Pervanadate mimics insulin action in rat adipocytes via activation of the insulin receptor tyrosine kinase

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Pervanadate [Peroxide(s) of Vanadate] Mimics Insulin Action in Rat Adipocytes via Activation of the Insulin Receptor Tyrosine Kinase†

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ABSTRACT: Both vanadate and hydrogen peroxide (H_2O_2) are known to have insulin-mimetic effects. We previously reported that the mixture of vanadate plus H_2O_2 results in the generation of a peroxide(s) of vanadate, which strongly enhances IGF-II binding to rat adipocytes (Kadota et al., 1987b). We now report that pervanadate mimics insulin in isolated rat adipocytes to (1) stimulate lipogenesis, (2) inhibit epinephrine-stimulated lipolysis, and (3) stimulate protein synthesis. The efficacy of pervanadate is comparable to that of insulin. However, it is 10^2–10^3 times more potent than vanadate alone. Exposure of intact rat adipocytes to pervanadate was found to activate the WGA-purified insulin receptor tyrosine kinase assayed with the exogenous substrate poly(Glu^{80}/Tyr^{20}) in a dose-dependent manner to a maximum of 1464% of control at 10^{-3} M compared with a maximum insulin effect of 1046% at 10^{-6} M. In contrast, in vitro assayed autophosphorylation of the WGA-purified extract was increased 3-fold after exposure of intact cells to insulin but not significantly increased after pervanadate. Furthermore, high concentrations of pervanadate (10^{-5} M) inhibited subsequent in vitro added insulin-stimulated autophosphorylation. In vitro addition of pervanadate to WGA-purified receptors could not stimulate autophosphorylation or exogenous tyrosine kinase activity and did not inhibit insulin-stimulated autophosphorylation. Labeling of intact adipocytes with [^{32}P]orthophosphate followed by exposure to 10^{-4} M pervanadate increased insulin receptor β-subunit phosphorylation (7.9 ± 3.0)-fold, while 10^{-7} M insulin and 10^{-4} M vanadate increased labeling (5.3 ± 1.8)- and (1.1 ± 0.2)-fold, respectively. Alkali treatment of SDS gels and phosphoamino acid analysis indicated that both phosphotyrosine and phosphoserine were increased by pervanadate and suggested that for an equivalent stimulation of exogenous kinase activity pervanadate augmented phosphotyrosine labeling to a greater extent than insulin. Vanadate but not pervanadate inhibited alkaline phosphatase catalyzed tyrosine dephosphorylation of the insulin receptor β-subunit. However, pervanadate inhibited dephosphorylation catalyzed by an endogenous rat hepatocyte derived phosphatase. We conclude that pervanadate is a novel potent insulin-mimetic agent which activates the insulin receptor kinase to an extent at least as great as insulin. Its mechanism of action appears to be related to a powerful inhibition of phosphotyrosine phosphatase activity with a specificity distinct from that of vanadate.

Vanadate and hydrogen peroxide (H_2O_2) are well documented to mimic the actions of insulin (Czech et al., 1974; Dubyak & Kleinzeller, 1980; May & de Haen, 1979; Schechter & Karlish, 1980). Recent interest in vanadate has increased since it has been demonstrated to increase the tyrosine kinase activity of the insulin receptor (Tamura et al., 1984), and it has been used successfully in short-term treatment of streptozotocin-diabetic rats (Heyliger et al., 1985; Meyerovitch et al., 1987). We recently demonstrated that a mixture of vanadate and H_2O_2 produced a synergistic effect to augment IGF-II (insulin-like growth factor II) binding to rat adipocytes and to activate the insulin receptor kinase (Kadota et al., 1987a). The efficacies of the mixture of vanadate and H_2O_2, of each agent alone, and of insulin to increase IGF-II binding correlated with their respective efficacies to activate the insulin receptor tyrosine kinase in an in situ[1] intact cell assay (Kadota et al., 1987a). We demonstrated that the synergistic insulin-like effect of vanadate mixed with H_2O_2 was due to the generation of a peroxide(s) of vanadate which we have termed pervanadate (Kadota et al., 1987b). Thus, addition of catalase abolished the synergism only if added at the same time as the vanadate and H_2O_2 but could not do so when added 10 min after mixing of the two agents (Kadota et al., 1987b). In this study, we document that pervanadate mimics classical insulin biological effects in adipocytes with maximal effects similar to that of insulin. Investigations into its mechanism of action indicate that it stimulates in situ tyrosine phosphorylation of the insulin receptor in adipocytes to as great an extent or more than does insulin.

Concomitant with enhanced tyrosine phosphorylation, pervanadate activated the insulin receptor kinase but with a slower time course than insulin. The inhibition of insulin receptor β-subunit tyrosine dephosphorylation by pervanadate and lack of in vitro stimulation of autophosphorylation or tyrosine kinase activity suggest that this insulin-mimetic agent acts via inhibition of specific tyrosine phosphatase(s).

Experimental Procedures

Materials. Porcine insulin was a gift from Connaught-Novo Laboratories (Willowdale, Ontario). Bovine serum albumin (fraction V), aprotinin, phenylmethanesulfonyl fluoride

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[1] In situ refers to incubations of intact live cells with the agents insulin, vanadate, or pervanadate. This has been previously referred to in some studies as in vivo (Klein et al., 1986).

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(PMSF),[2] *N*-acetyl-D-glucosamine, vanadate (sodium orthovanadate), the synthetic tyrosine kinase substrate poly(Glu/Tyr) (4:1), catalase, alkaline phosphatase (type VII from bovine intestine), and phosphoamino acid standards were purchased from Sigma Chemical Co. (St. Louis, MO). Wheat germ coupled to agarose and protein A–Sepharose CL-4B were from Pharmacia Fine Chemicals (Sweden). Collagenase (type I) was from Worthington Biochemical Corp. (Freehold, NJ). H_2O_2 was from BDH Chemicals Ltd. (Ville St. Laurent, Quebec). ATP was from Boehringer Mannheim (West Germany). Dithiothreitol was from Schwarz/Mann Biotech (Cleveland, OH). [γ-^{32}P]ATP (specific activity 289 Ci/mmol), [3-^{3}H]glucose (specific activity 10.8 Ci/mmol), [3,4,5-^{3}H]leucine (specific activity 140.5 Ci/mmol), Aquasol-II, and Econofluor were from New England Nuclear (Lachine, Quebec). Chemicals for electrophoresis were from Bio-Rad Laboratories (Mississauga, Ontario). Kodak X-OMAT AR films were purchased from Picker International (Montreal, Quebec).

Adipocyte Preparation. Male Sprague-Dawley rats (180–200-g body weight) were killed by cervical dislocation after overnight fasting. Epididymal fat tissues were cut into small pieces and digested for 1 h at 37 °C in KRB/HEPES buffer (118.5 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 24.9 mM $NaHCO_3$, and 30 mM HEPES, pH 7.4) containing 20 mg/mL bovine serum albumin, 5 mM glucose, and 1 mg/mL collagenase by the method of Rodbell (1964). The isolated adipocytes were separated from undigested tissue by filtration through nylon mesh and washed 3 times with the above buffer.

Preparation of Vanadate and Pervanadate Solutions. The vanadate solution was prepared as previously described (Kadota et al., 1987a) to avoid changes in pH and the generation of colored decavanadate (orange-yellow) or vanadyl ion, VO^{2+} (blue). The solution of pervanadate was prepared by mixing vanadate with H_2O_2, 10^{-3} M unless otherwise indicated, for 15 min at 22 °C. This was followed by the addition of catalase, 200 μg/mL, to remove residual H_2O_2. This procedure resulted in the generation of the peroxidized form of vanadate which is stable for 2 h without further addition of H_2O_2 (Kadota et al., 1987b). The concentration of pervanadate generated is denoted by the vanadate concentration added to the mixture.

Lipogenesis. Isolated rat adipocytes [(2.5–3.0) × 10^5 cells] were incubated with 0.3 μCi of [3-^{3}H]glucose for 15 min at 37 °C in the presence or absence of pervanadate, vanadate, H_2O_2, or insulin, at the concentrations indicated, in 1 mL of KRB/HEPES buffer, pH 7.4, containing 0.5 mM glucose and 1.5% bovine serum albumin. The reaction was terminated by adding 20 mL of Econofluor, and total ^{3}H incorporation into lipid was measured by β-scintillation counting (LKB, Model 1219). Results were corrected to 10^5 cells and expressed as percent of basal.

Inhibition of Lipolysis. Isolated rat adipocytes were incubated in KRB/HEPES buffer, pH 7.4, with or without 1 μM epinephrine in the presence or absence of pervanadate, vanadate, H_2O_2, or insulin at the concentrations indicated for 15 min at 37 °C. The reaction was terminated by the addition of ice-cold $KClO_4$ solution. The sample was neutralized with KOH and centrifuged and the supernatant assayed for glycerol by the method of Chernick (1969). Results were calculated as micromoles of glycerol per milliliter of packed fat cells (10.8 × 10^6 cells) and expressed as percent inhibition of epinephrine stimulation.

Protein Synthesis. Isolated rat adipocytes [(1–5) × 10^6 cells/mL] were incubated in KRB/HEPES buffer, pH 7.4, in the presence or absence of pervanadate, vanadate, H_2O_2, or insulin at the concentrations indicated in a total volume of 15 mL for 2 h at 37 °C. Subsequently, [3,4,5-^{3}H]leucine (final concentration 0.5 μCi/mL) was added and the incubation continued for 1 h. The reaction was terminated by removal of 500 μL which was added to 2 mL of 10% ice-cold trichloroacetic acid (TCA) as described (Marshall & Monzon, 1987). TCA-precipitable protein was separated by centrifugation at 800*g* for 15 min. The supernatant was aspirated and the pellet washed once with 10% TCA. The final pellet was resuspended in aqueous scintillation fluid (Aquasol-II) and counted in a β-counter (LKB, Model 1219). Results were corrected to 10^6 cells and expressed as percent of basal.

Solubilization and Lectin Purification of Insulin Receptors. Isolated rat adipocytes (2 × 10^7 cells) were incubated with or without the indicated concentrations of pervanadate, vanadate, H_2O_2, or insulin for 15 min at 37 °C in KRB/HEPES buffer, pH 7.4. The reaction was terminated by the addition of ice-cold solubilization buffer (1.0% Triton X-100, 4 mM EDTA, 2 mM NaF, 1 mM PMSF, 1 trypsin inhibitor unit/mL of aprotinin, and 30 mM HEPES, pH 7.6), followed immediately by freezing for 1 h at −70 °C. The cells were then thawed, homogenized, and allowed to stand for 1 h at 4 °C to continue solubilization. The fat cake was removed by centrifugation at 1800*g* for 10 min, and the cell extract was further centrifuged at 100000*g* for 1 h at 4 °C. The supernatant was collected and applied to a column (0.6 × 3.0 cm) of WGA–agarose. The column was washed with 100 mL of 50 mM HEPES buffer, pH 7.6, containing 150 mM NaCl and 0.1% Triton X-100, and insulin receptors were eluted by the above buffer supplemented with 0.3 M *N*-acetyl-D-glucosamine. ^{125}I-Insulin, iodinated to a specific activity of 180–200 Ci/g, was used to assay insulin binding to the lectin-purified extract as previously described (Kadota et al., 1987a).

Insulin Receptor Tyrosine Kinase Activity. Lectin-purified insulin receptor (10–20 fmol of insulin binding) was incubated with or without 10^{-7} M insulin for 30 min at 22 °C in 50 mM HEPES buffer, pH 7.6. The phosphorylation reaction was initiated by the addition of 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) in a total volume of 160 μL as described previously (Kadota et al., 1987a; Zick et al., 1983). After further incubation for 10 min at 22 °C, the reaction was terminated by spotting 80 μL of the reaction mixture onto Whatman 3 filter paper which was placed into 10% TCA containing 10 mM sodium pyrophosphate. After being extensively washed in the above solution, the paper was dried and placed in 20 mL of Aquasol-II in which radioactivity was determined by β-scintillation counting (LKB, Model 1219).

Insulin Receptor Autophosphorylation Assay. Lectin-purified insulin receptor (5–10 fmol of insulin binding) was incubated with or without 10^{-7} M insulin in a 50 mM HEPES buffer containing 8 mM $MnCl_2$, 10 mM $MgCl_2$, 270 μM dithiothreitol, and 10 μg/mL bovine serum albumin for 1 h at 4 °C, in a total volume of 90 μL. The phosphorylation reaction was initiated by the addition of 10 μL of diluted [γ-^{32}P]ATP (10 Ci/mmol) to a final concentration of 50 μM, and the reaction mixture was further incubated for 15 min at 4 °C. The reaction was terminated by adding 50 μL of 50

[2] Abbreviations: WGA, wheat germ agglutinin; PMSF, phenylmethanesulfonyl fluoride; KRB, Krebs–Ringer bicarbonate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NaF, sodium fluoride; TCA, trichloroacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

mM HEPES buffer, pH 7.4, containing 0.24% Triton X-100, 23 mM EDTA, 24 mM sodium pyrophosphate, 2 mM PMSF, and 24 mM ATP. The insulin receptor was immunoprecipitated by incubating with anti-insulin receptor antibody (140 μg of protein) for 4 h at 4 °C followed by incubation with protein A–Sepharose for 1 h at 4 °C. The immunoprecipitate was washed twice with 50 mM HEPES buffer, pH 7.6, containing 0.1% Triton X-100 and 0.1% SDS and once with the above buffer without SDS. ^{32}P incorporation into the insulin receptor β-subunit was detected by SDS–PAGE and radioautography. Densitometric scanning of the radioautographs was done with a Zeineh soft laser scanning densitometer (Model SL-504-XL).

In Situ ^{32}P Labeling of Adipocytes. Isolated adipocytes [(2–9) × 10^6 cells/mL] were incubated in a 10-mL volume of KRB buffer containing 25 mM HEPES, 1.5% bovine serum albumin, and 0.5 mCi/mL [^{32}P]orthophosphate, pH 7.4, for 2 h at 37 °C. Subsequently, 5 mL of 3 × 10^{-4} M pervanadate (pretreated with 200 μg/mL catalase), 3 × 10^{-4} M vanadate, or 3 × 10^{-7} M insulin was added for 15 min to 10-mL aliquots of ^{32}P-equilibrated cells. The reaction was stopped by the addition of 10 mL of 2.5× concentrated buffer containing 125 mM HEPES, 2.5% Triton X-100, 25 mM sodium pyrophosphate, 250 mM NaF, 10 mM EDTA, 10 TIU/mL aprotinin, 5 mM PMSF, and 5 mM sodium vanadate, pH 7.4. The tubes were immediately frozen at −70 °C for 1 h. Samples were then thawed, homogenized, and solubilized for 1 h at 4 °C, followed by centrifugation at 1800*g* for 10 min to remove the fat cake. Samples were centrifuged at 120000*g* for 1 h at 4 °C, and the supernatant was partially purified by passing it 6 times through a 2-mL WGA–Sepharose column. Columns were washed with 100 mL of 50 mM HEPES, 10 mM NaF, 10 mM sodium pyrophosphate, 4 mM EDTA, 2 mM sodium vanadate, and 0.1% Triton X-100, pH 6.0, buffer. Columns were centrifuged for 2 min at 500 rpm, washed with 4 mL of the same buffer at pH 7.6, and centrifuged again. Samples were eluted by adding 2 mL of 0.3 M *N*-acetylglucosamine, 1 mM PMSF, and 4 TIU/mL aprotinin to the above buffer, clamping the column for 30 min, and then centrifuging for 2 min at 500 rpm.

Insulin receptors were immunoprecipitated as described above for the autophosphorylation assay. ^{32}P incorporation into the receptor β-subunit was analyzed by SDS–PAGE on 7.5% gels according to the method of Laemmli (1970).

Phosphoamino Acid Analysis of the Insulin Receptor β-Subunit. Phosphoamino acid analysis was performed by a modification of the method described by Cooper (Cooper et al., 1983). Following SDS–PAGE, the gels were fixed for 30 min in 30% methanol/2% glycerol and dried. To extract labeled receptor, the radiolabeled 95-kDa band was excised and homogenized in 1 mL of 0.1 M NH_4HCO_3, 0.1% Triton X-100, and 5% 2-mercaptoethanol with carrier rabbit γ-globulin (5 μg/mL). The extract was subsequently precipitated with 10% TCA and was washed with ethanol/ether (1:1) at −20 °C. The protein was acid-hydrolyzed with 6 M HCl for 2 h at 100 °C, and the ^{32}P phosphoamino acids were purified by chromatography on Dowex AG1-X8. The phosphoamino acids were then separated by thin-layer electrophoresis (50 V/cm, 60 min) using precoated cellulose plastic sheets (20 cm × 10 cm). Radioactive amino acids were visualized by radioautography and identified by comparing to standards stained with 0.2% ninhydrin in acetone.

Preparation of Rat Hepatic Phosphoprotein Phosphatase. Microsomal membranes were prepared from freshly isolated rat livers using 0.25 M sucrose, 1 mM benzamidine, 1 mM PMSF, and 1 mM $MgCl_2$ in 5 mM Tris as described (Williams et al., 1984). Membranes were resuspended in 50 mM HEPES, pH 7.5, at a protein concentration of 10 mg/mL and stored at −70 °C. Aliquots were solubilized with equal volumes of 50 mM HEPES, pH 7.5, and 2% (w/v) Triton X-100 followed by shaking for 60 min at 4 °C. The samples were centrifuged at 40000*g* for 30 min, and the supernatants were concentrated 5-fold with an Amicon Centriprep 30 concentrator. These concentrated extracts were diluted 1:1 in the phosphotyrosine phosphatase assays.

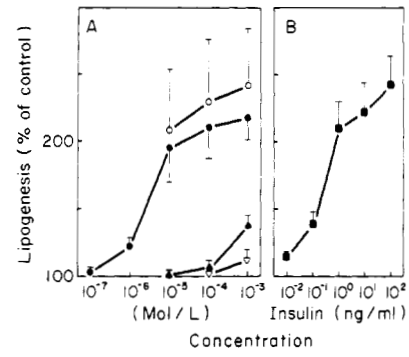

FIGURE 1: Stimulation of lipogenesis in rat adipocytes. Isolated adipocytes [(2.5–3.0) × 10^5 cells] from male Sprague-Dawley rats were incubated with 0.3 μCi of [3-^3H]glucose in the presence or absence of catalase-treated pervanadate prepared with 10^{-3} M H_2O_2 (○) or with 10^{-4} M H_2O_2 (●), vanadate (▲), catalase-treated H_2O_2 (▽), or insulin (■) for 15 min at 37 °C. The incorporation of ^3H into lipid was assayed as outlined under Experimental Procedures. Each value is the mean ± SE of three separate experiments. The mean incorporation of ^3H into lipid in control cells was 7.9 ± 1.23 nmol (10^5 cells)$^{-1}$ h^{-1}.

Dephosphorylation of the Insulin Receptor. Lectin-purified insulin receptor (6.0 fmol of insulin binding) was preincubated with 10^{-7} M insulin for 60 min at 4 °C. Phosphorylation was initiated by addition of [γ-^{32}P]ATP, and the insulin receptor was immunoprecipitated with anti-insulin receptor antibody and protein A–Sepharose as described above. After being washed, the immunoprecipitate was incubated with or without the indicated concentrations of vanadate, H_2O_2, or pervanadate in the presence or absence of either 15 units/mL alkaline phosphatase for 60 min at 4 °C or an equal volume, 50 μL, of rat hepatocyte phosphotyrosine phosphatase (as described above) for 20 min at 30 °C with vigorous shaking. This sample was washed twice with 50 mM HEPES buffer, pH 7.6, containing 0.1% Triton X-100 and subjected to SDS–PAGE followed by radioautography.

Results

Effects of Pervanadate, Vanadate, and Insulin on Lipogenesis. Previous studies indicated that pervanadate potently augmented IGF-II binding to isolated rat adipocytes as did insulin (Kadota et al., 1987a,b). In the present study, we have examined the effect of different concentrations of pervanadate, vanadate, and insulin on the incorporation of [3-^3H]glucose into the total lipid of rat adipocytes. Insulin stimulated lipogenesis in a dose-dependent manner, achieving levels of 220% and 240% of control at concentrations of 10 and 100 ng/mL, respectively (Figure 1B). Vanadate (10^{-3} M) and H_2O_2 (10^{-3} M) increased lipogenesis to 139% and 123% of control, respectively (Figure 1A). In contrast, pervanadate at 10^{-4} M and 10^{-3} M augmented lipogenesis to levels of 210% (10^{-4} M pervanadate prepared with 10^{-4} M H_2O_2) and to 240% (10^{-3} M pervanadate prepared with 10^{-3} M H_2O_2) of control, a degree of stimulation which compared to the levels attained with insulin (Figure 1A). Of note is the finding that the

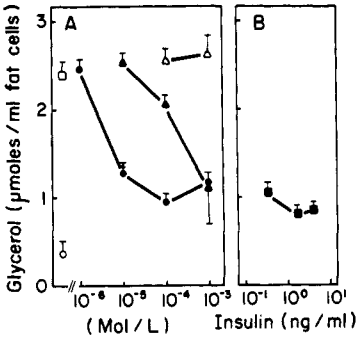

FIGURE 2: Inhibition of epinephrine-stimulated lipolysis in rat adipocytes. Isolated rat adipocytes were incubated with (□) or without (○) 1 μM epinephrine in the presence of (panel A) catalase-treated pervanadate (●), vanadate (▲), or catalase-treated H_2O_2 (△) or (panel B) insulin (■) for 15 min at 37 °C, and glycerol was assayed as described under Experimental Procedures. Basal and epinephrine-stimulated glycerol produced per milliliter of packed fat cells was 0.375 and 2.401 μmol, respectively.

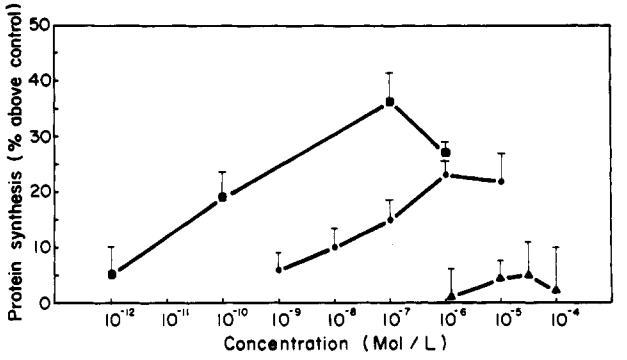

FIGURE 3: Stimulation of protein synthesis in rat adipocytes. Isolated rat adipocytes [(1–5) × 10^6 cells/mL] were incubated with 0.5 μCi/mL [3,4,5-^{3}H]leucine for 1 h at 37 °C after a 2-h preincubation at 37 °C with catalase-treated pervanadate (●), vanadate (▲), or insulin (■) at the concentrations indicated. The incorporation of [^{3}H]leucine into total protein was assayed and measured as described under Experimental Procedures. Values are mean ± SE of five (pervanadate), five (vanadate), and eight (insulin) experiments each performed in duplicate.

potency of pervanadate was 10^2–10^3 times that seen with vanadate.

Antilipolysis in Rat Adipocytes. One micromolar epinephrine added to rat adipocytes stimulated lipolysis by 540% over control (Figure 2A). Insulin, at 1.7 ng/mL, maximally inhibited epinephrine-stimulated lipolysis by 80% (Figure 2B). Vanadate, 10^{-4} and 10^{-3} M, inhibited lipolysis by 17% and 61%, respectively. The inhibition by pervanadate was 55% and 72% at 10^{-5} and 10^{-4} M, respectively (Figure 2A), while catalase alone was without effect. Thus, the effect of pervanadate was comparable to that seen with insulin, and the potency was approximately 10^2-fold greater than that of vanadate.

Stimulation of Protein Synthesis. Insulin has been well documented to have multiple anabolic actions. As a measure of a general anabolic effect in adipocytes, we tested the ability of insulin, vanadate, and pervanadate to stimulate protein synthesis by assessing [3,4,5-^{3}H]leucine incorporation into protein as described under Experimental Procedures. Insulin stimulated protein synthesis in a dose-dependent manner to a maximum of 36 ± 5.5% over control at 10^{-7} M. Pervanadate similarly augmented protein synthesis to a maximum of 23 ± 2.5% over control at 10^{-6} M (Figure 3). In contrast, vanadate had only minimal or no effect at 5 × 10^{-5} M (5.0 ± 6.0% above control). Higher concentrations of vanadate were found to inhibit protein synthesis (data not shown) as

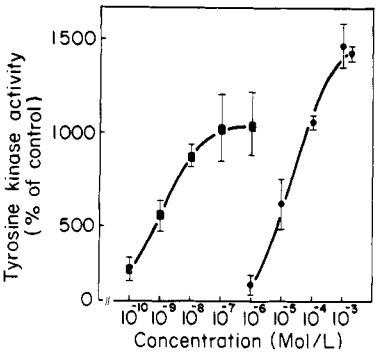

FIGURE 4: Dose–response of activation of the tyrosine kinase by pervanadate and insulin in rat adipocytes. Adipocytes were incubated with the indicated concentrations of pervanadate (●) or insulin (■) for 15 min at 37 °C. The reaction was terminated by adding ice-chilled solubilization buffer, and the cells were frozen at −80 °C for 1 h. Insulin receptors were partially purified by WGA–agarose chromatography, and receptor kinase activity toward the exogenous substrate poly(Glu/Tyr) (4:1) was determined as described under Experimental Procedures. Results were normalized to identical amounts of receptor. Each value is the mean ± SE of three separate experiments except the value at 2 × 10^{-3} M pervanadate (mean and range of two separate experiments). The mean ^{32}P incorporation into poly(Glu/Tyr) in the control receptor was 35.5 fmol of ^{32}P (fmol of insulin binding)$^{-1}$ (10 min)$^{-1}$.

Table I: Kinetics of Pervanadate-Stimulated Tyrosine Kinase Activity of the Insulin Receptor[a]

	control	insulin	pervanadate
V_{max} [fmol of ^{32}P incorporated (fmol of insulin binding)$^{-1}$ (10 min)$^{-1}$]	78.5	111.4	215.2
K_m (μM)	43.7	22.6	9.3

[a] Adipocytes were incubated with 10^{-3} M pervanadate, 10 ng/mL insulin, or no additions for 15 min at 37 °C. Tyrosine kinase activity of the lectin-purified insulin receptor was determined by exogenous substrate assay in the presence or absence of 5, 10, 25, 50, and 100 μM ATP. Linearity of Lineweaver–Burk analyses of three separate experiments was assessed by determining the correlation coefficients of the five points for control (r = 0.99), insulin (r = 0.99), and pervanadate (r = 0.97) stimulation, and V_{max} and K_m were determined from the intercepts.

reported previously in skeletal muscle (Clark et al., 1985).

Dose–Response and Kinetics of Tyrosine Kinase Activated by Pervanadate and Insulin. After the exposure of intact adipocytes to insulin for 15 min at 37 °C, the tyrosine kinase activity of the partially purified insulin receptor assayed with poly(Glu/Tyr) (4:1) was increased in a dose-dependent manner. At 10^{-7} and 10^{-6} M insulin, tyrosine kinase was maximally activated to 1028% and 1046% of control, respectively. Pervanadate also activated the receptor kinase in a dose-dependent manner (Figure 4). At 10^{-3} M pervanadate, the activation was 1464% of control. As the activation by 10^{-3} M vanadate alone and 10^{-3} M H_2O_2 alone was 132% and 158% of control, respectively (Kadota et al., 1987a), pervanadate's effect on tyrosine kinase was, like its other biological actions, far more potent than expected if the invididual effects of vanadate and H_2O_2 were additive. The level of maximal activation in most experiments was at least as great as that achieved by insulin. The kinetic study revealed that the activation of receptor kinase by pervanadate was, as with insulin, associated with both an increase in V_{max} and a decrease in K_m for ATP (Table I). We have previously employed immunoprecipitation to deplete insulin receptors from the WGA-purified cell extracts to show that the increased tyrosine kinase activity after pervanadate exposure of adipocytes was associated with the insulin receptor (Kadota et al., 1987a).

Time Course of Activation of Insulin Receptor Tyrosine Kinase by Pervanadate and Insulin. Previous studies have

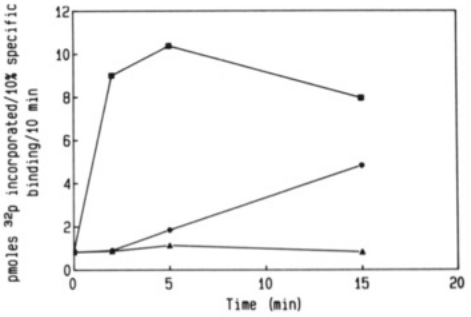

FIGURE 5: Time course of activation of the insulin receptor kinase in adipocytes by pervanadate and insulin. Adipocytes were incubated with 10^{-3} M pervanadate (●), 10^{-7} M insulin (■), or 10^{-3} M vanadate (▲) for the times indicated at 37 °C. The reaction was terminated and receptor tyrosine kinase activity determined as described in the legend to Figure 4. The results are from one of three separate experiments which all demonstrate similar time courses.

demonstrated that activation of the insulin receptor kinase after exposure of intact adipocytes to insulin occurs very rapidly, reaching a maximum after 1 min (Klein et al., 1986). However, activation after exposure to vanadate, a known phosphotyrosine phosphatase inhibitor, is much slower as is the stimulation of glucose transport. To gain insight into the mechanism of action of pervanadate, the time course of activation was assessed. Although more potent than vanadate, the time course of insulin receptor kinase activation by pervanadate was slower than that of insulin. Maximal activation was apparent at 2 min after insulin but was still increasing at 15 min after pervanadate (Figure 5).

Insulin Receptor Autophosphorylation after Exposure of Intact Adipocytes to Pervanadate and Insulin. The stimulative effect of pervanadate on insulin receptor β-subunit autophosphorylation was examined by using the same samples in which augmented tyrosine kinase activity was detected by the exogenous substrate assay above. Insulin, 10 ng/mL, caused a 3-fold increase in autophosphorylation. In contrast, incubating cells with pervanadate produced a minimal effect on autophosphorylation. In fact, the exposure of intact adipocytes to pervanadate inhibited the increase in autophosphorylation promoted by in vitro addition of insulin (Figure 6).

In Situ ^{32}P Labeling of Adipocytes. In view of the differences observed between the effects of insulin and pervanadate on autophosphorylation, we wished to investigate the mechanism of activation of the insulin receptor tyrosine kinase by pervanadate. Others have demonstrated that addition of insulin to ^{32}P-labeled intact cells resulted in increased labeling of the 95-kDa subunit of the insulin receptor on tyrosine and serine residues (Kasuga et al., 1982; Yu & Czech, 1986). Exposure of ^{32}P-labeled intact adipocytes to 10^{-7} M insulin, 10^{-4} M pervanadate, and 10^{-4} M vanadate for 15 min at 37 °C resulted in a stimulation of labeling of the 95-kDa β-subunit to (5.3 ± 1.8)-, (7.9 ± 3.00)-, and (1.1 ± 0.2)-fold of control, respectively (mean ± SE, $N = 3$). To assess the extent of phosphotyrosine labeling, the gels were washed with KOH and reexposed. While the control and vanadate-treated samples had no detectable ^{32}P-labeled band, both the insulin- and pervanadate-treated samples demonstrated alkali-resistant labeling of the β-subunit, indicating the presence of phosphotyrosine (Figure 7). In all experiments, labeling of the β-subunit before and after KOH treatment was greater with pervanadate by approximately 50% compared to insulin (Figure 7). It should be noted that at these concentrations of pervanadate and insulin, the activation of receptor tyrosine kinase activity assayed with exogenous substrate was similar (Figure 4).

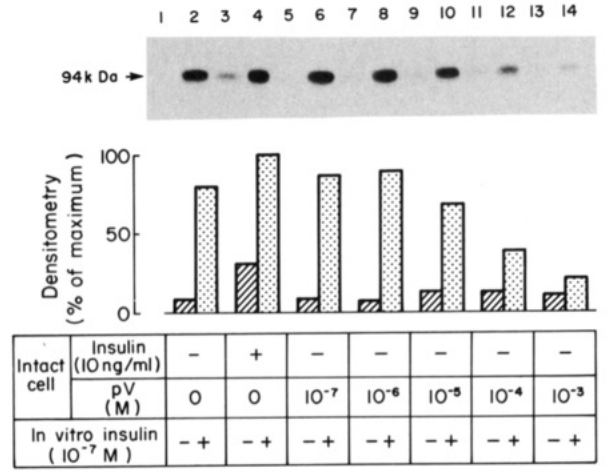

FIGURE 6: Effect of exposure of adipocytes to pervanadate on insulin receptor autophosphorylation. Isolated rat adipocytes were incubated with different concentrations of pervanadate (lanes 5–14), 10 ng/mL (1.7×10^{-9} M) insulin (lanes 3 and 4), or no additions (lanes 1 and 2) for 15 min at 37 °C. Insulin receptors were solubilized and partially purified by WGA chromatography. An aliquot of insulin receptor (5–10 fmol of insulin binding) was used in the autophosphorylation assay in the presence or absence of in vitro insulin followed by SDS–PAGE and radioautography as described under Experimental Procedures. The intensity of the 94-kDa band (β-subunit) was determined by densitometric scanning. The values are expressed as percent of maximum densitometry measured in arbitrary units.

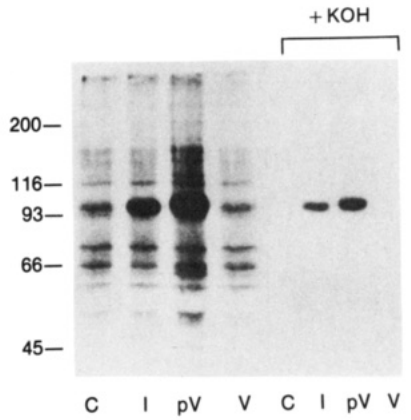

FIGURE 7: In situ ^{32}P labeling of the insulin receptor in rat adipocytes. Isolated rat adipocytes [(2–9) × 10^6 cells/mL] were incubated for 2 h at 37 °C in the presence of 0.5 mCi/mL [^{32}P]orthophosphate followed by the addition (final concentrations) for 15 min of buffer alone (C), 10^{-7} M insulin (I), 10^{-4} M catalase-treated pervanadate (pV), or 10^{-4} M vanadate (V). The reaction was stopped, and insulin receptors were prepared as described under Experimental Procedures. The receptors were immunoprecipitated and separated by SDS–PAGE. ^{32}P incorporation into the β-subunit of the insulin receptor is seen in the 95-kDa band on the radioautogram and was quantified by densitometry (see Results). Lanes 5–8 were washed in KOH before radioautography. Similar results were obtained in three separate experiments.

To further document the extent of amino acid phosphorylation suggested by the KOH treatment, the 95-kDa bands were excised, and the proteins were hydrolyzed in acid as described under Experimental Procedures. Separation of phosphoamino acids revealed that insulin and pervanadate increased the labeling of the β-subunit on both tyrosine and serine (Figure 8). Although quantitative comparison of phosphoamino acid labeling is not precise with this technique, the amount of phosphotyrosine isolated was more than 3-fold greater from pervanadate-treated as compared to insulin-treated cells (81 618 ± 721 vs 25 042 ± 21 cpm; mean ± SE, $N = 3$). We compared the relative amounts of phosphotyrosine and phosphoserine as fractions of the total phosphoamino acid

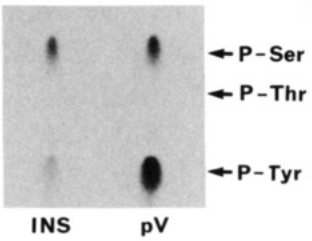

FIGURE 8: Phosphoamino acid analysis of the in situ ^{32}P-labeled insulin receptor subunit. The 95-kDa bands (seen in Figure 7, lanes 1–4) were excised from the gels. The proteins were eluted and hydrolyzed in HCl, and phosphoamino acids were separated by high-voltage electrophoresis on thin-layer cellulose plates as described under Experimental Procedures. The positions of phosphotyrosine and phosphoserine were indicated by the phosphoamino acid standards. The relative intensities of the spots seen on the radioautograms were quantified by cutting them out and counting in a β-scintillation counter (Model LKB 1219).

detected in each sample. After insulin stimulation, 42% of the total β-subunit ^{32}P amino acid was [^{32}P]tyrosine, while the pervanadate phosphotyrosine accounted for 64% of the label. Thus, pervandate appeared to stimulate more tyrosine phosphorylation of the insulin receptor β-subunit than insulin at these concentrations.

Lack of Effect on Insulin Receptor Autophosphorylation by in Vitro Pervanadate. Insulin receptors partially purified by WGA chromatography were directly incubated with different concentrations of pervanadate in the presence or absence of insulin, and autophosphorylation of the insulin receptor β-subunit was subsequently assayed. Under these conditions, insulin stimulated phosphorylation of the insulin receptor; however, no stimulation by pervanadate was observed (data not shown). We previously found a similar lack of stimulation of the tyrosine kinase activity toward exogenous substrate by in vitro addition of pervanadate to comparably purified insulin receptor preparations (Kadota et al., 1987b).

Effect of Pervanadate on Dephosphorylation of the Insulin Receptor. Vanadate is well documented to be an inhibitor of phosphotyrosine phosphatase (Pang et al., 1985). To test the possibility that pervanadate activates the tyrosine kinase in intact cells by potently inhibiting phosphotyrosine phosphatase, we first evaluated the effect of pervanadate on alkaline phosphatase catalyzed dephosphorylation of the lectin-purified insulin receptor. Alkaline phosphatase clearly dephosphorylated the ^{32}P-labeled insulin receptor β-subunit (Figure 9A, lanes 1 and 2). The inhibitory effect of increasing concentrations of vanadate and pervanadate on the extent of β-subunit dephosphorylation (Figure 9A, lanes 3–7 and 10–14) is summarized in panel B. Vanadate inhibited the dephosphorylation of the ^{32}P-labeled insulin receptor in a dose-dependent manner. In contrast, the inhibition by pervanadate was much less than that by vanadate alone. Also, 10^{-3} M H_2O_2 alone was without effect (Figure 9A, lanes 8 and 9). Since vanadate and pervanadate may have different specificities for various phosphoprotein phosphatases, we next tested their inhibitory effects on insulin receptor tyrosine dephosphorylation catalyzed by a crude preparation of phosphotyrosine phosphatase activity extracted from rat liver microsomes. In contrast to the above results, pervanadate powerfully inhibited tyrosine dephosphorylation (Figure 10). In this case, vanadate also inhibited dephosphorylation but with less efficacy, while H_2O_2 was without effect (Figure 10).

Discussion

We previously demonstrated that the apparent synergistic insulin-like effect of a mixture of vanadate and H_2O_2 to increase IGF-II binding in rat adipocytes was the result of the formation of a peroxidized form(s) of vanadate which we have termed pervanadate (Kadota et al., 1987a,b). In this study, we demonstrated that pervanadate has potent insulin-like effects to stimulate lipogenesis, inhibit epinephrine-stimulated lipolysis, and stimulate protein synthesis in rat adipocytes. The potency of pervanadate is comparable to that of insulin and is approximately 10^3-fold (lipogenesis) and 10^2-fold (antilipolysis) greater than that of vanadate alone.

Pervanadate, like insulin, also activated an exogenous tyr-

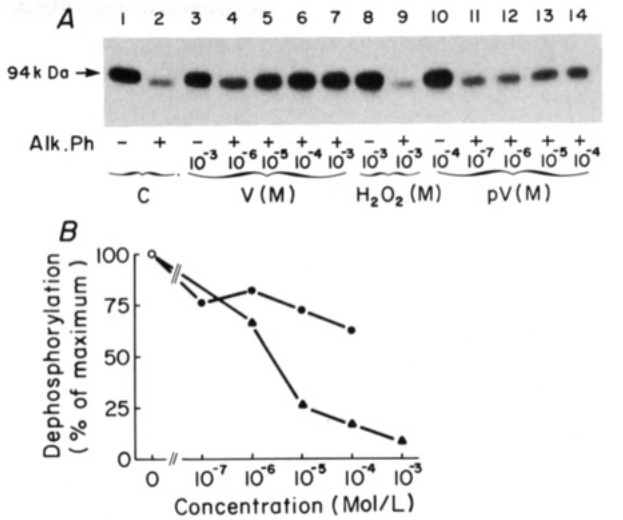

FIGURE 9: Effect of pervanadate on alkaline phosphatase catalyzed dephosphorylation of the lectin-purified insulin receptor. Lectin-purified insulin receptors were labeled with ^{32}P by incubating with insulin and [γ-^{32}P]ATP. Subsequently, the labeled receptors were incubated with the indicated concentrations of vanadate (lanes 3–7), 10^{-3} M H_2O_2 (lanes 8 and 9), the indicated concentrations of pervanadate (lanes 10–14), or no additions (lanes 1 and 2) in the presence or absence of 15 units/mL alkaline phosphatase for 60 min at 4 °C with vigorous shaking. The samples were subjected to SDS–PAGE and radioautography (panel A). After the density of the 94-kDa band was scanned, the inhibitory effect of increasing doses of vanadate (▲) and pervanadate (●) on the extent of β-subunit dephosphorylation by alkaline phosphatase was quantified and is depicted in panel B.

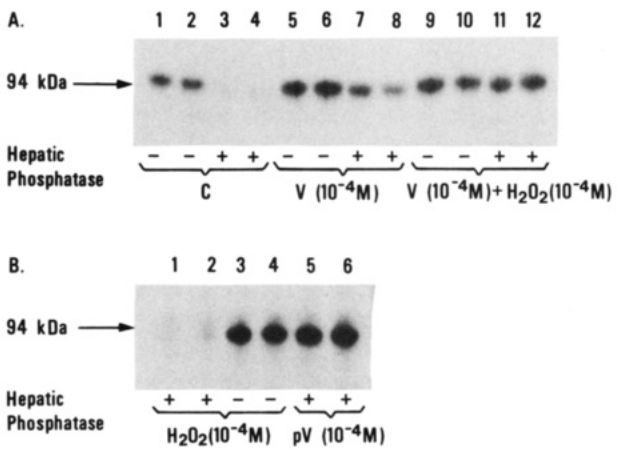

FIGURE 10: Effect of pervanadate on rat hepatocyte tyrosine phosphatase catalyzed dephosphorylation of the insulin receptor. Lectin-purified insulin receptors were labeled with ^{32}P by incubating with insulin and [γ-^{32}P]ATP. Fifty microliters of labeled receptor was incubated in the presence or absence of 50 μL of rat liver phosphotyrosine phosphatase activity, prepared as described under Experimental Procedures, for 20 min at 30 °C with vigorous shaking in the presence of (panel A) no additions (lanes 1–4), 10^{-4} M vanadate (lanes 5–9), or 10^{-4} M pervanadate (10^{-4} M vanadate plus 10^{-4} M H_2O_2 not treated with catalase) (lanes 9–12) and (panel B) 10^{-4} M H_2O_2 (lanes 1–4) or 10^{-4} M catalase-treated pervanadate (lanes 5 and 6). The samples were subjected to SDS–PAGE and radioautography.

osine kinase in intact rat adipocytes. This activated kinase is related to the insulin receptor, because more than 90% of this activity was immunoprecipitated by anti-insulin receptor antibody, but not by control IgG (Kadota et al., 1987a). The maximal effect of pervanadate on the tyrosine kinase was at least as great or slightly greater than that of insulin. These data are in agreement with and complementary to several recently published studies which indicate that a critical step in insulin action is the activation of the insulin receptor tyrosine kinase. Thus, diminished receptor kinase activity has been reported in some states of insulin resistance (Grigorescu et al., 1984; Caro et al., 1986; Freidenberg et al., 1987). Mutagenesis studies have demonstrated that amino acid substitutions in the insulin receptor β-subunit [e.g., Tyr-1150 and/or -1151[3] (Ellis et al., 1986) and Lys-1018 (Chan et al., 1987)] decreased or abolished tyrosine kinase activity and the ability of the mutated receptors to transmit the biological actions of insulin. Finally, it has been shown that the cytosolic accumulation in intact cells of kinase inhibitory antibodies prevents the biological effect of added insulin (Morgan & Roth, 1987).

The kinetic parameters of the tyrosine kinase activity were altered in the same manner by insulin and pervanadate; both increased the V_{max} and decreased the K_m for ATP. The K_m for poly(Glu/Tyr) (4:1) was not altered (data not shown). In a previous study, kinetic analysis of in vitro insulin-stimulated autophosphorylation was found to be associated with an increase in V_{max} but no change in K_m for substrate or ATP (White et al., 1984). In contrast, kinetic analysis of the tyrosine kinase activity toward exogenous substrate revealed that in vitro added insulin increased the V_{max} (Kasuga et al., 1983; Arsenis & Livingston, 1986), did not alter the K_m for poly(Glu/Tyr) (4:1) (Arsenis & Livingston, 1986) or another synthetic peptide substrate (Kasuga et al., 1983), and decreased the K_m for ATP (Arsenis & Livingston, 1986), similar to our results. Insulin activation of intact cells (in situ activation) has been associated with an increase in the V_{max} and no change in the K_m for substrate for the exogenous receptor kinase; however, the K_m for ATP was not studied (Yu & Czech, 1986). The reason for the apparent difference between insulin's effect on the K_m for ATP for autophosphorylation and for exogenous kinase activity remains to be determined.

The activation of the receptor tyrosine kinase by insulin is mediated by autophosphorylation of the β-subunit on tyrosine residues (Yu & Czech, 1986; Rosen et al., 1983). In previous studies, we and others have shown that insulin-induced activation of the receptor kinase toward exogenous substrate is accompanied by a parallel augmentation of β-subunit autophosphorylation activity (Klein et al., 1986; Khan et al., in press). Of interest in this study was the finding that, in contrast to the receptor tyrosine kinase assayed with exogenous substrate, exposure of cells to pervanadate only stimulated insulin receptor autophosphorylation to a minimal extent. This was markedly different from insulin which stimulated receptor autophosphorylation 3-fold. Although the cause of the marked disproportion between the activation of exogenous kinase and autophosphorylation by pervanadate was not immediately apparent, labeling of intact adipocytes with ^{32}P revealed a possible mechanism. We found that 10^{-4} M pervanadate stimulated ^{32}P incorporation into the 95-kDa insulin receptor β-subunit to an even greater extent than 10^{-7} M insulin while activating the exogenous kinase to a similar extent. A significant proportion of the ^{32}P labeling was KOH resistant (30% for both pervanadate and insulin), indicating labeling of phosphotyrosine. Phosphoamino acid analysis confirmed the presence of both phosphotyrosine and phosphoserine. These data indicate that activation of the receptor kinase by pervanadate as by insulin is associated with stimulation of β-subunit tyrosine phosphorylation. The greater extent of in situ phosphorylation at the higher concentrations of pervanadate (10^{-5} M) may explain in part the inhibitory effect on subsequent in vitro insulin-stimulated autophosphorylation (Figure 6). It is not clear, however, why basal autophosphorylation is not augmented after the exposure of intact cells to lower concentrations of pervanadate (10^{-7} and 10^{-6} M). In this circumstance, in vitro added insulin is able to stimulate further β-subunit tyrosine phosphorylation. This observation suggests that the mechanism of stimulation of phosphorylation by pervanadate is different from that induced by insulin.

To elucidate further the mechanism of action of pervanadate, it was added in vitro to solubilized WGA-purified adipocyte insulin receptors. In contrast to insulin, pervanadate did not significantly stimulate autophosphorylation (Figure 6) or exogenous tyrosine kinase activity (Kadota et al., 1987b). Vanadate itself has been documented to inhibit tyrosine phosphatases (Swarup et al., 1982). This may account for its weak insulin-mimetic effects. We found that pervanadate did not inhibit alkaline phosphatase catalyzed dephosphorylation of the insulin receptor. However, pervanadate was a potent inhibitor of insulin receptor tyrosine dephosphorylation catalyzed by an endogenous rat liver phosphoprotein phosphatase. In the latter case, pervanadate was more efficacious than vanadate. The lack of in vitro stimulation of the insulin receptor kinase, the slower time course of activation in intact cells as compared to insulin, and the inhibition of tyrosine dephosphorylation of the labeled receptor all strongly suggest that the mechanism of action of pervanadate involves primarily the inhibition of a specific phosphotyrosine phosphatase.

Recently, H_2O_2, which has been documented to have insulin-like effects (Czech et al., 1974; Little & de Haen, 1980), has been found to stimulate insulin receptor autophosphorylation and tyrosine kinase activity in intact rat adipocytes (Hayes & Lockwood, 1987). Whether pervanadate also acts as an oxidant and whether this contributes to its action remain to be determined. It should be noted that, as we have reported for the augmentation of IGF-II binding and the activation of the receptor tyrosine kinase activity by H_2O_2 (Kadota et al., 1987a), the maximal effect of H_2O_2 (10^{-3} M) to increase β-subunit ^{32}P labeling was 2-fold and less than that caused by insulin (Hayes & Lockwood, 1987) and pervanadate. H_2O_2 could not activate the kinase or stimulate autophosphorylation when added to partially purified receptors but could do so in whole cell homogenates (Hayes & Lockwood, 1987).

Since the data indicate that the tyrosine phosphorylation of the insulin receptor stimulated by pervanadate in intact cells is mediated by inhibition of phosphatase activity rather than by an activation of the autokinase reaction, we may explain the lack of stimulation of autophosphorylation after exposure of cells to pervanadate as opposed to insulin. A recent model of activation of growth factor receptors by their ligands involves the formation of dimers or oligomers as an initial step (Schlessinger, 1988). The approximation of the cytoplasmic domains of the receptors would permit cross-phosphorylation by the neighboring β-subunit. Insulin receptors have been found to exist in oligomeric forms, and it has been proposed that these may be induced by exposure of cells to insulin (Crettaz et al., 1984). This aggregation would permit in-

[3] The residue numbers are those of the proreceptor inferred from the cDNA sequence as determined by Ullrich et al. (1985).

creased cross-phosphorylation of β-subunits, i.e., autophosphorylation. Pervanadate may not induce such aggregation and thus may minimally increase the accessibility of β-subunits to phosphorylate each other. This hypothesis remains to be tested.

In summary, we demonstrated that pervanadate has potent insulin-like effects in rat adipocytes with respect to IGF-II binding, lipogenesis, antilipolysis, protein synthesis, and insulin receptor tyrosine kinase activity. It is 10^2–10^3 times more potent than vanadate. It stimulates tyrosine and serine phosphorylation of the insulin receptor β-subunit in intact adipocytes. It differs from insulin in that it does not bind to the receptor and does not act in vitro on solubilized receptors. It has phosphotyrosine phosphatase inhibitory activity as does vanadate but with different specificity. The potential utility of this compound as an insulin-mimicking agent remains to be determined.

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