The stimulation of tricarboxylic acid-cycle flux by α -adrenergic agonists in perfused rat liver

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Output of $^{14}\text{CO}_2$ from 1- ^{14}C -labelled glutamate, 2-oxoglutarate or octanoate and from 4-methyl-2-oxo[2- ^{14}C]pentanoate was increased by more than 100% after infusion of phenylephrine into perfused livers of fed rats. Infusion of ethanol or sorbitol raised 3-hydroxybutyrate/acetoacetate ratios and decreased the output of $^{14}\text{CO}_2$. Increases in $^{14}\text{CO}_2$ output induced by phenylephrine were observed in the presence or absence of ethanol or sorbitol and were accompanied by elevated 3-hydroxybutyrate/acetoacetate ratios under all conditions examined. Phenylephrine had no effect on total tissue ATP/ADP ratios in livers from fed or starved rats. The data suggest that phenylephrine-induced increases in tricarboxylic acid-cycle flux do not arise from lowered matrix NADH/NAD $^+$ or ATP/ADP ratios.

INTRODUCTION

The administration of α -adrenergic agonists to perfused rat liver results in a wide range of metabolic responses, which are mediated via an α_1 -receptor-linked Ca^{2+} -dependent mechanism (reviewed in Taylor *et al.*, 1983a; Reinhart *et al.*, 1984). One such response involves a stimulation of tricarboxylic acid-cycle activity. Enhanced rates of decarboxylation in the cycle have been inferred from experiments in which α -agonists promote $^{14}\text{CO}_2$ production from a variety of labelled substrates, including oleate, octanoate, proline, glutamate and 2-oxoglutarate (Sugden & Watts, 1983; Taylor *et al.*, 1983b; Haussinger & Sies, 1984).

Several groups have suggested that vasopressin- or α -agonist-induced increases in tricarboxylic acid-cycle flux may involve an increase in the activity of the mitochondrial enzymes 2-oxoglutarate dehydrogenase or isocitrate dehydrogenase after a rise in matrix free Ca^{2+} concentration (Denton *et al.*, 1980; Sugden & Watts, 1983; Ochs, 1984; Staddon & McGivan, 1984). Direct evidence for such a mechanism has not been obtained, and regulation by other control factors such as matrix NADH/NAD $^+$ or ATP/ADP ratios could also be involved (for a review see Williamson & Cooper, 1980).

In the present study we assess the possible involvement of matrix NADH/NAD $^+$ and ATP/ADP ratios in the control of tricarboxylic acid-cycle flux by α -adrenergic agonists. We examine the relationship between phenylephrine-induced increases in $^{14}\text{CO}_2$ production from a range of labelled cycle precursors and changes in the perfusate 3-hydroxybutyrate/acetoacetate and tissue ATP/ADP ratios in perfused rat liver. The data suggest that α -agonist-induced increases in tricarboxylic acid-cycle flux are unlikely to result from changes in matrix NADH/NAD $^+$ or ATP/ADP ratios.

EXPERIMENTAL

Animals and perfusions

Male Wistar-strain albino rats weighing 230–290 g were used in all experiments, and animals either were allowed free access to food or were starved for 48 h where

indicated. Animals were anaesthetized with sodium pentobarbitone (50 mg/kg body wt.), and the livers perfused with Krebs–Henseleit (1932) bicarbonate medium equilibrated with O_2/CO_2 (19:1) as described previously (Reinhart *et al.*, 1982). All livers were perfused with medium containing 1.3 mM added CaCl_2 at 35 °C and at a flow rate of 3.5 ml/min per g wet wt. of liver. In experiments where $^{14}\text{CO}_2$ output from labelled precursors was monitored, labelled compounds were continuously infused from the commencement of perfusion before further additions at 15 min of perfusion. All labelled compounds except [1- ^{14}C]octanoate were infused to give final concentrations of 200 μM and 8 nCi/ml. Octanoate was infused at a concentration of 5 μM and 5 nCi/ml.

Analytical procedures

Perfusate O_2 concentrations were continuously monitored with a Clark-type oxygen electrode as detailed by Reinhart *et al.* (1982). Effluent perfusate was assayed for glucose, lactate, pyruvate, ketone bodies and $^{14}\text{CO}_2$ as described by Taylor *et al.* (1983b). 1- ^{14}C - and 2- ^{14}C -labelled 4-methyl-2-oxopentanoate were prepared from labelled leucine as described by Tomera *et al.* (1983). Tissue ATP and ADP contents were assayed essentially as detailed by Denton & Halperin (1968), after freeze-clamping of livers perfused under control conditions or after 3 min of phenylephrine infusion.

Chemicals and materials

Phenylephrine, amino-oxyacetate, sorbitol and the glucose assay kit (510-A) were obtained from Sigma Chemical Co., St Louis, MO, U.S.A. Lactate dehydrogenase and 3-hydroxybutyrate dehydrogenase were from Boehringer, Mannheim, West Germany. [^{14}C]Leucine was obtained from the Commissariat à l'Énergie Atomique, Saclay, France. [^{14}C]Octanoate and -glutamate were supplied by New England Nuclear, Boston, MA, U.S.A. Hyamine hydroxide was from Packard Instrument Co., Downers Grove, IL, U.S.A. All other chemicals were of analytical grade.

Fig. 1. Effect of phenylephrine on $^{14}\text{CO}_2$ output from labelled tricarboxylic acid-cycle precursors

Livers of fed rats were perfused as described in the Experimental section. At 15 min of perfusion, phenylephrine was infused to give a final concentration of $2\ \mu\text{M}$. Production of $^{14}\text{CO}_2$ from [1- ^{14}C]octanoate (●), 2-oxo[1- ^{14}C]glutarate (□), [1- ^{14}C]glutamate (■), 1- ^{14}C -labelled 4-methyl-2-oxopentanoate (▲) or 2- ^{14}C -labelled 4-methyl-2-oxopentanoate (△) were monitored as described in the Experimental section. In each case, values for $^{14}\text{CO}_2$ output immediately before phenylephrine infusion were normalized to 100%, and all values are expressed relative to their respective controls. Control values for $^{14}\text{CO}_2$ output were 3.1 ± 0.2 , 386 ± 26 , 530 ± 42 , 706 ± 38 and 208 ± 24 ng-atoms/min for octanoate, 2-oxoglutarate, glutamate and 4-methyl-2-oxopentanoate labelled in the C-1 and C-2 positions respectively. Data shown are means for six independent experiments. For clarity, S.E.M. values are not shown, but were generally $< \pm 10\%$.

RESULTS AND DISCUSSION

Effect of phenylephrine on $^{14}\text{CO}_2$ production from tricarboxylic acid-cycle precursors

The generation of $^{14}\text{CO}_2$ from 1- ^{14}C -labelled glutamate, 2-oxoglutarate or octanoate, or from 2- ^{14}C -labelled 4-methyl-2-oxopentanoate, has been used previously to assess cycle flux in the perfused rat liver (Soboll *et al.*, 1981; Taylor *et al.*, 1983b; Tomera *et al.*, 1983; Haussinger & Sies, 1984). Phenylephrine increased $^{14}\text{CO}_2$ production from each of these by $> 100\%$ in perfused livers from fed rats (Fig. 1). Similar effects were obtained with 50 nM-adrenaline or -noradrenaline or with 2.5 m-units of vasopressin/ml (results not shown). Whereas phenylephrine-induced increases in $^{14}\text{CO}_2$ output from [1- ^{14}C]octanoate or 2- ^{14}C -labelled 4-methyl-2-oxopentanoate were maintained near-maximal throughout the period of phenylephrine infusion, increases in $^{14}\text{CO}_2$ output from [1- ^{14}C]glutamate or 2-oxo[1- ^{14}C]glutarate were transient and approached basal values within 4–5 min of phenylephrine infusion. Similar transient

increases in $^{14}\text{CO}_2$ generation from [1- ^{14}C]glutamate in response to α -agonists in perfused rat liver have been reported by Haussinger & Sies (1984). These authors suggested that a limited rate of entry of labelled glutamate or 2-oxoglutarate into the tissue could explain such transient phenomena. Prolonged increases in $^{14}\text{CO}_2$ output as observed with [1- ^{14}C]octanoate or 2- ^{14}C -labelled 4-methyl-2-oxopentanoate would therefore appear more useful experimentally. In particular, Tomera *et al.* (1983) have described a technique whereby $^{14}\text{CO}_2$ output obtained with 1- ^{14}C - or 2- ^{14}C -labelled 4-methyl-2-oxopentanoate may be used to calculate the fraction of total matrix acetyl-CoA oxidized by the tricarboxylic acid cycle. Thus $^{14}\text{CO}_2$ output from 1- ^{14}C -labelled 4-methyl-2-oxopentanoate is used to assess total acetyl-CoA generation from this substrate, whereas $^{14}\text{CO}_2$ generated from 2- ^{14}C -labelled 4-methyl-2-oxopentanoate reflects the further oxidation of this acetyl-CoA in the cycle. Treatment of the data shown in Fig. 1 indicates that approx. 30% of matrix acetyl-CoA is oxidized via the tricarboxylic acid cycle under basal conditions and that phenylephrine infusion increases the proportion of acetyl-CoA oxidation to around 80%.

However, it should be pointed out that rates of tricarboxylic acid-cycle flux determined with labels such as [1- ^{14}C]octanoate and 2- ^{14}C -labelled 4-methyl-2-oxopentanoate as used in the present study, or [U- ^{14}C]proline employed by Haussinger & Sies (1984), may be erroneously high. ^{14}C atoms from these labelled compounds enter the oxaloacetate pool and yield $^{14}\text{CO}_2$ only after subsequent oxaloacetate metabolism. Consequently, in experiments using these labels, a proportion of the $^{14}\text{CO}_2$ generated may arise from oxaloacetate decarboxylation to phosphoenolpyruvate rather than from decarboxylation in the tricarboxylic acid cycle. This could be an important consideration when assessing phenylephrine-induced changes in cycle flux, as this agent enhances flux from pyruvate to glucose in perfused livers from fed rats (W. M. Taylor, E. Van De Pol & F. L. Bygrave, unpublished work). The generation of $^{14}\text{CO}_2$ from [1- ^{14}C]glutamate or 2-oxo[1- ^{14}C]glutarate, which do not directly label the oxaloacetate pool, may therefore continue to serve as additional indicators of qualitative changes in tricarboxylic acid-cycle flux.

Effects of ethanol and sorbitol on phenylephrine-induced changes in octanoate oxidation, oxygen consumption and the 3-hydroxybutyrate/acetoacetate ratio

Considerable evidence now suggests that the mitochondrial matrix NADH/NAD $^+$ ratio plays a major role in the regulation of tricarboxylic acid-cycle flux (for a review see Williamson & Cooper, 1980). The elevation of matrix NADH/NAD $^+$ ratios after ethanol metabolism has been reported to diminish cycle flux in perfused rat liver (Williamson *et al.*, 1969). Similar findings were observed in the present study after infusion of ethanol or sorbitol in experiments where cycle flux was monitored by measuring [1- ^{14}C]octanoate conversion into $^{14}\text{CO}_2$ (Fig. 2, Table 1). In contrast with the effects of ethanol or sorbitol, however, phenylephrine infusion led to increased $^{14}\text{CO}_2$ output together with an elevated matrix NADH/NAD $^+$ ratio, as judged by the elevated 3-hydroxybutyrate/acetoacetate ratios observed. These effects of phenylephrine were observed in both the presence and the absence of ethanol or sorbitol. Thus, although ethanol or sorbitol infusion elevated the matrix NADH/NAD $^+$

Table 1. Effects of ethanol, sorbitol and amino-oxyacetate on phenylephrine-induced changes in $^{14}\text{CO}_2$ output from [1- ^{14}C]octanoate and 3-hydroxybutyrate/acetoacetate ratios

Livers of fed rats were perfused as described in Fig. 2, except that perfusions were extended to include amino-oxyacetate treatment. Phenylephrine was infused to a final concentration of $2\ \mu\text{M}$ at 15–20, 40–45 and 65–70 min of perfusion. Ethanol and amino-oxyacetate were continuously infused to final concentrations of 2.5 and 0.2 mM from 30 and 55 min of perfusion respectively. In a parallel series of experiments, ethanol was replaced by sorbitol (final concn. 10 mM), while all other additions remained unchanged. $^{14}\text{CO}_2$ output and 3-hydroxybutyrate/acetoacetate ratios were determined immediately before phenylephrine infusion and 3 min later during infusion. Data shown are means $\pm$ S.E.M. for six independent experiments: * $P < 0.01$ compared with –phenylephrine.

Addition		$^{14}\text{CO}_2$ output (ng-atoms/min)		3-Hydroxybutyrate/acetoacetate ratio	
		Phenylephrine... –	+	–	+
Ethanol Amino-oxyacetate					
–	–	2.8 ± 0.2	$7.6 \pm 0.3^*$	0.23 ± 0.03	0.81 ± 0.09
+	–	1.2 ± 0.1	$2.1 \pm 0.1^*$	1.28 ± 0.11	$2.65 \pm 0.16^*$
+	+	1.7 ± 0.1	$3.9 \pm 0.1^*$	0.85 ± 0.12	$1.9 \pm 0.15^*$
Sorbitol Amino-oxyacetate					
–	–	3.2 ± 0.2	$8.1 \pm 0.4^*$	0.21 ± 0.04	$0.83 \pm 0.10^*$
+	–	2.4 ± 0.2	$3.6 \pm 0.1^*$	0.60 ± 0.05	$1.5 \pm 0.12^*$
+	+	3.4 ± 0.3	$6.9 \pm 0.3^*$	0.10 ± 0.01	$0.43 \pm 0.04^*$

ratio and depressed overall tricarboxylic acid-cycle flux, the subsequent infusion of phenylephrine enhanced the cycle flux and further elevated the 3-hydroxybutyrate/acetoacetate ratios (Fig. 2, Table 1). Qualitatively similar effects of phenylephrine, ethanol and sorbitol were also observed in experiments where $^{14}\text{CO}_2$ output from [1- ^{14}C]glutamate or 2- ^{14}C -labelled 4-methyl-2-oxopentanoate were determined (results not shown).

Phenylephrine-induced increases in octanoate oxidation were also accompanied by increased rates of both oxygen consumption (Fig. 2) and glycolysis (results not shown). When ethanol or sorbitol was present, phenylephrine-induced increases in oxygen consumption were slightly enhanced compared with the effect of phenylephrine alone. Such increased rates of respiration in the presence of ethanol (Fig. 2) or sorbitol (results not shown) most probably reflect a compensatory increase in oxidative phosphorylation after inhibition of the glycolytic response by high cytoplasmic NADH/NAD $^+$ ratios (Soboll *et al.*, 1981; W. M. Taylor, E. Van De Pol & F. L. Bygrave, unpublished work). It is also clear, however, that rates of octanoate oxidation do not correlate with rates of oxygen consumption in the presence of ethanol or sorbitol (Fig. 2). Under those conditions a significant proportion of the reducing equivalents required for mitochondrial respiration are probably supplied by ethanol or sorbitol oxidation rather than from tricarboxylic acid-cycle activity (Williamson *et al.*, 1969; Berry, 1980; Berry *et al.*, 1983).

During ethanol metabolism NADH is generated in both cytoplasmic and intramitochondrial compartments, whereas NADH generated from sorbitol oxidation arises solely in the cytoplasm (Parrilla *et al.*, 1974; Berry, 1980). Data presented in Table 1 are also consistent with these sites of reducing equivalent generation. Amino-oxyacetate, a potent inhibitor of reducing-equivalent

Fig. 2. Effect of phenylephrine and ethanol on octanoate oxidation, oxygen uptake and 3-hydroxybutyrate/acetoacetate ratios

Livers of fed rats were perfused as described in Fig. 1. Phenylephrine (—) and ethanol (----) were infused for the times indicated to give final concentrations of $2\ \mu\text{M}$ and 2.5 mM respectively. Perfusate oxygen (continuous trace), 3-hydroxybutyrate/acetoacetate ratios (O) and $^{14}\text{CO}_2$ output from [1- ^{14}C]octanoate (●) were measured as described in the Experimental section. Data shown are from one of six experiments that gave similar results.

transfer from the cytoplasmic to the mitochondrial compartment via the malate/aspartate shuttle, greatly decreases the effects of sorbitol on 3-hydroxybutyrate/acetoacetate ratios and octanoate oxidation, whereas amino-oxyacetate had much less effect on the corresponding actions of ethanol. Data in Table 1 also show that, whereas amino-oxyacetate addition lowered matrix 3-hydroxybutyrate/acetoacetate ratios under all conditions examined, large increases in the ratio were still evident after phenylephrine infusion. This raises the possibility that phenylephrine-induced increases in reducing-equivalent generation in the mitochondrial matrix (including tricarboxylic acid-cycle flux) may largely account for the elevated 3-hydroxybutyrate/acetoacetate ratio observed in the presence of the α -agonist. From the data presented, then, it appears most unlikely that phenylephrine-induced increases in cycle flux could result from a lowered NADH/NAD⁺ ratio.

Phenylephrine-induced increases in respiration in perfused rat liver have recently been linked to enhanced cellular energy requirements (W. M. Taylor, E. Van De Pol & F. L. Bygrave, unpublished work). It could therefore be argued that tricarboxylic acid-cycle flux may be stimulated as a result of a decreased ATP/ADP ratio in the presence of phenylephrine. However, α -adrenergic agonists have been reported to increase (Titheradge *et al.*, 1979; Titheradge & Haynes, 1980) or have no effect on (Siess *et al.*, 1978) intramitochondrial ATP/ADP ratios in isolated hepatocytes. In addition, in the present study, phenylephrine had no significant effect on total tissue ATP/ADP ratios in perfused livers from fed or starved rats (3.8 ± 0.2 versus 3.8 ± 0.2 , mean $\pm$ S.E.M., $n = 6$, for control and phenylephrine treatment in fed and 1.6 ± 0.1 versus 1.5 ± 0.2 , mean $\pm$ S.E.M., $n = 5$, for control and phenylephrine treatment in starved animals, respectively).

Presumably, then, phenylephrine stimulates tricarboxylic acid-cycle flux by altering other controlling factors. Currently, much interest is focussed on the possible stimulation of isocitrate dehydrogenase or 2-oxoglutarate dehydrogenase by increased matrix free Ca²⁺ concentration (Ochs, 1984; Kleineke & Söling, 1985; McCormack, 1985). A definitive conclusion

regarding such a mechanism must await more detailed information on mitochondrial-matrix free Ca²⁺ concentrations.

REFERENCES

- Berry, M. N. (1980) *FEBS Lett.* **117**, Suppl. K106–K120
 Berry, M. N., Clark D. G., Grivell, A. R. & Wallace, P. G. (1983) *Eur. J. Biochem.* **131**, 205–214
 Denton, R. M. & Halperin, M. L. (1968) *Biochem. J.* **110**, 27–38
 Denton, R. M., McCormack, J. G. & Edgell, N. J. (1980) *Biochem. J.* **190**, 107–117
 Haussinger, D. & Sies, H. (1984) *Biochem. J.* **221**, 651–658
 Kleineke, J. & Söling, H. D. (1985) *J. Biol. Chem.* **260**, 1040–1045
 Krebs H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66
 McCormack, J. G. (1985) *FEBS Lett.* **180**, 259–264
 Ochs, R. S. (1984) *J. Biol. Chem.* **259**, 13004–13010
 Parrilla, R., Ohkawa, K., Lindros, K. O., Zimmermann, U. P., Kobayashi, K. & Williamson, J. R. (1974) *J. Biol. Chem.* **249**, 4926–4933
 Reinhart, P. H., Taylor, W. M. & Bygrave, F. L. (1982) *J. Biol. Chem.* **257**, 1906–1912
 Reinhart, P. H., Taylor, W. M. & Bygrave, F. L. (1984) *Biochem. J.* **223**, 1–13
 Siess, E. A., Brock, D. G. & Wieland, O. H. (1978) *Biochem. Soc. Trans.* **6**, 1139–1144
 Soboll, S., Heldt, H. W. & Scholz, R. (1981) *Hoppe-Seyler's Z. Physiol. Chem.* **362**, 247–260
 Staddon, J. M. & McGivan, J. D. (1984) *Biochem. J.* **217**, 477–483
 Sugden, M. C. & Watts, D. I. (1983) *Biochem. J.* **212**, 85–91
 Taylor, W. M., Reinhart, P. H. & Bygrave, F. L. (1983a) *Pharmacol. Ther.* **21**, 125–141
 Taylor, W. M., Reinhart, P. H. & Bygrave, F. L. (1983b) *Biochem. J.* **212**, 555–565
 Titheradge, M. A. & Haynes, R. C. (1980) *Arch. Biochem. Biophys.* **201**, 44–55
 Titheradge, M. A., Stringer, J. L. & Haynes, R. C. (1979) *Eur. J. Biochem.* **102**, 117–124
 Tomera, J. F., Kopito, R. R. & Brunengraber, H. (1983) *Biochem. J.* **210**, 265–268
 Williamson, J. R. & Cooper, R. H. (1980) *FEBS Lett.* **117**, Suppl. K73–K84
 Williamson, J. R., Scholz, R., Browning, E. T., Thurman, R. G. & Fukami, M. H. (1969) *J. Biol. Chem.* **244**, 5044–5054