Original article

HIV protease inhibitors activate the adipocyte renin angiotensin system

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Background: HIV-infected patients under antiretroviral therapy that includes HIV protease inhibitors (PIs) are prone to develop a complex metabolic syndrome including insulin resistance, lipodystrophy and hypertension. Whether hypertension and cardiovascular events could result from the adipocyte renin angiotensin system (RAS) overactivation has never been investigated.
Methods: Primary human adipocytes and 3T3-F442A murine adipocytes were incubated with lopinavir or atazanavir boosted with ritonavir, with or without the angiotensin II type-1 receptor (AT1R) blockers (ARBs), irbesartan or telmisartan, and the peroxysome proliferator-activated receptor-γ (PPAR-γ) regulators, rosiglitazone and GW9662. Adipose RAS activation and adipocyte functions were evaluated.
Results: The ritonavir-boosted PIs activated the adipose RAS in human and murine adipocytes as shown by the overexpression of AT1R protein, angiotensinogen messenger RNA and the amplified effect of angiotensin II on extracellular signal-regulated kinase 1/2 activity. ARBs prevented the PI effect on RAS activation (AT1R overexpression and signalling) and adipocyte functions (dedifferentiation, insulin resistance, oxidative stress and inflammation). Consistent with a role of PPAR-γ signalling in PI-induced RAS activation, the PPAR-γ agonist (rosiglitazone) normalized PI-induced AT1R overexpression and adipocyte dysfunction. Conversely, the PPAR-γ antagonist (GW9662) induced AT1R overexpression and reduced the beneficial effect of telmisartan on PI toxicity.
Conclusions: We report that two frequently prescribed PI combinations could activate the adipose RAS in cultured cells, in part through a PPAR-γ-dependant signalling pathway. Our data suggest a role for the adipose RAS in the development of hypertension in HIV-infected patients under PI treatment, and point out the potential use of ARBs to decrease PI adverse effects.

Introduction

Antiretroviral drug therapy has led to a major reduction in AIDS-related morbidity and mortality, turning HIV infection into a chronic disease in western countries. However, a lipodystrophic syndrome characterized by fat tissue dysfunction, metabolic complications and inflammation occurs in a large proportion of HIV-infected patients receiving antiretroviral therapy [1,2]. These disorders are associated with an increased risk of coronary heart disease [2,3] and hypertension [4–8]. HIV treatment with protease inhibitors (PIs) is independently associated with hypertension [5,7], insulin resistance [6] and lipodystrophy [7]. Cardiovascular events are now the third most common cause of death among HIV-infected patients in the US [9]. Understanding the pathophysiology of these alterations has become of primary importance.

It has been largely reported in non-HIV-infected patients that adipose tissue dysfunction, through its altered distribution and increased secretion of free fatty acids and adipokines, contributes to the pathogenesis of insulin resistance, endothelial dysfunction and the occurrence of a proinflammatory state, all of which promote progression of atherosclerosis [10,11] and hypertension [12]. The specific adipose renin angiotensin system (RAS) [13] is suspected to contribute to these metabolic and cardiovascular disorders [14–17]. RAS components produced by adipocytes (particularly angiotensin II) might be involved in adipose tissue

disorders through autocrine or paracrine mechanisms, and could represent a pathway through which lipodystrophy could be associated with hypertension [18].

Besides the ability of the angiotensin II type-1 receptor (AT1R) blockers (ARBs) to inhibit angiotensin II signalling through AT1R blockade, lipophylic ARBs are suspected to improve insulin resistance and hypertension through partial peroxisome proliferator-activated receptor-γ (PPAR-γ) agonistic properties [19–21]. The central role of PPAR-γ in blood pressure control is illustrated by the severe hypertension observed in lipodystrophic patients with PPAR-γ loss-of-function mutations [22–24] and by the deregulation of AT1R expression [25,26]. With regards to HIV antiretroviral therapy, *in vitro* and *ex vivo* studies have shown that PIs can induce PPAR-γ deficiency and deregulate adipocyte lipid storage and adipokine secretion [27–32]. Thus, PI-induced PPAR-γ dysfunction in adipose tissue might directly or indirectly participate in hypertension and cardiovascular disease occurring in HIV-infected patients on PI therapy.

To date, the involvement of the adipose RAS in complications related to antiretroviral therapy has not been evaluated. Therefore, we first determined *in vitro* the effects of PIs (lopinavir and atazanavir, both combined with ritonavir) on the adipose RAS in cultured adipocytes. Then, we tested the potential protective capacity of two ARBs (irbesartan and telmisartan) on PI-induced RAS activation. We also investigated whether defective PPAR-γ signalling might play a role in PI-induced RAS activation and adipocyte dysfunction.

Methods

Cell culture and treatment

Human subcutaneous preadipocytes from healthy participants (body mass index <25 kg/m^2; SP-F-1; ZenBio, Research Triangle Park, NC, USA) and murine 3T3-F442A preadipocytes were cultured and differentiated as previously described [27,29]. Once differentiated, human and murine adipocytes were incubated with the PIs and ARBs, as indicated, for 5 days. The PIs were used at concentrations close to their maximum concentration (C_{max}), that is, lopinavir 10 μM and atazanavir 5 μM, combined with a low concentration of ritonavir (2 μM), as described elsewhere [33]. Lopinavir and ritonavir were provided by Abbott Laboratories (Rungis, France). Atazanavir was provided by S Azoulay (Laboratoire de Chimie des Molécules Bioactives et Aromatiques UMR 6001, Université Nice Sophia Antipolis, Nice, France). The ARBs, irbesartan and telmisartan were used at 10 μM and 1 μM, respectively. Irbesartan (SR 47436) was provided by Bristol–Myers Squibb (Rueil Malmaison, France) and Sanofi-Synthélabo Recherche (Montpellier, France), and telmisartan (BIBR 277SE) by Boehringer Ingelheim Pharma GmbH & Co., KG (Ingelheim am Rhein, Germany). Murine adipocytes were treated with the PPAR-γ agonist rosiglitazone (10 μM) or the PPAR-γ antagonist GW9662 (1 μM), concomitantly with the antiretrovirals and/or ARBs for 5 days and 1 day, respectively. Treatment of GW9662-treated cells with antiretrovirals and/or ARBs was continued for 4 days. Rosiglitazone was provided by A Quignard-Boulange (Centre Biomedical des Cordeliers, INSERM U671-IFR58, Paris, France) and GW9662 by Sigma–Aldrich (Saint Louis, MO, USA).

Western blotting

Cell extracts, prepared as previously described [27], and 24-h culture supernatants were subjected to SDS-PAGE and western blotting. We used antibodies against AT1R (SC-1173), extracellular signal-regulated kinase (ERK) 1/2 (ERK 2, SC-154; p-ERK, SC-7383), PPAR-γ (SC-7196), and CAAT/enhancer binding protein-α (C/EBP-α, SC-61) from Santa Cruz Biotechnology (Santa Cruz, CA, USA). An antibody against adiponectin (MA1-054) was obtained from Affinity BioReagents (Golden, CO, USA). β-actin (A-5441; Sigma–Aldrich) or ERK 1/2 were immunoprobed as an index of the cellular protein content. Immune complexes were detected with a chemiluminescence kit (GE Healthcare, Saclay, France). Gel quantification was performed with the ChemiGenius2 image analyser and software (Ozyme, Saint Quentin en Yvelines, France).

Adipose RAS activation assays

Adipose RAS activation was evaluated by the effect of angiotensin II on the activation of ERK 1/2 [34]. Adipose cells in 6-well plates were depleted of fetal calf serum (FCS) for 16 h and stimulated with angiotensin II (100 nM) for 10 min. Whole cell lysates were prepared and submitted to SDS-PAGE and western blotting with antibodies against the total and activated forms of ERK 1/2. Signal was quantified by scanning densitometry.

Real-time PCR and reverse transcriptase PCR

The messenger RNA (mRNA) expression of angiotensinogen was determined using real-time PCR. The ribosomal 36B4 mRNA was used as an internal control. Total RNA was extracted using the RNeasy® mini kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer instructions. The complementary DNA was synthesized from a 1 μg sample of total RNA by using avian myeloblastosis virus reverse transcriptase (Promega Biosciences, San Luis Obispo, CA, USA). PCR reactions were carried out on a LightCycler® 2.0 system using FastStart DNA Master SYBR Green I fluorophore (Roche Diagnostics, Meylan, France) and mouse-specific primers. Angiotensinogen (NM_007428) forward

5′-AGGCGGGTTCTCTATCCAAG-3′ and reverse 5′-TGGAGTGACACCCAGAACAA-3; and 36B4 (NM_00747) forward 5′-GAGGAATCAGATGAGGA TATGGGA-3′ and reverse 5'-AAGCAGGCTGACTT GGTTGC-3'. The reverse transcriptase PCR products were analysed by electrophoresis through 2% agarose gel containing ethidium bromide. Gene expression was quantified using the comparative Ct method. All samples were run in triplicate. Results were normalized to 36B4 mRNA and expressed as percentage ±SEM of untreated control values.

Adipose cell dysfunction

Adipose cell dysfunction was evaluated by several means, including the alteration of the adipose cell differentiation status and response to insulin, and the presence of an oxidative stress and an inflammatory state, all events that are associated with lipodystrophy and diabetes [22] and linked with RAS overactivation [35].

Adipose cell differentiation was evaluated by Oil Red O staining [27], and by the protein expression on western blot of the adipogenic transcription factors PPAR-γ and C/EBP-α. Adiponectin protein expression was used as a marker of adipocyte differentiation and insulin sensitivity.

Insulin response was assessed by measuring the effect of insulin on glucose transport and lipogenesis in human and murine adipocytes incubated for 16 h in FCS-free culture medium. Glucose transport was measured by incubating the cells for 2 h in glucose transport solution (pH 7.6) containing HEPES 12.5 mM, NaCl 120 mM, KCl 5 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 1 mM, $NaHPO_4$ 1 mM, sodium pyruvate 2 mM and bovine serum albumin 2%. Insulin (100 nM) and a mix containing 0.2 mM 2-deoxy-glucose and 9.25 kBq 2-deoxy-D-[1-^{14}C]glucose (2.2 GBq/mmol, GE Healthcare) were added successively for 30 min and 5 min. Cells were washed with phosphate-buffered saline, solubilized for 30 min with 0.1% SDS, and counted. Insulin stimulation was expressed as the percentage ±SEM of the basal value.

Lipogenesis was assessed by measuring [U-^{14}C]glucose incorporation into lipids in FCS-starved cells. Insulin (100 nM) was added for 30 min. After a further 30 min incubation with 18.5 kBq/well of [U-^{14}C]glucose (11.2 GBq/mmol, GE Healthcare), the cells were harvested and labelled lipids were extracted with chloroform/methnol (1/2). After 30 min on ice, the lower phase was collected, dried by evaporation, and counted in scintillation fluid. Insulin stimulation was expressed as the percentage ±SEM of the basal value.

The production of reactive oxygen species (ROS) was assessed by measuring the oxidation of the CM-H_2DCFDA derivatives (5-[and 6]-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester, C6827; Molecular Probes, Eugene, OR, USA), and the reduction of nitroblue tetrazolium (NBT; Sigma–Aldrich) [36]. Cells were cultured and differentiated in 96-well plates, washed and incubated with CM-H_2D-CFDA (9 μM) or Hoechst 33258 (0.01 μg/ml; Sigma–Aldrich) for 20 min at 37°C in the dark. Fluorescence was quantified on a SPECTRA Fluor Plus plate reader (Tecan, Trappes, France) at 520 nm (CM-H_2DCFDA) and 460 nm (DNA). The results were expressed relative to the cellular DNA content and expressed as means ±SEM. Reduction of NBT was measured in murine or human adipocytes incubated for 90 min in culture medium containing 0.2% NBT. Dark-blue reduced NBT, dissolved in DMSO, was assessed at 560 nm. The results were normalized to the protein content and expressed as a percentage ±SEM of the basal value.

Interleukin (IL)-6 and monocyte chemotactic protein (MCP)-1 secretion was analysed in 24 h culture supernatant by the Luminex™ technology using a Procarta™ human analyte assay kit (Panomics, Ozyme) with the Bioplex 200 system (BioRad Laboratories, Inc., Marnes la Coquette, France). Acquired fluorescence was analysed by the Bio-Plex Manager 4.1 software (BioRad Laboratories). IL-6 and MCP-1 sensitivities of the tests were 0.05 pg/ml and 0.1 pg/ml, respectively. Results were normalized to the cellular protein level evaluated by the BioRad protein assay (BioRad Laboratories) in the corresponding culture well.

Statistical analyses

The experiments were repeated 3–10 times. Results are expressed as means ±SEM, and statistical significance was determined using ANOVA and the Kruskal–Wallis non-parametric test, followed by a Fisher protected least significant difference test for pairwise differences. *P*-values <0.05 were considered significant. Statistical analyses were carried out with StatView SAS software (version 5.0; SAS Institute, Inc., Cary, NC, USA).

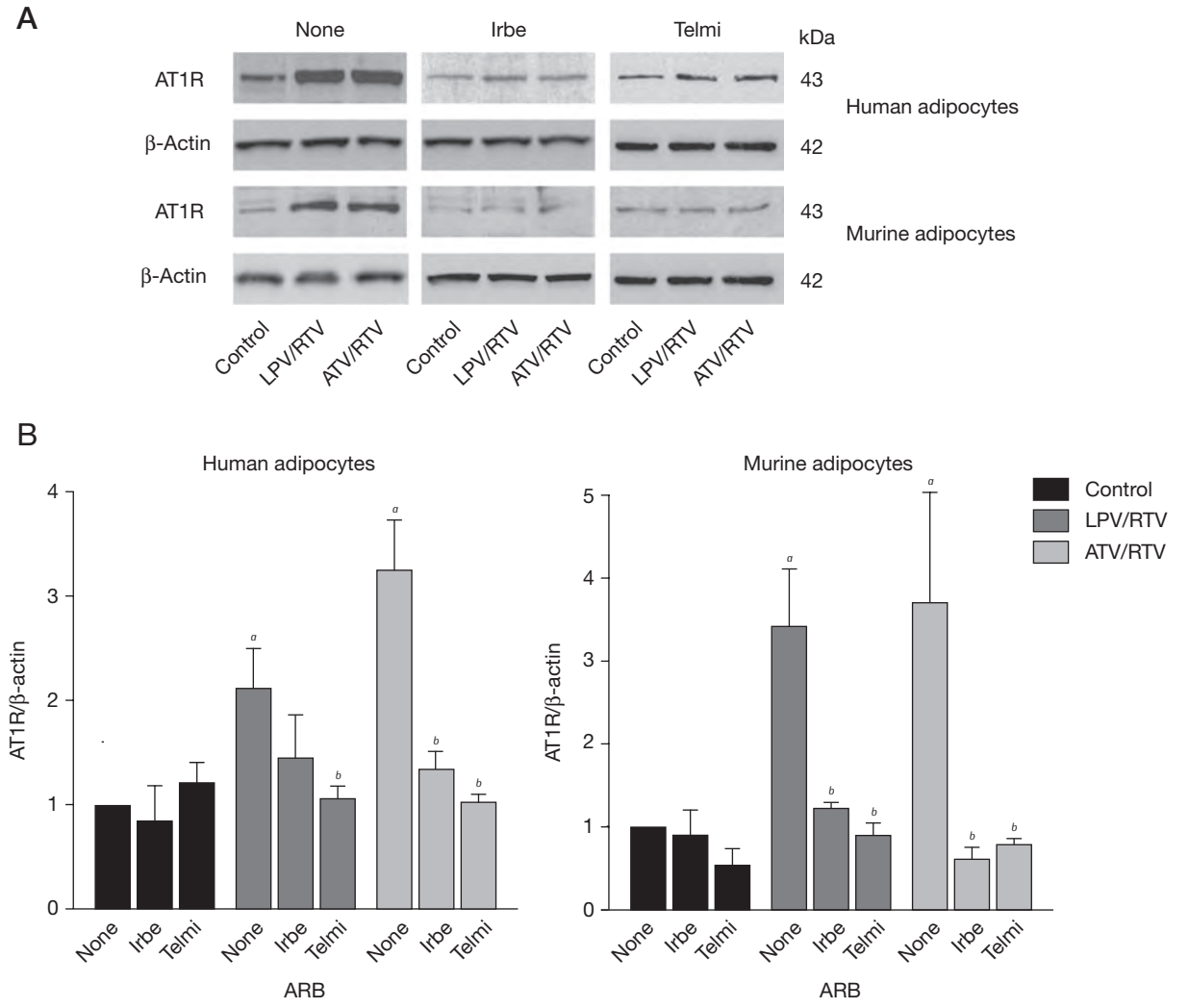
Results

PIs upregulate AT1R protein expression and induce adipose RAS activation

A 5-day treatment of human or murine adipocytes with lopinavir/ritonavir or atazanavir/ritonavir markedly increased (by 2–4-fold) AT1R protein expression (Figure 1A). Irbesartan and telmisartan, prevented PI-induced AT1R overexpression in both cell types, although they had no intrinsic effect (Figure 1A and 1B).

RAS activation was measured by the acute effect of angiotensin II on ERK 1/2 activation [37] and by the mRNA expression of angiotensinogen [16] (Figure 2). Angiotensin II (100 nM) induced a time-dependant increase in ERK 1/2 phosphorylation (FB

Figure 1. Protease inhibitors alter AT1R protein expression



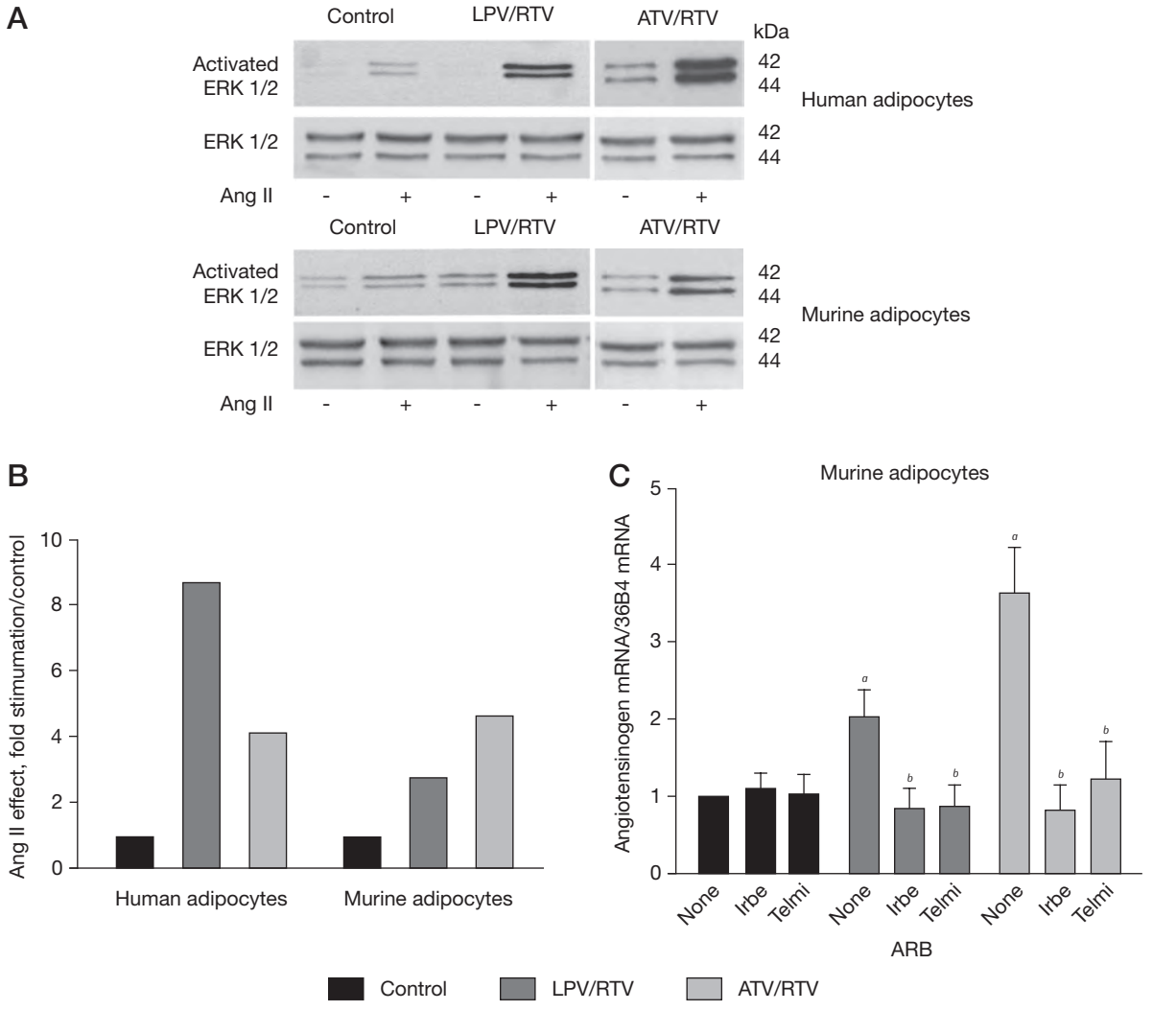
Human and murine adipocytes were treated with the protease inhibitors, lopinavir (LPV) or atazanavir (ATV) associated with ritonavir (RTV), in the presence or absence of irbesartan (Irbe) or telmisartan (Telmi) for 5 days. **(A)** Human or murine adipose cell lysates were subjected to SDS-PAGE and western blotting and revealed with antibodies directed against angiotensin II type-1 receptor (AT1R). β-Actin was used as an index of the cellular protein level. Representative blots (performed in triplicate) are shown. **(B)** The signals were quantified by scanning densitometry and expressed as means ±SEM relative to β-actin. $^{a}P<0.05$ versus control (untreated cells). $^{b}P<0.05$ versus the respective protease-inhibitor-treated cells without AT1R blockers (ARBs).

et al., data not shown), the maximal effect (2.5- and 4.5-fold increase in human and murine adipocytes, respectively) being obtained at 10 min (Figure 2A). Exposure of the cells for 5 days with lopinavir/ritonavir and with atazanavir/ritonavir markedly increased the acute effect of angiotensin II on ERK 1/2 activity in both human and murine adipocytes (by 2-8 fold; Figure 2A), a finding consistent with PI-induced adipose RAS activation. The two PI combinations also increased the mRNA expression of angiotensinogen, an effect that was markedly prevented by irbesartan and telmisartan (Figure 2B).

RAS blockers prevent PI-induced adipocyte dysfunction

AT1R overexpression and adipose RAS activation have been associated with various fat tissue diseases, including lipodystrophy and obesity [38,39]. We thus evaluated whether the ARBs that block PI action on RAS activation (Figures 1 and 2) could also prevent PI action on adipose cell differentiation and functions. As expected from our previous studies [40], the PI combinations lopinavir/ritonavir and atazanavir/ritonavir induced adipose cell dedifferentiation in human (Figure 3) and murine (Figure 4) adipocytes. The two PI combinations decreased cell lipid level by 40–60% (Figures 3A and 4A) and decreased

Figure 2. Protease inhibitors induce renin angiotensin system activation



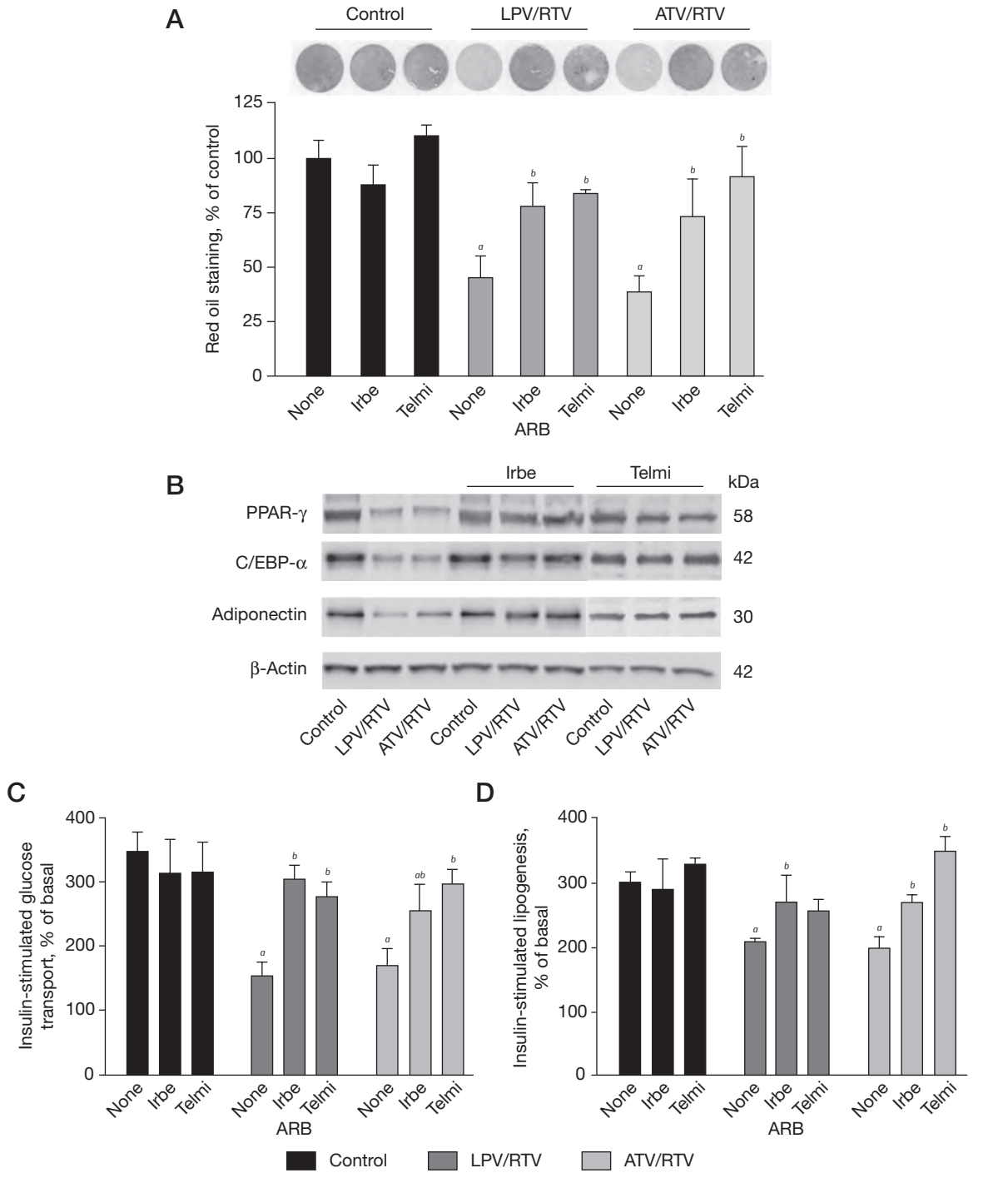
Human and murine adipocytes were incubated with lopinavir (LPV)/ritonavir (RTV) or atazanavir (ATV)/RTV for 5 days. **(A)** Cells were depleted of fetal calf serum for 16 h and stimulated with angiotensin II (Ang II; 100 nM) for 10 min. Cell lysates were prepared and submitted to SDS-PAGE and western blotting with antibodies against the total or activated forms of extracellular signal-regulated kinase (ERK) 1/2. Representative blots (performed in triplicate) are shown. **(B)** The signals were quantified, normalized to total ERK 1/2 and expressed relative to the Ang II effect in untreated cells. **(C)** Messenger RNA (mRNA) expression of angiotensinogen was evaluated by real-time reverse transcriptase PCR and expressed relative to 36B4 mRNA. The results are means ±SEM. $^{a}P<0.05$ versus control (untreated cells). $^{b}P<0.05$ versus the respective protease-inhibitor-treated cells without angiotensin II type-1 receptor blocker (ARBs). Irbe, irbesartan; Telmi, telmisartan.

protein expression of PPAR-γ, C/EBP-α and adiponectin (Figure 3B; FB *et al*., data not shown). Adipocyte response to insulin was also altered by the PIs (Figures 3 and 4). Lopinavir/ritonavir and atazanavir/ritonavir markedly decreased insulin activation of glucose transport and lipogenesis in human (Figure 3C and 3D) and murine (Figure 4B; FB *et al*., data not shown) adipocytes.

The ARBs, irbesartan and telmisartan, had no intrinsic effect on lipid storage, protein expression of adipose cell differentiation markers, or insulin response in human (Figure 3A, 3B, 3C and 3D; FB *et al*., data not shown) or murine (Figure 4A and 4B) adipocytes. However, irbesartan and telmisartan partially or totally prevented the PI adverse effects on lipid accumulation and insulin response in human (Figure 3) and murine (Figure 4) adipocytes.

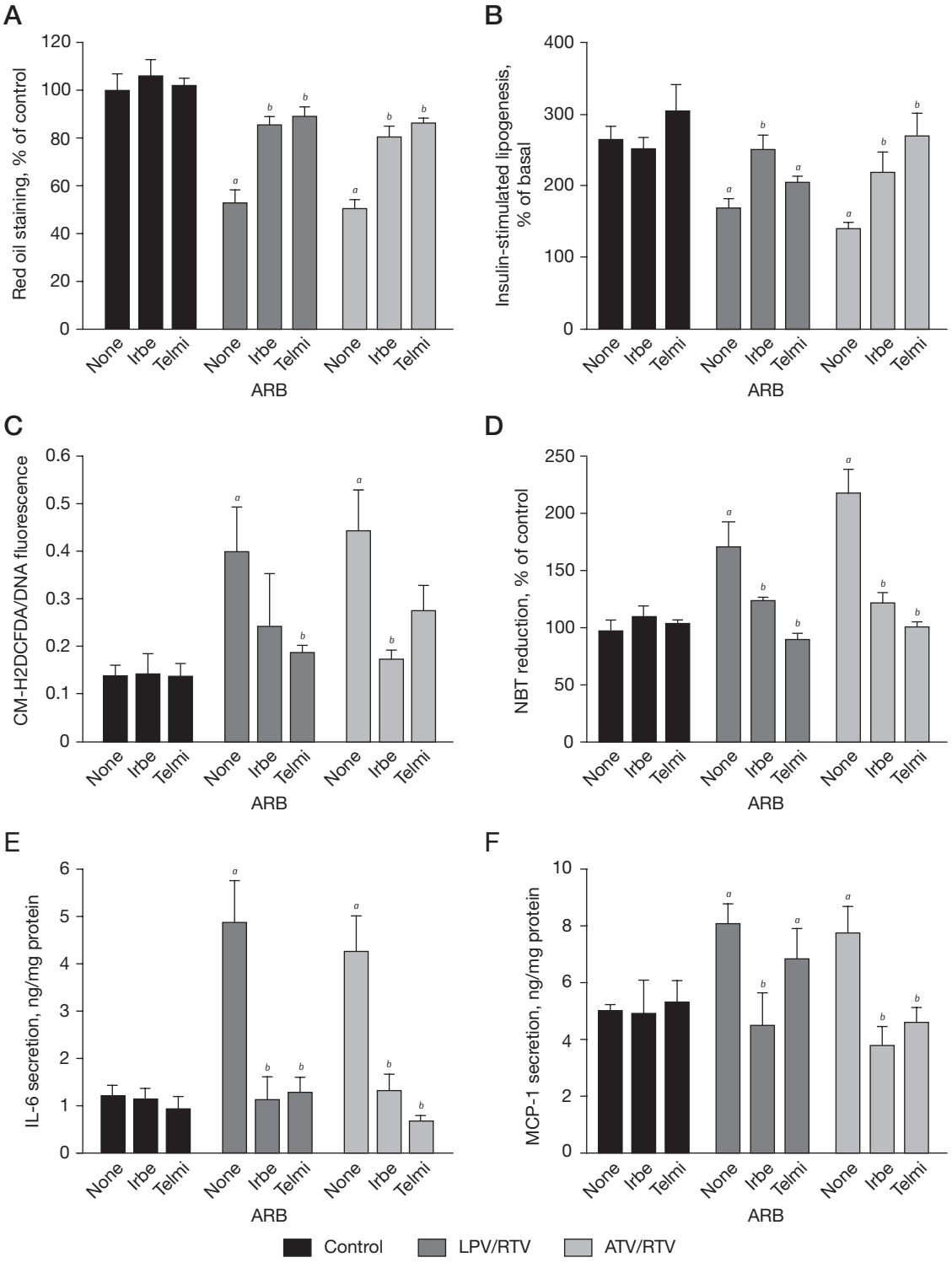
Consistent with PI-induced oxidative stress and inflammation [29,36], lopinavir/ritonavir and atazanavir/ritonavir increased ROS production, evaluated by CM-H_2DCFDA oxidation and NBT reduction (Figure 5A and 5B), and induced inflammation, evaluated by the secretion of IL-6 and MCP-1 (Figure 5C

Figure 3. ARBs prevent PI-induced adipocyte dysfunction in human adipocytes



Human adipocytes were incubated with the indicated protease inhibitor (PI) combination for 5 days, in the absence or presence of irbesartan (Irbe; 10 μM) or telmisartan (Telmi; 1 μM). **(A)** Cells cultured on glass coverslips were fixed, stained with Oil Red O and photographed. Red oil staining was dissolved in 10% SDS, assessed at 520 nm and normalized to the protein content evaluated in parallel. **(B)** Cell monolayers in 6-well plates were solubilized in Laemli sample buffer and submitted to SDS-PAGE and western blotting with the indicated antibodies. Representative blots (performed in triplicate) are shown. β-Actin was used as an index of the cellular protein level. **(C&D)** Control or PI- and /or angiotensin II type-1 receptor blocker (ARB)-treated or untreated human adipocytes were depleted of fetal calf serum for 16 h. Cells were then incubated with insulin (100 nM) for 30 min. Insulin stimulation of (C) glucose transport and (D) lipogenesis was measured as described in the *Methods*. Insulin stimulation was expressed as percentage of the basal value. All experiments shown were repeated 3–10 times. Results are means ±SEM. $^{a}P<0.05$ versus control (untreated cells). $^{b}P<0.05$ versus the respective PI-treated cells without ARB. ATV, atazanavir; C/EBP-α, CAAT/enhancer binding protein-α; LPV, lopinavir; PPAR-γ, peroxysome proliferator-activated receptor-γ; RTV, ritonavir.

Figure 4. ARBs prevent PI-induced dysfunction in murine adipocytes

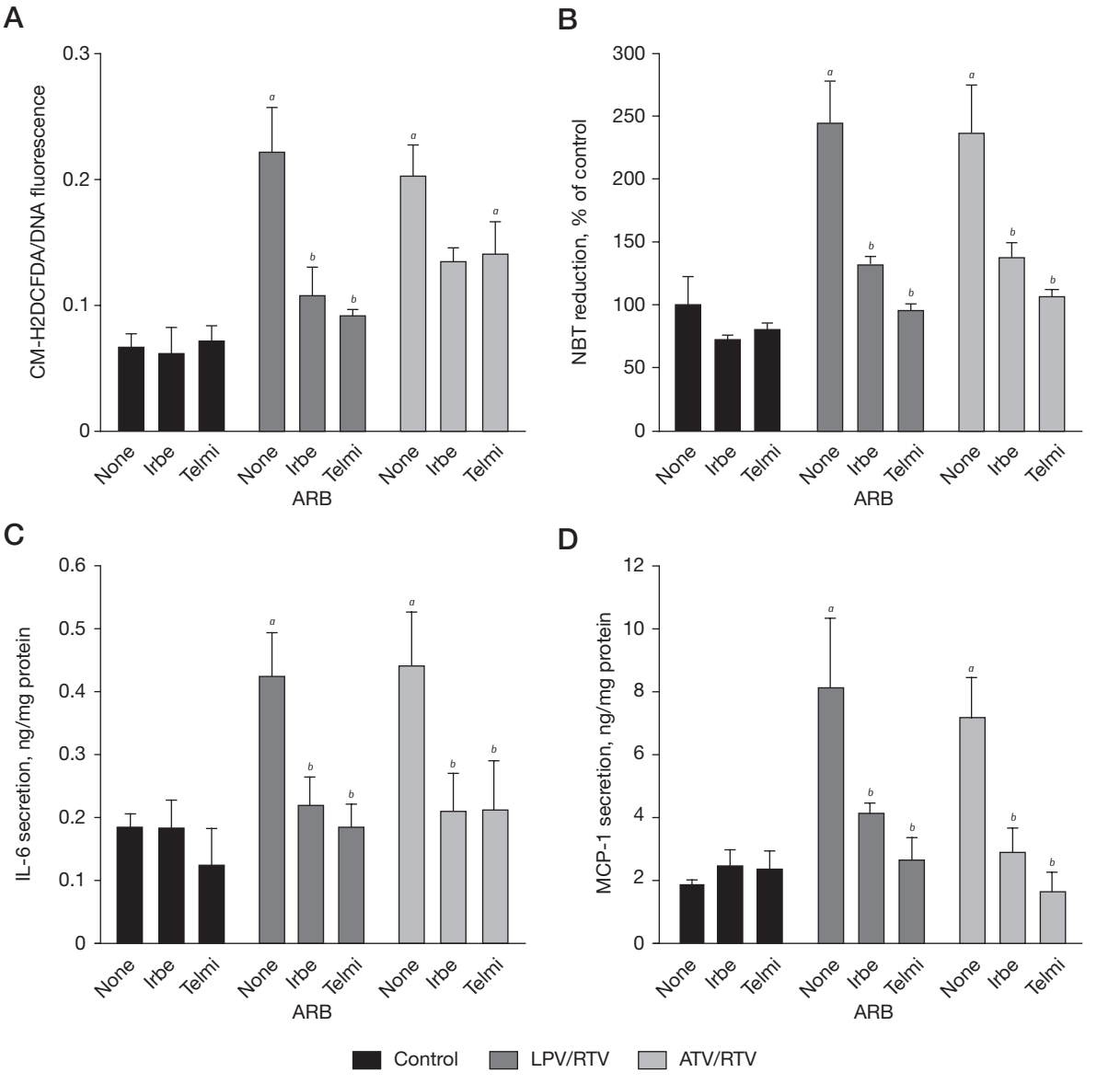


Murine adipocytes were incubated with the indicated protease inhibitors (PIs) and/or angiotensin II type-1 receptor blockers (ARBs) for 5 days. **(A)** Lipid accumulation, **(B)** insulin stimulation of lipogenesis, **(C&D)** reactive oxygen species production, **(E)** interleukin (IL)-6 secretion and **(F)** monocyte chemotactic protein (MCP)-1 secretion were measured as described in the *Methods*. Results are means ±SEM of 3–5 experiments performed in triplicate. $^{a}P<0.05$ versus control (untreated cells). $^{b}P<0.05$ versus the respective PI-treated cells without ARB. ATV, atazanavir; Irbe, irbesartan; LPV, lopinavir; NBT, nitroblue tetrazdum; RTV, ritonavir; Telmi, telmisartan.

and 5D). ROS production increased by 2–3-fold, IL-6 secretion by 2.5-fold and MCP-1 secretion by 4-fold in human adipocytes treated with lopinavir/ritonavir and atazanavir/ritonavir. No significant differences were observed according to the PI combinations. PIs also increased the secretion of IL-6 and MCP-1 in murine adipocytes (Figure 4C, 4D, 4E and 4F).

Irbesartan and telmisartan had no intrinsic effect on basal ROS production (Figure 5A and 5B) and cytokine secretion (Figure 5C and 5D), whereas they partially or totally prevented the PI effects on the oxidative and inflammatory status of human (Figure 5A, 5B, 5C and 5D) and murine (Figure 4C, 4D, 4E and 4F) adipocytes.

Figure 5. ARBs prevent PI-induced oxidative stress and inflammation in human adipocytes



Oxidative stress was measured in human adipocytes treated with the indicated protease inhibitors (PI) in the absence or presence of angiotensin II type-1 receptor blockers (ARBs). Reactive oxygen species production was assessed by two means: **(A)** in terms of the oxidation of CM-H_2DCFDA derivatives and normalized to the DNA content and **(B)** in terms of the reduction of nitroblue tetrazolium (NBT) as described in *Methods*. The secretion of **(C)** interleukin (IL)-6 and **(D)** monocyte chemotactic protein (MCP)-1 on 24-h cell culture supernatant was measured by the Luminex™ technology (Panomics, Ozyme, Saint Quentin en Yvelynes, France). Results are normalized to the cellular protein content and expressed as the mean ±SEM of 3–10 experiments performed in triplicate. $^{a}P<0.05$ versus control (untreated cells). $^{b}P<0.05$ versus the respective PI-treated cells without ARB. ATV, atazanavir; Irbe, irbesartan; LPV, lopinavir; RTV, ritonavir; Telmi, telmisartan.

PPAR-γ signalling pathway plays a role in PI-induced RAS activation

As shown here (Figure 3B) and in previous studies, some PIs can reduce PPAR-γ expression and intranuclear localization, pointing to an effect on PPAR-γ signalling [32]. Alternatively, PPAR-γ activation is suspected to play a role in RAS activation [41,42] by decreasing AT1R expression [24,25]. We thus examined the potential role of PPAR-γ signalling in PI-induced RAS activation by using reagents known to modulate PPAR-γ activity.

The potent PPAR-γ agonist, rosiglitazone, prevented the PI effect on AT1R protein expression, lipid accumulation and oxidative stress in murine adipocytes (Figure 6A and 6B) and human adipocytes (FB *et al*., data not shown). As previously observed [27], rosiglitazone could increase lipid accumulation (by 1.4-fold; Figure 6B, left). Rosiglitazone also reduced ROS production under basal conditions (Figure 6B, right), in line with its antioxidant properties [43]. Conversely, the irreversible PPAR-γ inhibitor GW9662 reduced the beneficial effect of telmisartan on PI-induced AT1R expression and oxidative stress (Figure 6C, left). Interestingly, GW9662 increased adipocyte ROS production by itself (Figure 6C, right), an effect that was not blocked by telmisartan. This might be explained by the protective role of PPAR-γ on oxidative cell status [23].

Discussion

This study provides the first evidence that two frequently prescribed HIV PI combinations (lopinavir or atazanavir associated with ritonavir) could activate the adipose RAS *in vitro*. This is indicated by the effect of PIs on AT1R expression and RAS activation. Moreover, RAS blockers prevented PI-induced RAS activation together with adipose cell dysfunction, including dedifferentiation, insulin resistance, oxidative stress and inflammation.

This overactivation of the adipose RAS might play a role in the development of hypertension in HIV-infected patients under PI therapy. Indeed, the prevalence of hypertension seems to be higher in HIV-infected patients as compared to the general population [5,6]. It has been shown that the duration of antiretroviral treatment and the use of PIs could increase the prevalence of hypertension [5,8]. In these studies, the link between lipodystrophy and the increased risk of hypertension has been consistently suggested [4–8]. Whether it is the abnormal fat distribution itself and/or the metabolic disturbances resulting from the lipodystrophy syndrome (insulin resistance or chronic low grade inflammation) and/or the direct PI toxicity that is involved in HIV-related hypertension has never been investigated.

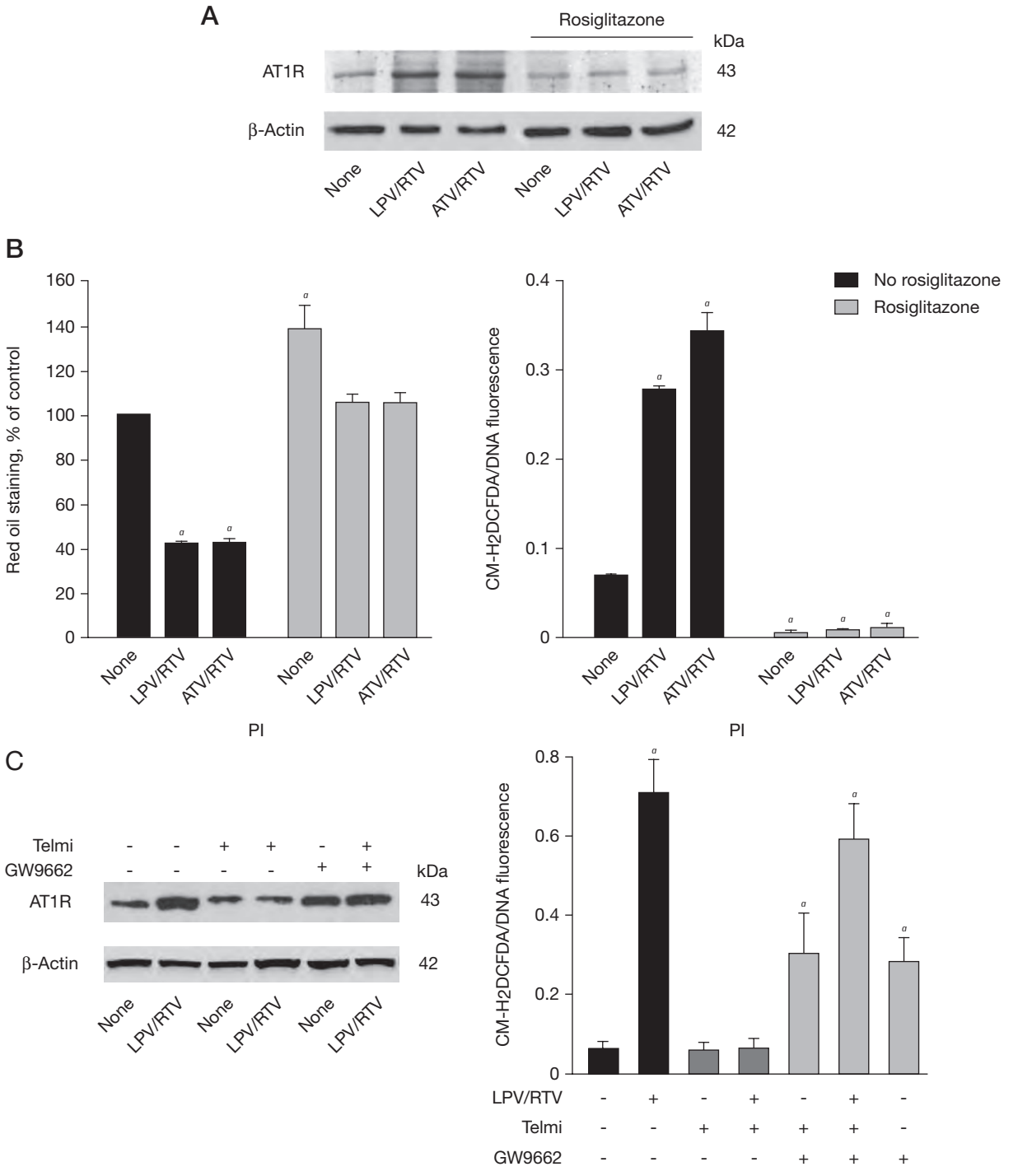
In the past decade, a role of the adipose RAS in the development of systemic hypertension, insulin resistance and diabetes mellitus has been suggested *in vitro* and *in vivo* [14,26,38,39]. Fat tissue dysfunction, including reduced uptake of serum lipids, increased lipolysis, oxidative stress, and secretion of proinflammatory cytokines, is one of the primary causes of these associated complications [11,12,22]. It is linked to hypertension and atherosclerosis in obesity [10,12,14–17,44,45] and in severe lipodystrophic syndromes [25], including HIV-related lipodystrophy [2,3,7,8]. Increased AT1R expression is also a feature of lipoatrophic mice with severe hypertension [25]. Thus, overactivation of the adipose RAS [14,26,38] provides another potential pathway through which fat cell dysfunction could lead to hypertension and cardiovascular disease in HIV-infected patients on PI therapy.

Numerous *in vitro* studies have reported that HIV antiretrovirals could induce severe dysfunctions in cultured adipocytes [40], including defective insulin signalling, oxidative stress and inflammation [28,29]. In the present study, we found that two PI combinations – lopinavir/ritonavir and atazanavir/ritonavir – decreased cell differentiation and insulin sensitivity, and promoted oxidative stress and inflammation in human and murine adipocytes. These results are in line with those of the only two previous studies that examined the adverse effects of these PI combinations on adipose cells and tissue [33,46]. In contrast to these latter studies, we observed no major differences between the two PI combinations. As atazanavir alone has no intrinsic effect on adipose cell function [29,36] (and data not shown), ritonavir was probably responsible in priority for the toxic effects.

We showed here that two PI combinations induced adipose RAS overactivation in cultured adipocytes. This was indicated by overexpression of AT1R, overstimulation of angiotensin II signalling and increased angiotensinogen mRNA expression. These findings are consistent with PI-induced activation of angiotensin II signalling and/or impaired AT1R desensitization, as reported in cells bearing mutant AT1R that fails to internalize [34,37,47].

We also observed that the ARBs could prevent PI effects on AT1R expression and activation. RAS blockers not only attenuated the PI action on RAS activation, but also protected adipocyte function, by partially or totally blocking the effects of PIs on lipid accumulation, expression of adipogenic factors, insulin responsiveness, ROS production and cytokine release, indicating that adipose RAS overactivation, insulin resistance, oxidative stress and inflammation are interrelated. These results are consistent with the ability of ARBs to improve insulin sensitivity in obese and/or hypertensive insulin-resistant rats [48] and to reduce adipocyte

Figure 6. Role of peroxysome proliferator-activated receptor-γ signalling in the beneficial effect of ARBs on PI-induced renin angiotensin system activation and adipocyte dysfunction



(A&B) Murine adipocytes were treated for 5 days with the indicated protease inhibitors (PIs), in the absence or presence of rosiglitazone (10 μM). (A) Angiotensin II type-1 receptor (AT1R) protein expression was analysed by western blot. (B) Lipid accumulation (left panel) and oxidative stress (right panel) were determined as described in *Methods*. Results are means ±SEM of three independent experiments performed in triplicate. **(C)** Murine adipocytes were incubated for 24 h with or without lopinavir (LPV)/ritonavir (RTV), GW9662 (1 μM) and/or telmisartan (Telmi; 1 μM), as indicated. The medium was changed and the treatment with LPV/RTV and/or Telmi was prolonged for 4 days. AT1R protein expression was determined by western blot (left panel) and reactive oxygen species production (right panel) by the oxidation of CM-H_2DCFDA. Representative blots (performed in triplicate) are shown. Results are means ±SEM of four experiments performed in triplicate. [a]$P<0.05$ versus untreated control cells. [b]$P<0.05$ versus untreated adipocytes. ARB, AT1R blocker; ATV, atazanavir.

size [49], in line with their ability to reduce fat mass in patients with the metabolic syndrome [50] and in diabetic rats [51]. Oxidative stress and inflammation are involved in the pathophysiology of diabetes and cardiovascular disease. Indeed, increased ROS production and IL-6 secretion has been linked to hypertension, insulin resistance and obesity, all of which are components of the metabolic syndrome [43]. Oxidative stress and inflammation are increased in HIV-infected patients on antiretroviral therapy [52] and can be induced by PIs in cultured cells [29,36]. The relationship between ROS and RAS is complex. ROS can activate RAS, and RAS can induce oxidative stress *in vivo* and *in vitro* by stimulating AT1R [35].

Many recent studies indicate that the beneficial effects of ARBs on insulin resistance and metabolic disorders might partly be mediated by their PPAR-γ agonistic activity [42,53]. Telmisartan and irbesartan behave as dual ARB/PPAR-γ modulators acting on two separate pathways, one by selectively blocking the AT1R-dependent proinflammatory, proatherogenic pathway, and the other through direct activation of the PPAR-γ pathway [19,20,54]. Our findings confirm previous data indicating that PPAR-γ deficiency might be a key factor in PI-induced adipose cell dysfunction [32,40]. Indeed, PPAR-γ activation by rosiglitazone prevented the PI effects on lipid accumulation and ROS production, whereas PPAR-γ blockade by GW9662 increased oxidative stress. PPAR-γ loss or dysfunction is observed in fat tissue disorders in HIV-related lipodystrophy [30,31] resulting from PI treatment [27]. Otherwise, dominant-negative and loss-of-function mutations in PPAR-γ can lead to severe hypertension [18,23,24]. Thus, PI-induced PPAR-γ dysfunction in adipose tissue might participate, via the adipose RAS activation, in hypertension and cardiovascular disease occurring in HIV-infected patients on PI therapy.

In summary, this *in vitro* study shows for the first time that PI-induced adipocyte dysfunction (lipid loss, insulin resistance, oxidative stress, inflammation) might be associated with adipose RAS activation. We suggest that the PIs that activate the adipose RAS might have deleterious effects on blood pressure control secondary to the overactivation of AT1R and associated insulin resistance, ROS overproduction and inflammation state. Clinical trials using RAS blockers should be performed to evaluate their potential advantageous metabolic effects beyond the blood pressure control in hypertensive HIV-infected patients with lipodystrophy and/or insulin resistance.

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