Regulation of ketogenesis, gluconeogenesis and the mitochondrial redox state by dexamethasone in hepatocyte monolayer cultures

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The effects of the glucocorticoid dexamethasone on fatty acid and pyruvate metabolism were studied in rat hepatocyte cultures. Parenchymal hepatocytes were cultured for 24 h with nanomolar concentrations of dexamethasone in either the absence or the presence of insulin (10 nM) or dibutyryl cyclic AMP (1 μ M BcAMP). Dexamethasone (1–100 nM) increased the rate of formation of ketone bodies from 0.5 mM-palmitate in both the absence and the presence of BcAMP, but inhibited ketogenesis in the presence of insulin. Dexamethasone increased the proportion of the palmitate metabolized that was partitioned towards oxidation to ketone bodies, and decreased the cellular [glycerol 3-phosphate]. The latter suggests that the increased partitioning of palmitate to ketone bodies may be associated with decreased esterification to glycerolipid. The $V_{\max}$ of carnitine palmitoyltransferase (CPT) and the affinity of CPT for palmitoyl-CoA were not affected by dexamethasone, indicating that the increased ketogenesis was not due to an increase in enzymic capacity for long-chain acylcarnitine formation. Dexamethasone and BcAMP, separately and in combination, increased gluconeogenesis. In the presence of insulin, however, dexamethasone inhibited gluconeogenesis. Changes in gluconeogenesis thus paralleled changes in ketogenesis. Dexamethasone decreased the [3-hydroxybutyrate]/[acetoacetate] ratio, despite increasing the rate of ketogenesis and presumably the mitochondrial production of reducing equivalents. The more oxidized mitochondrial NADH/NAD⁺ redox couple with dexamethasone is probably due either to an increased rate of electron transport or to increased transfer of mitochondrial reducing equivalents to the cytoplasm.

INTRODUCTION

In insulin-deficient states, glucocorticoid excess is associated with a rise in the concentration of ketone bodies in the blood and presumably in the rate of production of ketone bodies by the liver [1,2]. Glucocorticoids may increase the formation of ketone bodies by stimulating lipolysis in adipose tissue [3–5] and potentiating the lipolytic effects of catecholamines and growth hormone [5,6] or antagonizing the antilipolytic effects of insulin [7] thereby increasing the supply of non-esterified fatty acids, the main substrate for ketogenesis in liver. Glucocorticoids might also directly stimulate ketogenesis in liver.

Long-chain fatty acids taken up by the liver can either be esterified to glycerolipid or oxidized to acetyl-CoA, which is then converted into ketone bodies or further oxidized in the citrate cycle. In hepatocytes in suspension or in monolayer culture, glucocorticoids increase the activity of phosphatidate phosphohydrolase, which is involved in the formation of diacylglycerol [8,9]. The activity of glycerol-3-phosphate acyltransferase, the first enzyme involved in the esterification of fatty acyl-CoA to glycerolipid, is not affected by glucocorticoids [8,9]. The effects of glucocorticoids on the partitioning of fatty acids between the pathways of esterification and oxidation have not been studied previously.

Monolayer culture of parenchymal hepatocytes offers several advantages over freshly isolated hepatocytes for studying long-term as well as acute hormonal control of liver metabolism [10,11] in the absence of unknown

extrinsic factors. However, glucocorticoids are essential for the preservation of the activities of a variety of enzymes in hepatocytes cultured in serum-free medium, and the synthetic glucocorticoid dexamethasone is almost routinely used, at concentrations ranging from 10 nM to 10 μ M, in the maintenance of hepatocyte monolayer cultures [10].

The aims of the present study were to use this system, first to establish whether dexamethasone regulates the rate of ketogenesis and the partitioning of long-chain fatty acids between oxidation and esterification, and second to investigate the interaction of dexamethasone with insulin and dibutyryl cyclic AMP (BcAMP) in the control of fatty acid and pyruvate metabolism.

MATERIALS AND METHODS

Materials

Dexamethasone phosphate was a gift from Merck, Sharp and Dohme (Hoddesdon, Herts, U.K.). Pig insulin and BcAMP were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sodium [3-¹⁴C]pyruvate was from New England Nuclear (Boston, MA, U.S.A.). Sources of culture media and biochemicals were as described previously [12].

Hepatocyte monolayers

Parenchymal hepatocytes were isolated from male Wistar rats (180–200 g body wt.) by collagenase perfusion of the liver [13]. Cell viability estimated by

Abbreviations used: BcAMP, *N*⁶*O*²-dibutyryl-adenosine 3',5'-phosphate (dibutyryl cyclic AMP); CPT, carnitine palmitoyltransferase; G3P, glycerol 3-phosphate.

Trypan Blue exclusion was $> 90\%$, and cell yield per liver was 3×10^8 – 4×10^8 cells. The hepatocytes were cultured in monolayer either on micro-carriers (Table 1) or in flasks (Tables 2 and 3, and Figures).

For the monolayer cultures on micro-carriers, the hepatocytes were suspended in serum-free Minimum Essential Medium and incubated with collagen-coated dextran beads [12]. After 8 h, the micro-carriers with the hepatocyte monolayers were separated from the unattached cells and incubated in fresh medium at 37°C equilibrated with CO_2/air (1:19). The medium was changed again after 21 h and the experiments were performed at 22 h. For the monolayer cultures in flasks, the hepatocytes were suspended in Minimal Essential Medium containing 5% (v/v) heat-inactivated foetal bovine serum, and plated in 25 cm^2 culture flasks (10^6 cells/flask). For cell attachment 4 h was allowed, and the medium was then changed to serum-free Minimal Essential Medium containing the concentrations of dexamethasone, insulin and BcAMP shown. The change to serum-free medium containing the hormones was designated zero time. Duplicate flasks were used for each hormone concentration. The cultures were maintained at 37°C equilibrated with CO_2/air (1:19). The medium was changed at 21 h and experiments were performed between 22 and 24 h.

Palmitate and pyruvate metabolism

At 22 h the medium was supplemented with 0.5 mM-sodium palmitate, 0.5 mM-sodium $[3\text{-}^{14}\text{C}]$ pyruvate (0.2 Ci/mol), 1 mM-L-carnitine and 7.5 mg of bovine serum albumin/ml. After a 2 h incubation the medium was decanted and a sample was deproteinized with HClO_4 (0.33 M). The hepatocyte monolayers were rapidly rinsed twice with 0.1 M-phosphate-buffered 154 mM-NaCl (pH 7.4) and then solubilized either in 15 mM-NaOH for determination of glycogen and ^{14}C -labelled fatty acid or in buffer containing 100 mM-Tris, 100 mM-KCl, 20 mM-EDTA, 25 mM-NaF and Lubrol PX (0.05%, w/v), pH 7.9, for determination of enzyme activities.

The rate of palmitate uptake was determined from the decrease in [palmitate] in the medium during 2 h. Ketogenesis was determined from the increase in the concentrations of acetoacetate and D-3-hydroxybutyrate. The rate of pyruvate metabolism was determined from the decrease in the concentrations of pyruvate plus lactate in the medium. Gluconeogenesis and glycogen synthesis were determined from the rate of incorporation of $[3\text{-}^{14}\text{C}]$ pyruvate into glucose in the medium and cellular glycogen respectively. Preliminary studies established that the rates of palmitate and pyruvate metabolism were linear for 3 h under these conditions.

Metabolite and enzyme assays

Palmitate was assayed by a spectrophotometric enzymic method [14] in the untreated medium by using a kit (from Wako Chemicals G.m.b.H., Neuss, Germany). Pyruvate, lactate, acetoacetate and D-3-hydroxybutyrate in the perchlorate extracts were determined fluorimetrically [15]. The cellular glycogen content was determined, after acid extraction of the glycogen from the NaOH extract, by the amyloglucosidase method [16] fluorimetrically. Cellular glycerol-3-phosphate (G3P) was determined fluorimetrically, by using G3P dehydrogenase [17]. Carnitine palmitoyltransferase (CPT EC 2.3.1.21) activity

was assayed by a modification of the method used in [18], as described in [15]. The assay was performed at four palmitoyl-CoA concentrations (2.5, 5, 10, 20 μM) and the V_{max} and K_m for palmitoyl-CoA were determined by the Direct Linear Plot (P. R. Williams, Elsevier Biosoft, Cambridge, U.K.). Glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) activity was determined as described by Bergmeyer [19]. All spectrophotometric and fluorimetric assays were performed by using a centrifugal analyser (Cobas Bio 8326; Hofmann La Roche).

The incorporation of $[^{14}\text{C}]$ pyruvate into glucose in the medium was determined in the neutralized perchlorate extract as described previously [20]. The incorporation of $[^{14}\text{C}]$ pyruvate into glycogen was determined after trichloroacetic acid extraction of a sample of the cell extract. Glycogen carrier was added to the extract, which was then precipitated with ethanol [21] and the radioactivity determined. Glycogen recovery during ethanol precipitation was $> 90\%$. The incorporation of $[^{14}\text{C}]$ pyruvate into saponifiable fatty acid was determined as in [22].

Cellular protein was determined in the NaOH or Tris/Lubrol extracts, with bovine serum albumin as standard [23].

Expression of results

Metabolic rates determined during incubation of the intact hepatocyte monolayers with palmitate and pyruvate are expressed as nmol of substrate metabolized or product formed/h per mg of cell protein. Enzyme activities are expressed as munits/mg of cell protein, where 1 munit is the amount of enzyme converting 1 nmol of substrate/min at 30°C . Results are expressed as means $\pm$ S.E.M. of duplicate flasks from the numbers of hepatocyte culture preparations indicated. Statistical analysis was by Student's *t* test, either paired or unpaired.

RESULTS

Effect of carnitine on palmitate metabolism

The effects of L-carnitine on palmitate metabolism in isolated hepatocyte suspensions and hepatocytes maintained in monolayer culture on micro-carriers were examined. Carnitine (1 mM) caused a more marked stimulation of β -oxidation of palmitate, estimated from the conversion of $[U\text{-}^{14}\text{C}]$ palmitate into acid-soluble metabolites and CO_2 , in hepatocyte monolayers on micro-carriers (3-fold) than in freshly isolated hepatocytes (42%; Table 1). Hepatocytes cultured in monolayers on flasks for 24 h showed a similar stimulation of β -oxidation by carnitine (results not shown) to that of the cultures on micro-carriers (Table 1). The high rate of ketogenesis in the micro-carrier cultures incubated without carnitine, relative to palmitate β -oxidation, is probably due to the formation of ketone bodies from pyruvate [24]. Liver carnitine is not limiting under physiological conditions [25]. However, liver cells lose carnitine during collagenase perfusion of the liver [26] and when maintained in carnitine-free medium [27]. Carnitine biosynthesis in liver is dependent on an exogenous source of γ -butyrobetaine [28]. The present findings suggest that carnitine secretion or degradation exceeds biosynthesis in cultured hepatocytes. Throughout the rest of this study, palmitate metabolism and ketogenesis in hepatocytes cultured on flasks were determined in the presence of 1 mM-carnitine to ensure

Table 1. Effects of carnitine on palmitate metabolism and formation of ketone bodies in hepatocyte suspensions and micro-carrier cultures

Freshly prepared hepatocyte suspensions from fed rats (10^6 cells/ml) and hepatocytes precultured on micro-carriers for 22 h (~ 1 mg of cell protein/ml) were incubated with 0.5 mM-[U- 14 C]palmitate (0.3 Ci/mol) and 0.5 mM-pyruvate for 1 h in either the absence or the presence of 1 mM-L-carnitine. The incorporation of radioactivity into $^{14}\text{CO}_2$ and acid-soluble metabolites (ASM) was determined [24]. β -Oxidation represents the rate of incorporation of [U- 14 C]palmitate into CO_2 + ASM. Rates of palmitate metabolism and ketone-body formation are expressed as nmol of palmitate converted or nmol of acetoacetate + 3-hydroxybutyrate formed/h respectively. Rates are expressed per 10^6 cells for hepatocyte suspensions and per mg of cell protein for micro-carrier cultures. Values are means $\pm$ S.E.M. ($n = 4-5$). Values in parentheses refer to rates of palmitate metabolism in the presence of carnitine as a percentage of the corresponding values without carnitine. The hydroxybutyrate/acetoacetate ratio (BOH/ACAC) refers to the values at the end of the incubation.

	Hepatocyte suspensions (nmol/h per 10^6 cells)		Micro-carrier cultures (nmol/h per mg of protein)	
	Control	+ Carnitine	Control	+ Carnitine
[14 C]Palmitate conversion into:				
$^{14}\text{CO}_2$	10.3 ± 0.5	7.8 ± 0.4 (76)	3.5 ± 0.2	4.8 ± 0.2 (137)
[14 C]ASM	129 ± 9	193 ± 10 (150)	16.2 ± 0.5	69.7 ± 4.4 (430)
$^{14}\text{CO}_2$ + [14 C]ASM (β -oxidation)	140 ± 10	199 ± 10 (142)	19.7 ± 0.7	74.5 ± 4.6 (378)
Ketone-body formation	343 ± 20	613 ± 52	125 ± 9	280 ± 12
BOH/ACAC	1.05 ± 0.14	1.53 ± 0.25	0.92 ± 0.11	2.55 ± 0.14

that fatty acid entry into mitochondria is not limited by carnitine availability.

Effects of dexamethasone on ketogenesis

After cell attachment, the hepatocyte monolayers were cultured at the dexamethasone concentrations indicated (Fig. 1) either with or without insulin (10 nM) or BcAMP (1 μ M). After 22 h the medium was supplemented with

palmitate (0.5 mM), pyruvate (0.5 mM) and carnitine (1 mM) (see the Materials and methods section). The formation of ketone bodies (acetoacetate and 3-hydroxybutyrate) was determined after a further 2 h, during which time the decrease in [palmitate] was less than 25%. Dexamethasone (1–100 nM) increased ($P < 0.02$, paired t -test, $n = 5$) ketogenesis when present in the absence of other hormones or in the presence of BcAMP (Fig. 1). In the presence of insulin, however, dexamethasone had the reverse effect, and decreased ketogenesis ($P < 0.02$, at 100 nM-dexamethasone). Insulin (10 nM) had no significant effect on ketogenesis in the absence of dexamethasone, but inhibited ($P < 0.02$) ketogenesis in the presence of 10–100 nM-dexamethasone (Fig. 1). BcAMP increased the formation of ketone bodies ($P < 0.0001$) in both the absence and presence of dexamethasone (Fig. 1). When BcAMP was added after 21 h to monolayers precultured with or without dexamethasone, and ketogenesis was measured for 2 h, no significant stimulation of ketogenesis ($n = 4$; results not shown) by BcAMP was observed, indicating that the effects observed after 22–24 h exposure are not expressed acutely.

Ketogenesis in relation to palmitate uptake

Long-chain fatty acids are metabolized by the liver by two major pathways: (1) oxidation mainly to ketones and to a lesser extent to CO_2 (Table 1); and (2) esterification to glycerolipid. Freshly isolated hepatocytes or perfused liver from fed rats esterify more fatty acids than they oxidize, whereas the converse occurs in preparations from starved rats [29]. In cultured hepatocytes incubated without carnitine, fatty acid oxidation is less than 10% of the rate of esterification [30]. In the present study, the rate of ketogenesis in hepatocyte monolayers incubated with carnitine was expressed as a percentage of the total palmitate metabolized to obtain an estimate of the partitioning of palmitate towards oxidation to ketones. This calculation ignores the contribution of pyruvate to ketones, which in hepatocyte suspensions incubated with 0.5 mM-palmitate and 0.5 mM-

Fig. 1. Effects of dexamethasone, insulin and BcAMP on ketogenesis in hepatocyte monolayers

After cell attachment, the hepatocyte monolayers were incubated with the concentrations of dexamethasone indicated: ●, without other additions; ○, + 10 nM-insulin; □, + 1 μ M-BcAMP. After 22 h the medium was supplemented with 0.5 mM-palmitate and other additions as described in the text. The incubation was terminated after 2 h. The rate of formation of ketone bodies (acetoacetate + 3-hydroxybutyrate) after addition of palmitate is expressed as nmol/h per mg of cell protein. Values are means $\pm$ S.E.M. of duplicate flasks from five cultures.

Table 2. Effects of dexamethasone on fatty acid uptake and formation of ketone bodies in hepatocyte monolayers

Hepatocyte monolayers (in flasks) were cultured with the concentrations of dexamethasone indicated in either the absence or the presence of insulin (10 nM) or BcAMP (1 μ M). After 22 h the medium was supplemented with 0.5 mM-palmitate and 0.5 mM-pyruvate. The incubation was stopped after a further 2 h and the concentrations of palmitate, acetoacetate and 3-hydroxybutyrate were determined. Palmitate uptake is expressed as nmol/h per mg of protein. The formation of ketone bodies is expressed as a percentage of the rate of palmitate uptake, assuming that 1 mol of palmitate is equivalent to 8 mol of acetyl units and 1 mol of acetoacetate or hydroxybutyrate is assembled from 2 mol of acetyl units. The hydroxybutyrate/acetoacetate (HOB/ACAC) ratio refers to the concentrations at the end of the 2 h incubation. Values are means $\pm$ S.E.M. from duplicate flasks from four cultures. Statistics were by the unpaired *t* test (*P* < 0.05): ^arelative to the controls with no additions; ^brelative to the corresponding dexamethasone concentrations.

Addition	Dexamethasone (nM)	Palmitate uptake (nmol/h per mg)	Ketone-body formation (% of palmitate uptake)	HOB/ACAC
None	0	155 $\pm$ 19	49 $\pm$ 7	2.1 $\pm$ 0.2
	1	140 $\pm$ 11	57 $\pm$ 4	2.0 $\pm$ 0.2
	10	144 $\pm$ 10	50 $\pm$ 4	1.5 $\pm$ 0.1 ^a
	100	139 $\pm$ 5	64 $\pm$ 6	1.3 $\pm$ 0.1 ^a
10 nM-Insulin	0	138 $\pm$ 10	49 $\pm$ 3	2.0 $\pm$ 0.2
	1	132 $\pm$ 10	50 $\pm$ 4	1.9 $\pm$ 0.2
	10	124 $\pm$ 6	49 $\pm$ 3	1.4 $\pm$ 0.1 ^a
	100	115 $\pm$ 5 ^b	49 $\pm$ 3	1.3 $\pm$ 0.1 ^a
1 μ M-BcAMP	0	169 $\pm$ 13	60 $\pm$ 4	1.2 $\pm$ 0.1 ^a
	1	158 $\pm$ 16	65 $\pm$ 9	0.9 $\pm$ 0.1 ^{a,b}
	10	167 $\pm$ 15	80 $\pm$ 4	0.7 $\pm$ 0.1 ^{a,b}
	100	176 $\pm$ 20 ^b	76 $\pm$ 5	0.7 $\pm$ 0.1 ^{a,b}

pyruvate is about 10–18% of the total [24]. Since complete oxidation of palmitate to CO₂ accounts for less than 7% of β -oxidation (Table 1), it might be assumed that the rate of ketogenesis approximates the rate of β -oxidation of palmitate and that the palmitate metabolized not accountable for as ketone bodies largely represents esterified products. In hepatocyte monolayers incubated with 0.5 mM-palmitate and 1 mM-carnitine, ketogenesis accounted for between 49 and 80% of the total palmitate metabolized, depending on the hormonal conditions (Table 2). Increasing dexamethasone concentrations increased ketogenesis in proportion to total palmitate metabolism, in the absence of other additions and in the presence of BcAMP, but not in the presence of insulin (Table 2). This suggests that dexamethasone increased the partitioning of fatty acids towards β -oxidation and ketogenesis, as opposed to esterification, in the absence of insulin, but not in its presence.

Mitochondrial redox state

The [3-hydroxybutyrate]/[acetoacetate] ratio was decreased by dexamethasone and BcAMP, but unaffected by insulin (Table 2). The [3-hydroxybutyrate]/[acetoacetate] ratio normally reflects the mitochondrial NADH/NAD⁺ redox state [31]. The finding that the increased rates of ketogenesis (and presumably the production of reducing equivalents by β -oxidation) by dexamethasone and BcAMP were associated with a decrease in the [hydroxybutyrate]/[acetoacetate] ratio, rather than an increase, implies that the changes in redox state do not reflect the production of reducing equivalents.

Carnitine palmitoyltransferase

The activity of CPT was determined to establish whether the higher rates of ketogenesis with dexamethasone or BcAMP were associated with differences in activity of CPT. There was no effect on the *V*_{max} of CPT by either dexamethasone or BcAMP [control, 32.9 $\pm$ 1.1;

100 nM-dexamethasone, 31.7 $\pm$ 1.9; 1 μ M-BcAMP, 34.3 $\pm$ 1.9; 1 μ M-BcAMP + 100 nM-dexamethasone, 33.3 $\pm$ 3.9 (munits/mg of protein; means $\pm$ S.E.M., five flasks, from three cultures)]. The affinity of CPT for palmitoyl-CoA (*K*_m) was also unaffected by dexamethasone and BcAMP [control, 2.9 $\pm$ 0.3; 100 nM-dexamethasone, 3.7 $\pm$ 0.5; 1 μ M-BcAMP, 3.4 $\pm$ 0.2; 1 μ M-BcAMP + dexamethasone, 4.3 $\pm$ 0.7 (μ M-palmitoyl-CoA)]. The *K*_m of CPT for palmitoyl-CoA was higher in freshly isolated hepatocyte suspensions (14 $\pm$ 4 μ M-palmitoyl-CoA; means $\pm$ S.E.M., *n* = 4), suggesting that the CPT is in a more active form in the cultured cells.

Pyruvate incorporation into saponifiable fatty acid

Although dexamethasone and BcAMP did not affect the *V*_{max} or the affinity of CPT for palmitoyl-CoA, malonyl-CoA may inhibit CPT activity in the intact cell, and dexamethasone and BcAMP may relieve this inhibition. Since [malonyl-CoA] correlates with the rate of fatty acid synthesis in hepatocyte suspensions [32], the incorporation of [¹⁴C]pyruvate into saponifiable fatty acid was determined to establish whether there were significant changes in fatty acid synthesis. The rates of pyruvate incorporation into fatty acid were not significantly affected by the hormones [control, 12.3 $\pm$ 5.4; 100 nM-dexamethasone, 10.5 $\pm$ 4.4; insulin, 12.9 $\pm$ 5.9; insulin + dexamethasone, 11.9 $\pm$ 5.3; BcAMP, 14.3 $\pm$ 6.5; BcAMP + dexamethasone, 12.2 $\pm$ 5.2 (nmol of pyruvate incorporated into fatty acid/h per mg of protein; means $\pm$ S.E.M., four cultures)]. These rates of pyruvate incorporation into fatty acid are very low in comparison with the rates of total pyruvate metabolism or incorporation into glucose (Table 3). Palmitate inhibits fatty acid synthesis in freshly isolated hepatocytes [33] and cultured hepatocytes (results not shown). The lack of hormonal effects on pyruvate incorporation into fatty acid may be due to an over-riding inactivation of acetyl-CoA carboxylase by the exogenous palmitate.

Table 3. Effects of dexamethasone on pyruvate metabolism

Hepatocyte monolayers were cultured with the concentrations of dexamethasone indicated in either the absence or the presence of insulin (10 nM) or BcAMP (1 μ M). After 22 h, 0.5 mM-[3- 14 C]pyruvate, and 0.5 mM-palmitate were added to the medium and the incubation was terminated after 2 h. The rate of total pyruvate metabolism represents the decrease in pyruvate and lactate in the medium. Rates are expressed as nmol of pyruvate metabolized or incorporated into glucose or glycogen/h per mg of protein. Values are means $\pm$ S.E.M. of duplicate flasks from the numbers of cultures shown in parentheses. Values that are significantly different ($P < 0.05$) from the control with no additions (^a) or from the corresponding dexamethasone concentration (^b), paired (pyruvate metabolism and gluconeogenesis) or unpaired (glycogen synthesis), are shown.

Addition	Dexamethasone (nM)	Total pyruvate metabolized (nmol/h per mg of protein) (5)	Pyruvate conversion (nmol/h per mg of protein) into	
			Glucose (5)	Glycogen (3)
None	0	216 $\pm$ 8	102 $\pm$ 5	2.1 $\pm$ 0.3
	1	221 $\pm$ 16	131 $\pm$ 3	2.7 $\pm$ 0.3
	10	230 $\pm$ 14	139 $\pm$ 12 ^a	2.9 $\pm$ 0.7
	100	225 $\pm$ 15	141 $\pm$ 14 ^a	1.2 $\pm$ 0.1 ^a
10 nM-Insulin	0	210 $\pm$ 7	98 $\pm$ 7	2.6 $\pm$ 0.5
	1	200 $\pm$ 7	84 $\pm$ 11 ^{a,b}	3.3 $\pm$ 0.5
	10	182 $\pm$ 22 ^b	64 $\pm$ 2 ^{a,b}	6.9 $\pm$ 0.7 ^{a,b}
	100	172 $\pm$ 19 ^b	58 $\pm$ 6 ^{a,b}	6.8 $\pm$ 1.1 ^{a,b}
1 μ M-BcAMP	0	369 $\pm$ 9 ^a	261 $\pm$ 29 ^a	1.8 $\pm$ 0.4
	1	374 $\pm$ 29 ^{a,b}	305 $\pm$ 37 ^{a,b}	2.3 $\pm$ 0.5
	10	390 $\pm$ 29 ^{a,b}	296 $\pm$ 33 ^{a,b}	2.4 $\pm$ 0.6
	100	381 $\pm$ 31 ^{a,b}	344 $\pm$ 25 ^{a,b}	1.3 $\pm$ 0.2 ^a

Glycerol 3-phosphate

A hormonal effect on the partitioning of fatty acyl-CoA between esterification and mitochondrial metabolism may be due to changes in the activities of either the entry of fatty acids into mitochondria or the esterification to glycerolipid. The latter is regulated by changes in the activity of G3P acyltransferase or by the availability of G3P [34,35]. In the absence of insulin, dexamethasone decreased ($P < 0.001$, at 10 and 100 nM) the [G3P], whereas in the presence of insulin it had no

significant effect (Fig. 2). Insulin increased ($P < 0.002$) the G3P content in the presence of dexamethasone (10–100 nM), but not in its absence. BcAMP decreased ($P < 0.0005$) the [G3P] in the absence of dexamethasone and in its presence. The lower [G3P] with dexamethasone (10–100 nM) and BcAMP coincided with increased partitioning of fatty acids towards ketogenesis (Table 2).

In the absence of exogenous glycerol, G3P is derived from dihydroxyacetone phosphate through NAD-linked G3P dehydrogenase. Hormonal treatment caused no significant changes in G3P dehydrogenase activity [control, 176 $\pm$ 30; 100 nM-dexamethasone, 181 $\pm$ 25; insulin, 215 $\pm$ 23; insulin + dexamethasone, 199 $\pm$ 17; 1 μ M-BcAMP, 158 $\pm$ 11; BcAMP + dexamethasone, 181 $\pm$ 11 (munits/mg of protein; means $\pm$ S.E.M., three cultures)].

Pyruvate metabolism and gluconeogenesis

When the monolayers were incubated with 0.5 mM-pyruvate and 0.5 mM-palmitate, the [lactate] increased but the [pyruvate + lactate] decreased. Cytosolic pyruvate can be converted into lactate, or it can enter the mitochondria, where it is metabolized by carboxylation and decarboxylation. It may also be transaminated to alanine. There was no measurable increase in the [alanine] in the medium. It might be assumed, therefore, that the rate of decrease in [pyruvate + lactate] approximates to the rate of mitochondrial metabolism of pyruvate by carboxylation and decarboxylation. The rate of pyruvate metabolism was increased by BcAMP (Table 3). Gluconeogenesis, determined from the incorporation of [14 C]pyruvate into glucose, was increased by dexamethasone and BcAMP (Table 3). Insulin decreased gluconeogenesis only in the presence of dexamethasone (Table 3). These changes in gluconeogenesis paralleled the changes in ketogenesis (Fig. 1). The incorporation of

Fig. 2. Effects of dexamethasone, insulin and BcAMP on the glycerol 3-phosphate content of hepatocyte monolayers

For experimental details and key see Fig. 1. The G3P content was determined after 24 h exposure to the hormones and is expressed as nmol/mg of protein. Values are means $\pm$ S.E.M. of duplicate flasks from four cultures.

[^{14}C]pyruvate into glucose and glycogen, expressed as a percentage of the total pyruvate metabolized (control, 48%), was increased by dexamethasone (63%) and BcAMP (71%) (Table 3). The pyruvate metabolized that is not converted into glucose probably largely represents either pyruvate decarboxylation to acetyl-CoA, or diversion of triose phosphates to G3P, as opposed to fructose bisphosphate.

Glycogen synthesis and cellular glycogen

The incorporation of pyruvate into cellular glycogen did not parallel gluconeogenesis (Table 3) and was between 0.4 and 11% of the incorporation of label into glucose. Insulin and dexamethasone separately had no significant effect on the rate of pyruvate incorporation into glycogen, but together they exerted a synergistic stimulation (Table 3). Glycogen synthesis expressed as a percentage of gluconeogenic flux was increased 10-fold in the combined presence of dexamethasone and insulin relative to the control without hormones. During a 2 h incubation with pyruvate there was an increase in the glycogen content in hepatocyte monolayers cultured with insulin and dexamethasone (results not shown), indicating that the incorporation of labelled pyruvate into glycogen was associated with net glycogen deposition. Fig. 3 shows the cellular glycogen content at the end of the incubation with pyruvate. The differences in the glycogen content with the different hormonal treatments (Fig. 3) paralleled the incorporation of [^{14}C]pyruvate into glycogen (Table 3).

DISCUSSION

Hormonal control of ketogenesis and gluconeogenesis

Insulin deficiency in man causes a mild elevation of blood ketone-body concentrations in normal subjects,

Fig. 3. Effects of dexamethasone, insulin and BcAMP on cellular glycogen in hepatocyte monolayers

Hepatocyte monolayers were incubated for 22 h with the concentrations of dexamethasone indicated: ●, without other additions; ○, +10 nM-insulin; □, +1 μM -BcAMP. The medium was then supplemented with 0.5 mM-pyruvate and other additions as described in the text, and the glycogen content (nmol of glucosyl units/mg of cell protein) was determined after 2 h. Values are means $\pm$ S.E.M. of duplicate flasks from four cultures.

but a marked elevation in subjects made hypercortisol-aemic [1]. This could be interpreted in terms of effects of glucocorticoids and insulin on fatty acid mobilization from adipose tissue and changes in substrate supply to the liver [36]. The present study shows that dexamethasone has a direct ketogenic effect on parenchymal liver cells and increases the oxidation of palmitate to ketone bodies. It also increases gluconeogenesis. Insulin prevented both the ketogenic and the gluconeogenic effects of dexamethasone. These observations suggest that direct effects of glucocorticoids on liver contribute to the ketonaemic effects of glucocorticoid excess in insulin-deficient states.

Regulatory mechanisms involved in ketogenesis

The partitioning of fatty acids in liver between esterification to glycerolipid and mitochondrial oxidation can be regulated by changes in the activity of the esterification pathway or by changes in the entry of fatty acids into mitochondria as the carnitine ester. The ketogenic effects of dexamethasone and BcAMP were associated with (1) a decrease in G3P and (2) a more oxidized mitochondrial redox state, but not with an increase in the maximal activity of CPT or in the affinity of the enzyme for fatty acyl-CoA, suggesting that changes in CPT concentration or activity did not contribute to the stimulation of ketogenesis. Decreased glycerolipid formation with dexamethasone because of the low [G3P] may favour an increased partitioning of fatty acyl-CoA into mitochondria. Additional support for the involvement of G3P is that insulin prevented both the stimulation of ketogenesis and the decline in G3P with dexamethasone.

Glucocorticoids strongly induce phosphatidate phosphohydrolase activity in hepatocytes [8,9]. Whether this enzyme limits diacylglycerol formation, however, is uncertain. The present finding that dexamethasone decreased the metabolism of palmitate by pathways other than oxidation to ketones together with a decrease in the [G3P] suggests that in the absence of exogenous glycerol the cellular [G3P] may limit glycerolipid formation. *In vivo*, glycerol mobilized from adipose tissue may increase the hepatic G3P content and overcome the inhibition of glycerolipid formation. The increased activity of phosphatidate phosphohydrolase may be important when G3P is not limiting. The effects of glucocorticoids on glycerolipid formation may be analogous to the effects of thyroid hormones. Hyperthyroidism is associated with induction of phosphatidate phosphohydrolase in liver, but with a decrease in [G3P] and triacylglycerol synthesis [37].

Carnitine palmitoyltransferase

Theoretically, CPT activity can be regulated by: substrate availability ([fatty acyl-CoA] or [carnitine]); changes in the total amount of enzyme or enzyme located on the outer mitochondrial surface (CPT I); phosphorylation [18]; and [malonyl-CoA], an inhibitor of CPT [38]. Changes in [carnitine] are unlikely to have a physiological role, since the liver [carnitine] is higher than the K_m of CPT [25]. CPT activity was determined in detergent extracts, and therefore represents the activity on the outer and inner surfaces of the mitochondrial membrane. Since the V_{max} of CPT in the monolayers was unaffected by dexamethasone and BcAMP, the increased ketogenesis was not due to an

increase in total enzyme activity. The K_m of CPT for palmitoyl-CoA was also not affected by dexamethasone and BcAMP. The affinity of CPT for palmitoyl-CoA is increased by glucagon in freshly isolated hepatocytes [18], and decreased by insulin in cultured cells [15]. The higher affinity of CPT for palmitoyl-CoA in cultured cells than in freshly isolated cells suggests that the lack of activation by dexamethasone or BcAMP may be because the enzyme is in its fully activated state. Malonyl-CoA is unlikely to have an important regulatory role in CPT activity in this system, since the [malonyl-CoA] in liver cells normally correlates with the rate of fatty acid synthesis [32], but this was very low in the presence of palmitate and not further inhibited when ketogenesis was increased.

Control of [glycerol 3-phosphate]

G3P may limit esterification of fatty acids in hepatocyte suspensions [34,35,39]. If the decrease in [G3P] by dexamethasone and BcAMP observed in this study is important in the control of fatty acid esterification, it raises the question as to what mechanism(s) is involved in the regulation of [G3P]. In the absence of glycerol, hepatocytes form G3P from dihydroxyacetone phosphate. Since the activity of G3P dehydrogenase was not affected by the hormonal treatments, changes in G3P may be due either to changes in [dihydroxyacetone phosphate] or to changes in the cytosolic [NADH]/[NAD⁺] ratio. In hepatocyte suspensions, glucagon increases gluconeogenic flux and decreases the [G3P], [dihydroxyacetone phosphate] and [fructose 1,6-bisphosphate] by activation of fructose-bisphosphatase [40]. In hepatocyte micro-carrier cultures, glucagon increases gluconeogenesis from dihydroxyacetone [12], presumably by activation of a site after triose phosphate formation. Since a decrease in G3P in the present study was associated with increased gluconeogenesis, it may be due to activation of fructose bisphosphate or inactivation of phosphofructokinase. A decrease in G3P associated with increased gluconeogenesis is also observed in perfused livers from hyperthyroid rats [41].

Mitochondrial redox state

The present observation that dexamethasone and BcAMP, separately and in combination, decreased the [3-hydroxybutyrate]/[acetoacetate] ratio, and therefore the mitochondrial NADH/NAD⁺ redox state [31], despite increasing the rate of ketogenesis and presumably the production of reducing equivalents by β -oxidation, raises several questions regarding the cause of the redox changes and also the consequences. The more oxidized redox state could be due to: (i) increased transfer of reducing equivalents to the cytosol; or (ii) increased oxidation of NADH by the electron-transport chain. The NADH/NAD⁺ couple in the mitochondrial matrix is more reduced than in the cytosol [42], and the direction of flow of reducing equivalents depends on the relative poises of the NADH/NAD⁺ couples in the cytosol and matrix. During gluconeogenesis from pyruvate, the oxaloacetate formed by pyruvate carboxylation intramitochondrially is exported to the cytosol by either transamination to aspartate or reduction to malate. Efflux as malate provides a mechanism for transporting reducing equivalents to the cytosol for gluconeogenesis [43]. In hepatocyte monolayers incubated with palmitate

and pyruvate in the present study, the mitochondrial formation of NADH through β -oxidation (estimated from the rate of ketogenesis) was 110–500 nmol/h per mg of protein, and the cytosolic NADH requirement for gluconeogenesis (estimated from the incorporation of pyruvate into glucose + glycogen) was 65–345 nmol/h per mg of protein. Since dexamethasone and BcAMP increased both β -oxidation and gluconeogenesis, an increased transfer of reducing equivalents from the mitochondria to the cytosol may contribute to the observed changes in redox state. A more oxidized redox state in response to BcAMP and dexamethasone was also observed in the absence of pyruvate (L. Agius, M. H. Chowdhury & K. G. M. M. Alberti, unpublished work) indicating that the hormonal effects on the mitochondrial redox changes were not dependent on the presence of a gluconeogenic substrate. Theoretically, efflux of reducing equivalents as malate may exceed gluconeogenic flux if oxaloacetate formed from malate oxidation is converted into aspartate or if phosphoenolpyruvate formed from oxaloacetate is recycled to pyruvate by pyruvate kinase rather than converted into glucose. Efflux of reducing equivalents in excess of gluconeogenic flux would lead to a more reduced cytosolic redox state. The [lactate]/[pyruvate] ratios determined at the end of the 2 h incubation with pyruvate and palmitate were higher in the incubations with dexamethasone and BcAMP (results not shown). This suggests a more reduced cytosolic NADH/NAD⁺ state, but it is not unequivocal, since the lactate and pyruvate concentrations were not in a steady state. A more reduced cytosolic redox state would favour increased gluconeogenic flux.

Mitochondria isolated from dexamethasone- or glucagon-treated rats have increased rates of electron transport and transmembrane proton gradients. Some controversy exists whether these changes are due to stabilizing effects of the hormones on the isolated organelles or whether they reflect changes occurring *in vivo* [44,45]. The more oxidized mitochondrial redox state with dexamethasone may in part be due to an increased oxidation of NADH by the electron-transport chain. Although it is generally accepted that ketogenesis from long-chain fatty acids is regulated primarily at the level of entry of the fatty acids into mitochondria as the carnitine ester [46], there is increasing evidence that the reduction state of the mitochondrial NADH/NAD⁺ couple may regulate β -oxidation of fatty acids [47,48]. If the mitochondrial redox state imposes a constraint on β -oxidation in the cultured hepatocytes, the more oxidized redox state with dexamethasone and BcAMP may relieve the inhibition of β -oxidation.

Mechanism of action of dexamethasone

The effects of glucocorticoids on liver cells or hepatoma cell lines involve either cytosolic receptor-mediated regulation of gene transcription [49], or acute mechanisms independent of gene transcription [50]. The latter effects may be mediated by interaction of the glucocorticoids with membranes. Both stabilizing effects and permeability changes of membranes have been reported [50]. For the receptor-mediated mechanisms, the dexamethasone concentrations needed to elicit half-maximal stimulation are in the nanomolar range, whereas the membrane effects are in the micromolar range [50]. Throughout this study significant effects of dexamethasone on ketogenesis, gluconeogenesis and glycogen

synthesis were observed at nanomolar concentrations of the hormone. Higher dexamethasone concentrations were not used, because preliminary experiments showed that total cellular protein was decreased when the hepatocyte monolayers were cultured with $1\ \mu\text{M}$ rather than $100\ \text{nM}$ -dexamethasone, and because ketogenesis and gluconeogenesis were maximal at the lower concentration. This contrasts with the acute stimulation of gluconeogenesis by dexamethasone in freshly isolated hepatocytes, which increases progressively up to dexamethasone concentrations of $200\ \mu\text{M}$ [51,52]. In the present study the effects of dexamethasone and BcAMP on ketogenesis and gluconeogenesis were not due to acute mechanisms, since acute exposure (2 h) of hepatocyte monolayers (precultured for 22 h in the absence of hormones) to dexamethasone and BcAMP did not result in significant changes in ketogenesis and gluconeogenesis. It is likely that the ketogenic and gluconeogenic effects of dexamethasone observed in the present study and the acute gluconeogenic effects on freshly isolated cells [51,52] are mediated by different mechanisms.

In the absence of insulin, the effects of dexamethasone on ketogenesis, gluconeogenesis and the mitochondrial redox state were similar to the effects of BcAMP, suggesting that dexamethasone may increase [cyclic AMP]. The finding, however, that dexamethasone had opposite effects on ketogenesis and gluconeogenesis in the presence of insulin suggests that a rise in [cyclic AMP] is an unlikely explanation for the mechanism of action of dexamethasone. Synergistic stimulation by insulin and dexamethasone of the rate of glycogen synthesis and the cellular glycogen content in the hepatocyte monolayers has been reported previously for rat hepatocytes cultured with either low or high [glucose] [53–55]. In the present study, we used pyruvate as a glycogenic substrate at physiological [glucose] and observed a similar synergism of dexamethasone and insulin on glycogen synthesis. The effects of dexamethasone on ketogenesis and on the mitochondrial redox state have not been described previously. It is unlikely that the more oxidized mitochondrial redox state with dexamethasone is due to uncoupling of oxidative phosphorylation, firstly because it occurred at nanomolar concentrations of the hormone, and the membrane effects of glucocorticoids generally occur at higher concentrations, and secondly because it was associated with increased gluconeogenesis, suggesting increased ATP formation. The mechanism by which dexamethasone regulates mitochondrial properties warrants further study.

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