Oxidative stress impairs insulin but not platelet-derived growth factor signalling in 3T3-L1 adipocytes

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Activation of phosphatidylinositol 3-kinase (PI 3-kinase) is a common event in both insulin and platelet-derived growth factor (PDGF) signalling, but only insulin activates this enzyme in the high-speed pellet (HSP), and induces GLUT4 translocation. Recently, we have demonstrated that exposure of 3T3-L1 adipocytes to oxidative stress impairs insulin-stimulated GLUT4 translocation and glucose transport, associated with impaired PI 3-kinase translocation and activation in the HSP [Tirosh, Potashnik, Bashan and Rudich (1999) *J. Biol. Chem.* **274**, 10595–10602]. In this study the effect of a 2 h exposure to $\approx 30 \mu\text{M}$ H_2O_2 on insulin versus PDGF-BB signalling and metabolic effects was compared. PDGF-stimulated p85-associated PI 3-kinase activity in total cell lysates, as well as co-precipitation of the PDGF receptor, were unaffected by oxidative stress. Additionally, the increase in p85 association with the plasma-membrane lawns by PDGF remained intact following oxidation, whereas the insulin effect was decreased. PDGF significantly

increased protein kinase B (PKB) activity in early differentiated cells, and that of p70 S6-kinase in both early and fully differentiated 3T3-L1 adipocytes. Following oxidation the effect of PDGF on PKB and p70 S6-kinase activation remained intact, whereas significant inhibition of insulin-stimulated activation of those enzymes was observed. In accordance, in both early and fully differentiated cells, oxidative stress completely blunted insulin- but not PDGF-stimulated protein synthesis. In conclusion, oxidative stress impairs insulin, but not PDGF, signalling and metabolic actions in both early and fully differentiated 3T3-L1 adipocytes. This emphasizes compartment-specific activation of PI 3-kinase as an oxidation-sensitive step specifically leading to insulin resistance.

Key words: diabetes, hydrogen peroxide, insulin resistance, PI 3-kinase.

INTRODUCTION

Cellular responses to oxidative stress(es) appear to be extremely diverse, and may result in functional alterations of various biological processes [1,2]. Cell survival, the cell cycle, metabolism and gene regulation have all been shown to be affected by exposure to oxidative stresses, suggesting that oxidative stress is involved in the pathophysiology of diverse conditions, including degenerative, neoplastic, inflammatory and infectious diseases [1,3]. While explaining the growing interest in oxidative-stress biology, this diversity also raises the question of whether a certain cellular response to oxidative stress is specific, and can thus be linked to a defined alteration in cellular function.

Oxidative stress has been shown recently to impair insulin action [4–9], suggesting that it may play a role in the development and/or progression of insulin resistance. The decreased metabolic response of skeletal muscle and adipose tissues to acute insulin stimulation commonly occurs in aging, diabetes and sepsis. Within the insulin signal-transduction cascade, activation of phosphatidylinositol 3-kinase (PI 3-kinase) is central for most if not all of its metabolic effects [10,11]. This process occurs largely through the interaction of this enzyme with adapter proteins of the insulin receptor such as insulin receptor substrates (IRSs) 1 and 2 [10,12], which have been phosphorylated on tyrosine residues by the insulin receptor kinase. 3'-Phosphoinositides are

consequently generated, largely at the plasma membrane [13–15], and result in the recruitment and subsequent activation of downstream effectors [16]. These include proteins like the 3'-phosphoinositide-dependent kinase (PDK1) and protein kinase B (PKB), which bind 3'-phosphoinositides through their pleckstrin homology (PH) domains [17,18]. In recent studies we demonstrated that exposure of 3T3-L1 adipocytes to oxidative stress resulted in impaired metabolic response to insulin, which was associated with a reduced insulin-stimulated activation of PKB [6,7]. Yet, whether this effect of oxidative stress is confined to the insulin signal-transduction cascade, or represents a rather non-specific impairment in cellular capacity to respond to growth-factor stimulation, has not been determined.

In the present study, cellular response to acute insulin stimulation following oxidative stress was compared with acute stimulation with platelet-derived growth factor (PDGF). Similar to insulin, this growth factor also utilizes PI 3-kinase (as well as PKB and p70 S6 kinase) in transmitting its downstream signalling cascade and in producing certain metabolic effects, such as the stimulation of protein synthesis [19–21]. Yet, differently from insulin, this activation of PI 3-kinase occurs through direct interaction of this enzyme with the PDGF receptor. Consequently, most of the PI 3-kinase activity following PDGF is recoverable in the plasma-membrane fraction, whereas following insulin a significant amount of this activity is recoverable in an

Abbreviations used: PI 3-kinase, phosphatidylinositol 3-kinase; PKB, protein kinase B; PH, pleckstrin homology; PDGF, platelet-derived growth factor; IRS, insulin receptor substrate; DMEM, Dulbecco's modified Eagle's medium; HSP, high-speed pellet.

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intracellular compartment to which the IRS proteins localize [termed LDM or high-speed pellet (HSP) by different authors] [15,21–23]. By comparing the response of 3T3-L1 cells with these two agents we could demonstrate that oxidative stress impairs signalling events and metabolic effects of insulin, but not those induced by PDGF.

EXPERIMENTAL

Materials

Tissue-culture medium, serum and antibiotic solutions were obtained from Biological Industries (Beit-Haemek, Israel). Recombinant human insulin was from Novo Nordisk (Bagsvaerd, Denmark). Recombinant human PDGF-BB was purchased from PeproTech (Rocky Hill, NJ, U.S.A.). Crosstide, p70 ribosomal S6 kinase substrate peptide, anti-sheep IgG, anti-p85, anti-p70 S6 kinase, anti-phosphotyrosine (4G10) and anti-PKB α (PH domain) antibodies were from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Anti-phospho-specific PKB (Ser-473) and anti-phospho-Thr-421/Ser-424 p70 S6 kinase antibodies were from New England BioLabs (Beverly, MA, U.S.A.). Peroxidase-conjugated anti-rabbit IgG, anti-mouse IgG and [γ - 32 P]ATP were from Amersham Life Sciences (Little Chalfont, Bucks., U.K.). Protein G-Sepharose and Protein A-Sepharose were from Pharmacia Biotech. (Uppsala, Sweden). 2-Deoxy-[3 H]glucose was purchased from Nuclear Research Center-Negev (Dimona, Israel). [35 S]Methionine and [14 C]methylaminoisobutyric acid were from NEN (Boston, MA, U.S.A.). All other chemicals were obtained from Sigma (St. Louis, MO, U.S.A.).

Cell culture

3T3-L1 pre-adipocytes (American Type Culture Collection) were grown to confluence and were induced to differentiate in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose, as described previously [7]. Cells were used either 3 days (early differentiated) or 12 days (fully differentiated) following induction of differentiation. H₂O₂ was generated by adding 100 m-units/ml glucose oxidase (type II from *Aspergillus niger*, 20 000 units/g of solid in non-oxygen-saturated conditions; Sigma) to serum-free DMEM supplemented with 0.5% RIA-grade BSA. The addition of 100 m-units/ml glucose oxidase resulted in a medium H₂O₂ concentration that achieved a steady state of $30 \pm 7 \mu\text{M}$ after 15 min.

Cell lysates and Western blots

Cells grown in six-well plates (three wells per condition) were rinsed three times with PBS and incubated in the absence or presence of 100 nM insulin or 50 ng/ml PDGF-BB for 7 min. Cells were then scraped into 0.25 ml/well of ice-cold lysis buffer [50 mM Tris/HCl, pH 7.5, 0.1% (w/v) Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 10 mM sodium β -glycerophosphate, 5 mM sodium pyrophosphate, 1 mM sodium vanadate, 0.1% (v/v) 2-mercaptoethanol, 1 mM PMSF, 1 μM aprotinin, 1 μM leupeptin and 1 μM pepstatin A]. Lysates were shaken gently for 15 min at 4 °C, centrifuged (12 000 g, 15 min at 4 °C), supernatant collected and protein content determined using the BioRad Bradford procedure [24]. Laemmli buffer was added and samples were boiled for 10 min. Proteins were resolved by SDS/PAGE (7.5% or 10% gels) and subjected to Western blotting, followed by quantification using video densitometry analysis, as described in [4].

PKB and p70 S6 kinase activities

Cell lysates were prepared using the same lysis buffer as described above. Lysates were subjected to immunoprecipitation with either the anti-PKB α or anti-p70 S6 kinase antibodies and were assayed for kinase activities using Crosstide (GRPRTSSFAEG) or p70 ribosomal S6 kinase substrate peptide (KKRNRTLTK) as substrates, respectively, according to the manufacturer's instructions [25,26].

Protein-synthesis activity

[35 S]Methionine incorporation into proteins was assayed in six-well plates as described previously [27]. Briefly, following treatment without or with glucose oxidase (described above), cells were washed three times with PBS and were incubated for 1 h with methionine- and cysteine-free DMEM (Sigma, Rehovot, Israel) followed by an additional 1 h incubation in the same medium containing [35 S]methionine (16 $\mu\text{Ci/ml}$), either alone or in the presence of insulin or PDGF. Cells were then washed three times with ice-cold PBS containing 10 mM methionine and lysed in a lysis buffer containing 30 mM Tris/HCl (pH 7.5), 140 mM NaCl and 0.5% Nonidet P-40. Proteins were precipitated by the addition of ice-cold trichloroacetic acid [final concentration, 10% (w/v)] containing 10 mM methionine. The protein precipitates were then washed three times with ice-cold PBS containing 10% trichloroacetic acid and 10 mM methionine and then solubilized in 1 M NaOH at 37 °C for 30 min. Protein-associated radioactivity was determined using a β -counter.

Hexose-transport determinations

2-Deoxyglucose-uptake measurements were performed after 20 min of treatment with or without insulin or PDGF, as described previously [4]. Assays were performed for 10 min using 50 μM 2-deoxy[3 H]glucose (1 $\mu\text{Ci/ml}$). Non-specific uptake (less than 10% of the total) was determined in the presence of cytochalasin B (50 μM), and was subtracted from the total uptake.

Immunoprecipitation and PI 3-kinase assay

Cells were lysed as described above. For immunoprecipitation, 0.5 mg of cell-lysate protein was used, and assayed as described in [5]. PI 3-kinase activity was performed following the protocol described by Hadari et al. [28], using phosphatidylinositol and [γ - 32 P]ATP as substrates. The phosphorylated phosphatidylinositols were separated using TLC, and quantified by video densitometry.

Plasma-membrane lawn preparation

Plasma-membrane sheets were prepared as described by Chen et al. [29]. Differentiated 3T3-L1 adipocytes were washed with PBS and incubated for 1 min with 0.5 mg/ml poly-D-lysine, followed by three washes with hypotonic buffer (23 mM KCl, 10 mM Hepes, pH 7.5, 1.7 mM MgCl₂ and 1 mM EGTA). The cells were then covered with sonication buffer (3 $\times$ hypotonic buffer containing 1 mM dithiothreitol and 0.1 mM PMSF) and sonicated with a probe membrane disrupter. Following sonication, the plasma-membrane sheets were washed three times with sonication buffer, scraped, and were used for immunoblotting as described above.

Statistical analysis

Data are expressed as means $\pm$ S.E.M. Each treatment was compared with the control, and statistical significance between two groups was evaluated using the Student's *t* test. The criterion for significance was set at $P < 0.05$.

RESULTS

Oxidative stress does not impair the activation of PI 3-kinase by either insulin or PDGF, as assessed in total cell lysates

In order to study the effect of oxidative stress on the ability of PDGF or insulin to activate PI 3-kinase, 3T3-L1 adipocytes were exposed for 2 h to $27.4 \pm 0.8 \mu\text{M}$ H_2O_2 , generated continuously by addition of 100 m-units/ml glucose oxidase to the culture medium, as we described previously [4]. Under these conditions, the stimulation of PI 3-kinase activity by PDGF or by insulin were assessed in total cell lysates by immunoprecipitation of the p85 subunit of PI 3-kinase, followed by PI 3-kinase assay. As seen in Figure 1(A), PDGF stimulation of p85-associated PI 3-kinase activity was not decreased following oxidative stress. Similarly, under the same conditions, insulin-stimulated PI 3-

Figure 1 Effect of oxidative stress on insulin- or PDGF-induced PI 3-kinase activation and interactions

Fully differentiated 3T3-L1 adipocytes were exposed to oxidative stress (H_2O_2), rinsed and stimulated with either insulin (100 nM) or PDGF (50 ng/ml) for 7 min, after which cell lysates were prepared. Immunoprecipitation was then performed using anti-p85 antibody, as described in the Experimental section. (A) PI 3-kinase assay (using phosphatidylinositol and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as substrates) was performed on p85 immunoprecipitates (IP). Shown are autoradiographs of phosphoinositides separated by TLC representing three independent experiments. (B) Immunoprecipitates were subjected to Western-blot analysis followed by immunoblotting (IB) with anti-phosphotyrosine (anti PY) antibody. Shown is a representative blot representing two additional independent experiments, which yielded similar results.

kinase activity associated with p85 was unaffected by oxidative stress, when assessed in total cell lysates (Figure 1A), consistent with our previous publications [5,7]. To assess further the effect of oxidative stress on the capacity of either insulin or PDGF to stimulate the interaction between PI 3-kinase and tyrosine-phosphorylated proteins, p85 immunoprecipitates were subjected to Western-blot analysis using anti-phosphotyrosine antibodies. As expected, insulin stimulation of control cells resulted in increased co-precipitation of tyrosine-phosphorylated proteins of approx. 180 kDa, corresponding to the IRS1 and IRS2 proteins (Figure 1B). An increased co-precipitation of an additional band of 95 kDa could also be observed, corresponding to the insulin receptor. This degree of direct interaction between PI 3-kinase and the insulin receptor has been reported previously [30]. In contrast, PDGF stimulation resulted in increased association between p85 and a 205 kDa band, which corresponds to the PDGF receptor. Following oxidative stress, these interactions between PI 3-kinase and tyrosine-phosphorylated proteins stimulated by either insulin or PDGF remained intact, and even tended to be somewhat exaggerated (Figure 1B).

Oxidative stress impairs the activation of PKB and p70 S6 kinase by insulin but not by PDGF

PKB and p70 S6 kinase are two enzymes activated downstream of PI 3-kinase, yet their degrees of activation by either PDGF or insulin vary during the course of 3T3-L1 adipocyte differentiation [19]. Thus the effect of oxidative stress on insulin or PDGF stimulation of PKB or p70 S6 kinase activity has been studied in both early differentiated (3 days after induction of differentiation) as well as in fully differentiated 3T3-L1 adipocytes. Consistent with previous reports [19], in early differentiated cells insulin induced a significant increase in PKB Ser-473 phosphorylation (Figure 2A), associated with a 4-fold increase in kinase activity of anti-PKB α PH-domain immunoprecipitates (Figure 2B). Both processes revealed higher fold activation in fully differentiated cells (with PKB activation reaching 7-fold over basal levels; Figures 2C and 2D). In contrast, PDGF-stimulated PKB phosphorylation and activity could be observed largely in early differentiated cells, exceeding the effect of insulin (Figures 2A and 2B), and was nearly absent following full differentiation (Figures 2C and 2D). In both early and fully differentiated cells, insulin-stimulated PKB phosphorylation and activation were dramatically inhibited by exposure to H_2O_2 . In contrast, the stimulation of PKB following PDGF treatment was not impaired by oxidative stress, and was even enhanced. This vulnerability to oxidative stress of the insulin, but not of the PDGF, signalling cascade could be observed similarly in the activation pattern of p70 S6 kinase. Fully differentiated cells exhibited a lower basal and insulin- or PDGF-stimulated p70 S6 kinase activity, as compared with early differentiated cells (Figures 3A and 3B). Yet, at both differentiation levels oxidative stress completely abolished the insulin effect, while sparing the PDGF-mediated activation. In fully differentiated cells, the same pattern was observed in the Thr-421/Ser-424 phosphorylation of p70 S6 kinase (Figure 3C), with no effect on total p70 S6 kinase content (results not shown).

Oxidative stress impairs insulin- but not PDGF-stimulated metabolism

To assess whether these findings correlate with the effects of oxidative stress on downstream metabolic actions of insulin and PDGF, protein synthesis and glucose-uptake activities were evaluated. In early differentiated cells both insulin and PDGF

Figure 2 Oxidative stress impairs insulin, but not PDGF, stimulation of PKB phosphorylation and activation, in early and fully differentiated 3T3-L1 adipocytes

3T3-L1 adipocytes 3 days after induction of differentiation (early differentiated) or after full differentiation were exposed to oxidative stress for 2 h (H₂O₂) as described in the Experimental section. Cells were then rinsed and incubated for 7 min in the absence or presence of 100 nM insulin or 50 ng/ml PDGF, after which cell lysates were prepared. Western-blot analysis was performed, and Ser-473 phosphorylation of PKB was detected using specific antibodies in early (**A**) and fully differentiated cells (**C**). Shown are representative blots of three and five independent experiments for early and fully differentiated cells, respectively, yielding similar results. (**B**) and (**D**) Immunoprecipitation using anti-PKB α PH-domain antibody was performed on 400 μ g of cell-lysate protein, after which PKB kinase assay was performed as described in the Experimental section. (**B**) Early differentiated cells; (**D**) fully differentiated cells. Values are means $\pm$ S.E.M. from three independent experiments. * P < 0.05 as compared with non-stimulated control cells. § P < 0.01 as compared with insulin-stimulated control cells.

stimulated protein synthesis by 2.76 ± 0.12 -fold and 3.17 ± 0.26 -fold respectively (Figure 4A). Wortmannin (100 nM) and rapamycin (100 ng/ml) totally inhibited protein-synthesis activation by the two hormones, suggesting its dependency on PI 3-kinase and mTOR (mammalian target of rapamycin) activation, respectively. Insulin and PDGF stimulation of protein synthesis (Figures 4A and 4B) decreased during the course of differentiation, whereas insulin-stimulated glucose uptake (Figures 4C and 4D) increased dramatically. In agreement with the effects of oxidative stress described in Figures 2 and 3, exposure to H₂O₂ resulted in a complete reduction of insulin-stimulated protein synthesis in both early and fully differentiated cells (Figures 4A and 4B). Insulin-stimulated [¹⁴C]methylaminoisobutyric acid was not affected by oxidation (8.3 ± 0.35 and 8.4 ± 0.29 nmol/mg of protein per min in early differentiated non-oxidized and oxidized cells, respectively, and 2.2 ± 0.26 and 2.3 ± 0.21 nmol/mg of protein per min in fully differentiated non-oxidized and oxidized adipocytes, respectively), suggesting that system A amino acid transport does not become a rate-limiting step in protein synthesis following oxidative stress. Consistent with previous observations [7], oxidative stress, while significantly increasing basal glucose uptake, markedly inhibited insulin-stimulated glucose transport in differentiated 3T3-L1

adipocytes (Figure 4D) as well as in early differentiated cells (Figure 4C). In contrast with the induction of insulin resistance by oxidative stress, PDGF stimulation of protein synthesis in both early and fully differentiated cells was not inhibited, and even tended to be exaggerated in the oxidized cells as compared with the control.

Oxidative stress interferes with cellular redistribution of PI 3-kinase, which is induced by insulin but not by PDGF

The stimulation of PI 3-kinase activity by both insulin and PDGF involves cellular redistribution of this enzyme as compared with the basal, non-stimulated state. Following both stimuli, PI 3-kinase translocates from its cytosolic pool to the plasma membrane, whereas only following insulin can PI 3-kinase also be localized in intracellular pools. To assess whether oxidative stress differently affected insulin- versus PDGF-stimulated PI 3-kinase redistribution, plasma-membrane lawns were prepared and immunoblotted with anti-p85 antibodies. Figure 5 demonstrates that in control cells both insulin and PDGF stimulation resulted in an increased abundance of p85, which was associated with plasma-membrane lawn fractions. Oxidative stress, while resulting in a certain localization of p85

Figure 3 Oxidative stress impairs insulin but not PDGF stimulation of p70 S6 kinase activation and phosphorylation

Cells were treated as described in the legend for Figure 1, after which immunoprecipitation of p70 S6 kinase was performed from cell lysates (400 μ g of protein) of early (**A**) and fully (**B**) differentiated 3T3-L1 adipocytes. p70 S6 kinase assay was then performed on immunoprecipitates as described in the Experimental section, and results are presented as means $\pm$ S.E.M. from three independent experiments. * $P < 0.05$ as compared with unstimulated control cells. § $P < 0.01$ as compared with insulin-stimulated control cells. (**C**) Following Western-blot analysis of cell lysates of fully differentiated 3T3-L1 adipocytes, Thr-421/Ser-424 phosphorylation of p70 S6 kinase was detected using phospho-specific antibodies. Shown is a blot representing two additional experiments yielding similar results.

with plasma-membrane lawns, resulted in decreased insulin but exaggerated PDGF stimulation of p85 translocation.

Collectively, these data demonstrate that oxidative stress impairs insulin-signalling events and metabolic actions downstream of PI 3-kinase activation, while sparing the same processes induced following PDGF stimulation. This may be due to interference with insulin but not with PDGF stimulation of PI 3-kinase cellular redistribution.

DISCUSSION

In the present study we demonstrate that exposure to oxidative stress specifically impairs insulin-stimulated cell responses, by showing that PDGF signalling and metabolic effects remained intact. This observation occurs despite the fact that both insulin and PDGF utilize common signalling components, including PI 3-kinase, PKB and p70 S6 kinase, and result, albeit to different extents, in some common metabolic effects (protein synthesis and system A amino acid transport). Furthermore, despite abnormal capacity of insulin to induce cellular redistribution of PI 3-kinase following oxidative stress, PDGF displays the preserved potential to induce translocation of PI 3-kinase to the plasma membrane.

The observations obtained with 3T3-L1 adipocytes are not cell-type-specific, because studies utilizing L6 myotubes, a cell-culture model for skeletal muscle, yielded essentially similar results (A. Tirosh, A. Rudich, R. Potashnik and N. Bashan, unpublished work). These include impairment of insulin, but not

PDGF, stimulation of protein synthesis, PKB and p70 S6 kinase. The capacity of oxidative stress to specifically induce insulin resistance in cells representing targets for the metabolic actions of insulin may suggest a causative role for oxidation in the development or progression of insulin resistance in type 2 diabetes. This notion is consistent with several lines of evidence, including the documentation of reduced vitamin E levels as a risk factor for the development of type 2 diabetes [31], and improvement in insulin action following antioxidant supplementation [32].

The specificity of intracellular signals in response to different growth factors and environmental stresses remains largely an unsettled topic. Many signalling components are common to a variety of agents, which induce different cellular responses. In the case of insulin and PDGF, while both depend on activation of PI 3-kinase for inducing many of their effects, insulin is capable of stimulating glucose transport and metabolism in adipocytes, whereas PDGF has no similar effect [33]. On the other hand, both growth factors stimulate system A amino acid transport and protein synthesis in a PI 3-kinase-dependent manner [19,34]. These observations raise the question of which component of the insulin signal is responsible for its specific metabolic functions. The possibility that insulin and PDGF [35] or other growth factors [36,37] utilize different PI 3-kinase isoforms, or result in different levels of 3'-phosphoinositide production [13,15], has been raised to explain the specificity of their actions. Yet, the fact that insulin and PDGF result in the recovery of PI 3-kinase in

Figure 4 Effect of oxidative stress on the stimulation of protein synthesis, and glucose transport activity by insulin or PDGF, in early and fully differentiated 3T3-L1 adipocytes

3T3-L1 adipocytes were exposed to oxidative stress (H_2O_2), rinsed and stimulated by either insulin (100 nM) or PDGF (50 ng/ml). (A) and (B) In early and fully differentiated cells, respectively, protein synthesis was measured by the incorporation of [^{35}S]methionine for 1 h in the absence or presence of insulin or PDGF, as described in the Experimental section. (C) and (D) Glucose transport activity was measured in early and fully differentiated cells, respectively, using 2-deoxy[3H]glucose, after treating the cells for 20 min with or without insulin or PDGF. Values are means $\pm$ S.E.M. from at least four independent experiments performed in duplicate. * $P < 0.05$ as compared with unstimulated control cells. § $P < 0.01$ as compared with insulin-stimulated control cells. # $P < 0.05$ as compared with non-stimulated H_2O_2 -treated cells.

Figure 5 Effect of oxidative stress on PI 3-kinase content in plasma-membrane lawns of 3T3-L1 adipocytes following insulin or PDGF stimulation

Fully differentiated 3T3-L1 adipocytes were treated as described in the legend for Figure 1, after which plasma-membrane lawns were prepared, scraped and subjected to Western-blot analysis using anti-p85 antibodies as described in the Experimental section. Shown is a blot representing two additional independent experiments.

distinct cellular compartments has been described by various groups [21–23]. In particular, the unique capacity of insulin to activate PI 3-kinase in an intracellular compartment was commonly suggested as an explanation for its signal specificity, despite the ongoing debate about the exact nature of this compartment, and how exactly it relates to the actual location of 3'-phosphoinositide's production. Regardless of this, the data of this study largely supports the cellular redistribution of PI 3-kinase as an explanation for signal specificity, as described below.

Following oxidative stress, both insulin and PDGF normally stimulate PI 3-kinase, as assessed in total cell lysates (Figure 1A and [5,7]). However, the impairment in downstream signalling and metabolic actions of insulin was associated with defective PI 3-kinase cellular redistribution. This was evident by both

impaired insulin-stimulated targeting and activation of PI 3-kinase in the HSP [7] as well as impaired insulin-stimulated association of PI 3-kinase with plasma-membrane lawn preparations (Figure 5). Conversely, the preserved signalling and metabolic responses to PDGF stimulation following oxidative stress were associated with the preserved capacity of this growth factor to induce PI 3-kinase interaction with the plasma membrane. These observations suggest that normal cellular redistribution of PI 3-kinase is a required event in the signal-transduction cascade of both growth factors. Furthermore, while this process when induced by insulin appears to be oxidation-sensitive, PI 3-kinase cellular redistribution following PDGF is resistant to the effects of oxidative stress.

The exact identity of the target for oxidative stress within the insulin-signalling cascade remains unknown. The data presented in this study and a previous report [7] do not support an oxidation target directly on any of the signalling components studied. This assumption is based on the fact that in total cell lysate, PI 3-kinase shows normal capacity to interact with tyrosine-phosphorylated proteins such as IRSs and the PDGF receptor (Figure 1). The downstream effectors PKB and p70 S6 kinase, whose insulin-stimulated activation is impaired by oxidative stress, are activated normally by PDGF stimulation (Figure 2) or by heat shock [7]. Thus it may be speculated that a cellular 'translocation device' that is specific for the insulin signal is a target for oxidative stress. Interestingly, disruption of the actin-filament network in 3T3-L1 adipocytes and in L6 myotubes has been shown to impair insulin response in a very similar way to the impairment observed by oxidative stress, i.e. normal PI 3-kinase activation in total cell lysate but defective activation and localization in the HSP, which was associated

with impaired PKB and glucose-transport stimulation [38,39]. Whether elements of the cytoskeleton or a cytoskeleton-interacting protein(s) are the direct target for oxidative stress remains an intriguing question.

In conclusion, oxidative stress impairs insulin but not PDGF signalling, in association with disruption of the normal cellular redistribution of PI 3-kinase. Thus oxidative-stress-induced insulin resistance represents a specific cellular response of insulin-sensitive cells.

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