Biochemical Pharmacology, Vol. 19, pp. 2737–2743. Pergamon Press. 1970. Printed in Great Britain

THE INHIBITION OF MITOCHONDRIAL RESPIRATION BY β-BENZAL BUTYRIC ACID AND THE POSSIBLE RELATIONSHIP TO CHOLESTEROL BIOSYNTHESIS

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(*Received* 10 *March* 1970; *accepted* 14 *April* 1970)

Abstract—β-benzal butyric acid, an inhibitor of cholesterol biosynthesis, reduces in rat liver mitochondria the oxidation of all the intermediates of the tricarboxylic acid cycle and of pyruvate, but not that of succinate. The effect of the inhibition, which is particularly evident in the case of the oxidation of pyruvate to acetyl-coenzyme A, is correlated with the decreased biosynthesis of cholesterol, due to the reduced availability of the cytoplasmic acetyl-coenzyme A. β-benzal butyric acid does not act on the pyruvate oxidoreductase complex purified from pig heart muscle, nor on the NADH-oxidizing submitochondrial rat liver particles. 2,4-dinitrophenol partially releases in the liver mitochondria the inhibition of the oxygen uptake due to β-benzal butyric acid. A possible mode of action of β-benzal butyric acid on oxidative phosphorylations is postulated.

β-BENZAL BUTYRIC ACID (3-methyl-4-phenyl-3-butenoic acid, BBA) is known as an hypocholesterolemic agent, capable of lowering the pathological high levels of cholesterolemia.[1] Experimental evidence has indicated recently that BBA and other butyric acid derivatives act as noticeable inhibitors of sterol synthesis in the liver cell, by interfering with the initial steps of the biosynthetic pathway.[2–5]

This paper deals with the action of BBA upon the oxidative steps of the citric acid cycle, with particular regard to pyruvate oxidation, which, as known, provides the cell with the largest portion of the "active acetate", necessary for fatty acid and cholesterol biosynthesis.

EXPERIMENTAL

Preparation and incubation of rat liver mitochondria

Rat liver mitochondria were prepared according to Schneider[6] from female Sprague–Dawley rats (100–150 g), which were fasted for about 12 hr before sacrifice. The mitochondria (about 1 mg of protein N), suspended in 0·25 M sucrose solution, were incubated in Warburg flasks containing in a final volume of 2 ml the following components: KH_2PO_4–K_2HPO_4 buffer (pH 7·40), 30 μmoles; $MgSO_4$, 15 μmoles; cytochrome *c*, 0·022 μmoles; ATP, 3·5 μmoles; glucose, 35 μmoles; hexokinase (100 KM units[7]), in the side arm. Substrates were normally incubated in amounts corresponding to 20 μmoles, but in the case of pyruvate oxidation, 16 μmoles pyruvate + 4 μmoles fumarrate, as primer, were used. BBA was added in the amounts indicated in Tables 1–4. The addition of the single components was done in the above mentioned order; BBA, substrates and mitochondria were added last, in this sequence. NaOH was placed in

the center wall, and O_2 was used as gas phase. Incubation was carried out for 30 min at 30° at about 80 strokes/min.

Preparation and incubation of rat liver submitochondrial particles

Rat liver submitochondrial particles were prepared by a drastic sonication procedure according to Gregg,[8] and the oxygen uptake recorded by polarographic measurements at 25° in the following medium: Tris-HCl buffer (pH 7·40), 25 μmoles; NADH, 25 μmoles; potassium phosphate buffer (pH 7·40), 10 μmoles; bovine serum albumin, 0·2% (final concentration). Final volume of 2·5 ml.

Purification of the pyruvate–oxidoreductase complex

The pyruvate–oxidoreductase complex was extracted from pig heart muscle and purified according to Hayakawa *et al.*[9] The action of BBA was tested on the lipoamide dehydrogenase activity ($NADH_2$:lipoamide oxidoreductase, EC 1.6.4.3) and on the pyruvate dismutation activity (2 pyruvate + phosphate → acetylphosphate + CO_2 + lactate) of the enzyme complex, both measured as described by Hayakawa *et al.*[9] The units/mg protein of our preparation at step 5 of the purification procedure were 109 and 26, respectively for the lipoamide dehydrogenase and pyruvate dismutation activities.

Assays

Pyruvate determination. At the end of each incubation time, samples were treated with 0·6 ml of 6% perchloric acid (v/v), and the residual pyruvate determined enzymically according to Bücher *et al.*[10]

Citrate determination. The samples were treated with 1 ml of freshly prepared 5% tungstic acid (w/v). After spinning for few min, the citrate was determined on the supernatant fluid according to Ettinger *et al.*[11]

Glucose-6-phosphate determination for P/O ratio assay. When the P/O ratio was to be assayed, glucose-6-phosphate was determined following incubation, according to Hohorst, [12] after treating the medium with 0·6 ml of 6% perchloric acid (v/v).

Chemicals

BBA, provided by Istituto Biochimico Italiano (Milan, Italy), melted at 113°, and possessed a molar extinction coefficient of 15,600 at 246 mμ and four infrared maxima at 1700, 1250, 750 and 710 cm^{-1}. The elementary analysis was very close to the predicted values. Before using, BBA was converted into the sodium salt, by addition of appropriate amounts of diluted NaOH.

Cytochrome *c* (Fe, 0·33%), NADH, NAD, glucose-6-phosphate, CoA (90 per cent pure) were products of Biochemica "Boehringer" (Milan, Italy). ATP and hexokinase (grade III) were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Phosphate acetyltransferase (acetyl-CoA: orthophosphate acetyl-transferase, EC 2.3.1.8) was obtained from Biochemica "Boehringer" (Milan, Italy).

RESULTS

Experiments with rat liver mitochondria

The results of a typical experiment carried out with isolated rat liver mitochondria, tested with different substrates in the presence of BBA, are shown in Table 1. The oxidation of the Krebs cycle intermediates and of pyruvate was noticeably depressed,

even at concentrations of BBA as low as 0·1 μM; the most marked inhibition was obtained with pyruvate plus fumarate as substrates. Surprisingly, succinate oxidation was rather stimulated. Results were consistently similar throughout the whole series of experiments; moreover, all experimental points were statistically significantly different from controls.

TABLE 1. THE INHIBITION OF OXYGEN UPTAKE BY β-BENZAL BUTYRIC ACID (BBA) IN ISOLATED RAT LIVER MITOCHONDRIA*

Expt. no.	Substrate	BBA (μM)	O_2 uptake	Inhibition (%)	P/O
1	pyruvate	—	11·2	—	—
		100	0·4	96	—
		1·0	1·0	91	—
		0·1	1·0	91	—
2	α-oxoglutarate	—	11·2	—	2·7
		100	0·4	96	—
		1·0	3·5	69	0·7
		0·1	7.9	30	2·0
3	L-isocitrate	—	14·4	—	2·2
		100	0·7	95	—
		1·0	2·3	84	—
		0·1	3·0	79	0·2
4	fumarate	—	5·8	—	2·2
		100	1·5	75	—
		1·0	2·4	59	0·7
		0·1	4·2	28	1·2
5	succinate	—	14·7	—	1·3
		100	—	—	—
		1·0	20.1	+36	1·1
		0·1	18·9	+28	1·2

*Incubation with isolated rat liver mitochondria (about 1 mg of protein N) was carried out as described in the text for 30 min at 30°. In all the experiments BBA was pre-incubated for 15 min with the mitochondria, before adding the substrates. O_2 uptake expressed as μg atoms/mg protein N/30 min.

Pyruvate oxidation was examined more completely with assays of substrate and citrate after incubation. The results of Table 2 show that the high degree of inhibition of oxygen uptake during pyruvate oxidation was closely paralleled by a noticeable decrease of the pyruvate disappearance and of citrate production.

In order to obtain the most inhibitory effect, BBA needed to be pre-incubated with the mitochondria at least for 5 min, before adding the substrate. The preincubation time was not critical between 5 and 20 min. We have adopted a 15 min preincubation period as the most convenient (Tables 1 and 2). The degree of inhibition of oxygen uptake dropped consistently when the preincubation of the liver mitochondria with the inhibitor was carried out in the presence of equimolar amounts of substrate. Table 3 shows the results of this type of experiment with pyruvate plus fumarate, as substrates. Similar results were obtained with the Krebs cycle intermediates, and suggest a possible protective role of endogenous substrate *in vivo* towards the strong inhibitory effects of BBA (see Discussion).

Experiments with rat liver submitochondrial particles

We were unable to demonstrate any significant inhibition of NADH oxidation by

TABLE 2. EFFECT OF β-BENZAL BUTYRIC ACID (BBA) ON PYRUVATE OXIDATION AND CITRATE PRODUCTION IN ISOLATED RAT LIVER MITOCHONDRIA*

BBA (μM)	O_2 uptake †	pyruvate (μmoles)‡	citrate (μmoles)‡
—	11·2	−6·40	+1·80
100	0·8 (93)	−0·70 (89)	+0·10 (94)
1·0	1·0 (91)	−0·80 (87)	+0·10 (94)
0·1	1·5 (87)	−0·80 (87)	+0·11 (94)

*Experimental conditions were similar to those described in Table 1. Incubations carried out with pyruvate (16 μmoles) + fumarate (4 μmoles).
†Results expressed as μg atoms/mg protein N/30 min. Degree of inhibition between brackets.
‡Values represent the disappearance of pyruvate from or the increase of citrate in the whole incubation mixture. Degree of inhibition between brackets.

TABLE 3. THE PROTECTIVE ROLE OF SUBSTRATE UPON PYRUVATE OXIDATION BY ISOLATED RAT LIVER MITOCHONDRIA IN THE PRESENCE OF β-BENZAL BUTYRIC ACID (BBA)*

Preincubation	O_2 uptake†	citrate (μmoles)‡
none	10·2	+1·40
BBA (100 μM)	1·9 (81)	+0·20 (85)
BBA (100 μM) + pyruvate (100 μM)	7·3 (29)	+0·98 (30)

*Experiments were carried out exactly as described in Tables 1 and 2 and in the text, except that when preincubation was performed, the mitochondria were preincubated either with BBA only or with BBA and pyruvate together for 15 min at 30°, and then pyruvate (16 μmoles) + fumarate (4 μmoles) were added and normal incubation carried out, as reported in the text.
†Results expressed as μg atoms/mg protein N/30 min. Degree of inhibition between brackets.
‡Values represent the increase of citrate in the whole incubation mixture. Degree of inhibition between brackets.

submitochondrial rat liver particles, preincubated or not with BBA, as we should have had, if BBA were an inhibitor of the respiratory chain. Occasionally, a small inhibition, of the order of about 30 per cent was observed at BBA concentration values as high as 1 mM.

Experiments with the pyruvate oxidoreductase complex

None of the two enzymic activities of the pyruvate oxidoreductase complex tested, i.e. lipoamide dehydrogenase and pyruvate dismutation reaction, was inhibited to any considerable degree by BBA, even at concentrations capable of lowering markedly the mitochondrial oxidation of pyruvate, and even after 15 min of preincubation of the enzyme complex with the inhibitor.

Releasing of BBA inhibition with 2, 4-dinitrophenol

In order to explain the wide inhibitory effect of BBA on the mitochondrial respiration, it has been thought that BBA could act as an inhibitory agent of oxidative phosphorylations. If this supposition were correct, the oxygen uptake would not

probably be depressed by BBA, after uncoupling of the phosphorylative reactions. To test this hypothesis, experiments have been performed with 10 μM 2,4-dinitrophenol (DNP), as uncoupling agent, incubated with and without BBA. The results, which are reported in Table 4 and which were similar throughout the other experiments, confirmed that DNP is effective in restoring the oxidation of pyruvate to a considerable degree.

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

Additions	O_2 uptake†
none	10·6
BBA (100 μM)	1·4 (87)
DNP (10 μM)	14·7
BBA (100 μM) + DNP (10 μM)	7·8 (26)

*Experimental conditions were those described in Table 1. BBA was preincubated for 15 min at 30° with the mitochondria, before adding the substrates (pyruvate, 16 μmoles + fumarate, 4 μmoles) and 10 μM DNP.
†Results expressed as μg atoms/mg protein N/30 min. Degree of inhibition between brackets.

Experiments with other hypocholesterolemic agents

Experiments have been carried out on the effect of other hypocholesterolemic agents on the oxidation of the Krebs cycle intermediates and of pyruvate, under conditions exactly similar to those adopted in the studies carried out with BBA. The following compounds have been tested: α-hydroxy β-benzal butyric acid (2-hydroxy-3-methyl-4-phenyl-3-butenoic acid, HBBA)[3,4] β-benzal butyramide (3-methyl-4-phenyl-3-butenamide, BBAM)[4] and α-methyl γ-phenyl butyric acid (2-methyl-4-phenyl-butanoic acid, MPBA).[5] All the compounds tested were shown to act as inhibitors of these reactions, though the effect was not as strong as during BBA action.

DISCUSSION

The results presented in this work provide experimental evidence for the inhibitory effect of BBA and other butyric acid derivatives (HBBA, BBAM, MPBA) on the oxidation of pyruvate and of a number of intermediates of the tricarboxylic acid cycle in isolated rat liver mitochondria. Particularly, we have found that the oxidations of all the NAD-dependent substrates were markedly inhibited (Table 1). The oxygen uptake due to succinate was nevertheless rather stimulated in the presence of BBA.

The action of BBA on pyruvate oxidation is noteworthy, because of the well-known relationships that exist among intramitochondrial pyruvate oxidation, acetyl-CoA production and lipid biosynthesis in the cell. Evidently, a strong inhibition of the acetyl-CoA production might affect seriously the acetyl CoA-dependent first steps of sterol synthesis in the cell sap and endoplasmic reticulum.

It is well accepted now[13, 14] that the action of the citrate cleavage enzyme (ATP–citrate lyase, EC 4.1.3.8) on citrate is probably the most important way to obtain at the cytoplasmic level the acetyl-CoA moiety necessary for both fatty acid and choles-

terol biosynthesis. The output of citrate from the mitochondria is almost passive.[15] Now we have found that the intramitochondrial citrate formation was markedly depressed during the inhibition of pyruvate oxidation (Table 2). It is unlikely that such a marked inhibition takes place *in vivo* owing to the protective role that the endogenous pyruvate may exert towards BBA action, as indicated in Table 3. Yet, however, a moderate inhibition of pyruvate oxidation occurs in these conditions, of the order of about 30 per cent (Table 3), sufficient enough to cause a moderate lowering of the intramitochondrial citrate, which may prevent any citrate from passing outside from the mitochondria. In the case of substrate (citrate) shortage in the mitochondrion, the first need to be satisfied by the cell is that related to energy production, with detriment of new syntheses, if a competition arises. This seems to happen in the case of the moderate inhibition exerted by BBA upon the citrate production, since citrate serves both as an oxidizable substrate and as an acetyl-CoA precursor.

The protective rôle played by the various intermediates of the Krebs cycle and pyruvate itself towards the strong inhibitory action of BBA upon oxidations (which would take place in the absence of these protective compounds in a very noticeable degree), explain the complete atoxicity of the drug[16] and the absence of signs of cell damage. This protection does not hinder, on the other hand, the great potentiality of BBA to act as a noticeable hypocholesterolemic agent *in vivo*,[1] and as a powerful inhibitor of cholesterol synthesis *in vitro*,[2–5] presumably because the availability of the cytoplasmic acetyl-CoA is strongly reduced by the drug.

The experiments with the pyruvate oxidoreductase complex and with the submitochondrial particles have been carried out, in order to find out the site of action of BBA on the oxidative reactions. Since we have not succeeded in demonstrating any effect of the drug on these preparations, we have supposed that BBA could act as an inhibitor of oxidative phosphorylation. It is worthy mentioning, in this connection, that in those cases in which the oxygen uptake was not so severely inhibited as to prevent any correct P/O ratio evaluation, we have found that the P/O ratio was indeed depressed, if compared to the control values (Table 1). Namely, the phosphorylation was more inhibited than the respiration.

DNP, a phosphorylation uncoupling agent, seems to be effective in reducing the inhibition of the oxygen uptake brought about by BBA (Table 4). This result again let us reach the conclusion that BBA may interfere with some steps of the phosphorylative processes.

Acknowledgements—This work was partially carried out with grants from the Consiglio Nazionale delle Ricerche, Rome. Thanks are given to Drs. B. Frattini and U. Valcavi (Istituto Biochimico Italiano, Milan, Italy) for generous gifts of BBA and of the other butyric acid derivatives.

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