Stimulation of [1-¹⁴C]oleate oxidation to ¹⁴CO₂ in isolated rat hepatocytes by the catecholamines, vasopressin and angiotensin

A possible mechanism of action

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Adrenaline, noradrenaline, vasopressin and angiotensin increased ¹⁴CO₂ production from [1-¹⁴C]oleate by hepatocytes from fed rats but not by hepatocytes from starved rats. The hormones did not increase ¹⁴CO₂ production when hepatocytes from fed rats were depleted of glycogen *in vitro*. Increased ¹⁴CO₂ production from [1-¹⁴C]oleate in response to the hormones was observed when hepatocytes from starved rats were incubated with 3-mercaptopicolinate, an inhibitor of phosphoenolpyruvate carboxykinase. 3-Mercaptopicolinate inhibited uptake and esterification of [1-¹⁴C]oleate, slightly increased ¹⁴CO₂ production from [1-¹⁴C]oleate and greatly increased the [3-hydroxybutyrate]/[acetoacetate] ratio. In the presence of 3-mercaptopicolinate ¹⁴CO₂ production in response to the catecholamines was blocked by the α -antagonist phentolamine and required extracellular Ca²⁺. The effects of vasopressin and angiotensin were also Ca²⁺-dependent. The actions of the hormones on ¹⁴CO₂ production from [1-¹⁴C]oleate by hepatocytes from starved rats in the presence of 3-mercaptopicolinate thus have the characteristics of the response to the hormones found with hepatocytes from fed rats incubated without 3-mercaptopicolinate. The stimulatory effects of the hormones on ¹⁴CO₂ production from [1-¹⁴C]oleate were not the result of decreased esterification (as the hormones increased esterification) or increased β -oxidation. It is suggested that the effects of the hormones to increase ¹⁴CO₂ production from [1-¹⁴C]oleate are mediated by Ca²⁺-activation of NAD⁺-linked isocitrate dehydrogenase, the 2-oxoglutarate dehydrogenase complex, and/or electron transport. The results also demonstrate that when the supply of oxaloacetate is limited it is utilized for gluconeogenesis rather than to maintain tricarboxylic acid-cycle flux.

In addition to their actions to increase hepatic glycogenolysis and gluconeogenesis (for review, see Exton, 1979), the catecholamines (adrenaline and noradrenaline), vasopressin and angiotensin increase ¹⁴CO₂ production from [1-¹⁴C]oleate by hepatocytes from fed rats (Sugden *et al.*, 1980*a,b*; Watts & Sugden, 1982*a*). The stimulation of glycogenolysis and gluconeogenesis by these hormones is Ca²⁺-dependent, apparently mediated by an increase in intracellular [Ca²⁺] (for review, see

Williamson *et al.*, 1981). The actions of the hormones to increase ¹⁴CO₂ production from [1-¹⁴C]oleate are also Ca²⁺-dependent (Sugden *et al.*, 1980*a,b*) but whereas the mode of action of the hormones on glycogenolysis and gluconeogenesis is relatively well established as being at the level of Ca²⁺-mediated changes in activity of key enzymes for these processes, e.g. glycogen phosphorylase (see Williamson *et al.*, 1981), the mechanism(s) of their effects on fat oxidation is not known. For example, the effects of vasopressin on oleate metabolism have been correlated with increased glycogenolysis and glycolytic flux (Williamson *et al.*, 1980), possibly because of increased oxaloacetate availability (see Sugden *et al.*, 1980*a*). The present work sought to establish whether the stimulatory effects of the hormones on ¹⁴CO₂ production from [1-¹⁴C]oleate were related to their effects on glycogenolysis and consequently oxaloacetate availability, or whether

Abbreviations used: 3-MPA, 3-mercaptopicolinate; PEPCK, phosphoenolpyruvate carboxykinase (EC 4.1.1.32); OGDH, the 2-oxoglutarate dehydrogenase complex; NAD-ICDH, NAD⁺-linked isocitrate dehydrogenase (EC 1.1.1.41).

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their actions to increase oleate oxidation to CO_2 could be observed independently of their effects on glycogenolysis.

Experimental

Materials

All enzymes, coenzymes and substrates were from Boehringer Corporation, London W.5, U.K. [Arginine]vasopressin (grade VIII) was from Sigma (London) Chemical Co., Poole, Dorset, U.K. Adrenaline was from Antigen Ltd., Roscrea, Ireland. Noradrenaline was from Winthrop, Surbiton-on-Thames, Surrey, U.K. Phentolamine mesylate BP (Rogitine) was from CIBA, Horsham, Sussex, U.K. [$1\text{-}^{14}\text{C}$]Oleate was from Amersham International, Amersham, Bucks., U.K.

Animals

Female Albino Wistar rats (170–200 g) were subjected to a 12h-light/12h-dark cycle, the light period starting at 08:00h. Rats were allowed free access to standard rodent diet [BP Nutrition (U.K.), Witham, Essex, U.K.] or food was withdrawn for 24h. Water was supplied *ad libitum*. The glycogen contents of livers from fed or starved rats were 285.4 ± 15.3 (10) or 21.9 ± 2.1 (15) μmol of glucose/g wet wt. respectively.

Preparation of hepatocytes

Preparation of hepatocytes was commenced between 08:30 and 09:30h. Rats were anaesthetized with nembutal (60mg/kg body wt.; solution in 0.9% NaCl). Isolated hepatocytes were prepared as described by Berry & Friend (1969), modified as described by Krebs *et al.* (1974). Hepatocytes were suspended in Krebs & Henseleit (1932) bicarbonate buffer, pH 7.4, containing 2.5mM- CaCl_2 . In some experiments CaCl_2 was omitted. More than 90% of the cells excluded Trypan Blue. 3-MPA (added as the Na^+ salt, pH 7.4) did not decrease cell viability assessed by Trypan Blue exclusion or rates of gluconeogenesis from dihydroxyacetone (results not shown).

Glycogen depletion of hepatocytes *in vitro*

Hepatocytes prepared from fed rats were depleted of glycogen essentially as described by Williamson & Whitelaw (1978) by incubating for 1h in Krebs–Henseleit buffer containing 2.5% defatted albumin and 20 μM -dibutyl cyclic AMP. The cells were washed (three times) with Krebs–Henseleit buffer by centrifugation (75g, 1min) to remove dibutyl cyclic AMP, glucose and lactate. This procedure removes 90–95% of the cell glycogen (Williamson & Whitelaw, 1978), and accordingly rates of glucose release (see below) and lactate release [0.05 ± 0.1 ($n = 5$; mean $\pm$ S.E.M.) $\mu\text{mol}/\text{min}$ per g wet wt. of cells] are very low. Glycogen-depletion

decreased cell viability, assessed by Trypan Blue exclusion, by about 10%.

Experimental procedure

The incubation procedure and measurements of [$1\text{-}^{14}\text{C}$]oleate removal, oxidation to $^{14}\text{CO}_2$ and esterification were as described by Whitelaw & Williamson (1977). Acetoacetate, 3-hydroxybutyrate, glucose, pyruvate and lactate were determined in KOH-neutralized HClO_4 extracts by enzymic methods (Hohorst *et al.*, 1959; Williamson *et al.*, 1962; Slein, 1963). Rates of [$1\text{-}^{14}\text{C}$]oleate removal, esterification and oxidation, and production of metabolites were calculated from plots of the values at 20, 40 and 60min, which were generally linear.

Results are expressed as means $\pm$ S.E.M. with the number of hepatocyte preparations given in parentheses. Statistical significance of differences was assessed with Student's unpaired *t*-test, except where indicated.

Results

$^{14}\text{CO}_2$ production from [$1\text{-}^{14}\text{C}$]oleate by hepatocytes from fed or 24 h-starved rats

To investigate whether effects of the hormones were correlated with stimulation of glycogenolysis, the effects of the hormones on [$1\text{-}^{14}\text{C}$]oleate oxidation by hepatocytes from fed rats were compared with those found by using hepatocytes from fed rats after glycogen depletion *in vitro* (Williamson & Whitelaw, 1978) or hepatocytes from 24h-starved rats. In the absence of hormones, rates of $^{14}\text{CO}_2$ production from [$1\text{-}^{14}\text{C}$]oleate by untreated and glycogen-depleted hepatocytes from fed rats were similar, and greater than rates observed with hepatocytes from starved rats (Table 1). $^{14}\text{CO}_2$ production from [$1\text{-}^{14}\text{C}$]oleate by untreated hepatocytes from fed rats was increased by adrenaline, noradrenaline, vasopressin and angiotensin by 73%, 72%, 87% and 92% respectively (Table 1) in agreement with previous reports (Sugden *et al.*, 1980a,b; Watts & Sugden, 1982a). The hormones did not significantly increase $^{14}\text{CO}_2$ production from [$1\text{-}^{14}\text{C}$]oleate by glycogen-depleted hepatocytes or hepatocytes from starved rats. Glucose release by untreated hepatocytes from fed rats was 2.56 ± 0.21 ($n = 7$) $\mu\text{mol}/\text{min}$ per g wet wt. of cells in the absence of hormones (basal release) and was increased by about 50% on hormone addition, presumably due to activation of glycogenolysis. Rates of glucose release were 0.27 ± 0.05 (8) or 0.11 ± 0.01 (9) μmol of glucose/min per g wet wt. of glycogen-depleted or starved cells respectively, indicating that little if any glycogen was present in the cells. The hormones did not significantly increase glucose or lactate release from these cells (results not shown).

Oleate oxidation by hepatocytes from 24 h-starved rats in the presence of 3-MPA

As hormonal stimulation was not observed when the cells were depleted of glycogen *in vitro* or *in vivo* (starved cells), it was suggested that the ability of the hormones to exert their effects on oleate oxidation could be secondary to glycogenolysis and presumably flux of carbon from glucose 1-phosphate towards lactate and pyruvate. Since the K_m of pyruvate for pyruvate carboxylase (EC 6.4.1.1) is greater than the hepatic pyruvate concentration (Greenbaum *et al.*, 1971; Siess *et al.*, 1977) increased availability of lactate and pyruvate should increase the production of oxaloacetate. To determine whether the hormones increased ¹⁴CO₂ production from [1-¹⁴C]oleate because there was increased production of oxaloacetate (i.e. the effects of the hormones are secondary to their stimulation of glycogenolysis and glycolytic flux) or whether maintenance of a critical concentration of oxaloacetate was permissive for the hormone response, we examined the effects of the hormones on ¹⁴CO₂

production from [1-¹⁴C]oleate by hepatocytes from starved rats in the presence of 3-MPA. This latter compound inhibits PEPCK (DiTullio *et al.*, 1974) and thus utilization of endogenous oxaloacetate for gluconeogenesis.

Addition of 3-MPA inhibited glucose production from endogenous precursors by hepatocytes from starved rats by 66% [control ($n = 12$), $0.12 \pm 0.01 \mu\text{mol/min per g wet wt. of cells}$; 3-MPA treatment ($n = 12$), 0.04 ± 0.01 ; $p < 0.001$]. Glucose production from added lactate (4 mM) and pyruvate (1 mM) was similarly inhibited [control ($n = 15$), $0.95 \pm 0.04 \mu\text{mol/min per g wet wt. of cells}$; 3-MPA treatment ($n = 15$), 0.30 ± 0.02 ; $P < 0.001$]. In the presence of Ca²⁺, 3-MPA increased basal (no hormone) rates of oxidation of [1-¹⁴C]oleate to ¹⁴CO₂ by 31% (Table 2; $P < 0.01$). Addition of the hormones further increased ¹⁴CO₂ production and the hormone-stimulated rates of ¹⁴CO₂ production observed in the presence of 3-MPA and Ca²⁺ were about 50% greater than the basal rate (Table 2) and about 100% greater than the basal rate observed in the absence of 3-MPA.

Table 1. Effects of hormones on oxidation of [1-¹⁴C]oleate to ¹⁴CO₂ by hepatocytes from fed or 24 h-starved rats. For details see the Experimental section. The [1-¹⁴C]oleate concentration was 1 mM. The concentrations of the hormones were 3 μM (adrenaline and noradrenaline) or 10 nM (vasopressin and angiotensin). Values that are significantly different from those without addition of hormone are indicated by *** ($P < 0.001$).

Hormone addition	[1- ¹⁴ C]Oleate oxidized to ¹⁴ CO ₂ (nmol/min per g wet wt. of cells)		
	Fed rats		Untreated starved rats
	Untreated	Glycogen-depleted	
None	37.6 ± 4.6 (7)	37.3 ± 4.0 (10)	25.0 ± 1.6 (16)
Adrenaline	64.9 ± 7.0 (7)***	44.1 ± 4.7 (8)	25.9 ± 1.2 (13)
Noradrenaline	64.6 ± 5.0 (7)***	44.6 ± 4.2 (10)	25.8 ± 1.5 (10)
Vasopressin	70.2 ± 1.2 (5)***	44.1 ± 3.4 (7)	26.7 ± 1.7 (13)
Angiotensin	72.0 ± 1.0 (4)***	46.9 ± 5.1 (9)	24.3 ± 1.4 (8)

Table 2. Effects of 3-MPA on oxidation of [1-¹⁴C]oleate to ¹⁴CO₂ in hepatocytes from 24 h-starved rats in the presence and absence of CaCl₂.

For details see the Experimental section. The concentration of 3-MPA (Na⁺ salt, pH 7.4) was 1 mM. CaCl₂ when present was 2.5 mM. The concentrations of the hormones and [1-¹⁴C]oleate were given in the legend to Table 1. Values that are significantly different from those without addition of hormone are indicated by: ***, $P < 0.001$. Significance of differences between values obtained in the presence and absence of CaCl₂ are given by: †, $P < 0.05$; ††, $P < 0.01$; †††, $P < 0.001$.

Hormone addition	[1- ¹⁴ C]Oleate oxidized to ¹⁴ CO ₂ (nmol/min per g wet wt. of cells)			
	+CaCl ₂		-CaCl ₂	
	Control	+3-MPA	Control	+3-MPA
None	21.8 ± 1.3 (6)	28.6 ± 0.9 (6)	15.0 ± 1.0 (4)†	16.9 ± 1.0 (4)†††
Adrenaline	23.0 ± 1.0 (6)	44.0 ± 2.4 (6)***	16.0 ± 1.0 (3)††	18.5 ± 1.0 (4)†††
Noradrenaline	23.2 ± 1.0 (6)	45.1 ± 2.8 (6)***	15.4 ± 1.0 (3)††	18.9 ± 1.0 (4)†††
Vasopressin	23.3 ± 1.0 (6)	43.0 ± 1.5 (6)***	16.0 ± 1.0 (4)††	18.8 ± 1.1 (4)†††
Angiotensin	22.4 ± 1.0 (6)	42.7 ± 1.5 (6)***	16.9 ± 1.0 (4)††	17.2 ± 1.0 (4)†††

Effects of Ca^{2+} and phentolamine

As the response to the hormones of hepatocytes from 24 h-starved rats incubated with 3-MPA was analogous to that found with hepatocytes from fed rats without 3-MPA, the effects of omission of Ca^{2+} and the α -adrenergic antagonist phentolamine (which blocks the effects of the catecholamines on $^{14}\text{CO}_2$ production from 1- ^{14}C oleate by hepatocytes from fed rats) were investigated.

Basal rates of $^{14}\text{CO}_2$ production from [1- ^{14}C]oleate by hepatocytes from starved rats in the absence of Ca^{2+} were about 60% of those observed in the presence of Ca^{2+} (Table 2). 3-MPA did not significantly increase the basal rate of oxidation, so changes in cellular Ca^{2+} metabolism may be involved. The hormones did not increase $^{14}\text{CO}_2$ production, either in the presence or absence of 3-MPA, in the absence of Ca^{2+} .

Phentolamine did not affect the basal rate of $^{14}\text{CO}_2$ production in the absence of 3-MPA but slightly stimulated (15%; $P < 0.05$) $^{14}\text{CO}_2$ production in the presence of 3-MPA. Adrenaline and noradrenaline did not increase $^{14}\text{CO}_2$ production from [1- ^{14}C]oleate when phentolamine was included in the incubation medium (Table 3). Phentolamine did not affect the ability of vasopressin and angiotensin to increase oleate oxidation in the presence of 3-MPA (Table 3).

Metabolic fate of [1- ^{14}C]oleate in the presence and absence of 3-MPA

In the absence of 3-MPA the hormones significantly increased [1- ^{14}C]oleate esterification and slightly (4–6%) increased ketogenesis (Table 4). The increases in esterification ranged from 24 to 29%. The increases in fatty acid utilization were not the result of increased uptake of [1- ^{14}C]oleate (Table 4). In the presence of 3-MPA, the hormones increased both the oxidation of [1- ^{14}C]oleate to $^{14}\text{CO}_2$ (see Table 2) and its esterification to triacylglycerol (Table 4). The

increases in esterification were slightly greater than those observed in the absence of 3-MPA (30% for adrenaline, noradrenaline and vasopressin, and 39% for angiotensin). It is not known how the hormones increase esterification, but stimulation is not the result of increased flux through PEPCK since the increases are observed in the presence of 3-MPA. The increased oxidation of [1- ^{14}C]oleate to $^{14}\text{CO}_2$ observed in response to the hormones in the presence of 3-MPA (Table 2) is accompanied by decreased (9–12%) ketogenesis (Table 4) and so total oleate oxidation (to CO_2 and ketone bodies) is not increased. The hormones had no effect on [1- ^{14}C]oleate removal either in the presence or in the absence of 3-MPA (Table 4). The total recovery of oleate in the three fractions (esterified fats, ketone bodies and metabolic CO_2 ; see Tables 2 and 4) was less than 100% (about 66%), which implies that significant amounts of [1- ^{14}C]oleate taken up are neither esterified nor oxidized to ketone bodies or CO_2 . Presumably at least some of the 'missing' radioactivity is incorporated into other metabolites via exchange reactions.

3-MPA inhibited basal and hormone-stimulated esterification of oleate by 20–26% (Table 4). Inhibition of similar magnitude was observed in the absence of Ca^{2+} [control ($n = 7$), $0.11 \pm 0.01 \mu\text{mol/min per g wet wt. of cells}$; 3-MPA treatment ($n = 7$), 0.08 ± 0.01 ; $P < 0.05$]. The inhibition of esterification by 3-MPA is presumably the result of decreased availability of 3-glycerophosphate (Goodman, 1975), which limits the rate of esterification in glycogen-depleted hepatocytes (Declercq *et al.*, 1982). The changes in the relative rates of oleate oxidation to CO_2 or ketone bodies were accompanied by large and highly significant increases in [3-hydroxybutyrate]/[acetoacetate] (Table 4). This indicates that the mitochondrial $[\text{NAD}^+]/[\text{NADH}]$ is decreased. 3-MPA also slightly inhibited [1- ^{14}C]oleate uptake both in the absence and presence of hormones (Table 4).

Table 3. Effects of phentolamine on oxidation of [1- ^{14}C]oleate to $^{14}\text{CO}_2$ by hepatocytes from 24 h-starved rats in the presence and absence of 3-MPA

For details see the Experimental section. 3-MPA (Na^+ salt, pH 7.4) concentration was 1 mM. The phentolamine concentration was $30 \mu\text{g/ml}$. The concentrations of the hormones and [1- ^{14}C]oleate were as given in the legend to Table 1. Values that are significantly different from those without addition of hormone are indicated by: **, $P < 0.01$; ***, $P < 0.001$.

Hormone addition	[1- ^{14}C]Oleate oxidized to $^{14}\text{CO}_2$ (nmol/min per g wet wt. of cells)			
	Control	+ 3-MPA	+ Phentolamine	+ Phentolamine + 3-MPA
None	17.6 ± 0.7 (9)	21.0 ± 0.9 (9)	17.2 ± 0.9 (9)	24.2 ± 1.2 (9)
Adrenaline	18.7 ± 0.9 (9)	33.6 ± 1.9 (9)***	16.8 ± 1.3 (4)	22.7 ± 1.1 (9)
Noradrenaline	17.7 ± 1.1 (9)	33.8 ± 1.6 (9)***	16.8 ± 1.2 (4)	24.2 ± 1.0 (9)
Vasopressin	19.1 ± 2.0 (4)	35.5 ± 0.3 (4)***	20.4 ± 2.0 (4)	35.8 ± 0.4 (4)***
Angiotensin	18.5 ± 2.0 (4)	33.8 ± 0.4 (4)**	19.2 ± 2.0 (4)	34.3 ± 2.8 (4)**

Table 4. *Effects of hormones on the metabolism of [1-¹⁴C]oleate by hepatocytes from 24 h-starved rats in the presence and absence of 3-MPA*

For details see the Experimental section. The concentration of 3-MPA (Na⁺ salt, pH 7.4) was 1 mM. The concentrations of the hormones and [1-¹⁴C]oleate were as given in the legend to Table 1. Values that are significantly different from those without addition of hormone are indicated by: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Values that are significantly different from those without addition of 3-MPA are indicated by: †, $P < 0.05$; ††, $P < 0.01$; †††, $P < 0.001$. The significance of the effects of 3-MPA on [1-¹⁴C]oleate removal and ketone-body production and effects of the hormones on ketone-body production were assessed by Student's paired *t* test.

Hormone addition	Removal of [1- ¹⁴ C]oleate (μ mol/min per g wet wt. of cells)	Esterification of [1- ¹⁴ C]oleate (μ mol/min per g wet wt. of cells)	Ketone-body production (μ mol/min per g wet wt. of cells)	[3-Hydroxybutyrate]/ [acetoacetate]
Control				
None	0.61 $\pm$ 0.07 (5)	0.119 $\pm$ 0.006 (5)	1.14 $\pm$ 0.06 (9)	1.7 $\pm$ 0.1 (9)
Adrenaline	0.65 $\pm$ 0.07 (5)	0.146 $\pm$ 0.006 (5)*	1.20 $\pm$ 0.07 (9)	1.7 $\pm$ 0.1 (9)
Noradrenaline	0.66 $\pm$ 0.07 (5)	0.147 $\pm$ 0.006 (5)*	1.18 $\pm$ 0.06 (9)	1.7 $\pm$ 0.1 (9)
Vasopressin	0.66 $\pm$ 0.07 (5)	0.150 $\pm$ 0.005 (5)**	1.20 $\pm$ 0.07 (9)	1.7 $\pm$ 0.1 (9)
Angiotensin	0.65 $\pm$ 0.07 (5)	0.152 $\pm$ 0.004 (5)**	1.19 $\pm$ 0.07 (9)	1.7 $\pm$ 0.1 (9)
+ 3-MPA				
None	0.56 $\pm$ 0.08 (5)†	0.087 $\pm$ 0.004 (5)††	1.16 $\pm$ 0.06 (9)	2.9 $\pm$ 0.1 (9)†††
Adrenaline	0.56 $\pm$ 0.08 (5)†	0.112 $\pm$ 0.005 (5)††***	1.02 $\pm$ 0.06 (9)†***	3.1 $\pm$ 0.2 (9)†††
Noradrenaline	0.58 $\pm$ 0.08 (5)†††	0.113 $\pm$ 0.003 (5)†††***	1.04 $\pm$ 0.06 (8)†***	3.3 $\pm$ 0.2 (9)†††
Vasopressin	0.57 $\pm$ 0.07 (5)†††	0.111 $\pm$ 0.003 (5)†††**	1.03 $\pm$ 0.06 (9)†††***	3.1 $\pm$ 0.1 (9)†††
Angiotensin	0.58 $\pm$ 0.07 (5)†††	0.121 $\pm$ 0.004 (5)†††***	1.06 $\pm$ 0.07 (9)†††***	3.1 $\pm$ 0.1 (9)†††

Discussion

Oxidation of acetyl-CoA by the tricarboxylic acid cycle in liver may be limited by the availability of oxaloacetate for citrate synthesis (Lehninger, 1946; Siess *et al.*, 1981, 1982; Watts & Sugden, 1982b; for review, see Sugden & Williamson, 1982). Tricarboxylic acid cycle flux also depends on the energy state of the liver, since the activities of several enzymes of the cycle are regulated by [ADP]/[ATP] and [NAD⁺]/[NADH]. These include the decarboxylating enzymes of the cycle, NAD-ICDH and OGDH, which, in addition to activation by increased [ADP]/[ATP] and [NAD⁺]/[NADH], are activated by Ca²⁺ (for review, see Denton & McCormack, 1980). We have recently suggested (Sugden *et al.*, 1980a–c) that Ca²⁺-dependent increases in ¹⁴CO₂ production from [1-¹⁴C]oleate in response to the catecholamines and vasopressin might result from increased tricarboxylic acid cycle turnover. The aim of the present work was to gain insight into the mechanisms involved.

The hormones increased ¹⁴CO₂ production from [1-¹⁴C]oleate by hepatocytes from fed rats but did not increase ¹⁴CO₂ production if the hepatocytes were depleted of glycogen (Table 1). This suggested that the hormone effect was secondary to increased glycogenolysis and thus increased availability of oxaloacetate. However, the addition of 3-MPA to hepatocytes from starved rats (which did not contain glycogen) not only increased ¹⁴CO₂ production from [1-¹⁴C]oleate but also allowed the hormones to exert

their effects. The characteristics of the responses to the hormones found with starved hepatocytes were the same as those found with hepatocytes from fed rats in the absence of 3-MPA (Tables 2 and 3; see also Sugden *et al.*, 1980a,b). These observations indicate that the effects of the hormones on oxidation of [1-¹⁴C]oleate to ¹⁴CO₂ are not secondary to their effects on glycogenolysis since the hormone effects can be observed with starved hepatocytes, which do not contain glycogen.

CO₂ is produced by the tricarboxylic acid cycle at NAD-ICDH and OGDH. The hormones must therefore either increase the rate of production of [1-¹⁴C]acetyl-CoA from [1-¹⁴C]oleate (by increasing either mitochondrial uptake of oleoyl-CoA or β -oxidation) or increase flux through these enzymes (by inhibiting utilization of [1-¹⁴C]acetyl-CoA for ketogenesis or activating the tricarboxylic acid cycle). Available evidence suggests that with hepatocytes from starved rats in the presence of 3-MPA the hormones increase flux through NAD-ICDH and/or OGDH at the expense of ketogenesis. This conclusion is justified by the following observations. (1) The hormones stimulate oleate esterification both in the presence and in the absence of 3-MPA but stimulate oleate oxidation to CO₂ only in the presence of 3-MPA. It is therefore unlikely that the changes in oxidation are a consequence of, or related to, the changes in esterification. (2) The total rate of oleate oxidation (i.e. the combined rate of production of CO₂ and ketone bodies) is not increased by the hormones in the presence of 3-MPA (Tables

3 and 4), so it is unlikely that β -oxidation is increased. (3) The decreases in rates of ketogenesis observed with the hormones in the presence of 3-MPA are sufficient in magnitude to account for the increases in CO_2 production.

The question posed is how 3-MPA exerts its effect. 3-MPA inhibits both basal and hormone-stimulated esterification of oleate, but inhibition is not Ca^{2+} -dependent. The increases in the concentration ratio [3-hydroxybutyrate]/[acetoacetate] in response to 3-MPA are also observed both in the presence (Table 4) and in the absence (results not shown) of Ca^{2+} . In contrast the effects of the hormones on oleate oxidation to CO_2 are observed only in the presence of 3-MPA and Ca^{2+} . It seems likely that the permissive action of 3-MPA is related to increased availability of mitochondrial oxaloacetate secondary to inhibition of PEPCK in the cytoplasm (see Watts & Sugden, 1982b). In support of this, 3-MPA increases both cell [oxaloacetate] and [citrate] (Goodman, 1975) and itself increases $^{14}\text{CO}_2$ production from [1- ^{14}C]oleate (31%; Table 2). Importantly, the addition of exogenous precursors of oxaloacetate (e.g. lactate and pyruvate) was not necessary for the response to the hormones to be observed. The implication of this is that in hepatocytes from starved rats oxaloacetate is used preferentially for glucose formation rather than maintenance of cycle flux. Consequently the rate of utilization of available oxaloacetate for gluconeogenesis will determine the rate of tricarboxylic acid cycle turnover. Since in the presence of 3-MPA the hormone-stimulated rates of $^{14}\text{CO}_2$ production were about 50% greater than the basal rates, either the hormones further increase oxaloacetate availability, possibly by increasing pyruvate carboxylation (see Garrison & Haynes, 1975; Garrison & Borland, 1979; Titheradge *et al.*, 1979) or increase flux through NAD-ICDH and/or OGDH by influencing other metabolic parameters, e.g. $[\text{NAD}^+]/[\text{NADH}]$, $[\text{ADP}]/[\text{ATP}]$ or mitochondrial $[\text{Ca}^{2+}]$ (see above). 3-MPA could inhibit the activities of the enzymes via the decreased mitochondrial $[\text{NAD}^+]/[\text{NADH}]$; there is no evidence that 3-MPA decreases mitochondrial $[\text{Ca}^{2+}]$ or influences the $[\text{ADP}]/[\text{ATP}]$ ratio (Goodman, 1975). Our results are consistent with the idea that in the presence of 3-MPA the hormones activate NAD-ICDH and/or OGDH, whose activity is submaximal because of a decreased $[\text{NAD}^+]/[\text{NADH}]$ ratio rather than because of limitation of substrate. Transient hormone-induced changes in NADH fluorescence have been reported (e.g. Balaban & Blum, 1982) but the hormones did not decrease the steady-state [3-hydroxybutyrate]/[acetoacetate] ratio and so are unlikely to activate the enzymes by increasing $[\text{NAD}^+]/[\text{NADH}]$ (but see below). The hormones do not decrease the $[\text{ATP}]/[\text{ADP}]$ (Hems *et al.*,

1978; Titheradge & Haynes, 1980). The Ca^{2+} -dependency of the hormone effect suggests that, for the oxidation of [1- ^{14}C]oleate to $^{14}\text{CO}_2$, the primary action of the hormones may be activation of NAD-ICDH and/or OGDH by Ca^{2+} , an effect demonstrable (for OGDH at least) in the presence of end-product inhibition by NADH (McCormack & Denton, 1979).

Although the present results suggest that 3-MPA allows expression of the hormone responses because of increased oxaloacetate availability together with inhibition of NAD-ICDH and OGDH by a decreased $[\text{NAD}^+]/[\text{NADH}]$, other explanations are possible. The decreased mitochondrial $[\text{NAD}^+]/[\text{NADH}]$ would be expected to increase mitochondrial Ca^{2+} retention (Lehninger *et al.*, 1978) and the apparent lack of effect of the hormones with hepatocytes from starved rats in the absence of 3-MPA may thus reflect a change in cellular Ca^{2+} handling, which can be rectified by 3-MPA addition. It should also be noted that although the decreased $[\text{NAD}^+]/[\text{NADH}]$ produced by 3-MPA *in vivo* (Blackshear *et al.*, 1975; Watts & Sugden, 1982b) has been attributed to inhibition of utilization of reducing equivalents for gluconeogenesis (Blackshear *et al.*, 1975) a direct effect of 3-MPA to inhibit electron transport cannot be excluded. There is evidence that the catecholamines, at least, can stimulate uncoupler-dependent ATPase activity by an α -adrenergic, Ca^{2+} -dependent mechanism (Titheradge & Haynes, 1980) and such stimulation could indirectly influence flux through NAD-ICDH and/or OGDH via changes in $[\text{NAD}^+]/[\text{NADH}]$.

In conclusion, the results demonstrate that Ca^{2+} -dependent increases in $^{14}\text{CO}_2$ production from [1- ^{14}C]oleate by hepatocytes observed in response to adrenaline, noradrenaline, vasopressin and angiotensin are not dependent on hormone-induced increases in glycogenolysis. Hormone effects can be observed when glycogen is absent, provided that utilization of available oxaloacetate for phosphoenolpyruvate formation is inhibited. The effects of the hormones are not secondary to decreased esterification or increased β -oxidation. It is suggested that the hormones directly stimulate flux through the tricarboxylic acid cycle as a result of Ca^{2+} -dependent activation of NAD-ICDH, OGDH and/or electron transport. The results also demonstrate that when the supply of oxaloacetate is limited, it is utilized for gluconeogenesis rather than to maintain tricarboxylic acid cycle flux. This has important implications for the regulation of the tricarboxylic acid cycle and fuel utilization in liver in starvation.

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References

- Balaban, R. S. & Blum, J. J. (1982) *Am. J. Physiol.* **22**, C172–C177
- Berry, M. N. & Friend, D. S. (1969) *J. Cell Biol.* **43**, 506–520
- Blackshear, P. J., Holloway, P. A. & Alberti, K. G. M. M. (1975) *Biochem. J.* **148**, 353–362
- Declercq, P. E., Debeer, L. J. & Mannaerts, G. P. (1982) *Biochem. J.* **204**, 247–256
- Denton, R. M. & McCormack, J. G. (1980) *FEBS Lett.* **119**, 1–8
- DiTullio, N. W., Berkoff, C. E., Blank, B., Kostos, V., Stack, E. J. & Saunders, H. L. (1974) *Biochem. J.* **138**, 387–394
- Exton, J. H. (1979) *Biochem. Pharmacol.* **28**, 2237–2240
- Garrison, J. C. & Borland, K. (1979) *J. Biol. Chem.* **254**, 1129–1133
- Garrison, J. C. & Haynes, R. C., Jr. (1975) *J. Biol. Chem.* **250**, 2769–2777
- Goodman, M. N. (1975) *Biochem. J.* **150**, 137–139
- Greenbaum, A. L., Gumaa, K. A. & McLean, P. (1971) *Arch. Biochem. Biophys.* **143**, 617–663
- Hems, D. A., McCormack, J. G. & Denton, R. M. (1978) *Biochem. J.* **176**, 627–629
- Hohorst, H. J., Kreutz, F. H. & Bücher, T. (1959) *Biochem. Z.* **332**, 18–46
- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66
- Krebs, H. A., Cornell, N. W., Lund, P. & Hems, R. (1974) *Alfred Benzon Symp.* **6**, 718–743
- Lehninger, A. L. (1946) *J. Biol. Chem.* **164**, 291–306
- Lehninger, A. L., Vercesi, A. & Bababunmi, E. A. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1690–1694
- McCormack, J. G. & Denton, R. M. (1979) *Biochem. J.* **180**, 533–544
- Siess, E. A., Brocks, D. G., Lattke, H. L. & Wieland, O. (1977) *Biochem. J.* **176**, 899–906
- Siess, E. A., Kientsch-Engel, R. I. & Wieland, O. H. (1981) *Diabetologia* **21**, 328
- Siess, E. A., Kientsch-Engel, R. I. & Wieland, O. H. (1982) *Eur. J. Biochem.* **121**, 493–499
- Slein, M. W. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 117–123, Academic Press, New York and London
- Sugden, M. C. & Williamson, D. H. (1982) in *Metabolic Compartmentation* (Sies, H., ed.), pp. 287–315, Academic Press, London
- Sugden, M. C., Ball, A. J., Ilic, V. & Williamson, D. H. (1980a) *FEBS Lett.* **116**, 37–40
- Sugden, M. C., Tordoff, A. F. C., Ilic, V. & Williamson, D. H. (1980b) *FEBS Lett.* **120**, 80–84
- Sugden, M. C., Ball, A. J. & Williamson, D. H. (1980c) *Biochem. Soc. Trans.* **8**, 591–592
- Titheradge, M. A. & Haynes, R. C., Jr. (1980) *Arch. Biochem. Biophys.* **201**, 44–55
- Titheradge, M. A., Stringer, J. L. & Haynes, R. C., Jr. (1979) *Eur. J. Biochem.* **102**, 117–124
- Watts, D. I. & Sugden, M. C. (1982a) *Biochem. Soc. Trans.* in the press
- Watts, D. I. & Sugden, M. C. (1982b) *Biochem. Int.* **4**, 255–261
- Whitelaw, E. & Williamson, D. H. (1977) *Biochem. J.* **164**, 521–528
- Williamson, D. H. & Whitelaw, E. (1978) in *Regulatory Mechanisms of Carbohydrate Metabolism* (Esmann, V., ed.), pp. 151–160, Pergamon Press, Oxford and New York
- Williamson, D. H., Mellanby, J. & Krebs, H. A. (1962) *Biochem. J.* **82**, 90–96
- Williamson, D. H., Ilic, V., Tordoff, A. F. C. & Ellington, E. V. (1980) *Biochem. J.* **186**, 621–624
- Williamson, J. R., Cooper, R. H. & Hoek, J. B. (1981) *Biochim. Biophys. Acta* **639**, 243–295