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INSULIN-LIKE EFFECT OF VANADATE ON ADIPOCYTE GLYCOGEN SYNTHASE AND ON PHOSPHORYLATION OF 95,000 DALTON SUBUNIT OF INSULIN RECEPTOR

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Vanadate enhanced the state of activation of rat adipocyte glycogen synthase in a manner similar to that of insulin. No additional effect was observed when insulin and vanadate were added together. The effect of vanadate, like insulin, was reversed by incubation with epinephrine. Vanadate also enhanced the degree of phosphorylation of the 95,000 dalton subunit of insulin receptor, selectively on tyrosine residues, in the solubilized rat adipocyte insulin receptor system. This demonstrates that insulin and vanadate have similar initial actions on receptor phosphorylation and also act similarly on an intracellular event, namely the activation of glycogen synthase.

Dubyak and Kleinzeller (1) and Shechter and Karlish (2) in 1980 reported that vanadate producted an insulin-like stimulation of adipocyte glucose oxidation and 2-deoxyglucose transport. It has since been reported that vanadate, like insulin, also inhibited ACTH or isoproterenol induced lipolysis in adipocytes (3). Thus vanadate mimicked both the glucose transport independent and the glucose transport dependent actions of insulin. The role of an insulin mediator and of glucose 6-P in the effects of insulin on glycogen synthase was first elucidated in this laboratory (4). Since vanadate is known to enter adipocytes (1), the mechanism of its insulin-like effects may differ from that of lectins, and of anti-insulin receptor antibody, both of which act on the cell surface to produce the insulin mediator (5-8).

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We were therefore interested in determining 1) whether the insulin-like effects of vanadate extended to the activation of rat adipocyte glycogen synthase in the absence of glucose in the medium; and 2) whether the insulin-like action of vandate included an initial action on receptor phosphorylation similar to that of insulin.

In this communication we report that vanadate activates rat adipocyte glycogen synthase. We also demonstrate that vanadate enhances the phosphorylation of tyrosine residues on the 95,000 dalton subunit of the insulin receptor, suggesting that both vanadate and insulin have similar initial actions on the receptor and also a similar intracellular effect on glycogen synthase.

Materials

Collagenase was obtained from Worthington. Crystalline porcine insulin was obtained from NOVO Laboratories (Copenhagen) and assayed at 25 units/mg. Sodium orthovandate was from ICN. Wheat germ agglutinin coupled to agarose was from Miles. UDP-glucose, glucose 6-phosphate, phenylmethylsulfonyl fluoride (PMSF), Triton X-100, N-2-hydroxyethylpiperazine N'2-ethanesultonic acid (Hepes), N-acetyl glucosamine, protein A-Sepharose CL-4B and epinephrine bitartrate were from Sigma. All reagents for NaDodSO$_4$/polyacrylamide gel electrophoresis were from Bio-Rad. Labeled UDP-glucose was synthesized in our laboratory by published methods (9). [γ -^{32}P]ATP was a gift from Dr. G.E. Vandenhoff. Anti-rat insulin receptor antibody containing rabbit serum was a gift from Dr. S. Jacobs (Burroughs Wellcome Company, Research Triangle Park, North Carolina).

Methods

Preparation of isolated adipocytes. Adipocytes were prepared by standard methods from epididymal tissue of fed Sprague-Dawley rats weighing 140-180g (10). Experiments were performed in plastic tubes using Krebs-Ringer phosphate buffer pH 7.4 containing 3% (w/v) of bovine serum albumin (Fraction V, Sigma).

Assay of glycogen synthase. Extracts from adipocytes were prepared and assayed for glycogen synthase activity as described previously (11). The glycogen synthase activation state was expressed as the ratio of activity observed with low G6P (0.067 mM) to the activity observed with high G6P (6.7 mM).

Preparation of solubilized insulin receptor fraction. Adipocytes from epididymal tissue of 10 rats (ca. 10 ml of packed cells) were washed twice with Krebs-Ringer phosphate buffer containing 1% (w/v) of bovine serum albumin and were solubilized in 10 ml of 50 mM Hepes, pH 7.6, 1% Triton X-100 and 1 mM PMSF for 1 h at 4°C. The sample was centrifuged for 30 min at 200,000 x g. The infranate was applied on to a column of wheat germ agglutinin coupled to agarose (2 ml bed volume). After extensive washing with 50 mM Hepes buffer pH 7.6, containing 0.1% Triton X-100, the bound material was eluted with

buffer supplemented with 0.3 M N-acetylglucosamine in a final volume of 2 ml and used as the solubilized insulin receptor fraction.

NaDodSO$_4$ gel electrophoresis. NaDodSO$_4$ gel electrophoresis was performed according to Laemmli using 4% stacking and 6% resolving gels (12). After electrophoresis, the slab gels were stained with Coomassie Blue, destained, dried and autoradiographed with Kodak XAR-5 film for 3 days at -70°C.

Analysis of phosphoamino acids. The phosphoamino acids were analyzed by a modification of the method of Hunter and Sefton as described in the legend for Figure 3 (13).

High voltage paper electrophoresis. High voltage paper electrophoresis was performed in pH 3.5 buffer (pyridine/acetic acid/H_2O, 1:10:189) at 3.5 KV for 45 min at 12°C both in the first and second dimensions.

Results and Discussion

When isolated adipocytes were incubated with insulin, as described in the legend to Table 1, the state of activation of glycogen synthase was increased 2.1 fold over the control, confirming our previous report (11). Incubation of adipocytes with 1 mM vanadate under the same conditions, similarly increased the state of activation of glycogen synthase. The effect of vanadate on glycogen synthase was stable to desalting on Sephadex G-25, indicating that it represented altered phosphorylation of the enzyme. When cells were incubated with 1 mU/ml insulin together with 1 mM vanadate, no additional effect was observed, suggesting that insulin and vanadate act intracellularly by a similar mechanism.

Table 1

Effect of insulin and/or vanadate on fat cell glycogen synthase activation state

	Glycogen Synthase Activation State (%)	
additions	before Sephadex G25	after Sephadex G25
none (control)	9.6	9.0
insulin (1 mU/ml)	20.2	20.4
vanadate (1 mM)	18.2	17.4
insulin (1 mU/ml) + vanadate (1 mM)	19.7	

Adipocytes (5-6 x 10^5 cells/ml) were incubated with or without 1 mU/ml of insulin and/or 1 mM vanadate at 37°C for 20 min and then washed. Washed cells, collected by centrifugation were frozen in liquid N_2 and homogenized in 0.5 ml of a buffer containing 10 mM Tricine, pH 7.5, 100 mM KF, and 10 mM EDTA. Homogenates were centrifuged (8500 x g for 5 min) and infranate assayed for glycogen synthase before and after column chromatography on Sephadex G-25. These results represent one of three similar experiments.

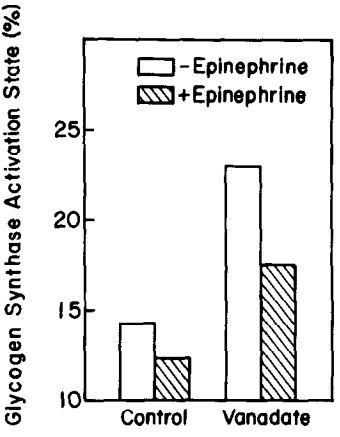

Figure 1 Effect of epinephrine on vanadate-stimulated glycogen synthase activation state Adipocytes (5-6 x 10^5 cells/ml) were incubated in 2 ml of Krebs Ringer phosphate buffer, pH 7.4, containing 1% bovine serum albumin in the presence or absence of 1 mM vanadate at 37°C for 30 min. The cells were then washed and incubated with or without 10^{-5}M epinephrine bitartrate at 37°C for 15 min and washed again. Washed cells were collected by centrifugation, frozen, homogenized and assayed for glycogen synthase activity as already described. Results represent one of two similar experiments.

The effect of vanadate on glycogen synthase was partially reversed by incubation of adipocytes with epinephrine, following prior incubation with vanadate and then extensive washing (Figure 1). This data confirms that the insulin-like effect of vanadate on glycogen synthase is due to decreased phosphorylation of the enzyme.

Kasuga et al. (14) have reported that insulin enhanced the phosphorylation of insulin receptors in both lymphocytes and hepatoma cells, and suggested that this was an early step in insulin action. In these whole cell experiments the phosphorylation of serine, and threonine, as well as tyrosine was enhanced (15). Kasuga et al. subsequently reported that preincubation of partially purified, detergent extracted insulin receptor with insulin, followed by incubation with [γ-^{32}P]ATP, and Mn^{++}, enhanced the phosphorylation of the 95,000 dalton subunit of the insulin receptor (16). In contrast to intact cells, only tyrosine residues were phosphorylated in this system (16).

Swarup et al. (17) reported that vanadate is a potent inhibitor of plasma membrane phosphotyrosine phosphatase. Machicao et al. have

recently reported that a highly purified insulin receptor preparation from human placenta contained phosphoprotein phosphatase activity that caused dephosphorylation of the 95,000 dalton subunit of the insulin receptor (18). This evidence prompted us to consider that vanadate might initiate its insulin-like effect by enhancing the degree of phosphorylation of the 95,000 dalton subunit, possibly through the inhibition of phosphotyrosine phosphatase. We therefore compared the effect of vanadate and of insulin on the phosphorylation of the 95,000 dalton subunit of the insulin receptor after solubilization of adipocyte insulin receptor with Triton X-100. When the receptor fraction prepared as described in Materials and Methods was incubated with 10 μM [γ-^{32}P]ATP for 10 min at 4°C in the presence of 2 mM $MnCl_2$ and 4.8 mM $MgCl_2$, following a preincubation with 1 mU/ml insulin for 1 hr at 4°C, the phosphorylation state of 95,000 dalton subunit of insulin receptor was enhanced relative to the control, confirming the previous report by Kasuga et al. (13) (Fig. 2). Preincubation of the

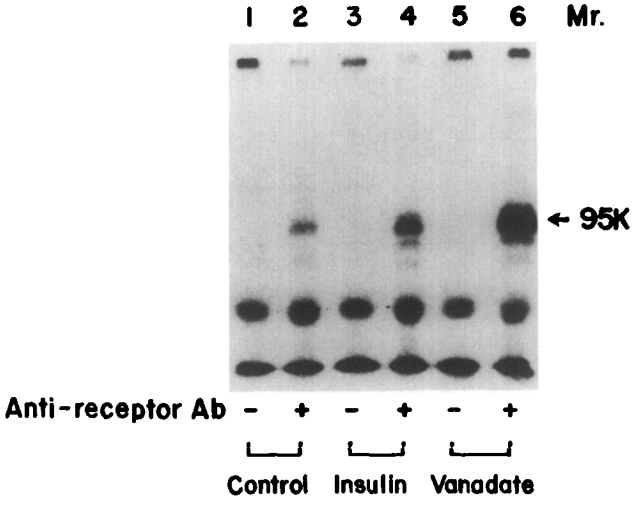

Figure 2 Effect of insulin and vanadate on the phosphorylation of insulin receptor in the solubilized insulin receptor system A 600 μl portion of the solubilized insulin receptor fraction prepared as described in Materials and Methods was preincubated with or without 1 mU/ml insulin or 1 mM vanadate in the presence of 85 mM Hepes buffer pH 7.6, 6.3 mM $MgCl_2$ and 0.004% (w/v) bovine serum albumin in a final volumne of 1 ml at 4°C for 1 hr. The phosphorylation reaction was begun by the addition of 250 μl of 30 μM [γ-^{32}P]ATP (66.7 μCi/nmole)

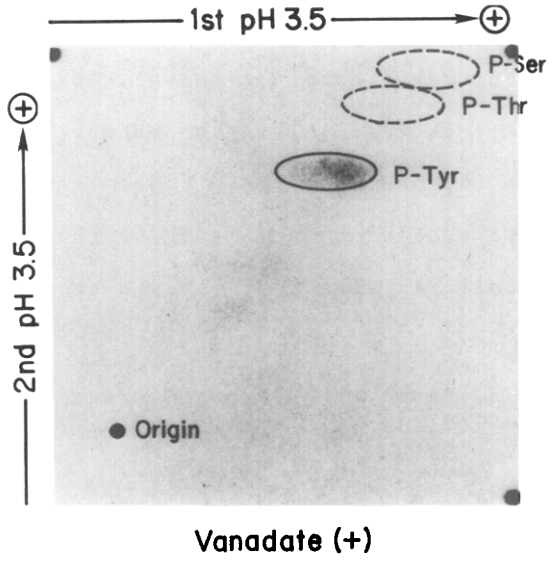

Figure 3 Identification of phosphoamino-acids in the 95,000 Mr phosphoprotein The 95,000-Mr band from the sample preincubated with vanadate, localized by autoradiography as shown in Figure 2 (lane 6), was cut out from the gel and hydrolyzed in 6N HCl for 2 h at 110°C. After lyophilization, the hydrolyzate was spotted on Whatman 3 MM paper. Electrophoresis was performed as described in Materials and Methods. Samples of phosphoserine (Sigma), phosphothreonine (Sigma) and phosphotyrosine (a gift from Dr. C. Schwartz) were added to all radioactive samples analyzed. Standards were located by ninhydrin staining. Radioactivity was located by autoradiography using Kodak XAR-5 film with 2 weeks exposure. Results represent one of two identical experiments.

same receptor fraction with 1 mM vanadate for 1 hr at 4°C greatly enhanced the phosphorylation state of the 95,000 dalton receptor subunit (Figure 2). Analysis of phosphorylated amino acids after acid hydrolysis of the 95,000 dalton subunit demonstrated that only

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and 10 mM $MnCl_2$ and the reaction was continued for 10 min at 4°C. Phosphorylation was stopped by addition of 500 µl of a solution containing of 34.8 mM Hepes buffer pH 7.6, 0.55% Triton X-100, 17.4 mM EDTA, 176 mM KF, 60.9 mM ATP, 17.4 mM sodium pyrophosphate and 34.8 mM sodium phosphate. The sample from each tube was divided into two equal parts and 10 µl of 0.2M PMSF added to each. Either rabbit anti-rat insulin receptor antibody or control rabbit serum was added to each tube to a final 1:200 dilution and the tubes were incubated for 18 hrs at 4°C. A 50 mg aliquot of protein A-Sepharose CL-4B was added to each tube and tubes were further incubated at 4°C for 4 hrs. The protein A-Sepharose CL-4B gel was washed extensively with 50 mM Hepes buffer pH 7.6 containing 0.1% Triton X-100. The samples were eluted from the gel with 1N acetic acid and lyophilized. The lyophilized samples were boiled for 5 min in 25 µl of "SDS solution" composed of 62.5 mM Tris HCl pH 6.8, 2% SDS, 2% mercaptoethanol and 0.1 M DTT before electrophoresis. Electrophoresis was performed as described in Materials and Methods. Results represent one of two similar experiments.

tyrosine phosphorylation was detectable with either vanadate (Figure 3), or insulin pretreatment (data not shown). Since vanadate has now also been shown to act intracellularly to enhance the activation state of glycogen synthase independently of glucose transport, it is tempting to speculate that phosphorylation of the receptor may influence the intracellular effect of insulin on glycogen synthase.

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