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IRS1 degradation and increased serine phosphorylation cannot predict the degree of metabolic insulin resistance induced by oxidative stress

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Abstract

Aim/hypothesis. Oxidative stress was shown to selectively induce impaired metabolic response to insulin, raising the possible involvement of alterations in Insulin-Receptor-Substrate (IRS) proteins. This study was conducted to assess whether oxidative stress induced IRS protein degradation and enhanced serine phosphorylation, and to assess their functional importance.
Methods. 3T3-L1 adipocytes and rat hepatoma cells (FAO) were exposed to micro-molar H_2O_2 by adding glucose oxidase to the culture medium, and IRS1 content, its serine phosphorylation and downstream metabolic insulin effects were measured.
Results. Cells exposed to oxidative stress exhibited decreased IRS1 (but not IRS2) content, and increased serine phosphorylation of both proteins. Total protein ubiquitination was increased in oxidized cells, but not in cells exposed to prolonged insulin treatment. Yet, lactacystin and MG132, two unrelated proteasome inhibitors, prevented IRS1 degradation induced by prolonged insulin but not by oxidative stress. The PI 3-kinase inhibitor LY294002 and the mTOR inhibitor rapamycin, but not the MEK1 inhibitor PD98059, could prevent IRS1 changes in oxidized cells. Rapamycin, which protected against IRS1 degradation and serine phosphorylation was not associated with improved response to acute insulin stimulation. Moreover, the antioxidant alpha lipoic acid, while protecting against oxidative stress-induced insulin resistance in 3T3-L1 adipocytes, could not prevent IRS1 degradation and serine phosphorylation.
Conclusion/interpretation. Oxidative stress induces serine phosphorylation of IRS1 and increases its degradation by a proteasome-independent pathway; yet, these changes do not correlate with the induction of impaired metabolic response to insulin. [Diabetologia (2003) 46:639–648]

Keywords IRS1 degradation, oxidative stress, H_2O_2, insulin resistance, serine-phosphorylation, lipoic acid, ubiquitination.

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Abbreviations: PI3-kinase, phosphatidylinositol 3'-kinase; mTOR, mammalian target of rapamycin; MEK1, mitogen activated protein kinase1; SH2, Src homology 2; PKB, protein kinase B; PKC, protein kinase C; ERK, extracellular signal-regulated kinase; GSK3, glycogen synthase kinase-3; JNK, c-Jun N-terminal kinase; PAGE, polyacrylamide gel electrophoresis; PDGF, platelet derived growth factor.

Oxidative stress has been shown in recent years to represent a causative factor for insulin resistance in cells in culture [1, 2, 3], and to accompany diabetes and insulin resistance states [4]. Cells exposed to oxidative stress show complex changes in protein function, localization, as well as in gene regulation [5, 6, 7]. Despite this diversity of cellular changes and the large number of proteins involved, specificity to the insulin signalling cascade had been suggested, since PDGF signalling and metabolic effects under the same conditions were unaltered in 3T3-L1 adipocytes [2]. One potential source for such specificity is the changes that Insulin Receptor Substrate (IRS) proteins undergo following oxidative stress, since these proteins are involved in insulin but

not in PDGF signalling. Previous studies suggested that oxidative stress alters insulin-mediated re-localization of IRS1 [7], but whether oxidative stress directly alters IRS proteins and what is the functional significance of these alterations is unknown.

The IRSs constitute one family of substrate proteins for the tyrosine kinase activity of the insulin receptor (and closely related receptors) [8]. Their tyrosine phosphorylation promotes their participation in transmitting the insulin signal, by inducing their interactions with protein-containing SH2 domain(s), like the p85 sub-unit of PI 3-kinase [9]. Cellular models for insulin resistance and in vivo studies suggested an association between insulin resistance and alterations in IRS proteins, and particular in IRS1 and IRS2 [10, 11, 12, 13]. A reduction in IRS1 expression has been reported in isolated adipocytes from insulin-resistant patients and Type 2 diabetic patients [10, 11], but not in skeletal muscle [12, 13]. Decreased IRS1 protein content was reported in cells in culture exposed to various inducers of insulin resistance, including TNF-α and chronic insulin [14, 15, 16]. Recent data suggests that prolonged exposure to insulin and TNF-α could result in increased degradation of IRS1 by the ubiquitin-proteasome system [17, 18, 19]. Conflicting reports are currently available regarding the involvement of the proteasome in determining IRS2 content under the same conditions [17, 19, 20]. Such an increase in proteasomal degradation of IRS1 is an attractive mechanism for insulin resistance, since increased ubiquitination of proteins is a common cellular mechanism for down-regulating various signalling processes [21].

In addition to decreased protein content of IRSs, increased phosphorylation of IRS proteins on serine/threonin residues has been reported as an additional alteration that accompanies the insulin-resistant state [22]. The primary sequence of IRS1 and IRS2 includes over 30 potential phosphorylation sites on serine and threonin residues, which could be the substrates for various kinases based on both the kinases' recognition motifs and on in vitro and in vivo assays. These include kinases like PKB and PKCs [23, 24], ERK1/2 [25], GSK3 [26], JNK [27], as well as the serine kinase activity of the p110 sub-unit of PI 3-kinase [28]. Some of these kinases were implicated in down-regulating the insulin signal, based on their activation by various inducers of insulin resistance. Moreover, some [29], (but not all [23]) of the phosphoserine residues might impair insulin receptor-mediated phosphorylation on adjacent tyrosine residues of IRS, resulting in impaired early insulin signalling events. Recently it was suggested that increased serine phosphorylation on specific sites on IRS1 proceeds its degradation [18, 30, 31].

The aim of this study was to evaluate the effect of oxidative stress on IRS1 protein content and its serine phosphorylation state, in relation to the induction of cellular insulin resistance. Our findings suggest that oxidative stress induces the serine phosphorylation of IRSs and the protein degradation of IRS1 in a LY294002 and rapamycin-sensitive, but lactacystin-resistant process. Yet, these IRS changes do not necessarily correlate with the induction of impaired metabolic actions of insulin.

Materials and methods

Chemicals. Tissue culture medium, serum and antibiotic solutions were obtained from Biological Industries (Beit-Haeemek, Israel). Recombinant human insulin was from Novo Nordisk (Bagsvaerd, Denmark). Polyclonal IRS1 antibodies (C-terminus), anti p85 and anti IRS2 were obtained from Upstate Biotechnology (Lake Placid, N.Y., USA). Alpha racemic lipoic acid was kindly provided by ASTA medica (Frankfurt, Germany). Phosphoserine antibodies were obtained from Chemicom International (Temecula, Calif., USA). Lactacystin and MG132 were purchased from Calbiochem (San Diego, Calif., USA). Alkaline phosphatase and all other chemicals were obtained from Sigma Chemical (St. Louis, Mo., USA).

Cell culture and treatment. 3T3-L1 pre-adipocytes (American Type Culture Collection) were grown to confluence in Dulbeco's Modified Eagle's Medium (DMEM) containing 25 mmol/l glucose, exactly as previously described [1]. Cells were used 11 to 12 days following differentiation induction when showing a greater than 90% adipocyte phenotype. Rat hepatoma Fao cells were grown to confluence in RPMI medium containing 5 mmol/l glucose with 10% foetal calf serum. Cells were serum deprived for 16 h by incubation in medium supplemented with 0.5% radioimmunoassay grade bovine serum albumin (BSA), with or without pre-treatment reagents. Cells were then rinsed three times in PBS, and exposed to fresh medium with or without glucose oxidase [type II from Aspergillus niger, 20 000 unit/g solid in non-oxygen saturated conditions, Sigma Chemical (St Louis, Mo.)], or with 100 nmol/l insulin.

Cell lysates and Western blot analysis. Lysates for immunoblots were prepared in a lysis buffer containing 50 mmol/l Tris-HCl pH 7.5, 0.1% (w/v) Triton X-100, 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mmol/l sodium orthovanadate, 50 mmol/l NaF, 10 mmol/l sodium β-glycerophosphate, 5 mmol/l sodium pyrophosphate, 0.1% (v/v) 2-mercaptoethanol, and protease inhibitors [a 1:1000 dilution of protease inhibitor cocktail (Sigma P-8340)]. Lysates were collected, gently shaken for 20 min. at 4°C, centrifuged (12 000 g, 15 min, 4°C), and the supernatant fraction was collected. Protein concentration was assessed using Bio-Rad reagents (Bio-Rad Laboratories, Munchen, Germany). Aliquots of 30–75 μg protein were resolved on 7.5% SDS-PAGE and subjected to Western blots followed by quantitation by video densitometry analysis [7].

H_2O_2 determination. H_2O_2 generated by glucose oxidase was measured by a colorimetric method based on production of ferrithiocyanate [32]. One ml aliquots of medium were collected and 0.1 ml TCA (50% w/v) was added. The samples were centrifuged at 10 000 g for 3 min, 0.2 ml of 10 mmol/l ferrous ammonium sulfate and 0.1 ml of 2.5 mol/l sodium thiocyanate were added to the supernatant. Absorption of the ferrithiocyanate complex was measured using a spectrophotometer at 480 nm, and compared to standard curves obtained from dilutions of a standard H_2O_2 solution.

Treatment with alkaline phosphatase. Lysates for alkaline phosphatase treatment were prepared in 25 mmol/l Tris-HCl pH 7.4, 2 mmol/l sodium orthovanadate, 0.5 mmol/l EGTA, 25 mmol/l NaCl, 10 mmol/l $MgCl_2$, and protease inhibitors [a 1:1000 dilution of protease inhibitor cocktail (Sigma P-8340)]. After three freeze-thaw cycles, the cells were centrifuged at 12 000 g for 30 min at 4°C and supernatant was collected. Aliquots (300 µg) were incubated with 600 units of alkaline phosphatase (Sigma P3681) for 1 h at 37°C. Following incubation, samples were boiled for 5 min. in Laemmli sample buffer (50 mmol/l Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 2% β-mercaptoethanol and 0.004% bromophenol blue), and separated by 7.5% SDS-PAGE .

Glucose transport. 2-Deoxyglucose (2DG) uptake measurements were performed as previously described [33].

Glycogen synthesis. Glycogen synthesis was measured, in 6-well plates, by assessing the amount of radioactivity in the glycogen fraction (precipitation in 70% ethanol) of cells incubated in the presence of (U-^{14}C) glucose. Following treatment cells were washed three times in PBS, and incubated with PBS containing 0.1 mmol/l (U-^{14}C) glucose (2 µCi /well) in the absence or presence of 100 nmol/l insulin, for 1 h. Cells were then washed, homogenized, and glycogen was precipitated on a Whatmann paper in 70% ethanol.

Statistical analysis. Data are expressed as means ± standard error (SE). Each treatment was compared to 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

Oxidative stress induces decreased protein content and increased serine phosphorylation of IRS1. 3T3-L1 adipocytes and rat hepatoma FAO cells were exposed to stable H_2O_2 concentrations, generated by adding glucose oxidase to the culture medium. Cells were exposed for 2 h to 40±5 µmol/l H_2O_2, after which the cellular content of IRS proteins was evaluated in total cell lysates by Western blot analysis. For comparison, cells were treated for 5 h with 100 nmol/l insulin, conditions previously shown to decrease IRS1 protein content in the two cell lines. Exposure to H_2O_2 resulted in a reduction in IRS1 immunoreactivity in both 3T3-L1 adipocytes, as well as in FAO cells (Fig. 1A,B). This decreased expression level was associated with retarded migration of the IRS1 band in the SDS-polyacrylamide gel, an effect that was more pronounced in the FAO cells. Densitometry analysis of four independent experiments yielding similar results, showed that these conditions of oxidative stress resulted in 45±11 and 37±5% decrease in IRS1 content in 3T3-L1 and FAO cells respectively, as compared to control cells ($p<0.05$). As expected, 5-h exposure to 100 nmol/l insulin also resulted in a 40±9 and 72±7% reduction in IRS1 content in 3T3-L1 adipocytes and FAO, respectively, as well as a retarded gel migration of the IRS1 band in both cell types.

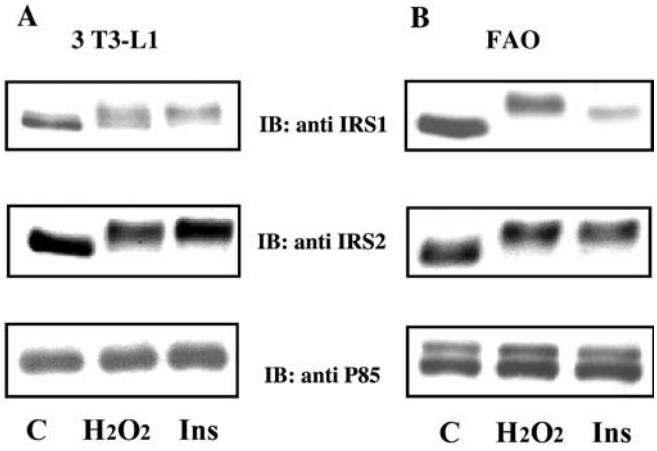

Fig. 1A, B. Effect of oxidative stress or prolonged insulin treatment on IRS1, IRS2 and PI 3-kinase in 3T3-L1 adipocytes and rat hepatoma Fao cells (FAO). Fully differentiated 3T3-L1 adipocytes (**A**) and FAO (**B**) cells were serum-starved for 16 h and treated with either glucose oxidase yielding a 40 µmol/l medium H_2O_2 concentration for 2 h (H_2O_2), or with 100 nmol/l insulin for 5 h (Ins). (**C**) represents control cells. Cells were then lysed, proteins were separated on 7.5% SDS-PAGE, transferred and immunoblotted using the indicated antibodies. Shown are blots representative of four independent experiments yielding similar results

To assess whether the effect of oxidative stress on IRS1 content was associated with enhanced degradation of other insulin signalling proteins, cell lysates were analyzed for the protein level of both IRS2 and the p85 subunit of PI 3-kinase, representing a closely related as well as a structurally unrelated protein to IRS1, respectively. While affecting IRS2 migration in the gel, oxidative stress caused only a non-striking 12±2 and 5±2% decrease in IRS2 protein content in 3T3-L1 and FAO cells, respectively (Fig. 1). Similarly, 5-h exposure to insulin had only a minor effect on IRS2 content in both cell types. The protein content of the p85 subunit of PI 3-kinase was unaltered by either oxidative stress or 5-h exposure to insulin (Fig. 1).

Since the decrease in IRS1 content was associated with retarded migration of the band during SDS PAGE, we further addressed the possibility that oxidative stress resulted in increased Ser/Thr phosphorylation of IRS1. FAO cell lysates were prepared without Ser/Thr phosphatase inhibitors, and treated in vitro for 1 h in the absence or presence of alkaline phosphatatse, and analyzed for IRS1 gel retardation as before. Alkaline phosphatase treatment completely reversed the retarded migration of the IRS1 band that was induced by either oxidative stress or insulin (Fig. 2A). Importantly, the intensity of the IRS1 band was still decreased as compared to control following this in vitro treatment (being 64±7 and 51±8% of control, in oxidized cells before and after alkaline phosphatase treatment). This verified that the decreased immunoreactivity of the IRS1 band following oxidative stress represented a true decrease in IRS1 content, and could

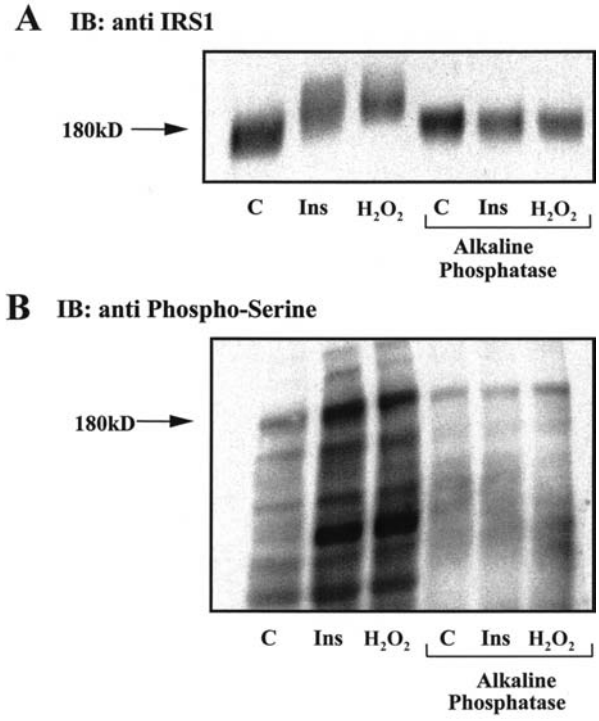

Fig. 2A, B. H_2O_2 and prolonged insulin treatment increase serine/threonine phosphorylation of IRS1. FAO cells were either incubated with no addition (**C**), with glucose oxidase (H_2O_2) or insulin (Ins). Cells were lysed in a lysis buffer devoid of Ser/Thr phosphatase inhibitors, and aliquots (300 µg) were incubated without or with 600 units of alkaline phosphatase for 1 h at 37°C, as described in Materials and Methods. Western blot analysis was performed using either IRS1 antibodies (**A**) or anti-phosphoserine antibodies (**B**). Shown are blots representative of four independent experiments yielding similar results

not be attributed to reduced affinity of the anti-IRS1 antibody to the serine/thronine hyper-phosphorylated IRS1 protein. To further establish that oxidative stress increased the serine phosphorylation of proteins in the cells, Western blot analysis of total cell lysates was carried out using anti phosphoserine antibodies. Lysates of cells exposed to oxidative stress exhibited an increased intensity of the signal generated with anti-phosphoserine antibodies, an effect which was reversible by in vitro treatment of the lysates with alkaline phosphatase (Fig. 2B). Strikingly, increased serine phosphorylation of an approximate 180 000 Mr band was noticeable in response to oxidative stress, potentially corresponding to IRS protein(s). Yet, we were unable to show any visible band when IRS1 immunoprecipitates were blotted using the same antibody. Interestingly, chronic insulin stimulation also resulted in enhanced serine phosphorylation of proteins in total cell lysates, in a pattern that was similar to that obtained by oxidative stress. Collectively these data suggest that oxidative stress specifically decreased IRS1 protein content, and was associated with increased serine phosphorylation.

To better characterize the effect of oxidative stress on IRS1 content and serine phopshorylation, the time- and dose-dependencies of these effects were studied. A reduction in IRS1 content could be observed already after 2 h of exposure to 40±5 µmol/l H_2O_2 (Fig. 3A). A decrease in IRS1 content was observed after 2 h exposure to as low as 40 µmol/l H_2O_2 (Fig. 3B). In both experiments, the effect on IRS1 serine phosphorylation, as evident by the retarded migration of the IRS1 band in the gel, coincided with the time and concentration change in IRS1 content.

Since the decrease in IRS1 content was induced after relatively short exposure to oxidative stress, we speculated that the major process underlying this phenomenon is enhancing protein degradation of IRS1. In order to exclude a major role for inhibition of translation of IRS1 transcripts, cells were exposed to oxidative stress in the presence of cycloheximide, an inhibitor of protein translation. The presence of 5 µg/ml cycloheximide for 2 h had no effect on IRS1 content in control cells, consistent with its half life of 20 h. Moreover, it did not alter the decrease in IRS1 content induced by oxidative stress (Fig. 3C), supporting the notion that oxidative stress decreased IRS1 content primarily by enhancing its protein degradation rate.

Oxidative stress-induced IRS1 degradation is PI 3-kinase/mTOR dependent. As shown above, oxidative stress induced a decrease in IRS1 (but not IRS2) protein content similar to the effect of prolonged exposure to insulin. Recent studies on the induction of IRS1 degradation by prolonged insulin have shown the involvement of the PI 3-kinase/mTOR signalling pathway, but not the MEK-ERK1/2 pathway in IRS1 degradation. To assess whether oxidative stress utilized similar signalling pathways as chronic insulin in inducing IRS1 degradation, FAO cells were incubated with pharmacological inhibitors 15 min. prior to, as well as during exposure to oxidative stress. Both LY294002, a PI 3-kinase inhibitor, as well as the mTOR inhibitor rapamycin, showed a similarly significant ($p<0.05$), but not full capacity to protect against oxidative stress–induced IRS1 degradation (Fig. 4A). In contrast, no effect on IRS1 content and gel migration was noted using the MEK inhibitor PD98059. Identical results regarding the effect of the three inhibitors on IRS1 degradation were obtained in 3T3-L1 adipocytes (data not shown). Consistent with recent reports on insulin-mediated IRS1 degradation, the PI3-kinase inhibitor prevented both the decrease in IRS1 content as well as its retarded migration in the gel (Fig. 4B). While rapamycin had a partial effect on insulin mediated IRS1 degradation, no similar effect was observed using PD98059. These data show the involvement of the PI 3-kinase/mTOR signalling pathway in mediating oxidative stress-induced IRS1 degradation and serine phosphorylation, similar but not

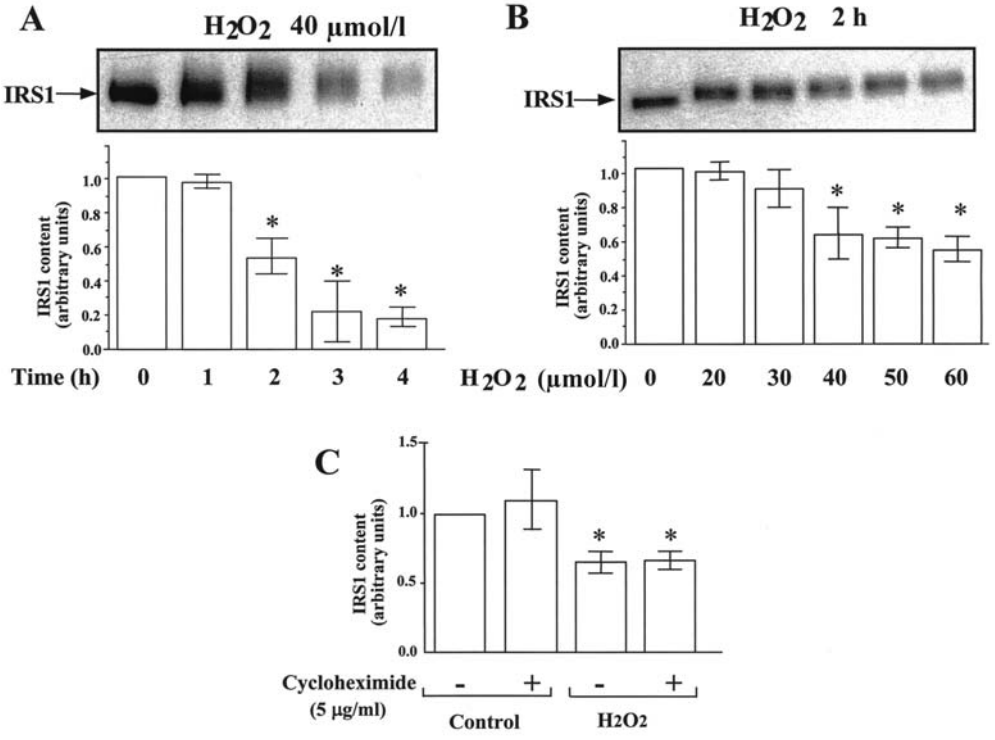

Fig. 3A–C. Time course and dose dependency of IRS1 changes induced by oxidative stress. (**A**) FAO cells were incubated with 100 mU/ml of glucose oxidase, which generated medium H_2O_2 concentrations of 40 ± 5 µmol/l H_2O_2 for the indicated time periods. (**B**) Cells were exposed to various glucose oxidase concentrations yielding the indicated medium H_2O_2 concentrations for 2 h. (**C**) Cells were incubated for 15 min prior to and during exposure to H_2O_2 without or with 5 µg/ml cycloheximide, a protein synthesis inhibitor. Following each of these experiments, cell lysates were prepared and analyzed for IRS1. Shown are representative blots (for A and B), as well as the results of densitometry analysis of three independent experiments, in which a value of 1 was assigned for IRS1 band intensity in control cells. * $p<0.05$ as compared to control cells

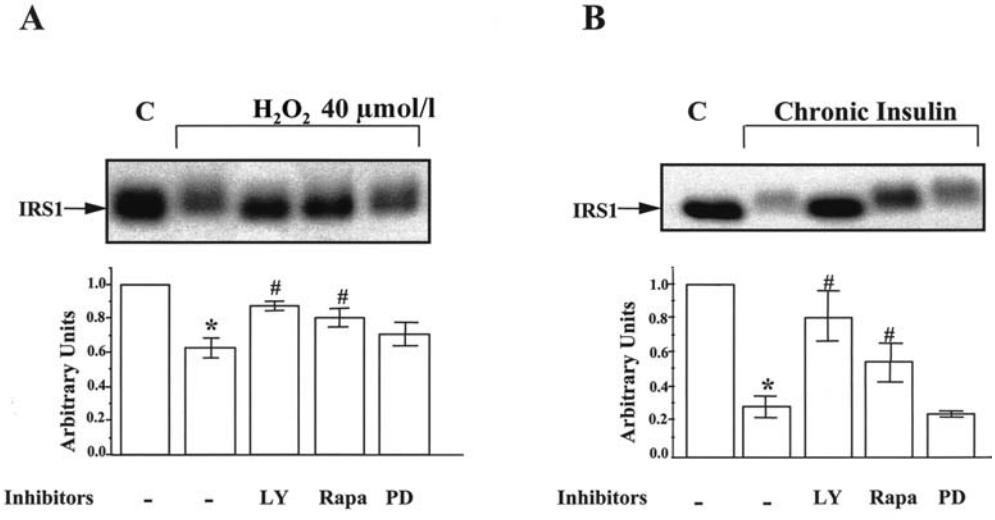

Fig. 4A, B. Effect of PI3-kinase, mTOR and MEK inhibitors on IRS1 changes induced by H_2O_2 or prolonged insulin. FAO cells were pretreated for 15 min prior to and then during glucose oxidase (H_2O_2), (**A**) or insulin treatment (**B**), with either vehicle (DMSO final concentration of 0.2%, C), 100 µmol/l LY294002 (LY), 150 nmol/l Rapamycin (Rapa), or 50 µmol/l PD98059 (PD). Cells were then analyzed for IRS1 by Western blot analysis. Shown are representative blots as well as the results of densitometry analysis of three independent experiments, in which a value of 1 was assigned for IRS1 band intensity in control cells. * $p<0.05$ as compared to control cells. # $p<0.05$ as compared to treated cells without inhibitors

identical to their involvement in IRS1 degradation induced by chronic insulin.

The role of the ubiquitin-proteasome system in IRS1 degradation following oxidative stress. Since protein degradation occurs in the cell through several pathways, the most prominent of which is the protein ubiquitination-proteasome pathway, we next assessed its involvement in the protein degradation of IRS1 induced by oxidative stress. Using immunodetection

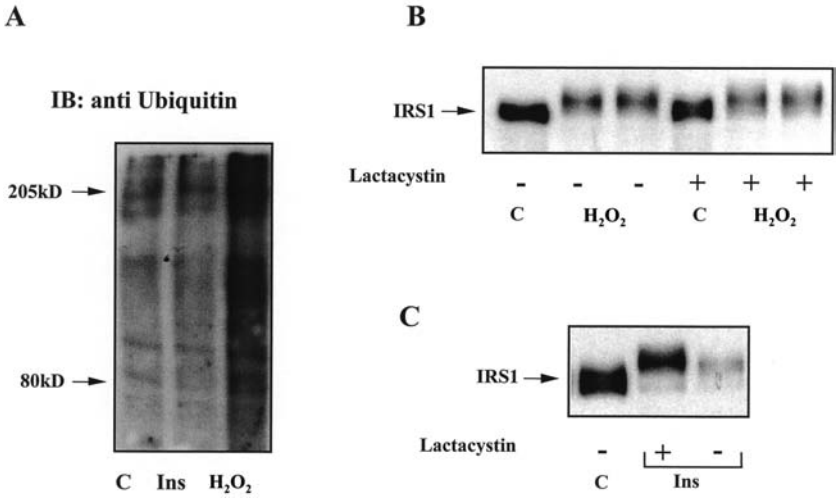

Fig. 5A, B. The involvement of the ubiquitin-proteasome system in IRS1 degradation induced by oxidative stress or prolonged insulin. FAO cells were exposed to prolonged insulin (Ins) or H_2O_2 generated by glucose oxidase (H_2O_2). Total cell lysates were separated on SDS-PAGE and blotted with anti-ubiquitin antibodies (**A**). FAO cells were incubated for 15 min prior to and during insulin or H_2O_2 treatment with 10 μmol/l lactacystin or vehicle (DMSO**,** final concentration 0.2%) (**B, C**)**.** IRS1 content in total cell lyastes was assessed by Western blot analysis. Shown are blots representative of three independent experiments yielding similar results

with anti-ubiquitine antibody, lysates of cells exposed to oxidative stress displayed increased total protein ubiquitination (Fig. 5A). This effect was not observed in lysates of cells exposed for 5 h to 100 nmol/l insulin. However, we could not detect a band when IRS1 was immunoprecipitated and blotted using the same anti-ubiquitine antibody (not shown). Addition of the proteasome inhibitor lactacystin could not prevent the decrease in IRS1 content (Fig. 5B), suggesting a lack of involvement of the proteasome protein degradation pathway in decreasing IRS1 content following oxidative stress. In contrast, lactacystin fully prevented IRS1 degradation when induced by insulin (Fig. 5C), consistent with recent reports. To exclude the possibility that the H_2O_2 decreased the bio-activity of lactacystin resulting in lack of effect in the cells co-exposed to oxidative stress, lactacystin was incubated for 2 h with 40 μmol/l H_2O_2. Catalase was then added to hydrolyse remaining H_2O_2, and the lactacystin containing medium was assessed for its capacity to inhibit IRS1 degradation by 5-h exposure to insulin. The effect of the lactacystin exposed to H_2O_2 was identical to fresh lactacystin added to the medium (data not shown). Collectively, these data confirm the bioactivity of lactacystin in these experiments. Similar results to those obtained with lactacystin were observed when using an alternative proteasome inhibitor, MG132. MG132 did not prevent oxidative stress-induced IRS1 degradation, while it prevented its degradation by chronic insulin exposure. Taken together these data argue against a role for the proteasome pathway in IRS1 degradation induced by oxidative stress, despite the observed increase in total protein ubiquitination.

The effect of IRS1 modifications by oxidative stress on insulin-mediated signalling and metabolism. We next assessed the effect of the alterations in IRS1 induced by oxidative stress on its capacity to bind PI 3-kinase in response to acute insulin stimulation. FAO cells were treated for 3 h with H_2O_2 or for 4 h with 100 nmol/l insulin, washed, and subsequently stimulated for 1 min with 100 nmol/l insulin. IRS1 was immunoprecipitated from total cell lysates, and immunopreciptates subjected to Western blot analysis. In non-oxidized cells, acute insulin stimulation induced an increase in the p85 regulatory subunit of PI 3-kinase that co-precipitated with IRS1 (Fig. 6A). Both chronic insulin as well as oxidative stress decreased the amount of p85 associated with IRS1 following acute insulin stimulation. Densitometry analysis showed a 52±2 and 48±3% decrease in the amount of p85 in the immunoprecipitates in oxidized and chronic insulin treated cells, respectively. The same blots were analyzed for the amount of IRS1 that was precipitated. Oxidative stress and chronic insulin treatments resulted in a 42±5 and 58±12% decrease in the amount of IRS1 immunoprecipitated from lysates (Fig. 6B), in agreement with the changes observed in total cell lysates (Fig. 1B). Thus, the decrease in p85 that co-precipitated with IRS1 (Fig. 6A) closely matched the reduction in cellular IRS1 content (Fig. 6B). Interestingly, under the same conditions, acute insulin-stimulated PKB/Akt serine 473 phosphorylation was almost completely blunted in cells exposed to oxidative stress (Fig. 6C), exceeding the effect observed on IRS1 content and interaction with p85. This finding suggests that the severity of the signalling defect induced by oxidative stress increases with down-stream propagation of the signal, or that the changes observed in IRS1 do not represent the actual rate-limiting step functionally responsible for im-

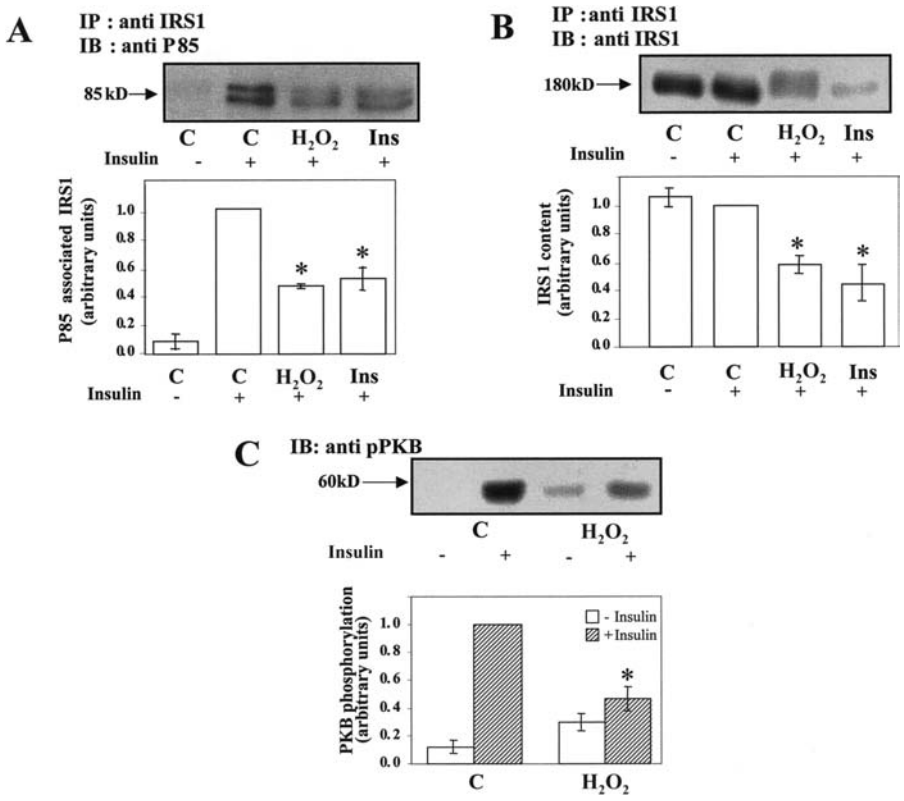

Fig. 6A–C. The effect of oxidative stress on the association of IRS1 with P85, and on PKB phosphorylation induced by acute insulin stimulation. FAO cells were treated with glucose oxidase (H_2O_2) for 3 h or 100 nmol/l insulin for 4 h, after which the cells were washed and further incubated for 1 min in the absence or presence of 100 nmol/l insulin. The cells were washed, lysed and IRS1 was immunoprecipitate from 500 μg protein and analyzed by Western blot using either the regulatory subunit of PI3 kinase (P85) (**A**), or IRS1 antibodies (**B**). In (**C**) lysates of control and oxidized cells were analyzed by Western blot using phospho-specific PKB antibody. Shown are blots representing four independent experiments as well as the results of densitometry analysis, in which a value of 1 unit was set to represent the content of either P85 (**A**), IRS1 (**B**) or pPKB (**C**) in non-oxidized cells in the presence of insulin. *p<0.05 compared with non-oxidized cells in the presence of insulin.

paired metabolic responses to insulin. To verify the latter possibility, we assessed whether changes in IRS1 induced by oxidative stress correlate with impaired metabolic response to acute insulin stimulation, using two complementary approaches. Firstly, we evaluated whether protecting against IRS1 degradation and serine phosphorylation induced by oxidative stress using rapamycin (Fig. 4A), would result in improved insulin mediated glycogen synthesis in FAO cells or insulin-stimulated glucose uptake in 3T3-L1 adipocytes. In FAO cells insulin increased glucose incorporation to glycogen, while oxidative stress blunted this effect (Fig. 7A). Surprisingly, while rapamycin protected against IRS1 degradation and serine phosphorylation (Fig. 4A), this was not associated with improved response to acute insulin stimulation (Fig. 7A). In 3T3-L1 adipocytes, exposure to H_2O_2 reduced insulin-stimulated glucose transport. Incubation with rapamycin, though preventing oxidative stress-induced IRS1 alterations, had no effect on the reduction of insulin-stimulated glucose transport (data not shown). Secondly, we evaluated whether preincubation with the antioxidant lipoic acid protected against IRS1 serine phosphorylation and degradation. 3T3-L1 adipocytes were pre-treated for 16 h with 200 μmol/l alpha lipoic acid, washed, and exposed for 2 h to oxidative stress. Using this protocol, lipoic acid pre-treatment prevented oxidative stress-induced reduction in insulin-stimulated glucose uptake and PKB phosphorylation (Fig. 7B). Yet, this was not associated with a protection against IRS1 degradation or increased serine phosphorylation (Fig. 7C). Densitometry analysis showed a 55±11 and 65±7% of control in oxidized cells in the absence or presence of lipoic acid, respectively. Collectively, these data suggest that the reduction in IRS1 content and/or its increased serine phosphorylation induced by oxidative stress cannot fully predict the degree of impairment in the metabolic response to insulin, nor the protective effect provided by an antioxidant.

Discussion

This study provides evidence that oxidative stress, concomitant to its ability to induce metabolic insulin resistance, also increases IRS1 serine phosphoryla-

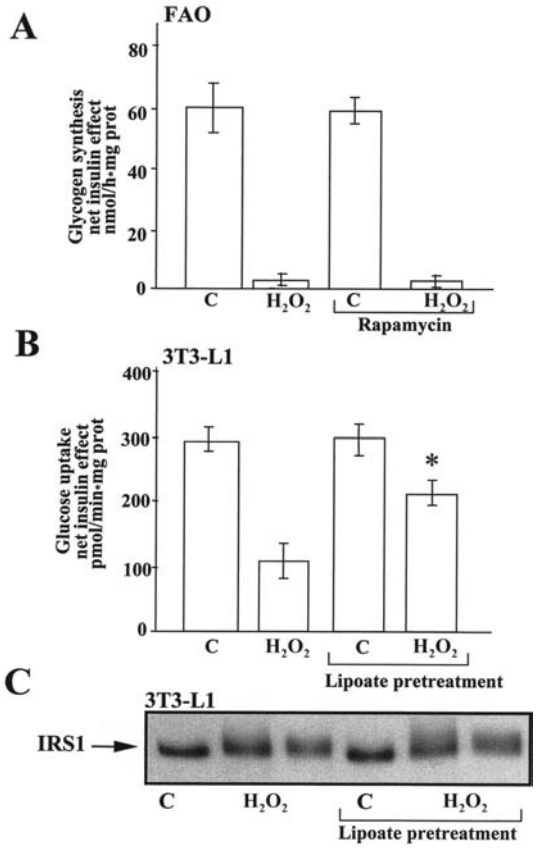

Fig. 7A–C. Different effects on oxidative stress-induced IRS1 changes and on the impaired metabolic response to insulin by rapamycin and alpha lipoic acid. (**A**) FAO cells were pretreated with 150 nmol/l rapamycin for 15 min prior to and during the treatment with H_2O_2 generated by glucose oxidase (H_2O_2). Incorporation of radiolabel glucose to glycogen in the absence or presence of insulin was measured. (**B**) 3T3-L1 adipocytes were treated for 16 h in serum-free (BSA supplemented) medium without or with 200 μmol/l racemic alpha lipoic acid, after which cells were washed and incubated for 2 h with H_2O_2. Thereafter cells were washed, and glucose uptake was measured without and after exposed for 20 min to 100 nmol/l insulin. The results (in **A** and **B**) represent the mean±SE of five independent experiments. *$p<0.05$ compared with net insulin effect in oxidized cells. (**C**) 3T3-L1 adipocytes were pre-treated with lipoic acid and exposed to H_2O_2, after which IRS1 content was evaluated by Western blot. Shown is a blot representing two additional experiments yielding similar results.

tion, and enhances its protein degradation in 3T3-L1 adipocytes and FAO cells. Both effects are sensitive to LY294002 and rapamycin (but not to PD98509), suggesting the involvement of a PI 3-kinase and/or mTOR dependent mechanism, respectively. In contrast to changes induced by prolonged exposure to insulin, the degradation of IRS1 was insensitive to proteasome inhibitors, despite an overall increase in cellular protein ubiquitination.

Both H_2O_2 and chronic exposure to insulin resulted in increased IRS1 serine phosphorylation. The mechanisms by which both conditions induce IRS1 serine phosphorylation are similar, but not identical. In both cases, an inhibitor of PI 3-kinase or of mTOR, but not of MEK1, prevented IRS1 changes suggesting that oxidative stress might activate components of the insulin signalling cascade (insulin mimicking effect) as was shown to occur with milimolar H_2O_2 [34]. Consistent with this notion is the finding, that both oxidative stress and chronic insulin had a differential effect on IRS1 compared with IRS2: while IRS1 protein content was largely decreased, IRS2 was relatively resistant to enhanced protein degradation [18, 30]. However, the magnitude of the inhibitory effect of each inhibitor differed: while in the case of prolonged insulin rapamycin had about 50% the potency in inhibiting IRS1 changes compared with LY29002, the two agents had a similar effect following oxidative stress. This could reflect a difference in the relative input of the various kinases involved. The serine phosphorylation and degradation of IRS1 following chronic insulin might be mediated directly by the serine kinase activity of the p110 subunit of PI 3-kinase [18, 19, 35]. In the case of oxidative stress, it seems that most of the input from PI 3-kinase is mediated by kinase(s) also inhibited by rapamycin such as p70 S6 kinase. Yet, neither rapamycin nor LY29002 completely prevented the reduction in IRS1 content following oxidative stress. This finding suggests that oxidative stress also affected IRS1 through input from additional kinases that are resistant to these inhibitors. Potential candidates include JNK and IKKbeta, both of which have been reported to be activated by oxidative stress, and have been implicated in phosphorylating IRS1 on Ser/Thr residues [4, 36, 37]. Different balance between kinases might result in a different set of phosphorylated Ser/Thr residues on IRS1 molecules, resulting in susceptibility to distinct degradation pathways. Indeed, our data suggests different contributions of the proteasome system in the enhanced protein degradation of IRS1 following oxidative stress compared with chronic insulin. While, as previously reported [17, 18, 19], chronic insulin-induced IRS1 degradation was inhibitable by two distinct inhibitors of the proteasome, oxidative stress–induced degradation was resistant to these agents. Several potential mechanisms could account for this difference. Assuming that under both conditions IRS1 becomes ubiquitinated, chronic insulin and oxidative stress might result in different lengths of the poly-ubiquitin chain. It seems that multiubiquitin chains are essential for proteolytic targeting, whereas mono-ubiquitination might only affect sub-cellular localization [38]. Alternatively, it is possible that oxidative stress inhibits proteasomal activity. Indeed, oxidative stress was previously shown to exert a bi-modal effect on the proteasomal degradation of cellular proteins, with enhanced proteasomal degradation at the early phase and a subsequent inhibition of the 26 subunit of the proteasome, resulting in accumulation of ubiquitinated proteins [39]. Supporting this notion is the finding that oxidative stress resulted in a

striking increase in total protein ubiquitination. Under these circumstances, other protein degradation systems that are less vulnerable to oxidative damage could predominate in degrading IRS1. These might include lysosomal proteases or Ca-dependent proteases, which were also previously suggested to participate in IRS1 degradation [40]. Thus, the protease involved in IRS1 degradation in response to oxidative stress has yet to be identified.

The role of IRS1 serine phosphorylation and degradation induced by oxidation in determining the metabolic response to acute insulin stimulation is questionable. In 3T3-L1 adipocyte, protection against oxidation-induced impairment in insulin-stimulated glucose uptake and PKB phosphorylation by lipoic acid pretreatment, had no similar protective effect on IRS1 content or serine phosphorylation [41]. Moreover, in FAO cells, rapamycin co-treatment, which prevented IRS1 changes induced by oxidative stress, could not protect the impairment in insulin-stimulated glycogen synthesis. Redundancy in IRS1 function had been documented in skeletal muscle of insulin resistant patients, in which a 50% decrease in insulin-stimulated IRS1-associated PI3-kinase activity did not manifest in impaired activation of PKB [42]. In this study, however, PKB/Akt phosphorylation in response to acute insulin stimulation was strikingly impaired, to a degree that exceeded the decrease in p85 that associated with IRS1. Previous studies have suggested that impaired sub-cellular localization of IRS1 and PI 3-kinase that were induced by oxidative stress, could contribute to impaired PKB/Akt activation by insulin, and to metabolic insulin resistance [7]. Our results suggest that at least in response to oxidative stress, alterations in the IRS1 protein itself cannot fully predict either the degree of decrease in PKB/Akt phosphorylation or the impaired metabolic response to insulin, in both 3T3-L1 adipocytes and FAO cells. These results suggest that signalling step(s) downstream of IRS1 become rate-limiting in the induction of metabolic insulin resistance by oxidative stress.

In conclusion, oxidative stress elicits serine phosphorylation of IRS1 and enhances its protein degradation, similar to prolonged insulin and other inducers of cellular insulin resistance. Yet, contrary to prolonged insulin, this process cannot be prevented by inhibitors of the proteasome degradation machinery. Defining the functional importance of these alterations in IRS1 that are induced by oxidative stress for the occurrence of impaired metabolic response to insulin remains pivotal for distinguishing an association compared with a causative relationship between the two.

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