Biochem. J. (1991) 276, 289–292 (Printed in Great Britain)

Peroxovanadates have full insulin-like effects on glycogen synthesis in normal and insulin-resistant skeletal muscle

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1. The insulin-like effects of orthovanadate (10 mM) and peroxides of vanadate (peroxovanadates, at 1 mM) on rates of lactate formation, glucose oxidation and glycogen synthesis were measured in incubated soleus-muscle preparations isolated from non-obese Wistar rats and lean (*fa/?*) or insulin-resistant obese Zucker (*fa/fa*) rats. 2. The stimulation of the rates of lactate formation and glucose oxidation by either orthovanadate or peroxovanadates was of similar magnitude to the stimulation by a maximally effective concentration of insulin (1000 μunits/ml). 3. Peroxovanadates, but not orthovanadate, maximally stimulated the rate of glycogen synthesis in incubated soleus muscles isolated from Wistar rats. 4. When soleus-muscle preparations were incubated in the presence of both insulin (1000 μunits/ml) and peroxovanadates (1 mM), this did not result in a synergistic increase in the rate of total glucose utilization as compared with either agent alone. 5. Soleus muscles isolated from obese (*fa/fa*) Zucker rats exhibited a decrease in response to a physiologically relevant concentration of insulin (100 μunits/ml). Peroxovanadates, at 1 mM, maximally stimulated the rate of glycogen synthesis in soleus muscles isolated from obese (*fa/fa*) Zucker rats. 6. The findings indicate that peroxovanadates are useful and important agents for investigating the mechanism of action of insulin in skeletal muscle.

INTRODUCTION

Skeletal muscle is the chief site of insulin-mediated glucose disposal [1]. In humans with type 2 (non-insulin-dependent) diabetes mellitus, hypertension or obesity, glucose metabolism in skeletal muscle is insensitive to the effects of normal blood concentrations of insulin [1,2]. A characteristic feature of human non-insulin-dependent diabetes mellitus is a decrease in the rate of non-oxidative glucose storage (i.e. mainly muscle glycogen synthesis) [3,4]. No agents, besides high levels of insulin and insulin-like growth factor-1, have been described that can acutely and fully overcome this form of insulin resistance.

Vanadium is a rare group-V trace element that exists in several valence states (from +II to +V) to affect a variety of biological processes. For example, orthovanadate (VO_4^{3-}, +V oxidation state) inhibits Na^+/K^+-ATPase [5]. Vanadate, which is converted into vanadyl (VO^{2+}, +IV) inside cells [6], stimulates the rate of glucose oxidation in adipocytes without a loss of cellular K^+ or inhibition of the rate of $^{86}Rb^+$ uptake [7]. Orthovanadate has qualitative insulin-like effects *in vitro* and can either maximally or partially stimulate glucose transport or glucose oxidation or glycogen synthase activity in adipocytes [6,7], skeletal muscle [8–10] or hepatocytes [8,11]. Administration of vanadium compounds in drinking water [12,13] is an effective means of normalizing blood glucose levels in rats made insulin-deficient with streptozotocin treatment (a model of insulin-dependent diabetes mellitus). Insulin resistance is a major determinant of elevated blood glucose levels in insulin-deficient pancreatectomized rats [14]. This form of insulin resistance largely consists of a decrease in the rate of insulin-mediated glycogen synthesis in skeletal muscle [14]. Vanadate normalizes blood glucose levels in pancreatectomized rats by correcting the defect in insulin-mediated muscle glycogen synthesis [14]. However, there are reports that orthovanadate at the previously given doses [13,14] is toxic [15]. Thus, if more potent insulin-mimetic vanadium compounds were identified, then perhaps these compounds could be administered at lower doses, which may solve the problem of toxicity. Interestingly, glycogen synthesis is only modestly stimulated by orthovanadate in skeletal muscle *in vitro* [8,10] and *in vivo* [9]. Therefore, we investigated the responsiveness of glycogen synthesis to various vanadium compounds in isolated soleus-muscle preparations. In the present study, the effects of insulin, orthovanadate (10 mM) and peroxides of vanadate (peroxovanadates; which are formed by reaction of orthovanadate with H_2O_2) (1 mM) on the rates of lactate formation, glycogen synthesis and glucose oxidation in incubated rat soleus-muscle preparations are reported. Peroxovanadates have potent insulin-like effects in isolated incubated rat adipocytes [16]. Also, we measured the effects of peroxovanadates on glucose metabolism in soleus muscle isolated from lean (*fa/?*) or obese Zucker (*fa/fa*) rats. The obese Zucker (*fa/fa*) rat is an insulin-resistant animal model.

MATERIALS AND METHODS

Animals

Male Wistar rats (Harlan–Olac, Bicester, Oxon., U.K.) were purchased at 5 weeks of age and were kept in the Department's animal quarters until experimentation. Lean and obese Zucker rats were purchased (Harlan–Olac) at 7–9 weeks of age. Rats were fed on a standard chow diet (supplied by SDS, Witham, Essex, U.K.; digestible carbohydrate 52%, protein 16%, fat 2%, non-digestible residue 30%, all by weight). The animals were housed in controlled conditions (23±1 °C; 12 h-light/12 h-dark cycle) and received food and water *ad libitum*, except for the 14 h period before isolation of muscles, when only food was withdrawn. Stripped soleus muscles were routinely prepared between 09:00 and 10:00 h.

Materials

All enzymes, biochemicals and radiochemicals were obtained from sources previously given [17,18]. Sodium orthovanadate and H_2O_2 were purchased from Sigma Chemical Co., Poole, Dorset, U.K. Peroxides of vanadate [19] were generated as

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previously described [20]. Briefly, this involved incubation of sodium orthovanadate with H_2O_2 in incubation medium at 20 °C. After 10 min, catalase (200 μg/ml) was added and the incubation was continued for another 10 min.

Isolation and incubation procedure

Strips of soleus muscle were isolated as originally described by Crettaz *et al.* [21] and tied under tension to stainless-steel clips. After a 30 min preincubation in Krebs–Ringer bicarbonate buffer (pH 7.4) containing 5.5 mM-glucose and 1.5% defatted BSA, muscles were transferred to flasks that contained identical medium plus 0.3 μCi of [U-^{14}C]glucose/ml and insulin or vanadate or peroxovanadates. After incubation for 60 min, muscles were removed, blotted and frozen in liquid N_2; the concentration of lactate in the incubation medium (spectrophotometric or 'net' [22]) or conversion into radiochemical lactate [16] and the rates of incorporation of [^{14}C]glucose into glycogen [23] were measured. It is important to note that the net rate of lactate formation, which is measured by a spectrophotometric assay, yields a measure of the rate of glycolysis from glucose potentially supplied from either muscle glycogen and/or the incubation medium. Radiochemical lactate formation is likely to be from glucose in the incubation medium. The methods for the measurement of glucose oxidation to $^{14}CO_2$ were those previously described [24]; measured $^{14}CO_2$ production rates in the present study were not corrected for any decrease in specific radioactivity of pyruvate owing to any glycogenolysis. Glycogen content was measured as previously described [24]. Statistical significance was determined with Student's *t* test.

RESULTS AND DISCUSSION

Wistar rats

The effect of insulin (1000 μunits/ml), orthovanadate (10 mM) and peroxovanadates (1 mM) on the rates of radiochemical and net lactate formation, glycogen synthesis and glucose oxidation are given in Table 1. Radiochemical lactate formation is a good index of the rate of glucose transport into muscle fibres [17]. Generally, the stimulation of the rates of radiochemical lactate formation and glucose oxidation by orthovanadate (10 mM) or peroxovanadates (1 mM) or a maximally effective concentration of insulin (i.e. 1000 μunits/ml) were similar (see Table 1). However, orthovanadate (10 mM) only partially stimulated the rate of glycogen synthesis (i.e. about 20% of the stimulation by insulin; see Table 1). In marked contrast, peroxovanadates maximally stimulated the rate of glycogen synthesis (Table 1). The content of glycogen was increased by both insulin (1000 μunits/ml) and peroxovanadates (1 mM) [μmol/g, means ± S.E.M.: control 22.3 ± 1.1 (12); insulin 30.1 ± 2.1 (8) ($P < 0.01$); peroxovanadate 26.3 ± 1.9 (11) ($P < 0.05$)]. The responses of glycogen synthesis to peroxovanadates (1 mM) or insulin (1000 μunits/ml) were equivalent (see Tables 1 and 2). Also, peroxovanadates stimulated glycogen synthesis in a concentration-dependent manner, with the concentration of peroxovanadates required for half-maximal stimulation being about 0.1 mM [25]. It was not possible to obtain concentration-dependent responses with orthovanadate *in vitro* (results not shown).

Incubation of isolated soleus muscles with both insulin (1000 μunits/ml) and peroxovanadates (1 mM) did not produce any further increase in the rate of total glucose utilization than with either agent alone (Table 2). The lack of a synergistic effect between insulin and peroxovanadates suggests that both agents utilize a common signal-transduction pathway in skeletal muscle. Alternatively, glycogen synthesis could be maximally stimulated by either insulin or peroxovanadates, and thus it is not possible to stimulate this process further. However, addition of a combination of sub-maximal concentrations of insulin (100 μunits/ml) and peroxovanadates (0.1 mM) did not increase the rate of glycogen synthesis compared with the corresponding insulin or peroxovanadate groups (Table 2). The rate of glucose utilization was increased by the combination of sub-maximal concentrations of insulin and peroxovanadates, and this was due to a significant effect on the rate of lactate formation (Table 2).

It is not known how vanadate produces its insulin-like effects, but the mechanism does not involve vanadate binding to the insulin receptor [26]. Vanadate may be transported into cells on an anion carrier and reduced to the vanadyl form, which is believed to be the insulin-mimetic agent [7]. Vanadate is an inhibitor of phosphotyrosine phosphatases [27]. It is suggested that activation of the tyrosine kinase of the β-subunits of the insulin receptor and tyrosine phosphorylation of uncharacterized intracellular proteins are important events in the transduction of the insulin signal. Peroxovanadates are also potent inhibitors of phosphotyrosine phosphatases [20]. Increased tyrosine phosphorylation of the insulin receptor or substrates of the insulin receptor by peroxovanadate-mediated inhibition of phosphotyrosine phosphatases may be the mechanism responsible for the effects of peroxovanadates, although this has yet to be proven.

Our results show that it is possible to activate maximally glycolysis and glucose oxidation, but not glycogen synthesis, with orthovanadate (Table 1). However, peroxovanadates fully activate all three processes. This may be evidence for the suggestion that the insulin receptor exists in at least three different conformational states [28], i.e. different conformational subsets of insulin receptors signal to different non-equilibrium processes in muscle. Alternatively, separate insulin-mediated mechanisms may exist in skeletal muscle for activation, by insulin, of the pathways of glucose transport/glycolysis or glycogen synthesis.

Table 1. Effects of orthovanadate, peroxovanadates and insulin on glucose oxidation, glycogen synthesis and lactate formation in stripped soleus-muscle preparations *in vitro*

All values are given as means ± S.E.M. for the numbers of observations given in parentheses. Statistical significance was determined by Student's *t* test. Significantly different increases from the control group are indicated by *** $P < 0.001$.

Treatment	Rates (μmol of 'glucosyl units'/h per g wet wt.) of:			
	$^{14}CO_2$ formation	[^{14}C]Glycogen synthesis	Net lactate formation	Radiochemical lactate formation
Control	0.48 ± 0.05 (23)	1.75 ± 0.11 (20)	4.04 ± 0.22 (23)	3.49 ± 0.33 (16)
Insulin (1000 μunits/ml)	0.59 ± 0.05 (23)	5.14 ± 0.21 (19)***	7.07 ± 0.36 (20)***	7.18 ± 0.53 (16)***
Orthovanadate (10 mM)	1.09 ± 0.16 (4)***	2.31 ± 0.31 (4)	11.41 ± 0.78 (4)***	8.14 ± 1.61 (4)***
Peroxovanadates (1 mM)	0.86 ± 0.07 (16)***	4.56 ± 0.43 (16)***	6.21 ± 0.63 (15)	6.85 ± 0.78 (16)***

Table 2. Combined effects of insulin and peroxovanadates on rates of glycogen synthesis, lactate formation and glucose utilization in isolated incubated rat soleus muscle

All values are given as means ± S.E.M. for the numbers of observations in parentheses. Peroxovanadate solutions were prepared as described in the Materials and methods section. Rates of glucose utilization were calculated as [(rates of lactate formation/2) + rate of glycogen synthesis]. This represents a realistic approximation, since the rate of glucose oxidation is small in the stripped soleus-muscle preparation. Significantly different increases (Student's t test) from the control group are indicated by ** $P < 0.01$, *** $P < 0.001$. Significantly different changes between the combination of insulin and peroxovanadates and the corresponding insulin or peroxovanadate groups are indicated by $^{a}P < 0.05$ and $^{b}P < 0.05$ respectively.

Treatment	Rates (μmol of 'glucosyl units'/h per g wet wt.)		
	[^{14}C]Glycogen synthesis	Net lactate formation	Glucose utilization
Control	2.08 ± 0.31 (9)	3.43 ± 0.31 (9)	5.50 ± 0.50 (9)
Insulin (100 μunits/ml)	4.43 ± 0.11 (5)***	4.91 ± 0.19 (5)**	9.35 ± 0.43 (5)***
Peroxovanadates (0.1 mM)	3.69 ± 0.49 (5)**	5.46 ± 0.10 (5)***	9.15 ± 0.43 (5)***
Peroxovanadates (0.1 mM) plus insulin (100 μunits/ml)	4.65 ± 0.40 (50)***	6.41 ± 0.48 (5)***^{ab}	11.00 ± 0.76 (5)***^{ab}
Insulin (1000 μunits/ml)	5.95 ± 0.57 (4)***	7.40 ± 0.59 (4)***	12.10 ± 0.41 (4)***
Peroxovanadate (1 mM)	6.51 ± 0.91 (4)***	6.29 ± 0.93 (3)**	12.34 ± 2.02 (3)**
Peroxovanadate (1 mM) plus insulin (1000 μunits/ml)	5.99 ± 0.72 (4)***	5.86 ± 0.19 (4)***	11.85 ± 0.56 (4)***

Table 3. Effects of insulin and peroxovanadates on rates of lactate formation, glucose oxidation and glycogen synthesis in incubated soleus-muscle preparations isolated from lean and obese Zucker rats

All the values are presented as means ± S.E.M. for four separate incubations. Soleus muscles were incubated in the absence or presence of insulin (100 or 1000 μunits/ml) or peroxovanadates (1 mM). The statistical significance of the difference (Student's t test) between control and treated muscles is indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Significantly different changes from the corresponding lean group are denoted by $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$.

	Rates (μmol/h per g wet wt.)					
	Glycogen synthesis		Glucose oxidation		Lactate formation	
	Lean	Obese	Lean	Obese	Lean	Obese
Control	0.96 ± 0.07	0.87 ± 0.06	0.33 ± 0.06	0.18 ± 0.02^{a}	3.25 ± 0.29	3.29 ± 0.12
Insulin (100 μunits/ml)	4.36 ± 0.63***	1.41 ± 0.10***^{c}	0.63 ± 0.05**	0.29 ± 0.07^{b}	6.29 ± 0.36***	3.84 ± 0.83^{a}
Insulin (1000 μunits/ml)	5.24 ± 1.02***	3.93 ± 0.24***	0.85 ± 0.16*	0.21 ± 0.02^{c}	8.39 ± 1.11***	4.62 ± 0.47^{a}
Peroxovanadates (1 mM)	5.49 ± 2.00***	4.33 ± 0.28***	0.71 ± 0.12*	0.31 ± 0.03**^{b}	9.31 ± 0.40***	6.40 ± 0.21**^{c}

Lean and obese Zucker rats

A characteristic feature of non-insulin-dependent diabetes mellitus is a decrease in insulin-stimulated rates of non-oxidative glucose disposal in skeletal muscle, which is largely reflected as a decline in the rate of glycogen synthesis [3]. The present study has demonstrated that peroxovanadates are novel, in that the magnitude of stimulation of glycogen synthesis in muscle by peroxovanadates or insulin is the same. Therefore, could peroxovanadates ameliorate the decreased responsiveness of glycogen synthesis in insulin-resistant skeletal muscle? To answer this question, soleus muscles were isolated from genetically obese Zucker (*fa/fa*) rats and incubated with peroxovanadates (1 mM). The obese Zucker (*fa/fa*) rat exhibits marked insulin resistance *in vivo* [29]. Compared with soleus muscles isolated from lean littermates (*fa/?*), the sensitivities of glycogen synthesis, glucose oxidation and lactate formation to insulin are significantly decreased in incubated soleus muscle isolated from obese Zucker (*fa/fa*) rats [30]. The effects of insulin (100 and 1000 μunits/ml) and peroxovanadates (1 mM) on rates of glucose oxidation, lactate formation and glycogen synthesis in incubated soleus muscle isolated from lean (*fa/?*) and obese Zucker (*fa/fa*) rats are given in Table 3. The response of glucose oxidation, glycolysis and glycogen synthesis to a physiologically relevant concentration of insulin (100 μunits/ml; Table 1) was significantly decreased in soleus muscles isolated from obese (*fa/fa*) Zucker rats as compared with responses in incubated soleus muscle isolated from lean Zucker (*fa/?*) rats (Table 1). In order to stimulate these processes, this required a high concentration of insulin (1000 μunits/ml). Peroxovanadates significantly increased the rates of glycogen synthesis, lactate formation and glucose oxidation in incubated soleus-muscle preparations isolated from both lean (*fa/?*) and obese (*fa/fa*) Zucker rats (Table 1). However, the peroxovanadate-mediated stimulation of rates of lactate formation and glucose oxidation in soleus muscles isolated from obese Zucker rats was significantly lower than the responses in soleus muscle isolated from lean littermates. Nevertheless, the stimulation of glycogen synthesis by peroxovanadates in soleus muscle from obese (*fa/fa*) Zucker rats was equivalent to the response of a maximally effective concentration of insulin. Peroxovanadates, at 1 mM, significantly increased the content of glycogen in incubated soleus-muscle preparations isolated from obese (*fa/fa*) Zucker rats [μmol/g, means ± S.E.M.; control 23.9 ± 0.5 (4); insulin (1000 μunits/ml) 29.6 ± 0.4 (4) ($P < 0.001$);

peroxovanadates 28.4 ± 1.1 (4) ($P < 0.01$)]. These results demonstrate that peroxovanadates can acutely correct the decreased responsiveness of glycogen synthesis to insulin in soleus muscle isolated from obese (*fa/fa*) Zucker rats. Also, in a preliminary experiment we observed that peroxovanadates, at a sub-maximal concentration (i.e. 0.1 mM; see [25]), stimulated the rate of glycogen synthesis by 40% in incubated soleus muscles isolated from obese Zucker rats (B. Leighton & E. A. Foot, unpublished work). However, further studies are required to determine the precise EC_{50} value for insulin and peroxovanadates in soleus muscles isolated from lean and obese Zucker rats. Also, the investigation of the effects of combinations of sub-maximal and maximal concentrations of insulin and peroxovanadates in insulin-resistant skeletal muscle is warranted.

In conclusion, we have demonstrated that peroxovanadates maximally stimulate the rates of lactate formation, glucose oxidation and glycogen synthesis in isolated incubated soleus-muscle preparations. Furthermore, peroxovanadates are more potent insulin-mimetic agents than is orthovanadate [25]. The results from the present study suggest that peroxovanadates may be utilized as biochemical tools to investigate the mechanism of action of insulin.

We thank Professor E. M. Southern for provision of facilities.

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Received 5 December 1990/22 January 1991; accepted 30 January 1991