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POST-TRANSLATIONAL REDUCTION OF CYTOCHROME P450IIE BY CCl_4, ITS SUBSTRATE

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The molecular mechanism of cytochrome P450IIE reduction by CCl_4 was reexamined by measuring its enzyme activity, immunoreactive protein contents, and mRNA levels. Aniline hydroxylase and the amounts of immunoreactive P450IIE were rapidly decreased in a time-dependent manner after a single dose of CCl_4. No changes were observed in the amounts of immunoreactive P450IIC and P450IA despite significant decreases in their catalytic activities. However, the decreases in P450IIE enzyme activity and immunoreactive protein by CCl_4 were not accompanied by a decline in its mRNA level. The data thus suggested a post-translational reduction of P450IIE by CCl_4, probably due to specific destruction of the P450IIE protein by its own substrate rather than heme moiety.

Microsomal ethanol-inducible cytochrome P450 (P450IIE) is one of the cytochromes P450 well characterized with respect to its substrate specificity, regulation, gene structure, and induction mechanism. It is responsible for the primary metabolism of variety of substrates including ethanol, acetone, propanol, ether, benzene, CCl_4, clinically used drugs, and certain potent carcinogens such as nitrosamines (1). It can be induced by most of these substrates and pathophysiological conditions such as starvation and diabetes. The multiple mechanisms of P450IIE induction are in contrast to that of transcriptional activation observed with other major forms of cytochromes P450 by their respective inducers (2). Despite numerous reports on the multiple mechanisms of P450IIE induction (3-9), the mechanism of its reduction by substrate, for instance, CCl_4, has not been elucidated on a molecular level. Earlier studies (10-12) suggested that a rapid decrease in cytochromes P450 after CCl_4 treatment was due to destruction of the heme moiety of cytochrome P450. In order to determine whether P450IIE suppression by CCl_4 was related to transcriptional inhibition, we have reexamined the fate of P450IIE by using polyclonal antibodies and a cDNA probe for P450IIE. In this communication, we demonstrate evidence of

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specific reduction of P450IIE protein and enzyme activity by CCl_4 which is not accompanied by a concomitant decrease in its mRNA, indicating a post-translational destruction causing inhibition of the enzyme by its substrate.

MATERIALS AND METHODS

Animal Treatment - Male outbred Sprague-Dawley rats (5 per group, weighing 150 - 170 grams) were obtained from Charles River Breeding company and kept in a 12 hour light-dark cycle with food and water *ad libitum* in accordance with NIH guidelines. After a single intraperitoneal injection of CCl_4 (2 g/kg body weight diluted in corn oil, 1:1), the animals were sacrificed at the times as indicated. Several tissues including liver, lung, and kidney were immediately excised and processed as described.

Preparation of Microsomes and Enzyme Assays - Microsomal fractions from the tissues of the control and CCl_4-treated animals were prepared by differential centrifugation as described previously (3). Aniline hydroxylase activity was determined by measuring p-aminophenol formation as detailed (3). Pentoxyresorufin O-dealkylase and ethoxyresorufin O-deethylase activities were measured by the method detailed (13).

Immunoblot Analyses - Microsomal proteins from the control and treated animals were separated on SDS-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membranes. Immunoblot analyses were performed as previously described (3,5) using specific polyclonal antibodies against P450IIE (5) and polyclonal antibodies against P450IA and P450IIC which were either purchased from the Oxford Biomedicals (Oxford, MI) or kindly provided by Dr. Frank Gonzalez (National Cancer Institute).

Northern mRNA Blot Analyses - Cytoplasmic RNA from each tissue were prepared by the acid guanidine isothiocyanate-phenol-chloroform method (14). Total cytoplasmic RNA separated on 0.66 M formaldehyde/1% agarose gels were transferred to Gene Screen membranes by the method of Davis *et al.* (15) and hybridized with [^{32}P]-labeled cDNA probes of P450IIE (3) or β-actin (kindly supplied by Dr. Shioko Kimura, National Cancer Institute). The details of Northern blot analysis followed by autoradiography were carried out as described (5). Other methods and chemicals not mentioned were described elsewhere (3,5,8).

RESULTS

Inhibition of P450-Mediated Catalytic Activities by CCl_4 - In order to investigate the mechanism of P450IIE inhibition by CCl_4, the levels of P450IIE mediated enzyme activity and immunoreactive protein content were measured after a single injection of CCl_4. The activity of P450IIE-mediated aniline hydroxylase was reduced in a time-dependent manner (Table 1). At 2 h after CCl_4 administration, the activity was 57% of the control and only 14% of the control after 24 h exposure to CCl_4. The activities of pentoxyresorufin O-dealkylase (P450IIC-related) and ethoxyresorufin-O-deethylase (P450IA-related) were also suppressed in a time-dependent manner by CCl_4 (Table 1).

Differential Effects of CCl_4 on the Amounts of Immunoreactive P450s - The immunoreactive amounts of P450IIE (M_r 52,000 daltons on SDS-polyacrylamide gel

Table 1. Effect of CCl_4 on aniline hydroxylase and alkoxyresorufin-O-dealkylase in rat hepatic microsomes

CCl_4 Treatment	Aniline hydroxylase Activity[1] (% Control)	Pentoxyresorufin-O-dealkylase Activity[2] (% Control)	Ethoxyresrufin-O-deethylase Activity[2] (% Control)
Time 0h	0.35 ± 0.04 (100)	41.5 ± 7.2 (100)	49.1 ± 7.0 (100)
2h	0.15 ± 0.05 (43)	48.1 ±17.9 (118)	9.3 ± 6.3 (19)
8h	0.12 ± 0.02 (34)	32.4 ±14.7 (78)	2.9 ± 1.9 (6)
24h	0.05 ± 0.02 (14)	16.0 ± 3.4 (39)	13.1 ± 1.9 (27)

Data are presented as means ± SEM, n=5.
[1] nmoles p-aminophenol formed/min/mg protein at 37°C.
[2] pmoles resorufin formed/min/mg protein at 25°C.

electrophoresis) was rapidly decreased in CCl_4-treated rats within 2 h of exposure and remained same at 24 h (Fig. 1). The decline in the P450IIE content generally correspond to the inhibitory pattern of P450IIE enzyme activity by CCl_4 (Table 1). The inhibition of renal P450IIE by CCl_4 was also observed although the extent of inhibition was smaller (maximal 50% inhibition at 24 h) than that observed in hepatic tissue (data not shown). In contrast, the changes or reductions in the immunoreactive

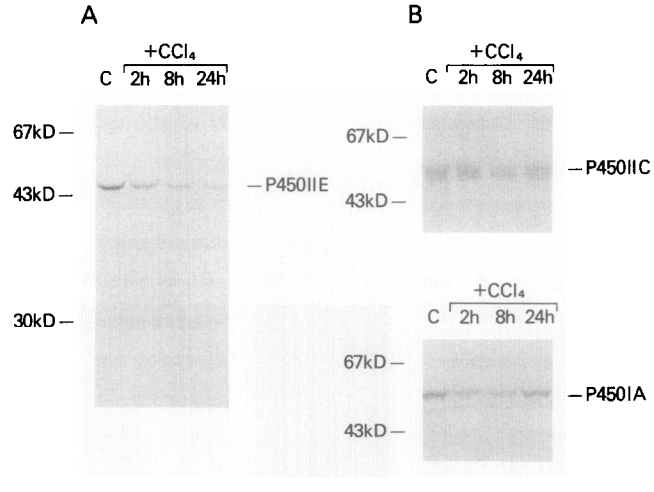

Fig. 1. Western immunoblot analyses of P450IIE, P450IIC and P450IA in rat liver microsomes after CCl_4 treatment. Equal amounts of microsomal proteins (30 µg/well for P450IIE and 15 µg for P450IIC and P450IA) from control and CCl_4-treated animals for different times as indicated were subjected to immunoblot analyses using specific polyclonal antiserum against cytochrome P450IIE (A) or P450IIC (B, top) or P450IA (B, bottom).

amounts of other cytochromes P450 (P450IIC and P450IA, M_r 53,000 and 54,000 daltons, respectively) were not so apparent as the inhibitions of the corresponding catalytic activities (Fig. 1 and Table 1). These data thus suggest that the inhibition of P450IIE by CCl_4 shown here could be due to the destruction of the enzyme molecule by the substrate and that the inhibition of the other two P450s without apparent changes in their protein content might be due to possible conversion to apoenzymes after destruction of heme molecule by CCl_4 as previously suggested (10-12).

No Changes in P450IIE mRNA Level by CCl_4 - The molecular mechanism of the decreases in P450IIE-mediated catalytic activity and immunoreactive protein by CCl_4 was further studied by Northern blot analysis using a cDNA probe specific for P450IIE (Fig. 2). When equal amounts of cytosolic RNA were analyzed as evidenced by the comparable levels of ribosomal RNA subunits (Fig. 2B), no apparent changes in the level of P450IIE mRNA (1.8 kb in size) were observed after from CCl_4 treatment (Fig. 2A). As a positive control, actin mRNA (1.8 kb) level was also measured. A slight decline in actin mRNA level was observed during early stages of 2 h and 8 h after CCl_4 treatment while a significant increase was observed at 24 h, similar to the results reported earlier (16,17). The post-translational destruction of P450IIE by CCl_4 without changes in its mRNA level was further supported by nuclear run-off transcription analysis where no differences in the rate of transcription were observed (data not shown). Our data therefore suggest that inhibition of P450IIE catalytic activity by CCl_4 may be due to concurrent, specific destruction of P450IIE protein by its substrate via metabolism to free radicals as proposed (18).

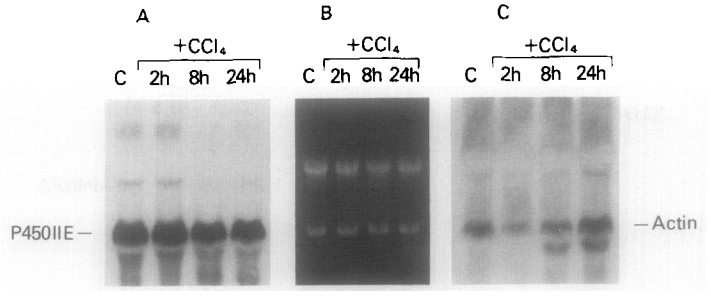

Fig. 2. Northern mRNA blot analyses for hepatic P450IIE mRNA after CCl_4 treatment. Total cytosolic RNA (20 μg/well) was isolated from the same two groups as described in Fig. 1 legend and subjected to Northern mRNA blot analyses using [^{32}P]-labeled cDNA probes for P450IIE (A) and β-actin (C). Panel B (middle) represents the ethidium bromide-staining pattern of the typical gel showing 28S and 18S ribosomal RNA subunits.

DISCUSSION

The detrimental effects of CCl_4 are believed to be dependent upon its metabolism by cytochromes P450, especially by P450IIE (19). The ensuing toxicological conditions can be aggravated by ethanol drinking or other conditions where P450IIE can be induced (19,20). Our data demonstrated that there was a rapid, specific decline in the amounts and catalytic activity of P450IIE without changes in its mRNA level, indicating that CCl_4 treatment does not alter the P450IIE gene expression as compared to the transcriptional regulation of several other proteins (16,17). The post-translational inhibition of P450IIE by CCl_4 further suggests that the decrease in P450IIE content could be due to either a decrease in the rate of P450IIE protein synthesis or an increase in the rate of its degradation. Although we are not clear at this moment, the latter seems more likely based on the earlier study (11,12) measuring the turnover rate of P450s. If so, it indicates a specific *in situ* destruction of P450IIE by the free-radical metabolites locally generated, which may render P450IIE more susceptible to enzymatic and/or non-enzymatic degradation. Furthermore, our data of the post-translational inhibition of P450IIE by its substrate provide additional mechanism of P450IIE regulation along with at least five or six distinct mechanisms already observed on P450IIE regulation (3-9,21).

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