Vanadate Augments Insulin-Stimulated Insulin Receptor Kinase Activity and Prolongs Insulin Action in Rat Adipocytes

Evidence for Transduction of Amplitude of Signaling Into Duration of Response

I. GEORGE FANTUS, FERHAAN AHMAD, AND GUY DERAGON

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/43/3/375/360644/43-3-375.pdf by guest on 27 March 2025

Vanadate, a protein tyrosine phosphatase inhibitor, preserves insulin-stimulated lipogenesis after removal of insulin. To investigate the mechanism of this action of vanadate, lipogenesis was studied in isolated rat adipocytes exposed to vanadate for 60 min followed by insulin for 15 min at 37°C. Vanadate (10–50 μM) prolonged insulin-stimulated lipogenesis. The half-time ($t_{1/2}$) of the decay in insulin (0.34 nM)-stimulated lipogenesis after removal of insulin by washing in pH 7.0 followed by pH 7.6 buffer was 21 min in the absence and 59 min in the presence of vanadate. During these conditions, vandate did not alter insulin binding nor the removal of insulin by the series of washes. In contrast to lipogenesis, the $t_{1/2}$ of the decay in insulin receptor tyrosine kinase (IRK) activity, assayed with the artificial substrate Poly[Glu:Tyr] (4:1), was not significantly prolonged by vanadate (6 vs. 6.8 min). However, insulin-stimulated IRK activity was markedly augmented by vanadate to 319 ± 19% of insulin alone, associated with a similar augmentation of phosphotyrosine incorporation into the insulin receptor β-subunit determined by Western blotting with antiphosphotyrosine antibodies. To determine the relationship between prolongation of lipogenesis and the increase in IRK, adipocytes were exposed to 17.2 nM insulin to activate the IRK to the same extent as insulin (0.34 nM) plus vanadate (maximum activation). During these two conditions, the decay of lipogenesis was similar and after stimulation with 17.2 nM insulin was not prolonged by vanadate. We conclude that vanadate prolongs insulin action at insulin concentrations that do not maximally activate the IRK by augmenting IRK activity. The data suggest that the amplitude of the insulin signal, the IRK, is transduced into duration of response, lipogenesis. Thus activation of receptor above that required for a maximum biological response results in prolongation of action. ***Diabetes*** **43:375–83, 1994**

From the Protein and Polypeptide Hormone Laboratory, Department of Medicine, Royal Victoria Hospital and McGill University, Montreal, Quebec; and Department of Medicine, Mount Sinai Hospital and Banting and Best Diabetes Center, University of Toronto, Toronto, Ontario, Canada.

Address correspondence and reprint requests to Dr. I.G. Fantus, Department of Medicine, Mount Sinai Hospital, 600 University Avenue, Suite 780, Toronto, Ontario M5G 1X5, Canada.

Received for publication 23 November 1992 and accepted in revised form 4 November 1993.

IGF-II, insulin-like growth factor II; STZ, streptozocin; BSA, bovine serum albumin; PMSF, phenylmethylsulfonyl fluoride; WGA, wheat germ agglutinin; $t_{1/2}$, half-time; KRBH, Krebs-Ringer bicarbonate–HEPES buffer; KRH, KRBH without $NaHCO_3$; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; FBS, fetal bovine serum; ANOVA, analysis of variance; IRK, insulin receptor tyrosine kinase.

During the past decade, insulin-mimetic vanadium compounds such as sodium orthovanadate (vanadate) have been the object of increasing attention both as a means of exploring the mechanism of action of insulin and as potential therapeutic agents in the management of diabetes (1–3). The insulin-like activity of vanadate was first observed in 1979 and 1980 when studies reported that this compound stimulates glucose uptake, glucose oxidation, and potassium uptake in rat adipocytes in vitro (4–6). Since then, vanadate has been documented to mimic the actions of insulin in several different tissues. In adipocytes, stimulation of insulin-like growth factor II (IGF-II) binding (7), lipogenesis (8,9), glycogen synthase (10), protein synthesis (9), and inhibition of lipolysis (8,11) have all been demonstrated. Clark et al. (12) have observed increases in glucose transport, glycolysis, glycogen synthesis, and glucose oxidation in skeletal muscle. In hepatocytes, vanadate has been shown to stimulate glycogen synthesis and lipogenesis (13) and inhibit protein degradation (14).

The importance of tyrosine phosphorylation appears relevant to the postulated mechanism of the action of vanadate. Insulin activates its receptor by binding to the externally oriented α-subunit and stimulating autophosphorylation of the cytosolic portion of the β-subunit on tyrosine residues (15–18). The tyrosine-autophosphorylated β-subunit is then capable of phosphorylating and

altering the function of endogenous substrates thereby transmitting its intracellular biological effects (19). The second consideration is that vanadate is a potent phosphotyrosine phosphatase inhibitor (20–22), possibly because of its structural resemblance to phosphate (23). One may therefore speculate that phosphotyrosine phosphatase inhibition leads to augmented autophosphorylation and activity of tyrosine kinases such as the insulin receptor (7,10), the epidermal growth factor receptor (20), and pp60v-src (24).

Several studies have now demonstrated that vanadate added to the drinking water of streptozocin (STZ)-induced diabetic rats normalizes blood glucose, produces weight gain, stimulates glucose transport into muscle and liver, and improves cardiac performance (25–33). Reversal of hyperglycemia has been demonstrated in two mouse models of non-insulin-dependent diabetes mellitus, *ob/ob* and *db/db* mice (34) and the *fa/fa* rat (35). Very low serum concentrations of vanadate (<15 μM) have been effective in the studies cited above (26). In contrast, significant acute insulin-mimetic activity in isolated rat adipocytes is generally observed at higher concentrations (200 μM to 1 mM) (8–10).

To resolve this apparent discrepancy between in vitro and in vivo studies, we previously investigated the effects of low concentrations of vanadate (<200 μM) on insulin binding, processing, and action in rat adipocytes (36). Vanadate increased insulin binding that was associated with a significant increase in sensitivity to insulin. At 200 μM, vanadate was also observed to preserve a large fraction of insulin-stimulated lipogenesis 30 min after removal of insulin. These interactions of vanadate with the biological activity of insulin were independent of the insulin-mimetic behavior of vanadate alone.

In this study, we examined in greater detail the effect of vanadate on the maintenance of the biological action of insulin. We demonstrate a dose-dependent effect of vanadate to prolong insulin-stimulated lipogenesis subsequent to the removal of insulin. The ability of insulin (0.34 nM) to stimulate its receptor kinase was enhanced threefold in the presence of vanadate. However, the rate of decay of the kinase activity was not altered. Maximum stimulation of the kinase by insulin 17.2 nM (100 ng/ml) resulted in a decay of lipogenesis similar to that observed with 0.34 nM insulin plus vanadate, and vanadate did not prolong insulin action further. These data support the hypothesis that the amplitude of the hormonal signal, i.e., the receptor kinase, in the face of spare receptors is reflected in the rate of decay of the biological response and provides another mechanism to explain the ability of low concentrations of vanadate to lower blood glucose in vivo.

RESEARCH DESIGN AND METHODS

Porcine insulin was a gift from Nordisk (Gentofte, Denmark). Aprotinin, bovine serum albumin (BSA), HEPES, *N*-acetyl-D-glucosamine, phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate (vanadate), Triton X-100, antiphosphotyrosine (P-tyr) antibody, and the synthetic tyrosine kinase substrate (Poly[Glu/Tyr], 4:1) were purchased from Sigma (St. Louis, MO). Wheat germ agglutinin (WGA) coupled to agarose and protein A-sepharose CL-4B were obtained from Pharmacia (Uppsala, Sweden). Collagenase (type I) was from Worthington (Mississauga, Ontario). [^{32}P]ATP (specific activity 289 Ci/mmol), [3-^{3}H]glucose (specific activity 10.8 Ci/mmol), and Aquasol-II were supplied by DuPont Canada-New England Nuclear (Lachine, Quebec). Anti-insulin receptor β-subunit (anti-960) antibody was a gift from Dr. B. Posner (McGill University, Montreal).

Preparation of adipocytes. Adipocytes were isolated as described previously (36,37) with minor modifications. Male Sprague-Dawley rats weighing 160–220 g were killed by cervical dislocation. Epididymal fat pads were removed and placed into Sarstedt 50 ml polypropylene centrifuge tubes containing Krebs-Ringer bicarbonate–HEPES buffer (KRBH) (in mM: 118.5 NaCl, 4.7 KCL, 2.5 $CaCl_2$, 1.2 KH_2PO4, 1.2 $MgSO_4$, 29.2 $NaHCO_3$, and 30.6 HEPES) supplemented with 20 mg/ml BSA, 5 mM glucose, and 1 mg/ml collagenase at pH 7.4. The tissue was finely minced and bubbled with 95% O_2-5% CO_2 for 5 min and then allowed to shake gently for 1 h at 37°C. The digested tissue was filtered through nylon mesh (1,000 μm), centrifuged at 500 rpm, and washed four times in the buffer described above without collagenase.

Effect of various doses of vanadate on maintenance of insulin-stimulated lipogenesis. Isolated adipocytes were incubated in the presence of vanadate (0–100 μM) for 1 h at 37°C. Lipogenesis was maximally stimulated by exposure to 1.7 nM (10 ng/ml) insulin during the final 15 min, after which lipogenesis was assayed as described below without or with removal of all hormone by a series of washes. The cell suspension was centrifuged and the infranatant aspirated. The adipocytes were washed twice and incubated in glucose- and insulin-free KRH buffer (KRBH without $NaHCO_3$), pH 7.0, supplemented with 1 mM pyruvate and 1.5% BSA for a total of 20 min at 37°C. The cells were subsequently washed twice and incubated for an additional 10 min at 37°C in the KRBH buffer at pH 7.6. Vanadate concentrations were maintained at constant levels through all incubations, washes, and the lipogenesis assay.

After preincubation with vanadate, exposure to insulin, and repeated washes, lipogenesis activity was assayed as described by Moody et al. (38). The adipocytes (5×10^5 cells) were suspended in KRBH (pH 7.6) with 1.5% BSA, 0.5 mM glucose, and [3-^{3}H]glucose (0.33 μCi) at 37°C. The incubation was terminated after 15 min by the addition of 15 ml Econofluor and followed by vigorous shaking at 22°C. After overnight extraction at 22°C, radioactivity was measured in an LKB β-scintillation counter. Counts were converted to nanomoles of glucose incorporated into lipid per 10^5 cells and expressed as a percentage of the control value.

Effect of vanadate on reversibility of insulin-stimulated lipogenesis. To determine the time course of decay of insulin-stimulated lipogenesis, a protocol similar to that described above was followed. Isolated adipocytes were incubated in the absence or presence of 100 μM vanadate for 60 min at 37°C and for an additional 15 min with or without 0.34 nM (2 ng/ml) insulin, a concen-

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/43/3/375/360644/43-3-375.pdf by guest on 27 March 2025

tration that consistently resulted in maximum insulin-stimulated lipogenesis but did not result in maximum receptor occupancy (36). The cell suspension was centrifuged, and the infranatant was aspirated. The adipocytes were then washed twice and incubated in glucose- and insulin-free KRH (pH 7.0) with 1.5% BSA for various times (0–120 min) at 37°C. The cells were subsequently washed twice and stabilized for 10 min at 37°C in KRBH buffer (pH 7.6) after which lipogenesis was assayed exactly as described above. Exposure to 100 μM vanadate was maintained through all incubations and washes and through the lipogenesis assay in adipocytes initially preincubated with vanadate.

Solubilization and lectin purification of insulin receptors. To investigate the effect of vanadate on insulin-stimulated receptor kinase activity, isolated adipocytes (2×10^7 cells) were preincubated with or without 100 μM vanadate for 60 min at 37°C and then with 0.34 nM insulin for 15 min as described above. In an additional set of samples, 100 μM vanadate was added 1 min before the termination of the incubation. The cell suspension was centrifuged, and the infranatant was aspirated. The adipocytes were then washed twice and incubated in glucose- and insulin-free KRH (pH 7.0) with 1 mM pyruvate and 1.5% BSA for various times (0–50 min) at 37°C. The cells were subsequently washed twice and stabilized at 37°C in KRBH buffer (pH 7.6) for an additional 10 min, except for the 10-min sample that was re-equilibrated at pH 7.6 for 8 min after 2 min at pH 7.0. At the times indicated, ice-cold solubilization buffer (1.0% Triton X-100, 4 mM EDTA, 2 mM NaF, 1 mM PMSF, 1 trypsin inhibitor U/ml of aprotinin, and 30 mM HEPES, pH 7.6) was added, and the cells were immediately frozen for 1 h at −70°C (9). The adipocytes were subsequently thawed, homogenized, and solubilized for an additional 1 h at 4°C. After removal of the fat cake by centrifugation at 1,800 *g* for 10 min, the cell extract was centrifuged further at 100,000 *g* for 1 h at 4°C. The supernatant was applied to a 1 ml column of WGA-agarose, which was then washed with 50 ml of 50 mM HEPES buffer (at pH 6.0) containing 150 mM NaCl and 0.1% Triton X-100 followed by 25 ml of the same buffer at pH 7.6. This pH 7.6 buffer, supplemented with 0.3 M *N*-acetyl-D-glucosamine, was used to elute the insulin receptor. ^{125}I-labeled insulin, iodinated to a specific activity of ~1.1–1.2 mCi/mmol, was used to determine insulin binding to the lectin-purified extract as described previously (9).

Insulin receptor tyrosine kinase (IRK) activity. A procedure similar to that reported previously was followed (7,9). Lectin-purified insulin receptor (10–20 fmol of insulin binding) was placed in a buffer at 22°C containing 50 mM HEPES, 2 mM $MnCl_2$, 15 mM $MgSO_4$, 2.5 mg/ml Poly[Glu/Tyr] (4:1), and 5 μM [γ-^{32}P]ATP (5 μCi/tube, pH 7.6) in a total volume of 160 μl. After 10 min, the reaction was terminated by spotting 100 μl of the reaction mixture on a Whatman no. 3 filter paper, which was then placed in 10% trichloroacetic acid containing 10 mM sodium pyrophosphate. After extensive washing in this solution, the paper was dried and placed in 20 ml of Aquasol-II for determination of radioactivity in an LKB β-scintillation counter.

P-tyr antibody immunoblots. The above-described protocol for the assessment of the effect of 100 μM vanadate on insulin-stimulated receptor tyrosine kinase activity was followed for determination of the extent of in situ insulin receptor tyrosine phosphorylation stimulated by insulin. Thus one set of adipocytes was incubated with and without insulin alone for 15 min, one set preincubated with vanadate for 60 min, and a third set with vanadate added 1 min before the termination of the incubation.

The reaction was stopped by rapid freezing of the adipocytes after addition of an ice-cold solution of 1% Triton X-100, 50 mM HEPES (pH 7.6), 4 mM EDTA, 1 trypsin inhibitor U/ml aprotinin, 20 mM NaF, and 1 mM PMSF (final concentrations). The cells were thawed and homogenized with the addition to all samples of 1 mM vanadate. A second set of experiments was performed with the modification of stopping the reaction by immediate freezing of the samples in liquid nitrogen. The above solubilization buffer supplemented with 1 mM vanadate was added to all samples that were subsequently thawed and solubilized. Insulin receptors were partially purified as above, and an aliquot was submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (9).

After electrophoretic transfer of proteins from the gels to nitrocellulose membranes, the latter were washed and blocked with 50 ml of phosphate-buffered saline (PBS), pH 7.4, containing 10% fetal bovine serum (FBS) for 1 h at 22°C. The solution was replaced with 50 ml of a 1:1,000 dilution of affinity purified anti-P-tyr antibody in PBS (pH 7.4), containing 10% FBS and incubated for 2 h at 22°C as described (39). The membranes were washed and incubated with 50 ml of ^{125}I-labeled goat anti-mouse antibody (25,000 cpm/100 μl) in PBS containing 10% FBS for 1 h at 22°C. The membranes were washed, dried, and fixed. Labeled phosphotyrosine-containing proteins were visualized by autoradiography and intensities of the 95 kD bands determined by densitometry. Receptor content was determined by immunoblotting with antibodies against the 960 region of the β-subunit. The above procedure used for the anti-P-tyr immunoblots was modified by using a solution of PBS (pH 7.4), with 3% Carnation milk for blocking followed by incubation with 1:500 dilution of anti-960 antibody in PBS with 3% Carnation milk for 2 h at 22°C. The incubation with labeled ^{125}I-labeled goat anti-rabbit antibody was also conducted in PBS, 3% milk. Radioactivity was quantified as above by densitometry of exposed films.

Dissociation of insulin. To determine whether vanadate had any effect to alter the rate of insulin dissociation from its receptor during the wash procedure, adipocytes were incubated with ^{125}I-labeled insulin (0.34 nM) for the identical times and under the conditions described above. The cells were exposed or not exposed to 100 μM vanadate for 60 min followed by addition of labeled hormone for 15 min. Total cell-associated radioactivity was determined by centrifugation through oil as described previously (36), and nonspecific binding was determined in the presence of excess 0.9 μM unlabeled insulin. After the 15-min incubation with ^{125}I-labeled insu-

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/43/3/375/360644/43-3-375.pdf by guest on 27 March 2025

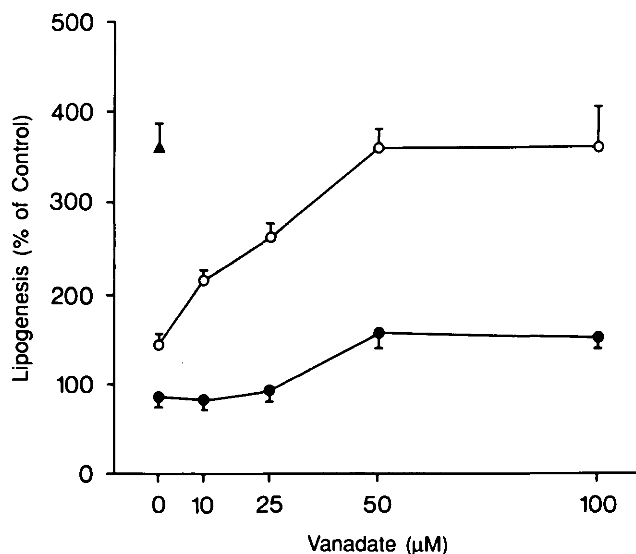

FIG. 1. Dose-dependent effect of vanadate on the maintenance of insulin-stimulated lipogenesis in rat adipocytes. Freshly isolated rat adipocytes were incubated in the absence and presence of the indicated concentrations of vanadate for 1 h at 37°C. The cells were further treated (○) or not treated (●) with 1.7 nM insulin for 15 min. The cells were then washed for 20 min in KRH buffer with 1 mM pyruvate and 1.5% BSA at pH 7.0 to remove cell-associated insulin and for an additional 10 min in KRBH and 1.5% BSA at pH 7.6. Lipogenesis was subsequently assayed as described in METHODS and is expressed as a percentage of that measured in the absence of insulin and vanadate before the wash (100%). Maximum lipogenesis in the presence of 1.7 nM insulin, i.e., without washing, are represented (▲). Data are mean ± SE of 4–10 separate experiments each performed in triplicate. The residual insulin-stimulated lipogenesis after 30 min of wash was significantly augmented by vanadate (10 μM, $P < 0.01$; 25 μM, 50 μM, and 100 μM, $P < 0.001$).

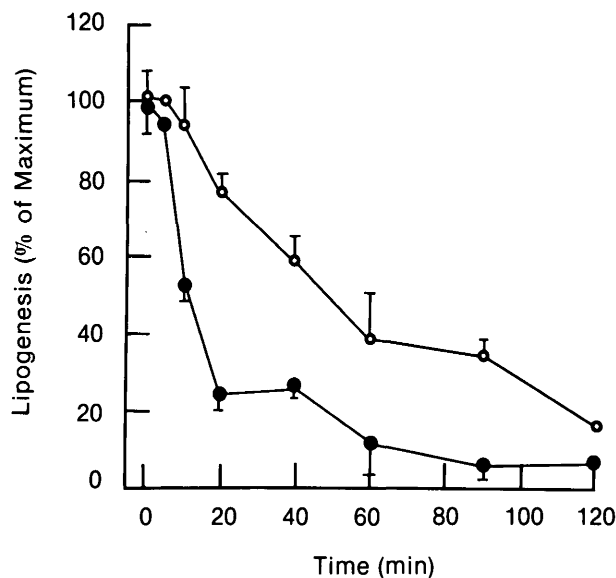

FIG. 2. Effect of vanadate on the decay of insulin-stimulated lipogenesis. Freshly isolated rat adipocytes were incubated in the absence (●) or presence (○) of 100 μM vanadate for 1 h at 37°C. Insulin (0.34 nM) or buffer alone was added for an additional 15 min. The adipocytes were then washed for the various times indicated (0–120 min) in insulin-free KRH at pH 7.0 and for an additional 10 min in KRBH at pH 7.6 as described in METHODS. Lipogenesis was subsequently assayed and is expressed as percentage of insulin-stimulated lipogenesis before the wash. In all experiments, basal activity in the absence or presence of vanadate has been subtracted so that only insulin-stimulated activity is shown. Data are means ± SE of 3–6 separate experiments each performed in triplicate. The rates of decay in the absence and presence of vanadate were significantly different ($P = 0.03$). The $t_{1/2}$ of the decay in insulin-stimulated lipogenesis was increased by vanadate from 21 to 59 min. Note that the time plotted indicates the duration of the pH 7.0 wash, and the 10-min equilibration time in pH 7.6 buffer was added to determine the $t_{1/2}$ values.

lin, aliquots of the adipocytes were washed as described for various times in glucose and insulin-free KRH (pH 7.0) and 1.5% BSA, then re-equilibrated for 10 min in KRBH (pH 7.6). Residual specific cell-associated radioactivity was plotted versus total time of the washes as a percentage of that determined before washing ($t = 0$).

Statistical analysis. Data are presented as means ± SE. Significant differences for individual values were determined by paired or unpaired Student's *t* test (two-tailed). In each study of the decay rate (lipogenesis and tyrosine kinase activity), within- and between-study parameters were analyzed using a repeated measures analysis of variance (ANOVA). The $t_{1/2}$ (half-time) values were determined by direct graphic analysis of the decay curves without assumptions. Statistical significance was accepted at the level of $P < 0.05$.

RESULTS

Dose-dependent prolongation of insulin action by vanadate. Using the experimental protocol described in METHODS, preincubation of rat adipocytes with vanadate resulted in a significant inhibition of the loss of insulin-stimulated lipogenesis after a 30-min wash to remove insulin (Fig. 1). Insulin alone stimulated lipogenesis to 361 ± 29% of control adipocytes. This decreased after the wash procedure to 130 ± 15%. Preexposure to vanadate resulted in a dose-dependent prolongation of insulin action observed with as little as 10 μM and reaching maximum at 50 μM (361 ± 20% after wash, Fig. 1). Note that vanadate itself had only a small insulin-mimetic effect at these concentrations (a maximum of 140 ± 12%) and that the stimulation by vanadate was subtracted from the insulin plus vanadate value in every experiment. The residual insulin-stimulated portion of lipogenesis was significantly greater in the presence of vanadate than in its absence at all concentrations (10–100 μM, Fig. 1).

Effect of vanadate on the rate of decay of insulin-stimulated lipogenesis. To determine the effect of vanadate on the prolongation of insulin action, adipocytes were incubated in the presence or absence of 100 μM vanadate and then incubated for 15 min with 0.34 nM insulin. The subsequent wash time was varied from 0 to 120 min in pH 7.0 after which a 10-min incubation in pH 7.6 buffer was performed to re-equilibrate the cells before the assay. The decay of insulin-stimulated lipogenesis was significantly prolonged in the presence of vanadate ($P = 0.03$, repeated measures ANOVA; Fig. 2). The $t_{1/2}$ of the decay in insulin action in control cells was 21 min, whereas that in the presence of vanadate was increased to 59 min. As in the previous set of experiments, the insulin-mimetic effect of vanadate itself was

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/43/3/375/360644/43-3-375.pdf by guest on 27 March 2025

measured and subtracted at all times in every experiment.

Effect of vanadate on IRK activity. The documented inhibitory effect of vanadate on protein phosphotyrosine phosphatase lead us to postulate that the observed prolongation of insulin action by vanadate may be mediated by an alteration of IRK activity. As described in METHODS, the cells had received one of three separate treatments: *1*) no exposure to vanadate at any time, *2*) preexposure to 100 μM vanadate for 60 min, and *3*) exposure to 100 μM vanadate 1 min before freezing the adipocytes. An additional protocol was included in some experiments in which vanadate was absent during the incubations and washes (treatment 1) but added after freezing the sample, i.e., during homogenization and receptor preparation. The addition of vanadate at this stage yielded the same results as treatment 1 (data not shown).

In the presence of vanadate (treatment 2), the ability of insulin to stimulate its receptor kinase was enhanced more than threefold compared with cells not exposed to vanadate (treatment 1, 319.0 ± 18.7% of insulin alone). When vanadate was added 1 min before the freezing (treatment 3), the IRK activity was also increased compared with insulin treatment alone and approached that of the cells pretreated for 60 min with vanadate (242 ± 29% of insulin alone (Fig. 3). This suggested a very rapid effect of vanadate to inhibit tyrosine dephosphorylation.

In contrast to the effect of vanadate on the decay of insulin-stimulated lipogenesis, the $t_{1/2}$ of the decay in receptor tyrosine kinase activity after removal of insulin was not altered by vanadate ($P = 0.77$, repeated measures ANOVA). Thus the $t_{1/2}$ of deactivation was 6 min after insulin alone and 6.8 min after insulin plus vanadate. Note that, because the decay of IRK activity was very rapid, the $t_{1/2}$ values determined under these conditions should be considered semiquantitative.

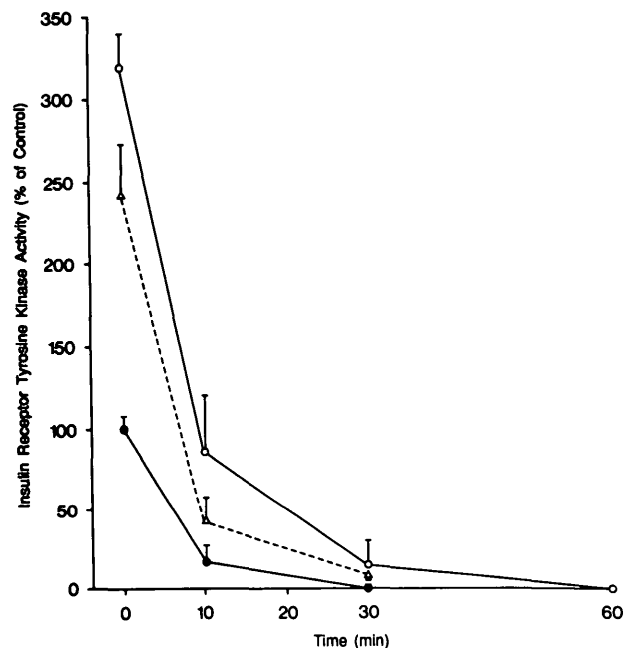

FIG. 3. Effect of vandate on IRK activity. Freshly isolated rat adipocytes were incubated in the absence (●) or presence (○) of 100 μM vanadate for 1 h at 37°C. Insulin (0.34 nM) or buffer alone was added for an additional 15 min. Vanadate (100 μM) was added to a third set of adipocytes (△) 1 min before the termination of the incubations. The cells were then washed in insulin-free KRH at pH 7.0 for 0–50 min and for an additional 8–10 min in KRBH at pH 7.6. At the times indicated, the reactions were terminated by the addition of ice-cold solubilization buffer and immediate freezing to −70°C as described in METHODS. Cells were homogenized and solubilized, insulin receptors were partially purified, and tyrosine kinase activity was assayed. Data are means ± SE of 3–6 separate experiments each performed in triplicate and expressed as a percentage of insulin-stimulated tyrosine kinase activity assayed before the wash. The decay rate in the presence and absence of vanadate was not significantly different. The $t_{1/2}$ of the decay in IRK activity was 6 min in control sample, 6 min with vanadate added for 1 min, and 6.8 min with vanadate added for 1 h.

Vanadate enhances in situ insulin-stimulated insulin receptor phosphotyrosine content. To correlate the augmentation of insulin-stimulated tyrosine kinase activity by vanadate with receptor tyrosine autophosphorylation in the intact cells, the adipocytes were treated with the identical three protocols as described above, and the reaction stopped without washing ($t = 0$). The P-tyr content of the β-subunit corrected for receptor concentration was not altered by vanadate alone (Fig. 4). Insulin (0.34 nM) increased P-tyr to 137% of control adipocytes. In the presence of vanadate (60-min preincubation), insulin increased P-tyr to 372% and with vanadate added 1 min before the termination of the assay, P-tyr content was 289% of control adipocytes (Fig. 4).

To determine that the augmented insulin-stimulated P-tyr incorporation in the presence of in vivo vanadate was not caused simply by inhibition of insulin receptor dephosphorylation during solubilization, the experiments were repeated (treatments 1 and 2), and the reactions were terminated by freezing of the cells in liquid nitrogen. All samples were subsequently thawed and solubilized in a buffer containing 1 mM vanadate. Stimulation of P-tyr incorporation into the B-subunit by 0.34 nM insulin was 156% in the absence and 345% in the presence of 100 μM vanadate (densitometry, arbitrary units: control 0.9, insulin 1.4, vanadate 1.1, vanadate plus insulin 3.8; data not shown).

Effect of insulin concentration on the decay of insulin-stimulated lipogenesis. The lack of increase of the $t_{1/2}$ of the decay in IRK activity but markedly augmented activity in the presence of vanadate plus insulin led us to test whether the increased amplitude of the signal was an important factor that determined the duration of lipogenesis. To increase the IRK activity to approximately the level observed with 0.34 nM insulin plus vanadate, adipocytes were incubated with 17.2 nM insulin. Insulin dose-response curves demonstrated that this concentration of insulin activates the IRK to a similar extent, which is at maximum (9). The decay in lipogenesis was assayed in the presence and absence of vanadate as described; 20 min after removal of 0.34 nM insulin, the presence of vanadate significantly increased lipogenesis (Table 1). Insulin alone at 17.2 nM resulted in a similar rate of decay as 0.34 nM insulin in the presence of vanadate. Furthermore, the addition of vanadate did not result in any prolongation of insulin action after stimulation with 17.2

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/43/3/375/360644/43-3-375.pdf by guest on 27 March 2025

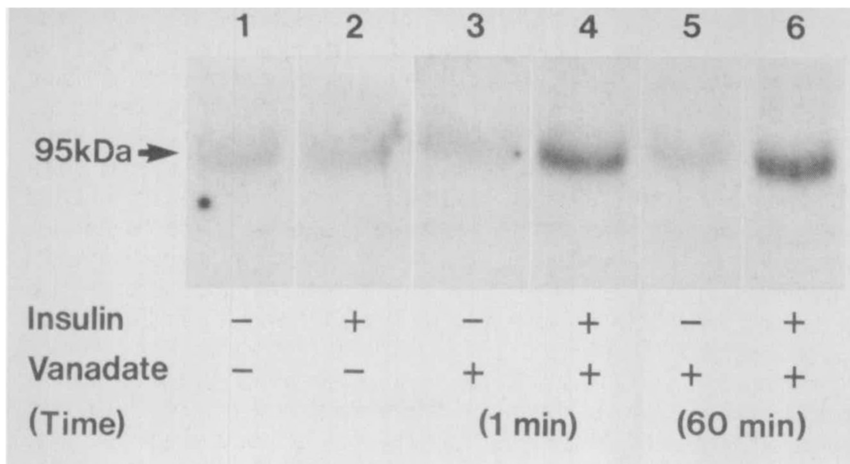

FIG. 4. Effect of vanadate on phosphotyrosine incorporation into the insulin receptor β-subunit. Adipocytes were incubated in the absence or presence of vanadate for 1 min or 60 min and with and without insulin for 15 min as described in Fig. 3. At the end of the incubation, the reaction was terminated by the addition of ice-cold stopping solution and immediate freezing to −70°C. Cells were thawed, homogenized, and receptors solubilized. Insulin receptors were immunoprecipitated and separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and immunoblotted with antiphosphotyrosine antibodies as described in METHODS. The intensities of the 95 kD bands were determined by densitometry and corrected for receptor content determined by immunoblotting a second membrane with anti-β-subunit antibody as described (not shown). The effect of vanadate on P-tyr incorporation was similar to that observed on tyrosine kinase activity. The relative intensities in arbitrary units were: 2.03 (*lane 1*), 2.79 (*lane 2*), 1.87 (*lane 3*), 5.41 (*lane 4*), 1.73 (*lane 5*), and 6.43 (*lane 6*). Similar results were obtained in two separate experiments.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/43/3/375/360644/43-3-375.pdf by guest on 27 March 2025

nM insulin at 20, 30, or 40 min after removal of insulin (Table 1).

Lack of effect of vanadate on insulin removal. Because we (36) and others (40) demonstrated previously that under certain conditions vanadate can increase insulin binding, the prolongation of biological response was possibly associated with an additional effect of vanadate to inhibit the removal of insulin from the adipocytes by the series of washes. Incubation of the adipocytes with or without exposure to 100 μM vanadate followed by ^{125}I-labeled 0.34 nM insulin for 15 min at 37°C followed by washing, as described for the biological responses, revealed that vanadate did not alter the removal of insulin (Fig. 5). Thus after 10 min (2 min, pH 7.0, and 8 min, pH 7.6) of washing 12.6 ± 2.05 and 13.5 ± 2.39% of initial ^{125}I-labeled insulin remained cell-associated in the absence and presence of vanadate, respectively. After 30 min of washing, these values were 3.8 ± 0.95 and 4.9 ± 1.54%. Note that vanadate did not increase the specific cell-associated ^{125}I-labeled insulin before washing. Insulin binding was 7.3 ± 0.26%/ 5 × 10^5 adipocytes in the absence and 7.1 ± 0.81%/ 5 × 10^5 adipocytes in the presence of vanadate. This is consistent with the lower concentration of vanadate and higher concentration of insulin used in these experiments compared with our earlier studies (36).

TABLE 1
Effect of insulin concentration on the decay of insulin action

Vanadate (100 μM)	Insulin (nM)	Insulin-stimulated lipogenesis (% of initial insulin)		
		20 min	30 min	40 min
Without	0.34	39.8 ± 18	26.5 ± 14	ND
With		95.5 ± 26*	70.8 ± 23*	ND
Without	17.2	98.5 ± 29	76.9 ± 24	55.7 ± 20
With		57.7 ± 14†	57.8 ± 24†	43.8 ± 13†

Data are means ± SE (n = 4) and expressed as the percentage of insulin-stimulated activity measured at the end of incubation before removal of hormone. Freshly isolated rat adipocytes were incubated in the presence and absence of 100 μM vanadate for 60 min in KRBH buffer. Insulin (0.34 or 17.2 nM) was then added for 15 min. Lipogenesis was assayed immediately, or the cells were washed and incubated for an additional 10, 20, or 30 min in KRH (pH 7.0) followed by 10 min in KRBH (pH 7.6). Lipogenesis was then assayed as described in METHODS. Vanadate significantly increased lipogenesis with 0.34 nM insulin but not with 17.2 nM insulin. ND, not determined.
*$P < 0.05$ in the presence compared with absence of vanadate.
†P = NS in the presence compared with absence of vanadate.

DISCUSSION

It has been postulated that the insulin-mimetic effects of vanadate are mediated by its inhibition of phosphotyrosine phosphatase activity, thereby leading to an augmentation of tyrosine phosphorylation and tyrosine kinase activity of the insulin receptor (1,2). We observed that, after removal of insulin, lipogenesis remained significantly higher in the presence of 200 μM vanadate (36). In this study, a dose-dependent effect of vanadate to prolong insulin-stimulated lipogenesis in rat adipocytes was demonstrated. This effect on prolongation was maximum at 50 μM and was significant at vanadate concentrations as low as 10 μM, the range measured in serum of diabetic rats treated with oral vanadate (26). The $t_{1/2}$ of the decay in lipogenesis was increased from 21 to 59 min by vanadate after stimulation by 0.34 nM insulin. Because high concentrations of vanadate were demonstrated previously to increase insulin binding and insulin sensitivity (36,40), the possibility that the prolongation of lipogenesis was related to an increase in residual cell-associated insulin required examination. During the conditions of these experiments, i.e., lower vanadate concentration and higher insulin concentration, no significant effect of vanadate on insulin binding was noted. Furthermore, a similar amount of insulin was removed by the series of washes in the presence and absence of vanadate. After the 30-min wash, 3.8 ± 0.95% (control) and 4.9 ± 1.54% (with vanadate) of the initial insulin remained cell-associated. This amount of cell-bound insulin would be expected in incubations

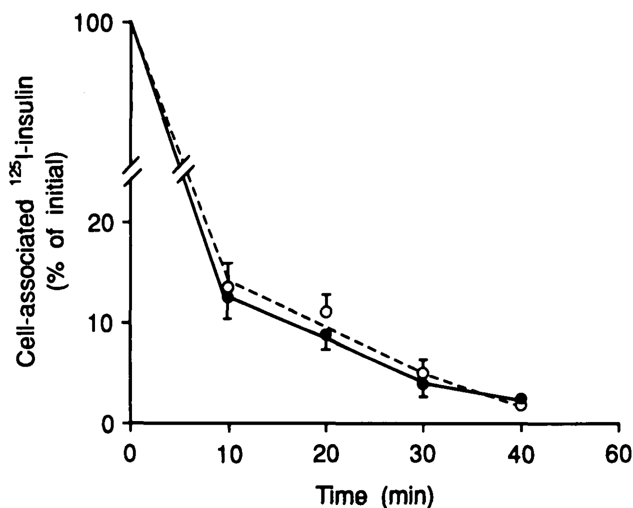

FIG. 5. Effect of vanadate on insulin removal by repeated washing. Freshly isolated rat adipocytes were incubated in the absence (●) and presence (○) of 100 μM vanadate for 1 h at 37°C and 0.34 nM ^{125}I-labeled insulin subsequently added for 15 min with and without excess 0.9 μM unlabeled insulin. Aliquots were removed (t = 0) and centrifuged through oil to determine total and nonspecific cell-associated ^{125}I-labeled insulin. The remaining cells were washed for various times from 2 to 30 min in pH 7.0 KRH buffer followed by 10 min in pH 7.6 KRBH as described in METHODS. Specific cell-associated ^{125}I-labeled insulin was determined by subtracting nonspecific from total cell-bound radioactivity and plotted as percentage of specific cell-bound ^{125}I-labeled insulin at t = 0 versus total time of washing. Data are means ± SE of 5 (t = 0, 10, or 20 min) or 3 (t = 30 or 40 min) separate experiments performed in duplicate. Specific ^{125}I-labeled insulin bound at t = 0 was 7.3 ± 0.26%/5 × 10^5 adipocytes in the absence and 7.1 ± 0.81% in the presence of vanadate.

containing medium insulin concentrations of ~6.9 pM (control) and 8.6 pM (vanadate) and could not account for the extent of stimulation of lipogenesis at this time either in the absence or presence of vanadate (Fig. 2) (36). Previous investigators have reported a significant delay in the decay of insulin-stimulated glucose transport in comparison to dissociation of hormone (41,42).

Several studies have questioned the mechanism of vanadate action. A number of investigators have demonstrated insulin-like actions in vitro (43,44) and in vivo (33,34) without IRK activation. To determine whether this prolongation effect was related to IRK activity, we assessed the effect of vanadate. In contrast to lipogenesis, vanadate did not prolong the decay of insulin-stimulated IRK activity; $t_{1/2}$ of decay was 6 min in the absence and 6.8 min in the presence of vanadate. However, IRK activity was markedly augmented in the presence of vanadate. Furthermore, this increase correlated with an increase in phosphotyrosine content of the insulin receptor β-subunit isolated from intact cells. The latter finding is consistent with an inhibitory effect of vanadate on a tyrosine phosphatase that dephosphorylates the insulin receptor (22). However, the return to basal activity of both IRK activity and lipogenesis indicates that this inhibitory effect is partial. This may explain the apparent lack of prolongation of the IRK. Thus, dephosphorylation of the receptor in rat adipocytes has been demonstrated by Mooney and Anderson (45) to be very rapid with a $t_{1/2}$ of 21 s. vanadate (1 mM) slowed dephosphorylation by 25% so that the rate of dephosphorylation in the presence of vanadate would still be rapid in comparison with the rate of dissociation of the hormone from its receptor. The latter is in the range of several minutes in rat adipocytes (36). Therefore, under the conditions of our experiments the rate of decay of IRK activity was probably determined by the rate of insulin dissociation from its receptor rather than dephosphorylation. To confirm the rapid dephosphorylation rate, the effect of vanadate added 1 min before the termination of the insulin incubation was determined. The augmentation of IRK activity and receptor P-tyr content was close to that observed with preincubation with vanadate for 60 min. A similar time course and relationship to hormone dissociation of the decay in IRK activity in rat adipocytes was noted by Klein et al. (46).

In view of these results, we considered the possibility that the prolongation of insulin-stimulated lipogenesis by vanadate was secondary to the absolute increase in IRK. It has been suggested that, in the presence of spare receptors, stimulation by hormone concentrations greater than that required to achieve a maximum biological response results in a prolonged duration of action (47,48). Haring et al. (41) reported that the decay in insulin-stimulated glucose transport correlated with the activating concentration of insulin. We found that the rate of decay of lipogenesis after stimulation by 17.2 nM insulin was slower than that observed with 0.34 nM insulin and similar to that observed after stimulation with 0.34 nM insulin in the presence of vanadate. The similar decay rates were associated with a similar extent of stimulation of IRK activity. Furthermore, vanadate did not alter the decay rate with 17.2 nM insulin, which results in maximum IRK activation. Our data support the hypothesis that the augmentation of IRK activity by vanadate results in the transduction of the amplitude of the signal, i.e., IRK, into duration of the final metabolic response, lipogenesis. These results also are consistent with vanadate acting via a partial inhibition of protein tyrosine phosphatase activity. The mechanism of insulin signaling (16,19,49) and the deactivation process (42,48) involve other biochemical events distal to the receptor. It is likely that the concentration or activity of an intermediary signaling molecule is regulated by the IRK and does not manifest spareness. The rapid loss of IRK activity in relation to lipogenesis suggests that this molecule has a longer decay time than the IRK and may not be regulated by tyrosine dephosphorylation.

Clearly, vanadate lowers circulating glucose concentrations in rodent models of diabetes. The relative importance of the direct insulin-mimetic effects of vanadate compared with the enhancement of insulin sensitivity and prolongation of insulin action is not clear. In the hypoinsulinemic STZ-injected rat model, the latter may be significant.

In summary, this study shows that vanadate prolongs insulin-stimulated lipogenesis in isolated rat adipocytes. This effect is apparent at a concentration of 10 μM and is maximal at 50 μM. This action appears to be secondary to a marked increase in IRK activity in the presence of vanadate, despite the fact that the half-life of IRK activity

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/43/3/375/360644/43-3-375.pdf by guest on 27 March 2025

is not altered by this agent during these conditions. The data are consistent with a mechanism of action of vanadate involving partial inhibition of protein tyrosine phosphatase and supports the hypothesis that in the presence of spare receptors the amplitude of signaling is transduced into duration of biological response.

ACKNOWLEDGMENTS

This work was supported by a grant from the Medical Research Council of Canada (MT-7658) to I.G.F. I.G.F. was a recipient of a Chercheur Boursier Scholarship from the Fonds de la Recherche en Santé du Québec. F.A. was supported by a Medical Research Council (Canada) summer studentship.

The authors thank Dr. J. Miller for help with the statistical analysis and B. Baubinas for secretarial assistance.

REFERENCES

1. Shechter Y: Insulin-mimetic effects of vanadate: possible implications for future treatment of diabetes. *Diabetes* 39:1–5, 1990
2. Posner BI, Shaver A, Fantus IG: Insulin mimetic agents: vanadium and peroxovanadium compounds. In *New Antidiabetic Drugs*. Bailey CJ, Flatt PR, Eds. London, Smith-Gordon, 1990, p. 107–18
3. Shechter Y, Meyerovitch J, Amir S: The use of post-binding agents in studying insulin action and its relation to experimental diabetes. *Biochem Pharmacol* 37:1891–96, 1988
4. Tolman EL, Barris I, Burns M, Pansini A, Patridge R: Effects of vanadium on glucose metabolism in vitro. *Life Sci* 25:11159–64, 1979
5. Dubyak GR, Kleinzeller A: The insulin-mimetic effects of vanadate in isolated rat adipocytes. *J Biol Chem* 255:5306–12, 1980
6. Shechter Y, Karlish SJD: Insulin-like stimulation of glucose oxidation in rat adipocytes by vanadyl (IV) ions. *Nature* 284:556–58, 1980
7. Kadota SI, Fantus IG, Deragon G, Hersh B, Guyda HJ, Posner BI: Stimulation of IGF-II receptor binding and insulin receptor kinase activity in rat adipocytes: effects of vanadate and H_2O_2. *J Biol Chem* 262:8252–56, 1987
8. Duckworth WC, Solomon SS, Liepnieks J, Hamel FG, Hand S, Peavy DE: Insulin-like effects of vanadate in isolated rat adipocytes. *Endocrinology* 122:2285–89, 1988
9. Fantus IG, Kadota SI, Deragon G, Foster B, Posner BI: Pervanadate (peroxides of vanadate) mimics insulin action in rat adipocytes via activation of the insulin receptor tyrosine kinase. *Biochemistry* 28:8864–71, 1989
10. Tamura S, Brown TA, Whipple JH, Fujita-Yamaguchi Y, Dubler RE, Cheng K, Larner J: A novel mechanism for the insulin-like effect of vanadate on glycogen synthase in rat adipocytes. *J Biol Chem* 259:6650–58, 1984
11. Degani HM, Gochin M, Karlish SJD, Shechter Y: Electron parameagnetic resonance studies and insulin-like effects of vanadium in rat adipocytes. *Biochemistry* 20:5795–99, 1981
12. Clark A, Fagan JM, Mitch WE: Selectivity of the insulin-like actions of vanadate on glucose and protein metabolism in skeletal muscle. *Biochem J* 232:273–76, 1985
13. Jackson TK, Salhanick AI, Sparks JD, Sparks CE, Bolognini M, Amatruda JM: Insulin-mimetic effects of vanadate in primary cultures of rat hepatocytes. *Diabetes* 37:1234–40, 1988
14. Seglen PO, Gordon PB: Vanadate inhibits protein degradation in isolated rat adipocytes. *J Biol Chem* 256:7699–7701, 1981
15. Morgan DO, Ho L, Korn LJ, Roth RA: Insulin action is blocked by a monoclonal antibody that inhibits the insulin receptor kinase. *Proc Natl Acad Sci USA* 83:328–32, 1986
16. Rosen OM: After insulin binds. *Science* 237:1452–58, 1987
17. Tornqvist HE, Gunsalus JR, Nemenoff RA, Frackelton AR, Pierce MW, Avruch J: Identification of the insulin receptor tyrosine residues undergoing insulin-stimulated phosphorylation in intact rat hepatoma cells. *J Biol Chem* 263:350–59, 1988
18. White MF, Shoelsen SE, Keutmann H, Kahn CR: A cascade of tyrosine autophosphorylation in the B-subunit activates the phosphotransferance of the insulin receptor. *J Biol Chem* 263:2969–80, 1988
19. Sun XJ, Rothenberg P, Kahn CR, Backer JM, Araki E, Wilden PA, Cahill DA, Goldstein BJ, White MF: Structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. *Nature* 352:73–77, 1991
20. Swarup G, Cohen S, Garbers DL: Inhibition of membrane phosphotyrosyl-protein phosphatase activity by vanadate. *Biochem Biophys Res Commun* 107:1104–109, 1982
21. Swarup G, Speeg KV Jr, Cohen S, Garbers DL: Phosphotyrosyl-protein phosphatase of TCR-2-cells. *J Biol Chem* 257:7298–301, 1982
22. Mooney RA, Bordwell KL: Differential dephosphorylation of the insulin receptor and its 160 kDa substrate (pp160) in rat adipocytes. *J Biol Chem* 267:14054–60, 1992
23. Nechay BR, Nanninga LB, Nechay PSE, Post RL, Granthan JJ, Macara IG, Kubena LF, Phillips TD, Nielsen FFH: Role of vanadium in biology. *Fed Proc* 45:123–32, 1986
24. Brown DJ, Gordon JA: The stimulation of pp60v-src kinase activity by vanadate in intact cells accompanies a new phosphorylation state of the enzyme. *J Cell Biol* 259:9580–86, 1984
25. Heyliger CE, Tahiliani AG, McNeill JH: Effect of vanadate on elevated blood glucose and depressed cardiac performance of diabetic rats. *Science* 227:1474–77, 1985
26. Meyerovitch J, Farfel Z, Sack J, Shechter Y: Oral administration of vanadate normalizes blood glucose levels in streptozotocin-treated rats: characterisation and mode of action. *J Biol Chem* 262:6658–62, 1987
27. Brichard SM, Okitolonda W, Henquin JC: Long-term improvement of glucose homeostasis by vanadate treatment in diabetic rats. *Endocrinology* 123:2048–53, 1988
28. Gil J, Miralpeix M, Cameras J, Bartrons R: Insulin-like effects of vanadate on glucokinase activity and fructose 2,6-bisphosphate levels in the liver of diabetic rats. *J Biol Chem* 263:1868–71, 1988
29. Blondel O, Bailbe D, Portha B: In vivo insulin resistance in streptozotocin-diabetic rats: evidence for reversal following oral vanadate treatment. *Diabetologia* 32:185–90, 1989
30. Bendayan M, Gingras D: Effect of vanadate administration on blood glucose and insulin levels as well as on the exocrine pancreatic function in streptozotocin-diabetic rats. *Diabetologia* 32:561–67, 1989
31. Ramanadham S, Mongold JJ, Brownsey RW, Cros GH, McNeill JH: Oral vanadyl sulfate in treatment of diabetes mellitus rats. *Am J Physiol* 257:H904–11, 1989
32. Pederson RA, Ramanadham S, Buchan AMJ, McNeill JH: Long-term effects of vanadyl treatment on streptozotocin-induced diabetes in rats. *Diabetes* 38:1390–95, 1989
33. Venkatesan N, Avidan A, Davidson MB: Antidiabetic action of vanadyl in rats independent of in vivo insulin-receptor kinase activity. *Diabetes* 40:492–98, 1991
34. Meyerovitch J, Rothenberg P, Shechter Y, Bonner-Weir S, Kahn CR: Vanadate normalizes hyperglycemia in two mouse models of non-insulin-dependent diabetes mellitus. *J Clin Invest* 87:1286–94, 1991
35. Brichard SM, Pottier AM, Henquin JC: Long-term improvement of glucose homeostasis by vanadate in obese hyperinsulinemic *fa/fa* rats. *Endocrinology* 125:2510–16, 1988
36. Fantus IG, Ahmad F, Deragon G: Vanadate augments insulin binding and prolongs insulin action in rat adipocytes. *Endocrinology* 127:2716–25, 1990
37. Rodbell M: Metabolism of isolated fat cells: effects of hormones in glucose metabolism and lipolysis. *J Biol Chem* 239:375–80, 1964
38. Moody AJ, Stan MA, Stan M: A simple free fat cell bioassay for insulin. *Horm Metab Res* 56:12–16, 1974
39. Torossian K, Nower P, Schwartz T, Fantus IG: Phorbol esters inhibit insulin-induced receptor down-regulation in cultured human lymphocytes: association with diminished insulin receptor autophosphorylation. *Biochem J* 290:151–58, 1993
40. Marshall S, Garvey WT, Monzon R: Shunting of insulin from a retroendocytotic pathway to a degradative pathway by sodium vanadate. *J Biol Chem* 262:12005–12, 1987
41. Haring HU, Biermann E, Kemmler W: Relation of insulin receptor occupancy and deactivation of glucose transport. *Am J Physiol* 242:E234–40, 1982
42. Ciaraldi TP, Olefsky JM: Relationship between deactivation of insulin-stimulated glucose transport and insulin dissociation in isolated rat adipocytes. *J Biol Chem* 255:327–30, 1980
43. Strout HV, Saperstein R, Salter EE: The insulin-mimetic effect of vanadate is not correlated with insulin receptor tyrosine kinase activity, nor phosphorylation in mouse diaphragm in vivo. *Endocrinology* 124:1918–24, 1989
44. Green A: The insulin-like effect of sodium vanadate on adipocyte glucose transport is mediated at a post-insulin-receptor level. *Biochem J* 238:663–69, 1986
45. Mooney RA, Anderson DL: Phosphorylation of the insulin receptor in permeabilized adipocytes is coupled to a rapid dephosphorylation reaction. *J Biol Chem* 264:6850–57, 1989
46. Klein HH, Freidenberg GR, Kladde M, Olefsky JM: Insulin activation of insulin receptor tyrosine kinase in intact rat adipocytes: an in vitro system to measure histone kinase activity of insulin receptors

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/43/3/375/360644/43-3-375.pdf by guest on 27 March 2025

activated in vivo. *J Biol Chem* 261:4691–97, 1986
47. Gliemann J, Gammeltoft S, Vinten J: Time course of insulin receptor binding and insulin-induced lipogenesis in fat cells. *J Biol Chem* 250:3368–74, 1975
48. Roth J, Grunfeld C: Mechanism of action of peptide hormones and catecholamines. In *Williams Textbook of Endocrinology*. Wilson JD, Foster DW, Eds. Philadelphia, Saunders, 1985, p. 76–122
49. deVries-Smits, AMM, Burgering BMT, Leevers SJ, Marshall CJ, Bos JL: Involvement of p21ras in activation of extracellular signal-regulated kinase 2. *Nature* 357:602–604, 1992

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/43/3/375/360644/43-3-375.pdf by guest on 27 March 2025