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Stimulation by α-adrenergic agonists of Ca^{2+} fluxes, mitochondrial oxidation and gluconeogenesis in perfused rat liver

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Glucose output from perfused livers of 48h-starved rats was stimulated by phenylephrine (2μM) when lactate, pyruvate, alanine, glycerol, sorbitol, dihydroxyacetone or fructose were used as gluconeogenic precursors. Phenylephrine-induced increases in glucose output were immediately preceded by a transient efflux of Ca^{2+} and a sustained increase in oxygen uptake. Phenylephrine decreased the perfusate [lactate]/[pyruvate] ratio when sorbitol or glycerol was present, but increased the ratio when alanine, dihydroxyacetone or fructose was present. Phenylephrine induced a rapid increase in the perfusate [β-hydroxybutyrate]/[acetoacetate] ratio and increased total ketone-body output by 40–50% with all substrates. The oxidation of [1-^{14}C]octanoate or 2-oxo[1-^{14}C]glutarate to $^{14}CO_2$ was increased by up to 200% by phenylephrine. All responses to phenylephrine infusion were diminished after depletion of the hepatic α-agonist-sensitive pool of Ca^{2+} and returned toward maximal responses after Ca^{2+} re-addition. Phenylephrine-induced increases in glucose output from lactate, sorbitol and glycerol were inhibited by the transaminase inhibitor amino-oxyacetate by 95%, 75% and 66% respectively. Data presented suggest that the mobilization of an intracellular pool of Ca^{2+} is involved in the activation of gluconeogenesis by α-adrenergic agonists in perfused rat liver. α-Adrenergic activation of gluconeogenesis is apparently accompanied by increases in fatty acid oxidation and tricarboxylic acid-cycle flux. An enhanced transfer of reducing equivalents from the cytoplasmic to the mitochondrial compartment may also be involved in the stimulation of glucose output from the relatively reduced substrates glycerol and sorbitol and may arise principally from an increased flux through the malate–aspartate shuttle.

Ca^{2+} ions appear to be involved in the mechanism of action of α-adrenergic agonists in liver in both the fed and starved states (for reviews see: Exton, 1981; Williamson *et al.*, 1981; Taylor *et al.*, 1983). Although it is generally agreed that α-adrenergic agonists enhance glycogenolysis through a Ca^{2+}-dependent activation of phosphorylase *b* kinase, such clearly defined regulatory site(s) have not been elucidated for the stimulation of the gluconeogenic pathway by these agonists. Proposed sites of action of α-adrenergic agonists include pyruvate carboxylase (Garrison & Borland, 1979; Williamson *et al.*, 1981), phosphoenolpyruvate carboxykinase (Merryfield & Lardy, 1982) and pyruvate kinase (Garrison *et al.*, 1979, 1981). It has also been suggested that these agonists may stimulate gluconeogenesis from reduced substrates such as glycerol and sorbitol in hepatocytes, by promoting the exchange of reducing equivalents from the cytoplasmic to the mitochondrial compartment (Kneer *et al.*, 1979; Yip & Lardy, 1981). A Ca^{2+}-dependent activation of the mitochondrial glycerol phosphate dehydrogenase was purported to play a major role in this exchange mechanism (Yip & Lardy, 1981). However, α-adrenergic agonists have also been reported to stimulate gluconeogenesis by a mechanism apparently independent of Ca^{2+} (Kneer *et al.*, 1979).

Previously we described a method for depleting the α-adrenergic-sensitive Ca^{2+} pool in the perfused rat liver (Reinhart *et al.*, 1982*a*). In the present study this technique is employed to examine further the role of Ca^{2+} in the stimulation of gluconeogenesis by α-adrenergic agonists in the perfused liver of starved

rats. This study provides insights into the general role of Ca^{2+} and examines the possible role of mitochondrial respiration and transfer of reducing equivalents across the mitochondrial inner membrane.

Experimental

Animals and perfusions

Wistar-strain albino rats (200–280g body wt.) were starved for 48h before use. Rats were anaesthetized with sodium pentobarbitone (50mg/kg), and the livers perfused with Krebs–Henseleit (1932) bicarbonate medium equilibrated wtih O_2/CO_2 (19:1) and modified to contain 1.3mM added $CaCl_2$ unless otherwise indicated. All other perfusion details were as described previously (Reinhart *et al.*, 1982*a*).

Perfusate Ca^{2+} and oxygen determinations

Perfusate Ca^{2+} and oxygen concentrations were continuously monitored wtih a Radiometer F2112 Ca^{2+}-selective electrode and Clark-type oxygen elctrode, respectively, as detailed by Reinhart *et al.* (1982*a*). Lag times for Ca^{2+} and oxygen responses were determined as described previously (Reinhart *et al.*, 1982*b*).

Other analytical procedures

Effluent perfusate was assayed for glucose by the glucose oxidase/peroxidase method, and lag times for glucose output response were measured with [^{3}H]inulin, both as described by Reinhart *et al.* (1982*b*). In some experiments, larger volumes of perfusate (7ml) were collected in graduated centrifuge tubes, centrifuged to remove contaminating erythrocytes, and the supernatants (6ml) mixed with 0.2ml of 2M-$HClO_4$ containing 5mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]. Samples were left on ice for 10min and then neutralized with 0.2ml of 2M-KOH, and the resulting $KClO_4$ was removed by centrifugation at 2000*g* for 5min. Portions were taken for assay of pyruvate, lactate, acetoacetate and β-hydroxybutyrate, essentially as described by Bergmeyer (1974). Where relatively large volumes of perfusate were required for these multiple assays, the values obtained necessarily represented the average attained over the time of sampling (7ml corresponding to 10–15s sampling time), and corrections for response lag times were not made.

Production of $^{14}CO_2$ from labelled substrates was estimated from perfusate samples (1ml) taken by syringe from either the inflow or outflow cannulae close to the liver. Perfusate samples were injected into sealed vials containing 2ml of 100mM-sodium phosphate/citric acid buffer (pH3.0), and fitted with centre wells containing filter paper impregnated with 0.1ml of Hyamine hydroxide. After 15min, the filter papers were transferred to scintillation vials containing 10ml of scintillant {0.6% (w/v) butyl-PBD [5-(biphenyl-4-yl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] in 2-methoxyethanol/toluene (2:3, v/v)} and their radioactivity was determined in a Beckman LS 300 scintillation counter. Samples of perfusion medium taken before passage through the liver were used for background correction and specific-radioactivity determination.

Chemicals and materials

Phenylephrine, noradrenaline hydrochloride, adrenaline bitartrate, [Arg^8]vasopressin, amino-oxyacetate and the glucose assay kit (510-A) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Ca^{2+}-electrode membranes (F2002) and filling solution (S43316) were obtained from Radiometer, Copenhagen, Denmark. Lactate dehydrogenase and β-hydroxybutyrate dehydrogenase were from Boehringer, Mannheim, West Germany. Hyamine hydroxide, [1-^{14}C]octanoate and 2-oxo[1-^{14}C]-glutarate were supplied by New England Nuclear, Boston, MA, U.S.A. All other chemicals were of analytical grade.

Results

Although the glycogen contents in livers of starved rats are reported to be low (Exton & Park, 1967; Hems & Whitton, 1973) and therefore unlikely to contribute significantly to glucose output by the liver, it was important to establish this point at the outset of the present experiments. Use was made of the finding that the transaminase inhibitor amino-oxyacetate, which blocks the malate–aspartate shuttle (Borst, 1963), inhibits gluconeogenesis from lactate (Arinze *et al.*, 1973; Rognstad, 1980).

Data in Fig. 1 show that the presence of 0.2mM-amino-oxyacetate essentially abolished the increase in glucose output induced by lactate infusion in livers of 48h-starved rats. In addition, glucose output in the absence of added lactate was very low (cf. that observed in the perfused liver of fed rats; Reinhart *et al.*, 1982*a*,*b*), and was also decreased by approx. 40% by amino-oxyacetate. Moreover, phenylephrine-induced increases in glucose output in either the presence or the absence of lactate were largely prevented by the transaminase inhibitor. The maximal rate of glucose output induced by phenylephrine in the absence of lactate was only 5–10% of that observed in the fed rat (see Reinhart *et al.*, 1982*b*), and substantial increases in glucose output were only induced by phenylephrine in the presence of lactate. In other control experiments amino-oxyacetate was shown to have little effect on basal oxygen consumption by the liver and to inhibit only slightly phenylephrine-induced in-

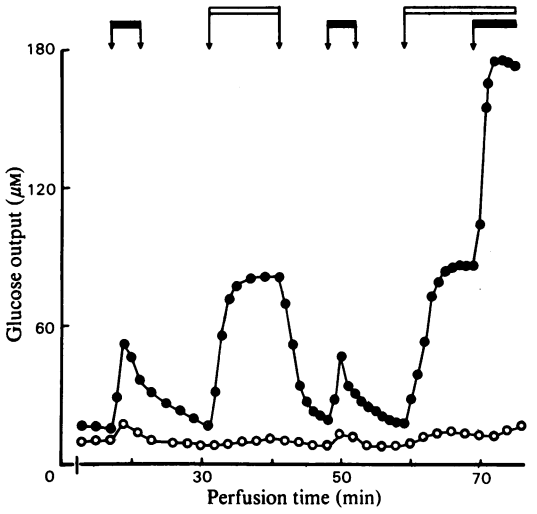

Fig. 1. *Effects of lactate, amino-oxyacetate and phenylephrine on glucose output from perfused liver*
Livers of 48h-starved rats were perfused with Krebs–Henseleit buffer as described in the Experimental section in either the presence (○) or the absence (●) of 0.2mM-amino-oxyacetate. Phenylephrine (▬) and L(+)-lactate (▭) were infused for the times indicated to give final concentrations of 2μM and 2.5mM respectively. Effluent samples were collected, contaminating erythrocytes removed by centrifugation, and samples of supernatant assayed for glucose. Data shown are from a typical experiment of three experiments performed in the presence of amino-oxyacetate and four experiments in its absence.

creases in oxygen uptake and Ca^{2+} efflux ($138 \pm 21\mu M$ versus $106 \pm 18\mu M$, and $20.6 \pm 2.1\mu M$ versus $19.8 \pm 2.0\mu M$ respectively; ±S.E.M., $n = 3$). Finally, we showed that glycogenolysis is apparently insensitive to amino-oxyacetate, since phenylephrine-induced glucose output from perfused livers of fed rats was only slightly inhibited by the transaminase inhibitor ($628 \pm 54\mu M$ versus $562 \pm 41\mu M$, $n = 4$).

We would thus conclude that the substrate- and hormone-induced increase in glucose output observed in Fig. 1 and in the data reported below for the livers of 48h-starved rats reflects that arising from gluconeogenesis.

Effect of phenylephrine on Ca^{2+} efflux, oxygen uptake and glucose output

Livers perfused with 2.5mM-lactate were treated with a maximally effective concentration of phenylephrine (2μM) and showed rapid changes in Ca^{2+} efflux, oxygen uptake and glucose output, as detailed in Fig. 2. Phenylephrine-induced increases in Ca^{2+} efflux, oxygen uptake and glucose output were

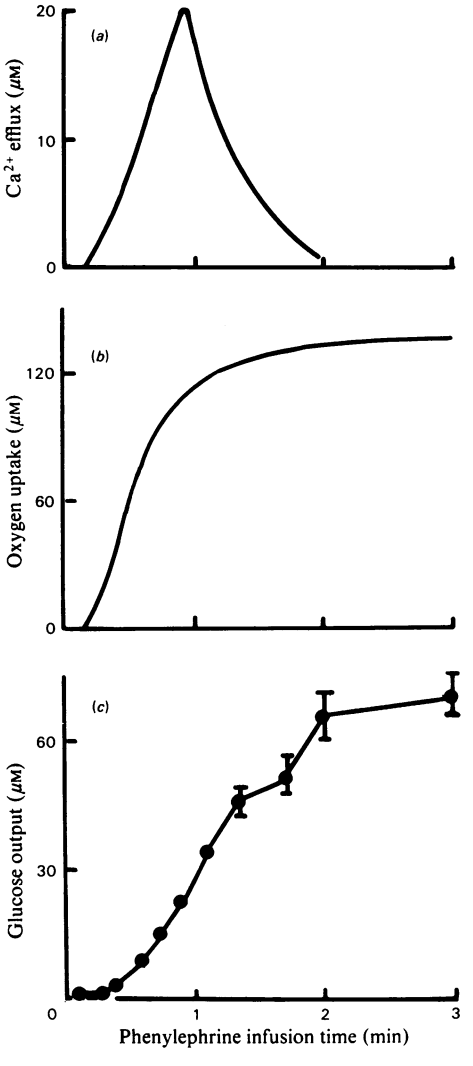

Fig. 2. *Time course for the effect of phenylephrine on Ca^{2+} efflux, oxygen uptake and glucose output*
Livers of 48h-starved rats were perfused as described in Fig. 1 in the presence of 2.5mM-lactate for 15min, and then phenylephrine (2μM final concn.) was infused. Perfusate Ca^{2+} (*a*), oxygen uptake (*b*) and glucose output (*c*) were monitored as described in the Experimental section. Increases in these during the initial 3min of phenylephrine infusion are shown. Lag times for Ca^{2+}, oxygen and glucose responses were determined separately for each experiment and data shown were corrected for these. Just before phenylephrine infusion, basal oxygen uptake was $382 \pm 38\mu M$ ($n = 8$), basal glucose output was $84 \pm 9\mu M$ ($n = 5$) and perfusate Ca^{2+} was 1.3mM. Data shown for Ca^{2+} efflux and oxygen uptake are continuous traces from one of six experiments that gave similar results. Variation between experiments was small, with S.E.M. values less than 10% of mean. Glucose-output data are the means ± S.E.M. for five separate experiments.

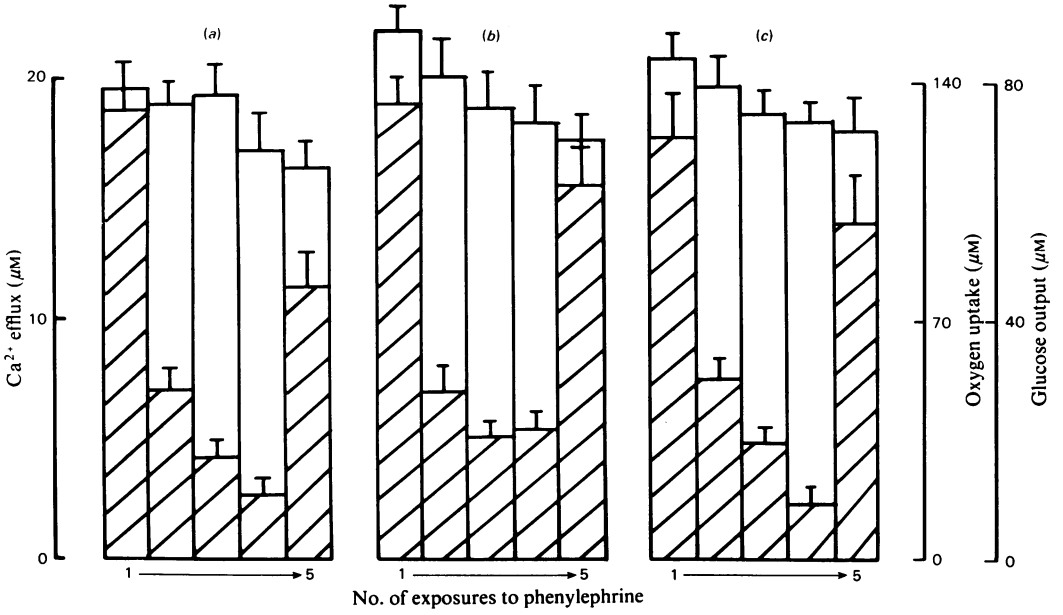

Fig. 3. *Dependence on Ca^{2+} of phenylephrine-induced increases in Ca^{2+} efflux, oxygen uptake and glucose output*
Livers were initially perfused for 15 min as described in Fig. 2. Phenylephrine (2 μM) was then infused in five pulses each of 4 min duration and at 4 min intervals except the last, which was given 8 min after the fourth pulse. In some experiments (hatched columns) the 1.3 mM-$CaCl_2$ present in the perfusion medium (administered by infusion syringe) was removed 4 min before the commencement of the phenylephrine infusions. In those experiments, 1.3 mM-$CaCl_2$ was re-administered for 2 min, 6 min before the fifth phenylephrine infusion. All other experiments were performed with 1.3 mM-$CaCl_2$ present throughout the entire perfusion (open columns). Perfusate Ca^{2+} (*a*), oxygen uptake (*b*) and glucose output (*c*) were determined as described in the Experimental section and the maximal increase induced by each phenylephrine infusion is shown. Basal values for oxygen uptake and glucose output were 345 ± 54 μM and 87 ± 11 μM respectively at a perfusate Ca^{2+} concentration of 1.3 mM, and 341 ± 50 μM and 84 ± 12 μM respectively in experiments where the $CaCl_2$ infusion was terminated and perfusate Ca^{2+} decreased to approx. 6 μM just before infusion of phenylephrine. These basal values remained essentially unchanged throughout the experimental period. Data shown are the means ± S.E.M. for five separate experiments.

evident after 9.5 ± 0.8, 10.5 ± 1.1 and approx. 20 s respectively. The increase in Ca^{2+} efflux was transient, with a maximal increase of 21.7 ± 2.3 μM being attained after 75 s of phenylephrine infusion. Ca^{2+} efflux declined thereafter and reached basal values again by 2.5 min (Fig. 2). In contrast with the transient effect on Ca^{2+} efflux, phenylephrine-induced increases in oxygen uptake and glucose output were maintained near maximal values until the α-agonist infusion was discontinued (results not shown). Near-maximal increases in oxygen uptake and glucose output were achieved after 1.5 and 2 min of phenylephrine infusion respectively (Fig. 2). The responses in terms of Ca^{2+} efflux and oxygen consumption to phenylephrine seen here are virtually identical with those seen with perfused livers from fed rats (Reinhart *et al.*, 1982*a*,*b*).

The next set of experiments involved utilizing conditions that lead to the depletion of the α-adrenergic-agonist-sensitive pool of Ca^{2+} in the perfused liver of the fed rat and were designed to test the hypothesis that intracellular Ca^{2+} is essential for α-adrenergic-agonist-induced increases in gluconeogenesis. The procedure involves perfusing the liver without added Ca^{2+} in the medium and to infuse phenylephrine by repeated pulses (see Fig. 5 of Reinhart *et al.*, 1982*a*). As shown in that paper, four such pulses, each of 90 s duration and given at 5 min intervals, were sufficient essentially to deplete the liver of the α-adrenergic-sensitive intracellular Ca^{2+} pool.

Data in Fig. 3(*a*) show that when the livers of 48 h-starved rats, perfused with buffer containing no added Ca^{2+}, were given four repeated pulses of phenylephrine, the maximum rate of Ca^{2+} efflux was decreased from 20 μM to approx. 2 μM, reflecting a loss of phenylephrine-sensitive intracellular Ca^{2+}. The response to the fifth pulse, given after a 2 min re-infusion of 1.3 mM-$CaCl_2$ to the liver, was approx. 70% of that which followed the first pulse. A parallel diminution in oxygen uptake and gluconeogenic responses was observed after the loss of intra-

Table 1. *Dependence on Ca^{2+} of phenylephrine-induced increases in glucose output*

Livers were initially perfused for 15 min as described in Fig. 2, except that the gluconeogenic substrates (each at 2.5 mM final concn.) were present as shown. Phenylephrine was administered for 4 min, and after a further 4 min the 1.3 mM-$CaCl_2$ in the perfusion medium (administered by infusion syringe) was removed. The liver was then infused with five successive pulses of phenylephrine (2 μM) each of 4 min duration as described in Fig. 3. A 2 min pulse of 1.3 mM-$CaCl_2$ was administered 6 min before the final phenylephrine infusion. Samples of perfusate were taken for glucose assay, and maximal increases in glucose output induced by phenylephrine were determined before $CaCl_2$ removal (+1.3 mM-Ca^{2+}) during the fourth infusion of the α-agonist in buffer containing no added Ca^{2+} (Ca^{2+}-depleted) and during the last infusion of phenylephrine, 4 min after a 2 min re-administration of 1.3 mM-Ca^{2+} (Ca^{2+} re-added). For each substrate the rate of basal glucose output was determined after the initial 15 min perfusion and was essentially unchanged throughout the period of perfusion. Data shown are means ± S.E.M. for at least four separate experiments.

	Glucose output (μM)			
		Phenylephrine-induced increase in output		
Substrate	Basal output	+1.3 mM-Ca^{2+}	Ca^{2+}-depleted	+1.3 mM-Ca^{2+} re-added
Alanine	33 ± 5	68 ± 4.8	10 ± 2.7	57 ± 6
Pyruvate	41 ± 6	33 ± 5.7	15 ± 3.0	28 ± 4
Lactate	83 ± 7	93 ± 7.7	12 ± 2.3	71 ± 9
Glycerol	71 ± 6	81 ± 7.4	22 ± 6.8	53 ± 8
Dihydroxyacetone	152 ± 24	67 ± 9.0	23 ± 8.0	59 ± 11
Sorbitol	78 ± 11	114 ± 15	13 ± 3.9	92 ± 13
Fructose	177 ± 34	57 ± 11	17 ± 5.0	38 ± 8
None	14 ± 4	16 ± 2.1	3 ± 0.8	7 ± 2

cellular Ca^{2+} (Figs. 3*b* and 3*c*); these responses also were largely restored after a short re-infusion of 1.3 mM-$CaCl_2$. Fig. 3 also shows that in control experiments, where 1.3 mM-$CaCl_2$ was continuously present, responses to repeated pulses of phenylephrine were only slightly diminished.

Stimulation of glucose output by phenylephrine was also observed with a range of gluconeogenic substrates, as shown in Table 1. Increases in glucose output ranged from 28% and 31% with fructose and dihydroxyacetone as substrate to 160% and 180% with sorbitol and alanine as substrate. Basal glucose output was substantially higher with fructose or dihydroxyacetone than with the other substrates, and consequently the absolute increases in glucose output induced by phenylephrine with fructose or dihydroxyacetone approached those observed with the other substrates. With all substrates tested, increases in glucose output induced by phenylephrine were substantially decreased when livers were perfused under conditions which deplete the α-agonist-sensitive Ca^{2+} pool (Table 1). In these Ca^{2+}-depletion experiments, increases in glucose output in response to phenylephrine approached maximal values again when 1.3 mM-$CaCl_2$ was re-infused for 2 min before phenylephrine treatment. Data similar to those shown for phenylephrine in Table 1 were also obtained with 50 nM-adrenaline, 50 nM-noradrenaline and 2.5 munits of vasopressin/ml (results not shown). It should be noted that, although glucose output was still enhanced by adrenaline or noradrenaline at higher concentrations of hormone, oxygen consumption was inhibited at catecholamine concentrations greater than 0.5 μM.

Thus, and by analogy with information gained earlier from experiments with fed rats (Reinhart *et al.*, 1982*a*), the present experiments provide strong evidence that intracellular Ca^{2+} is required for phenylephrine-induced stimulation of gluconeogenesis.

Effect of phenylephrine on perfusate [lactate]/[pyruvate] and [β-hydroxybutyrate]/[acetoacetate] ratios

Since it has been suggested that α-adrenergic agonists may increase gluconeogenesis from relatively reduced substrates by enhancing the transfer of reducing equivalents from the cytoplasmic to the mitochondrial compartment (Kneer *et al.*, 1979; Yip & Lardy, 1981), the effects of phenylephrine on perfusate [lactate]/[pyruvate] and [β-hydroxybutyrate]/[acetoacetate] ratios, indicators of the cytoplasmic and mitochondrial [NADH]/[NAD^+] ratios respectively, were examined with a range of gluconeogenic substrates.

Changes in [lactate]/[pyruvate] ratios were evident 30–40 s after phenylephrine infusion, to near-maximal values by 90 s (Fig. 4). The [lactate]/[pyruvate] ratio was decreased by phenylephrine when the relatively reduced substrates glycerol or sorbitol were employed and the initial ratios were relatively high (Fig. 4). With all other substrates tested, phenylephrine induced an increase in the

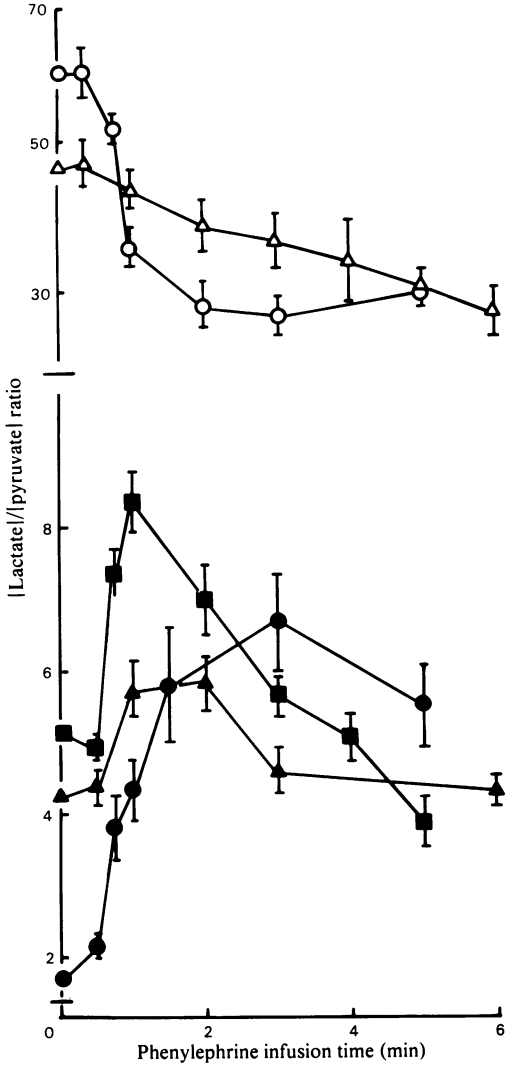

Fig. 4. *Effect of phenylephrine on the [lactate]/[pyruvate] ratio*

Livers were initially perfused for 15 min as described in Fig. 2, except that lactate was replaced by the gluconeogenic substrates (○) glycerol, (△) sorbitol, (■) dihydroxyacetone, (▲) fructose or (●) alanine, each at a final concentration of 2.5 mM. Phenylephrine (2 μM) was then infused for 6 min. Perfusate lactate and pyruvate were assayed as described in the Experimental section. Data shown are the means ± S.E.M. for five separate experiments.

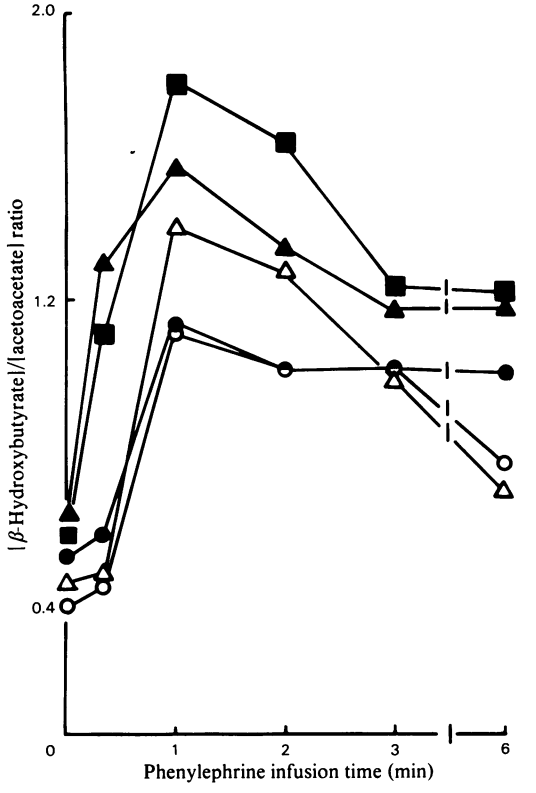

Fig. 5. *Effect of phenylephrine on the [β-hydroxybutyrate]/[acetoacetate] ratios*

Samples of perfusate obtained as in Fig. 4 were assayed for β-hydroxybutyrate and acetoacetate as described in the Experimental section. Substrates were (○) glycerol, (△) sorbitol, (■) dihydroxyacetone, (▲) fructose and (●) alanine, each at a final concentration of 2.5 mM. Data shown are the means of five separate experiments. For simplicity, S.E.M. values are not shown; however, they never exceeded 0.2 and were generally less than 0.1.

[lactate]/[pyruvate] ratio. Where fructose or dihydroxyacetone was employed, the increase in the [lactate]/[pyruvate] ratio was transient and had returned close to initial values by 6 min of phenylephrine administration.

[β-Hydroxybutyrate]/[acetoacetate] ratios were increased by phenylephrine irrespective of the substrate employed (Fig. 5). The ratio increased within 20–30 s of phenylephrine infusion, was near maximal by 90 s and decreased slightly thereafter, but was still significantly above initial values at the end of the phenylephrine infusion (6 min). Effects similar to those described for phenylephrine in Figs. 4 and 5 were also obtained after perfusion with adrenaline, noradrenaline or vasopressin (results not shown). However, effects of phenylephrine on ([lactate]/[pyruvate] and [β-hydroxybutyrate]/[acetoacetate] ratios were not observed after livers were perfused under conditions that deplete the α-agonist-sensitive Ca^{2+} pool as described by Reinhart *et al.* (1982*a*) and used in Fig. 3 and Table 1 (results not shown).

Effect of agents that alter intracellular redox ratios on glucose output

The transaminase inhibitor, amino-oxyacetate, blocks the mitochondrial malate–aspartate shuttle and thereby diminishes the transfer of reducing equivalents between the cytoplasmic and mitochondrial compartments. Data presented in Fig. 6 and Table 2 indicate that amino-oxyacetate (0.2mM) inhibits basal glucose output from glycerol and sorbitol by 34% and 55% respectively. Moreover, phenylephrine-induced increases in glucose output from glycerol or sorbitol were inhibited by this agent by 66% and 75% respectively. Amino-oxyacetate, however, had little effect on either basal or phenylephrine-induced glucose output from fructose or dihydroxyacetone.

Phenazine methosulphate, an agent that rapidly re-oxidizes reduced nicotinamide nucleotides, and dinitrophenol, an uncoupler of oxidative phosphorylation, both increased glucose output from glycerol and sorbitol. With glycerol as substrate, phenazine methosulphate (30μM) and dinitrophenol (25μM) increased glucose output from 73 ± 8 to 116 ± 13 and 134 ± 18μM ($n = 4$) respectively. With sorbitol, however, dinitrophenol was much less effective, and variable responses were obtained (average increase of 15%), whereas phenazine methosulphate was still an effective activator (112 ± 10 versus 198 ± 16μM, $n = 4$). Phenazine methosulphate and dinitrophenol both inhibited glucose output from fructose or dihydroxyacetone and increased total pyruvate and lactate outputs (results not shown).

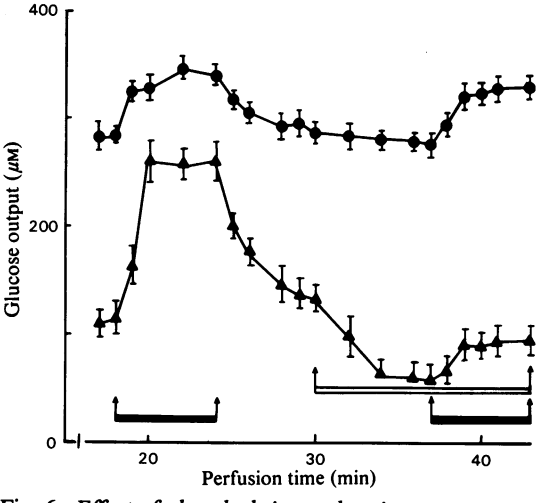

Fig. 6. *Effect of phenylephrine and amino-oxyacetate on glucose output*

Livers were initially perfused for 15min as described in Fig. 2, except that 2.5mM-fructose (●) or 2.5mM-sorbitol (▲) was used as gluconeogenic substrate. Phenylephrine (2μM, ▬) or amino-oxyacetate (0.2mM, ▭) was then administered as indicated and perfusate samples were taken for glucose determination. Data shown are the means ± S.E.M. for four separate experiments.

Effect of phenylephrine on ketogenesis and oxidation of [1-^{14}C]octanoate and 2-oxo[1-^{14}C]glutarate

The rate of oxidation of tracer amounts of [1-^{14}C]octanoate to $^{14}CO_2$ has been used as a measure of tricarboxylic acid-cycle flux in the perfused rat liver (Soboll *et al.*, 1981). In the present study, tricarboxylic acid-cycle flux was also monitored by following $^{14}CO_2$ generation from 2-oxo-[1-^{14}C]glutarate and gave results similar to those obtained with [1-^{14}C]octanoate. Relatively low rates of $^{14}CO_2$ production were observed in the absence of gluconeogenic substrates when rates of ketone-body output were relatively high (see Table 3). For example, rates of octanoate oxidation were increased from a basal value of 0.38 ± 0.09ng-atom/min ($n = 3$) to 0.80 ± 0.11 or 1.5 ± 0.20ng-atom/min ($n = 4$) on addition of sorbitol or fructose respectively. Rates of ketogenesis were correspondingly decreased on addition of sorbitol or fructose (Table 3).

Table 2. *Effect of phenylephrine and amino-oxyacetate on glucose output*

Livers were perfused as described in Fig. 6 with the gluconeogenic substrates shown, each at a final concentration of 2.5mM. Perfusate samples were taken just before and during infusion of phenylephrine or amino-oxyacetate (final concns. 2μM and 0.2mM respectively). Maximal changes in glucose output induced by these agents were determined as described in the Experimental section. Data shown are means ± S.E.M. for four separate experiments.

	Glucose output (μM)			
Substrate	Additions ... None	Phenylephrine	Amino-oxyacetate	Amino-oxyacetate + phenylephrine
Sorbitol	107 ± 10	254 ± 16*	59 ± 10*	97 ± 11
Glycerol	70 ± 9	146 ± 19*	46 ± 7*	71 ± 13
Fructose	281 ± 11	338 ± 14*	273 ± 9	324 ± 13
Dihydroxyacetone	196 ± 14	251 ± 18*	185 ± 16	253 ± 20

* $P < 0.01$ compared with no additions.

The effect of phenylephrine on [1-^{14}C]octanoate oxidation is shown in Fig. 7(*a*). A rapid increase in octanoate oxidation was observed within 10–15 s of phenylephrine infusion when either fructose or sorbitol was present. Maximal increases were observed between 30 and 60 s of α-agonist infusion, and rates of oxidation declined thereafter. Some variability in this latter portion of the phenylephrine response was observed. In some experiments rates of oxidation were maintained at approx. 70% of the maximal value, whereas in others the rate fell to approx. 10–20% above the basal value at the completion of the phenylephrine infusion. Essentially similar data were obtained when [1-^{14}C]-octanoate was replaced with 2-oxo[1-^{14}C]glutarate (Fig. 7*b*). Effects of phenylephrine on oxidation of [1-^{14}C]octanoate or 2-oxo[1-^{14}C]glutarate were inhibited by over 80% after livers were perfused under conditions which deplete the α-agonist-sensitive Ca^{2+} pool (results not shown).

Table 3. *Effect of phenylephrine on total ketone-body output*

Perfusate samples were taken immediately before and 2 min after infusion of phenylephrine (2 μM) as described in Fig. 4. Ketone bodies were determined as described in the Experimental section. Data shown are the means ± S.E.M. for five separate experiments.

Substrate	Additions	Total ketone-body output (μM) None	Phenylephrine
None		103 ± 5.8	111 ± 6.1
Alanine		29 ± 1.4	48 ± 6.4*
Glycerol		38 ± 1.0	55 ± 1.4*
Dihydroxyacetone		39 ± 4.3	55 ± 3.8*
Fructose		34 ± 2.5	51 ± 2.2*
Sorbitol		36 ± 1.4	51 ± 1.5*

* $P < 0.01$ compared with respective control.

Ketone-body output was measured during gluconeogenesis from a range of substrates shown in Table 3. Phenylephrine induced a rapid increase in ketogenesis, with maximal increases of 40–50% being observed within 2 min of α-agonist infusion. In

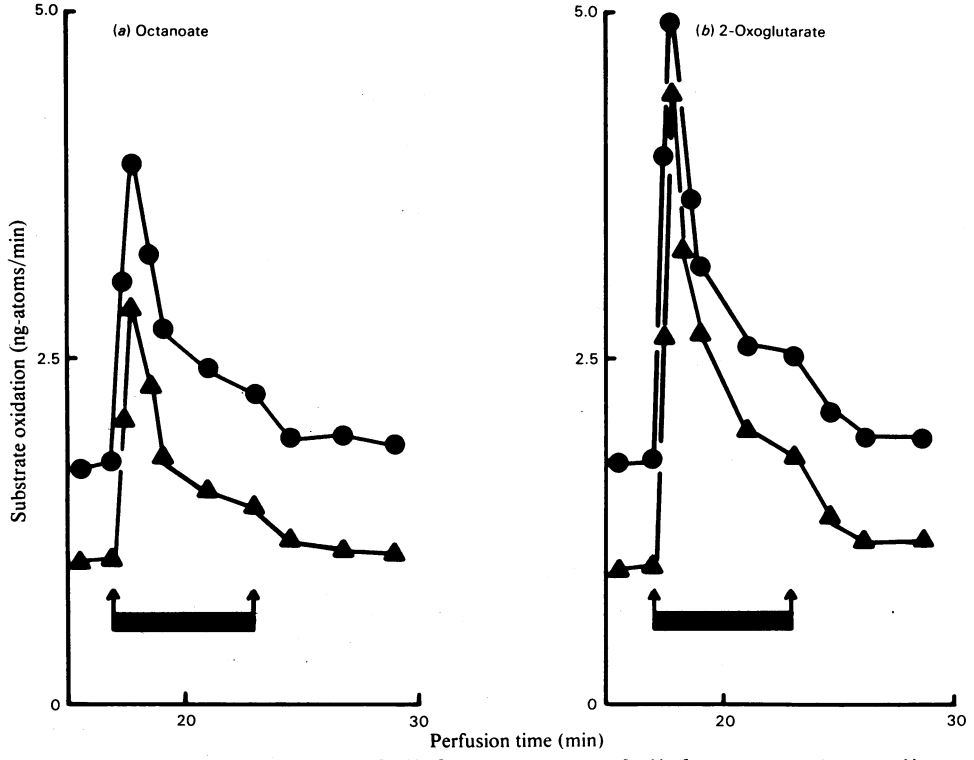

Fig. 7. *Effect of phenylephrine on [1-^{14}C]octanoate or 2-oxo[1-^{14}C]glutarate oxidation to $^{14}CO_2$*

Livers were perfused as described in Fig. 6, except that 2-oxo[1-^{14}C]glutarate (53.6 Ci/mol) or [1-^{14}C]octanoate (25.1 Ci/mol) was continuously infused to give final concentrations of 0.20 and 0.55 μM respectively. The substrates fructose (●) or sorbitol (▲) were used at final concentrations of 2.5 mM and phenylephrine (▬) was infused at a final concentration of 2 μM as indicated. Samples of perfusate were assayed for $^{14}CO_2$ as described in the Experimental section. Data shown are from one experiment of four, each of which gave similar results.

the absence of gluconeogenic substrates, ketone-body output was elevated and phenylephrine-induced increases were not significant under those conditions. Phenylephrine did not affect ketone-body output after livers had been perfused under conditions that deplete the α-agonist-sensitive Ca^{2+} pool (results not shown).

Discussion

Among the major points revealed in this study of α-agonist-induced gluconeogenesis in the perfused liver of starved rats are (*a*) the obligatory role of Ca^{2+} in the gluconeogenic response, (*b*) the variety of metabolic events seemingly associated with the gluconeogenic response and which are also Ca^{2+}-dependent, and (*c*) the rapidity with which the responses are seen. Of particular interest in this regard was the observation that ketogenesis and tricarboxylate-cycle activity are each increased by α-agonists concomitant with enhanced rates of gluconeogenesis.

An obligatory role for Ca^{2+} in α-agonist-induced gluconeogenesis could be deduced from experiments in which the α-agonist-sensitive intracellular pool of Ca^{2+} was manipulated by the administration of phenylephrine through repeated pulses to the liver perfused with bicarbonate buffer containing no added Ca^{2+} (Fig. 3, Table 1). In this way it could be shown that the smaller the α-agonist-induced Ca^{2+} efflux (and presumably the greater the depletion of the α-agonist-sensitive Ca^{2+} pool), the smaller the gluconeogenic response. It was noteworthy that the Ca^{2+}-dependence of the α-agonist-induced increase in glucose output was observed with all gluconeogenic substrates tested (Table 1 and Fig. 3).

An additional feature of our experimental approach (Reinhart *et al.*, 1982*a*), hitherto apparently not undertaken in studies on gluconeogenesis, was the ability to measure rapid and continuous changes in Ca^{2+} fluxes across the plasma membrane and in whole-tissue respiration; such respiration is attributable in large part to mitochondria (Reinhart *et al.*, 1982*b*). In this way we were able to show that such changes precede phenylephrine-induced increases in glucose output. Indeed the time-course and magnitude of Ca^{2+} efflux induced by phenylephrine in these perfusions with livers from starved rats were virtually identical with the phenylephrine-induced Ca^{2+} efflux previously shown to precede glycogenolysis in perfused livers of fed rats (Reinhart *et al.*, 1982*a*). The important conclusion can thus be drawn that such changes in Ca^{2+} efflux are independent of the nutritional status of the experimental animal. The findings also suggest that altered Ca^{2+} fluxes may be linked to the initial events in the mechanism of action of α-adrenergic agonists in rat liver.

The important role of Ca^{2+} in the responses to α-adrenergic agonists in rat liver was further highlighted by the findings that the changes induced by phenylephrine in the redox state of the cytoplasm (Fig. 4) and mitochondria (Fig. 5), as well as in ketogenesis (Table 3) and the oxidation of [1-^{14}C]octanoate or 2-oxo[1-^{14}C]glutarate (Fig. 7), were all considerably diminished after depletion of the α-agonist-sensitive Ca^{2+} pool. As with gluconeogenesis (Fig. 3, Table 1), re-infusion of livers with 1.3 mM-$CaCl_2$ restored all of these responses.

A requirement for Ca^{2+} in the stimulation of gluconeogenesis by α-adrenergic agonists has been reported previously and a number of possible sites for Ca^{2+} action have been suggested. Tolbert & Fain (1974) reported a Ca^{2+}-dependent catecholamine-induced increase in glucose output from a range of gluconeogenic substrates with rat hepatocytes. However, in those studies, basal rates of gluconeogenesis were markedly decreased by the omission of Ca^{2+}. This is in contrast with the present study and earlier studies with perfused liver (Friedmann & Rasmussen, 1970; Sugano *et al.*, 1980) or hepatocytes (Kneer *et al.*, 1979), where Ca^{2+} omission did not cause appreciable changes in the basal rates of gluconeogenesis. More recently, Garrison *et al.* (1979, 1981) have suggested that a cytoplasmic Ca^{2+}-dependent protein kinase may catalyse the phosphorylation and subsequent inhibition of pyruvate kinase in response to catecholamines. In addition, an activation of phosphoenolpyruvate carboxykinase, indirectly mediated by Ca^{2+}, and involving the release of mitochondrial Fe^{2+}, has been proposed by Merryfield & Lardy (1982). It has also been argued that Ca^{2+} may regulate pyruvate carboxylase activity (Williamson *et al.*, 1981).

A Ca^{2+}-sensitive exchange of reducing equivalents between cytoplasmic and mitochondrial compartments involving an increased mitochondrial glycerol phosphate dehydrogenase activity has been proposed to account for the Ca^{2+}-dependent increase in gluconeogenesis from reduced substrates such as glycerol and sorbitol in hepatocytes prepared from 24 h-starved rats (Kneer *et al.*, 1979; Yip & Lardy, 1981). Concentrations of amino-oxyacetate that block the malate–aspartate shuttle reportedly had no effect on noradrenaline-induced glucose output from these relatively reduced substrates (Kneer *et al.*, 1979).

Some of the findings reported by Kneer *et al.* (1979) and Yip & Lardy (1981) differ substantially from the data obtained with perfused livers as documented in the present study. The different results obtained may in part reflect differences between the hepatocytes and perfused liver preparations used, as well as differences in the time of α-agonist treatment and the techniques employed to deplete the relevant Ca^{2+} pool(s). In this regard, it is

noteworthy that hepatocyte preparations have previously been reported to become depleted of malate and aspartate, and consequently exhibit low malate–aspartate-shuttle activities (Cederbaum *et al.*, 1977). In addition, in relatively long-term experiments such as those of Kneer *et al.* (1979), inhibition of reducing-equivalent transfer by amino-oxyacetate may be by-passed as glycerol phosphate concentrations rise and flux through the mitochondrial glycerophosphate dehydrogenase increases (Williamson *et al.*, 1971).

In our hands amino-oxyacetate significantly decreased basal glucose output from sorbitol and glycerol and effectively blocked the phenylephrine-induced increases in glucose output observed with these reduced substrates. Data in the present study then indicate that α-adrenergic agonists may increase gluconeogenesis from glycerol and sorbitol by promoting the removal of cytoplasmic reducing equivalents by the operation of the malate–aspartate shuttle, although some involvement of the glycerol phosphate–dihydroxyacetone phosphate shuttle cannot be ruled out. The mechanism whereby α-agonists may stimulate the malate–aspartate shuttle is unknown, but may be related either to changes in total amounts of shuttle intermediates or to a change in the energization of the mitochondrial inner membrane, which may in turn affect the electrogenic exchange of aspartate for glutamate.

Other data presented are consistent with the suggestion that glucose output from glycerol and sorbitol may be enhanced by an increase in the rate of oxidation of cytoplasmic nicotinamide nucleotides. The electron acceptor phenazine methosulphate, and to a lesser extent dinitrophenol, an uncoupler of oxidative phosphorylation, enhanced glucose output from both of these reduced substrates. In addition, phenylephrine-induced increases in glucose output with glycerol or sorbitol were accompanied by a decrease in the [lactate]/[pyruvate] ratio and an increase in the [β-hydroxybutyrate]/[acetoacetate] ratio.

This study also shows an α-agonist-induced elevation of the [β-hydroxybutyrate]/[acetoacetate] ratio (the mitochondiral redox ratio) with every substrate tested. Sugano *et al.* (1980) reported that noradrenaline increased the total cellular reducing equivalents in perfused liver of starved rats with either pyruvate of lactate as substrate, and presented some evidence for a mitochondrial site of reducing-equivalent generation. In studies with hepatocytes where lactate was the substrate, Siess *et al.* (1978) demonstrated an increase in the mitochondrial redox ratio and a lowered mitochondrial 2-oxoglutarate content in response to catecholamines. Our studies of the conversion of [1-^{14}C]-octanoate or 2-oxo[1-^{14}C]glutarate into $^{14}CO_2$ suggest that α-adrenergic agonists rapidly stimulate tricarboxylic acid-cycle flux. We also observed a 40–50% increase in total ketone-body output. Sugden *et al.* (1980) have reported the stimulation of conversion of [1-^{14}C]oleate into $^{14}CO_2$ in rat hepatocytes after administration of adrenaline or vasopressin. Data from the present study and that of Sugden *et al.* (1980) suggest that α-agonists may increase the mitochondrial redox ratio mainly as a result of an increased rate of β-oxidation of fatty acids and an increased flux through the tricarboxylic acid cycle. A significant contribution of cytoplasmic reducing-equivalent transfer to the mitochondrial compartment may only occur when the initial cytoplasmic [NADH]/[NAD$^+$] ratio is relatively high, for example when glycerol or sorbitol is employed.

Although Ca^{2+} has been reported to stimulate both isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase (McCormack & Denton, 1981), and a Ca^{2+}-dependent inhibition of acetyl-CoA carboxylase has been observed in hepatocytes treated with α-adrenergic agonists (Ly & Kim, 1981), it is not known if these effects are involved in the stimulation of tricarboxylic acid-cycle flux and ketogenesis described in the present study.

We also report the Ca^{2+}-dependent activation by α-adrenergic agonists of glucose output from fructose and dihydroxyacetone, substrates which enter the gluconeogenic pathway at the triose phosphate level. Since these changes were observed in the absence of significant changes in glycolysis (lactate + pyruvate output unchanged), α-adrenergic agonists may stimulate the gluconeogenic pathway at other site(s) located between the triose phosphates and glucose. A Ca^{2+}-dependent inhibition of fructose 6-phosphate 2-kinase, and lowered concentration of the activator of phosphofructokinase, fructose 2,6-bisphosphate, have been described in hepatocytes after treatment with adrenaline (Richards & Uyeda, 1982). Thus α-agonists may also inhibit phosphofructokinase and decrease the rate of cycling of fructose phosphates. However, such a proposed action may be limited in the starved state, as phosphofructokinase activity and the cycling of fructose phosphate are already extremely low in starved-rat liver (Kummel, 1982; Van Schaftingen *et al.*, 1980). Consequently, a future study should examine the possibility that α-adrenergic agonists may stimulate fructose 1,6-bisphosphatase or glucose 6-phosphatase activities.

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