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Elevated adiponectin prevents HIV protease inhibitor toxicity and preserves cerebrovascular homeostasis in mice

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ABSTRACT

HIV protease inhibitors are key components of HIV antiretroviral therapies, which are fundamental in the treatment of HIV infection. However, the protease inhibitors are well-known to induce metabolic dysfunction which can in turn escalate the complications of HIV, including HIV associated neurocognitive disorders. As experimental and epidemiological data support a therapeutic role for adiponectin in both metabolic and neurologic homeostasis, this study was designed to determine if increased adiponectin could prevent the detrimental effects of protease inhibitors in mice. Adult male wild type (WT) and adiponectin-overexpressing (ADTg) mice were thus subjected to a 4-week regimen of lopinavir/ritonavir, followed by comprehensive metabolic, neurobehavioral, and neurochemical analyses. Data show that lopinavir/ritonavir-induced lipodystrophy, hypoadiponectinemia, hyperglycemia, hyperinsulinemia, and hypertriglyceridemia were attenuated in ADTg mice. Furthermore, cognitive function and blood–brain barrier integrity were preserved, while loss of cerebrovascular markers and white matter injury were prevented in ADTg mice. Finally, lopinavir/ritonavir caused significant increases in expression of markers of brain inflammation and decreases in synaptic markers in WT, but not in ADTg mice. Collectively, these data reinforce the pathophysiologic link from metabolic dysfunction to loss of cerebrovascular and cognitive homeostasis; and suggest that preservation and/or replacement of adiponectin could prevent these key aspects of HIV protease inhibitor-induced toxicity in clinical settings.

1. Introduction

Combination HIV antiretroviral therapy restricts viral replication, raises CD4 cell counts, prevents opportunistic infections, and improves/extends the lifespan and healthspan of people living with HIV/AIDS [1]. In spite of these revolutionary effects, it is well known that these drugs, particularly HIV protease inhibitors, have significant metabolic complications, fostering the development of dyslipidemia, insulin resistance, and lipodystrophy [2,3]. This iatrogenic sequela undermines patient health and limits ART compliance [4], and can also predispose patients to cognitive impairment and other neurologic complications [5–9]. Thus, these metabolic co-morbidities must be clinically managed to preserve quality of life and maintain self-care independence for people living with HIV/AIDS. Unfortunately, however, current pharmacologic strategies have produced only limited success in clinical settings [10]. For example, the insulin-sensitizing drug metformin reduces insulin resistance in HIV patients [11,12], but does not improve hyperlipidemia [12] and may actually accelerate lipodystrophy [13]. Thiazolidinediones (TZDs) show a similar dichotomous pattern with improvement in insulin sensitivity [11,12], but increased hyperlipidemia [11,12] and accelerated bone loss [14]. Tesamorelin, a recently approved synthetic human growth hormone-releasing hormone (hGHRH) analogue designed to treat HIV lipodystrophy, has been shown to decrease abdominal fat accumulation, improve glucose homeostasis [15], and even preserve cognition in adults with mild cognitive impairment [16]. However, studies have also shown that hGHRH can decrease subcutaneous fat mass, as well as induce arthralgia and edema [17]. Thus, new therapeutic approaches to significantly and successfully mitigate metabolic co-morbidities in HIV patients are needed.

In this context, remedies that prevent lipodystrophy and/or replicate adipocyte function in the face of lipodystrophy could provide novel and complementary strategies to maintain metabolic and neurologic function in people living with HIV/AIDS. Adipocytes orchestrate aspects of physiology via secretion of adipokines [18]; and in particular, adiponectin may be fundamental for optimal health. In terms of metabolic homeostasis, adiponectin is known to modulate glucose and fatty acid metabolism, inflammation, and vascular tone [19]. Adiponectin

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also has both vasculoprotective and neuroprotective properties [20–22], and indeed, hypoadiponectinemia predicts cognitive impairment [23] and decreased hippocampal volume [24] in humans. In specific relation to HIV, serum adiponectin levels are decreased in HIV patients [25–27], and correlate inversely with cognitive dysfunction in mice treated with HIV protease inhibitors [28]. Indeed, adiponectin administration has been shown to mitigate protease inhibitor-induced dyslipidemia in mice [29], suggesting that hypoadiponectinemia may drive, at least in part, the metabolic derangements associated with HIV protease inhibitors. Collectively these data support investigation into adiponectin-based therapies in the context of HIV antiretroviral therapy to support metabolic function and curtail the development of HIV-associated neurocognitive disorders. To determine the ability of adiponectin to prevent the adverse metabolic and neurologic effects of protease inhibitors, adult male wild type (WT) and adiponectin-overexpressing (ADTg) mice were subjected to a clinically relevant regimen of lopinavir/ritonavir, followed by comprehensive metabolic, neurobehavioral, and biochemical analyses.

2. Materials and methods

2.1. Animal treatments

The Institutional Animal Care and Use Committee at the Pennington Biomedical Research Center approved all experimental protocols, which were compliant with NIH guidelines on the use of experimental animals. ADTg mice on a C57Bl/6 background were generated as described in [30]. These mice express an aP2 promoter-driven transgene encoding a truncated form of adiponectin, leading to chronic, ~2 to 2.5-fold induction of secretion of oligomeric adiponectin complexes [30]. 6–8 month-old male ADTg and wild-type (WT) littermate control mice generated from local breeding colonies were housed in standard caging with 12:12 light:dark cycle and ad libitum access to food and water. Lopinavir/ritonavir (Kaletra®, Abbott Laboratories), was diluted in a vehicle of 10% ethanol/15% propylene glycol, and mice received daily administration of vehicle or lopinavir/ritonavir at 150/37.5 mg/kg via daily oral gavage for 4 weeks as previously described [28,31,32]. The dose was devised based on dosing guidelines for daily oral lopinavir/ritonavir in adult HIV patients (800/200 total mg or 10/2.5 mg/kg), and on body surface area (BSA) normalization factors [33], which translate 10 mg/kg in humans to approximately 125 mg/kg in mice. Previous UPLC-MRM-MS measurements of serum lopinavir 4 h after intraperitoneal injection of combined lopinavir/ritonavir show that that serum lopinavir in mice (3–18 μg/ml) approximates Abbott's reported C_{max} for lopinavir of 9.8 + 3.7 μg/ml 4 h after administration to adult HIV-positive patients [31].

Body weight and composition (measured using a Bruker minispec LF90 time domain NMR analyzer, Bruker Optics, Billerica MA) were measured regularly throughout lopinavir/ritonavir exposure. Fasting blood glucose was measured in tail blood using a glucometer (Ascensia Elite, Bayer, Mishawaka, IN). After cognitive testing, all mice were humanely euthanatized after a brief (6 h) fast, and blood, cerebral spinal fluid (CSF), and brain were collected. Data were compiled from 3 separate cohorts of mice.

2.2. Fear conditioning memory task

Each mouse was individually evaluated for fear conditioning using an automated, video-based fear conditioning system (Med-Associates, St. Albans, VT) as described previously [32,34]. The apparatus consists of a "startle chamber" used on days 1 and 2, which is an 8 × 15 × 15-cm acrylic and wire mesh cage located within a custom designed 90 × 70 × 70 ventilated sound-attenuating chamber, and the unique context is reinforced with an anise-based scent applied to each cage before testing. Animal movement within the apparatus results in displacement of an accelerometer (model U321AO2; PCB Piezotronics, Depew, NY, USA). Acquisition of fear conditioning on day 1 consists of 5 min acclimation to the startle chamber, followed by five consecutive 30 s auditory stimuli (85 db, 4 kHz) co-terminating with a mild footshock (0.5 mA × 1 s), with 30 s recovery periods between tones. On day 2, mice return to the same chambers, but no stimuli are applied to evaluate freezing responses to context. On day 3, mice are placed in an entirely separate chamber located in a different room to remove all contextual cues, and after 5 min habituation, a continuous tone (85 db, 4 kHz) is applied for 5 min. Freezing behavior is recorded as a measure of memory of the conditioned response to the tone.

2.3. Measurement of blood–brain barrier permeability

Sodium fluorescein (NaF, 376 Da) was used to assess the BBB permeability using established protocols [35]. Briefly, mice received an intravenous injection of PBS (150 μL) containing sodium fluorescein (NaF, 2 mg/mL; Sigma). Exactly 30 min later, blood was collected from the right atrium, mice were immediately perfused with 15 mL ice-cold PBS, and brain tissues were collected and kept at 4 °C. Weighed sections of the cerebral cortex and samples of serum were homogenized in 0.5 M borate buffer (pH 10) and centrifuged at 800 ×*g* for 15 min at 4 °C. Supernatants were mixed with ethanol and then centrifuged (15,000 ×*g*) for 20 min at 4 °C. Supernatant brain and serum Na-F concentrations were measured with a fluorimeter at 460 nm excitation and 515 nm emission within a linear range of standards of known concentrations and tissue/serum ratio of fluorescence was determined.

2.4. Clinical chemistry

Whole blood was collected by cardiac puncture of terminally anesthetized mice, and plasma was collected and either analyzed immediately or aliquoted and stored at −80 °C. Levels of total cholesterol, triglycerides, and non-esterified fatty acids (NEFA) in plasma were measured calorimetrically using commercially available kits (Wako Chemicals, Richmond, VA). Adiponectin and insulin levels in plasma and CSF were evaluated by ELISA in accordance with the manufacturer's assay protocol (R&D Systems, Minneapolis, MN).

2.5. Western blot

Tissue samples were homogenized and processed for Western blot with chemiluminescence as described in previous reports [36]. Blots were processed using the following primary antisera: anti-claudin-5 (1:400, Abcam Inc., Cambridge, MA), anti-ZO-1 (1:100, Abcam Inc.), anti-occludin (1:8000, Abcam Inc.), anti-MMP2 (1:1000, Abcam Inc.), anti-MMP-9 (1:1000, Abcam Inc.), anti-synapsin 1 (1:10,000, Thermo Fisher Scientific, Pittsburg, PA), anti-phospho(S553)-synapsin 1 (1:10,000, Abcam Inc.), anti-synapse associated protein 97 (1:2500, Abcam Inc.), anti-GFAP (1:5000, Abcam Inc.); anti-Iba-1 (1:500, Wako Chemicals USA Inc., Richmond, VA), and anti-tubulin (1:1000, Wako Chemicals USA Inc.). To ensure accurate quantification across multiple blots, samples from all treatment groups (vehicle and lopinavir/ritonavir in both WT and ADTg mice) were included in each individual blot. Data were first calculated as a ratio of expression over tubulin expression, which was included as an internal loading control, and then expression in lopinavir/ritonavir-treated mice was calculated and presented as percent expression in vehicle-treated mice.

2.6. Luxol fast blue stain

For histological examination, hemibrains were rapidly collected from mice and immersed in 10% neutral buffered formalin for 24–48 h and then processed for paraffin embedding. 6 μm mid-sagittal sections containing the anterior (genu) corpus callosum at the level of the lateral septal nuclei, medial to the lateral ventricle were selected, and following standard dewaxing and rehydration, tissue sections were immersed

overnight in Luxol fast blue solution (Solvent Blue 38, Sigma) at 27 °C. Sections were then immersed in 95% ethanol to remove excess stain before being rinsed in deionized water. Differentiation was initiated with immersion in 0.05% aqueous lithium carbonate followed by differentiation in 95% ethanol (with 100 μL per 300 mL of acetic acid) and washing in 95% ethanol only. After washing, sections were counterstained with hematoxylin and eosin, washed, dehydrated, and cover slipped. Slides were scanned using a Hamamatsu NanoZoomer Digital Slide Scanning System (Hamamatsu City, Japan) at 2×–40× magnification. The average pixel intensity from the anterior region (genu) of the corpus callosum was measured using Image J. To control for unavoidable variations in processing/washing, LFB staining is expressed as the ratio of pixel intensity in the corpus callosum relative to background staining in the stratum oriens of the hippocampus (CA1).

2.7. Statistical analyses

All data were analyzed using Prism software (GraphPad Software, Inc., La Jolla, CA), and are displayed as mean ± standard error of measurement. Body weight and composition were analyzed with 2-way repeated measures ANOVA to determine main effects of drug treatment and duration, followed by planned Bonferroni post-hoc comparisons to determine differences between in groups (vehicle/WT, vehicle/ADTg, lopinavir/ritonavir/WT and lopinavir/ritonavir/ADTg). All other data were analyzed by 1-way ANOVA followed by Tukey's Multiple Comparison post-hoc tests to determine specific differences between groups. Statistical significance for all analyses was accepted at $p < 0.05$, and *, **, and *** represent $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

3. Results

3.1. Body composition and serum markers of metabolic syndrome in lopinavir/ritonavir-treated WT and ADTg mice

Previous studies from our laboratory and others have clearly shown that exposure of mice to lopinavir/ritonavir (Kaletra®, Abbott Laboratories, Abbott Park, IL), a protease inhibitor cocktail commonly used in clinical settings to manage HIV, produces severe metabolic derangement and neurocognitive dysfunction [31,28]. To determine if elevated adiponectin can prevent protease inhibitor-induced metabolic dysfunction, a 4-week regimen of lopinavir/ritonavir or vehicle was given to 6–8 month old, male WT and adiponectin-overexpression ADTg C57BL/6 mice. Body weight and body composition measurements taken throughout treatment demonstrated that lopinavir/ritonavir administration caused modest but significant decreases in body weight in WT mice, but not in ADTg mice (Table 1). Furthermore, lopinavir/ritonavir-treated WT mice lost significant amounts of total body fat during the 4-week exposure (Table 1). Conversely, lopinavir/ritonavir-induced fat loss was attenuated in ADTg mice as compared to WT mice (Table 1), although lopinavir/ritonavir did cause modest fat loss in ADTg mice. Along with loss of adipose tissue, circulating adiponectin was significantly decreased in lopinavir/ritonavir-treated WT mice as compared to vehicle-treated mice (Table 1). However, lopinavir/ritonavir treatment did not significantly lower serum adiponectin in ADTg mice (Table 1). In light of recent data documenting adiponectin receptor expression in the brain [37], and studies indicating a role for CNS adiponectin in energy homeostasis, we evaluated the effects of lopinavir/ritonavir on cerebrospinal fluid (CSF) levels of adiponectin. As previously reported [38], CSF levels of adiponectin were considerably lower (~1000 fold) than serum levels in both WT and ADTg mice, and CSF adiponectin was significantly increased in ADTg mice as compared to WT mice (Table 1). Interestingly, however, lopinavir/ritonavir treatment did not significantly decrease CSF adiponectin in either WT or ADTg mice (Table 1), suggesting that decreased central activities of adiponectin are unlikely to participate in the metabolic effects of lopinavir/ritonavir.

To determine the effects of elevated adiponectin on lopinavir/ritonavir-induced metabolic syndrome, measures of fasting blood glucose and serum insulin were conducted at the end of the 4-week exposure period. While adiponectin overexpression did not affect glucose or insulin levels in vehicle-treated mice (Table 1), lopinavir/ritonavir treatment significantly increased both fasting blood glucose and serum insulin in WT mice, but not in ADTg mice (Table 1). Hyperlipidemia is also a well-established side-effect of protease inhibitor treatment in both mice and humans [39,31,28], and thus levels of bioactive serum lipids were documented in WT and ADTg mice following vehicle and lopinavir/ritonavir treatment. Basal levels of cholesterol in vehicle-treated mice were significantly lower in ADTg mice as compared to WT mice (Table 1). Furthermore lopinavir/ritonavir significantly increased cholesterol levels in WT mice as compared to vehicle-treated WT mice (Table 1). While lopinavir/ritonavir administration to ADTg significantly increased total cholesterol relative to vehicle, ADTg mice displayed significantly lower levels of cholesterol compared to WT mice in response to lopinavir/ritonavir (Table 1). Similarly, basal levels of triglycerides in mice were significantly lower in vehicle-treated ADTg mice as compared to vehicle-treated WT mice, and a robust, greater than 2-fold increase in serum triglycerides was detected in WT mice given lopinavir/ritonavir (Table 1). While lopinavir/ritonavir did increase triglyceride levels in ADTg mice, triglyceride levels in ADTg mice were less than half of that observed in WT mice following lopinavir/ritonavir (Table 1). Finally, levels of LDL cholesterol and circulating non-esterified fatty acids (NEFA) were not significantly affected by lopinavir/ritonavir or adiponectin (data not shown).

Table 1
Effects of lopinavir/ritonavir (L/R) on metabolic dysfunction in WT and ADTg mice. Male C57BL/6 (WT) and adiponectin transgenic (ADTg) mice were given daily administration of 10% ethanol/15% propylene glycol (vehicle) or 150 mg lopinavir/37.5 mg ritonavir/kg (L/R) for 28 days and body weight, body composition, and serum markers of metabolic syndrome were measured as described in the Materials and methods. Data are means ± S.E.M. generated from 20 to 36 mice per group, and were analyzed by 1-way ANOVA followed by planned Tukey posttests to determine the effects of L/R in WT and ADTg mice. *, **, and *** indicate statistically significant ($p < 0.05$, $p < 0.01$, $p < 0.001$, respectively) differences caused by lopinavir/ritonavir in either WT or ADTg mice, while #, ##, and ### indicate significant ($p < 0.05$, $p < 0.01$, $p < 0.001$, respectively) differences in ADTg mice as compared to identically treated WT mice.

	WT		ADTg	
	Vehicle	L/R	Vehicle	L/R
Body weight (%initial)	100.4 ± 1.4	94.1 ± 0.8**	98.1 ± 1.1	94.5 ± 0.8
% Body fat (%initial)	105.4 ± 2.1	80.6 ± 1.8***	105.2 ± 3.0	91.3 ± 4.0* #
Serum adiponectin (μg/ml)	23.8 ± 8.6	11.1 ± 1.0**	44.5 ± 8.0 ###	37.1 ± 7.0 ###
CSF adiponectin (ng/ml)	21.0 ± 4.3	23.1 ± 6.6	67.9 ± 9.9 ###	64.2 ± 9.1 ###
Fasting blood glucose (mg/dl)	155.4 ± 3.2	182.6 ± 5.2***	154.9 ± 8.9	162.4 ± 6.1 #
Fasting insulin (ng/ml)	0.567 ± 0.04	0.970 ± 0.08***	0.527 ± 0.1	0.467 ± 0.06 ###
Total cholesterol (mg/dl)	92.6 ± 2.1	117.0 ± 3.6***	80.3 ± 4.1 #	98.6 ± 4.1* ##
Triglycerides (mg/dl)	47.6 ± 3.6	107.5 ± 8.6***	31.8 ± 3.5 #	50.4 ± 6.5* ###

3.2. Cognitive performance and blood barrier integrity in lopinavir/ritonavir-treated mice

We have previously shown that chronic administration of combined lopinavir/ritonavir causes significant impairments in memory performance in mice [31,28]. To determine if elevated adiponectin can preserve cognitive function, vehicle- and lopinavir/ritonavir-treated WT and ADTg mice were evaluated using the fear conditioning assay as described in the Materials and methods. No significant differences in behavioral responses across groups were observed on day 2 (Fig 1A), indicating that all mice retained the basic memory of the conditioned context. However, differences in freezing behavior were observed on the third day of the fear conditioning test, when the "tone test" conducted in an entirely novel environment provides a measure of associative learning. Specifically, freezing behavior in response to the tone was dramatically decreased in lopinavir/ritonavir-treated WT mice as compared to vehicle-treated WT mice (Fig. 1B), suggesting impaired memory of the tone cue. However, lopinavir/ritonavir-induced memory impairment was completely prevented in ADTg mice (Fig. 1B). To ensure that intact freezing behavior in ADTg mice was not secondary to enhanced fear or anxiety responses, vehicle- and lopinavir/ritonavir-treated WT and ADTg mice were also evaluated in the Open Field maze. Neither lopinavir/ritonavir nor elevated adiponectin significantly affected overall ambulation (total distance traveled, mean and peak velocity) nor anxiety related behavior (freezing, time/entries in center of field) in the open field (data not shown), suggesting that the deficits in freezing behavior in lopinavir/ritonavir-treated WT mice reflect impaired cued-memory formation.

To determine the effects of lopinavir/ritonavir and adiponectin on the integrity of the blood brain barrier, we evaluated an additional cohort of mice with regard to brain uptake of sodium fluorescein (NaF) 30 min after intravenous injection as described in the Materials and methods. There was no intrinsic difference in blood brain barrier permeability between vehicle-treated WT and ADTg mice (Fig. 2). However, lopinavir/ritonavir significantly increased NaF transfer into the cerebral cortex of WT mice, but not ADTg mice (Fig. 2). As fluorescein is a substrate for the transport proteins OAT-3 and MRP2 [40,41], it is possible that ritonavir-induced MRP2 inhibition [42,43] facilitated brain uptake of NaF in lopinavir/ritonavir-treated mice. However, lopinavir/ritonavir-induced NaF brain uptake was prevented in treated ADTg mice, and there is no evidence that adiponectin directly affects the activity of any ATP-dependent drug efflux pump and no difference in NaF uptake between vehicle-treated WT and ADTg mice. Thus, these data suggest that lopinavir/ritonavir disrupts, while adiponectin preserves, the integrity of the blood brain barrier in mice.

3.3. Markers of cerebrovascular and brain injury in lopinavir/ritonavir-treated mice

In light of the observed detrimental effects of lopinavir/ritonavir on blood brain barrier permeability in WT mice, markers of cerebrovascular and blood brain barrier homeostasis were evaluated in brain tissues. Histopathological manifestations representative of leukoaraiosis were measured in Luxol fast blue (LFB)-stained tissue sections, as loss of LFB staining in the corpus callosum and other white matter tracks has been shown to be consistent with the MRI findings of white matter inflammatory lesions [44]. There were no baseline differences in overall LFB staining intensity of vehicle-treated WT and ADTg mice (Fig. 3A and B). However, lopinavir/ritonavir significantly decreased LFB intensity in the anterior corpus callosum of WT mice, but not ADTg mice (Fig. 3A

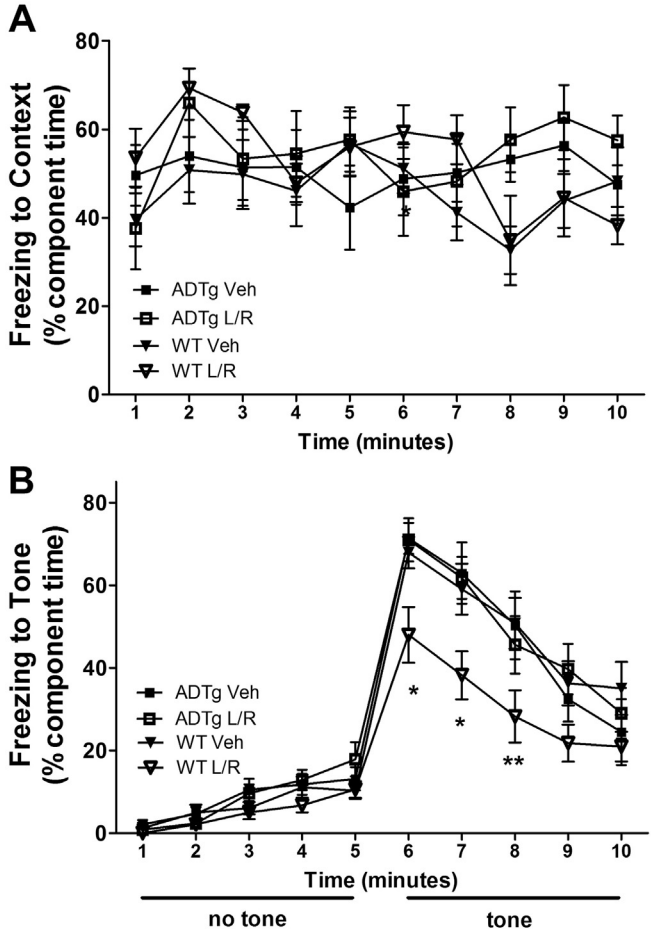

Fig. 1. Elevated adiponectin preserves cognitive function in mice following exposure to lopinavir/ritonavir. Male C57BL/6 (WT) and adiponectin transgenic (ADTg) mice were given daily administration of 10% ethanol/15% propylene glycol (vehicle) or 150 mg lopinavir/37.5 mg ritonavir/kg (L/R) for 28 days, after which mice were evaluated for memory performance using the fear conditioning assay as described in the Materials and methods. Freezing behavior was recorded on day 2 (A) and day 3 (B) of the 3-day test to document memory of the context and the conditioned auditory cue, respectively. Experiments were conducted in 12–20 animals per group over 2 separate cohorts. Data are means ± S.E.M. of composite freezing behavior, and were analyzed by 2-way ANOVA. *and ** indicate significant ($p < 0.05$, $p < 0.01$, respectively) decreases in freezing behavior following the conditioned stimulus (tone cue) in lopinavir/ritonavir-treated WT mice as compared to vehicle-treated WT mice.

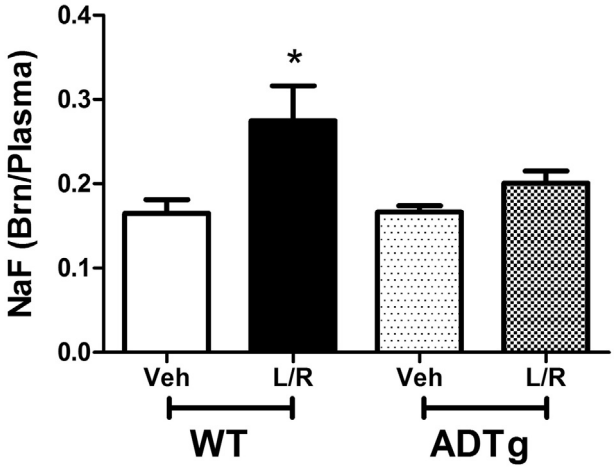

Fig. 2. Elevated adiponectin prevents blood–brain barrier permeability in mice following exposure to lopinavir/ritonavir. Male C57BL/6 (WT) and adiponectin transgenic (ADTg) mice were given daily administration of 10% ethanol/15% propylene glycol (vehicle) or 150 mg lopinavir/37.5 mg ritonavir/kg (L/R) for 28 days, after which mice sodium fluorescein (NaF) was administered intravenously, and NaF levels in brain and plasma were determined after 30 min as described in the Materials and methods. Data are means ± S.E.M. of NaF expressed as the ratio of NaF in brain over plasma levels, were generated from 7 to 10 mice per group, and *indicates significantly ($p < 0.05$) increased NaF partition into brain fractions from lopinavir/ritonavir-treated WT mice as compared to vehicle-treated WT mice.

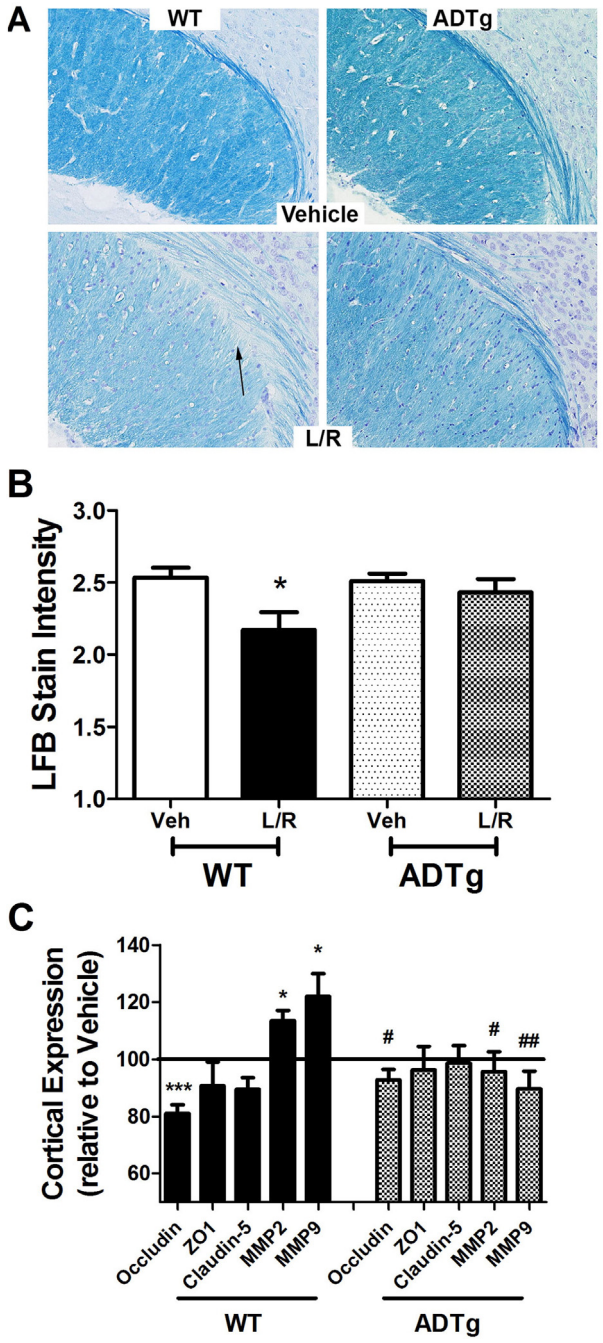

Fig. 3. Elevated adiponectin preserves cerebrovascular homeostasis in mice following exposure to lopinavir/ritonavir. Male C57BL/6 (WT) and adiponectin transgenic (ADTg) mice were given daily administration of 10% ethanol/15% propylene glycol (vehicle) or 150 mg lopinavir/37.5 mg ritonavir/kg (L/R) for 28 days, after which markers of cerebrovascular integrity were evaluated in tissue prepared from the frontal cortex as described in the Materials and methods. (A) Representative images of Luxol fast blue (LFB) staining in anterior genu of the corpus callosum of lopinavir/ritonavir- and vehicle-treated WT and ADTg mice from which quantitative measures were drawn. The arrow highlights decreased LFB intensity in WT mice following treatment with lopinavir/ritonavir. (B) Quantification of LFB intensity in the corpus callosum of WT and ADTg mice. Data are means ± S.E.M. of LFB staining expressed as the ratio of pixel intensity in the genu of the corpus callosum relative to background staining in the stratum oriens of the hippocampus (CA1). Data were generated from 9 to 13 mice per group, and *indicates significantly ($p < 0.05$) decreased LFB intensity in the corpus callosum in lopinavir/ritonavir-treated WT mice as compared to vehicle-treated WT mice. (C) Expression of the tight junction proteins occludin, ZO-1, and claudin-5; and the matrix metalloproteinases MMP2 and MMP9. * and *** indicate significant ($p < 0.05$ and 0.001, respectively) changes in expression in lopinavir/ritonavir-treated WT mice as compared to vehicle, while # and ## depict significant ($p < 0.05$, $p < 0.01$, respectively) changes in expression in lopinavir/ritonavir-treated ADTg mice as compared to lopinavir/ritonavir-treated WT mice.

and B). Disruption to cerebrovascular homeostasis was further evaluated by measuring the expression of tight junction proteins ZO-1 and occludin, as well as the matrix metalloproteinases MMP2 and MMP9. All Western blot analyses were conducted using brain tissue isolated from the frontal cerebral cortex as cortical injury has been shown to be caused by PI exposure in mice [28], and also to perturb performance in the fear conditioning task in rodents [45]. Data show that lopinavir/ritonavir administration to WT mice significantly decreased expression of occludin and increased expression of MMP2 and MMP9 (Fig. 3C). Conversely, lopinavir/ritonavir treatment did not affect expression of tight junction proteins or matrix metalloproteinases in ADTg mice (Fig. 3C).

Analyses of inflammation/reactive gliosis and synaptic density in vehicle and lopinavir/ritonavir-treated mice were based on quantification of expression of specific brain proteins by Western blot, as described previously [28]. The expression of astrocyte/microglial markers and toll-like receptors (TLR2 and TLR4) was evaluated to determine the effects of elevated adiponectin on lopinavir/ritonavir-induced inflammation and reactive gliosis. Lopinavir/ritonavir treatment significantly increased the expression of astrocytic glial fibrillary acidic protein (GFAP) and microglial/macrophage Iba-1 in WT but not ADTg mice (Fig. 4A). Likewise, expression of TLR2 and TLR4 was increased in WT mice following lopinavir/ritonavir administration, but not ADTg mice (Fig. 4A). Deficits in synaptic density were based on decreased expression of the post-synaptic protein synapse associated protein 97 (SAP97) and total and phosphorylated forms of the pre-synaptic protein synapsin 1 (SYN1). Expression of SAP97 and total SYN1 was similar in all groups (Fig. 4B), but phosphorylated SYN1 expression was significantly reduced in WT mice treated with lopinavir/ritonavir (Fig. 4B). However, SYN1 phosphorylation was completely preserved in ADTg mice given lopinavir/ritonavir (Fig. 4B).

4. Discussion

Data in this manuscript show that elevated adiponectin prevents metabolic and neurologic dysfunction caused by HIV protease inhibitors in mice. Specifically, adiponectin overexpression diminished lopinavir/ritonavir-induced loss of subcutaneous adipose, prevented hyperinsulinemia, hypertriglyceridemia, and hypoadiponectinemia; and preserved cognitive function and cerebrovascular/brain integrity. Collectively, these data suggest that ART-induced lipodystrophy and the resulting hypoadiponectinemia may drive metabolic dysfunction in people living with HIV/AIDS, which could then facilitate the development of HIV-associated neurocognitive disturbances in these individuals. This scenario is in overall agreement with clinical studies indicating a role for lipodystrophy in metabolic complications and the development of cardiovascular/hepatic injury and premature aging in people living with HIV/AIDS [46]. Furthermore, increased neurologic complications are well-established in HIV patients with metabolic compromise [7–9,47], and HIV-associated neurocognitive disorders have been shown to correlate with cardiovascular disease, hypertension, and cholesterolemia; but not with CD4 cell counts, viral load, CNS penetration of ART, hepatitis C infection, or alcohol abuse [48]. As recent clinical trials using neuroprotective or anti-inflammatory drugs for treatment of HIV-associated neurocognitive disorders have generally proven unsuccessful [49], there is a critical need to develop novel and innovative therapies to preserve neurologic function in HIV patients. Data in this manuscript raise the exciting possibility that preservation of circulating levels of adiponectin could not only mitigate key aspects of ART-induced metabolic syndrome but also decrease the incidence and/or severity of HIV-associated neurocognitive disorders.

Since the widespread availability of ART, HIV-related mortality has been reduced by 50–80% [50]. However, individuals taking ART face a disproportionate risk of developing of metabolic disturbances, including lipodystrophy (fat loss and/or redistribution), hypertriglyceridemia/hypercholesterolemia, and insulin resistance; most notably

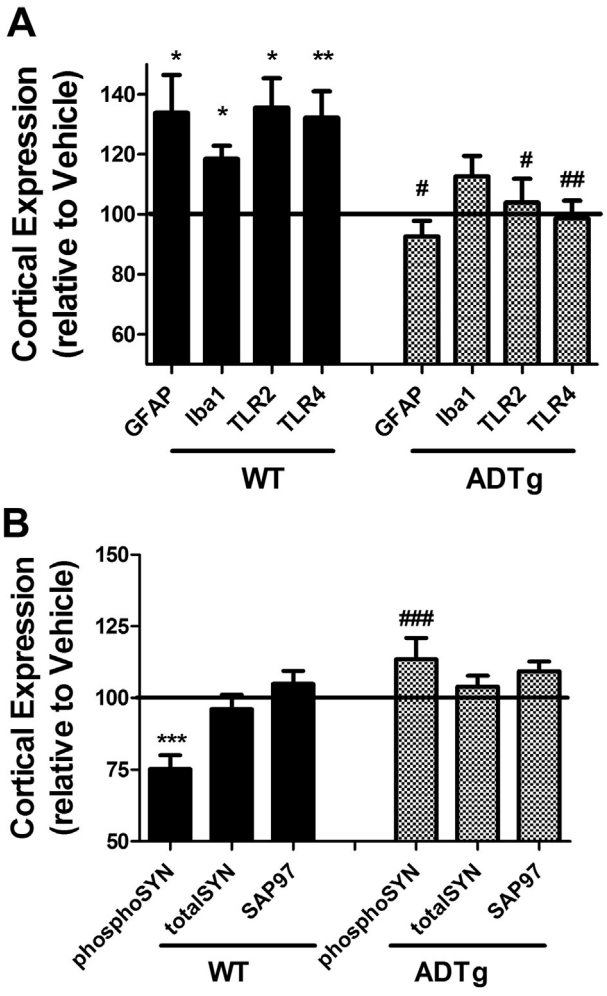

Fig. 4. Elevated adiponectin prevents brain inflammation and preserves synaptic density in mice following exposure to lopinavir/ritonavir. Male C57BL/6 mice were treated daily with vehicle or lopinavir/ritonavir (150/37.5 mg/kg body weight) for 28 days, after which markers of inflammation/reactive gliosis and synaptic density, and were evaluated in tissue homogenates prepared from the frontal cortex as described in the Materials and methods. Data depict mean ± SEM expression in lopinavir/ritonavir-treated mice presented as % vehicle (100% line) on graph. Data were obtained from 12 to 20 mice/group, and were analyzed by 1-way ANOVA. (A) Expression of the glial markers GFAP and Iba-1 and toll-like receptors (TLR2 and TLR4). * and ** indicate significant ($p < 0.05$, $p < 0.01$, respectively) increases in markers of inflammation in WT mice treated with lopinavir/ritonavir relative to vehicle-treated mice, while # and ## depict the significant reversal in GFAP, TLR2, and TLR4 expression in lopinavir/ritonavir-treated ADTg mice. (B) Expression of the post-synaptic marker synapse associated protein 97 (SAP97), the pre-synaptic protein synapsin 1, and phosphorylated synapsin 1. *** indicates the significant ($p < 0.001$) decrease in phosphorylated synapsin 1 expression in lopinavir/ritonavir-treated WT mice relative to vehicle-treated WT mice, while ### depicts the significant ($p < 0.001$) reversal in phosphorylated synapsin 1 expression in lopinavir/ritonavir-treated ADTg mice as compared to lopinavir/ritonavir-treated WT mice.

with regimens containing HIV protease inhibitors and/or nucleoside reverse transcriptase inhibitors [51,52,2]. While our data show that combined lopinavir/ritonavir treatment reliably reproduces this pattern of lipodystrophy and metabolic impairment in mice [28,32], whether lopinavir, ritonavir, or the combination of both drives metabolic impairment it not known. Ritonavir is included in many ART regimens because its inhibition of hepatic cytochrome P450-3A4 profoundly boosts the action of co-administered drugs [53]. However, adverse effects related to altered metabolism of medications and/or food additives are also possible, and it is thus possible that ritonavir-mediated disruption of the metabolism of the ethanol and/or propylene glycol components of the vehicle, or even bile acid metabolism [54], could participate in some of the observed metabolic effects. Regardless, the incidence of ART-induced metabolic complications is very likely to increase given the increased availability of ART worldwide and the increasing number and lifespan of people living with HIV and AIDS. Collectively, these trends reflect a growing public health crisis, as metabolic syndrome disrupts homeostasis and increases inflammation, and puts particularly aging HIV-infected patients at a higher risk of cardiovascular disease, frailty, and neurocognitive impairment even in the context of sustained viral suppression [46,55]. Thus, these metabolic co-morbidities must be clinically remediated to preserve healthspan and maintain self-care independence for people living with HIV/AIDS. While the exact sequela of ART-induced metabolic dysfunction is not completely clear, clinical and experimental data suggests that lipodystrophy might be an initiating factor, precipitating the further development of insulin resistance and dyslipidemia [56,3,57]. HIV-associated lipodystrophy is generally described as a combination of fat loss in limbs and central fat accumulation, representing a heterogeneous group of disorders typified by abnormal adipose tissue distribution, utilization, and metabolism [58]. The underlying mechanism whereby lipodystrophy precipitates systemic metabolic decline is likely follow loss of key aspects of adipocyte function, specifically loss of insulin sensitivity and derangements in adipokine release. Thus, therapies that preserve adipocyte health and/or maintain adipokine levels in the context of lipodystrophy could strategically preserve overall health and function in HIV patients.

Adiponectin levels are decreased in HIV-positive patients with lipodystrophy [25,3,20] and correlation inversely with insulin resistance [59], indicating that adiponectin could be a key therapeutic target in HIV patients. Data in this manuscript show that elevated adiponectin partially preserved adipose tissue depletion in HIV protease inhibitor-treated mice, and that this partial preservation of adipose was associated with essentially complete preservation of glucose/insulin homeostasis and circulating lipids. Furthermore, elevated adiponectin completely prevented neurologic decline in lopinavir/ritonavir-treated mice, supporting existing data showing that adiponectin receptor signaling can support neurologic health [60–62,20,21]. As adiponectin was detected in CSF, protective actions on neurologic function could include direct actions on neurons. While the exact role of adiponectin in the brain is still subject to debate, adiponectin receptors are expressed in brain cells [37,63,64], and peripheral adiponectin has been shown to activate signaling pathways in the hypothalamus [65,66]. However, our data indicate that CSF levels of adiponectin were not significantly affected by lopinavir/ritonavir treatment (Table 1), suggesting that loss of central adiponectin is unlikely to participate in the detrimental effects of lopinavir/ritonavir. Conversely, it is highly possible that elevated circulating adiponectin could indirectly improve CNS health by mitigating metabolic dysfunction and/or preserving vascular homeostasis [67,68]. Indeed, our data show preservation of blood–brain barrier integrity and cerebrovascular homeostasis in mice with elevated adiponectin, which is consistent with multiple lines of evidence linking adiponectin to microvascular health [22,69]. Based on these data, one can hypothesize that lipodystrophy contributes to HIV-related brain injury by undermining cerebrovascular health and blood brain barrier integrity. In support of this hypothesis, published studies show a strong relationship of cardiovascular disease to neurocognitive impairment in HIV patients [47,70], and indeed show that cognitive impairment correlates better with cardiovascular disease indices than with conventional risk factors for dementia, including hepatitis C infection, alcohol abuse, CD4 cell counts, or viral load [48]. In terms of potential mechanisms, data indicate that lipodystrophy/lipoatrophy in HIV patients drives vascular aging, including endothelial inflammation, carotid intima thickness, arterial stiffness, and vascular smooth muscle calcification and senescence [71–73]. Vascular aging involving arterial stiffness, endothelial changes and blood–brain barrier dysfunction can drive neuronal injury via chronic hypoperfusion and loss of neurovascular coupling [74–76]. While lipodystrophy could accelerate vascular aging through multiple mechanisms, adiponectin deficiency is known to increase inflammation and endothelial dysfunction [77–79]. Likewise,

adiponectin deficiency results in increased brain infarctions and neurological deficits following ischemia/reperfusion, while adiponectin replacement reduces cerebral infarction size in both wild-type and adiponectin-deficient mice [80]. Evaluated collectively, these data indicate that the neurologically damaging effects of HIV lipodystrophy and the protective effects of adiponectin could intersect at the cerebrovascular compartment. This physiologic scenario is especially significant because it suggests that attenuation of HIV-associated neurocognitive disorders could be achieved with drugs that need not penetrate into the brain.

This manuscript complements a growing body of literature providing proof of concept data on the benefit of adiponectin replacement therapy in multiple disease states. For example, injection of recombinant forms of adiponectin has been reported to improve insulin sensitivity and alleviate hyperlipidemia [81], as well as preserve metabolic function in experiential models of high-fat/sucrose diets [67], leptin deficiency [82], and ritonavir-induced hyperlipidemia in mice [29]. A critical caveat to these studies, however, is whether or not recombinant adiponectin is able to recapitulate the physiologic actions of endogenous adiponectin, which is released into the circulation as full-length trimers, hexamers, and high-molecular weight multimers. As the metabolic stabilizing effects of adiponectin are generally attributed to the high molecular weight species [83,84], it is likely that the lack of multimerization and/or post-translational modifications of bacterially produced adiponectin compromises its in vivo efficacy. Overall, the potential for a safe and effective adjunctive therapy to reduce the prevalence/severity of HIV-associated neurocognitive decline would be tremendously beneficial in clinical settings, as essentially all anti-inflammatory and/or neuroprotective agents that have been investigated as potential adjuvant therapies have proven ineffective in clinical trials [49]. Thus, these data provide strong support for the development of adiponectin-based therapies as adjunctive regimens to bolster both metabolic and neurologic function in clinical settings of HIV infection.

Transparency Document

The Transparency document associated with this article can be found, in the online version.

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