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STUDY OF THE INCREASED GLYCERYL TRINITRATE METABOLISM AFTER PRETREATMENT WITH PHENOBARBITAL IN RAT LIVER*

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Abstract—The increase of the metabolism of glyceryl trinitrate by phenobarbital pretreatment was studied in the soluble fraction of rat liver homogenate. These *in vitro* studies indicate that phenobarbital pretreatment results in an increase of glyceryl trinitrate metabolism of about 20 and 43 per cent of the control value, with and without addition of exogenous glutathione (GSH) respectively. In connection with the activity of organic nitrate reductase and of the GSH generating system, it was shown that the increased formation of inorganic nitrite in the phenobarbital pretreated group was accompanied by a faster disappearance of NADPH.

In order to study further the GSH generating system, the activity of glucose-6-phosphate dehydrogenase and glutathione reductase was determined in the soluble fraction of rat liver homogenates: in phenobarbital pretreated rats, a 53 per cent increase of glucose-6-phosphate dehydrogenase and a 30 per cent increase of glutathione reductase were found. These results indicate that the enhancement of glyceryl trinitrate metabolism by phenobarbital pretreatment is due to the increase of both the organic nitrate reductase and the activity of the GSH generating system. The increase of glyceryl trinitrate metabolism can be completely inhibited by pretreatment with DL-ethionine or actinomycin D.

HEPPEL and Hilmoe[1] observed that glyceryl trinitrate reacts with reduced glutathione (GSH) to form inorganic nitrite and oxidized glutathione (GSSG). The degradation of glyceryl trinitrate is catalyzed by organic nitrate reductase with reduced glutathione in the soluble fraction of rat liver homogenate.[2] The oxidized glutathione formed is regenerated to the reduced form by glutathione reductase, with NADPH as a hydrogen donor.[3]

It was previously reported that phenobarbital pretreatment increased the activity of the glyceryl trinitrate metabolizing enzyme in rat liver.[4–6] *In vivo* results obtained in the rabbit were consistent with such an increase in enzymatic activity.[7]

The present study was carried out in order to obtain further information on the mechanism of the increase in glyceryl trinitrate metabolism after pretreatment with phenobarbital in rats.

METHODS

Animals and drug administration. Male rats weighing approximately 120 g were used throughout the experiments. Some rats were pretreated by intraperitoneal injection of sodium phenobarbital 100 mg/kg daily for 3 days. Control rats were treated

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similarly with 0·9% saline. DL-Ethionine 100 mg/kg or actinomycin D 1 μg/rat was administered daily by intraperitoneal injection for 3 days.

Preparation of the soluble fraction from the rat liver homogenate. All rats were sacrificed on the 4th day. Livers were removed, washed and 25 per cent homogenates were prepared in 0·25 M sucrose and 0·067 M phosphate buffer (pH 7·4). The homogenate was centrifuged at 9000 *g* for 20 min in a Servall refrigerated centrifuge. The supernatant fraction was decanted and centrifuged at 105,000 *g* for 75 min in a Beckman Spinco ultracentrifuge.

Enzyme assays. The metabolism of glyceryl trinitrate was determined in the soluble fraction as described by Needleman *et al.*;[4] the incubations were carried out aerobically at 37° for 10 min, in a medium containing 0·067 M phosphate buffer (pH 7·4), 4·4 mM glyceryl trinitrate, 8 mM GSH, 8 mM KCN and 0·5 ml tissue preparation in a final volume of 1 ml.

The reaction was stopped by addition of 2 ml of 5% mercuric chloride. Inorganic nitrite formation was assumed to parallel the disappearance of glyceryl trinitrate, as in our experimental conditions the denitration of glyceryl trinitrate proceeded only to the stage of glyceryl dinitrate.[6] Inorganic nitrite was determined by measuring the color complex formed in the presence of 0·2% sulfanilamide and 0·1% N-(1-naphtyl)-ethylenediamine dihydrochloride. Samples were read after 10 min at 538 nm.[8] Since glyceryl trinitrate has been shown to undergo a non-enzymatic reaction with GSH, blank assays were incubated without tissue.[1]

The activity of the GSH generating system was determined by measuring NADPH disappearance at 340 nm as described by Needleman *et al.*[2] The incubations were carried out at 25° in 0·1 M phosphate buffer (pH 7·4) containing 1·26 mM glyceryl trinitrate, 4 mM GSH, 0·35 mM NADPH, 1·9 mg protein (liver soluble enzyme), 20 mM nicotinamide, 0·3 mM KCN, 1 mM EDTA, and 0·02% bovine serum albumin in a final volume of 3·5 ml. Aliquots of 0·1 ml were taken at 10, 15, 20 and 30 min for the determination of inorganic nitrite.

The activity of glucose-6-phosphate dehydrogenase was assayed by the spectrophotometric measurement of NADPH formation at 340 nm with glucose-6-phosphate as the substrate by the method of Löhr *et al.*[9]

Glutathione reductase activity was determined with oxidized glutathione as the substrate by the spectrophotometric measurement of NADPH at 340 nm as described by Horn.[10]

Protein concentration was measured by the method of Lowry *et al.*[11] using bovine albumin as a standard.

RESULTS

The effect of phenobarbital pretreatment on the metabolism of glyceryl trinitrate in the soluble fraction of rat liver homogenate. When incubating glyceryl trinitrate with the soluble fraction of liver, the amount of nitrite formed was found to be proportional to time during a 10 min period. There was also a linear relationship between nitrite formation and enzyme concentration. The incubations were carried out both with and without exogenous GSH, to see whether phenobarbital treatment influenced the activity of organic nitrate reductase or the activity of the GSH generating system, or both. In the incubation without addition of GSH, the soluble fraction of liver, as described in Methods, was used. Since with addition of GSH to the incubation

TABLE 1. THE EFFECT OF PHENOBARBITAL ON THE METABOLISM OF GLYCERYL TRINITRATE IN THE SOLUBLE FRACTION OF RAT LIVER HOMOGENATE

Pretreatment	Liver wt / Body wt (%)	Rate of metabolism*			
		Without glutathione		With glutathione	
		Per mg protein	Total activity	Per mg protein	Total activity
Saline	4·8 ± 0·1	0·082 ± 0·004	48·52 ± 2·01	0·688 ± 0·044	398 ± 21
Phenobarbital	6·1 ± 0·2‡	0·117 ± 0·004‡	86·03 ± 3·01‡	0·817 ± 0·025†	602 ± 23‡

* The rate of metabolism is expressed in μmole NO_2^- formed/10 min and total activity is calculated on the basis of whole liver wt.
Data presented are mean values in 19 animals ±S.E.M.
† Significantly different from control value ($P < 0·01$).
‡ Significantly different from control values ($P < 0·001$).
Experimental conditions are described under Methods.

mixture, the rate of metabolism was much higher, the soluble fraction was diluted 10 times. The amount of GSH, 8 mM, used in our incubation mixture, was found to be sufficient to obtain the maximum rate of glyceryl trinitrate metabolism in our experimental conditions. As it can be seen in Table 1, the rate of metabolism, expressed per milligram of protein, was significantly higher in the phenobarbital-treated group than in the controls, both with and without addition of GSH. When the activity is expressed per whole liver, the difference of the metabolic rate is even larger, as the liver weight increased about 25 per cent after phenobarbital treatment.

Addition of exogenous GSH increased the rate of metabolism about 8-fold both in the control and in the phenobarbital-treated group. However, it is of interest to note that in the absence of exogenous GSH, the rate of metabolism in the phenobarbital-treated group increased about 43 per cent (expressed as per milligram of protein) while with exogenous GSH added, the rate of metabolism in pretreated rats was only 19 per cent above the control value.

NADPH disappearance and nitrite formation. In the assay system coupling organic nitrate transformation with glutathione reductase, a comparison of the rate of NADPH disappearance and nitrite formation was made. Blanks containing the reaction mixture with enzyme but without glyceryl trinitrate were also carried out; they showed virtually no disappearance of NADPH. As shown in Fig. 1, phenobarbital pretreatment resulted in an increase of inorganic nitrite formation and this was accompanied by a faster rate of NADPH disappearance.

Glucose-6-phosphate dehydrogenase. Table 2 shows the effect of phenobarbital on

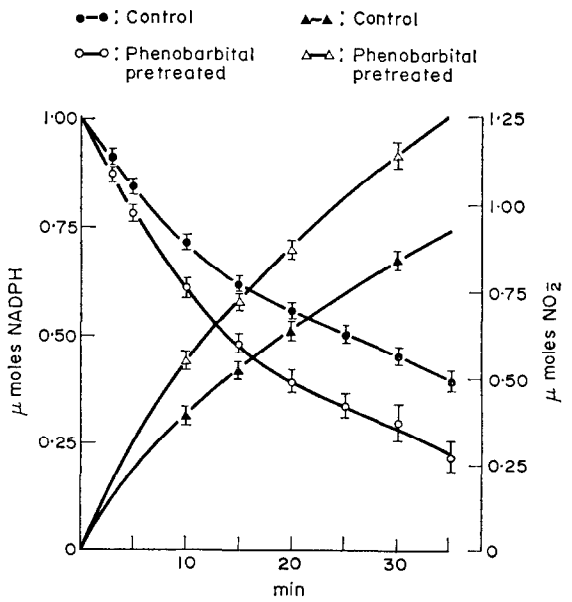

FIG. 1. The effect of phenobarbital pretreatment on the disappearance of NADPH in the denitration of glyceryl trinitrate. NADPH disappearance was followed at room temperature by direct readings at 340 nm. Aliquots of incubation mixture were taken at the indicated times for the analysis of inorganic nitrite. Experimental conditions are described under Methods. The data presented are the mean values in 15 animals ± S.E.M., expressed in μmole of NADPH and inorganic nitrite.

the activity of glucose-6-phosphate dehydrogenase in the soluble fraction of rat liver homogenate. Phenobarbital pretreatment resulted in a significantly increased activity of glucose-6-phosphate dehydrogenase of about 50 per cent above the control value when calculated as per milligram of protein. The total activity for the whole liver was even more increased.

TABLE 2. THE EFFECT OF PHENOBARBITAL PRETREATMENT ON THE ACTIVITY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE IN RAT LIVER

	Glucose-6-phosphate dehydrogenase (μmole NADPH produced/min)	
Pretreatment	Per mg protein	Total activity*
Saline	0·049 ± 0·002	30·1 ± 1·2
Phenobarbital	0·075 ± 0·004†	56·8 ± 3·0†

Data presented are mean values in 9 animals ±S.E.M.
* Total activity is calculated on the basis of whole liver wt.
† Significantly different from control values ($P < 0·001$).
The assay method is described under Methods.

Glutathione reductase. The results are summarized in Table 3. Phenobarbital pretreatment significantly increased the activity of glutathione reductase in the soluble fraction of rat liver homogenate. The activity expressed as per milligram of protein shows an increase of about 30 per cent in the phenobarbital treated group. When it is calculated on the basis of total liver weight, phenobarbital pretreatment resulted in an increase of about 70 per cent of the activity of glutathione reductase.

TABLE 3. THE EFFECT OF PHENOBARBITAL PRETREATMENT ON THE ACTIVITY OF GLUTATHIONE REDUCTASE IN THE SOLUBLE FRACTION OF LIVER HOMOGENATE

	Glutathione reductase activity (μmole NADPH oxidized/min)	
Pretreatment	Per mg protein	Total activity*
Saline	0·069 ± 0·004	40·21 ± 2·21
Phenobarbital	0·089 ± 0·006†	68·63 ± 4·71‡

Data presented are mean values in 17 animals ±S.E.M.
* Total activity is calculated on the basis of whole liver wt.
† Significantly different from control value ($P < 0·005$).
‡ Significantly different from control value ($P < 0·001$).
The assay method is described under Methods.

Inhibition of phenobarbital induced increase in glyceryl trinitrate metabolism by inhibitors of protein synthesis. The effect of inhibitors of protein synthesis on the phenobarbital-induced glyceryl trinitrate metabolism was studied, and the results are shown in Table 4. Pretreatment with DL-ethionine which blocks protein synthesis by decreasing ATP levels in liver,[12] did prevent completely the increase of glyceryl trinitrate metabolism after phenobarbital treatment. This inhibitory effect was found

TABLE 4. THE EFFECT OF DL-ETHIONINE AND ACTINOMYCIN-D ON THE PHENOBARBITAL-INDUCED INCREASE IN METABOLISM OF GLYCERYL TRINITRATE

Pretreatment	Rate of metabolism*	
	Without glutathione	With glutathione
Saline	0·065 ± 0·004	0·616 ± 0·038
Phenobarbital	0·113 ± 0·009	0·813 ± 0·059
Ethionine	0·065 ± 0·005	0·599 ± 0·046
Ethionine + phenobarbital	0·063 ± 0·005	0·593 ± 0·040
Actinomycin D	0·059 ± 0·004	0·608 ± 0·041
Actinomycin D + phenobarbital	0·060 ± 0·003	0·594 ± 0·032

* The rate of metabolism is expressed in μmole NO_2^- formed/10 min per mg protein.
Data presented are mean values of 8 determinations ±S.E.M.
† Significantly different from control value ($P < 0·01$).
‡ Significantly different from control value ($P < 0·001$).
Experimental conditions are described under Methods.

in the experiments with and without addition of GSH to the incubation mixture. The effect of phenobarbital on the metabolism of glyceryl trinitrate was also blocked by pretreatment with actinomycin D. This inhibitor is known to bind to DNA and block the DNA-directed synthesis of messenger RNA which is required for protein synthesis.[13] The inhibitory effect of actinomycin D was observed in the incubations with and without addition of GSH. The two inhibitors had no significant effects on the activity of the enzyme of rats that had not received the phenobarbital pretreatment.

DISCUSSION

The hepatic metabolism of glyceryl trinitrate is known to be dependent on the activity of organic nitrate reductase in the presence of reduced glutathione.[1,2] Rall and Lehninger[3] demonstrated the specificity of glutathione reductase for GSSG in the presence of NADPH.

The results of the present investigation indicate that the increase of glyceryl trinitrate metabolism by phenobarbital pretreatment is due to the increase in the activity of organic nitrate reductase and of the GSH-generating system. This view is supported by the *in vitro* studies in which incubations of glyceryl trinitrate with and without addition of GSH were carried out. By the addition of a sufficient amount of GSH to the incubation mixture, the influence of phenobarbital pretreatment on the GSH generating system can be eliminated; the increase of glyceryl trinitrate metabolism seen in these conditions in the phenobarbital-pretreated group, can be attributed to the increased organic nitrate reductase.

The fact that phenobarbital has a more pronounced effect on the preparations with no GSH added than on those with GSH added suggests that the phenobarbital treatment provokes also an increase in the activity of the GSH generating system.

The finding that the increased formation of nitrite was accompanied by a faster oxidation of NADPH, can also be related to the enhanced activities of organic nitrate reductase and of the GSH-generating system. Needleman *et al.*[2] have reported that the disappearance of NADPH has a stoichiometric relationship with the formation of

inorganic nitrite; in our experiments however, NADPH disappearance was not stoichiometric with nitrite formation.

In phenobarbital treated rats, there was an increase in glutathione reductase of about 30 per cent above the control value. The activity of glucose-6-phosphate dehydrogenase was found to be increased about 50 per cent which is in agreement with other reports.[14,15]

Since phenobarbital pretreatment increases liver weight, the increase of the enzyme activities was greater in the total liver than per mg of protein. Thus total activity could be more significant, when dealing with *in vivo* effects.

It is probable that the increase of glyceryl trinitrate metabolism by phenobarbital depends on the induction of enzyme synthesis, because pretreatment with inhibitors of protein synthesis, DL-ethionine[12] or actinomycin D[13], were able to inhibit completely phenobarbital-induced glyceryl trinitrate metabolism without affecting basal glyceryl trinitrate metabolism.

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