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Vanadate fully stimulates insulin receptor substrate-1 associated phosphatidyl inositol 3-kinase activity in adipocytes from young and old rats

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Abstract Vanadate stimulates adipocyte 2-deoxyglucose transport and GLUT-4 translocation to the membrane through an insulin receptor-independent but wortmannin-inhibitable pathway. Vanadate stimulates PI 3-kinase in anti-IRS-1 immunoprecipitates and the binding between IRS-1 and the p85α subunit of PI 3-kinase. In insulin-resistant adipocytes from old rats vanadate fully stimulates IRS-1-associated PI 3-kinase, but partially activates glucose uptake. We conclude that: (a) vanadate stimulates 2-deoxyglucose uptake using a pathway that converges with that of insulin at the level of PI 3-kinase; and (b) adipocytes from old rats are defective in the insulin pathway at steps located both upstream and downstream of PI 3-kinase.

Key words: Rat adipocyte; Vanadate; Aging; Insulin resistance; Glucose transport; Phosphatidyl inositol-3-kinase

1. Introduction

The insulin-mimetic agent vanadate has been shown to restore normoglycemia in streptozotocin-treated diabetic rats [1,2] and in experimental animals with type II diabetes [3–6] after oral administration, and to improve insulin sensitivity in the skeletal muscle of normal [7], obese [8], and aged rats [9], an effect resulting from stimulation of glucose uptake and metabolism by tissues. In rat adipocytes and other intact cellular systems vanadate is able to stimulate hexose uptake [10,11], glucose oxidation [11,12], and glycogen synthase activity [13], as well as to inhibit lipolysis [14]. Concerning the mode of action of vanadate (5+ oxidation state), it has been concluded that its effects are not mediated by the stimulation of insulin receptor autophosphorylation and kinase activity [6,11,14,15], but the point of convergence with the insulin signal remains to be elucidated. A recent report has shown that vanadate stimulates a membranous non-receptor tyrosine kinase in rat adipocytes that could mediate its effects on glucose uptake and lipolysis [16]. If vanadate can bypass the early events of the insulin-dependent cascade, it could be used as a powerful tool to investigate which steps in this cascade are impaired in insulin-resistant cells.

Activation of phosphatidyl inositol 3-kinase (PI 3-kinase) is a necessary step in insulin stimulation of glucose uptake and glucose transporter 4 (GLUT-4) translocation and its inhibition by wortmannin is known to block these effects [17–22]. Vanadate also stimulates glucose uptake in rat adipocytes [10,11,14,23] and L6 myotubes [20], and wortmannin has been shown to partially block its effect on myotubes suggesting the existence of PI 3-kinase-dependent and -independent pathways in the mechanism of vanadate action.

In the present work we analyzed the vanadate effect on complex formation between the p85α subunit of PI 3-kinase and insulin receptor substrate-1 (IRS-1), and the PI 3-kinase activity associated with it, as well as the GLUT-4 translocation and glucose uptake in isolated adipocytes from 3-month-old mature (not juvenile) rats, and explored whether it can stimulate 2-deoxyglucose uptake in insulin-resistant adipocytes from 24-month-old rats, which we have previously shown to be extremely insulin-insensitive both in vivo [24] and in vitro [25,26].

2. Materials and methods

2.1. Materials

Cytochalasin B was purchased from Aldrich Chemical (Wisconsin, USA). Porcine insulin, sodium orthovanadate, wortmannin, quercetin, phosphatidyl inositol, and protein A-Sepharose were from Sigma Chemical (Missouri, USA). 2-Deoxy-D[1-^{3}H]glucose, [γ-^{32}P]ATP, and the ECL method for Western blot were purchased from Amersham (UK). Specific polyclonal antibodies for the GLUT-4 protein were prepared as in [27]. Anti-IRS-1, anti-p85α, and monoclonal 4G10 anti-phosphotyrosine antibodies were from UBI (New York, USA). Polyclonal antibodies for the C-terminal domain of IR β-subunit were from Santa Cruz Biotechnology (USA).

2.2. Animals

Male adult (3 months; body weight 408 ± 29 g) and old (24 months; body weight 622 ± 22 g) Wistar rats fed ad libitum on a standard laboratory chow and water were used throughout this study. The characteristics of the two groups of rats have been previously reported [24,25]. Adipocyte size was similar in both groups of animals [25].

2.3. Isolation of fat cells and determination of glucose uptake

Adipocytes were prepared by the collagenase method according to [28]. Determination of glucose transport was based on previously published procedures [29] with some modifications. Briefly, adipocytes were washed three times in 1% bovine serum albumin/KRP buffer, and subsequently incubated for 10 min in the same medium containing 3% bovine serum albumin in a proportion of 1 ml of cells per 3 ml

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Abbreviations: IR, insulin receptor; IRS-1, insulin receptor substrate-1; GLUT-4, glucose transporter 4; PI 3-kinase, phosphatidyl inositol 3-kinase; KRP, Krebs-Ringer phosphate; PM, plasma membrane; LDM, low density microsomes

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of medium. 50 μM cytochalasin B or ethanol was added, and cells were further incubated in the presence or absence of 160 nM insulin or 1 mM Na_3VO_4 for 20 min. Wortmannin (1 μM) or quercetin (100 μM) was added 10 min before insulin or vanadate. Reaction was started by addition of 4 μCi of 2-deoxy-D[1-^{3}H]glucose (2 mM) and after 6 min the medium was aspirated and cells were washed four times with cold PBS. Aliquots of the cellular suspension were counted for radioactivity, and carrier-mediated transport activity was calculated by subtracting the amount of radioactivity incorporated in the presence of cytochalasin B, which was always lower than 30% of total basal incorporation. Under the reaction conditions used, incorporation of 2-deoxyglucose was linear up to 10 min in cells preincubated with 160 nM insulin, in agreement with data in [29]. Cells were counted after staining with osmium tetroxide and the diameter was determined as described in [25].

2.4. Isolation of adipocyte membrane fractions

Adipocytes incubated as described above were lysed in homogenization buffer (10 mM Tris-HCl, pH 7.4, 100 mM sucrose, 1 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, 2 mM Na_3VO_4, 10 μg/ml trypsin inhibitor, 1 mM PMSF) and centrifuged at $8000\times g$ for 30 min. Pellets were used for the preparation of plasma membrane and the supernatant was further centrifuged in order to sediment the low density microsome (LDM) fraction as reported in [30]. Whole membrane fraction was obtained by centrifugation of cell lysates at $200\,000\times g$ for 70 min.

2.5. Measurement of immunoreactive GLUT-4 protein

10 μg of protein from purified membrane fractions was resolved by 10% SDS-PAGE and then transferred to nitrocellulose membranes. Membranes were blocked in PBS containing 5% fat skimmed dry milk for 1 h and subsequently incubated for 3 h with the GLUT-4-specific antibody (dilution 1:3000) at room temperature. After washing the membranes with 0.05% Tween 20 in PBS, goat anti-rabbit peroxidase-conjugated IgG was added and immunolabeled bands were visualized by the ECL method. GLUT-4 protein was quantitated by scanning densitometry of autoradiographs whose exposure was in the linear range.

2.6. Determination of PI 3-kinase and association between IRS-1 and p85α

Isolated adipocytes preincubated with 1.6 μM insulin or 1 mM Na_3VO_4 for 10 min were homogenized as indicated above in homogenization buffer supplemented with 1% NP-40. Equal amounts of protein (1 mg) were incubated overnight with anti-IRS-1 antibodies (2 μg/ml) and subsequently 50 μl of protein A-Sepharose was added. Pellets were washed as described in [31] and incubated in 15 mM HEPES, pH 7.4, in presence of 50 μM adenosine, 0.2 mM Na_3VO_4, 0.1 mg/ml phosphatidyl inositol, 5 mM $MgCl_2$, and 50 μM [γ-^{32}P]ATP (50 μCi) for 20 min. Lipid extraction and separation in silica gel plates was performed as in [31]. PI 3-P was localized using a mixture of phosphoinositides as markers, and ^{32}P incorporation was visualized and quantitated with a phosphorimager BAS 150. Association of p85α to IRS-1 was determined after electrophoretic separation of anti-IRS-1 immunoprecipitates and subsequent Western blotting with anti-p85α antibodies.

2.7. Insulin receptor phosphorylation

50 μg of adipocyte plasma membrane protein was resolved by electrophoresis and transferred to nitrocellulose. Membranes were blotted with anti-phosphotyrosine and anti-IR β-subunit antibodies respectively.

3. Results

As demonstrated in Table 1, preincubation of adipocytes from young mature rats with 1 mM vanadate stimulates the initial rate of 2-deoxyglucose uptake twofold, an effect similar to that elicited by 160 nM insulin in these cells, and correspondingly lower than that observed in small adipocytes from juvenile rats [32]. Quercetin, an insulin receptor kinase inhibitor, suppressed the insulin effect on glucose uptake without altering the stimulatory action of vanadate confirming that both effectors act through pathways differing, at least, at the early events as previously reported [6,11,14,15].

Stimulation of glucose transport by insulin [33] and vanadate [23] in rat adipocytes is known to be mainly due to the recruitment of GLUT-4 carriers from an endocellular microsomal pool to the plasma membrane. Fig. 1 shows that 1 mM vanadate and 160 nM insulin induce a similar increase in the amount of GLUT-4 associated with the plasma membrane fraction of young rat adipocytes (2.8 ± 0.3- and 3.4 ± 0.4-fold stimulation respectively), in good agreement with their effect on 2-deoxyglucose uptake. In both cases a decrease in GLUT-4 in the LDM pool was observed (not shown). Quercetin did not modify the vanadate induced translocation of GLUT-4 to the plasma membrane, but prevented that induced by insulin (Fig. 2A,B) supporting further the existence of two separate stimulatory pathways for both effectors. Moreover, Fig. 1C shows that the effect of vanadate is not mediated by the IR. Thus, in contrast to insulin, vanadate neither alters the phosphotyrosine content of IR β-subunit nor induces a decrease in IR amount in plasma membrane due to internalization.

Activation of PI 3-kinase has been identified as a crucial step for the stimulation of glucose uptake and GLUT-4 translocation by insulin in rat adipose cells, 3T3-L1 adipocytes and L6 myotubes [17–22]. Moreover, it has been suggested that tyrosine phosphorylation of IRS-1 in response to insulin leads to the association, activation, and targeting to intracellular GLUT-4 containing vesicles, of PI 3-kinase [34]. As shown in Table 1, the inhibitor of the PI 3-kinase catalytic activity wortmannin completely abolishes the stimulation of 2-deoxyglucose uptake by insulin or vanadate in young rat adipocytes suggesting that the signaling pathways of both agents converge at the level of PI 3-kinase. Fig. 2A,B shows that wortmannin also inhibits the translocation of GLUT-4 to the plasma membrane in response to vanadate or insulin. Moreover,

Table 1
Stimulation of 2-deoxyglucose uptake in adipocytes from 3- and 24-month-old rats

Experimental conditions	Age	
	3 months	24 months
Insulin	2.18 ± 0.07 ($n=39$)	1.25 ± 0.04[a] ($n=21$)
insulin+quercetin	1.34 ± 0.14[d] ($n=5$)	n.d.
Insulin+wortmannin	0.96 ± 0.08[d] ($n=4$)	n.d.
Vanadate	1.95 ± 0.08 ($n=17$)	1.48 ± 0.03[b,c] ($n=8$)
Vanadate+quercetin	1.86 ± 0.15 ($n=6$)	1.38 ± 0.03[b,c] ($n=4$)
Vanadate+wortmannin	1.04 ± 0.12[e] ($n=8$)	1.13 ± 0.06[e] ($n=4$)

Adipocytes were incubated in the presence or absence of 160 nM insulin or 1 mM vanadate, and 2-deoxyglucose uptake was determined as indicated in Section 2. Wortmannin (1 μM) or quercetin (100 μM) was added 10 min before insulin or vanadate where indicated. Data are expressed as the quotient between 2-deoxyglucose uptake in the presence and in the absence of effectors, for each rat age, and represent mean ± S.E. Basal 2-deoxyglucose uptake values were 0.9 ± 0.1 and 1.1 ± 0.15 nmol of 2-deoxyglucose/min/10^5 cells (P = NS) for young and old rat adipocytes, respectively. Values in parentheses indicate the number of different experiments corresponding to each experimental condition.
[a] $P<0.0001$ vs. 3 months; [b] $P<0.0005$ vs. 3 months; [c] $P<0.01$ vs. insulin; [d] $P<0.0005$ vs. insulin; [e] $P<0.0002$ vs. vanadate; n.d. = not determined.

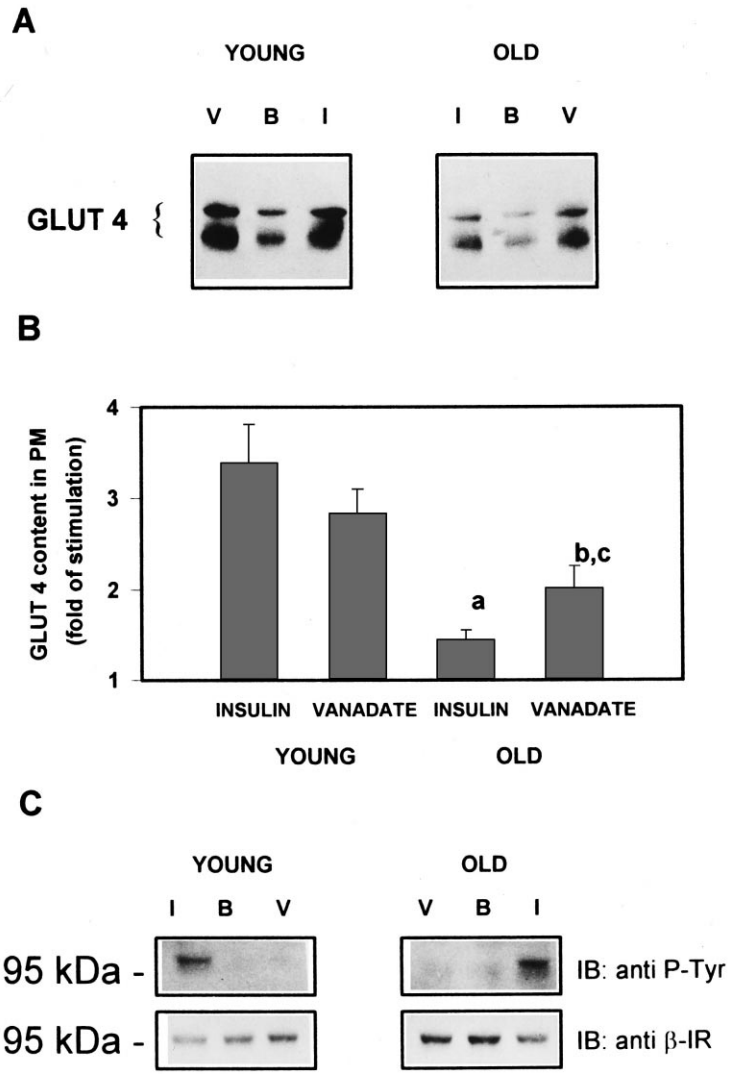

Fig. 1. GLUT-4 translocation and IR phosphorylation. A: 50 μg of plasma membrane protein from adipocytes incubated in the absence (B) or presence of 160 nM insulin (I) or 1 mM vanadate (V) was resolved by 7.5% SDS-PAGE and GLUT-4 was detected by Western blotting as indicated in Section 2. The figure shows two representative experiments for young and old rat adipocytes respectively. B: Seven autoradiographs like those in A were quantitated by densitometric scanning. The figure represents the increase in GLUT-4 in the plasma membrane as fold stimulation ± S.E., elicited by preincubation of adipocytes with insulin or vanadate. a: $P < 0.002$ vs. young; b: $P = 0.05$ vs. young; c: $P = 0.05$ vs. insulin. C: Plasma membrane proteins were resolved as in A. Phosphotyrosine content of IR β-subunit was detected by immunoblot with anti-phosphotyrosine antibodies (upper part), and the amount of receptors in each case was estimated by immunoblot with anti-IR antibodies (lower part) as described in Section 2.

as shown in Fig. 2C, vanadate 1 mM stimulates the PI 3-kinase activity in anti-IRS-1 immunoprecipitates similarly to insulin, and both effectors promote the association of p85α, the regulatory subunit of PI 3-kinase, to IRS-1 (Fig. 2D).

Since vanadate activates PI 3-kinase and its association to IRS-1, GLUT-4 translocation, and 2-deoxyglucose uptake, it seems interesting to investigate whether it can overcome the insulin insensitivity of these cellular events in insulin-resistant cells. Therefore, we explored the effect of vanadate in isolated adipocytes from old rats, a cell type previously reported to be very insensitive to insulin [24–26]. As can be seen in Fig. 3A, 1 mM vanadate stimulates more than twofold the PI 3-kinase activity of anti-IRS-1 immunoprecipitates from old rat adipocytes, an effect similar to that elicited in young rat cells by both vanadate and insulin. In contrast, insulin causes only a weak activation of this activity in adipocytes from old rats (1.2-fold), a fact that correlates well with the low effect of the hormone on glucose uptake and GLUT-4 translocation in these cells (Table 1 and Fig. 1A,B). Fig. 3B also shows that 1 mM vanadate promotes the association of p85α to IRS-1 to the same extent in adipocytes from young and old rats, whereas insulin fails to induce this association in the insulin-resistant adipocytes. The effects of vanadate on cells from aged animals also take place without stimulating IR phosphorylation and internalization (Fig. 1C). These data might indicate that in adipocytes from old rats the insulin signal cascade is fully functional downstream of the stimulation of IRS-1 binding to p85α and subsequent activation of PI 3-kinase activity, but is defective at an earlier step. However, as shown in Table 1 and Fig. 1A,B, in adipocytes from old rats vanadate stimulates only partially both GLUT-4 translocation to the plasma membrane and 2-deoxyglucose uptake, suggesting that another signaling impairment exists downstream of PI 3-kinase. Wortmannin fully abolished the effect

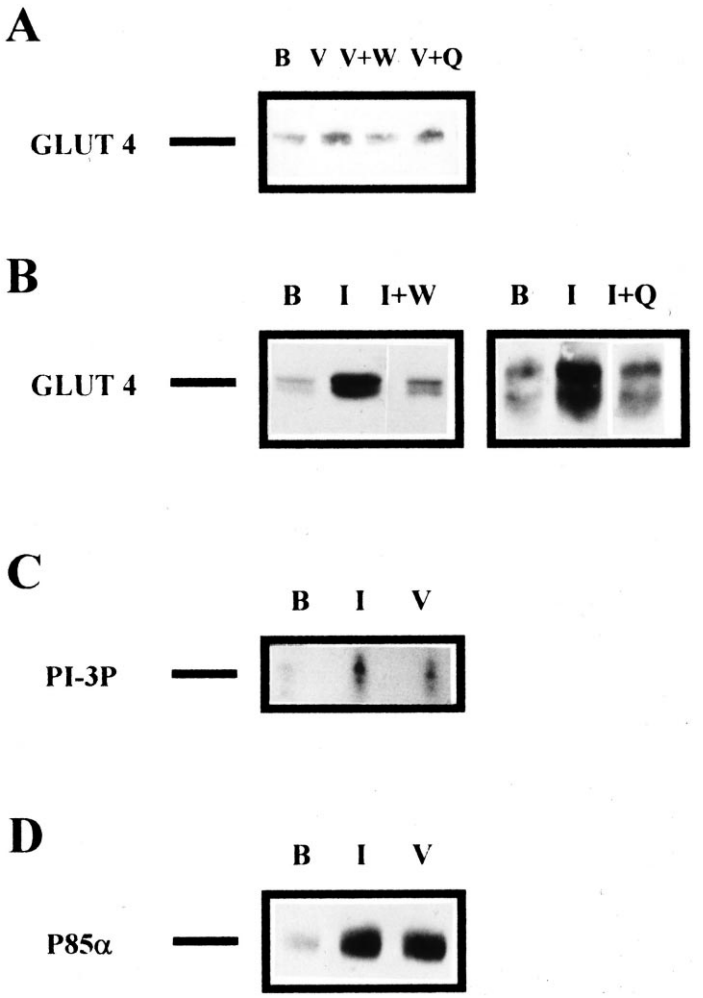

Fig. 2. Effect of vanadate and insulin on PI 3-kinase activity associated with IRS-1 and inhibition by wortmannin and quercetin of their effects on GLUT-4 translocation. A: Effect of preincubation with wortmannin or quercetin on GLUT-4 translocation induced by vanadate in adipocytes from young animals. B: without effectors; V: 1 mM vanadate; V+W: 1 mM vanadate+1 μM wortmannin; V+Q: 1 mM vanadate+100 μM quercetin. B: Effect of preincubation with wortmannin or quercetin on GLUT-4 translocation induced by insulin in adipocytes from young animals. B: without effectors; I: 160 nM insulin; I+W: 160 nM insulin+1 μM wortmannin; I+Q: 160 nM insulin+100 μM quercetin. C: Adipocytes from young rats were incubated in the absence (B) or presence of insulin (I) and vanadate (V), and PI 3-kinase activity was determined in anti-IRS-1 immunoprecipitates using phosphatidyl inositol as substrate (see Section 2). The lipid product PI 3-P was extracted and separated by TLC and the ^{32}P incorporation was detected by phosphorimager and autoradiography. D: 100 μg of protein from adipocytes preincubated without effectors (B), or in presence of insulin (I) or vanadate (V), was immunoprecipitated with anti-IRS-1 antibodies and separated by SDS-PAGE. Proteins were transferred to nitrocellulose and p85α was detected by blotting the membranes with anti-p85α antibodies.

of vanadate in these cells (Table 1) indicating that it is mediated through activation of PI 3-kinase.

Looking for changes in the signal pathway downstream of PI 3-kinase we focused our attention in the study of the GLUT-4 content and its distribution between membrane pools in adipocytes from adult and old rats. By studying by Western blot analysis (not shown) the relative amounts of GLUT-4 in PM and LDM fractions we found that under basal, unstimulated, conditions the equilibrium distribution of GLUT-4 is shifted to the plasma membrane as demonstrated by $[GLUT\text{-}4]_{LDM}/[GLUT\text{-}4]_{PM}$ ratios of 7.9 ± 0.8 ($n = 5$) and 3.0 ± 0.5 ($n = 9$) for young and old rat adipocytes respectively ($P < 0.0002$). Since basal glucose uptake rates are similar for both cell populations, this difference could be interpreted as consequence of a decrease in the amount of carriers in the LDM fraction available for translocation. In fact, the [GLUT-4] in LDM from old rat cells was estimated to be $46 \pm 18\%$ ($n = 6$) of that observed in fat cells from young animals. However, determination of GLUT-4 in whole membrane fractions showed only a $25 \pm 10\%$ ($n = 6$) decrease in adipocytes from old rats, suggesting that another membrane pool is sequestering some carriers in the aged animals.

4. Discussion

Vanadate induces metabolic effects similar to those elicited by insulin in rat adipocytes and other insulin-sensitive cells, but the molecular mechanism involved remains unclear. In the present work we studied the molecular events induced by vanadate that lead to the stimulation of 2-deoxyglucose transport in isolated adipocytes. Our data indicate that 1 mM vanadate and 160 nM insulin are equally potent stimulators of glucose uptake and GLUT-4 translocation to the plasma membrane. However, whereas the insulin effect is abolished by the insulin receptor phosphotransferase inhibitor quercetin, vanadate action appears to be quercetin-insensitive and does

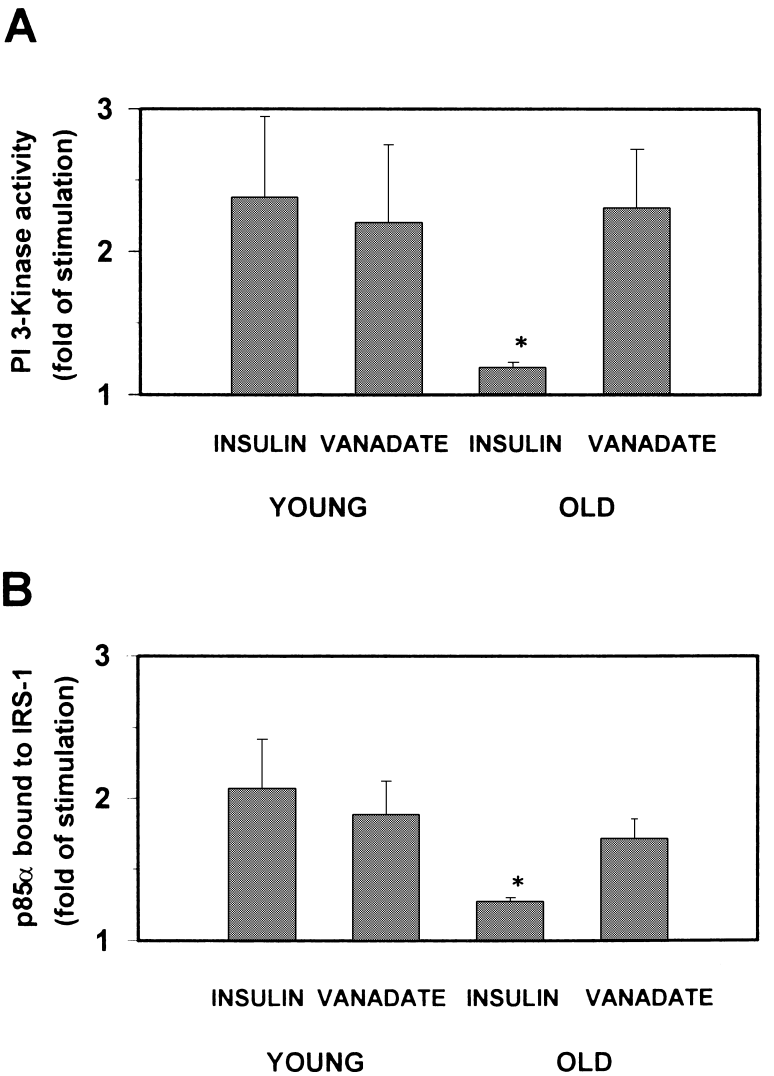

Fig. 3. Stimulation by insulin and vanadate of PI 3-kinase activity and p85α association to IRS-1 in adipocytes from young and old rats. A: Adipocytes from 3- and 24-month-old rats were preincubated in the absence or presence of insulin or vanadate and PI 3-kinase activity was determined in anti-IRS-1 immunoprecipitates as indicated in Fig. 2C. Data represent the mean value ± S.E. of the stimulatory effect elicited by insulin and vanadate from seven different experiments performed separately with young and old rat adipocytes respectively. $*P < 0.05$ vs. 'young+insulin' and 'old+vanadate'. B: Association of p85α with IRS-1 in adipocytes from young and old rats was determined as indicated in Fig. 2D. Data show the stimulation of association over basal, induced by insulin or vanadate, and are the mean ± S.E. of seven and five independent experiments performed with young and old rat adipocytes respectively. $*P < 0.05$ vs. 'young+insulin' and 'old+vanadate'.

not involve IR phosphorylation and internalization as previously reported [6,11,14,15]. In contrast, the effect of vanadate and that of insulin were totally suppressed by preincubation with the PI 3-kinase inhibitor wortmannin, suggesting the involvement of this enzyme in the signaling pathway. In a previous study with L6 myotubes, wortmannin was shown to inhibit only ~60% of vanadate-activated glucose transport, suggesting that it acts through PI 3-kinase-dependent and -independent pathways [20]. In contrast, our data here demonstrate that in rat adipose cells the effect of vanadate on glucose transport is mediated by stimulation of PI 3-kinase with no evidence of PI 3-kinase-independent pathways, as is the case for insulin.

Recent evidence supports that the acute redistribution of intracellular glucose transporters to the plasma membrane in response to insulin in rat adipocytes and 3T3-L1 adipose cells is related to the formation of complexes containing IRS-1 bound to PI 3-kinase, which are targeted to the GLUT-4-containing vesicles [34,35]. A very recent report has concluded that vanadate stimulates PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates from adipose cells [36]. Our data in this work show for the first time that vanadate increases the PI 3-kinase activity in anti-IRS-1 immunoprecipitates from rat adipocytes, as well as the association of the p85α regulatory subunit of the enzyme with IRS-1, as reported for insulin. Whether vanadate acts by stimulation of IRS-1 phosphorylation through inhibition of a vanadate sensitive tyrosine phosphatase [37] remains to be elucidated. Recently, it has been described that vanadate stimulates in vitro the autophosphorylation and kinase activity of a plasma membrane non-receptor protein tyrosine kinase activity in rat adipocytes that associates with the p85α subunit of PI 3-kinase [16], postulating a mechanism independent of IRS-1 for vanadate action on glucose uptake. However, it is well known that membrane tyrosine kinase receptors that bind directly PI 3-kinase after ligand stimulation, such as PDGF receptor, do not stimulate

glucose uptake and it seems unlikely that this vanadate-sensitive kinase could do it. However, it might phosphorylate IRS-1 in response to vanadate leading to the binding and activation of PI 3-kinase as reported here.

The former data indicate that vanadate shares part of the insulin pathway leading to activation of glucose uptake, but differs at the early molecular events, making it a valuable tool to localize which steps in the insulin pathway are impaired in insulin-resistant cells. Using this approach to the study of the insulin-resistant adipocyte from 24-month-old rats we demonstrate here that the formation of complexes containing IRS-1 bound to p85α, as well as the PI 3-kinase activity associated to IRS-1, are fully stimulated by vanadate in these cells, in contrast to the poor effect elicited by insulin. These data would indicate that the impaired insulin action associated with aging is likely due to a defective early step in the insulin pathway. However, the fact that vanadate activates only partially the GLUT-4 translocation and glucose transport in adipose cells from old rats suggests that an additional defective step in the pathway, downstream of PI 3-kinase, impairs the vanadate and insulin action. Among the early molecular events in the insulin signal cascade that could be impaired in aged rats, autophosphorylation of insulin receptor and stimulation of its kinase activity have been reported to be decreased in vitro in adipocytes [25,26], and in vivo in liver and muscle [38]. Carvalho et al. [38] also reported a progressive decrease with aging of IRS-1 tyrosine phosphorylation in response to insulin that strongly correlates with the decreased phosphorylation of insulin receptor. A defective association between IRS-1 and the p85α regulatory subunit of PI 3-kinase due to intrinsic changes in any of both proteins seems unlikely since vanadate elicits a normal stimulation of PI 3-kinase, and the association of both molecules. Finally, changes in the number and affinity of insulin receptors during aging have been previously ruled out [26].

Concerning the steps downstream of PI 3-kinase altered in insulin-resistant adipocytes from old rats, there is evidence of decreases in the number and the translocation of either cytochalasin B binding sites [39], or specific glucose carriers [40,41], in aged rats. These data are in agreement with our findings indicating a 25% lower amount of GLUT-4 in adipocytes from old animals, a decreased number of carriers in LDM available for translocation, as well as a shift to the plasma membrane in the equilibrium distribution of these carriers under basal conditions.

In summary, we have shown that in rat adipocytes vanadate stimulates the IRS-1 associated PI 3-kinase activity, and that wortmannin fully inhibits the translocation of GLUT-4 to the plasma membrane, and the increase of 2-deoxyglucose uptake, suggesting a critical role for this activity in the vanadate pathway. In insulin-resistant adipocytes from 24-month-old rats, despite a normal stimulation of IRS-1 binding to p85α and PI 3-kinase activity, vanadate does not elicit a full activation of glucose uptake probably due to the changes observed in the number and distribution of GLUT-4 carriers that contribute to the well characterized insulin resistance of these cells.

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