

Indinavir and Nelfinavir Inhibit Proximal Insulin Receptor Signaling and Salicylate Abrogates Inhibition: Potential Role of the NFκB Pathway

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ABSTRACT

The molecular basis of insulin resistance induced by HIV protease inhibitors (HPIs) remains unclear. In this study, Chinese hamster ovary cells transfected with high levels of human insulin receptor (CHO-IR) and 3T3-L1 adipocytes were used to elucidate the mechanism of this side effect. Indinavir and nelfinavir induced a significant decrease in tyrosine phosphorylation of the insulin receptor β -subunit. Indinavir caused a significant increase in the phosphorylation of insulin receptor substrate-1 (IRS-1) on serine 307 (S307) in both CHO-IR cells and 3T3-L1 adipocytes. Nelfinavir also inhibited phosphorylation of Map/ERK kinase without affecting insulin-stimulated Akt phosphorylation. Concomitantly, levels of protein tyrosine phosphatase 1B (PTP1B), suppressor of cytokines signaling-1 and -3 (SOCS-1 and -3), Src homology 2B (SH2B) and adapter protein with a pleckstrin homology domain and an SH2 domain (APS) were not altered significantly. When CHO-IR cells were pre-treated with sodium salicylate (NaSal), the effects of indinavir on tyrosine phosphorylation of the IR β -subunit and phosphorylation of IRS-1 at S307 were abrogated. These data suggest a potential role for the NF κ B pathway in insulin resistance induced by HPIs. *J. Cell. Biochem.* 114: 1729–1737, 2013. © 2013 Wiley Periodicals, Inc.

KEY WORDS: HIV PROTEASE INHIBITORS; INSULIN RESISTANCE; SALICYLATE

After 30 years ago, HIV infection remains a major threat globally in particular in the southern part of sub-Saharan Africa [Dieffenbach and Fauci, 2011]. While HIV vaccine development is a promising solution, the treatment of HIV/AIDS is still of major concern.

The most potent HIV treatment is highly active antiretroviral therapy (HAART). This has increased longevity but has also increased the incidence of various metabolic disorders such as insulin resistance

and lipodystrophy, which are associated with type 2 diabetes mellitus and cardiovascular disease respectively [Barbaro, 2002; Florescu and Kotler, 2007; Stein, 2007]. It has emerged that HIV protease inhibitors (HPIs), one of the components of HAART drug therapy, play a critical role in inducing the side effects. These drugs impair insulin-regulated metabolism in adipose tissue [Gougeon et al., 2004].

Previous studies reported that HPI drugs at plasma concentrations (10 μ M) affect distal steps in the insulin signaling pathway [Murata

Abbreviations: HAART, highly active antiretroviral therapy; HPIs, HIV protease inhibitors; CHO-IR, Chinese hamster ovary cells transfected with high levels of human insulin receptor; IR, insulin receptor; IRS-1, insulin receptor substrate-1; S307, serine 307; PTP1B, protein tyrosine phosphatase 1B; SOCS-1 and -3, suppressor of cytokines signaling-1 and -3; SH2-B, Src homology 2-B; APS, adapter protein with a pleckstrin homology domain and an SH2 domain; NF κ B, nuclear factor kappa B; I κ B, inhibitor kappa B; IKK β , I κ B kinase β ; GLUT4, glucose transporter 4; NaSal, sodium salicylate, Map/Erk Kinase; mitogen-activated/Extracellular signal-regulated protein kinase.

Conflicts of interest: nothing to declare.

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et al., 2002]. Specifically, the drugs inhibit glucose transporter 4 (GLUT4) at the cell membrane, preventing the uptake of glucose into cells, resulting in hyperglycemia. However, the precise mechanism whereby the drugs induce insulin resistance and consequent hyperglycemia is still uncertain [Murata et al., 2002; Noor et al., 2002; Rudich et al., 2003, 2005].

In this study, we used indinavir, a prototypic HPI, to analyze the effects on the insulin signaling pathway in Chinese hamster ovary cells transfected with high levels of human insulin receptor (CHO-IR) and differentiated murine 3T3-L1 adipocytes. 3T3-L1 adipocytes have a similar morphology and physiology to human adipocytes. The cells also express additional proteins that are not present in CHO-IR cells, such as the adapter protein with a pleckstrin homology domain and an SH2 domain (APS), and the glucose transporter GLUT4 [Ahn et al., 2004].

This is the first study in which insulin receptor-transfected cells have been used to investigate the effects of HPIs. We found that indinavir (50 μ M) inhibited insulin signaling at a proximal level in the insulin signaling cascade. In particular, indinavir inhibited tyrosine phosphorylation of the insulin receptor β -subunit and its substrate, insulin receptor substrate-1 (IRS-1). This was associated with increased phosphorylation of IRS-1 on serine 307 (S307).

In order to identify the pathway involved, CHO-IR cells were treated with sodium salicylate (NaSal, 5 mM) which inhibits the upstream activator of nuclear factor kappa B (NF κ B), the I κ B kinase β (IKK β), resulting in decreased phosphorylation of IRS-1 on S307. Salicylates abrogated the effects of indinavir, implicating the involvement of the NF κ B pathway.

MATERIALS AND METHODS

MATERIALS

CHO-IR cells and 3T3-L1 pre-adipocytes were used as previously described [Ahmed and Pillay, 2003]. Media and other tissue culture supplies were purchased from Sigma-Aldrich Co. and Lonza Group Ltd. Indinavir was donated by Merck & Co. and NaSal was obtained from Sigma-Aldrich Co. Nelfinavir was obtained from the NIH Aids reagents program. The antibodies used in the study were: mouse monoclonal anti-phosphotyrosine (PY20; BD Transduction), rabbit polyclonal: anti-insulin receptor β -subunit, anti-protein tyrosine phosphatase 1B (PTP1B), anti-suppressor of cytokines signaling-1 (SOCS-1) and anti-SOCS-3, goat polyclonal anti-actin, mouse anti-goat horseradish peroxidase (HRP)-conjugated secondary antibody and goat anti-rabbit HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.), rabbit polyclonal: anti-IRS-1, anti-phosphoIRS-1 at S307, and anti-APS (Cell Signaling Technology, Inc.), goat anti-mouse HRP-conjugated secondary antibody (Thermo Fisher Scientific, Inc.), and rabbit polyclonal anti-Src homology 2-B (SH2B, produced in the authors' laboratory) [Kotani et al., 1998].

CELL CULTURE

CHO-IR cells were cultured in Ham's F12 mixture culture medium and incubated at 37°C in a 5% CO₂ incubator. 3T3-L1 pre-adipocytes were cultured and differentiated as previously described [Ahn et al., 2004].

SAMPLE PREPARATION

CHO-IR cells (1×10^5 cells/ml) were cultured in six-well plates. 3T3-L1 adipocytes were differentiated and grown to full confluence in 3.5 cm dishes [Mehra et al., 2007] and serum-starved for 16 h. Prior to aspirating the serum-free medium (SFM) from the cells, the cells were stimulated with insulin (10 ng/ml) for 5 min. The cells were lysed in lysis buffer [50 mM HEPES, pH 7.5, 1% NP40, 10% glycerol, 50 mM NaCl, protease inhibitor and phosphatase inhibitor (Roche)] and mixed with 5 \times Laemmli buffer (25 mM Tris, pH 6.8, 2% SDS, 0.002% bromophenol blue, 10% glycerol, and 5% 2-mercaptoethanol) prior to boiling at 100°C for 5 min. The experiment was repeated using cells pre-treated with NaSal (5 mM) for 1 h prior to indinavir (100 μ M) treatment for 16 h in SFM. NaSal, indinavir, and nelfinavir were prepared as previously described [Yuan et al., 2001; Vernochet et al., 2005]. The impact of the solvent and cell viability were monitored in preliminary experiments. The concentrations used did not affect cell viability.

IMMUNOBLOT/WESTERN BLOT ANALYSIS

Immunoblotting was performed as previously described [Ahmed and Pillay, 2003]. Phosphorylation of IR β -subunit and IRS-1 was detected using anti-PY20 antibody. The proteins were visualized using chemiluminescent reagent (Pierce) and quantified by ChemImager software (Fluorchem). The membrane was stripped with a stripping buffer (62.5 mM Tris, pH 6.8, 2% SDS and 100 mM of 2-mercaptoethanol) at 50°C for 15 min to reprobe with the other antibodies listed in the materials section. Anti-actin was used as a loading control for cellular protein. The section of the membrane containing the protein was cut into a horizontal strip and used for probing and reprobing. Membranes were probed and stripped up to twice in total only.

STATISTICAL ANALYSIS

Data are expressed as the mean \pm SEM of at least triplicate experiments. Statistical comparisons were made using Student's *t*-test and factorial-ANOVA.

RESULTS

EFFECT OF HPIs ON THE PHOSPHORYLATION OF INSULIN RECEPTOR β -SUBUNIT AND IRS-1

CHO-IR cells and 3T3-L1 adipocytes were pre-treated with indinavir for 16 h prior to stimulation with a submaximal insulin concentration (10 ng/ml; 1.7 nM) for 5 min (Figs. 1 and 2). In CHO-IR cells, samples pre-treated with indinavir and stimulated with insulin showed a significant decrease in tyrosine phosphorylation of the insulin receptor β -subunit ($P=0.02$; Fig. 1a) and in the phosphorylation of IRS-1 ($P=0.0002$; Fig. 1b). In 3T3-L1 adipocytes under the same conditions, there was a significant decrease in tyrosine phosphorylation of the insulin receptor β -subunit ($P=0.003$; Fig. 2a) and IRS-1 ($P=0.0002$; Fig. 2b), as compared with cells stimulated with insulin only.

The expression levels of total insulin receptor β -subunit and IRS-1 were also examined to determine if the decreased tyrosine phosphorylation was due to changes in protein expression. There were no significant differences in expression levels of insulin

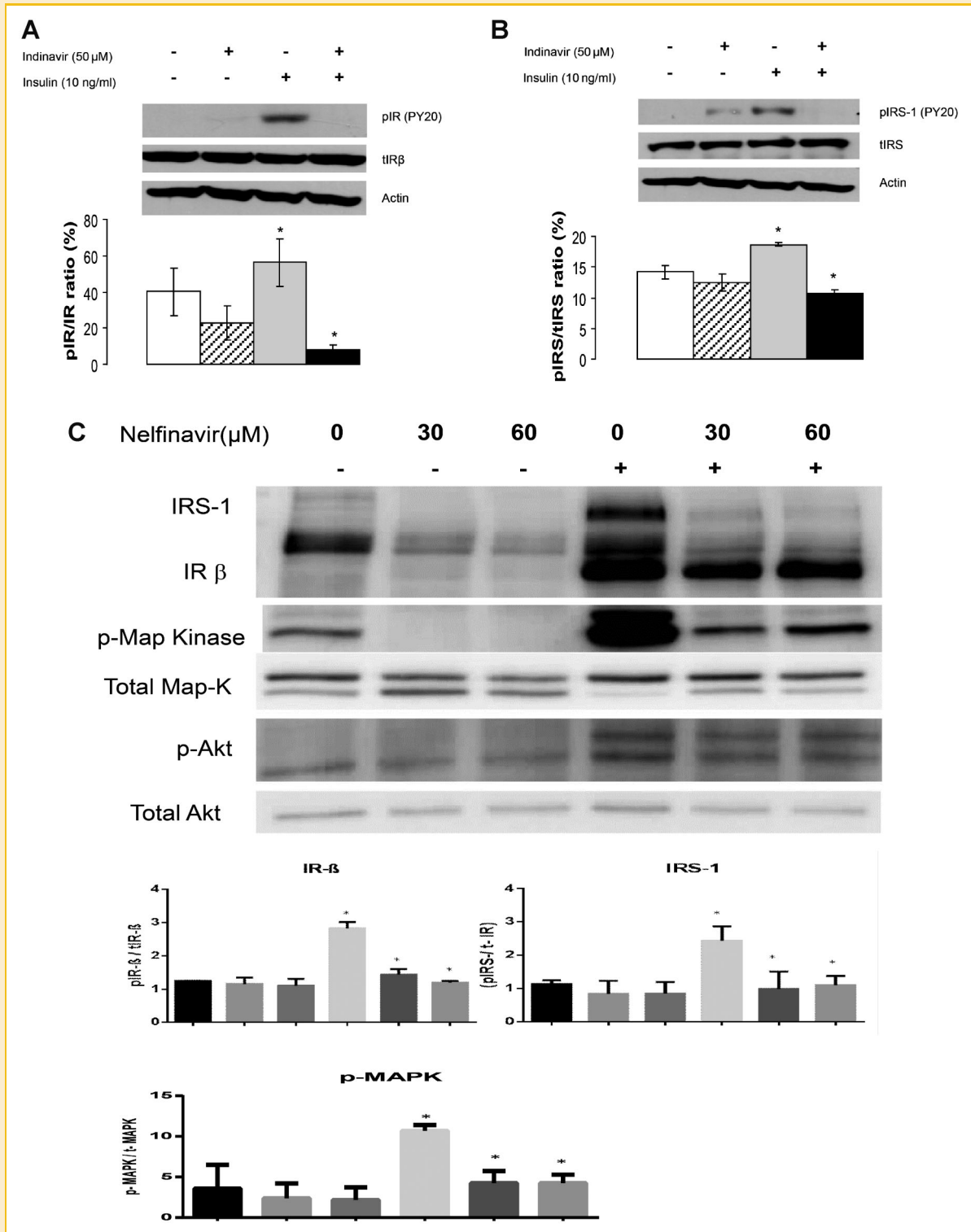


Fig. 1. Inhibition of insulin-stimulated tyrosine phosphorylation by indinavir and nelfinavir in CHO-IR cells. a: CHO-IR cells were treated with indinavir (50 μ M) for 16 h followed by insulin stimulation. The cells were then lysed and the lysates subjected to immunoblotting with anti-phosphotyrosine PY20 antibody (upper panels). The blots were then stripped and reprobbed with anti-IR β -subunit (a), anti-IRS-1 (b) and anti-actin (lower panels). Bound antibodies were visualized by chemiluminescence. Chemiluminescence was quantified by real-time imaging. The band intensity of the proteins from five independent experiments was normalized to the band intensity of total IR or IRS protein. Data are shown as the mean \pm SEM (n = 5). pIR (PY20), tyrosine phosphorylation of IR; tIR β , total insulin receptor β -subunit; pIRS-1 (PY20), tyrosine phosphorylation of IRS-1; tIRS-1, total IRS-1. (c) Cells were treated with nelfinavir as indicated and analyzed by anti-phosphotyrosine immunoblotting and blotting for phosphor-Mapkinase and phosphoAkt and total Mapkinase and Akt. The quantification of the chemiluminescence is illustrated in the graphs from three independent experiments. ** $P < 0.05$, using Student's *t*-test. pIRS-1 (PY20), tyrosine phosphorylation of IRS-1; tIRS-1, total IRS-1; pIR (PY20), tyrosine phosphorylation of IR; tIR β , total insulin receptor β -subunit.

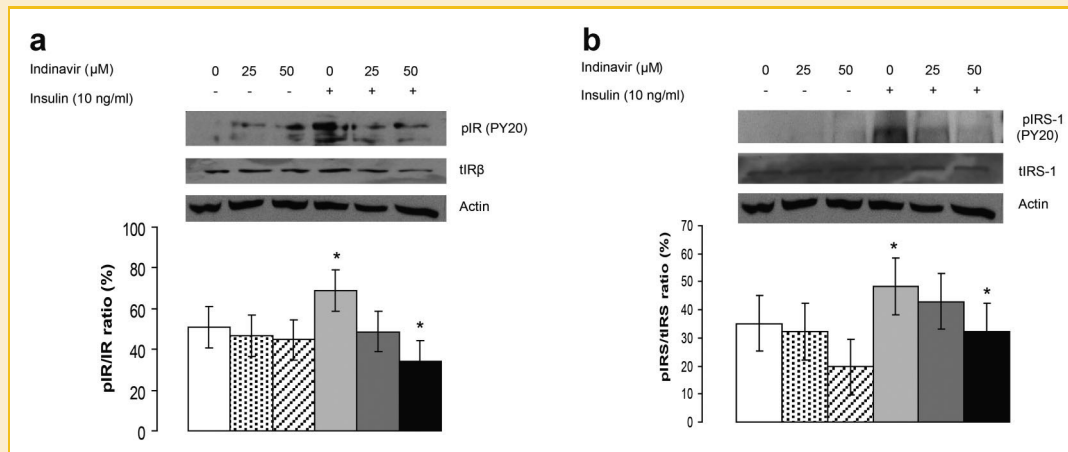


Fig. 2. Inhibition of insulin-stimulated tyrosine phosphorylation by indinavir in 3T3-L1 adipocytes. 3T3-L1 adipocytes were treated with varying concentrations of indinavir for 16 h before and after insulin stimulation. The cells were then lysed and the lysates subjected to immunoblotting with anti-phosphotyrosine PY20 antibody (upper panels). The blots were then stripped and reprobbed with anti-IR β -subunit (a), anti-IRS-1 (b) and anti-actin (lower panels). Bound antibodies were visualized by chemiluminescence. Chemiluminescence was quantified by real-time imaging. The band intensity of the proteins from six independent experiments was normalized to the band intensity of total IR or IRS protein. Data are shown as the mean \pm SEM ($n = 6$). pIR PY20, tyrosine phosphorylation of IR; tIR β , total insulin receptor β -subunit; pIRS-1 (PY20), tyrosine phosphorylation of IRS-1; tIRS-1, total IRS-1. * $P < 0.05$, using Student's t -test. pIRS-1 (PY20), tyrosine phosphorylation of IRS-1; tIRS-1, total IRS-1; pIR (PY20), tyrosine phosphorylation of IR; tIR β , total insulin receptor β -subunit.

receptor β -subunit and IRS-1 among the samples, indicating that the decreased signal observed (Figs. 1 and 2) was due to a decrease in tyrosine phosphorylation rather than in total protein levels. In addition, the expression level of actin was also examined as a loading control for cellular protein. The change in IRS-1 phosphorylation in Figure 1b was not significant when normalized to protein levels.

Based on these results, two possible hypotheses could be invoked: viz. (1) the drug inhibits the insulin receptor kinase directly or (2) the drug effects are indirect, and involve a regulator of tyrosine phosphorylation.

In order to test these hypotheses, the blots were stripped and reprobbed with antibodies against phosphotyrosine regulatory proteins of insulin signaling, such as phosphoIRS-1 at S307, PTP1B, SOCS-1 and -3, SH2-B, and APS.

We performed additional experiments with nelfinavir, a newer protease inhibitor (Fig. 1c,d). Similarly to indinavir, nelfinavir also inhibited insulin-stimulated tyrosine phosphorylation of the receptor beta-subunit and IRS proteins by greater than 50%. Interestingly, nelfinavir also inhibited insulin-stimulated Map Kinase activation by more than 50% whilst leaving Akt activation largely intact.

EFFECT OF HPIs ON REGULATORY PROTEINS IN THE INSULIN SIGNALING PATHWAY

There was a significant increase in the level of phosphorylated S307 on IRS-1 in both cell lines, CHO-IR cells ($P = 0.001$; Fig. 3a) and 3T3-L1 adipocytes ($P = 0.004$; Fig. 4a), treated with indinavir and then stimulated with insulin, compared to cells stimulated with insulin alone. These results were not accompanied by a change in the level of total IRS-1 (Figs. 3a and 4a).

The expression levels of PTP1B, SOCS-1 and -3, and SH2B in CHO-IR cells after pre-treatment with indinavir were not significantly different (Fig. 3b-e). In 3T3-L1 adipocytes, pre-treatment with indinavir did not significantly alter the expression levels of PTP1B, SOCS-1 and -3, SH2B and APS (Fig. 4b-f).

EFFECTS OF INDINAVIR AFTER NaSal PRE-TREATMENT

CHO-IR cells were used to examine the effect of NaSal on indinavir treatment, as the cells have a high level of insulin receptor expression, which facilitates more sensitive detection of changes in signaling proteins than in 3T3-L1 adipocytes.

Cells pre-treated with NaSal prior to treatment with indinavir showed a significant increase in insulin-stimulated tyrosine phosphorylation of the insulin receptor β -subunit ($P = 0.002$; Fig. 5a) when compared to cells treated with indinavir and insulin only. In fact, insulin-stimulated tyrosine phosphorylation was restored to the levels seen in the presence of insulin only, without indinavir. This indicates that NaSal blocked the effects of indinavir to inhibit tyrosine phosphorylation of the insulin receptor β -subunit.

A slight, but statistically insignificant, increase in insulin-stimulated tyrosine phosphorylation of IRS-1 was also observed after NaSal treatment, when compared to cells treated with indinavir and insulin alone (Fig. 5b).

NaSal also decreased phosphorylation of IRS-1 at S307 twofold, although this was not statistically significant compared to cells treated with indinavir followed by insulin (Fig. 5c). These results suggest that NaSal treatment abrogated the effects of indinavir to inhibit tyrosine phosphorylation of the insulin receptor β -subunit and IRS-1 in parallel with a decrease in phosphorylation of IRS-1 at S307 (Fig. 5a,c).

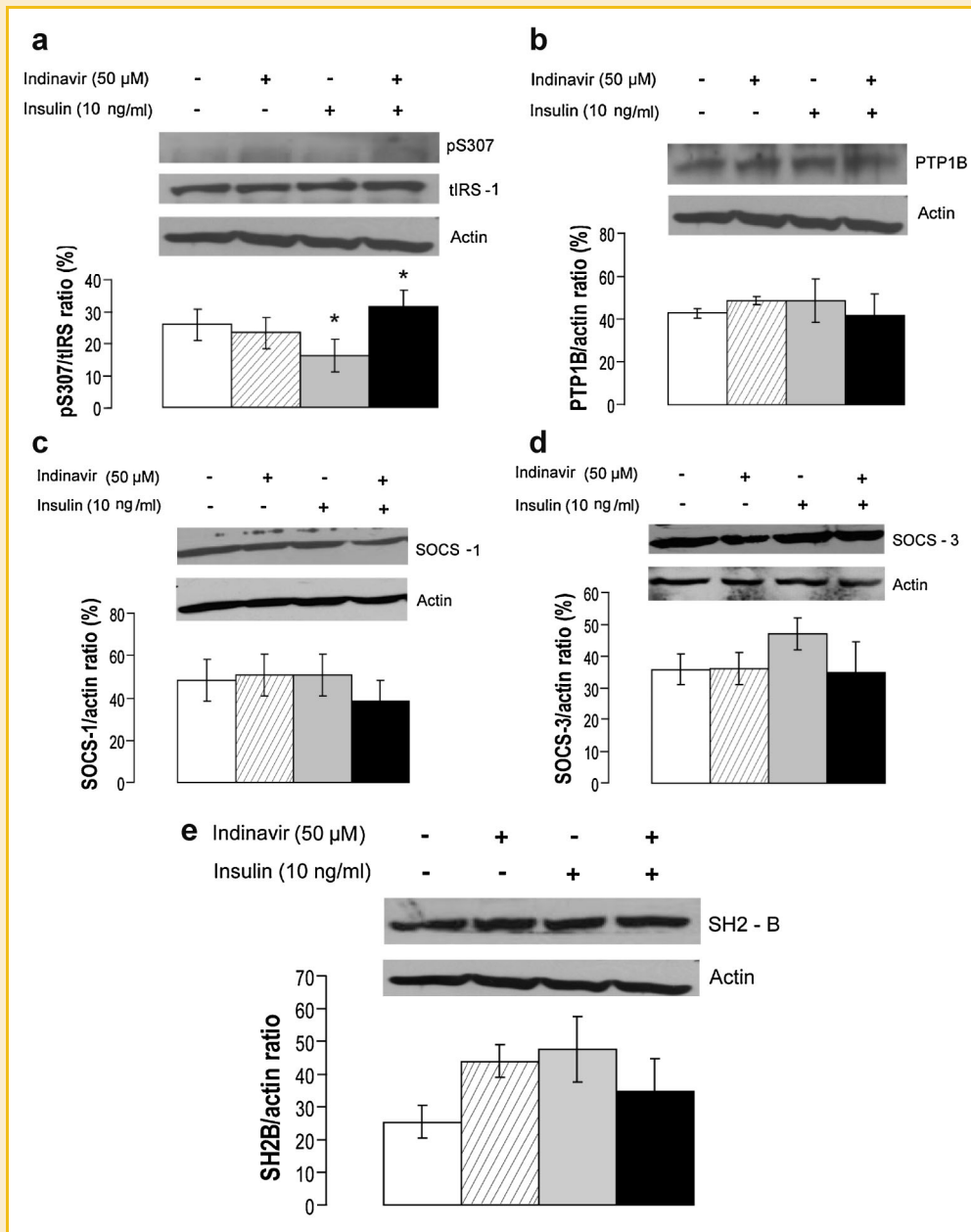


Fig. 3. Effects of indinavir on regulatory proteins in the insulin signaling pathway in CHO-IR cells. CHO-IR cells were treated with indinavir (50 μ M) for 16 h followed by insulin stimulation. The cells were then lysed and the lysates subjected to immunoblotting with antibodies against: (a) pS307 (b) PTP1B (c) SOCS-1 (d) SOCS-3 (e) SH2B (upper panels). The blots were then stripped and reprobbed with anti-actin or anti-IRS (lower panels). Bound antibodies were visualized by chemiluminescence. Chemiluminescence was quantified by real-time imaging. The band intensity of the proteins from five independent experiments was normalized to the band intensity of actin or total IRS protein. Data are shown as the mean \pm SEM ($n = 5$). pS307, phosphorylated serine at residue 307; tIRS-1, total IRS-1; PTP1B, protein tyrosine phosphatase 1B; SOCS-1, suppressor of cytokines signaling 1; SOCS-3, suppressor of cytokines signaling-3; SH2B, Src homology 2B. * $P < 0.05$, using Student's *t*-test. pS307, phosphorylated serine at residue 307; PTP1B, protein tyrosine phosphatase 1 B; SH2B, Src homology 2B; SOCS-1, suppressor of cytokines signaling-1; SOCS-3, suppressor of cytokines signaling-3.

DISCUSSION

The effects of HPIs have been examined in numerous studies [Wan Iryani et al., 2009]. The majority of studies have largely focussed on downstream events in the insulin signaling pathway. HPIs, including indinavir, were found to inhibit glucose uptake by inhibiting GLUT4 at the cell surface [Murata et al., 2002; Noor

et al., 2002] without any effect on insulin receptor phosphorylation using insulin concentrations of 1 μ M. There was no evidence of any upstream effect using this supraphysiological concentration of insulin. There is a paucity of studies that have examined proximal steps in the insulin signaling cascade, including insulin binding to the receptor and activation of the intrinsic tyrosine kinase activity and subsequent tyrosine phosphorylation of

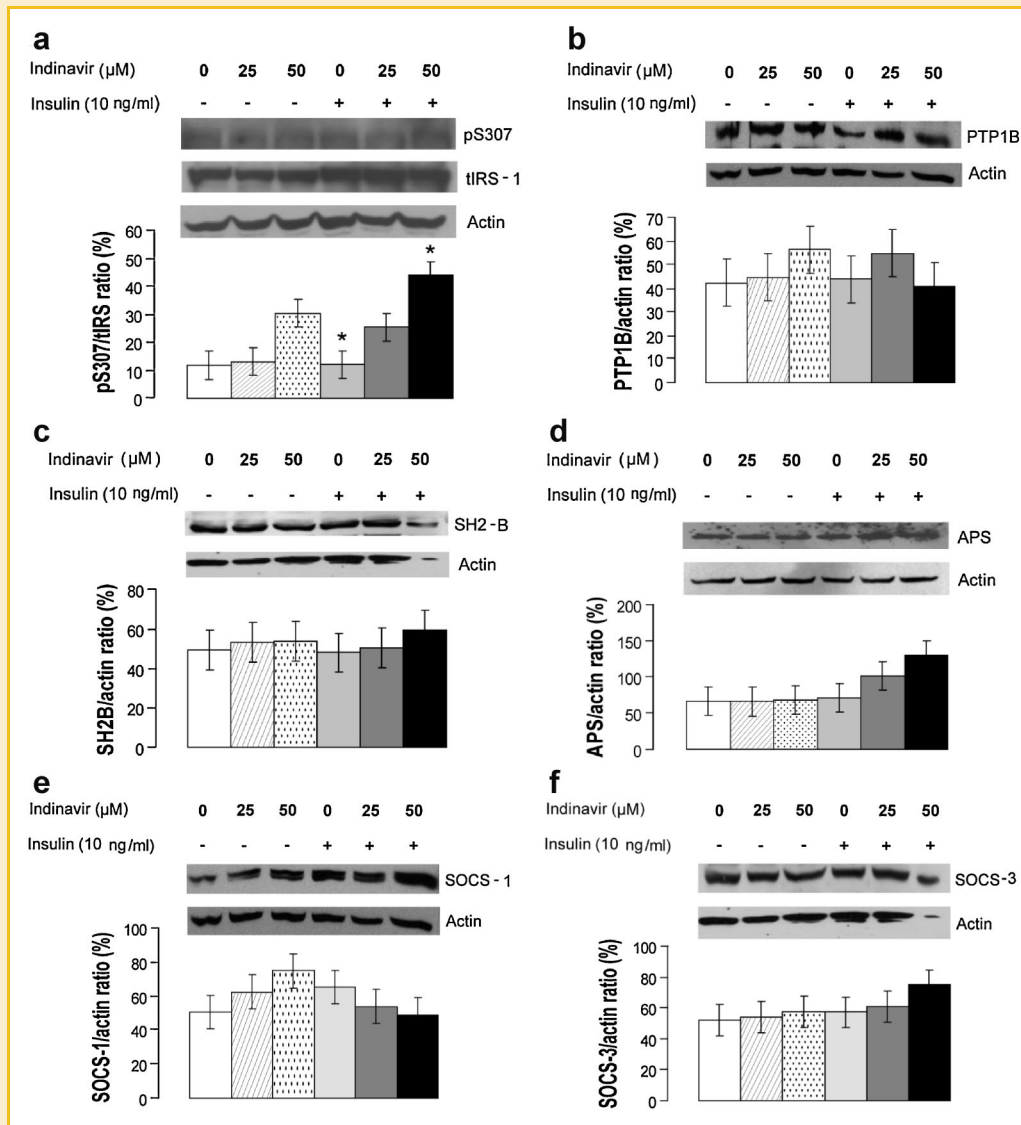


Fig. 4. Effects of indinavir on regulatory proteins in the insulin signaling pathway in 3T3-L1 adipocytes. 3T3-L1 adipocytes were treated with different concentrations of indinavir for 16 h before and after insulin stimulation. The cells were then lysed and the lysates subjected to immunoblotting with antibodies against: (a) pS307, (b) PTP1B, (c) SOCS-1, (d) SOCS-3, (e) SH2B, (f) APS (upper panels). The blots were then stripped and reprobed with anti-actin or anti-IRS-1 (lower panels). Bound antibodies were visualized by chemiluminescence. Chemiluminescence was quantified by real-time imaging. The band intensity of the proteins from six independent experiments was normalized to the band intensity of actin or total IRS-1 protein. Data are shown as the mean \pm SEM ($n = 6$). pS307, phosphorylated serine at residue 307; tIRS-1, total IRS-1; PTP1B, protein tyrosine phosphatase 1 B; SOCS-1, suppressor of cytokines signaling-1; SOCS-3, suppressor of cytokines signaling-3; SH2B, Src homology 2B; APS, adapter protein with a pleckstrin homology domain and an SH2 domain. * $P < 0.05$, using Student's t -test. pS307, phosphorylated serine at residue 307; PTP1B, protein tyrosine phosphatase 1 B; SH2B, Src homology 2 B.

substrate, where direct or indirect drug-induced inhibition may occur.

In this study, for the first time, CHO-IR cells were used to analyze the effects of HPIs on the insulin receptor β -subunit phosphorylation and tyrosine kinase activation. The results reveal that indinavir (50 μM) treatment for 16 h inhibited tyrosine phosphorylation of insulin receptor β -subunit and IRS-1 in the insulin signaling pathway. These are early events following insulin binding to the insulin receptor. The same effect was also observed in 3T3-L1 adipocytes. In a prior investigation using HepG2 hepatoma cells, it

was reported that indinavir (100 μM) treatment for 48 h decreased tyrosine phosphorylation of the IRS-1 protein but not phosphorylation of the insulin receptor itself [Schutt et al., 2000]. The effects of indinavir may be different in different cell lines depending on the experimental model and duration of exposure [Ben-Romano et al., 2003; Noor et al., 2006; Kovsan et al., 2009]. However, it would be technically difficult to analyze insulin receptor beta subunit phosphorylation in 3T3-L1 adipocytes at physiological insulin concentrations because of the level of expression, hence the rationale for using CHO-IR cells in this study. The indinavir

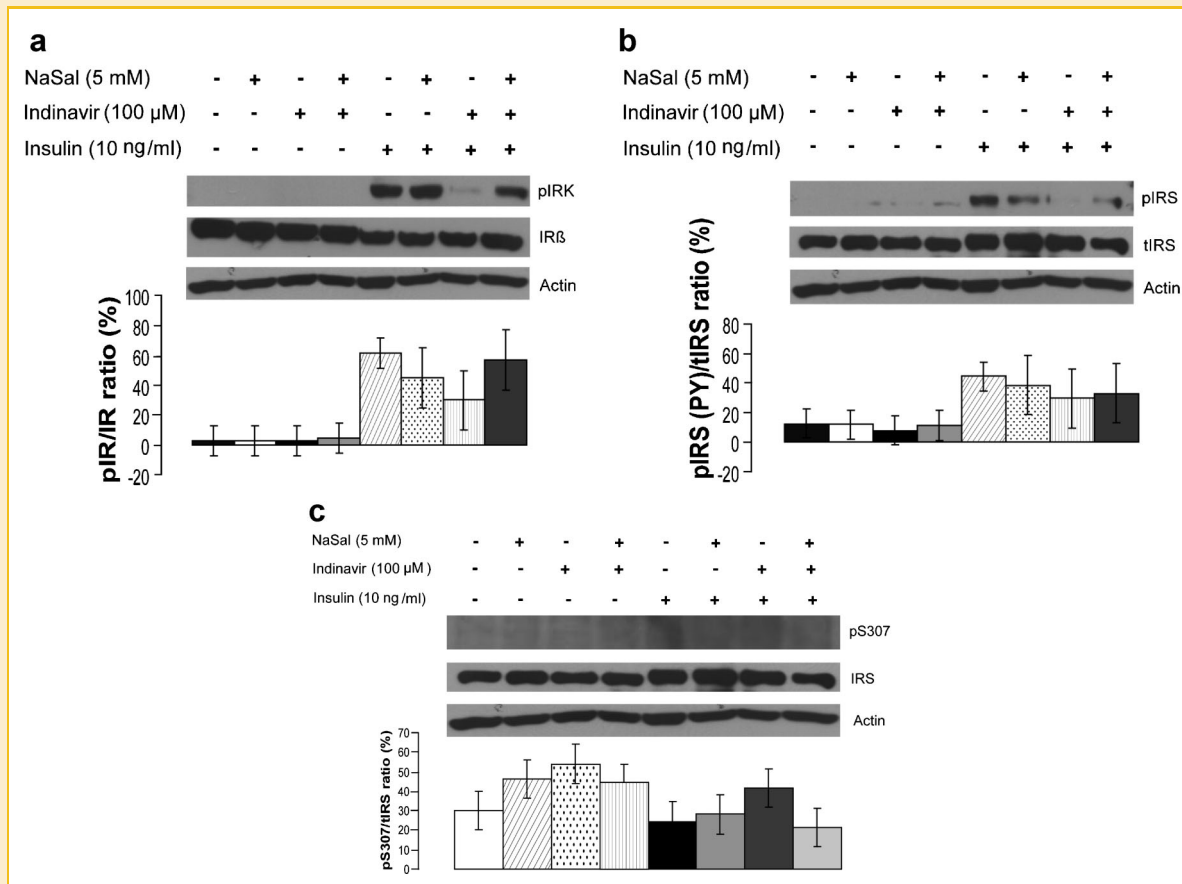


Fig. 5. Abrogating effects of indinavir by sodium salicylate. CHO-IR cells were pre-treated with NaSal (5 mM) for 1 h prior to treatment with indinavir (100 μM) for 16 h. The cells were then stimulated with insulin for 5 min and then lysed and the lysates subjected to immunoblotting with antibodies against: (a) and (b) anti-phosphotyrosine PY20 antibody and (c) pS307 (upper panels). The blots were then stripped and reprobed with anti-IR, anti-IRS-1 and anti-actin (lower panels). Bound antibodies were visualized by chemiluminescence. Chemiluminescence was quantified by real-time imaging. The band intensity of the proteins from three independent experiments was normalized to the band intensity of total IR or IRS-1 protein. Data are shown as the mean ± SEM (n = 3). pIR PY20, tyrosine phosphorylation of IR; pIRS-1 PY20, tyrosine phosphorylation of IRS-1; tIRβ, total insulin receptor β-subunit; pS307, phosphorylated serine at residue 307 of IRS-1; tIRS-1, total IRS-1. *P < 0.05, using factorial-ANOVA. NaSal, sodium salicylate; pIRS-1 (PY20), tyrosine phosphorylation of IRS-1; tIRS-1, total IRS-1.

concentration used in this study was higher than reported plasma concentrations (11 μM) [Lin, 1999]. A previous study reported that indinavir accumulated in adipose tissue [Vernochet et al., 2005]. Therefore, in vivo drug levels in plasma do not reflect the actual drug concentration in tissue which may be higher than the circulating levels [Ranganathan and Kern, 2002]. The effects of indinavir observed in the present study are unlikely to be due to cellular toxicity because in a previously reported study, indinavir was not toxic to cells even at concentrations of 100 μM [Germinario and Colby-Germinario, 2003]. Therefore, the level of indinavir used in the observation was within the range (0–100 μM). We did not observe any toxic effects on either CHO cells or adipocytes at these concentrations.

In a cellular context ex vivo, the effects of indinavir may only be apparent at submaximal insulin concentrations, which are more typical of physiological circulating insulin concentrations. For this reason, we have used a submaximal concentration of 10 ng/ml (1.7 nM) insulin to stimulate cells even though the maximal

activation of the insulin receptor tyrosine phosphorylation is observed at levels approximately at least 10-fold higher than 10 ng/ml, that is, 100 ng/ml (17 nM). In other words, even though the maximal activation of insulin receptor tyrosine phosphorylation is observed at much higher levels of insulin in CHO-IR cells, the pharmacological effects of insulin being used at maximal concentrations in a cell line that overexpresses insulin receptors at such a high level may mask the inhibitory effects of indinavir. This difference is important when comparing our results with those of Murata et al. [2002]. In this article, the authors have used a maximal and supraphysiological concentration of insulin (1 μM) to stimulate the cells. In all of our studies, we used 10 ng/ml (1.7 nM), which is 1,000-fold less and more similar to a physiological concentration. We have also found that when we used 100 ng/ml (17 nM), we did not observe any effects on the insulin receptor kinase (data not shown).

The inhibition of tyrosine phosphorylation of the insulin receptor kinase and its substrate, IRS-1, implies that indinavir and nelfinavir

interact at a very proximal step in the insulin signaling pathway. Interestingly, nelfinavir showed a significant effect on Map kinase phosphorylation, whilst leaving Akt phosphorylation largely intact. There are two possible mechanisms to explain this. The drug may act directly to inhibit the insulin receptor kinase or the drug may affect other proteins that regulate the intrinsic tyrosine kinase activity. In order to address this hypothesis, the blots were stripped and reprobed with antibodies against regulatory proteins of insulin receptor signaling such as Serine 307 phosphorylated IRS-1 (phosphoIRS-1 at S307), PTP1B, SOCS-1 and -3, SH2B and APS.

The protein expression of PTP1B in both cell lines before and after pre-treatment with indinavir was not significantly different. PTP1B, a tyrosine phosphatase, may negatively regulate insulin signaling by binding to the tyrosine phosphorylated proteins, insulin receptor β -subunit and IRS-1 [Cheng et al., 2002] and catalyzing dephosphorylation. However, a previous study suggested that total PTPase activity, but not the activity of PTP1B, is associated with the inhibition of insulin-stimulated glucose transport in adipose tissue [Wu et al., 2003].

The protein expression of SOCS-1 and -3 in CHO-IR cells, and SOCS-3 in 3T3-L1 adipocytes was not significantly different before and after treatment with indinavir. However, the protein expression of SOCS-1 in 3T3-L1 adipocytes before insulin stimulation, was significantly increased as a result of the indinavir treatment. This suggests that increased expression of SOCS-1 could potentially play a regulatory role in 3T3-L1 adipocytes.

SOCS-1 and -3 are adapter proteins that can bind to the insulin receptor β -subunit or IRS-1 or IRS-2 to inhibit downstream signaling. Increased SOCS-1 and -3 expression have been reported to induce insulin resistance by preventing tyrosine phosphorylation and subsequent activation of IRS-1 and -2 proteins [Carper et al., 2008]. In addition, SOCS-1 and -3 have been reported to negatively regulate the insulin signaling pathway, without modifying the insulin receptor tyrosine kinase activity [Emanuelli et al., 2001].

Moreover, SOCS-1 and -3 expression can be induced by TNF- α [Emanuelli et al., 2001]. However, this cytokine is released from immune cells and adipose tissue and not from CHO-IR cells. Thus, there was no change in SOCS-1 and -3 protein expression in CHO-IR cells following treatment with indinavir. Similarly, the protein expression of SOCS-3 was also not significantly altered in 3T3-L1 adipocytes treated with the drug. However, there was an alteration in SOCS-1. Therefore, the current investigation indicated that in CHO-IR cells, these proteins are not involved in decreasing phosphorylation of insulin receptor β -subunit. However, SOCS-1 may play a role in 3T3-L1 adipocytes.

SH2B (aka SH2B1) is an adapter protein involved in the signaling pathway mediated by insulin tyrosine kinases and Janus kinase. Deletion of the adapter protein gene in mice results in severe obesity, infertility, and leptin and insulin resistance [Maures et al., 2007]. This protein has also been shown to regulate the tyrosine phosphorylation state of the insulin receptor [Ahmed and Pillay, 2001]. However, the expression of SH2B in CHO-IR cells and 3T3-L1 adipocytes was not significantly altered after treatment with indinavir.

APS (aka SH2B2) and SH2B have similar roles, and these proteins may delay dephosphorylation of the insulin receptor kinase and IRS-

1 and -2 in the insulin signaling pathway [Ahmed and Pillay, 2001, 2003]. The adapter protein, APS, however, binds the insulin receptor with a higher affinity than SH2B [Ahmed and Pillay, 2001, 2003]. CHO-IR cells lack endogenous APS protein [Ahmed et al., 2000]. Therefore, only this protein could be analyzed in 3T3-L1 adipocytes [Ahn et al., 2004]. The protein expression of APS in 3T3-L1 adipocytes treated with indinavir was not significantly different before and after treatment with indinavir.

Indinavir treatment resulted in a significant increase in phosphorylation of IRS-1 at S307 in both CHO-IR cells and 3T3-L1 adipocytes. Serine phosphorylation of IRS-1 is known to inhibit phosphorylation of the insulin receptor. Indinavir may induce pro-inflammatory cytokines such as TNF- α or unknown cytokines in the cells, which activate IKK β . IKK β is a serine kinase that regulates the activation of NF κ B, a critical activator of genes involved in inflammation and immunity. Prior to activation, NF κ B is bound to the inhibitor κ B (I κ B). The activated IKK β phosphorylates the NF κ B-I κ B complex, which stimulates the conjugation of I κ B with ubiquitin and the subsequent degradation of I κ B protein in the proteasome, leading to the nuclear translocation of NF κ B [Tergaonkar, 2006].

Activation of IKK β also induces serine phosphorylation of IRS-1, as IRS-1 is a substrate of IKK β . This phosphorylation blocks interactions with the insulin receptor β -subunit, decreases tyrosine phosphorylation, and inhibits insulin action in the cells [Aguirre et al., 2002; Gao et al., 2003].

High doses of salicylate (4–10 g/day), including NaSal and aspirin, are known to reduce blood glucose concentrations in diabetic patients via inhibition of NF κ B and its upstream activator IKK β [Yuan et al., 2001]. In the present study, NaSal was used for the first time in the context of cells treated with HPIs. In CHO-IR cells treated with HPIs, our findings reveal that the drug inhibits the effects of indinavir on insulin receptor tyrosine phosphorylation even at 100 μ M. One hundred micromolar of indinavir was used to obtain the maximal effect of the drug on insulin receptor tyrosine phosphorylation. A significant increase of tyrosine phosphorylation of insulin receptor β -subunit in cells pre-treated with NaSal prior to treatment with HPI drug was observed and this is consistent with the known effects of NaSal on the activation of IKK β . NaSal also increased tyrosine phosphorylation of IRS-1 slightly and decreased serine phosphorylation of IRS-1 by twofold. This was associated with a decreased inhibition of tyrosine phosphorylation in response to indinavir, that is, a blockade of the effects of indinavir. Based on these results, we can hypothesize that HPIs may induce insulin resistance via the NF κ B pathway.

In conclusion, PTP1B, SOCS-1 and -3, SH2B1 and APS, the insulin signaling regulatory proteins, were not altered after indinavir treatment indicating that changes in the expression of these proteins are unlikely to play a role in the insulin resistance induced by antiretroviral protease inhibitors. However, a lack of expression does not exclude a role. However, we show for the first time that NaSal, possibly acting by blocking IKK β , abrogates the effect of indinavir on tyrosine phosphorylation of the insulin receptor β -subunit by decreasing phosphoIRS-1 S307. We conclude, therefore, that HPIs inhibit insulin receptor tyrosine phosphorylation indirectly via the NF κ B pathway as a secondary effect involving a regulator of tyrosine phosphorylation, viz. the level of

phosphorylation of IRS-1 at S307. Salicylates may therefore provide a novel therapy for HPI-induced insulin resistance and lipodystrophy. These effects of anti-retroviral drugs are but one example of the complex effects that protease inhibitors may have, including effects on the endoplasmic reticulum and oxidative stress and the alteration of adipokines [Hruz, 2011].

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