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INHIBITION OF UREOGENESIS IN ISOLATED RAT LIVER CELLS BY A NON-STEROIDAL ANTI-INFLAMMATORY DRUG (BUTIBUFEN)*

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Abstract—The effect of a non-steroidal anti-inflammatory drug (butibufen) on ureogenesis in isolated rat hepatocytes has been studied. Butibufen at 0 4 mM, and particularly at 2 mM, strongly inhibited urea synthesis The drug at these concentrations also inhibited markedly carbamoylphosphate synthetase activity In addition, 2 mM butibufen lowered ATP concentrations of the cells and enhanced oxygen consumption in isolated liver mitochondria The results suggest that the inhibition by 0 4 mM butibufen on carbamoylphosphate synthetase activity can account for the entire inhibition of ureogenesis, whereas the decreased cellular ATP concentration at 2 mM butibufen might be at least partly responsible for low carbamoylphosphate synthesis and thus, for reduced urea production The decrease in ATP levels probably results from uncoupling effects of butibufen on oxidative phosphorylation

Non-steroidal anti-inflammatory drugs (NSAID) are widely used in the treatment of rheumatoid arthritis and other rheumatic diseases. The *p*-substituted phenylalkanoic acids occupy a prominent position among the more recently synthesized NSAID. One of them, butibufen (2-(4-isobutyl phenyl) butyric acid) has proved to be clinically useful [1–3]. It is absorbed very rapidly and is metabolized in the liver [4].

We have studied the metabolic effects of this drug on isolated liver cells and have found that it decreases their ability to synthesize urea. This result appears to be unrelated to a gross hepatotoxicity of the drug and we have looked for a possible biochemical mechanism for this effect.

In this paper we present evidence indicating that butibufen action on urea synthesis results from inhibition of carbamoylphosphate synthetase (EC 2.7.2.5) At a higher concentration butibufen also decreases ATP levels and this factor might also be responsible for part of the inhibition of urea synthesis.

MATERIALS AND METHODS

Animals. Male Wistar rats maintained on stock laboratory diet and weighing 200–250 g were used Rats were fasted for 24 hr before experiments

Chemicals. Hexokinase, glucose-6-phosphate dehydrogenase, pyruvate kinase and NADP were purchased from Sigma Chemical Company (St. Louis, Missouri, U.S.A.) Collagenase was obtained from Boehringer GmbH (Mannheim, F.R.G.). Butibufen was generously donated by the laboratory Juste S.A.Q.F (Madrid, Spain). All other reagents were of analytical grade.

Isolation and incubation of hepatocytes. Isolated hepatocytes were prepared from 24-hr starved rats as described in [5], with the modifications suggested in [6]. The cells (70–100 mg wet wt) in a final volume of 4 ml Krebs–Henseleit bicarbonate buffer pH 7.4 [7] containing the appropriate substrates, were incubated at 37° in 25 ml Erlenmeyer flasks in a shaking bath for 30 min; the flask atmosphere contained 95% O_2 : 5% CO_2. More than 85 per cent of the cells excluded trypan blue at the end of the incubation. The reactions were stopped with perchloric acid (2% w/v final concentration) and the precipitate was removed by centrifugation. The supernatant was used for the assays.

Preparation of mitochondria and oxygen consumption Mitochondria were obtained from livers of rats fasted overnight as described in [8]. Oxygen consumption was measured polarographically with a Clark type oxygen electrode [9]. The incubation medium contained 0.25 M sucrose, 0.01 M K_2HPO_4, 5 mM sodium succinate and 0.01 M Tris–HCl pH 7.4 An aliquot (0.05 ml) of the mitochondrial suspension was added to 1.95 ml of this medium and the oxygen uptake measured with continuous magnetic stirring.

Biochemical determinations. Carbamoylphosphate synthetase and ornithine-carbamoyl transferase (EC 2.1 3.3) were assayed in whole rat liver homogenates in the presence and absence of butibufen according to [10], and citrulline was determined colorimetrically [11]. Urea was also measured colorimetrically [12] on perchloric acid extracts, and ATP was determined enzymatically [13] on neutralized perchloric acid extracts Protein was determined by the biuret method [14] using bovine serum albumin as standard

Statistical analysis of data was carried out using Student's *t*-test

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RESULTS

Figure 1 shows the dose–response curve of butibufen concentration vs percentage of the cells that excluded trypan blue at the end of incubation As can be seen, the highest concentration of butibufen that did not affect cell viability was 2 mM; we therefore used this as the maximum concentration in the experiments on metabolism

Figure 2 shows as a dose–response curve, the inhibition produced by butibufen concentrations ranging between 0 1 and 2 mM. Urea production by hepatocytes was linear with time of incubation (not shown)

Preliminary studies in our laboratory show that other non-steroidal anti-inflammatory drugs, e g. ibuprofen (2-(isobutyl phenyl)-propionic acid) and sulindac (Z-5-fluoro-methyl-1-((4-(methyl-sulfinyl)phenyl)methylene) - 1H - indene - 3 - acetic acid), have effects similar to butibufen but to a lesser degree, and they not affect cell viability at the same concentrations. In the following experiments we have used butibufen at concentrations of 0 4 and 2 mM which are equivalent to *c.* 2 and 10 times the therapeutic plasma concentrations

Table 1 shows urea synthesis of isolated hepatocytes in a given time in the absence or presence of different combinations of exogenous nitrogen sources Addition of butibufen inhibited urea production in the absence of a nitrogen source and the effect was concentration-dependent As shown also in Table 1, butibufen at 0 4 mM had little or no effect on endogenous urea synthesis when only ornithine, aspartate or glutamate were present.

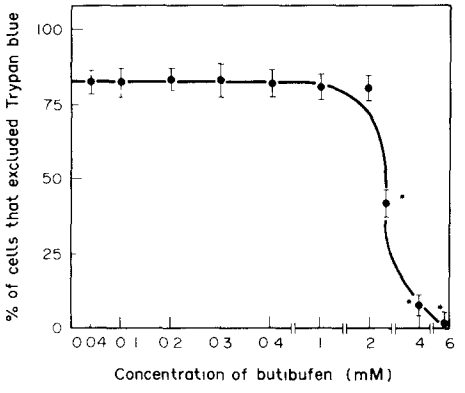

Fig 1 Effect of different concentrations of butibufen on the ability of isolated hepatocytes to exclude trypan blue Liver cells were incubated in the absence and presence of various concentrations of butibufen for 30 min at 37° The results are expressed in a semilogarithmic scale as the mean ± S D of seven experiments, * $P < 0.001$

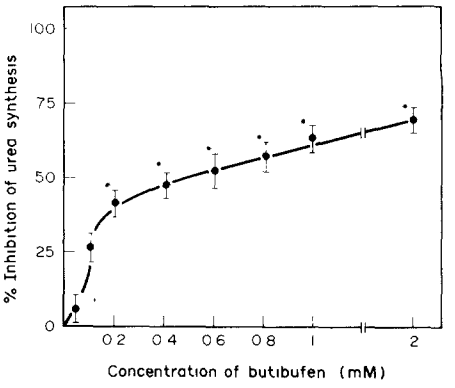

Fig 2 Dose–response curve of inhibitory effect of butibufen on urea synthesis by isolated hepatocytes after 30 min incubation in 10 mM NH_4Cl and 10 mM ornithine, at 37° The results are the mean ± S D of five experiments, † $P < 0.05$, * $P < 0.001$

The marked inhibition (*c.* 90% at 2 mM of the drug) observed in hepatocytes in the presence of high concentrations of ammonia (Table 1) could be due to a decrease in the activity of carbamoylphosphate synthetase induced by butibufen; on this point, Table 3 shows that carbamoylphosphate synthetase in liver homogenates is inhibited markedly by the addition of butibufen Under the same conditions, ornithine-carbamoyl transferase activity was not affected by the drug. Since two molecules of ATP are required per molecule of carbamoylphosphate synthesized by the enzyme, the influence of butibufen on ATP levels has also been studied. The drug (at 2 mM) decreased by at least half the concentration of the nucleotide in isolated hepatocytes (Table 2) On the other hand, butibufen clearly increased oxygen consumption by isolated mitochondria in the absence of ADP (state IV respiration) (Fig. 3)

Table 1 Effect of butibufen on urea synthesis in isolated rat hepatocytes

Substrate	No butibufen	0 4 mM butibufen (nmol urea/min/g wet wt)	2 mM butibufen
None	107 ± 10	90 ± 3 (16)**	46 ± 4 (57)*
NH_4Cl	271 ± 37	131 ± 6 (52)*	33 ± 10 (88)*
Ornithine	260 ± 24	235 ± 41 (6)	—
Aspartate	113 ± 15	115 ± 28 (0)	—
Glutamate	180 ± 19	181 ± 14 (0)	—
NH_4Cl + ornithine	497 ± 48	272 ± 42 (45)*	68 ± 41 (86)*
NH_4Cl + ornithine + aspartate	701 ± 52	295 ± 43 (57)*	206 ± 95 (71)*
NH_4Cl + ornithine + glutamate	680 ± 63	308 ± 66 (55)*	154 ± 74 (77)*

All substrate concentrations used were 10 mM The results are expressed as the mean ± S D of seven rats *$P < 0.001$, **$P < 0.01$ Numbers in parenthesis represent the degree of inhibition as percentage of appropriate controls

Table 2. Effect of butibufen on ATP levels in isolated rat hepatocytes

Substrate	No butibufen	0 4 mM butibufen (nmol ATP/g wet weight)	2 mM butibufen
None	1 90 ± 0 25	1 53 ± 0.21 (19)**	0 69 ± 0 05 (64)*
NH_4Cl	1 86 ± 0 24	1 39 ± 0 14 (25)**	0.44 ± 0 05 (76)*
NH_4Cl + ornithine + aspartate	1 80 ± 0 14	1 52 ± 0 15 (16)**	0 86 ± 0 15 (53)*
NH_4Cl + ornithine + glutamate	1 79 ± 0 10	1 55 ± 0 19 (15)**	0 92 ± 0 17 (49)*

All substrate concentrations used were 10 mM The results are expressed as the mean ± S.D of five rats; *P < 0 001; **P < 0.05 Numbers in parenthesis represent the degree of inhibition as percentage of appropriate controls.

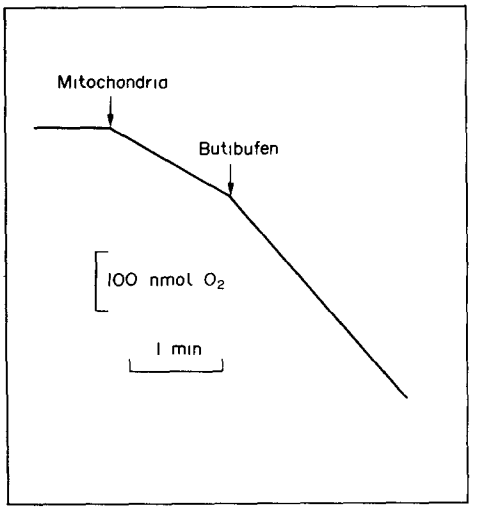

Fig 3 Stimulation of State IV respiration by butibufen. The experimental conditions were as described in Methods, using 4 mM succinate as substrate The respiratory control ratio (RCR, rate in presence of ADP/rate in the absence of ADP) was *c.* 4. The arrows show the point of addition of mitochondria (10 mg protein) or butibufen (10^{-4} M) Rates of oxygen consumption are expressed as nmol O_2/min and are the mean of four separate determinations differing less than 10 per cent

DISCUSSION

Butibufen clearly decreased ureogenesis by isolated hepatocytes when exogenous ammonia was present; however, the drug at 0.4 mM had no effect on urea synthesis if ornithine, aspartate or glutamate alone were added (Table 1). These results suggest an inhibitory effect of butibufen on the first step of the urea cycle, i.e. the reaction catalyzed by carbamoylphosphate synthetase, since ornithine, aspartate and glutamate are amino acids which enter into the cycle after the carbamoylphosphate synthetase step.

This decrease in ureogenesis is associated with an inhibition of carbamoylphosphate synthetase activity. When the data from Tables 1 and 3 are compared, it appears that inhibition of urea synthesis by 0.4 mM butibufen is due to a direct inhibition of carbamoylphosphate synthetase activity. Indeed, 0 4 mM butibufen produced only a small decrease in ATP levels in isolated liver cells. On the other hand, at the higher concentrations, the inhibition of carbamoylphosphate synthetase activity alone seems insufficient to explain the greater inhibition of ureogenesis, a lower availability of ATP in the intact cell (Table 2), which could further decrease the velocity of synthesis of carbamoylphosphate, might also contribute to this effect.

Since butibufen increased the oxygen consumption in isolated liver mitochondria, as shown in Fig. 3, a possible mechanism for the reduction in ATP levels could be that the drug is behaving as a weak uncoupler of oxidative phosphorylation, as has been shown for other NSAID [15, 16]. Indeed, butibufen shares some of the structural features of other standard uncouplers, i e an aromatic ring associated with a weak acidic group If this was one of the mechanisms of inhibition of ureogenesis it would be expected that addition of substrates which could produce ATP by an independent mechanism of oxidative phosphorylation would impair the butibufen effect. Indeed, addition of glucose diminished the drug effect on ureogenesis (data not shown)

Inhibition of ureogenesis by 2 mM butibufen in the presence of NH_4Cl and ornithine is also decreased by aspartate and glutamate (Table 1) These amino acids participate in the malate–aspartate shuttle which transfers reducing equivalents into mito-

Table 3 Effect of butibufen on carbamoylphosphate synthetase (CPS) and ornithine transcarbamylase (OTC) activities in whole rat liver homogenate

	None	Butibufen 0 4 mM	2 mM
CPS (μmol citrulline/hr/g liver)	580 ± 37	261 ± 31*	163 ± 41**
OTC (μmol citrulline/hr/g liver)	12,320 ± 209	11,850 ± 184†	12,860 ± 195†

The homogenates were pre-incubated with the drug for 30 min The activities were assayed as described in Methods Results are expressed as mean ± S.D of five rats *P < 0 001, **P < 0 001, †N S

chondria [17, 18] Studies by Williamson *et al.* [19] indicate that the rate of entry of glutamate into mitochondria is an important regulator of the malate–aspartate cycle, but that the affinity of the carrier system for glutamate is poor ($K_m = 6$ mM) Because such a carrier would not be saturated at normal glutamate concentrations in liver (*c* 2 mM in fasted rat liver [20]), addition of glutamate or aspartate would increase the efficiency of this mechanism, providing higher levels of intramitochondrial NADH for the electron transport chain, thereby resulting in increased synthesis of ATP by means of the residual oxidative phosphorylation The effect of glutamate could also be due to increased intramitochondrial acetylglutamate levels resulting in a higher carbamoylphosphate synthetase activity

The potential clinical implications of our study deserve consideration In this study butibufen has been used at concentrations two to ten times greater than the therapeutic plasma levels of the drug. Therefore a substantial inhibition of carbamoylphosphate synthetase activity and of ureogenesis might occur during the course of treatment with butibufen This could have noxious effects, particularly in patients with reduced functional hepatic reserve

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