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INDUCTION OF RAT LIVER MICROSOMAL ENZYMES BY CYCLOHEXIMIDE

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Abstract—Administration of cycloheximide into rats induced certain enzymes of rough and smooth microsomes. NADPH cytochrome *c* reductase of smooth microsomes and glucose-6-phosphatase of rough and smooth microsomes attained peak activities 2 hr after cycloheximide treatment. Cytochrome P-450 and cytochrome b_5 of the rough microsomes were highest 6 hr after the antibiotic injection. On the other hand, cycloheximide reduced ATPase activity in the smooth microsomes. All the components studied except NADPH cytochrome *c* reductase of the smooth microsomes approached control values 24 hr after the treatment. The responses of rough and smooth microsomes toward cycloheximide were different.

Xenobiotics such as phenobarbital and 3-methylcholanthrene are known to induce microsomal enzymes in mammalian liver [1–6]. Inhibitors of protein synthesis like puromycin [2], cycloheximide [7, 8], actinomycin D [9], ethionine [10] and thioacetamide [11] have been shown to reduce the levels of microsomal enzymes. However, there are reports that cycloheximide stimulates protein synthesis [7, 12, 13] and this stimulation is both time- and dose-dependent [13]. Cycloheximide, besides altering protein synthesis, also affects many other biochemical functions [14–17]. With nonlethal doses of cycloheximide the biochemical alterations are apparently followed by a compensatory process of recovery [17].

In the present study, the effect of cycloheximide at a nonlethal dose of 2 mg/kg body weight [17] on hepatic microsomal enzymes has been investigated at different time intervals after the antibiotic treatment. The microsomes have been separated into rough and smooth fractions to study their differential responses because chemicals such as phenobarbital are known to exert a greater influence on the smooth microsomes than on the rough [18, 19].

MATERIALS AND METHODS

Animals. Female Wistar rats weighing 250 ± 25 g (approximately 75 days old) were used in these studies. The rats belonged to an inbred colony maintained in our animal house for over 10 years. The animals were maintained on a nutritionally sufficient laboratory diet and water was supplied *ad lib.*

Preparation of microsomal fractions. The rats were starved for 24 hr prior to death but they had free access to water. Cycloheximide, dissolved in 0.9% NaCl, was injected intraperitoneally at a dose of 2 mg/kg body weight. All injections were given at the same time of day (9:00 a.m.). The rats were killed by cervical dislocation at different time intervals after the injections. Liver was collected and immediately chilled in ice. The fractionation of hepatic rough and smooth microsomes was carried out in a sucrose–caesium chloride density gradient [20]. The rough and smooth microsomes were collected separately, resuspended in Tris–KCl–$MgCl_2$ (TKM) buffer, pH 7.55 [21], and were again centrifuged at 105,000 *g* for 30 min. The pellets were collected, suspended in a known volume of TKM buffer and distributed into small aliquots. The aliquots were frozen in liquid nitrogen and stored at −20° until use. The aliquots were never stored for more than a week.

Assays. Glucose-6-phosphatase (G-6-Pase) was assayed according to the method of Dallner [22]. The inorganic phosphate liberated was estimated by the method of Fiske and Subbarow [23] as modified by Stainton [24]. Adenosine triphosphatase (ATPase) was measured by the method of Quigley and Gotterer [25]. The inorganic phosphate released by the enzyme was determined [23, 24].

Cytochrome P-450 was estimated according to the method of Omura and Sato [26] as modified by Beaufay *et al.* [27]. The difference spectrum between CO-treated and nontreated samples was used in the computation of cytochrome P-450 employing the constant

$$\Delta\varepsilon\ 450\text{–}490\ \text{nm} = 91\ \text{cm}^{-1}\,\text{mM}^{-1}.$$

Cytochrome b_5 was estimated according to the procedure of Klingenberg [28] as modified by Beaufay *et al.* [27]. The difference spectrum between the sample as such and as reduced by 63 μM NADH was recorded between 400 and 430 nm. The concentration of cytochrome b_5 was calculated from the difference

$$\Delta\varepsilon\ 424\text{–}405\ \text{nm} = 160\ \text{cm}^{-1}\,\text{mM}^{-1}.$$

NADPH cytochrome *c* reductase was assayed by the method of Lu *et al.* [29]. The spectra were taken for the first 4 min when the rate of cytochrome *c* reduction was found to be constant and proportional to the amount of the reductase present. One unit of reductase is defined as the amount of the enzyme catalyzing the reduction of 1 nmole cytochrome *c*/min under these conditions.

A Hitachi Perkin–Elmer double beam spectrophotometer was used for the spectral analyses. Protein was estimated by the method of Lowry *et al.* [30]. Data were analyzed statistically using Student's *t*-test.

Table 1. Effect of cycloheximide on microsomal protein content

Microsomal fraction	Protein content after cycloheximide treatment				
	0 hr	2 hr	6 hr	12 hr	24 hr
Rough	6.44 ± 0.16*	7.16 ± 0.11†	5.00 ± 0.27†	5.44 ± 0.81‡	5.10 ± 0.21†
Smooth	5.93 ± 0.39	5.86 ± 0.32‡	5.41 ± 0.70‡	5.38 ± 0.25‡	5.53 ± 0.54‡

* Values are expressed as mg protein/g of liver; mean ± S.E. (from five experiments each).
† Significantly different ($P < 0.05$) from the control.
‡ Difference from the control not significant ($P > 0.05$).

RESULTS

The effect of cycloheximide on protein content of the microsomal fractions is shown in Table 1. There is a statistically significant increase in the rough microsomal protein at 2 hr after cycloheximide treatment. At 6 hr and 24 hr post-treatment the protein value is lower than that of the control. The protein level of the smooth microsomes remains unchanged following the antibiotic treatment. The rough and the smooth microsomes show a significant increase in G-6-Pase activity after cycloheximide treatment (Fig. 1). The increase in the rough microsomes is nearly 2½ times and 2 times in the smooth microsomes ($P < 0.01$), in comparison with the controls. The enzyme activity declines after 2 hr and this reduction is more in the smooth microsomes. G-6-Pase levels in rough and smooth microsomes are more or less the same as in the respective controls 24 hr after the antibiotic treatment. The effect of cycloheximide on the levels of total ATPase is shown in Fig. 2. In the controls, the enzyme activity of the smooth microsomes is over 2-fold that of the rough microsomes. ATPase activity of the smooth microsomes declines steadily until 12 hr after cycloheximide treatment. Although there is an increase in the smooth microsomal ATPase activity between 12 and 24 hr ($P < 0.01$), it still remains below the control value at the end of 24 hr. A significant reduction in ATPase activity of the rough microsomes is observed only at 12 hr ($P < 0.01$).

The effects of cycloheximide on the levels of three "mixed-function oxidases," *viz.* NADPH cytochrome *c* reductase, cytochrome P-450 and cytochrome b_5 are shown in Figs. 3, 4 and 5 respectively. In general, the smooth microsomes have higher levels of these enzymes than the rough microsomes in intact animals. NADPH cytochrome *c* reductase increases by about 2-fold in the smooth microsomes 2 hr after cycloheximide treatment ($P < 0.01$) (Fig. 3). There is a fall in the enzyme activity after 2 hr, and by 6 hr the activity is reduced to nearly 65 per cent of that of the control. This is followed by another increase ($P < 0.05$) which brings the enzyme activity 24 hr after the antibiotic treatment nearly on a par with that at 2 hr. NADPH cytochrome *c* reductase activity of the rough microsomes also changes after cycloheximide treatment with the result that the enzyme level is lower than that of the control 12 hr after the antibiotic administration (Fig. 3). The enzyme activity rises between 12 and 24 hr after cycloheximide treatment. However, these changes are not statistically significant.

Cytochrome P-450 is present in slightly higher quantities in the smooth microsomes than in the rough microsomes of the control rats ($P > 0.05$) (Fig. 4). The

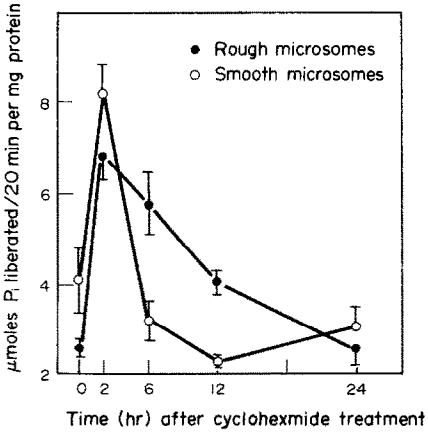

Fig. 1. Effect of cycloheximide on glucose-6-phosphatase activity of rat liver rough and smooth microsomes as a function of time. The cycloheximide treatment, the preparation of microsomes and the enzyme assay were as described in Materials and Methods. Values are means ± S.E. from five or six animals.

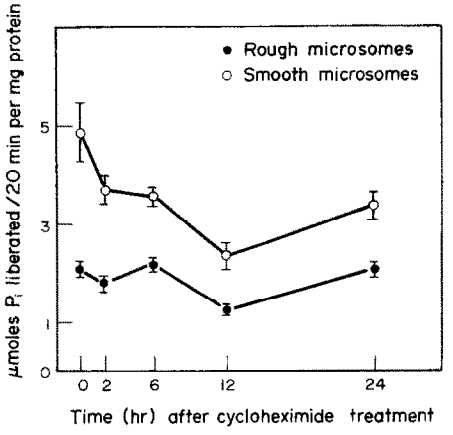

Fig. 2. Effect of cycloheximide on adenosine triphosphatase activity of rat liver smooth and rough microsomes as a function of time. Experimental details are given in Materials and Methods. Values are means ± S.E. from five or six animals.

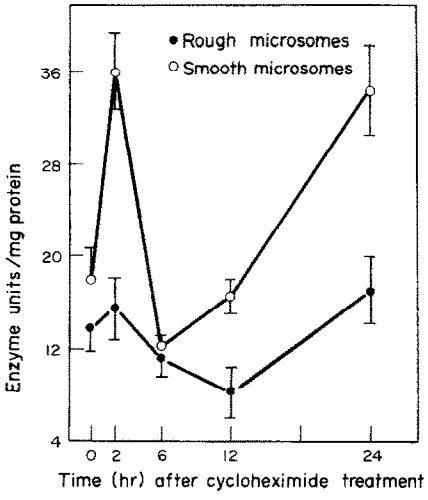

Fig. 3. Influence of cycloheximide on NADPH cytochrome *c* reductase activity of rat liver rough and smooth microsomes as a function of time. Experimental details are given in Materials and Methods. Values are means ± S.E. from five or six animals.

pattern of changes in cytochrome P-450 is similar in both smooth and rough microsomes following cycloheximide administration. The enzyme activity increases between 2 and 6 hr after the antibiotic treatment, and it declines after 6 hr. However, only the increase of cytochrome P-450 at 6 hr in the rough microsomes is statistically significant ($P < 0.01$). At the end of 24 hr the treated and the control groups have similar cytochrome P-450 activities. The changes in cytochrome b_5 due to cycloheximide administration are similar to those noted in the case of cytochrome P-450 (Fig. 5). The maximum amount of cytochrome b_5 is observed 6 hr after the antibiotic treatment in both smooth and rough microsomes.

DISCUSSION

Cycloheximide alters the level of microsomal enzymes just as it affects other biochemical functions

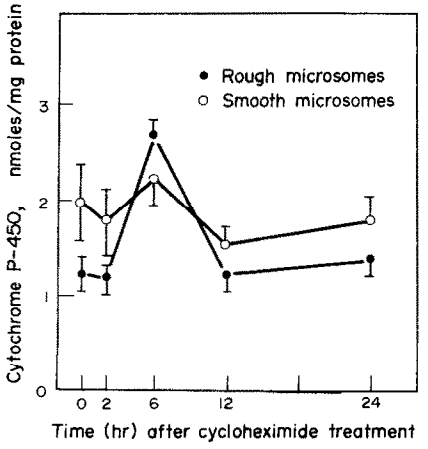

Fig. 4. Effect of cycloheximide on cytochrome P-450 activity of rat liver smooth and rough microsomes as a function of time. Details of the experiments are given in Materials and Methods. Values are means ± S.E. from five or six animals.

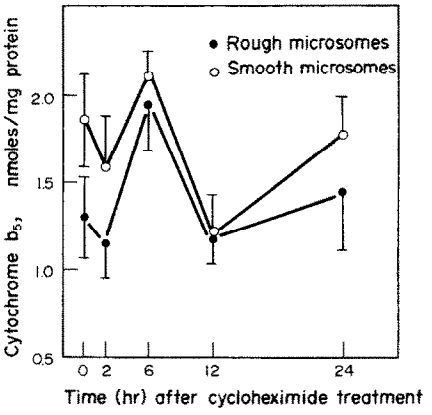

Fig. 5. Influence of cycloheximide on cytochrome b_5 activity of rat liver rough and smooth microsomes as a function of time. Experimental details are given in Materials and Methods. Values are means ± S.E. from five or six animals.

[14–17]. Jondorf *et al.* [7] have shown that rat liver microsomal drug-metabolizing enzymes are selectively inhibited by *in vivo* application of cycloheximide although protein synthesis is enhanced by the antibiotic. Our results, on the other hand, show that certain microsomal enzymes are augmented after cycloheximide treatment. The peak activities for different enzymes are at different time intervals after antibiotic administration. There are also differences between smooth and rough microsomes with respect to responses toward cycloheximide.

A number of enzymes are involved in drug metabolism. Many of these enzymes are, in turn, induced by drugs and other foreign substances. Cytochrome P-450 is a key enzyme, among what are known as the "mixed-function oxidases", involved in the oxidation of drugs. It has been reported that there is a reduction in microsomal cytochrome P-450 at 12 hr after cycloheximide treatment and that normal activity is restored at 24 hr [8]. In our experiments the induction of cytochrome P-450 is apparent in rough microsomes at 6 hr after the antibiotic injection ($P < 0.01$). At 24 hr both fractions have control levels of cytochrome P-450. Our results for 12 hr and 24 hr conform to those of Williams and Karler [8]. It is likely that they [8] have missed the induction process since it appeared 6 hr after the antibiotic treatment. Another difference between our studies and those of Williams and Karler [8] is that we separated the microsomes into rough and smooth fractions. Phenobarbital has been shown to enhance microsomal cytochrome P-450 more prominently in the rough microsomes than in the smooth [19], which conforms to our results. There is an increase in cytochrome b_5 in both the smooth and the rough microsomes at 6 hr after cycloheximide administration. However, the increase is not statistically significant ($P > 0.05$). Remmer and Merker [18] observed an increase in cytochrome b_5 which is more prominent in rough microsomes after phenobarbital treatment in rabbits. On the other hand, Eriksson [19] has reported that there is no significant increase in cytochrome b_5 after phenobarbital treatment in rats. Although cytochrome b_5 has been implicated in drug oxidations, its precise function is not clear [31].

NADPH cytochrome *c* reductase activity of the smooth microsomes doubles 2 hr after cycloheximide treatment. This is followed by a reduction in the enzyme activity, with the result that at 6 hr it is lower than the control level. There is a second increase in the enzyme activity which at 24 hr is comparable to what it was at 2 hr. Williams and Karler [8] noted a slight increase in the level of NADPH cytochrome *c* reductase at 12 hr after cycloheximide treatment and no difference from the control value at 24 hr. The dose of cycloheximide used by them [8] and in the present study is the same. So, the differences in the results may be either because they [8] did not assay the enzyme earlier than 12 hr or because their study was on male Sprague–Dawley rats and ours was on female Wistar rats. Gillette [32] has shown that the capacity to metabolize drugs is different in male and female rats.

Glucose-6-phosphatase increases considerably in rough and smooth microsomes after cycloheximide treatment. The enzyme activity is nearly twice at 2 hr after the antibiotic injection in comparison with the control. Thereafter G-6-Pase activity declines and returns to control value by 24 hr. Cycloheximide has been shown to reduce glycogen of the endoplasmic reticulum [16]. The increase in G-6-Pase after the antibiotic treatment may be correlated with the glycogen breakdown. On the other hand, it has been reported that phenobarbital suppresses G-6-Pase activity of the microsomal fractions [6, 19]. ATPase activity of the smooth microsomes declines more than that of the rough microsomes after cycloheximide treatment. Phenobarbital lowers the level of Mg^{2+}-dependent ATPase of rat liver microsomes [19, 32] and rabbit liver microsomes [18].

It may be surmised from our data that cycloheximide induces components of the mixed-function oxidase system and the microsomal marker enzyme, glucose-6-phosphatase. The induction of the microsomal enzymes is time-dependent and takes place in spite of the known inhibitory effect of cycloheximide on protein synthesis [13, 33].

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