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INDUCTION BY PHENOBARBITAL OF REDUCED NICOTINAMIDE-ADENINE DINUCLEOTIDE PHOSPHATE (NADPH) CYTOCHROME c REDUCTASE IN REGENERATING RAT LIVER*

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Abstract—The administration of a single dose of phenobarbital (100 mg/kg) to rats resulted in a 60 per cent increase in the activity of NADPH cytochrome c reductase within 48 hr after the inducer. The imposition of the competitive stress of partial hepatectomy of animals 1 hr after phenobarbital treatment prematurely terminated the inductive process and reduction of NADPH cytochrome c reductase activity ensued thereafter, reaching control non-induced levels by 49 hr. Partial hepatectomy lowered the basal level of cytochrome b_5 by 30 per cent at 24–72 hr after the operation, but no alteration in the level of cytochrome b_5 was produced by phenobarbital in either sham-operated or partially hepatectomized rats. The rate of incorporation of [4,5-^{3}H]L-leucine into trypsin-solubilized microsomal protein at 2, 4, 6 and 9 hr after treatment with phenobarbital was equivalent to that of untreated sham-operated rats; whereas, at 25 hr after the inducer a significant increase in the rate of incorporation of leucine into the trypsin-solubilized microsomal protein of sham-operated animals was obtained. No significant change in the incorporation of leucine into microsomal protein was observed in the livers of partially hepatectomized rats in either the presence or absence of phenobarbital. Measurement of the loss of microsomal radioactivity following labeling with [^{14}C-guanidino]L-arginine revealed a similar rate of decay at 25–73 hr after administration of phenobarbital in the livers of sham-operated and partially hepatectomized animals.

THE INFLUENCE of hepatic cellular proliferation induced by partial hepatectomy on the regulation of microsomal mixed function oxidase activity has been described in a number of reports. The basal level of drug-metabolizing enzymes in general appears to be lower in regenerating liver than in the normal organ 1–7 days following hepatectomy.[1–3] Furthermore, the responsiveness to phenobarbital of mixed function oxidase enzymes from the livers of partially hepatectomized rats is similar to that of normal livers at times when the regenerative process is essentially complete.[4] During the relatively early period following partial hepatectomy, however, the requirement for DNA synthesis predominates over the ability of the liver to respond functionally to the inducer by formation of mixed function oxidase enzymes, and a delay occurs in the onset of elevated enzyme activity.[5–7]

In view of the relative importance of NADPH cytochrome c reductase to the microsomal mixed oxidase scheme[8,9] and the possible rate-limiting role of this enzyme in electron transport,[10,11] the effect of partial hepatectomy on induction of

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NADPH cytochrome c reductase by phenobarbital was investigated. The results demonstrate that: (a) the induced synthesis of NADPH cytochrome c reductase by phenobarbital is terminated prematurely in regenerating liver, and (b) differences occur between normal and regenerating liver in the rate of synthesis but not in the rate of degradation of microsomal protein following exposure to phenobarbital.

MATERIALS AND METHODS

Materials. Cytochrome c (type VI), NADH and NADPH were purchased from Sigma Chemical Co., and phenobarbital from Merck & Company, Inc. [^{14}C-guanidino]L-arginine (25 mc/m-mole) was obtained from Amersham-Searle, and [4,5-^{3}H]L-leucine (55 c/m-mole) was purchased from New England Nuclear Corporation.

Preparation of animals. Male Sprague–Dawley rats (Charles River Laboratories, Inc.) weighing 180–220 g were used in all experiments. Animals were housed over corn cob bedding and alternating periods of 12 hr dark and 12 hr light were maintained. Purina rat chow and water were available *ad lib.*

Partial hepatectomies and sham operations were performed under ether anesthesia between 9:00 and 11:00 a.m. according to the method of Higgins and Anderson.[12]

Phenobarbital sodium was dissolved in 0·9% sodium chloride and injected intraperitoneally at a dose of 100 mg/kg 1 hr before partial hepatectomy or sham operation; control animals received an equivalent volume (approximately 1·0 ml) of 0·9% sodium chloride.

[4,5-^{3}H]L-Leucine was diluted in 0·9% sodium chloride and injected intraperitoneally at a level of 50 μc/100 g 1 hr before sacrifice at 2, 4, 6, 9 and 25 hr after administration of phenobarbital or saline. [^{14}C-guanidino]L-arginine was dissolved in 0·9% sodium chloride and injected intraperitoneally at a level of 5 μc/100 g immediately after partial hepatectomy or sham operation and groups of animals were sacrificed at 24, 36, 48 and 72 hr later.

Preparation of liver microsomes. Rats were killed by decapitation and livers were perfused *in situ* through the portal vein with 20 ml of ice-cold 0·9% sodium chloride and removed. Only the right and caudate lobes were used from sham-operated rats. Livers were immediately minced and expressed through a syringe into a beaker at 4°; all subsequent operations were carried out at this temperature. An aliquot weighing 2·5 g was homogenized in 0·25 M sucrose with a Teflon glass homogenizer and the final concentration was adjusted to 25 per cent (w/v). Homogenates were centrifuged at 10,000 *g* for 20 min and the overlying layer of fat was removed. The supernatant fluid was then centrifuged at 100,000 *g* for 60 min, the resulting microsomal pellet was washed once with 0·1 M phosphate buffer, pH 7·4, and the pellet was resuspended in the same buffer. Trypsin-solubilized microsomal proteins were prepared by the method of Omura *et al.*[13] A concentration of 0·6 mg trypsin/g wet weight of liver was used.

Assays. NADPH cytochrome c reductase (NADPH:cytochrome c oxidoreductase, EC 1.6.2.3) was assayed by a modification of the method of Omura *et al.*[13] The assay mixture (total volume 1·0 ml) contained 1×10^{-4} M NADPH, 5×10^{-5} M cytochrome c, 0·1 M phosphate buffer, pH 7·4, and approximately 50 μg of microsomal protein. Substrate concentrations were found to be saturating under the experimental conditions employed. Activity was measured at 550 nm in a Gilford multiple sample

absorbance recording spectrophotometer with a water-jacketed cuvette chamber maintained at 37°.

Cytochrome b_5 was determined from the spectral difference between NADH reduced and oxidized samples.[14]

Samples for liquid scintillation counting were dissolved in 1·0 ml of Soluene (Packard Instrument Co.) and mixed with 20 ml of Liquifluor (New England Nuclear Corporation) containing 30% (v/v) absolute ethanol. Radioactivity was determined with a Packard Tri-Carb liquid scintillation spectrometer at 60 per cent efficiency for ^{14}C and 15 per cent efficiency for ^{3}H.

Protein was determined by the method of Lowry *et al.*[15]

RESULTS

The activity of rat liver NADPH cytochrome c reductase was measured in normal and partially hepatectomized animals at various times after the administration of a single dose of 100 mg/kg of phenobarbital (Fig. 1). Maximal elevation of reductase activity in sham-operated rats occurred at 49 hr after injection of the barbiturate inducer. The imposition of the stress of partial hepatectomy in phenobarbital-treated animals prematurely terminated the induced increase in NADPH cytochrome c reductase activity. No significant change in the basal levels of enzyme activity occurred in the livers of partially hepatectomized animals throughout the experimental period employed.

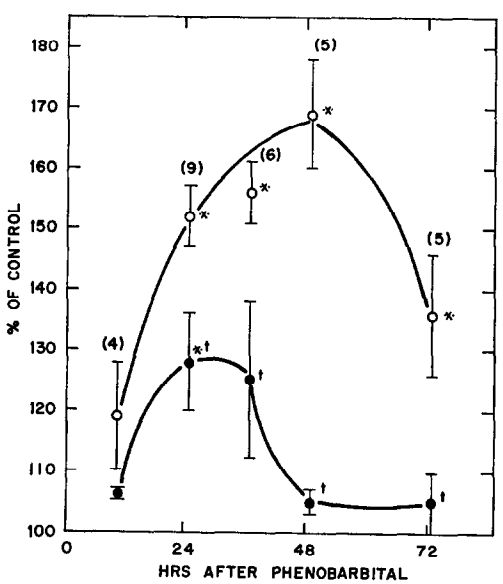

FIG. 1. NADPH cytochrome c reductase activity after phenobarbital treatment of sham-operated and partially hepatectomized animals. The data are expressed as the per cent of saline-injected sham (○—○) or partially hepatectomized (●—●) controls. Numbers in brackets indicate the number of sham-operated or partially hepatectomized rats. Asterisks (*) indicate statistical difference ($P < 0.05$) with respect to saline-injected controls. Daggers (†) indicate statistical difference ($P < 0.05$) with respect to the per cent change for phenobarbital-treated sham-operated animals. Control levels for saline-injected sham-operated and partially hepatectomized controls were 55 ± 1 and 61 ± 1 nmoles/min/mg protein respectively.

Microsomal levels of cytochrome b_5 remained unchanged throughout the interval studied in both sham-operated and partially hepatectomized rats following phenobarbital treatment (Table 1). However, the basal level of cytochrome b_5 decreased significantly, by approximately 30 per cent, at 24–72 hr following partial hepatectomy. These data confirm the previous observations of von der Decken and Hultin.[16]

TABLE 1. CYTOCHROME b_5 LEVELS AFTER PHENOBARBITAL TREATMENT OF SHAM-OPERATED AND PARTIALLY HEPATECTOMIZED ANIMALS*

Treatment	Cytochrome b_5 levels (nmoles/mg protein) after				
	11 hr†	25 hr	37 hr	49 hr	73 hr
Saline, sham	0·29 ± 0·01	0·32 ± 0·02	0·30 ± 0·01	0·30 ± 0·03	0·35 ± 0·01
Phenobarbital, sham	0·32 ± 0·01	0·32 ± 0·03	0·33 ± 0·03	0·34 ± 0·01	0·40 ± 0·02
Saline, partially hepatectomized	0·32 ± 0·01	0·22 + 0·02‡	0·21 ± 0·02‡	0·20 + 0·01‡	0·22 ± 0·01‡
Phenobarbital, partially hepatectomized	0·32 ± 0·01	0·25 ± 0·02	0·21 ± 0·02	0·21 ± 0·02	0·27 ± 0·02

* Rats received a single intraperitoneal injection of either phenobarbital (100 mg/kg) or an equivalent volume of 0·9% sodium chloride 1 hr before partial hepatectomy or sham operation, and cytochrome b_5 levels were measured at the indicated times thereafter. Each value represents the mean ± S.E. of results obtained from the separate analyses of microsomal preparations from four to five rats.

† Time after phenobarbital or 0·9% sodium chloride.

‡ Statistically significant ($P < 0·05$) from saline-injected sham-operated rats.

Since the induction of activity of NADPH cytochrome c reductase in partially hepatectomized rats was not sustained for the duration of the experimental period after the administration of phenobarbital, and NADPH cytochrome c reductase is a component of the endoplasmic reticulum, possible alterations in the rate of synthesis and degradation of the protein fraction of microsomes were estimated. Table 2 presents the results of studies measuring the rate of synthesis of trypsin-solubilized microsomal protein by pulse labeling of this fraction with [4,5-^{3}H]L-leucine. Phenobarbital treatment did not alter the rate of incorporation of leucine into microsomes of either sham-operated or partially hepatectomized rats 2–9 hr following exposure to the barbiturate; however, increased utilization of leucine occurred in the livers of sham-operated rats at 25 hr after the administration of inducer. Increased incorporation of leucine into trypsin-solubilized microsomal protein 25 hr after phenobarbital was not observed in the livers of partially hepatectomized animals. Similar results were obtained by measuring the rate of incorporation of [4,5-^{3}H]L-leucine into partially purified (20-fold) NADPH cytochrome c reductase after fractionation on Sephadex G-100.[17]

The rate of loss of radioactivity of total microsomal protein labeled with [^{14}C-guanidino]L-arginine following injection of phenobarbital was measured (Fig. 2). The decay of radioactivity from the microsomal fraction of sham-operated rats treated with phenobarbital was 45 per cent that of sham-operated animals not exposed to the inducer. Superimposition of the stress of partial hepatectomy did not substantially alter the response to phenobarbital. Removal of approximately two-thirds of the liver

TABLE 2. INCORPORATION OF [4,5-^{3}H]L-LEUCINE INTO TRYPSIN-SOLUBILIZED MICROSOMAL PROTEIN AFTER PHENOBARBITAL TREATMENT OF SHAM-OPERATED AND PARTIALLY HEPATECTOMIZED RATS*

Treatment	Leucine incorporation (counts/min/mg protein) after				
	2 hr†	4 hr	6 hr	9 hr	25 hr
Saline, sham	1250 ± 50	960 ± 100	1060 ± 50	1290 ± 80	900 ± 60
Phenobarbital, sham	1200 ± 220	1000 ± 170	1100 ± 110	1340 ± 90	1710 ± 260‡
Saline, partially hepatectomized	1430 ± 210	1170 ± 170	1200 ± 150	1530 ± 30	1630 ± 150
Phenobarbital, partially hepatectomized	1600 ± 150	1110 ± 320	1480 ± 210	1130 ± 140	2000 ± 50

* Rats received a single intraperitoneal injection of either phenobarbital (100 mg/kg) or an equivalent volume of 0·9% sodium chloride 1 hr before partial hepatectomy or sham operation. [4,5-^{3}H]L-Leucine (50 μc/100 g) was injected intraperitoneally at the indicated times, animals were killed 1 hr later and the microsomal fraction was isolated, digested with trypsin and the radioactivity of this fraction determined. Each value represents the mean ± S.E. of results obtained from the separate analyses of microsomal preparations from three to four rats.

† Time after phenobarbital or 0·9% sodium chloride.

‡ Statistically significant ($P < 0·05$) from corresponding saline-injected rats.

resulted in a corresponding decrease in initial total radioactivity at 24 hr after partial hepatectomy. This finding suggests that the uptake and dilution by cellular pools of labeled amino acid were similar in intact and regenerating liver. The virtually identical turnover rates of microsomal protein of saline-treated sham-operated and partially hepatectomized controls also appears to exclude a non-specific aberrant effect of the operative procedures.

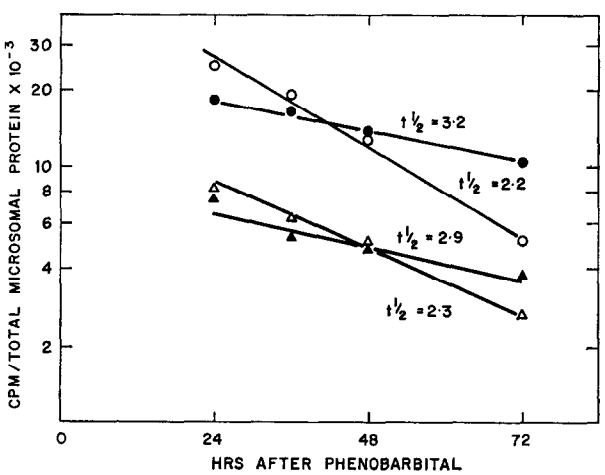

FIG. 2. Decay of total radioactivity of microsomes after phenobarbital treatment of sham-operated and partially hepatectomized animals. Rats received a single intraperitoneal injection of either phenobarbital (100 mg/kg) or an equivalent volume of 0·9% sodium chloride 1 hr before partial hepatectomy or sham operation. Animals were injected intraperitoneally with [^{14}C-guanidino]L-arginine (5 μc/100 g) immediately after the operation and were sacrificed at the indicated times thereafter. Each point represents the mean of the separate analyses of microsomal preparations from two rats. Saline, sham operation (○); phenobarbital, sham operation (●), saline, partial hepatectomy (△); phenobarbital, partial hepatectomy (▲).

DISCUSSION

NADPH cytochrome c reductase is considered to be an essential and possibly rate-limiting component of the microsomal electron transport scheme.[8–11] This enzyme is a constituent of the endoplasmic reticulum and shows synthetic and degradative properties similar to the membrane to which it is bound both in the presence and absence of treatment with phenobarbital.[13,17–20] Since the imposition of liver cell proliferation by partial hepatectomy was shown to delay the induced synthesis of microsomal drug-metabolizing enzymes,[5–7] it was of interest to determine the influence of such proliferation on the activity of NADPH cytochrome c reductase. The results suggest that the endoplasmic reticulum of the regenerating liver following administration of phenobarbital is at least initially functionally similar to that of sham-operated animals with respect to elevation of enzyme activity. However, the increase in the activity of NADPH cytochrome c reductase in response to inducer is a transient event in partially hepatectomized rats; the induced increase in enzyme activity is prematurely terminated in these animals and activity declines to non-induced control levels by 49 hr. In contrast, the administration of phenobarbital to sham-operated rats resulted in a relatively sustained elevation of enzyme activity.

An increase in the rate of synthesis of microsomal protein was observed at 25 hr after phenobarbital; this was accompanied by an approximately 50 per cent increase in the retention of radioactivity in protein at 25–73 hr after inducer in sham-operated animals. Since [^{14}C-guanidino]L-arginine was used in the latter experiments, reutilization of labeled protein was minimal;[18] however, under the conditions of the study (i.e. a single dose of phenobarbital) the attainment of steady state conditions was impossible. Findings of a similar increase in the half-life of hepatic endoplasmic reticulum during and following multiple injections of phenobarbital have been reported.[17,20] Thus, the findings represent only an estimate of the turnover of certain microsomal proteins following exposure to the barbiturate. Although the labeling of microsomal proteins may not be unequivocally related to the phenobarbital-induced elevation of NADPH cytochrome c reductase, the lack of effect of phenobarbital treatment on levels of cytochrome b_5 supports the contention that alterations in microsomal protein are specifically related to induction of mixed function oxidase enzymes and are in agreement with previous studies.[13,17,21,22] The absence of increased incorporation of [^{3}H]L-leucine into liver microsomes of sham-operated rats relatively early in the inductive process (i.e. prior to 25 hr after phenobarbital) is in agreement with the results of Shuster and Jick[20] following a single injection of phenobarbital. However, these results are contrary to those reported by Kuriyama *et al.*,[17] who observed an increase in the rate of synthesis of microsomal protein 5–7 hr after intravenous injection of phenobarbital into fasted rats maintained on a synthetic protein diet. This discrepancy may be a result of differences in route of administration of inducer or the nutritional status of the animals or both.

The requirement for liver cell replication imposed by partial hepatectomy produced an abortive induction of NADPH cytochrome c reductase. The regulatory signals responsible for this phenomenon are unknown; however, these studies, as well as those in which mixed function oxidase activity was measured,[5,7] indicate that the requirement for replication takes precedent over the functional response of the tissue to inducers of drug-metabolizing enzymes. It is possible that an alteration in the integrity of the endoplasmic reticulum occurs during the initial stages of liver regener-

ation that is responsible for the differences in enzyme induction in replicating and non-replicating liver cells. Dissolution of the endoplasmic reticulum has been observed by electron microscopy during the early period after partial hepatectomy.[23] Decreased quantities of rough and smooth endoplasmic reticulum coupled with an increase in the amount of free polyribosomes 24–72 hr after partial hepatectomy have also been reported.[6,24,25] Since NADPH cytochrome c reductase activity is elevated in both rough and smooth endoplasmic reticulum after a single injection of phenobarbital,[21] the reported alterations in hepatic ultrastructure following partial hepatectomy could theoretically result in a more labile environment for this enzyme. Thus, subcellular perturbations could result in the inability of replicating liver cells to sustain phenobarbital-induced reductase activity.

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