Non-insulin-like action of sodium orthovanadate in the isolated perfused liver of fed, non-diabetic rats

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Summary. Vanadium compounds exert insulin-like effects on isolated rat adipocytes and skeletal muscle and improve glucose homeostasis in diabetic rats and mice. However, reports on metabolic actions of vanadium in the liver are still contradictory. Thus, the acute effect of sodium orthovanadate infusion on net glucose production was measured in isolated perfused livers of non-fasting, non-diabetic rats. Continuous infusion (0.2 ml/min; 90 min) of vanadate (10–500 $\mu\text{mol/l}$) rapidly increased hepatic glucose ($p < 0.001$), but not cyclic AMP output, reaching peak values after 20 min. The cumulative glucose release displayed concentration dependence with a maximal net effect of 394.3 $\mu\text{mol}/100$ g body weight

and an apparent half-maximal effective vanadate concentration of 19.6 $\mu\text{mol/l}$. The glycogenolytic response to vanadate was almost completely blocked by 100 mU/l insulin ($p < 0.005$), by 0.1 mmol/l indomethacin ($p < 0.05$) and in the absence of Ca^{2+} ($p < 0.001$). These results indicate that sodium orthovanadate stimulates glycogenolysis in livers of fed, non-diabetic rats by a Ca^{2+} -dependent mechanism, which may involve the release of prostaglandins.

Key words: Vanadate, isolated liver perfusion, glycogenolysis, prostaglandins, insulin resistance.

The trace element vanadium has been shown to stimulate glucose transport, glucose oxidation, and potassium uptake as well as to increase the activity of glycogen synthase in isolated rat adipocytes [1–3] and skeletal muscle [4]. These actions are partly attributable to vanadate-dependent inhibition of phosphatases [5] or to increased binding of insulin to its target cells possibly due to enhanced tyrosine phosphorylation of the insulin receptor by vanadate [6]. Alternatively, vanadate could act directly by increasing gene expression of the glucose transporters as shown for GLUT 1 in NIH3T3 mouse fibroblasts [7] and for GLUT 4 in skeletal muscle of streptozotocin-diabetic rats [8]. Moreover, vanadate or vanadyl treatment has been shown to improve glucose homeostasis in vivo in several models of diabetes such as insulin-deficient rats [9, 10], after partial pancreatectomy [11], in insulin-resistant fa/fa rats [12], spontaneously diabetic BB rats [13], and in Aston ob/ob mice [14].

In contrast to its apparently beneficial action on peripheral glucose utilization the effect of vanadium compounds on hepatic carbohydrate metabolism remains controversial. Previously, stimulation of glycogen phosphorylase activity with concomitant inactivation of glycogen synthase by sodium vanadate (0.1–5.0 mmol/l) has been observed in hepatocytes isolated from fed, fasted and

streptozotocin-treated rats [15, 16]. These results are, however, in contrast to an earlier report indicating that vanadate enhances glycogen synthesis in rat hepatocytes [3]. Chronic treatment with sodium orthovanadate revealed different results for hepatic glycogen content in vivo in that liver glycogen was not altered in fa/fa rats [12], but increased two-fold in ob/ob mice [14].

The aim of the study was to determine the net effect of sodium orthovanadate on hepatic glucose metabolism in vitro in the isolated perfused liver of non-fasting, non-diabetic rats. This technique allows hepatic glucose production to be studied independently of counterregulatory hormones and influences due to alterations of peripheral carbohydrate metabolism.

Materials and methods

Experimental animals

Male Sprague-Dawley rats (Him:OFA/SPF, Tierforschungsinstitut Himberg, Himberg, Austria) were kept on a constant 12-h day-night cycle for at least 3 days before the experiment and had free access to standard laboratory rat chow and tap water. The animals of different experimental groups were matched for body weight (range: 220–280 g).

Fig. 1. Time-course of net glucose production of isolated rat livers. Sodium orthovanadate (in $\mu\text{mol/l}$ 0.5: Δ , 5: $\blacktriangle$, 10: $\circ$, 500: $\bullet$) was infused continuously during 90 min. Subsequently glucagon (1 nmol/l) was infused over 30 min to deplete the residual glycogen pools in all experiments. Data are given as means $\pm$ SD of four experiments in each group

Materials

The perfusion medium consisted of Krebs-Ringer buffer (in mmol/l: 143 Na^+ , 6 K^+ , 2.5 Ca^{2+} , 1 Mg^{2+} , 125.5 Cl^- , 25 HCO_3^- , 1 H_2PO_4^- , 1 SO_4^{2-}), 5 mmol/l D(+)-glucose, and 2% dialysed bovine serum albumin (BSA, fraction V; Boehringer Mannheim, Mannheim, FRG). Dialysed aged human erythrocytes were added to give a haematocrit of approximately 20%. Nominally Ca^{2+} -free perfusion media were defined by isomolar replacement of Ca^{2+} by Na^+ . Since these media still contained measurable amounts of Ca^{2+} , ethyleneglycol-bis-(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA, final concentration: 0.5 mmol/l) was added to nominally Ca^{2+} -free media in one group of experiments to reduce the perfusate free Ca^{2+} concentration to below 1 nmol/l [17]. Sodium orthovanadate (Na_3VO_4 ; Sigma, St. Louis, Mo., USA) was dissolved in distilled water prior to dilution in perfusion buffer. Human insulin (Actrapid MC) and glucagon (Novo, Copenhagen, Denmark) were diluted in the perfusion buffer.

Methods

The experiments were started before 10.00 hours in non-fasting rats, to provide for full hepatic glycogen stores [18]. The animals were anaesthetised by intraperitoneal injection of 80 mg/kg body weight thiopental (Sanabo, Vienna, Austria) and their livers were prepared as described previously [19]. The isolated liver (37°C) was perfused in anterograde direction with oxygenated (95% O_2 /5% CO_2) perfusion medium in a non-recirculating system at a constant flow rate (ranging from 6.6 to 7.0 ml/min). After 45 min of equilibration hormones and vanadium compounds were constantly admixed to the main perfusate at a rate of 0.2 ml/min (Perfusor V; Braun Melsungen, Melsungen, FRG). At timed intervals samples were collected from the influent and from the effluent perfusate in ice-chilled tubes.

As in glycogen-rich livers perfused with 5 mmol/l D-glucose hepatic glucose uptake is completely blocked [18], any induced increase in glucose output (e.g. by orthovanadate or glucagon) indicates glycogenolysis [19].

The viability of the liver was checked by normal gross appearance and uniform red colour as well as by constant release of lactate, and by the activities of aspartate aminotransferase (< 5 U/l) and alanine aminotransferase (< 5 U/l). In addition, glucagon (1 nmol/l) was infused at the end of some experiments to prove intact hormonal responsiveness and to estimate the residual glycogen pool [20].

Glucose concentrations were determined enzymatically (Glucose liquiUV; Human GmbH, Taunusstein, FRG). Hepatic glycogen concentrations were estimated enzymatically after precipitation and hydrolysis (1 mol/l HCl) of freeze-clamped livers and expressed as μmol glycosyl units per g liver. The activities of aspartate aminotransferase EC 2.6.1.1. and alanine aminotransferase EC 2.6.1.2. were determined using a standard automated analysis for Hitachi Systems 747 (Boehringer Mannheim).

For measurements of cyclic AMP, the diluted (1:10, volume/volume) perfusate samples were prepared by adsorption on Sep Pack C18 columns (Waters, Milford, Mass., USA), evaporation, and subsequent lyophilisation. After reconstitution in assay buffer [21] cyclic AMP concentrations were estimated by RIA (Amersham Int., Amersham, Bucks, UK).

For determination of calcium and vanadium aliquots of samples (2 ml) were transferred into a porcelain crucible, evaporated to dryness at 90°C and subsequently ashed in a furnace at 700°C for 48 h. The residue was dissolved in 2 ml of 6 mol/l hydrochloric acid and the remaining carbon oxidized by evaporation with 0.5 ml of hydrogen peroxide. The resulting clear solution was diluted with doubly distilled water and analysed for calcium (detection limit $\pm 2 \times \text{SD}$: $0.032 \mu\text{mol/l}$) and vanadium (detection limit $\pm 2 \times \text{SD}$: $0.295 \mu\text{mol/l}$) by inductively-coupled plasma-atomic emission spectroscopy (spectrometer ARL 3520 ICP; Ecublens, Switzerland; [22]).

Statistical analysis

Hepatic production rates of glucose and cyclic AMP were calculated from the trans-hepatic concentration difference times flow rate related to 100 g body weight (bw) or g liver wet weight. In addition, the incremental glucose release was estimated from the area under the time curve by using the trapezoidal rule.

All data are presented as means $\pm$ SD. The statistical significance of differences between the experimental groups were calculated by two-tailed, paired or unpaired Student's *t*-test or analysis of variance (Kruskal-Wallis test) as appropriate.

Results

Vanadium concentrations

The concentrations of vanadium in the effluent medium after 40 min of perfusion as measured by atomic emission spectroscopy were: $617.0 \pm 364.4 \mu\text{mol/l}$ at 500 $\mu\text{mol/l}$, $45.6 \pm 0.6 \mu\text{mol/l}$ at 50 $\mu\text{mol/l}$, $31.4 \pm 28.7 \mu\text{mol/l}$ at 10 $\mu\text{mol/l}$ and $3.03 \pm 0.59 \mu\text{mol/l}$ at 5 $\mu\text{mol/l}$ (means $\pm$ SD of 2–7 different experiments).

Hepatic net glucose production

At low concentrations of sodium orthovanadate (0.5–5 $\mu\text{mol/l}$) hepatic glucose output was not significantly different from basal glucose production rates prior to orthovanadate infusion (Fig. 1). Increasing orthovanadate concentration in the influent perfusate to 10 $\mu\text{mol/l}$ resulted in a transient stimulation of the hepatic glucose production, which turned to baseline values within 45 min (Fig. 1). Higher concentrations of orthovanadate (50 and 500 $\mu\text{mol/l}$) induced a rapid increase of hepatic glucose output with maximal values at 25 and 20 min. In contrast

Table 1. Hepatic glycogen levels before and after infusion of sodium orthovanadate (50 $\mu\text{mol/l}$) or glucagon (1 nmol/l). The mean values ($\pm$ SD) of the three groups were significantly different ($p < 0.05$)

	Duration of perfusion (min)	Hepatic glycogen concentration ($\mu\text{mol glycosyl/g liver}$)	Number of experiments
Control	0	145.8 ± 72.0	5
Vanadate	90	47.7 ± 40.7	5
Glucagon	30	26.4 ± 23.2	4

Fig. 2. Concentration-dependence of the vanadate effect on hepatic net glucose release within 90 min (area under the curve). Data are presented as means $\pm$ SD of four experiments in each group and were fitted to simple saturation kinetics

to the effect of 10 $\mu\text{mol/l}$ orthovanadate, the elevation of glucose production induced by 50 and 500 $\mu\text{mol/l}$ orthovanadate lasted up to 90 min. During the subsequent infusion of 1 nmol/l glucagon, hepatic glucose output showed a second peak within 15 min, which was approximately two-fold higher than that in the presence of 500 $\mu\text{mol/l}$ orthovanadate (Fig. 1). In parallel, a reduction by 67 % (orthovanadate) and 82 % (glucagon) was seen in hepatic glycogen stores ($p < 0.05$) after infusion of 50 $\mu\text{mol/l}$ orthovanadate or 1 nmol/l glucagon (Table 1).

The cumulative glucose production within 90 min (area under the time curve) of orthovanadate infusion displayed a concentration-dependence with a maximal net effect of $394.3 \mu\text{mol}/100 \text{ g bw}$ and an apparent half-maximal effective orthovanadate concentration of $19.6 \mu\text{mol/l}$ as calculated from simple saturation kinetics (Fig. 2).

Antagonistic effect of insulin

When the livers were simultaneously infused with insulin (25–100 mU/l) the increase of hepatic glucose production by 50 $\mu\text{mol/l}$ orthovanadate was inhibited in a concentration-dependent manner (Fig. 3A). In the presence of 100 mU/l insulin the orthovanadate effect was completely blocked. Similarly, the cumulative net glucose release was reduced by 33 % (NS), 85 % ($p < 0.01$) and 92 % ($p < 0.005$) in the presence of 25, 50 and 100 mU/l insulin, respectively (Fig. 3B) suggesting a half-maximal inhibitory effect at approximately 30 mU/l insulin.

Fig. 3(A, B). Insulin-dependent inhibition of hepatic glucose production induced by vanadate. **(A)** Insulin (in mU/l 25: ○, 50: □, 100: △), sodium orthovanadate (50 $\mu\text{mol/l}$: ●, ○, □, △) and glucagon (1 nmol/l: ●, ○, □, △) were infused continuously from -5 to 90, 0–90, and 90–120 min, respectively. **(B)** Cumulative glucose release within the first 90 min (area under the curve) of the experiments presented in A. * $p < 0.01$, ** $p < 0.005$ vs control

Mechanism of orthovanadate-induced hepatic glycogenolysis

Cyclic AMP concentrations in the effluent perfusate were not altered by 50 $\mu\text{mol/l}$ orthovanadate, but increased 11-fold following 1 nmol/l glucagon (Table 2). The orthovanadate-induced stimulation of glucose production was not inhibited by perfusing the livers with nominally Ca^{2+} free medium (Fig. 4). However, the effect of orthovanadate was almost completely abolished, when EGTA (0.5 mmol/l) was added to further reduce the free Ca^{2+} concentration in the perfusion medium. The corresponding area under the curve was lower ($p < 0.05$) in the presence of EGTA than its absence (71.8 ± 18.1 vs $389.9 \pm 258.5 \mu\text{mol/g bw}$). Subsequent infusion of 1 nmol/l glucagon increased hepatic glucose production even in the absence of Ca^{2+} (Fig. 4).

To determine the potential role of prostaglandins in orthovanadate (50 $\mu\text{mol/l}$) induced glycogenolysis, cyclooxygenase activity was inhibited by 0.1 mmol/l indomethacin [23] in a series of experiments. In this context indo-

Table 2. Concentrations of cyclic AMP in the effluent perfusate. Isolated rat livers ($n = 4$) were infused with 50 $\mu\text{mol/l}$ sodium orthovanadate (0–90 min) and subsequently with 1 nmol/l glucagon (95–120 min). Data are given as means $\pm$ SD of four experiments

Time (min)	Perfusate cyclic AMP concentration (pmol/l)
0	88.6 $\pm$ 35.4
10	181.3 $\pm$ 154.3
20	107.2 $\pm$ 18.2
95	135.9 $\pm$ 46.2 ^a
110	468.3 $\pm$ 294.8
120	987.5 $\pm$ 394.7 ^{b,c}

^a $p < 0.05$, ^b $p < 0.025$ vs cyclic AMP level at $t = 0$ min; ^c $p < 0.05$ vs cyclic AMP level at $t = 95$ min

methacin impaired the orthovanadate-induced hepatic glucose production rates (Fig. 5) and reduced ($p < 0.05$) the cumulative hepatic glucose release within 90 min by 58 % (137.5 ± 127.5 vs 324.6 ± 57.5 $\mu\text{mol/g bw}$) suggesting an involvement of arachidonate metabolites in the glycogenolytic action of orthovanadate.

Discussion

The present study demonstrates that low (micromolar) orthovanadate concentrations induce Ca^{2+} -dependent glucose production and thereby decrease glycogen content in isolated livers of non-fasting, non-diabetic rats. This acute effect of orthovanadate can be completely blocked by insulin. Partial inhibition by indomethacin suggests that prostaglandins are, at least partly, involved in the glycogenolytic response to orthovanadate.

Recently, sodium metavanadate, has been shown to inhibit glucose output from livers of fed rats with a maximal effect (50–60 % suppression) at extremely low concentrations of 0.5 to 1 $\mu\text{mol/l}$ [24]. This finding could be explained by enhancement of glycogen synthesis as reported for rat hepatocytes [3]. Using sodium orthovanadate (0.5 $\mu\text{mol/l}$) we observed only a small, but insignificant decrease of glucose production in isolated perfused rat livers between 45–60 min, while micromolar concentrations of orthovanadate in excess of 10 $\mu\text{mol/l}$ stimulated hepatic glucose release. Even at high orthovanadate levels lactate output and low activities of aspartate aminotransferase and alanine aminotransferase in the hepatic effluent remained constant indicating no hepatotoxic effect of vanadium in concentrations up to 500 $\mu\text{mol/l}$. Bosch et al. [15] demonstrated that millimolar concentrations of vanadate exerted a short-term (20 min) glycogenolytic effect in freshly isolated hepatocytes. These authors found inactivation by sodium vanadate of glycogen synthase activity in hepatocytes of fed and fasted rats due to increased phosphorylation of the enzyme's 88 kDa subunit as estimated by incorporation of ^{32}P and subsequent immunoprecipitation [15]. Activation of glycogen phosphorylase with concomitant inhibition of glycogen synthase by 2 mmol/l sodium orthovanadate has also been detected in isolated hepatocytes of streptozotocin-diabetic rats [16]. Despite this similarity to the action of glucagon, orthovanadate does not increase cyclic AMP production as has been shown by others [25]. That finding is in contrast to

Fig. 4. Calcium-dependence of the glycogenolytic effects of sodium orthovanadate (50 $\mu\text{mol/l}$; 0–90 min) and glucagon (1 nmol/l; 90–120 min). Isolated rat livers were perfused without added Ca^{2+} resulting in low Ca^{2+} levels (Ca^{2+} : 1.14 ± 0.83 mmol/l) both with ($\circ$) and without 0.5 mmol/l EGTA ($\bullet$)

Fig. 5. Effect of indomethacin (0.1 mmol/l, –10–90 min) on glucose production stimulated by sodium orthovanadate (50 $\mu\text{mol/l}$, 0–90 min) or glucagon (1 nmol/l, 90–120 min). Time-course of hepatic glucose production in the absence (control: $\bullet$; $n = 4$) or in the presence of indomethacin ($\circ$; $n = 6$)

previous reports on the activation of adenylate cyclase in rat fat cells [26] and rat liver slices [27] using millimolar concentrations of metavanadate. The lack of any glycogenolytic effect of orthovanadate in the absence of physiological Ca^{2+} concentrations indicates that orthovanadate stimulates hepatic glycogen phosphorylase by the calcium-dependent pathway that does not involve cyclic AMP, which is similar to the mechanism reported for epinephrine-induced glycogenolysis [28, 29].

In contrast to this acute effect of orthovanadate in vitro in perfused livers of non-diabetic rats, in vivo experiments revealed that chronic oral treatment with vanadium compounds normalizes the elevated blood glucose levels in various models of diabetes and insulin resistance [9–14]. In streptozotocin-diabetic rats the hypoglycaemic effect of vanadate has been explained not only by an insulin-like action of vanadate on peripheral glucose disposal, but also by a restoration of the effects of insulin on hepatic glucose

production [30, 31]. Long-term (12 weeks) oral treatment with sodium orthovanadate decreased the activities of phosphorylase *a* and total phosphorylase without alteration of glycogen synthase activity in livers of db/db-, but not of non-diabetic mice [32]. This could be in keeping with the finding that hepatic glycogen content was two- and four-fold increased in ob/ob mice [14] and in streptozotocin-diabetic rats [33], respectively, by vanadate pretreatment. Venkatesan et al. [34] reported that oral administration of vanadyl sulphate for 9–12 days augmented insulin-stimulated peripheral glucose utilization significantly and to a lesser extent insulin-mediated suppression of (hepatic) glucose production during submaximal hyperinsulinaemic clamps in streptozotocin-diabetic rats, whereas vanadyl did not affect glucose metabolism in non-diabetic rats. However, in another study liver glycogen levels of vanadate-treated fa/fa rats were not different from those of untreated fa/fa rats [12].

Interestingly the blood concentrations of vanadium required for successful oral vanadate treatment of diabetic animals (20.8 [12], 22.0 [33], 23.6 [10] and 26.8 $\mu\text{mol/l}$ [14]) are close to the half-maximal effective vanadium concentration (19.6 $\mu\text{mol/l}$) calculated for hepatic glycogenolysis in the present study. Any absence of an effect of vanadate on hepatic glucose production and hepatic glycogen stores in vivo may therefore be interpreted as an equilibrium between the glycogenolytic action of orthovanadate and its previously reported insulin-like effects including the restoration of decreased gluconeogenic enzyme activities and the stimulation of glucokinase in hepatocytes isolated from diabetic rats [35, 37].

Stimulation of hepatic glycogenolysis may also be mediated by prostaglandins (e.g. PGE_2 and PGD_2), which are released from non-parenchymal liver cells such as Kupffer cells and to lesser extent by endothelial cells [38, 40]. In this context it is notable that indomethacin was shown to partially block orthovanadate-induced glycogenolysis. This is in keeping with our previous observation that orthovanadate increases the production of PGE_2 in rabbit colon [41].

The finding that acute infusion of orthovanadate stimulates glucose production in perfused livers from fed, non-diabetic rats clearly does not exclude beneficial actions of vanadium compounds in livers from fasting and diabetic animals or of chronic vanadate administration in vivo, which may result in normalization of hepatic glucose production. Nevertheless, the glycogenolytic effect of orthovanadate in rat liver in vitro should be taken into account if hypoglycaemic peripheral vanadate action is considered to be of potential therapeutic use in the treatment of diabetes mellitus.

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