ORIGINAL RESEARCH

HIV Protease Inhibitor Ritonavir Impairs Endothelial Function Via Reduction in Adipose Mass and Endothelial Leptin Receptor-Dependent Increases in NADPH Oxidase 1 (Nox1), C-C Chemokine Receptor Type 5 (CCR5), and Inflammation

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BACKGROUND: Cardiovascular disease is currently the leading cause of death in patients with human immunodeficiency virus on combination antiretroviral therapy. Although the use of the protease inhibitor ritonavir has been associated with increased prevalence of cardiovascular disease, the underlying mechanisms remain ill-defined. Herein, we tested the hypothesis that ritonavir-mediated lipoatrophy causes endothelial dysfunction via reducing endothelial leptin signaling.

METHODS AND RESULTS: Long-term (4 weeks) but not short-term (3 days) treatment with ritonavir reduced body weight, fat mass, and leptin levels and induced endothelial dysfunction in mice. Moreover, ritonavir increased vascular NADPH oxidase 1, aortic H$_2$O$_2$ levels as well as interleukin-1[β], GATA3 (GATA binding protein 3), the macrophage marker (F4/80), and C-C chemokine receptor type 5 (CCR5) expression. Reactive oxygen species scavenging with tempol restored endothelial function, and both NADPH oxidase 1 and CCR5 deletion in mice protected from ritonavir-mediated endothelial dysfunction and vascular inflammation. Remarkably, leptin infusion markedly improved endothelial function and significantly reduced vascular NADPH oxidase 1, interleukin-1[β], GATA3, F4/80, and CCR5 levels in ritonavir-treated animals. Selective deficiency in endothelial leptin receptor abolished the protective effects of leptin infusion on endothelial function. Conversely, selective increases in endothelial leptin signaling with protein tyrosine phosphatase deletion blunted ritonavir-induced endothelial dysfunction.

CONCLUSIONS: All together, these data indicate that ritonavir-associated endothelial dysfunction is a direct consequence of a reduction in adiposity and leptin secretion, which decreases endothelial leptin signaling and leads to a NADPH oxidase 1-induced, CCR5-mediated reduction in NO bioavailability. These latter data also introduce leptin deficiency as an additional contributor to cardiovascular disease and leptin as a negative regulator of CCR5 expression, which may provide beneficial avenues for limiting human immunodeficiency virus infection.

Key Words: CCR5 ● HIV ● leptin ● lipoatrophy ● NADPH oxidase ● ritonavir

Over the past few decades, the introduction of combination antiretroviral therapy (cART) has led to a profound suppression of human immunodeficiency virus-1 (HIV-1) replication and increased the longevity of patients with HIV, which now approaches that of the general population. As a consequence, the spectrum of diseases related to HIV has shifted from opportunistic AIDS-related diseases towards...
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long-term age-related complications. Notably, atherosclerosis-associated cardiovascular disease (CVD) has markedly increased in patients with HIV (PWH) in the post-cART era, and CVD is currently the leading cause of death in PWH on cART.\(^3\)\(^{-}\)\(^7\) A current limitation to the prevention and treatment of accelerated atherosclerosis in PWH is our limited knowledge of the underlying mechanisms, which have been almost exclusively obtained through clinical observations.\(^4\)

Despite our limited knowledge regarding the cause of CVD in PWH, certain classes of antiretroviral drugs such as the HIV protease inhibitors have been strongly implicated in the process, and the contribution of the protease inhibitor ritonavir was pointed out by several studies. Indeed, ritonavir has been shown to have a small but statistically significant effect on the progression of carotid-wall thickness,\(^8\) to be associated with increased risk of myocardial infarction,\(^9\) and to increase cardiovascular mortality of heart failure in PWH.\(^10\) More recently, the D:A:D (Data Collection on Adverse Events of Anti-HIV Drugs) study, a 7-year surveillance study involving a cohort of patients from all over the world, further highlighted the potential contribution of ritonavir by indicating that ritonavir-boosted darunavir use was associated with increased risk of CVD, with an incidence rate ratio of 1.59.\(^11\) Ritonavir is currently a major component of second-line cART regimens\(^12\)\(^{-}\)\(^14\) but also a compound considered for the treatment of several forms of cancer,\(^15\) including multiple myeloma,\(^16\) for the treatment of hepatitis C,\(^17\) and more recently for the treatment of adults hospitalized with severe coronavirus 2019.\(^18\) Therefore, it remains critical to understand the mechanisms whereby ritonavir could promote CVD.

Previous clinical and basic science reports have associated ritonavir with lipoatrophy and dyslipidemia\(^9\)\(^{-}\)\(^21\) and showed that acute in vitro exposure of vessels or endothelial cells to ritonavir impairs endothelial function via increasing oxidative stress and reducing NO bioavailability.\(^22\)\(^{-}\)\(^23\) Despite numerous studies, the underlying mechanisms whereby ritonavir impairs endothelial function, a major precursor and contributor to CVD, remain poorly defined in an in vivo setting. Herein, we treated C57bl/6 mice with ritonavir for 4 weeks to test the hypothesis that ritonavir-mediated lipoatrophy impairs endothelial function via reducing leptin-mediated decrease in endothelial oxidative stress and inflammation.

**METHODS**

The data that support the findings of this study are available from the corresponding author on reasonable request.

**Experimental Animals**

Male and female C57BL/6 mice (33 male and 10 female mice), males deficient in NADPH oxidase 1 (Nox1\(^{-}\)\(^/-\) mice, gift from Dr. DW. Stepp, Vascular Biology Center, Augusta University) (N=8), C-C chemokine receptor type 5 (CCR5\(^{-}\)\(^/-\) mice, B6.129P2-Ccr5tm1Kuz/J Jackson Laboratory) (N=8), and males with selective deletion of either Ptp1b (protein tyrosine phosphatase 1B) (N=7) or leptin receptor (LepR) (N=7), in endothelial cells (EC) have been used at 8 to 14 weeks of age. Inducible endothelial Ptp1b- and LepR-deficient mice
have been generated by crossing Cdh5-CreERT2 mice (from R. Adams, Max-Planck-Institute) with Ptp1b\textsubscript{flox/}\textsubscript{flox} and LepR\textsubscript{flox/flox} respectively (Jackson Laboratory). Endothelial-specific deletion of Ptp1b or LepR was induced via activation of the endothelial-specific Cre recombinase in 6- to 8-week-old mice by 5 for Ptp1b or 14 for LepR consecutive daily intraperitoneal injections of tamoxifen (0.1 mL of a 20 mg/mL solution in corn oil). Animals receiving tamoxifen injections (+/−) were compared with vehicle (corn oil)-treated animals (+/+).

All animals were fed standard mouse chow, and tap water was provided ad libitum. Mice were housed in an American Association of Laboratory Animal Care–approved animal care facility at Augusta University. Augusta University Institutional Animal Care and Use Committee approved all protocols (IACUC protocol #2011-0108).

**Ritonavir and Leptin Treatments**
Mice were submitted to daily intraperitoneal injections of either ritonavir (5 mg/kg for 4 weeks) or vehicle as previously described. After 3 weeks of ritonavir treatment, mice were separated into 2 groups and treated with either leptin (0.3 mg/kg per day, ProSpec, Israel) or vehicle via subcutaneous osmotic mini-pumps (ALZET, Cupertino, CA; model 1007D, 0.5 μL/h) for 7 more days, as previously described by our group. A short-term ritonavir treatment (5 mg/kg for 3 days) was also used to test the potential direct effects of ritonavir on endothelial function.

**Statistical Analysis**
All data are presented as mean±SEM. P<0.05 were considered significant. Differences in means between 2 groups for nonrepeated variables were compared by the Student’s t test. Differences in means among groups and treatments were compared by 2-way ANOVA with repeated measures, when appropriate. Tukey test was used as the post hoc test (GraphPad).

Detailed description of the methods used is available in Data S1. The sequence of the primers is included in Table S1.

**RESULTS**

**Ritonavir Induces Endothelial Dysfunction Via Reducing Leptin Biosynthesis**
Following 4 weeks of ritonavir treatment, male mice exhibited a lipoatrophic phenotype characterized by a significant reduction in body weight (Figure 1A), fat mass (Figure 1B), and leptin levels (Figure 1F). As reported in Figure 1B through 1E, fat mass reduction affected gonadal, subcutaneous, and perivascular adipose depots. Lean mass, glycemia, and plasma lipids levels remained intact in ritonavir-treated mice (Table S2). While investigating the effects of ritonavir treatment in endothelial function, we reported that ritonavir markedly reduced acetylcholine- but not sodium nitroprusside–induced relaxation of the aortic rings (Figure 1G and 1H), which supports a dysfunction at the levels of the endothelium. In female mice, 4 weeks of ritonavir treatment reduced body weight, fat mass, and impaired endothelial function to a similar extent as in males (Figure S1A through S1C), suggesting that ritonavir-mediated metabolic and vascular alterations are not sex-specific. Treatment of male animals with ritonavir for 3 days did not reduce body weight, fat mass or leptin levels, nor impair endothelial function (Figure S1D through S1G). These latter data rule out direct effects of ritonavir on endothelial function and support the contribution of ritonavir-induced lipoatrophy to endothelial dysfunction. To further test the contribution of fat mass reduction to endothelial dysfunction, we investigated whether restoration of the levels of the adipokine leptin improved endothelial function. As reported in Figure 1G, leptin treatment markedly improved endothelial function despite further reducing body weight (Figure 1A through 1F), suggesting that decreases in leptin levels mediates endothelial dysfunction in ritonavir-treated animals.

**Ritonavir-Induced Endothelial Dysfunction and Inflammation are Nox1-Dependent**
While investigating the mechanisms whereby ritonavir impairs endothelial function, we revealed a reduced NO bioavailability reflected by a complete abolition of ACh-mediated relaxation in l-NG-nitro arginine methyl ester–treated aortic rings in both control and ritonavir-treated animals (Figure 2A). Reactive oxygen species are a major cause of reduced NO bioavailability. Therefore, we repeated the relaxation response curve to ACh in the presence of the reactive oxygen species scavenger tempol, which fully restored endothelial function in ritonavir-treated mice and revealed that ritonavir-mediated endothelial dysfunction involves oxidative stress (Figure 2B). Concomitantly, ritonavir increased Nox1, NoxA1 transcript expression without altering NoxO1, Nox2, and Nox4 expression (Figure 2C). Ritonavir also increased aortic H$_2$O$_2$ levels (Figure 2D) and induced vascular inflammation as reflected by marked increases in aorta interleukin 1β (IL-1β), GATA3 (GATA binding protein 3), F4/80, and CCR5 expression as well as in CCR5 ligand, C-C motif chemokine ligand 5 (CCL5) (Figure 2E). The crucial role of Nox1 in ritonavir-mediated endothelial dysfunction and vascular inflammation was demonstrated by reporting that Nox1 deficiency protected mice from ritonavir-mediated endothelial dysfunction (Figure 2F) and increases
in vascular IL-1β, GATA3, F4/80, CCR5, and CCL5 (Figure 2E) without blunting ritonavir-mediated decreases in body weight and fat mass (Figure S2A and S2B). The potential contribution of endothelial Nox1 to vascular inflammation was investigated via overexpression of Nox1, NoxA1, and NoxO1 in EC. This approach revealed that high Nox1 expression in EC is associated with a 5-fold increase in CCR5 expression (Figure 2G).

Figure 1. Ritonavir induces endothelial dysfunction via reducing leptin secretion.
Body weight (A), percentage of fat mass (B), gonadal fat depot (C), subcutaneous fat depot (D), PVAT, (E) leptin plasma levels (F), and concentration response curves to ACh (G) and SNP (H) in aortic rings from control (Ctrl, vehicle-treated) and ritonavir-treated mice (ritonavir, 5 mg/kg per day for 4 weeks, ip) in the presence or absence of leptin treatment (0.3 mg/kg per day for 1 week, via osmotic mini-pump). Data are presented as mean±SEM. N=5 to 8; *P<0.05 vs Ctrl; †P<0.05 vs Ctrl and ritonavir. ACh indicates acetylcholine; BW, body weight; Ctrl, control; PVAT, perivascular adipose tissue; and SQF, subcutaneous fat.
Figure 2. Ritonavir-induced endothelial dysfunction and inflammation are Nox1-dependent.

CRC to ACh in aortic rings in the presence of l-NAME (100 μmol/L) (A) or tempol (100 μmol/L) (B). Real-time PCR quantification of aortic NADPH oxidases subunits (C). Aortic H₂O₂ levels measured by Amplex Red (D) from control (Ctrl, vehicle-treated) and ritonavir-treated mice (ritonavir, 5 mg/kg per day for 4 weeks, ip) in the presence or absence of leptin treatment (0.3 mg/kg per day for 1 week, via osmotic mini-pump). Real-time PCR quantification of inflammatory markers (E) and CRC to ACh (F) in aortic segments from Nox1-deficient mice (Nox1−/−) treated or not with ritonavir. G, CCR5 gene expression in human umbilical vein endothelial cells transduced with Nox1/NoxA1/NoxO1. Data are presented as mean±SEM. Gene expression data are presented as Min. to Max. N=3 to 8; *P<0.05 vs Ctrl; †P<0.05 vs Ctrl and ritonavir. ACh indicates acetylcholine; Ctrl, control; CCL5, C-C motif chemokine ligand 5; CCR5, C-C chemokine receptor 5; CRC, concentration response curves; IL1-β, interleukin 1-β; F4/80, the macrophage marker F4/80; GATA3, GATA binding protein 3; IL1-β, interleukin 1-β; l-NAME, l-NG-nitro arginine methyl ester; NADPH, reduced nicotinamide adenine dinucleotide phosphate; Nox1, NADPH oxidase 1; and PCR, polymerase chain reaction.
Ritonavir-Induced Endothelial Dysfunction and Vascular Inflammation Are CCR5-Dependent

As ritonavir-mediated increases in Nox1 led to CCR5 overexpression, we investigated CCR5 contribution to endothelial dysfunction and vascular inflammation. CCR5 deficiency did not protect mice from ritonavir-mediated reductions in body weight and fat mass (Figure S3A and S3B) but protected mice from endothelial dysfunction (Figure 3A) to a similar extent as Nox1 deletion (Figure S4) and increases in IL-1β, GATA3, and F4/80 (Figure 3B). As reported in Figure 3C, CCR5 deficiency also protected from ritonavir-mediated increases in Nox1 and NoxA1.

Leptin Treatment Decreases Nox1 Expression and Vascular Inflammation

As leptin treatment restored endothelial function, we investigated whether leptin would also reduce Nox1 and CCR5 expression as well as vascular inflammation. We also assessed whether Nox1 and CCR5 deletion, as well as ritonavir treatment affect vascular leptin signaling. As reported in Figure 4A through 4C, leptin treatment blunted ritonavir-mediated increases in aortic

![Figure 3](image_url)

Figure 3. Ritonavir-induced endothelial dysfunction and vascular inflammation are CCR5 dependent.
Concentration response curves to ACh in aortic rings (A), real-time polymerase chain reaction quantification of aortic inflammatory gene markers (B), and aortic reduced nicotinamide adenine dinucleotide phosphate oxidases subunits (C) from control (Ctrl, vehicle treatment) and CCR5-deficient mice (CCR5−/−) in the presence or absence of ritonavir treatment (ritonavir, 5 mg/kg per day for 4 weeks, ip). Vascular reactivity data are presented as mean±SEM. Gene expression data are presented as Min. to Max. N=3 to 8; *P<0.05 vs Ctrl; †P<0.05 vs Ctrl and ritonavir. ACh indicates acetylcholine; CCL5, C-C motif chemokine ligand 5; CCR5, C-C chemokine receptor 5; F4/80, the macrophage marker F4/80; GATA3, GATA binding protein 3; IL1-β, interleukin 1-β; and Nox1, NADPH oxidase 1.
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Nox1 and NoxA1, in aortic H$_2$O$_2$ production, as well as in IL-1β, GATA3, F4/80, CCR5, and CCL5 transcript expression. We also showed that vascular leptin receptor expression remained intact with Nox1 and CCR5 deletion as well as ritonavir treatment (Figure 4D).

Protective Effects of Leptin Require Endothelial Leptin Signaling Activation

To investigate the origin of the protective effects of leptin, we repeated the ritonavir experiments in inducible endothelial leptin receptor–deficient mice as well as in inducible endothelial Ptp1b–deficient mice that present increased endothelial leptin signaling. As reported in Figure 5A and 5B, endothelial leptin deficiency blunted the protective effects of leptin treatment on endothelial function as well as on Nox1 and NoxA1. However, increases in endothelial leptin signaling with Ptp1b deletion blunted ritonavir-mediated endothelial dysfunction and increases in Nox1 and NoxA1 expression. Neither endothelial leptin receptor nor Ptp1b deletion affected ritonavir-mediated decrease in body weight and fat mass (Figure S5).
Lastly, we investigated whether ritonavir-mediated endothelial dysfunction could result in increased vascular adrenergic contractility. As reported in Figure 6A, ritonavir increased vascular contractility to phenylephrine. Both leptin treatment (Figure 6A) and Nox1 deficiency (Figure 6B) restored vascular contractility. In addition, i-NG-nitro arginine methyl ester abolished the protective effects of leptin treatment and Nox1 deletion (Figure 6C and 6D), indicating that leptin treatment and Nox1 deletion reduced contractility via restoration of NO bioavailability. Remarkably, ritonavir treatment, leptin infusion, and Nox1 deletion did not affect general vascular contractility because none of these treatments altered vascular constriction in response to vascular smooth muscle cell depolarization (KCl-mediated constriction, Figure 6E).
DISCUSSION

In the present study, we investigated the mechanisms whereby ritonavir, a protease inhibitor associated with CVD in numerous clinical studies,8 –11 impairs endothelial function, in an in vivo setting. We identified for the first time that ritonavir-mediated reduction in adipose mass causes endothelial dysfunction via reducing the secretion of the adipokine leptin and lessening the activation of endothelial leptin signaling. We extended our findings by providing the first demonstration that reduction in endothelial leptin signaling induces an increase in Nox1 expression, which elevates vascular CCR5 levels, promotes vascular inflammation, reduces NO bioavailability, and consequently leads to impaired endothelium-dependent relaxation (mechanisms summarized in Figure 7). Relevant to these observations are the roles of fat mass, endothelial leptin receptor, Nox1, and CCR5 in ritonavir-associated endothelial dysfunction.

Reduction in fat mass and adipokines secretion have been among the first side effects reported in PWH...
on protease inhibitors; however, no study had investigated the cardiovascular consequences of alterations in adipose tissue endocrine function. Herein, in agreement with previous clinical studies, we report that ritonavir reduced adipose mass and the secretion of the adipokine leptin. We demonstrated that reduction in leptin levels and its consequent decrease in endothelial leptin signaling are the causes of endothelial dysfunction. Indeed, we showed that leptin supplementation markedly improved endothelial function, and while selective endothelial leptin receptor deficiency abolished the protective effects of leptin supplementation, selective increases in endothelial leptin signaling protected against ritonavir-mediated endothelial dysfunction. In contradiction to our results, previous in vitro studies in EC in culture and ex vivo experiments in isolated arteries support direct toxic effects of ritonavir on EC. However, our current in vivo study, added to the work by others, minimizes the contribution of the latter mechanism and supports ritonavir-mediated decreases in adipose mass as a primary cause of endothelial dysfunction. Indeed, while toxic effects of ritonavir would have been expected to induce endothelial dysfunction after only a few days of exposure, endothelial function remained intact after 3 days of ritonavir treatment, which did not reduce adipose mass. In addition, ritonavir treatment did not induce endothelial dysfunction in healthy volunteers, in the absence of changes in adipose mass. While these results provide strong arguments to support the contribution of ritonavir-associated fat mass reduction to endothelial dysfunction, these results also raised the question of the fat depot responsible for the decreases in leptin levels. The subcutaneous adipose tissue is the adipose depot preferentially affected by protease inhibitor. Indeed, PWH on protease inhibitors do commonly present with reduced limb and face subcutaneous fat mass but with intact or increased visceral fat mass. In the present mouse study, ritonavir similarly reduced subcutaneous, visceral, as well as perivascular adipose tissue. Thus, based on the reports that the subcutaneous adipose tissue is the main source of leptin, we could speculate that the reduction in subcutaneous fat mass causes endothelial dysfunction. However, our data do not rule out the potential contribution of a localized decrease in leptin mediated by a reduction in perivascular adipose tissue. Therefore, further investigations are required to identify the primary fat depot responsible for the maintenance of EC homeostasis.

Dyslipidemia has been presented as another key contributor to ritonavir-mediated endothelial dysfunction. However, the present study as well as work by others do also challenge this concept. Endothelial dysfunction was not associated with significant increases in cholesterol and plasma triglycerides in our ritonavir-treated mice, and reduction in plasma cholesterol and lipid levels did not restore endothelial function in PWH on ritonavir treatment. Moreover, despite raising triglyceride levels, liponavir/ritonavir treatment did not induce endothelial dysfunction in healthy volunteers exhibiting no alteration in body mass index.
Further evidence to support the contribution of adipose mass reduction and reduced leptin levels in endothelial dysfunction are provided by recent observations in obese PWH. Indeed, recent clinical evidence reports no negative impact of increased adiposity on carotid intima-media thickness and flow-mediated dilatation in PWH on cART.\cite{45-47} and large epidemiology studies suggest no increased risk for myocardial infarction among higher body mass index patients on cART.\cite{9,48,49} Remarkably, higher plasma levels of the adipocyte-derived hormone leptin have been associated with healthier arterial structure and function in PWH on cART.\cite{50} Together with studies reporting that leptin treatment improves metabolic function in patients and animals on cART,\cite{51-53} these data indicate that preserving adequate leptin levels may represent an avenue to limit metabolic and cardiovascular disorders in patients on cART.

Several studies have correlated high leptin levels with increased prevalence of CVD\cite{54} and associated excess leptin with NO synthase inhibition, oxidative stress, vascular inflammation, and increases in release of endothelin-1.\cite{55-57} However, very few studies have investigated the potential cardiovascular consequences of reduced leptin levels. Consistent with previous work by our group in a mouse model of congenital lipodystrophy,\cite{28} we reported that reduced endothelial leptin signaling activation elevates Nox1 expression and impairs endothelial dysfunction via reactive oxygen species–dependent mechanisms. Interestingly, we made the new observation that elevated vascular Nox1 levels result in the increased expression of a major regulator of inflammation, CCR5, its ligand, CCL5 as well as GATA3, F4/80, and IL-1β, which are respective markers of T cells, macrophages, and inflammation in general. The role of Nox1 as a driver of inflammation and vascular dysfunction was demonstrated by reporting that Nox1 deletion prevented ritonavir-mediated increases in CCR5, GATA3, and F4/80 and abolished ritonavir-induced endothelial dysfunction and increased vascular adrenergic contractility. Further arguments in support of Nox1 were provided by showing that increases in Nox1 expression in EC elevated CCR5 expression. Remarkably, similarly to Nox1 deletion, CCR5 deletion protected from ritonavir-mediated endothelial dysfunction and vascular inflammation, without altering adipose mass. CCR5, which is expressed in immune and vascular cells, including EC,\cite{58} plays a crucial role in atherogenesis through the regulation of chemotraction of immune cells and infiltration into the inflammatory site.\cite{59} The role of CCR5 in the control of endothelium-dependent relaxation is, however, unknown. Although our study did not enable us to delineate the mechanisms whereby CCR5 deletion restores NO bioavailability, it presents, for the first time, CCR5 as a regulator of endothelium-dependent relaxation.

Based on the reduced expression of Nox1, GATA3, F4/80, and IL-1β with CCR5 deletion, we can speculate that CCR5 deficiency increases NO bioavailability via reducing macrophage- and T cell–derived reactive oxygen species production. However, we cannot rule out a potential direct role of CCR5 in the control of endothelial nitric oxide synthase function. Therefore, additional experiments are required to investigate the contribution of CCR5 signaling to EC homeostasis.

CCR5 function is not limited to chemoattraction and the progression of atherosclerosis. CCR5 is also a main co-receptor for HIV involved in the entry of the virus into the cell as well as in the spread of the virus.\cite{60} Therefore, the novel observation that leptin downregulates CCR5 expression via Nox1-dependent mechanisms could potentially have important implications beyond the regulation of endothelial function. Leptin-mediated decreases in CCR5 expression can potentially limit the spread of the viral infection and may contribute to explaining the slower HIV disease progression in PWH with high body mass index,\cite{61} as well as the better immune reconstitution over time in obese PWH.\cite{62} Although our study demonstrates that leptin-mediated CCR5 regulation is Nox1 dependent, additional studies will be required to delineate the molecular mechanisms whereby leptin downregulates Nox1 expression and whereby Nox1 controls CCR5 expression.

A limitation of the current study is the lack of measurement of blood pressure. Hypertension, which affects 35% of PWH on cART, is both a consequence of and a contributor to endothelial dysfunction.\cite{63} In addition, lipodystrophy has been identified as a contributor to hypertension in PWH on cART. Therefore, one can speculate that ritonavir-associated endothelial dysfunction could induce hypertension or that ritonavir-associated lipodystrophy induces hypertension, which causes endothelial dysfunction. However, our results do suggest that ritonavir-mediated alterations in endothelial function are independent of blood pressure. Indeed, while leptin has been associated with vascular inflammation, increases in sympathetic activity, and blood pressure,\cite{64,65} leptin treatment improved endothelial function and reduced vascular adrenergic contractility as well as vascular inflammation, which do not appear compatible with elevations in blood pressure. However, additional studies are required to confirm this hypothesis.

In conclusion, the present study provides the first evidence that endothelial dysfunction associated with the use of the protease inhibitor ritonavir is a direct consequence of a decrease in adipose mass and leptin secretion, which reduces endothelial leptin signaling activation and triggers an increase in CCR5 expression and inflammation via Nox1-dependent mechanisms. Taken together, these findings highlight the crucial role...
played by the adipose tissue in the control of cardiovascular function and indicate that excessive reduction in fat mass associated with protease inhibitor, chemotherapy, and anorexia likely primes the cardiovascular system for more severe alterations.

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Author contributions: TBN and EBC participated in the conception, design of the work, acquisition of the data, analysis and interpretation of the data, and redaction of the manuscript. TCK participated in the acquisition of the data and SK generated the mouse models.

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Disclosures
None.

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Data S1.

Supplemental Methods

Metabolic characterization

Body weight as well as body composition analyzed by nuclear magnetic resonance spectroscopy (EchoMRI)\textsuperscript{28} were measured at baseline and at the end of the treatment. After 4 weeks of treatment, mice were anesthetized (isoflurane 5\%) and euthanized via decapitation, in accordance with our approved animal protocol. Gonadal, subcutaneous and perivascular adipose tissue (PVAT) depots were collected, dried and weighed. Trunk blood was collected for plasma isolation and non-fasting blood glucose (glucometer, AlphaTRAK, Abbott, USA), plasma leptin, cholesterol, and triglycerides levels determined using ELISA kit from R&D System (Minneapolis, MN, USA) respectively.

Vascular function studies

Thoracic aortas were dissected surgically, cleaned of surrounding fat, cut in four rings and mounted on a wire myograph (DMT) as described previously\textsuperscript{29, 30}. Briefly, two tungsten wires were inserted into the lumen of the arteries and fixed to a force transducer and a micrometer. Arteries were bathed in a physiological salt solution and arterial viability was determined with a potassium-rich solution (40 mmol/l). Endothelium-dependent and independent relaxations were respectively tested with concentration response curves (CRC) to acetylcholine (ACh, Sigma Aldrich, MO); (0.1 nmol/l to 100 μmol/l) and sodium nitroprusside (SNP, Sigma Aldrich, MO-USA); (0.1 nmol/l to 10 μmol/l) in vessels preconstricted with serotonin (5HT, 0.1-1 μM, Sigma Aldrich, MO). CRCs to ACh were repeated in the presence of the unspecific nitric oxide synthase (NOS) inhibitor, N-nitro-
l-arginine methyl ester [l-NAME; 100 μmol/L, Sigma Aldrich, MO-USA], or tempol (100 μmol/l; SOD mimetic, Sigma Aldrich, MO-USA). CRCs to ACh and SNP are presented as percent of 5HT-induced constriction. The individual CRCs were fitted by nonlinear regression analysis. pD2 (defined as the negative logarithm of the EC50 values) and maximal response (Emax) were determined.

**Hydrogen Peroxide (H$_2$O$_2$) Measurement**

H$_2$O$_2$ was measured by Amplex Red (Thermo Fisher Scientific, NH-USA) in thoracic aortas as previously described.$^{28,66}$

**Human Umbilical Endothelial Cells (HUVEC)**

In order to analyze whether Nox1 regulates CCR5 expression, HUVECs were transfected with Nox1/NoxO1/NoxA$^{28,67}$. 24h after transfection, CCR5 gene expression was analyzed by quantitative real-time RT-PCR as described below.

**Real-time PCR**

Total aortic mRNA was extracted (Trizol Plus, Invitrogen, Carlsbad, Calif) and the concentration established with a NanoDrop 1000 (NanoDrop Technologies, Wilmington, Del). Complementary DNA was generated by RT-PCR with SuperScript III (Thermo Fisher Scientific, NH-USA). Reverse transcription was performed at 50 °C for 50 min; the enzyme was heat inactivated at 85 °C for 5 min, and real-time quantitative RT-PCR was performed with the SYBR-Green Supermix (Bio-Rad Laboratories, Hercules, Calif). Genes analyzed were Nox family members (Nox1, Nox2, Nox4, NoxA1 and NoxO1), as
well as Interleukin 1β (IL-1β), C-C chemokine receptor type 5 (CCR5), chemokine ligand 5 (CCL5), GATA binding protein 3 (GATA3), F4/80, leptin receptor and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which was used as housekeeping gene. The sequence of the primers is included in Supplemental Table S1.
## Supplemental Tables

### Table. S1. Primers sequence

| Gene       | Primer 1 sequence | Primer 2 sequence          |
|------------|-------------------|----------------------------|
| Nox1       | FW- 5' CATGGCCTGGGTGGGATTGT 3' | RV- 5' TGGGAGCGATGAAAAAGCGAAGGA 3' |
| Nox2       | FW- 5' CAAGATGGAGGTGGGACAGT 3' | RV- 5' GCTTATCACAGGCACAAGCA 3' |
| Nox4       | FW- 5' TGTTGCAATGGGAGGTGGGACAGT 3' | RV- 5' AAAACCCTCGAGGCAAAAGAT 3' |
| NoxA1      | FW- 5' ACGGTGGATGTTCTGTGTGA 3' | RV- 5' AAGCATGGCCTCCACATAGG 3' |
| NoxO1      | FW- 5' ACACGTCGGGGGCATACTGGTC 3' | RV- 5' GGCTGCCTCTGGGTGTTGGGATA 3' |
| CCL5       | FW- 5' AGATCTCTGCAGCTGCCTCA 3' | RV- 5' GGAGCAGCTTGCTGCTGTAG 3' |
| CCR5       | FW- 5' GGTTCCTGAAAGCGGCTGTAAATA 3' | RV- 5' CTGTTGGCAGTCAGCCACATC 3' |
| F4/80      | FW-5' CCTGCTGTGTCGTGCTGTTC 3' | RV- 5' GCCGTCTGTTGGTCAGTCTTGGTC 3' |
| IL-1β      | FW- 5' TCACAGACGACATCAACAAG 3' | RV- 5' CCACCGTATCAGTATCAGT 3' |
| GATA3      | FW- 5' TTTACCCTCAGGCTTCATCTCTCTC 3' | RV- 5' TGCACCTGACTTGGAGGCACCTCT 3' |
| Leptin receptor | FW- 5' TGATTTGTTTGATTTATGTTT 3' |
| Gene   | Forward Primer  | Reverse Primer   |
|--------|----------------|-----------------|
| GADPH  | FW - 5' ACCCAGAAGACTGTGGATGG 3' | RV - C5' ACATTGGGGGTAAGGAACAC 3' |

FW: Forward; RV: reverse.
### Table. S2. Metabolic parameters

| Parameter             | Control   | Ritonavir |
|-----------------------|-----------|-----------|
| Lean Mass (%)         | 78.8 ± 1.1| 77.3 ± 1.0|
| Glucose (mg/dL)       | 158.0 ± 3.9| 164.0 ± 2.3|
| Triglycerides (mg/dL) | 216.4 ± 15.1| 242.1 ± 13.3|
| Cholesterol (mg/dL)   | 144.6 ± 10.7| 129.8 ± 4.79|

Lean mass (%) and glucose (mg/dL), Triglycerides (mg/dL) and cholesterol (mg/dL) plasma levels in control and ritonavir treated mice (5mg/kg/day for 4 weeks).
Fig. S1. Ritonavir-induced endothelial dysfunction is sex-independent and requires lipoatrophy. Body weight (A), percentage of body fat (B), and concentration response curves to acetylcholine (C) in aortic rings from control (Ctrl, vehicle treatment) and ritonavir treated female mice (Ritonavir, 5mg/kg/day for 4 weeks, i.p). Body weight (D), percentage of body fat (E), plasma leptin levels (F) and concentration response curves to acetylcholine in aortic rings from control (G) (Ctrl, vehicle treatment) and ritonavir treated male mice (Ritonavir, 5mg/kg/day for 3 days, i.p). Data are presented as mean ± S.E.M. N=4-8; *P<0.05 vs Ctrl; †P<0.05 vs Ctrl and Ritonavir.
Fig. S2. Nox1 deficiency does not affect ritonavir-induced reduction in fat mass
Body weight (A) and percentage of body fat (B) in Nox1 deficient mice (Nox1-/-) treated with vehicle or ritonavir (Ritonavir, 5mg/kg/day for 3 days, i.p). Data are presented as mean ± S.E.M. N=3-6; *P<0.05 vs Ctrl or Nox1-/- without ritonavir treatment.
Fig. S3. CCR5 deficiency does not affect ritonavir-induced reduction in fat mass. Body weight (A) and percentage of body fat (B) in CCR5 deficient mice (CCR5−/−) treated with vehicle or ritonavir (Ritonavir, 5mg/kg/day for 3 days, i.p). Data are presented as mean ± S.E.M. N=3-6; *P<0.05 vs Ctrl or Nox1−/− without ritonavir treatment.
Fig. S4. Endothelial leptin signaling does not affect ritonavir-induced reduction in fat mass. Body weight (A and C) and percentage of body fat (B and D) in endothelial leptin receptor deficient mice (LepR \(^{EC-/}\), A and B) or endothelial Protein tyrosine phosphatase 1B deficient mice (Ptp1B \(^{EC-/}\), C and D) treated with vehicle (Ctrl) or ritonavir (5mg/kg/day for 4 weeks, i.p.). Data are presented as mean ± S.E.M. N=4; *P<0.05 vs LepR \(^{EC-/}\) or Ptp1B \(^{EC-/}\) without ritonavir treatment.
Fig. S5. Ritonavir induces endothelial dysfunction via Nox1 and CCR5-dependent mechanisms. Concentration response curves to acetylcholine in aortic segments from control (Ctrl, vehicle treated), Nox1 (Nox1−/−) and CCR5 deficient mice (CCR5−/−) treated or not with ritonavir (Ritonavir, 5mg/kg/day for 4 weeks, i.p) Data are presented as mean ± S.E.M. N=3-7; *P<0.05 Ritonavir vs Ctrl, Nox1−/− and CCR5−/−; †P<0.05 Ctrl vs Nox1−/− and CCR5−/− treated with ritonavir.