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EFFECTS OF GLUCAGON ON THE REDOX STATES OF CYTOCHROMES IN MITOCHONDRIA IN SITU IN PERFUSED RAT LIVER

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The effects of glucagon on the respiratory function of mitochondria in situ were investigated in isolated perfused rat liver. Glucagon at the concentrations higher than 20 pM and cyclic AMP (75 μM) stimulated hepatic respiration, and shifted the redox state of pyridine nucleotide (NADH/NAD) in mitochondria in situ to a more reduced state as judged by organ fluorometry and β-hydroxybutyrate/acetoacetate ratio. The organ spectrophotometric study revealed that glucagon and cyclic AMP induced the reduction of redox states of cytochromes $a(a_3)$, b and $c+c_1$. Atractyloside (4 μg/ml) abolished the effects of glucagon on these parameters and gluconeogenesis from lactate. These observations suggest that glucagon increases the availability of substrates for mitochondrial respiration, and this alteration in mitochondrial function is crucial in enhancing gluconeogenesis.

Glucagon is known to stimulate gluconeogenesis in the liver. Because the initial steps of gluconeogenesis in rat liver take place in mitochondria (1), it seems important to elucidate the metabolic effects of glucagon on mitochondria for a better understanding of glucagon action in the liver. Although some functional changes have been reported in mitochondria isolated from glucagon treated liver (2-4), it is still controversial whether or not the changes in isolated mitochondria are artefact (5,6). The effects of glucagon on respiration and the redox state of pyridine nucleotide in perfused rat liver (7,8) and in isolated hepatocytes (9) have been also reported, but none of these reports showed the data about the effects of glucagon on cytochromes. Recently, Kobayashi and his coworkers established

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a computed scanning spectrophotometric method for perfused organs (10) which is sensitive enough to detect the changes in the redox states of cytochromes $a(a_3)$, b and $c+c_1$ in mitochondria functioning in intact cells (in situ). In the present study, by using this new technique in combination with organ fluorometry (11,12) and an oxygen electrode, it is shown for the first time that glucagon increases the reduced form of cytochromes $a(a_3)$, b and $c+c_1$ as well as NAD in mitochondria in situ.

Materials and Methods

Liver Perfusion: Male Wistar rats weighing about 140-180 g were used. The livers were perfused with Krebs-Henseleit buffer saturated with oxygen and carbon dioxide mixture (95%:5%) at a constant rate of 30-35 ml/min in a flow-through system as described previously (13). The liver under perfusion was then isolated, placed on a plastic holder and allowed to equilibrate by perfusing it for about 30 min before starting the experiments.
Measurement of Hepatic Oxygen Consumption: The oxygen concentration in the effluent leaving the liver was monitored polarographically with a Clark type oxygen electrode. The rate of oxygen consumption was estimated from the difference in oxygen concentrations in the inflow fluid and in the effluent.
Redox States of Cytochromes and Pyridine Nucleotide: With a quartz fiber system connected to the newly devised scanning spectrophotometer (10), a small area of the perfused liver surface was illuminated by monochromatic light which was scanned from 370 nm to 700 nm once every second. Ten sequential spectra of reflexion light were averaged, and recorded automatically. Simultaneously, the difference spectra were computed against the basal control spectrum obtained before the addition of glucagon or other agents (10). The changes in the redox states of cytochromes were estimated by the differences in the optical densities (ΔOD) at 605 nm (a_α), 560 nm (b_α), 550 nm ($[c+c_1]_\alpha$) and 520 nm ($[c+c_1]_\beta$) (14). The redox state of the pyridine nucleotides in the perfused liver was monitored continuously with an organ fluorometer as described elsewhere (11,12). The fluorescence intensity from the resting organ was scaled arbitrarily as 100%.
Determination of Metabolites: The concentration of glucose in the effluent fluid was measured by the glucose oxidase-peroxidase method (Boehringer-Mannheim Corp., Germany). Lactate, pyruvate, ß-hydroxybutyrate and acetoacetate were determined enzymatically (15-18).
Chemicals: Glucagon was obtained from Novo Industry (Copenhagen, Denmark). Cyclic AMP and atractyloside were obtained from Sigma Chemical Co. (St. Louis, Missouri). All other chemicals were of reagent grade.

Results

Effects of Glucagon on Hepatic Respiration and the Redox States: When glucagon (1.4×10^{-9} M) was added to the perfusion medium, it caused an increase in the hepatic oxygen consumption and in fluorescence intensity (Fig. 1A). These effects were demonstrable when the concentrations of glucagon were higher than 2×10^{-11} M. In addition, the hormone induced slight reduction of cytochromes $a(a_3)$, b and $c+c_1$ as shown in Fig. 1B, when

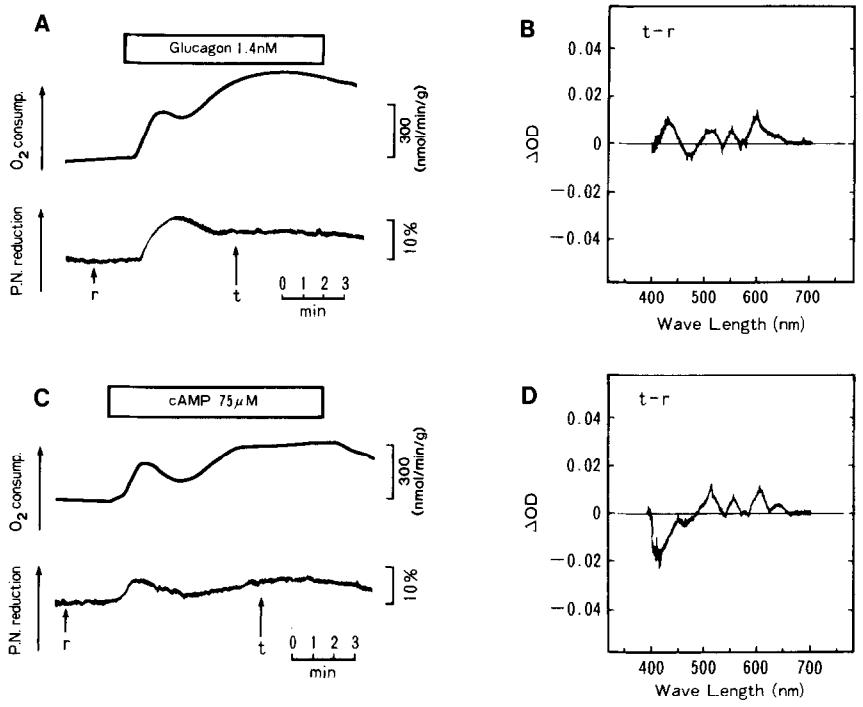

Fig. 1 Effects of glucagon and cyclic AMP on metabolic parameters. Livers from fed rats were perfused without added substrate. Effects of 1.4 nM glucagon (A, B) and 75 μM cyclic AMP (C, D) on the hepatic oxygen consumption, the redox state of pyridine nucleotides (P.N.) (A, C) and difference spectra (B, D) obtained at time t against the spectra at time r in Fig. 1A and 1C are shown. The basal respiratory rates were 2.46 and 2.60 μmol O_2/min/g wet liver in experiments for glucagon and cyclic AMP, respectively. The dilution of these agents on infusion was about 1:400. Infusion of 10% lactose solution (vehicle for glucagon) or physiological saline at the same rate as these agents caused no changes in any of these parameters (data not shown). cAMP, cyclic AMP.

the concentrations of glucagon were higher than 5×10^{-10} M. The effects of glucagon on these parameters were highly reproducible and mimicked by 7.5×10^{-5} M cyclic AMP (Fig. 1C,1D).

Effects of Glucagon on Metabolites in the Effluent: The concentrations of lactate and pyruvate in the effluent declined gradually following the addition of glucagon (Fig. 2A). The lactate/pyruvate ratio, which represents the NADH/NAD ratio in cytosol, increased markedly after a lag period of about 5 min. On the other hand, the ß-hydroxybutyrate/acetoacetate ratio, which represents the NADH/NAD ratio in mitochondria, increased without any appreciable lag period after the addition of glucagon (Fig. 2B).

Effects of Atractyloside on Glucagon Action: The addition of atractyloside to the perfusate (4 μg/ml) resulted in an inhibition of hepatic respiration

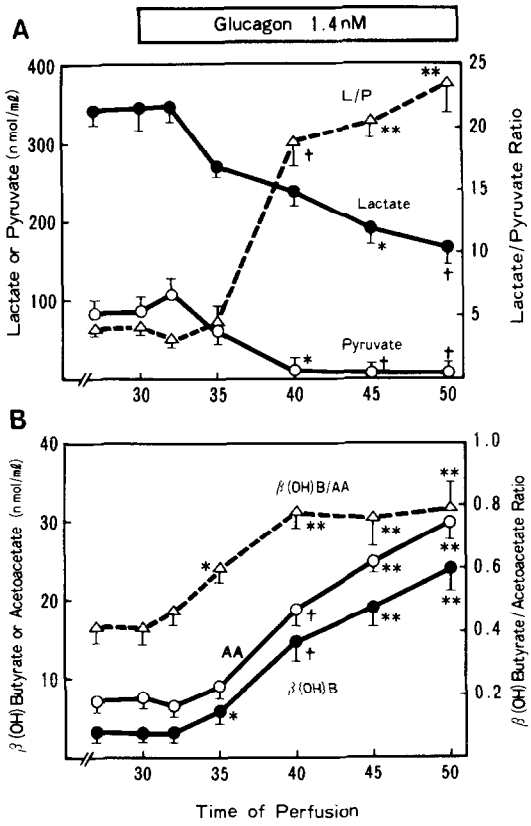

Fig. 2 Effects of glucagon on cytosolic and mitochondrial oxidation-reduction couples in the effluent.

Liver perfusion was carried out as described in the legend for Fig. 1. A. Effects of glucagon (1.4 nM) on lactate, pyruvate and the lactate/pyruvate ratio (L/P). B. Effects of glucagon (1.4 nM) on ß-hydroxybutyrate, acetoacetate and the ß-hydroxybutyrate/acetoacetate ratio (ß(OH)B/AA). Points and vertical bars indicate means and SEMs from four experiments. The control perfusions without glucagon showed no significant change in any of these parameters, and the results were omitted from the figure for simplicity. *p<0.05, +p<0.01, **p<0.005.

and a marked reduction of the pyridine nucleotide redox state (Fig. 3A). The redox states of cytochromes showed no significant changes (data not shown). When atractyloside was present, all the effects of glucagon on respiration, pyridine nucleotide(s) and cytochromes were completely abolished (Fig. 3). The effects of atractyloside on glucagon-induced stimulation of glucose output from the perfused liver were investigated to determine whether or not the inhibitory effect of atractyloside was related to the effects of glucagon on glycogenolysis and/or gluconeogenesis. When livers from fed rats were perfused with a buffer containing no substrate, atractyloside did not inhibit the glucagon-induced stimulation of hepatic

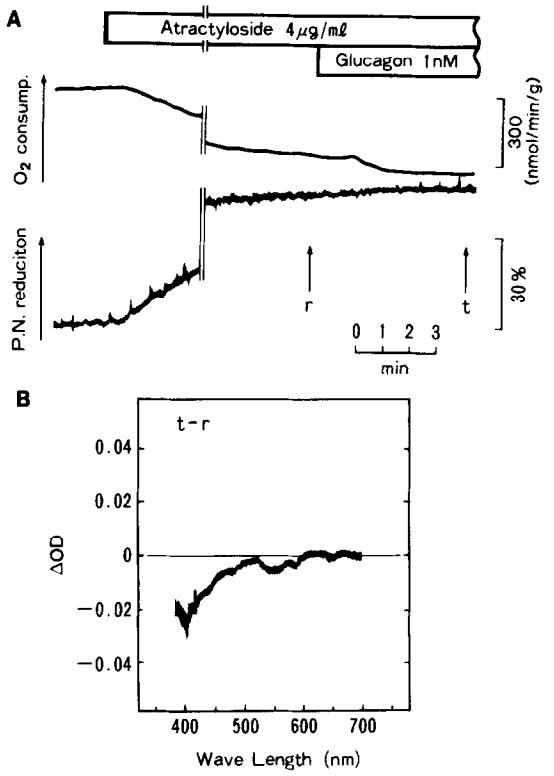

Fig. 3 Effects of glucagon on the metabolic parameters in the presence of atractyloside.

Liver perfusion and the presentation of the results are carried out as described in the legend for Fig. 1. The final concentrations of atractyloside and glucagon were indicated in the figure. The dilution of these agents on infusion was about 1:500. The basal respiratory rate before adding atractyloside was 1.95 μmol O_2/min/g wet liver.

glucose output (glycogenolysis) as shown in Table 1. However, when livers from 48h-fasted rats were perfused in the presence of 5 mM lactate as substrate, atractyloside inhibited significantly the glucagon-induced stimulation of glucose output (gluconeogenesis).

Discussion

The validity of the organ fluorometry and spectrophotometry used in the present system has been shown in our previous studies (10-12). The present study clearly demonstrated that glucagon reduces the redox states of cytochromes a(a_3), b and c+c_1. This report is the first one in which the effects of glucagon on the redox states of mitochondrial cytochromes were examined without disrupting the cells, excluding the artefactual modifications during and after the isolation procedure of mitochondria. Moreover,

Table 1

Effects of Atractyloside on Glucagon-induced Glucose Output.

		No. of expts	Glucose output	
			Basal[a]	Glucagon-induced[b]
Fed	control	3	0.73±0.15[c]	15.5±2.1
	atractyloside	3	0.87±0.07	14.6±1.9
Fasted	control	5	0.51±0.03	4.1±0.6
	atractyloside	4	0.68±0.08	0.5±0.2[+]

In experiments with fed-rat livers, they were perfused without substrate. Glucagon (1.4 nM) was added 30 min after the beginning of the perfusion. In experiments with fasted-rat livers, livers from 48h-fasted rats were perfused without substrate for the initial 30 min, then lactate (5 mM) was added. Glucagon (1.4 nM) was added 20 min later. Atractyloside (4 µg/ml) or saline (control) was added 10 min before the addition of glucagon under both conditions.

a Basal glucose output represents the mean glucose output (µmol/min/g wet liver) during 5 min immediately before the addition of glucagon.
b Net increase in glucose output (µmol/g wet liver) during 10 min following glucagon addition (basal glucose output has been subtracted).
c Values indicate mean±SEM.
+ $p<0.005$ vs fasted control (glucagon-induced).

the effects of the hormone on respiratory activity and the redox states of pyridine nucleotide(s) were examined simultaneously in the same system, whereby analysis of the function of mitochondria *in situ* is feasible. The reductive shift of pyridine nucleotide redox state induced by glucagon seems to represent in part, at least, an increase in the reduced form of mitochondrial NAD, as has been proposed previously (19), because the hormone also increased the ß-hydroxybutyrate/acetoacetate ratio in the effluent in a similar time course as that of fluorescence intensity (Fig. 2). Therefore, the present observation indicates that glucagon induced the reduction of mitochondrial NAD and cytochromes in the face of the stimulation of respiration.

The glucagon-induced reduction of NAD and cytochromes does not seem to be due to a limited supply of oxygen, because in our preliminary experiments, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), an uncoupler, induced oxidation of pyridine nucleotide and cytochromes whereas it stimulated oxygen consumption to the same extent as glucagon. Alternatively, the combination of metabolic responses observed in mitochondria *in situ* after the glucagon addition seems to indicate that glucagon increases the

availability of substrates for mitochondrial respiration, because only the shift from a steady state near the state 2 (substrate limited state) toward the state 3 will provoke the same events in mitochondria (14). This can be the mechanism by which glucagon treatment increases state 3 respiration (2) and calcium uptake (3) by isolated mitochondria.

Recently, Richard and his coworkers demonstrated that glucagon stimulates hepatic gluconeogenesis by decreasing the concentration of fructose-2,6-bisphosphate (20,21). In the present study, however, when the glucagon-induced transition of mitochondrial function was inhibited by atractyloside, the gluconeogenic action of glucagon was also inhibited strongly, while the glycogenolytic effect remained unaffected (Table 1). This observation indicates that without the stimulation by glucagon of the substrate entry into mitochondria, the regulation of gluconeogenesis by fructose-2,6-bisphosphate is no more elicitable. In this sense, the reductive shift of redox states of the members of mitochondrial respiratory chain may be a fundamental mechanism by which glucagon stimulates hepatic gluconeogenesis. Although the mechanism by which glucagon stimulates substrate entry into mitochondria was not clarified in the present study, it may be somehow related to the adenine nucleotide translocase system as has been proposed for glucocorticoid action by Kimura and Rasmussen (22), because atractyloside inhibited the effects of glucagon on mitochondria. The present system is shown very useful in monitoring the dynamic function of mitochondria *in situ*.

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