Downregulation of Bone Morphogenetic Protein Receptor Axis During HIV-1 and Cocaine-Mediated Pulmonary Smooth Muscle Hyperplasia

Implications for HIV-Related Pulmonary Arterial Hypertension

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Objective—Our previous findings support an additive effect of cocaine to HIV infection in the development of pulmonary arteriopathy through enhanced proliferation of human pulmonary smooth muscle cells. We now examined the role of antiproliferative bone morphogenetic protein receptor (BMPR) axis in HIV protein and cocaine-mediated pulmonary smooth muscle hyperplasia.

Approach and Results—Stimulation of BMPR axis resulted in attenuation of synergistic increase in the proliferation of human pulmonary arterial smooth muscle cells in response to cocaine and HIV protein, transactivator of transcription (Tat). Interestingly, an increase in mRNA but decrease in protein levels of BMPR with correlated decrease in the activation of Sma- and MAD-related family protein 1/5/8 and Id1 gene expression was observed on combined treatment with cocaine and Tat when compared with the untreated cells at all time points tested. Although longer exposure to either cocaine or Tat alone also resulted in a significant decrease in the BMPR protein expression, the abrogation on combined treatment was still significantly more when compared with that of the monotreatments. Significant increase in mRNA but downmodulation of BMPR protein expression was also observed in the lung extracts from HIV-infected intravenous drug users (HIV+IVDU) when compared with that from HIV-infected non-IVDUs (HIV) or uninfected IVDUs (IVDU). Furthermore, significant decrease in BMPR protein expression was also observed in HIV or IVDUs when compared with normal controls that correlated with in vitro findings on chronic exposure to cocaine or HIV protein alone.

Conclusions—Simultaneous exposure of pulmonary smooth muscle cells to viral protein(s) and cocaine exacerbates downregulation of BMPR axis that may result in enhanced pulmonary vasculature aberrations in HIV+IVDUs.

Key Words: bone morphogenetic proteins • gp-120 • HIV-Tat • Nef protein • pulmonary vascular remodeling • smooth muscle proliferation cocaine

Pulmonary arterial hypertension is one of the most common noninfectious complications of HIV infection,1 with ≈1000× higher incidence in HIV-infected patients compared with the general population.2 The probability of survival reduces to one half in the individuals who develop HIV-related pulmonary arterial hypertension (HRPAH) compared with HIV-infected individuals without PAH.3 Despite major clinical advances in therapy for the past few years, the prognosis of HRPAH remains poor and is similar to that of some advanced cancers. Furthermore, although it is evident from other case reports that the abuse of cocaine and other stimulants is a possible risk factor in the development of PAH,4–6 intravenous drug use (IVDU) was found to be one of the major risk factors for HIV infection in the patients with HRPAH.7 Our recent study showing enhanced pulmonary vascular remodeling in HIV-infected lung tissues from intravenous heroin and cocaine abusers indicates that IVDU and HIV-1 potentially act in concert to cause pulmonary arteriopathy.8 However, it is still not clear how illicit drugs and HIV infection either alone or in combination can cause the vascular dysregulation associated with increased pulmonary vascular resistance and cardiac dysfunction.

The possibility of direct HIV infection of pulmonary vasculature cells leading to HRPAH development is unlikely because HIV-1 RNA or DNA is not found in the pulmonary vessels of human lung tissues.9 Studies demonstrate that the direct action of HIV proteins released by the infected lymphocytes and macrophages plays a major role in the development of HRPAH.10 Recently, we11 showed that pulmonary vascular remodeling develops in the presence of...
HIV-1 proteins without an active infection, leading to pulmonary hypertension in a noninfectious HIV-transgenic rat model. The pulmonary arterial smooth muscle cells (PASMCs) are key players in the pathogenesis of all forms of PAH vascular remodeling. The exposure of PASMCs to viral proteins and growth factors after damage to the endothelial monolayer leads to smooth muscle hypertrophy and proliferation. However, the cellular and molecular mechanisms underlying the thickening of blood vessels are poorly defined.

Although bone morphogenetic protein receptor (BMPR)-2 mutations have been associated with familial PAH, many studies suggest that a critical reduction in the expression of BMPRs may be important in the pathogenesis of PAH. BMP-2 or BMP-4 on binding to BMPR negatively regulates SMAD signaling and proliferation. The BMP ligands bind to heteromeric complexes of BMPR-1A or BMPR-1B with BMPR-2 resulting in the phosphorylation of regulatory Smad and MAD-related family proteins (SMAD). Activated regulatory SMADs: SMAD1/5/8 then form complex with SMAD4 that translocate to the nucleus and regulate the transcription of BMP/SMAD-responsive antiproliferative genes.

HIV-1 protein: transactivator of transcription (Tat), is actively secreted by infected cells and acts as an angiogenic and oncogenic factor by promoting growth, migration, and production of growth factors in various cell types. In our previous findings, we have shown that cocaine synergizes with HIV-Tat to promote proliferation of PASMCs. In this study, we partially define the mechanism(s) mediating this increased proliferation and enhanced pulmonary vascular remodeling in HIV-infected IVDUs by examining the alterations in the antiproliferative BMP/BMPR axis. We here report significantly more attenuation in the BMPR protein expression in PASMCs on combined treatment with HIV-Tat and cocaine compared with either treatment alone, concomitant with abrogation of BMP downstream signaling and antiproliferative Id1 gene expression. Furthermore, to the best of our knowledge, we found that this is the first report demonstrating significant downmodulation of BMPR expression in the lungs from HIV-infected IVDUs compared with that from HIV-infected non–drug users or uninfected IVDUs. Some of the results of these studies have been previously reported in the form of abstract.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

BMP-2 Stimulation and BMPR-2 Overexpression Result in Attenuation of Tat- and Cocaine-Mediated Synergistic Increase in Proliferation of PASMCs

We reported earlier that cocaine or Tat treatment alone significantly increases PASMC proliferation, and this effect is further enhanced on simultaneous cocaine and Tat exposure. Because BMPs on binding to BMPR negatively regulate PASMC growth and proliferation, we next wanted to elucidate whether cocaine- and Tat-mediated enhanced proliferation of PASMCs involves alterations in this antiproliferative BMPR axis. To begin with, we exposed the cells to cocaine in the presence or absence of Tat for 2, 3, and 6 days and saw a gradual increase in the PASMC proliferation on cocaine or Tat treatment alone when compared with that of the untreated cells. As expected, combined treatment with cocaine and Tat resulted in synergistic increase in cell proliferation, with maximum fold increase observed overnight when compared with either of the monotreatments (Figure 1A). We then tested whether stimulation with BMP-2 could prevent the cocaine- and Tat-mediated enhanced SMC proliferation. As represented in Figure 1B, BMP-2 could completely prevent an increase in cell proliferation induced by either cocaine or Tat treatment alone as early as 48 hours after treatment at all concentrations tested. However, pretreatment with BMP-2 could significantly reduce but was not able to completely abrogate the synergistic increase in cell proliferation after combined treatment with cocaine and Tat, even at higher concentrations and longer exposure (Figure 1B and 1C). Furthermore, Western blot analysis of proliferation marker, proliferating cell nuclear antigen (Figure 1D), also confirmed the inability of BMP-2 to prevent completely the cocaine-Tat–triggered proliferation. This could be because of the alterations in the BMPR expression on combined treatment with Tat and cocaine, resulting in lower availability of receptors for ligand binding. Therefore, to next explore the importance of BMPR in cocaine-Tat–mediated SMC proliferation, we overexpressed BMPR-2 by transiently transfecting human PASMCs (HPASMCs) with BMPR-2 expression vector. BMPR-2 overexpression was first confirmed by Western blot analysis of transfected cells (Figure 1 in the online-only Data Supplement). Cell proliferation analysis showed significant reduction in proliferation of cells transfected with BMPR-2 expression vector on treatment with Tat and cocaine when compared with that of the treatment of mock-transfected cells (Figure 1E). The fold increase in the cell proliferation of mock-transfected cells in response to cocaine and Tat treatment was comparable with that of obtained on treatment of untransfected control cells with cocaine and Tat. Importantly, BMP-2 treatment of BMPR-2 overexpressing cells could completely abrogate the cocaine-Tat–mediated enhanced proliferation. These findings clearly confirm the involvement of BMPR axis dysfunction in Tat- and cocaine-mediated augmentation of PASMC proliferation.

Combined Treatment of PASMCs With Tat and Cocaine Results in Increased BMPR mRNA Expression

The above mentioned findings led us to investigate the expression of heteromeric BMP receptor complex in relation to
Figure 1. Attenuation of transactivator of transcription (Tat) and cocaine mediated increased proliferation of human pulmonary arterial smooth muscle cells (HPASMCs) on the activation of bone morphogenetic protein (BMP)/BMP receptor (BMPR) axis. A, HPASMCs (3×10^3/well) were seeded in 96-well plate. After 48 hours, the medium was replaced with 0.1% serum containing medium followed by cocaine (1 μmol/L) with or without Tat (25 ng/mL) treatment for indicated time periods followed by MTS cell proliferation assay. All values are mean±SD of 3 independent experiments. *P<0.01, **P<0.001 vs control, #P<0.01 vs cocaine, $P<0.001 vs Tat. Cell proliferation analyses of cocaine- and Tat-treated HPASMCs in the presence or absence of pretreatment with BMP-2 (10, 50, or 100 ng/mL) at 48 hours (B) and at 2 to 6 days (C) after treatment. All values are mean±SD of 3 independent experiments. *P<0.001 vs control, #P<0.001 vs cocaine, $P<0.001 vs Tat, @P<0.001 vs combined cocaine and Tat. D, Quiescent HPASMCs were treated with combined- or mono-treatment of cocaine and Tat in the presence or absence of 10- or 50-ng/mL BMP-2 for 48 hours followed by protein extraction. Western blot was used to determine the cell proliferation using proliferating cell nuclear antigen (PCNA) as the marker. Lower, The densitometric analysis of blots from 3 independent experiments (mean±SEM). *P<0.001 vs control, #P<0.01, ##P<0.001 vs cocaine, $P<0.001 vs Tat, @P<0.001 vs combined cocaine and Tat. E, BMPR-2 overexpression reduced significantly the cocaine- and Tat-mediated smooth muscle proliferation. The HPASMCs plated in 96 wells were serum-starved for 24 hours before transfection with BMPR-2 expression vector. At 24 hours after transfection, cells were treated with combined treatment of cocaine and Tat in the presence or absence of BMP-2 (10 ng/mL) for 48 hours followed by MTS assay. All values are mean±SD of 3 independent experiments. *P<0.001 vs control, #P<0.001 vs cocaine and Tat.
Tat- and cocaine-mediated increase in proliferation. The HIV-Tat protein has been found to repress the BMPR-2 gene expression in monocyte cell line, resulting in inhibition of BMP responsive downstream signaling. Similar to these findings, we also observed a significant decrease in the BMPR-2 mRNA expression on analysis of total RNA isolated from HPASMCs treated with Tat (Figure 2A). In addition, we also found a decrease of BMPR-2 mRNA in cells exposed to only cocaine for 1, 2, 3, or 6 days. However, cells treated with both Tat and cocaine showed a significant increase in BMPR-2 mRNA when compared with that of the untreated cells at all time intervals tested. In addition, expression of both BMPR-1A and BMPR-1B also decreased after treatment with cocaine alone, whereas Tat treatment did not result in significant changes at any of the time points tested (Figure 2B and 2C). Interestingly, similar to the effect on BMPR-2 expression, combined treatment with both Tat and cocaine resulted in significant increase in BMPR-1A and BMPR-1B expressions (Figure 2B and 2C) when compared with that of untreated control with higher difference in the expression observed at 24 hours after treatment compared to later time points.

**Combined Treatment With Tat and Cocaine Results in Greater Attenuation of BMPR Protein Expression Compared With That of Monotreatments**

After observing an increase in the mRNA levels of BMPRs in cocaine and Tat treated HPASMC, the protein expression was determined by Western blot analysis at 2, 3, or 6 days after treatment. We observed a significant decrease in the BMPR-2 protein levels after simultaneous exposure to both Tat and cocaine compared with that of untreated cells at all time points tested (Figure 3). On the contrary, although not significant, a trend toward increase in the BMPR-2 (Figure 3A) expression was observed after 48-hour treatment with Tat alone. However, a downward trend in BMPR-2 expression was seen on treatment with cocaine alone when compared with that of untreated control. Nevertheless, a significant reduction in BMPR-2 expression was observed as the cocaine or Tat exposure time increased from 2 to 6 days. However, this cocaine or Tat monoculture-mediated attenuation of BMPR protein levels on longer exposure was still significantly less when compared with that observed on the combined treatment.

Furthermore, decrease in BMPR-1A and BMPR-1B was also observed on combined treatment with Tat and cocaine at all time points tested (Figure 3B and 3C). Similar to BMPR-2 findings, a significant increase in BMPR-1B (Figure 3C) expression was observed after 48-hour treatment with Tat alone. Interestingly, an increase in BMPR-1B protein expression was also found on treatment of cells with cocaine alone when compared with that of the untreated control (Figure 3C). BMPR-1B has been reported earlier to be involved in the enhanced proliferation of PASMC isolated from patients with primary pulmonary hypertension in response to BMP-2/BMP-7 ligands, and we also observed significantly higher secretion of BMP-2 and BMP-7 ligands in response to cocaine or Tat monoculture compared with that of untreated control (Figure 2 in the online-only Data Supplement). However, just like BMPR-2 expression, cocaine or Tat monoculture for 6 days also resulted in significant reduction of BMPR-1A and BMPR-1B protein expressions when compared with that of the untreated controls with the protein levels still significantly higher when compared with that of the combined treatment.

**Cocaine Exposure Results in the Repression of BMPR-Mediated Downstream Signaling in HIV-Tat–Treated PASMCs**

The binding of ligands to BMPR results in phosphorylation of regulatory SMADs (SMAD1/5/8) followed by their complex formation with co-SMAD4. The nuclear translocation of this complex then leads to the regulation of BMP/SMAD-responsive antiproliferative genes. As illustrated in Figure 4A, combined treatment of cells with cocaine and Tat for 2, 3, or 6 days resulted in significant repression in the levels of p-SMAD1/5/8 when compared with that of the untreated cells. Initially, at 48 hours after treatment, HIV-Tat exposure resulted in significant increase of p-SMAD1/5/8 expression.
However, this increase in activation of SMAD1/5/8 came back to control levels at 3 days after treatment followed by significant decrease on longer exposure of 6 days. Interestingly, cocaine alone exhibited a significant reduction in the phosphorylation of SMAD1/5/8 when compared with that of the untreated control at 2 and 6 days after treatment. However, similar to BMPR protein expression, levels of p-SMAD1/5/8 on combined treatments were still significantly less when compared with that of the monotreatments at 3 and 6 days after treatment.

It has been reported earlier that addition of BMP-2 activates SMAD1/5/8 and inhibits growth factor–stimulated proliferation of human pulmonary SMCs. Likewise, prestimulation with BMP-2 caused significant increase in p-SMAD1/5/8 level in cells exposed or unexposed to cocaine or Tat when compared with that of the non–BMP-2-stimulated cells. Also, the presence of BMP-2 could rescue the reduction in the levels of p-SMAD1/5/8 on combined treatment with cocaine and Tat (Figure 4B). Furthermore, the antiproliferative effect of BMP-2 seen in Figure 1 could be reversed in cells lacking SMAD1/5/8 in case of monotreatments and combined cocaine/Tat treatments, as observed in HPASMCs transiently transfected with siRNA against SMAD1/5/8 (Figure III in the online-only Data Supplement).

Alterations in the activation of SMAD1/5/8 were further confirmed by analysis of its communoprecipitation with SMAD4. As illustrated in Figure 4C, combined treatment of cells with cocaine and Tat for 48 hours resulted in significant repression in the levels of p-SMAD1/5/8–SMAD4 complex when compared with that of the untreated cells. Treatment with Tat alone caused a significant increase, whereas cocaine alone exhibited a significant reduction in the levels of SMAD1/5/8–SMAD4 complex when compared with that of the untreated control. Interestingly, contrasting effect was seen on the transforming growth factor (TGF)-β–mediated regulatory SMAD–co-SMAD (p-SMAD2/3–SMAD4) complex formation when SMAD4 immunoprecipitated cell lysate was independently probed with p-SMAD2/3. Combined treatment of cells with cocaine and Tat or treatment with cocaine alone resulted in significant increase in p-SMAD2/3–SMAD4 complex formation compared with that of the untreated cells, whereas Tat alone caused significant reduction in the p-SMAD2/3–SMAD4 complex levels (Figure 4C).

Next, to assess the effect of cocaine and Tat on the downstream BMPR target gene, total RNA from 48-hour–treated HPASMCs was evaluated for the levels of antiproliferative Id1 mRNA by real-time reverse transcriptase-polymerase chain reaction. Concomitant with the levels of p-SMAD1/5/8,
Figure 4. Abrogation of bone morphogenetic protein receptor (BMPR)–mediated downstream signaling on exposure of transactivator of transcription (Tat)–treated human pulmonary arterial smooth muscle cells (HPASMCs) to cocaine. A, Cellular extracts of HPASMCs treated with cocaine (1 μmol/L) in the presence or absence of Tat (25 ng/mL) for 2, 3, and 6 days were analyzed for phosphorylated (p)-Sma- and MAD-related family proteins (SMAD)1/5/8 and total SMAD1/5/8 protein expression by Western blot. Histogram represents densitometric analysis of 3 independent experiments. Values are mean±SEM. *P<0.05, **P<0.01, ***P<0.001 compared with that of the untreated control, #P<0.05, ##P<0.01 compared with that of the cocaine treatment alone, $P<0.05, $$$P<0.01 compared with that of the Tat treatment alone. B, Cellular extracts of HPASMCs treated with cocaine (1 μmol/L) in the presence or absence of Tat (25 ng/mL) for 48 hours stimulated with or without BMP-2 (10 ng/mL) 30 minutes before cell lysis were analyzed for phosphorylated (p)-Sma- and MAD-related family proteins (SMAD)1/5/8 and total SMAD1/5/8 protein expression by Western blot. Histogram represents densitometric analysis of 3 independent experiments. Values are mean±SEM. *P<0.05, **P<0.01, ***P<0.001 compared with that of the untreated control, #P<0.05 compared with that of the cocaine treatment alone, $P<0.01 compared with that of the Tat treatment alone. C, Immunoprecipitation (IP) of protein extract from cells treated with cocaine in the presence or absence of Tat for 48 hours using total SMAD4 antibody followed by immunoblotting (IB) with antibodies against p-SMAD1/5/8 or p-SMAD2/3. Graphs (lower) represent densitometric analysis of complex formation (mean±SEM) from 3 independent experiments. *P<0.05, **P<0.01, ***P<0.001 compared with that of the untreated control. D, Decreased antiproliferative Id1 target gene expression in HPASMCs on combined treatment with Tat and cocaine for 48 hours. Quantitative analysis of BMP target gene Id1 was performed by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) using the SYBR green detection method. All values are mean±SD of ≥3 independent experiments. *P<0.001 compared with that of the untreated control, #P<0.001 compared with that of the cocaine alone. E, Upregulation of antiproliferative Id1 gene in cocaine-and Tat-treated HPASMCs on BMPR-2 overexpression. Serum-starved, BMPR-2–transfected HPASMCs were treated with cocaine and Tat for 48 hours followed by stimulation with BMP-2 (10 ng/mL). Total RNA was then isolated after 30 minutes for RT-PCR analysis of Id1. All values are mean±SD of 3 independent experiments. *P=0.001 compared with the treated vs untreated, #P>0.001 compared with the combined cocaine–Tat treatment, $P<0.05, $$$P<0.001 compared with BMP-2 treatment.
both combined treatment of cells with cocaine and Tat and cocaine treatment alone expressed lower Id1 mRNA levels when compared with that of the untreated cells. Furthermore, the decrease in the Id1 mRNA expression on treatment with both cocaine and Tat (Figure 4D) was significantly more than cocaine treatment alone. Overexpression of BMPR-2 in HPASMCs prevented the cocaine- and Tat-mediated decrease in Id1 mRNA expression (Figure 4E) both in the absence and in the presence of BMP-2 stimulation. Overall, these results demonstrate clearly that cocaine and Tat together negatively affect BMPR downstream signaling in HPASMCs that could lead to increased proliferation.

**IVDU Is Associated With Increase in BMPR mRNA Expression in HIV-Infected Lungs When Compared With That of Lung Tissues From HIV-Infected Non–Drug Users or Uninfected IVDUs**

Downmodulation of BMPR expression and downstream signaling pathways in response to HIV-Tat and cocaine treatment led us to explore the possible implication of this phenomenon in vivo. We evaluated BMPR expression in human lung tissues from HIV-infected IVDUs (HIV+IVDUs) that demonstrated enhanced pulmonary vascular remodeling when compared with that from the HIV-infected non–drug users (HIV) or uninfected IVDUs (IVDU), as reported in our previous study.8 Similar to our in vitro findings on BMPR mRNA expression in Tat- and cocaine-treated HPASMCs, human lungs from HIV+IVDU group demonstrated significant increase in BMPR-2, BMPR-1A, and BMPR-1B mRNA expressions (Figure 5A) when compared with that from HIV group, IVDU group, or normal controls as seen by real-time reverse transcriptase-polymerase chain reaction analysis. However, lungs from HIV or IVDU group did not show any changes in BMPR-2, BMPR-1A, and BMPR-1B mRNA expressions when compared with that of the normal controls.

**IVDU Is Associated With Greater Downmodulation of BMPR Protein Expression in HIV-Infected Lungs When Compared With That of Lung Tissues From HIV-infected Non–Drug Users or Uninfected IVDUs**

Western blot analysis of total lung extract revealed significant loss of BMPR-2 protein expression in HIV+IVDU group when compared with that of HIV or IVDU groups (Figure 5B). However, when compared with the normal group, a significant reduction in BMPR-2 protein expression was also observed in the protein extracts from IVDU or HIV group. Similarly, HIV+IVDU group had lower BMPR-1A and BMPR-1B protein expression when compared with that of either HIV or IVDU groups. Maximum expression of BMPR-1A and BMPR-1B receptors was observed in the protein extract from normal lung tissues.

The loss of BMPR-2 expression in HIV+IVDU group was further confirmed by immunohistochemical analysis on paraffin-embedded lung sections from selective individuals. As shown in the representative images in Figure 5C and Figure 4A in the online-only Data Supplement, HIV+IVDU group had remarkably reduced BMPR-2 expression in the smooth muscle lining of the thickened arterial wall, whereas BMPR-2 staining was distinctly seen in the arterial smooth muscle lining of lung sections from HIV or IVDU groups. However, BMPR-2 staining in the vascular wall of HIV or IVDU group lung sections was notably less when compared with that of the uninfected non-IVDU normal lung sections with maximum expression observed in case of normal lungs. The absence of staining in the negative control (Figure IVB in the online-only Data Supplement) validates the positive BMPR-2 expression in the representative sections.

**Chronic Exposure of PASMCs to HIV-Tat in the Presence of Cocaine Results in Greater Reduction in BMPR Protein Expression When Compared With That of the Other HIV Proteins**

Although we observed a decrease in the BMPR protein expression in human lungs of HIV group, we speculated that there may be an involvement of other HIV proteins in addition to Tat in downmodulating BMPR in HIV-infected individuals. Hence, we chronically exposed HPASMCs to either HIV protein: Nef or gp-120 in the presence or absence of cocaine. As illustrated in Figure 6, chronic exposure to either Nef or gp-120 demonstrated significant reduction in BMPR-2, BMPR-1A, and BMPR-1B protein expressions when compared with that of the untreated cells. In case of BMPR-2 and BMPR-1A, a trend toward decrease in expression was observed in Nef-treated or gp-120–treated cells when compared with that of the Tat-treated cells (Figure 6A). However, maximum loss of BMPR expression was observed only on combined treatment with cocaine and Tat when compared with that of the all other treatments. These results correlate with our in vivo findings of reduced protein expression of BMP receptors in lungs from HIV or IVDU groups. Overall, our in vitro findings showing enhanced impairment of BMP signaling in cocaine and HIV-Tat–treated HPASMCs correlate with increased loss of BMPR expression in the lungs from HIV-infected IVDUs when compared with that of the lungs exposed to either HIV or IVDU alone.

**Discussion**

In this study, we demonstrate that the synergistic increase in the proliferation of pulmonary SMCs in response to combined treatment with HIV-Tat and cocaine involves attenuation in the protein levels of BMPR-2, BMPR-1A, and BMPR-1B expressions. Consistent with the downregulation of BMPR expression, we observed significant decrease in the phosphorylation of SMAD1/5/8 and expression of antiproliferative Id1 gene. Furthermore, increase in the proliferation of HPASMCs in response to cocaine and Tat treatment was found to be diminished in cells overexpressing BMPR-2. In addition, we offer for the first time in vivo evidence of significantly more decrease in the expression of both type II and type I BMPRs in the lung tissues from HIV+IVDUs with enhanced pulmonary arteriopathy when compared with that of the HIV or IVDU groups, as shown in our previous findings.8 Various studies on human lung tissues from idiopathic or heritable PAH have shown that impairment in BMPR-2 expression may contribute critically to the pathogenesis of PAH, irrespective of mutations in the gene.13,19 This indicates
that other environmental factors could negatively affect BMP signaling predisposing SMCs to enhanced proliferation associated with PAH. Furthermore, reduction in BMPR expression and downregulation of SMAD signaling has also been reported in the monocrotaline and chronic hypoxia-induced animal models of PAH.\textsuperscript{20,21} Similar to these findings, in this study, we observed a significant decrease in the BMPR-2 and downward trend in BMPR-1A and BMPR-1B protein levels after simultaneous exposure of HPASMCs to both Tat and cocaine when compared with that of the untreated cells at early time points. However, chronic treatment with both Tat and cocaine later resulted in significant decrease in the protein expression of all BMPRs. Nevertheless, chronic treatment for 6 days with either Tat or cocaine alone also resulted in significant decrease in BMPR protein levels when compared with that of the untreated controls. Chronic treatment of HPASMCs with other viral proteins, such as Nef or gp-120, in the presence or absence of cocaine also led to significant decrease in the BMPR expression. However, maximum decrease in BMPR protein levels at this time point was observed on simultaneous treatment with both HIV-Tat and cocaine.

On the contrary to the protein expression, combined treatment with cocaine and Tat resulted in significant increase in the mRNA expressions of BMPR-2, BMPR-1A, and BMPR-1B. Nevertheless, cocaine monotherapy also led to a decrease in BMPR-2, BMPR-1A, and BMPR-1B mRNA expressions, and this correlated with the decrease in protein expression observed after longer exposure. HIV-Tat monotherapy

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\caption{Increase in mRNA and decrease in protein expression of bone morphogenetic protein receptors (BMPRs) in human lung tissues from HIV-infected intravenous drug users (IVDUs). A, Total RNA was extracted from frozen human lung tissues from HIV-infected individuals with (HIV+IVDU group; \(n=4\)) or without IVDU (HIV group; \(n=4\)) and uninfected individuals with (IVDU group; \(n=3\)) or without IVDU (normal group; \(n=3\)). Quantitative analysis of BMPR-2, BMPR-1A, and BMPR-1B expressions was performed by real-time reverse transcriptase-polymerase chain reaction using the SYBR green detection method. Mean±SD, *\(P<0.05\) compared with that of the normal, \#\(P<0.05\) compared with that of the IVDU, $\$P<0.05$ compared with that of the HIV group. B, Expression of BMPR-2, BMPR-1A, and BMPR-1B was analyzed by Western blot of total protein extract obtained from frozen lung tissues. Upper represents the Western blot image and lower represents the densitometric analysis of the immunoblots. Mean±SEM, *\(P<0.05\) compared with that of the normal, \#\(P<0.05\) compared with that of the IVDU, $\$P<0.05$ compared with that of the HIV. C, Representative photomicrographs of BMPR-2 immunohistochemistry on paraffin-embedded lung sections from normal, HIV±IVDUs are shown. Original magnification ×100, scale bar, 100 µm.}
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showed decrease in the BMPR-2 mRNA expression with no significant alterations in the BMPR-1A and BMPR-1B mRNA expression at all time intervals. Our ex vivo findings on human lungs from HIV+IVDUs also demonstrated decrease in BMP protein expression with increase in mRNA expression. A number of proteins have previously been shown to have decreased expression in the presence of elevated corresponding mRNA. This may be because of involvement of feedback regulation of BMP mRNA expression in response to significant increase in the secretion of BMP ligands by PASMCs on treatment with Tat or cocaine alone and in response to no change in ligands on combined treatment (Figure II in the online-only Data Supplement). However, the changes in BMPR mRNA expression in response to the BMP ligands did not correspond to the changes at the protein level. It could be that enhanced mRNA expression reflect a negative feedback loop coupled with post-transcriptional targeting of mRNA and regulation of protein expression by microRNAs or by ubiquitinated degradation of receptor proteins, which is a focus of our ongoing studies.

BMP ligands are known to have a higher affinity for BMPR-1A and BMPR-1B than for BMPR-2. Distinct downstream signaling cascades are known to be activated depending on the expression level of ligands and receptors, ligand–receptor affinity, or the presence of preformed type I/type II receptor complexes. Binding of ligands to preformed receptor complexes leads to the activation of canonical SMAD-dependent signaling, whereas ligand-induced receptor complex formation results in the activation of non–SMAD-dependent mitogen-activated protein kinase signaling. The increased expression of BMP ligands on treatment with Tat or cocaine (Figure II in the online-only Data Supplement) may have led to first binding of ligands to type I receptors and then recruitment of type II receptor BMPR-2, resulting in activation of mitogen-activated protein kinase–dependent proliferative signaling. Given that we did not observe significant reduction in expression of BMPRs on exposure to only Tat or cocaine at 2 days after treatment, the above explanation may fit our findings of enhanced proliferation of PASMCs on treatment with either Tat or cocaine alone. Interestingly, we also observed an increase in the protein levels of BMPR-1B on exposure to either Tat or cocaine alone at 2 days after treatment. Takeda et al previously reported enhanced expression of BMPR-1B in PASMCs isolated from primary pulmonary hypertension patients when compared with that of the control cells. They also demonstrated involvement of BMPR-1B in the stimulation of mitosis of primary pulmonary hypertension PASMC through mitogen-activated protein kinase pathway in response to BMP-2 and BMP-7. In addition, we are currently investigating the role of other inhibitory factors, such as SMAD6, SMAD7, or BMP and activin membrane-bound inhibitor, that may also alter the downstream signaling cascade and may change the cell fate in response to cocaine and Tat treatment.

Yang et al earlier found reduction in phosphorylation of SMAD1/5 in the pulmonary arterial walls of patients with familial and idiopathic PAH with and without underlying BMPR-2 mutations. Likewise in this study, reduction in the expression of BMPRs was accompanied with the inhibition of SMAD1/5/8 phosphorylation and BMP/SMAD responsive Id1 gene expression on combined treatment with Tat and cocaine. Interestingly, treatment with cocaine alone also resulted in the reduction of SMAD1/5/8 activation and Id1 gene expression at early time point but without any changes in the BMPR-2 and BMPR-1A expression in the presence of significant increase in the BMPR-1B expression at 2 days after treatment. Cocaine has high affinity for o1 receptors, and binding results in translocation of these receptors to plasma membrane where they are known to activate other receptors or kinase(s), including Src family kinase. In addition, Src tyrosine kinase is known to interact with cytosolic terminal of BMPR-2 and is negatively regulated by BMP signaling. However, it is not known whether activation of Src family kinase can conversely inhibit BMP signaling through interaction with BMPR-2 or by crosstalk with SMAD1/5/8. Furthermore, the enhanced levels of phosphorylated SMAD2/3 complexed with co-SMAD4 when compared with the phosphorylated...
SMAD1/5/8 in the presence of cocaine suggest activation of an alternative TGF-β receptor-SMAD2/3-dependent pathway on cocaine treatment that may have competitively inhibited the binding of co-SMAD4 with phosphorylated SMAD1/5/8. Possibility of activation of this alternative pathway in the presence of cocaine is further supported by earlier evidence of phosphorylation of TGF-β type II receptor by Src during oncogenic signaling in mammary epithelial cells. Moreover, increased expression of phosphorylated SMAD2 in small pulmonary arteries of patients with idiopathic PAH has been shown earlier, indicating that a failure of BMPR-2/SMAD1, 5 signaling leads to increased signaling via TGF-β/ALK-5/SMAD2, 3 axis. In addition, SMAD1 is known to physically interact with SMAD3, and this may prevent the phosphorylation of SMAD3.19

We demonstrated earlier that Tat- and cocaine-mediated proliferation of PASMCs is associated with activation of platelet-derived growth factor-β (PDGF) receptor and could be blocked by a PDGF tyrosine kinase inhibitor, imatinib.8 Interestingly, PDGF is able to antagonize the BMP and TGF signaling pathways in vascular SMCs by repressing the SMAD protein expression, and deficiency in BMPR activity is associated with enhanced activity of PDGF signaling pathway.32 In our previous findings, we reported enhanced pulmonary vascular remodeling and PDGF expression in HIV+IVDUs when compared with that of lungs from HIV-infected non–drug users or uninfected IVDUs. Now in our current findings, we demonstrate significant greater loss of BMPR-1A, BMPR-1B, and BMPR-2 expressions in these human lung tissues from HIV-infected IVDUs when compared with lungs tissue from the HIV or IVDU group. Therefore, it may be that interplay between PDGF and BMP signaling pathways in response to illicit drugs and HIV proteins results in exacerbated vascular remodeling in the lungs from HIV-infected IVDUs.

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Disclosures

None.

References

This article focuses on the alterations in antiproliferative bone morphogenetic protein receptor axis during HIV protein(s) and cocaine-mediated pulmonary smooth muscle hyperplasia. Our study indicates significantly more attenuation in the bone morphogenetic protein receptor expression and downstream signaling pathway on combined exposure with HIV protein(s) and cocaine when compared with any 1 exposure. Understanding the effect of viral–cocaine interactions on this antiproliferative pathway, it will be critical subsequently for the development of novel therapeutic strategies aimed at abrogating pulmonary arterial hypertension associated with HIV and drug abuse, in particular, and all types of arteriopathy in general.
Downregulation of Bone Morphogenetic Protein Receptor Axis During HIV-1 and Cocaine-Mediated Pulmonary Smooth Muscle Hyperplasia: Implications for HIV-Related Pulmonary Arterial Hypertension
Pranjali Dalvi, Amy O'Brien-Ladner and Navneet K. Dhillon

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MATERIAL AND METHODS

Cell Culture and Treatments
Primary human pulmonary arterial smooth muscle cells (HPASMCs) (ScienCell research laboratories, Carlsbad, CA) were grown in smooth muscle cell media (ScienCell research laboratories) supplemented with 2% fetal bovine serum (FBS), smooth muscle cell growth supplements and penicillin/streptomycin. At 80% confluency, cells were serum starved for 48h followed by treatment with recombinant HIV-Tat (25ng/ml) (University of Kentucky) in the presence or absence of cocaine (1 µM) for 2, 3 or 6 days. Cells were alternatively treated for 6 days with Nef (10ng/ml, Prospec, East Brunswick, NJ) or gp-120CM (100ng/ml, Protein Sciences Corporation, Meriden, CT) with or without cocaine. The concentration of cocaine or HIV-proteins used was based on our previous published findings.

Human lung tissues and sections
Frozen human lung tissues and the paraffin-embedded lung sections obtained at the time of autopsy from HIV-infected individuals with (HIV+IVDU group, n=4) or without IVDU (HIV group, n=4) and uninfected individuals with (IVDU group, n=3) were from the Manhattan HIV Brain Bank (R24MH59724; U01MH083501; New York, NY). Normal un-infected archival controls (normal group, n=3) were from National Disease Research Interchange (NDRI, Philadelphia, PA). The IVDUs were mainly heroin and/or cocaine abusers. The clinical, demographic and pathological characteristics of the lung samples and more details of these human subjects are included in our previous report.

MTS cell proliferation assay
HPASMCs (3X10^3 cells/well) seeded in 96 well plate for 48h, were treated with cocaine and/or Tat containing 0.1% serum medium in the presence or absence of BMP-2 (10, 50 or 100 ng/ml) for 2, 3 or 6 days. Change in cell proliferation as a response to different treatments was measured by CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer’s instructions.

Transfection of HPASMCs with BMPR2 expression plasmid
HPASMCs were transiently transfected with PCMV6-XL5-BMPR2 plasmid (Origene Technologies, Inc., Rockville, MD) using GeneJuice transfection reagent (Novagen, EMD Chemicals Inc., Philadelphia, PA) according to the manufacturer’s instructions. To confirm the increase in protein expression, transfected cells were lysed with RIPA lysis buffer and analyzed for BMPR-2 over-expression by western blot. For proliferation assay, cells were serum starved for 24h at 24h post-transfection followed by 48h treatment with cocaine (1µM) and Tat (25ng/ml) and MTS assay as described above. In another set of experiments, serum starved, transfected cells were treated with cocaine and Tat for 48h followed by stimulation with BMP-2 (10ng/ml) for 30min before the extraction of total RNA for Id1 mRNA analysis. Mock transfection control was done using empty PCMV6-XL5 vector under same treatment conditions as the BMPR2 expression plasmid.

Real time RT-PCR analysis
Total RNA was extracted with Trizol® reagent (Invitrogen, Carlsbad, CA) as per manufacturer’s instructions from quiescent HPASMCs treated with cocaine and/or Tat for 1, 2, 3 and 6 days and also from frozen human lung tissues. Quantitative analysis of BMPR-2,-1A and -1B and that of BMP target gene Id1 was carried out by Real-Time RT-PCR using the SYBR Green detection method as described earlier.

Western Blot Analysis
Cells treated with cocaine and/or Tat for 2, 3 or 6 days were lysed using radioimmunoprecipitation assay (RIPA) lysis buffer (Cell Signaling Technology, Danvers, MA) and then used for Western blot analysis as described earlier. Total cellular extract from frozen human lung tissues was also probed for BMPR expression by Western blot analysis. Western blots were probed with antibodies against PCNA (Cell signaling, Beverly, MA), BMPR-1A, BMPR-1B, BMPR-2, SMAD1/5/8 (Santa Cruz Biotechnology, Santa Cruz, CA) and phosphorylated-SMAD1/5/8 (p-SMAD1/5/8) (Millipore, Billerica, MA). The horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Millipore, Billerica, MA) were used with the enhanced chemiluminescence system for detection (Thermo Scientific, Rockford, Illinois). The NIH imageJ software was used for densitometric analysis of immunoblots.

**Co-immunoprecipitation**

Quiescent HPASMCs treated with cocaine and/or Tat for 48 hours were lysed to obtain protein extract as mentioned above. Total cellular extract (100 µg) was then incubated overnight at 4°C with 2µg of anti-SMAD-4 (Santa Cruz Biotechnology, Santa Cruz, CA) followed by immunoprecipitation using protein A/G agarose beads (Thermo Scientific) as per manufacturer's instructions. Levels of p-SMAD1/5/8-SMAD4 complex was compared with p-SMAD2/3-SMAD4 complex by resolving 50µg of immuno-precipitated protein on SDS-PAGE followed by Western blot analysis using antibodies against p-SMAD1/5/8 and p-SMAD2/3 from Santa Cruz Biotechnology.

**Immunohistochemical analysis of human lung sections**

The paraffin-embedded lung sections from normal, HIV, IVDU and HIV+IVDU individuals were immunostained using primary antibody against BMPR-2, as described previously. The reddish-brown coloration as an indicator of positive staining was obtained using ImmPACT NovaRED peroxidase substrate (Vector laboratories, Burlingame, CA).

**Statistical Analysis**

Statistical analysis was performed using one-way analysis of variance with a post-hoc Bonferroni test for multiple comparisons. Two-sided p-values were calculated for the analysis of all cell culture experiments using STATA 13 software (StataCorp LP, College Station, TX, USA) and results were judged statistically significant when the Bonferroni corrected P values were less than 0.05. A non-parametric Wilcoxon Rank Sum test at 0.05 level was used to determine statistical significance of human tissue studies with no control for multiple comparisons due to the limited sample size.

**References**

Down-regulation of BMPR Axis during HIV-1 and Cocaine-mediated Pulmonary Smooth Muscle Hyperplasia: Implications for HRPAH.

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SUPPLEMENTARY MATERIAL
Supplementary Methods:

**ELISA:**
Quiescent primary human pulmonary arterial smooth muscle cells (HPASMCs) (ScienCell research laboratories, Carlsbad, CA) were treated with cocaine (1µM) in the presence or absence of recombinant HIV-Tat (25ng/ml) (University of Kentucky) daily for 12 days. Supernatant fluid was then collected from these cells at various time intervals for analysis of BMP-2, -4 and -7 levels by ELISA (Quantikine® R&D Systems, Minneapolis, MN) as per manufacturer’s instructions.

**Transient transfection with siRNA against SMAD1, 5, 8:**
HPASMCs were transfected with siRNA against SMAD1, 5, 8 (Ambion life technologies), using HiPerFect transfection reagent (Qiagen, Valencia, CA) as per manufacturer’s instructions. After 24h, cells were serum starved in 0.1% serum containing medium for 48h followed by cocaine and/or Tat treatment in presence or absence of 10ng/ml BMP-2. After 2 days of treatment, cell proliferation was measured by MTS assay.
Supplementary figures and figure legends:

Supplementary Figure I. Western blot representing increased BMPR-2 expression in BMPR-2 over-expressed cells compared to untreated control. Quiescent HPASMCs were transiently transfected with PCMV6-XL5-BMPR2 plasmid (1µg and 2µg) using GeneJuice transfection reagent (Novagen, EMD Chemicals Inc., Philadelphia, PA) according to the manufacturer’s instructions. At 72h post-transfection cells were lysed followed by analysis of protein extract by Western blot.
Supplementary Figure II: Secretion of BMP ligands by pulmonary smooth muscle cells on treatment with Tat and/or cocaine. Quiescent HPASMCs were treated with cocaine (1μM) and/or Tat (25ng/ml) daily for 12 days. Cell supernatants collected at specified intervals were analyzed for BMP-2 (A), BMP-4 (B) and BMP-7 (C) by ELISA. All values are mean ± SD of at least three independent experiments, *p<0.05  ** p<0.01, *** p<0.001 compared to untreated control.
Supplementary Figure III: Reversal of the anti-proliferative effect of BMP-2 in cells lacking SMAD1/5/8. HPASMCs were transiently transfected with siRNA against SMAD1,5,8 followed by serum starvation in 0.1% FBS containing medium for 48h. After this, cocaine and/or Tat treatment was carried out in the presence or absence of 10ng/ml BMP-2 for 48hrs followed by cell proliferation assay. Scrambled (scrm) siRNA was also used as a transfection control. All values are mean ±SD of three independent experiments. *p<0.001 compared to untreated control, #p<0.001 compared to cocaine alone, $p<0.001 compared with Tat alone, @p<0.001 compared to combined cocaine-Tat treatment, ¨p<0.001 compared with BMP-2 and cocaine treated cells, ¢p<0.001 compared with BMP-2 and Tat treated cells, ®p<0.001 compared with BMP-2 and cocaine+Tat treated cells.
Supplementary Figure IV: Down-modulation of BMPR-2 expression in the lungs from HIV infected +/- intravenous drug users (IVDU). A. Representative photomicrographs of distal vessels showing BMPR-2 immunohistochemistry on paraffin-embedded lung sections from Normal, HIV+/-IVDUs. B. Representative images from negative control section (no primary antibody) showing proximal and distal vessels. Original magnification 100X, Scale bar: 100µm.