Effect of treatment of rats with dexamethasone *in vivo* on gluconeogenesis and metabolite compartmentation in subsequently isolated hepatocytes

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Hepatocytes prepared from rats treated with dexamethasone for 2 or 3 h and maintained in the presence of $10\mu\text{M}$ -dexamethasone in the preparation and incubation buffers showed significantly elevated rates of gluconeogenesis compared with those prepared from control animals. Dexamethasone treatment also increased the sensitivity of the cells to glucagon and the catecholamines. Analysis of the concentrations of metabolites in the gluconeogenic pathway indicated that dexamethasone decreased the intracellular concentration of pyruvate and increased those of phosphoenolpyruvate, acetyl-CoA and citrate, suggesting a stimulation of the reaction(s) converting pyruvate into phosphoenolpyruvate. This was substantiated by analysis of the pattern of metabolites found in the mitochondrial compartment after digitonin fractionation of the cells. Inclusion of 3-mercaptopycolinate in the incubation enhanced the effect of the hormone on the distribution of metabolites. Thus, in the absence of an effect of the steroid at the level of phosphoenolpyruvate carboxykinase or pyruvate kinase, dexamethasone treatment still increased the formation of malate, aspartate and citrate from pyruvate, indicating a stimulation in the intact cell of pyruvate carboxylase. It is suggested that the stimulation of pyruvate carboxylase is a result of a general activation of mitochondrial function, with an increase in the intramitochondrial concentrations of acetyl-CoA and ATP, a decrease in glutamate and an enhanced intramitochondrial [ATP]/[ADP] ratio.

It is clearly established that glucocorticoids play a major role in the control of gluconeogenesis (Long *et al.*, 1940; Haynes, 1962; Rinard *et al.*, 1969; Exton *et al.*, 1970, 1972, 1973). In the starved or diabetic animal, adrenalectomy decreases the rate of glucose synthesis by the liver and markedly lowers the degree of stimulation by glucagon and adrenaline (Exton *et al.*, 1972, 1973, 1976; Friedmann *et al.*, 1967). Replacement treatment with glucocorticoids, either *in vivo* or *in vitro*, enhances the rate of gluconeogenesis in both liver slices (Haynes, 1962; Rinard *et al.*, 1969) and the perfused liver (Exton *et al.*, 1972, 1973, 1976) and restores the sensitivity to glucagon and catecholamines (Friedmann *et al.*, 1967; Exton *et al.*, 1972). Analysis of the concentrations of the intermediary metabolites in the gluconeogenic pathway have shown that adrenalectomy results in an increase in the tissue concentration of lactate and pyruvate and a decrease in phosphoenolpyruvate, whereas treatment with glucocorticoids produces the oppo-

site effects and normalizes the metabolite pattern (Rinard *et al.*, 1969; Exton *et al.*, 1973, 1976). From these data it has been suggested that a major site of action of the steroid hormones residues at the level of the conversion of pyruvate into phosphoenolpyruvate, either at the pyruvate carboxylase reaction (Rinard *et al.*, 1969) or, in the absence of any effect of the steroids on the total pyruvate carboxylase activity of the tissue, at the level of phosphoenolpyruvate carboxykinase (Exton *et al.*, 1976). Although the induction of phosphoenolpyruvate carboxykinase by glucocorticoids is firmly established (Foster *et al.*, 1966; Barnett & Wicks, 1971; Gunn *et al.*, 1975; Krone *et al.*, 1975, 1976), several reports suggest that this may not account for the earliest effects of glucocorticoids on gluconeogenesis. Gunn *et al.* (1975) have shown that in the normal animal glucocorticoids stimulate gluconeogenesis before any effect on phosphoenolpyruvate synthesis occurs. Similarly, in the adrenalectomized animal, the restoration of gluconeogenesis after glucocorticoid treatment occurs before that of phosphoenolpyruvate carboxykinase

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(Exton *et al.*, 1973). In contrast, the studies by Haynes and co-workers (Adam & Haynes, 1969; Wakat & Haynes, 1977) and those in the preceding paper (Martin *et al.*, 1984) have suggested that alterations in the rate of mitochondrial pyruvate carboxylation may be important in explaining the earliest effects of glucocorticoids on gluconeogenesis. The onset of this response correlates temporally with the enhancement of gluconeogenesis (Rinard *et al.*, 1969; Wakat *et al.*, 1977), does not require the induction of pyruvate carboxylase and appears to be mediated by alterations in the intramitochondrial concentrations of effector molecules (Martin *et al.*, 1984).

The purpose of the present study was twofold: firstly, to determine whether treatment of the normal fed rat with glucocorticoids over the short term results in a direct stimulation of gluconeogenesis which is independent of the circulating concentrations of glucagon and the catecholamines; secondly, to examine metabolite concentrations in both the intact cell and the mitochondrial compartment of digitonin-treated cells to establish whether a stimulation of pyruvate carboxylation occurs *in vivo* after dexamethasone treatment and, if so, the possible mechanisms underlying the change.

Experimental

Materials

Collagenase (type IV), bacitracin, dexamethasone acetate, adrenaline bitartrate, digitonin and the enzymes and substrates used in the metabolite assays were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). [2-¹⁴C]Pyruvate was obtained from Amersham International (Amersham, Bucks., U.K.). Dexamethasone sodium phosphate and glucagon were gifts from Merck, Sharp and Dohme (Hoddesdon, Herts., U.K.) and Novo Laboratories (Basingstoke, Hants, U.K.) respectively. 3-Mercaptopicolinate was given by Smith, Kline and French (Philadelphia, PA, U.S.A.) and silicone oil AR 200 by Wacker Chemicals Ltd. (Walton-on-Thames, Surrey, U.K.). All other chemicals were AnalaR grade from BDH Chemicals (Enfield, Middx., U.K.).

Methods

Fed male Sprague-Dawley rats weighing 180–240 g were injected with dexamethasone acetate (15 μ mol/100 g) or 0.9% NaCl intraperitoneally 2 or 3 h before the preparation of the hepatocytes as indicated. After the appropriate time, the animals were anaesthetized with sodium pentobarbital (60 mg/kg), and hepatocytes were prepared as described previously (Chisholm *et al.*, 1983). Batches of collagenase were carefully screened in terms of metabolic function and Trypan Blue exclusion,

and only those producing cells of which over 85% excluded the dye were used. Dexamethasone sodium phosphate (final concn. 10 μ M) was included in both the perfusion medium and all further media used for the preparation and incubation of hepatocytes from the steroid-treated animals. The preparation of the hepatocytes took approx. 1 h, after which they were resuspended to a final concentration of 120–140 mg wet wt./ml in Krebs–Ringer bicarbonate buffer, pH 7.4 (Krebs & Henseleit, 1932), containing 1.5% (w/v) gelatin, 2 mM-glucose and 0.1% (w/v) bacitracin. Except where indicated, 2 ml portions of the cell suspensions were preincubated in 25 ml Erlenmeyer flasks, gassed with O₂/CO₂ (19:1), for a period of 30 min before the addition of a pyruvate/lactate mixture (final concns. 2.5 mM of each). When the rate of gluconeogenesis was to be determined, [2-¹⁴C]pyruvate (sp. radioactivity 50 mCi/mol) replaced the unlabelled pyruvate in the pyruvate/lactate mixture. Glucagon and adrenaline were added simultaneously with the pyruvate/lactate mixture where appropriate, to the final concentrations indicated. The incubation was continued for a further 15 min after the addition of the substrate. The rate of gluconeogenesis from [2-¹⁴C]pyruvate was determined as described previously (Chisholm *et al.*, 1983). The concentrations of metabolites were determined at the end of the incubation period by using the digitonin fractionation procedure described by Siess *et al.* (1976). The final concentration of digitonin was 3 mg/ml, and AR 200 silicone oil was used instead of a mixture of AR 200 and AR 20. For the determination of the total cellular metabolite concentrations, the digitonin was omitted from the fractionation buffer and the intact hepatocytes were sedimented through oil in an Eppendorf micro-centrifuge. In experiments where 3-mercaptopicolinate was used in the incubation, this was added after the first 15 min of the preincubation period. The intramitochondrial concentrations of metabolites were calculated by assuming a mitochondrial volume of 0.166 ml/g dry wt. of cells (Siess *et al.*, 1976). There was no difference in the intramitochondrial volumes of mitochondria isolated from control and dexamethasone-treated animals (Martin *et al.*, 1984). Metabolites were analysed in neutralized HClO₄ extracts of the cells and mitochondrial fractions. Citrate was assayed as described by Passonneau & Brown (1974), malate as described by Goldberg & Passonneau (1974), pyruvate by the method of Passonneau & Lowry (1974), aspartate by the method of Williamson & Corkey (1969) and phosphoenolpyruvate by the method of Czok & Lamprecht (1974). Other metabolites were measured as described previously (Chisholm *et al.*, 1983).

Results are expressed as means $\pm$ S.E.M. for the

numbers of different cell preparations given in parentheses. Statistical analysis of results was performed by a paired Student's *t* test.

Results and discussion

Effect of dexamethasone treatment of the rat on gluconeogenesis in subsequently isolated hepatocytes

Previous studies have indicated that glucocorticoids play a permissive role in the regulation of gluconeogenesis (Friedmann *et al.*, 1967; Exton *et al.*, 1972). Eigler *et al.* (1979) have demonstrated that infusion of cortisol into the intact dog to produce a physiological elevation in the plasma concentration of cortisol, which in itself did not stimulate gluconeogenesis, potentiated the action of both the catecholamines and glucagon on glucose synthesis. Similarly, Stumpo & Kletzien (1981) have shown that, in primary cultures of hepatocytes, dexamethasone potentiated the action of glucagon to stimulate gluconeogenesis, although the steroid alone had only a minimal effect over the first 5 h of treatment. In the preceding paper (Martin *et al.*, 1984) and earlier studies, it has been postulated that the enhancement of mitochondrial metabolism observed after treatment of the rat for 3 h is important in the stimulation of gluconeogenesis and may account for the earliest effects of the steroids on glucose synthesis (Adam & Haynes, 1969; Wakat & Haynes, 1977; Allan *et al.*, 1983; Martin *et al.*, 1984). Therefore the effect of dexamethasone treatment of the rat on the rate of glucose synthesis by subsequently isolated hepatocytes has been examined to determine whether, under the conditions of hormone treatment used in the mitochondrial studies, a stimulation of the pathway actually occurs either directly or as a result of a change in the sensitivity of the cells to

physiological concentrations of glucagon or catecholamines. The results are shown in Table 1. Treatment of the rat with dexamethasone for 2 h *in vivo* before the preparation of the hepatocytes and 1½ h *in vitro* during the preparation and incubation resulted in a 44% increase over the basal rate of gluconeogenesis in hepatocytes prepared from control animals and incubated in the absence of dexamethasone. The effect of dexamethasone treatment was maintained when the hepatocytes were incubated in the presence of both submaximal and maximal concentrations of glucagon and adrenaline. The combination of the glucocorticoid with either of the other two gluconeogenic hormones potentiated their effects, although for glucagon this was only significant at the lowest concentration. Therefore the results indicate that glucocorticoids have a direct effect on gluconeogenesis in the normal fed rat under our experimental conditions, while at the same time they increase both the sensitivity and the magnitude of the response to both glucagon and catecholamines. The magnitude of the response to dexamethasone was considerably greater than that reported in the primary culture system and more rapid in time of onset (Stumpo & Kletzien, 1981), although it was comparable with results of the study *in vitro* by Wakat *et al.* (1977).

Table 2 shows the effect of dexamethasone treatment on the sensitivity of the cells to glucagon and adrenaline. The results indicate that the permissive effect of dexamethasone occurs in the normal fed rat in addition to that observed in the starved adrenalectomized animal (Friedmann *et al.*, 1967; Exton *et al.*, 1972) and that the sensitivity to both glucagon and adrenaline was significantly increased ($P < 0.05$). There was an approx. 5-fold decrease in the concentration of either hormone required to achieve a maximal response, and the con-

Table 1. *Effect of dexamethasone treatment of the rat on gluconeogenesis in isolated hepatocytes*

Dexamethasone (15 µmol/100 g body wt.) or vehicle was administered intraperitoneally 2 h before the preparation of the hepatocytes; 10 µM-dexamethasone was included in all buffers used in the isolation procedure and in the subsequent incubations of hepatocytes prepared from the dexamethasone-treated animals. The incubation and measurement of gluconeogenesis were as described in the Experimental section. Both adrenaline and glucagon produced significant stimulations of gluconeogenesis in the hepatocytes prepared from control and dexamethasone-treated animals ($P < 0.01$): * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for difference from the corresponding flask in the absence of dexamethasone ($n = 9$). Results are expressed in nmol/min per g dry wt. of cells.

Additions	Control		+ Dexamethasone	
	Gluconeogenesis	Stimulation	Gluconeogenesis	Stimulation
None	553 ± 51	—	796 ± 88**	—
Adrenaline (15 nM)	601 ± 54	42 ± 6	945 ± 108**	149 ± 24***
Adrenaline (30 nM)	620 ± 56	61 ± 11	1041 ± 126**	245 ± 40**
Adrenaline (100 nM)	702 ± 60	143 ± 18	1236 ± 129***	423 ± 45***
Glucagon (80 pM)	741 ± 67	182 ± 43	1145 ± 126***	349 ± 43***
Glucagon (200 pM)	973 ± 92	413 ± 48	1352 ± 177*	556 ± 88
Glucagon (1 nM)	1089 ± 104	529 ± 48	1447 ± 191*	651 ± 102

Table 2. *Effect of dexamethasone treatment of the rat on the sensitivity of the cells to glucagon and adrenaline*. The experimental protocol was as in Table 1, except that the animals were treated with dexamethasone for 3 h before the preparation of the hepatocytes. The results are expressed as means $\pm$ S.E.M. for six and ten different cell preparations in the experiments with adrenaline and glucagon respectively; eight concentrations of hormone were used for each determination: * $P < 0.05$, ** $P < 0.01$, for difference from controls.

Hormone	Concn. of hormone required for half-maximal stimulation		Concn. of hormone required for maximal stimulation	
	Control	+ Dexamethasone	Control	+ Dexamethasone
Glucagon	$0.125 \pm 0.069 \text{ nM}$	$0.008 \pm 0.002 \text{ nM}^*$	$17.40 \pm 3.00 \text{ nM}$	$3.70 \pm 1.20 \text{ nM}^{**}$
Adrenaline	$0.177 \pm 0.078 \text{ } \mu\text{M}$	$0.032 \pm 0.005 \text{ } \mu\text{M}^*$	$6.17 \pm 0.94 \text{ } \mu\text{M}$	$1.95 \pm 0.41 \text{ } \mu\text{M}^{**}$

centration required to elicit a half-maximal response was decreased 3-fold and 16-fold for adrenaline and glucagon respectively.

Effect of dexamethasone treatment on metabolite contents in isolated hepatocytes

Although the above results indicate that glucocorticoids have an effect on gluconeogenesis which is independent of the altered sensitivity to glucagon and adrenaline, the evidence in favour of this being via an increase in the rate of mitochondrial pyruvate carboxylation is complicated by the change in sensitivity of the liver to glucagon and adrenaline. With the exception of the time of onset, the characteristics of the mitochondrial response after administration of glucocorticoids *in vivo* are indistinguishable for those described previously for glucagon and the catecholamines (for review see Claus & Pilgis, 1981), and therefore it is unclear whether the enhancement of metabolism in the isolated organelles is the result of a direct action of the glucocorticoids or a change in the sensitivity of the cells to the circulating plasma concentrations of other gluconeogenic hormones. Therefore we have examined the effect of dexamethasone on the contents of metabolites in cells incubated for 15 min with lactate and pyruvate to determine whether the alterations in the pattern of gluconeogenic intermediates on hormone treatment are consistent with a stimulation of pyruvate carboxylation. The results are shown in Table 3. Except for the intracellular concentration of pyruvate, the values found for the other metabolites in the hepatocytes prepared from the control rats are comparable with those reported previously by Siess *et al.* (1977). The lower value obtained for pyruvate probably reflects the lower substrate concentration used in the present study, 2.5 mM-pyruvate plus 2.5 mM-lactate as opposed to 11.9 mM-lactate. In agreement with the study by Rinard *et al.* (1969), using liver slices prepared from starved adrenalectomized rats, and those by Exton *et al.* (1973, 1976), using perfused livers from either starved adrenalectomized or diabetic adrenalecto-

mized rats, dexamethasone treatment resulted in a decrease in the cellular concentration of pyruvate and an increase in phosphoenolpyruvate, indicating a stimulation of the reaction(s) converting pyruvate into phosphoenolpyruvate. No significant change was apparent in the total contents of glutamate, malate or the adenine nucleotides. However, there was a significant increase in the intracellular concentration of both acetyl-CoA ($P < 0.05$) and citrate.

Effect of dexamethasone treatment on the mitochondrial metabolite contents

The change in pattern of metabolites in the whole cells after glucocorticoid treatment indicates an enhanced reaction in the sequence converting pyruvate into phosphoenolpyruvate. Although this may suggest a stimulation of pyruvate carboxylase activity, the results could also be interpreted in terms of a stimulation of phosphoenolpyruvate carboxykinase or an inhibition of pyruvate kinase, both of which have been reported (Foster *et al.*, 1966; Barnett & Wicks, 1971; Gunn *et al.*, 1975; Krone *et al.*, 1975, 1976; Johnson *et al.*, 1981). To distinguish between these three possibilities, the concentrations of metabolites in the mitochondrial fractions of cells prepared from control animals and animals treated with dexamethasone were measured. The results are shown in Table 4. The concentrations of metabolites in the mitochondrial fraction of control cells determined in this study are comparable with those reported previously by Siess *et al.* (1977, 1978), the major exception being the intramitochondrial concentrations of ATP and ADP, which are closer to the values reported previously by Bryla *et al.* (1977) and Titheradge *et al.* (1979). Dexamethasone treatment significantly decreased the intramitochondrial concentration of pyruvate ($P < 0.01$) and increased those of malate and citrate. It also resulted in a significant increase in the intramitochondrial concentration of ATP ($P < 0.01$), with no change in the ADP content, and thus increased the intramitochondrial [ATP]/[ADP] ratio, in agreement with the known effects

Table 3. *Effect of dexamethasone treatment on the concentrations of metabolites in isolated hepatocytes*

Dexamethasone or vehicle was administered to the rat 3 h before the preparation of hepatocytes as described in the Experimental section. The preincubation step was omitted and glucose synthesis was measured over the first 15 min of the incubation. The rates for cells prepared from control and dexamethasone-treated animals were 768 ± 49 and 1025 ± 52 (12) nmol of glucose/min per g dry wt. of cells respectively. The metabolites were measured as described in the Experimental section after centrifugation through silicone oil at the end of the 15 min incubation period: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for difference from controls.

Metabolite	Concentration (nmol/g dry wt. of cells)	
	Control	+ Dexamethasone
Pyruvate	446 ± 36 (10)	$328 \pm 34^*$ (10)
Malate	1302 ± 119 (10)	1509 ± 104 (10)
Phosphoenolpyruvate	744 ± 49 (10)	$857 \pm 68^{**}$ (10)
Citrate	2085 ± 108 (10)	$2724 \pm 171^{***}$ (10)
Glutamate	12913 ± 745 (8)	12282 ± 703 (8)
Acetyl-CoA	234 ± 19 (7)	$293 \pm 29^*$ (7)
ATP	9960 ± 384 (8)	10016 ± 346 (8)
ADP	1242 ± 228 (8)	1381 ± 363 (8)

Table 4. *Effect of dexamethasone treatment on the concentration of metabolites in the mitochondrial fraction of isolated hepatocytes*

For details see Table 3: * $P < 0.05$, ** $P < 0.001$, *** $P < 0.001$ for difference from controls.

	Control		+ Dexamethasone	
	Amount (nmol/g dry wt. of cells)	Intramitochondrial concn. (mM)	Amount (nmol/g dry wt. of cells)	Intramitochondrial concn. (mM)
Pyruvate	124 ± 9 (10)	0.748	$94 \pm 5^{***}$ (10)	0.568
Malate	239 ± 18 (10)	1.436	$304 \pm 13^*$ (10)	1.832
Phosphoenolpyruvate	305 ± 19 (10)	1.839	$368 \pm 18^*$ (10)	2.217
Citrate	1295 ± 65 (10)	7.796	$1542 \pm 117^*$ (10)	9.283
Glutamate	2163 ± 75 (8)	13.020	$1777 \pm 131^*$ (8)	10.700
Acetyl-CoA	139 ± 25 (7)	0.839	$197 \pm 30^*$ (7)	1.186
ATP	1597 ± 108 (8)	7.960	$1994 \pm 89^{**}$ (8)	9.939
ADP	690 ± 143 (8)	3.090	648 ± 122 (8)	3.709
[ATP]/[ADP]	2.31		3.08	

of the other gluconeogenic hormones on adenine nucleotide metabolism (Siess *et al.*, 1977, 1978; Bryla *et al.*, 1977; Titheradge *et al.*, 1979). The intramitochondrial concentrations of acetyl-CoA and phosphoenolpyruvate were also significantly elevated ($P < 0.05$), whereas that of glutamate was decreased.

The changes in metabolite concentrations in the mitochondrial fraction after steroid administration are therefore consistent with an activation of mitochondrial metabolism and in increased flux through pyruvate carboxylase, the increased formation of oxaloacetate being reflected in an increase in the intramitochondrial concentrations of malate and citrate and a decrease in the concentration of pyruvate. The elevated concentrations of acetyl-CoA and ATP and the elevated [ATP]/[ADP] ratio provide a possible mechanism for the

stimulation of pyruvate carboxylase (Keech & Utter, 1963; Seubert *et al.*, 1968; Stucki *et al.*, 1972; von Glutz & Walter, 1976) and confirm the conclusions drawn from studies with isolated mitochondria (Adam & Haynes, 1969; Martin *et al.*, 1984). The major difference between the conclusions drawn from the present study and that with the isolated organelles (Martin *et al.*, 1984) resides in the importance of glutamate. Glutamate has been reported to be an effective inhibitor of pyruvate carboxylase, with a K_i of approx. 5 mM (Scrutton & White, 1974), and it is apparent from the data in Table 4 and the results of Siess *et al.* (1977, 1978) that the intramitochondrial concentration in the hepatocytes is sufficient to impose severe restraint on the enzyme. Dexamethasone treatment decreased the intramitochondrial concentration of glutamate, and this may have been

Table 5. *Effect of 3-mercaptopycolinate on the concentrations of metabolites in the intact cell and the mitochondrial fractions of cells prepared from control and dexamethasone-treated animals*

The experimental protocol was as described in Fig. 1. The final concentration of 3-mercaptopycolinate was 500 μ M. The rates of glucose production in control and dexamethasone-treated hepatocytes were 643 ± 79 and 1000 ± 97 ($P < 0.01$) nmol/min per g dry wt. of cells in the absence of inhibitor and 104 ± 9 and 116 ± 16 nmol/min per g dry wt. of cells in its presence respectively. The results are expressed in nmol/g dry wt. of cells and are the means $\pm$ S.E.M. for six different cell preparations: * $P < 0.05$, ** $P < 0.01$, for difference from respective controls.

Metabolite	No 3-mercaptopycolinate				+ 3-Mercaptopycolinate			
	Cells		Mitochondria		Cells		Mitochondria	
	Control	+ Dexamethasone	Control	+ Dexamethasone	Control	+ Dexamethasone	Control	+ Dexamethasone
Pyruvate	491 $\pm$ 45	344 $\pm$ 57**	180 $\pm$ 27	139 $\pm$ 18*	1261 $\pm$ 70	1047 $\pm$ 35*	293 $\pm$ 35	258 $\pm$ 26
Citrate	1714 $\pm$ 204	2498 $\pm$ 257*	1044 $\pm$ 134	1409 $\pm$ 139**	3723 $\pm$ 347	5766 $\pm$ 399**	1704 $\pm$ 121	2414 $\pm$ 148**
Malate	1017 $\pm$ 108	1115 $\pm$ 75	220 $\pm$ 25	256 $\pm$ 25*	5757 $\pm$ 1104	9933 $\pm$ 1554*	697 $\pm$ 126	1108 $\pm$ 112*
Aspartate	1772 $\pm$ 236	1957 $\pm$ 222	176 $\pm$ 34	217 $\pm$ 39	5379 $\pm$ 867	11027 $\pm$ 1163**	234 $\pm$ 25	316 $\pm$ 21*

important in augmenting the effects of both acetyl-CoA and the increased [ATP]/[ADP] ratio of pyruvate carboxylase.

Effect of 3-mercaptopycolinate on metabolite concentrations in control and dexamethasone-treated hepatocytes

To substantiate the claim that the increased flux through pyruvate carboxylase after dexamethasone treatment of the rat is a result of a direct activation of pyruvate carboxylase itself and is independent of alterations in the gluconeogenic pathway distal to the enzyme, 3-mercaptopycolinate was used to inhibit gluconeogenesis at the level of phosphoenolpyruvate carboxykinase. The cells were incubated with 3-mercaptopycolinate for 15 min before the addition of the gluconeogenic substrates. It was found that increasing the concentration of the inhibitor resulted in a progressive decline in glucose synthesis and diminished the effect of hormone treatment, until, at a final concentration of 500 μ M-3-mercaptopycolinate, the rate of gluconeogenesis in the control was lowered to approx. 15% of the initial activity and the effect of dexamethasone was completely abolished.

Table 5 shows the effect of dexamethasone on the concentrations of metabolites in both the whole cell and the mitochondrial fraction in the presence and absence of 500 μ M-3-mercaptopycolinate. In the absence of inhibitor, the glucocorticoid significantly increased the rate of glucose synthesis by the cells ($P < 0.01$), and this was reflected in a decrease in the total cellular and mitochondrial pyruvate concentrations. Malate and aspartate showed a small but insignificant increase in both the intact cell and the mitochondrial compartment, whereas the concentration of citrate was significantly elevated ($P < 0.05$). As shown by Jensen *et al.* (1983), treatment of the cells with 3-mercaptopycolinate

increased the intracellular concentrations of pyruvate, citrate, malate and aspartate, as a consequence of the decreased utilization of oxaloacetate by phosphoenolpyruvate carboxykinase. Despite the lowered rate of gluconeogenesis and the absence of any hormone effect on glucose synthesis from [2- 14 C]pyruvate, the effect of dexamethasone treatment on the pattern of metabolites was still apparent and even enhanced. The steroid increased the utilization of pyruvate within the cell and enhanced the production of malate, aspartate and citrate in both the tissue as a whole and the mitochondrial fraction. Thus, in the absence of an effect of the glucocorticoid at the level of phosphoenolpyruvate carboxykinase or pyruvate kinase, the change in metabolite pattern is evidence in favour of a stimulation of the pyruvate carboxylase reaction *in vivo*.

In conclusion, the data presented above indicate that glucocorticoids are capable of stimulating the gluconeogenic pathway in the normal fed rat both directly and as a result of increasing the sensitivity of the cells to physiological concentrations of glucagon and adrenaline. The mechanism behind the stimulation of glucose synthesis by dexamethasone can be explained, in part at least, by an increased rate of pyruvate carboxylation within the mitochondria. This study corroborates the conclusions drawn from studies using the isolated organelles and suggests that the activity of pyruvate carboxylase is restrained within the mitochondria by the intramitochondrial concentrations of effector molecules, and that glucocorticoids, in common with other gluconeogenic hormones, relieve this restraint by decreasing the intramitochondrial concentration of glutamate and increasing those of acetyl-CoA and ATP.

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