Atorvastatin Reduces the Proadhesive and Prothrombotic Endothelial Cell Phenotype Induced by Cocaine and Plasma From Cocaine Consumers In Vitro

Claudia G. Sáez, Karla Pereira-Flores, Roberto Ebensperger, Olga Panes, Teresa Massardo, Patricia Hidalgo, Diego Mezzano, Jaime Pereira

Objective—Cocaine consumption is a risk factor for vascular ischemic complications. Although endothelial dysfunction and accelerated atherosclerosis have been observed in cocaine consumers, the mechanisms underlying their pathogenesis are not fully understood. This study aimed at identifying the effects of atorvastatin in relation to a proadhesive and prothrombotic phenotype induced by cocaine and plasma from chronic cocaine users on endothelial cells.

Approach and Results—Human umbilical vein endothelial cells were exposed to either cocaine or platelet-free plasma (PFP) from chronic cocaine consumers in the presence or absence of 10 μmol/L of atorvastatin. Atorvastatin significantly reduced the enhanced platelet adhesion that was induced by cocaine and PFP from chronic cocaine consumers, as well as the release of the von Willebrand factor. Atorvastatin also avoided striking alterations on cell monolayer structure triggered by both stimuli and enhanced NO reduction because of cocaine stimulation through disrupting interactions between endothelial nitric oxide synthase (eNOS) and caveolin-1, thus increasing eNOS bioavailability. Cocaine-increased tissue factor–dependent procoagulant activity and reactive oxygen species generation were not counteracted by atorvastatin. Although monocyte chemoattractant protein-1 levels were not significantly higher than controls either under cocaine or PFP stimulation, atorvastatin completely avoided monocyte chemoattractant protein-1 release in both conditions. Platelets stimulated with cocaine or PFP did not express P-selectin, glycoprotein IIb/IIIa, or CD40L and failed to adhere to resting human umbilical vein endothelial cell.

Conclusions—Cocaine and patient plasma equally induced a proadhesive and prothrombotic phenotype in endothelial cells, except for von Willebrand Factor release, which was only induced by PFP from chronic cocaine consumer. Atorvastatin improved endothelial cell function by reducing cocaine-induced and PFP from chronic cocaine consumer–induced effects on platelet adhesion, cell architecture, and NO production. (Arterioscler Thromb Vasc Biol. 2014;34:00-00.)

Key Words: atherosclerosis ■ cocaine

Cocaine consumption, which is considered to be one of the most prevalent addictions worldwide, is associated with accelerated atherosclerosis, greater risks of cardiovascular disease, and fatal ischemic events. The mechanisms underlying the deleterious vascular effect of cocaine use involve vasoconstriction-vasospasm, endothelial dysfunction, and thrombogenesis. The pathogenesis of cocaine-induced arterial obstruction is probably multifactorial although evidence provided by studies using experimental animal models and observations in cocaine abusers suggest that hemostatic system activation may play an important role, being that it promotes thrombus formation. Findings from the available literature and our own data strongly support the notion that endothelial cell (EC) damage is a major phenomenon observed in chronic cocaine consumers. Evidence of endothelial dysfunction in cocaine users has been observed in different model systems. In an ex vivo study, for instance, we identified several significantly elevated endothelial dysfunction markers in chronic cocaine users, especially circulating ECs. After the patients underwent a short period of cocaine withdrawal, some markers diminished to normal values, but circulating ECs and monocyte chemoattractant protein-1 (MCP-1) levels remained elevated. The latter suggests that EC impairment induced by cocaine consumption persists even beyond the acute effect of the drug.

In vitro studies using human aortic ECs showed that cocaine induced a significant increase in tissue factor (TF) expression...
Platelet activation has also been implicated as a possible mechanism by which cocaine promotes thrombus formation. Chronic cocaine consumption is associated with elevations of CD40L, neutrophil-activating protein-2 (CXCL7), and Regulated on Activation, Normal T Cell Expressed and Secreted (eNOS) reduce intracellular calcium mobilization and alter the endothelium of chronic cocaine consumers might be exposed to a variety of bioactive mediators besides cocaine or its metabolites. On the basis of this finding, we also examined whether platelet-free plasma (PFP) from chronic cocaine consumers can induce alterations in ECs similar to those provoked by cocaine.

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

Results
As previously mentioned, adhesion of platelets to an activated endothelium is a fundamental step during atherogenesis. Statins, however, improve several EC functions, including endothelial function by increasing NO bioavailability after 3 pic effects of atorvastatin have been demonstrated to improve endothelial function by increasing NO bioavailability after 3 days of administration effectively. This is achieved without affecting low-density lipoprotein-cholesterol or cholesterol levels in patients with cardiovascular risk or established coronary artery disease. In the endothelium, atorvastatin downregulates endothelin-1, CD40, and vascular cell adhesion molecule-1 expression, and increases NO as well as eNOS levels. In patients with coronary artery disease, atorvastatin reduces CD40L expression and improves activation markers in platelets.

The main objective of this work was to evaluate whether atorvastatin can modify the enhanced adhesive and procoagulant phenotype induced by cocaine in human ECs. Our finding showed that activation markers derived from blood cells persist even after short-term abstinence suggests that the endothelium of chronic cocaine consumers might be exposed to a variety of bioactive mediators besides cocaine or its metabolites. On the basis of this finding, we also examined whether platelet-free plasma (PFP) from chronic cocaine consumers can induce alterations in ECs similar to those provoked by cocaine.

Atorvastatin Reduced Enhanced Platelet Adhesion to HUVEC Induced by Cocaine and Moderately Decreased the Exposure of VWF on Cell Surface
HUVECs exposed to cocaine either in the presence or in the absence of atorvastatin were coincubated with resting platelets and evaluated by immunostaining for VE-cadherin or VWF for ECs and glycoprotein Ibx for platelets. As observed in Figure 1A, the number of platelets adhered to EC increased dramatically after 5 hours of cocaine exposure when compared with HUVEC stimulated with the vehicle (220±73.54 versus 10.2±1.07 platelets/100 EC; P=0.0025). Cocaine also induced changes in cell architecture, resulting in a loss of the cells’ bricked shape by forming spaces between groups of cells. When atorvastatin was added together with cocaine, it completely abolished platelet adhesion to HUVEC (8.57±1.81 platelets/100 EC; P=0.00156) and restored cell shape and monolayers similar to untreated cells. Stimulated cells were tested...
for signs of apoptosis with propidium iodide and incorporation or caspase 3 cleavage products, which showed no differences from control cultures (data not shown). The quantification of platelets adhered to HUVEC is shown in Figure 1B. The number of platelets adhered to cocaine-treated HUVEC was ≈200-fold higher than vehicle-treated cells and atorvastatin reduced the number of adhered platelets to <10 platelets per 100 ECs. Figure 1C shows that cocaine induced the expression of significant amounts of VWF on HUVEC surfaces when compared with control cells. Although atorvastatin significantly reduced cocaine-induced platelet adhesion, it only moderately diminished VWF expression. Under our experimental conditions, cocaine failed to increase VWF release to the supernatant, even in cells treated for 24 hours with cocaine (data not shown). A higher concentration of cocaine (100 μmol/L) or cotreatment with atorvastatin did not modify the amount of VWF released by untreated cells (Figure 1D).

**Increased Platelet Adhesion and VWF Exposure and Secretion by HUVEC Supplemented With Plasma From Chronic Cocaine Consumers Are Hindered by Atorvastatin**

Figure 2A shows a representative picture of HUVEC supplemented with PFP from patients (PFP-CP) and controls (PFP-NP) with and without atorvastatin. As observed, a larger number of platelets adhered to cells supplemented with PFP-CP than those with PFP-NP. Quantification (Figure 2B) showed that HUVEC treated with PFP-CP adhered ≫2-fold of the number of platelets bound to cells exposed to PFP-NP (22.37±4.09 versus 8.74±2.83 platelets/100 EC; \( P = 0.0040 \), respectively). When atorvastatin was added, it significantly reduced platelet adhesion induced by PFP-CP (22.37±4.09 versus 3.46±0.98 platelets/100 EC, respectively; \( P = 0.0002 \)). Furthermore, treatment only with PFP-CP induced changes in cell shape toward a fibroblast-like phenotype, which was completely restored to normal by atorvastatin (Figure 2A).

Just like cocaine, PFP-CP supplementation significantly increased VWF expressed on the cell surface. The latter was significantly reduced by atorvastatin to levels comparable with HUVEC supplemented with PFP-NP (Figure 2C). Unlike observations in cocaine-treated cells, increased levels of secreted VWF were found in the supernatants of HUVEC supplemented with PFP-CP when compared with those treated with PFP-NP (188±76.43 versus 77.06±25.61 UI/dL, respectively; \( P = 0.0403 \); Figure 2D). To discard the differential contribution of ADAMTS13 coming from the plasmas used for supplementation, we determined the activity of this protease in patients and controls. As shown in Figure 1 in
the online-only Data Supplement, there were no significant differences in ADAMTS13 activity between chronic cocaine consumers and their respective control subjects (107.7±7.218 versus 102.1±6.997% of normal pooled plasmas). Although atorvastatin significantly reduced VWF on cell surfaces, it only partially diminished its release to the supernatant from cells stimulated with PFP-CP for 5 hours (104.8±51.67% UI/dL), as shown in Figure 2D. However, in HUVEC incubated for 24 hours, atorvastatin significantly reduced VWF secretion when compared with PFP-CP (77.09±33.08 versus 256.0±59.07% UI/dL respectively; P=0.036; not shown).

Platelets Stimulated With Either Cocaine or PFP-CP Did Not Adhere to Healthy HUVEC

Chronic cocaine consumers showed elevated levels of several platelet activation markers as reported by our group. Thus, we investigated whether cocaine or patient PFP would activate platelets, which could lead to increased adhesion to untreated ECs.

As shown in Figure 3A and 3B, neither cocaine nor plasma stimulation increased P-selectin exposure in platelets, as assessed by flow cytometry. No significant differences were observed between untreated or treated platelets even with a higher concentration of cocaine. Moreover, atorvastatin did not affect P-selectin exposure in platelets. Platelets stimulated with TRAP exposed large amounts of P-selectin (42.0±5.04%), denoting platelet activation capability under assay conditions (Figure 3B). In addition, stimulated platelets did not exhibit CD-40 L or activation of glycoprotein IIb/IIIa because they did when activated by thrombin (data not shown). Despite these results, platelets incubated with cocaine exhibited reduced NO content when compared with untreated cells (0.158±0.01 versus 0.195±0.03 arbitrary fluorescence units (AFU), respectively; P=0.0156), as well as with PFP-CP when compared with PFP-NP (0.263±0.015 versus 0.331±0.016 AFU, respectively; P=0.005; Figure 3C and 3D).

Atorvastatin, completely avoided decreases in NO in platelets stimulated by cocaine (0.181±0.02 versus 0.158±0.01 AFU, respectively; P=0.0313; Figure 3C). Adhesion of platelets stimulated with cocaine or PFP to untreated HUVEC showed <10 platelets per 100 HUVECs adhered under all experimental conditions, as observed in Figure 3E.
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Atorvastatin Counteracts MCP-1 Levels Released by HUVEC Stimulated With Cocaine or With PFP From Chronic Cocaine Consumers

MCP-1 participates in the development of atherosclerotic lesions by promoting the recruitment of monocytes at vascular inflammation sites. In HUVEC, MCP-1 is synthesized and released by inflammatory cytokines and platelet adhesion. Statins were reported to reduce MCP-1 levels in vivo and in several in vitro models. As observed in Figure 4A and 4B, exposure of HUVECs to cocaine or PFP-CP resulted in a slight (not significant) increase in levels of released MCP-1. Nevertheless, atorvastatin significantly reduced, such MCP-1 levels from 128±20.87 to 93±7.23% (P=0.031) in cocaine-treated cells and from 149±51.03% to 107±34.95% (P=0.039) in PFP-CP–treated cells (Figure 4B).

Cocaine and PFP From Chronic Cocaine Users Increased TF-Dependent Procoagulant Activity

The loss of antithrombotic properties and increased procoagulant activity (PCA) by damaged/activated endothelia is another characteristic feature of endothelial dysfunction. Because thrombin generation is a key phenomenon in the

Figure 3. Adhesion of platelets stimulated with cocaine or platelet-free plasma (PFP) to healthy human umbilical vein endothelial cells (HUVECs). A and B, Quantification analysis of flow cytometry for P-selectin exposure on platelets stimulated with cocaine in the presence or absence of atorvastatin (A) or with PFP of chronic cocaine consumers (PFP-CP) or of its noncocaine control (PFP-NP) with or without atorvastatin (B). P-selectin in platelets stimulated with TRAP is shown as control. C and D, Quantification of nitric oxide content in washed platelets stimulated with cocaine (C) or PFP (D). The presence or absence of atorvastatin is specified. L-NNA incubated platelets were used as control. C, *P=0.0156 between unstimulated and cocaine-stimulated platelets; **P=0.0313 between platelets stimulated with cocaine in the presence or absence of atorvastatin; n=6. D, *P=0.005 between PFP-NP and PFP-CP; n=5. E, Representative microphotographs of platelets adhered to HUVEC stimulated under different conditions. Control (unstimulated), cocaine or TRAP stimulated, shows HUVEC immunostained for VE-cadherin and platelets for glycoprotein Ibα (GPIbα). PFP-NP and PFP-CP show photographs of HUVEC immunostained for von Willebrand factor (VWF) and platelets for GPIbα taken with a double cab 418/580 nm filter. Bar, 10 μm. AFU indicates arbitrary fluorescence units.
pathogenesis of vascular ischemic complications, we assessed TF-PCA in HUVEC stimulated by cocaine or PFP-CP.

Figure 5A shows that cocaine induced a significant increase in TF-PCA when compared with untreated cells (150.8±27.7% versus 100% of control; P=0.0238). However, as shown in Figure 5B, the increased PCA of cells stimulated with PFP-CP did not reach significance with respect to cells incubated with PFP-NP (224.2±92.88% versus 100%, respectively), which is probably because of the variability of the data. Atorvastatin neither prevent PCA increases induced by cocaine (119.8±28.95 versus 150.8±27.7%, respectively; P=0.156) nor affect the PCA of cells treated with PFP-CP (178.2±62.57%, respectively; P=0.219) as observed in Figure 5A and 5B.

Atorvastatin Avoided the Reduction of NO Induced by Cocaine or by Plasma From Chronic Cocaine Consumers, but Did Not Modify Reactive Oxygen Species Generation

Considering that one of the best known pleiotropic effects of statins is to improve NO bioavailability, we studied whether atorvastatin can modify NO and reactive oxygen species (ROS) in HUVEC stimulated with PFP-CP. In agreement with reports based on other experimental models,7,10 NO significantly decreased in HUVEC stimulated with cocaine when compared with untreated cells (0.297±0.013 versus 0.583±0.133 AFU, respectively; P=0.0225). Atorvastatin completely re-established the NO content to levels similar to those found in untreated cells (0.523±0.083 versus 0.583±0.133 AFU, respectively; P=0.0313; Figure 6A). Cells coincubated with atorvastatin and cocaine for 5 hours showed a tendency to increase eNOS levels without affecting their phosphorylation states (Figures IIA–IIC in the online-only Data Supplement). However, atorvastatin addition reduced caveolin-1 protein levels beyond those observed in control cells (Figure IID in the online-only Data Supplement) and induced a marked effect on the subcellular redistribution of eNOS and caveolin-1 toward a reduced degree of interaction (Figure III in the online-only Data Supplement). Similarly, HUVEC stimulated with PFP-CP showed a significant reduction in NO when compared with cells treated with PFP-NP (0.370±0.062 versus 0.638±0.104 AFU, respectively; P=0.0225). Atorvastatin impairs reductions in NO induced by PFP-CP (0.490±0.057 versus 0.370±0.062 AFU, respectively; P=0.0244) although levels did not reach those of HUVECs treated with PFP-NP (0.370±0.062 versus 0.638±0.104 AFU; respectively; Figure 6C).

HUVEC treated for 5 hours with cocaine generated significantly more ROS than untreated cells (8.01±0.323 versus 2.75±0.19 AFU, respectively; P=0.0014) as observed in Figure 6B. Although HUVEC stimulated with PFP-CP showed a tendency to generate more ROS, they did not reach significant levels (17.77±10.59 versus 6.617±2.401 AFU, respectively; P=0.109; Figure 6D). Unexpectedly, atorvastatin did not change ROS generation levels either in cocaine-stimulated cells or in PFP-CP–supplemented cells (Figure 6E).

Discussion

Cocaine is one of the most frequently used illicit drugs associated with severe cardiovascular events. The mechanisms involved in the deleterious effects of cocaine on the vasculature are undoubtedly multifactorial, yet the loss of the endothelium’s protective functions seems to be a common denominator. In this regard, actions taken toward improving ED could have an effect on the management of cardiovascular complications related to drug addiction. Here, we confirmed the protective action of atorvastatin by inhibiting proadhesive and prothrombotic changes in ECs induced by cocaine or by plasma from chronic cocaine consumers.

We observed that ECs exposed to cocaine displayed a notable proadhesive phenotype, enhancing significantly the binding of platelets under static conditions. The most remarkable observation of this study was the complete
inhibition of platelet adhesion to cocaine-treated ECs by atorvastatin. Similarly, when HUVECs were incubated with PFP-CP, the binding of platelets was enhanced and then significantly reduced by atorvastatin. Under both experimental conditions, the increase in binding of platelets was associated with enhanced VWF deposited on the surfaces of ECs. However, atorvastatin was more effective in reducing VWF of cells stimulated with PFP-CP than those stimulated with cocaine. The results observed from cocaine stimulation were in agreement with those reported by Hobbs et al.29 in different tissue-derived EC lines stimulated with cocaine and several metabolites. The authors showed that cocaine induces the release of VWF under flow, thus forming strings on the cell surface to which platelets adhere. In our study, we used atorvastatin to inhibit the adhesion of platelets to HUVEC stimulated with cocaine and observed the same response when HUVEC were exposed to PFP-CP from chronic cocaine consumers. The effect of plasma was exclusive of PFP-CP because PFP from noncocaine consumers did not induce either VWF release or platelet adherence. Our previous report showed that chronic cocaine users have elevated levels of several platelet14 and ECs2 activation markers that remain in circulation beyond the half-life of cocaine metabolites. On the basis of latter observation, our results on PFP stimulation and atorvastatin inhibition are of particular interest because these conditions may replicate the environment to which vascular endothelial and leukocytes are exposed. Although atorvastatin was observed to reduce both significantly, platelet adhesion and VWF expression on the cell surfaces of HUVEC treated with PFP-CP, it only moderately diminished VWF in the case of cells stimulated with cocaine although it significantly reduced the number of platelets adhered. Patients and the control group did not show any differences in ADAMTS13 activities. As a result, the enzyme activity in plasma did not account for the higher VWF found in cell supernatants of HUVEC stimulated with PFP-CP. Additional studies are required to disclose the presence of other proteases in PFP-CP (but not in PFP-NP) that could cleave VWF34 and other factors within PFP-CP that would induce higher ADAMTS13 released by ECs.

Platelets express many ligands and receptors that, on activation, enable interactions between them, as well as with leukocytes and ECs. In addition to membrane glycoproteins and P-selectin, platelets express various integrins that can also interact with molecules lying on ECs, such as intercellular adhesion molecule.35 We showed that platelets from chronic cocaine patients after recent consumption seem activated, as reflected by elevated levels of platelet activation markers in circulation.34 Hence, we evaluated whether activation of platelets, similar to that of HUVEC stimulation with cocaine, could be sufficient to promote increased adhesion to nonstimulated cells. Cocaine and PFP induced a significant decrease in platelet NO content, which did not result in increased adhesion to nonstimulated HUVECs. Independent of the stimulation conditions, neither cocaine nor PFP induced the expression of P-selectin or CD40-L or the activation of glycoprotein IIb/IIIa. These results suggest that under our experimental conditions, endothelial activation is required for providing the adequate environment for platelet adhesion rather than solely platelet activation.

Figure 5. Tissue factor–dependent procoagulant activity in human umbilical vein endothelial cells (HUVECs) stimulated with cocaine and chronic cocaine plasma increased (PFP-CP). A, Quantification and statistics of procoagulant activity (PCA) of HUVEC stimulated with cocaine in the presence or absence of atorvastatin, expressed as the percentage of pmol of activated factor X; *P=0.0238; n=6. B, Quantification and statistics of PCA in HUVEC stimulated with PFP-CP with or without atorvastatin compared with cells stimulated with its noncocaine control (PFP-NP); n=6.
Increased TF expression along with a reduced TF pathway inhibitor has been reported in response to cocaine. Nevertheless, whether this has consequences on TF-dependent PCA had not been studied until now. We found that only cocaine induced a significant increase in TF PCA in HUVEC, which unexpectedly was not abolished by atorvastatin under our experimental conditions. Although there are no data specifically on the effect of atorvastatin on TF-dependent PCA associated with cocaine, it has been shown that statins reduce monocyte TF levels and activity in other experimental settings. Additional investigations are needed to clarify the lack of atorvastatin effectiveness in lowering TF PCA induced by cocaine.

One of the best known pleiotropic effects of statins is that of increasing NO production. In HUVEC, both cocaine and PFP-CP similarly decreased NO content by ≈57% when compared with untreated or PFP-NP–supplemented cells. In addition, as expected, atorvastatin effectively impeded this reduction. Although eNOS levels and bioavailability have been reported to be involved in the mechanisms by which cocaine diminishes NO release in bovine coronary artery ECs, the mechanism through which atorvastatin impedes NO reduction is still unknown. Our results showed that atorvastatin impeded the reduction of eNOS induced by cocaine to some extent and reduced caveolin-1 protein levels even to lower level than control cells. These results agreed with reports showing that atorvastatin decreases caveolin-1 expression in bovine ECs treated with low-density lipoprotein-cholesterol. The effect of atorvastatin was shown to be independent from extracellular low-density lipoprotein-cholesterol.

In addition, atorvastatin strongly modified eNOS subcellular localization by visibly diminishing its association to caveolin-1. Although the dispersed distribution of eNOS in control cells and the changes observed on cocaine stimulation are in agreement with results reported for bovine aortic ECs by He et al, the association between eNOS and caveolin-1, as well as the dramatic effect of atorvastatin on cocaine-treated ECs, has not been evidenced before.

These results showed that the addition of atorvastatin to cocaine-treated cells for 5 hours triggers cell dynamic changes...
that result in a disruption of the interactions between eNOS and caveolin-1 at the cell membrane, most probably causing increased eNOS bioavailability. It may be possible that atorvastatin would target rapid mechanisms involved in protein mobilization at the subcellular level, which might be reflected in changes of active eNOS at later times of culture. In other cell systems, eNOS is also regulated by its membrane location-sequestration into caveolae, specifically by its interaction with caveolin-1 (among others).

The effect of atorvastatin on caveolin-1 could account, at least in part, for the mechanism involved in the prevention of NO reduction induced by cocaine. In human aortic ECs, cocaine has been reported to decrease NO production along with increasing the expression of endothelin-1 (as well as its receptor) and enhancing monocyte adhesion. The specific inhibition of the ET-1 receptor has been observed to amend the reduction of NO and monocyte adhesion partially, suggesting a possible mechanism by which cocaine induces endothelial damage.

Whether the effect of atorvastatin involves endothelin-1 or its receptor remains to be studied. Cocaine, not PFP-CP, significantly induced ROS production after 5-hour stimulation, which was a phenomenon that was not affected by atorvastatin. It has been reported that pretreatment of human aortic ECs with atorvastatin reduces ROS liberation induced by tumor necrosis factor-α." However, in our experimental model, cotreatment with atorvastatin did not show the same results and possibly denoting differences in experimental designs.

A remarkable observation was the effect of atorvastatin in recovering cell monolayer structure, which was altered by cocaine and PFP-CP stimulation. The consequences involved in cytoarchitecture changes induced by cocaine and PFP and the capacity of atorvastatin to prevent these alterations completely warrant further studies aiming to identify the specific pathways involved in this phenomenon.

In summary, our results showed that ECs exposed to cocaine and to PFP from cocaine consumers exhibit comparable changes, resulting in a prothrombotic phenotype. Atorvastatin improved most of the damages induced by both stimuli, except for PCA and ROS generation. Although several preventive effects of atorvastatin may occur in response to increase NO bioavailability, the exact signaling pathways involved in cocaine-related stimulation of ECs are still under investigation.

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Disclosures
None.

References
Cocaine consumption is associated with a high risk of vascular ischemic events affecting several areas. Vasconstriction-vasospasm and accelerated atherosclerosis have been observed in the pathogenesis of such vascular ischemic events, which are two phenomena that aim at the vascular endothelium as a common target. In this context, we took advantage of the beneficial pleiotropic effects of statins in an attempt to ameliorate the deleterious effects of cocaine or plasma from cocaine consumers on endothelial function. In this report, we showed that atorvastatin inhibited platelet adhesion and reduced most of the endothelial dysfunction markers induced by cocaine and plasma from cocaine users. Because damage/activation of endothelial cells seems to play an important role in the pathogenesis of cocaine-induced vascular damage, these results open an alternative and novel therapeutic strategy to be explored in the comprehensive management of cocaine addiction.
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Supplemental Material

Materials and Methods

Patients

This study was approved by the Medical Ethics Committee of the Pontificia Universidad Católica de Chile and University of Chile Clinical Hospital. Written informed consent was obtained from all volunteers. Cocaine dependence was defined based on the Structured Clinical Interview for DSM-IV (SCID). Exclusion criteria were neurological or other disorders not related to drug abuse or associated with vascular damage. Blood samples were obtained from patients seeking treatment for cocaine addiction and were recruited at the time of admission to a rehabilitation clinic. All individuals declared recent cocaine consumption and tested positive for cocaine metabolites on urine toxicology tests on the day of sampling. Controls were age and gender-matched healthy individuals tested for absence of substance abuse other than nicotine.

Blood collection and preparation of plasma samples. Blood collection was performed by atraumatic puncture of the antecubital vein with a 19-gauge needle without venous stasis and drawn into citrate-containing tubes (Vacutainer, Becton Dickinson). Platelet-free plasma (PFP) was obtained by two centrifugations, each at 2600 rpm for 15 min and an additional spin at 11000 rpm for 5 min. Aliquots of PFP from chronic cocaine consumer (PFP-CP) or from its corresponding non-cocaine consumer (PFP-NP) were immediately frozen at -80º C, until further use.

ADAMTS-13 activity

ADAMTS-13 activity was measured by FRETS-VWF73 assay (1). Briefly, pooled human plasma (a range of 0–6 μl as a standard), or 4 μl of each test plasma, was diluted in 100 μl of assay buffer in a 96-well white plate at RT. Then, 100 μl of 4 μmol/l FRETS-VWF73 in the assay buffer, was added to each well. Fluorescence was measured at 30°C in a Wallac 1420 ARVO multilabel counter (PerkinElmer Japan,
Yokohama, Japan) equipped with a 340-nm excitation filter and a 450-nm emission filter. Fluorescence was measured every 5 min. The reaction rate was calculated by linear regression analysis of fluorescence over time from 0 to 60 min.

**Human umbilical vein endothelial cells (HUVEC) preparation and experimental design.** Endothelial cells from human umbilical cord veins were prepared according to the method previously described. Confluent cells at passage two were plated onto 1% gelatin covered glass coverslip in a 24-well culture plates and grown to reach subconfluence in complete endothelial growth medium (M199 medium, Gibco), FBS 3% (Gibco), endothelial cell growth medium (#c39210-PromoCell-Germany). Then, cells were starved for 16 h, cultured only with M199 medium and incubated for 5h with M199 with cocaine-HCl 10 μM (obtained from the National Institute on Drug Abuse Drug Supply Program, Division of Neuroscience and Behavioral Research, USA), or 10% PFP, without extra growth factors, in the presence or absence of atorvastatin-Ca (10 μM) (Pfizer). When HUVEC were treated with PFP, 5000 UI/mL sodium heparin was added. After incubation, the cell supernatant was collected and centrifuged at low speed to clear cell debris. The remaining supernatant was used for VWF and MCP-1 determination.

**Measurement of VWF and MCP-1 in culture supernatants.** VWF from the supernatants of cocaine or plasma supplemented HUVEC was measured by sandwich-type ELISA, using a capture monoclonal antibody (VWF1, kindly provided by Dr. Robert R. Montgomery, Milwaukee, WI) and a peroxidase-conjugated rabbit antibody for detection (Dako Corp., California). The VWF released by HUVEC when supplemented with cocaine consumers plasma was calculated by subtracting the VWF value of the plasma supplemented media after culture from its corresponding pre-cultured one.
MCP-1 was determined by ELISA (BD Biosciences, San José, CA) in supernatants of untreated, cocaine or plasma supplemented treated HUVEC. MCP-1 levels released by HUVEC treated with patient's plasma was determined by subtracting the levels obtained in plasma supplemented media after incubation from the levels in the same plasma supplemented media before addition to HUVEC.

**Tissue Factor dependent Procoagulant activity (TF-PCA).** The procoagulant activity (PCA) of the cells was measured by FXa generation assay without adding external tissue factor. Therefore, the amount of Factor Xa generated is a response of the intrinsic content of TF of the cells. TF-PCA was measured in lysates of HUVEC treated with cocaine or with plasma supplemented media, as reported, 3 aliquots of 100 µL, in duplicate, of lysate were transferred to microtiter plates and adding 20 µL of each 1U/mL FVIIa (American Diagnostica, Stamford, CT), 1 U/mL FX (Aniara, Mason, OH), 25 mM CaCl₂ and 40 µL of 1mg/mL FXa chromogenic substrate (Biophen CS11; Aniara, Mason, OH). Absorbance at 620 nm was subtracted from the maximal absorbance at 405 nm, which was continuously monitored under agitation at 37º C. This value corresponded to the maximal generation of FXa, expressed as pmol/mg of protein.

**Platelet preparation and experimental design.** Platelet rich plasma (PRP) was prepared from whole blood with ACD-A solution (0.13 M dextrose, sodium citrate 85mM, Fenwal International Inc, USA), 1 µM prostaglandin E₁ (PGE₁) and 12 mM theophylline, by centrifugation at 200 g for 9 min at 4º C. The PRP was removed and further centrifuged at 1500 g for 9 min. The pellet was suspended in buffer Tyrode (137 mM NaCl, 2mM KCl, 2mM MgCl₂, 12mM NaHCO₃, 0,35mM Na₂HPO₄, 1%BSA, 5,5mM glucose, 56nM PGE₁, pH 6,2) and centrifuged twice at 200 g for 9 min, recovering the supernatant. Then, the supernatant was centrifuged at 1500 g for 9 min at 4º C and the pellet containing the washed platelets was suspended in Tyrode buffer without PGE₁
and finally centrifuged at 150g for 5 min to remove nucleated cells. Platelet purity was verified by microscopic (Nikon Optiphot, Nikon Corporation, Japan) observation of an aliquot stained with propidium iodide (0.75 mM propidium iodide, sodium citrate 4.7 mM, NP-40 3x10^-4% v / v) aiming for the absence of nucleated cells. The number of platelets was assessed with a Coulter Counter Z1 (Beckam Coulter Inc, USA).

**Evaluation of NO content in platelets and HUVEC.** The media from HUVEC seeded in a 96 well plaque was replaced for Earl’s buffer (150 mM NaCl, 5mM KCl, 5 mM MgCl_2, 35 mM CaCl_2 and 30 mM HEPES) and incubated with 4,5-diaminofluorescein diacetate (5 μM DAF-2DA; Merck, Darmstadt, Germany) for 5h at 37º C under mild agitation in darkness. As for HUVEC, washed platelets (1x10^7 / μL) in Tyrode’s buffer without PGE_1, were incubated with 5 μM DAF-2DA for 45 min 37º C under mild agitation, in darkness. After removing the HUVEC media and washing the platelets once, cocaine or 10% PFP was added in the presence of 0.1m M L-arginine and 1mM of CaCl_2 and incubated for 45min at 37 ºC under mild agitation. As control, L-NNA was used. For HUVEC and platelets, the reaction was ended by adding 100 μL of stop solution (1% EDTA and 3.7% formaldehyde in phosphate saline buffer). Both preparations were evaluated by reading in a LS50B Fluorimeter (Perkin-Elmer) under a 495 nm filter for excitation and 530 nm for emission and the results expressed as arbitrary fluorescence units (AFU).

**ROS Measurements.** The initial protocol was similar to NO measurement, but cells were incubated with 100 μM dichlorofluorescein for 2 h at 37º C with mild agitation. After washing, cocaine or 10% PFP in buffer Earls was added for 5 h for HUVEC. Absorbance was determined in a LS50B Fluorimeter (Perkin-Elmer) using 495nm filter for excitation and 530nm for emission. As for NO measurements, continuous readings, starting at 1 min after the stimulation, for every 5 min were performed before concluding that 5 h was the representative time of stimulation for all the samples.
Platelet activation by flow cytometry. Washed platelets (1x10⁶ total) were incubated with PFP in buffer Tyrode or with cocaine in buffer Tyrode plus 1mM CaCl₂ for 5, 15, 30 and 45 min at 37º C under mild agitation. After incubation, the reaction was stopped by cooling the sample on ice, then centrifugated at 2500 rpm for 3 min. Platelets were suspended in PBS and incubated with Phycoerythrin (PE) conjugated antihuman CD62, CD40-L or PAC-1 (all from BD Biosciences, USA) and fluorescein isothiocyanate (FITC) conjugated CD61 antibody (BD Biosciences, USA) or antihuman IgG (isotype control, BD Biosciences). Samples were then incubated for 25 min at room temperature, in the dark. Then, samples were washed at 2500rpm for 3 min, suspended in PBS and analyzed using a FACSCanto II flow cytometer. Population CD61 positive (20000 events) were selected and CD62 positive cells percent were registered.

Adhesion assays under static conditions. HUVEC at passage two or three were grown on a coverslips until subconfluence. After starvation period of 16 h cells were incubated with M199 supplemented with cocaine 10 µM or the different plasmas for 5 h. Then, the media was removed and the cells were gently rinsed with M199 before the addition of 1x10⁷ non activated washed platelets and co-cultured for an additional 45 min. The media with non-adhered platelets was removed and the coverslips were washed three times with PBS. The HUVEC with the attached platelets were fixed with formaldehyde (Merck) 3.7% v/v in saline buffer and permeabilized with 0.01% (v/v in saline buffer) Triton X-100 (Sigma-Aldrich) when specified. The preparations were immunostained as described afterward.

Immunocytochemistry. After blocking with 1% normal goat serum for 2 h at room temperature, cells and platelets were identified with specific antibodies. The presence of VWF, VE-cadherin was visualized by indirect immunofluorescence with anti-VWF (Dako Corp., California), anti-VE-cadherin (Cayman Chemical). To identify platelets,
anti-GPIbα antibody was used (SZ2 clone, Beckman Coulter). eNOS and caveolin-1 were detected using specific anti-human antibodies (BD Transduction Laboratories). Appropriate mouse or rabbit Alexa 488 or 555 (Invitrogen) secondary antibodies were used to recognize primary antibodies. Samples were evaluated using an Olympus BMX60 fluorescent microscope (Zeiss Axiovert 200 M and LSM 5 Pascal Laser-Scanning Confocal; Zeiss, Oberkochen, Germany) and the number of platelets attached to endothelial cells (EC) was quantified in a minimum of 5 independent fields for each experiment with Image J software (NIH) and the results expressed as number of platelets / 100 EC.

**Western blotting analysis**

HUVEC were cultured in 10 cm dishes until 90% confluence and treated with medium supplemented with 10% PFP from chronic cocaine consumer (PFP-CP) or from its corresponding non-cocaine consumer (PFP-NP), 10µM cocaine, vehicle, with or without co-stimulation with Atorvastatin 10µM for 5 hours. 50µg protein of each condition was separated by electrophoresis and then transferred onto a PVDF membrane. Membranes were blocked with 5% skim milk, 2% BSA and incubated with primary antibody for p-eNOS, eNOS and caveolin-1 (BD Transduction Laboratories), pAKT (Cell Signaling), AKT1/2 and ERK2 (Santa Cruz Biotechnology) overnight at 4°C. After washing with PBS-tween, membranes were incubated with HRP-conjugated secondary antibody (Santa Cruz Biotechnologies). Proteins were detected with chemiluminescence kit (PerkinElmer) in a ChemiDoc-It Imaging System and each protein band was quantified by densitometry and analyse using ImageJ.

**Statistical Methods**

Results were expressed as mean ± SEM unless otherwise specified. All qualitative data was representative of at least 3 independent experiments. Statistics analyses were performed with GraphPad Prism 6. The Mann-Wittney Rank Sum test was used
for unpaired non-parametric analyses and the Wilcoxon Signed Rank test for differences between paired samples. Significant differences was set at p<0.05.

References

Figure I

% ADAMTS 13 activity

Controls    Cocaine Consumers
Figure II

A

% of control
eNOS T/ERK T

Cocaine 10 μM
Atorvastatin 10 μM

- - + +

140 kDa-eNOS T/ERK T
42 kDa-

145 kDa-peNOS/ERK T
42 kDa-

B

% of control
peNOS/ERK T

Cocaine 10 μM
Atorvastatin 10 μM

- - + +

140 kDa-eNOS T/ERK T
42 kDa-

145 kDa-peNOS/ERK T
42 kDa-

C

% of control
pAKT/AKT

Cocaine 10 μM
Atorvastatin 10 μM

- - + +

60 kDa-pAKT
55 kDa-

25 kDa-pAKT
42 kDa-

D

% of control
caveolin 1/ERK T

Cocaine 10 μM
Atorvastatin 10 μM

- - + +

60 kDa-pAKT
55 kDa-

25 kDa-pAKT
42 kDa-
Figure III

Control

Cocaine

Cocaine + Atorvastatin
Supplemental Figure Legends

Supplemental Figure I
ADAMTS13 activity in chronic cocaine consumer patients and in age and gender matched controls. ADAMTS-13 activity was measured in eighteen patients and controls by FRETS-VWF73 assay. The reaction rate was calculated by linear regression analysis of fluorescence over time from 0 to 60 min and the results are expressed as percentage of normal pooled plasmas.

Supplemental Figure II
Effect of Atorvastatin on Cocaine-induced changes of eNOS, peNOS, pAKT and caveolin-1 in HUVEC. A. eNOS protein levels in HUVEC treated with cocaine in the presence or absence of 10 µM atorvastatin expressed as percentage of control cells and normalized against total ERK. *p=0.0286. B and C. peNOS and pAKT in HUVEC treated with cocaine in the presence or absence of atorvastatin expressed as percentage of control cells and normalized against total ERK and total AKT respectively. peNOS between control and cocaine treated cells: *p=0.0286 *p=0.0500 for pAKT. D. Caveolin-1 levels in HUVEC treated with cocaine in the presence or absence of atorvastatin expressed as percentage of control cells and normalized against total ERK; n=3. *p=0.0286 between control and atorvastatin cells.

Supplemental Figure III