Original article

Impact of darunavir, atazanavir and lopinavir boosted with ritonavir on cultured human endothelial cells: beneficial effect of pravastatin

*Martine Auclair[1,2,3], Pauline Afonso[1,2,3], Emilie Capel[1,2,3], Martine Caron-Debarle[1,2,3], Jacqueline Capeau[1,2,3,4]**

[1]INSERM, UMR_S 938, CDR Saint Antoine, F-75012, Paris, France
[2]Sorbonne Universités UPMC Univ Paris 06, UMR_S 938, F-75012, Paris, France
[3]ICAN, Institute of Cardiometabolism and Nutrition, Paris, France
[4]AP-HP, Hopital Tenon, Department of Biochemistry, F-75020, Paris, France

*Corresponding author e-mail: jacqueline.capeau@inserm.fr

Background: HIV-infected patients administered long-term ritonavir-boosted protease inhibitors (PIs) are at a greater risk for developing cardiovascular diseases. Endothelial dysfunction is an initiating event in HIV-associated atherosclerosis. Cultured endothelial cells can be used as a model to compare the endothelial toxicity of different PIs.
Methods: We compared the effect of darunavir (DRV), darunavir/ritonavir (DRV/r), lopinavir/ritonavir (LPV/r) and atazanavir/ritonavir (ATV/r), used at clinically relevant concentrations, on human coronary artery endothelial cell vascular function, oxidative stress, inflammation and senescence, and studied the effect of pravastatin on PI-induced alterations.
Results: Vascular endothelial cell function, evaluated by the expression of endothelial nitric oxide synthase and the production of nitric oxide and endothelin-1, was unaffected by DRV or DRV/r, but altered by LPV/r or ATV/r. DRV or DRV/r did not alter, or mildly induced oxidative stress and inflammation (phosphorylation of p65/RelA-NFκB, secretion of IL-6 and IL-8), while ATV/r and LPV/r induced a marked increase. Secretion of sICAM or sVCAM, indicative of altered cell integrity, was not or weakly altered by DRV or DRV/r, but increased by 2–3-fold by LPV/r or ATV/r. Similar results were observed regarding senescence markers: SA-β-galactosidase activation and overexpression of phospho-p53, $p16^{ink4}$, $p21^{WAF-1}$ and prelamin A. Pravastatin could, in part, reverse PI-induced adverse effects.
Conclusions: Ritonavir-boosted PIs differentially induced vascular endothelial cell dysfunction, reactive oxygen species production, inflammation and senescence with no effect or a mild effect of DRV/r, an intermediate effect of ATV/r, and a stronger effect of LPV/r. Statins could, in part, protect the cells from PI-induced endothelial dysfunction.

Introduction

Protease inhibitors (PIs) are widely used to control HIV infection; however, PI-based therapies are known to increase cardiovascular risk in HIV-infected patients [1–4]. Among currently used PIs, lopinavir (LPV) boosted with ritonavir (LPV/r) has been associated with a greater risk of cardiovascular disease [4,5]. Atazanavir (ATV) has not been associated with an increased risk of myocardial infarction in the D:A:D study [6] but, boosted with ritonavir (ATV/r), it exerted a more atherogenic lipid profile than nevirapine in treatment-naive patients [7]. Up to now, no data are available on a potential cardiovascular risk exerted by darunavir (DRV). DRV-based therapies are considered as safe and well-tolerated at the metabolic level [8–10]. In addition to PI-based therapies, the increased risk of premature myocardial infarction has been attributed to HIV infection-related factors (CD4 nadir, CD8 level, HIV viral load) and to other classic cardiovascular risk factors [3–5,11–13].

Antiretroviral therapy may promote premature cardiovascular disease through endothelial dysfunction either indirectly, via PI-induced metabolic disturbances [5,14–17] or directly, via alterations of vascular endothelial cells [18–22]. The endothelium normally exerts a number of vasoprotective effects such as vasodilation, suppression of smooth muscle cell growth and inhibition of inflammatory responses. Many of these effects are largely mediated by nitric

oxide (NO), the most potent endogenous vasodilator that opposes the effect of endothelium-derived vasoconstrictors such as endothelin-1. A defect in the production or activity of NO leads to endothelial dysfunction, which is an early marker for atherosclerosis and can be detected *in vivo* before structural changes to the vessel are apparent [23]. *In vitro*, a number of endothelial functions can also be evaluated as the production of NO and endothelin-1, oxidative stress and the release of proinflammatory cytokines. Regarding PIs, it has been previously shown that a short-term treatment with ritonavir (RTV; 24–72 h), alone or in association with LPV, directly altered endothelial cell function [22,24] by triggering oxidative stress in human [25] and porcine endothelial cells [26–28]. Otherwise, long-term treatment with RTV or LPV/r induced inflammation and premature senescence in cultured human endothelial cells together with oxidative stress [29]. The treatment with the combination ATV/r brought controversial data: a 4-week treatment of healthy subjects with ATV/r or LPV/r did not impair endothelial function [30,31], whereas switching to ATV/r treatment did not improve endothelial dysfunction in HIV-infected patients [32]. To our knowledge, the impact of ATV/r or DRV/r on cultured human endothelial cells has never been evaluated.

The risk of premature cardiovascular events might also result from the accelerated biological ageing imposed by antiretroviral therapy and/or HIV itself [33–35]. Indeed, vascular endothelial cell dysfunction is a feature of the human physiological ageing process [36,37], and syndromes of premature ageing are associated with precocious cardiovascular disease. The most striking findings are observed in progeroid syndromes linked to molecular alterations in the prelamin A maturation process [38–41], in which alteration of vascular cells is obvious. Importantly, some PIs, but not all, can induce the accumulation of farnesylated prelamin A by inhibiting the metalloprotease ZPMSTE24, involved in the prelamin A maturation process. The ability of the different PIs to inhibit this enzyme decreased from LPV, to RTV and then to ATV, DRV being devoid of any effect even at high concentrations [42,43].

Our objective was to compare the long-term effect of DRV (used alone or in combination with boosting concentration of RTV), LPV/r or ATV/r on cultured human endothelial cells. We evaluated the possible role of farnesylated prelamin A accumulation, by treating the PI-treated cells with pravastatin, since statins inhibit the synthesis of the farnesyl anchor and therefore decrease the accumulation of farnesylated prelamin A [44], as previously shown in endothelial cells treated with LPV/r or RTV [29].

Methods

Cell culture and treatment

Human coronary artery endothelial cells (HCAECs; PromoCell, Heidelberg, Germany) were cultured as previously described [29]. They were exposed during 20 days to clinically relevant concentrations of DRV (11.8 μmol/l), DRV/r (11.8/0.8 μmol/l) [45], LPV/r (15.9/1.4 μmol/l) [46], ATV/r (7.4/1.3 μmol/l) [47] or to the solvent (0.1% dimethyl sulfoxide [DMSO]). The treatment with pravastatin (25 μmol/l) was performed during the last 3 days. DRV was obtained from Janssen-Cilag (Issy-les-Moulineaux, France) and the other PIs purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Western blotting

Whole cell lysates were subjected to SDS/PAGE and western blotting. We used antibodies against endothelial nitric oxide synthase (e-NOS; SC-653), endothelin-1 (ET-1, SC-21625) and prelamin A (SC-6214) from Santa Cruz Biotechnology, Inc. Antibodies against NF-κB p65/RelA (#3987) and phospho-NF-κB p65/RelA (ser 536; 3033) were from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies against p53 (clone DO-1, ab80645) and anti-phospho(S15)-p53 (ab38497) were from Abcam (Cambridge, UK). Antibodies against $p16^{INK4}$ (551144) and $p21^{WAF-1}$ (556431) were from BD Biosciences (San Jose, CA, USA). Beta-actin (A-5441; Sigma-Aldrich, Saint Louis, MO, USA) was used as an index of the cellular protein content.

NO production

NO production was assessed with the cell-permeant NO indicator 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM; D23844; Molecular Probes, Life Technologies, Carlsbad, CA, USA). Cells were cultured in 96-well plates, washed and incubated with DAF-FM (12.5 μmol/l) or Hoechst 33258 (0.01 mg/ml) in DMEM without FBS for 30 min at 37°C in the dark. Quantification was performed with a plate fluorescence reader (Infinite M200; Tecan-France, Trappes, France) at 515 nm (DAF-FM) and 460 nm (Hoechst 33258), respectively. NO production was also indirectly determined by e-NOS protein expression.

Oxidative stress and inflammation

The production of reactive oxygen species was indirectly measured by the oxidation of CM-H_2DCFDA derivatives (5-[and 6]-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester) and the reduction of nitroblue tetrazolium (NBT) as described [29]. Secretion of interleukin (IL)-6 and IL-8 was analysed in 24-h culture supernatants by Luminex®

technology [29]. Inflammation was also determined by the protein expression of the phosphorylated form (serine 536) of the p65/RelA subunit of NF-κB.

Senescence

Cell senescence was evaluated by the senescence-associated (SA)-β-galactosidase activity, the protein expression of the senescence markers phospho-p53, $p21^{WAF-1}$ and $p16^{INK4}$, and the accumulation of prelamin A, as described previously [29,34].

Statistical analysis

The experiments were repeated 3–8×. Results are expressed as mean ±SEM. Statistical significance was determined using ANOVA and the Kruskal–Wallis non-parametric test, followed by a Fisher protected least significant difference test for pair-wise differences. *P*-values were calculated relative to cells cultured with 0.1% DMSO. *P*-values of less than 0.05 were considered significant.

Results

Endothelial cell dysfunction

DRV used alone or in combination with ritonavir (DRV/r) did not decrease the protein expression of e-NOS (Figure 1A) or the production of NO by endothelial cells, measured by the fluorescent NO indicator DAF-FM diacetate (Figure 1B). In addition, DRV or DRV/r did not alter the basal level of cellular or secreted ET-1 (Figure 1A).

LPV/r activated all markers of endothelial cell vascular dysfunction. It decreased e-NOS protein expression (by 3-fold) and increased cellular and secreted ET-1 by 2–3-fold (Figure 1A and 1B). ATV/r also adversely modified vascular endothelial cell functions: its effect was consistently lower than that of LPV/r and higher than that of DRV/r (1.5–2-fold).

Regarding the release of the adhesion molecules sICAM and sVCAM (Figure 1C), which are markers of altered endothelial cell integrity, DRV had no effect on the secretion of sICAM or sVCAM, whereas DRV/r moderately increased their secretion (1.5-fold increase), compared with the effect of LPV/r (4–6-fold increase) or ATV/r (3–4-fold increase).

Regarding oxidative stress evaluated by CM-H_2DCFDA oxidation and NBT reduction, DRV did not alter the oxidative stress markers (Figure 2A), while DRV/r moderately increased CM-H_2DCFDA oxidation (by 2-fold), and had no effect on NBT reduction. By contrast, LPV/r or ATV/r increased the two markers of oxidative stress by, respectively, 6.5- and 5-fold (CM-H_2DCFDA) and 3-fold (NBT).

With regard to inflammation, DRV or DRV/r did not modify the phosphorylation state of the p-65/RelA subunit of NF-κB as compared with control or DMSO-treated cells (Figure 2B). In addition, DRV or DRV/r had a low, or had no effect on the secretion of IL-6 and IL-8 (Figure 2C). LPV/r markedly increased p65/RelA NF-κB phosphorylation (by 3-fold) and cytokine secretion (by 3–4-fold). ATV/r also increased inflammation but in most cases at a lower level than LPV/r (Figure 2B and 2C).

Endothelial cell senescence

DRV or DRV/r had no effect on the cell cycle arrest markers phospho-p53, $p21^{WAF-1}$ and $p16^{INK4}$, and did not induce prelamin A accumulation (Figure 3A). The combination DRV/r moderately increased SA-β-galactosidase activity (by 1.6-fold), whereas LPV/r or ATV/r had a stronger effect (3.2- and 2.8-fold increase; Figure 3B), consistent with the LPV/r- and ATV/r-increased protein expression of cell cycle arrest markers and prelamin A (Figure 3A).

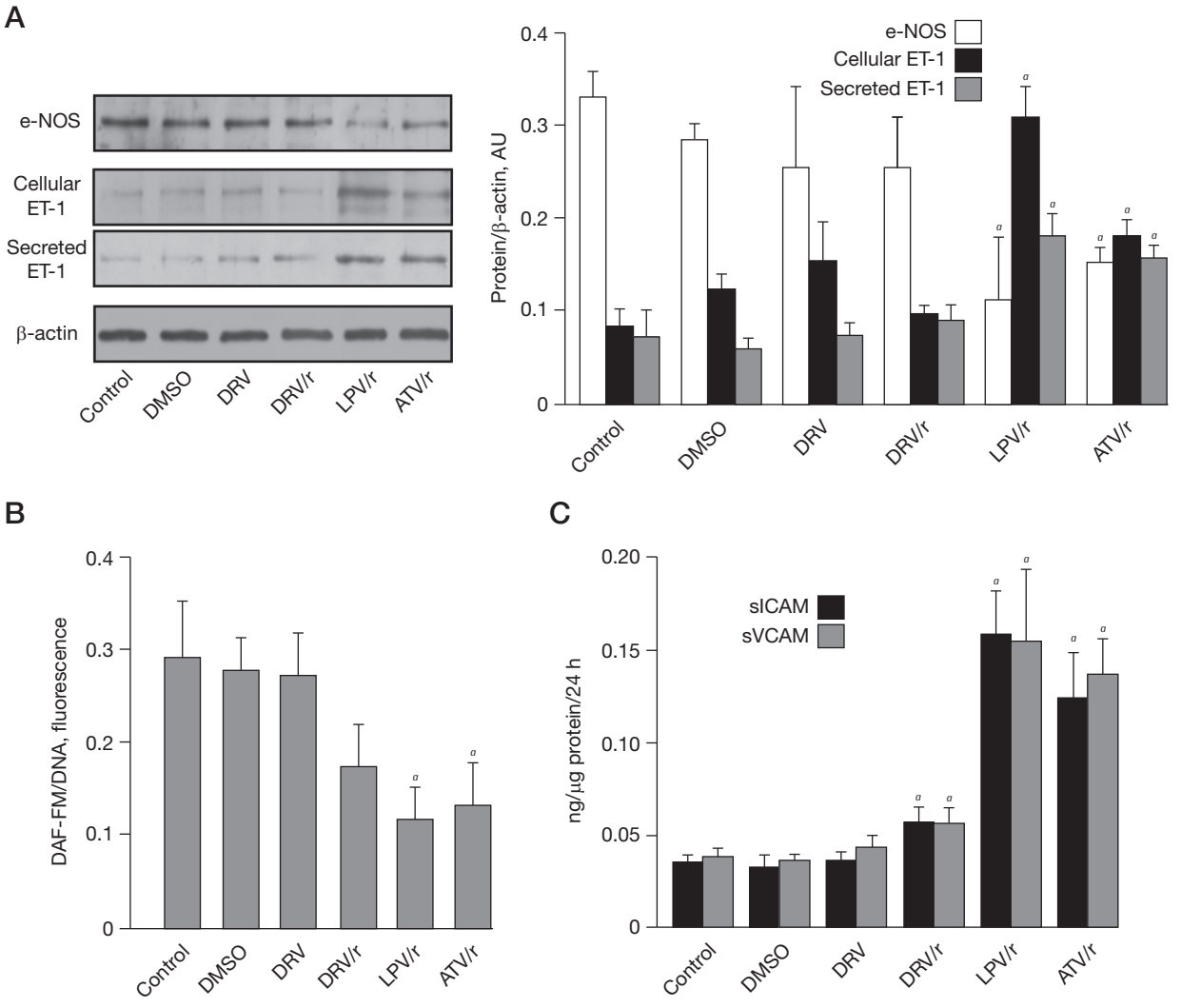
Effect of pravastatin on PI-induced endothelial cell dysfunction and senescence

We then studied the effect of pravastatin on PI-induced endothelial cell dysfunctions. PI-induced HCAEC dysfunction was improved by pravastatin, as shown by the normalized production of NO, sICAM or sVCAM in LPV/r- or ATV/r-treated cells (Figure 4A). In addition, pravastatin decreased the effect of LPV/r and ATV/r on oxidative stress markers by 1.8–2-fold, and on inflammation and senescence markers by 1.6–3-fold (Figure 4B and 4C).

Discussion

We compared the effect of DRV, DRV/r, LPV/r and ATV/r on cultured human endothelial cells. All drug combinations were used at concentrations near to the maximum concentration measured in the patients' serum [45–47]. The PI combinations differentially affected endothelial functions and induced senescence. A continuous incubation of HCAECs for 20 days with LPV/r or ATV/r decreased e-NOS protein expression and NO production, caused the secretion of ET-1, and induced oxidative stress and inflammation. The ability of PIs to enhance the secretion of IL-6 and IL-8 led us to evaluate their ability to increase the phosphorylation of the NF-κB subunit, p65/Rel A on serine 536. The phosphorylation of p65/Rel A, though mediated independently of IκBα, is important for the expression of IL-6 and IL-8 [48,49]. LPV/r and ATV/r also altered endothelial cell integrity, as shown by the increased release of sICAM-1 and sVCAM-1, and induced senescence. PI-induced endothelial cell dysfunction and senescence could regress in the presence of pravastatin.

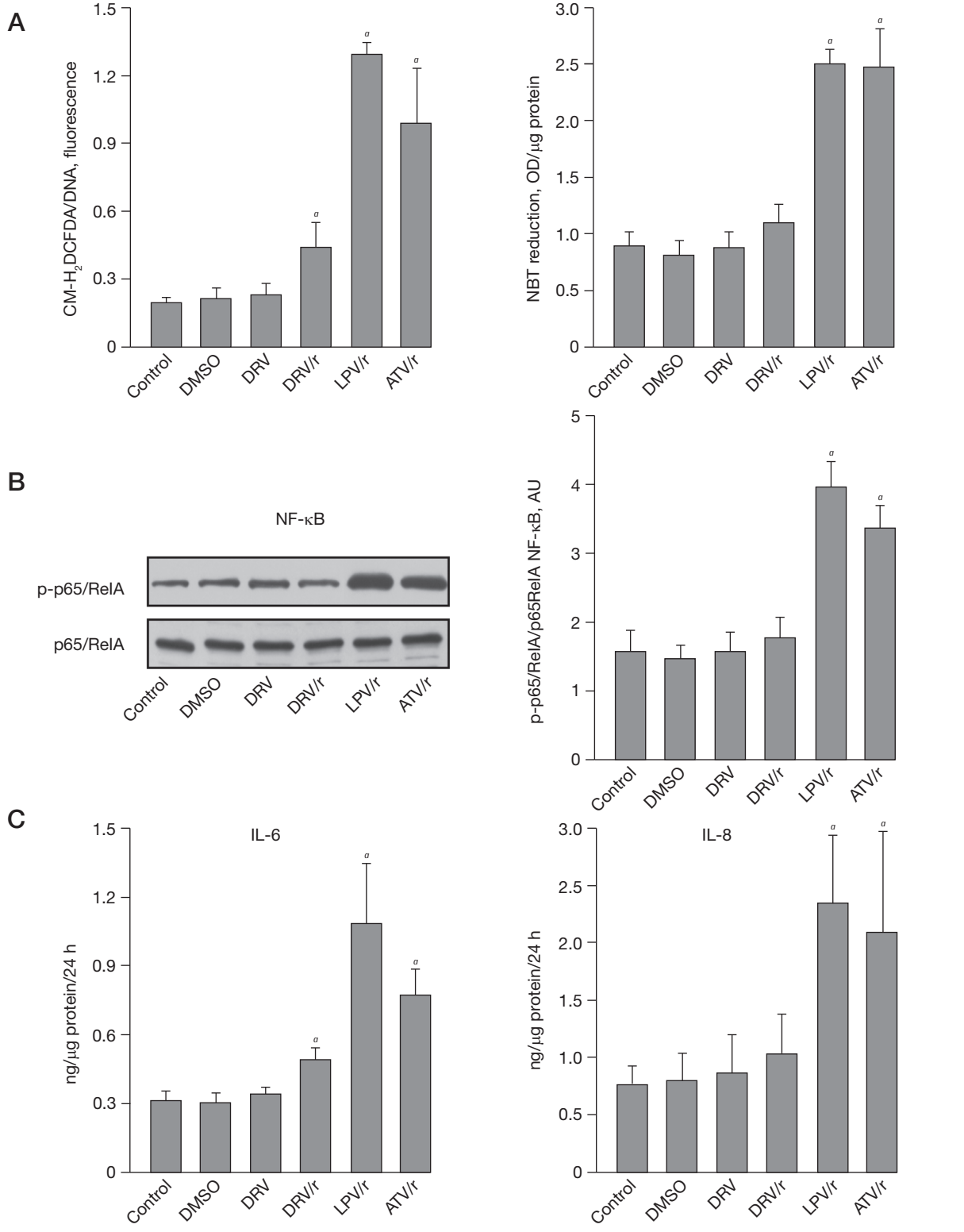
Figure 1. PI effect on endothelial cell functions



Human coronary artery endothelial cells were cultured for 20 days with the indicated protease inhibitors (PIs). **(A)** Protein expression of cellular and/or secreted endothelial nitric oxide synthase (e-NOS), endothelin-1 (ET-1) and β-actin. Representative blots (performed in triplicate) are shown. Quantification was performed relative to β-actin and expressed as arbitrary units (AU). **(B)** Nitric oxide (NO) production was evaluated by the fluorescence of 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM) normalized to DNA (Hoechst 33258 fluorescence). **(C)** Soluble ICAM and VCAM was evaluated in 24 h culture supernatant by Luminex® technology and expressed as ng/µg protein/24 h. Results are the mean ±SEM of 3–5 experiments. $^{a}P<0.05$ versus dimethyl sulfoxide (DMSO)-incubated cells. ATV/r, ritonavir-boosted atazanavir; DRV/r, ritonavir-boosted darunavir; LPV/r, ritonavir-boosted lopinavir.

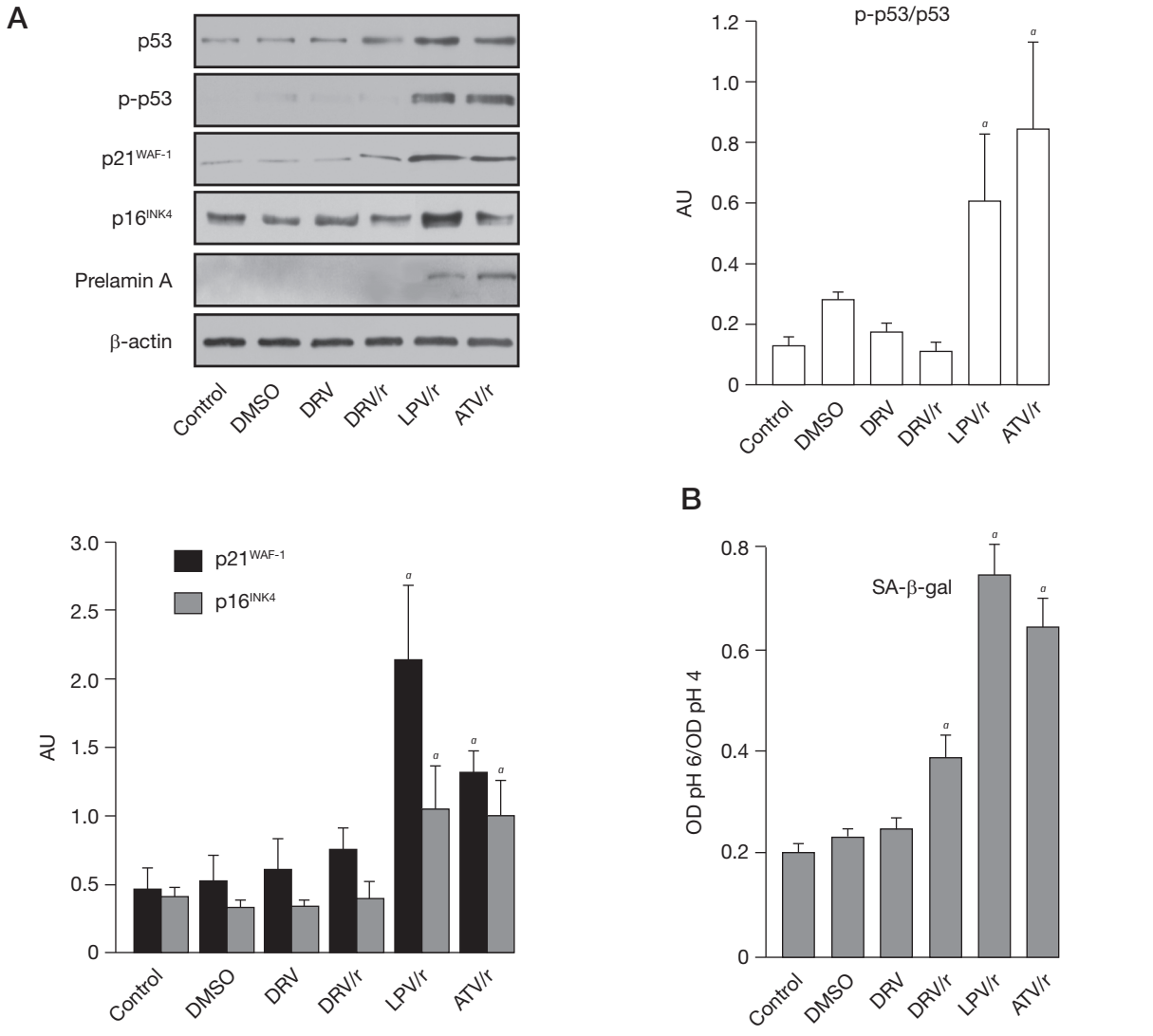
A 20-day treatment of HCAECs with DRV had no incidence on endothelial cell function. When DRV was associated with a low concentration of RTV (DRV/r), it exerted no, or little effect on endothelial function (1.5–2-fold change of some, but not all, markers). This low-grade toxicity could probably result from the toxicity of the booster, even at this low concentration [50]. Even if we have not directly tested the toxicity of RTV used alone at low concentrations, it can be expected that its toxicity will increase with increasing concentrations, becoming important at 7.5 µmol/l, as previously reported [29]. The higher toxicity of ATV/r compared with DRV/r on endothelial cell function may be explained in part by a higher impact of the booster, which was used at a 1.6-fold higher concentration in ATV/r versus DRV/r (1.3 versus 0.8 µmol/l). Indeed, RTV used alone in porcine and human coronary artery endothelial cells (24 h, 15–30 µmol/l) can decrease e-NOS expression [27], and increase oxidative stress [21,24,28]. Increased ET-1 release and decreased NO production was suspected to mediate the adverse effects of HIV drugs on endothelial cell function [20,25,51,52].

Figure 2. PI effect on markers of oxidative stress and inflammation



Human coronary artery endothelial cells were cultured for 20 days with the indicated protease inhibitors (PIs). **(A)** Reactive oxygen species production was assessed by the oxidation of CM-H_2DCFDA derivatives or the reduction of nitroblue tetrazolium (NBT). Results are normalized to the DNA or protein content, respectively, and expressed as mean ±SEM of 3–10 experiments. **(B)** The activation of NF-κB was evaluated by the phosphorylation of the p65/RelA subunit. Quantification was performed versus total p65/RelA NF-κB and expressed as arbitrary units (AU). Representative blots (performed in triplicate) are shown. **(C)** The secretion of interleukin (IL)-6 and IL-8 in 24 h culture supernatants was measured by Luminex® technology. Results are normalized to the cell protein content and are the mean ±SEM of 3–8 experiments performed in triplicate. [a]$P<0.05$ versus dimethyl sulfoxide (DMSO)-incubated cells. ATV/r, ritonavir-boosted atazanavir; DRV/r, ritonavir-boosted darunavir; LPV/r, ritonavir-boosted lopinavir.

Figure 3. PI effect on senescence markers

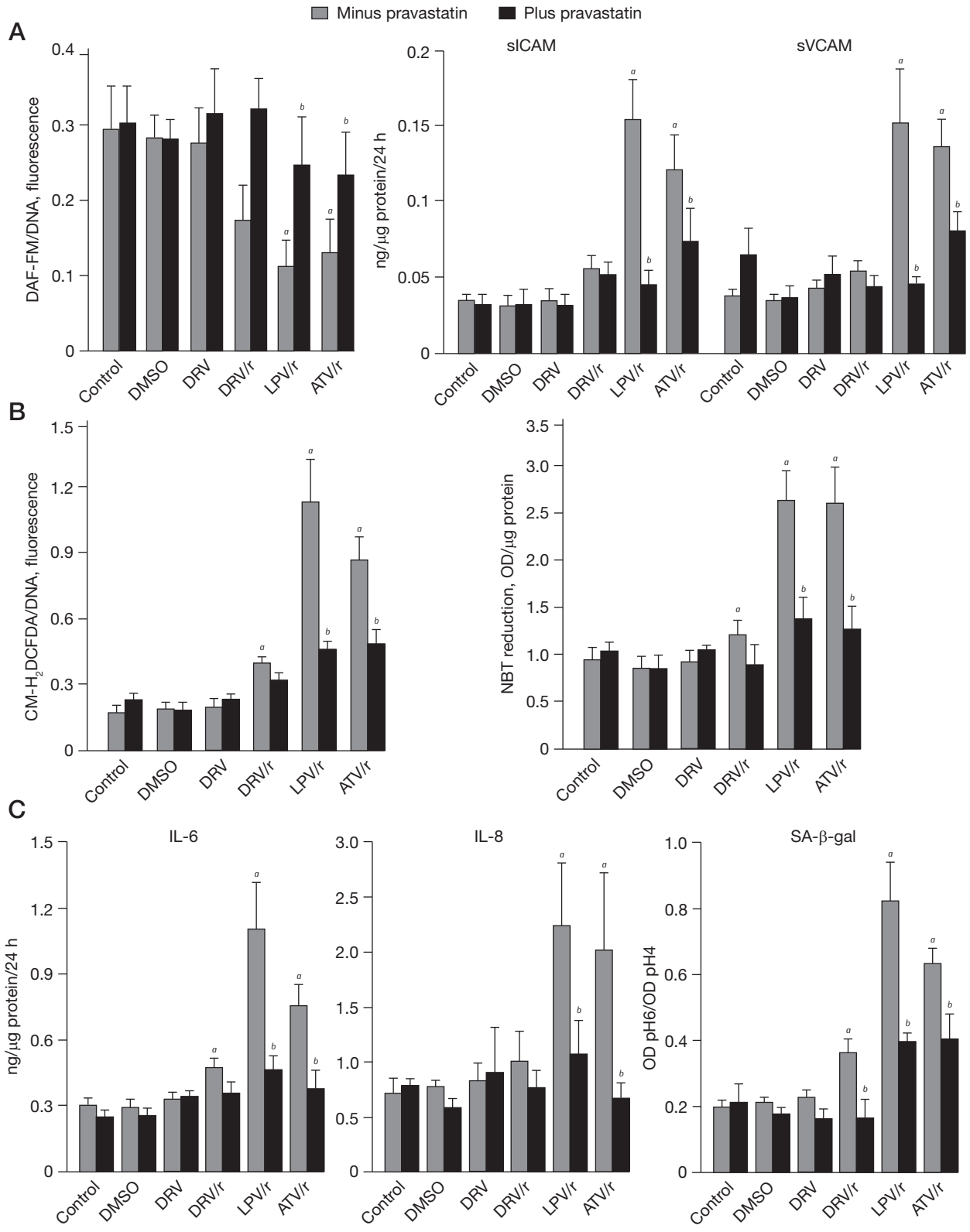


Human coronary artery endothelial cells were cultured for 20 days with the indicated protease inhibitors (PIs). **(A)** Senescence was evaluated by the phosphorylation state of p53 (pp53/p53) and the expression of $p21^{WAF-1}$, $p16^{INK4}$ and prelamin A. Quantification was performed relative to β-actin. Results are expressed as arbitrary units (AU) and are the mean ±SEM of 3–8 experiments. Representative blots (performed in triplicate) are shown. **(B)** Senescence-associated β-galactosidase (SA-β-gal) activity. Blue X-gal staining was evaluated at 630 nm. Results are the mean ±SEM of 3–8 experiments performed in triplicate. $^{a}P<0.05$ versus dimethyl sulfoxide (DMSO)-incubated cells. ATV/r, ritonavir-boosted atazanavir; DRV/r, ritonavir-boosted darunavir; LPV/r, ritonavir-boosted lopinavir.

However, an effect linked to ATV is also possible, since in the absence of RTV it can promote senescence of human mesenchymal stem cells [53]. Moreover, ATV, as LPV and RTV, could inhibit ZMPSTE24, even if the level of inhibition was lower than observed with the two other PIs, while DRV was devoid of any inhibitory effect [54]. This *in vitro* toxicity contrasts with the *in vivo* clinical data since treatment with ATV or ATV/r was not associated with an increased risk of myocardial infarction in HIV-infected patients [6]. It could be proposed that the increased level of free bilirubin generally observed in patients receiving ATV might exert beneficial anti-oxidant effects. Indeed, diabetic patients with Gilbert syndrome and increased bilirubin level presented with a lower prevalence of vascular complications as well as reduced levels of markers of oxidative stress and inflammation as compared to diabetic patients without Gilbert syndrome [55].

Otherwise, LPV/r markedly altered endothelial cell function: it impaired NO production, increased the secretion of ET-1 and adhesion molecules, and induced oxidative stress and inflammation. It also induced premature senescence. These results agree with our previous studies [29], even if a shorter incubation time (20- versus 30-day)

Figure 4. Effect of pravastatin on PI-induced endothelial cell dysfunctions



Human coronary artery endothelial cells were cultured for 20 days with the indicated protease inhibitors (PIs). Pravastatin (25 μmol/l) was added for the last 3 days of incubation. **(A)** Nitric oxide (NO) production was evaluated with the fluorescent indicator 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM), and sICAM and sVCAM secretion by Luminex® technology. **(B)** Oxidative stress was evaluated as in the footnote of Figure 2A. **(C)** Inflammation (interleukin [IL]-6 and IL-8) and senescence (SA-β-galactosidase [SA-β-gal] activity) markers were evaluated as in the footnotes of Figures 2 and 3, respectively. The results are the mean ±SEM of four separate experiments performed in triplicate. [a]$P<0.05$ versus dimethyl sulfoxide (DMSO)-incubated cells. [b]$P<0.05$ versus the respective control or PI-treated cells.

and other concentrations of LPV (15.9 versus 10.0 μmol/l) and RTV (1.4 versus 2.0 μmol/l) have been tested.

Statins are lipid-lowering drugs widely used for the treatment and prevention of cardiovascular disease. They display additional cholesterol-independent or pleiotropic effects on various aspects of cardiovascular disease, including improving endothelial function, decreasing vascular inflammation and enhancing plaque stability [56]. Statins have also been shown to decrease oxidative stress [57]. In the present study, a beneficial effect of pravastatin was observed on all PI-induced endothelial cell dysfunctions; vascular dysfunction, inflammation, oxidative stress and also on PI-induced senescence. This effect could be related to the ability of statins to decrease the PI-induced accumulation of farnesylated prelamin A, by impeding the synthesis of the farnesyl anchor, and therefore decreasing prelamin A toxicity and ability to induce cell senescence [29,58]. In our previous study we also reported the beneficial effect of an anti-oxidant treatment [29]. These data led us to propose that the PI-induced farnesylated prelamin A accumulation is the initial toxic event leading to increased oxidative stress, inflammation and to senescence. The ability of the different PI combinations to alter endothelial cell functions, as shown here, correlated with their efficiency to inhibit ZMPSTE24 [54].

This study has limitations. We have not evaluated human endothelial cell samples from HIV-infected patients treated with these PI combinations. However, in our previous study senescence markers have been detected in peripheral blood mononuclear cells from HIV-infected patients under ritonavir-boosted PIs, and their level was lower when the patients were co-treated with statins [29].

In conclusion, we report here that DRV/r, ATV/r and LPV/r differentially affected vascular endothelial cell function, cell integrity and induced senescence. The effect of each PI combination on endothelial cells might in part result from the concentration of the ritonavir boost and from their ability to inhibit ZMPSTE24. Even if these *in vitro* studies cannot be translated directly to the clinics, they suggest that even at boosting concentrations, RTV could adversely affect endothelium. Whether another CYP3A4 inhibitor, such as cobicistat, structurally related but devoid of any inhibitory effect on the HIV protease, will exert or not an effect on ZMPSTE24 is difficult to infer and requires additional experiments. Importantly, adding a statin to long-term PI-treated cells could diminish endothelial cell dysfunction and delay senescence, which is important in the clinical use.

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Disclosure statement

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