A NEW INHIBITOR OF ADENINE NUCLEOTIDE TRANSLOCASE IN MITOCHONDRIA: AGARIC ACID

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SUMMARY: The effect of agaric acid (∝ cetyl citric acid) on the transport of ADP and ATP has been examined in rat liver mitochondria. Respiration stimulated by ADP is progresively inhibited by agaric acid; maximal inhibition is attained at 40 μM agaric acid. ATPase activity is inhibited 30% by 20 μM agaric acid. The exchange of adenine nucleotides is competitively inhibited by agaric acid.

The adenine nucleotide carrier is the more thoroughy studied anion carrier in the inner mitochondrial membrane (for review see ref.1) An important part of these studies has been made with the specific inhibitor, atractyloside which is a diterpene glucoside that contains three negative charges (2); the hydrophobic diterpene nucleus undoubtedly plays an important role in its inhibitory effect. Other antagonist ligands like carboxyatractyloside and bongkrekic acid posses a significant hydrophobicity. Since other hydrophobic compounds such as acyl-CoA derivatives also inhibit the adenine nucleotide transport (3), it has been assumed that there is an important relation between the lipid enviroment and the mobility of the carrier in the mitochondrial membrane (4). The hydrophilic portion of the inhibitors is also essential for full inhibitory activity of these compounds which suggests that the ADP carrier posseses a polar site which may undergo ionic interaction with the adenine nucleotides (4).

These observations prompted us to examine, on some mitochon-

drial functions the effect of agaric acid , i.e. citrate covalently bound to a saturated alkyl chain of 16 carbons (5). The results of this work indicate that agaric acid inhibits the ADP stimulated respiration without affecting State 4 respiration. Agaric acid has no effect on the DNP (2,4-dinitrophenol) stimulated respiration, but it inhibits the DNP-stimulated ATPase, apparently this is due to the interference of agaric acid with the adenine nucleotide carrier, since agaric acid inhibits competitively adenine nucleotide exchange.

MATERIALS AND METHODS

Rat liver mitochondria were isolated by differential centrifugation of liver homogenates as previously described by Schneider et al (6), in 0.25 M sucrose-1 mM EDTA (ethylene diamine tetra acetate) adjusted to pH 7.3 with tris-base; submitochondrial particles were prepared by suspending rat liver mitochondria in the above medium and sonicating for six periods of 60 seconds at the maximum setting of a MSE sonicator. Unbroken mitochondria were removed by centrifugation for 10 min at 8 000 x g; the supernatant was centrifugated at 100 000 x g for 60 min; the pellet is refered to as submitochondrial particles . Exchange of ADP and ATP was measured by Millipore filtration according to Winkler and Lehninger (7) after incubation of the mitochondria in the conditions indicated in the Results section. Respiration rates were determined polarographically as described under Results. DNP-stimulated ATPase activity was followed by measuring the amount of phosphate liberated from ATP according to the method of Sumner (8) after stopping the reaction with 5% trichloroacetic acid, final concentration. Uptake of inorganic phosphate (Pi), by mitochondria was measured by incubation of mitochondria in mixture that contained 10 mM ^{32}Pi; 20 mM tris-HCl pH 7.3; 125 mM sucrose EDTA and 2 mg of mitochondrial protein; after one min of incubation at 25^{o} an aliquot of 0.2 ml was filtered in Millipore filter of 0.45 μ, and the amount of radioactivity in the filter that contained the mitochondria was estimated. Mitochondrial protein was measured according to the method of Murphy and Kies (9); the labeled compunds were obtained from Amersham Searle; agaric acid was obtained from Sigma Chemical.

RESULTS

Figure 1 shows the effect of agaric acid (AA) on the respiration of mitochondria and submitochondrial particles. Agaric acid inhibits almost completely the State 3 respiration with succinate as substrate (Fig 1A). Fig. 1B shows that agaric acid does not affect State 4 respiration nor

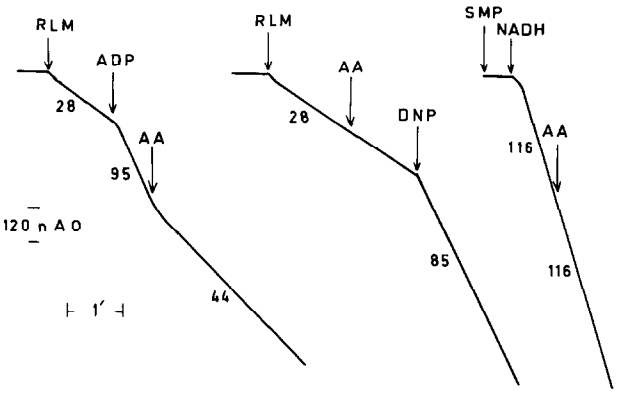

Figure 1. Effect of Agaric Acid on Respiration of Mitochondria (AandB) and on the Aerobic Oxidation of NADH by Submitochondrial Particles (C). The mixture (final volume 3 ml) contained 125 mM sucrose-1mM EDTA-tris pH 7.3; 10 mM succinate-tris pH 7.3 (except in C); 10 mM phosphate-tris, pH 7.3 and 20 mM tris-HCl pH 7.3. At zero time 4.5 mg of mitochondrial protein (RLM) were added to the medium in A and B; in C, submitochondrial particles (SMP) (4mg protein) were added; 1.6 mM ADP, 20 μM agaric acid (AA), 100 μM 2,4-dinitrophenol (DNP) and 1 mM NADH were added to the mixture at the indicated points. The results are expressed as natoms oxygen consumed per min per mg protein. Temperature 25°

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the DNP-stimulated oxidation of succinate; also agaric acid does not affect the aerobic oxidation of NADH by submitochondrial particles (Fig 1C). These experiments indicate that the inhibition of State 3 respiration by AA is not due to modifications of the uptake of succinate by the mitochondria or to an alteration of the functioning of the electron transport chain.

The inhibition of mitochondrial State 3 respiration by various concentrations of agaric acid is shown in Figure 2; half-maximal inhibition is attained at a concentration of 20 μM AA. According to these results the inhibition of respiration in mitochondria could be due to inhibition of the phosphate or adenine nucleotide carrier; however no effect of agaric acid on the uptake of inorganic phosphate was observed. In a one min incubation period, control mitochondria took up 18 μmoles of phosphate per mg of protein while 16 μmoles of phosphate per mg pro-

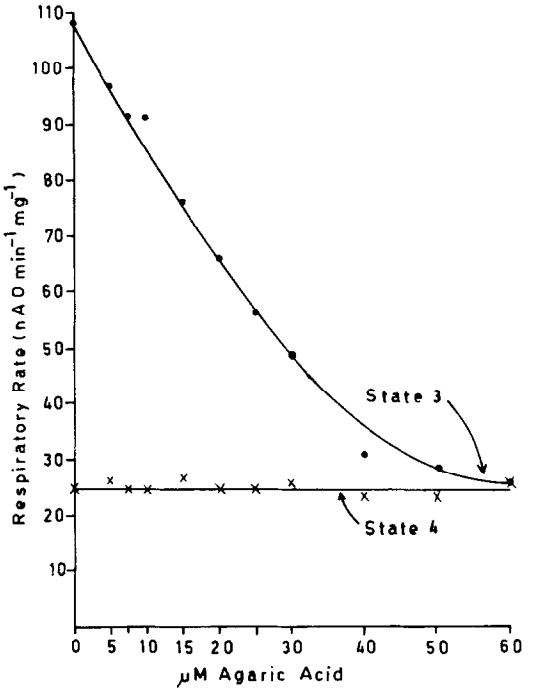

Figure 2. Effect of Increasing Concentrations of Agaric Acid on State 3 and State 4 Respiration. Respiration rates of rat liver mitochondria (4.5 mg) were measured polarographically in a medium similar to that described in Figure 1. Agaric acid was added as indicated.

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tein were detected in mitochondria incubated with 40 μM AA.

The inhibition of State 3 respiration (Fig 1A) by agaric acid suggested that agaric acid could have an atractyloside-like effect, thus the effect of AA on the DNP-stimulated ATPase activity was studied. Figure 3 shows that this activity is inhibited progressively by increasing concentrations of agaric acid. In the same range of concentrations atractyloside is slightly more effective in inhibiting the DNP-stimulated ATPase activity.

A Lineweaver-Burk plot of the effect of agaric acid on the exchange of ADP in mitochondria shows that 40 μM AA increases the Km for ADP from 10.4 to 71 μM with no change in the Vmax (Fig 4A) which

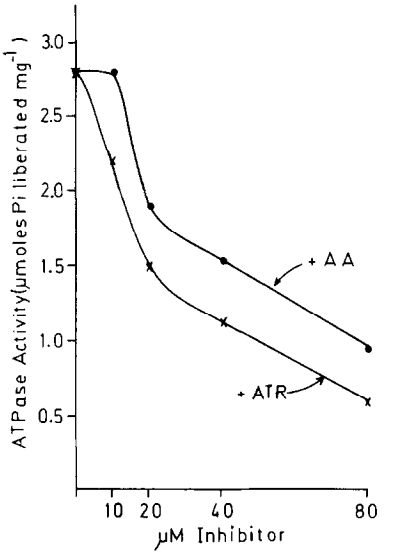

Figure 3. Effect of Agaric Acid and Atractyloside on the 2,4-dinitrophenol Stimulated ATPase Activity. The mixture (final volume 1.5 ml) contained 125 mM sucrose, 0.5 mM EDTA, 20 mM tris-HCl pH 7.3, 7.2 mM ATP, 4 mg of mitochondrial protein, 0.1 mM DNP and the indicated concentrations of agaric acid and atractyloside (ATR). After an incubation period of 10 min the reaction was stopped. Temperature 25°

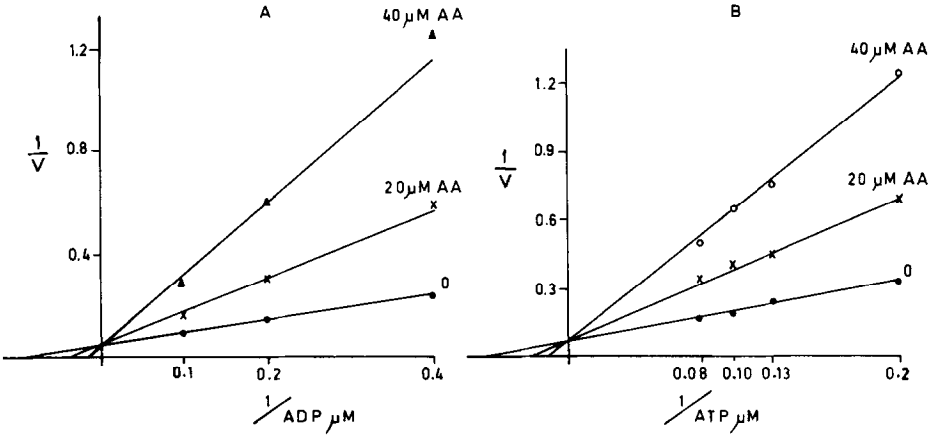

Figure 4. Double-Reciprocal Plot of the Exchange of ADP (A) and ATP (B) in the Presence of Agaric Acid. Adenine nucleotide exchange was measured as described in Methods; 2 mg of mitochondrial protein were incubated in mixtures that contained 125 mM sucrose-EDTA; 20 mM tris-HCl pH 7.3 and the indicated concentrations of [adenosine-8-^{14}C]ADP, [adenosine-8-^{14}C]ATP ($3x10^4$ cpm respectively) and agaric acid. After 1 min of incubation an aliquot of 0.2 ml was filtered and its radioactivity measured. Temperature 25° .

suggest that there is a competition between agaric acid and the nucleotide. Figure 4B shows also that AA acts as a competitive inhibitor of the exchange of ATP.

DISCUSSION

The results of this paper suggest that agaric acid at the concentration of 40 μM is a powerful inhibitor of oxidative phosphorylation. The fact that the respiration of submitochondrial particles and the DNP-stimulated oxigen uptake are not affected by agaric acid indicates that the mechanism through which this reagent blocks State 3 respiration may be related to an inhibitory effect on the adenine nucleotide translocase. This conclusion was substantiated when it was observed that agaric acid inhibited competitively the exchange of ADP and ATP.

With respect to the mechanism through which agaric acid inhibits adenine nucleotide exchange, it may be suggested that the citrate moiety of agaric acid interacts with the carrier; this interaction would be estabilized by hydrophobic interactions between the alkyl chain of AA and the hydrophobic phase of the membrane. In this respect it should be acknowledged that citrate at concentrations of 10 mM does not affect adenine nucleotide transport (data not shown).

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