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Studies on swelling-shrinkage and oxidative phosphorylation of liver mitochondria of rat fed on 3'-methyl-4-dimethylaminoazobenzene

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Studies on swelling-shrinkage and oxidative phosphorylation of liver mitochondria of rat fed on 3'-methyl-4-dimethylaminoazobenzene*

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Abstract

The swelling-shrinkage and oxidative phosphorylation of rat liver mitochondria affected by 3'-methy1-DAB feeding were observed in correlation with function by the method mentioned, and the following results were obtained: 1. By feeding 3'-methy1-DAB the swelling-shrinkage ability of rat liver mitochondria showed a remarkable alteration reducing in the amplitude. It reduced gradually during the days of feeding, reached the minimum value on 30th day and restored gradually thereafter (in Case 1). 2. ADP/O ratio also decreased by feeding the carcinogen reached the minimum point on 30th day and increased on 38th day showing the similar tendency in the decrease of the swelling-shrinkage amplitude (in Case 1). 3. The mitochondria from the hepatoma, which was induced by 3'-methy1DAB feeding, showed a lower amplitude in swelling-shrinkage with the dropped ADP/O ratio compared with those of mitochondria from liver tissue neighbouring the tumor. 4. The mechanism in the reduction of swelling-shrinkage ability has been discussed in the relation with fatty acid composition of mitochondria which is reported elsewhere. 5. From the above results it is deduced that lowered ability for swellingshrinkage with the reduced oxidative phosphorylation will be somehow related to the mechanism of cancer induction.

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STUDIES ON SWELLING-SHRINKAGE AND OXIDATIVE PHOSPHORYLATION OF LIVER MITOCHONDRIA OF RAT FED ON 3'-METHYL-4-DIMETHYL-AMINOAZOBENZENE

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According to the reports of ARCOS *et al.*[1,2], the liver mitochondria from the rat fed with several chemical carcinogens reduce their swelling ability, the high amplitude swelling[3], as observed *in vitro* by light absorption at 520 mμ. It is generally believed that the swelling of mitochondria observed by that method is partly related to the functioning state of electron transport system[3]. But the method is inadequate to clarify any minute changes in the functioning state of the mitochondria. For this reason we tried to observe the swelling-shrinkage changes of mitochondria by 90° light-scattering, designated low amplitude[3], by which the minute changes occurring in the volume of mitochondria geared to the physiological functioning state of respiration can be checked[3-6].

In the present paper, it is reported that the rat liver mitochondria affected by feeding 3'-methyl-4-dimethyl-aninoazobenzene (3'-methyl-DAB) reduce their ability for swelling-shrinkage in amplitude with close correlation to the reduction in oxidative phosphorylation, as revealed by observing them under 90° light-scattering in the cuvette for oxymeter which has been designed and constructed by UTSUMI[6].

MATERIALS AND METHODS

Albino male rats weighing between 180 and 220 g were conditioned prior to the experiments by feeding the polished rice for a week in the first and for a month in the second experiment. The dye feeding groups were given 0.6 g of 3'-methyl-DAB per 100 g of polished-rice *ad libitum* and the control groups were fed on rice without carcinogen. In each experiment, from 6 to 10 rats were killed by decapitation and liver mitochondria were isolated by a modified method of HOGEBOOM described previously[9]. The sucrose used in the isolation contained 1 mM Tris-HCl buffer (pH 7.4) and 0.05 mM ethylenediamine tetraacetate

(EDTA). Final mitochondrial pellet was suspended in one ml of 0.25 M sucrose as to represent 2 g tissue equivalent.

The oxygen consumption and swelling-shrinkage change of the mitochondria were measured using an apparatus[6,7] which can record simultaneously. The oxygen consumption was recorded by the rotating platinum electrode, while the swelling-shrinkage change was 90° light-scattering at 520 or 650 mμ. The basic medium in the first experiment was composed of 0.05 M sucrose, 0.02 M KCl, 5 mM K-phosphate buffer (pH 7.4) and 0.1 mM EDTA, and that in the second added with 1 mM $MgCl_2$. The reaction was started by the addition of mitochondrial suspension at 25°C in total volume of 2 ml. The amount of mitochondria used in first experiments was 0.2 g tissue equivalent of liver and in second experiments was adjusted to show the constant level of scattering which was about 3 mg protein in intact mitochondria. In this case the mitochondrial content per tissue equivalent during the feeding decreased transiently but the extent of its scattering intensity per mg protein was not changed. Reaction was observed by the subsequent addition of 10 mM Na-succinate, 100 μM ADP and 0.16 mM Na-oleate, respectively, and then high amplitude swelling was recorded by a large amount of oleate. The ADP/O ratio was calculated from the amount of consumed oxygen by addition of ADP, and respiratory control index was indicated by the ratio between the rates of respiration in the presence of ADP and in its absence[8]. The degree of respiratory release with oleate was the ratio between the rates of oxygen consumption in the presence of 0.16 mM oleate and in state after phosphorylation of added ADP. The scattering was adjusted to read 100 per cent on the chart paper by showing initial 90° light-scattering level by addition of mitochondria to the basic medium.

The ATPase activity was tested as described in the previous paper[9], and the medium was composed of 0.1 M sucrose, 0.02 M KCl, 0.05 mM EDTA, 5m M Tris-HCl buffer (pH 7.4), 3m M ATP and 0.16 mM oleate. Total volume was 2 ml and reaction time was 10 minutes at 25°C.

The rats were placed on the carcinogen-containing diet for 50 days, followed by another day of a semisynthetic diet, then the tumor mass became visible in the liver on 8th month. Both mitochondria from the tumor and non-tumor tissue separated from the livers of 4 rats were isolated under the identical conditions, then simultaneous measurements of oxygen consumption, 90° light-scattering change and relative fluorescence intensity were taken using a modified apparatus[7].

ADP and ATP were obtained from Shigma Chem. Co., and other chemicals were of reagent grade. Na-oleate (pH 7.4) was prepared from oleic acid obtained from Tokyo Kasei Co. The distilled water used to make up the solution was deionized. Mitochondrial protein was measured by Kjeldahl technique[10].

RESULTS

The mitochondria isolated from the rat liver of control group on 1st day showed the typical swelling-shrinkage changes coupled with the changes of oxygen consumption on addition of various reagents as shown in Fig. 1. The endogenous oxygen consumption and the swelling (decrease in 90° light-scattering) of mitochondria were observed to a slight extent and these were accelerated on addition of succinate. The oxygen consumption was enhanced by about 3 folds on addition of ADP, the respiratory control index was 3.1, and the shrinkage (increase in 90° light-scattering) was induced. After the phosphorylation of added ADP to ATP, the velocity of oxygen consumption returned to a steady state, and the shrinkage reversed to swelling. ADP/O ratio was 2.1. On addition of oleate, the respiration was released, followed by a fall-off and there occurred a remarkable reversal of mitochondrial swelling. The degree of respiratory release was 2.1 of the initial velocity and the percent reversal of swelling was 123%. A large amount of oleate promoted the drastic swelling (high amplitude).

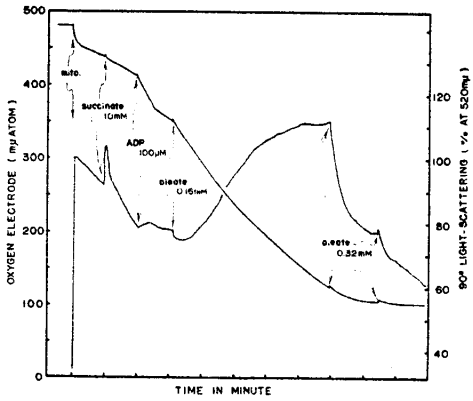

Fig. 1. The records of oxygen electrode and 90° light-scattering of rat liver mitochondria on the first day in control group. The reaction mixture contained 0.05 M sucrose, 0.02 M KCl, 5 mM K-phosphate buffer (pH 7.4) and 0.1 mM EDTA. Reaction started by the addition of mitochondria (0.2 g tissue equivalent), total volume, 2 ml and temperature at 25°C. Other additions were as in Fig. Upper trace refers to oxygram and lower trace to 90° light-scattering. Time moved from left to right.

The results of the first experiment are shown in Table 1. During the dye feeding, the ADP/O ratio of the mitochondria decreased progressively, attaining the minimum rate (1.6) on the 30th day, and increased again on the 38th day (2.0), thus the recovery of ADP/O ratio to normal level was observed despite the continuous feeding of the 3'-methyl-DAB. Proportional to the ability of phosphorylation, the respiratory control was minimized on the 30th day (2.0), which was followed by the recovery on the 38th day (2.3). On the countrary, the ADP/O ratio of the control group mitochondria remained unchange during the experimental period though the respiratory control was slightly increased.

The rate of respiratory release with oleate of mitochondria during the dye feeding proved to be about 2 folds on early period (6th day), but decreased

gradually to 1.7 (30th day) and recovered to normal as in the case of respiratory control. In contrast, the rates in control groups hardly changed throughout the experiments.

Table 1 Alterations of the oxidative phosphorylation and of the 90° light-scattering changes with oleate on succinate linked system of mitochondria from rat liver during feeding of 3'-methyl-DAB and non-feeding groups. The condition and the reaction mixture were described in the text and Fig. 1. Details of the calculations related to respiration were in the text and the rate of swelling was represented by the decreased light-scattering from the initial scattering level to the addition of oleate (for 3 minutes, induced by Pi and succinate) and the shrinkage with oleate (0.16 mM) was represented by the percent of increased light-scattering (for 4 minutes) after the treatment with oleate.

Condition in Exp. 1 Days	ADP/O Ratio	Respiratory Control Index	Rate of Respiratory Release with Oleate	% Change in Light-Scattering		% Reversal of Swelling
				Swelling	Shrinkage with Oleate	
Control-1	2.1	3.1	2.1	26	32	123
Control-10	2.1	3.3	2.1	30	15	50
Control-21	2.1	3.7	2.2	25	14	56
Control-43	2.0	3.6	2.6	23	12	52
Fed-DAB-6	2.0	3.8	4.7	28	26	93
Fed-DAB-13	1.9	3.6	3.9	23	20	87
Fed-DAB-20	1.8	3.3	2.7	23	9	39
Fed-DAB-30	1.6	2.0	1.7	18	4	22
Fed-DAB-38	2.0	2.3	1.9	23	9	39

The extent of the spontaneous and succinate-induced swelling did not differ much between the dye-fed and the non-fed rat liver mitochondria but the swelling decreased slightly on 30th day in the dye-fed group. However, when the ability of mitochondrial shrinkage was measured by addition of 0.16 mM oleate to the 0.2 g tissue equivalent of the liver mitochondria, the extent of shrinkage during feeding of 3'-methyl-DAB was found to be of a particular significance to the fact that the shrinkage decreased successively to 30th day accompanied by increase on 38th day as shown in Fig. 2 and Table 1. This fact can be represented more clearly by the per cent reversal of the swelling as indi-

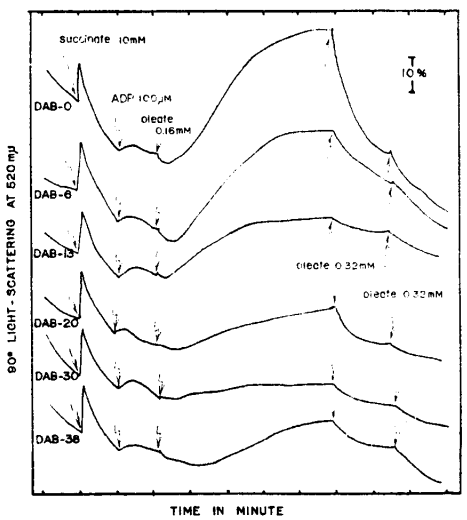

Fig. 2. Alteration in 90° light-scattering of rat liver mitochondria during feeding of 3'-methyl-DAB. Details are given in Fig. 1.

cated in Table 1, which was reduced by about 80% on 30th day proportional to the alteration of respiratory release with oleate, phosphorylation and respiratory control. In addition, the extent of the swelling induced by a large amount of oleate was proportionate to the extent of the ability of shrinkage with oleate in a small amount as shown in Fig. 2. On the other hand, the percent swelling of the control mitochondria was a half of that on 1st day, but constant level was observed later.

In the second experiment, ADP/O ratio of the dye-fed mitochondria was reduced transiently as in the former experiments although the minimum rate was observed a little early (on about 3rd week), and respiratory control was also decreased transiently, as shown in Table 2. Percent swelling of mitochondria

Table 2 Alterations of structure and function of rat liver mitochondria during feeding of 3'-methyl-DAB. Incubation mixture was as in Fig. 1 except with 1 mM $MgCl_2$. The reaction started by the addition of the mitochondria (about 3 mg protein) showing the constant level of initial light-scattering. Other measuring details are in Table 1 and the text. Control was the avarage in 3 cases (on 1st, 15th, 31st days) of control groups. ATPase was indicated as formed μmoles Pi/mg protein/hour.

Condition in Exp. 2 Days	ADP/O Ratio	Respiratory Control Index	% Change in Light-Scattering				Oleate-induced ATPase Activity	mg Protein of mitochondria/1g Tissue Equivalent
			% Swelling	% Reversal of Swelling with Oleate				
				80 μM	160 μM	240 μM		
Control	2.4	3.8	18	100	122	118	7.1	1.7
Fed-DAB-9	2.3	3.4	18	89	128	71	9.1	1.4
Fed-DAB-16	1.7	2.5	16	91	100	32	6.0	0.9
Fed-DAB-23	1.7	3.0	18	71	100	96	—	—
Fed-DAB-29	2.4	4.1	18	60	140	100	12.2	1.4

with succinate was not altered throughout the dye-feeding, but the reversal of swelling induced by oleate was proportional to respiratory control. The drastic swelling induced by a large amount of oleate diminished transiently parallel to the lessening of its shrinkage by a small amount of oleate during dye feeding and to oleate induced latent ATPase activity. On the countrary, the control groups tested on 1st, 15th and 31st days showed no alteration in the abilities and activities of the above phenomena.

As shown in Fig. 3, the ADP/O ratio of the mitochondria from tumor tissue was lower (1.7) than that from the non-tumor tissue (2.0) with succinate as substrate, and the respiratory control was 3.1 and 3.0 respectively. The extent of scattering changes of the mitochondria from tumor tissue induced by Pi, succinate and ADP was lowered less as compared to that from non-tumor tissue, but the pattern of the swelling-shrinkage changes was similar. However, the degree of oleate-induced shrinkage and swelling of tumor mitochondria was

less than that of non-tumor mitochondria, especially drastic swelling was never observed when oleate in a large amount was added to tumor mitochondria. The trace of relative fluorescence, which shows the oxidation and reduction of pyridine nucleotides, revealed the identical pattern in both cases. Namely, the ADP-induced oxidation of pyridine nucleotides was brought about clearly and the reduction was induced by phosphorylation of ADP to ATP, but

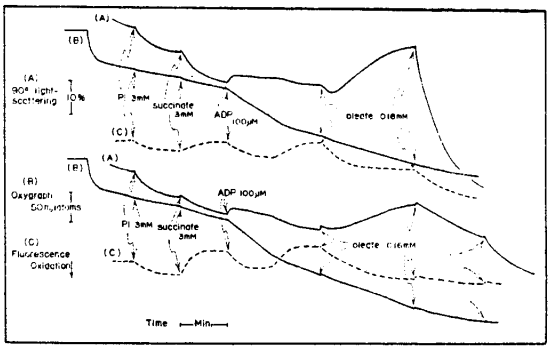

Fig. 3. The oxygen consumption, 90° light-scattering at 650 mμ and relative fluorescence intensity of reduced pyridine nucleotides of mitochondria isolated from the tumor and non tumor tissue in livers of rats fed 3'-methyl-DAB. Reaction mixture contained 0.1 M sucrose, 0.02 M KCl, 50 μM EDTA, 10 mM Tris-HCl buffer (pH 7.4) and about 2 mg protein mitochondria. Other additions are shown in Fig. Upper traces indicate the records of non-tumor and lower traces of tumor tissue mitochondria. Details are given in the text.

the relative extinction of pyridine nucleotides fluorescence induced by succinate was higher in the tumor mitochondria than that of non-tumor ones. Rate of the intensity of oxidation induced by oleate was much lower in the tumor mitochondria as compared with a steady level of the intensity in the non-tumor ones.

DISCUSSION

Mitochondria manifest a mechanochemical change which is coupled to its energy transfer reaction[3-6, 9, 11], especially the change measured by 90° light-scattering (low amplitude) is closely correlated to the oxidative phosphorylation. In the present experiment, the measurement by 90° light-scattering was carried out to detect the structural alteration of rat liver mitochondria during 3'-methyl-DAB feeding, and ADP/O ratio and respiratory control, the determining factors of the intactness of the mitochondria, were measured to detect the functional alteration. In addition, the effect of oleate, known to be a kind of endogenous uncoupling factor[9], was observed to find out the alterations of the mitochondrial function and structure.

During feeding of 3'-methyl-DAB, the capacity of oxidative phosphorylation was decreased transiently parallel with the decrease in respiratory control and in respiratory release extent with uncoupler. Similar alteration was also observed in the oleate-induced latent ATPase activity. These alterations suggest that the function of the mitochondria was affected obviously by the dye feeding in an earlier period, and the control mechanism in energy yielding reaction was slightly modified.

It is of a particular significance that in the first experiment fatty acid-induced scattering changes, shrinkage (in low amplitude) and swelling (in high amplitude), decreased successively in an earlier period of the dye feeding comparing to the extent of substrate-induced swelling which demonstrated well the normal extent of swelling. A similar pattern of the swelling ability (in high amplitude) was demonstrated by ARCOS *et al.* [1], in their study on mitochondria of the liver from rat fed on 4'-methyl-DAB, and the decreased swelling ability was further observed on the mitochondria after feeding of 4-DAB[12],[13].The transient property of the scattering changes by the same concentration of oleate to the tissue equivalent mitochondria, however, may probably be due to the difference in the mitochondrial concentration[9]. Several authors[1,14] reported the decrease of the mitochondrial content from the liver of rat fed on carcinogenic dye. As the matter of fact, the mitochondria per tissue equivalent fell in parallel with the decrease of phosphorylating ability, but the extent of scattering per mg mitochondrial protein showed the constant level. With the results that amount of mitochondria was adjusted in the second experiment, the transient decrease of scattering changes induced by oleate was also observed during feeding of 3'-methyl-DAB and occurred in parallel with the decrease of the phosphorylating activities. Thus, it is suggested that the structural alteration of mitochondria occurred during the dye feeding related to the functional alterations.

The structural alteration suggests the change in the structural rigidity of the mitochondrial menbrane. The composition of fatty acids in mitochondrial menbrane would contribute the flexibility and permeability of the menbrane. RICHARDSON and TAPPEL[15] reported on fish and rat liver mitochondria that the ingredient of unsaturated fatty acids in mitochondrial menbrane is correlated to the swelling. Another expreiments[16,17] carried out together with the present experiment demonstrated that the fatty acid component was altered in the mitochondria from rat liver fed on the dye, particularly palmitic acid and linolenic acid. The decreased oleate-induced scattering changes were observed in parallel with the decrease of the unsaturated fatty acid component ($C_{18:2}$) and the increase of saturated one ($C_{16:0}$). It is likely that the oleate-induced scattering changes is correlated to the increase of saturated fatty acids in the mitochondrial menbrane during feeding of 3'-methyl-DAB which would favor a

more rigid and less flexible menbrane.

The mitochondria from tumor tissue in the liver of rat fed on 3'-methyl-DAB showed the decrement of ADP/O ratio and resistance to the scattering changes, especially to high amplitude swelling with oleate as compared with one from the non-tumor tissue. The resistance to swelling was demonstrated in Ehrlich ascites tumor mitochondria[5,18] and in hepatoma mitochondria[19,20]. The decreasing extent of the swelling on the tumor mitochondria may differ from the one observed on the mitochondria in ealier periods of the 3'-methyl-DAB feeding. These evidences, however, suggest that a gradual increase in structural rigidity leads finally to the emergence of tumor and alters the control mechanism of cell metabolism during 3'-methyl-DAB feeding.

SUMMARY

The swelling-shrinkage and oxidative phosphorylation of rat liver mitochondria affected by 3'-methyl-DAB feeding were observed in correlation with function by the method mentioned, and the following results were obtained:

1. By feeding 3'-methyl-DAB the swelling-shrinkage ability of rat liver mitochondria showed a remarkable alteration reducing in the amplitude. It reduced gradually during the days of feeding, reached the minimum value on 30th day and restored gradually thereafter (in Case 1).

2. ADP/O ratio also decreased by feeding the carcinogen reached the minimum point on 30th day and increased on 38th day showing the similar tendency in the decrease of the swelling-shrinkage amplitude (in Case 1).

3. The mitochondria from the hepatoma, which was induced by 3'-methyl-DAB feeding, showed a lower amplitude in swelling-shrinkage with the dropped ADP/O ratio compared with those of mitochondria from liver tissue neighbouring the tumor.

4. The mechanism in the reduction of swelling-shrinkage ability has been discussed in the relation with fatty acid composition of mitochondria which is reported elsewhere.

5. From the above results it is deduced that lowered ability for swelling-shrinkage with the reduced oxidative phosphorylation will be somehow related to the mechanism of cancer induction.

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