Malic enzyme levels are increased by the activation of NADPH-consuming pathways: detoxification processes

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The administration to rats of either *t*-butyl hydroperoxide or phenobarbital, compounds that are metabolized through detoxification processes, produces an increase in specific activity of the NADPH-consuming enzymes, glutathione reductase and NADPH-cytochrome *c* reductase. These compounds also produce a very significant increase in the specific activity of malic enzyme. Immunoprecipitation with a specific antibody for malic enzyme indicates that specific activity changes are the result of corresponding changes in the amounts of enzyme protein present. The administration of 1,3-bis(chloroethyl(-1-nitrosourea (a glutathione reductase inhibitor) together with *t*-butyl hydroperoxide abolishes any stimulation of malic enzyme activity. These results indicate that an increase in NADPH consumption induces the synthesis of malic enzyme. Alternatively, a protection of enzyme degradation cannot be rigorously excluded.

Malic enzyme *NADP+/NADPH* t-*Butyl hydroperoxide* *Phenobarbital* *Glutathione reductase* *NADPH-cytochrome* c *reductase* *1,3-Bis(chloroethyl)-1-nitrosourea*

1. INTRODUCTION

The participation of malic enzyme in the generation of reducing equivalents as NADPH has been demonstrated experimentally under a number of different conditions [1–3]. In many of these situations, the activity of malic enzyme and the rate of fatty acid synthesis are highly correlated in several animal systems [4–6]. This correlation between fatty acid synthesis and the activity of malic enzyme, one of whose functions include that of providing NADPH for 'de novo' fatty acid synthesis, could suggest that the activity of malic enzyme could be regulated by the NADPH/$NADP^+$ ratio through a mechanism involving changes in the NADPH requirement. In this way, an increase or decrease in NADPH requirement would respectively produce the increase or decrease in the amount of malic enzyme.

Here, we have studied the effect of changes in the flux through different NADPH-consuming pathways on the synthesis and activity of malic enzyme. We have examined firstly the effect of xenobiotics compounds [*t*-butyl hydroperoxide (t-BHP) and phenobarbital (PB)] which are metabolized through detoxification processes which use the NADPH:glutathione peroxidase-glutathione reductase system [7] and NADPH-cytochrome P-450 reductase [8] and secondly the effect of administration of 1,3-bis(chloroethyl)-1-nitrosourea (BCNU) an inhibitor of glutathione reductase [9] in conjunction with that of t-BHP.

The results show that an increase in the consumption of NADPH is paralleled by an increase in the synthesis, hence in specific activity of malic enzyme and that when the increase in NADPH consumption is prevented, the malic enzyme level does not change.

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2. MATERIALS AND METHODS

2.1. *Animals*

Female rats of the Wistar strain weighing 150–250 g were used. They were maintained on a standard laboratory diet. Rats described as fasted-refed were deprived of food for 48 h and then allowed free access for the next 48 h to a carbohydrate-rich diet.

2.2. *Treatment*

The following compounds were administered: t-BHP, 25 $mg \cdot kg^{-1}$ i.p. daily for 3 days; PB, 70 $mg \cdot kg^{-1}$ i.p. daily for 4 days; BCNU, 40 $mg \cdot kg^{-1}$ i.p. daily for 3 days.

2.3. *Chemicals*

All enzymes and coenzymes were purchased from Sigma (USA) and Boehringer (FRG). BCNU was obtained from Bristol Laboratories (USA). All other chemicals were of the highest purity available from commercial sources.

2.4. *Preparation of tissue extracts*

Rats were killed by cervical dislocation and the liver and adipose tissue immediately removed and homogenized in 7 vols ice-cold medium containing 0.25 M sucrose, 1 mM EDTA, 1 mM DTT and 15 mM Tris-HCl, pH 7.4. The homogenate was centrifuged at 800 × *g* for 10 min. The supernatant fraction (supernatant 1) was then centrifuged for 10 min at 8200 × *g* and the supernatant obtained was recentrifuged for 15 min at 11500 × *g*. The postmitochondrial supernatant fraction was centrifuged at 105000 × *g* for 60 min and the pellet (microsomal fraction) resuspended in the buffer described above.

2.5. *Measurements of enzyme activities*

Malic enzyme was assayed by the method of Ochoa et al. [10]. Glutathione reductase was determined as described by Wöthington and Rosemeyer [11]. NADPH-cytochrome *c* reductase was assayed by the method of Vermilion and Coon [12]. Enzyme activities are expressed as nmol of $NADPH \cdot mg\ protein^{-1} \cdot min^{-1}$. All assays were carried out in supernatant 1, except for hepatic NADPH-cytochrome *c* reductase, whose activity was determined in the microsomal fractions. Proteins were determined by the method of Lowry et al. [13].

2.6. *Purification of malic enzyme*

The enzyme was purified from fasted-refed rat liver. The method used was basically that of F-Lobato et al. [14] but in the last step of the process the L-malato-AH Sepharose 4B affinity chromatography column was replaced by a column of NADP-agarose (5 ml) equilibrated with 50 mM Tris-HCl, 10% glycerol. Under these conditions, the enzyme was bound to the column and eluted at the end of a 0–0.02 mM linear gradient of NADP made in equilibration buffer.

A single protein band was obtained when samples (25–50 μg) were analysed by 7.5% (w/v) polyacrylamide gel electrophoresis in the presence of SDS by the method of Maizel [15].

2.7. *Preparation of antibodies*

Antiserum to the enzyme was prepared by pure enzyme inoculation of female New Zealand rabbits by the technique of Chauser [16]. The γ-globulin fraction was obtained by two consecutive precipitations with ammonium sulfate (40% saturation), followed by dialysis against 10 mM sodium phosphate buffer, pH 7.2, containing 15 mM NaCl.

2.8. *Immunoprecipitation of the malic enzyme activity*

The supernatant obtained by centrifugation at 105000 × *g* was used in all the immunoprecipitation assays. Increasing volumes of antiserum were added to a fixed amount of malic enzyme (50 mU) from crude extract, the mixture adjusted to a constant volume with 150 mM NaCl and Triton X-100 added to a final concentration of 1.7% (w/v). The mixtures were allowed to react for 1 h at 37°C, overnight at 4°C and then centrifuged at 12000 × *g* for 10 min at 4°C. Samples of the supernatants were assayed for malic enzyme activity.

3. RESULTS

3.1. *Effect of the variation of NADPH consumption, caused by the administration of xenobiotic substances, on the enzymatic activities of malic enzyme, glutathione reductase and NADPH-cytochrome c reductase of rat liver*

In our experiments, NADPH consumption has been increased by the administration of different substances which are metabolized through detoxification processes which use NADPH: (i) t-BHP, whose metabolism is mediated by the glutathione peroxidase-glutathione reductase system [7] and (ii) PB, which is metabolized through NADPH-cytochrome P-450 reductase [8].

At the same time, inhibition of NADPH-consuming pathways has been carried out using BCNU, an inhibitor of glutathione reductase [9].

In table 1 are summarized the specific activities of malic enzyme, glutathione reductase and NADPH-cytochrome *c* reductase of rat liver when the animals were treated with the above compounds. As shown, the administration of t-BHP produces a significant increase in the specific activities of the NADPH-consuming enzymes glutathione reductase and NADPH-cytochrome *c* reductase. Similar but more marked results are obtained when PB is used. However, in (t-BHP + BCNU)-treated rats, the specific activity of NADPH-cytochrome *c* reductase is not increased and that of glutathione reductase is markedly diminished, as expected, due to the inhibitory effect of BCNU on glutathione reductase activity [9]. At the same time, it can be observed that BCNU prevents the increase in specific activity of malic enzyme that is produced by t-BHP.

Fig.1a represents immunoprecipitation of the hepatic malic enzyme of rats treated as described above. The results show that the amount of malic enzyme present is proportional to the enzymatic activity found under these situations since the gra-

Table 1

Effects of t-BHP, t-BHP + BCNU and PB on specific activities of malic enzyme, glutathione reductase and NADPH-cytochrome *c* reductase in rat liver and adipose tissue

Tissue	Treatment	Malic enzyme	Glutathione reductase	NADPH-cytochrome *c* reductase
Liver	control	8.9 ± 0.35 (19)	57.3 ± 7.6 (9)	115.3 ± 7.1 (7)
	t-BHP	15.5 ± 1.8[a] (7)	65.6 ± 4.3[a] (6)	152.2 ± 8.8[a] (6)
	t-BHP + BCNU	5.9 ± 2.7[ab] (5)	21.5 ± 5.7[ab] (5)	125.5 ± 9.8[ab] (5)
	PB	28.3 ± 1.3[a] (6)	80.8 ± 3.2[a] (6)	208.0 ± 12.2[a] (6)
Adipose tissue	control	172.9 ± 23.1 (10)	53.9 ± 2.9 (7)	13.9 ± 0.9 (9)
	t-BHP	305.5 ± 63.3[a] (4)	66.4 ± 9.8[a] (4)	13.1 ± 0.7 (4)
	t-BHP + BCNU	55.3 ± 11.5[ab] (4)	4.6 ± 1.3[ab] (4)	7.9 ± 1.3[ab] (4)
	PB	279.6 ± 32.7[a] (4)	56.0 ± 1.7 (4)	19.7 ± 7.4[a] (4)

[a] $p < 0.05$

[b] Compared with t-BHP-treated rats

Enzyme activities were measured as indicated in section 2. Results are given in mU/mg protein as means ± SE for the number of animals in parentheses

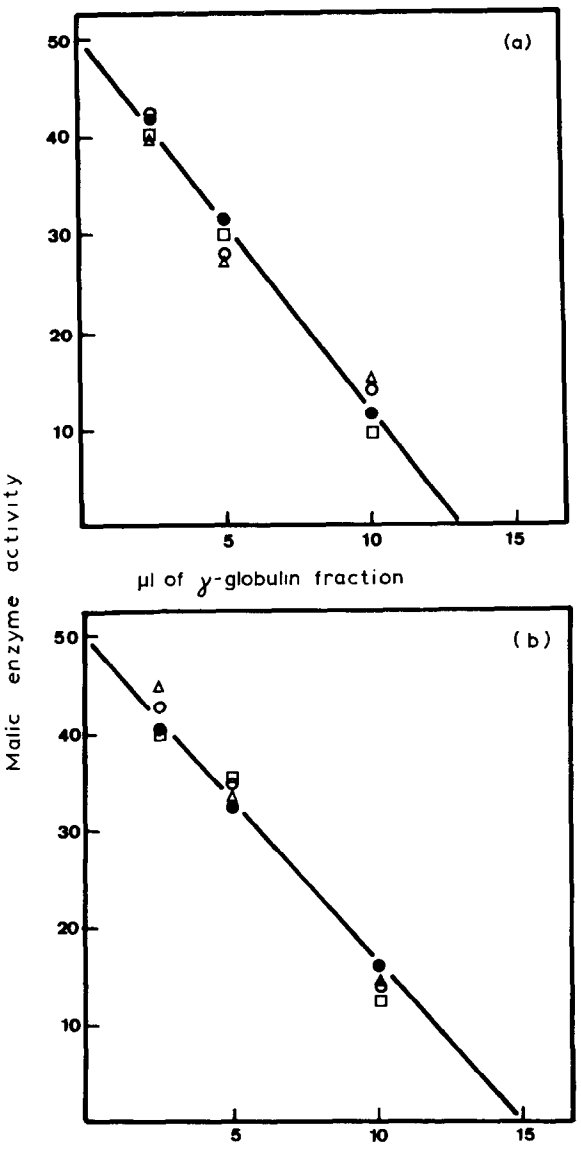

Fig.1. Immunoprecipitation of malic enzyme of liver (a) and of adipose tissue (b). A fixed amount of malic enzyme (50 mU) was incubated with increasing volumes of antiserum as described in section 2. Enzyme activity remaining in the supernatant is represented as mU. Each data point corresponds to 3 individual experiments. (○) Control, (△) t-BHP-treated rats, (●) (t-BHP + BCNU)-treated rats, (□) PB-treated rats.

dients remain constant under the different conditions assayed. These results indicate that the increase in specific activity of this enzyme by t-BHP and PB is caused by an increase in synthesis of the enzyme or its protection against degradation.

3.2. *Effect of the variation of NADPH consumption on enzyme activities in adipose tissue*

The effects of xenobiotic substances on enzymatic activities in adipose tissue are listed in table 1 and fig.1b. As shown, the specific activity of NADPH-cytochrome *c* reductase is lower than in liver; this presumably relates to the fact that liver has a major detoxification capacity. The specific activity of the enzyme in adipose tissue is increased slightly by PB and its activity is significantly reduced by BCNU. In this tissue, however, the specific activity of glutathione reductase is only increased by t-BHP. This effect is due to the fact that t-BHP is metabolized through GSH oxidation [7] as also occurs in liver. At the same time, as expected, BCNU produces a great decrease in activity of this enzyme. This result represents the specific effect of BCNU on glutathione reductase.

With respect to malic enzyme, the behaviour of this enzyme in adipose tissue parallels that found in liver: t-BHP and PB produce a significant increase in its specific activity and BCNU abolishes the action of t-BHP.

Fig.1b shows the immunoprecipitation of malic enzyme in adipose tissue using the same conditions described for liver. As can be seen, the activity of malic enzyme is dependent upon the amount of protein present. As considered previously, protection by xenobiotics against degradation of malic enzyme remains a possibility. These results again indicate that t-BHP and PB are likely to induce synthesis of this enzyme and that BCNU prevents this effect.

4. DISCUSSION

Taking into account our results, we suggest that if an increase in NADPH requirement is common to most metabolic conditions which cause an induction of malic enzyme, then another metabolic situation which also involves increased consumption of NADPH will produce induction of this enzyme as well.

In these experiments, we have produced an increase of NADPH requirement with the administration of substances which are metabolized through detoxification processes which use NADPH: t-BHP and PB. When these substances

were administered, there was an increase of glutathione reductase and NADPH-cytochrome *c* reductase, both NADPH-consuming enzymes. These results were more significant in liver (table 1). At the same time, these substances produced a very significant increase of malic enzyme activity in both tissues assayed (table 1). This corresponds with the increase of enzymatic protein (fig.1a,b). Consequently, these results are in support of t-BHP and PB, which produce the increase of NADPH requirements, producing an increase in the amount of malic enzyme.

This result is strongly supported by the BCNU effect. The administration of BCNU at the same time as t-BHP prevents the increase of NADPH requirements that takes place with t-BHP alone (this is due to the inhibition of glutathione reductase). When both compounds are administered together the increase of malic enzyme is prevented, probably due to a decrease in NADPH consumption caused by BCNU.

The major effect found in adipose tissue could be due to the strong inhibition of NADPH-consuming enzymes by BCNU: it produces a decrease of one order of magnitude in glutathione reductase and NADPH-cytochrome *c* reductase activity in (t-BHP + BCNU)-treated rats is 50% of the activity seen in rats treated with t-BHP alone.

BCNU could have another effect, since the increase of GSSG under these conditions could produce the inhibition of protein synthesis [17]. To establish that this effect was not responsible for the results found, we have measured the effect of BCNU on malic enzyme of fasted-refed rats. In this situation there is an increase in the activity of this enzyme and BCNU does not affect the NADPH consuming pathway (fatty acid synthesis). In this case, BCNU does not prevent the malic enzyme induction (control, 8.9 ± 0.35; fasted-refed rats, 22.1 ± 4.6; fasted-refed rats + BCNU, 20.2 ± 3.04).

All these results support, to a great extent, the theory that the increase of NADPH requirements could be a factor implicated in the induction of malic enzyme.

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