

# Differential effects of IRS1 phosphorylated on Ser307 or Ser632 in the induction of insulin resistance by oxidative stress

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## Abstract

**Aims/hypothesis** Induction of stress kinases leading to serine hyperphosphorylation of IRS1 may link oxidative stress to insulin resistance. The aim of this study was to investigate the roles of the phosphorylated serine residues Ser307 and Ser632, two sites implicated in the inhibition of IRS1 function in insulin signalling.

**Materials and methods** Fao hepatoma cells were exposed to an H<sub>2</sub>O<sub>2</sub>-generating system, and antibodies against the two phosphorylated serine residues were used for immunoprecipitation, immunoblot and immunofluorescence analyses.

**Results** Exposure to ~50 μmol/l H<sub>2</sub>O<sub>2</sub> for 2 h resulted in IRS1 phosphorylation on both Ser307 and Ser632, concomitant with activation of inhibitor kappa kinase β (IKKβ) and c-Jun kinase (JNK). Immunoprecipitation studies revealed that the maximum overlap between phospho (p) Ser307-IRS1 and pSer632-IRS1 was 20%, and confocal microscopy suggested distinct localisations of IRS1 molecules phosphorylated on either site. Although pSer307-IRS1 showed

decreased insulin-induced tyrosine phosphorylation and interaction with phosphatidylinositol 3-kinase (PI3K) in response to insulin, pSer632-IRS1 molecules were normally tyrosine-phosphorylated and exhibited typical associated PI3K activity. Salicylic acid and SP600125 partially inhibited IKKβ and JNK, respectively, which indicated distinct roles for these two kinases in the phosphorylation of IRS1 at the two serine sites. Protection against oxidation-mediated impairment in insulin-induced phosphorylation of protein kinase B/Akt and in glycogen synthesis was achieved only by combining salicylic acid and SP600125.

**Conclusions/interpretation** These results suggest that pSer307-IRS1 and pSer632-IRS1 may define two minimally overlapping pools of IRS1 in response to oxidative stress, contributing differentially to insulin resistance. A combination of stress kinase inhibitors is required to protect against insulin resistance and IRS1 hyperphosphorylation induced by oxidative stress.

**Keywords** c-Jun kinase · Glycogen synthesis · Hydrogen peroxide · IKKβ · Inhibitor kappa kinase β · Stress kinases

## Abbreviations

IKKβ inhibitor of kappa B kinase β  
JNK c-Jun kinase  
PI3K phosphatidylinositol 3-kinase  
PKB protein kinase B

## Introduction

The diabetic and obese states are increasingly being shown to induce stress responses in tissues relevant to metabolic

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regulation. Stress signalling cascades are signalling pathways involving serine/threonine ‘stress kinases’, which may be activated in response to diverse conditions and cues, including inflammatory cytokines and endoplasmic reticulum stress, both of which are implicated in the pathogenesis of insulin resistance. Once activated, stress kinases can impinge upon the insulin signalling cascade, leading to insulin resistance [1, 2]. One mechanism by which this may occur is through stress kinase-induced serine/threonine phosphorylation of the insulin receptor substrate (IRS) proteins [3–5]. Specifically, IRS1 Ser307 has been shown to be a site phosphorylated by several kinases, including mTOR [6–9], inhibitor of kappa B kinase  $\beta$  (IKK $\beta$ ) [10, 11] and *c-Jun* kinase (JNK) [9, 12–14]. This rendered IRS1 molecules phosphorylated on this site less capable of becoming tyrosine-phosphorylated in response to insulin, hence having decreased capacity to interact with phosphatidylinositol 3-kinase (PI3K). Elevated levels of phospho (p)-Ser307-IRS1 have been reported in animal models of insulin resistance [15], and the equivalent Ser312 site in human IRS1 has been demonstrated to be hyperphosphorylated in insulin-resistant subjects [16]. Phosphorylation of IRS1 on Ser632 has also been implicated in insulin resistance. Hyperosmolar stress induced pSer632 hyperphosphorylation [7], and an increase in the phosphorylation state of the human equivalent Ser636 was present in myotubes from patients with type 2 diabetes [17], their insulin-resistant offspring [16] and in healthy subjects infused with TNF- $\alpha$  [18].

The wealth of information suggesting a role for serine/threonine hyperphosphorylation of IRS molecules in the induction of insulin resistance suggests that inhibition of the responsible kinases may constitute a viable approach to the relief of insulin resistance. This approach is particularly attractive for the stress kinases, which are not traditionally viewed as intrinsic components of the insulin signalling cascade. Hence, their inhibition is unlikely to impair normal signal propagation. Defining which stress kinase inhibitor(s) could prevent insulin resistance, and in response to which type of physiologically relevant inducers, is of interest, as it sheds light both on basic mechanisms leading to insulin resistance and on potential therapeutic interventions.

Oxidative stress is increasingly implicated as a common mechanism mediating the pathogenesis of insulin resistance in response to potential inducers, including inflammatory cytokines, endoplasmic reticulum stress and nutrient excess [1, 19, 20]. Evidence exists for increased systemic oxidative stress and for oxidative stress at the adipose tissue level of obese persons [2, 21] and in the livers of persons with hepatic steatosis [22], a close surrogate of insulin resistance. Using cellular model systems, it has been shown by others and us that oxidative stress impairs insulin action [23–26], associated with serine phosphorylation of IRS1

[27]. Intriguingly, overall serine/threonine hyperphosphorylation did not correlate with the degree of insulin resistance at the metabolic level [27]. In parallel, oxidative stress is known to activate several stress signalling cascades, including JNK and the IKK $\beta$ -nuclear factor kappa B signalling cascades [1]. Here we examined: (i) the role of these kinases in the induction of insulin resistance and IRS1 phosphorylation of Ser307 and Ser632 by oxidative stress; (ii) the significance of phosphorylation of these specific residues for IRS1 function; and (iii) whether insulin resistance can be relieved by pharmacological inhibitors of these stress kinases.

## Materials and methods

### 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). Polyclonal IRS1 antibodies (C-terminus) and anti-p85 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). Polyclonal pSer307-IRS1 and pSer632-IRS1 were generated as described previously [6]. Polyclonal phospho-*c-Jun*, monoclonal p-JNK, polyclonal JNK1/2 and monoclonal IKK $\beta$  antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Polyclonal p-I $\kappa$ B and pSer473-protein kinase B (PKB)/Akt antibodies were purchased from Cell Signaling (Danvers, MA, USA). SP600125, a reversible ATP-competitive inhibitor of JNK, was purchased from Biomol (Plymouth Meeting, PA, USA). Peroxidase-conjugated anti-rabbit IgG, anti-mouse IgG and [ $\gamma$ - $^{32}$ P]ATP were from Amersham Life Sciences (Little Chalfont, Bucks, UK). Protein A-Sepharose was from Pharmacia Biotech (Uppsala, Sweden). U- $^{14}$ C-glucose was purchased from Nuclear Research Center Negev (Dimona, Israel). Salicylate and all other chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA).

### Cell culture and treatment

Rat hepatoma Fao cells were grown to confluence in RPMI medium containing 5 mmol/l glucose with 10% foetal calf serum. Before experiments, cells were deprived of serum for 16 h by incubation in medium supplemented with 0.5% (w/v) radioimmunoassay grade BSA. Cells were then rinsed three times in PBS, exposed to fresh medium with or without glucose oxidase [24, 28], and rinsed again before stimulation with 100 nmol/l insulin. The amount of H $_2$ O $_2$  in the medium was measured spectrophotometrically as described previously [29].

## Glycogen synthesis

Incorporation of U-<sup>14</sup>C-glucose into glycogen in Fao cells was measured as described previously [24, 27].

## Whole-cell lysates, post-nuclear internal membrane preparations and western blots

After treatment, cells were thoroughly washed with ice-cold PBS and scraped into 0.2 ml of ice-cold lysis buffer (50 mmol/l Tris-HCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mmol/l NaCl, 1 mmol/l EGTA, 1 mmol/l sodium vanadate, 1 mmol/l NaF and protease inhibitors [1:1,000 dilution of protease inhibitor cocktail Sigma P-8340]). Lysates were shaken gently for 15 min at 4°C and centrifuged (12,000×g, 15 min at 4°C), the supernatant was collected, and protein content was determined (BCA method; Pierce, Rockford, IL, USA). Post-nuclear internal membrane fractions were prepared from three 10-cm dishes of confluent Fao cells per treatment. Cells were homogenised in 2.5 ml fractionation buffer (255 mmol/l sucrose, 20 mmol/l HEPES, pH 7.4, 1 mmol/l EDTA, 0.2 mmol/l sodium vanadate with a 1:1,000 dilution of protease inhibitor cocktail). Nuclei and debris were sedimented by centrifugation at 1,000×g for 5 min, after which the supernatants were subjected to two rounds of centrifugation (30,000×g for 30 min then 250,000×g for 90 min). Pellets were resuspended in fractionation buffer to 5 mg of protein/ml. Laemmli buffer was added and samples were boiled for 5 min. Protein samples were resolved by 7.5–10% SDS-PAGE and subjected to western blotting, followed by quantitation using video densitometry analysis, as described previously [28, 30].

## Immunoprecipitation and PI3K assay

Cells were lysed in ice-cold deoxycholate based buffer, as described above. For immunoprecipitation, 0.5 mg protein of cell lysate was used, and the PI3K assay was performed following the protocol described by Hadari et al. [31] using phosphatidylinositol and [ $\gamma$ -<sup>32</sup>P]ATP as substrates, as reported previously [28, 30]. The phosphorylated phosphatidylinositols were separated using thin-layer chromatography and quantified by video densitometry.

## Fluorescence microscopy

After cell treatments, Fao cells grown on 15-mm diameter glass coverslips were fixed with 4% (v/v) paraformaldehyde in PBS for 25 min (first 5 min on ice) then rinsed, and excess paraformaldehyde was quenched with 100 mmol/l glycine for 10 min. Cells were then permeabilised with 0.1% (v/v) Triton X-100 in PBS for 20 min and blocked for 30 min with

PBS containing 5% goat serum (v/v), as described previously [32]. The following dilutions in blocking buffer of the primary antibodies were used: anti-IRS1, 1:100; anti-pSer307-IRS1, 1:150; anti-pSer632-IRS1, 1:150. After 1 h of incubation at room temperature, cells were rinsed with PBS and incubated for an additional 1 h in the dark with Alexa555-conjugated anti-rabbit IgG secondary antibody at a dilution of 1:750. Cell monolayers were washed further with PBS and mounted onto glass slides using fluorescent mounting medium (DakoCytomation, Carpinteria, CA, USA). For confocal fluorescence microscopy, cells were examined with a Zeiss LSM 510 laser scanning confocal microscope with ×40 and ×100 oil immersion objectives. Acquisition parameters were adjusted to exclude saturation of the pixels, and to exclude background fluorescence determined by incubating cells only with secondary antibody.

## Statistical analysis

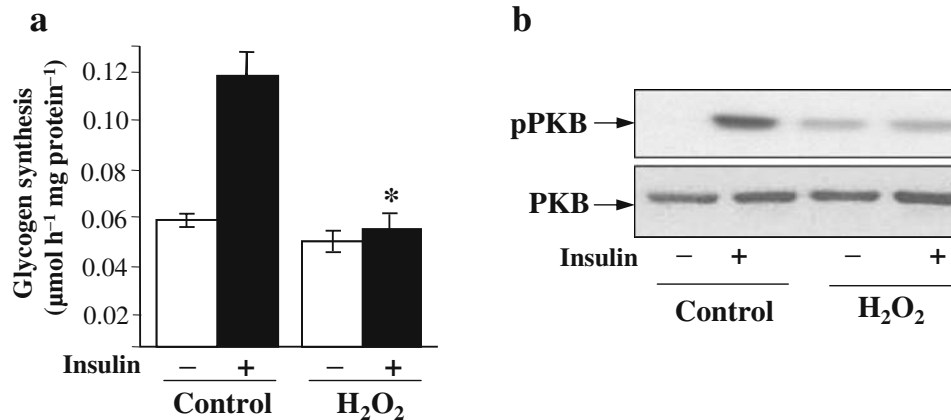
Data are expressed as mean±SEM. Each treatment was compared with a control, and differences between pairs of groups were evaluated using Student's *t*-test. The criterion for significance was set at *p*<0.05. When multiple groups were compared, ANOVA was used for analysis.

## Results

IRS1 phosphorylated on Ser307 or Ser632 in response to oxidative stress represents seemingly distinct pools of IRS1 protein

The Fao hepatoma cell line was used as a cellular model of liver cells, as Fao cells respond acutely to insulin stimulation with a metabolically relevant increase in glycogen synthesis. Cells were exposed to oxidative stress by 2 h of incubation in the presence of glucose oxidase, continuously generating submillimolar H<sub>2</sub>O<sub>2</sub> concentrations (see **Materials and methods** for details), as we have described previously [24, 28]. Insulin-stimulated glycogen synthesis (Fig. 1a) was markedly impaired after exposure to the H<sub>2</sub>O<sub>2</sub>-generating system. Insulin-stimulated activation of PKB/Akt is thought to participate in mediating insulin's action on glycogen synthesis, and, consistently with the metabolic insulin resistance, oxidative stress impaired insulin-stimulated Ser473 phosphorylation of PKB/Akt (Fig. 1b). This signalling effect of oxidative stress seen in Fao cells was consistent with our previous observations in 3T3-L1 adipocytes [30, 33].

Serine hyperphosphorylation of IRS1 was observed in a time-dependent manner in response to oxidative stress, as indicated by retarded migration of the IRS1 band (Fig. 2a, upper blot). This effect was associated at 2 h of exposure with a decrease of up to 20% in total IRS1 content, which



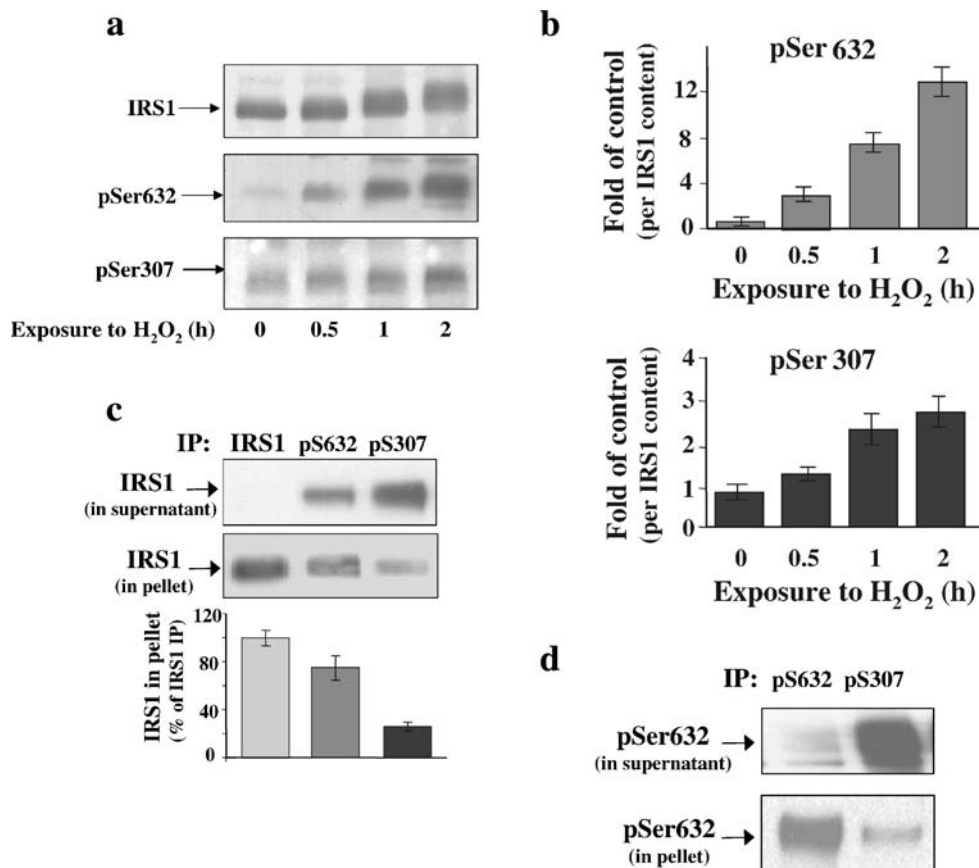
**Fig. 1** Oxidative stress induces resistance to the acute metabolic and signalling effects of insulin. **a** Fao hepatoma cells were starved of serum overnight then incubated for 2 h in the absence or presence of 50 μmol/l H<sub>2</sub>O<sub>2</sub> generated by the addition of glucose oxidase (100 mU/ml) to the glucose-containing medium. Cells were rinsed, and then stimulated with or without 100 nmol/l insulin for 60 min, during which incorporation of glucose into glycogen was measured as described in *Materials and methods*. Results are mean ± SEM of at

least four independent experiments performed in duplicate. \**p*<0.05 vs insulin-stimulated control cells. **b** PKB/Akt Ser473 phosphorylation was measured after exposure to oxidative stress as described above, without or with insulin stimulation for 10 min. Lysates were prepared and western blot analysis was performed using anti-pSer473-PKB/Akt or anti-PKB/Akt antibodies. Blots representative of six independent experiments are shown

was further augmented by prolonging exposure to oxidative stress, as we have reported previously [27]. To assess specific pSer sites on IRS1, implicated previously in mediating insulin resistance, we used phosphoserine IRS1 antibodies whose specificity was demonstrated using site-specific Ser→Ala IRS1 mutants [6]. Oxidative stress increased serine phosphorylation of IRS1 on both Ser307 and Ser632 (Fig. 2a, lower blots; quantified and presented as the ratio of pSer-IRS1 to total IRS1 in Fig. 2b). To determine the relative amounts of IRS1 molecules phosphorylated on both Ser307 and Ser632, lysates of oxidised cells were subjected to immunoprecipitation using anti-IRS1, anti-pSer307 and anti-pSer632 antibodies, followed by immunoblotting with anti-IRS1 antibody (equal efficiency of the immunoprecipitate was confirmed by immunoblotting of supernatants, and was found to be >90% in all cases). Of the entire pool of IRS1 molecules, approximately 70% were phosphorylated on Ser632, but only 20% on Ser307 (Fig. 2c). To evaluate the degree of overlap between the two phosphoserine sites, immunoprecipitation with the two phosphoserine IRS1 antibodies was performed. We were unsuccessful in detecting pSer307-IRS1 by immunoblotting after immunoprecipitation with either anti-pSer307-IRS1 or anti-pSer632-IRS1 antibody. However, pSer-IRS1 immunoprecipitates immunoblotted with anti-pSer632-IRS1 revealed that the maximal amount of pSer632-IRS1 molecules also phosphorylated on Ser307 was 18±2% (Fig. 2d). This finding suggests that, in response to oxidative stress, IRS1 phosphorylation on Ser632 and on Ser307 may represent minimally overlapping pools of IRS1 proteins.

Serine phosphorylation of IRS1 was previously suggested to regulate the cellular localisation of IRS proteins and to subject hyperphosphorylated IRS1 molecules to enhanced protein degradation. To assess whether the small overlap between pSer632-IRS1 and pSer307-IRS1 is reflected in differential cellular localisation, we used indirect immunofluorescence. Figure 3 demonstrates the immunofluorescence findings, which are qualitatively consistent with the western blot analysis shown in Fig. 2. Total IRS1 protein tended to be decreased in oxidised cells (compare panels b and a in Fig. 3), as we have described previously [27]. However, the amount of both pSer632-IRS1 and pSer307-IRS1 was increased (Fig. 3; compare panel d with panel c and panel f with panel e, respectively). Using higher magnification, total IRS1 protein exhibited diffuse cytosolic and punctate staining (Fig. 3g). Remarkably, whereas pSer307-IRS1 exhibited more pronounced perinuclear staining, pSer632-IRS1 was more discernible in the cell periphery (Fig. 3i and h, respectively).

An acute effect of insulin has been shown to relocalise IRS1 from the membrane fraction to the cytosol ([30, 34, 35] and Fig. 4a, upper blot). Interestingly, in response to oxidative stress, pSer632-IRS1 displayed a similar effect, whereas pSer307-IRS1 failed to dissociate from a post-nuclear internal membrane preparation following acute insulin stimulation (Fig. 4a, middle and lower blots, respectively). Combined, these data suggest that the two pSer sites of IRS1 may define spatially distinct pools of IRS1 proteins in response to oxidative stress.



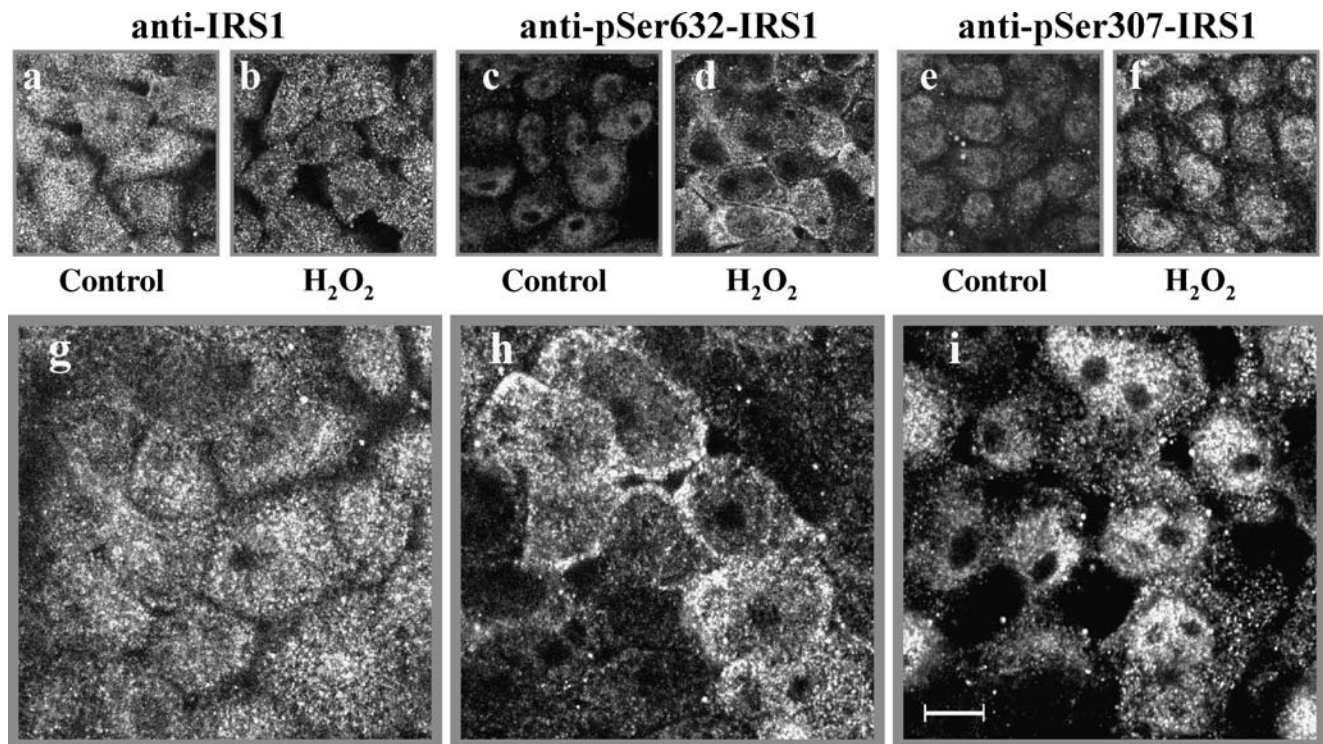
**Fig. 2** Oxidation induces IRS1 phosphorylation on Ser307 and Ser632, representing two minimally overlapping pools of IRS1 molecules. **a** Fao cells were treated as described in the legend of Fig. 1, lysed and analysed by western blotting using anti-IRS1, anti-pSer307-IRS1 and anti-pSer632-IRS1 antibodies. **b** Densitometry was used to determine the ratios of pSer632-IRS1 and pSer307-IRS1 to total IRS1; a value of 1 was set for the ratio between the band intensities in non-oxidised cells (time 0). Values are mean  $\pm$  SEM of four independent experiments. **c** Lysates were prepared from oxidised Fao cells, and 500  $\mu$ g total lysate protein was subjected to immunoprecipitation with

anti-IRS1, anti-pSer307-IRS1 or anti-pSer632-IRS1 antibodies. Supernatants (*upper blot*) or immunoprecipitates (*lower blots*) were subjected to western blot analysis using anti-IRS1 antibodies. Representative blots are shown, and below the blots are indicated the mean  $\pm$  SEM densitometry values derived from three independent experiments. **d** Supernatants (*upper*) or immunoprecipitates (*lower*) of pSer632-IRS1 and pSer307-IRS1 were subjected to immunoblotting using anti-pSer632 antibodies. This experiment was performed twice with similar results

Phosphorylation of IRS1 on Ser307 but not on Ser632 in response to oxidative stress impairs insulin-stimulated tyrosine phosphorylation of IRS1 and interaction with PI3K

Figure 4a suggests that in response to oxidative stress Ser307 phosphorylation signifies an attenuated response of IRS1 molecules to the acute action of insulin on IRS1. Thus, we next assessed whether, in addition to determining a unique cellular localisation, oxidative stress-induced phosphorylation on either Ser307 or Ser632 differentially affected the ability of IRS1 proteins to undergo tyrosine phosphorylation and to interact with PI3K in response to insulin. We have shown previously that insulin-stimulated tyrosine phosphorylation of IRS1 proteins and interaction with PI3K are not impaired in response to oxidative stress when assessed in total cell lysates [28, 30]. However,

impaired activation of PI3K was observed in a specific cellular compartment, the low-density microsomal fraction, potentially explaining the defect in downstream signal propagation to PKB/Akt [30]. Total IRS1 proteins, pSer632-IRS1 or pSer307-IRS1 proteins were immunoprecipitated using the respective antibodies, and in each immunoprecipitate we determined IRS1 protein, its tyrosine phosphorylation, interaction with the p85 regulatory subunit of PI3K, and IRS1-associated PI3K activity (Fig. 4b). As shown in the upper panel, pSer307-IRS1 represented a small fraction of total IRS1 protein in oxidised cells (similar to the data shown in Fig. 2c). Nevertheless, insulin-stimulated tyrosine phosphorylation of pSer307-IRS1, its interaction with p85, and the associated PI3K activity were markedly decreased. In contrast, pSer632-IRS1 molecules displayed a high capacity to undergo insulin-induced



**Fig. 3** Differential cellular localisation of pSer307-IRS1 and pSer632-IRS1 in oxidised Fao cells revealed by indirect immunofluorescence. Fao cells were treated as described above, fixed, permeabilised and immunostained for IRS1 (a, b, g), pSer632-IRS1 (c, d, h) or pSer307-IRS1 (e, f, i), followed by laser confocal microscopy analysis as described in [Materials and methods](#). Repre-

sentative images from three independent experiments are shown of a single focal plane set to the middle height of the cells (in the z-axis). Sections g, h and i are enlarged images of IRS1, pSer632-IRS1 and pSer307-IRS1 immunofluorescence, respectively, after exposure of the cells to oxidative stress. Scale bar = 10  $\mu$ m

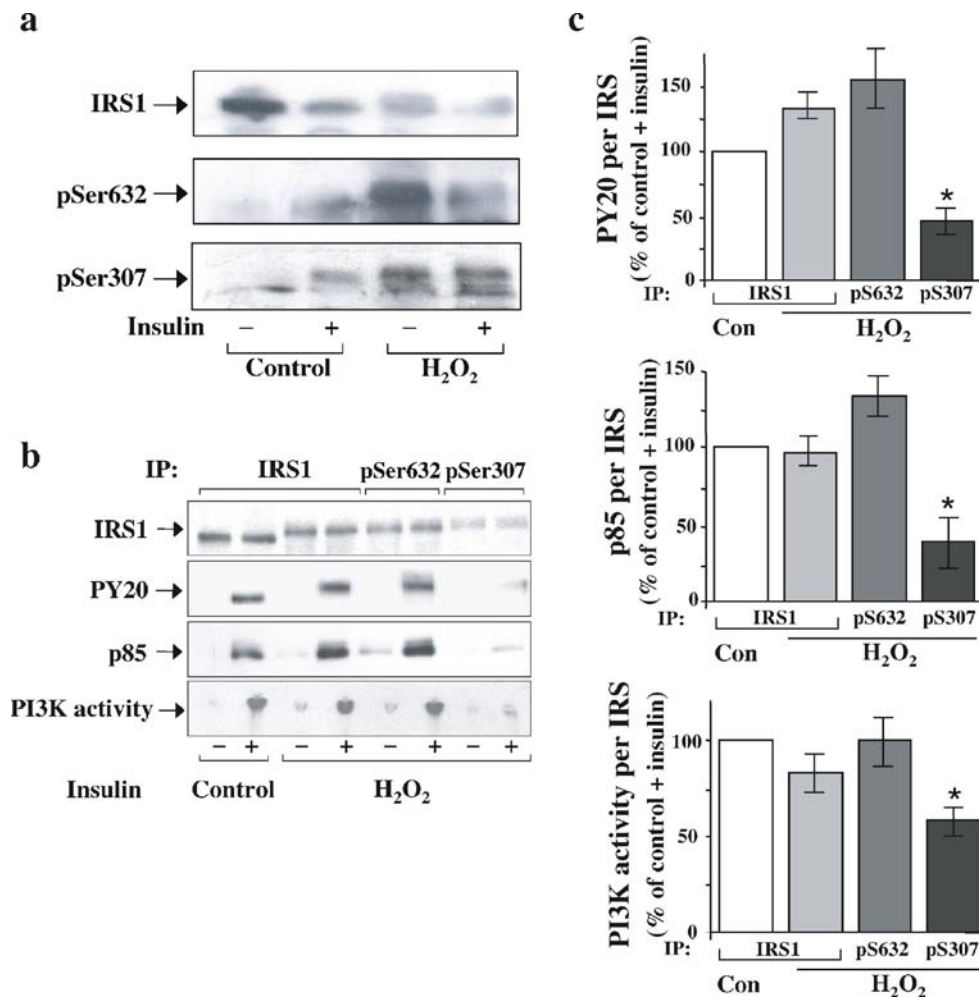
tyrosine phosphorylation and to interact with PI3K. Figure 4c demonstrates the ratio between the phosphotyrosine or p85 bands and IRS1, and the associated PI3K activity (upper, middle and lower panels, respectively). In comparison with total IRS1 immunoprecipitated from oxidised cells, immunoprecipitates of pSer307-IRS1 demonstrated 50% less IRS1 tyrosine phosphorylation, p85 association with IRS1 content, and PI3K activity. No similar decrease was observed with pSer632-IRS1. These data suggest that, in response to oxidative stress, phosphorylation on Ser307, but not on Ser632, can result in impaired signalling capacity in a subset of IRS1 molecules.

Stress kinases inhibitors differentially affect IRS1 phosphorylation on Ser307 and Ser632, and only combined inhibition of stress kinases relieves metabolic insulin resistance

The spatial and functional data described above suggest a differential input of stress kinases in the phosphorylation of Ser307 and Ser632 of IRS1 in response to oxidative stress. To begin assessing the potential contributions of IKK $\beta$  and JNK, we first assessed the time-dependent activation of these stress kinases in response to oxidative stress. The total

amount of IKK $\beta$  or of JNK1/2 was unchanged by up to 2 h of exposure to oxidative stress (lower panels of Fig. 5a and b, respectively). Oxidative stress-induced activation of IKK $\beta$ , assessed as the degree of phosphorylation of its substrate, I $\kappa$ B, revealed a gradual effect over time (Fig. 5a, upper blot). Similarly, JNK was activated, as indicated by the increase in its phosphorylated form, but the effect was maximal after 1 h of exposure to H<sub>2</sub>O<sub>2</sub> (Fig. 5b, upper blot). Figure 5c demonstrates in a quantitative manner the dynamic of oxidative stress-induced activation of JNK and IKK $\beta$ , revealing that both kinases are activated in a time-dependent manner which largely coincides with the increase in Ser307 and Ser632 phosphorylation of IRS1 (Fig. 2a,b).

To examine the role of each of these kinases in IRS1 phosphorylation, we used established pharmacological inhibitors. Using antibodies against the JNK substrate phospho-c-Jun and the IKK $\beta$  substrate phospho-I $\kappa$ B, 48% inhibition of JNK and 65% diminution of IKK $\beta$  activity was observed with SP600125 and salicylic acid, respectively (Fig. 6a). Using these inhibitors, the contributions of IKK $\beta$  and JNK1/2 to IRS1 phosphorylation on Ser307 and Ser632 in response to oxidative stress were estimated. IRS1 phosphorylation on Ser307 was significantly inhibited by salicylic acid (Fig. 6b), probably reflecting a role for IKK $\beta$ .



**Fig. 4** IRS1 molecules phosphorylated on Ser307 but not on Ser632 exhibit impaired insulin-induced tyrosine phosphorylation, interaction with PI3K and release from internal membranes **a** Postnuclear internal membrane fractions were prepared as described in [Materials and methods](#) from control or oxidised cells stimulated or not stimulated with 100 nmol/l insulin for 5 min. Equal protein contents from each preparation were resolved by SDS-PAGE and assessed for the amount of IRS1, pSer632-IRS1 or pSer307-IRS1 using the respective antibodies. This experiment was repeated twice with similar results. **b** Fao cells were treated as above before cell lysates were prepared, and 500 µg protein was subjected to immunoprecipitation using anti-IRS1, anti-pSer632-IRS1 or anti-pSer307-IRS1. Immunoprecipitates were

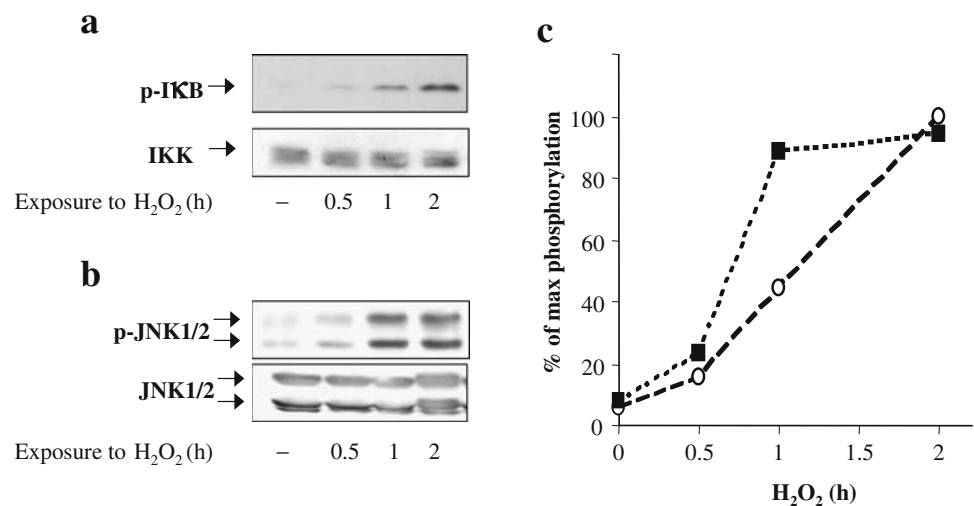
subjected to immunoblotting using anti-IRS1, anti-phosphotyrosine (PY20) antibodies, or antibodies directed against the regulatory subunit of PI3K (p85). Alternatively, PI3K activity was measured in immunoprecipitates (*bottom panel*), as described in [Materials and methods](#). Blots shown are representative of three independent experiments yielding similar results. **c** Densitometry analysis of the ratio between the phosphotyrosine, p85 bands, or the PI3P generated in the PI3K assay and the respective IRS1 band. Densitometry values from three independent experiments were used; 100% was assigned to the ratio in IRS1 immunoprecipitate from insulin-stimulated control cells. \* $p < 0.05$  vs IRS1 immunoprecipitates from oxidised cells. *Con* control cells

Importantly, salicylic acid did not fully inhibit oxidative stress-induced IRS1 Ser307 phosphorylation, indicating incomplete inhibition of IKK $\beta$  (as suggested in Fig. 6a) and/or involvement of other, salicylic acid-insensitive serine kinases. Consistent with this notion was the finding that a decrease of nearly 20% in oxidation-induced Ser307 phosphorylation of IRS1 was observed with LY294002, rapamycin or PD98059 (data not shown). Surprisingly, SP600125 had minimal effect on IRS1 phosphorylation on Ser307, suggesting incomplete inhibition of JNK or lack of involvement of this kinase in Ser307 phosphorylation in

response to oxidative stress. Consistently, the combination of salicylic acid with SP600125 was without significant additional inhibitory effect on Ser307 phosphorylation compared with salicylic acid alone (Fig. 6b).

The sensitivity to salicylic acid of Ser632 phosphorylation of IRS1 was similar to that of Ser307 phosphorylation (Fig. 6c), suggesting a contribution of a salicylic acid-sensitive kinase, potentially IKK $\beta$ . However, in contrast to Ser307 phosphorylation, the phosphorylation of the Ser632 site was highly sensitive to SP600125. Furthermore, the combined use of salicylic acid and SP600125 inhibited IRS1

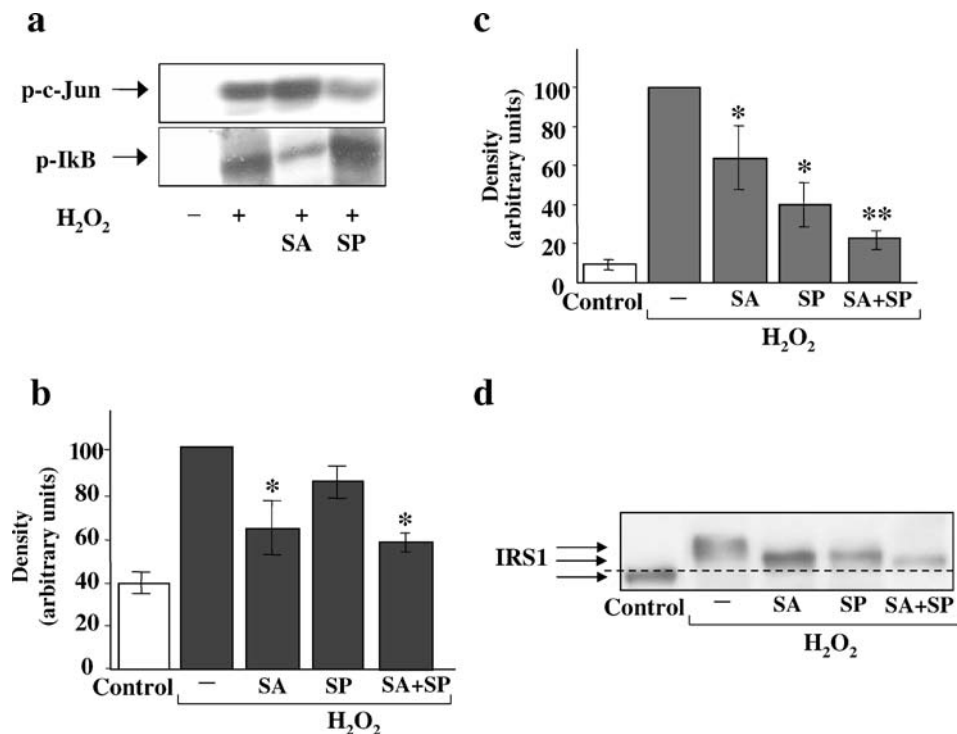
**Fig. 5** Activation of IKK $\beta$  and JNK by oxidative stress. Fao cells were exposed to H<sub>2</sub>O<sub>2</sub> generated by glucose oxidase for 0.5–2 h, after which cells were washed, lysed and subjected to western blot analysis using anti-phospho-IkappaB (pIkB), anti-IKK $\beta$ , anti-p-JNK1/2 or anti-JNK1/2. **a, b** Representative blots. **c** Results of densitometry analysis of the phosphoprotein bands over time presented as percentage of maximal intensity. These experiments were repeated twice with identical results. *Filled squares, p-JNK; empty circles, p-IkB*



Ser632 phosphorylation almost completely in response to oxidative stress. The role of salicylic acid and SP600125-insensitive kinases in total IRS1 phosphorylation in response to oxidative stress can be deduced from Fig. 6d, depicting marked but incomplete restoration of normal IRS1 migration in the gel. Importantly, the combination of salicylic acid with

SP600125 was more efficient than either individual inhibitor alone in preventing the retarded gel migration of IRS1.

Finally, the ability of combined treatment with salicylic acid and SP600125 to relieve insulin resistance at the level of PKB/Akt activation and at a metabolic level was studied. Insulin-stimulated phosphorylation of PKB/Akt was signifi-



**Fig. 6** Effects of SP600125 and salicylate on Ser632 and Ser307 phosphorylation of IRS1. Fao cells were preincubated for 60 min with 10 mmol/l salicylate (SA) and/or for 30 min with 40  $\mu$ mol/l SP600125 (SP), before and during 2 h of exposure to oxidative stress (H<sub>2</sub>O<sub>2</sub>). Cells were washed, lysed and subjected to western blot analysis. **a** Representative blots exposed to anti-phospho-c-Jun and anti-pIkB. Blots were exposed to **b** anti-pSer307-IRS1 or **c** anti-pSer632-IRS1, followed by

densitometry analysis. Results are mean  $\pm$  SEM of values derived from seven independent experiments yielding similar results, in which a value of 100% was assigned to the band intensity in oxidised cells. \* $p$ <0.05 and \*\* $p$ <0.01 vs oxidised cells. A representative blot of membrane exposed to anti-IRS1 antibody is shown in **d**. The *dashed line* denotes the gel migration of the IRS1 band in non-oxidised cells



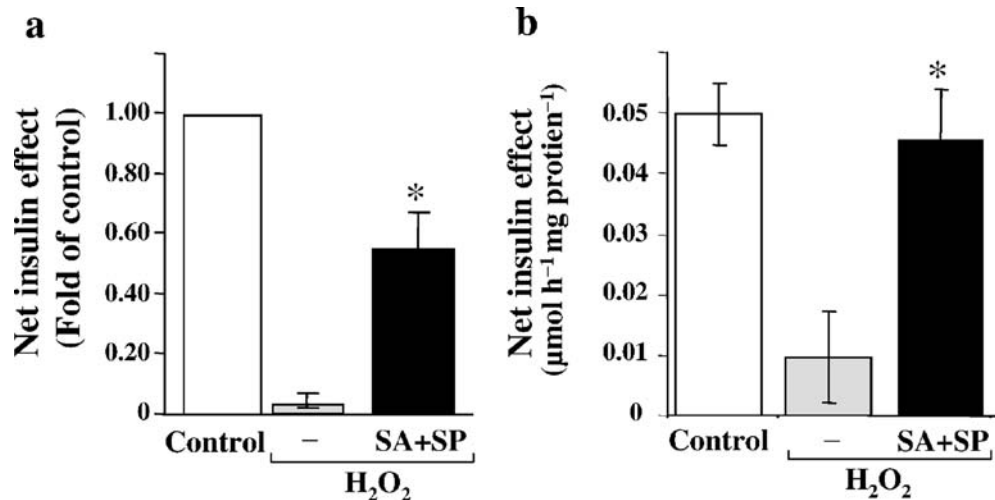
cantly increased by the combined use of salicylic acid with SP600125, amounting to over 50% of the response in control cells (Fig. 7a). To correlate these findings to a metabolic endpoint, we measured insulin-stimulated glycogen synthesis (Fig. 7b), demonstrating that the combined treatment of the cells with salicylic acid and SP600125 resulted in significant ( $p < 0.05$ ) prevention of the impairment of insulin-induced glycogen synthesis induced by oxidative stress. These results suggest the capacity of combined stress-signalling inhibition with salicylic acid and SP600125 to protect against the induction of insulin resistance by oxidative stress.

## Discussion

The present study suggests that phosphorylation of IRS1 on Ser307 or on Ser632 in response to oxidative stress defines two minimally overlapping pools of IRS1 molecules that are distinct spatially and functionally. The small pool of IRS1 molecules phosphorylated on Ser307 responds poorly to insulin and fails to be released from internal membranes. Its participation in mediating insulin resistance in response to oxidative stress is further supported by the finding that a combination of pharmacological stress kinase inhibitors can decrease Ser307 phosphorylation of IRS1 vis à vis partial restoration of insulin-stimulated PKB/Akt phosphorylation and glycogen synthesis.

It is over a decade since the initial demonstrations that phosphorylation of IRS on Ser/Thr residues modulates its

ability to undergo tyrosine phosphorylation and hence to function as a key mediator in propagating insulin receptor signalling [3]. Our understanding of the complexity of this regulatory mode has been steadily increasing. IRS polypeptides have dozens of Ser/Thr residues in their primary sequence, which are potential substrates for various Ser/Thr kinases. Some of these are kinases that are also intrinsic to the insulin signalling cascade, such as PKB/Akt, mTOR and PKC $\zeta$ . These can mediate the physiological feed-forward and shutdown mechanisms of the insulin signal [5], but additionally can act as modulators of insulin signal transduction when activated by other stimuli and/or extracellular cues. Other kinases not traditionally viewed as an integral part of the insulin signalling cascade, such as the stress kinases JNK1/2 and IKK $\beta$ , mediate crosstalk between stress signalling and the insulin signalling cascade [1]. In particular, phosphorylation of IRS by these kinases tends to decrease insulin signalling efficiency and thus generate a state of insulin resistance. Further understanding of the role of Ser phosphorylation of IRS molecules will require the assignment of specific IRS Ser residues to biological outcomes, and identification of the IRS1 kinases responsible. For example, in cell lines treated with inducers of insulin resistance, including TNF- $\alpha$ , chronic insulin and phorbol 12-myristate-13-acetate (PMA), it was suggested that pSer307-IRS1 mediates insulin resistance, but the kinases implicated in IRS1 phosphorylation were JNK1, IKK $\beta$  or mTOR [6, 12, 36]. Nevertheless, consistent with its inhibitory role in insulin signal propagation, pSer307-



**Fig. 7** Combined incubation with SP600125 and salicylate rescues oxidised cells from insulin resistance. **a** Fao cells were preincubated for 60 min with 10 mmol/l salicylate (SA) and for 30 min with 40  $\mu\text{mol/l}$  SP600125 (SP), and were then exposed to oxidative stress ( $\text{H}_2\text{O}_2$ ) in the absence or presence of the two inhibitors for 2 h. Cells were then washed and further incubated for 10 min in the absence or presence of 100 nmol/l insulin. The cells were washed, lysed and subjected to western blot analysis using anti-pSer473-PKB/Akt antibodies. Results are shown of densitometry analysis of seven

independent experiments in which a value of 1 was assigned to the net insulin effect on PKB/Akt phosphorylation in control cells. **b** Fao cells were treated with inhibitors under the oxidation conditions described above, followed by incubation for 60 min without or with 100 nmol/l insulin, during which glycogen synthesis was measured as described in **Materials and methods**. Results are the mean  $\pm$  SEM of the net insulin effect in five independent experiments. \* $p < 0.05$  vs the net insulin effect in oxidised cells

IRS1 was increased in muscle of patients with type 2 diabetes and in insulin-resistant patients [13, 37].

The functional consequence of IRS1 phosphorylation on Ser632 is variable and may depend on the stimulus that triggers it. Phosphorylation of this site by rho kinase is required for normal transmission of the insulin signal [38]. In contrast, however, Ser 632 phosphorylation was suggested to play a negative role in signal propagation: it is increased in obesity in muscle of patients with type 2 diabetes and their insulin-resistant offspring, as well as after TNF administration [16–18]. In addition, it was demonstrated recently that nutrients can induce the phosphorylation of this site, and this phosphorylation negatively regulates the association between IRS1 and the PI3K [39].

Our results demonstrate an inhibitory role of pSer307-IRS1 in the insulin signal in response to oxidative stress, as these molecules exhibited diminished insulin-induced tyrosine phosphorylation, interaction with p85, and associated PI3K activity (Fig. 4b,c). They also failed to be released from a membrane fraction in response to acute insulin stimulation (Fig. 4a). In contrast, pSer632-IRS1 seemed to participate normally in insulin signal propagation after oxidative stress. The finding that the two phosphoserine sites also define distinct cellular locations may be significant: various studies using biochemical and cell imaging approaches demonstrated that, in response to insulin stimulation, IRS1 is mobilised between cellular compartments and domains. Tyrosine-phosphorylated IRS1 molecules may specifically accumulate in low-density microsomal fractions [30, 34, 35], potentially representing F-actin-rich sites in which they may serve to dock and activate PI3K [32, 34]. Activating PI3K in such defined locations may be a specific effect of insulin, since it does not seem to occur in response to other stimuli, such as platelet-derived growth factor [33, 34, 40]. We and others have suggested that this unique ability of insulin to mobilise IRS1 and target PI3K may be impaired in response to oxidative stress, resulting in decreased activation of downstream signalling mediators and metabolic end-points [30, 33, 41]. Since the majority of IRS1 molecules were phosphorylated on Ser632, it is not surprising that insulin-stimulated tyrosine phosphorylation and interaction with PI3K assessed in total cell lysates was normal after oxidative stress. However, pSer307-IRS1 molecules are retained in response to insulin stimulation in an internal membrane pool and have poor ability to propagate the insulin signal to PKB/Akt and to metabolic outcomes. The combination of spatial and functional characteristics of this small pool of IRS1 molecules renders them critical for the mechanism of insulin resistance in response to oxidative stress. Assigning such a potent function to a rather small fraction of IRS1 molecules is consistent with previous estimates that even under normal conditions insulin engages only a low percentage of the entire cellular IRS1 pool [35].

With the worldwide surge in the incidence of type 2 diabetes, there is a pressing need to discover new therapeutic approaches to the treatment of insulin resistance. The stress kinases link to Ser/Thr phosphorylation of IRS molecules is a molecular mechanism mediating the association between inflammation, endoplasmic reticulum stress, oxidative stress and insulin resistance, providing an attractive point for novel therapeutic intervention. Salicylates at high doses are known to improve glucose intolerance clinically, and have been shown to do so at least in part by inhibiting IKK $\beta$ -mediated phosphorylation of IRS1 [42]. Pharmacological inhibitors of other stress kinases, including JNK, are being developed and are at various stages of testing for clinical use. Here we show that significant prevention of signalling and metabolic insulin resistance induced by oxidative stress can be achieved by the combined action of salicylates and SP600125 (Fig. 7). Stress sensing involves the activation of several parallel and interrelated signalling cascades, and rarely activates single proteins with IRS1 Ser/Thr kinase activity. The results of this study suggest that combined pharmacological inhibition of such kinases, rather than an attempt to target an individual stress kinase, may hold promise in relieving insulin resistance.

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