Reactive Oxygen Species/Hypoxia-Inducible Factor-1 α /Platelet-Derived Growth Factor-BB Autocrine Loop Contributes to Cocaine-Mediated Alveolar Epithelial Barrier Damage

Lu Yang¹, Xufeng Chen², Samantha M. Simet³, Guoku Hu^{1,3}, Yu Cai³, Fang Niu³, Yeonhee Kook³, and Shilpa J. Buch³

¹School of Medicine, University of Electronic Science and Technology of China, Chengdu, China; ²Department of Emergence, the First Affiliated Hospital of Nanjing Medical University, Nanjing, China; and ³Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, Nebraska

Abstract

Abuse of psychostimulants, such as cocaine, has been shown to be closely associated with complications of the lung, such as pulmonary hypertension, edema, increased inflammation, and infection. However, the mechanism by which cocaine mediates impairment of alveolar epithelial barrier integrity that underlies various pulmonary complications has not been well determined. Herein, we investigate the role of cocaine in disrupting the alveolar epithelial barrier function and the associated signaling cascade. Using the combinatorial electric cell-substrate impedance sensing and FITC-dextran permeability assays, we demonstrated cocaine-mediated disruption of the alveolar epithelial barrier, as evidenced by increased epithelial monolayer permeability with a concomitant loss of the tight junction protein zonula occludens-1 (ZO-1) in both mouse primary alveolar epithelial cells and the alveolar epithelial cell line, L2 cells. To dissect the signaling pathways involved in this process, we demonstrated that cocaine-mediated induction of permeability factors, platelet-derived growth factor (PDGF-BB) and vascular endothelial growth factor, involved reactive oxygen species (ROS)-dependent induction of hypoxia-inducible factor (HIF)-1 α . Interestingly, we demonstrated that ROS-dependent induction of another transcription factor, nuclear factor erythroid-2-related factor-2, that did not play a role in cocaine-mediated barrier

dysfunction. Importantly, this study identifies, for the first time, that ROS/HIF-1 α /PDGF-BB autocrine loop contributes to cocaine-mediated barrier disruption via amplification of oxidative stress and downstream signaling. Corroboration of these cell culture findings *in vivo* demonstrated increased permeability of the alveolar epithelial barrier, loss of expression of ZO-1, and a concomitantly increased expression of both HIF-1 α and PDGF-BB. Pharmacological blocking of HIF-1 α significantly abrogated cocaine-mediated loss of ZO-1. Understanding the mechanism(s) by which cocaine mediates barrier dysfunction could provide insights into the development of potential therapeutic targets for cocaine-mediated pulmonary hypertension.

Keywords: cocaine; alveolar epithelium; reactive oxygen species; hypoxia-inducible factor-1 α ; platelet-derived growth factor-BB

Clinical Relevance

Inhibiting hypoxia-inducible factor-1 α -mediated autocrine signaling could be considered as novel therapeutic strategies for reversing cocaine-mediated alveolar epithelial barrier dysfunction.

Among all the illicit drugs, cocaine is one of the most commonly used psychostimulants, and has been implicated in the development of serious health complications, including those associated respiratory conditions

(1–3). Regardless of the route of administration, cocaine abusers have been diagnosed with a variety of acute and chronic respiratory and pulmonary complications, including pulmonary

infections, airway and interstitial disease, respiratory failure, pulmonary hypertension, and edema (4–6). In the respiratory system, pulmonary epithelium acts as a barrier to limit the access of

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Correspondence and requests for reprints should be addressed to Shilpa J. Buch, Ph.D., Durham Research Center, Room 8011, 985880 Nebraska Medical Center, Omaha, NE 68198-5880. E-mail: sbuch@unmc.edu

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various toxic inhaled stimuli to the subepithelial tissue, thereby playing a key role in maintaining lung homeostasis. More recently, in addition to the pulmonary macrophages, alveolar epithelial cells have also been found to play important roles in the defense and pathogenesis of infection. Disruption of the epithelial barrier has been shown to contribute to the development of various pulmonary complications, such as chronic obstructive pulmonary disease and acute lung injury (7, 8). It has been well recognized that cocaine-related deaths in drug addicts correlates with increased pulmonary edema, likely due to epithelial barrier breach (9). In keeping with this observation, the focus of the current study was to examine whether exposure of alveolar epithelial cells *in vitro* to exogenous cocaine could lead to disruption of epithelial barrier integrity, leading, in turn, to pulmonary inflammation.

The involvement of oxidative stress in cocaine-induced tissue damage and cellular toxicity has been reported in both human and animal models (10, 11). Reactive oxygen species (ROS), such as the superoxide radical and hydrogen peroxide, have been recognized to impair the alveolar epithelial barrier, resulting in enhanced inflammation and increased susceptibility to pulmonary infections (12). In fact, cocaine-mediated oxidative stress has been implicated in damage of other tissues, such as the liver and kidneys (13). Cocaine has also been demonstrated to cause generation of ROS in pulmonary endothelial cells, thereby contributing to pulmonary vascular leakage (14). Pulmonary systems have evolved elaborate mechanisms that ensure proper balance between the pro-oxidant and antioxidant molecules to control the redox status of normal lung function, as well as to defend against constant oxidative stress (15). Nuclear factor erythroid-2-related factor (NRF) 2 is a transcription factor that is expressed predominantly in the epithelium and alveolar macrophages, and that has a protective role in the lung through activation of the antioxidant response element-regulated antioxidant genes (16). Another transcription factor, hypoxia-inducible factor (HIF)-1 α , known to mediate gene expression under hypoxia, is redox sensitive under normoxic conditions, and plays an important pathophysiological role in regulating changes in the permeability of the pulmonary vasculature that are associated with elevated levels of ROS (17).

Impairment of barrier permeability by growth factors has been reported in various pathologies (18, 19). Previous studies have indicated that vascular endothelial growth factor (VEGF)- and platelet-derived growth factor (PDGF-BB)-mediated impairment of barrier integrity is directly associated with their direct effects on redistribution and down-regulation of the tight junction proteins (TJPs), including zonula occludens-1 (Zo-1) and occludin (20, 21). Interestingly, the expression of both VEGF and PDGF-BB can be regulated by HIF-1 α in response to ROS, and both of the growth factors have also been implicated in triggering pulmonary barrier dysfunction (22, 23). Importantly, significant increases in expression of PDGF-BB and VEGF have been reported in lung tissues from cocaine abusers (24, 25), suggesting, thereby, their potential role in cocaine-mediated pulmonary impairment. In the present study, we sought to investigate the effect of cocaine on alveolar epithelial barrier function and to dissect the mechanism(s) involved in this process. Understanding the detailed molecular pathways in cocaine-mediated alveolar epithelial barrier disruption could provide insights into potential therapeutic targets for improving the treatment for cocaine-mediated pulmonary complications.

Method and Materials

Animals

C57BL/6N mice were purchased from Charles River Laboratories (Wilmington, MA). All animal procedures were performed according to the protocols approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center (Omaha, NE).

Cell Culture

Primary lung alveolar epithelial cell line L2 cells were purchased from ATCC (Manassas, VA). Cells were cultured as monolayers in Ham's F12 medium supplemented with 10% FBS and antibiotics. Primary mouse alveolar epithelial cells were prepared from C57BL/6 mice as reported previously (26), with some modification (*see the online supplement for details*).

Epithelial Barrier Function

Primary alveolar epithelial cells were grown on Vitrogen 30-coated 8EW10⁺ culture

ware (Sigma-Aldrich, St. Louis, MO) and incubated at 37°C in a CO₂ incubator until cells were grown to confluence. The confluent monolayers were exposed to cocaine for up to 48 hours to monitor the barrier functional change using the electric cell-substrate impedance sensing (ECIS) (Applied Biophysics, Troy, NY). FITC Dextran (70 k; 1 mg/ml; Sigma, St. Louis, MO) was used to detect changes in monolayer permeability (BioTek, Winooski, VT).

Reactive Oxidative Species Detection

The Image-iT LIVE Green ROS Detection Kit (Invitrogen, Carlsbad, CA) was used to estimate total cellular ROS in live L2 cells. Change in fluorescence intensity was represented in arbitrary units. MitoSOXTM Red (ThermoFisher Scientific, Waltham, MA) was used to measure the mROS superoxide. All ROS experiments shown are representative of three independent experiments.

Small Interfering RNA Transfection

Small interfering RNA (siRNA) targeted against Nrf-2 or HIF-1 α mRNA were obtained from Thermo Scientific Dharmacon RNAi Technologies (ThermoFisher Scientific). siRNA transfection protocols were followed according to the manufacturer's instructions. Knockdown efficiencies of the proteins were determined by Western blotting.

Western Blot Analysis

Treated cells or lung tissue were lysed using the Mammalian Cell Lysis kit (Sigma). Proteins were separated by electrophoresis and transferred to polyvinylidene difluoride membranes. The blots were incubated with the corresponding primary antibodies overnight at 4°C for detection of Zo-1, claudin-5, occludin (1:800; Invitrogen), Nrf-2, HIF-1 α , PDGF-BB (1:500; Santa Cruz Biotechnology, Dallas, TX), VEGF (1:1,000; Cell Signaling, Danvers, MA), and β -actin (1:2,000; Invitrogen). All of the Western blots were repeated at least three times.

ELISA Analysis

PDGF-BB or VEGF protein levels in conditioned media from L2 cells were examined using the rat PDGF-BB Quantikine or rat VEGF Quantikine ELISA kit (R&D Systems, Minneapolis, MN), respectively. ELISA was performed according to the manufacturers' instructions. Samples were

analyzed for PDGF-BB and VEGF protein in triplicate determined in three independent experiments.

Reverse Transcription and RT-PCR

The quantitative PCR primers for rat were obtained from SABiosciences (Frederick, MD). Total RNA was extracted with Trizol reagent (Invitrogen) according to the manufacturer's instructions and our previous report (27). The primers used in this study are listed in Table E1 in the online supplement.

Immunohistochemistry Staining

Lung sections were used to detect Zo-1 (1:50; Invitrogen) and Nrf-2, HIF-1 α (1:100; Santa Cruz Biotechnology) expression. Slides were mounted with Pro-Long Gold Anti-fade reagent (ThermoFisher Scientific) to visualize the nuclei. The immunofluorescent images were captured using confocal microscopy (see the online supplement for details).

Statistical Analysis

Statistical analysis was performed using one-way ANOVA with a *post hoc* Student's *t* test. Results were judged statistically significant at a *P* value less than 0.05 by ANOVA.

Results

Cocaine-Mediated Permeability Change in Pulmonary Epithelial Cells

In our study, we first evaluated whether cocaine directly alters alveolar epithelial barrier integrity. To determine that, alveolar epithelial cell line L2 cells were treated with cocaine (1–100 μ M) for 24 hours and the effect of cocaine on the L2 monolayer permeability change was examined after cocaine exposure. As shown in Figure 1A, cocaine treatment caused a significant increase in the permeability of FITC-dextran in a dose-dependent manner. To confirm the cocaine-mediated alveolar epithelial “leak,” we isolated the mouse primary alveolar epithelial cells and grew them in the specialized ECIS chamber as confluent monolayers. The real-time resistance changes further demonstrated that 10- μ M cocaine exposure significantly increased the barrier permeability (Figure 1B) without changing cell viability compared with control (Figure E1). As such, 10 μ M were used in the subsequent experiments. Because the diminished barrier function correlates well

with the level of TJP expression in cells, we further examined the expression of Zo-1, occluding, and claudin-5 in cocaine-treated L2 cells. Our results indicate that there was a significant loss of Zo-1, but not of other examined TJPs, after 24-hour cocaine exposure (Figure 1C). To further validate the result, the level of Zo-1 was examined by immunofluorescence staining in the primary epithelial cells grown on the ECIS chamber, and, as expected, cocaine treatment significantly reduced the Zo-1 expression compared with control (Figure 1D). Taking together, these results demonstrate that cocaine disrupts lung alveolar epithelial barrier integrity and decreases Zo-1 expression.

ROS Is Essential for Cocaine-Induced Barrier Disruption

Oxidative stress has been demonstrated to play an important role in cocaine-mediated barrier dysfunction (27). We sought to determine whether cocaine-mediated alveolar epithelial barrier disruption involves ROS production. We found that cocaine rapidly induced the formation of ROS as early as 30 minutes and maintained a high level after 24 hours after treatment (Figure 2A). However, cocaine-mediated ROS production cannot be fully inhibited by pretreating L2 cells with two well characterized nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase inhibitors—the apocynin or diphenyleneiodonium (Figure 2B), indicating that NADPH oxidase is not the only source of ROS generation in cocaine-treated alveolar epithelial cells; however, the nonselective ROS scavenger, phenyl-*N*-t-butyl nitron (PBN), totally inhibited cocaine-induced ROS generation. As a major site of ROS production, mitochondrial ROS (mROS) were thought to exclusively cause cellular damage; thus, we also monitored the mROS by Mitosox Red. From the flow cytometric analysis, cocaine exposure causes rapid ROS generation in the mitochondria, which is totally inhibited by pretreating cells with mitochondria-targeted antioxidant, mitoTEMPO (Figure 2C). We next studied the effects of ROS depletion on cocaine-mediated alveolar barrier disruption. In our results, cocaine-mediated alveolar monolayer barrier permeability change could not be significantly reversed by either apocynin or mitoTEMPO. However, cocaine-mediated permeability change was totally abolished by PBN pretreatment

(Figure 2D). We further detected that pretreatment with PBN significantly reversed the loss of Zo-1 caused by cocaine (Figure 2E). Taken together, these results suggest an essential contribution of oxidative stress to lung alveolar epithelial alterations elicited by cocaine.

Regulation of ROS-Dependent Activation of Nrf-2 and HIF-1 α in Cocaine-Exposed L2 Cells

Elevated ROS generation has been shown to affect signal transduction and gene regulation by HIF-1 α and Nrf2; however, the involvement of Nrf-2 and HIF-1 α in cocaine-mediated alveolar barrier disruption has never been explored before. Our results indicate that Nrf-2 and the Nrf-2-induced antioxidant enzymes (Figure E1) increased at a much earlier stage after cocaine exposure, followed by a significant decrease at the end point of the measurements; however, the level of HIF-1 α persistently increased upon cocaine treatment (Figure 3A). By using different ROS inhibitors, we also revealed that the cocaine-mediated Nrf-2 up-regulation was mainly dependent on activation of NADPH oxidase, as apocynin significantly blocked cocaine-induced Nrf-2 expression, whereas both of the NADPH oxidase-dependent ROS and mROS contributed to HIF-1 α generation in cocaine-exposed L2 cells (Figure 3B). We further investigated the regulation and cross-talk of Nrf2 and HIF-1 α . The siRNA approach was used to knock down the expression of either Nrf-2 or HIF-1 α in L2 cells. The knockdown efficiency was examined and quantified, and the optimal concentration of siRNA for the rest of the studies was then chosen (Figure E3). Our results indicate that knockdown of Nrf-2 attenuated cocaine-mediated induction of HIF-1 α at an early stage, whereas no significant change was obtained after 12-hour cocaine exposure between the control and Nrf-2 knockdown groups (Figure 3C). In keeping with these findings, knockdown of Nrf-2 also had no effect on cocaine-mediated permeability changes (Figure 3D). However, knockdown of HIF-1 α successfully reversed cocaine-induced barrier disruption, as shown in Figure 3E; meanwhile, the cocaine-mediated Zo-1 dysregulation was also reversed by knockdown of HIF-1 α (Figures 3F and 3G). These results indicate that ROS-induced endogenous Nrf-2 antioxidant signaling

Figure 1. Cocaine-mediated permeability change in pulmonary epithelial cells. (A) Cocaine increased the permeability of alveolar epithelial cell line L2 cells in a dose-dependent manner, with maximal response at 100 μ M. (B) Electronic cell-substrate impedance sensing (ECIS) assay analysis confirmed cocaine-mediated increase of barrier permeability in a dose-dependent manner in mouse primary alveolar epithelial cells. (C) Exposure of L2 cells to cocaine resulted in a time-dependent decrease in expression of zona occludens proteins (Zo-1) but not occludin or claudin-5. The analysis data are shown at the bottom. (D) Primary epithelial cells grown in the ECIS chamber were treated with cocaine for 48 hours and stained with antibodies specific for Zo-1. Arrows indicate cocaine treatment significantly reduced the Zo-1 expression compared with control. All the data are presented as mean $\pm$ SD of at least three individual experiments. * P < 0.05, ** P < 0.01 versus control group. Ctrl, control; FITC, fluorescein isothiocyanate; TJPs, tight junction proteins.

failed to protect cocaine-disrupted lung alveolar barrier integrity; however, HIF-1 α has a potential contribution to cocaine-mediated alveolar barrier disruption.

HIF-1 α -Dependent PDGF and VEGF Expression under Cocaine Exposure

To further study the HIF-1 α -mediated transcriptional signaling under cocaine exposure, we transfected L2 cells with an HIF-1-responsive luciferase reporter and

negative control plasmids. As expected, cocaine treatment led to a significant increase of HIF-1 α activation compared with nontreated cells (Figure 4A). Because HIF-1 α is a transcription factor that is implicated in the induction of VEGF and PDGF-BB, both of which are known barrier permeant factors, we investigated the link between HIF-1 α with PDGF and VEGF in cocaine-exposed alveolar epithelial cells. We evaluated the expression

levels of PDGF-BB and VEGF for possible cocaine-induced changes in L2 cells. Our results indicate that cocaine increased the expression of both PDGF-BB and VEGF in L2 cells (Figures 4B–4D). Intriguingly, HIF-1 α siRNA significantly abrogated cocaine-mediated up-regulation of PDGF-BB and VEGF expression compared with control siRNA-transfected cells (Figures 4E and 4F). To further confirm that HIF-1 α binds to the putative

Figure 2. Reactive oxygen species (ROS) are essential for cocaine-induced barrier disruption. (A) Cocaine-mediated ROS formation in L2 cells assessed by 2',7'-dichlorodihydrofluorescein diacetate. Rapid formation of ROS was induced by cocaine as early as 30 minutes and maintained a high level after 24 hours. (B) Effects of apocynin and diphenyleneiodonium (DPI) (nicotinamide adenine dinucleotide phosphate reduced oxidase inhibitors) and phenyl-*N*-t-butyl nitron (PBN) (nonselective ROS scavenger) on cocaine-induced ROS formation in L2 cells. (C) Cocaine-mediated generation of ROS in the mitochondria was monitored by Mitosox Red in L2 cells. Pretreating L2 cells with mitochondria-targeted antioxidant, mitoTEMPO, resulted in amelioration of cocaine-mediated induction of mitochondrial ROS. (D) Effect of apocynin, mitoTEMPO, and PBN on cocaine-mediated disruption of alveolar monolayer barrier permeability. (E) Pretreatment of PBN resulted in amelioration of cocaine-mediated Zo-1 disruption. All the data are presented as mean $\pm$ SD of at least three individual experiments. * P < 0.05, ** P < 0.01, *** P < 0.001 versus control group; # P < 0.05, ## P < 0.01 versus cocaine-treated group. DCF, dichlorofluorescein.

promoter of PDGF-BB and VEGF in L2 cells, we resorted to the chromatin immunoprecipitation assay to reveal active sites on their promoters that were accessible to HIF-1 α (Figure 4G). Our results reveal prominent bands with cocaine exposure, whereas no bands were evident in the untreated or IgG controls, thereby indicating the specific binding of HIF-1 α to the hormone response element region on the PDGF-BB and VEGF promoters in cocaine-exposed L2 cells (Figure 4H).

Inhibition of PDGF-BB, not VEGF, Abolished Cocaine-Mediated Alveolar Epithelial Barrier Damage

To determine whether cocaine-mediated disruption of alveolar epithelial integrity was

specifically due to the presence of VEGF and PDGF in cocaine-exposed culture medium, we pretreated L2 cells with VEGF- or PDGF-BB-neutralizing antibodies before cocaine exposure. Interestingly, the result of ECIS assay indicate that the cocaine-induced permeability change was significantly attenuated by pretreatment with the inhibitory anti-PDGF-BB antibody, but not anti-VEGF antibody (Figure 5A), and this was further validated by FITC-dextran permeability assay (Figure 5B). We further examined the ROS generation in the presence of PDGF-BB- or VEGF-neutralizing antibodies in cocaine-treated L2 cells. As shown in Figure 5C, PDGF-BB-neutralizing antibody significantly inhibited cocaine-mediated

ROS generation, whereas VEGF antibody had little effect. The protective effect of anti-PDGF-BB antibody was also observed by examining the level of Zo-1 expression in cocaine-treated cells (Figure 5D), indicating that cocaine-mediated alveolar epithelial barrier disruption exerts its action mainly via PDGF-BB rather than VEGF.

The ROS/HIF-1 α /PDGF Autocrine Loop Involved in Cocaine-Mediated Alveolar Epithelial Barrier Damage

The autocrine of PDGF-BB signaling was found to modulate the brain endothelial permeability change and contribute to cocaine-mediated blood-brain barrier damage. We successfully detected that exogenous PDGF-BB-recombinant protein

Figure 3. Regulation of ROS-dependent activation of Nrf-2 and hypoxia-inducible factor (HIF)-1α in cocaine-exposed L2 cells. (A) Cocaine-mediated, time-dependent induction of Nrf-2 and HIF-1α protein expression by Western blot. Cells were incubated with 10 μM cocaine at various time points (0–24 h) and processed for protein isolation and Western blot analysis. (B) The expression level of Nrf-2 and HIF-1α under cocaine exposure in the presence or absence of apocynin, mito-TEMPO, and PBN was determined by Western blot along with intensity quantification. (C) Nrf-2 small interfering RNA (siRNA), but not nonspecific siRNA, significantly attenuated cocaine-mediated induction of HIF-1α expression at 3 hours but not at 12 hours. (D and E) The effect of Nrf-2 and HIF-1α siRNA on cocaine-mediated permeability change was determined by FITC-dextran permeation assay. (F) HIF-1α siRNA, but not scrambled siRNA, inhibited cocaine-mediated loss of Zo-1 expression. (G) Representative immunofluorescent images of cells under cocaine exposure in the presence of scrambled siRNA or HIF-1α siRNA. Arrows indicate loss of Zo-1 and barrier disruption. Scale bars, 20 μm. All the data are presented as mean ± SD of at least three individual experiments. * $P < 0.05$, ** $P < 0.01$ versus control group; # $P < 0.05$ versus cocaine-treated group. n.s., not significant. Nrf-2, nuclear factor erythroid 2-related factor 2.

stimulated ROS generation as well as loss of TJP Zo-1 in a dose-dependent manner (Figures 6A and 6B). This indicates that cocaine-induced PDGF-BB alone might

form an autocrine loop to amplify the cocaine-initiated ROS and HIF-1α pathways and contribute to the disruption of epithelial barrier. To test this hypothesis,

we first validated the expression of PDGF receptors (PDGFRs) in L2 cells. As shown in Figure 6C, PDGFR-α is highly expressed in L2 cells. We pretreated L2 cells with

Figure 4. HIF-1 α -dependent platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) expression under cocaine exposure. (A) Cocaine-mediated activation of HIF-1 α was determined by luciferase assays in the presence of negative control reporter or HIF reporter. (B) Representative blots show the cocaine-mediated up-regulation of both VEGF and PDGF-BB at both 24 and 48 hours. (C and D) Cocaine-mediated up-regulation of PDGF-BB and VEGF protein level in the supernatant fluids was determined by ELISA. (E and F) The expression level of PDGF-BB and VEGF in L2 cells under cocaine exposure in the presence of scrambled siRNA or HIF-1 α siRNA was determined by Western blot analysis. (G) A schematic showing the hormone response element (HRE) promoter and the target region tested in chromatin immunoprecipitation assays in the left panel. (H) Cocaine-mediated up-regulation of specific binding of HIF-1 α to the HRE region on the PDGF-BB and VEGF promoters was determined by chromatin immunoprecipitation assays. All the data are presented as mean $\pm$ SD of at least three individual experiments. * P < 0.05, ** P < 0.01 versus control group.

STI-571 (imatinib), a tyrosine kinase inhibitor known as a PDGFR blocker, before cocaine exposure. Our results revealed that cells pretreated with STI-571 significantly reduced the ROS level after 24-hour cocaine exposure (Figure 6D). We also detected that blocking of PDGFRs reversed cocaine-induced loss of Zo-1. In contrast, cocaine-induced

expression of HIF-1 α and PDGF-BB was significantly blocked by pretreated cells with PDGFR inhibitor (Figure 6E). The permeability assay was also performed in the presence of STI-571 upon cocaine treatment. As shown in Figure 5F, cocaine-mediated permeability increase was significantly inhibited by blocking the PDGFRs in L2 cells, thereby underscoring

the role of the ROS/HIF-1 α /PDGF-BB autocrine loop in this process.

Cocaine Disrupts Pulmonary Epithelial Barrier *In Vivo*

We next wanted to validate the cocaine-mediated alveolar epithelial barrier disruption *in vivo*. To achieve that, we used Sulfo-NHS-Biotin (ThermoFisher Scientific) as a

Figure 5. Inhibition of PDGF-BB, but not VEGF, abolished cocaine-mediated alveolar epithelial barrier damage. (A) Effect of cocaine on permeability in the absence or presence of anti-PDGF-BB or anti-VEGF antibody using ECIS assay. (B) Effect of cocaine on permeability in the absence or presence of anti-PDGF-BB antibody, anti-VEGF antibody, or control IgG using FITC-dextran permeability assay. (C) Cocaine-mediated ROS generation in the presence or absence of anti-PDGF-BB antibody, anti-VEGF antibody, or control IgG was determined by DCF-diacetate (DA) assay. (D) Western blotting demonstrating that the down-regulation of Zo-1 under cocaine exposure could be inhibited by the presence of anti-PDGF-BB antibody but not anti-VEGF antibody or control IgG. All the data are presented as mean $\pm$ SD of at least three individual experiments. * $P < 0.05$, ** $P < 0.01$ versus control group; # $P < 0.05$, ## $P < 0.01$ versus cocaine-treated group. Ab, antibody.

paracellular tracer molecule to access the lung epithelial barrier function after saline or cocaine injection. Biotins are membrane-impermeable reagents that will penetrate into the intercellular space if the epithelial barrier structure/function is disrupted. As shown in Figure 7A, cocaine administration resulted in the distribution of biotin tracer into the barrier, whereas biotin penetration was retained in mice with saline administration. We also performed bronchoalveolar lavage (BAL) to evaluate the alveolar barrier integrity. The increased alveolar epithelial barrier permeability was evidenced by an

increase in the total cell number and total protein concentration in BAL fluid from mice administered cocaine (Figures 7B and 7C). This result indicates that cocaine directly causes the leakage of lung alveolar epithelial barrier, and possibly induced pulmonary edema and inflammatory response *in vivo*. The expression of several targets in the lung homogenates from cocaine- versus saline-treated mice were also assessed by Western blotting. Compared with saline group, cocaine administration resulted in increased expression of HIF-1 α and PDGF-BB; however, the level of tight

junction Zo-1 decreased significantly in cocaine-treated mice (Figures 7D and 7E). Interestingly, we observed no significant change of Nrf-2 in cocaine-treated animals *in vivo*. To further validate that HIF-1 α played a key role in cocaine-mediated barrier disruption, we used a well established HIF-1 α inhibitor YC-1 to elucidate the role of HIF-1 α in this process. As expected, and as demonstrated by immunostaining, cocaine-mediated induction of HIF-1 α was significantly abrogated in mice pretreated with YC-1. In addition, cocaine-mediated impairment

Figure 6. The ROS/HIF-1 α /PDGF autocrine loop involved in cocaine-mediated alveolar epithelial barrier damage. (A) PDGF-BB-induced, dose-dependent generation of ROS using DCF-DA assay. (B) Representative Western blotting of PDGF-BB-mediated, dose-dependent down-regulation of Zo-1. (C) Representative RT-PCR image of PDGF receptor (PDGFR)- α and PDGFR- β expression in L2 cells. (D) Pretreatment of cells with the PDGFR blocker STI-571 resulted in amelioration of cocaine-mediated induction of ROS production. (E) Western blot analysis showing that STI-571 inhibited cocaine-induced up-regulation of PDGF-BB and HIF-1 α and suppressed cocaine-mediated down-regulation of Zo-1. (F) Pretreatment of cells with STI-571 resulted in amelioration of cocaine-mediated permeability change. All the data are presented as mean $\pm$ SD of at least three individual experiments. * P < 0.05, ** P < 0.01 versus control group; # P < 0.05, ## P < 0.01 versus cocaine-treated group.

of barrier integrity, as evidenced by Zo-1 expression in lung epithelial cells, was also blocked in mice pretreated with YC-1 compared with the untreated group (Figure 7F).

Discussion

Drug abuse represents a serious medical and social problem facing the current

generation, both nationally and globally. Cocaine abuse is associated with a myriad of vascular complications, including, but not limited to, stroke, hypertension, and respiratory failure. In fact, increasing respiratory complications, including inflammation and proteinaceous edema, have been associated with cocaine abuse (6, 9). Alveolar epithelial barrier plays a key role in maintaining a tight barrier that is impermeable to influx of toxic inhalants.

In keeping with the role of cocaine in mediating pulmonary edema, the present study was aimed to examine the effect of cocaine on the alveolar epithelial barrier integrity and signaling pathways involved in this process. Herein, we provide causal evidence that cocaine mediates alterations in the structure and function of alveolar epithelial barrier both *in vitro* and in the *in vivo* models. TJPs are intercellular junctions that are crucial for epithelial

Figure 7. Cocaine disrupts the pulmonary epithelial barrier *in vivo*. (A) Representative image of cocaine-mediated penetration of biotins in the mouse lung epithelial barrier. *Arrows* indicate retained biotin in the epithelial barrier. (B and C) Quantification of cell count and total proteins in the bronchoalveolar lavage fluid (BALF) showing cocaine-mediated increase of total cell number and total protein concentration. (D) Western blot analysis showing down-regulated expression of Zo-1 and up-regulation of HIF-1 α and PDGF-BB expression in the cocaine-injected mice, whereas no significant change of Nrf-2 expression was observed. (E) Representative image of immunofluorescence staining demonstrating cocaine-mediated Zo-1 loss and up-regulated HIF-1 α expression, whereas pharmacological blocking of HIF-1 α by YC-1 significantly abrogated cocaine-mediated loss of Zo-1. Scale bar, 20 μm . (F) A schematic summary for the molecular mechanism underlying cocaine-mediated disruption of alveolar epithelial barrier integrity. All the data are presented as mean $\pm$ SD of at least three individual experiments. * $P < 0.05$, ** $P < 0.01$ versus control group.

adhesion and barrier functions in a wide variety of tissues and organs (28). It has been reported that the permeability barrier in terminal airspaces is primarily due to the presence of TJPs between the alveolar epithelial cells (29). Down-regulation or redistribution of the TJPs leads to changes

in barrier integrity. For example, Mazzon and Cuzzocrea (30) reported that, in a mouse model of acute lung inflammation, altered expression of the TJP Zo-1 correlated with epithelial barrier disruption. Similar to these reports, we also observed that exposure of alveolar epithelial cells to

cocaine resulted in a significant decrease in the expression of Zo-1 and barrier functioning, thereby implicating the effect of cocaine on barrier structure and function.

Accumulating evidence indicates that cocaine exposure results in generation and

transmission of redox signals in various tissues (13). In fact, previous findings from our laboratory identified the role of cocaine-mediated ROS in endothelial barrier dysfunction, which, in turn, contributes to leaky blood–brain barrier (31). Furthermore, ROS is also implicated as a mediator of epithelial injury, as it is known to impair several epithelial cell functions via elicitation of signal cascades (32, 33). Our findings clearly demonstrate that exposure of L2 alveolar epithelial cells to cocaine resulted in a time-dependent induction of NADPH oxidase–dependent ROS and mROS, which was critical for cocaine-mediated disruption of barrier permeability. Mitochondria have recently been proposed as critical targets for cocaine-induced toxicity (34). In fact, earlier studies have demonstrated depressed mitochondrial respiration and increased mROS in animals treated with cocaine (35), which lends credence to our findings.

Induction of ROS is tightly controlled with the inducible antioxidant program that, in turn, is responsive to oxidative stress, with the transcription factor, Nrf-2, playing a predominant role in regulating the antioxidant gene expression in various cells (36). In addition, similar to Nrf2, HIF-1 α signaling has also been shown to be responsive to redox signaling, and plays a key role in modulating epithelial barrier integrity (37). Increasing evidence indicates a possible regulation and cross-talk between Nrf2 and HIF-1 α in response to enhanced generation of ROS in the cells (26, 38, 39). In fact, it has been shown by Malec and colleagues (38) that the ROS-dependent induction of Nrf-2 is important for sustained induction of HIF-1 α in the lung adenocarcinoma cells. In contrast, another study by Sullivan and colleagues (40) demonstrated that loss of Nrf-2 resulted in increased ROS, resulting in further stabilization of HIF-1 α and a concomitant increase in intracellular ROS in cancer cells. Unlike the cancer cells, our results indicate that, in alveolar epithelial cells, Nrf-2–mediated antioxidant effect actually prevented induction of HIF-1 α during the short-term cocaine treatment; however, no significant difference was observed after 24-hour exposure to cocaine. More interestingly, knocking down Nrf-2 had no effect on cocaine-mediated barrier permeability change. In contrast, blocking HIF-1 α significantly reversed cocaine-

induced barrier disruption. These findings suggest that Nrf-2–mediated antioxidant signaling could be involved as a protective response early during cocaine exposure, and that sustained ROS, with its downstream signaling cascade, could lead to persistent induction of HIF-1 α , eventually contributing to alveolar epithelial barrier disruption. In keeping with our findings on Nrf2, an earlier report also indicated that depletion of Nrf-2 gene failed to alter cocaine-induced behavior or neurotoxicity (41). Taken together, our findings suggest that Nrf-2, although important for short-term protection against oxidative stress, is not actually functionally involved in cocaine-induced disruption of epithelial barrier.

Based on the transcription factor binding analyses, although the PDGF-BB promoter has several putative HIF-response elements, direct involvement of HIF-1 α in the regulation of PDGF gene expression in lung alveolar epithelial cells is not clear. Our study demonstrates, for the first time, that both PDGF-BB and VEGF are transcriptionally regulated by HIF-1 α in cocaine-exposed lung alveolar epithelial cells using the chromatin immunoprecipitation assay. We also uncovered a new mechanism demonstrating presence of autocrine loop involving ROS/HIF-1 α /PDGF-BB that contributes to cocaine-mediated impairment of epithelial barrier permeability. Inhibition of PDGF-BB activity by neutralizing antibody, but not that of VEGF, significantly abolished cocaine-mediated barrier permeability changes, as well as ROS production. Exposure of epithelial cells to the recombinant protein, PDGF-BB, resulted in generation of ROS, leading, in turn, to persistent increases in the expression of HIF-1 α . Intriguingly, exposure of cells to the PDGFR antagonist blocked cocaine-mediated increase of HIF-1 α and PDGF-BB, thereby suggesting that PDGF-BB is not only an HIF-1 α downstream target, but is also involved in the autocrine signaling needed to maintain the high levels of ROS and sustained expression of HIF-1 α in lung epithelial cells after exposure to cocaine. Similar to our findings, ROS/HIF-1 α –dependent induction of PDGF-BB has also been shown to play an important role in human immunodeficiency virus-1–mediated pulmonary endothelial dysfunction (22). Autocrine and paracrine regulation of PDGF-BB–mediated pulmonary barrier impairment have emerged as important

pathways controlling pulmonary arterial hypertension (42). These findings are in concordance with our previous report demonstrating that cocaine-mediated release of PDGF-BB leads to autocrine activation of the PDGF-BB/PDGF- β axis, which then culminates in a breach of the blood–brain endothelial cells (19, 31). An earlier report has also indicated that HIF-1 α and PDGF-BB cooperate with Akt signaling to mediate autocrine regulation that promotes angiogenesis in endothelial cells (43). It is worth mentioning that, similar to our results, an earlier study also reported that short-term cocaine treatment had no effect on alveolar epithelial barrier integrity (44). Taken together, our findings underpin the important role of the ROS/HIF-1 α /PDGF-BB autocrine loop in modulating the pulmonary dysfunction in response to cocaine.

Cocaine-mediated alveolar epithelial barrier permeability alteration was also assessed in an *in vivo* model. Consistent with our *in vitro* findings, a significant increase of biotin diffusion into the intercellular space of alveolar epithelial barrier was observed in cocaine-administrated mice in comparison with the saline control group. Treatment of mice with HIF-1 α inhibitor resulted in significant restoration of Zo-1 expression, thereby underpinning the role of HIF-1 α signaling in cocaine-mediated disruption of lung barrier integrity. In addition, mice that were administered cocaine demonstrated increased cell counts and total protein concentration in the BAL fluid, thereby indicating a loss of epithelial barrier function and increased inflammatory milieu in the lung. In keeping with this finding, increased inflammation has been observed in cocaine abusers compared with healthy control subjects (9, 44). Our results indicate that loss of epithelial barrier function could contribute to alveolar inflammatory response, leading to pulmonary complications observed in cocaine abusers. It is also in agreement with a recent study investigating the role of cocaine in accelerating human immunodeficiency virus–mediated pulmonary endothelial and smooth muscle dysfunction via induction of PDGF-BB *in vivo* (25).

In summary, our study reveals a novel and detailed molecular mechanism involved in cocaine-mediated epithelial barrier

disruption both *in vitro* and in *in vivo* model systems. Our findings unravel an important role of cocaine-induced oxidative stress in mediating this process. Autocrine regulation involving the ROS/HIF-1 α /PDGF-BB pathway was critical for

augmenting the ROS cascade and for sustaining the induction of PDGF-BB, leading ultimately to a cocaine-mediated breach of the alveolar epithelial barrier. Inhibiting autocrine signaling by either the antioxidant cocktail or HIF-1 α inhibitors

could be considered as novel therapeutic strategies for reversing cocaine-mediated alveolar epithelial barrier dysfunction. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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