INDUCTION OF HEPATIC CYTOSOLIC DT DIAPHORASE IN RATS TREATED WITH *TRANS*–STILBENE OXIDE

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1. Introduction

The flavoprotein DT diaphorase (NAD(P)H:oxidoreductase, EC 1.6.99.2) is a highly dicoumarol-sensitive enzyme catalyzing the oxidation of NADH and NADPH by various quinones and redox dyes [1–3]. The physiological electron acceptor for DT diaphorase has not yet been established. However, it has been suggested that the enzyme serves as a quinone reductase in connection with conjugation of hydroquinones during detoxification, as well as in biosynthetic processes such as the vitamin K-dependent γ-carboxylation of glutamyl residues in prothrombin synthesis (reviewed [3]).

Treatment of rats with polycyclic aromatic hydrocarbons, potent inducers of the aryl hydrocarbon monooxygenase system [4,5], is known to increase hepatic DT diaphorase [6–11]. The induction of DT diaphorase is due to an increase in the amount of the enzyme [12]. The coinduction of DT diaphorase and aryl hydrocarbon monooxygenase [3,9] has drawn attention to a possible linkage between the genetic regulation of these activities.

trans-Stilbene oxide has been suggested to represent a new class of inducers of drug-metabolizing enzymes in the rat [13–16]. This compound preferentially induces epoxide hydratase [13–16] and glutathione *S*-transferases [16], whereas cytochrome P450 linked monooxygenase reactions are only moderately affected [13–17]. Because of the specificity of this inducer, the level of DT diaphorase in TSO-treated rats has been investigated in order to obtain information about a possible coupling of the regulatory gene(s) of DT diaphorase and the aryl hydrocarbon monooxygenase system.

Abbreviations: MC, 3-methylcholantrene; MTT, 3-(4,5-dimethyl-thiazolyl-2)-2,5-diphenyl tetrazolium bromide; TSO, *trans*-stilbene oxide

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2. Experimental

2.1. *Chemicals*

TSO was purchased from EGA-chemie (Steinheim/Albuch) and was ≥97% pure by NMR and IR. Agarose C was obtained from Pharmacia (Uppsala). 2,6-Dichlorophenolindophenol, dicoumarol, menadione, MTT (3-(4,5-dimethylthiazolyl-2)-2,5-dipheynl tetrazolium bromide) NADH, Triton X-100 were bought from Sigma Chemical Co (St Louis, MO). All other chemicals were of analytical grade.

2.2. *Animals and preparations*

Male Sprague-Dawley rats weighing 180–200 g were used in all experiments. The rats were kept on a standard laboratory diet and starved 16 h before sacrifice. Some of the animals were injected i.p. with various amounts of TSO dissolved in 1 ml sunflower oil, as indicated in the figure legends. The rats were decapitated 24 h after the last injection. The 105 000 × *g* supernatant fraction was prepared from 20% (w/v) liver homogenates as in [12].

2.3. *Assays*

DT diaphorase activity was measured as in [12] employing NADH as electron donor and 2,6-dichlorophenolindophenol as electron acceptor. The enzyme activity was completely inhibited by 10^{-5} M dicoumarol. Protein was determined according to [18] with bovine serum albumin as standard.

2.4. *Immunological methods*

Antiserum raised against cytosolic DT diaphorase from rats treated with MC was obtained as in [12]. Rocket immuno-electrophoresis and fused rocket immuno-electrophoresis were performed in 1% (w/v) agarose C containing 1% (v/v) antiserum against DT diaphorase as in [12]. The plates were stained for menadione-mediated NADH–MTT reductase activity [12]. The activity staining was totally inhibited by preincubation of the plates with 10^{-3} M dicoumarol.

3. Results and discussion

In fig.1. is shown the time course of induction of cytosolic DT diaphorase after administration of TSO to rats. No significant increase in enzyme activity is observed within the first 24 h after injection of the inducer. This lag period is much longer than that observed for the induction of cytosolic DT diaphorase by treatment of rats with MC [3,9,19]. As early as 12 h after a single injection of MC, a 3-fold enhancement of enzyme activity was found [3,9,19].

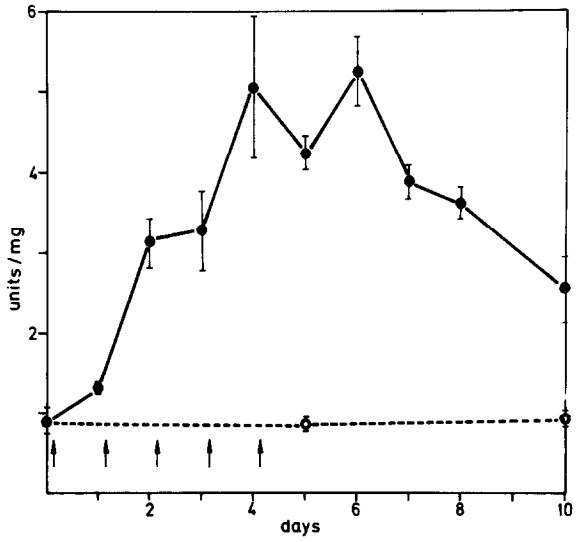

Fig.1. Time course of the changes in cytosolic DT diaphorase brought about by treatment of rats with TSO. Rats were injected once daily for 1–5 days, as indicated by the arrows, with 400 mg TSO/kg body weight and killed 24 h after the final injection. DT diaphorase activity was measured as in section 2, in duplicates. Each time-point represents the average (± SD) of the individual values obtained with a group of 3 rats. (●——●) Rats injected with TSO; (○——○) rats injected with sunflower oil.

Whether this time discrepancy in enzyme induction is the result of differences in the rate of distribution of these inducers or due to a different induction mechanism remains to be determined.

Maximal DT diaphorase activity, 4–5-fold higher than the control value, was obtained after 4–5 days of TSO-treatment of rats (fig.1). After the final injection with TSO the enzyme activity decreases slowly; the level of DT diaphorase in the cytosol is still 2.5-fold higher than that of controls 6 days after cessation of the treatment. Both the time course of induction of DT diaphorase by TSO and the slow return to control levels after the final injection of the inducer to rats are very similar to those observed for other enzymatic activities, such as epoxide hydratase [16] and glutathione *S*-transferase(s) [16].

In fig.2 the effect of different doses of TSO on cytosolic DT diaphorase is shown. The increase in enzyme activity did not entirely level off at higher doses of the inducer (400 mg/kg), an observation also made in the cases of epoxide hydratase [16] and glutathione *S*-transferase(s) [16].

An enhanced enzyme activity in animals treated with an inducer does not a priori indicate an increase in the amount of enzyme. Recently, a sensitive method for determining concentrations of hepatic DT diaphorase was developed in this laboratory [12]. The technique is based on rocket immuno-electro-

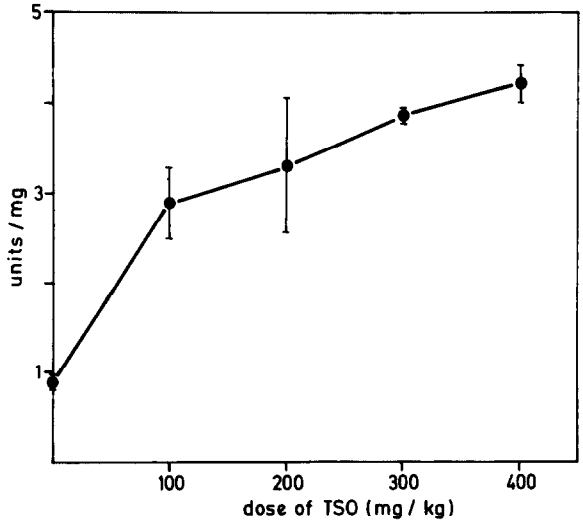

Fig.2. Response of cytosolic DT diaphorase to different doses of TSO. Rats were injected once daily for 5 days with various doses of TSO and killed 24 h after the last injection. For further details see fig.1 and section 2.

phoresis with antibodies raised against purified cytosolic DT diaphorase. Analysis of cytosols from control and TSO-treated rats with this technique is shown in fig.3. The peak heights of known amounts of purified DT diaphorase (fig.3A) were determined and used as a standard curve (fig.3B). By determining the corresponding peak heights of cytosols from induced and non-induced animals the content of DT diaphorase was found to be 2.6 μg/mg cytosolic protein and 0.5 μg/mg cytosolic protein, respectively. This 5.2-fold higher specific content of DT diaphorase in TSO-treated rats correlates well with the 4.7-fold higher specific activity observed in cytosols from induced rats. For comparison, the cytosol from MC-treated rats was also analyzed (fig.3). In agreement with [12] the extent of induction of DT diaphorase by MC was the same whether based on enzyme content or enzyme activity. Thus, as in the case of MC-treated animals, the enhanced DT diaphorase observed in these TSO-treated rats is the result of an increase in enzyme concentration rather than altered enzyme properties.

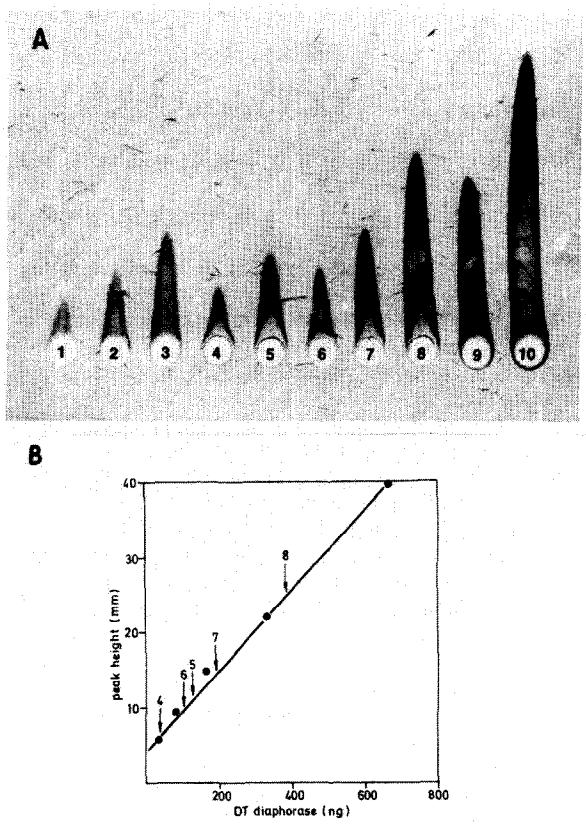

Fig.3. Rocket immuno-electrophoresis of cytosolic DT diaphorase from control and TSO-treated rats. The rocket immuno-electrophoresis was performed as in section 2. The precipitates were detected with menadione-mediated tetrazolium reductase activity as described. (A) Wells 1–3 and 9,10 contained known amounts of purified DT diaphorase: 1, (33.5 ng); 2, (83.8 ng); 3, (167 ng); 9, (335 ng); 10, (670 ng). Wells 4,5 contained cytosol from control rats, 105 and 210 μg protein, respectively, with spec. act. 0.9 units/mg. Wells 6,7 contained cytosol from TSO-treated rats (400 mg TSO/kg, 5 days) 37 and 75 μg protein, respectively, with spec. act. 4.2 units/mg. Well 8 contained 140 μg cytosol protein from MC-treated rats (20 mg MC/kg, 3 days) with spec. act. 4.6 units/mg. (B) Peak heights in (A) from wells 1–3 and 9,10 were employed as standard points in the determination of the concentration of DT diaphorase in cytosols from control, TSO- and MC-treated rats. The arrows indicate the peak heights from respective wells in (A). The concentration of DT diaphorase was determined to 0.5, 2.6 and 2.8 μg/mg cytosol protein in control, TSO- and MC-treated rats, respectively.

The nature of the assay for cytochrome P450, which measures the amount of heme contained in this protein, strongly suggests that this cytochrome is also increased in amount by TSO treatment; while disc-gel electrophoretic patterns support the same conclusion in the case of epoxide hydratase (J. S. and J. W. de P., unpublished). In addition, inhibitors of RNA and protein synthesis prevent the induction of epoxide hydratase by TSO [14]. Finally, quantitative immuno-electrophoresis has demonstrated that administration of TSO increases the amounts of glutathione *S*-transferases A–C in rat liver cytosol [20].

Cytosolic DT diaphorase has been shown to consist of several different antigenic forms, one of which is preferentially induced by MC-treatment of rats [12]. The results from analysis of cytosols from TSO-induced animals with fused rocket immuno-electrophoresis indicate that the same antigenic form is induced by TSO (not shown).

The relatively minor increase in microsomal cytochrome P450 content observed in TSO-treated rats is not accompanied by a 2 nm spectral shift in the peak absorption of the reduced CO-complex of the cytochrome [13–17], in contrast to that found in microsomes from MC-induced animals [4,5]. The rate of metabolism of benzo[a]pyrene and other polycyclic aromatic hydrocarbons is known to increase drastically in MC-treated rats [4,5]. Some increase in the overall metabolism of benzo[a]pyrene has also been observed in TSO-induced rats [15,17]. The metabolite pattern of benzo[a]pyrene after monooxygenation in microsomes from TSO-treated rats [17] is

quite different from that obtained with microsomes from control and MC-treated rats [21,22]. Additional studies on substrate specificity also support the conclusion that the forms of cytochrome P450 induced by TSO and MC are not the same [23].

The induction of cytosolic DT diaphorase by TSO-treatment of rats was found to be of the same magnitude as that observed in MC-treated animals of the same age [19]. This response of DT diaphorase to TSO- and MC-treatment of rats indicates that the induction of DT diaphorase and that of cytochrome P448-catalyzed aryl hydrocarbon monooxygenase system are under separate genetic control. Similar genetic differences have been reported in the mouse [24]. Their results suggest that there are two loci which regulate DT diaphorase induction by MC, one gene being the same as or closely linked to a gene regulation aryl hydrocarbon monooxygenase induction, the other unlinked and located on another chromosome.

Acknowledgements

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