to vascular remodeling associated with PAH.

To test our hypothesis, we evaluated the effects of HIV-1 and cocaine on pulmonary endothelial cell (pEC) and SMC function with a focus on platelet-derived growth factor (PDGF)-BB. PDGF-BB, along with its receptor, is an important mediator in the pathogenesis of pulmonary vascular remodeling associated with PAH (26–29), and is known to be up-regulated with HIV infection (30, 31). Imatinib mesylate, which inhibits PDGF signaling, has been demonstrated to reverse the pulmonary vascular remodeling in animal models and improve the hemodynamics in some patients (32–34). We chose to use HIV protein, Trans-activator of transcription (Tat), to investigate the effect of HIV-1 on vascular remodeling in vascular cell culture model systems. Tat is secreted by HIV-infected cells, can be taken up by other noninfectable neighboring cells lining the vasculature (35–37), and has been shown to possess the capability to function as both an oncogenic and angiogenic factor (38–42). Finally, to add clinical relevance and substantiate our *in vitro* findings, we examined human lung sections from individuals with a history of HIV infection with and without IVDU history.

MATERIALS AND METHODS

Cell Culture and Treatments

Monocyte-derived macrophage (MDM) cultures were obtained from peripheral blood mononuclear cells by incubation in a macrophage differentiation medium, as described previously (25). Macrophages were inoculated with HIV_{Bal} virus at a multiplicity of infection of 0.01 for 4 hours at 37°C. Cells were then extensively washed and replenished with fresh medium with or without 1 μ M cocaine for 5 days. The concentration of cocaine used was based on our previous reports (25). Primary human pulmonary arterial endothelial cells (HPAECs) (ScienCell Research Laboratories, Carlsbad, CA) were serum starved (0.5% FBS) overnight, followed by treatment with Tat (25 ng/ml) and/or cocaine (1 μ M) for mRNA analysis at 24 hours after treatment (*see the online supplement*). T/G human aorta–vascular SMCs (T/G HA-VSMCs), acquired from American Type Culture Collection (Rockville, MD), or primary human pulmonary arterial SMCs (HPASMCs) (ScienCell Research Laboratories), were stimulated with cocaine and/or recombinant Tat protein under serum-starved conditions, and proliferation was determined by Cell Proliferation Assay (Promega, Madison, WI) (*see MATERIALS AND METHODS in the online supplement for further details*).

Endothelial Permeability Assay

An *in-vitro* vascular permeability assay kit from Millipore (Billerica, MA) was used according to the manufacturer's instructions. HPAECs grown on collagen-coated inserts were treated with Tat (25 ng/ml) and/or cocaine (1 μ M) containing cell basal medium for 6 hours, followed by measurement of FITC-dextran permeability across monolayers.

Immunocytochemistry and Immunohistochemical Analysis

Immunocytochemical analysis on paraformaldehyde-fixed HPAEC cultures and immunohistochemical or immunofluorescence staining of paraffin-embedded lung sections was performed as previously described (21, 30). Quantitative analysis of staining was performed using Image J software (National Institutes of Health, Bethesda, MD). Further details are provided in the online supplement.

TABLE 1. A SUMMARY OF CLINICAL, DEMOGRAPHIC, AND PATHOLOGIC CHARACTERISTICS OF VARIOUS LUNG SAMPLES

Patient	Age/Sex		Pulmonary Vascular Changes*	ARV Drugs
	(Yr/M or F)	Plasma Viral Load/CD4 Count		
Normal	43/F	NA	None (–)	NA
Normal	54/M	NA	None (–)	NA
Normal	26/M	NA	None (–)	NA
Uninfected/IVDU	48/F	NA	None (–)	NA
Uninfected/IVDU	48/M	NA	Moderate medial hypertrophy of small and medium vessels (++)	NA
Uninfected/IVDU	50/M	NA	Rare but significant arteriopathy (+/+++)	NA
HIV infected/non-IVDU	51/M	49,346/76	None (–)	Kaletra, trizivir
HIV infected/non-IVDU	54/M	111,980/1	None (–)	Fuseon, trizivir
HIV infected/non-IVDU	39/M	—/6	None (–)	None
HIV infected/non-IVDU	49/M	493,381/2	None (–)	None
HIV infected/IVDU	45/F	—/6	Moderate medial hypertrophy, endothelial proliferation in the vasculature, neointima formation, narrowing of lumen (++)	None
HIV infected/IVDU	37/M	—/8	Significant thickening of medium vessels, hypervascularity (+++)	Sustiva, epivir, viread
HIV infected/IVDU	61/M	17,802/83	Significant medial hypertrophy of small, medium to large vessels, with significant reduction in lumen, endothelial proliferation, and hypervascularity (+++)	None
HIV infected/IVDU	47/M	683,333/1	Moderate medial hypertrophy of medium vessels, endothelial proliferation (++)	d4t, 3TC, sustiva
HIV infected/IVDU	51/M	89,138	Mild vascular remodeling of medium to large vessels, endothelial proliferation (+)	Videx, viramune, kaletra

Definition of abbreviations: ARV, antiretroviral; F, female; HIV, human immunodeficiency virus; IVDU, intravenous drug use; M, male; NA, not applicable.

* Semiquantitative score for vascular remodeling is shown in parentheses.

Western Blot Analysis

HIV-infected or uninfected MDMs or Tat-treated HPAECs/HPASMCs, in the presence or absence of cocaine treatment, were lysed using the mammalian cell lysis buffer (Sigma, St. Louis, MO), and then used for Western blot analysis as described previously (43). Primary antibody details are provided in the online supplement.

Transfection of Pulmonary SMCs with Small Interfering RNA

For PDGF receptor (PDGFR) inhibition in HPASMCs, silencer-select predesigned and validated small interfering (si) RNA duplexes targeting β -chain of PDGFR were obtained from Applied Biosystems (Carlsbad, CA). Cells were also transfected with silencer-select negative control siRNA for comparison. HPASMCs were transfected with

5 or 10 nM siRNA using HiPerfect transfection reagent (Qiagen, Valencia, CA), as instructed by the manufacturer. The transfected cells were then serum starved (0.1% FBS containing media) for 1 day, followed by 48-hour treatment with or without cocaine and/or Tat.

Human Lung Tissues and Sections

Frozen human lung tissues and paraffin-embedded sections from HIV-infected individuals with or without IVDU and uninfected individuals with IVDU obtained at the time of autopsy were from the Manhattan HIV Brain Bank (R24MH59724; U01MH083501; New York, NY), and normal, uninfected archival control data were from the National Disease Research Interchange (Philadelphia, PA). The subjects with IVDU used in this study were mainly heroin and/or

Figure 1. Cocaine potentiates the expression of platelet-derived growth factor (PDGF)-BB in human immunodeficiency virus (HIV)-infected human macrophages and Trans-Activator of Transcription (Tat)-treated pulmonary endothelial cells (pECs), but not in Tat-treated pulmonary smooth muscle cells (SMCs). Real-Time RT-PCR analysis of PDGF-B chain in (A) HIV_{BaI}-infected monocyte-derived macrophages (MDMs), (C) Tat (25ng/ml)-treated primary human pulmonary arterial endothelial cells (HPAECs), and (E) Tat-treated human pulmonary arterial SMCs (HPASMCs), in the absence or presence of cocaine (1 μ M). The data represent means ($\pm$ SD) of three or more independent experiments done in triplicates. Western blot analysis of PDGF-BB in cellular extracts from (B) HIV_{BaI}-infected MDMs in the absence or presence of cocaine, (D) HPAECs, and (F) HPASMCs treated with or without cocaine and/or Tat. The blots were reprobed with human β -actin antibodies. Representative Western blot images (upper panel) are shown with histograms (lower panel), showing the average densitometric analysis of the PDGF-BB band normalized to corresponding β -actin band from three experiments. Statistically significant differences are shown as * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, treatment versus control, and # $P < 0.05$, ## $P < 0.01$, HIV+cocaine versus cocaine or HIV alone; Tat+cocaine versus cocaine or Tat alone.

cocaine abusers. The slides were reviewed blindly by pathologists. No hemodynamic parameters were available for any of the individual samples. The clinical data described in Table 1 were obtained in accordance with Health Insurance Portability and Accountability Act regulations.

QuantiGene-Plex Analysis and Real-Time RT-PCR

RNA data from frozen lung tissues were quantified using the QuantiGene Plex 2.0 assay system (Panomics, Inc., Fremont, CA) according to manufacturer's instructions, and signal was reported as mean fluorescence intensity. Quantitative analysis of PDGF-B chain or TJP

mRNA in cellular extracts was done by real-time RT-PCR using the SYBR Green detection method, as described previously (20).

Statistical Analysis

Statistical analysis was performed using two-way ANOVA with a *post hoc* Student's *t* test for parametric data, or Wilcoxon's rank-sum test for nonparametric multiple comparisons. Exact two-sided *P* values were calculated for all analyses using SAS 9.2 software (SAS Institute, Inc., Cary, NC). Because human tissue studies involve limited sample sizes, an unadjusted type I error rate of 5% was used for determining statistical significance.

Figure 2. Down-regulation in the expression of tight junction proteins (TJPs) in pulmonary arterial endothelial cells on exposure to HIV-Tat and/or cocaine. HPAECs grown in six-well plates were serum starved (0.5% FBS) overnight, followed by treatment with Tat (25 ng/ml) and/or cocaine (1 μ M) for mRNA analysis by real-time RT-PCR at 24 hours after treatment. Values represent fold decrease of (A) claudin (CLDN)-4, (B) TJP-1, (C) TJP-3, and (D) occludin (OCLN) mRNA on treatment with Tat and/or cocaine compared with untreated cells. Means ($\pm$ SD) are shown; **P* < 0.05, ***P* $\leq$ 0.01, ****P* $\leq$ 0.001, compared with untreated cells. (E) HPAECs grown on coverslips, treated with cocaine (1 μ M) and/or Tat (25 ng/ml) for 24 hours, respectively, were immunostained for TJP-1. Control represents untreated cells. (F) Quantitative analysis of TJP-1 immunofluorescence staining. The TJP-1 staining images were acquired from three independent experiments performed on endothelial monolayers stimulated with cocaine and/or Tat. Graph represents the average fluorescent units present as a percentage of untreated control cells (**P* $\leq$ 0.001, treatment versus control; #*P* $\leq$ 0.01, cocaine +Tat versus cocaine or Tat alone.). (G) Effect of cocaine (1 μ M) and Tat (25 ng/ml) on barrier function of HPAECs as assessed by FITC-dextran. Cells were grown on collagen-coated Transwell filters. Confluent monolayers were incubated for 6 hours with cocaine and Tat, followed by treatment with the FITC-dextran for 5 minutes. The fluorescence in the lower compartment was then measured and expressed as percentage of basal fluorescence. The values shown are means ($\pm$ SD) of three independent experiments (**P* $\leq$ 0.05, ***P* < 0.01 treatment versus control; #*P* < 0.05 Cocaine +Tat versus Tat, ##*P* < 0.01 Cocaine +Tat versus cocaine).

Figure 3. Increased proliferation of SMCs with Tat, cocaine, or PDGF-BB. The SMCs grown in 96 wells were starved in 0.1% serum-containing media for 48 hours before treatment. (A) Exposure of quiescent human aorta-vascular SMCs (T/G HA-VSMC) to Tat and cocaine results in synergistic or additive increase in proliferation of cells compared with cells treated with Tat or cocaine alone. Means ($\pm$ SD) are shown, and are representative of three independent experiments; * $P < 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, treated versus untreated cells; # $P \leq 0.05$, compared with Tat (25 ng/ml) treatment alone; and \$ $P \leq 0.05$, compared with Tat (50 ng/ml) treatment alone. (B) Increased proliferation of primary HPASMCs with Tat (25 ng/ml), cocaine (1 μ M), or PDGF-BB (100 ng/ml) treatment with further enhancement on simultaneous treatment with cocaine and Tat or either cocaine or Tat with PDGF-BB. The data shown are means ($\pm$ SD), and are representative of three independent experiments, in triplicates. * $P < 0.05$, compared with untreated cells; # $P \leq 0.01$, compared with cocaine treatment alone; \$ $P \leq 0.01$, compared with PDGF-BB treatment alone.

RESULTS

Cocaine Exposure Augments the Expression of PDGF-BB Associated with HIV-Infection/Tat Treatment in Macrophages and pECs

Our previous studies have demonstrated increased expression of PDGF-PDGFR axis with simian/HIV infection (30, 31, 44).

Because cocaine use has been reported to be associated with incidence of PAH (17, 18), we sought, in the present study, to examine whether cocaine might modulate expression of PDGF-BB, an important mediator of vascular remodeling (28), in HIV-infected macrophages. For this purpose, we exposed both uninfected and HIV_{bal}-infected MDMs to 1 μ M cocaine; total RNA collected from these cells was then assessed for PDGF-BB by real-time RT-PCR. As shown in Figure 1A, HIV infection of MDMs or cocaine exposure alone, on the one hand, resulted in a small, but significant, increase in the expression of PDGF-B chain mRNA. On the other hand, exposure of HIV-infected MDMs to cocaine led to a significant enhancement of PDGF-B chain expression at Day 5 after treatment compared with HIV-infected cells not treated with cocaine or cocaine-treated, uninfected cells.

We then assessed changes in the PDGF-B chain expression at the protein level by Western blot analysis of cellular extracts after treatment. As expected, treatment of uninfected human MDMs with cocaine alone or HIV-infection alone induced greater expression of PDGF-BB protein compared with untreated control (Figure 1B). In addition, in keeping with our findings at the transcriptional level, Western blot analysis further supported our hypothesis of an additive effect of cocaine and HIV, as exposure of HIV-infected MDMs to cocaine resulted in significant augmentation of PDGF-BB expression in the cell lysate when compared with either treatment alone (Figure 1B).

Because circulating PDGF-BB can be released by endothelial cells and SMCs, as well as by mononuclear cells (29), we next tested the expression of PDGF-BB in human pECs and SMCs in response to the viral protein Tat and/or cocaine by real-time RT-PCR and Western blot analysis. As shown in Figure 1C, treatment of primary HPAEC (or pECs) with 1 μ M cocaine for 24 hours resulted in a significant increase of PDGF-B chain mRNA expression, with further enhancement when cells were exposed to cocaine in the presence of Tat. Similarly, densitometric analysis of Western blots also demonstrated increased expression of PDGF-BB by simultaneous treatment of cocaine and Tat compared with either treatment alone (Figure 1D).

Treatment of quiescent HPASMCs (or pulmonary SMCs, pSMCs) with Tat or simultaneous treatment with Tat and cocaine resulted in significant increase of PDGF-B chain compared with untreated control, as analyzed by real-time RT-PCR (Figure 1E). We could not, however, demonstrate further enhancement in PDGF-B chain expression after simultaneous Tat and cocaine treatment compared with either treatment alone. In addition, we observed a significant increase in the expression of PDGF-BB protein in quiescent HPASMCs treated with Tat compared with control, as evaluated by Western blot analysis (Figure 1F). Consistent with our RNA findings, treatment with both Tat and cocaine did not result in augmentation of PDGF-BB expression when compared with Tat treatment alone (Figure 1F). From all these findings, we conclude that both HIV infection of MDMs, and the exposure of pECs to the viral protein, Tat, result in the augmentation of the PDGF-BB expression in the presence of cocaine. However, the combined effect of HIV and cocaine does not result in increased PDGF-BB expression in pSMCs.

Cocaine or Tat Exposure Decreases Expression of TJP1s in pECs

Because endothelial alterations have been observed to precede the development of muscularization of pulmonary arteries in the animal model of PAH (45), we next examined the direct effects of cocaine and Tat on endothelial dysfunction. HPAECs were treated with or without cocaine (1 μ M) and or Tat (25 ng/ml) for 24 hours, after which we analyzed expression of various TJPs by real-time RT-PCR. As shown in Figures 2A–2D, exposing cells

to either cocaine and/or Tat resulted in a significant reduction of claudin (CLDN)-4, TJP-1, TJP-3, and occludin (OCLN) mRNA levels at 24 hours compared with untreated cells. Treatment with both cocaine and Tat resulted in enhanced reduction in the expression of TJP-3 and OCLN as compared with cocaine or Tat treatment alone, but this reduced expression was not statistically significant.

Endothelial integrity is not only affected by the change in expression of TJPs, but also by the redistribution of TJPs within the cell (46). With this phenomenon in mind, we next assessed the distribution of TJP in cocaine- and/or Tat-treated and untreated endothelial cells by immunofluorescence staining. In untreated control cells (Figure 2E), TJP-1 was clearly visible as a uniform and continuous structure corresponding to the cell–

Figure 4. Involvement of PDGF signaling in HIV-Tat- and cocaine-induced proliferation of SMCs. (A) HIV-Tat and cocaine induces expression and activation of PDGF-β receptor (PDGF-βR) in pulmonary SMCs (pSMCs) as shown in the representative Western blot image and by densitometric analysis of the signal intensity. Quiescent HPASMCs were incubated with Tat (25 ng/ml) and cocaine (1 μM) for 24 hours, followed by extraction of protein and sequential immunoblotting with antibodies specifically directed to the PDGF-βR, phosphorylated active form of PDGF-βR, and finally reprobated with β-actin antibody for normalization. * $P \leq 0.05$ versus control; # $P \leq 0.05$ versus cocaine or Tat treatment. (B) Imatinib mesylate reverses the effect of Tat and cocaine on the proliferation of SMCs. Quiescent HPASMCs were pretreated with 1 μM imatinib mesylate, followed by treatment with Tat (25 ng/ml) and cocaine (1 μM) for 48 hours. Means ($\pm$ SD) are shown, and are representative of three independent experiments. * $P \leq 0.01$, treated versus untreated cells; # $P \leq 0.001$ versus cocaine plus Tat treatment. (C) Evaluation of PDGF-βR knockdown by Western blot analysis of whole-cell lysates from HPASMCs transfected with either PDGF-βR small interfering (si) RNA (5 nM and 10 nM) or negative siRNA control (10 nM) in the absence or presence of cocaine and Tat treatment. Blot is representative of three independent experiments, with histogram (lower panel) showing the average densitometric analysis of PDGF-βR normalized to β-actin; * $P < 0.01$, treatment versus control; # $P < 0.01$, treatment versus cocaine plus Tat treatment. (D) PDGF-βR siRNA inhibited cocaine- and Tat-mediated induction of smooth muscle proliferation. Cells were transfected, treated, and analyzed for proliferation by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay, as described in MATERIALS AND METHODS. All values are means ($\pm$ SD). * $P \leq 0.001$, treatment versus control; # $P \leq 0.001$, treatment versus cocaine plus Tat-treated, untransfected cells.

cell borders. However, exposure of HPAECs to cocaine or Tat for 24 hours resulted in dramatic destruction of the TJP-1 at the periphery of cells. Interestingly, almost complete loss of TJP-1 from cell membrane was observed in cells treated with both cocaine and Tat when compared with either treatment alone. Quantitative evaluation of TJP-1 immunofluorescence staining demonstrated an approximately 71% loss of TJP-1 staining intensity after 24-hour exposure to both cocaine and Tat (Figure 2F), whereas a 35 and 55% loss of TJP-1 staining was seen with cocaine or Tat treatment alone, respectively.

Furthermore, functionality of the additive effect of cocaine and Tat treatment on attenuation of TJP-1 expression in endothelial cells was tested by monitoring the permeability of HPAECs to FITC-dextran. Exposure of HPAECs to either cocaine or Tat for 6 hours resulted in a 156 and 175% increase in the monolayer permeability, respectively (Figure 2G). However, treatment with both cocaine and Tat resulted in 241% increase in permeability, which was significantly more when compared with either treatment alone (Figure 2G). We conclude, therefore, that cocaine exposure can exacerbate the pulmonary endothelial dysfunction caused by HIV proteins, such as Tat.

Cocaine Exposure Synergizes with HIV-Tat to Promote Proliferation of pSMCs

One of the key pathological features of PAH is the increased proliferation of pulmonary VSMCs (7, 47). Thus, to see the direct effect of Tat and cocaine on proliferation of SMCs, we first treated quiescent T/G HA-VSMCs with various concentration of cocaine (100 nM, 1 μ M, 10 μ M) and/or Tat (10, 25, 50 ng/ml). As shown in Figure 3A, treatment with cocaine and Tat

for 48 hours resulted in a significant increase in proliferation of cells, as measured by cell proliferation assay. Interestingly, the simultaneous treatment of cells with 1 μ M cocaine and 25 ng/ml of Tat resulted in a synergistic increase, whereas exposure to 1 μ M cocaine and 50 ng/ml Tat resulted in an additive increase in cell proliferation compared with corresponding cocaine or Tat treatment alone. Because these findings were based on VSMC line, we next confirmed these findings in primary human pSMCs. Treatment of quiescent HPASMCs with cocaine (1 μ M) and Tat (25 ng/ml) for 48 hours resulted in a significant increase in the proliferation of these cells (Figure 3B). Simultaneous exposure of HPASMCs to cocaine and Tat resulted in a further increase in the proliferation of cells when compared with either treatment alone. As expected, treatment of pSMCs with PDGF-BB (100 ng/ml) resulted in enhanced proliferation of cells compared with untreated cells (Figure 3B). In addition, exposure of cells to cocaine or Tat in the presence of PDGF-BB demonstrated significant increases in the proliferation of cells compared with either treatment alone. Therefore, the simultaneous exposure of SMCs to Tat and cocaine, or to a soluble host factor, such as PDGF-BB, results in the enhanced proliferation of SMCs compared with either treatment alone.

Involvement of PDGF-PDGFR Axis in Cocaine-Mediated and Tat-Mediated Enhanced Proliferation of pSMCs

As mentioned previously here, whereas PDGF-BB is released by macrophages, endothelial cells, and SMCs, PDGFRs are mainly expressed in SMCs of pulmonary arteries in patients with PAH (29). To examine whether cocaine and Tat could increase PDGF- β receptor (PDGF- β R) expression on pSMCs,

Figure 5. Down-modulation of TJPs in the lungs from HIV-infected individuals with a history of intravenous drug use (IVDU). Expression of TJPs was analyzed by QuantiGene multiplex analysis of RNA extracted from frozen lung tissues of uninfected normal ($n = 3$), uninfected individual with IVDU ($n = 3$), HIV-1-infected individual without IVDU ($n = 4$), and HIV-1-infected individual with IVDU (HIV+IVDU; $n = 5$). Values represent the levels of (A) CLDN4, (B) TJP-1, (C) TJP-3, and (D) OCLN in various groups. Mean fluorescence intensity (MFI) obtained for each target was normalized with MFI of β -actin from the same sample. (E) Immunofluorescence demonstrated decreased expression of TJP-1 in HIV+IVDU compared with uninfected control. Paraffin-embedded lung sections were stained using human anti-TJP-1 primary antibody. Images were captured using confocal microscopy. Original magnification, 40 $\times$.

thus rendering them more responsive to PDGF-BB, we first examined the expression and activation of PDGF- β R in response to Tat and/or cocaine. Western blot analysis of cell lysate using antibodies against PDGF- β R and phosphorylated PDGF- β R (activated form) revealed significant increases in the expression of both forms of PDGF- β R in HPASMCs on exposure to Tat (25 ng/ml) and cocaine (1 μ M), as shown by densitometric analysis (Figure 4A).

To confirm further the involvement of PDGF-BB-PDGFR axis in Tat- and/or cocaine-mediated enhanced proliferation of SMCs, we pretreated pSMCs with or without 1 μ M imatinib mesylate, a tyrosine kinase inhibitor (Novartis, Basel, Switzerland) for 60 minutes, followed by Tat and cocaine treatment for 48 hours. The concentration of imatinib mesylate used was based on earlier studies showing inhibition of PDGF-specific signaling without any cytotoxic effects at 1 μ M (48-51). As shown in Figure 4B, imatinib could significantly inhibit the Tat- and cocaine-induced proliferation of HPASMCs. Because imatinib is not a specific inhibitor of PDGF signaling, we next used siRNA against PDGF- β R to transfect HPASMCs as an alternative approach to specifically inhibit PDGF signaling. First, to evaluate the efficiency of PDGF- β R siRNA in HPASMCs, expression of PDGF- β R was tested by Western blot analysis of the transfected cells. As illustrated in Figure 4C, transfection of HPASMCs with PDGF- β R siRNA resulted in knockdown of PDGF- β R expression, whereas transfection with negative control siRNA demonstrated significant increases in the receptor expression in response to cocaine and Tat treatment compared with untransfected cells treated with both cocaine and Tat. Furthermore, pSMCs transfected with PDGF- β R siRNA did not demonstrate enhanced proliferation of cells in the presence of Tat and cocaine (Figure 4D), therefore suggesting the involvement of PDGF signaling in Tat- and cocaine-mediated increases in proliferation.

IVDU Is Associated with Greater Down-Regulation of TJPs in HIV-Infected Lungs Compared with Lung Tissues that Were HIV Infected or IVDU Alone

To obtain a better understanding of the interactions between HIV and drug abuse that might escalate the development of pulmonary vascular remodeling, we evaluated the integrity of pulmonary endothelium in lungs from uninfected normal (normal; $n = 3$), uninfected/IVDU (IVDU; $n = 3$), HIV-1-infected/non-IVDU (HIV; $n = 4$), and HIV-1-infected/IVDU (HIV+IVDU; $n = 5$) individuals (Table 1). Using QuantiGene multiplex system, we quantitated the expression of TJPs in total mRNA isolated from frozen human lung tissues obtained from each individual in these groups. As shown in Figures 5A-5D, mRNA analysis suggested moderately significant down-regulation of CLDN4, TJP-1, TJP-3, and OCLN expression in HIV+IVDU tissues compared with normal or IVDU groups, except that no significant decrease was observed in TJP1 expression when compared with lung tissues from IVDU group. Down-modulation in the expression of CLDN4, TJP3, and OCLN in the HIV+IVDU group was found to be statistically significant when compared with HIV group. No significant alterations in TJP expression were observed in IVDU or HIV groups when compared with normal healthy lung tissue controls.

The decrease in the expression of TJPs was further confirmed by immunofluorescence analysis on the lung sections from HIV+IVDU and normal control. In uninfected normal control sections, blood vessels of all sizes demonstrated continuous staining patterns with TJP-1 antibody along the endothelial lining (Figure 5E, upper panel). Sections from HIV+IVDU, however, demonstrated weak or no expression of TJP-1, as seen in Figure 5E (lower panel). In conclusion, these results confirm an increase in endothelial damage of pulmonary vessels in the lungs from HIV+IVDU compared with tissues exposed to HIV or IVDU alone.

Figure 6. Representative images showing pulmonary vasculature from each individual within normal, IVDU, HIV⁺, and HIV+IVDU groups. Lungs were characterized for endothelial and SMC markers by immunohistochemistry for factor VIII (red color), and α -smooth muscle actin (SMA; brown color) on paraffin-embedded lung sections. Scale bars, 100 μ m.

Human Lung Sections Offer Evidence of IVDU Contribution to HIV-1-Related Pulmonary Vascular Remodeling

We next examined the histology of archival human lung sections for pulmonary vascular lesions in all of the groups described previously here. Table 1 shows the pulmonary vascular changes found in the various groups, including CD4 count and plasma viral load in HIV-infected individuals. As shown in the representative factor VIII and α -SMA immunostained lung sections from each individual in Figure 6, two of the three individuals in the IVDU group were found to have mild to moderate medial smooth muscle hypertrophy in small, nonmuscular arteries. Interestingly, none of the individuals from the HIV group showed any signs of pulmonary arteriopathy when compared with other groups, whereas all five lung sections from the HIV+IVDU group were confirmed by smooth muscle staining to have a moderate to significant medial hypertrophy (Figure 6, Table 1). Both, nonmuscular arteries and proximal, medium-sized arteries had severe medial hypertrophy in lung sections from the HIV+IVDU group. Concentric intimal thickening was also seen in the moderate and large vessels of two individuals within this group, comprising both muscle-specific actin staining and endothelial cell-specific factor VIII staining.

IVDU Contributes to Greater Up-Regulation of PDGF-BB in HIV-Infected Lungs Compared with Lung Tissues from Uninfected or IVDU Alone

Next, we performed immunohistochemical analysis of PDGF-BB on paraffin-embedded human lung sections from individuals with HIV with and without IVDU. As shown in Figure 7A, immunostaining demonstrated a strong increase in the expression of PDGF-BB in the lung sections from the HIV+IVDU group, especially in the large and small vessels with medial hypertrophy, lesions showing concentric intimal thickening, and in the perivascular macrophages within or surrounding the lesions. However, we found little expression of PDGF-BB in the thickened medial layer of vascular lesions in the sections from the IVDU group, whereas lung parenchyma, including smooth muscle layer of large vessels in the lung sections from the HIV group, had positive expression of PDGF-BB. Quantitative analysis of PDGF-BB-stained images showed statistically significant increases in the PDGF-BB staining in the HIV+IVDU group when compared with IVDU or HIV groups (Figures 7B and 7C), with an approximately twofold increase in the expression when whole-lung parenchyma (Figure 7B) was considered. However, when PDGF-BB staining was compared in the medial or intimal layer of blood vessels within the groups, an approximately sixfold increase was observed in the intensity of PDGF-BB staining

Figure 7. Expression of PDGF-BB in lungs of individuals from normal, IVDU, HIV₁, and HIV+IVDU groups. (A) Representative photomicrographs of immunohistochemistry on paraffin-embedded lung sections from each group are shown. Original magnification, 20 $\times$; scale bar, 100 μ m. (B and C) Quantitative analysis of PDGF-BB-stained lung sections from HIV-infected individuals with or without history of IVDU compared to normal controls. Mean staining intensity in the entire lung parenchyma (A) and in the arterial wall (B) obtained from three different lung sections within each group is shown. Data represent means ($\pm$ SD); * $P < 0.001$, treatment versus normal; # $P < 0.05$, ### $P < 0.001$, HIV+IVDU versus IVDU or HIV alone.

Figure 8. Model showing possible changes in the pulmonary arterial walls of HIV-infected cocaine users.

(Figure 7C) in the HIV+IVDU group compared with lung sections from the HIV group.

DISCUSSION

In this study, we offer *in vitro* findings that substantiate an additive role of HIV infection and cocaine exposure in vascular cell dysfunction, with alteration in TJP expression of pECs and enhanced proliferation of pSMCs through modulation of the PDGF-BB-PDGFR axis. Furthermore, our findings include clinical evidence of advanced pulmonary arteriopathy, with disrupted endothelial layer and medial hypertrophy in the lung tissues from HIV-positive individuals with a history of IVDU.

The possibility of direct HIV infection of pulmonary vasculature cells, such as endothelial cells and SMCs (two important vascular cell types), leading to HIV-PAH development, is highly unlikely (52–55). As such, it is believed that either the direct effect of circulating HIV proteins on the vascular components and/or the indirect effect of proinflammatory markers and growth factors released by infected lymphocytes and macrophages can lead to the development of HIV-PAH (56, 57). Endothelial dysfunction of the pulmonary vasculature plays a key role in the progression of PAH (45). In this regard, various viral proteins, including Tat, have been implicated in endothelial dysfunction (58–61). Glycoprotein-120, an HIV-1 protein involved in the entry of virus into cells, has been shown to stimulate the secretion of endothelin-1 in macrophages and lung endothelial cells (59,

60). The negative factor protein of HIV has also been found to be present at the site of pulmonary vascular lesions in patients with HPAH, and also in simian HIV-infected macaques (58, 61). Tat is known to act as an angiogenic and oncogenic factor (38–42) by promoting growth, migration, and production of growth factors in various cell types (38, 62, 63). In addition, Tat is known to induce actin–cytoskeleton changes, including apoptosis of pulmonary microvascular endothelial cells through caspase activation (64), leading to induction of vascular permeability (65, 66).

Cocaine has also been shown to cause endothelial dysfunction through its direct effect on microvascular endothelial cells and through indirect effects via the release of proinflammatory cytokines (21, 67–69). In the present study, we demonstrated down-modulation in the expression of TJPs in pECs on exposure to Tat and/or cocaine. We furthermore observed enhanced loss of immunoreactivity of peripheral TJP-1, resulting in enhanced permeability of endothelial monolayer, in the presence of Tat and cocaine compared with either treatment alone. Our evidence supports the plausibility of HIV-1 and cocaine working in concert to elicit pulmonary endothelial dysfunction, which may account for the higher incidence of HIV-PAH in individuals with HIV and IVDU. These findings were further supported by our demonstration of down-modulation of TJPs in the lungs of patients with pulmonary arteriopathy, again with greater loss in those with HIV infection *and* a history of IVDU as compared with those with HIV infection or IVDU alone. To be sure, our findings regarding human subjects have limitations due to small sample size and con-

founding variables associated with patient lung tissues, but even so, this work may offer a realistic perspective into the clinical nature of pulmonary arteriopathy development in the presence of HIV with and without IVDU.

Animal-based studies suggest endothelial injury as an initiating step in the development of pulmonary arteriopathy associated with PAH, followed by the proliferation of VSMCs, leading to medial hypertrophy (45). In addition, studies on lung biopsy samples from patients with PAH have also highlighted the alteration of pulmonary endothelium (70). Injury to the endothelium allows increased penetration of viral proteins, stimulants, and growth factors into the subendothelium, followed by the enhanced exposure of these factors to SMCs. Here, we report that the simultaneous exposure of SMCs to both cocaine and Tat results in a synergistic, or additive, increase in the proliferation of SMCs, although independent treatment with cocaine or Tat also increased the proliferation of SMCs. Furthermore, histological evidence demonstrated at least moderate pulmonary arteriopathy with presence of smooth muscle hyperplasia in all lung tissue samples from patients with HIV with a history of IVDU.

SMCs are particularly sensitive to mitogenic and chemotactic properties of PDGF-BB, a potential mediator of medial hypertrophy and intima formation associated with PAH (71). Previous studies provide direct evidence for the involvement of PDGF in the pathogenesis of pulmonary vascular remodeling in animal models (26, 27), and imatinib mesylate, which inhibits PDGFR signaling, has been demonstrated to reverse the pulmonary vascular remodeling in animal models and improve the hemodynamics in some patients (32, 34). Studies by Humbert and colleagues (28, 29, 72) have demonstrated increased expression of PDGF in lung biopsies from patients with primary PAH or with HIV-PAH. Similar to these findings, we too found enhanced expression of PDGF-BB in the HIV+IVDU group, particularly in SMCs of arteries showing medial hypertrophy, in comparison with SMCs from vascular lesions in the normal or IVDU groups. An increase in the expression of PDGF-BB with HIV infection is consistent with our earlier findings demonstrating an overexpression of PDGF- β R axis *in vitro* in HIV-infected macrophages and *in vivo* in the lungs from simian HIV-infected macaques (30, 31, 44). However, the present study goes further by demonstrating that cocaine potentiates the expression of PDGF-B chain in HIV-infected primary macrophages and Tat-treated pECs. Furthermore, our present study demonstrates enhanced activation and expression of PDGF- β R in Tat- and cocaine-treated pSMCs. In addition, we found that the increase in proliferation of pSMCs on simultaneous exposure to Tat and cocaine was blocked on pretreatment of cells with PDGFR tyrosine kinase inhibitor. Thus, our *in vitro* and histological findings suggest that enhanced paracrine and autocrine activation of PDGF- β R in pulmonary arterial SMCs of HIV-infected samples exposed to cocaine may play a role in potentiating smooth muscle hyperplasia in these tissues.

Our previous studies have demonstrated enhanced virus replication in macrophage cultures upon introduction of exogenous PDGF-BB (31). When applied to the case of enhanced PDGF-BB expression in cocaine-exposed, HIV-infected lungs, these previous findings of ours would seem to suggest a further increase in the secretion of viral proteins by infected cells in HIV-positive IVDU lungs as well. Because we have further demonstrated, again in previous work, that cocaine itself exacerbates viral replication in HIV-infected macrophages (25), we conclude here that exposure to cocaine increases virus and viral proteins, such as Tat, glycoprotein-120, and negative factor, in the circulation, resulting in potentiation of the effects of HIV-1.

Based on data presented here and in previous reports, we speculate (Figure 8) that HIV-infected cells, such as macrophages, and endothelial cells exposed to viral proteins, such as Tat, are made more potent sources of PDGF-BB by the presence of cocaine. In addition, we speculate that viral protein(s) and cocaine act in concert to initiate pulmonary vascular injury at the endothelial level through loss of TJPs. We suggest that the nature of the relationship of HIV infection with PDGF, each driving the other, and increased viral replication in the presence of cocaine, allows for a constant source of viral proteins and PDGF. Subsequent to these alterations, increased penetration of viral protein(s), cocaine, and PDGF-BB ensues, allowing the enhanced stimulation, proliferation, and migration of pSMCs (Figure 8). In summary, the interactions between HIV-1 infection and cocaine helps to maintain an environment that promotes arteriopathy, thus likely contributing to the enhanced incidence of clinical HIV-PAH in individuals who are infected with HIV with a history of IVDU.

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