The effects of orthovanadate on fatty acid synthesis in isolated rat hepatocytes

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Extracellular Ca^{2+} stimulated fatty acid synthesis in isolated rat hepatocytes. Orthovanadate (0.2–2.0 mM), an inhibitor of Ca^{2+} -dependent ATPases, stimulated fatty acid synthesis in both the presence and the absence of extracellular Ca^{2+} . Insulin stimulated fatty acid synthesis only in the presence of extracellular Ca^{2+} . The contribution of extracellular Ca^{2+} to insulin stimulation of fatty acid synthesis is discussed.

Vanadium occurs in mammalian tissues in oxidation states of +4 and +5 (Macara, 1980). In oxidation-state +5 [orthovanadate (VO_4^{3-}) and metavanadate (VO_3^-)] it is an inhibitor of ($\text{Na}^+ + \text{K}^+$)-dependent ATPases and Ca^{2+} -dependent ATPases (Cantley *et al.*, 1977; O'Neal *et al.*, 1979). In isolated rat adipocytes orthovanadate stimulated hexose transport and the oxidation of [$\text{U}-^{14}\text{C}$]-glucose to the same extent as insulin (Dubyak & Kleinzeller, 1980; Shechter & Karlsh, 1980). Clausen *et al.* (1981) showed that the stimulation of hexose transport by vanadate in adipose tissue and muscle correlated with an increase in free [Ca^{2+}] in the cytosol.

Fatty acid synthesis in isolated rat hepatocytes incubated with added Ca^{2+} is stimulated in the short term by insulin (Müller *et al.*, 1976; Geelen *et al.*, 1978) and vasopressin (Assimakopoulos-Jeannet *et al.*, 1981). When Ca^{2+} is omitted from the incubation medium the stimulation by both insulin (L. Agius & W. J. Vaartjes, unpublished work) and vasopressin (Assimakopoulos-Jeannet *et al.*, 1981) is decreased. An increase in the cytosolic free [Ca^{2+}] has been suggested to be part of the mechanism of action of both vasopressin (Blackmore *et al.*, 1979) and insulin (Davis *et al.*, 1981). A key question is whether an increase in the cytosolic free [Ca^{2+}] is a prerequisite for the stimulation of fatty acid synthesis. If this were the case vanadate would be expected to stimulate fatty acid synthesis. The present work addresses itself to the following questions. Does vanadate affect fatty acid synthesis? What is the influence of extracellular [Ca^{2+}] and how do these effects compare with those of insulin and vasopressin? The results show that vanadate stimulated glycolysis and fatty acid syn-

thesis but not glycogen mobilization. The rate of fatty acid synthesis was influenced by extracellular [Ca^{2+}], but whereas insulin stimulated fatty acid synthesis only in the presence of Ca^{2+} , vanadate stimulated fatty acid synthesis in both the presence and the absence of Ca^{2+} . The possible implications of these findings are discussed.

Experimental

Chemicals

Sodium orthovanadate was from BDH Chemicals (Poole, Dorset, U.K.). Crystalline bovine insulin was from Lilly Laboratories (Indianapolis, IN, U.S.A.). [Arginine]vasopressin (grade VII; 160 m-units/mg), collagenase and coenzymes were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), other enzymes were from Boehringer (Mannheim, Germany). $^3\text{H}_2\text{O}$ was from The Radiochemical Centre (Amersham, Bucks., U.K.).

Rats

Albino Wistar lactating rats fed standard laboratory chow *ad libitum* were used between 14 and 17 days after giving birth (body wt. 300–330 g). Preliminary studies showed that the rates of fatty acid synthesis were appreciably higher in hepatocytes from lactating rats, compared with hepatocytes from male rats of the same strain and body weight. Studies *in vivo* have established that insulin stimulates fatty acid synthesis in liver of lactating rats by 175–220% (Agius & Williamson, 1980; Agius *et al.*, 1981). Because of the better yield of hepatocytes, the high basal rate of fatty acid synthesis and the consistent stimulation by insulin hepatocytes from lactating rats were used in the present study.

Hepatocytes were prepared between 09:30 and 11:00h by collagenase digestion of the liver essentially as described by Seglen (1976). Glucose (20mM) was included in the perfusion medium to minimize hepatic glycogenolysis and the cells were maintained at 37°C throughout. The cells were preincubated for 20min in Krebs–Henseleit (1932) buffer containing 0.5mM-Ca²⁺ and 20mM-glucose. They were then filtered (100µm, mesh), centrifuged and washed once in Ca²⁺-free Krebs–Henseleit buffer and twice in Ca²⁺ free buffer containing 5mM-glucose and 2% (w/v) defatted bovine serum albumin. They were suspended in the latter medium and filtered (60µm mesh). More than 90% of the cells excluded Trypan Blue.

The cells were incubated in Krebs–Henseleit buffer with (2.5mM) or without added CaCl₂, containing 5mM-glucose, 2% bovine serum albumin ³H₂O (0.25mCi/ml) and other additions as indicated, in a total volume of 2.0ml in 25-ml glass Erlenmeyer flasks. The final cell concentration was 14–21mg dry wt./ml. The flasks were gassed with O₂/CO₂ (19:1), stoppered and incubated at 37°C with constant shaking. Under these conditions rates of fatty acid synthesis were linear for up to 90min. At the end of the incubation 500-µl portions were added to 2.0ml of 5% (w/v) HClO₄ for determination of metabolites [pyruvate and L-lactate (Hohorst *et al.*, 1959) and glucose (Slein, 1963)]. The rest of the flask contents were deproteinized with 0.4ml of 20% (w/v) HClO₄ and then saponified and the saponified fatty acids were extracted as described by Stansbie *et al.* (1976).

Statistics were by Student's *t* test.

Results and discussion

In the present study, the basal rate of fatty acid synthesis in hepatocytes prepared from lactating rats varied between 10.8 and 29.2µmol of ³H₂O/h per g wet wt. in the presence of 2.5mM-Ca²⁺. These values compare well with the rate of fatty acid synthesis in liver of lactating rats measured *in vivo* (15.4 ± 3.3µmol of ³H₂O/h per g) (mean ± S.D.; range 11.6 to 19.0; Agius & Williamson, 1980) and with a previous study (Benito & Williamson, 1978) on hepatocytes from lactating rats. Because of the variation in basal rates of fatty acid synthesis, results of representative experiments are shown in Fig. 1 and Table 1. The effects of insulin and vanadate shown in these experiments were observed consistently. In the presence of added Ca²⁺, insulin (85nM) stimulated fatty acid synthesis between 15 and 63% [controls (*n* = 11), 20.6 ± 1.4; insulin treatment (*n* = 11), 27.8 ± 1.8µmol of ³H₂O/h per g; means ± S.E.M.; *P* < 0.005]; vanadate (1mM) stimulated fatty acid synthesis between 18 and 57% and vanadate (10mM) inhibited fatty acid synthesis between 45 and 75% [controls (*n* = 7), 20.2 ± 2.7;

Fig. 1. Effects of Ca²⁺ (2.5 mM), orthovanadate (0.2–10 mM) and insulin (85 nM) on fatty acid synthesis in rat hepatocytes

For experimental details see the text. Open columns indicate incubations in which Ca²⁺ was omitted, hatched columns indicate incubations with 2.5mM-Ca²⁺. Results are means ± S.E.M. for triplicate incubations in one representative experiment, expressed as µmol of ³H₂O incorporated into saponifiable fatty acid/h per g wet wt. of hepatocytes. **P* < 0.05; ***P* < 0.005 relative to the corresponding Ca²⁺-free or Ca²⁺-containing control.

vanadate (1mM) treatment (*n* = 7), 30.5 ± 2.3, *P* < 0.01; vanadate (10mM) treatment (*n* = 4), 9.0 ± 0.7, *P* < 0.01]. In the absence of added Ca²⁺, vanadate (1mM) stimulated fatty acid synthesis between 21 and 51% [controls (*n* = 4), 14.5 ± 0.6; vanadate treatment (*n* = 4), 19.6 ± 0.3, *P* < 0.005], whereas insulin had no effect [controls (*n* = 8), 13.1 ± 1.1; insulin treatment (*n* = 8), 13.7 ± 1.1]. In the absence of other additions fatty acid synthesis was lower (18–44%) in the absence of added Ca²⁺ than in its presence [–Ca²⁺ (*n* = 10), 12.9 ± 1.2; +Ca²⁺ (*n* = 10), 19.8 ± 1.7, *P* < 0.005]. In two preparations of hepatocytes from fed male rats fatty acid synthesis was stimulated by 1mM-vanadate and inhibited by 10mM-vanadate (results not shown).

The effects of Ca²⁺, insulin and various concentrations of vanadate are shown in Fig. 1. Concentrations of 0.2–1.0mM-vanadate stimulated fatty acid synthesis in both the absence and the presence of added Ca²⁺. In the presence of Ca²⁺ the stimulation by vanadate was similar to that by insulin, whereas in the absence of Ca²⁺, insulin did not stimulate fatty acid synthesis. At concentrations of 5 and 10mM, vanadate inhibited fatty acid synthesis, and the incubation was greater in the

Table 1. *Effects of orthovanadate (1 mM), insulin (85 nM) and vasopressin (2.5 m-units/ml) on release of glucose, accumulation of lactate and pyruvate and fatty acid synthesis in rat hepatocytes*

For experimental details see the text. Unless otherwise stated the medium contained 2.5 mM-Ca²⁺. 'Total glucose equivalents' represents: glucose released + $\frac{1}{2}$ (lactate plus pyruvate accumulated) + $\frac{1}{2}$ (two-carbon unit incorporation into fatty acids). 'Glycolysis' represents the sum of the last two factors. Results are means $\pm$ S.E.M. for triplicate incubations in one representative experiment: * $P < 0.05$; ** $P < 0.005$ relative to the control values (with Ca²⁺ and no other addition).

Additions	None	Vanadate	Insulin	Vanadate + insulin	Vasopressin	Vanadate + vasopressin	Insulin + vasopressin	-Ca ²⁺ + vasopressin
Glucose release (μ mol/h per g wet wt.)	29.1 $\pm$ 3.5	19.4 $\pm$ 1.4*	23.4 $\pm$ 6.1	24.2 $\pm$ 0.9	49.8 $\pm$ 0.6**	41.0 $\pm$ 1.2*	42.1 $\pm$ 1.9*	21.7 $\pm$ 2.0
Lactate + pyruvate accumulation (μ mol/h per g wet wt.)	8.4 $\pm$ 2.0	22.8 $\pm$ 3.5**	11.4 $\pm$ 2.3	24.3 $\pm$ 0.8**	22.7 $\pm$ 1.8**	36.8 $\pm$ 0.7**	26.1 $\pm$ 0.8**	6.4 $\pm$ 1.9
[Lactate]/[pyruvate]	4.19 $\pm$ 0.04	4.24 $\pm$ 0.27	4.72 $\pm$ 0.06	4.58 $\pm$ 0.43	4.95 $\pm$ 0.19	4.25 $\pm$ 0.08	4.93 $\pm$ 0.11	3.05 $\pm$ 0.37
Fatty acid synthesis (μ mol of ³ H ₂ O/h per g wet wt.)	29.2 $\pm$ 0.7	38.4 $\pm$ 0.9**	33.7 $\pm$ 1.2	38.8 $\pm$ 1.1**	32.4 $\pm$ 0.9*	35.3 $\pm$ 0.4**	34.9 $\pm$ 1.3*	18.1 $\pm$ 0.4**
Total glucose equivalents (μ mol/h per g wet wt.)	45.8 $\pm$ 4.0	44.2 $\pm$ 3.2	43.5 $\pm$ 5.4	53.1 $\pm$ 1.0	75.2 $\pm$ 1.7**	74.6 $\pm$ 1.2**	70.2 $\pm$ 1.9**	32.9 $\pm$ 0.9
Glycolysis glucose release	0.60 $\pm$ 0.05	1.48 $\pm$ 0.13**	0.88 $\pm$ 0.08*	1.19 $\pm$ 0.05*	0.51 $\pm$ 0.02	0.82 $\pm$ 0.03*	0.67 $\pm$ 0.03	0.54 $\pm$ 0.11
								0.53 $\pm$ 0.01

absence of Ca²⁺. Clumping of the cells, which also assumed non-spherical shapes, was observed with 10 mM-vanadate, as was reported by Seglen & Gordon (1981). Vanadate has been shown to activate adenyl cyclase (Schwabe *et al.*, 1979) and to increase the cyclic AMP concentration in rat liver slices (Catalan *et al.*, 1980). Inhibition of fatty acid synthesis in liver *in vitro* by dibutyryl cyclic AMP and hormones that increase the cyclic AMP concentration is well documented (Harris, 1975). The inhibition with 5 and 10 mM-vanadate may be related to an increase in cyclic AMP concentration.

To establish whether the changes in rates of fatty acid synthesis are associated with changes in glycogenolysis and glycolysis, the rates of accumulation of glucose, lactate and pyruvate were determined and an estimate of glycogenolysis expressed as 'total glucose equivalents' was determined from: the rate of glucose release + $\frac{1}{2}$ (the accumulation of lactate and pyruvate) + $\frac{1}{2}$ (the incorporation of two-carbon units into fatty acid). ³H₂O incorporation was converted into two-carbon equivalents by multiplying by 1.2 (Jungas, 1968). Tricarboxylic acid-cycle activity was ignored. Table 1 shows that insulin or vasopressin did not produce an additional stimulation of fatty acid synthesis in the presence of vanadate. Insulin and vanadate did not affect 'total glucose equivalents' mobilized. Vanadate decreased glucose release but increased the accumulation of lactate and pyruvate and hence glycolysis. Insulin increased the ratio 'glycolysis'/glucose release, but did not affect the accumulation of lactate and pyruvate (Table 1). Vasopressin stimulates glycogenolysis, glucose release and the accumulation of lactate and pyruvate (Hems *et al.*, 1975; Stubbs *et al.*, 1976; Williamson *et al.*, 1980). In the present study both insulin and vanadate decreased the stimulation of glucose release by vasopressin (37% and 43% respectively; $P < 0.01$). Vanadate increased ($P < 0.005$) the accumulation of lactate and pyruvate and hence the glycolytic rate in the presence of vasopressin. In the presence of vanadate alone, the lactate/pyruvate ratio (and presumably the cytosolic NADH/NAD⁺ state) was the same as in the control but lower than the ratio in the presence of insulin or vasopressin. With vasopressin and vanadate the lactate/pyruvate ratio was lower than with vasopressin alone (Table 1), suggesting that the higher glycolytic rate may be related to the more oxidized cytosolic NADH/NAD⁺ state.

The present study confirms a stimulatory effect of extracellular Ca²⁺ on fatty acid synthesis and shows a requirement for extracellular Ca²⁺ for the stimulation of fatty acid synthesis by insulin. This is not unequivocal evidence for the involvement of extracellular Ca²⁺ in the intracellular events of insulin action, since Ca²⁺ may influence insulin binding to the receptor (Cuatrecasas, 1971). However, we have

observed an inhibition of glucose release by insulin in both the absence and the presence of extracellular Ca^{2+} , in the same experiments where insulin stimulated fatty acid synthesis only in the presence of Ca^{2+} (results not shown). Studies with adipocytes showed that extracellular Ca^{2+} is required for the stimulatory effect of insulin on protein synthesis, but not for the stimulation of hexose transport or the inhibition of lipolysis (Jacobs & Krahl, 1973; Fain & Butcher, 1976), suggesting that the requirement for Ca^{2+} may not be related to insulin binding. At concentrations of 0.2–2.0 mM, vanadate stimulated glycolysis and fatty acid synthesis and this was not associated with an increase in glycogen mobilization. In the absence of added Ca^{2+} vanadate did not affect the [lactate]/[pyruvate] ratio (results not shown). The stimulation of fatty acid synthesis by vanadate in the absence of added Ca^{2+} could be related to an increase in cytosolic Ca^{2+} by inhibition of Ca^{2+} uptake by the endoplasmic reticulum (Clausen *et al.*, 1981). This raises the question whether during insulin-stimulation of fatty acid synthesis, extracellular Ca^{2+} could be the main source of the increase in cytosolic Ca^{2+} as recently proposed by Davis *et al.* (1981). It is suggested that vanadate in the concentration range 0.2–2.0 mM is a useful tool for further studies on the relationship between cytosolic Ca^{2+} levels and fatty acid synthesis. Although the present finding that vanadate stimulates fatty acid synthesis in isolated hepatocytes may not necessarily have physiological implications, it is of interest that intraperitoneal administration of vanadate to rats (160 $\mu\text{mol}/100\text{g}$ body wt.) resulted in a stimulation of fatty acid synthesis measured *in vivo*, in both liver and white adipose tissue (L. Agius, unpublished work).

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