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Role of RNA in Induction of Hepatic Microsomal Mixed Function Oxidases

(α-amanitin/RNA polymerase/phenobarbital/microsomal enzymes)

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ABSTRACT **Induction of hepatic microsomal cytochrome P-450 and ethylmorphine *N*-demethylase activity by phenobarbital requires *de novo* synthesis of mRNA. Inhibition of RNA synthesis by α-amanitin given up to 8 hr after phenobarbital administration substantially inhibits this induction. However, beyond 8 hr after phenobarbital administration, RNA synthesis is not required for induction of these hepatic microsomal systems. Thus, mRNAs for cytochrome P-450 and ethylmorphine *N*-demethylase appear to be stable. Furthermore, these experiments reveal that the lag period for RNA synthesis approximates the length of the lag period for induction of the hepatic microsomal enzyme systems.**

Of the numerous agents that induce hepatic microsomal drug-metabolizing enzymes (1, 2), the prototype is phenobarbital, which stimulates the synthesis of several of these enzyme systems simultaneously. Attempts have been made to elucidate the molecular mechanisms of action of phenobarbital (PB) (3, 4). A serious objection to these studies is the large dose of actinomycin D employed to inhibit DNA-dependent RNA synthesis. This dose of actinomycin D (1 mg/kg of body weight) needed to demonstrate a requirement of RNA synthesis for drug-mediated induction of hepatic microsomal enzymes (3) produces undesirable secondary effects (5–7) and indeed ultimately proves lethal, being twice the mean lethal dose (8). Even at this high dose of actinomycin D RNA synthesis is only partially inhibited (5). The RNA fraction that is antibiotic-resistant is probably nonribosomal RNA, since actinomycin D in a dose as low as 0.3 mg/kg of body weight completely blocks synthesis of rRNA in rat liver (9). Recent studies of another eukaryotic system (10) also demonstrated that very high doses of actinomycin D do not completely inhibit the synthesis of mRNA.

Nebert and Gelboin (4) employed mammalian cell cultures such as hamster embryo cells to study induction of microsomal hydroxylating enzymes. However, these cultures do not respond to PB. Moreover, in cell cultures and less frequently in intact animals, actinomycin D, with or without such hepatic microsomal enzyme inducers as polycyclic hydrocarbons (4) or adrenocorticosteroids (11, 12) can produce a greater increase in the enzyme activity than that observed with the inducer alone. This phenomenon is designated the paradoxical effect of actinomycin D.

Studies in our laboratory (13, 14) and elsewhere (15, 16) established that α-amanitin, a toxic peptide isolated from poisonous mushrooms *Amanita phalloides*, inhibits RNA synthesis by binding to nucleoplasmic RNA polymerase, form II. We (17, 18) subsequently showed that this toxin, when administered to rats in a dose as low as 300 μg/kg of body weight inhibits RNA synthesis without mortality. Synthesis of both ribosomal and nonribosomal RNA is inhibited *in vivo* in rat liver (17–20); however, inhibition of ribosomal RNA (rRNA) synthesis is transient. Complete recovery of rRNA synthesis occurs within 2 hr; the bulk of the messenger RNA (mRNA) synthesis remains inhibited within this period (16, 17). We exploited selective inhibition of nonribosomal mRNA synthesis by amanitin to investigate the role of mRNA in induction of hepatic microsomal enzymes. The effect of α-amanitin on the induction of cytochrome P-450 and ethylmorphine *N*-demethylase by PB was studied. Parallel studies were performed on the effect of the toxin on the nucleoplasmic DNA-dependent RNA polymerase (form II) at various times after a single injection of PB.

Abbreviation: PB, phenobarbital.

MATERIALS AND METHODS

Animals and Their Treatments. Male Sprague–Dawley rats weighing 80–120 g were obtained from Charles River Laboratories and maintained on Purina Chow and tap water *ad libitum*. Groups of three rats received either phenobarbital (100 mg/kg) dissolved in 0.9% NaCl, or 0.9% NaCl alone intraperitoneally. Rats receiving PB were also given α-amanitin in 0.9% NaCl (0.5 mg/kg) at 8, 12, or 22 hr after PB. In two experiments, an additional group of rats received amanitin 90 min prior to PB treatment.

Reagents. [^{14}C]UTP (384 mCi/mmol) was obtained from New England Nuclear Corp. α-Amanitin was a gift from Prof. Th. Wieland. In some experiments, the toxin obtained commercially from Henley and Co., New York, was used. ATP, GTP, CTP, UTP, and dithiothreitol were purchased from Calbiochem.

Isolation of Microsomes. Microsomes were isolated as described previously (21). After decapitation, livers were removed, weighed, and homogenized in 2 volumes of cold 0.02 M Tris·HCl buffer (pH 7.4) containing 1.15% KCl. The homogenate was centrifuged at 9000 × *g* for 20 min in a Sorvall Centrifuge. The supernatant was then centrifuged for 1 hr at 78,000 × *g* (av) in a Spinco rotor 30. The microsomal pellet was suspended in Tris–KCl buffer in a volume equal to twice the original liver weight.

Determination of Cytochrome P-450 Content and Ethylmorphine N-Demethylase Activity. Cytochrome P-450 content was determined in an Aminco–Chance dual wavelength spectrophotometer, as described by Omura and Sato (22).

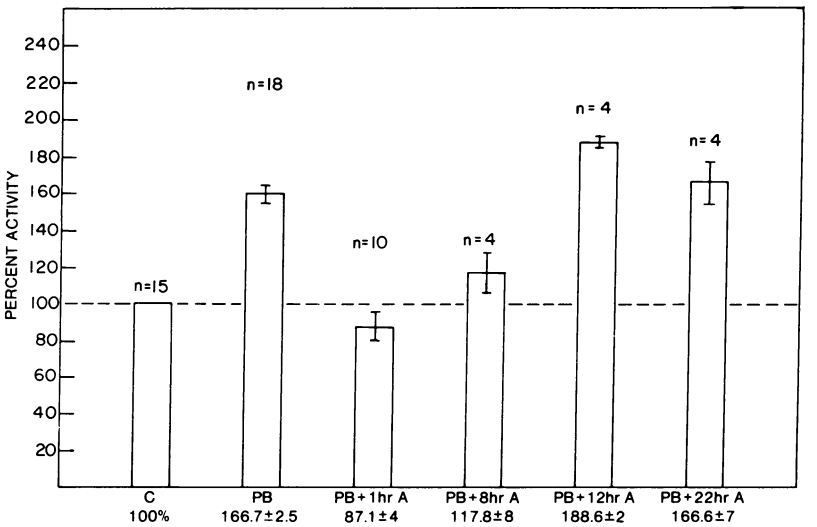

FIG. 1. Effect of α-amanitin on the induction of cytochrome P-450 content by phenobarbital. Animals were given phenobarbital intraperitoneally in a dose of 100 mg/kg of body weight and sacrificed 24 hr later. Control animals received 0.9% NaCl. Test animals were treated with α-amanitin (500 μg/kg of weight) via the jugular vein at different time intervals after PB administration, as indicated in the figure. (PB + 1hr A = amanitin 1 hr after PB; PB + 8hr A = amanitin 8 hr after PB, etc). Cytochrome P-450 content was determined in the isolated microsomes as described in the text and expressed as nmol/mg of protein. Experiments were performed in triplicate and the data expressed as mean values with standard errors. The number of experiments (n) for each time point is given above the bars. The horizontal broken line indicates the control activity, C, that is given a value of 100.

Ethylmorphine N-demethylase activity was determined in a 3.0-ml reaction mixture consisting of 0.5 ml of microsomal suspension, 0.5 ml of 0.3 M Tris·HCl buffer (pH 7.4) containing 1.15% KCl, 1 ml of 15 mM ethylmorphine, and 1 ml of NADPH generating system (3 μmol of NADP, 25 μmol of $MgCl_2$, 50 μmol of glucose-6-phosphate and 2 units of glucose-6-phosphate dehydrogenase, EC 1.1.1.49). The N-demethylation of ethylmorphine by the microsomal enzyme was estimated by measuring the amount of formaldehyde according to the method of Nash (23).

Estimation of Protein. Protein was determined by a modification of the procedure developed by Gornall *et al.* (24). The reaction mixture consisted of 4.5 ml of Biuret Solution, 0.1 ml of microsomal suspension, and 0.4 ml of 1.2% sodium cholate in a total volume of 5.0 ml and read at 540 nm in a Gilford model 2000 spectrophotometer. Bovine-serum albumin was used as the standard.

Isolation of Nuclei. Livers were removed, minced well, and homogenized at 4° in 11 volumes (w/v) of 2.3 M sucrose containing 15 mM $MgCl_2$ and 0.25 mM spermine (18, 25). Homogenization was carried out with four up-and-down strokes in a Teflon-glass homogenizer (0.015–0.020-inch pestle clearance). After filtration through two layers of cheesecloth, the homogenate was centrifuged at 40,000 × g for 65 min to sediment nuclei. Nuclei were washed once with 0.25 M sucrose containing 2 mM $MgCl_2$ and finally suspended in the same media (1 ml/g of original wet weight of liver).

RNA Polymerase Assay. RNA polymerase (EC 2.7.7.6) was assayed essentially as described by Jacob *et al.* (14, 26). Incubations were carried out in high ionic media in the presence and absence of α-amanitin. The residual activity in the presence of amanitin represents the nucleolar RNA polymerase. Nucleoplasmic RNA polymerase activity was estimated by subtracting the activity in the presence of amanitin from the activity in its absence (27, 28).

RESULTS

Twenty-four hours after a single injection of PB, cytochrome P-450 content and ethylmorphine N-demethylase activity increased by 67 ± 2.5% and 51 ± 6.8%, respectively. The percent increase in these systems after PB administration was calculated for each experiment and the result expressed as mean values of several experiments with standard errors. The effect of α-amanitin on the induction of cytochrome P-450 and ethylmorphine N-demethylase is shown in Figs. 1 and 2. When the rats were treated with amanitin 1 hr after PB treatment and sacrificed 23 hr later (24 hr after PB treatment) induction of both cytochrome P-450 and ethylmorphine N-demethylase was prevented. However, if the rats were treated with amanitin 8 hr after PB, induction of cytochrome P-450 was not blocked. Based on the results with cytochrome P-450, we used fewer time points for experiments with ethylmorphine N-demethylase. The induction of this enzyme was also unaffected when amanitin was given 12 or more hours after a single dose of PB. However, given at shorter intervals after PB administration, amanitin blocked PB-induced elevations of ethylmorphine N-demethylase activity.

In order to correlate the enzyme induction with mRNA synthesis, the effect of amanitin on nucleoplasmic RNA polymerase activity (form II) was measured at various times after a single injection of PB. We previously demonstrated that inhibition of nonribosomal mRNA synthesis by amanitin persists for at least 1 hr (13, 14) with a partial or no restoration at later time periods. Since little induction of hepatic microsomal drug-metabolizing enzymes occurs during the first 1 hr (2), we wondered whether inhibition of mRNA synthesis, as measured by the activity of RNA polymerase II, was sustained during PB induction. To test this possibility, a single

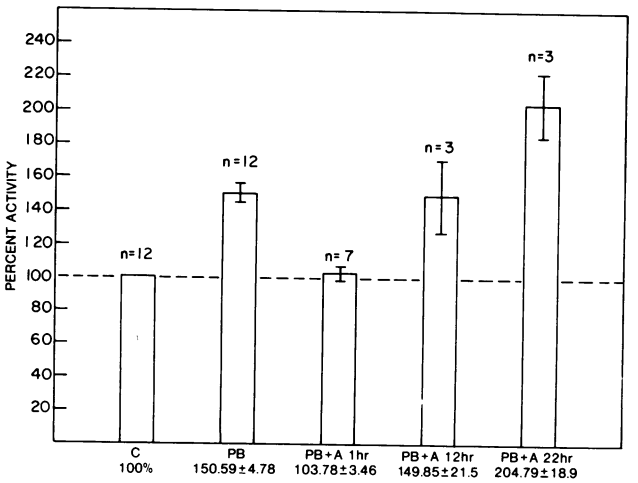

FIG. 2. Effect of α-amanitin on the induction of ethylmorphine *N*-demethylase activity by phenobarbital. The modes of treatment of animals with PB and amanitin are identical with those described in the legend to Fig. 1 with the exception that 8-hr time point is deleted in this set of experiments. Ethylmorphine *N*-demethylase activity was determined as described in the text and expressed as nanomoles formaldehyde formed per min/mg of protein. Experiments were performed in triplicate and the data expressed as mean values with standard errors. The number of experiments for each time point is given above the bars. The horizontal broken line indicates the control activity which is given a value of 100.

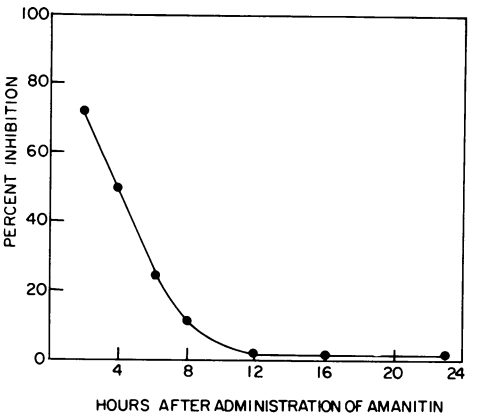

FIG. 3. Effect of α-amanitin on the nucleoplasmic RNA polymerase (form II) activity. α-Amanitin was given intravenously in a dose of 500 μg/kg of body weight 1 hr after the intraperitoneal injection of phenobarbital (100 mg/kg) and the animals were sacrificed at different time intervals as indicated. The nucleoplasmic RNA polymerase activity was determined in triplicate as described in the text and expressed as picomoles UMP incorporated per mg of DNA with standard error. The enzyme activity of the control (untreated with amanitin) animals was given a value of 100 and the activities of the treated animals were expressed in relation to this.

dose of amanitin (500 μg/kg of body weight) was given at 1, 8, 12, 18, 20, and 22 hr after PB administration; animals were killed 23, 16, 12, 6, 4, and 2 hr after amanitin administration, respectively. The results are shown in Fig. 3. The activity of RNA polymerase II was obtained by subtracting the activity at high salt with Mn^{2+} in the presence of amanitin, from the activity under the same conditions without toxin (27, 28). The RNA synthesized under these conditions is predominantly "(A+U)-rich" RNA (29) which includes the mRNA. RNA polymerase II activity was inhibited by approximately 70, 50, and 20% in rats sacrificed 2, 4, and 6 hr, respectively, after treatment with amanitin; enzyme activity was restored completely at later times.

DISCUSSION

The present studies demonstrate that for induction of hepatic microsomal enzymes by phenobarbital to occur RNA synthesis is required for 6–8 hr after PB administration. Complete inhibition of cytochrome P-450 induction by amanitin administered 1 hr after a single injection of PB, despite almost total restoration of RNA synthesis within 8 hr after the toxin is given (Fig. 3), shows that to achieve maximal stimulation RNA synthesis should be allowed to continue for at least several hours after PB administration. Since RNA synthesis is almost recovered 8 hr after administration of amanitin, rats treated with the toxin 1 hr after PB had the drug present for almost 16 hr after restoration of RNA synthesis. Under these conditions absence of even a slight increase in cytochrome P-450 content indicates an absolute requirement for RNA synthesis during the first few hours after PB administration for induction to occur. Furthermore, if RNA synthesis is blocked during the first 8 hr after PB administration, even the extended presence of PB beyond this period does not suffice to permit enzyme induction to occur. Since the first significant induction of cytochrome P-450 also occurs only about 12 hr after PB administration (2), *de novo* mRNA synthesis in response to PB precedes induction of microsomal drug metabolizing enzymes. The lag for both RNA synthesis and hepatic microsomal enzyme induction could arise from several causes. Possibly PB must be converted to an active metabolite before exerting its inducing effects; conversion of PB to an active metabolite might require several hours. Alternatively, in a system analogous to that of estradiol and its interaction with receptor sites (30, 31), PB may first form a complex with its cytoplasmic receptor sites. This complex may then be transported to the nucleus where it may interact with the chromatin–RNA polymerase complex. These events could account for the observed lag.

When administered at a dose of 300 μg/kg of body weight α-amanitin can inhibit *in vivo* the synthesis of all RNA species (17–20). However, inhibition of rRNA synthesis lasts only for 1 hr; rRNA synthesis is restored thereafter (19, 20). Consequently, inhibition of hepatic microsomal enzyme induction after administration of amanitin 1 hr after PB may be due to inhibition of total RNA synthesis. This possibility can be tested by studying induction in animals receiving amanitin prior to PB administration. In two such experiments, α-amanitin was given 1.5 hr before PB; sacrifice occurred 24 hr after PB. At the time of PB administration, rRNA synthesis had almost completely recovered from inhibition by amanitin; however, nonribosomal RNA, including mRNA, was still inhibited by almost 80% (Fig. 3). Under these conditions, prevention* of cytochrome P-450 induction strongly suggested that induction of hepatic microsomal enzymes by PB depends on synthesis of mRNA. A small but consistent stimu-

* The specific activities of cytochrome P-450 (nmol/mg of protein) in control, PB-treated and amanitin pre-treated samples were 0.85, 1.43, and 0.86, respectively (mean of two experiments).

lation of nucleoplasmic RNA polymerase activity occurred 24 hr after PB administration. Such stimulation has also been observed by other workers (32). Under these conditions, PB does not stimulate either nucleolar rRNA synthesis (33) or RNA polymerase activity (34). Thus PB and probably other inducers of hepatic microsomal enzymes appear to stimulate specifically *de novo* synthesis of mRNA for certain hydroxylating enzymes. Continued induction of microsomal enzymes, despite inhibition of mRNA synthesis by amanitin at any time after 8 hr of PB treatment (Figs. 1 and 2) suggests that mRNAs for these enzymes are stable. The mRNAs for enzymes inducible by such other compounds as steroids are also stable (35). α-Amanitin has been used to elucidate the mechanism of induction of such enzymes as tyrosine transaminase by cortisol (36) and by cyclic AMP (37). The present experiments demonstrate how a specific inhibitor of RNA synthesis can serve as a tool to understand another biological process.

Finally, twin studies revealed genetic differences in the induction response of normal, otherwise nonmediated human subjects to PB, as reflected by the extent of changes in plasma antipyrine half-life of each twin before and after PB (38). Recent confirmation of genetic regulation of large interindividual differences of induction came from studies in cultured human lymphocytes; interindividual variations in induction of aryl hydrocarbon hydroxylase activity by 3-methylcholanthrene were under control of alleles at a single genetic locus (39). The present studies on relationships between RNA synthesis and induction of hepatic microsomal enzymes may provide better insight into the molecular basis for these two examples of genetically controlled differences in the induction response in man.

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