Ligand-Independent Activation of Platelet-Derived Growth Factor Receptor β during Human Immunodeficiency Virus–Transactivator of Transcription and Cocaine-Mediated Smooth Muscle Hyperplasia

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Abstract

Our previous study supports an additive effect of cocaine to human immunodeficiency virus infection in the development of pulmonary arteriopathy through enhancement of proliferation of pulmonary smooth muscle cells (SMCs), while also suggesting involvement of platelet-derived growth factor receptor (PDGFR) activation in the absence of further increase in PDGF-BB ligand. Redox-related signaling pathways have been shown to regulate tyrosine kinase receptors independent of ligand binding, so we hypothesized that simultaneous treatment of SMCs with transactivator of transcription (Tat) and cocaine may be able to indirectly activate PDGFR through modulation of reactive oxygen species (ROS) without the need for PDGF binding. We found that blocking the binding of ligand using suramin or monoclonal IMC-3G3 antibody significantly reduced ligand-induced autophosphorylation of Y1009 without affecting ligand-independent transphosphorylation of Y934 residue on PDGFR β in human pulmonary arterial SMCs treated with both cocaine and Tat. Combined treatment of human pulmonary arterial SMCs with cocaine and Tat resulted in augmented production of superoxide radicals and hydrogen peroxide when compared with either treatment alone. Inhibition of this ROS generation prevented cocaine- and Tat-mediated Src activation and transphosphorylation of PDGFR β at Y934 without any changes in phosphorylation of Y1009, in

addition to attenuation of smooth muscle hyperplasia. Furthermore, pretreatment with an Src inhibitor, PP2, also suppressed cocaine- and Tat-mediated enhanced Y934 phosphorylation and smooth muscle proliferation. Finally, we report total abrogation of cocaine- and Tat-mediated synergistic increase in cell proliferation on inhibition of both ligand-dependent and ROS/Src-mediated ligand-independent phosphorylation of PDGFR β .

Keywords: pulmonary arterial hypertension; transactivator of transcription; cocaine; platelet-derived growth factor receptor; reactive oxygen species

Clinical Relevance

This report, on the partial involvement of ligand-independent activation of platelet-derived growth factor receptor (PDGFR) in a synergistic induction of smooth muscle cell proliferation upon combined treatment with cocaine and transactivator of transcription, suggests that alternate mechanisms of PDGFR axis activation may be relevant in pulmonary vascular remodeling. This could be the reason that therapies based on inhibiting ligand-induced PDGFR have not been completely successful.

Human immunodeficiency virus (HIV)–related pulmonary arterial hypertension (HRPAH) is one of the most critical noninfectious complications of HIV

infection, as it is significantly associated with morbidity and mortality in these patients (1). Despite antiretroviral therapy, 1 in 200 patients with acquired immune

deficiency syndrome still develop HRPAAH (2, 3), which is a roughly 1,000 times higher incidence when compared with PAH in the general population (4). However, the

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disease may be even more under-recognized than previously described, as recent estimates based on echocardiographic measurements indicate elevated pulmonary arterial systolic pressures in approximately 35% (5, 6), with evidence of pulmonary hypertension in 2.6% (7) of asymptomatic patients infected with HIV. Furthermore, many studies suggest HRPAAH to be more common in intravenous drug users (IVDUs) (2, 3, 8, 9). A recent report by Quezada and colleagues (3) suggesting 10% prevalence of HRPAAH in the Spanish cohort also found association of PAH with intravenous drug use. Previous studies from our laboratory showing enhanced pulmonary vascular remodeling in HIV-infected human lung tissues or simian immunodeficiency virus-infected macaque lungs exposed to intravenous cocaine and/or opioids abuse (10, 11) further indicate that intravenous drug use and HIV-1 potentially act in concert to cause pulmonary arteriopathy.

Cocaine is one of the most commonly used illicit drugs in the United States (12), and is associated with high blood pressure, vasoconstriction, microvascular injury, platelet activation, and atherosclerosis. Chronic intravenous use of cocaine in the absence of viral infection has been associated with PAH and other cardiopulmonary complications (13–16). Abnormal smooth muscle cell (SMC) proliferation is considered to play a key role in vascular remodeling associated with PAH (17); however, specific mechanisms underlying the drug abuse- and HIV infection-mediated arteriopathy are at their infancy. Our previous findings support an independent effect of cocaine and HIV proteins on the enhancement of pulmonary SMC proliferation (10). Although both abuse of cocaine and HIV proteins have been observed to be an independent risk factor of vascular injury, we reported that the combination of cocaine and HIV protein: transactivator of transcription (Tat) exposure results in a synergistic increase in the proliferation of pulmonary SMCs (10).

Platelet-derived growth factor (PDGF) has been implicated in the abnormal proliferation and migration of vascular SMCs (18). Previous studies provide direct evidence for the involvement of PDGF in the pathogenesis of pulmonary vascular remodeling in animal models (19, 20) and in lung biopsies from patients with idiopathic pulmonary hypertension or with HRPAAH (21).

In our previous findings we demonstrated enhanced phosphorylation of PDGF receptor β (PDGFR β) in the pulmonary SMCs (pSMC) of remodeled vessels in lungs obtained from HIV-infected IVDUs (10). Furthermore, in our cell culture studies, we observed significant augmentation in PDGFR β activation on simultaneous exposure of primary human pulmonary arterial SMCs (HPASMCs) to Tat and cocaine compared with either treatment alone. However, when we looked at the ligand expression, we could not demonstrate further enhancement in PDGF-B expression in HPASMCs after simultaneous Tat and cocaine treatment compared with either treatment alone (10). Therefore, in this study, we sought to question if cocaine and Tat could indirectly activate PDGFR β independent of extracellular PDGF binding in addition to their direct effect on PDGF expression and ligand-dependent receptor activation. This may aid in designing improved treatment modalities to abrogate PAH, because the available drugs aimed at blocking the binding of growth factors to its receptor may not be sufficient to prevent the activation of downstream signaling pathways involved in SMC proliferation.

PDGF receptors belong to the receptor tyrosine kinase (RTK) family and binding of PDGF to its receptors results in dimerization and autophosphorylation of specific tyrosine residues, leading to binding and activation of downstream signaling substrates containing a Src homology domain (22). In addition, these growth factor RTKs are also known to be phosphorylated by other non-RTKs, such as Src, in a ligand-independent manner (23). One of the key mediators in ligand-independent transactivation of growth factor receptors, including PDGFR, are reactive oxygen species (ROS) (24). We and others have demonstrated induction of oxidative stress on exposure of vascular cell types to various HIV proteins, including Tat, and on exposure to illicit drugs, such as cocaine and opioids (11, 25, 26). Therefore, in this study, we explored the possibility of ROS-mediated ligand-independent activation of PDGFR in HPASMCs on combined treatment with cocaine and Tat.

Our study shows an additive increase in the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent ROS generation on simultaneous treatment of SMCs with cocaine and Tat. Furthermore, we provide conclusive

evidence demonstrating the role of this augmented ROS generation in Src-mediated ligand-independent phosphorylation of PDGFR β at Y934. In addition, we provide evidence that this indirect activation of PDGFR not requiring the ligand binding is partially involved in cocaine- and Tat-mediated smooth muscle hyperplasia.

Materials and Methods

Cell Culture and Treatments

Primary HPASMCs (ScienCell Research Laboratories, Carlsbad, CA) were grown in SMC media (ScienCell Research Laboratories) supplemented with 2% FBS, SMC growth supplements, and penicillin/streptomycin. At 80% confluence, cells were serum starved for 48 hours followed by treatment with cocaine (1 μ M) in the presence or absence of recombinant HIV-Tat (25 ng/ml; University of Kentucky, Lexington, KY) for variable time points. The concentration of cocaine and Tat was based on our previously published findings (27). Cells were treated with antioxidant cocktail (0.2 mM L-ascorbate, 0.5 mM glutathione, and 3.5 μ M α -tocopherol) (28) 30 minutes before or 48 hours after Tat and cocaine treatment.

Cell Proliferation Assay

HPASMCs seeded in 96-well plates (3×10^3 cells/well) were allowed to grow for 24 hours, followed by replacement of medium with 0.1% FBS-containing medium for 48 hours. Quiescent cells were treated with Tat and cocaine in the presence or absence of antioxidant cocktail, suramin (0.5, 1 μ M), IMC-3G3 (10, 20 μ g/ml), and PP2 (10, 20 μ M) for 48 hours. The total number of cells was later measured by CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI).

Western Blot

Cells treated with cocaine and/or Tat for various time periods were lysed using RIPA buffer (Santa Cruz Biotechnology, Santa Cruz, CA), followed by Western blot, as described previously (29). Blots were probed with antibodies against Src, phosphorylated (p)-PDGFR β (Y1009), and PDGFR β (Santa Cruz Biotechnology), p-PDGFR β (Y934) (Invitrogen, Grand Island, NY), p-Src, p-Akt, and p-phosphoinositide-3 kinase (PI3K; Cell Signaling Technology, Danvers, MA). Monoclonal IMC-3G3 antibody was kindly provided by

Dr. Nick Loizos, ImClone Systems Corporation (Bridgewater, NJ). ImageJ software (National Institutes of Health, Bethesda, MD) was used for densitometric analysis.

Immunofluorescence

HPASMCs treated with cocaine-Tat and/or suramin (0.5 μ M) for 24 hours were fixed with 4% paraformaldehyde, permeabilized with methanol, and blocked with 5% goat serum, followed by

immunostaining using p-PDGFR β (Y1009), p-PDGFR β (Y934), and PDGFR β primary antibodies and AlexaFluor 488 rabbit secondary antibody.

Measurement of ROS

To evaluate the cocaine- and Tat-mediated increase in hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\cdot-}$) generation, Amplex Red assay and cytochrome c reduction assay were conducted, respectively, as described

previously (11). Quantification of total cellular oxidative stress was measured using dichlorodihydrofluorescein (DCF) (28) in cells treated with apocynin (25 and 50 μ M), suramin (0.5 μ M), or IMC-3G3 (10 μ g/ml) for 10 minutes followed by cocaine and Tat treatment for 1 hour.

Transfection of HPASMCs

Cells were transfected with small interfering RNA (siRNA) against p22^{phox} or Src using

Figure 1. Cocaine- and transactivator of transcription (Tat)-mediated ligand-independent activation of platelet-derived growth factor receptor (PDGFR) β . Western blot (A and B) and immunofluorescence (C) analysis of PDGFR β phosphorylation in human pulmonary arterial smooth muscle cells (HPASMCs) treated with cocaine (1 μ M) and/or Tat (25 ng/ml) in the presence or absence of suramin (0.5 μ M) (A and C) or the monoclonal antibody IMC-3G3 (10 μ g/ml) (B). Total and phosphorylated PDGFR β at Y934 and Y1009 was examined at 24 hours after treatment. Graphs represent densitometry analysis of the ratio of phosphorylated to total PDGFR β , both first normalized to β -actin. Original magnification: $\times 40$. All values are mean $\pm$ SEM of at least three independent experiments. * P < 0.05, ** P < 0.001 compared with control; @ P < 0.05, @@ P < 0.001 compared with cocaine and Tat treatment.

Figure 2. Cocaine- and Tat-mediated synergistic increase of pulmonary smooth muscle proliferation partially involves ligand-independent activation of PDGFR β . 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) proliferation assay in HPASMCs treated with cocaine and/or Tat in the presence or absence of suramin (A) or monoclonal antibody IMC-3G3 (B) at 48 hours after treatment. All values are mean $\pm$ SD of three independent experiments. * P < 0.001 compared with control, # P < 0.05, ## P < 0.001 compared with cocaine, \$ P < 0.001 compared with Tat, @ P < 0.001 compared with cocaine and Tat treatment.

HiPerFect transfection reagent (Qiagen, Valencia, CA) per the manufacturer's instructions. Cells transfected with scrambled siRNA were used as negative control. At 48 hours after transfection, cells were treated with cocaine and/or Tat followed by DCF assay or Western blot at indicated time intervals. To check the transfection efficiency, quantitative analysis of p22^{phox} messenger RNA (mRNA) was performed by real-time RT-PCR, as described previously (30). Western blot for total Src was performed to confirm the knockdown of Src expression.

Statistical Analysis

Statistical analysis was performed using one-way ANOVA with a *post hoc* Bonferroni test for multiple comparisons using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA). Results were judged statistically significant when Bonferroni-corrected P values were less than 0.05.

Results

Involvement of Ligand-Independent Activation of PDGFR β in Cocaine- and Tat-Mediated Increase of pSMC proliferation

First, to determine if cocaine- and Tat-mediated PDGFR β stimulation is partly or

completely independent of extracellular growth factor ligand, we pretreated HPASMCs with suramin, which blocks the ligand binding, followed by cocaine and Tat treatment for 24 hours. Total cellular lysates were later used for Western blot analysis of autophosphorylation of ligand-dependent Y1009 residue and transphosphorylation of ligand-independent Y934 residue on intracellular domains of PDGFR β . As shown in Figure 1A, pretreatment with suramin resulted in abrogation of cocaine- and Tat-mediated phosphorylation of PDGFR β at Y1009, whereas PDGFR β transphosphorylation at Y934 was not affected. In corroboration,

Figure 3. Significant increase in oxidative stress on combined treatment with cocaine and Tat compared with either treatment alone. (A) Intracellular hydrogen peroxide (H₂O₂) measured by Amplex Red assay and (B) superoxide radicals measured by cytochrome c reduction assay in cocaine- and Tat-treated HPASMCs in the presence or absence of the vascular endothelial growth factor receptor inhibitor BD1047 (10⁻⁷ nM), or α receptor inhibitor SU5416 (0.1 μ M) at 1 hour after treatment. (C) Dichlorodihydrofluorescein (DCF) assay at 1 hour after treatment with cocaine and Tat in the presence or absence of apocynin (25 or 50 μ M). (D) DCF assay after transient transfection of cells with siRNA^{p22^{phox}}, followed by cocaine and Tat treatment for 1 hour. All values are mean $\pm$ SD of three independent experiments. * P < 0.05, ** P < 0.01, *** P < 0.001 versus control; # P < 0.001 compared with cocaine; \$ P < 0.001 compared with Tat; @ P < 0.05, @@ P < 0.001 compared with combined cocaine and Tat. ROS, reactive oxygen species; Scrm., scrambled; siRNA, small interfering RNA; SOD, superoxide dismutase.

immunofluorescence staining of p-Y1009 residue was remarkably decreased on suramin exposure of cocaine- and Tat-treated cells, whereas staining of p-Y934 remained unchanged (Figure 1C). To further determine if cocaine- and Tat-mediated PDGFR phosphorylation requires ligand-dependent dimerization of α/β chains, HPASMCs were treated with cocaine and Tat in the presence or absence of monoclonal antibody, IMC-3G3, which specifically blocks receptor dimerization by blocking its ligand-binding domain. As illustrated in Figure 1B, IMC-3G3 significantly prevented the cocaine- and Tat-mediated augmentation of Y1009 phosphorylation, while not affecting the increase in the transphosphorylation of Y934 in response to cocaine and Tat treatment when compared with untreated control. Next, we investigated the involvement of these phosphorylation sites in cocaine- and Tat-mediated enhanced SMC proliferation. We observed that suramin or IMC-3G3 pretreatment could attenuate, but not completely abrogate, the cocaine-Tat-mediated increase in cell proliferation (Figures 2A and 2B). Overall, these results indicate that, in addition to ligand-dependent activation of PDGFR β at Y1009, cocaine and Tat are able to phosphorylate PDGFR β at Y934 without the requirement of PDGF binding, and this activation may be partially responsible for smooth muscle hyperplasia observed upon combined treatment.

Enhanced Oxidative Stress in pSMCs on Combined Treatment with Cocaine and Tat Compared with That of Monotreatments

As mentioned previously here, ROS is known to activate RTKs independent of its ligand (24), and, furthermore, both cocaine (31) and Tat (32) are known to cause oxidative stress. Therefore, we first analyzed the effect of cocaine and Tat treatment on ROS generation in pulmonary SMCs. As shown in Figure 3A, treatment of HPASMCs with either cocaine or Tat caused a significant increase in H_2O_2 generation compared with untreated cells. However, H_2O_2 levels were further augmented on combined treatment with cocaine and Tat when compared with monotreatments. Cocaine is known to interact with σ receptor (33), and Tat is shown to bind and activate vascular endothelial growth factor receptor

(VEGFR)-2 (34) to facilitate entry into cells. To confirm that ROS generation occurs specifically due to the interaction of cocaine and Tat with SMCs, we pretreated the cells with σ (BD1047) and VEGFR (SU5416) inhibitors before cocaine and Tat treatment, followed by measurement of oxidative stress. As expected, inhibition of σ receptor or VEGFR caused a significant decrease in H_2O_2 generation as compared with that generated by the dual effect of cocaine and Tat without inhibitors (Figure 3A). On the one hand, pretreatment with catalase prevented the cocaine- or Tat-mediated enhanced H_2O_2 production; on the other hand, the presence of superoxide dismutase had no significant effect on H_2O_2 production compared with single or combined cocaine and Tat exposure.

Similarly, we also evaluated the production of superoxide radicals on cocaine and Tat treatments in HPASMCs (Figure 3B). Similar to H_2O_2 generation, superoxide production also increased significantly on cocaine or Tat treatments compared with the untreated control, although the maximum effect was seen in cases of combined treatment. This effect was substantially reduced in the presence of BD1047 or SU5416.

To further determine the source of ROS, cocaine- and Tat-exposed HPASMCs were treated with apocynin, a well-known NADPH oxidase inhibitor, followed by DCF assay to measure ROS. Exposure of cells to cocaine and Tat in the presence of 25 or 50 μ M apocynin resulted in abrogation of cocaine- and Tat-mediated ROS generation

Figure 4. Oxidative stress-dependent cocaine- and Tat-mediated PDGFR β (Y934) phosphorylation and enhanced HPASMC proliferation. (A) HPASMCs were pretreated with antioxidant cocktail 30 minutes before 48-hour treatment of cocaine and/or Tat. (B) Cells were post-treated with antioxidant cocktail after 48-hour treatment with cocaine and Tat, and analyzed for changes in cell proliferation by MTS assay after 2 days. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control; # $P < 0.001$ compared with cocaine; \$ $P < 0.001$ compared with Tat; @ $P < 0.001$ compared with combined cocaine and Tat treatment. (C) DCF assay at 1 hour after treatment of HPASMCs with cocaine and Tat in the presence or absence of IMC-3G3 (10 μ g/ml) or suramin (0.5 μ M). * $P < 0.001$ compared with control; # $P < 0.001$ compared with cocaine; \$ $P < 0.001$ compared with Tat. (D) Western blot analysis of PDGFR β phosphorylation at Y934 and Y1009 in cocaine- and Tat-treated HPASMCs pretreated with antioxidant cocktail at 24 hours after treatment. Graphs represent densitometry analysis of the ratio of phosphorylated to total PDGFR β , first normalized to β -actin. * $P < 0.01$, ** $P < 0.001$ compared with control; @ $P < 0.001$ compared with cocaine and Tat. All values are mean $\pm$ SEM of at least three independent experiments.

Figure 5. Cocaine- and Tat-mediated ROS generation results in ligand-independent activation of Src. Western blot analyses of phosphorylated Src in cocaine- and Tat-treated HPASMCs in the presence or absence of antioxidant cocktail for indicated time periods (A) and suramin (0.5 μ M) for three hours (B). Values are mean $\pm$ SEM of two (A) or three (B) independent experiments. * $P < 0.05$, ** $P < 0.01$ compared with control; @ $P < 0.05$ compared with cocaine and Tat (C + T). Aox, antioxidant cocktail.

without any effect of apocynin itself on ROS production at the concentrations used (Figure 3C).

Due to the nonspecificity of apocynin (35), we next silenced the common, critical subunit of NADPH oxidase-p22^{phox}, with siRNA^{p22^{phox}}. The siRNA transfection efficiency was first confirmed by quantifying the mRNA expression of p22^{phox} in transfected and untransfected cells (see Figure E1A in the online supplement). Interestingly, an increase in p22^{phox} mRNA was detected in untransfected cells treated with cocaine and Tat when compared with the untreated control. Hence, we compared the effect of combined treatment and monotreatments on p22^{phox} expression, and found significant up-regulation in p22^{phox} expression on combined treatment when compared with cocaine or Tat treatment alone (Figure E1B). Concomitant to the apocynin findings, we observed significant reduction in cocaine- and Tat-mediated enhanced generation of ROS in cells transfected with siRNA^{p22^{phox}} (Figure 3D) when compared with untransfected cocaine- and Tat-treated cells. ROS production was unaltered in cocaine- and Tat-treated cells transfected with scrambled siRNA. Overall, these results indicate significant increase in NADPH oxidase-mediated ROS generation after cocaine and Tat exposure in SMCs.

Augmented Oxidative Stress Is Partially Responsible for Cocaine- and Tat-Mediated Ligand-Independent Activation of PDGFR β and Smooth Muscle Hyperplasia

To elucidate whether cocaine- and Tat-mediated increase in pSMC proliferation is due to the augmented production of ROS that was observed in these cells, HPASMCs were treated with both cocaine and Tat in the presence or absence of antioxidant cocktail, followed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. As illustrated in Figures 4A and 4B, both pre- and post-treatment with antioxidant cocktail resulted in the attenuation of cocaine- and Tat-mediated synergistic increase in HPASMCs proliferation.

After confirming a partial role of oxidative stress in cocaine- and/or Tat-mediated augmented proliferation of SMCs, we next determined if cocaine- and Tat-

induced oxidative stress is a downstream effect of PDGF-B binding to its receptor, or if it is a direct effect of cocaine and Tat independent of ligand binding. To test this, we treated the cells with cocaine and Tat in the presence of suramin or IMC-3G3, followed by DCF assay. As shown in Figure 4C, pretreatment with suramin or IMC-3G3 did not alter the cocaine- and Tat-induced oxidative stress, indicating that cocaine- and Tat-mediated ROS generation was independent of PDGF-PDGFR axis activation.

Furthermore, to see if enhanced ROS generation in HPAMCs on simultaneous exposure to both cocaine and Tat is involved in ligand-independent activation of PDGFR β , phosphorylation of Y934 and Y1009 was examined by Western blot analyses of protein extracts from cells treated with cocaine and Tat for 24 hours in the presence or absence of antioxidant cocktail. A significant attenuation in the cocaine- and Tat-mediated increase in ligand-independent transactivation of PDGFR β was observed in presence of antioxidants, as indicated by a drop in the phosphorylation of Y934 when compared with combined treatment in the absence of antioxidants (Figure 4D). However, no alteration in the autophosphorylation of Y1009 by cocaine and Tat treatment was observed in the presence of antioxidants, as shown in Figure 4D. As anticipated, phosphorylation of PDGFR β was absent in the negative control cells treated with antioxidants alone.

ROS-Mediated Ligand-Independent Activation of PDGFR β in Response to Combined Treatment with Cocaine and Tat Involves Src Induction

Based on previous studies, ROS is known to activate Src (36), and Y934 of PDGFR β has been reported to be a substrate of Src kinase (22). Given that we saw significant increase in ROS as well as phosphorylation of PDGFR β at Y934 upon cocaine and Tat treatment, we next examined Src activation in HPASMCs exposed to combined treatment of cocaine and Tat for 1, 3, and 6 hours. Western blot analysis showed increase in the phosphorylation of Src as early as 1 hour after treatment compared with untreated cells (Figure 5A). Maximum increase in p-Src expression was observed after 3 hours of combined treatment. Pretreatment of cocaine- and Tat-treated HPASMCs with antioxidant cocktail

Figure 6. Cocaine- and Tat-mediated enhanced PDGFR β activation at Y934 is Src mediated. (A) Western blot analyses of phosphorylated PDGFR β at Y-934 and Y-1009 in cocaine- and Tat-treated HPASMCs pretreated with PP2 (10 μ M). Values are mean $\pm$ SEM of four independent experiments. (B) Western blot analyses of phosphorylated PDGFR β in cocaine- and Tat-treated HPASMCs transfected with siRNA^{Src} or scrambled siRNA (Scrm.). Values are mean $\pm$ SEM of three independent experiments. Graphs represent densitometry analysis of the ratio of phosphorylated to total PDGFR β , first normalized to β -actin. * P < 0.05, ** P < 0.001 compared with control; @ P < 0.001 compared with cocaine and Tat.

abrogated the p-Src expression to the level of untreated control or cells exposed to antioxidants only, therefore suggesting ROS-dependent induction of Src. In addition, we observed that cocaine- and Tat-mediated Src activation was unaffected by blocking PDGF binding to its receptor using suramin (Figure 5B), indicating that ROS-mediated Src activation on exposure to both cocaine and HIV-Tat is

independent of PDGF-PDGFR axis induction.

Next, to examine if this Src induction is involved in cocaine- and Tat-mediated transphosphorylation of PDGFR β at Y934, we inhibited Src using a well-known inhibitor of Src family kinase, PP2. As seen in Figure 6A, inhibition of Src significantly down-regulated the expression of cocaine- and Tat-mediated phosphorylation of

Figure 7. Abrogation of cocaine- and Tat-mediated synergistic increase in pulmonary smooth muscle cells (pSMC) proliferation on inhibition of both ligand-dependent and ligand-independent phosphorylation of PDGFR. (A) Western blot analysis of phosphorylated phosphoinositide-3 kinase (p-PI3K) and p-Akt in HPASMCs pretreated with PP2 (10 μ M) and/or suramin (0.5 μ M) for 20 minutes, followed by cocaine and/or Tat treatment for 30 hours. Values are mean $\pm$ SEM of two independent experiments. (B) MTS cell proliferation assay showing complete abrogation of cocaine- and Tat-mediated HPASMC proliferation on

PDGFR β at Y934, whereas ligand-induced phosphorylation at Y1009 was unaffected. The phosphorylation of both Y934 and Y1009 in the case of cells treated with PP2 alone was comparable to that of untreated cells. To further selectively inhibit Src activation, HPASMCs were transiently transfected with siRNA against Src, followed by cocaine and Tat treatment. Western blot analyses of Src levels in the transfected cells confirmed the knockdown of total Src expression, as shown in Figure E2. Similar to the findings with PP2 treatment, loss of Src expression in cocaine-Tat-treated transfected cells failed to phosphorylate PDGFR β at Y934 when compared with untransfected cells or cells transfected with scrambled siRNA exposed to cocaine and Tat (Figure 6B). In addition, no alteration in augmented autophosphorylation of Y1009 was observed in cocaine-Tat-treated cells transfected with siRNA^{src} when compared with cocaine-Tat-exposed untransfected or scrambled siRNA transfected cells.

Inhibition of Both Ligand-Dependent and Ligand-Independent Activation of PDGFR β Abrogates Cocaine- and Tat-Mediated Increase in pSMC Proliferation

PDGFR β is known to activate the PI3K/Akt downstream signaling pathway, which is one of the important drivers of cell proliferation and survival. Therefore, we confirmed the activation of this pathway as a consequence of increased cocaine-Tat-mediated PDGFR β activation. Figure 7A shows significant increases in PI3K and Akt activation, respectively, on cocaine and Tat treatment compared with untreated HPASMCs. Interestingly, inhibiting the activation of PDGFR β , either at Y1009 or Y934, using suramin or PP2, respectively, partially inhibited the activation of Akt and PI3K, whereas simultaneous inhibition of both ligand-dependent and -independent phosphorylation of PDGFR β resulted in complete inhibition of cocaine- and Tat-induced Akt and PI3K phosphorylation.

Finally, we determined the effect of blocking both ligand-dependent and ROS/Src-mediated ligand-independent

activation of PDGFR β on cocaine- and Tat-mediated enhanced smooth muscle proliferation. We simultaneously pretreated the cells with suramin and PP2, followed by cocaine and Tat treatment for MTS proliferation assay. As seen in Figure 7B, combined treatment with both the inhibitors could completely abrogate the cocaine-Tat-induced pSMC proliferation. However, treatment with PP2 alone could not completely prevent the cocaine-Tat-induced enhanced cell proliferation as we saw on pretreatment with suramin alone (Figure 2A).

Discussion

The present study contributes toward understanding our previous, consistent findings showing enhanced pulmonary vascular dysfunction in IVDUs infected with HIV compared with uninfected non-drug users or uninfected IVDUs (10, 11, 28, 37). Here, we add new significant mechanistic details to support our earlier observation of uncontrolled proliferation of pulmonary SMCs associated with vascular remodeling in the presence of HIV proteins and illicit drugs. The prime outcome of this study is that stimulation of HPASMCs with both cocaine and Tat results in ligand-independent activation of PDGFR β via ROS-mediated Src activation.

Stimulation of PDGFR downstream signaling has been implicated in proliferation and migration of vascular SMCs (38). Previously, we reported significant augmentation in the activation of PDGFR β and a consequent additive increase in the proliferation of HPASMCs, in spite of absence of further increase in PDGF-BB ligand, on simultaneous treatment with HIV-Tat and cocaine compared with either treatment alone (10). In this study, we demonstrate that the PDGFR β -dependent cocaine-Tat-mediated uncontrolled cell proliferation is only partially driven by ligand binding, and involves alternate noncanonical PDGFR activation mechanism(s) that do not require PDGF binding. We show significant attenuation, but not complete abrogation, of cell

proliferation on blocking ligand binding and ligand-induced dimerization of PDGFR using suramin or IMC-3G3. Inhibition of ligand binding significantly ameliorated autophosphorylation of PDGFR β at Y1009, but did not show any effect on Y934 phosphorylation, thereby suggesting Y934 phosphorylation independent of ligand binding or dimerization of PDGFR. Although IMC-3G3 monoclonal antibody is known to specifically block ligand binding domain of PDGFR α , the inhibition of PDGFR β (Y1009) activation may be affected by preventing the PDGF-B-induced heterodimerization of the α and β chains and associated phosphorylation of PDGFR β at Y1009 (39).

Our findings are consistent with earlier reports of activation of RTKs via ligand-independent mechanisms. Many stimuli, such as heat shock, hypoxia (40), or G protein-coupled receptor activation (41), and agents, such as deoxycholic acid, are known to cause ligand-independent activation of growth factor receptors (42). Lysophosphatidic acid has previously been reported to cause ligand-independent activation of PDGFR β (43). PDGFR α has been reported to be activated by ROS upon treatment of cells with growth factors from other than the PDGF family (44). Likewise, we now document involvement of ROS in cocaine- and Tat-mediated ligand-independent activation of PDGFR β at Y934. HIV-1 proteins are known to induce oxidative stress, thereby disrupting the balance of oxidants/antioxidants in favor of pathogenesis (32). Similar to our earlier reports of enhanced levels of ROS in pulmonary endothelial cells on exposure to viral proteins and/or opioids (11, 45), we found an additive increase in the generation of ROS in HPASMCs treated with cocaine and Tat compared with monotreatments. Our observation is supported by previous finding (46) showing additive increase in oxidative stress in rat hippocampal cells on exposure to cocaine and Tat. Binding of cocaine to its σ -1 receptor results in translocation of these receptors to plasma membrane, followed by activation of other receptors or kinase(s), including Src family kinase (47), which are known to generate

Figure 7. (Continued). pretreatment with both suramin and PP2. Values are mean $\pm$ SD of three independent experiments; * P < 0.05, ** P < 0.01, *** P < 0.001 compared with control; # P < 0.001 compared with cocaine; \$ P < 0.001 compared with Tat; @ P < 0.05, @@ P < 0.01, @@@ P < 0.001 versus cocaine and Tat; ^a P < 0.05 compared with suramin + cocaine + Tat treatment; ^b P < 0.05, ^{bb} P < 0.001 compared with PP2 + cocaine + Tat treatment.

ROS (48). HIV-Tat is known to bind VEGFR-2, and activation of this receptor results in ROS generation (45). Consistent with this, we observed attenuation in cocaine- and Tat-mediated oxidative stress and enhanced proliferation of SMCs in the presence of VEGFR and σ receptor antagonists.

ROS are known to stimulate non-RTKs, including Src (36). Furthermore, activated Src is known to phosphorylate Y934 residue within the kinase domain of PDGFR β (22). Here, we demonstrate reduction in cocaine- and Tat-mediated activation of Src in the presence of antioxidants and reduced receptor activation at Y934, with no change in phosphorylation of Y1009 on Src inhibition. Hansen and colleagues (22) showed increased mitogenic activity resulting from Src-induced phosphorylation of Y934. This fits our observation that blocking Y934 along with Y1009 by using PP2 and suramin, respectively, brings back pSMC proliferation to the levels comparable to control cells, even in the presence of

cocaine and Tat. Although we investigated phosphorylation of only Y934 to look for ROS/Src-dependent ligand-independent effects of cocaine and Tat, Saito and colleagues (49) demonstrated H₂O₂-mediated ligand-independent PDGFR β activation at Y1021 in vascular SMC isolated from the thoracic aorta of rats. Therefore, inhibition of Src in cocaine- and Tat-treated HPASMCs using PP2 may have prevented activation of other ROS/Src ligand-independent sites, including Y1021. Given that various other tyrosine sites are also phosphorylated in the intracellular domain of PDGFR β on binding of PDGF-B (50), pretreating cells using suramin is also expected to block other ligand-mediated autophosphorylation sites. Furthermore, in light of the fact that PDGF-B can also lead to heterodimerization of PDGFR α and β chains, the role of PDGFR α in enhanced smooth muscle proliferation cannot be ignored, more so when PDGFR α can be activated indirectly on stimulation of ROS and Src kinases in the absence of ligand binding (51). However, it is clearly evident from our study that ligand-independent

phosphorylation of PDGFR β plays an important role in cocaine- and Tat-mediated enhanced pSMC proliferation.

The recent advances made in providing better therapeutic regimen for PAH have not been able to cover the broad spectrum of the complexity of this disease. The paucity of lucid information on understanding the mechanism of pSMC proliferation and migration has hindered development of an effective therapy regimen. The therapies based on inhibiting ligand-induced PDGFR have not been completely successful (52). This report on the involvement of ligand-independent activation of PDGFR in cocaine- and Tat-mediated enhanced proliferation pSMCs, suggests that alternate mechanisms of PDGFR axis activation should be targeted at designing potential PAH therapies in future, especially for IVDUs infected with HIV. ■

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