Kinetic analysis of short-term effects of α-agonists on gluconeogenesis in isolated rat hepatocytes

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Received 9 November 1984

Isolated hepatocytes from fasted rats were perifused with glycerol as gluconeogenic substrate. Stimulation of gluconeogenesis with phenylephrine (10^{-5} M) as α-adrenergic agonist consisted of two distinct phases. The first phase was a transient stimulation of gluconeogenesis and was accompanied by transient changes in cytosolic and mitochondrial redox state; this phase was abolished by the transaminase inhibitor aminooxyacetate. The second phase was a stable stimulation of less magnitude, without change in redox state and insensitive to addition of aminooxyacetate. It is concluded that the first phase is due to a transient enhancement of flux through the malate/aspartate shuttle and that the stable phase is probably due to a stimulation of mitochondrial glycerol-3-phosphate dehydrogenase and glycerol kinase.

Gluconeogenesis *α-Agonist* *Phenylephrine* *Malate-aspartate shuttle* *Hepatocyte* *Catecholamine*

1. INTRODUCTION

α-Adrenergic agents are known to stimulate gluconeogenesis in hepatocytes. The mechanism is cyclic AMP-independent and is believed to be related to changes in the cellular concentration of free Ca^{2+} [1–3]. The magnitude of the stimulation is commonly found to be 30–50% [4–8] although higher values have been reported [3]. Several sites of action of catecholamines have been proposed: between pyruvate and phospho*enol*pyruvate at the pyruvate carboxykinase step [9]; at the pyruvate kinase step [10] as in the case of glucagon [11]; between fructose 1,6-bisphosphate and fructose 6-phosphate [7]; and, with reduced substrates, at the malate/aspartate shuttle [3] or the glycerol phosphate shuttle [7,12].

To investigate the characteristics of the α-adrenergic stimulation of hepatic gluconeogenesis we have used the perifusion technique [11–14] as an approach to quantify the effects of phenylephrine (10^{-5} M) on flux through the individual enzymes of the gluconeogenic pathways.

Unexpectedly, we found that the stimulation of gluconeogenesis by α-adrenergic agonists consisted of two distinct phases: a rapid transient stimulation followed by a phase of stable stimulation which was less than that in the initial phase. We report in this paper some characteristics of the biphasic response of rat hepatocytes to phenylephrine with glycerol as gluconeogenic substrate.

2. MATERIALS AND METHODS

Hepatocytes were isolated from livers of 24-h fasted male Wistar rats (200–250 g), by the method of Berry and Friend [15] as modified [14]. Liver cells were perifused by the method of Van der Meer and Tager [13] with the modifications described in [11,14]. Glucose, lactate, pyruvate, acetoacetate and 3-hydroxybutyrate were determined in the perifusate by standard enzymic pro-

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

cedures [16]. For 3-hydroxybutyrate, the concentration of which was very low in the perifusate, the NADH formed in the 3-hydroxybutyrate dehydrogenase reaction was measured in a Luminometer (Packard).

Phenylephrine (Sigma, St. Louis, MO) was diluted in Krebs-bicarbonate solution and was infused with a pump to reach a final concentration of 10^{-5} M in the perifusion chamber. To ensure an exact initial concentration of 10^{-5} M, we injected 120 nmol phenylephrine directly into the perifusion chamber at the same time as the infusion of phenylephrine was started. The dead time between the perifusion chamber and the perifusate sampling (30 s) was taken into account in calculating the time course of the changes in metabolites.

3. RESULTS

When perifused hepatocytes were stimulated with 10^{-5} M phenylephrine glucose formation from glycerol (2.5 mM) showed a biphasic stimulation. The first phase was transient with a maximal stimulation of about 40% of the basal rate and was followed by a second stable phase in which the stimulation was only about 15% (fig.1A). Concomitantly, the α-adrenergic stimulation resulted in a stable stimulation of oxygen uptake of 15% (fig.1B) which was completely transient if EGTA (5 mM) was added just before hormone infusion in order to decrease extracellular Ca^{2+} (not shown). Injection of Krebs-bicarbonate instead of hormone had no effect (not shown).

A similar biphasic stimulatory effect on glucose formation was observed when vasopressin (5×10^{-9} M) was infused instead of phenylephrine and the characteristics of this effect were the same as with phenylephrine (not shown).

The metabolism of reduced substrates like glycerol is controlled by the rate of removal of cytosolic reducing equivalents [12,17]. Fig.1C shows the changes in the lactate/pyruvate ratio, which reflects changes in the cytosolic redox state [18]. Between 1 and 6 min after hormone stimulation there was a transient decrease in the lactate/pyruvate ratio after which it remained stable at the same level as before phenylephrine infusion. At the same time the 3-hydroxybutyrate/acetoacetate ratio, an indicator of the mitochondrial redox state [18] showed a transient increase during the first 5 min; after that time the ratio remained

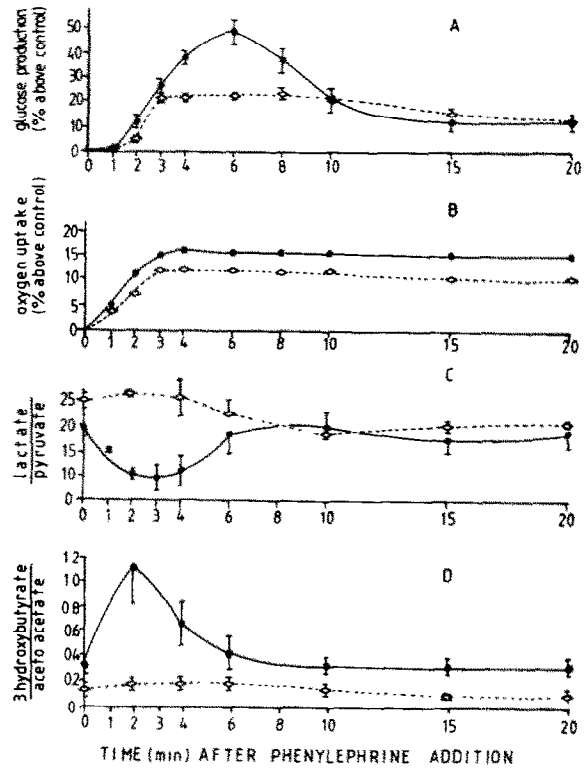

Fig.1. Time course of the effects of phenylephrine on perifused rat hepatocytes. Liver cells (240 mg) from 24-h fasted rats were perifused with Krebs-bicarbonate and glycerol (2.5 mM). Phenylephrine (10^{-5} M) was added at zero time without (●—●) or with (◇---◇) aminooxyacetate (0.3 mM). (A) Stimulation of glucose formation. The rate of glucose formation was calculated from the measured glucose in the perifusate and was 4.8 ± 0.25 μmol·min^{-1}·g^{-1} dry wt without and 3.5 ± 0.49 μmol·min^{-1}·g^{-1} dry wt with aminooxyacetate. The values after phenylephrine addition are expressed as the percentage increase with respect to the control rate. (B) Stimulation of oxygen consumption. Oxygen concentration in the perifusate was monitored with a Clark electrode. The values are percentage stimulation of oxygen uptake by the cells with respect to the basal rate. The basal rate was 14.7 ± 0.10 and 13.2 ± 0.5 μmol O_2·min^{-1}·g^{-1} dry wt without and with aminooxyacetate, respectively. (C) Lactate/pyruvate ratios in the perifusate. (D) 3-Hydroxybutyrate/acetoacetate ratios in the perifusate. Results are expressed as means ± SE of 3 separate experiments. In panel B SE bars are not shown because they are less than 1% of the stimulation.

stable at the same value as before stimulation (fig.1D). Addition of oleate (0.1 mM) or octanoate (0.2 mM) together with glycerol, which leads to an increase in the mitochondrial and cytosolic redox states, resulted in a decrease in glucose production of about 55%. In this case too phenylephrine stimulation involved a biphasic stimulation of glucose production of about 25% for the transient phase and about 10% for the subsequent stable stimulation, and the same transient changes in the cytosolic and mitochondrial redox state indicator metabolites were observed as with glycerol alone (not shown).

The more rapid removal of reducing equivalents from the cytoplasm to the mitochondria observed during the transient phase of hormonal stimulation can be due to stimulation of either the malate/aspartate shuttle or the 3-glycerolphosphate shuttle. Indeed, it is known that inhibition of the malate/aspartate shuttle by addition of a transaminase inhibitor leads to partial inhibition of gluconeogenesis from reduced substrates in the absence of hormones [3,7]. However, there are some discrepancies in the literature concerning the sensitivity to the transaminase inhibitor aminooxyacetate of the hormonal effect. Kneer et al. [7] found that the α-adrenergic stimulation in long incubations is insensitive to inhibition of the malate/aspartate shuttle whereas Taylor et al. [3] found a decrease in the hormonal response when a transaminase inhibitor was added in perfused livers with a short exposure to this hormone.

To test the importance of the malate/aspartate shuttle in the two different phases of the stimulation, aminooxyacetate (0.3 mM) was added in order to block this shuttle by inhibiting the transamination steps. The data in fig.1C and D clearly indicate that in the presence of aminooxyacetate the lactate/pyruvate and 3-hydroxybutyrate/acetoacetate ratios did not change after hormone stimulation. Furthermore, glucose production showed an increase of only about 20% (fig.1A), which, in fact, represented the second, stable stimulatory phase observed without inhibitor.

Thus, the first transient phase is accompanied by transient redox state changes; this phase and the accompanying redox changes are aminooxyacetate sensitive. The second phase is stable, without changes in the redox state and aminooxyacetate insensitive.

4. DISCUSSION

Our results show clearly that the α-adrenergic stimulation of gluconeogenesis by phenylephrine with glycerol as substrate consists of two distinct phases, a transient phase followed by a stable phase in which the magnitude of the stimulation is less than in the first phase. Similar results, i.e., a transient followed by a stable stimulation, were also obtained with other gluconeogenic substrates: lactate/pyruvate, dihydroxyacetone or proline (not shown). To our knowledge this is the first time that such a transient effect on gluconeogenesis of α-adrenergic stimulation has been reported in the presence of a physiological concentration of extracellular Ca^{2+} [19].

The transient stimulation of glucose production is not the consequence of a glycogenolytic effect of phenylephrine. Firstly, the glycogen content in the liver of the fasted rat is low [20,21] and secondly glycogenolysis is insensitive to the transaminase inhibitor aminooxyacetate [3] which in our experiments abolished the transient stimulation.

The fact that the first transient phase of stimulation of glucose production was accompanied by transient changes in redox state suggests that there is a connection between these two phenomena. The cytosolic redox state controls the rate of reduced substrate metabolism [12,17]; for instance, fatty acid addition decreases the rate of gluconeogenesis from glycerol and oxidizing agents increase it [3]. It has been suggested that α-adrenergic agonists increase gluconeogenesis from reduced substrates by enhancing the transfer of reducing equivalents from the cytoplasm to the mitochondrion [3,7]. Our results show that this is only the case in the first, transient phase of stimulation. The clear suppression by aminooxyacetate of the transient phase indicates that the increase in the rate of removal of cytosolic reducing equivalents is caused by an enhanced flux through the malate/aspartate shuttle as suggested by Taylor et al. [3] and not through the α-glycerolphosphate shuttle as suggested by Berry et al. [12] and Kneer et al. [7].

The second stimulatory phase is entirely different: it is stable, of less magnitude than the first phase and aminooxyacetate insensitive. In agreement with Kneer et al. [7] the malate/aspartate shuttle is certainly not involved. In principle activation of mitochondrial α-glycerolphosphate

dehydrogenase by the hormone could explain the stimulation of glucose production as implied by Berry et al. [12] and Kneer et al. [7]. However, in that case a decrease in lactate/pyruvate ratio would be expected. Since no change in the cytosolic redox state was observed in the stable phase, another step must have been activated by the hormone in addition to mitochondrial α-glycerolphosphate dehydrogenase.

With reduced substrates the flux through pyruvate kinase is low, and, in addition, not only glucose production, but also the rate of formation of lactate plus pyruvate increases during the stable phase (from 0.70 ± 0.15 to 0.84 ± 0.12 μmol lactate plus pyruvate$\cdot min^{-1} \cdot g^{-1}$ dry wt; $n = 3$). Therefore, inhibition of pyruvate kinase, as proposed by Chang and Exton [10], cannot be involved with reduced substrates. On the other hand, these results indicate that there is an increased flux through glycerol kinase.

The transient changes in the mitochondrial and cytosolic redox states and in glucose production are not exactly in phase (fig.1); when glucose production is highest, both ratios are already back to the control values. This could be explained by a lag phase due to a build-up of several intermediates.

The question arises as to why the malate/aspartate shuttle is transiently increased upon α-adrenergic stimulation. There are at least two possible mechanisms. Firstly, transient changes in the concentration of intermediates affecting the rate of the shuttle could be involved as has been shown for 2-oxoglutarate during proline metabolism [22]. Secondly, a transient change in membrane potential caused by a stimulation of mitochondrial respiration could have a direct effect on flux through the shuttle.

ACKNOWLEDGEMENTS

The authors are grateful to Fred Meijer for helpful discussions and Wendy van Noppen for her help in the preparation of this manuscript. This study was supported in part by a grant from the Netherlands Organization for the Advancement of Pure Research (ZWO) under the auspices of the Netherlands Foundation for Chemical Research (SON). X.M.L. is a fellow of the Fondation pour la Recherche Médicale.

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