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ROLE OF ZINC AS AN ACTIVATOR OF MITOCHONDRIAL FUNCTION IN RAT LIVER

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Abstract—The effects of zinc on the enzymes of hepatic mitochondria were investigated in rats that had been given zinc sulfate (10 mg Zn^{2+}/100 g body wt) p.σ. Administration of zinc caused a marked elevation of succinate dehydrogenase, glutamate dehydrogenase, cytochrome *c* oxidase and ATPase activities, whereas it did not cause significant changes in pyruvate carboxylase, malate dehydrogenase and isocitrate dehydrognease activities. The effect of zinc as a function of time was greatest on succinate dehydrogenase. Zinc also produced a marked elevation of ATP concentration in the hepatic cytosol and a corresponding increase in ATPase activity in the hepatic mitochondria. Zinc content of the inner membrane of mitochondria was raised significantly by administration of zinc. The removal of zinc by washing in 10 mM EDTA caused a significant decrease of the increased succinate dehydrogenase activity caused by administration of zinc, while it did not lower ATPase activity. The addition of zinc in amounts of 10–10^3 ng Zn^{2+} per mg protein produced a significant increase in succinate dehydrogenase activity in the inner membrane of mitochondria, whereas ATPase activity was elevated significantly at 10^3–10^4 ng Zn^{2+} per mg protein, indicating that zinc activated succinate dehydrogenase more sensitively than ATPase. The present investigation suggests that zinc taken up by hepatic mitochondria stimulates the electron transport system and oxidative phosphorylation and, as a result, increases the ATP concentration in the hepatic cytosol.

It is well known that zinc is essential for the growth of man and many animals [1]. Supplemental zinc in a diet can accumulate markedly in the livers of animals [2, 3]. Zinc, accumulated in the liver cells, causes an increase in metallothionein in the cytosol of liver cells [4, 5]. It is uncertain, however, whether zinc taken up by liver binds to the subcellular organelles and affects the cellular metabolic systems.

Earlier publications from this laboratory have indicated that liver is a target organ of zinc and that the metal taken up by liver cells is stored by binding to the cytosolic proteins [6]. Zinc also accumulated in the mitochondria and markedly raised succinate dehydrogenase activity in that organelle [7]. The present study was undertaken, therefore, to investigate the effects of zinc on mitochondrial enzymes in liver after a single oral administration of zinc sulfate to rats. We found that zinc plays a role as an activator of mitochondrial function in rat liver.

MATERIALS AND METHODS

Young male Wistar rats weighing 100–200 g were obtained from the Nippon Bio Supp. Center Co., Tokyo. The animals were fed commercial laboratory chow and distilled water freely until use. Zinc sulfate was dissolved in distilled water to a concentration of 10 mg as Zn^{2+} per ml. This solution (1.0 ml/100 g body wt) was orally administered to rats fasted for 2 hr before the experiments. The animals were bled by cardiac puncture 3, 12 and 24 hr after zinc administration. Malonic acid or 2,4-dinitrophenol was dissolved in distilled water to concentrations of 0.5 mg/ml and 0.1 mg/ml. These solutions (1.0 ml/100 g) were intraperitoneally injected in rats administered zinc (10 mg/100 g) 11 hr before. The rats bled 1 hr after drug injection.

The livers were perfused with ice-cold 0.25 M sucrose solution and frozen immediately, cut into small pieces, suspended 1:4 in 0.25 M sucrose solution, and homogenized in a Potter–Elvehjem homogenizer with a Teflon pestle. The homogenate was spun at 600 *g* in a refrigerated centrifuge for 10 min and the supernatant fraction was spun at 5,500 *g* for 12 min to obtain the mitochondrial fraction. The 5,500 *g* supernatant fraction was further spun at 105,000 *g* for 60 min and its supernatant fraction (cytosol) was collected. Mitochondrial preparations for enzyme analysis were resuspended in ice-cold distilled water. The cytosol was immediately used to determine ATP concentration.

The inner membrane fractions of mitochondria were prepared by the method of Okamoto [8]. Mitochondrial preparations resuspended in ice-cold mannitol solution (0.21 M mannitol, 0.07 M sucrose and 0.1 mM EDTA) were washed six times by centrifugation for 8 min at 8,500 *g*. Washed mitochondria were resuspended in ice-cold mannitol solution containing 2% digitonin by stirring for 15 min at 4°. The inner membrane fraction of mitochondria was prepared from the pellets obtained by centrifugation for 12 min at 12,000 *g*, and this fraction was washed once with ice-cold mannitol solution. In separate experiments, the pellet of inner membrane was stirred, in ice-cold mannitol solution containing 10 mM EDTA, for 10 min at 4°, to remove zinc. This suspension was

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washed by centrifugation with ice-cold mannitol solution. The fractions of inner membrane of mitochondria for enzyme analysis were resuspended in ice-cold distilled water.

Succinate dehydrogenase activity was measured after incubation for 15 min at 37° of the reaction mixture in a final volume of 1.0 ml containing 50 mM potassium phosphate buffer (pH 7.4), 0.1% 2-(*p*-iodophenyl) - 3 - (*p* - nitrophenyl) - 5 - phenyltetrazolium chloride, 50 mM sodium succinate, 2.5 mM sucrose and the protein (5–10 μg), with or without the appropriate additions of zinc (10^{-5}–10^{-3} μg Zn^{2+} per mg protein) [9]. After the addition of 10% trichloroacetic acid (1 ml) and the extraction with ethyl acetate (4 ml), the absorbance was measured at 490 nm. Enzyme activity was expressed as absorbance at 490 nm per min per mg protein.

ATPase activity in the reaction mixture was measured after incubation for 10 min at 37° in a final volume of 1.0 ml containing 5 mM ATP, 5 mM $MgSO_4$, 20 mM potassium phosphoenolpyruvate, 32 μg pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40), 20 mM Tris–H_2SO_4 (pH 7.4) and the protein (50–60 μg) [10], with or without the appropriate additions of zinc (1–10^6 ng Zn^{2+} per mg protein). Trichloroacetic acid (50%, 0.1 ml) was added, and the supernatant fraction (0.5 ml) after centrifugation was mixed with 3.25 ml of water, 1 ml of 2.5% ammonium molybdate in 5 N H_2SO_4, and 0.25 ml of aminonaphthol sulfonate solution. The mixture was incubated at 30° for 10 min, and the absorbance was measured at 660 nm. Enzyme activity was expressed as nmoles of phosphate released per min per mg protein.

All of the enzyme assays described below were carried out under optimal conditions with protein of 1–15 μg. Glutamate dehydrogenase and isocitrate dehydrogenase activities were assayed at 37° according to the methods of King [11] and Bernt and Bergmeyer [12] respectively. Malate dehydrogenase, pyruvate carboxylase and cytochrome *c* oxidase activities were measured at 25° according to the methods of Siegel and Bing [13], Barden *et al.* [14], and Orii and Okunuki [15] respectively.

The enzymatic determination of adenosine-5′-triphosphate (ATP) in the cytosol with 3-phosphoglycerate kinase, PGK (ATP:3-phospho-D-glycerate-1-phosphotransferase, EC 2.7.2.3), was measured by the method of Jaworek *et al.* [16].

Protein concentration was determined by the method of Lowry *et al.* [17].

Zinc content of the inner membrane fraction of mitochondria was determined by atomic absorption spectrophotometry after digestion with nitric acid and was expressed as the amount of zinc (ng) per mg protein of the inner membrane fraction.

The significance of the difference between values was estimated by Student's *t*-test. P values of less than 0.05 were considered to indicate statistically significant differences.

RESULTS

Effect of zinc administration on activities of enzymes in mitochondria. The effect of zinc administration on various enzymes in hepatic mitochondria was examined after a single oral dose of zinc sulfate (10 mg Zn^{2+}/100 g) in rats as shown in Table 1. Of a number of enzymes tested, only succinate dehydrogenase activity was increased significantly 3 hr after zinc administration. Glutamate dehydrogenase, succinate dehydrogenase, cytochrome *c* oxidase and ATPase activities were elevated significantly 12 hr after zinc administration, while malate dehydrogenease, isocitrate dehydrogenase and pyruvate carboxylase activities were not altered significantly. Of the increased enzymes, only ATPase activity was enhanced significantly even at 24 hr after zinc administration.

The effects of increasing doses of zinc on succinate dehydrogenase and ATPase activities in hepatic mitochondria are shown in Fig. 1. The rats were killed 12 hr after a single oral dose of zinc (5, 10 and 20 mg Zn^{2+}/100 g). All the doses of zinc caused a

Table 1. Changes of the activities of various enzymes in the hepatic mitochondria of rats after a single oral dose of zinc sulfate*

Enzyme	Activity			
	Hours after zinc administration			
	0	3	12	24
Pyruvate carboxylase†	108.9 ± 18.9	106.7 ± 2.1	99.0 ± 10.2	101.9 ± 4.3
Glutamate dehydrogenase†	112.7 ± 7.9	137.7 ± 11.1	231.0 ± 26.0‡	89.9 ± 5.6
Succinate dehydrogenase§	67.3 ± 1.9	99.9 ± 4.5‡	94.8 ± 1.5‡	65.4 ± 1.9
Malate dehydrogenase‖	1.76 ± 0.13	1.82 ± 0.12	1.95 ± 0.13	1.80 ± 0.12
Isocitrate dehydrogenase†	120.4 ± 2.2	126.3 ± 2.6	107.8 ± 2.5	118.0 ± 3.1
Cytochrome *c* oxidase¶	35.7 ± 3.7	38.6 ± 1.5	46.1 ± 2.0‡	35.0 ± 5.4
ATPase‖	5.42 ± 0.13	6.23 ± 0.46	8.38 ± 0.30‡	7.52 ± 0.26‡

* Zinc (10 mg/100 g) was administered orally. Each value is the mean ± S.E.M. of five animals.
† Expressed as nmoles per min per mg protein.
‡ $P < 0.01$, compared with the control zero time.
§ Expressed as 10^{-2} × absorbance at 490 nm per min per mg protein.
‖ Expressed as μmoles per min per mg protein.
¶ Expressed as sec^{-1} per cuvette per mg protein.

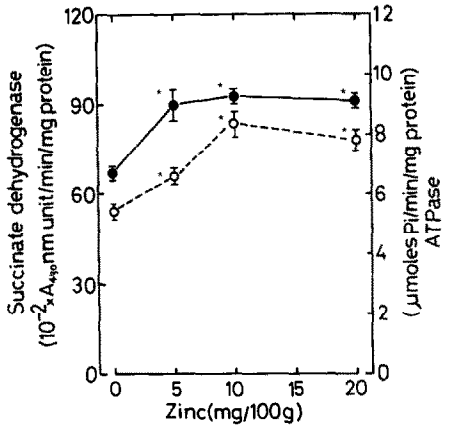

Fig. 1. Effects of increasing doses of zinc on succinate dehydrogenase and ATPase activities in the hepatic mitochondria of rats. The rats were killed 12 hr after a single oral dose of zinc sulfate (5, 10 and 20 mg Zn^{2+}/100 g). Each point is the mean of five animals. Vertical lines represent the S.E.M. Key: (*) $P < 0.01$, compared with the control; (—●—) succinate dehydrogenase; and (---○---) ATPase.

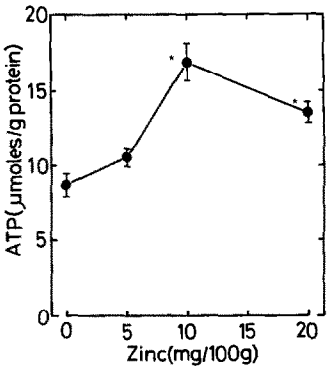

Fig. 3. Effects of increasing doses of zinc on ATP concentration in the hepatic cytosol of rats. The rats were killed 12 hr after a single oral dose of zinc sulfate (5, 10 and 20 mg Zn^{2+}/100 g). Each point is the mean of five animals. Vertical lines represent the S.E.M. Key: (*) $P < 0.01$, compared with the control.

significant increase in succinate dehydrogenase and ATPase activities.

Effect of zinc administration on ATP concentration in cytosol. The change of ATP concentration in the hepatic cytosol after a single oral dose of zinc sulfate (10 mg Zn^{2+}/100 g) in rats is shown in Fig. 2. ATP concentration was increased markedly 12 hr after zinc administration. This increase was observed even at 24 hr after zinc administration.

The effects of increasing doses of zinc on ATP concentration in hepatic mitochondria are shown in Fig. 3. The rats were killed 12 hr after a single oral dose of zinc (5, 10 and 20 mg Zn^{2+}/100 g). Zinc administration produced a slight but not significant increase in ATP concentration at the lowest dose (5 mg Zn^{2+}/100 g). With higher doses, the effect was remarkable, but the effect of 20 mg Zn^{2+}/100 g was significantly less than that of 10 mg Zn^{2+}/100 g.

Effect of inhibitors on succinate dehydrogenase and ATPase activities in mitochondria. The effects of malonic acid and 2,4-dinitrophenol on succinate dehydrogenase and ATPase activities in hepatic mitochondria are shown in Table 2. The administration of malonic acid (0.5 mg/100 g) and 2,4-dinitrophenol (0.1 mg/100 g) in the control rats produced a marked reduction of succinate dehydrogenase and ATPase activities. Although the inhibitory effects by malonic acid were also seen in the zinc-treated rats, these enzymes' activities, decreased by malonic acid, were significantly restored by the administration of zinc (10 mg Zn^{2+}/100 g). On the other hand, the restoring effects of zinc were not observed in the case of 2,4-dinitrophenol administration.

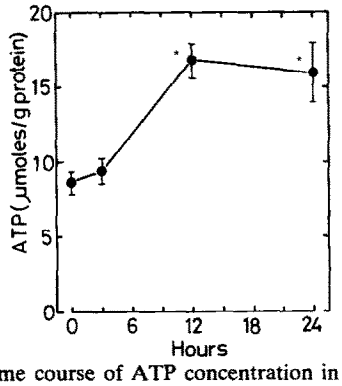

Fig. 2. Time course of ATP concentration in the hepatic cytosol of rats after a single oral dose (10 mg/100 g) of zinc sulfate. Each point is the mean of five animals. Vertical lines represent the S.E.M. Key: (*) $P < 0.01$, compared with the control zero time.

Effect of EDTA treatment on zinc content and on succinate dehydrogenase and ATPase activities in inner membrane of mitochondria. The time courses of zinc content and of succinate dehydrogenase and ATPase activities in the inner membrane fraction of hepatic mitochondria after a single oral dose of zinc sulfate are shown in Table 3. At 3 hr after the administration of zinc (10 mg/100 g), zinc content and succinate dehydrogenase activity in the inner membrane fraction were increased significantly but ATPase activity was not. Significant increases in zinc content and in the activities of the two enzymes, however, were observed 12 hr after zinc administration. Of these, that of ATPase activity continued for another 12 hr. Then, when the inner membrane fraction was washed with 10 mM EDTA solution, the zinc content and succinate dehydrogenase activity in the inner membrane fraction, obtained from both control rats and zinc-treated rats, were decreased significantly (Table 4). ATPase activity in the inner membrane fraction of those rats, however, was not changed significantly by 10 mM EDTA treatment.

Effects of zinc addition on succinate dehydrogenase and ATPase activities in inner membrane of mitochondria. The effects of Zn^{2+} addition on succinate dehydrogenase and ATPase activities in the inner membrane fraction of hepatic mitochondria in normal rats are shown in Fig. 4. Succinate dehydrogenase activity was clearly increased by addition of zinc in the range of 10^1–10^3 ng Zn^{2+} per mg protein of inner membrane. The maximum effect was observed

Table 2. Effects of malonic acid and 2,4-dinitrophenol on succinate dehydrogenase and ATPase activities in the hepatic mitochondria of rats after a single oral dose of zinc sulfate*

Treatment	Succinate dehydrogenase [$10^{-2} \times A_{490} \cdot min^{-1} \cdot (mg\ protein)^{-1}$]	ATPase [$\mu moles\ P_i \cdot min^{-1} \cdot (mg\ protein)^{-1}$]
Control		
None	67.3 ± 1.9	5.43 ± 0.20
Malonic acid	37.8 ± 2.9†	2.32 ± 0.28†
2,4-Dinitrophenol	49.8 ± 2.3†	3.18 ± 0.18†
Zinc		
None	94.8 ± 1.5	8.38 ± 0.30
Malonic acid	47.3 ± 1.3†,‡	4.52 ± 0.15†,‡
2,4-Dinitrophenol	50.5 ± 2.1†	3.42 ± 0.18†

* Zinc (10 mg/100 g) was administered orally, and 11 hr later the rats received a single intraperitoneal injection of malonic acid (0.5 mg/100 g) or 2,4-dinitrophenol (0.1 mg/100 g). The rats were killed 12 hr after zinc administration. Each value is the mean ± S.E.M. of five animals.

† $P < 0.01$, compared with the "none" group.

‡ $P < 0.01$, compared with the control malonic acid group.

Table 3. Changes of zinc content and of succinate dehydrogenase and ATPase activities in the liver membrane fraction of hepatic mitochondria in rats after a single oral dose of zinc sulfate*

Hours after zinc administration	Zinc content (ng/mg protein)	Succinate dehydrogenase [$10^{-2} \times A_{490} \cdot min^{-1} \cdot (mg\ protein)^{-1}$]	ATPase [$\mu moles\ P_i \cdot min^{-1} \cdot (mg\ protein)^{-1}$]
0	117 ± 5	53.7 ± 3.5	9.98 ± 0.27
3	149 ± 5†	87.8 ± 4.3†	10.90 ± 0.53
12	146 ± 2†	77.5 ± 2.7†	14.75 ± 0.35†
24	127 ± 12	47.0 ± 5.4	13.13 ± 1.25†

* Zinc (10 mg/100 g) was administered orally. Each value is the mean ± S.E.M. of five animals.

† $P < 0.01$, compared with the control zero time.

Table 4. Effect of EDTA treatment on zinc content and on succinate dehydrogenase and ATPase activities in the inner membrane fraction of hepatic mitochondria in rats after a single oral dose of zinc sulfate*

Treatment	Zinc content (ng/mg protein)	Succinate dehydrogenase [$10^{-2} \times A_{490} \cdot min^{-1} \cdot (mg\ protein)^{-1}$]	ATPase [$\mu moles\ P_i \cdot min^{-1} \cdot (mg\ protein)^{-1}$]
Control			
None	118 ± 4	53.7 ± 3.5	9.98 ± 0.27
EDTA	102 ± 3†	35.8 ± 2.3†	10.21 ± 0.40
Zinc			
None	148 ± 5	77.5 ± 2.7	14.70 ± 0.35
EDTA	132 ± 2†	58.5 ± 4.3†	14.68 ± 0.17

* Rats were killed 12 hr after a single oral dose of zinc (10 mg/100 g). The inner membrane fraction prepared from hepatic mitochondria was washed with 10 mM EDTA solution by stirring for 10 min at 4°. Each value is the mean ± S.E.M. of five animals.

† $P < 0.01$, compared with the "none" group.

at 10^3 ng Zn^{2+} per mg protein. With the higher levels of zinc, the enzyme activity rapidly began to decrease.

On the other hand, ATPase activity was not elevated significantly by addition of 10^1 and 10^2 ng Zn^{2+} but was by 10^3 and 10^4 ng Zn^{2+} per mg protein. This enzyme activity was markedly reduced by 10^6 ng Zn^{2+} per mg protein.

DISCUSSION

It has been recently found that succinate dehydrogenase activity in hepatic mitochondria markedly increases in rats after giving them a single oral dose of zinc sulfate [7]. However, the effect of zinc on other enzymes in hepatic mitochondria remains to be established. In the present study, we examined the effects of zinc orally administered on the activities of pyruvate carboxylase, glutamate dehydrogenase, succinate dehydrogenase, malate dehydrogenase, isocitrate dehydrogenase, cytochrome *c* oxidase and ATPase in hepatic mitochondria of rats. Of these enzymes, the activities of glutamate dehydrogenase, succinate dehydrogenase, cytochrome *c* oxidase and ATPase were clearly increased by zinc administration. Thus, zinc administration largely stimulated

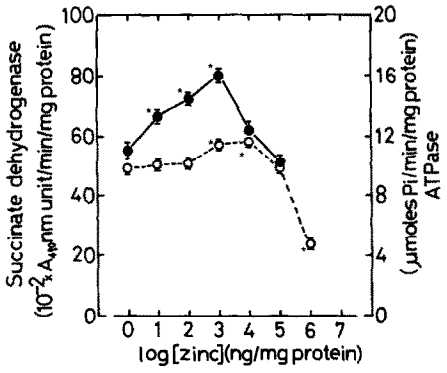

Fig. 4. Effect of zinc addition on succinate dehydrogenase and ATPase activities in the inner membrane fraction of hepatic mitochondria in rats. Zn^{2+} was added in the incubation mixture (see Materials and Methods) to concentrations of $1–10^6$ ng per mg protein of the inner membrane prepared from hepatic mitochondria of normal rats. The addition of 1 ng Zn^{2+}/mg protein had no effect on the activation of the two enzymes in comparison with the values of no addition. Each point is the mean of five animals. Vertical lines represent the S.E.M. Key: (*) $P < 0.01$, compared with the values of no addition; (—●—) succinate dehydrogenase; and (---○---) ATPase.

electron transport and oxidative phosphorylation in hepatic mitochondria. Furthermore, the metal administration produced a marked elevation of ATP concentration in hepatic cytosol. These results suggest that zinc may play a role as an activator in the mitochondrial function of rat liver.

We also examined the effects of malonic acid and 2,4-dinitrophenol on the effect of zinc in hepatic mitochondria. It is well known that malonic acid is an inhibitor of electron transport and 2,4-dinitrophenol is an inhibitor of oxidative phosphorylation. Both inhibitors produced remarkable reductions of succinate dehydrogenase and ATPase activities in the hepatic mitochondria of control rats. It was found, however, that the decreases in the activities of these enzymes by malonic acid were significantly restored by zinc administration, while the metal administration had no effect on the inhibition by 2,4-dinitrophenol. From these results, it was suggested that zinc may have a more sensitive effect on the electron transport system than on oxidative phosphorylation in the hepatic mitochondria of rats. The mechanisms of interaction of zinc and inhibitors on mitochondrial function, however, remain to be elucidated.

The time course study of zinc content, succinate dehydrogenase activity and ATPase activity in the inner membrane of hepatic mitochondria after zinc administration indicates that zinc which has accumulated in mitochondrial inner membrane first elevates succinate dehydrogenase activity and, later on, enhances ATPase activity. The treatment with EDTA caused a reduction of zinc content and a corresponding fall in succinate dehydrogenase activity in the inner membrane, while ATPase activity was not altered significantly. These findings suggest that zinc may activate more directly succinate dehydrogenase than ATPase in the inner membrane of hepatic mitochondria.

Furthermore, the studies of Zn^{2+} addition to a mitochondrial inner membrane fraction confirm that zinc directly activates succinate dehydrogenase. The levels of zinc that were found in the inner membrane fraction of hepatic mitochondria in rats orally administered zinc sulfate could clearly enhance succinate dehydrogenase activity, but not ATPase activity.

It was reported that concentrations lower than 4 μM Zn^{2+} caused respiratory stimulation in coupled mitochondria isolated from rat liver [18]. Conversely, zinc ions were reported to be effective inhibitors of the respiratory chain of mitochondria; the inhibition site by 1–10 μM Zn^{2+} was between cytochromes *b* and c_1, while ATPase was not inhibited [19]. Thus, the *in vitro* effect of zinc on mitochondrial function in rat liver has not been fully solved. The *in vivo* effect of zinc on hepatic mitochondria is also little understood.

The primary findings, that oral administration of zinc sulfate in rats produces an activation of mitochondrial function and a corresponding production of ATP in the liver cells, suggest a physiological significance of zinc in the regulation of cell function.

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