Regulation of oxidative degradation of L-lysine in rat liver mitochondria

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The generation of $^{14}CO_2$ from [1-^{14}C]lysine by hepatic mitochondria through the saccharopine pathway is controlled by intramitochondrial concentrations of lysine, 2-oxoglutarate and NADPH. Mitochondria, isolated from rats pre-treated with glucagon, exhibited higher activities of L-lysine:2-oxoglutarate reductase, saccharopine dehydrogenase and 2-aminoadipate aminotransferase. The flux through this pathway is stimulated in liver mitochondria after glucagon treatment. Multiple regulation of lysine oxidation in liver mitochondria confirms a complex mechanism of 'mitochondrial activation' by glucagon.

INTRODUCTION

Hepatic L-lysine degradation occurs principally through the saccharopine pathway, located within the mitochondrial matrix [1]. The formation of saccharopine and its cleavage to 2-aminoadipic semialdehyde and glutamic acid are catalysed by lysine:2-oxoglutarate reductase (LOGR; EC 1.5.1.8) and saccharopine dehydrogenase (SADH; EC 1.5.1.9) respectively. The oxidation of 2-aminoadipic semialdehyde to 2-aminoadipic acid and subsequent steps of the pathway, transamination and oxidation, yield acetyl-CoA as a product of mitochondrial lysine oxidation. This catabolic route of lysine metabolism is summarized in Scheme 1.

Among liver enzymes involved in the catabolism of amino acids, LOGR is one of several which adapts to the level of protein and amino acids in the diet [2–4]; for LOGR this adaptation is due to an increased synthesis of LOGR protein [2]. Shinno et al. [5] have demonstrated induction of hepatic LOGR in rats and in isolated hepatocytes by glucagon, alloxan and glucocorticoid treatments.

However, enzyme induction cannot occur sufficiently rapidly to account for the increased degradation of amino acids observed after a single protein load [6]. A short-term stimulation of hepatic catabolism has been associated with an increased secretion of glucagon [7]; glucagon rapidly activates several oxidative reactions and stimulates amino acid catabolism [8–10]. We therefore investigated the short-term effects of glucagon on the activities of enzymes involved in the degradation of lysine and on measured rates of lysine oxidation in isolated liver mitochondria.

MATERIALS AND METHODS

Animals

Male Hooded–Lister (Rowett strain) rats weighing 200±20 g were given free access to food (standard stock diet containing 21% protein), with a cycle of 12 h light (07:00–19:00 h) and 12 h dark. The animals were not fasted before the experiments, which began at 10:00 h. Glucagon (80–100 mg/100 g body wt.) in 0.9% NaCl (pH 2.8) was injected intraperitoneally; control rats were injected with the same volume of saline alone. Animals were killed by cervical dislocation 30 min later.

Preparation of mitochondria and mitochondrial incubations

Liver mitochondria were isolated in 0.3 M mannitol/1 mM EDTA, pH 7.6, by differential centrifugation as described by Myers and Slater [11]. Mitochondria were suspended in 0.3 M mannitol at 20 mg of protein/ml as measured by the biuret procedure [12].

Incubations were carried out at pH 7.6 in 2 ml of basal medium, containing 60 mM KCl, 25 mM $KHCO_3$, 25 mM triethanolamine/HCl, pH 7.6, 10 mM NaCl, 5 mM $MgCl_2$, 5 mM K_2HPO_4, 2 mM ADP, 1 mM EGTA plus 5 mM sodium succinate, 5 mM sodium isocitrate, 0.5 mM L-[1-^{14}C]lysine and 0.5 mM 2-oxoglutarate, with additions as indicated in the Tables and Figures. Reactions were started by adding approx. 2 mg of mitochondrial protein per sample. Incubations were carried out in sealed 25 ml Erlenmeyer flasks with inserted wells containing 0.25 ml of a mixture of methanol/phenethylamine (1:1, v/v) to trap CO_2. The rate of CO_2 production was linear up to 45 min: routinely, reactions were terminated after 20 min with 1 ml of 1 M $HClO_4$, and the flasks were shaken for an additional 60 min to ensure complete release of $^{14}CO_2$.

Absorbed $^{14}CO_2$ was counted for radioactivity after mixing the well contents with 4 ml of scintillation liquid (Atomlight from Du Pont–NEN). For the estimation of non-enzymic lysine oxidation, $HClO_4$ was added to a flask before the mitochondria, and the mixture was incubated for 60 min. The value of the blank was usually less than 10% of the control value, and for the calculation of mitochondrial oxidation activity was subtracted from the control values.

Disruption of isolated mitochondria

For the enzymic assays, isolated mitochondria were disrupted by solubilization in the detergent Nonidet P-40 or sonicated at a mitochondrial protein concentration of 20 mg/ml; the optimal concentration of detergent for spectrophotometric assays was 0.2% (v/v). Isolated mitochondria suspended in 0.3 M mannitol at a final protein concentration of 20 mg/ml were sonicated for 6×10 s with 20 s intervals by using an MSE Soniprep 150 instrument.

Measurement of enzyme activities

LOGR was determined spectrophotometrically at 340 nm. The assay medium was 100 mM Hepes, pH 7.8, 7.5 mM 2-oxoglutaric acid, 0.125 mM NADPH, 0.5 mg of mitochondrial protein and 25 mM lysine. The reaction was started by addition of lysine.

SADH was assayed by the formation of NADH monitored at 340 nm in a medium consisting of 50 mM Tris/HCl, pH 9.4,

Abbreviations used: LOGR, lysine:2-oxoglutarate reductase (EC 1.5.1.8); SADH, saccharopine dehydrogenase (EC 1.5.1.9).
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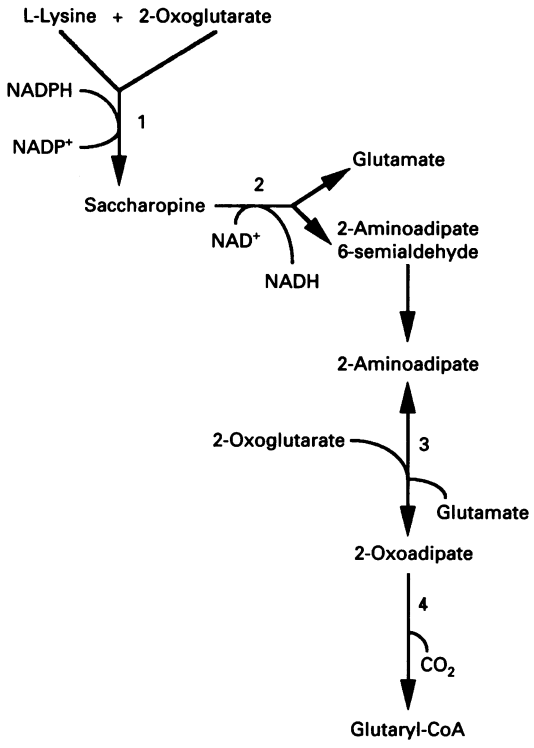

Scheme 1 Oxidative degradation of L-lysine in mammalian mitochondria

Enzymes: 1, LOGR; 2, SADH; 3, 2-aminoadipate aminotransferase; 4, 2-oxoadipate dehydrogenase.

1.8 mM NAD^+, 2 mM saccharopine and 1 mg of mitochondrial protein. The reaction was started by addition of saccharopine. Both LOGR and SADH activities were assayed with mitochondria solubilized by Nonidet P-40. The addition of known inhibitors of respiratory-chain activity, rotenone (2 μM), antimycin A (5 μg) or KCN (1 mM), did not affect the rate or linearity of NADPH consumption or of NADH production in either of the above assays.

2-Aminoadipate aminotransferase (EC 2.6.1.39) was assayed by the formation of 2-oxoglutaric acid. Sonicated mitochondria were incubated in a medium of 150 mM potassium phosphate buffer, pH 7.8, 2 mM 2-oxoadipic acid and 20 mM glutamic acid. The reaction was initiated by adding mitochondria (2 mg), and after 10 min was terminated by addition of 1 M $HClO_4$. Protein was removed by centrifugation, and neutralized supernatants were used for determination of 2-oxoglutaric acid with glutamic dehydrogenase as described in [13]. In the control incubations 2-oxoadipic acid was omitted.

Energy-dependent nicotinamide nucleotide transhydrogenase activity was measured with sonicated mitochondria essentially as described by Ernster and Lee [14] in a medium of 50 mM Tris/HCl, pH 8.0, 5 mM $MgCl_2$, 1 mM KCN, 2 μM rotenone, 50 mM ethanol, 0.15 mM NAD^+, 0.2 mM $NADP^+$, 10 units of yeast alcohol dehydrogenase and 5 mM ATP.

Citrate synthase (EC 4.1.3.7) activity in mitochondrial preparations was measured in the presence of 0.2% Nonidet P-40 by using Ellman reagent as originally described by Srere [15]. Lactate dehydrogenase (EC 1.1.1.27), malate dehydrogenase (EC 1.1.1.37) and NADP-linked isocitrate dehydrogenase (EC 1.1.1.42) in isolated mitochondria were assayed spectrophotometrically in 100 mM Hepes, pH 7.8, with 0.2% Nonidet P-40.

Table 1 Enzymic activities of the lysine-degradation pathway in liver mitochondria from control and glucagon-treated rats

Values are means ± S.D. for the numbers of experiments in parenthesis. Statistically significant differences: $^*P < 0.01$, $^{**}P < 0.05$, compared with the control value.

Enzyme	Activity (nmol/min per mg of protein)		Stimulation (%)
	Control	Glucagon	
LOGR	2.17 ± 0.46 (11)	4.49 ± 0.99* (11)	107
SADH	2.71 ± 0.56 (10)	4.87 ± 1.16* (10)	80
2-Aminoadipate aminotransferase	115.7 ± 13.2 (5)	141.3 ± 12.4** (5)	23

All enzymic assays and mitochondrial incubations were performed at 30 °C. The Ca^{2+} content in isolated mitochondria was determined by a colorimetric method with Arsenazo III as an indicator [16].

Chemicals

2-Aminoadipic acid, 2-oxoadipic acid, 2-oxoglutaric acid, saccharopine, *threo*-D(+)-isocitric acid, CoA (lithium salt), rotenone and 2,4-dinitrophenol were supplied by Sigma Chemical Co. (Poole, Dorset, U.K.). L-[1-^{14}C]Lysine (specific radioactivity 56 mCi/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.), and 2-phenethylamine was from Fisons Laboratory Supplies (Loughborough, Leics., U.K.).

RESULTS

After a single injection of glucagon, plasma glucose concentration was elevated by 55% and the synthesis of citrulline in isolated mitochondria was increased 2–3-fold. These criteria were used to confirm the effectiveness of glucagon treatment (results not shown). Table 1 summarizes the stimulatory effects of glucagon on the mitochondrial activities of three enzymes involved in the saccharopine pathway of lysine oxidation. The activities of LOGR and SADH were stimulated to a similar extent (80–107%) in glucagon-treated animals, whereas 2-aminoadipate aminotransferase was stimulated by only 25%. To investigate whether short-term glucagon treatment also enhanced the oxidative flux of lysine in isolated rat liver mitochondria, we measured the production of CO_2 from [1-^{14}C]lysine.

The results of Figure 1 characterise the conditions *in vitro* for the optimal activity of lysine oxidation in isolated liver mitochondria. Without 2-oxoglutarate, the oxidation of lysine was markedly decreased; the low rate of oxidation observed may occur though the pipecolic acid pathway [17]. The omission of isocitric acid, ADP or succinic acid, or the addition of dinitrophenol or malonic acid, significantly diminished the rate of lysine oxidation, suggesting the importance of energy and NADPH for maximal activity. The energy requirement was further confirmed in incubations in which ADP and succinic acid were replaced with 4 mM ATP. This suggests the importance of energy-dependent transhydrogenase activity for NADPH generation. The requirement for ATP could be explained by the generation of NADPH through the energy-linked transhydrogenase reaction. NADPH for lysine oxidation may also be generated independently of this reaction, through NADP-linked isocitrate dehydrogenase, as indicated by the sustaining effect of isocitric acid on rates of lysine oxidation.

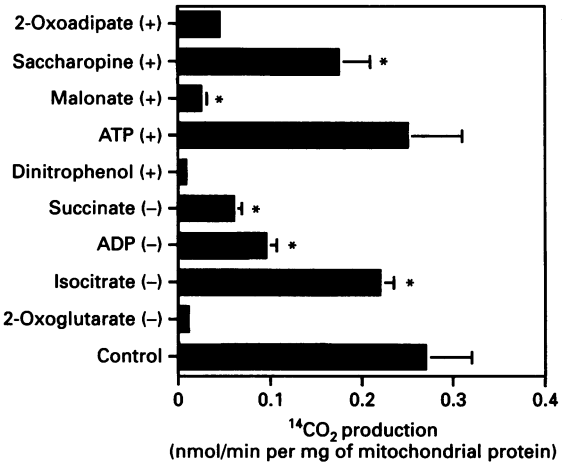

Figure 1 Effects of addition (+) or omission (−) of the specified metabolites on $^{14}CO_2$ production from L-[1-^{14}C]lysine in isolated rat liver mitochondria

Results are expressed in nmol of CO_2/min per mg of mitochondrial protein and represent means ± S.D. of three separate experiments: *significantly different ($P < 0.01$) from control values. Concentrations of compounds added to the incubation medium were: ATP, 3 mM; malonate, 10 mM; saccharopine, 1.5 mM; 2-oxoadipate, 5 mM. Composition of control medium and incubation conditions are described in the Materials and methods section.

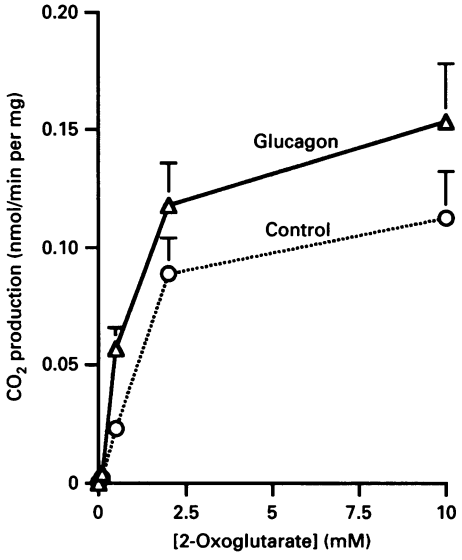

Figure 2 Effect of 2-oxoglutarate on the production of CO_2 from L-[1-^{14}C]lysine by isolated mitochondria of control and glucagon-treated rats

Incubation conditions were as described in the Materials and methods section. Results are means ± S.D. for three separate experiments. At each 2-oxoglutarate concentration the activity was higher in mitochondria isolated from glucagon-injected rats ($P < 0.05$).

The addition of unlabelled saccharopine and 2-oxoadipic acid significantly diluted the specific radioactivity of $^{14}CO_2$ generated, confirming lysine oxidation through the saccharopine pathway. In the presence of 10 mM 2-oxoglutaric acid we measured the effect of lysine concentration on its oxidation in isolated liver mitochondria. The apparent K_m for lysine was 0.55 ± 0.07 mM (mean ± S.D., $n = 4$) in both control and glucagon-treated rats. We subsequently measured the effect of 2-oxoglutarate on lysine oxidation when included at a concentration close to that *in vivo*, i.e. 0.5 mM [18,19]. Figure 2 illustrates the higher rate of lysine oxidation in glucagon-injected rats. The stimulatory effect of glucagon was more pronounced (approx. 200%) at low (0.2 mM and 0.4 mM) concentrations of 2-oxoglutaric acid; at concentrations of 2-oxoglutaric acid of 2 mM and above, glucagon caused approx. 40% stimulation of lysine oxidation. It is known that a single glucagon injection activates ATP-dependent transhydrogenase in submitochondrial particles [20]. Indeed, in sonicated mitochondria isolated from rats treated with glucagon, we detected a doubled activity of transhydrogenase (control 3.6 ± 2.4, glucagon 7.6 ± 3.8 nmol/min per mg of protein; means ± S.D., $n = 7$). The higher activity of transhydrogenase indicates that this reaction increases the intramitochondrial pool of NADPH rather than the NADP-dependent isocitrate dehydrogenase, the activity of which was the same in control (126 ± 16 nmol/min per mg of protein) and glucagon-injected rats (117 ± 9 nmol/min per mg of protein; means ± S.D., $n = 3$).

DISCUSSION

Short-term effects of glucagon on enzyme activities within the lysine-oxidation pathway

It is well documented that a single injection of glucagon stimulates the activities of three mitochondrial dehydrogenases: NAD-dependent isocitrate dehydrogenase, the 2-oxoglutarate dehydrogenase complex, and succinate dehydrogenase [21]. We have now demonstrated the short-term activation of LOGR and SADH by glucagon. It has been suggested that glucagon, by increasing intramitochondrial Ca^{2+} concentration, activates NAD-dependent isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase [22]. Indeed, we have detected a 30% increase in intramitochondrial Ca^{2+} concentration in samples from glucagon-injected rats (results not shown). However, we did not observe any effect of added (0.1–2 mM) Ca^{2+} ions on the activities of LOGR or SADH measured in isolated hepatic mitochondria from control or treated rats (results not shown).

The stimulatory effect of glucagon on mitochondrial function has been postulated to occur as a result of changes in mitochondrial volume [23]. However, since we measured LOGR and SADH in the presence of the non-ionic detergent Nonidet P-40, it is unlikely that mitochondrial volume changes were responsible for the observed stimulatory effects on enzyme activity. Moreover, as suggested recently [24], changes in mitochondrial matrix volume are probably a result of glucagon action, rather than a mediator of it.

It is also possible that glucagon treatment might affect the disruption of mitochondria and that this, rather than any real effect on enzyme activity, might give rise to our observations. To examine this possibility we measured, in parallel with the measurements of LOGR and SADH, the activities of citrate synthase, lactate dehydrogenase and malate dehydrogenase. The mean values of citrate synthase activity in mitochondria of control and glucagon-treated rats (nmol/min per mg of protein; means ± S.D., $n = 3$) were 162 ± 8 and 166 ± 14 respectively, of lactate dehydrogeanse 424 ± 44 and 426 ± 71 respectively, and of malate dehydrogenase 2230 ± 320 and 2410 ± 410 respectively. As the activity of citrate synthase, a mitochondrial matrix enzyme, did not differ significantly between mitochondria of glucagon-treated and control rats, we suggest that our results are unlikely to have arisen from an unequal disruption of mitochondria from treated and control animals. The equal activity of lactate dehydrogenase, a cytoplasmic enzyme, suggests that there

was no difference in the purity of the mitochondrial preparations from control and treated rats. Finally, the similarity in malate dehydrogenase activity suggests that there was no difference in the leakage of mitochondrial protein during the preparation of the mitochondria, as observed for mitochondrial isoenzymes of malate and NADP-dependent isocitrate dehydrogenases [25].

The oxidative decarboxylation of 2-oxoadipic acid is analogous to the oxidation of 2-oxoglutaric acid, and could be catalysed by the 2-oxoglutarate dehydrogenase complex as shown recently [26]. Our results on CO_2 production from [1-^{14}C]lysine in the presence of saturating amounts of 2-oxoglutarate confirm the presence of 2-oxoadipic dehydrogenase activity in parallel with that of the 2-oxoglutarate dehydrogenase complex; this enzyme has not yet been isolated from any mammalian species, but its existence is also supported by clinical reports of 2-oxoadipic-aciduria in humans [27].

Some mitochondrial enzymes are bound in complexes, and the presence of a multienzyme complex has been reported for mitochondrial NAD-dependent isocitrate dehydrogenase and the 2-oxoglutarate dehydrogenase [28]. Markovitz and Chuang [29] observed that LOGR and SADH activities purified from baboon and bovine liver are associated as a bifunctional enzyme. However, according to the data of Noda and Ichihara [30], rat hepatic LOGR and SADH may be separated. It is at present unclear whether these are real species differences, or whether the discordant results reflect different purification procedures. Nevertheless, it is possible that glucagon may alter the submitochondrial organization of certain dehydrogenases, causing specific channelling of substrates through enzymic complexes.

Metabolic consequences of glucagon on the oxidative degradation of lysine in rat liver mitochondria

There are at least three links between increased lysine oxidation and the metabolic changes in liver already known to be brought about by glucagon. First, one of the mechanisms by which glucagon stimulates gluconeogenesis is by changing the concentrations of several key metabolites [31]. For example, glucagon causes increased utilization of 2-oxoglutarate by an increase in the activities of 2-oxoglutarate dehydrogenase [32] and alanine aminotransferase [33]. In the present study, the higher oxidative flux of lysine observed after glucagon treatment is another metabolic route by which the utilization of 2-oxoglutarate is increased. Such a decrease in 2-oxoglutarate concentration by glucagon is in concordance with a recent report showing the modulation of phosphoenolpyruvate carboxykinase activity by physiological concentrations of 2-oxoglutarate [34].

Secondly, an increase in LOGR activity over 3 h after high doses (30 mg/100 g body wt.) of glucagon has previously been interpreted as evidence of enzyme induction [35]. Our data suggest that the increased activities of LOGR and SADH are part of the short-term activation of mitochondrial function by glucagon. Although we do not have quantitative evidence that increased lysine oxidation resulting from glucagon treatment enhances the intramitochondrial pool of acetyl-CoA and glutamic acid, we speculate that these two metabolites, being substrates for *N*-acetylglutamate synthetase (EC 2.3.1.1), may be responsible for the increase in *N*-acetylglutamate concentration [36] which could then lead to higher citrulline production.

Lastly, after glucagon treatment increased mitochondrial ATP may increase the activity of the ATP-linked transhydrogenation reaction. Indeed, we have demonstrated *in vitro* the link between elevated intramitochondrial NADPH production through the transhydrogenase and the oxidative degradation of lysine. In sonicated mitochondria we monitored the stimulation of NADH oxidation only after sequential addition of all three metabolites: 2-oxoglutarate, L-lysine and ATP (results not shown).

Physiological relevance of glucagon action on lysine oxidation

The experimental approaches used in the present study are similar to those used by others [37,38]. It is worth emphasizing that such glucagon treatment mimics the postprandial increase in glucagon secretion observed after a high-protein meal [39]. Interesting serial studies published recently on the effects of a single protein meal on postprandial changes in the oxidation of glycine [40] and glutamine [41] and urea synthesis [42] correlate well with our observation of increased lysine oxidation in rat liver mitochondria after glucagon injection. It appears that glucagon release triggered by a protein-containing meal [43] is an important physiological mediator of rapid changes not only in liver carbohydrate metabolism but also in the metabolism of amino acids. We suggest that there are multiple actions of glucagon on hepatic mitochondrial metabolism that result in its final physiological effects that are observed as increased synthesis of glucose, ketone bodies and urea.

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