Pentoxifylline Reduces Tumor Necrosis Factor- α and HIV-Induced Vascular Endothelial Activation

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Abstract

Untreated HIV infection is associated with endothelial dysfunction and subsequent cardiovascular disease, likely due to both direct effects of the virus and to indirect effects of systemic inflammation on the vasculature. We have recently shown that treatment with the antiinflammatory agent pentoxifylline (PTX) improved *in vivo* endothelial function and reduced circulating levels of the inflammatory markers vascular cell adhesion molecule-1 (VCAM-1) and interferon-gamma-induced protein (IP-10) in HIV-infected patients. To delineate the mechanisms underlying this therapeutic effect, we tested whether clinically relevant concentrations of PTX suppress VCAM-1 or IP-10 release in cultivated human lung microvascular endothelial cells. Indeed, we found that tumor necrosis factor (TNF)- α -induced VCAM-1 was reduced with concentrations of PTX in the low nanomolar range, comparable to plasma levels in PTX-treated groups. We also investigated the effect of HIV proteins and found that HIV transactivator of transcription (HIV-Tat) and HIV-envelope-derived recombinant gp120 enhanced TNF- α -induced VCAM-1 gene expression in lung microvascular and coronary macrovascular endothelial cells, respectively. In addition, PTX and a NF- κ B-specific inhibitor reduced this enhanced VCAM-1 gene induction in microvascular and macrovascular endothelial cells. These results provide novel insights in how the antiinflammatory agent PTX can directly reduce HIV-associated proinflammatory endothelial activation, which may underlie vascular dysfunction and coronary vascular diseases.

Introduction

CARDIOVASCULAR DISEASE is a leading cause of death in those infected with HIV.¹ Population-based data suggest that HIV-infected patients have an increased risk of atherosclerotic disease compared to the general population.^{2–4} Although the induction of dyslipidemia by combined antiretroviral therapy (cART) has been proposed as one possible explanation for the increased risk of cardiovascular events in HIV-positive patients, recent studies⁵ have suggested that untreated HIV infection may contribute more to the development of cardiovascular disease than cART-related effects.^{6–8} Specifically, increased systemic inflammation in untreated HIV infection has been associated with increased activation of the coagulation system, cardiovascular events, and overall mortality.

It is widely accepted that endothelial activation and dysfunction are involved in the early development of athero-

sclerotic disease. Proinflammatory cytokines, including tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ), can induce both endothelial dysfunction and atherosclerosis.^{9,10} Whereas the effects of TNF- α in endothelial cells are primarily mediated through NF- κ B and secondarily through the p38 MAPK pathways,^{11–13} the effects of IFN- γ are signaled primarily through the JAK-STAT pathways.¹⁴ However, little is known about whether HIV viral proteins can directly contribute to atherosclerosis. The HIV envelope protein gp120 and the HIV transactivating protein Tat, which are secreted from HIV-infected cells, can activate endothelial cells.^{15,16} Therefore, it is likely that HIV infection can cause endothelial activation and dysfunction by both direct (Tat and gp120) and indirect (release of cytokines from mononuclear cells) means, thus promoting atherogenesis or atherogenic plaque progression.

To assess the endothelial mechanism of early atherogenic events in HIV-infected patients, flow-mediated dilation

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(FMD) as an *in vivo* marker for endothelial function was employed. In these studies, salsalate (a salicylic acid-generating drug) and pentoxifylline (PTX) were demonstrated to improve endothelial function in HIV-infected persons not receiving cART.^{17–19} While salsalate treatment was based on the initially description of its correlate sodium salicylate as an NF- κ B signal transduction inhibitor,²⁰ PTX was originally discovered as a PDE-1 inhibitor and later demonstrated to also inhibit NF- κ B.^{21–24} In addition to improving FMD values, treatment with PTX reduced soluble levels of vascular cell adhesion molecule-1 (VCAM-1) and interferon-gamma-induced protein (IP-10) after 8 weeks of treatment.¹⁸ Soluble VCAM-1 is a surrogate marker for the transmembrane form of the endothelial adhesion molecule VCAM-1, which mediates leukocyte adhesion and is linked to endothelial dysfunction and to the early onset of atherosclerosis.²⁵ The chemokine IP-10 has been previously described to promote two key processes involved in atherosclerosis, namely Th1 immune system activation and endothelial apoptosis.^{26,27}

In the present study, we tried to determine whether we could identify the cellular mechanisms underlying the beneficial effects observed in our clinical PTX treatment trial. Thus, we investigated the role of the HIV-1 proteins gp120 and Tat in combination with proinflammatory cytokines in the presence and absence of PTX in both primary human lung microvascular endothelial cells (HLMVECs) and human coronary arterial endothelial cells (HCAECs). We chose the first cell type because the lung has the highest number of endothelial cells of all organs and is thus likely to have contributed greatly to the observed reduction of sVCAM-1 and IP-10 levels in our PTX trial.^{17,18} The second endothelial cell type was used because of its physiological relevance to HIV-associated cardiovascular disease. Our *in vitro* data demonstrate that HIV proteins can enhance TNF- α -induced endothelial cell activation as measured by induction of VCAM-1 and that PTX can reduce this proinflammatory activation.

Materials and Methods

Reagents

Recombinant Tat and gp120 proteins were purchased from ImmunoDiagnostics (Woburn, MA). VCAM-1-PE antibody was purchased from Abcam. IKK inhibitor SC-514 was purchased from Calbiochem (La Jolla, CA). All other reagents including PTX and cytokines were obtained from Sigma (St. Louis, MO).

Cell culture

HLMVECs and HCAECs were obtained from Lonza (Allendale, NJ) and maintained in culture medium consisting of EMB-2, 5% FBS, 0.4% hydrocortisone, 1.6% hFGF, 1% VEGF, 1% IGF-1, 1% ascorbic acid, 1% hEGF, 1% GA-100, and 1% heparin. All primary cell cultures were maintained at 37°C in 5% CO₂ and 95% air. Culture medium was changed every 2 days.

In vitro assays

For analysis of VCAM-1 and IP-10 gene expression and protein release, 4.0×10^4 HLMVECs between passages 3 and 6 were seeded in each well of 12-well tissue culture plates. After 2 days culture medium was changed, and after a further 17 h

reagents were added without changing the medium again. After the indicated time points, the supernatant was harvested for IP-10 analysis and the cells were either removed from the plate using 5 mM EDTA in phosphate-buffered saline (PBS) for staining analysis or lysed and the RNA harvested using a NucleoSpin RNA isolation kit (Clontech Laboratories, Mountain View, CA) for reverse transcriptase polymerase chain reaction (RT-PCR).

Real-time qRT-PCR analysis

Total RNA was extracted and reactions were performed in duplicate on 96-well optical reaction plates with one-step SYBR Green PCR master mix (iScript One-Step RT-PCT Kit with SYBR Green, Bio-Rad Laboratories, Hercules, CA). The real-time PCR reaction and expression quantification was performed in a Chromo4 Opticon analyzer instrument (Bio-Rad, Hercules, CA). Then 50 ng of total RNA was added to each well and the thermal cycling conditions were as follows: 50°C for 10 min (cDNA synthesis), 95°C for 5 min (reverse transcriptase inactivation), followed by 42 cycles of 95°C for 10 s and 58°C for 30 s. Primers used for the RT-PCR include CTCAGTCTCAGCACCATGA (FW) and CAAATTTGGC TTGCAGGAAT (RE) for IP-10 as well as TGTGAGATC TCCCCTGGAC (FW) and GAATTGGTCCCCTCACTCCT (RE) for VCAM-1. To compare the levels of gene expression between the experimental groups, a second derivative calibration method for relative quantification was employed as described previously.¹² The amount of the target gene transcript was normalized to eF1 α (elongation factor alpha), and measured relative to a calibrator. The target gene expression was computed by the $2^{-\Delta\Delta C_t}$ method, where C_t is the crossing point or turning point of the fluorescence curve calculated by analyzer instrument software, ΔC_t of target or calibrator is the C_t of the target gene subtracted from the C_t of the housekeeping gene, and $\Delta\Delta C_t$ is ΔC_t (unknown target gene) – ΔC_t (calibrator).

Immunofluorescence and MetaMorph analysis

For immunofluorescence of surface VCAM-1 expression, HLMVECs were plated at 4.0×10^4 cells per well of a four-well chambered slide (Nunc) and cultured for 2 days. Cells were treated with or without different factors and incubated for 6 h then washed three times with wash buffer [0.5% bovine serum albumen (BSA), 0.05% Tween 20 in PBS], and nonspecific antigens were blocked with universal blocking solution (Dako, Carpinteria, CA). The samples were incubated with a 1:100 dilution of rabbit antihuman VCAM-1 antibody (clone H-276; Santa Cruz Biotechnology, Santa Cruz, CA) in a moist chamber for 16 h at 4°C, followed by three washes in PBS and incubation with 1:200-diluted Alexa Fluor 488-conjugated goat antirabbit IgG (Invitrogen, Carlsbad, CA) for 1 h in a moist chamber at 37°C. Cells were then treated with 10 μ g/ml DAPI for 5 min and the cells were mounted under CC-mount (Sigma, St. Louis, MO) and analyzed on a Nikon Eclipse (TE200S) inverted fluorescence microscope. For the negative control reaction some wells were either incubated with nonimmune isotype-matched control IgG or omitted primary antibody. About six images per well were captured with a 20 $\times$ objective using Voxx image software in a blinded fashion and quantitative intensity (expression) data were obtained by MetaMorph Imaging software

(Molecular Devices, Sunnyvale, CA). Values for VCAM-1 staining were normalized to the number of nuclei for every image in both treated and untreated groups. Data were presented as mean $\pm$ SEM of each treatment normalized to unstimulated cells performed in triplicate and repeated independently two additional times.

Flow cytometry

After seeding of HLMVECs or HCAECs, treatment, and subsequent harvesting as described above, cells were incubated with a 1:10 dilution of anti-VCAM-1-PE (Abcam) for 1 h at 4°C then washed three times with PBS and fixed with 4% PFA for 5 min at RT. FACS analysis was performed using a FACS Calibur II (Beckton Dickinson) and analyzed using CellQuest software.

Statistical analysis

Comparisons were made using both two-tailed and paired Student's *t*-tests as appropriate. A *p*-value <0.05 was considered significant.

Results

Pentoxifylline reduces TNF- α -induced VCAM-1 at low concentrations consistent with those observed in the serum of treated subjects

We questioned whether PTX could reduce TNF- α -induced VCAM-1 or IFN- γ -induced IP-10 gene activation in human HLMVECs at concentrations found in individuals treated with PTX (median plasma PTX concentrations were between 270 and 340 nM).¹⁹ For TNF- α (20 ng/ml) and IFN- γ (40 ng/ml), we employed concentrations previously used to stimulate endothelial cells.^{12,28} Pentoxifylline substantially inhibited the expression level of TNF- α -induced VCAM-1 in a dose-dependent manner starting at a concentration as low as 8 nM (Fig. 1A). In contrast, IFN- γ -induced expression of the chemokine IP-10 was reduced by PTX only at much higher concentrations (50 and 250 μ M) than those achieved in PTX-treated patients (Fig. 1B). Therefore, we focused on the role of TNF- α -induced VCAM-1 production in the following experiments.

HIV transactivator of transcription (HIV-Tat) enhances TNF- α -induced VCAM-1 expression in HLMVECs

To explain the effects of reduced sVCAM-1 levels in serum of PTX-treated HIV-infected individuals, we first tested whether secreted HIV proteins can enhance TNF- α -induced VCAM-1 production and whether this induction was reversible with PTX. We cotreated cells with HIV-Tat²⁹ and TNF- α to mimic the inflammatory state found in patients with HIV.^{30–32} As shown in Fig. 2A, exposure of HLMVECs to recombinant Tat significantly increased the gene expression level of VCAM-1 in combination with TNF- α . Importantly, while we used the concentration of Tat resulting in the highest increase (100 ng/ml), a dose dependency revealed significantly more VCAM-1 induction at a lower concentration of 50 ng/ml Tat in the presence of TNF- α (Supplementary Fig. S1A; Supplementary Data are available online at www.liebertonline.com/aid). This finding was also confirmed by VCAM-1 protein expression levels using flow cytometry (Fig. 2B). We also

FIG. 1. Pentoxifylline reduces tumor necrosis factor (TNF)- α -induced vascular cell adhesion molecule-1 (VCAM-1) at low concentrations. Human lung microvascular endothelial cells (HLMVECs) were incubated for 6 h with either (A) TNF- α (20 ng/ml) or (B) interferon (IFN)- γ (40 ng/ml) in the absence or presence of increasing concentrations of pentoxifylline (PTX). Total RNA was isolated and qRT-PCR was performed using primers specific for (A) VCAM-1 or (B) interferon-gamma-induced protein (IP-10). Relative gene expression levels were normalized to the endogenous control (EF1 α) compared to target genes. All experiments were performed at least three times.

confirmed this finding with the highest TNF- α concentration used in Fig. 1A and immunofluorescence staining (Fig. 2C), which revealed no VCAM-1 protein expression in untreated HLMVECs (Fig. 2Ci), some VCAM-1 expression in TNF- α -treated cells (Fig. 2Cii), and an enhancement when Tat

FIG. 2. HIV transactivator of transcription (HIV-Tat) enhances TNF- α -induced VCAM-1 expression in HLMVECs. HLMVECs were treated with low TNF- α concentrations (1 ng/ml) alone or in combination with HIV-Tat (100 ng/ml) for 6 h. **(A)** qRT-PCR analysis of relative gene expression levels of VCAM-1. **(B)** Flow cytometry analysis of VCAM-1 surface expression. Inset depicts a typical example of VCAM expression (black dotted line HIV-Tat + TNF- α vs. gray dotted line TNF- α only). **(C)** Treatment with a high TNF- α concentration (20 ng/ml) alone or in combination with HIV-Tat (100 ng/ml) with or without treatment with PTX (200 nM) for 6 h. Immunofluorescence pictures captured for VCAM-1 staining in (i) vehicle-treated, (ii) TNF- α only-treated, (iii) HIV-Tat and TNF- α -treated, and (iv) HIV-Tat and TNF- α and PTX-treated endothelial cells. **(D)** Quantification of VCAM-1 staining was determined using MetaMorph image analysis software. All experiments were performed three times.

and TNF- α were combined (Fig. 2Ciii). In agreement with Fig. 1, the numbers of VCAM-1 expressing cells and the overall expression level in individual cells were reduced upon treatment with PTX (Fig. 2Civ; for quantification using MetaMorph image software see also Fig. 2D).

gp120 enhances TNF- α -induced VCAM-1 expression in HCAECs

To confirm the pathophysiological significance of our findings in human lung microvascular endothelial cells, we extended our studies to HCAECs. While Tat did not significantly increase TNF- α -induced VCAM-1 production in HCAECs (data not shown), gp120 strongly enhanced TNF- α -induced VCAM-1 production in HCAECs at both mRNA (Fig. 3A) and protein (Fig. 3B) levels. Once again, we used the maximal concentration of gp120 (100 ng/ml) although gp120 in a comparable dose dependency as observed with Tat

already showed a robust enhancement of TNF- α -induced VCAM-1 expression at 50 ng/ml (Supplementary Fig. S1B).

Pentoxifylline and IKK inhibition reduce Tat- and gp120-induced upregulation of VCAM-1

TNF- α signals primarily through the NF- κ B pathway,³³ which is also inhibited by PTX. Therefore, we compared the effect of an IKK inhibitor with PTX on the upregulation of VCAM-1. HLMVECs and HCAECs were treated with TNF- α and Tat or gp120, respectively, in the absence and presence of 200 nM PTX or 100 nM of the IKK inhibitor SC-514 (IKKI), two concentrations that we had established to maximally reduce TNF- α -induced VCAM expression (Fig. 1A and data not shown). After 6 h cells were either harvested for RNA, with the levels of VCAM-1 RNA determined by RT-PCR (Fig. 4A and C), or were stained for surface VCAM-1 and analyzed by flow cytometry (Fig. 4B and D). In both cell types the RNA

FIG. 3. gp120 enhances TNF- α -induced VCAM-1 expression in HCAECs. HCAECs were treated with TNF- α (1 ng/ml) alone or in combination with gp120 (100 ng/ml) for 6 h and then analyzed for **(A)** relative VCAM-1 gene expression levels and **(B)** surface expression of VCAM-1 as determined by flow cytometry. Inset depicts a typical example of VCAM expression (black dotted line HIV-Tat + TNF- α vs. gray dotted line TNF- α only). Values were expressed as mean fluorescence intensity (FI) and normalized to the untreated control group.

levels and surface VCAM-1 decreased upon treatment with PTX or the IKK inhibitor, although the effect on mRNA was much more pronounced with the IKK inhibitor. In contrast, surface VCAM-1 reduction was similar upon treatment with PTX or IKK inhibitor; the reductions were not as dramatic as with mRNA levels. Although the time course of VCAM-1 expression in endothelial cells is known to be between 4 and 12 h, we also repeated these experiments with 12 h of treatment and had very similar results (data not shown). We further confirmed the effect of PTX on VCAM-1 expression using a lower (50 ng/ml) concentration of Tat and gp120 (Supplementary Fig. S2A and C). However, the higher Tat and gp120 concentrations displayed borderline or no significant synergies with TNF- α , respectively, and inhibition with PTX was not significant any more (Supplementary Fig. S2B and D).

Discussion

This study shows for the first time that secreted HIV proteins strongly augment endothelial production of the proinflammatory adhesion molecule VCAM-1 in microvascular and arterial macrovascular endothelial cells when combined with the inflammatory cytokine TNF- α . These *in vitro* results suggest that HIV proteins, in concert with the proinflammatory cytokines found in HIV-infected patients, can synergistically increase endothelial inflammation in adult coronary and microvascular endothelial cells, similar to what has been previously shown for the HIV protein Tat in umbilical cord endothelial cells.²⁹ Surprisingly, we found Tat enhanced TNF- α -induced VCAM-1 induction only in human lung microvascular endothelial cells, whereas gp120 enhanced TNF- α only in human coronary arterial endothelial cells. In all cases the observed effects were significantly reduced upon treatment with the NF- κ B inhibitors SC-514 and PTX.^{21–24,34,35} This response seems to be specific for TNF- α , as we also tested the combination of IFN- γ and Tat with and without PTX. These results were negative in two ways: first, there was no significant additional upregulation of VCAM-1 by the combination of Tat and IFN- γ , and second, PTX treatment did not result in lower VCAM-1 expression as compared to IFN- γ alone (data not shown). Together, these findings

highlight the direct link between HIV infection, inflammation, and vascular dysfunction on the level of the endothelium and emphasize the significance of our previous finding that PTX can reduce vascular dysfunction in the endothelium.¹⁹

To address the mechanism of reduction of soluble VCAM-1 levels in PTX-treated HIV-infected patients not on anti-retroviral therapy, we have tested for VCAM-1 induction in human lung microvascular endothelial cells, as these are the most common type of endothelial cell in humans. Our data can explain the PTX-dependent reduction of sVCAM-1, which is a surrogate marker for endothelial VCAM-1 production, in HIV-positive individuals.^{36,37} To test our hypothesis that cooperation between HIV and inflammatory cytokines may be a mechanism leading to HIV-associated cardiovascular disease, we assessed for a potential HIV and TNF- α synergy in HCAECs. In this study we focused on the effect of HIV proteins on VCAM-1 since it was upregulated in the *in vivo* trials and is specific for endothelial cells, whereas other TNF-regulated inflammatory proteins, such as ICAM-1 and IL-6, are also expressed in other cell types. Although we do not anticipate different regulation with the majority of these genes based on our finding that PTX predominately inhibits the NF- κ B pathway, we cannot verify this with all proteins. Indeed, our preliminary data show that ICAM-1 exhibits the same enhanced expression with HIV proteins as VCAM-1 (data not shown). Further studies into the specifics of TNF-regulated inflammatory proteins and the differences between endothelial expression and that of other cell types are needed.

Surprisingly, Tat did not significantly enhance TNF- α -induced VCAM-1 expression in HCAECs under conditions we applied for microvascular endothelial cells. However, gp120 strongly enhanced TNF- α -induced VCAM-1 expression in HCAECs, which is in line with a previous study demonstrating that HIV gp120 and TNF- α synergistically reduce eNOS expression.³⁸ Interestingly, gp120 had no effect by itself or on the TNF- α -induced VCAM-1 expression in microvascular HLMVECs.

TNF- α signals primarily through the NF- κ B pathway^{33,39} and PTX inhibits NF- κ B signal transduction.^{22,23} Therefore, we addressed the role of NF- κ B for enhanced TNF- α -induced VCAM-1 expression in HCAECs using a more specific IKK

FIG. 4. Tat- and gp120-enhanced TNF- α -induced upregulation of VCAM-1 displays differential sensitivity to PTX and IKK inhibitors. HLMVECs were treated with TNF- α (1 ng/ml) in combination with HIV-Tat (100 ng/ml) with/without PTX (200 nM) or IKKI (100 nM) for 6 h and then analyzed for **(A)** relative VCAM-1 gene expression levels and **(B)** surface expression of VCAM-1 as determined by flow cytometry. HCAECs were also treated with TNF- α (1 ng/ml) and gp120 (100 ng/ml) with/without PTX (200 nM) or IKKI (100 nM) for 6 h and then analyzed for **(C)** relative VCAM-1 gene expression levels and **(D)** surface expression of VCAM-1 as determined by flow cytometry. Values were expressed as % inhibition of VCAM-1 as compared to the HIV-Tat + TNF or gp120 + TNF samples, respectively.

inhibitor. The IKK inhibitor was stronger than PTX when it came to blocking VCAM-1 upregulation on gene expression, whereas surface expression levels were similar in PTX- and IKKI-treated cells. This could be explained by the possibility that surface expression also is dependent on several regulatory steps including protein translation, trafficking, and protein stability. Alternatively, this discrepancy could be explained by differential kinetics between TNF- α -induced VCAM-1 upregulation and PTX or IKKI inhibition of mRNA. If VCAM-1 upregulation occurs prior to the effects of PTX or IKKI, there would still be a small amount of VCAM-1 expressed on the surface before inhibition of transcription, and this amount would be consistent between all samples treated with TNF- α and Tat or gp120.

Upon treatment with PTX, VCAM-1 inhibition occurred at concentrations as low as 8 nM, whereas IP-10 inhibition required more than 25,000-fold higher concentrations, at $\sim 250 \mu\text{M}$ (Fig. 1). One possible reason for the difference in sensitivity to PTX in TNF- α - and IFN- γ -treated cells is that IFN- γ signals through the JAK-STAT pathway rather than the NF- κB signaling pathway.¹⁴ Although we did not observe a reduction of IP-10 production at physiologically relevant concentrations of PTX here, IP-10 was significantly reduced in patients.¹⁹ This discrepancy may be because we tested only endothelial cells in this study and, unlike VCAM-1, IP-10 is produced by multiple cell types; therefore, the *in vivo* reduction of IP-10 levels may be due to a reduction in other cell types. Of note, our dose-response curve in human lung

microvascular endothelial cells shows PTX to work at a much lower dose than reported in human umbilical cord vein endothelial cells.^{40,41} Again, it could be a property of lung microvascular endothelial cells to metabolize PTX differently than other endothelial cells. Finally, the PTX-dependent inhibition of VCAM-1 expression was incomplete, suggesting that there is at least one other pathway in addition to NF- κ B-dependent signaling that may contribute to the upregulation of VCAM-1. In this context the previously described ability of the HIV-Tat protein to induce oxidative and inflammatory pathways in the endothelium is an important finding.¹⁶ One potential pathway could involve MAP kinases, which have been shown to be activated by HIV-Tat.^{42,43} These are significant because perturbation of MAP kinase signaling has been shown to be significant in HIV infection; for example, increased signaling can increase LPS-mediated HIV-1 transcytosis across the blood-brain barrier,⁴⁴ a possible key step in the development of HIV-associated dementia.

VCAM-1 has been identified as a novel prognostic marker for cardiovascular diseases^{8,45,46} and is suggested to promote processes of atherosclerosis by recruiting monocytes and T cells to the sites of the beginning plaques.²⁵ It may be that infected monocytes and T cells then further contribute to vascular dysfunction by increasing the local concentration of Tat and gp120. Inflammation is a prerequisite because endothelial activation by Tat or gp120 alone was low in comparison to that induced by TNF- α (Fig. 3). Importantly, very high concentrations of Tat and gp120 showed reduced synergy with TNF- α and inhibition with PTX (Supplementary Fig. S2A and C), indicating that in extremely high viremia PTX could be less protective against HIV-1-induced endothelial dysfunction. Since antiretroviral therapy in the absence of anti-inflammatory therapy only partially restores endothelial function,⁴⁷ adding PTX may provide additive benefits by controlling residual inflammation and may thus further improve endothelial function in patients receiving antiretroviral therapy. These findings also have implications for the treatment of lung abnormalities as well as cardiovascular disease in the HIV-positive population. HIV-infected patients exhibit increased incidence of lung disorders such as pulmonary edema, ARDS, and emphysema.^{48–51} Interestingly, the development of ARDS can be predicted by the levels of sVCAM-1 in the serum, much like cardiovascular disease.⁵² Therefore, it is likely that treatment with PTX will not only decrease the severity of cardiovascular risk in HIV patients but may also reduce the risk of pulmonary disorders.

Together, our *in vitro* data in combination with our *in vivo* clinical studies can lead to a more complete mechanistic understanding of the links among HIV infection, inflammation, and endothelial activation and dysfunction. These results may subsequently lead to the development of novel anti-inflammatory approaches to reduce cardiovascular events in HIV-infected patients.

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Author Disclosure Statement

No competing financial interests exist.

References

1. Sackoff JE, Hanna DB, Pfeiffer MR, and Torian LV: Causes of death among persons with AIDS in the era of highly active antiretroviral therapy: New York City. *Ann Intern Med* 2006;145:397–406.
2. Freiberg MS, Chang CC, Skanderson M, McGinnis K, Kuller LH, Kraemer KL, Rimland D, Goetz MB, Butt AA, Rodriguez Barradas MC, Gibert C, Leaf D, Brown ST, Samet J, Kazis L, Bryant K, and Justice AC: The risk of incident coronary heart disease among veterans with and without HIV and hepatitis C. *Circ Cardiovasc Qual Outcomes* 2011; 4:425–432.
3. Obel N, Thomsen HF, Kronborg G, Larsen CS, Hildebrandt PR, Sorensen HT, and Gerstoft J: Ischemic heart disease in HIV-infected and HIV-uninfected individuals: A population-based cohort study. *Clin Infect Dis* 2007;44:1625–1631.
4. Triant VA, Lee H, Hadigan C, and Grinspoon SK: Increased acute myocardial infarction rates and cardiovascular risk factors among patients with human immunodeficiency virus disease. *J Clin Endocrinol Metab* 2007;92:2506–2512.
5. El-Sadr WM, Grund B, Neuhaus J, Babiker A, Cohen CJ, Darbyshire J, Emery S, Lundgren JD, Phillips A, and Neaton JD: Risk for opportunistic disease and death after reinitiating continuous antiretroviral therapy in patients with HIV previously receiving episodic therapy: A randomized trial. *Ann Intern Med* 2008;149:289–299.
6. Jong E, Louw S, van Gorp EC, Meijers JC, ten Cate H, and Jacobson BF: The effect of initiating combined antiretroviral therapy on endothelial cell activation and coagulation markers in South African HIV-infected individuals. *Thromb Haemost* 2010;104:1228–1234.
7. Baker JV and Lundgren JD: Cardiovascular implications from untreated human immunodeficiency virus infection. *Eur Heart J* 2011;32(8):945–951.
8. Skiest DJ, Su Z, Havlir DV, Robertson KR, Coombs RW, Cain P, Peterson T, Krambrink A, Jahed N, McMahon D, and Margolis DM: Interruption of antiretroviral treatment in HIV-infected patients with preserved immune function is associated with a low rate of clinical progression: A prospective study by AIDS Clinical Trials Group 5170. *J Infect Dis* 2007;195:1426–1436.
9. Bowie AG and O'Neill LA: Vitamin C inhibits NF-kappa B activation by TNF via the activation of p38 mitogen-activated protein kinase. *J Immunol* 2000;165:7180–7188.
10. Koga M, Kai H, Yasukawa H, Kato S, Yamamoto T, Kawai Y, Kusaba K, Seki Y, Kai M, Egashira K, Kataoka Y, and Imaizumi T: Postnatal blocking of interferon-gamma function prevented atherosclerotic plaque formation in apolipoprotein E-knockout mice. *Hypertens Res* 2007;30:259–267.
11. Clauss M, Sunderkotter C, Sveinbjornsson B, Hippenstiel S, Willuweit A, Marino M, Haas E, Seljelid R, Scheurich P, Suttorp N, Grell M, and Risau W: A permissive role for tumor necrosis factor in vascular endothelial growth factor-induced vascular permeability. *Blood* 2001;97:1321–1329.
12. Rajashekhar G, Grow M, Willuweit A, Patterson CE, and Clauss M: Divergent and convergent effects on gene expression and function in acute versus chronic endothelial activation. *Physiol Genomics* 2007;1:104–113.
13. Zhang Y, Herbert BS, Rajashekhar G, Ingram DA, Yoder MC, Clauss M, and Rehman J: Premature senescence of highly proliferative endothelial progenitor cells is induced by tumor necrosis factor-alpha via the p38 mitogen-activated protein kinase pathway. *FASEB J* 2009; 23:1358–1365.

14. Jaruga B, Hong F, Kim WH, and Gao B: IFN-gamma/STAT1 acts as a proinflammatory signal in T cell-mediated hepatitis via induction of multiple chemokines and adhesion molecules: a critical role of IRF-1. *Am J Physiol Gastrointest Liver Physiol* 2004;287:G1044–1052.
15. Chi D, Henry J, Kelley J, Thorpe R, Smith JK, and Krishnaswamy G: The effects of HIV infection on endothelial function. *Endothelium* 2000;7:223–242.
16. Toborek M, Lee YW, Pu H, Malecki A, Flora G, Garrido R, Hennig B, Bauer HC, and Nath A: HIV-Tat protein induces oxidative and inflammatory pathways in brain endothelium. *J Neurochem* 2003;84:169–179.
17. Gupta SK, Johnson RM, Mather KJ, and Dube MP: Anti-Inflammatory Treatment with Pentoxifylline Improves HIV-Related Endothelial Dysfunction: A Pilot Study. 15th Conferences on Retroviruses and Opportunistic Infections, 2008.
18. Gupta SK, Johnson RM, Saha C, Mather KJ, Greenwald ML, Waltz JS, Rehman J, and Dube MP: Improvement in HIV-related endothelial dysfunction using the anti-inflammatory agent salsalate: A pilot study. *AIDS* 2008;22:653–655.
19. Gupta SK, Johnson RM, Mather KJ, Clauss M, Rehman J, Saha C, Desta Z, and Dube MP: Anti-inflammatory treatment with pentoxifylline improves HIV-related endothelial dysfunction: A pilot study. *AIDS* 2010;24:1377–1380.
20. Kopp E and Ghosh S: Inhibition of NF-kappa B by sodium salicylate and aspirin. *Science* 1994;265:956–959.
21. Zhang M, Xu YJ, Saini HK, Turan B, Liu PP, and Dhalla NS: TNF-alpha as a potential mediator of cardiac dysfunction due to intracellular Ca2+-overload. *Biochem Biophys Res Commun* 2005;327:57–63.
22. Chen YM, Tu CJ, Hung KY, Wu KD, Tsai TJ, and Hsieh BS: Inhibition by pentoxifylline of TNF-alpha-stimulated fractalkine production in vascular smooth muscle cells: Evidence for mediation by NF-kappa B down-regulation. *Br J Pharmacol* 2003;138:950–958.
23. Ji Q, Zhang L, Jia H, and Xu J: Pentoxifylline inhibits endotoxin-induced NF-kappa B activation and associated production of proinflammatory cytokines. *Ann Clin Lab Sci* 2004;34:427–436.
24. Tozawa K, Sakurada S, Kohri K, and Okamoto T: Effects of anti-nuclear factor kappa B reagents in blocking adhesion of human cancer cells to vascular endothelial cells. *Cancer Res* 1995;55:4162–4167.
25. Dansky HM, Barlow CB, Lominska C, Sikes JL, Kao C, Weinsaft J, Cybulsky MI, and Smith JD: Adhesion of monocytes to arterial endothelium and initiation of atherosclerosis are critically dependent on vascular cell adhesion molecule-1 gene dosage. *Arterioscler Thromb Vasc Biol* 2001;21:1662–1667.
26. Schwarz JB, Langwieser N, Langwieser NN, Bek MJ, Seidl S, Eckstein HH, Lu B, Schomig A, Pavenstadt H, and Zohlnhofer D: Novel role of the CXC chemokine receptor 3 in inflammatory response to arterial injury: Involvement of mTORC1. *Circ Res* 2009;104:189–200.
27. Feldman ED, Weinreich DM, Carroll NM, Burness ML, Feldman AL, Turner E, and Xu H, Alexander HR Jr: Interferon gamma-inducible protein 10 selectively inhibits proliferation and induces apoptosis in endothelial cells. *Ann Surg Oncol* 2006;13:125–133.
28. Salvucci O, Basik M, Yao L, Bianchi R, and Tosato G: Evidence for the involvement of SDF-1 and CXCR4 in the disruption of endothelial cell-branching morphogenesis and angiogenesis by TNF-alpha and IFN-gamma. *J Leukoc Biol* 2004;76:217–226.
29. Dhawan S, Puri RK, Kumar A, Duplan H, Masson JM, and Aggarwal BB: Human immunodeficiency virus-1-tat protein induces the cell surface expression of endothelial leukocyte adhesion molecule-1, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 in human endothelial cells. *Blood* 1997;90:1535–1544.
30. von Sydow M, Sonnerborg A, Gaines H, and Strannegard O: Interferon-alpha and tumor necrosis factor-alpha in serum of patients in various stages of HIV-1 infection. *AIDS Res Hum Retroviruses* 1991;7:375–378.
31. Arditi M, Kabat W, and Yagev R: Serum tumor necrosis factor alpha, interleukin 1-beta, p24 antigen concentrations and CD4+ cells at various stages of human immunodeficiency virus 1 infection in children. *Pediatr Infect Dis J* 1991;10:450–455.
32. Basaran Y, Basaran MM, Babacan KF, Ener B, Okay T, Gok H, and Ozdemir M: Serum tumor necrosis factor levels in acute myocardial infarction and unstable angina pectoris. *Angiology* 1993;44:332–337.
33. Rajan S, Ye J, Bai S, Huang F, and Guo YL: NF-kappaB, but not p38 MAP kinase, is required for TNF-alpha-induced expression of cell adhesion molecules in endothelial cells. *J Cell Biochem* 2008;105:477–486.
34. Futakuchi M, Ogawa K, Tamano S, Takahashi S, and Shirai T: Suppression of metastasis by nuclear factor kappaB inhibitors in an in vivo lung metastasis model of chemically induced hepatocellular carcinoma. *Cancer Sci* 2004;95:18–24.
35. Hasebe Y, Thomson LR, and Dorey CK: Pentoxifylline inhibition of vasculogenesis in the neonatal rat retina. *Invest Ophthalmol Vis Sci* 2000;41:2774–2778.
36. Nikolopoulou A, Tousoulis D, Antoniadis C, Petroheilou K, Vasiliadou C, Papageorgiou N, Koniari K, Stefanadi E, Latsios G, Siasos G, and Stefanadis C: Common community infections and the risk for coronary artery disease and acute myocardial infarction: Evidence for chronic over-expression of tumor necrosis factor alpha and vascular cells adhesion molecule-1. *Int J Cardiol* 2008;130:246–250.
37. Gattegno L, Bentata-Peyssare M, Gronowski S, Chaouche K, and Ferriere F: Elevated concentrations of circulating intercellular adhesion molecule 1 (ICAM-1) and of vascular cell adhesion molecule 1 (VCAM-1) in HIV-1 infection. *Cell Adhes Commun* 1995;3:179–185.
38. Jiang J, Fu W, Wang X, Lin PH, Yao Q, and Chen C: HIV gp120 induces endothelial dysfunction in tumour necrosis factor-alpha-activated porcine and human endothelial cells. *Cardiovasc Res* 2010;87:366–374.
39. Lucas R, Juillard P, Decoster E, Redard M, Burger D, Donati Y, Giroud C, Monso-Hinard C, De Kesel T, Buurman WA, Moore MW, Dayer JM, Fiers W, Bluethmann H, and Grau GE: Crucial role of tumor necrosis factor (TNF) receptor 2 and membrane-bound TNF in experimental cerebral malaria. *Eur J Immunol* 1997;27:1719–1725.
40. Taha H, Grochot-Przeczek A, Was H, Kotlinowski J, Kozakowska M, Marek A, Skrzypek K, Lackowska B, Balcerczyk A, Mustafa S, Dulak J, and Jozkowicz A: Modulation of inflammatory response by pentoxifylline is independent of heme oxygenase-1 pathway. *J Physiol Pharmacol* 2009;60:3–12.
41. Hoie EB, McGuire TR, Leuschen PM, and Zach TL: Pentoxifylline inhibits tumor necrosis factor-alpha induced synthesis of complement component C3 in human endothelial cells. *Biol Pharm Bull* 2004;27:1670–1673.
42. Fu J, Meng X, He J, and Gu J: Inhibition of inflammation by a p38 MAP kinase targeted cell permeable peptide. *Med Chem* 2008;4:597–604.

43. Li JC and Lau AS: A role for mitogen-activated protein kinase and Ets-1 in the induction of interleukin-10 transcription by human immunodeficiency virus-1 Tat. *Immunology* 2007;121:337–348.
44. Dohgu S and Banks WA: Lipopolysaccharide-enhanced transcellular transport of HIV-1 across the blood-brain barrier is mediated by the p38 mitogen-activated protein kinase pathway. *Exp Neurol* 2008;210:740–749.
45. Postadzhiyan AS, Tzontcheva AV, Kehayov I, and Finkov B: Circulating soluble adhesion molecules ICAM-1 and VCAM-1 and their association with clinical outcome, troponin T and C-reactive protein in patients with acute coronary syndromes. *Clin Biochem* 2008;41:126–133.
46. Silvestro A, Brevetti G, Schiano V, Scopacasa F, and Chiariello M: Adhesion molecules and cardiovascular risk in peripheral arterial disease. Soluble vascular cell adhesion molecule-1 improves risk stratification. *Thromb Haemost* 2005;93:559–563.
47. Torriani FJ, Komarow L, Parker RA, Cotter BR, Currier JS, Dube MP, Fichtenbaum CJ, Gerschenson M, Mitchell CK, Murphy RL, Squires K, and Stein JH: Endothelial function in human immunodeficiency virus-infected antiretroviral-naive subjects before and after starting potent antiretroviral therapy: The ACTG (AIDS Clinical Trials Group) Study 5152s. *J Am Coll Cardiol* 2008;52:569–76.
48. Diaz PT, Clanton TL, and Pacht ER: Emphysema-like pulmonary disease associated with human immunodeficiency virus infection. *Ann Intern Med* 1992;116:124–128.
49. Petrache I, Diab K, Knox KS, Twigg HL 3rd, Stephens RS, Flores S, and Tudor RM: HIV associated pulmonary emphysema: A review of the literature and inquiry into its mechanism. *Thorax* 2008;63:463–469.
50. Mehta NJ, Khan IA, Mehta RN, and Sepkowitz DA: HIV-Related pulmonary hypertension: analytic review of 131 cases. *Chest* 2000;118:1133–1141.
51. Afessa B Green B: Clinical course, prognostic factors, and outcome prediction for HIV patients in the ICU. The PIP (Pulmonary complications, ICU support, and prognostic factors in hospitalized patients with HIV) study. *Chest* 2000;118:138–145.
52. Agouridakis P, Kyriakou D, Alexandrakis MG, Prekates A, Perisinakis K, Karkavitsas N, and Bouros D: The predictive role of serum and bronchoalveolar lavage cytokines and adhesion molecules for acute respiratory distress syndrome development and outcome. *Respir Res* 2002;3:25.

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