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Lopinavir co-induces insulin resistance and ER stress in human adipocytes

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ABSTRACT

HIV-protease inhibitors (PIs) markedly decreased mortality of HIV-infected patients. However, their use has been associated with occurence of metabolic abnormalities the causes of which are not well understood. We report here that lopinavir, one of the most prescribed PI, dose-dependently co-induced insulin resistance and ER stress in human adipocytes obtained from differentiation of precursor cells.

Insulin resistance was subsequent to IRS1 phosphorylation defects and resulted in a concentration-dependent decrease of glucose uptake. The major ER stress pathway involved was the phosphorylation of eIF2-α. Salubrinal, a selective eIF2-α dephosphorylation inhibitor, induced insulin resistance by targeting IRS1 phosphorylation at serine 312 and acted synergistically with LPV when both drugs were used in combination.

This study points out the key role of eIF2-α phosphorylation in the development of PI-associated insulin resistance and ER stress. Thus, this protein represents a promising therapeutic target for development of new PIs devoid of adverse metabolic effects.

Introduction

Treatment of patients with HIV-protease inhibitors (PIs) has considerably improved the survival and the quality of life of HIV-infected patients, but their use is frequently hindered by an array of metabolic adverse events [1]. Indeed, most patients treated with PIs develop lipodystrophy, hyperlipidemia [2] and metabolic disturbances, such as reduction of insulin-stimulated glucose uptake [3]. As a result, insulin resistance develops in treated HIV patients leading to type 2 diabetes [4].

Insulin resistance is multifactorial [5] and may result from modifications of phosphorylations in the insulin signalling pathway, loss of the glucose transporter Glut4, insulin receptor (IR) and Peroxisome proliferator-activated receptor γ (PPARγ). Recently, a novel concept of organelle disturbance affecting the endoplasmic reticulum (ER) was implicated in disorders related to the adipose tissue [6,7]. The ER is the main site for fatty acid and protein synthesis and protein folding. Impairment of the ER function produces a stress-driven cellular response which aims to circumvent it. Such ER stress response (or Unfolded Protein Response: UPR) is a conserved evolutionary mechanism occurring in physio-pathological situations to reduce the disequilibrium between ER load and its folding capacity [8]. UPR triggers different responses involving the activation of the 78 kDa glucose-regulated/binding immunoglobulin protein (GRP-78 or BiP) [9] as a common initial stimulus. This leads to the phosphorylation of the alpha subunit of the eukaryotic initiation factor 2 (eIF2-α) on serine 51 and results in a transient attenuation of protein biosynthesis within the ER, thus preventing further accumulation of unfolded proteins. However, it may also lead to cellular apoptosis, as a final protective mechanism for cells [8].

The association of lopinavir (LPV) with ritonavir (RTV) is a widely effective PI combination and one of the most prescribed in developed countries [10]. PIs induce UPR and facilitate insulin resistance in a number of murine and human cellular models [11–13]. Because of their important role in glucose homeostasis [5] and as direct pharmacological targets of PIs [14], adipocytes play a determinant role in the development of drug-induced insulin resistance. Therefore we aimed to determine by which mechanisms LPV impairs the insulin sensitivity of human adipocytes, and a possible link to UPR activation. We used hMADS cells, a specific model of human adipocytes, as they display on a long term basis stable expression of specific adipocytes markers, lypolitic response to β-adrenoreceptor agonists, as well as secretion of adipokines [15,16]. In this model, LPV impaired insulin signalling by decreasing the phosphorylation of insulin receptor substrate 1 (IRS1). Concomitantly, PIs activated UPR by phosphorylating eIF2-α. Salubrinal (Sal), a selective eIF2-α dephosphorylation inhibitor [17], decreased IRS1 phosphorylation at serine 312, therefore mimicking LPV effects. It synergistically maximized LPV-induced insulin resistance at low concentrations.

These results shed new light on the cellular mechanisms by which HIV-PIs modify insulin signalling in human adipocytes and underline eIF2-α phosphorylation as a determinant of PIs-associated metabolic disorders.

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Materials and methods

Reagents. LPV was obtained through the AIDS Research and Reference Reagent Program (National Institute of Health, Bethesda, MA, USA) and used as previously described [14]. Cell culture media were purchased from LONZA (Levallois-Perret, France) and fetal calf serum (FCS) from Dutscher S.A. (Brumath, France). Salubrinal was obtained from VWR International SAS (Fontenay-sous-bois, France). All the other products were purchased from Sigma–Aldrich Chimie (Saint Quentin, France).

Cell culture. hMADS cells were grown and differentiated into adipocytes as described previously [15]. After maximal differentiation (typically 90% of cells differentiated 3 weeks after induction) cells were placed for 48 h in Dulbecco's modified Eagle's medium (DMEM) with 0.2% bovine serum albumin (BSA) before experiments. Differentiated hMADS cells will further be referred as hMADS adipocytes.

Preparation of cell extracts. hMADS adipocytes were incubated for 48 h with LPV followed by a 15-min exposure to insulin (10^{-7}M), then rinsed in ice-cold phosphate buffered saline (PBS) and solubilized in stop buffer (50 mM Hepes, pH 7.2, 150 mM NaCl, 10 mM ethylene-diamine-tetra-acetate, 10 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1% Triton X-100 (v/v) in presence of Complete™ protease inhibitor cocktail (Roche)).

Western blotting. Equal amounts of protein were resolved by 7.5 or 10% SDS–PAGE under reducing conditions and transferred to Immobilon–P membranes (Millipore Corp., Bedford, MA). Forty or thirty micrograms of proteins were used for insulin signalling or UPR measurements, respectively.

For immunoblotting assays, the detecting antibodies were: mouse anti-phophotyrosine (Upstate), rabbit anti-IRS1 (Upstate), rabbit anti-phospho-IRS1 (Ser307 for mouse or Ser312 for human) (Upstate), rabbit anti-insulin receptor (Santa Cruz), mouse anti-KDEL motif (Stressgen) to detect BiP [18], rabbit anti-phospho-eIF2-α (Ser51) (Cell Signaling Technology) and mouse anti-β-tubulin I (Sigma).

The bound primary antibody was then detected by horseradish peroxidase-conjugated secondary antibody and visualized by an electrochemical luminescence (ECL) detection kit (Millipore).

Chemiluminescence was observed and quantified using a Fujifilm LAS 3000 imager.

Intensity of the bands was measured using the Fujifilm Multi Gauge software.

RNA extraction and polymerase chain reaction (PCR) analysis. Total RNA was extracted using TRI-Reagent kit (Euromedex, France) according to manufacturer's instructions. CHOP-10 expression was analysed using real time PCR after reverse transcription (RT) performed using Superscript II reverse transcriptase (Invitrogen). Reactions were carried out on an ABI Prism 7000 (Applied Biosystem). For each PCR runs two master mixes containing SYBR green I PCR Master Mix Plus (Applied Biosystem) and specific primers were prepared. The first one was to amplify CHOP-10 contained 300 nM of the following primers: Forward: 5′-CATACATCACCACACCTGAAAGC-3′ and reverse: 5′-CCAAAGGAGAAAGGCAATGACT-3′. The second was to amplify 18S (used as an internal control) contained 100 nM of the following primers: forward: 5′-GCCCGAAGCGTTTACTTTGA-3′, reverse: 5′-TCCATTATTCCTAGCTGCGGTATC-3′. Gene expression was quantified using the comparative C_t method.

Glucose transport. Glucose transport was studied on hMADS adipocytes starved overnight in DMEM containing 0.2% BSA. Cells were then incubated in Krebs–Ringer phosphate buffer containing 0.2% BSA [3] for 4 h at 37 °C followed by treatment for 45 min with insulin (100 nM) and 2-Deoxy-D-[2,6^{3}H] glucose (Amersham, Piscataway, NJ, USA) was added during the last 10 min (100 μM of 2-Deoxy-D-glucose containing 0.5 μCi/ml of the tritiated form). Glucose uptake was stopped by washing the cells with ice-cold PBS followed by cell lysis in PBS containing 1% triton.

Statistical analysis. Results are shown as mean ± standard error of the mean (SEM) with the number of experiments indicated. Continuous variables and their change from control conditions were analysed with Student *t*-test using Micrococal Origin 6.0 (Micrococal Software, Northampton, MA). Probabilities values <0.05 were considered as statistically significant.

Results and discussion

We previously showed that both LPV and RTV impaired adipocyte differentiation, while in fully differentiated hMADS adipocytes they accumulated into lipid droplets [14]. LPV or RTV treatment of hMADS adipocytes, neither induced any morphologic change as compared to control nor modified the Glycerol 3-phosphate dehydrogenase (GPDH) activity indicating that metabolic changes were unlikely to result from an alteration of the differentiation state of hMADS adipocytes (see Supplemental data).

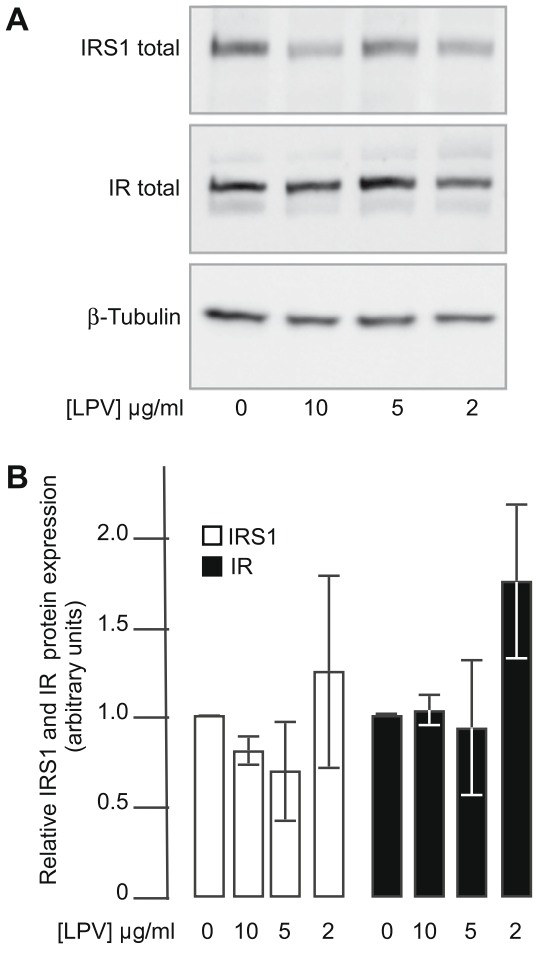

Fig. 1. Lack of significant LPV effect on IRS1 and IR expression. (A) Lysates from hMADS adipocytes treated or not with LPV (2–10 μg/ml) were analysed by Western blots using anti-IRS1 (upper panel), anti-IR (medium panel) and anti-β-tubulin I (lower panel) antibodies. Typical autoradiographs are shown. (B) Quantification of IRS1 (white histograms) and IR (black histograms) expression levels. Histograms represent signals normalised to the β-tubulin I signal. Control condition: cells with no LPV treatment. Mean ± SEM obtained from [LPV] = 10 μg/ml ($n = 5$) and others concentrations ($n = 3$) independent experiments are shown. p values were >0.05 (NS).

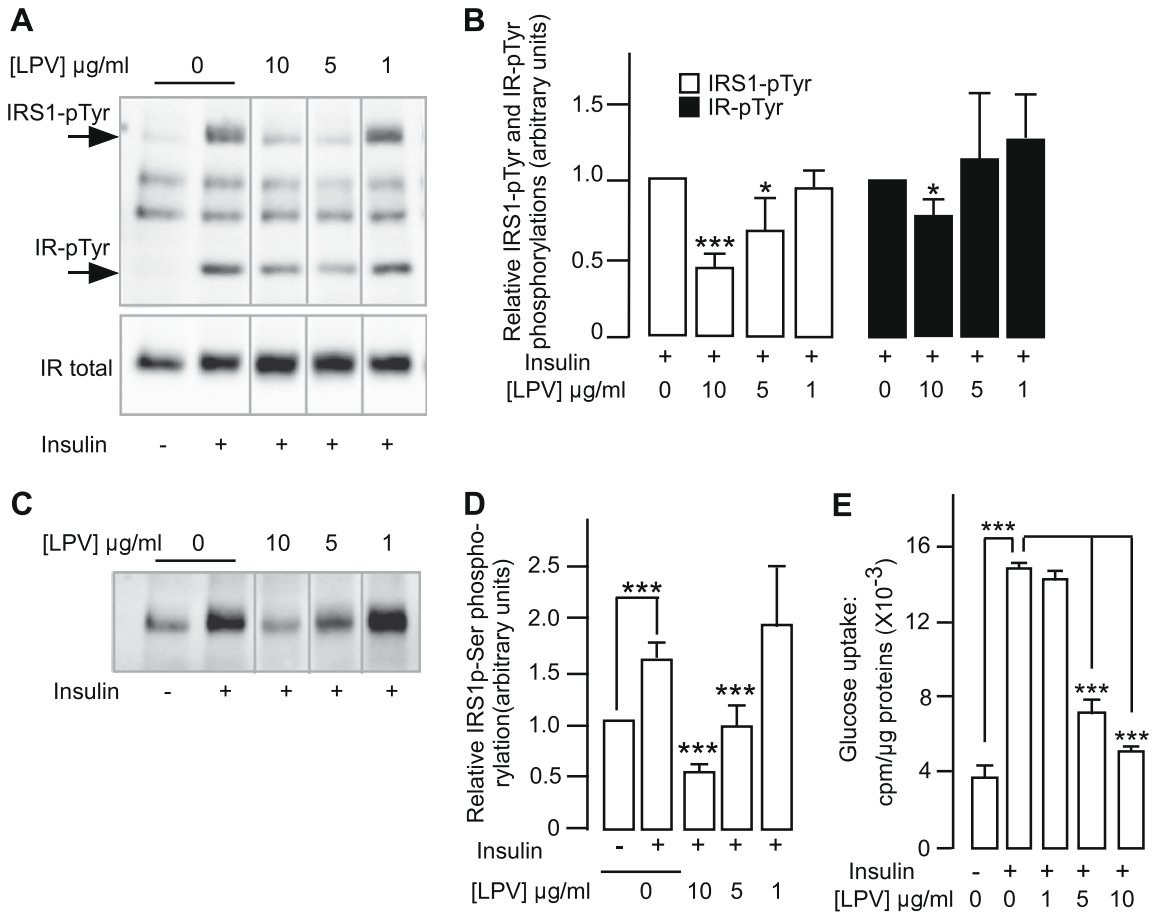

Fig. 2. LPV dose-dependently decreases insulin signalling in hMADS adipocytes. hMADS adipocytes were treated for 48 h with LPV (1–10 µg/ml). Cell lysates were prepared before and after insulin stimulation. (A) Cell lysates were analysed by Western blots using anti-phosphotyrosine (upper panel), and anti-IR (lower panel) antibodies. All bands are issued from the same autoradiogram. (B) Quantification of IRS1-PY and IR-PY phosphorylation levels. The histograms represent the phosphorylation signals normalised to the IR signal for cells stimulated with insulin (100 nM). Control condition corresponds to cells with no LPV. Mean ± SEM obtained from 4 independent experiments are shown. $*$ indicates $p < 0.05$ and $***$ indicates $p < 0.001$ versus control condition. (C) Cell lysates were analysed by Western blots using anti-IRS1 phosphoserine 312. (D) LPV decreased insulin-induced phosphorylation of IRS1 on serine 312. The histograms represent the phosphorylation signals normalised to the IR signal. Control condition corresponds to cells with no insulin and LPV treatment and was referred as 1. Mean ± SEM obtained from 3 independent experiments are shown. $***p < 0.001$. (E) LPV dose-dependently decreased the insulin-stimulated glucose uptake. hMADS adipocytes were treated for 48 h with LPV (1–10 µg/ml). Cells were then incubated in the glucose transport solution (4 h). Insulin-induced uptake of 2-Deoxy-D-[2, 6 ^{3}H] glucose was measured as described in Materials and methods and normalised to the amount of proteins present in each well. The experiment presented is representative of 2 independent experiments with determinations performed in triplicate. ($***p < 0.001$).

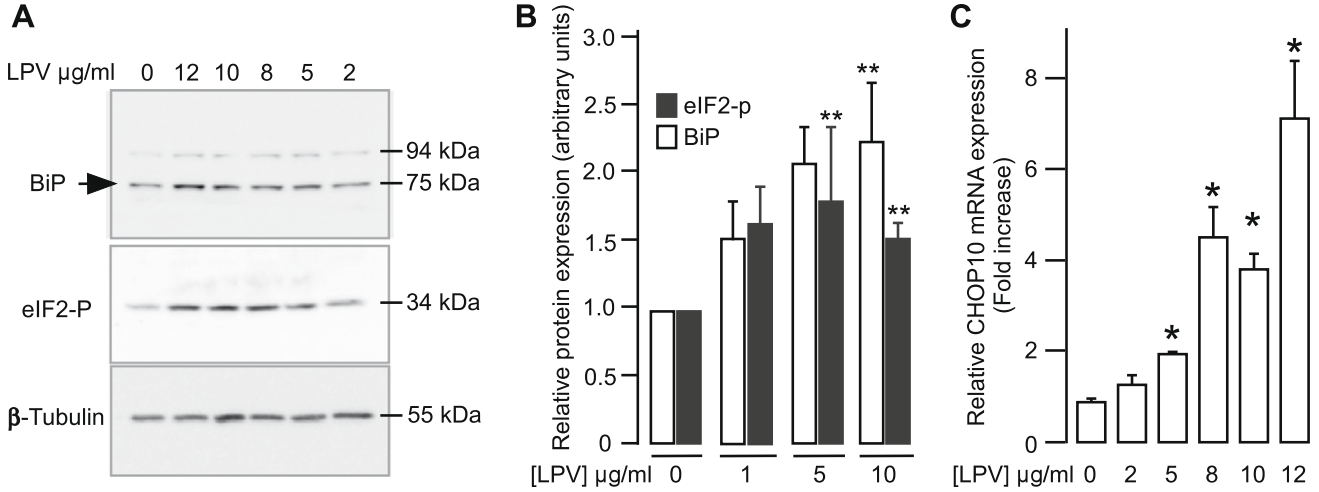

Fig. 3. Induction of ER stress by LPV. (A) hMADS adipocytes were treated for 48 h with various doses of LPV. Expression of BiP, Tubulin and phosphorylation of eIF2-α were assessed from cell lysates by Western blots using specific antibodies. (B) Quantification of BiP expression levels and of eIF2-α phosphorylation levels. The histograms represent the signals normalised to the β-tubulin I signal. Control condition corresponds to cells with no LPV. Mean ± SEM obtained from 3 independent experiments are shown. $**$ indicates $p < 0.01$. LPV augments BIP expression and eIF2 phosphorylation. (C) hMADS adipocytes were treated for 48 h with various doses of LPV. CHOP-10 mRNA expression was analysed by real time PCR and normalised for the expression of 18S RNA. The results represent the mean ± SEM of 3 independent experiments carried out in duplicate. $*p < 0.05$ for comparison of LPV treated cells to cells treated with vehicle (DMSO).

We then checked the effects of LPV on insulin signalling. While incubation of hMADS adipocytes with increasing doses of LPV (2–10 μg/ml) did not induce significant changes of IRS1 and insulin receptor expression (Fig. 1), similar concentrations inhibited significantly the insulin-induced phosphorylation of IRS1 on tyrosines (Fig. 2A) and serine 312 (Fig. 2C) residues, in a dose dependent manner. Phosphorylation of the insulin receptor was merely affected for the highest dose of LPV only (Fig. 2A and B). At 10 μg/ml LPV, the remaining insulin-induced phosphorylation of IRS1 on tyrosines and serine 312 was below 50% of that of untreated cells, suggesting an important impact of LPV on insulin signalling (Fig. 2B and D). As a consequence, LPV dose-dependently blunted insulin-induced glucose uptake. A 90% inhibition was observed at a concentration of 10 μg/ml ($p < 0.001$, Fig. 2E). These observations identify IRS1 phosphorylation defects as the main LPV targets in the insulin signalling pathway leading to LPV-induced insulin resistance.

Basal IRS1 phosphorylation at serine 312 was detectable in control hMADS adipocytes, increased upon insulin application (Fig. 2C and D) and decreased dose-dependently with LPV treatment ($p < 0.001$; Fig. 2C and D), independently from a possible modification of IRS1 protein expression (Fig. 1). Studies carried out in murine cell models show that various cytokines promote phosphorylation of IRS1 on serine, blunting the transduction of the insulin signal [19]. We never noticed such an increase in response to LPV treatment in our cells (Figs. 2 and 4C). Therefore, in human adipocytes, a decrease rather than an increase in IRS1 phosphorylation at serine 312, seems to be involved in insulin resistance development. For example, rapamycin decreases both phosphorylation of IRS1 at serine 312 and insulin sensitivity [20]. Modulation of IRS1 phosphorylation at serine 312 appears therefore capital to impair insulin signalling.

Beside its effect on insulin signalling, LPV dose-dependently increased expression of the 75 kDa protein GRP-78 or BiP ($p < 0.01$, Fig. 3A and B). This induced phosphorylation of eIF2-α (Fig. 3A and B) indicating that LPV up-regulates an UPR pathway involved in translation attenuation. It further increased

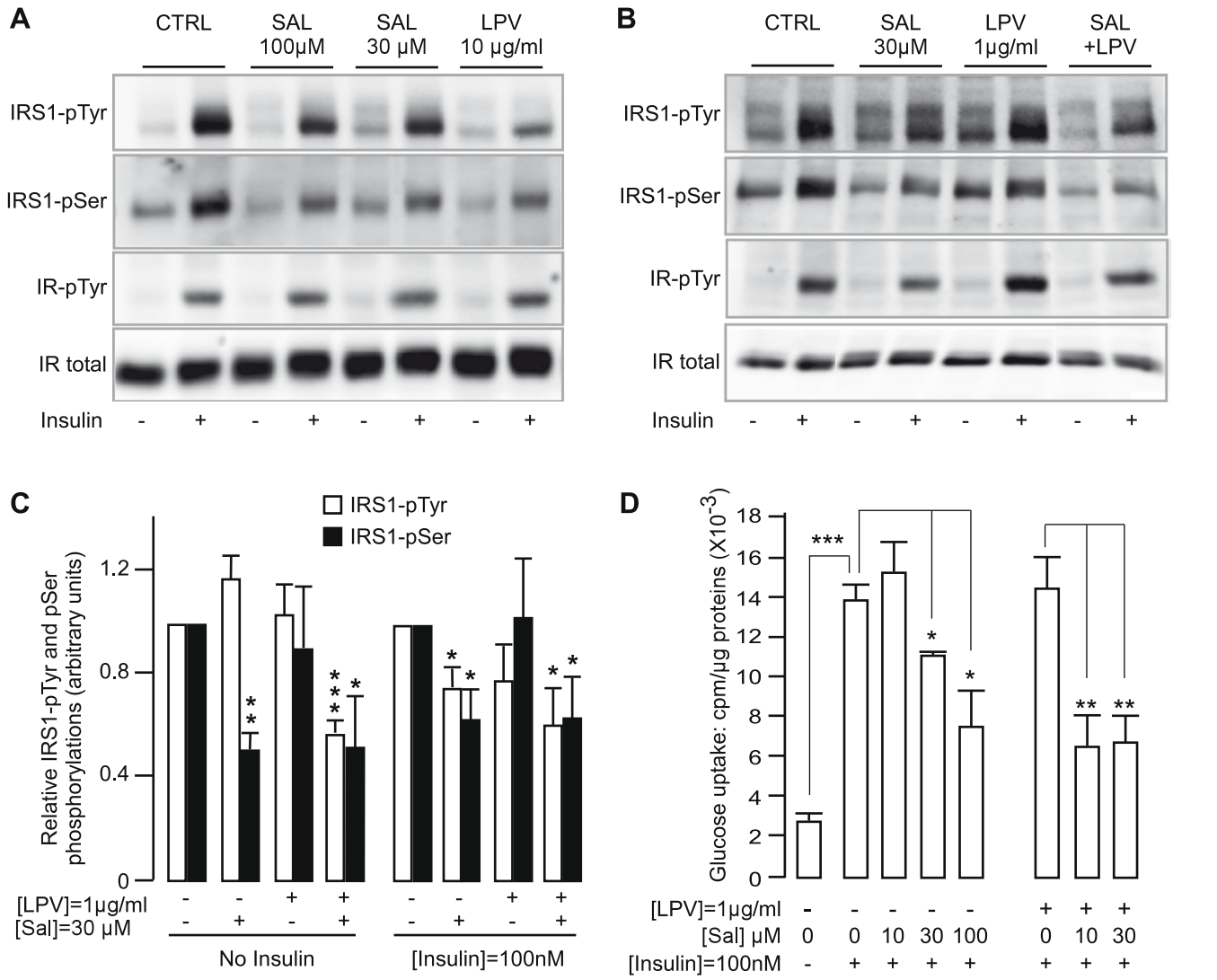

Fig. 4. Salubrinal decreases insulin signalling and glucose uptake in hMADS adipocytes. (A and B) hMADS adipocytes were incubated for 48 h in culture medium with 0.2% BSA and then treated for 48 h with different doses of salubrinal or with LPV as indicated. Treatment with both compounds was performed with salubrinal (30 μM) and LPV (1 μg/ml) (B). Insulin (100 nM) was added for 15 min. Cell lysates were analysed by Western blots using anti-phosphotyrosine (P-Tyr), anti-IRS1-phosphoserine, and anti-insulin receptor (IR) antibodies. (C) Quantification of IRS1-PY and IRS1-P Ser 312 phosphorylation levels. The histograms represent the phosphorylation signals normalised to the IR signal for cells stimulated with or without insulin (right and left panel, respectively). Control condition corresponds to cells with no LPV and no salubrinal. Mean ± SEM obtained from 3 independent experiments are shown except for [LPV] = 1 μg/ml ($n = 5$). $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ versus control condition. (D) hMADS adipocytes were treated for 48 h with salubrinal (10–100 μM), with LPV (1 μg/ml), or simultaneously with the two compounds. Insulin was added in presence of 2-Deoxy-D-[2, 6 ^{3}H] glucose. Glucose uptake was normalised to the amount of proteins present in each well. The experiment presented is representative of 2 independent experiments with determinations performed in triplicate. ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$). LPV and Salubrinal had synergistic effects as compared to LPV or salubrinal alone.

transcription of UPR associated genes such as CHOP-10 (Fig. 3C). Long-term UPR responses, assessed by analysis of X-box binding protein 1 expression, were not significantly changed by LPV, as compared to control (data not shown), making it an unlikely candidate for the major UPR induced by LPV. Our observations fit with the previously reported ER stress induced by PIs in various human and murine cell models [12,13]. They elicit UPR consistently associated with an increase in GRP-78 and CHOP-10 expression in the murine 3T3-L1 model of adipocytes [12].

We compared the LPV-induced insulin resistance and UPR and found that they were positively and highly significantly related to LPV concentrations (see Supplemental data). The implication of UPR in the induction of LPV-induced insulin resistance was assessed pharmacologically using salubrinal: an inhibitor of eIF2-α dephosphorylation [17]. Salubrinal (100–30 μM) decreased basal IRS1 phosphorylation at serine 312, but did not significantly affect basal phophorylations IRS1 on tyrosines (Fig. 4C and Supplemental Fig. 3). Thus the prime effect of salubrinal was to alter IRS1 phosphorylation at serine 312. In insulin-stimulated cells, both phosphorylation at serine 321 and at tyrosines were affected (Fig. 4A and B and Supplemental Fig. 3) indicating that salubrinal affects IRS1 in a general manner. As a consequence, salubrinal alone (30–100 μM) induces insulin resistance as observed through the significant decrease in insulin-stimulated glucose transport ($p < 0.05$) (Fig. 4D).

The importance of this process in response to eIF2-α phosphorylation was evaluated by measuring LPV-induced insulin resistance, in cells pretreated with salubrinal. At a concentration of LPV devoid of significant effect on insulin signalling pathway (1 μg/ml, Figs. 2 and 4B and C), salubrinal (30 μM) worsened the insulin resistance as it dramatically reduced IRS1 phosphorylation on both serine and tyrosine residues. Again, the insulin-stimulated glucose uptake was dramatically decreased: when cells were treated with concentrations of salubrinal and LPV that do not separately alter glucose transport (Fig. 4D), a striking decrease in glucose uptake occurred. Therefore, these two drugs act synergistically to aggravate insulin resistance.

These results indicate that phosphorylation of eIF2-α is a key element leading to the development of LPV-induced insulin resistance in hMADS adipocytes by impairing preferentially IRS1 phosphorylation at serine 312. It remains to determine if this defect results from a decrease in phosphorylation or an increase in phosphatase activity.

While previously described as a protective agent against ER stress-mediated apoptosis [17,21], in our model, salubrinal induced, rather than protected from, LPV-induced insulin resistance. A similar observation has been made in a pancreatic β-cell model [22], indicating that both adipocytes and pancreatic β-cells tolerate poorly an uncontrolled eIF2-α phosphorylation. Whether potentiation of LPV effect by salubrinal results from convergence of ER stress and other distinct pathways such as disturbance in oxidative metabolism [23] remains to be established.

In conclusion, LPV alters the differentiation and cytokine production [14] of human adipocytes, induces an UPR, decreases insulin-stimulated glucose uptake and facilitates the development of insulin resistance. We identified eIF2-α phosphorylation as a cornerstone for impairing insulin signalling in response to LPV and/or to salubrinal. The identification of such a target is an important step to help design new drugs devoid of adverse pharmacological effects, therefore minimizing the overall increase of cardio-metabolic risk associated with HIV-PIs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.05.148.

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