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SHORT COMMUNICATIONS

Effect of the polyflavane P13 on rat liver mitochondria: protection against ageing and modification of transition temperature

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Roots of *Poterium spinosum*, used in traditional Middle-East medicine to cure diabetes, contain polyflavanes, the Centre de Recherches Pierre Fabre isolated one type that was named P13. Coordinated studies in different laboratories put forward various physiological activities of P13: it protected against hypertension and hypoxia, and it modified cationic exchanges linked to action potentials [1, 2]. Biochemical investigations showed that P13 bound to membranes, and that it inhibited ATPases, when these enzymes were freely accessible [3, 4].

We report here that P13 protects mitochondrial functions during ageing, and evidences are presented that P13 can induce structural modifications in membranes as detected by a rise of the mitochondrial transition temperature.

MATERIALS AND METHODS

Mitochondria were isolated from adult female rat livers according to standard procedures, by differential centrifugation in 0.25 M sucrose 1 mM EGTA (pH 7.5). Oxygen uptake was measured with a Clark electrode (YSI) in the following respiratory medium: 24 mM glycylglycyn, 9.6 mM $MgCl_2$, 60 mM KCl, 87 mM sucrose, pH 7.4. Oxygen concentrations given in [5] were used for calculations. All preparations used had a ratio of respiration with ADP to respiration without ADP (RCR) over three. These preparations kept a good phosphorylating activity for 18–24 hr, this led us to estimate the protecting effect of P13 on mitochondria 48 hr after their isolation. Mitochondria kept for 24 hr in ice, were found more suitable to detect the effect of P13 on transition temperature.

Fluorescence emissions were measured with a double beam FICA 55 spectrofluometer corrected for excitation and emission intensities. Reference and assay mitochondrial suspensions were studied either in separate experiments or alternatively in a similar experiment. Temperature was increased in a stepwise or in a continuous manner, but never more than 0.5°/min. Mitochondria (0.5 mg protein/ml) were dispersed in a suspension of 2(9-anthroyl) palmitic acid 2.10^{-6} M in the medium used for mitochondria preparation. The fluorescent probe 2(9-anthroyl) palmitic acid (2AP) was synthetized according to [6].

The polyflavane P13 used was a purified polymer of 3, 3', 4, 4', 5, 7 hexahydroxyflavane with an average mol. wt of 3000 daltons [4].

RESULTS AND DISCUSSION

Increasing P13 concentrations led to a progressive inhibition of active respiration, without noticeable effects on the ADP/O values, as shown in Fig. 1. For doses over 80 μg/mg protein, a progressive loss of respiratory control was moreover noted. We tested the effect of low P13 concentrations on ageing of mitochondria, i.e. the effect on the progressive loss of the phosphorylating activity of preparations kept in ice (Table 1).

There was a good protecting effect of P13 on the mitochondrial membrane integrity, since both the ADP/O value and the respiratory control ratio (RCR) were maintained.

Addition of P13 to mitochondrial suspensions in the

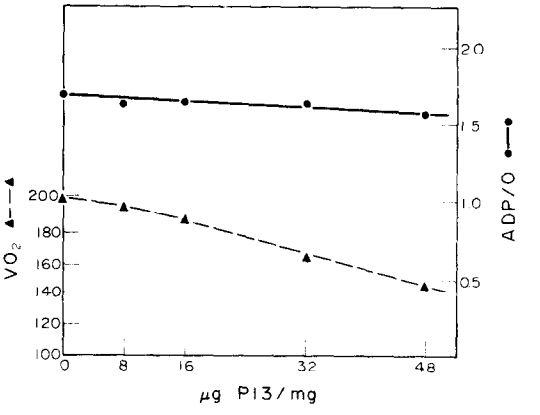

Fig. 1. P13 activity on rat liver mitochondria active respiration (VO_2 = natg O_2/min/mg protein, dotted line, right scale) and on phosphorylation (ADP/O = moles of ADP transformed per oxygen atomgram consumed, full line, left scale). P13 was added in the respiratory medium before mitochondria.

Table 1. Effect of P13 on rat liver mitochondria kept in ice, respiration (natg O_2/min/mg protein), RCR (active respiration/controlled respiration), and ADP/O (moles of ADP transformed per oxygen atomgram consumed). This experiment was repeated four times, after 48 hr the P13 protected samples had a ADP/O differing by less than 10 per cent from the initial values (these values ranged from 1.56 to 1.9 according to the preparations).

Mitochondria	Respiration (VO_2) +ADP	−ADP	RCR	ADP/O
Freshly prepared without P13	148	30	4.9	1.56
after 48 hr without P13	72	36	2.0	1.13
after 48 hr with 2 μg P13/mg proteins	133	29	4.6	1.49
after 48 hr with 4 μg P13/mg protein	120	32	3.8	1.48

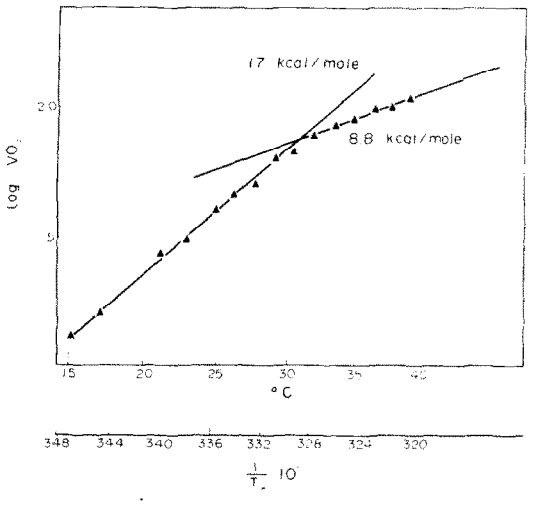

Fig. 2. Arrhenius plots of respiration by rat liver mitochondria kept 24 hr in ice with 5 μg P13/mg protein. Activation energies are indicated. log VO_2 = decimal logarithm of VO_2 (respiration: natg O_2/min/mg protein).

respiratory medium (containing 9.6 mM Mg^{2+}) resulted in a time and concentration dependent increase of optical density (at 520 nm), which was stabilized 1 min after addition. This increase reached a maximum of about 25 per cent of the initial value for 150 μg P13 per mg of protein. With mitochondria in sucrose, the maximum OD increase was one tenth of what was observed in respiratory medium; this agreed with the noted cations mediated fixation of the polyflavane [3]. This result indicated that fixation of P13 on the membrane induced a contraction of mitochondria.

The contraction was easily observed for inhibitory P13 concentrations. We tried to detect membrane modifications with concentrations not detrimental to oxidative phosphorylation, and under conditions used for experiments on ageing. Transition temperatures were used as physical indicators of membrane state. Mitochondria were kept in 0.25 M sucrose at 0° for 24 hr, alone or with 5 μg P13/mg protein. Then, the temperature dependence of either active respiration with succinate as substrate, or emission intensity of fluorescent probe 2AP were followed (Figs 2 and 3).

The transition temperature observed by fluorescence were in the range 19°–23° for mitochondria alone, and 27°–32° for mitochondria with P13. By respiration measurements, these values were respectively in the range 19°–23° and 26°–31°. The transition temperatures and the activation energies we measured with freshly prepared or aged mitochondria without P13, were in agreement with published values obtained by respiration measurements or ESR spectroscopy [7, 8].

The relationship between transition temperatures and molecular organisation of lipids is rather clear for membranes of fatty acids auxotroph bacteria, but not in mammalian membranes [9, 10]. However, it is generally admitted that an elevation of the transition temperature of membranous systems indicates a loss in fluidity, a greater cohesion of the systems.

Membrane rigidification together with the contraction of mitochondria in the presence of P13 correlates with the observation that P13 protects mitochondria against passive loss of Mg^{2+}, Ca^{2+} and proteins during ageing [3]. Such effects on membranes may be a key to the understanding of the multiple pharmacological properties of the polyflavane from *Poterium spinosum*.

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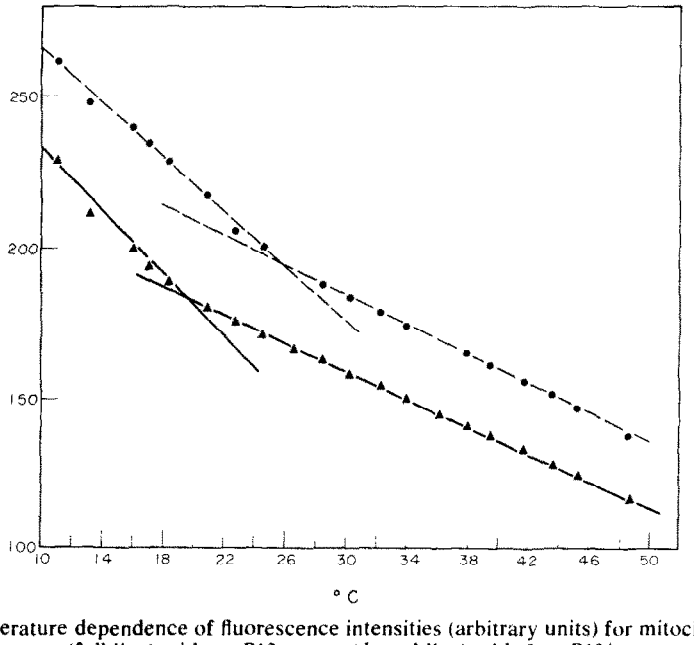

Fig. 3. Temperature dependence of fluorescence intensities (arbitrary units) for mitochondria kept 24 hr in ice: reference (full line) without P13, assay (dotted line) with 5 μg P13/mg protein. Fluorescent probe 2-AP 2.10^{-6} M; excitation 385 nm; emission 440 nm.

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Effect of dexamethasone treatment on *N*,*N*-dimethylaniline demethylation and *N*-oxidation in pulmonary microsomes from pregnant and fetal rabbits

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In a previous report [1] we looked at the effects of pregnancy and the administration of certain steroids to nonpregnant adult rabbits on *N*,*N*-dimethylaniline (DMA) metabolism by rabbit liver and lung microsomes. In that study, we showed that microsomal DMA *N*-oxidase and demethylase activities in lung were higher in pregnant rabbits than in adult nonpregnant controls. We are reporting here the effects of animal pretreatment with dexamethasone on pulmonary microsomal DMA demethylase and *N*-oxidase activity in pregnant and fetal rabbits. In this study, we wanted to determine if the elevated levels of drug metabolism that we saw in pulmonary microsomes from pregnant rabbits would be affected by steroid treatment. Our effort was also directed toward determining if fetal lung drug metabolism would be affected by steroid treatment of the pregnant doe, and, if so, at what stages of gestation. We used the steroid dexamethasone, which is known to affect lung maturation [2, 3], and which we had shown to have a stimulatory effect on DMA metabolism in pulmonary microsomes from adult nonpregnant rabbits [1].

Dutch Belt rabbits (Arrow Farms, Statesville, NC) were used in this study. The rabbits, housing conditions, and microsomal preparations were the same as reported previously [4].

N,*N*-dimethylaniline was obtained from Fisher Scientific Co. (Pittsburgh, PA) *N*,*N*-dimethylaniline *N*-oxide (DMAO) was prepared by the method of Craig and Purushothaman [5]. Solutions of DMA and DMAO were made up as described previously [1]. Dexamethasone acetate was obtained from Sigma Chemical Co. (St. Louis, MO). Emulphor EL 620 (polyethoxylated vegetable oil) was supplied by GAF Corp. (New York, NY). All other chemicals were reagent grade and were obtained from commercial sources.

Dexamethasone acetate was administered to pregnant rabbits in three daily s.c. doses or a single s.c. dose of 2 mg/kg in Emulphor–ethanol–water (3:3:4). Control rabbits received Emulphor–ethanol–water (3:3:4) in a dose of 0.5 ml/kg (the same as treated animals). The animals were killed on day 4. In one set of experiments, pregnant rabbits were treated with dexamethasone on day 21 or days 21, 22 and 23 of pregnancy and then killed on day 24 of pregnancy. In the other set of experiments, the steroid was administered to the rabbits on days 24, 25 and 26 of pregnancy and the rabbits were killed on day 27.

The amount of protein in the microsomal fractions was measured by the method of Lowry *et al.* [6]. The methods for assaying DMA demethylase and *N*-oxidase activities have been reported previously [4].

The experimental data were analyzed statistically utilizing two-sided Student's *t*-tests.

The effect of dexamethasone acetate pretreatment of animals on DMA *N*-oxidation and demethylation in pulmonary microsomes from 24-day pregnant rabbits and 24-day fetal rabbits is shown in Table 1. Dexamethasone treatment had no effect on DMA demethylase or *N*-oxidase activities in pulmonary microsomes of the pregnant rabbits. Treatment of pregnant does with dexamethasone had little or no effect on the low microsomal DMA metabolism seen in the 24-day fetal rabbit lung. In an earlier report [7], we showed the first appearance of measurable DMA demethylase and *N*-oxidase activities in fetal pulmonary microsomes at about 24 days of gestation.

We have shown previously that maternal pulmonary microsomes from 27- to 28-day pregnant rabbits have DMA *N*-oxidase and demethylase activities about 50 per cent above control levels [1]. Dexamethasone pretreatment of pregnant does did not alter these elevated enzyme activities in the maternal lung microsomes of the 27-day pregnant rabbits (Table 1). However, these data show that dexamethasone treatment caused significant increases in DMA *N*-oxidase and demethylase activities in pulmonary microsomes of 27-day fetal rabbits; pulmonary demethylase activity was increased 2.8 times and *N*-oxidase was increased 1.5 times the control activity (fetal rabbits from untreated pregnant rabbits).

In a previous study [1] we showed that treatment of adult nonpregnant rabbits with dexamethasone caused significant increases in DMA demethylase and *N*-oxidase activity in pulmonary microsomes. However, the treatment of pregnant rabbits with dexamethasone, as seen in these experiments, had no further effect on the already elevated levels of these enzymes.

In the experiments with fetal rabbits, it was shown that treatment of the pregnant rabbit by dexamethasone caused