# Inhibition of Increasing Effect of Vanadate on Glycogen Content and Lipoprotein Lipase Activity in Fat Pads by 5-*N*,*N*-Hexamethylene Amiloride

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**Sodium orthovanadate (vanadate) increased the glycogen content in isolated rat fat pads in a dose-dependent manner up to 2 mM. Biochanin A, a specific inhibitor of tyrosine kinases, inhibited the increasing effect of vanadate or insulin on both glycogen content and lipoprotein lipase (LPL) activity in fat pads. The increasing effect of vanadate on glycogen content was not decreased by the replacement of $Na^+$ with choline ion in the incubation medium. 5-*N*,*N*-Hexamethylene amiloride, a potent inhibitor of the $Na^+/H^+$ exchange system, showed a 50%-inhibition of the vanadate-increased LPL activity and glycogen content at 25 and 80 $\mu$M, respectively, suggesting that mechanisms of the inhibition differ in part between the vanadate actions. Furthermore, a similar inhibitory profile of the vanadate-increased glycogen content was observed with incubation in the presence or absence of $Na^+$ in the medium. These results suggest that the activation of the $Na^+/H^+$ exchange system by vanadate is not involved in an increase in the glycogen content in fat pads.**

**Keywords** sodium orthovanadata; glycogen content; lipoprotein lipase activity; fat pad; sodium ion; amiloride; hexamethylene amilorid

## Introduction

Sodium orthovanadate (vanadate) shows various biological actions such as insulin-mimetic effects,[1-3] the activation of the $Na^+/H^+$ exchange system,[4] inhibitions of adenosine triphosphatases (ATPases) and phosphatases,[5] and the modulation of lipoprotein lipase (LPL) activity.[6] LPL hydrolyzes triacylglycerides in very low density lipoproteins and chylomicrons to release fatty acids for use in metabolic processes.[7] Our recent reports show that vanadate stimulates an increase in and release of LPL activity by incubation with isolated rat fat pads but not with adipocytes.[8,9] These stimulations markedly decreased in the presence of amiloride or absence of $Na^+$ in the incubation medium, suggesting that the activation of the $Na^+/H^+$ exchange system is involved in the action of vanadate. Glycogen synthase in adipocytes is activated by vanadate *via* the stimulatory phosphorylation of an insulin receptor,[2] but detailed mechanisms still remain to be elucidated. We investigated whether or not vanadate increases both glycogen content and LPL activity in fat pads through a similar mechanism.

The present paper shows that the activation of the $Na^+/H^+$ exchange system is not involved in an increase in glycogen content in fat pads by vanadate.

### Materials and Methods

**Chemicals** The sources of chemicals used in this work were as follows: vanadate ($Na_3VO_4$) and glucose C-test kit from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), amiloride and biochanin A from Sigma Chemical Co. (St. Louid, MO), amyloglucosidase (1,4-α-D-glucan glucohydrolase, *Aspergillus niger*) from Aldrich Chemical Co. (Milwaukee, Wis), intralipos from Green Cross Co. (Osaka, Japan). All other chemicals used were of analytical grade.

**Animals** Male Wistar rats, weighing 200—220 g, were fed on a commercial pellet diet *ad libitum*, given free access to water for 1 week and starved for 24 h before use.

**Synthesis of Amiloride Analog** *N*-Amidino-3-amino-5-*N*,*N*-hexamethylene-6-chloropyrazinecarboxamide (hexamethylene amiloride) was synthesized according to the method of Cragoe *et al.*[10]

**Preparation of Fat Pads and Determination of Glycogen Content** Epididymal adipose tissues were quickly removed from rats killed under ether anaesthesia and cut into small pieces (30—40 mg) with scissors. The fat pads (6 pieces, 200 mg) were preincubated with inhibitors dissolved in dimethylsulfoxide at 37 °C for 15 min in 2 ml of Krebs–Ringer bicarbonate buffer containing 5 mM glucose and 2% bovine serum albumin, pH 7.4, and further incubated with vanadate for 105 min. The fat pads incubated were dissolved in 1 ml of 30% KOH at 100 °C for 10 min, and precipitated by the addition of 1.25 ml of 95% ethanol containing 1.6% $BaSO_4$, as described previously.[11] The precipitates were suspended in 0.5 ml of deionized water, mixed with 1.25 ml of 95% ethanol, and heated at 80 °C for 20 min. After cooling and centrifugation, glycogen content of the precipitates was determined by the enzymatic method using a combination of amyloglucosidase and glucose C-test kit.[11] Results are expressed in terms of μg glycogen/g fat pads.

**Determination of LPL Activity** The fat pads incubated with vanadate were homogenized and centrifuged to prepare crude LPL solution, and LPL activity of the supernatant was determined as described previously.[9] Briefly, the activated intralipos (0.05 ml) was added to the mixture of 0.2 ml of the enzyme solution and 0.25 ml of 30 mM Tris–HCl buffer, pH 8.5, containing 2% bovine serum albumin in the presence or absence of 1 M NaCl and incubated at 37 °C for 30 min. Free fatty acids (FFA) produced were determined by the method of Duncombe.[12] The LPL activity is calculated from values reduced in the presence of 1 M NaCl and expressed as μM FFA produced/h/g fat pads. Results represent mean ±S.E. of four or five observations for two separate experiments.

## Results and Discussion

The incubation of fat pads with vanadate increased the glycogen content in a dose-dependent manner up to 2 mM and maintained a steady-state level up to 10 mM (Fig. 1). This profile was similar to that of the vanadate-increased LPL activity in fat pads.[9] A significant inhibition by biochanin A, a specific inhibitor of tyrosine kinases,[13] was observed with the increasing effect of vanadate or insulin on both glycogen content and LPL activity, suggesting that vanadate as well as insulin increases both glycogen content and LPL activity through a biochanin A-sensitive process, probably stimulatory phosphorylation (Table I). The increasing effect of vanadate on glycogen content, in contrast to that on LPL activity,[9] was not decreased by replacement of $Na^+$ with choline ion in the incubation medium, showing no requirement of extracellular $Na^+$ for the action of vanadate (data not shown).

Next, we investigated whether or not the activation of the $Na^+/H^+$ exchange system is involved in the increasing effect of vanadate on glycogen content. Amiloride inhibits the stimulation of tyrosine kinase activities of the receptors

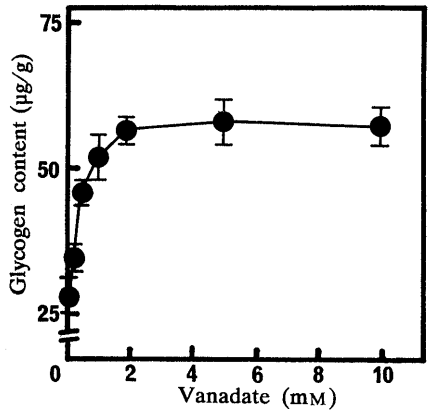


Fig. 1. Dose-Response Curve for Increase in Glycogen Content in Fat Pads by Vanadate

The fat pads were incubated with vanadate at the indicated concentrations at 37 °C for 120 min.

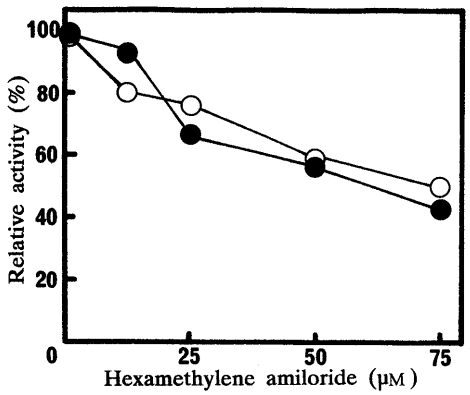


Fig. 2. Inhibition of Increasing Effects of Vanadate on Glycogen Content by Hexamethylene Amiloride in the Presence or Absence of $Na^+$

Results are expressed as percentage of net increase by incubation with vanadate (2 mM) at the indicated concentrations of the inhibitor with $Na^+$ (○) or without (●) in the incubation medium.

TABLE I. Increasing Effects of Vanadate or Insulin on Glycogen Content and LPL Activity in Fat Pads Preincubated with or without Biochanin A

| | LPL activity (μM FFA/h/g) | | Glycogen content (μg/g) | |
|---|---|---|---|---|
| Biochanin A | (−) | (+) | (−) | (+) |
| Control | 5.2±0.2 | 5.5±0.3 | 17± 4 | 14±3 |
| Vanadate (2 mM) | 11.4±1.6 | 6.8±1.0[a)] | 39± 5 | 19±3[a)] |
| Insulin (6 nM) | 7.1±0.5 | 4.8±0.5[a)] | 80±10 | 14±2[a)] |

The fat pads were preincubated with or without biochanin A (100 μg/ml) for 15 min and further incubated with vanadate or insulin for 105 min. *a)* $p<0.05$ compared to vanadate- or insulin-treated group without biochanin A.

TABLE II. Inhibitory Effects of Amiloride and Its Analog on Vanadate-increased LPL Activity and Glycogen Content in Fat Pads

| | $IC_{50}$[a)] (μM) | | Ratio (B)/(A) |
|---|---|---|---|
| | LPL activity (A) | Glycogen content (B) | |
| Amiloride | 250 | 330 | 1.3 |
| Hexamethylene amiloride | 25 | 80 | 3.2 |

*a)* Concentration required for 50%-inhibition calculated from each inhibitory curve.

for insulin and growth factors as well as the activation of the $Na^+/H^+$ exchange system,[14,15] but hexamethylene amiloride shows a rather preferential inhibition of the latter.[16] Table II shows the concentration required for 50%-inhibition ($IC_{50}$) which is calculated from each inhibitory curve. The $IC_{50}$ ratios of an increase in glycogen content to that in LPL activity were calculated to be 1.3 for amiloride and 3.2 for hexamethylene amiloride. The $IC_{50}$ ratio of hexamethylene amiloride shows that the $IC_{50}$ of an increase in glycogen content is higher than that of an increase in LPL activity, suggesting that mechanisms of the inhibition differ in part between the vanadate actions. Furthermore, a similar inhibitory profile of the vanadate-increased glycogen content was observed with the incubation in the presence or absence of $Na^+$ in the medium (Fig. 2). These results suggest that the activation of the $Na^+/H^+$ exchange system is not involved in the increasing effect of vanadate on glycogen content. In the study on Chinese hamster lung fibroblasts (CCL39 cells), vanadate activated the $Na^+/H^+$ exchange system through a pertussis toxin-sensitive pathway, probably due to the stimulation of phospholipase C *via* the activation of G protein.[17] If the activation of G protein is coupled with a biochanin A-sensitive process, there is a possibility that vanadate activates the $Na^+/H^+$ exchange system *via* the process. However, further detailed mechanisms are still unknown.

In conclusion, vanadate appears to show the increasing effect on both glycogen content and LPL activity in fat pads through separated mechanisms after the stimulation of a biochanin A-sensitive process.

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