Effect of the Plasticizer Di-(2-ethylhexyl)phthalate on Oxidative Phosphorylation in Rat Liver Mitochondria: Modification of the Function of the Adenine Nucleotide Translocator

Shinichi KORA, Mineo SADO* and Hiroshi TERADA**

*Technical R & D Division, Terumo Corp., * 2656-1, Obuchi, Fuji, 417, Japan and Faculty of Pharmaceutical Sciences, University of Tokushima, ** Shomachi-1, Tokushima, 770, Japan*

(Received April 23, 1988)

The effect of di-(2-ethylhexyl)phthalate (DEHP) on oxidative phosphorylation of isolated rat liver mitochondria was investigated. DEHP at concentrations of 20–1000 μM had no effect on state 4 respiration, but at 40 μM , DEHP decreased the rate of state 3 respiration by about 20%. Although DEHP had no effect on electron transport through the respiratory chain, it decreased the rate of adenosine triphosphate (ATP) synthesis. Its inhibition of ATP synthesis showed a similar concentration dependence to that of state 3 respiration. Furthermore, DEHP at 40 μM inhibited the uptake of [^3H]adenosine diphosphate into mitochondria. DEHP also retarded the action of cationic uncoupling agents, which are known to modify the 29000-dalton protein involved in adenine nucleotide exchange. These results suggest that DEHP affects the activity of adenine nucleotide exchange and consequently partially decreases the rate of state 3 respiration. The action of DEHP on the 29000-dalton protein involves a protective effect against mitochondrial damage induced by hydrophobic cations or heavy metal cations.

Keywords — di-(2-ethylhexyl)phthalate; oxidative phosphorylation; mitochondrion; adenine nucleotide translocator; cationic uncoupler; phthalic acid ester; plasticizer

Introduction

Di-(2-ethylhexyl)phthalate (DEHP) is a plasticizer commonly added to the polyvinyl chloride plastics used in many kinds of medical equipment, such as blood containers. When used as a plasticizer in plastic containers, DEHP is known to dissolve in the contents of the containers, and consequently its stability and biological activities, especially in blood, have attracted much attention.¹⁾ It is reported to have a protective effect against hemolysis occurring during preservation of erythrocytes for transfusion.²⁾ In this case, it was suggested to act on the erythrocyte membrane, since it inhibited deterioration of membrane structure associated with erythrocyte crenation and microvesicle formation. It is also reported to inhibit platelet function.³⁾

The action of DEHP on energy transducing membranes, such as those of mitochondria should also be important. There have been some studies on this subject,⁴⁾ but with discrepant results: Takahashi^{4a)} reported that 1 mM DEHP (1.0–1.4 $\mu\text{mol}/\text{mg}$ of mitochondrial

protein) had no effect on the rate of state 3 respiration. Melnick and Schiller^{4b,c)} obtained similar results with DEHP at concentrations of 0.1 and 1.0 mM DEHP (67 and 670 nmol/mg protein). On the other hand, Inouye *et al.*^{4d)} observed that 1 mM DEHP (625 nmol/mg protein) reduced the rate of state 3 respiration to 81% of the control level. Furthermore, analogs of DEHP, such as di-*n*-butylphthalate and mono-(2-ethylhexyl)phthalate were reported to accelerate state 4 respiration.^{4c)} Thus, since details of the action of DEHP on energy transducing membranes are uncertain, we investigated the mechanism of action of DEHP on mitochondria. Our results provide a clue to the action mechanism of DEHP on other biomembranes such as those of erythrocytes and platelets.

Materials and Methods

DEHP was purchased from Kyowa Hakko Kogyo Co., Tokyo, (Japan). The amount of contaminating mono-(2-ethylhexyl)phthalate in DEHP was below 0.1 ppm as judged by gas-liquid chromatographic analysis. A solution of

500 mM DEHP in ethanol was used as the stock solution. A cationic cyanine dye, 2,2'-[3-[2-(3-butyl-4-methyl-2-thiazolin-2-ylidene)ethylidene]-propenylene]bis[3-butyl-4-methylthiazolinium iodide] (Tri-S-C₄(5)) was a gift from Nippon Kankoshikiso Research Laboratory, Okayama (Japan). Other reagents were commercial products of reagent grade.

Rat liver mitochondria were isolated from adult male Wistar rats (about 250 g of body weight) by the method of Myers and Slater.⁵⁾

Consumption of oxygen in the reaction medium due to respiration of mitochondria was measured polarographically with a Clark-type oxygen electrode (Yellow Springs, type 5331). The incubation medium consisted of 200 mM sucrose, 2 mM MgCl₂, 1 mM ethylenediamine-tetraacetic acid two sodium (Na₂EDTA), and 10 mM potassium phosphate buffer, pH 7.4. Succinate (10 mM, sodium salt) was used as a respiratory substrate with rotenone (0.5 μ g/ml). Incubation was carried out at 25 °C in a reaction vessel of 2.53 ml volume. The final concentration of mitochondria was 0.7 mg protein/ml, protein being determined by the biuret method⁶⁾ in the presence of 1% sodium dodecyl sulfate.

The activity of the electron transport chain was measured by the method of Lee *et al.*,⁷⁾ with succinate as the substrate and ferricyanide as an artificial electron acceptor. The rate of ferricyanide reduction was monitored in terms of the decrease in absorbance at 420 nm in a Shimadzu UV-3000 spectrophotometer. The rate of adenosine triphosphate (ATP) synthesis was determined by measuring the change in pH of the medium by the method of Nishimura *et al.*⁸⁾

The effect on the transport of inorganic phosphate (Pi) was examined by monitoring the osmotic behavior of mitochondria associated with the entry of ammonium phosphate in a medium containing 150 mM ammonium phosphate, 100 μ M Na₂EDTA, 1 mg/ml rotenone and 15 mM Tris-HCl buffer, pH 7.2.⁹⁾ The amount of mitochondria was 0.7 mg protein/ml in a total volume of 3 ml. The degree of Pi transport into mitochondria was determined by measuring the decrease in the absorbance of mitochondrial suspension at 540 nm at 25 °C in a Shimadzu UV-3000 spectrophotometer.

The activity of adenine nucleotide translocator (AdNT) was assayed by measuring the rate of [³H]adenosine diphosphate ([³H]ADP) uptake. The incubation medium consisted of 200 mM sucrose, 2 mM MgCl₂, 1 mM Na₂EDTA, 10 mM succinate (sodium salt), 6 μ g/ml oligomycin, 2 μ g/ml rotenone, and 10 mM Tris-HCl buffer, pH 7.4. Since the rate of transport of ADP is reported to be very fast at physiological temperatures,¹⁰⁾ the reaction was carried out in test tubes immersed in ice-water. Five minutes after the addition of DEHP (40 μ M) to a suspension of mitochondria at 1 mg protein/ml in a total volume of 15 ml, [³H]ADP (0.5 μ Ci/ml, final concentration 1 mM) was added to the mitochondrial suspension. After 10 min, the incubation mixture was removed from the ice-bath and 30 μ M carboxyatractyloside (CATR) was promptly added. The mixture was centrifuged at 13000 rpm for 1 min in an Eppendorf-type centrifuge (model KM-15000A, Kubota), and the resulting mitochondrial pellet was washed twice with the washing medium (incubation medium containing 30 μ M CATR). The pellet was solubilized in 500 μ l of 4% sodium dodecyl sulfate, and its radioactivity was measured in a liquid scintillation counter (Aloka model LSC-700).

Fig. 1. Effects of DEHP on State 3 and 4 Respirations of Mitochondria

Isolated rat liver mitochondria (0.7 mg protein/ml) were incubated in the medium described in the text. After addition of DEHP, the respiratory rate (V_{ox}) in state 4 (●) was measured for 2 min. Then ADP (10 nmol) was added, and the V_{ox} in state 3 (○) was determined.

Results

First we examined the effect of DEHP on mitochondrial respiration. The effects of DEHP on state 4 and 3 respirations are shown in Fig. 1. DEHP at up to 1 mM had no effect on state 4 respiration. At 40 μM , it inhibited state 3 respiration by more than about 20%, but at higher concentrations, state 3 respiration increased, reaching the original level at about 200 μM DEHP. When 40 μM DEHP was added before the addition of ADP, the rate of state 3 respiration decreased time-dependently, and it became constant about 1 min after the addition of DEHP (data not shown). In this case also, inhibition of state 3 respiration was about 20%. Preincubation of mitochondria with 40 μM DEHP before the addition of respiratory substrate (succinate) had no effect on the rate of state 4 respiration (data not shown).

As shown in Fig. 2, DEHP inhibited ATP synthesis similarly to state 3 respiration: it was maximally inhibitory at between 40 and 100 μM , where it caused 20 to 25% inhibition, and was progressively less inhibitory at higher concentrations.

To see whether DEHP had any effect on electron transfer in the respiratory chain, we next measured the rate of reduction of ferricyanide with succinate as the substrate in the presence of rotenone to inhibit nicotinamide adenine dinucleotide (NADH) oxidation, and cyanide to

Fig. 2. Effect of DEHP on the Rate of ATP Synthesis

Two minutes after the addition of DEHP to incubated mitochondria, ADP (10 nmol) was added. The rate of ATP synthesis was determined from the pH change of the medium during state 3 respiration.⁸⁾

Fig. 3. Effect of DEHP on the Rate of Electron Transport

The rate of electron transport was determined with ferricyanide as an artificial electron acceptor. The rate of reduction of ferricyanide was determined by monitoring the decrease in absorbance at 420 nm. (●), control; (○), with 20 μM DEHP; (□), with 100 μM DEHP.

block cytochrome c oxidase. Under these conditions, the reduction rate represents electron transfer from succinate dehydrogenase to cytochrome c. As shown in Fig. 3, neither 20 nor 100 μM DEHP had any effect on the reduction rate.

The partial inhibitions of state 3 respiration and ATP synthesis might be due to inhibition of the transport of either Pi or ADP into mitochondria. Thus, we first examined the effect of DEHP on the Pi-transport by monitoring decrease in the optical absorbance at 540 nm associated with swelling of mitochondria as a result of Pi-transport. DEHP was found not to have any effect up to 1 mM, indicating that the partial inhibition of state 3 respiration is not due to the

TABLE I. Effect of DEHP on [^3H]ADP Uptake into Mitochondria

	ADP uptake (nmol/mg protein)
Control	0.78
DEHP (40 μM)	0.33

Fig. 4. Effect of DEHP on the Uncoupling Induced by Tri-S-C₄(5)

Two minutes after the addition of DEHP (final concentration 40 μM), tri-S-C₄(5) (final concentration 80 μM) was added.

inhibition of the Pi-transport. Next we examined the effect on the translocation of ADP across the mitochondrial membrane mediated by AdNT. As shown in Table I, DEHP at 40 μM reduced the [³H]ADP uptake; the value 10 min after the initiation of ADP transport was about 50% of that in the absence of DEHP. This partially inhibited ADP transport was completely inhibited by addition of CATR.

If, as suggested from the results in Table I, DEHP acts on AdNT in the mitochondrial membrane, it should have effects on the actions of reagents that influence the function of AdNT. The divalent hydrophobic cationic cyanine dye (tri-S-C₄(5)) and (*o*-phenanthroline)₂-Cu²⁺ complex (Cu(OP)₂) are thought to be uncouplers of oxidative phosphorylation by acting on AdNT,¹¹⁾ unlike weakly acidic uncouplers, which dissipate the ΔpH by protonophoric actions.¹²⁾ As shown in Fig. 4, 80 μM tri-S-C₄(5) accelerated state 4 respiration, with the characteristic effect of causing progressive release of state 4 respiration. In the presence of 40 μM DEHP, this release of respiration was significantly retarded. Cu(OP)₂ showed a similar uncoupling effect, and again DEHP inhibited the

Fig. 5. Effect of DEHP on the Uncoupling Induced by Cd²⁺

Two minutes after the addition of DEHP (final concentration 40 μM), CdCl₂ (final concentration 2 μM) was added.

uncoupling action (data not shown). Furthermore, the thiol-reactive metal cations Cd²⁺ and Ag⁺¹³⁾ were shown to uncouple oxidative phosphorylation, probably by interaction with AdNT (H. Terada *et al.* unpublished results). DEHP also inhibited the uncoupling induced by Cd²⁺, as shown in Fig. 5.

Discussion

In the present study we found that DEHP at 40–100 μM (57–140 nmol/mg protein) partially inhibited oxidative phosphorylation by inhibiting ADP transport mediated by AdNT, and thus inhibiting state 3 respiration and ATP synthesis. However, it had no effect on the respiratory chain. These findings are consistent with those of Inouye *et al.*,^{4d)} although the effective concentration range for these effects was very different in our study and theirs. This difference may be related to the difference of experimental conditions.

Tager *et al.*,¹⁴⁾ using an inhibitor titration method, estimated the contribution of each step in oxidative phosphorylation to the control of the rate of mitochondrial oxygen uptake. They concluded that state 3 respiration is controlled by a number of steps, including those involving AdNT, dicarboxylate carrier, and cytochrome c oxidase. Our results show that DEHP has no

effect on electron transfer through the respiratory chain, and hence, that neither the dicarboxylate carrier nor cytochrome c oxidase can be a site of action of DEHP. On the other hand, [^3H]ADP uptake was partially inhibited by 40 μM DEHP, consistent with the results in heart mitochondria reported by Bell and Hubert.¹⁵⁾

In this connection it is interesting that DEHP retarded the action of the cationic uncouplers tri-S-C₄(5), Cu(OP)₂, Cd²⁺ and Ag⁺. Tri-S-C₄(5) and Cu(OP)₂ are suggested to induce uncoupling by modifying the state of a sulfhydryl group(s) in the 29000-dalton protein in the mitochondrial membrane.¹¹⁾ Possibly this protein is an AdNT.¹¹⁾ The heavy metal cations Cd²⁺ and Ag⁺ inhibit the cross linking of 29000-dalton protein induced by Cu(OP)₂ (H. Terada *et al.* unpublished results), so their site of action may be the 29000-dalton protein. Thus, from the above results, DEHP could interact with AdNT either directly or indirectly.

Hydrophobic cations such as tri-S-C₄(5) and Cu(OP)₂, and heavy metal cations such as Cd²⁺ and Ag⁺ cause mitochondrial damage under extreme conditions due to chemical modification of the 29000-dalton protein.¹²⁾ Thus, the plasticizer DEHP may protect mitochondrial function by retarding the uncoupling actions of these cations. Since AdNT is thought to be very sensitive to perturbation of the integrity of phospholipid membrane structure,¹⁶⁾ DEHP probably does not act on AdNT directly, but probably affects the state of phospholipid bilayers, resulting in the modification of the function of AdNT.

In preliminary experiments, we found that DEHP also protects mitochondria from Ca²⁺ damage. Since the features of Ca²⁺-uncoupling associated with mitochondrial damage are very similar to those of uncoupling by hydrophobic and heavy metal cations,¹⁷⁾ AdNT seems to be involved in the action of Ca²⁺.¹⁸⁾ The effect of DEHP on mitochondrial damage by Ca²⁺ will be reported elsewhere.

At present, it is not clear why the inhibitory effects of DEHP were reversed at higher concentrations. However, Kramer¹⁹⁾ reported that the activity of AdNT in a reconstituted system showed a biphasic dependence on the cholesterol/phospholipid molar ratio of the liposomes.

Thus, the effects of DEHP on AdNT may be based on its action on membrane lipids, resulting in alteration of the physico-chemical properties of phospholipid bilayers and of lipid-protein interactions.

The protective effect of DEHP on the mitochondrial membrane may provide a clue to explain the inhibitory effect of the plasticizer on hemolysis occurring during blood-bank storage of erythrocytes.

Acknowledgements The authors thank Mr. Yasuo Shinohara for helpful discussions, and also thank Mr. Yosuke Fukui and Mr. Haruhiko Koike for technical assistance.

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