Activation of hepatic glycogen phosphorylase *b in vivo* by sodium sulphate in normal (Wistar) and phosphorylase *b* kinase-deficient (*gsd/gsd*) rats

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1. Sulphate ions have been known for some years to enhance the activity of hepatic glycogen phosphorylase *b in vitro*. 2. Here we report that intravenous injections of 4.92 mmol of Na_2SO_4 /kg body wt. to rats induced marked hepatic glycogenolysis *in vivo*, accompanied by polyuria, glycosuria and a mild hyperglycaemia. These effects were observed both in normal (Wistar) rats and in *gsd/gsd* rats that lacked hepatic phosphorylase kinase. 3. In both rat strains the activity of glycogen phosphorylase in liver extracts was enhanced by pretreatment of the animals with Na_2SO_4 , but in phosphorylase kinase-deficient livers the enhancement was solely in phosphorylase *b* activity, whereas both the *a* and *b* forms of the enzyme were activated in normal livers. 4. Hepatic glycogenolysis was also induced by perfusing rat livers, both normal and *gsd/gsd*, with 25 mM- Na_2SO_4 . Under these conditions both the rat strains showed only enhanced activities of glycogen phosphorylase *b*. This suggested that the increased activity of phosphorylase *a* in the extracts of normal livers after Na_2SO_4 administration *in vivo* was due to a hormonally mediated conversion of the *b* form into the *a* form. 5. The activation of glycogen phosphorylase *b* was stable to dilution and appeared to be due to a long-lasting structural change in the enzyme or very tight binding of an activator.

INTRODUCTION

The rate of glycogenolysis in liver or muscle is generally thought to depend on the fraction of glycogen phosphorylase in the active (*a*) form, but it has been known for many years that the *b* form of the muscle isoenzyme can be activated *in vitro* by AMP or IMP (Green *et al.*, 1942), and that phosphorylase *b* has complex allosteric control mechanisms (Graves & Wang, 1972). The activity of the *b* form of the liver isoenzyme is also stimulated weakly by AMP or IMP, and a number of other substances are known to activate the enzyme and to enhance stimulation by nucleotides *in vitro*. Na_2SO_4 (Appleman *et al.*, 1966), NaF (Stalmans & Hers, 1975) and certain organic solvents (Uhing *et al.*, 1979) all act in this way. The aim of the present work was to see whether Na_2SO_4 would stimulate glycogenolysis *in vivo*, and whether this could be attributed to an action on glycogen phosphorylase *b*. The demonstration of such a mechanism would suggest the possibility of alternative physiologically significant activation mechanisms for glycogen phosphorylase.

The simplest system in which to study the role of phosphorylase *b* is a tissue that lacks phosphorylase kinase and so cannot form phosphorylase *a*. There have been a number of studies of this kind on the phosphorylase kinase-deficient mouse, which lacks phosphorylase kinase in its muscles but not in its liver (Danforth & Lyon, 1964; Rahim *et al.*, 1980; Griffiths, 1981), but the role of phosphorylase *b* in the liver is less clear. In the *gsd/gsd* strain of rat phosphorylase kinase is absent from the liver, but not from the muscles (Malthus & Clark, 1977). These animals do not break down their hepatic glycogen in response to normal

hormonal stimuli (Blackmore & Exton, 1981); only ischaemia (Clark *et al.*, 1982; Lutaya *et al.*, 1983) or cyanide poisoning (Conaglen *et al.*, 1984) have been shown to induce glycogenolysis. If the mechanism of action of sulphate ions on hepatic glycogenolysis is indeed via phosphorylase *b*, it should be possible to induce the effect in the phosphorylase kinase-deficient rat liver. We therefore extended the study to *gsd/gsd* rats.

MATERIALS AND METHODS

Animals

Normal Wistar rats were obtained and hepatic phosphorylase *b* kinase-deficient rats (*gsd/gsd*) were bred as previously reported (Lutaya *et al.*, 1983). They were used at 200–250 g body wt. and were anaesthetized by intraperitoneal injection of sodium pentobarbitone (60 mg/kg), which induced deep anaesthesia in both strains of rat within 10–15 min. Na_2SO_4 solution (4.92 mmol/kg; 1.23 M) was then injected into the jugular vein; control animals were given an equivalent dose of NaCl solution. The animals were kept for 30 min in a cabinet at 37 °C, and urine was collected. The liver was then rapidly exposed. A blood sample was taken from the hepatic portal vein and a freeze-clamped sample was taken from the liver with liquid- N_2 -cooled tongs. Groups of six rats that did not receive any treatment were used as controls for the effect of NaCl and Na_2SO_4 injection on hepatic glycogenolysis. The basal glycogen content was measured in freeze-clamped liver samples taken from these control rats 10–15 s after death and compared with glycogen contents 30 min after injection of NaCl or Na_2SO_4 respectively.

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Liver perfusion

Rats were anaesthetized by the same procedure, and aerobic liver perfusion was performed by a method similar to that of Hems *et al.* (1966). The perfusion medium was that of Krebs & Henseleit (1932) but the 1.18 mM-MgSO₄ was replaced with 1.18 mM-MgCl₂; 2% (w/v) bovine serum albumin, washed rat erythrocytes (5%, v/v) and 5 mM-glucose were also added. When the glucose output had become stable (usually after about 45 min), 25 mM-Na₂SO₄ was infused at the time shown. As the glucose output of each liver varied, the results have been normalized to show the increase above this basal output.

For enzyme assays the livers were perfused for 30, 60 or 90 min. Samples taken after 30 min perfusion were used as zero-time controls. Na₂SO₄ or NaCl was infused and, after the stated time, the livers were freeze-clamped between liquid-N₂-cooled tongs, rapidly weighed, pulverized and homogenized with 3.5 vol. of ice-cold pH 7.0 buffer containing 50 mM-glycylglycine and 100 mM-NaF. Phosphorylase activity in the homogenate was immediately measured by the methods described below.

Chemicals

All reagents were purchased from BDH Chemicals, Poole, Dorset, U.K., except the following: [U-¹⁴C]glucose 1-phosphate was purchased from The Radiochemical Centre, Amersham, Bucks., U.K., and enzymes were purchased from either Boehringer Corp., London W.5, U.K., or Sigma Chemical Co., Poole, Dorset, U.K.

Assays

Glucose and glycogen were assayed in groups of five rats by the methods described by Lutaya *et al.* (1983), except where noted. Liver biopsies were extracted by the

method of Lush *et al.* (1979). Phosphorylase *a* was assayed by the method of Lutaya *et al.* (1983); total phosphorylase (*a*+*b*) was measured in the presence of 20 mM-AMP and 20% (v/v) 1,2-dimethoxyethane by the method of Kobayashi & Graves (1982), and phosphorylase *b* activity was taken to be the difference between these two values. Statistical significance was tested by Student's *t* test.

The sulphate content of the rat liver water space was estimated in each strain of rats by using [U-¹⁴C]sucrose and [³⁵S]sulphate, both obtained from Amersham International. The water space of the liver was determined in four *gsd/gsd* rats and four normal rats by using [U-¹⁴C]sucrose. The intracellular sulphate content was then measured after injection of [³⁵S]sulphate into a further four rats from each strain. In these experiments Buffalo rats were substituted for Wistars as the control strain.

RESULTS

Results of the experiments on anaesthetized rats are given in Table 1. Intravenous injection of Na₂SO₄ caused rapid glycogenolysis in both Wistar and *gsd/gsd* rats, whereas control animals, which received an equivalent dose of NaCl, showed no hepatic glycogen breakdown. The concentration of glucose in liver extracts also rose sharply over the 30 min; again, control animals showed on change. At 30 min after Na₂SO₄ administration, the concentrations of liver glucose in Wistar and *gsd/gsd* rats were 6- and 15-fold higher than in the control animals.

In the Wistar rats there was a marked enhancement of phosphorylase activity during the glycogenolysis, as shown in Table 1. Surprisingly, both phosphorylase *b* and phosphorylase *a* activities increased by about

Table 1. Effects of injection of Na₂SO₄ (4.92 mmol/kg) on normal (Wistar) and phosphorylase kinase-deficient (*gsd/gsd*) rats

Animals were treated and assays performed as described in the text. All values are shown as means $\pm$ S.E.M. (*n* = 5). *P* values refer to the significance of the differences between the mean values for Na₂SO₄-treated experimental animals and their respective NaCl-treated controls.

	Treatment . . .	4.92 mmol of NaCl/kg	<i>P</i>	4.92 mmol of Na ₂ SO ₄ /kg
	Rats			
Liver phosphorylase <i>a</i> (μ mol of glucose/min per g of liver)	Wistar <i>gsd/gsd</i>	8.6 $\pm$ 0.6 0.83 $\pm$ 0.35	0.018	13.5 $\pm$ 0.7 1.40 $\pm$ 0.53
Liver total phosphorylase <i>a</i> + <i>b</i> (μ mol of glucose/min per g of liver)	Wistar <i>gsd/gsd</i>	17.5 $\pm$ 0.6 22.7 $\pm$ 0.8	0.0009 (0.0887%) 0.009 (0.922%)	22.3 $\pm$ 0.5 27.6 $\pm$ 1.0
Hepatic glycogenolysis (μ mol of glucose/min per g of liver)	Wistar <i>gsd/gsd</i>	0.5 $\pm$ 1.0 0.5 $\pm$ 1.3	0.018 0.003	3.1 $\pm$ 0.4 10.5 $\pm$ 0.6
Liver glucose (μ mol/g of liver)	Wistar <i>gsd/gsd</i>	8.6 $\pm$ 0.6 6.0 $\pm$ 0.4	2.7 $\times 10^{-5}$ 1.8 $\times 10^{-6}$	53.0 $\pm$ 3.0 91.0 $\pm$ 1.0
Urine output (ml/30 min)	Wistar <i>gsd/gsd</i>	0 0		2.1 $\pm$ 0.4 3.2 $\pm$ 0.3
Glucosuria (μ mol of glucose/ 30 min per g of liver)	Wistar <i>gsd/gsd</i>	0 0		1.8 $\pm$ 0.2 2.9 $\pm$ 0.2
Blood glucose (mmol/l)	Wistar <i>gsd/gsd</i>	5.6 $\pm$ 0.2 4.4 $\pm$ 0.3	0.025 0.004	7.4 $\pm$ 0.3 7.1 $\pm$ 0.2

Fig. 1. Effect of sulphate perfusion on glucose output from (a) (□, ■) normal (Wistar) and (b) (○, ●) phosphorylase kinase-deficient (*gsd/gsd*) rat livers in the presence (□, ○) and absence (■, ●) of 25 mM- Na_2SO_4

Livers were perfused and enzymes assayed as described in the text. The values shown are the means for three perfusions, except for the later points in (a), where only two preparations were studied. The error bars show the S.E.M.

1.5-fold. The increases in both the *a* and *b* activities were statistically significant ($P < 0.002$ and 0.02 respectively; $n = 5$). In *gsd/gsd*-rat livers the much lower phosphorylase *a* activity did not change significantly, whereas the

1.2-fold increase in phosphorylase *b* activity was statistically significant ($P < 0.02$, $n = 5$).

There was marked polyuria in both strains of rat 10–15 min after Na_2SO_4 injection; no urine was passed by control rats injected with NaCl . The urine contained glucose in both cases, and both strains showed a mild hyperglycaemia.

It was not clear from these results whether the increase in phosphorylase *a* activity in the normal rat livers was due to phosphorylase kinase-mediated conversion of the *b* into the *a* forms or to a direct effect on the *a* form of the enzyme. We therefore studied the effect of sulphate infusion in the isolated perfused liver. The effect on glucose output is shown in Fig. 1. In both cases there was a significant rise in glucose output from the livers after NaSO_4 was added to the perfusion medium. The changes in phosphorylase activity are shown in Table 2. In the perfused livers there was no significant rise in phosphorylase *a* activity, but in both normal and phosphorylase kinase-deficient livers there was a sustained and significant rise in phosphorylase *b* activity.

The hepatic intracellular sulphate concentration, measured by using [^{35}S]sulphate, was 8.2 ± 0.3 mM in the *gsd/gsd* rats and 8.0 ± 0.2 mM in the normal rats. The hepatic extracellular sulphate concentration was 8.1 ± 0.7 mM in the *gsd/gsd* rats and 7.5 ± 0.6 mM in the normal rats, showing that Na_2SO_4 equilibrated across the cell membrane.

DISCUSSION

The original intention of these experiments was to induce hepatic glycogenolysis without formation of phosphorylase *a*. This was accomplished in the phosphorylase kinase-deficient livers, which underwent marked glycogenolysis and showed elevated glucose contents with no significant formation of phosphorylase *a*. In these livers the activity of phosphorylase *a* was so low that it could have had little glycogenolytic effect, so the glycogenolysis was probably due to phosphorylase *b*. However, we did not perform experiments to eliminate the possibility that hydrolytic glycogenolysis was taking place.

Table 2. Effect of perfusion with 25 mM- Na_2SO_4 on phosphorylase activity in normal (Wistar) and phosphorylase kinase-deficient (*gsd/gsd*) rat livers

Livers were perfused and enzymes were assayed as described in the text. At zero time the perfusion medium was supplemented with either 25 mM- NaCl or 25 mM- Na_2SO_4 . All values are shown as means $\pm$ S.E.M. for n rats. P values refer to the significance of the differences between each mean value and the enzyme activity after 30 min perfusion, before addition of NaCl or Na_2SO_4 .

Duration of perfusion . . .	Phosphorylase <i>a</i> activity ($\mu\text{mol/g}$ of liver)					Total phosphorylase (<i>a</i> + <i>b</i>) activity ($\mu\text{mol/g}$ of liver)				
	+ 25 mM- NaCl			+ 25 mM- Na_2SO_4		+ 25 mM- NaCl			+ 25 mM- Na_2SO_4	
	30 min	60 min	90 min	60 min	90 min	30 min	60 min	90 min	60 min	90 min
Wistar rats	9.0 ± 1.0	7.6 ± 1.2	7.7 ± 2.2	11.8 ± 1.4	12.5 ± 1.9	21.7 ± 1.1	18.6 ± 1.2	19.0 ± 1.4	28.5 ± 1.4	32.6 ± 2.0
<i>P</i>	—	0.5	0.6	0.2	0.2	—	0.4	0.5	0.03	0.02
<i>n</i>	6	3	3	3	3	6	3	3	3	3
<i>gsd/gsd</i> rats	1.8 ± 0.2	1.5 ± 0.3	1.1 ± 0.3	2.5 ± 0.7	2.1 ± 0.3	20.3 ± 0.5	20.0 ± 0.9	22.5 ± 0.5	24.5 ± 0.9	28.7 ± 1.7
<i>P</i>	—	0.7	0.3	0.3	0.4	—	0.4	0.04	0.02	0.01
<i>n</i>	6	3	3	3	3	6	3	3	3	3

In the normal Wistar rats there was a rise in the activity of both forms of liver phosphorylase after infusion of Na_2SO_4 . The alteration in activity of phosphorylase *a* could have been due to a direct action on phosphorylase *a* itself, or to a conversion of phosphorylase *b* into the active *a* form. The latter mechanism would have been mediated by phosphorylase *b* kinase and so would not have occurred in the *gsd/gsd* livers. Effects due to extra-hepatic neural or hormonal stimuli acting on phosphorylase *b* kinase were eliminated in the perfusion experiments shown in Table 2. No significant activation of phosphorylase *a* occurred here: although the activity of phosphorylase *a* was higher in the sulphate-treated normal livers than in those treated with chloride, the difference was not statistically significant. Nevertheless, the errors were larger in these perfusion experiments, so the possibility of conversion of phosphorylase *b* into the *a* form cannot be ruled out.

The rate of glycogenolysis induced in Wistar rats by infusion of sulphate (3.1 μmol of glucose/min per g of liver; see Table 1 for s.e.m. values) can be accounted for by the observed increase in phosphorylase *a* activity (4.9 μmol of glucose/min per g of liver). In *gsd/gsd* rats, on the other hand, the glycogenolysis was more marked (10.5 μmol of glucose/min per g of liver) and exceeded the increase in total phosphorylase activity (4.9 μmol of glucose/min per g of liver). However, the untreated *gsd/gsd* rats had a high phosphorylase activity (22.7 μmol of glucose/min per g of liver), 96% of which was in the *b* form. As the glycogen content is stable in these livers, this phosphorylase *b* is presumably kept inactive by the absence of the allosteric activators AMP and IMP or by the presence of allosteric inhibitors such as ATP and glucose 6-phosphate, and possibly by a low concentration of the substrate P_i (Iles *et al.*, 1985). If Na_2SO_4 infusion altered the balance between these allosteric modulators or increased the concentration of P_i , in addition to the observed increase in the activity of phosphorylase *b*, then the enhanced glycogenolysis could be explained. An effect of this kind might have occurred in the normal livers, although it need not be postulated to account for the present results.

The marked glycogenolysis induced by relatively low concentrations of sulphate ions was unexpected. Stalmans & Hers (1975) found that about 200–300 mM-sulphate was necessary for half-maximal activation of phosphorylase *b in vitro*, whereas 8 mM was found in the

hepatic cell water of both rat strains in the present experiments.

The statistically significant rise in total hepatic phosphorylase activity in both normal rats (27%) and *gsd/gsd* rats (22%) was also surprising. It was stable to 38-fold dilution of the homogenates before the assay, and, since the concentration of Na_2SO_4 in the liver water was only 8 mM, it is unlikely that the residual sulphate in the assay medium would have been sufficient to activate the enzyme. As the period of the experiment was too short for significant protein synthesis, it seems probable that sulphate treatment induced a long-lasting activation of phosphorylase *b in situ*.

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