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Insulin-Like and Non-Insulin-Like Selenium Actions in 3T3-L1 Adipocytes

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Abstract In insulin-sensitive 3T3-L1 adipocytes, selenium stimulates glucose transport and antilipolysis and these actions of selenium, like insulin actions, are sensitive to wortmanin, an inhibitor of phosphatidylinositol-3-kinase (PI3K). Selenium stimulates PI3K activity that is sustained up to 24 h. Selenium after 5–10 min increases tyrosine phosphorylation of selective cellular proteins, but after 24 h overall tyrosine phosphorylation is increased. Tyrosine phosphorylation of insulin receptor substrate 1 is detected when enriched by immunoprecipitation with anti-PI3K antibody. Selenium, however, does not stimulate insulin receptor tyrosine kinase activity. Selenium also increases phosphorylation of other insulin signaling proteins, including Akt and extracellular signal regulated kinases. Selenium-stimulated glucose transport is accompanied by increases in glucose transporter-1 content in the plasma membrane. These data are consistent with similar selenium action in glucose transport in 3T3-L1 fibroblasts expressing mainly GLUT1. In chronic insulin-induced insulin resistant cells, selenium unlike insulin fully stimulates glucose transport. In summary, selenium stimulates glucose transport and antilipolysis in a PI3K-dependent manner, but independent of insulin receptor activation. Selenium exerts both insulin-like and non-insulin-like actions in cells. J. Cell. Biochem. 88: 719–731, 2003.

Key words: selenium; insulin signaling; phosphatidylinositol-3-kinase; glucose transport; antilipolysis

Selenium is an essential trace element and its dietary supplementation has been popularized for its beneficial effects in general health [Rayman, 2000]. Selenium has a variety of functions in cells, but its anti-oxidant function has been best known [Rayman, 2000]. Recently, selenium supplementation was reported to reduce the risk of cancer [Clark et al., 1996] and selenium deficiency was also linked with loss of immunocompetence [Rayman, 2000]. Moreover, selenium has been reported to exert insulin-like cellular functions both in vivo and in vitro. Addition of selenium as sodium selenate to isolated primary rat adipocytes stimulated glucose transport [Ezaki, 1990]. In streptozotocin-induced diabetic rats, sodium selenate was shown to improve glucose homeostasis when administered orally [McNeill et al., 1991] and intraperitoneally [Becker et al., 1996; Battell et al., 1998]. In these rats, selenium partly reversed abnormal expression of liver glycolytic (i.e., glucokinase and pyruvate kinase) and gluconeogenic (phosphoenolpyruvate carboxykinase) enzymes [Becker et al., 1996]; and glucose-6-phosphate dehydrogenase and fatty acid synthase [Berg et al., 1995]. These studies strongly suggest beneficial effects of selenium on glucose homeostasis in diabetic rats. Yet, underlying mechanisms for insulin-like actions of selenium are unclear.

When insulin binds to its receptor, the insulin receptor gets activated via tyrosine autophosphorylation and tyrosine phosphorylates other cellular substrates [Virkamaki et al., 1999]. Insulin receptor substrates 1/2 (IRS-1/2), the major substrates for the insulin receptor, contain multiple potential tyrosine phosphorylation sites and function as docking proteins [White, 1998]. Tyrosine phosphorylated IRS-1/2 interact with the SH2 domains of the p85 regulatory subunit of phosphatidylinositol-3-

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kinase (PI3K) and activate the p110 catalytic subunit of PI3K [Backer et al., 1992; Keller and Lienhard, 1994; Quon et al., 1994]. PI3K activation is reported to be crucial for most insulin actions [Virkamaki et al., 1999]. Recently, the serine/threonine protein kinase Akt1 was discovered as a molecule downstream of PI3K [Burgering and Coffer, 1995; Franke et al., 1995; Kohn et al., 1995; Didichenko et al., 1996] and reported to mediate some insulin-induced cellular responses including glucose transport and antilipolysis [Virkamaki et al., 1999].

Employing insulin-sensitive 3T3-L1 adipocytes, we investigated effects of selenium on two major metabolic functions regulated by insulin, glucose transport and antilipolysis, and on intracellular insulin signaling pathways. Here, we report for the first time that selenium, independently of the insulin receptor, stimulates PI3K in insulin-sensitive cells and this PI3K activity mediates insulin-like cellular actions of selenium. We also report that selenium exerts both similar and dissimilar regulation of insulin signaling pathways when compared with insulin actions.

RESEARCH DESIGN AND METHODS

Materials

Monoclonal antibodies to phosphotyrosines and antiserum to the p85 regulatory subunit of PI3K were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY); anti-phosphoERK(Thr^{202}/Tyr^{204}) and anti-phosphoAkt(Ser^{473}) antibody from Cell Signaling (Beverly, MA); anti-GLUT4 antibody from Biogenesis (Sandown, NH). Anti-GLUT1 antiserum was a gift of Dr. Amira Klip at the Hospital for Sick Children (Toronto, Canada); anti-IRS-1 antiserum, a gift of Dr. Tom Maassen (Leiden, The Netherlands). 2-[1,2-^{3}H]deoxy-D-glucose (2-DG, 26.2 Ci/mmol) and [γ-^{32}P]ATP (26.2 Ci/mmol) were purchased from NEN Life Science Products, Inc. (Boston, MA). Glycerol assay kit and other chemicals were from Sigma (St. Louis, MO), unless specified otherwise.

Cell Culture and Glucose Uptake

Both 3T3-L1 fibroblasts (preadipocytes) and 3T3-L1 adipocytes were used for study. 3T3-L1 fibroblasts were grown and differentiated into adipocytes as previously described [Heart et al., 2000]. Cells in 24-well plates were serum-starved for 5 h and treated with sodium selenate for various times. Cells were then placed in buffer A (1.47 mM K_2HPO_4, pH 7.4, 140 mM NaCl, 1.7 mM KCl, 0.9 mM $CaCl_2$, 0.9 mM $MgSO_4$, 0.1% BSA) [Heart et al., 2000] and incubated with sodium selenate or insulin for 20 min at 37°C. Next, radioactive 2-DG (0.2 mM, 0.5 μCi) was added for 10 min. Cell-associated radioactivity was measured and normalized for protein content as previously described [Heart et al., 2000]. Non-specific 2-DG uptake measured in the presence of 10 μM cytochalasin B accounted for ~10% of basal 2-DG uptake and was subtracted from all experimental data.

Enzymatic Assays for PI3K and Insulin Receptor Tyrosine Kinase

3T3-L1 adipocytes were incubated with sodium selenate or insulin and solubilized cell lysates were used for subsequent studies [Heart et al., 2000]. For insulin receptor tyrosine kinase activity, immunocapture assay was performed in 96-well microtiter plates (Dynatech; Oxnard, CA) as previously described [Heart et al., 2000]. Briefly, insulin receptors were captured from cell lysates (100 μg each) and assayed for tyrosine kinase activity with poly(Glu:Tyr=4:1) as a substrate [Heart et al., 2000].

For PI3K assay, cell lysates (500 μg each) were immunoprecipitated with either anti-phosphotyrosine antibody or anti-PI3K p85 antibody. These immunoprecipitates were then assayed with phosphatidylinositol (0.2 mg/ml) as a substrate [Kumakura et al., 1998]. Phosphatidylinositol-3-phosphate, a reaction product, was excised from thin layer plates and counted for its radioactivity in a liquid scintillation β counter.

Subcellular Fractionation

Cells in 150 mm dishes were preincubated with sodium selenate for various times or insulin for 30 min. Cells were next homogenized in homogenization buffer followed by subcellular fractionation as previously described [Heart et al., 2000]. The plasma membrane and low density microsomes (50 μg each) were analyzed for their GLUT1 and GLUT4 contents by Western blot analysis.

Western Blot Analysis

Following appropriate treatments of cells with sodium selenate or insulin, cells were solubilized as described above. Cell lysates (50 μg) were then resolved by SDS–PAGE followed by

electrophoretic transfer of proteins onto Hybond-P membranes (Amersham, Buckinghamshire, UK). The membrane was then probed with appropriate antibodies and signal was detected by hydrogen peroxide-enhanced chemiluminescence method as previously described [Heart et al., 2000].

Lipolysis

Cells in 12-well plates were treated for 2 h with sodium selenate or insulin. During the last 1 h of treatment, cells were placed in KRB buffer containing 2% bovine serum albumin and isoproterenol at 10 nM was added to induce lipolysis [Dandekar et al., 1998]. Media were collected and assayed for glycerol released from cells by using Triglyceride Reagent (Sigma). Glycerol content was normalized for protein content.

Statistical Analysis

Data are expressed as mean ± SEM. The significance of the differences among different treatments was evaluated using the Student's paired *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Selenium Stimulates Glucose Transport in a Dose- and a Time-Dependent Manner and This Selenium Action is Sensitive to Wortmanin

Selenium at 25–50 μM significantly stimulated glucose transport (Fig. 1A). At 250 μM, selenium-stimulated glucose transport reached maximum and this level was similar to that obtained by 100 nM insulin (Fig. 2A). For a time course study, cells were treated with 250 μM selenium for 0–24 h and 2-DG uptake was measured. Selenium significantly stimulated glucose transport after 10 min treatment, reaching maximum after 0.5–1 h (Fig. 1B). Maximal glucose transport was sustained after prolonged selenium treatment up to 24 h.

To determine whether effects of selenium and insulin on glucose transport are additive in these cells, we performed a study at submaximal and maximal concentrations of these agents. When cells were treated with submaximal concentrations of selenium and insulin together, 2-DG uptake was fully additive whereas at their maximal concentrations, 2-DG uptake was only partially additive (Fig. 2A). It should be noted that differences in the fold effect

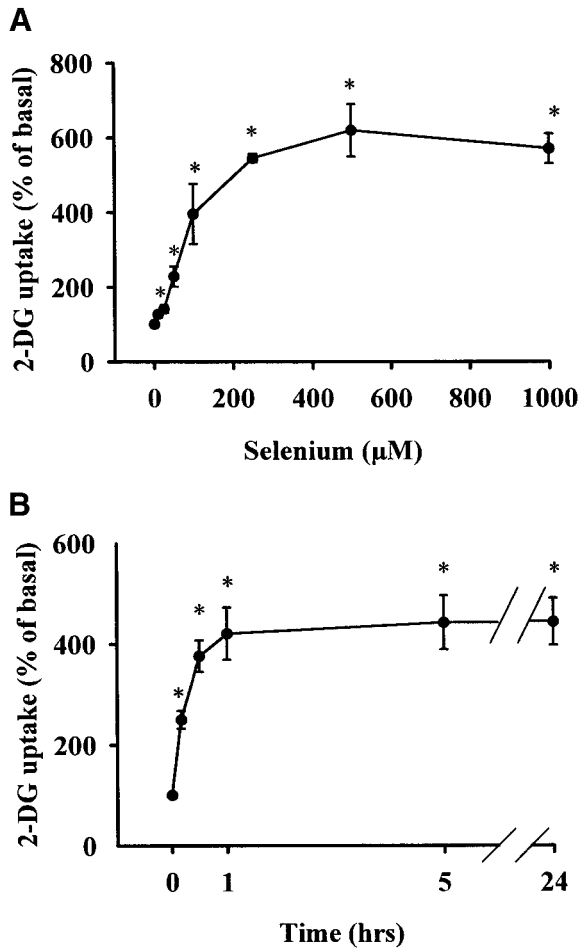

Fig. 1. **A**: Selenium stimulates glucose uptake in a dose- and a time-dependent manner in 3T3-L1 adipocytes. A: Dose response. Cells were pretreated for 20 min with sodium selenate at 0, 10, 25, 50, 100, 250, 500, and 1,000 μM and treated with [^{3}H]2-DG for additional 10 min. Following cell lysis in 0.05% SDS, cell-associated radioactivity was measured in a β-counter. Data are presented as % of basal and mean ± SEM of three to six experiments. The basal 2-DG uptake was 3.13 ± 0.26 nmol 2-DG uptake/mg protein/10 min. *, $P < 0.05$ versus basal by Student's paired *t*-test. **B**: Time course. Cells were pretreated with sodium selenate at 250 μM for 0, 10, 30 min, 1, 5, and 24 h and 2-DG uptake was measured as described in A. Data are presented as % of basal and mean ± SEM of four experiments. *, $P < 0.05$ versus basal by Student's paired *t*-test.

of selenium on glucose transport in Figures 1 and 2 is mainly due to the differences in basal glucose transport (data not shown). Sodium sulfate used as a control element for sodium selenate failed to stimulate 2-DG uptake in these cells (at 250 μM, 94 ± 8.2% of basal 2-DG uptake was obtained).

Insulin stimulation of glucose transport in insulin-sensitive cells such as 3T3-L1 adipocytes is mediated by activation of PI3K

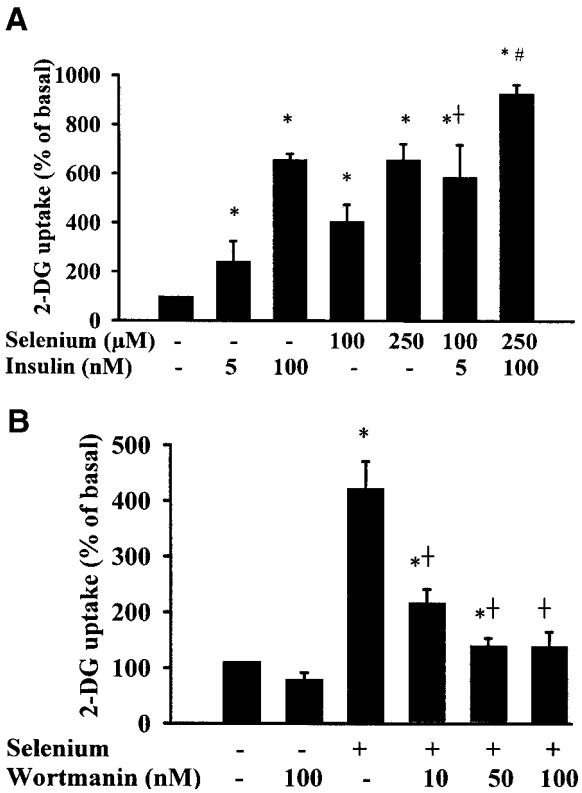

Fig. 2. **A**: Effects of selenium and insulin on glucose transport in 3T3-L1 adipocytes. Cells were pretreated for 20 min with sodium selenate and/or insulin. Following incubation with [^{3}H]2-DG for additional 10 min, cell-associated 2-DG uptake was measured as described in Fig. 1A. Data are presented as % of basal and mean ± SEM of three experiments. *, $P < 0.05$ versus basal; +, $P < 0.05$ versus 100 μM selenium or 5 nM insulin; #, $P < 0.05$ versus 250 μM selenium or 100 nM insulin by Student's paired *t*-test. **B**: Selenium-stimulated glucose transport is inhibited by wortmanin. Cells were pretreated for 1 h with varying doses of wortmanin and treated for 20 min with 250 μM sodium selenate. Following 10 min incubation with [^{3}H]2-DG, glucose transport was measured as described in Fig. 1A. Data are presented as % of basal and mean ± SEM of four experiments. *, $P < 0.05$ versus basal; +, $P < 0.05$ versus selenium by Student's paired *t*-test.

[Virkamaki et al., 1999]. To determine whether selenium stimulation of glucose transport depends on PI3K activity like insulin action, we employed PI3K inhibitor wortmanin. Selenium stimulation of 2-DG uptake was inhibited by wortmanin in a dose-dependent manner and wortmanin at 50–100 nM abolished this selenium action (Fig. 2B). As expected, wortmanin at these concentrations abolished insulin-stimulated glucose transport (data not shown). Wortmanin at 100 nM decreased basal 2-DG uptake by 20–30%. These data suggest that selenium is a potent stimulator of glucose transport and this selenium action is mediated by PI3K in 3T3-L1 adipocytes.

Selenium Increases Tyrosine Phosphorylation of Cellular Proteins Without Activation of Insulin Receptor Tyrosine Kinase

Insulin binds to and activates the insulin receptor, which then tyrosine phosphorylates cellular substrates such as IRS-1/2 [Virkamaki et al., 1999]. Tyrosine phosphorylated IRS-1/2 interacts with PI3K, resulting in activation of the latter. To determine effects of selenium on tyrosine phosphorylation of cellular proteins, we employed Western blot analysis with anti-phosphotyrosine antibody. As expected, insulin increased tyrosine phosphorylation of the insulin receptor and IRS-1/2 (Fig. 3A, left panel). In contrast, selenium did not alter tyrosine phosphorylation of these proteins. Selenium, however, increased tyrosine phosphorylation of a cellular protein(s) at 50–60 kDa (p50-60) as early as after 5–10 min and their phosphorylation was continuously increased. After 24 h, selenium caused increases in tyrosine phosphorylation of other additional cellular proteins (Fig. 3A, left panel).

Next, we examined phosphotyrosine proteins associated with PI3K following selenium treatment of cells. We reasoned that selenium-induced tyrosine phosphorylation of IRS-1/2 and/or other proteins might be low, below a detectable level in total cell lysates, but might be visualized when enriched by immunoprecipitation. Thus, we immunoprecipitated cell lysates with anti-PI3K p85 antibody and examined phosphotyrosine proteins associated with PI3K. In these immunoprecipitates, we found three phosphotyrosine proteins including the one (marked with an asterisk) at expected molecular weight for IRS-1/2 following selenium treatment of cells (Fig. 3A, right upper panel). This protein was subsequently identified as IRS-1 by Western blot analysis with anti-IRS-1 antibody (Fig. 3A, right lower panel).

To investigate whether selenium alters insulin receptor catalytic activity resulting in phosphorylation of IRS-1/2 and other cellular proteins, we next performed an enzyme assay for the insulin receptor tyrosine kinase. Selenium failed to increase insulin receptor kinase activity whereas insulin greatly stimulated receptor tyrosine kinase activity (Fig. 3B).

Selenium Stimulates PI3K Activity

Since selenium stimulation of glucose transport was sensitive to wortmanin, we examined

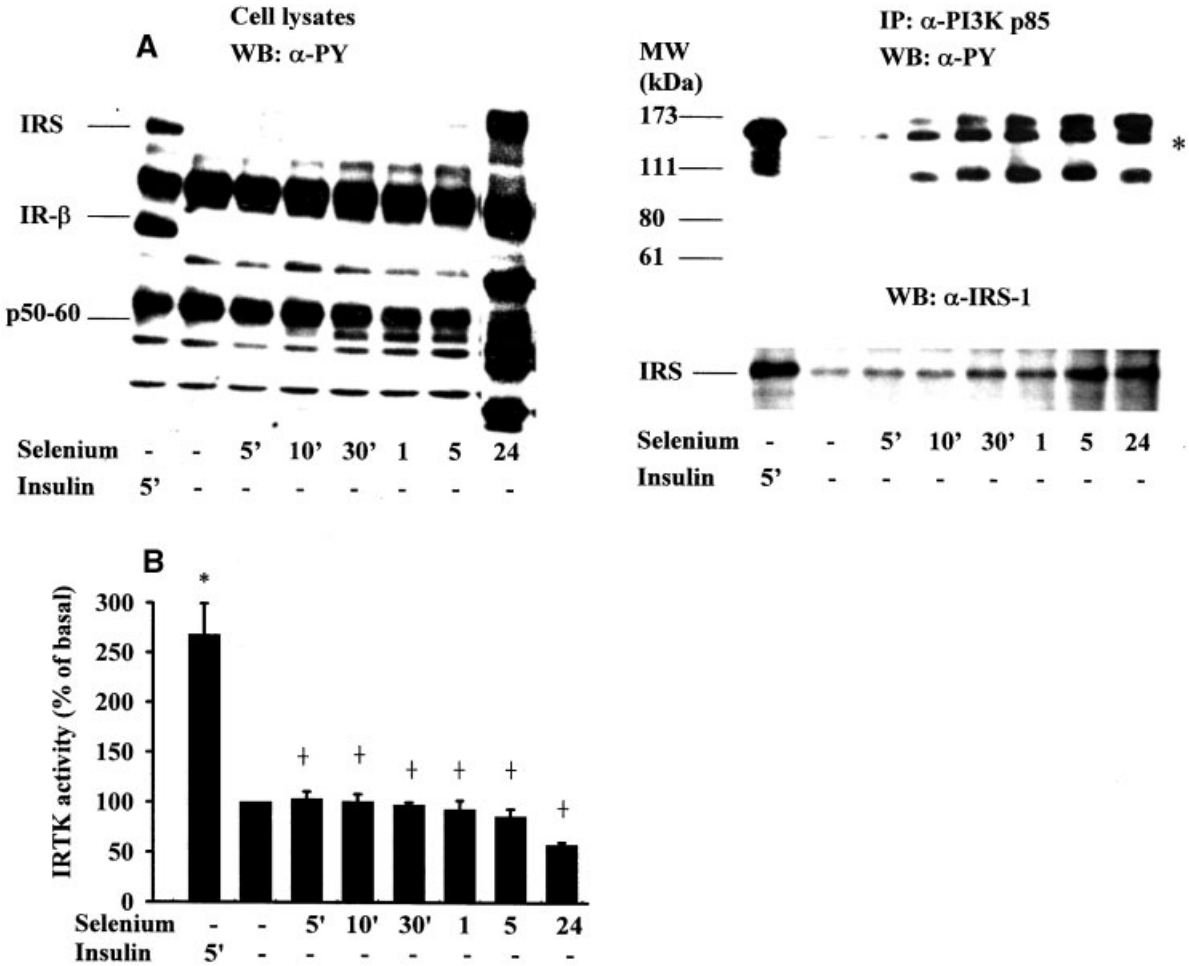

Fig. 3. **A**: Selenium increases tyrosine phosphorylation of cellular proteins. Cells were treated with 250 μM sodium selenate for various times or with 100 nM insulin for 5 min. Cells were then solubilized in 1% NP-40 lysis buffer [Heart et al., 2000] and resolved by Western blot analysis with anti-phosphotyrosine antibody (α-PY; **left panel**). In **right panel**, cell lysates were first immunoprecipitated with anti-PI3K p85 antibody (α-PI3K p85) and resolved by Western blot analysis with α-PY (**upper one**) or anti-IRS-1 antibody (α-IRS-1; **lower one**). A representative experiment is shown. IR-β, insulin receptor β subunit; IRS, insulin receptor substrates; IP, immunoprecipitation antibody; WB, Western blotting antibody. *, IRS-1. **B**: Selenium does not activate the insulin receptor tyrosine kinase. Cells were pretreated with 250 μM sodium selenate for various times and solubilized in 1% NP-40 lysis buffer. Insulin receptors in these cell lysates were immobilized and enzyme assays were performed in reaction mixture containing poly (Glu:Tyr=4:1) (PGT) and [^{32}P]ATP as previously described [Heart et al., 2000]. In parallel, cells were treated for 5 min with 100 nM insulin. Insulin receptor tyrosine kinase activity is presented as % of basal and mean ± SEM of four experiments. Basal activity was 3.73 ± 0.32 nmol Pi incorporated/mg PGT/mg protein/h. *, $P < 0.05$ versus basal; +, $P < 0.05$ versus insulin by Student's paired *t*-test.

whether selenium indeed stimulates PI3K activity in 3T3-L1 adipocytes. In the case of insulin stimulation of PI3K, PI3K associates with tyrosine phosphorylated IRS-1/2, resulting in its activation [Virkamaki et al., 1999]. In 3T3-L1 adipocytes, we observed that selenium increased tyrosine phosphorylation of various cellular proteins including IRS-1 (Fig. 3A). We hypothesized that selenium, like insulin, increased PI3K activity by its association with phosphotyrosine proteins. Thus, we measured PI3K activity associated with phosphotyrosine proteins following selenium treatment of cells (Fig. 4). At 5–10 min, selenium weakly increased PI3K activity and its activity was further increased up to 24 h. As expected, insulin greatly increased PI3K activity. These data suggest that selenium treatment of cells increases PI3K associated with phosphotyrosine proteins in 3T3-L1 adipocytes. Unlike insulin stimulation of PI3K that is transient, selenium exerted prolonged stimulation of PI3K up to 24 h.

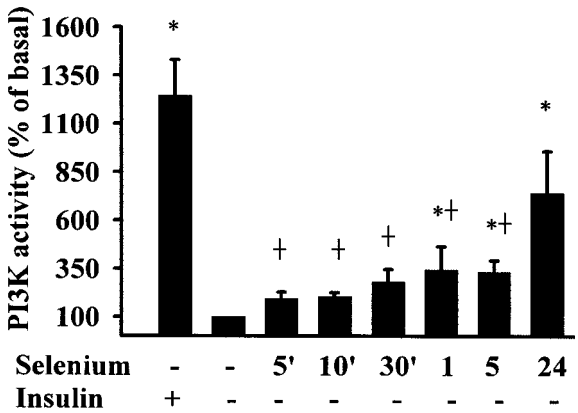

Fig. 4. Selenium stimulates PI3K activity in 3T3-L1 adipocytes. Cells were treated with 250 μM sodium selenate for various times followed by cell lysis. Solubilized cell lysates were next immunoprecipitated with either α-PY (**A**) or α-PI3K p85 (**B**). These immunoprecipitates were measured for their PI3K activities in reaction mixtures containing phosphatidylinositol (PI) and [^{32}P]ATP [Heart et al., 2000]. In parallel, cells were treated for 5 min with 100 nM insulin. Data are presented as % of basal and are mean ± SEM of three to four experiments. Se, sodium selenate; Ins, insulin. *, $P < 0.05$ versus basal; +, $P < 0.05$ versus insulin by Student's paired *t*-test.

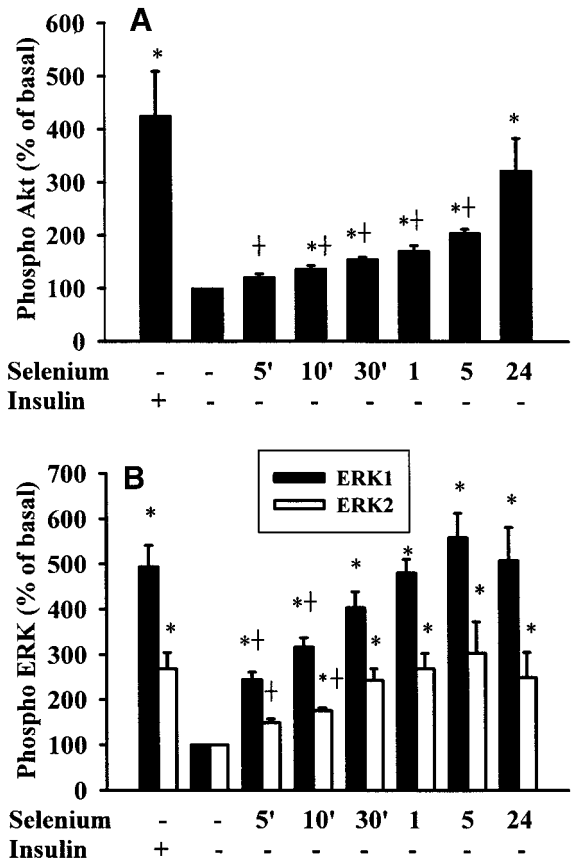

Fig. 5. Selenium stimulates phosphorylation of cellular proteins in insulin signaling pathways: Akt (**A**) and ERK1/2 (**B**). Cells were treated with 250 μM sodium selenate for various times and solubilized in 1% NP-40 lysis buffer. These cell lysates were next resolved by Western blot analysis with anti-phosphoAkt antibody or anti-phosphoERK antibody as described in Research Design and Methods. In parallel, cells were treated with 100 nM insulin for 5 min. Band intensities were quantified by a laser densitometer. Data are presented as % of basal and are mean ± SEM of three and four experiments, respectively. *, $P < 0.05$ versus basal; +, $P < 0.05$ versus insulin by Student's paired *t*-test (A). *, $P < 0.05$ versus corresponding basal; +, $P < 0.05$ versus corresponding insulin by Student's paired *t*-test (B).

Selenium Increases Phosphorylation of Various Serine/Threonine Kinases Present in Insulin Signaling Pathways

Since selenium stimulated PI3K activity in 3T3-L1 adipocytes, we next investigated phosphorylation/activation of Akt, which resides downstream of PI3K in insulin signaling pathways. Phosphorylation of Akt at serine 473 (Ser^{473}) is required for its full activation by growth factors [Kohn et al., 1996a,b], thus we performed Western blot analysis with antibodies against phosphorylated Ser^{473} (Fig. 5A). Selenium significantly increased Akt phosphorylation as early as 5–10 min that continued to increase up to 24 h. Wortmanin completely abolished Akt phosphorylation by selenium (data not shown). These data suggest that selenium stimulates Akt phosphorylation/activation and this selenium action is downstream of PI3K activation as in insulin signaling pathways.

Next, we investigated phosphorylation/activation of ERK following selenium treatment of cells. In 3T3-L1 adipocytes, the insulin signaling pathway for PI3K activation was reported to be distinct from that for ERK activation [Cheatham et al., 1994]. To examine effects of selenium on ERK activation in these cells, we employed antibodies to phosphorylated ERK at threonine 202 and tyrosine 204 (anti-phosphoERK). Phosphorylation of ERK at these sites is required for its full activation by growth factors [Marshall, 1995]. As expected, insulin stimulated ERK1/2 phosphorylation that peaked after 5 min (Fig. 5B) and declined after 30 min (data not shown). In these cells, selenium like insulin stimulated ERK1/2, but unlike insulin this selenium action continued to increase up to 24 h (Fig. 5B). These data are consistent with the previous report [Stapleton et al., 1997)]. Taken together, these studies suggest that

selenium increased phosphorylation of Akt and ERK with different kinetics from insulin action.

Selenium Increases Glucose Transporter 1 (GLUT1) Content in the Plasma Membrane

Insulin stimulates glucose transport mainly by increasing GLUT4 content in the plasma membrane [Virkamaki et al., 1999]. In 3T3-L1 adipocytes, insulin also increased GLUT1 content in the plasma membrane [Heart et al., 2000]. Here, we investigated whether selenium stimulation of glucose transport is accompanied by GLUT1/4 trafficking. Selenium after 30 min significantly increased GLUT1 content in the plasma membrane, reaching maximum after 5–24 h (Fig. 6A). Increases in GLUT1 contents in the plasma membrane were accompanied by concomitant decreases in its contents in the low density microsomes (Fig. 6B). In contrast to GLUT1, selenium did not significantly increase GLUT4 content in the plasma membrane (Fig. 6). Insulin increased both GLUT4 and GLUT1 contents in the plasma membrane with their concomitant decreases in low density microsomes (Fig. 6). Selenium treatment did not alter total GLUT1/4 contents (data not shown).

To confirm our finding that selenium did not affect GLUT4 translocation in 3T3-L1 adipocytes, we have analyzed insulin-regulated aminopeptidase (IRAP) contents in the same subcellular fractions. IRAP was previously reported to reside in the same vesicle as intracellular GLUT4 storage pool and translocate to the plasma membrane with GLUT4 following insulin treatment of cells [Virkamaki et al., 1999]. Selenium did not significantly alter IRAP contents in the plasma membrane and low density microsomes whereas insulin increased IRAP content in the plasma membrane with its concomitant decrease in low density microsomes (data not shown). These data further support that selenium stimulates glucose transport mainly by GLUT1 movement.

Selenium Fully Stimulates Glucose Transport in Both 3T3-L1 Fibroblasts and Insulin Resistant 3T3-L1 Adipocytes

Our data suggest that selenium stimulates glucose transport via GLUT1 translocation, independently of activation of the insulin receptor. To ascertain these findings, we employed 3T3-L1 fibroblasts expressing GLUT1, but not insulin-responsive GLUT4, and few insulin receptors. In these cells, selenium fully stimulated glucose transport as in 3T3-L1 adipocytes and its dose response was similar to that in 3T3-L1 adipocytes (Fig. 7A). In these cells, insulin at 100 nM stimulated glucose transport less than twofold. To further investigate differential actions of selenium and insulin in glucose

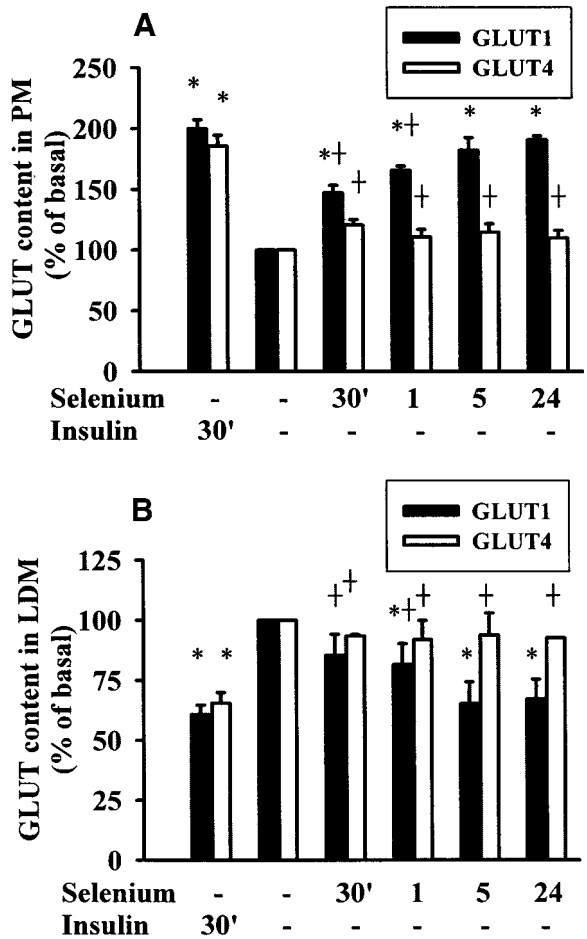

Fig. 6. Effects of selenium on GLUT1/4 trafficking in 3T3-L1 adipocytes. Cells were treated with 250 μM sodium selenate for various times and their subcellular fractions were prepared as previously described [Heart et al., 2000]. These subcellular fractions including the plasma membrane and low density microsomes were next resolved by Western blot analysis with anti-GLUT1 antibody or anti-GLUT4 antibody. In parallel, cells were treated for 30 min with 100 nM insulin and their subcellular fractions were analyzed. **A**: GLUT contents in the plasma membrane; (**B**) GLUT contents in the low density microsomes. Basal GLUT contents were taken as 100%. Data are presented as % of basal and are mean ± SEM of four to five experiments. PM, plasma membrane; LDM, low density microsomes. *, $P < 0.05$ versus corresponding basal; +, $P < 0.05$ versus corresponding insulin by Student's paired *t*-test (A). *, $P < 0.05$ versus corresponding basal; +, $P < 0.05$ versus corresponding insulin by Student's paired *t*-test (B).

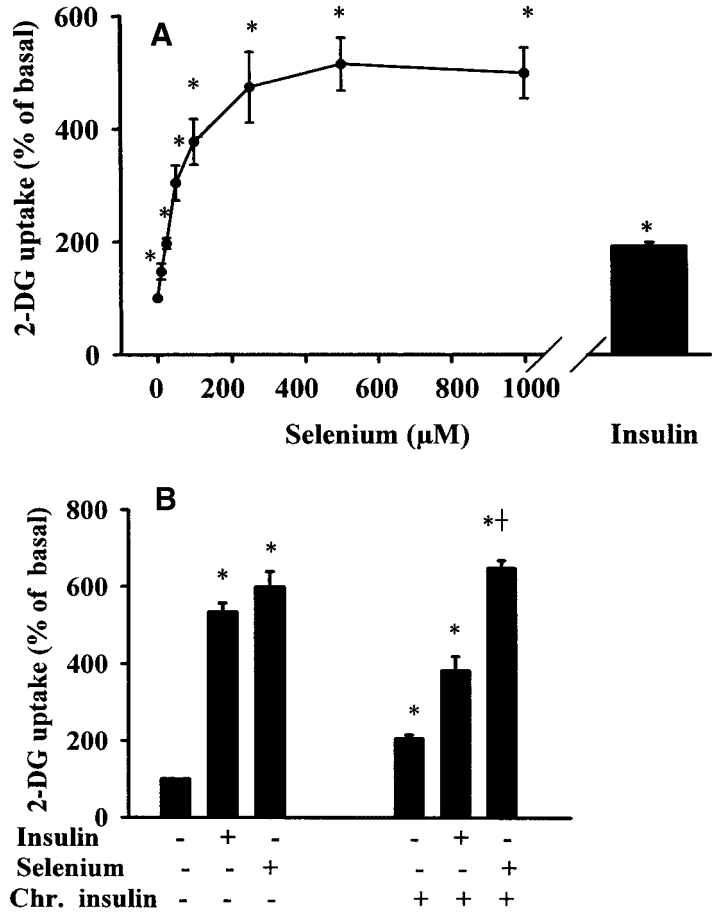

Fig. 7. Selenium fully stimulates glucose transport in 3T3-fibroblasts (**A**) and chronic insulin-treated insulin resistant 3T3-L1 adipocytes (**B**). A: 3T3-L1 fibroblasts were pretreated for 20 min with sodium selenate at 0, 10, 25, 50, 100, 250, 500, and 1,000 μM and glucose transport was measured as previously described in Fig. 1. For insulin action, cells were treated with 100 nM insulin. B: 3T3-L1 adipocytes were pretreated with 10 nM insulin for 16 h. Cells were next treated with either 100 nM insulin or 250 μM selenium and glucose transport was measured. Data are presented as % of basal and are mean ± SEM of three experiments. *, $P < 0.05$ versus basal by Student's paired *t*-test (A). *, $P < 0.05$ versus basal; +, $P < 0.05$ versus insulin after chronic insulin treatment by Student's paired *t*-test (B).

transport, we induced insulin resistance by chronic insulin treatment of 3T3-L1 adipocytes. In these cells, selenium fully stimulated glucose transport as in normal 3T3-L1 adipocytes whereas insulin failed to fully stimulate glucose transport as previously reported (Fig. 7B) [Kozka et al., 1991; Janez et al., 2000]. These data further support our previous finding that selenium stimulates glucose transport mainly via GLUT1 and independently of the insulin receptor.

Selenium Stimulates Antilipolysis in a PI3K-Dependent Manner

In fat cells, β agonists stimulate lipolysis, generating free fatty acid and glycerol [Rondinone et al., 2000]. Insulin inhibits lipolysis and this counter-regulation of β agonist-stimulated lipolysis (i.e., antilipolysis) is another major metabolic function of insulin [Virkamaki et al., 1999]. Here, we examined effects of selenium on antilipolysis. In 3T3-L1 adipocytes, isoproterenol at 10 nM stimulated lipolysis threefold as assessed by glycerol release (Fig. 8). Selenium at 250 μM inhibited isoproterenol-stimulated lipolysis (i.e., stimulated antilipolysis) 30–40%, which was similar to that achieved by 100 nM insulin (Fig. 8A). Submaximal concentrations of selenium or insulin inhibited lipolysis 20% (Fig. 8A). When these agents were added together at submaximal concentrations, there was further decrease in lipolysis, reaching the level similar to those obtained by maximal concentrations of either agent alone. At their

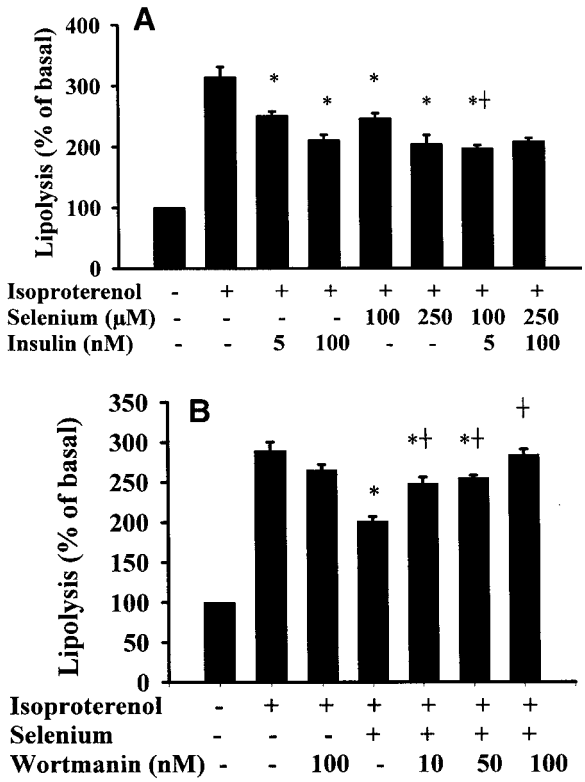

Fig. 8. **A**: Selenium inhibits isoproterenol-induced lipolysis in 3T3-L1 adipocytes. Cells were treated for 2 h with sodium selenate or insulin. During the last 1 h of treatment, 10 nM isoproterenol was added. Next, glycerol released into culture media was measured as described in Research Design and Methods. Data are presented as % of basal and mean ± SEM of four to six experiments. Basal glycerol release was 0.84 ± 0.08 mmol glycerol/mg protein/h. *, $P < 0.05$ versus isoproterenol; +, $P < 0.05$ versus 5 nM insulin or 100 μM selenium by Student's paired *t*-test. **B**: Selenium action in isoproterenol-induced lipolysis is inhibited by wortmanin. Cells were pretreated for 1 h with wortmanin at varying concentrations and treated for 2 h with 250 μM sodium selenate. Next, isoproterenol-induced lipolysis was measured as described in A. Data are presented as % of basal and mean ± SEM of three to five experiments. *, $P < 0.05$ versus isoproterenol; +, $P < 0.05$ versus isoproterenol plus selenium by Student's paired *t*-test.

maximal concentrations, there was no further decrease in lipolysis [Rondinone et al., 2000]. Sodium sulfate did not affect isoproterenol-induced lipolysis (at 250 μM, 91.8±7.0% of isoproterenol-induced lipolysis was obtained).

Next, we investigated the role of PI3K in this selenium action. Insulin-induced antilipolysis was previously reported to be mediated by the PI3K/Akt pathway [Kitamura et al., 1998; Virkamaki et al., 1999]. In 3T3-L1 adipocytes, wortmanin inhibited selenium-induced antilipolysis in a dose-dependent manner (Fig. 8B). As expected, insulin-induced antilipolysis was also completely abolished at 100 nM wortmanin (data not shown). Wortmanin at 100 nM did not significantly affect isoproterenol-induced lipolysis (Fig. 8B). Taken together, these data suggest that selenium action in antilipolysis is mediated by a mechanism involving PI3K like insulin action.

DISCUSSION

In the present study, we investigated both insulin-like and non-insulin-like actions of selenium in insulin-sensitive 3T3-L1 adipocytes. In these cells, selenium as sodium selenate stimulated glucose transport and antilipolysis, two major metabolic functions regulated by insulin, in a PI3K-dependent manner, but independently of the insulin receptor. Our finding that selenium per se stimulates PI3K activity further supports the crucial role of PI3K in these insulin-like actions of selenium in cells. Selenium also stimulated Akt that is downstream of PI3K in insulin signaling pathway. Previously, Akt has been implicated in insulin stimulation of antilipolysis [Kitamura et al., 1998] and glucose transport [Kohn et al., 1996a,b]. However, the weak ability of selenium to stimulate PI3K and Akt, together with distinct time courses compared to that for glucose transport suggests that the PI3K/Akt pathway may not be the major mechanism underlying selenium stimulation of glucose transport and antilipolysis. It should also be noted that overall selenium action in insulin signaling pathways is distinct from insulin action.

Our observation that selenium stimulation of glucose transport is PI3K-dependent was interesting since many extracellular signals can stimulate PI3K activity, but not all of them stimulate glucose transport like insulin [Virkamaki et al., 1999]. In fact, PI3K activity has been reported to be necessary for, but not sufficient for all insulin actions, suggesting that there may be additional specific molecules (or compartmentalization) required for specific functions of insulin [Heller-Harrison et al., 1996; Pessin et al., 1999; Virkamaki et al., 1999]. Thus, we further investigated underlying mechanisms for selenium stimulation of PI3K. In the case of insulin action, PI3K gets associated with phosphotyrosine proteins, mainly via tyrosine phosphorylated IRS-1, resulting in its activation [Virkamaki et al.,

1999]. Thus, we measured phosphotyrosine protein-associated PI3K activity following selenium stimulation of cells. We found that selenium indeed increased PI3K activity associated with phosphotyrosine proteins. Unlike insulin action, however, PI3K activity stimulated by selenium was small when compared with that by insulin. Moreover, unlike transient insulin activation of PI3K, selenium-stimulated PI3K activity continued to increase up to 24 h.

Next, we investigated whether IRS-1 was involved in selenium stimulation of PI3K as in the case of insulin action. Our data indicated that IRS-1 was indeed tyrosine phosphorylated by selenium and became associated with PI3K. The level of IRS-1 tyrosine phosphorylation by selenium was much lower than that by insulin. In fact, we could not detect IRS-1 phosphorylation by selenium when the total cell lysates were examined. It should be noted that there were two other phosphotyrosine proteins that were associated with PI3K. Taken together, these data suggest that selenium stimulates PI3K at least in part by a similar mechanism involving IRS-1 as in insulin action. Unlike insulin action, however, selenium stimulation of PI3K was sustained and continued to increase up to 24 h. A much smaller degree of phosphotyrosine protein (i.e., IRS-1)-associated PI3K activity following selenium treatments when compared with insulin actions suggests that other additional mechanism may be operative in selenium stimulation of PI3K in these cells.

The first action of insulin in cells is to bind to the insulin receptor and activate its tyrosine kinase activity [Virkamaki et al., 1999]. To study whether selenium activates the insulin receptor that in turn tyrosine phosphorylates IRS-1 in 3T3-L1 adipocytes, we performed insulin receptor enzyme assays following selenium treatment of cells. Employing a sensitive immunocapture assay, we failed to detect activation of the insulin receptor by selenium. These data suggest that selenium promotes tyrosine phosphorylation of IRS-1 and other cellular proteins via a mechanism independent of activation of the insulin receptor. In this regard, a cellular kinase JAK2 has been implicated to phosphorylate IRS-1 in growth hormone signaling without activation of the insulin receptor [Kim et al., 1998]. Our data are also consistent with the previous report in rat primary adipocytes [Ezaki, 1990]. In other studies, however, selenium was reported to increase tyrosine phosphorylation of the insulin receptor [Pillay and Makgoba, 1992; Stapleton et al., 1997]. In these studies, the activity of the insulin receptor tyrosine kinase was not measured. In this regard, it is noteworthy that insulin-mimetic effects of zinc were reported to be associated with tyrosine autophosphorylation of the insulin receptor, but neither with tyrosine phosphorylation of IRS-1 nor association of PI3K with IRS-1 [Tang and Shay, 2001]. Yet, this zinc stimulation of glucose transport was dependent on PI3K, suggesting insulin receptor/IRS-1-independent mechanisms for PI3K activation [Tang and Shay, 2001].

In our study, we used sodium sulfate as control element since it belongs to the same VIa group in the periodic table. Sodium sulfate, unlike sodium selenate, did not affect 2-DG uptake and isoproterenol-induced lipolysis, arguing against non-specific general effects of selenium. In addition, vanadate is a better-studied element mimicking insulin actions. Selenium and vanadium belong to the 4th period of the periodic table of the elements and stimulate glucose transport. Unlike selenate, vanadate stimulates glucose transport in PI3K-independent, and insulin receptor and IRS-1 phosphorylation-independent manner [Fantus and Tsiani, 1998; Goldwaser et al., 2000]. These data suggest that insulin-mimetic effects of these two elements are not due to non-specific effects of elements and are mediated by distinct mechanisms.

Next, we investigated how selenium alters tyrosine phosphorylation of cellular proteins. Protein phosphorylation is a balanced net result of actions of both protein kinases and protein phosphatases. Some cellular proteins such as p50-60 and IRS-1 are phosphorylated at early times whereas most other cellular proteins are phosphorylated after prolonged selenium treatment of cells. Kinetics of early and sustained (and further increased at later times) protein phosphorylation by selenium may suggest involvement of both kinases and phosphatases in selenium actions in cells.

The ability of selenium to stimulate PI3K independently of insulin receptor activation prompted us to investigate other intracellular signaling pathways utilized by the insulin receptor, either downstream or independent of PI3K. The Akt/GSK-3 axis in insulin signaling is downstream of the PI3K pathway and plays a

role in both glycogen synthesis and cell survival [Virkamaki et al., 1999]. In 3T3-L1 adipocytes, selenium stimulated phosphorylation of both Akt (Fig. 5A) and GSK-3 (data not shown) with a prolonged time course, similar to that for PI3K activation. Together with wortmanin sensitivity of these selenium actions, our data suggest that Akt/GSK-3 phosphorylation by selenium is downstream of PI3K as in insulin signaling pathways. We also found that p70 S6 kinase downstream of PI3K, but independent of the Akt/GSK-3 axis, in insulin signaling is also phosphorylated by selenium with a prolonged time course and this selenium action was sensitive to wortmanin as in insulin signaling (data not shown). In insulin signaling, ERK phosphorylation was previously reported to be independent of the PI3K pathway [Cheatham et al., 1994]. As in other intracellular signaling pathways, selenium stimulated ERK phosphorylation with a prolonged time course and this selenium action was not sensitive to wortmanin. Taken together, these data suggest that selenium triggered most known intracellular signaling pathways operative in insulin action, but with distinct kinetics and potencies. Sustained phosphorylation by selenium of various intracellular signaling pathways may provide a clue for additional cellular effects of selenium in cells following prolonged selenium treatments.

In 3T3-L1 adipocytes, we found that selenium stimulation of glucose transport was accompanied by translocation of GLUT1 from the intracellular storage pool (i.e., low density microsomes) to the plasma membrane. However, selenium slightly, if at all, increased GLUT4 content in the plasma membrane. This was further confirmed by the inability of selenium to affect IRAP movements since GLUT4 and IRAP reside in the same intracellular pool and move to the plasma membrane following insulin stimulation of cells (data not shown). In chronic insulin-treated insulin resistant 3T3-L1 adipocytes, selenium, unlike insulin, fully stimulated glucose transport. In these insulin resistant cells, GLUT4 translocation by subsequent insulin treatment of cells was decreased as well as total GLUT4 content [Kozka et al., 1991]. These studies further suggest that actions of selenium and insulin in glucose transport are different and selenium-induced glucose transport is mainly mediated via GLUT1. This interpretation is consistent with our observation that selenium fully stimulated glucose transport in 3T3-L1 fibroblasts expressing only GLUT1. In contrast, Ezaki [1990] reported that selenium at 1 mM stimulated translocation of both GLUT1 and GLUT4. Presently, reasons for this discrepancy are unclear. It should also be noted that selenium stimulation of glucose transport reached its maximum after 30 min although GLUT1 content in the plasma membrane after 30 min did not yet reach a maximal level. These data suggest that maximal glucose transport does not require maximal GLUT1 content in the plasma membrane and/or there may be an additional mechanism operative in selenium stimulation of glucose transport such as activation of intrinsic activity of GLUT1 and perhaps GLUT4 present in the plasma membrane.

At submaximal concentrations, effects of selenium and insulin on glucose transport were fully additive whereas at maximal concentrations they were only partially additive. Unlike primary adipocytes expressing mainly GLUT4, 3T3-L1 adipocytes express both GLUT1 and GLUT4 with a molar ratio of 3:1 [Tordjman et al., 1990]. In 3T3-L1 adipocytes, insulin mobilizes both GLUT1 and GLUT4 from intracellular storage pools to the plasma membrane, suggesting contribution of both transporters in insulin-mediated glucose transport. Given the primary role of GLUT1 in selenium action and the role of both GLUT1/4 in insulin action, it is likely that glucose transport is not fully additive when both agents are present at their maximal concentrations. Moreover, similar degrees of glucose transport by maximal doses of insulin and selenium despite of insulin effects on both GLUT1 and 4 (compared with selenium effects on GLUT1 only) further suggest another level of GLUT regulation in the plasma membrane, perhaps differential GLUT activation in the plasma membrane by insulin and selenium. In the case of antilipolysis, effects of selenium and insulin at submaximal concentrations were additive whereas at their maximal concentrations their effects were similar to those achieved by either agent alone. These data suggest that selenium and insulin exert antilipolysis via a common mechanism.

In summary, selenium stimulates two major metabolic functions regulated by insulin in PI3K-dependent manner, but in the insulin receptor-independent manner. In addition, selenium stimulates insulin signaling pathways with different kinetics from insulin action.

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