

# Nelfinavir-induced insulin resistance is associated with impaired plasma membrane recruitment of the PI 3-kinase effectors Akt/PKB and PKC- $\zeta$

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## Abstract

**Aims/hypothesis.** Chronic exposure of 3T3-L1 adipocytes to the HIV protease inhibitor nelfinavir induces insulin resistance, recapitulating key metabolic alterations of adipose tissue in the lipodystrophy syndrome induced by these agents. Our goal was to identify the defect in the insulin signal transduction cascade leading to nelfinavir-induced insulin resistance.

**Methods.** Fully differentiated 3T3-L1 adipocytes were exposed to 30  $\mu\text{mol/l}$  nelfinavir for 18 h, after which the amount, the phosphorylation and the localisation of key proteins in the insulin signalling cascade were evaluated.

**Results.** Insulin-induced interaction of phosphatidylinositol 3'-kinase (PI 3-kinase) with IRS proteins was normal in cells treated with nelfinavir, as was IRS-1-associated PI 3-kinase activity. Yet insulin-induced phosphorylation of Akt/protein kinase B (PKB), p70S6

kinase and extracellular signal-regulated kinase 1/2 was significantly impaired. This could not be attributed to increased protein phosphatase 2A activity or to increased expression of phosphoinositide phosphatases (SHIP2 or PTEN). However, insulin failed to induce translocation of the PI 3-kinase effectors Akt/PKB and protein kinase C- $\zeta$  (PKC- $\zeta$ ) to plasma membrane fractions of nelfinavir-treated adipocytes.

**Conclusions/interpretation.** We therefore conclude that nelfinavir induces a defect in the insulin signalling cascade downstream of the activation of PI 3-kinase. This defect manifests itself by impaired insulin-mediated recruitment of Akt/PKB and PKC- $\zeta$  to the plasma membrane.

**Keywords** 3T3-L1 adipocyte · HIV protease inhibitor · Insulin resistance · Nelfinavir · PKB · PKC- $\zeta$  · Plasma membrane

Received: 3 December 2003 / Accepted: 13 February 2004

Published online: 28 May 2004

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**Abbreviations:** ERK, extracellular signal-regulated kinase · HPI, HIV protease inhibitor · MAP, mitogen-activated protein · MEK, MAP/ERK kinase · PDK, phosphoinositide-dependent kinase · PI 3-kinase, phosphatidylinositol 3'-kinase · PKB, protein kinase B · PKC, protein kinase C · PP2A, protein phosphatase 2A · PTEN, 3' phosphoinositide phosphatase · SHIP2, SH2 containing 5'-phosphoinositide phosphatase

## Introduction

Normal metabolic and endocrine function of adipose tissue is increasingly recognised as playing a major role in systemic insulin resistance accompanying common conditions such as Type 2 diabetes and obesity (for review see [1]). However, defining the function of adipose tissue in relation to whole-body fuel metabolism, as well as the molecular mechanisms underlying adipocyte insulin resistance, has proven to be a complex task: systemic insulin resistance accompanies both over-abundance of adipose tissue (obesity) [1], as well as conditions in which adipose tissue mass is lacking (lipodystrophy) [2]. At the molecular level, our understanding of the insulin signalling network appears to unravel an ever-increasing complexity. Currently it is even uncertain whether multiple differ-

ent signalling defects can result in the common endpoint of abnormal insulin response, or whether a relatively common signalling defect accounts for the majority of cases of adipocyte insulin resistance. Consequently, there is a great need to characterise in molecular terms the potential alterations leading to insulin resistance in a variety of conditions and models, and through that to allow mapping of the key steps in the insulin signalling cascade that may go awry and thus should be targeted therapeutically.

Recently, a new form of drug-induced insulin resistance has been identified in HIV-positive patients treated with a cocktail of anti-retroviral agents known as HAART. Metabolic side-effects may occur in up to 60% of HAART-treated patients, depending on host factors (presence of "traditional" risk factors for Type 2 diabetes) and on the specific agents used in the cocktail [3, 4]. Further clinical and pharmacological studies have identified the HIV protease inhibitors as a class of agents that may independently contribute to the development of peripheral insulin resistance [5], as well as to impaired pancreatic beta cell function [6]. Yet adipose tissue alterations appear to lie at the centre of this metabolic syndrome, since it is frequently associated with adipose tissue redistribution, i.e. lipodystrophy of subcutaneous "peripheral" adipose tissue, but an increase in abdominal adipose mass (central adiposity) [5, 7, 8]. Furthermore, impaired glucose tolerance and dyslipidaemia frequently occur, with elevated circulating levels of non-esterified fatty acids signifying abnormal regulation of adipocyte lipid metabolism [6]. Thus, a striking clinical similarity exists between common forms of the insulin resistance syndrome and this newly identified drug-induced metabolic syndrome. This similarity suggests that cellular processes leading to the latter may also shed light on the former.

Currently, several mechanisms have been proposed for the alterations in adipose tissue induced by HIV protease inhibitors. These include induction of adipocyte apoptosis [9], interference with terminal adipocyte differentiation [9, 10, 11], direct inhibition of the insulin-responsive glucose transporter GLUT4 [12, 13, 14] and interference with intracellular insulin signals leading to the deregulation of glucose and lipid metabolism [15]. None of these cellular mechanisms is necessarily mutually exclusive of the others [16, 17]. For example, nelfinavir, saquinavir and indinavir all acutely inhibit GLUT4-mediated glucose uptake, but only nelfinavir induced insulin resistance after an 18-h incubation [16]. Indeed, we have recently reported that differentiated 3T3-L1 adipocytes that were exposed to the HIV protease inhibitor nelfinavir for 18 h exhibited enhanced basal lipolysis and impaired responsiveness to insulin [15]. Insulin-stimulated glucose uptake was severely reduced, attributed to decreased capacity of insulin to recruit GLUT4 to the adipocyte plasma membrane.

In the present study, we aim to further characterise the molecular events leading to nelfinavir-induced adipocyte insulin resistance. Our data indicate that a signalling defect occurs downstream of PI 3-kinase activation, but upstream of the PI 3-kinase effectors Akt/protein kinase B (PKB) and protein kinase C- $\zeta$  (PKC- $\zeta$ ) (and p70S6 kinase). Furthermore, greatly diminished insulin-induced recruitment of PI 3-kinase effectors to the plasma membrane may underlie nelfinavir-induced insulin resistance in adipocytes.

## Materials and methods

*Materials and reagents.* Tissue culture medium, serum and antibiotic solutions were obtained from Biological Industries (Beit-Haemek, Israel). Recombinant human insulin was from Novo Nordisk (Bagsvaerd, Denmark). Anti p85, anti IRS-1, anti-phosphotyrosine (4G10), anti-protein phosphatase 2A (PP2A) antibodies and Ser/Thr Phosphatase Assay Kit 1 were supplied by Upstate Biotechnology (Lake Placid, N.Y., USA). Anti-Akt/PKB- $\beta$  was a kind gift from Dr D. Alessi (University of Dundee, Scotland, United Kingdom) and was used as described previously [18]. Anti Na/K ATPase was a kind gift from Dr I. Sakler (Ben-Gurion University, Beer-Sheva, Israel). We procured peroxidase-conjugated anti rabbit IgG, anti-mouse IgG, and [ $\gamma$ - $^{32}$ P] ATP from Amersham Life Sciences (Buckingham, United Kingdom). Protein A-Sepharose was from Pharmacia Biotech (Uppsala, Sweden). Anti-phospho p70S6 Kinase (Thr421/Ser424) antibody, anti-Akt/PKB (against the carboxy-terminal sequence of mouse Akt/PKB), anti-phosphoSer473 Akt/PKB, anti-phosphatase and tensin homolog (PTEN) were provided by Cell Signaling (Beverly, Mass., USA). Anti-PDK1 was from Transduction Laboratories (San Diego, Calif., USA). Anti-SHIP2 antibodies were prepared as previously described [19]. We sourced anti-PKC- $\zeta$  from Santa Cruz Biotechnology (Santa Cruz, Calif., USA), and obtained calyculin A, okadaic acid and all other chemicals from Sigma (St. Louis, Mo., USA). Nelfinavir was supplied by Roche Pharmaceuticals (Tel Aviv, Israel).

*Cell culture and treatments.* 3T3-L1 pre-adipocytes (American Type Culture Collection) were grown in DMEM and differentiated exactly as previously described [15, 16, 20]. Fully differentiated cells (10–12 days after induction of differentiation) were incubated in serum-free DMEM supplemented with 0.5% RIA-grade BSA, with or without 30  $\mu$ mol/l of nelfinavir, for 18 h. The final nelfinavir medium concentration was achieved by the appropriate dilution of a 100 mmol/l stock solution prepared in 100% ethanol. Final ethanol concentrations of up to 0.04% were found to have no measurable effects on the parameters measured in this study.

*Cell lysates and western blots.* Cells (one or two wells of a six-well plate per condition) were rinsed three times with PBS and incubated in the absence or presence of 100 nmol/l insulin for 7 min. Cells were then scraped into 0.25 ml/well of ice cold lysis buffer with the following constituents: 50 mmol/l Tris-HCl, pH 7.5, 0.1% [v/v] Triton X-100, 1 mmol/l EDTA, 1 mmol/l EGTA, 50 mmol/l NaF, 10 mmol/l sodium  $\beta$ -glycerophosphate, 5 mmol/l sodium pyrophosphate, 1 mmol/l sodium vanadate, 0.1% (v/v) 2-mercaptoethanol and inhibitors (1:1000 dilution of protease inhibitor cocktail [Sigma]). Lysates were shaken gently for 20 min at 4 °C and then centrifuged (12,000 g, 15 min at 4 °C). After this, supernatants were col-

lected and protein content determined using the Bio-Rad Bradford procedure [21]. Proteins were resolved by SDS/PAGE (7.5% or 10% gels) and subjected to western blot analyses, followed by quantification using video densitometry analysis. In each experiment the intensity of the band derived from control cells was assigned a value of 1 arbitrary unit, and the intensity of all treatment groups was expressed as a fold value of control.

**Immunoprecipitation and PI 3-kinase assay.** Cells were lysed as described above. For immunoprecipitation, 0.5 mg of cell-lysate protein was used and assayed as described in [16]. PI 3-kinase activity was measured following the protocol described by Hadari et al. [22], using phosphatidylinositol and [ $\gamma$ - $^{32}$ P] ATP as substrates. The phosphorylated phosphatidylinositols were separated using thin-layer chromatography, and quantified by video densitometry.

**Plasma membrane preparation.** Following treatment with or without 30  $\mu$ mol/l nelfinavir, cells (four 10-cm plates per condition) were rinsed three times with PBS and incubated for 7 or 20 min with or without 100 nmol/l insulin in freshly prepared medium supplemented with 0.5% bovine serum albumin (RIA grade). Cells were then washed twice with ice-cold PBS, scraped into 2 ml/plate ice cold buffer A (20 mmol/l HEPES pH 7.4, 255 mmol/l sucrose, 1 mmol/l EDTA, 0.2 mmol/l sodium vanadate and inhibitors [1:1000 dilution of protease inhibitor cocktail]) and immediately homogenised using 20 strokes of a teflon-glass homogeniser. Cell homogenates were centrifuged for 10 min (1000 g) to spin down unbroken cells and nuclei. Supernatants were centrifuged for 20 min (16,000 g), after which the pellet was resuspended in buffer B (buffer A without sucrose), loaded on a 1.12 mol/l sucrose cushion and centrifuged for 1 h (100,000 g, spin-out buckets). The interphase, which consists of the plasma membrane, was resuspended in buffer B and centrifuged for 30 min (30,000 g). The pellet of this last centrifugation was collected as the plasma membrane fraction.

**Protein phosphatase 2A activity.** Following treatment with or without 30  $\mu$ mol/l nelfinavir, cells were lysed in 50 mmol/l Tris-HCl, pH 7.0 containing 100  $\mu$ mol/l  $\text{CaCl}_2$ . After this PP2A activity was measured using the Ser/Thr Phosphatase Assay Kit 1, following the instructions of the manufacturer.

**Statistical analysis.** Data are expressed as means  $\pm$  SEM. Each treatment was compared with the control, and statistical significance between two groups was evaluated using the Student's *t* test.

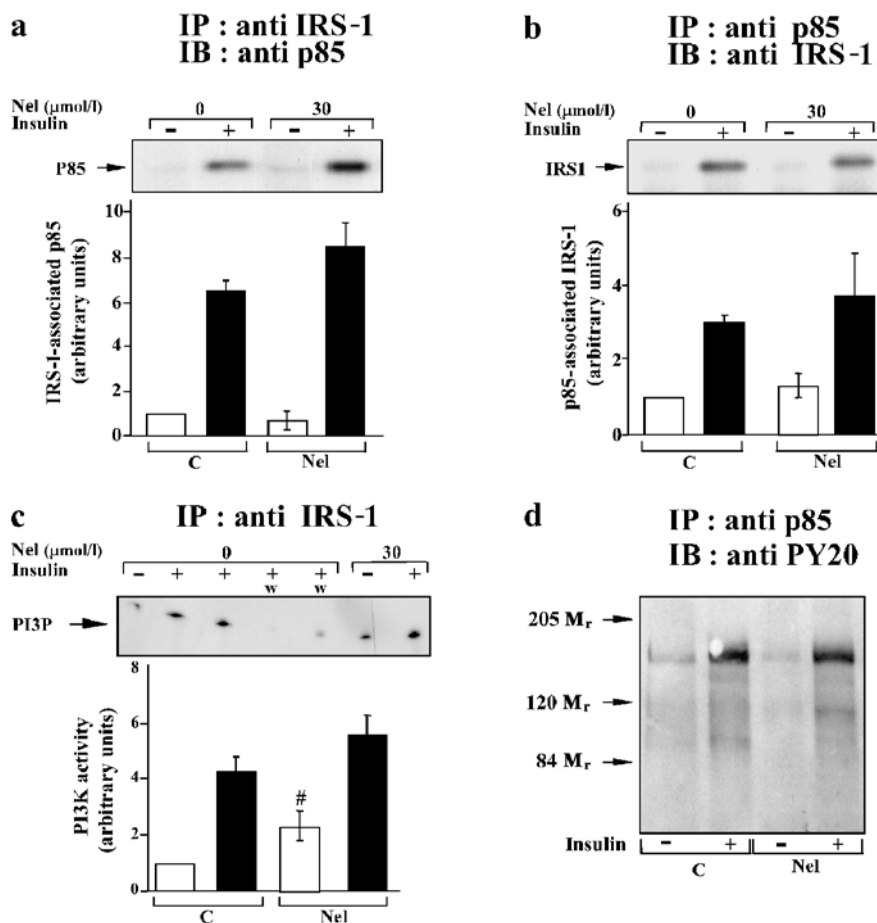
A *p* value of less than 0.05 was considered statistically significant.

## Results

**Nelfinavir impairs propagation of the insulin signal downstream of PI 3-kinase.** We have previously shown that 18 hours' exposure of 3T3-L1 adipocytes to the HIV protease inhibitor nelfinavir impairs their metabolic response to insulin. Basal glucose uptake was increased, while insulin-stimulated 2-deoxyglucose uptake was markedly reduced. The latter effect was attributed to an impairment of the capacity of insulin to normally stimulate recruitment of GLUT4 glucose transporters to the plasma membrane [15].

To begin characterising the defect in the insulin signal transduction cascade leading to nelfinavir-induced insulin resistance, we first assessed the activation of PI 3-kinase. Insulin-stimulated PI 3-kinase activation is largely achieved by interaction of the regulatory subunit of this enzyme, p85, with phosphotyrosine moieties on insulin receptor substrates, particularly IRS proteins. For this reason, insulin-induced interaction between PI 3-kinase and IRS-1 was assessed by reciprocal immunoprecipitation. In control cells, insulin greatly increased the amount of p85 that co-precipitated with IRS-1 (Fig. 1a), as well as the amount of IRS-1 that co-precipitated with p85 (Fig. 1b). Following treatment of differentiated 3T3-L1 adipocytes with 30  $\mu$ mol/l nelfinavir, these responses to insulin were maintained and even tended to be exaggerated, confirming normal insulin-induced IRS-1-p85 interaction. Consistently, when IRS-1-associated PI 3-kinase activity was measured in an *in vitro* kinase assay, control and nelfinavir-treated adipocytes responded with a similar net increase in PI 3-kinase activity in response to insulin (Fig. 1c). Yet basal IRS-1-associated PI 3-kinase activity was significantly greater in cells treated with nelfinavir than in control cells (*p*=0.03). To assess whether nelfinavir treatment significantly impaired the interaction of p85 with tyrosine phosphorylated proteins other than IRS-1, p85 was immunoprecipitated and the resulting pellets were assessed by phosphotyrosine immunoblots (Fig. 1d). Nelfinavir treatment altered the recovery of several minor phosphotyrosine bands (~95 and ~120  $M_r$ ) that co-precipitated with p85 in response to insulin. The intensity of the ~120  $M_r$  band was increased, while that of the ~95  $M_r$  band was decreased. The identity of those proteins is beyond the scope of the present study, yet this blot verifies that nelfinavir did not alter the intensity of the major phosphotyrosine bands of ~180  $M_r$ , which correspond to the IRS proteins (IRS-1 and IRS-2). Taken together, these results demonstrate that while nelfinavir may alter the interaction between PI 3-kinase and minor phosphotyrosine partners, it did not cause a gross reduction in the insulin-induced interaction between PI 3-kinase and its major interacting proteins, the IRSs.

Akt/PKB is a PI 3-kinase effector required for insulin-stimulated GLUT4 translocation and glucose uptake activity. Following activation of PI 3-kinase and the ensuing increase in cellular PI-3,4,5-P3 and PI-3,4-P2 content, Akt/PKB is activated by translocation to the cellular membrane, where it is phosphorylated by phosphoinositide-dependent kinases (PDKs). In control cells, insulin robustly stimulated the phosphorylation of Akt/PKB on Ser473 (Fig. 2a, lower blot). Following nelfinavir treatment, this process was inhibited by 80 $\pm$ 4%, while the total amount of Akt/PKB was unaltered (Fig. 2a, upper blot). Similarly, impaired insulin-stimulated phosphorylation of Akt/PKB on Thr308 was observed using a phospho-

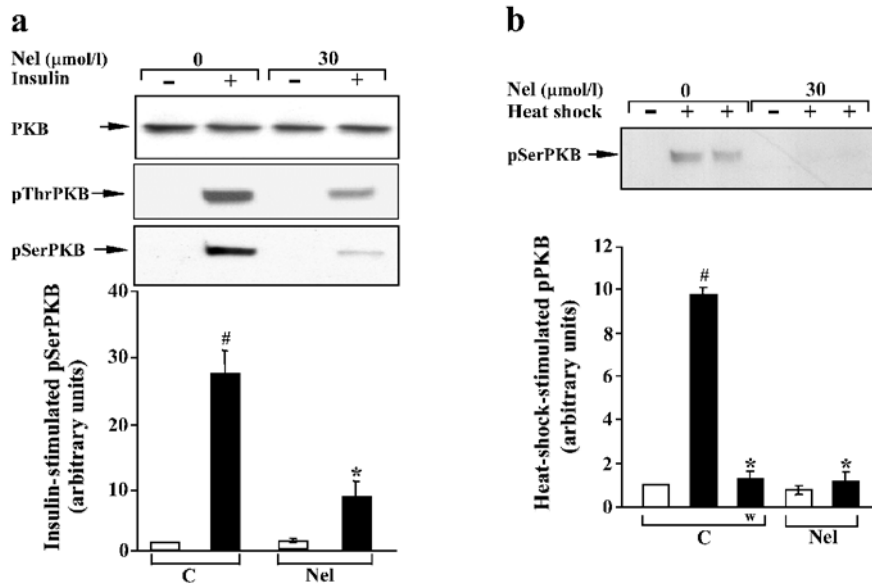


**Fig. 1.** Normal activation of PI 3-kinase through interaction with IRS in nelfinavir-treated 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were incubated for 18 h without or with 30 μmol/l nelfinavir (Nel), after which cells were thoroughly washed in PBS and treated for 7 min without or with 100 nmol/l insulin. Cell lysates were prepared as described in Materials and methods, and 500 μg protein were subjected to immunoprecipitation using the indicated antibodies, followed by western blot analysis and immunodetection of the indicated co-precipitated protein (**a**, **b**, **d**), or by an in vitro PI 3-kinase activity assay (**c**) (see Materials and methods). The blots (**a–d**) and densitometry analyses (**a**, **b**, **c**) are representative of at least three experiments. A value of 1 was assigned to the intensity of the band observed in control (C) cells in the absence of insulin. W: cells incubated with 250 nmol/l wortmannin 30 min before and during insulin stimulation. White bars, without insulin treatment; black bars, with insulin treatment. IP, immunoprecipitation; IB, immunoblot. # $p=0.03$  compared to control cells without insulin

specific antibody (Fig. 2a, middle blot). Exposure of 3T3-L1 adipocytes to heat shock stimulus also resulted in increased Akt/PKB Ser473 phosphorylation in control cells, in a PI 3-kinase-dependent (wortmannin-sensitive) process (Fig. 2b). Similar to insulin, heat-shock-induced Ser473 phosphorylation of Akt/PKB was nearly fully inhibited in cells treated with nelfinavir. Interestingly, a degree of Ser473 phosphorylation of Akt was discernable in control cells in re-

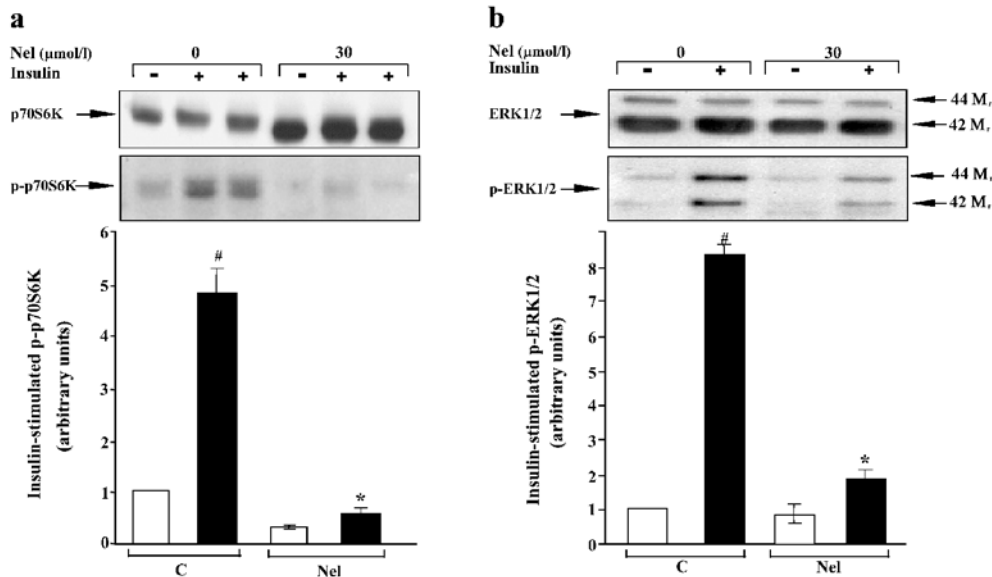
sponse to stimulation with platelet-derived growth factor-BB (50 μg/l) for 7 min. Nelfinavir treatment had no effect on this Akt phosphorylation induced by platelet-derived growth factor (data not shown). However, it is not clear whether this represents selectivity of nelfinavir to the insulin signalling cascade or the response of a sub-population of cells within the cell culture [23]. Consistent with the latter possibility is the finding that 3T3-L1 pre-adipocytes are resistant to the effects of nelfinavir, whereas their differentiated counterparts are not [16].

To determine whether nelfinavir also affected the insulin-mediated activation of other downstream Ser/Thr kinases, we assessed the phosphorylation of p70S6 kinase and the ERK (extracellular signal-regulated kinase) 1/2 MAP (mitogen-activated protein) kinases. The former is phosphorylated and activated by insulin in a manner that is dependent on PI 3-kinase [24], whereas the ERK MAP kinases are largely considered to be activated in a process that is independent of PI 3-kinase [25]. Despite a marked increase in the total content of p70S6 kinase in nelfinavir-treated cells, insulin-mediated phosphorylation of this protein on Thr421/Ser424 was blunted (Fig. 3a). These findings lead us to propose that nelfinavir induced cellular alterations that impaired the normal propagation of signals between PI 3-kinase activation and its downstream effectors. Surprisingly however, insulin-medi-



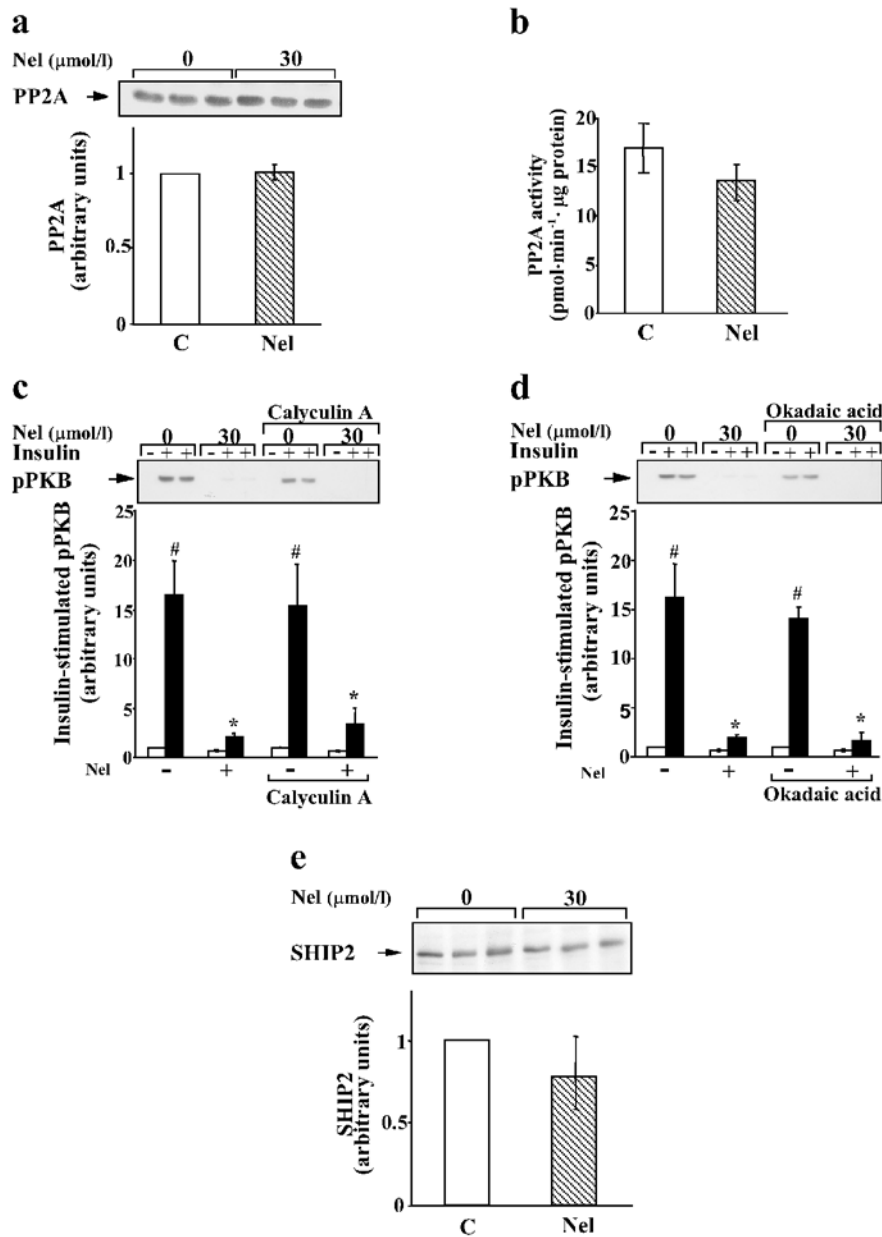
**Fig. 2.** Nelfinavir treatment impaired insulin and heat-shock-stimulated Akt/PKB phosphorylation. Treatment with nelfinavir (Nel) was as described in Figure 1, after which cells were exposed to 100 nmol/l insulin for 7 min (a) or to 44 °C for 20 min in the absence or presence of 250 nmol/l wortmannin (w) (b). Treated cells were washed and lysates were prepared, followed by western blot analyses using anti-total (PH domain) Akt/PKB, pThr308 or pSer473 Akt/PKB antibodies, as indicated. The blots

and densitometry analyses shown are representative of three experiments using anti pSer473 PKB antibodies, with a value of 1 assigned to the intensity of the band observed in control (C) cells in the absence of insulin. Cells incubated with 250 nmol/l wortmannin 30 min before and during exposure to heat shock: w. White bars, without insulin treatment; black bars, with insulin treatment. #  $p < 0.01$  compared with control cells without insulin; \*  $p < 0.05$  compared with insulin-stimulated control cells



**Fig. 3.** Nelfinavir treatment (Nel) impaired insulin stimulated p70S6 kinase and ERK 1/2 phosphorylation. Whole-cell lysates were prepared from insulin-treated and untreated cells as described in Figure 2. Western blot analysis using anti total and phospho-Thr421/Ser424 p70S6 kinase (a), and p-ERK1/2 or ERK1/2 (b) were performed. The blots and densitometry analyses (for p-P70S6 kinase and for p-ERK 1/2) are representative of four experiments with a value of 1 assigned to the intensity of the band observed in control (C) cells in the absence of insulin. White bars, without insulin treatment; black bars, with insulin treatment. #  $p < 0.05$  compared with control cells without insulin; \*  $p < 0.05$  compared with insulin-stimulated control cells

ated phosphorylation of ERK1/2 was also impaired (Fig. 3b). The nelfinavir concentrations that induced impaired phosphorylation of Akt/PKB, p70S6 kinase and ERK1/2 were similar, with significantly reduced phosphorylation observed with as low as 20 to 30  $\mu\text{mol/l}$  nelfinavir (data not shown). Based on these results, we next addressed two potential cellular alterations that would explain the observed impairment in insulin-mediated phosphorylation of kinases downstream of PI 3-kinase, as well as in the MEK (MAP/ERK kinase)–ERK signalling pathway.



**Fig. 4.** Lack of involvement of PP2A and SHIP2 in nelfinavir-induced insulin resistance. **a.** After treatment with or without nelfinavir (Nel), 40  $\mu\text{g}$  total cell lysate protein were subjected to SDS electrophoresis and immunoblot analysis using anti-PP2A antibodies. **b.** We used 5  $\mu\text{g}$  lysate protein (preparation, see Materials and methods) to measure PP2A activity with a commercial phosphatase activity kit. PP2A-specific activity was calculated using Pi as standard. After treatment with or without nelfinavir for 18 h (**c, d**), cells were rinsed and incubated for 30 min without or with 25  $\mu\text{mol/l}$  calyculin A (**c**) or 1  $\mu\text{mol/l}$  okadaic acid (**d**). Cells were then treated with or without insulin (7 min, 100 nmol/l), after which lysates were prepared and analysed for Akt/PKB Ser473 phosphorylation by western blot analysis using phospho-specific antibodies. **e.** We subjected 40  $\mu\text{g}$  total cell lysate protein to SDS electrophoresis and immunoblot analysis using anti-SHIP2 antibodies. Blots (**a, c, d, e**) and densitometry analyses are representative of four experiments in triplicate. For **b**, PP2A activity was measured in eight samples in duplicate. White bars, without insulin treatment; black bars, with insulin treatment; <sup>#</sup> $p < 0.05$  compared to control cells (C) without insulin; <sup>\*</sup> $p < 0.05$  compared to insulin-stimulated control cells

*Increased PP2A content and activity, or increased expression of phosphoinositide phosphatases cannot explain the impairment in insulin-stimulated Akt/PKB phosphorylation.* PP2A is a family of Ser/Thr protein phosphatases that are the major phosphatases of Akt/PKB, p70S6 kinase, as well as ERK1/2 [26, 27]. Hence, a potential cellular mechanism to explain the results described above is an enhanced rate of dephosphorylation of these kinases by PP2A. Moreover, increased PP2A activity was recently implicated in several models of insulin resistance in which normal PI 3-kinase, but reduced Akt/PKB activation were observed [26, 28, 29, 30]. Different complementary ways of assessing a potential role of PP2A in nelfinavir-induced insulin resistance were utilised. PP2A expression (Fig. 4a) and total cellular activity (Fig. 4b) were measured, and were unaltered in nelfinavir-treated cells versus controls.

To determine whether PP2A functionally contributed to the insulin-resistant state induced by nelfinavir, we assessed whether pharmacological inhibition of PP2A could improve the deficient insulin-stimulated Akt/PKB phosphorylation observed. Following treatment with nelfinavir, 3T3-L1 adipocytes were treated with either vehicle or the PP2A inhibitors calyculin A (Fig. 4c) or okadaic acid (Fig. 4d). Using a commercial kit to determine PP2A activity, we found that 1  $\mu\text{mol/l}$  okadaic acid and 25  $\mu\text{mol/l}$  calyculin A inhibited PP2A activity by 84% and 89% respectively. In non-insulin-stimulated cells these concentrations of the two inhibitors had no significant effect on the basal level of Akt/PKB Ser473 phosphorylation. Both calyculin A (Fig. 4c) and okadaic acid at 3 nmol/l (not shown) or 1  $\mu\text{mol/l}$  (Fig. 4d) failed to restore the normal response of Akt/PKB to insulin stimulation, which was impaired in nelfinavir-treated cells. Collectively, these findings argue against a significant contribution of PP2A to the insulin resistance induced by nelfinavir in 3T3-L1 adipocytes.

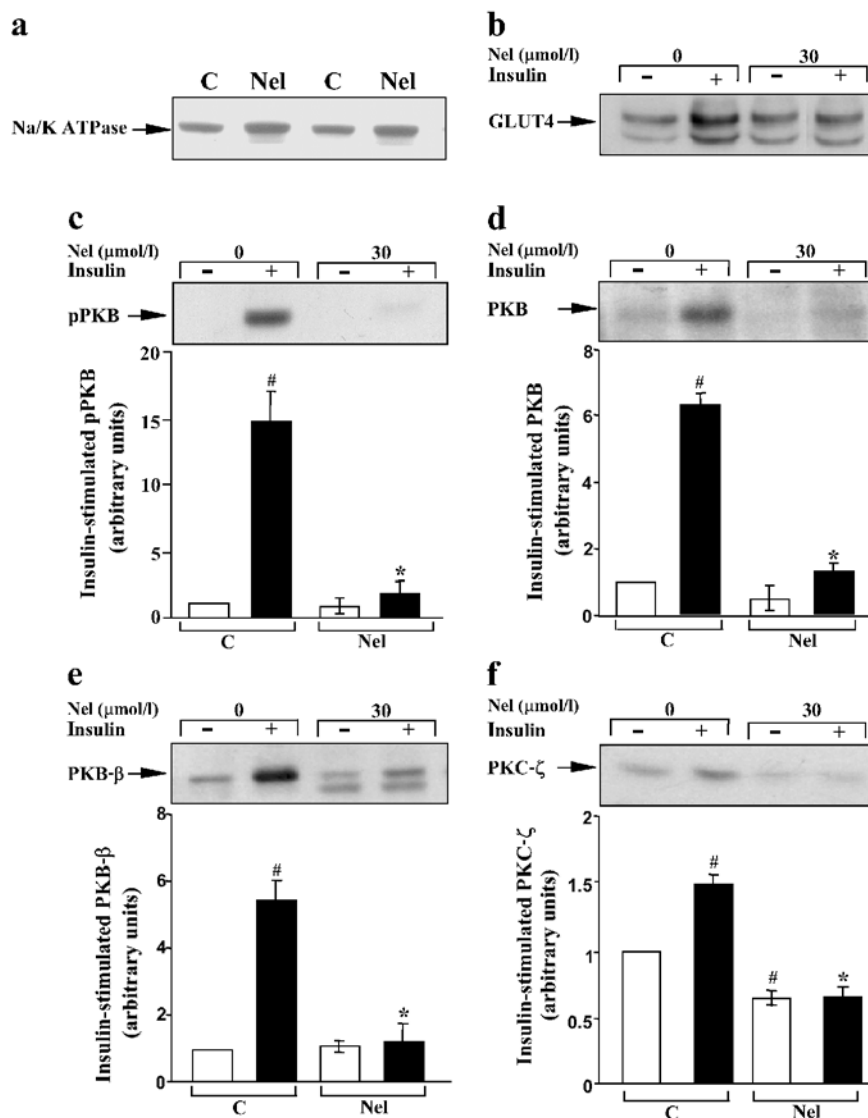
Increased expression of the SH2 containing 5'-phosphoinositide phosphatase (SHIP2) or of the 3'-phosphoinositide phosphatase (PTEN) could provide an additional cellular mechanism(s) capable of explaining normal PI 3-kinase activation but impaired Akt/PKB as well as ERK1/2 phosphorylation [31, 32, 33, 34]. Indeed, increased SHIP2 expression was recently observed in animal models of insulin resistance [35]. A breakpoint between PI 3-kinase and Akt/PKB and atypical PKCs is caused by a SHIP2-mediated decrease in the cellular PI-3,4,5,-P3 : PI-3,4,-P2 ratio [33], whereas impaired ERK1/2 activation could arise from competition of SHIP2 with Sos on its binding to Grb2, leading to decreased insulin-mediated ras activation [34]. However, protein levels of SHIP2 were not altered in nelfinavir-treated cells (Fig. 4e). Similarly, nelfinavir treatment did not increase the protein expression of PTEN (data not shown). Though not excluding the possibility that increased SHIP2 or PTEN activities play a role in nelfinavir-induced insulin resistance, SHIP2 or PTEN overexpression is unlikely to be the cause of this phenomenon.

*Insulin-stimulated recruitment of Akt/PKB and of PKC- $\zeta$  to the plasma membrane is impaired by nelfinavir.* As mentioned above, recruitment of both Akt/PKB and PKC- $\zeta$  to the plasma membrane is a necessary step in their activation, since at this locale these proteins meet their immediate upstream kinase PDK1 [36, 37]. To determine whether insulin-mediated recruitment of these two PI 3-kinase effectors to the plasma membrane is impaired in nelfinavir-treated cells, plasma membranes were isolated by sub-cellular fractionation. To verify that plasma membrane fraction recovery in nelfinavir-treated and control cells was comparable, the amount of Na/K ATPase in equally loaded amounts of plasma membrane proteins

was assessed. Na/K ATPase immunoreactivity was increased in nelfinavir-treated plasma membranes (Fig. 5a), suggesting that HPI treatment did not decrease plasma membrane fraction recovery. As expected to occur in the plasma membrane fraction of 3T3-L1 adipocytes, 20 min of insulin stimulation resulted in an approximately three-fold increase in the amount of GLUT4 in control cells (Fig. 5b). Yet this response to insulin was blunted in nelfinavir-treated cells, consistent with our previous findings in plasma membrane lawns [15]. Nelfinavir treatment also seemed to increase plasma membrane GLUT4 content in the basal state. Yet the plasma membrane GLUT4 : Na/K ATPase ratio was not significantly different in nelfinavir-treated cells from in control cells.

Consistent with the observations in total cell lysates (Fig. 2a), insulin stimulation resulted in a robust increase in Akt/PKB phosphorylated on Ser473 in the plasma membrane of control, but not of nelfinavir-treated cells (Fig. 5c). Yet in contrast to total cell lysates, in which the total protein content of Akt/PKB was unaltered by insulin or by nelfinavir (Fig. 2a), insulin induced, in the plasma membrane fraction of control, but not of nelfinavir-treated cells, a six-fold increase in the amount of Akt/PKB detected using an antibody that recognises all isoforms of this protein (Fig. 5d). Since the  $\beta$  isoform of PKB (or Akt2) was recently linked to insulin stimulation of GLUT4 translocation in adipocytes [38, 39, 40], we also assessed its recruitment to the plasma membrane in response to insulin using an isoform-specific antibody. In control cells, insulin stimulation resulted in a nearly six-fold increase in Akt2/PKB- $\beta$  plasma membrane content (Fig. 5e). In cells treated with nelfinavir, this effect of insulin was greatly impaired. In nelfinavir-treated cells the anti-Akt2/PKB- $\beta$  antibody also reproducibly (in five independent experiments) detected an additional band of a molecular weight lower than that of Akt2/PKB- $\beta$  (Fig. 5e). The identity of this band is unknown, but there was no increase in its intensity following insulin stimulation.

Similar to these findings with Akt/PKB, insulin stimulation in control cells was associated with a 1.5-fold ( $p=0.02$ ) increase in the amount of PKC- $\zeta$  in the plasma membrane (Fig. 5f). Nelfinavir-treated cells had a 40% lower content of this enzyme in the plasma membrane fraction in the basal conditions ( $p<0.01$ ). Importantly, insulin treatment failed to result in further recruitment of the enzyme to the plasma membrane fraction. Since the total amount of PDK1 was not altered by nelfinavir treatment (data not shown), these data suggest that the impaired insulin-stimulated Akt/PKB phosphorylation seen in total cell lysates of nelfinavir-treated adipocytes is a result of impaired cellular relocation of Akt/PKB in response to acute insulin stimulation.



**Fig. 5.** Plasma membrane recruitment of Akt/PKB and of PKC- $\zeta$  is impaired in nelfinavir-treated cells. Nelfinavir-treated (Nel) and control cells (C) were rinsed three times with PBS and stimulated for 7 min (a, c–f) or 20 min (b) without or with 100 nmol/l insulin. Plasma membrane fractions were then prepared (see Materials and methods), and 50  $\mu$ g protein were subjected to SDS electrophoresis and blotted with anti-Na/K-ATPase (a), anti-GLUT4 (b), anti-phospho-Ser473 Akt/PKB (c), anti total (PH domain) Akt/PKB (d), anti-Akt2/PKB- $\beta$  (e) or anti PKC- $\zeta$  (f) antibodies. Each panel contains a representative blot of two (a, b) or five (c–f) independent experiments with similar results. White bars, without insulin treatment; black bars, with insulin treatment. #  $p < 0.02$  compared with control cells without insulin; \*  $p < 0.05$  compared with insulin-stimulated control cells

## Discussion

The first proposed mechanism to explain HPI-induced insulin resistance was the acute inhibition of glucose transport through GLUT4 [13]. Additional mechanisms have since been proposed, suggesting

that “multiple hits” may affect the normal function of various cell types exposed to these agents. In the present study we aimed at characterising the insulin signalling defect that is induced in adipocytes exposed to the HIV protease inhibitor nelfinavir for 18 h. We show that insulin-induced interaction between PI 3-kinase and IRS proteins is intact, as well as PI 3-kinase activity as assessed in vitro in IRS-1 immunoprecipitates. However, insulin-induced Ser phosphorylation of Akt/PKB, and p70S6 kinase are markedly impaired. The reduction in Akt/PKB phosphorylation may be due to a failure of insulin to promote its translocation to the plasma membrane, a process required for its phosphorylation and activation. Furthermore, plasma membrane recruitment of PKC- $\zeta$  is also perturbed. The study therefore identifies an insulin signalling defect induced in nelfinavir-treated adipocytes and “located” between PI 3-kinase activation and the translocation of Akt/PKB and PKC- $\zeta$  to the plasma membrane. The latter may lead to the impaired GLUT4 translocation previously reported [15].



A defect in the transmission of the insulin signal between PI 3-kinase and its downstream effectors Akt/PKB and PKC- $\zeta$  was observed in several cellular models of insulin resistance. These include insulin resistance induced by high glucose [28], palmitate and ceramide [26, 30], hyperosmolarity [29], actin cytoskeleton disruption [41] and oxidative stress [20]. In the case of palmitate and ceramide, it was proposed that increased PP2A activity underlies this signalling defect. This proposition was based on either a demonstrated increase in PP2A expression and/or activity, or on the capacity of okadaic acid or calyculin A to reverse the signalling defect [26]. None of these seem to apply in nelfinavir-treated adipocytes (Fig. 4a–d).

In the case of insulin resistance induced by oxidative stress, abnormal activation of PI 3-kinase in the LDM fraction (due to impaired cellular re-distribution of PI 3-kinase and IRS-1) was proposed as the underlying mechanism [20]. Here, we demonstrate that following nelfinavir treatment, insulin-induced cellular redistribution of Akt/PKB and of PKC- $\zeta$  was impaired. Thus, both conditions are characterised by an impaired capacity of signalling molecules to be normally relocated in response to insulin and to be activated in a specific cellular locale, a process deemed necessary for signal propagation. The possibility that intracellular generation of reactive oxygen species mediates nelfinavir-induced insulin resistance is currently being studied in our laboratory.

Insulin-induced recruitment of Akt/PKB and PKC- $\zeta$  to the plasma membrane depends on generation of PI 3-kinase lipid products [36, 37, 42]. It is, therefore, plausible that nelfinavir treatment affects the normal accumulation of PI-3,4,5-P3 and/or of PI-3,4-P2 in response to insulin, leading to impaired recruitment of PI 3-kinase effectors to the plasma membrane. The fact that p85/IRS interaction is maintained in nelfinavir-treated cells and that *in vitro* PI 3-kinase assay reveals normal activation by insulin (Fig. 1a–d) does not rule out the possibility that the enzyme is not activated normally within the cell. Such a discrepancy between PI 3-kinase activity as assessed in an *in vitro* kinase assay or *in vivo* has been previously reported following exposure to calmodulin antagonists [43]. Moreover, assuming that our data reflect the *in vivo* situation, i.e. that PI 3-kinase is activated normally, it is possible that modulation of 3-PIP content occurs by enhanced degradation. This is exemplified by recent papers demonstrating that overexpression of SHIP2, which decreases the PI-3,4,5-P3 : PI-3,4-P2 ratio, leads to impaired activation of Akt/PKB and aPKC and to metabolic insulin resistance [33, 44]. Overexpression of other PIP phosphatases, either of the 3' position [32] or of the 5' position of the inositol ring [45], was also shown to induce insulin resistance. However, the protein content both of SHIP2 and of PTEN is not increased in nelfinavir-treated cells (Fig. 4e). These findings do not exclude the possibility

that activation and/or altered cellular localisation, without changes in protein content, occurred in nelfinavir-treated cells.

If increased PP2A activity or SHIP2 expression are not responsible for nelfinavir-induced insulin resistance, the mechanism for the impaired insulin-stimulated phosphorylation of ERK1/2 remains unexplained. Though ERK activation downstream of ras and MEK are largely seen as an insulin signalling arm independent of PI 3-kinase, cross-talk between PI 3-kinase and ras pathways is well described in the literature [46]. Moreover, few reports have challenged the dichotomous “2 arms view” of the insulin signalling cascade, which consists of mitogenic (i.e. ras–MEK–ERK) versus metabolic (PI 3-kinase–Akt/PKB) linear signalling pathways. Inhibition of PI 3-kinase has been reported to also affect ERK1/2 both in 3T3-L1 adipocytes and in primary adipocytes [46, 47]. Whether the insulin signalling defect that occurs between PI 3-kinase activation and the recruitment of its downstream effectors to the plasma membrane also results in impaired ERK activation remains to be further explored.

Studies on humans and animal models of insulin resistance have demonstrated defects in the insulin signalling cascade at levels as proximal as the insulin receptor expression level and its tyrosine kinase activity [48, 49, 50]. If insulin resistance is a result of such a proximal signalling defect, what is the physiological relevance of an additional breakage point further downstream, like the one described in this study?

We believe that the literature to date does not rule out a role for insulin signalling defect(s) downstream of PI 3-kinase. Normal insulin-induced Akt/PKB activation in skeletal muscle of diabetic patients was observed despite a significant decline in PI 3-kinase activation [51], suggesting that a dramatic (>50%) decrease in proximal signalling events may be required to render them rate-limiting for propagation of the insulin signal. In addition, a recent study demonstrated that the impairment of Akt/PKB and PKC activation in tissues of insulin-resistant rats was larger than the defect observed in more proximal insulin signalling events (IRS-1 tyrosine phosphorylation) [35]. This finding suggests that multiple hits, both proximal and more distal, occur *in vivo* within the insulin signalling cascade, resulting in metabolic insulin resistance. We believe that transmission of the insulin signal between PI 3-kinase and its downstream effectors may represent a “sensitive” cellular process, which is possibly impaired in various states of insulin resistance, including that induced by nelfinavir.

*Acknowledgements.* This study was supported by a grant (no. 568/02) from the Israel Science Foundation. We are thankful to Dr Eva Degerman (Lund University, Sweden) for the initial studies on PP2A, and to Evgeniya Malyarevskaya for excellent technical assistance. R. Ben-Romano and A. Rudich contributed equally to this work.

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