Hormonal control of pyruvate dehydrogenase activity in rat liver

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1. A detailed study of the control of liver pyruvate dehydrogenase activity by various hormones was carried out with perfused liver and isolated hepatocytes. 2. Vasopressin produced a significant increase in the enzyme activity in fed rats, and the time course and sensitivity of the response was similar to that of glycogen phosphorylase *a*. The enzyme from starved animals was resistant to hormonal activation. The possible factors involved in the above effects are discussed. 3. Angiotensin and phenylephrine also increased pyruvate dehydrogenase activity, and the magnitude of the response was of the same order as that to vasopressin by the liver enzyme. 4. The effects of these hormones on pyruvate dehydrogenase activity were critically dependent on extracellular Ca^{2+} , thus suggesting a role for this ion in the mechanism of action of the hormones. 5. Insulin did not appear to have a role in the control of the enzyme activity, as shown by its lack of effect on the enzyme. 6. Glucagon, in contrast with previous reports, produced a rapid, transient and significant increase in pyruvate dehydrogenase activity. 7. The physiological importance of the above effects is discussed.

Several studies indicate that the potent and rapid stimulation of hepatic glycogenolysis by vasopressin is associated with an increase in hepatic glycolysis. Thus the net loss of glycogen caused by vasopressin is more than sufficient to account for the glucose released (Hems & Whitton, 1973; Hems *et al.*, 1978a), and phosphorylase is activated at very low concentrations of vasopressin without any glucose release (Hems *et al.*, 1976, 1978a). The absence of a significant effect of the hormone on lactate production (Hems & Whitton, 1973; Hems *et al.*, 1978a), the increase in oxygen uptake (Hems *et al.*, 1978a) and lack of stimulation of fatty acid synthesis (Ma & Hems, 1975; Hems *et al.*, 1975) suggest that there may be increased pyruvate oxidation with an increase in pyruvate dehydrogenase activity. A rapid stimulatory effect of vasopressin on pyruvate dehydrogenase has been shown in the perfused liver (Hems *et al.*, 1978b). It has been suggested that the activation may be via the stimulation of pyruvate dehydrogenase phosphatase, by an increase in the intra-mitochondrial concentration of Ca^{2+} (Hems *et al.*, 1978b).

The present paper further characterizes the activation of pyruvate dehydrogenase in hepatocytes exposed to vasopressin and reports that angiotensin and phenylephrine, which are also thought to increase cytoplasmic concentration of Ca^{2+} , also activate pyruvate dehydrogenase. Some preliminary studies of this work have been reported (Denton *et al.*, 1981).

Materials and methods

Animals

Albino Porton–Wistar rats (180–220 g) were fed on a standard PRD diet (Williams Lilli Co., Surrey, U.K.). Some rats were starved for 48 h from about 10:00 h before use.

Chemicals

Chemicals were of the highest grade commercially available. Most enzymes and substrates for assays were obtained from C. F. Boehringer Corp. (London) Ltd., Lewes, Sussex, U.K. CoA (sodium salt), L-phenylephrine hydrochloride and [8-arginine]vasopressin were purchased from Sigma (Kingston-upon-Thames, Surrey, U.K.). Radioactive chemicals were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Insulin (glucagon-free) was the highest grade

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commercial ox preparation from Burroughs Wellcome and Co., Dartford, Kent, U.K., and glucagon (insulin-free) was kindly given by Eli Lilly, Indianapolis, IN, U.S.A. Angiotensin (synthetic [5-valine] analogue) was kindly provided by the Medical Research Council National Institute of Biological Standards (Hampstead, London N.W.3, U.K.). Arylamine acetyltransferase was prepared in our laboratory from pigeon liver, essentially by the method of Tabor *et al.* (1953). *p*-(*p*-Aminophenylazo)benzenesulphonic acid was kindly given by Dr. R. M. Denton (University of Bristol, Bristol, U.K.).

Perfusion of rat liver

Livers were perfused with 60 ml of bicarbonate-buffered medium containing 5 mM-glucose (except when livers from starved rats were being perfused), albumin and washed rat erythrocytes, gassed with O₂/CO₂ (19:1) (Hems & Whitton, 1973). After a period of about 50 min, a control liver sample was removed and immediately freeze-clamped at the temperature of liquid N₂. The various effectors were added and a second liver sample was taken after 5 min.

Preparation and incubation of hepatocytes

Isolated hepatocytes were prepared by perfusion of the liver with collagenase essentially as described by Krebs *et al.* (1974) with minor modifications (Hems *et al.*, 1978a). Viability of hepatocyte preparations was assessed in several ways (Hems *et al.*, 1978a; Oviasu, 1980).

Cells were incubated in duplicate, as described elsewhere (Hems *et al.*, 1978a). In most cases, cells were incubated for a period of time before the addition of agonists. The incubations were stopped by rapidly freezing either a 0.5 ml sample immediately in liquid-N₂-cooled tubes for phosphorylase assay, or the cell pellet after centrifugation of a 1 ml sample for the assay of pyruvate dehydrogenase.

Extraction and assay of pyruvate dehydrogenase

Pyruvate dehydrogenase was assayed by a method based on that of Coore *et al.* (1971). Frozen samples of liver were extracted at 0–4°C and assayed for the enzyme activity as described by Hems *et al.* (1978b). The enzyme activity obtained by this procedure is referred to as the 'initial' enzyme activity. For the determination of 'total' pyruvate dehydrogenase activity, samples were taken from livers that were perfused with and without hormone. These 'whole' liver samples were left in the perfusion cabinet at 37°C for 30 min, after which they were frozen in liquid N₂. The frozen liver samples were then extracted and assayed for the enzyme activity as described.

Preliminary studies have shown that this treatment, of severing the liver samples from their blood supply and incubating them under hypoxic conditions for a period of 30 min, brought about complete activation of the enzyme complex (Oviasu, 1980). It should be emphasized that it was the 'whole' liver samples that were incubated, and not the homogenized extracts, and as such inactivation by lysosomal proteinases would be prevented. The endogenous amounts of phosphatase, Mg²⁺ and Ca²⁺ were found to be adequate for the conversion process.

Enzyme activity is expressed as μmol of acetyl-CoA produced/min per g wet wt. of liver at 30°C (unless otherwise stated), or as a percentage of the total enzyme activity.

The procedure for the assay of pyruvate dehydrogenase activity in isolated rat hepatocytes was essentially as described for whole liver, except that the extraction of the cell pellet was in 300 μl of 100 mM-sodium phosphate buffer, pH 7.3, which contained 1% Triton X-100 in addition to the compounds detailed by Hems *et al.* (1978b). The extract was suspended in a Gillette syringe and ten strokes were applied to break the cells, after which they were frozen in liquid N₂ and thawed. Samples of the extract were then assayed for pyruvate dehydrogenase activity.

Glycogen phosphorylase a assay

Glycogen phosphorylase *a* was assayed with [¹⁴C]glucose 1-phosphate, in conditions which specifically exclude activity of the *b* form (Stalmans & Hers, 1975; Hems *et al.*, 1976). The enzyme activity in isolated hepatocytes was determined essentially as described by Hems *et al.* (1976) with minor modifications (Hems *et al.*, 1978a).

Results and discussion

Comparison of vasopressin effects in hepatocytes and perfused liver

The proportion of pyruvate dehydrogenase in the active form was significantly increased in both perfused livers and isolated hepatocytes from fed rats (Table 1). A 2-fold increase in enzyme activity was observed in perfused livers, and about 50% increase occurred in isolated hepatocytes. Although the increase in enzyme activity caused by vasopressin was less in isolated hepatocytes, the sensitivity of the enzyme from both systems to vasopressin was similar. In fact, activation of pyruvate dehydrogenase in perfused livers and isolated hepatocytes was observed over the entire range of concentrations tested, 200 pM–50 nM, with maximum activation occurring at 20 nM-vasopressin (Fig. 1). The fact that vasopressin increased the

Table 1. *Effects of vasopressin and other hormones on pyruvate dehydrogenase and phosphorylase a activities*

(a) Livers were perfused as described in the text. A control liver sample was removed after 50 min. Vasopressin (50 nM) was then added to the perfusion media and a second liver sample taken after 5 min. (b) Isolated hepatocytes were preincubated in bicarbonate-buffered saline containing 5 mM-glucose, after which vasopressin (20 nM), phenylephrine (20 μ M), angiotensin (90 nM) or insulin (10 munits/ml) were added to appropriate vials for 5 min. Results are given as mean $\pm$ S.E.M. for the numbers of observations in parentheses. Total pyruvate dehydrogenase activities were 1.99 ± 0.14 , 2.06 ± 0.15 and 1.91 ± 0.08 μ mol/min per g wet. for perfused livers from fed and starved rats and for isolated hepatocytes respectively, and there was no effect of hormone on total activity. $**P < 0.01$, $*P < 0.05$ versus appropriate control; $\dagger P < 0.05$ versus fed 'normal' control.

	Pyruvate dehydrogenase activity (initial, as % of total)						Phosphorylase <i>a</i> activity (μ mol/min per g wet wt.)	
	No hormone	Vasopressin	Phenylephrine	Glucagon	Angiotensin	Insulin	No hormone	Vasopressin
(a) Perfused liver from:								
Fed rats (4)	26 $\pm$ 2	50 $\pm$ 7*	—	—	—	—	5.2 $\pm$ 0.15	14.4 $\pm$ 2.6*
48 h-starved rats (4)	9 $\pm$ 1†	11 $\pm$ 2	—	—	—	—	2.5 $\pm$ 0.72†	10.8 $\pm$ 2.9*
							(μmol/min per g dry wt. of cells)	
(b) Isolated hepatocytes from:								
Fed rats (4–9)	25 $\pm$ 1	35 $\pm$ 3*	36 $\pm$ 5*	40 $\pm$ 4**	31 $\pm$ 2*	27 $\pm$ 2	28.5 $\pm$ 6.5	57.1 $\pm$ 6.7*

activity of pyruvate dehydrogenase in perfused livers as well as in isolated hepatocytes indicates that its effect is a direct one on the liver cells and not secondary to the vasoconstrictor effect of the hormone. The concentrations of vasopressin that elicit changes in pyruvate dehydrogenase activity correspond to those that occur in blood in 'stressed' animals (for review, see Hems & Whitton, 1980), and as such its effect on the enzyme could be of physiological importance *in vivo*.

The time course of the vasopressin effect on pyruvate dehydrogenase was similar to that of phosphorylase a in isolated hepatocytes (Fig. 2). Maximal stimulation of the two enzymes occurred between 1 and 2 min exposure to vasopressin. The half-maximal stimulation of pyruvate dehydrogenase and phosphorylase a activities occurred at about 2 nM- and 1 nM-vasopressin respectively (Oviasu, 1980), thus indicating that the sensitivity of phosphorylase to vasopressin (which is well documented; Hems *et al.*, 1976; Stubbs *et al.*, 1976; Keppens *et al.*, 1977; Van de Werve *et al.*, 1977) and that of pyruvate dehydrogenase in the present study are similar. This observation would be consistent with the increase in aerobic oxidative carbohydrate metabolism observed in the presence of the hormone (Hems & Whitton, 1973; Hems *et al.*, 1976, 1978a,b).

Effect of starvation on vasopressin effect in perfused livers

The proportion of pyruvate dehydrogenase in the active form was significantly decreased in livers from 48h-starved animals, compared with that from fed rats (Table 1). This result is in agree-

ment with those of other groups, who have shown that starvation decreases the active form of pyruvate dehydrogenase complex in various organs (Wieland *et al.*, 1971; Kerbey *et al.*, 1976; Siess & Wieland, 1976; Baxter & Coore, 1978). Starvation had no effect on the total enzyme activity. In marked contrast with the fed state, where a doubling in pyruvate dehydrogenase activity is observed in response to vasopressin, very little or no increase occurs in the liver enzyme from starved rats (Table 1). Consistent with this result is the observation that when hepatocytes from fed rats are treated with vasopressin in the presence of oleate as substrate, no increase in enzyme activity is observed, whereas the response is normal in the presence of glucose as substrate (Oviasu, 1980). The reason for the lack of an effect of vasopressin on the enzyme in livers from starved animals is not very clear, and further studies are required in order to establish the basis for the different responses.

Control of pyruvate dehydrogenase activity by other hormones (and agonists)

Apart from vasopressin, angiotensin and the α -adrenergic agonist phenylephrine also increase pyruvate dehydrogenase activity in isolated hepatocytes (Table 1). The effects were similar to that of vasopressin, although the magnitudes of the responses were slightly less. The mechanism of action of the above peptide hormones on hepatic glycogenolysis is similar to that of α -adrenergic agents (at least in cells containing normal Ca^{2+} concentrations), in that it is independent of cyclic AMP (Assimakopoulos-Jeannet *et al.*, 1977; Kep-

Fig. 1. Effect of vasopressin on pyruvate dehydrogenase activity in perfused liver (●) and isolated hepatocytes (○) from fed rats

Rat livers were perfused and hepatocytes prepared as described in the text. Pyruvate dehydrogenase assay in samples from perfused livers were carried out at 25°C. Results are means $\pm$ S.E.M. (bars) for three to seven preparations and are shown as increases in enzyme activity caused by the hormone (control value 0.11–0.26 μ mol/min per g wet wt.). Enzyme assays in hepatocytes were carried out at 30°C and the results are means $\pm$ S.E.M. (bars) for six cell preparations, expressed as the increment over control activity (0.45 ± 0.03 μ mol/min per g wet wt.). All values were statistically significant at $P < 0.01$ or $P < 0.05$ compared with appropriate control values.

pens *et al.*, 1977). The observation that all three agents produce similar effects on the enzyme could suggest that such effects are brought about by a similar mechanism, and results in the subsequent section provide evidence to support this.

Insulin had no significant effect on pyruvate dehydrogenase activity (Table 1). In contrast with previous observations (Mukherjee & Jungas, 1975; Claus & Pilkis, 1977) was the finding that glucagon (5 nM) causes a transient and significant increase in the enzyme activity in hepatocytes incubated with glucose (Table 1; Oviasu, 1980). Since the effects of glucagon are mediated mainly via an increase in cyclic AMP concentration, and the hormone activates pyruvate dehydrogenase, it is probable that cyclic AMP could also increase the enzyme

Fig. 2. Time course of vasopressin effect on pyruvate dehydrogenase and glycogen phosphorylase activities in isolated hepatocytes from fed rats

Hepatocytes were prepared as described in the text, and incubated in the presence of 5 mM-glucose for 30 min. Pyruvate dehydrogenase and phosphorylase *a* activities were measured after various times in response to 20 nM-vasopressin. Results are expressed as either an increase in pyruvate dehydrogenase activity caused by vasopressin, total activity being 1.8 μ mol/min per g wet wt. of cells and control activity being about 30% of the total activity, or the increase in phosphorylase *a* activity by hormone, control activity being 26.6 ± 1.3 μ mol per g dry wt. of cells. Results are means $\pm$ S.E.M. (bars) for three to eight cell preparations. All 1, 2, 5 and 10 min values were significant ($P < 0.05$), as assessed by the paired two-tailed *t* test.

activity. Consistent with this hypothesis is the observation that the activity of the enzyme increases in the presence of this 'second messenger' (Table 2).

Mechanism of action of hormones

The effects of vasopressin, angiotensin and the α -agonist phenylephrine on hepatic glycogenolysis are dependent on Ca^{2+} (Stubbs *et al.*, 1976; Keppens *et al.*, 1977; Table 2). The likely site of Ca^{2+} action is phosphorylase *b* kinase, which is stimulated by the cation, thus resulting in an increase in phosphorylase *a* (for review, see Whitton, 1981). The stimulation of pyruvate dehydrogenase phosphate phosphatase in mammalian systems by Ca^{2+} is also well documented (Linn *et al.*, 1969; Denton *et al.*, 1972; Roche & Cate, 1977). It is therefore possible that the above hormones, which also activate pyruvate dehydrogenase, could do so via changes in the mitochondrial free $[\text{Ca}^{2+}]$. It has been reported that increases in the extramitochondrial $[\text{Ca}^{2+}]$ within the physiological range activate pyruvate dehydrogenase in coupled heart and adipose-tissue mitochondria (Denton *et al.*, 1980; McCormack & Denton, 1980).

Table 2. *Effect of Ca^{2+} depletion on the activation of pyruvate dehydrogenase and phosphorylase a by hormones in hepatocytes from fed rats*

Isolated hepatocytes were washed, resuspended and preincubated in either the presence or absence of Ca^{2+} . In addition to omission of Ca^{2+} , one group of cells was preincubated in the presence of EGTA (0.5 mM). Glucose (10 mM) was present in all incubations. After 30 min, vasopressin (20 nM), phenylephrine (20 μM), glucagon (5 nM) and cyclic AMP (10 μM) were added for an additional 5 min period, and samples were taken for the determination of pyruvate dehydrogenase and phosphorylase a activities. Results are expressed as increments over control enzyme activities. Control pyruvate dehydrogenase activities are expressed as a percentage of the total enzyme activity (1.08 $\mu\text{mol/min per g wet wt.}$) and are $31 \pm 2\%$, $40 \pm 4\%$ and $29 \pm 5\%$ for 'normal' Ca^{2+} , no added Ca^{2+} and no added Ca^{2+} plus EGTA (0.5 mM) respectively. Control phosphorylase a activities are 29.9 ± 5.3 , 31.3 ± 4.2 and $14.1 \pm 2.1 \mu\text{mol/min per g dry wt.}$, for 'normal' Ca^{2+} , no added Ca^{2+} and no added Ca^{2+} plus EGTA (0.5 mM) respectively. Results are means $\pm$ S.E.M. for three to seven cell preparations. * $P < 0.05$ as assessed by paired two-tailed t test.

Additions	Increase in enzyme activity					
	Pyruvate dehydrogenase (as % of total activity)			Phosphorylase a ($\mu\text{mol/min per g dry wt.}$)		
	'Normal' Ca^{2+}	No added Ca^{2+}	No added Ca^{2+} + EGTA (0.5 mM)	'Normal' Ca^{2+}	No added Ca^{2+}	No added Ca^{2+} + EGTA (0.5 mM)
Vasopressin	$15 \pm 2^*$	$8 \pm 2^*$	0 ± 1	$35.6 \pm 3.7^*$	1.1 ± 2.6	-1.4 ± 2.3
Phenylephrine	$12 \pm 3^*$	2 ± 2	3 ± 1	$32.5 \pm 4.3^*$	$12.7 \pm 2.6^*$	$15.7 \pm 3.3^*$
Glucagon	$24 \pm 4^*$	$9 \pm 3^*$	4 ± 2	$46.5 \pm 5.2^*$	$37.5 \pm 2.4^*$	$37.9 \pm 5.2^*$
Cyclic AMP	$11 \pm 2^*$	6 ± 3	3 ± 2	$36.2 \pm 4.9^*$	$33.2 \pm 3.0^*$	$30.6 \pm 5.5^*$

The effect of Ca^{2+} depletion on the stimulation of hepatic pyruvate dehydrogenase by vasopressin, phenylephrine, glucagon and cyclic AMP were assessed by omitting Ca^{2+} from the incubation medium. Phosphorylase activity under the above conditions was studied in parallel. The hormonal effects were decreased in all cases, but significant increases in pyruvate dehydrogenase activity owing to vasopressin and glucagon were still apparent (Table 2). In the absence of added extracellular Ca^{2+} , as well as in the presence of 0.5 mM-EGTA, the effects of all agents, including those of vasopressin and glucagon, were abolished (Table 2). All hormones tested produced significant increases in phosphorylase a activity in cells containing normal Ca^{2+} . In the absence of Ca^{2+} , the effects of vasopressin were abolished, whereas those brought about by the other agents were still apparent, although the magnitude was less (Table 2). These results are in general agreement with those of Keppens *et al.* (1977), Whitton *et al.* (1977), and Hems *et al.* (1978a). The results obtained lend some support to the earlier hypothesis that the effects of vasopressin, angiotensin and phenylephrine on pyruvate dehydrogenase activity are dependent on Ca^{2+} ions. In view of the conflicting reports in the literature regarding the initial flux of Ca^{2+} during the activation of cytosolic phosphorylase b kinase and mitochondrial pyruvate dehydrogenase phosphate phosphatase (Exton, 1979; Babcock *et al.*, 1979; Denton *et al.*, 1980; Goldstone *et al.*, 1983; Sies *et al.*, 1983), it is obvious that an investigation of the

subcellular distribution of Ca^{2+} in parallel with the activities of pyruvate dehydrogenase and glycogen phosphorylase in the presence of these hormones is required in order to clarify this area of controversy.

The increases in pyruvate dehydrogenase activity reported in the presence of vasopressin could also involve alterations in the amount of some metabolites known to modify the activity of pyruvate dehydrogenase kinase. However, under the same conditions where vasopressin produced a significant increase in pyruvate dehydrogenase activity, there was a decrease in the amount of pyruvate released from isolated hepatocytes. The mitochondrial redox state, as assessed by the β -hydroxybutyrate/acetoacetate ratio, in response to vasopressin in hepatocyte suspensions would suggest that the effects of the hormone on pyruvate dehydrogenase were not mediated via alterations in pyruvate dehydrogenase kinase activity, as no decrease was observed (Oviasu, 1980).

Results from the present study indicate that increases in pyruvate dehydrogenase activity in response to vasopressin, angiotensin and phenylephrine are mediated mainly via alterations in pyruvate dehydrogenase phosphate phosphatase activity.

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