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THE MODE OF ACTION OF β-BENZAL BUTYRIC ACID, AN HYPOCHOLESTEROLEMIC DRUG, IN AFFECTING MITOCHONDRIAL RESPIRATION

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Abstract—β-benzal butyric acid, an inhibitor of cholesterol biosynthesis and of mitochondrial respiration, induces a release of pyridine nucleotides from the mitochondria, without inducing mitochondrial swelling. The addition of NAD to the incubation system results in a restoration of the oxidative functions. EDTA and substrates are effective in reducing the inhibition produced by β-benzal butyric acid. The protection given by substrates is related to the physiological role that they play in preventing dangerous side-effects, which could arise during the use of the drug.

β-BENZAL BUTYRIC ACID (3-methyl-4-phenyl-3-butenoic acid, BBA), a drug which has been found effective in lowering the pathological high levels of cholesterolemia,[1,2] was reported to inhibit the mitochondrial oxidation of NAD-dependent substrates.[3] The site of the inhibition was tentatively located at an early stage of the oxidative phosphorylation.

Experiments are reported in this paper with the aim of clarifying the mechanism of action of this inhibition. The results allow us to ascribe the inhibition of the mitochondrial respiration to a release of NAD from the mitochondria following pre-incubation with BBA.

EXPERIMENTAL

Preparation and incubation of rat liver mitochondria

Rat liver mitochondria were prepared according to Schneider[4] from female Sprague–Dawley rats (100–150 g), which were fasted for about 12 hr before sacrifice. The mitochondria (6–8 mg protein), suspended in 0·25 M sucrose solution, were incubated in Warburg flasks containing in a final volume of 2 ml the following components: K_2HPO_4–KH_2PO_4 buffer (pH 7·4), 30 μmoles; tris–HCl buffer (pH 7·4), 40 μmoles; $MgSO_4$, 15 μmoles; ATP, 3·5 μmoles; hexokinase, 100 KM units;[5] sucrose, 300 μmoles. Pyruvate (16 μmoles), plus fumarate (4 μmoles) as primer, was used as substrate. BBA and other material were added in the amounts indicated in the Tables.

Since BBA need to be pre-incubated with mitochondria,[3] in order to obtain the most inhibitory effect, double side-armed Warburg flasks were used. Mitochondria were left in the main compartment with BBA and Tris buffer for 10 min at 30°, before carrying out the incubation. This was done by adding the content of the two side-arms (hexokinase and glucose in the first, and other constituents of the medium in the second). The "control" flasks were treated similarly, except that the mitochondria

were left in the main compartment with the Tris buffer alone, for the preincubation period. Incubation was carried out for 20 min at 30°, by using O_2 as the gas phase.

Glucose-6-phosphate determination

When P/O ratio had to be assayed, glucose-6-phosphate was determined enzymically following incubation, according to Hohorst,[6] after treatment of the medium with 6% perchloric acid and neutralization with 5 M K_2CO_3.

Mitochondrial NAD content determination

NAD content was evaluated in the mitochondria after preincubation (with and without BBA) for 10 min at 30°, in the same experimental conditions and BBA concentration used when the oxygen uptake was estimated, except that the amount of mitochondria was increased 10-fold (60–80 mg protein). After preincubation, the mitochondria were centrifuged for 5 min at 35,000 *g*, washed with 10 ml of 0·25 M sucrose and again centrifuged. NAD was then extracted from the carefully drained mitochondrial pellet with 3 ml of 1·6 M perchloric acid, and determined enzymically by alcohol dehydrogenase assay,[7] after neutralization of the extract with 3 M K_2HPO_4 and 6 M KOH.

Mitochondrial swelling

According to Tedeschi and Harris,[8] the optical density (*E*) of a diluted mitochondrial suspension is inversely dependent on the mitochondrial volume, so that, when a swelling process occurs, the *E* of the mitochondrial suspension decreases. We have employed this method in order to detect whether BBA would act as a swelling agent for mitochondria.

A Beckman DK-2 spectrophotometer equipped with recording apparatus was used at the wavelength of 520 mμ. Three ml of a diluted suspension of mitochondria in 0·25 M sucrose, containing about 100 μg protein/ml, were pipetted in a spectrophotometric cuvette of 1 cm light path; BBA was then added, and the E recorded for 20 min. Alternatively, the mitochondria were first preincubated as usual with or without BBA, diluted in order to obtain a protein concentration of about 100 μg/ml, and their optical density successively recorded.

Chemicals

Potassium pyruvate, sodium fumarate and EDTA were products of Fluka, Buchs, Switzerland. ATP, NAD, NADP, alcohol dehydrogenase and glucose-6-phosphate dehydrogenase were purchased from Biochemia "Boheringer" (Milan, Italy). Hexokinase (grade III) was obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). BBA was kindly provided by Istituto Biochimico Italiano (Milan, Italy).

RESULTS

Relieving by 2,4-dinitrophenol (DNP) of the inhibition of oxygen consumption

2,4-DNP shows different effects on the BBA-dependent inhibition of oxygen consumption. As shown in Table 1, DNP is effective in relieving a relatively small degree of inhibition, as obtained with 10 μM BBA. When the concentration of BBA is raised to 100 μM (90 per cent inhibition), DNP is ineffective.

TABLE 1. THE EFFECT OF 2,4-DINITROPHENOL (DNP) ON THE INHIBITION OF THE MITOCHONDRIAL RESPIRATION PRODUCED BY β-BENZAL BUTYRIC ACID (BBA)*

Expt. no.	Addition (final concentrations)	O_2 uptake
1	none†	3·9
	BBA (10 μM)	1·7 (57)
	BBA (10 μM) + DNP (30 μM)	2·7 (32)
2	none†	4·5
	BBA (10 μM)	1·0 (77)
	BBA (10 μM) + DNP (30 μM)	2·3 (49)
3	none†	4·5
	BBA (100 μM)	0·6 (87)
	BBA (100 μM) + DNP (30 μM)	0·7 (85)
	none†	4·0
	BBA (100 μM)	0·2 (95)
	BBA (100 μM) + DNP (30 μM)	0·4 (90)

* Isolated rat liver mitochondria (6–8 mg protein) incubated, as described in the text, for 20 min at 30° in O_2. The BBA concentration in the preincubation medium of 1 ml was 20 and 200 μM, respectively. DNP was added at the starting of the reaction. O_2 uptake is expressed as μg atoms/hr/mg protein. Degree of inhibition between brackets.

† The controls with DNP alone did not show any significant variation of the respiratory rate.

Restoration of mitochondrial respiration by NAD

Owing to the fact that BBA inhibits the mitochondrial oxydation of NAD-dependent substrates, we decided to test the effect of adding the coenzyme to the incubation medium. As shown in Table 2, NAD is effective in restoring the oxydative capacities of mitochondria, independently from the degree of inhibition. The phosphorylative properties of the mitochondria are also partially restored.

TABLE 2. RESTORATION OF MITOCHONDRIAL RESPIRATION BY NAD*

Expt. no.	Addition (final conc.)	O_2 uptake	P/O
1	none	3·9	2·6
	BBA (10 μM)	1·7 (57)	2·0
	BBA (10 μM) + NAD (1 mM)	3·7 (5)	2·3
2	none	3·9	2·6
	BBA (100 μM)	0·8 (80)	1·1†
	BBA (100 μM) + NAD (1 mM)	4·1	2·5

* Experimental conditions as described in Table 1. NAD was added at the starting of the reaction. O_2 uptake is expressed as μg atoms O_2/hr/mg protein. Degree of inhibition between brackets.

† Results may be not completely correct, owing to low O_2 uptake.

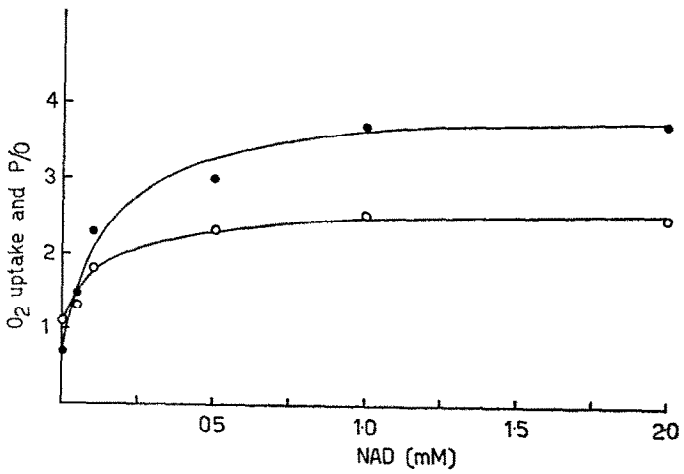

FIG. 1. The relationship between restoration of BBA-inhibited mitochondrial respiration and NAD concentration in the incubation system.
Mitochondria (6–8 mg protein) were preincubated with 200 μM BBA for 10 min at 30°; NAD was then added, together with the other components of the incubation medium. O_2 uptake is expressed as μg atoms O_2/hr/mg protein.
–●–●–●–●–●–, O_2 uptake.
–O–O–O–O–O–, P/O.

As reported in Fig. 1, a relation apparently exists between the restoration of the respiration and the NAD concentration in the incubation system. More precisely, the respiratory rate increases rapidly on adding NAD, so that, at 0·1 mM NAD concentration, the inhibition is reduced to 38 per cent. The phosphorylation concomitantly increases.

Decrease of NAD content after exposure of mitochondria to BBA

The NAD content of BBA-treated and control mitochondria is reported in Table 3. After exposure of mitochondria to BBA, under the same experimental condition used when oxygen uptake was estimated (Tables 1 and 2), 80 per cent of NAD was lost. The NAD was recovered in the supernatant, after the mitochondria were sedimented from the preincubation system. Owing to the great dilution of the NAD content in the supernatant, this determination is not as accurate as that carried out on the mitochondria, and therefore represents an approximate way to indicate that NAD leaks out from the mitochondria under the effect of BBA.

Failure of BBA in inducing mitochondrial swelling

As reported by Hunter and Ford,[9] preincubation of mitochondria with a variety of chemically different substances, like inorganic phosphate, arsenate, metal ions used in low concentration, surface active agents, results in a loss of the pyridine nucleotide content of the mitochondria, this phenomenon being always accompanied by a mitochondrial swelling. However, by testing the suspension of mitochondria treated with BBA for a similar effect, no significant evidence of a mitochondrial swelling was obtained.

TABLE 3. THE DECREASE OF THE PYRIDINE NUCLEOTIDE CONTENT OF BBA-TREATED MITOCHONDRIA

Expt. no.	Preincubation	NAD content *
1	Control	3·5
	BBA (200 μM)	0·8 (77)
2	Control	2·8
	BBA (200 μM)	0·7 (75)
3	Control	3·4
	BBA (200 μM)	0·7 (79)

Mitochondria (60–80 mg protein) were preincubated for 10 min at 30°, in a final volume of 10 ml, with 2 μmoles of BBA.
* NAD content is expressed as μmoles/mg of mitochondrial protein.

As shown in Fig. 2, no decrease in the E at 520 mμ appeared within 20 min, when BBA was directly added in the spectrophotometer cuvette. Moreover, when mitochondria, preincubated as usual with or without BBA, were tested, those treated with BBA showed a slightly lower extinction, as compared to the controls. If a well known swelling agent, like inorganic phosphate (P_i) is now added, both the suspensions showed an immediate decrease of their extinction, which was faster for the BBA-treated sample.

Protection of mitochondrial respiration by EDTA

EDTA has been found to protect mitochondria against the loss of NAD due to aerobic incubation[10] and against various swelling agents, except organic solvents, detergents and lysolecithin;[11] EDTA has also been found to favour NAD reincorporation into mitochondria.[12] Probably it stabilizes directly the membrane, by

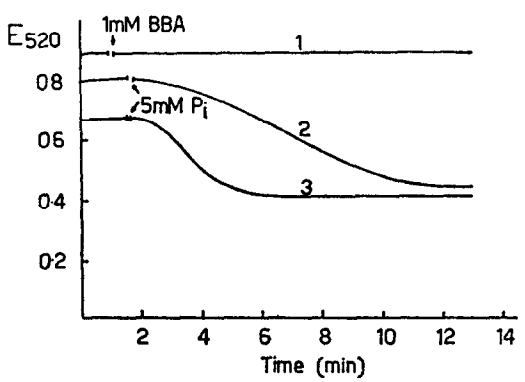

FIG. 2. The action of BBA on the mitochondrial swelling. Mitochondrial concentration (in 0·25 M sucrose) was 400 μg protein in 3 ml cuvette with 1 cm of light path.
1. Non-preincubated mitochondria.
2. Mitochondria preincubated without BBA.
3. Mitochondria preincubated with 200 μM BBA.

virtue of its complexing capacities.[11] As shown in Table 4, the EDTA, added to the preincubation medium, is also effective in protecting the mitochondria from the action of BBA.

Protection of mitochondrial respiration by substrate

It is known from earlier work[3] that substrates protect mitochondrial respiration from the inhibition due to BBA. In the present paper additional data are given, which show the evident physiological role that the endogenous substrates play in preventing the undesirable side-effects, which could arise during the pharmacological use of BBA.

TABLE 4. THE PROTECTION OF MITOCHONDRIAL RESPIRATION BY EDTA

Preincubation	O_2 uptake	P/O
Control	3·2	2·6
BBA (200 μM)	0·6 (81)	0·8
BBA (200 μM) + EDTA (2 mM)	2·3 (28)	1·7

Experimental conditions as described in Table 1. EDTA was present in the preincubation medium. O_2 uptake expressed as μg atoms O_2/hr/mg protein. Degree of inhibition between brackets.

The protection by substrates was studied by preincubating the mitochondria with BBA together with physiological amounts of substrates. At the end of the preincubation, pyruvate (16 μmoles) and fumarate (4 μmoles) were added as usual, together with the other components of the preincubation mixture. The data reported in Table 5 show that protection is exerted not only by the NAD-dependent substrates, as pyruvate and α-oxoglutarate, but by succinate too.

Experiments have also been carried out in order to show how the inhibition given by the increasing amounts of BBA varies at fixed concentrations of protective substrates. The data of Table 6 indicate that the BBA concentration may be raised up to 5 mM, before obtaining a 50 per cent inhibition, when 0·5 mM pyruvate is added to the preincubation medium.

TABLE 5. THE PROTECTION OF MITOCHONDRIAL RESPIRATION BY OXIDATIVE SUBSTRATES

Preincubation	O_2 uptake	P/O
Control	4·0	2·3
BBA (20 μM)	1·9 (53)	1·5
BBA (20 μM) + pyruvate (20 μM)	3·2 (20)	2·1
BBA (20 μM) + α-oxoglutarate (20 μM)	2·9 (27)	2·0
BBA (20 μM) + succinate (20 μM)	2·8 (30)	2·2

Experimental conditions similar to those reported in Table 1. The substrates tested for the protective effect were present in the preincubation medium. The incubation was carried out, as usual, with 16 μmoles of pyruvate + 4 μmoles of fumarate, as substrates. O_2 uptake is expressed as μg atoms O_2/hr/mg of protein. Degree of inhibition between brackets.

TABLE 6. THE PROTECTION OF MITOCHONDRIAL RESPIRATION BY 0·5 mM SUBSTRATE, AT INCREASING BBA CONCENTRATIONS

Preincubation	O_2 uptake	P/O
Control	3·6	2·6
Pyruvate (0·5 mM) + BBA (20 μM)	3·4 (4)	2·4
Pyruvate (0·5 mM) + BBA (200 μM)	3·1 (14)	2·3
Pyruvate (0·5 mM) + BBA (2 mM)	2·4 (33)	2·3
Pryuvate (0·5 mM) + BBA (5 mM)	1·7 (52)	1·1

The experimental conditions were similar to those reported in Table 1. Preincubation was carried out as described in Table 5, except that increasing concentrations of BBA were used. O_2 uptake is expressed as μg atoms O_2/hr/mg protein. Degree of inhibition between brackets.

DISCUSSION

The data reported in this paper indicate that the inhibition of mitochondrial oxidation of NAD-dependent substrates is due to a BBA-induced loss of the mitochondrial NAD. This finding explains the effectiveness of 2,4-DNP to relieve moderate inhibitions of respiration, while it is without effect when more severe inhibition is produced. A mild inhibition in fact indicates that a fraction of mitochondria still retains NAD and oxidative capacities: in this case DNP can act by increasing the respiration rate, owing to its uncoupling effect. On the other hand, when nearly all the mitochondria have lost their NAD content, as in the case of a severe inhibition of respiration, no DNP-dependent stimulation may be expected.

The BBA-dependent diffusion of NAD from the mitochondria, and the experimental evidence that external NAD, added to the preincubation system, can join the respiratory chain, and can restore the oxidative functions of mitochondria, show that BBA makes the inner membrane quite permeable to pyridine nucleotides, and probably to other constituents of the matrix space. The nature of the alteration at the inner membrane level is obscure. BBA does not seem to act as a swelling agent *per se*, and presumably makes the membrane somewhat less resistant, thus favouring the effect of swelling agents (Fig. 2). The action of BBA on the membrane is counteracted by the stabilizing effect of EDTA; mitochondria stored in hypotonic sucrose, supplemented with EDTA, are reported to be very resistant to loss of pyridine nucleotides.[13]

The protection given by NAD-dependent substrates may be due to their property of maintaining the pyridine nucleotides in the reduced state, and therefore tightly bound in the mitochondria.[14] Consequently, reduced NAD is not expected to diffuse readily out from the mitochondria, under the action of BBA. The protection given by succinate could be mediated also by an action on the pyridine nucleotides, through the "reversal" of the oxidative phosphorylation, through which succinate reduces rapidly NAD.[15]

In the experiments carried out on the protective role of substrates, we have tried to reproduce *in vitro*, in the preincubation medium, a substrate concentration as near as possible to the physiological levels. The pyruvate concentration in liver and other tissues may vary between 0·04 and 0·5 mM,[16] so that we have chosen a 0·5 mM concentration as roughly representative of the maximal protection given by the endogenous substrates *in vivo*, in order to test how much the BBA concentration could be

raised, without impairing too severely the oxidative functions of the mitochondria. Conclusion is given that the pharmacological effects of this hypocholesterolemic agent are not at all impaired by this action at the mitochondrial level, which is efficiently counteracted by the amount of endogenous substrates.

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