Okadaic acid inhibits insulin-induced glucose transport in fetal brown adipocytes in an Akt-independent and protein kinase C ζ-dependent manner

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Abstract In the present study we have investigated the effect of increased serine/threonine phosphorylation of insulin receptor substrates-1 and -2 (IRS-1 and IRS-2) by okadaic acid pretreatment on brown adipocyte insulin signalling leading to glucose transport, an important metabolic effect of insulin in brown adipose tissue. Okadaic acid pretreatment before insulin stimulation decreased IRS-1 and IRS-2 tyrosine phosphorylation in parallel to a decrease in their sodium dodecyl sulfate–polyacrylamide gel electrophoresis mobility. IRS-1/IRS-2-associated p85α and phosphatidylinositol (PI) 3-kinase enzymatic activity were partly reduced in brown adipocytes pretreated with okadaic acid upon stimulation with insulin. Furthermore, insulin-induced glucose uptake was totally abolished by the inhibitor in parallel with a total inhibition of insulin-induced protein kinase C (PKC) ζ activity. However, activation of Akt/PKB or p70 S6 kinase ($p70^{s6k}$) by insulin remained unaltered. Our results suggest that downstream of PI 3-kinase, insulin signalling diverges into at least two independent pathways through Akt/PKB and PKC ζ, the PKC ζ pathway contributing to glucose transport induced by insulin in fetal brown adipocytes.

Key words: Insulin signaling; Okadaic acid; Serine/threonine phosphorylation; Insulin receptor substrate; Glucose transport; Protein kinase C ζ; Insulin resistance

1. Introduction

Brown adipose tissue is the main tissue involved in non-shivering thermogenesis in mammalian newborns and is responsible for heat production associated with the expression of the mitochondrial uncoupling protein-1 (UCP1) [1]. Differentiation of brown adipose tissue also encompasses an adipogenic program related to lipid synthesis and its accumulation results in a multilocular fat droplets phenotype [2], with glucose being the main lipogenic substrate [3,4]. Insulin promotes glucose uptake into muscle and adipose tissues through translocation of the Glut4 glucose transporter from an intracellular pool to the plasma membrane [5]. An impairment of the ability of insulin to stimulate glucose uptake in these tissues contributes to the development of type 2 diabetes, hypertension and cardiovascular disease [6]. Over the past two decades, considerable progress has been made defining the upstream signaling mechanisms regulating insulin-induced glucose uptake into the cells. It is well known that after insulin stimulation, insulin receptor substrate-1 and -2 (IRS-1 and IRS-2) are phosphorylated on several tyrosine residues, most of them being located in YXXM or YMXM motifs [7,8]. This tyrosine phosphorylation step is crucial, since it allows the interaction of IRS proteins with SH2 domain-containing proteins leading to the activation of multiple signalling pathways. Among these, the activation of phosphatidylinositol 3-kinase (PI 3-kinase) has been shown to be essential for insulin-induced Glut4 translocation and glucose uptake [9–13]. Hence, abnormalities in these phosphorylations are likely to lead to alterations in insulin action.

In addition to tyrosine, phosphorylation increased serine and, to a lesser extent, threonine phosphorylation of either the insulin receptor β-subunit or IRS-1 have been associated with reduced insulin signalling, resulting in an insulin-resistant state [14–18]. These results prompted us to investigate in our cell model of brown adipocytes whether the elevation of serine/threonine phosphorylation of IRS-1 and IRS-2 by okadaic acid, a serine/threonine phosphatase inhibitor, could interfere with the signalling pathways emerging downstream of these molecules. Furthermore, we analyzed the effect of this inhibitor in insulin-induced glucose uptake, one of the main metabolic effects of this hormone in brown adipose tissue.

2. Materials and methods

2.1. Materials

Fetal calf serum (FCS) and culture media were from Imperial Laboratories (Hampshire, UK). Insulin, okadaic acid and anti-mouse IgG-agarose were from Sigma (St. Louis, MO, USA). Protein A-agarose was from Roche Molecular Biochemicals. For IRS-1 and IRS-2 immunoprecipitations, polyclonal antibodies were the generous gift of Dr. M.F. White (Joslin Diabetes Center, MA, USA). The anti-insulin receptor β-subunit monoclonal antibody and the pY20 anti-Tyr(P) antibody were purchased from Santa Cruz Biotechnology (Palo Alto, CA, USA). The antibodies against Akt1 and Akt2 were purchased from Upstate Biotechnology (Lake Placid, NY, USA). The anti-phospho $serine^{493}$ Akt, total Akt and anti-phospho $serine^{424}$/$threonine^{421}$ $p70^{s6k}$ antibodies were purchased from New England Biolabs (Beverly, MA, USA). The anti-protein kinase C (PKC) ζ antibody was purchased from Gibco BRL (Life Technologies). [γ-^{32}P]ATP (3000 Ci/mmol) and 2-deoxy-D[1-^{3}H]glucose (11.0 Ci/mmol) were from Amersham (Aylesbury, UK). All other reagents used were of the purest grade available.

2.2. Cell culture

Brown adipocytes were obtained from interscapular brown adipose tissue of 20-day-old fetuses of Wistar rats and isolated by collagenase dispersion as described [19]. Cells were plated at 5×10^6 cells/100 mm

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Abbreviations: IRS, insulin receptor substrate; PI, phosphatidylinositol; FCS, fetal calf serum; PBS, phosphate buffered saline

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or 1–1.2×10^6 cells/60 mm tissue culture plates in MEM supplemented with 10% FCS to allow cell attachment to the plastic surface of the plates. After 4–6 h of culture at 37°C, cells were rinsed twice with phosphate buffered saline (PBS) and 80% of the initial cells were attached. Cells were maintained for 20 h in a serum-free medium supplemented with 0.2% (w/v) bovine serum albumin (BSA) to assure inhibition of the intrinsic mitogenic competence of fetal cells and quiescence. At this time, cells were treated for 5 min with 10 nM insulin, or preincubated for 30 min with 1 μM okadaic acid followed by treatment for 5 min with 10 nM insulin, or in the absence of growth factors as a control for cellular quiescence.

2.3. Immunoprecipitations

Quiescent fetal brown adipocytes (after 20 h of serum deprivation) were treated with insulin or okadaic acid plus insulin in MEM as indicated and lysed at 4°C in buffer containing 10 mM Tris/HCl, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 100 μM Na_3VO_4, 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride, pH 7.6 (lysis buffer). After protein content determination, equal amounts of protein were immunoprecipitated at 4°C with the corresponding antibodies and the immune complexes were collected on protein A-agarose or antimouse Ig-agarose beads. Immunoprecipitates were washed three times with lysis buffer analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by Western blotting as previously described [20].

2.4. In vitro autophosphorylation assay

The insulin receptor autophosphorylation was measured in vitro as described [21]. The anti-Tyr(P) immune complexes were incubated in kinase buffer containing 20 mM HEPES, 3 mM $MnCl_2$, 10 mM $MgCl_2$, and 20 μCi of [γ-^{32}P]ATP (in a final concentration of 5 mM) for 15 min at room temperature. The complexes were washed twice with cold PBS and then resuspended in 2× SDS–PAGE sample buffer and analyzed by SDS–PAGE. The separated proteins were dried in the gel and the incorporation of [^{32}P]phosphate into proteins was visualized by autoradiography and quantitated by scanning laser densitometry (Molecular Dynamics).

2.5. PI 3-kinase activity

PI 3-kinase activity was measured in the anti-IRS-1 and anti-IRS-2 immunoprecipitates by in vitro phosphorylation of PI as described [22].

2.6. PKC ζ activity

Fetal brown adipocytes either untreated or stimulated were extracted with lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1 mM EGTA, 1 μM PMSF, 25 μg/ml leupeptin and 25 μg/ml aprotinin) and immunoprecipitated with an anti-PKC ζ antiserum. Immune complexes were washed five times with ice-cold lysis buffer with 0.5 M NaCl and two times with kinase buffer (35 mM Tris, pH 7.5, 10 mM Mg Cl_2, 0.5 mM EGTA, 1 μM Na_3VO_4). The kinase reaction was performed in buffer containing 1 μCi of [γ-^{32}P]ATP, 60 μM ATP and 1 μg of MBP as a substrate for 30 min at 30°C and was terminated by the addition of 4× SDS–PAGE sample buffer followed by boiling for 5 min at 95°C. Samples were resolved in 12% SDS–PAGE; and gels were dried out and subjected to autoradiography.

2.7. Measurement of the glucose transport

Glucose transport was measured in triplicate dishes from three independent experiments as previously described [20].

2.8. Protein determination

Protein determination was performed by the Bradford dye method [23], using the Bio-Rad reagent and BSA as the standard.

3. Results

3.1. Effect of okadaic acid on insulin-induced IRS-1, IRS-2 and insulin receptor tyrosine phosphorylation in fetal brown adipocytes

Insulin stimulates tyrosine phosphorylation of IRS-1/IRS-2 in primary fetal brown adipocytes at physiological doses [24]. Our first aim in this paper was to investigate whether serine/threonine phosphorylations modulate the levels of tyrosine phosphorylation of these IRS in fetal brown adipocyte primary cultures. Quiescent cells (20 h serum-deprived) were stimulated for 5 min with 10 nM insulin or preincubated for 40 min with 1 μM okadaic acid, a serine/threonine phosphatase inhibitor, and subsequently stimulated with 10 nM insulin for a further 5 min. Then, cell lysates were immunoprecipitated with the corresponding antibodies against IRS-1 and IRS-2 and the immune complexes were analyzed by Western blot with the anti-Tyr(P) antibody. As shown in Fig. 1, insulin-induced tyrosine phosphorylation of IRS-1 was signifi-

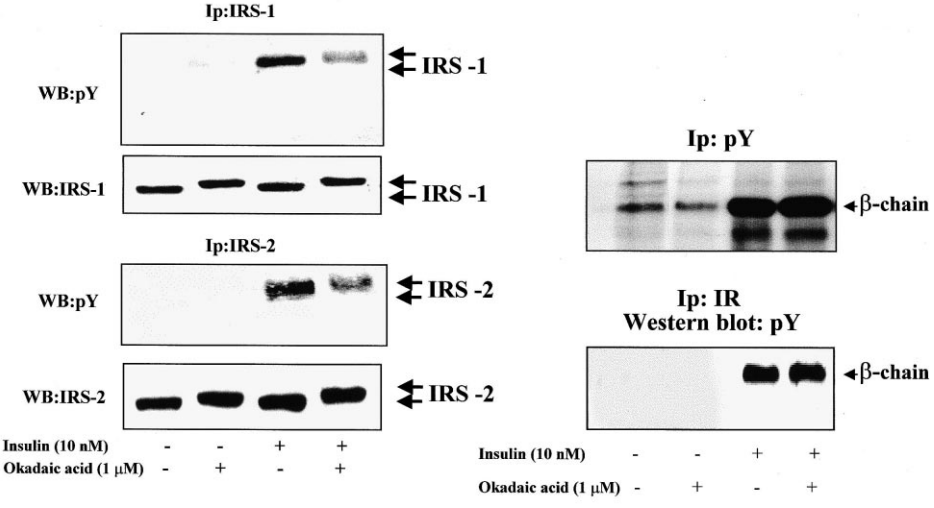

Fig. 1. Effect of okadaic acid on insulin-induced tyrosine phosphorylation of IRS-1/2 and insulin receptor β-chain in fetal brown adipocytes. Left panel: 20 h serum-starved fetal brown adipocytes were incubated for 5 min with 10 nM insulin or preincubated for 40 min with 1 μM okadaic acid followed by treatment with 10 nM insulin for a further 5 min. Cells were then lysed and immunoprecipitates, prepared with anti-IRS-1 and anti IRS-2 antibodies, were submitted to SDS–PAGE and analyzed by Western blotting with the anti-Tyr(P) antibody or with the anti-IRS-1 and anti-IRS-2 antibodies. Right panel: cells were cultured as described above. The anti-Tyr(P) immunoprecipitates were assayed for in vitro autophosphorylation or the anti-IR β-chain immunoprecipitates were analyzed by Western blotting with the anti-Tyr(P) antibody. All the results shown are representative of at least three independent experiments.

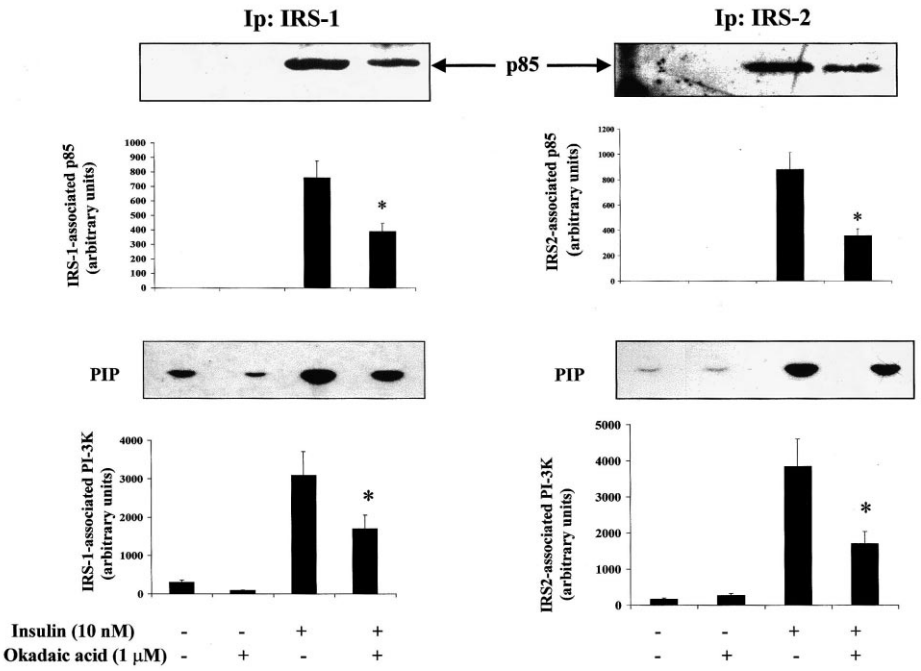

Fig. 2. Effect of okadaic acid on the association of IRS-1 and IRS-2 with the p85α subunit of PI 3-kinase and PI 3-kinase activity in fetal brown adipocytes. Upper panel: 20 h serum-starved fetal brown adipocytes were stimulated as described in Fig. 1. Cells were then lysed and the anti-IRS-1 or anti-IRS-2 immunoprecipitates were analyzed by SDS–PAGE followed by Western blotting with the anti-p85α antibody. Representative experiments are shown. Results are the mean ± S.E.M. ($n = 3$) from three independent experiments and are expressed as arbitrary units of IRS-1/IRS-2-associated p85α. Statistical analysis by Student's paired t-test between values in the presence of insulin plus okadaic acid versus insulin is represented by an asterisk; $^*P < 0.01$. Lower panel: quiescent fetal brown adipocytes were cultured as described above. The immunoprecipitates (prepared with the anti-IRS-1 or anti-IRS-2 antibodies) were washed and immediately used for an in vitro PI 3-kinase assay. Results are expressed as arbitrary units of PI 3-kinase activity and are the mean ± S.E.M. ($n = 3$) from three independent experiments. Statistical analysis by Student's paired t-test between values in the presence of insulin plus okadaic acid versus insulin is represented by an asterisk; $^*P < 0.01$.

cantly reduced in cells pretreated with 1 μM okadaic acid as compared with non-pretreated cells. Interestingly, a slight shift in the electrophoretic mobility of IRS-1, which indicates a serine/threonine phosphorylation [15], was observed in insulin-stimulated cells that had been pretreated with the inhibitor. Insulin-induced tyrosine phosphorylation of IRS-2 was also markedly reduced by okadaic acid pretreatment, the electrophoretic mobility shift being slightly higher than that of IRS-1. Furthermore, the reblotting of the membranes with the corresponding anti-IRS-1 and anti-IRS-2 antibodies revealed equal amounts of immunoprecipitated proteins under all the experimental conditions.

To evaluate the effect of okadaic acid pretreatment on tyrosine autophosphorylation of the insulin receptor β-subunit, serum-deprived brown adipocytes were stimulated as described above. Then, lysates were subjected to immunoprecipitation with the anti-Tyr(P) antibody and an in vitro assay of insulin receptor autophosphorylation was performed. As shown in Fig. 1, the presence of 10 nM insulin caused a marked increase in the tyrosine autophosphorylation of the 95 kDa band, which corresponds with the M_r of the β-subunit of the insulin receptor, little phosphorylation being observed in control cells either in the absence or presence of okadaic acid. The level of tyrosine phosphorylation of the 95 kDa band did not change significantly when brown adipocytes were pretreated with 1 μM okadaic acid before insulin stimulation. The same results were obtained by direct immunoprecipitation with the anti-insulin receptor antibody followed by Western blotting with the anti-Tyr(P) antibody.

3.2. *Effect of okadaic acid on insulin-induced IRS-1/IRS-2-associated p85α subunit and PI 3-kinase activity in fetal brown adipocytes*

Our next aim was to analyze the effect of okadaic acid in the insulin signalling downstream IRSs. In the case of the brown adipocyte insulin/IGF-I receptor, PI 3-kinase is stimulated by the interaction of the p85α regulatory subunit with tyrosine-phosphorylated IRS-1 and IRS-2 [24]. To study whether this interaction is affected by okadaic acid, we prepared soluble cell lysates after the incubation of cells in the absence or presence of 1 μM okadaic acid before insulin (10 nM) stimulation, as described above. They were immunoprecipitated with the anti-IRS-1 and anti-IRS-2 antibodies and analyzed by Western blotting with the anti-p85α antibody. As shown in Fig. 2, control cells did not show any p85α bound to either IRS-1 or IRS-2, regardless of the pretreatment with okadaic acid. After an addition of 10 nM insulin for 5 min there was a marked increase in the amount of p85α bound to both IRS proteins. However, when cells were pretreated with 1 μM okadaic acid before the stimulation with insulin, there was a significant reduction in the amount of p85α bound to IRS-1 and IRS-2.

Next, we wanted to demonstrate if the reduction of the amount of IRS-1/IRS-2-associated p85α subunit of PI 3-kinase in the presence of okadaic acid had an effect on the activation of its enzymatic activity. As shown in Fig. 2 (bottom), there was a significant inhibition of insulin-stimulated IRS-1 and IRS-2-associated PI 3-kinase activity in cells that

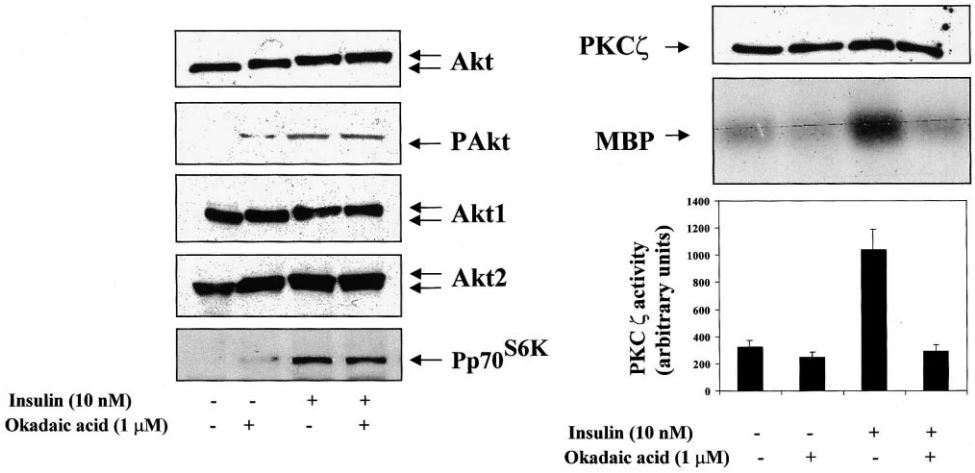

Fig. 3. Effect of okadaic acid on insulin-induced Akt, $p70^{s6K}$ and PKC ζ activation in fetal brown adipocytes. Left panel: quiescent brown adipocytes were stimulated as described in Fig. 1. Cell lysates were analyzed by SDS–PAGE followed by Western blotting with the corresponding antibodies against total Akt, Akt1, Akt2, phospho-Akt and phospho-$p70^{s6k}$ antibodies. Representative experiments are shown. Right panel: cells were cultured as described above. Cell lysates were either analyzed by Western blotting with the anti-PKC ζ antibody or immunoprecipitated with the anti-PKC ζ antibody and immediately assayed for PKC ζ activity. The corresponding autoradiograms were quantitated by scanning densitometry. Results are expressed as arbitrary units of PKC ζ activity and are the mean ± S.E.M. ($n = 3$) from three independent experiments. Statistical analysis by Student's paired *t*-test between values in the presence of insulin plus okadaic acid versus insulin is represented by an asterisk; $*P < 0.01$.

have been pretreated with okadaic acid before insulin stimulation.

3.3. Effect of okadaic acid on insulin-induced glucose transport in brown adipocytes

PI 3-kinase has been implicated as one of the key signal transducers in insulin/IGF-I-induced glucose uptake in brown adipose tissue [25,26]. To determine the effect of okadaic acid on insulin-induced glucose transport, brown adipocytes were pretreated with various doses (0.25–1 μM) of okadaic acid, stimulated for 10 min with 10 nM insulin and then incubated for a further 5 min in the presence of 2-deoxy-D[1-^{3}H]glucose. As shown in Table 1, okadaic acid inhibited insulin-induced glucose transport in a dose-dependent manner. Interestingly, total inhibition of insulin-induced glucose transport was elicited at 1 μM okadaic acid concentration, which was the dose that partly inhibited IRS-associated PI 3-kinase activity. Furthermore, glucose transport of the control cells was reduced in the presence of the serine phosphatase inhibitor.

Table 1
Dose–response of okadaic acid on glucose transport in brown adipocytes during insulin stimulation

Treatment	None	Insulin (10 nM)
None	2.20 ± 0.23	6.02 ± 0.74[a]
Okadaic acid (0.25 μM)	2.08 ± 0.82	4.51 ± 0.97
Okadaic acid (0.5 μM)	1.75 ± 0.36	2.65 ± 0.56[b]
Okadaic acid (1 μM)	1.33 ± 0.18	1.94 ± 0.12[b]

Brown adipocytes were stimulated as described in Fig. 1. Control cells were incubated in serum-free medium. Deoxyglucose transport was measured as described in Section 2. Results are the mean ± S.E.M. ($n = 3$) from three independent experiments and are expressed as disintegrations/min/μg of protein. Statistical analysis by Student's paired *t*-test between values in the presence of insulin versus control is represented by [a] and the differences between values in the presence of insulin plus okadaic acid versus insulin is represented by [b]. [a,b]$P < 0.01$.

3.4. Okadaic acid inhibits insulin-induced PKC ζ activation, but not Akt or $p70^{s6k}$ activation, in fetal brown adipocytes

Finally, we examined whether Akt/PKB, $p70^{s6k}$ and the atypical PKC ζ isoenzyme, downstream targets of PI 3-kinase, act in the signalling pathway by which glucose transport is activated upon insulin stimulation in brown adipocytes. Quiescent brown adipocytes were preincubated with okadaic acid and subsequently stimulated with insulin as described above. Cell lysates were analyzed by Western blotting with the total Akt, the isoform-specific and the anti-phospho Akt antibodies. As shown in Fig. 3, okadaic acid alone increased Akt/PKB phosphorylation in fetal brown adipocytes as compared to control cells. Upon insulin stimulation, Akt/PKB was highly serine phosphorylated, this effect not being precluded by the pretreatment with okadaic acid. In addition, the serine/threonine phosphorylation of $p70^{s6k}$ in response to insulin, determined by Western blotting with an anti-phospho $p70^{s6k}$ antibody, remained unaffected by the presence of the inhibitor.

The atypical PKC ζ isoenzyme is expressed in fetal brown adipocytes and its enzymatic activity was analyzed in the anti-PKC ζ immune complexes. As shown in Fig. 3, the level of MBP phosphorylation in the presence of okadaic acid was similar to that observed in non-treated brown adipocytes. Interestingly, PKC ζ activation induced in the presence of 10 nM insulin was inhibited by $>90\%$ when okadaic acid was present.

4. Discussion

Insulin resistance is a state in which target cells fail to respond to ordinary levels of circulating insulin [27]. Previous studies have reported that serine/threonine phosphorylation of the insulin receptor itself by activation of PKC [28] and overexpression of H-ras oncogen [18] resulted in a decreased tyrosine phosphorylation and insulin resistance. Furthermore

serine/threonine phosphorylation of IRS-1 by addition of the serine/threonine phosphatase inhibitor okadaic acid [14], activation of cellular stress pathways by tumor necrosis factor α (TNFα) [17] and more recently by activation of the MAP kinase pathway [29] also resulted in a decreased tyrosine phosphorylation. These results, together with our previous published data, prompted us to investigate in our cell model whether the elevation of serine/threonine phosphorylation of IRS-1 and IRS-2 upon okadaic acid pretreatment impairs insulin signalling leading to glucose uptake in brown adipocytes.

The treatment of brown adipocytes with okadaic acid markedly reduces the insulin stimulatory action on both IRS-1 and IRS-2 tyrosine phosphorylation. In parallel, IRS-1 and, to a greater extent, IRS-2 had a decreased electrophoretic mobility in SDS–PAGE suggesting an increased phosphoserine/phosphothreonine content of these proteins. However, this insulin-resistant state occurs without detectable changes in the insulin receptor β-subunit tyrosine kinase activity. In comparison, TNFα pretreatment of brown adipocytes, which led to insulin resistance on glucose transport and also on the adipogenic-related gene expression, markedly reduces the insulin receptor β-subunit tyrosine autophosphorylation [30]. Thus, these results suggest that different phosphoserine/phosphothreonine elevating agents (i.e. okadaic acid and TNFα) might have specific targets within the brown adipocyte signalling complex.

The activation of PI 3-kinase is an essential requirement for complete brown adipocyte differentiation [13]. The fact that tyrosine phosphorylated IRS-1 and IRS-2 strongly associate with and activate PI 3-kinase [24] indicates that both IRS proteins equally contribute to propagating this signalling cascade. In the present paper, we have shown that insulin-induced IRS-1/IRS-2-association with p85α is significantly reduced by okadaic acid pretreatment. Consequently, these cells showed a parallel inhibition of PI 3-kinase enzymatic activity. These results indicate that the elevation of serine/threonine phosphorylation of IRS-1/IRS-2, which decreases their tyrosine phosphorylation, impairs the PI 3-kinase signalling pathway involved in brown adipocyte differentiation. Interestingly, inhibition of adipogenic differentiation of brown adipocytes with TNFα resulted in an inhibition of insulin-induced IRS-2-associated PI 3-kinase activity, PI 3-kinase associated to IRS-1 remaining unaltered [30]. Hence, IRS-1 and IRS-2 are subjected to different phosphoserine/phosphothreonine negative controls in brown adipocytes. Consequently, the specific pathways which impair the insulin signalling cascade induced by different phosphoserine/phosphothreonine elevating agents merits further investigation.

It is well known that PI 3-kinase is required for the movement of glucose transporters to the cell membrane in brown adipose tissue [26] In fetal brown adipocytes, glucose transport increased significantly upon stimulation with physiological doses of insulin/IGF-I, this effect being PI 3-kinase-dependent [13,25]. However, controversial results of the role of okadaic acid on insulin-induced glucose transport in adipose cells have been published. An inhibitory effect was proposed in 3T3L1 adipocytes in parallel with a decreased IRS-1 tyrosine phosphorylation and PI 3-kinase activity [14]. In comparison, okadaic acid provoked insulin-like effects on glucose transport and Glut4 translocation in rat white adipocytes [31]. The pretreatment of brown adipocytes with okadaic acid totally inhibited insulin-induced glucose uptake in a dose-dependent manner, although 40–50% remnant PI 3-kinase activity was found. These results prompted us to elucidate the PI 3-kinase downstream targets involved in glucose uptake induced in response to insulin. Among these, the activation of PKC ζ, blunted by PI 3-kinase inhibitors [13], was also inhibited by okadaic acid pretreatment. However, activation of other PI 3-kinase targets such as Akt/PKB or $p70^{s6k}$ were not affected by the inhibitor. In fact, although previous reports indicate that transient expression of constitutive active Akt/PKB results in the activation of GLUT4 translocation [32,33], it seems that only a relatively small fraction of insulin-stimulated GLUT4 translocation is inhibited by a kinase-inactive form of Akt/PKB in rat adipocytes [33]. Also, studies performed in 3T3L1 with a double mutant (T308AA, S473A) as a dominant negative inhibitor, suggest that Akt/PKB is not required for insulin-stimulated glucose transport [34]. Finally, okadaic acid elicits insulin-like effects and subsequently activates PKC ζ by itself in white adipocytes [35]. However, in this paper we show that okadaic acid completely blocks insulin-induced PKC ζ activity in parallel to glucose uptake in brown adipocytes. Consequently, we propose the requirement of the PI 3-kinase/PKC ζ pathway, but not the PI 3-kinase/Akt pathway, for the insulin action on glucose uptake in brown adipocytes.

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