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Biochemical Pharmacology, Vol. 27, pp. 1983–1985

0006-2952/78/0801-1983$02.00/0

Role of cytochrome P-450 in CCl_4-induced microsomal lipid peroxidation

(*Received* 3 *November* 1977; *accepted* 8 *February* 1978)

Although CCl_4 is considered to be activated to free radicals in the microsomal NADPH-dependent electron transport pathway before it acts as a stimulant of lipid peroxidation[1–3], no definite proof exists on which step—NADPH–cytochrome *c* reductase or cytochrome P-450—is involved in the activation. Slater and Sawyer[4] suggested that the former is involved, while Recknagel and Glende[3] pointed out the latter. Recently, Sipes *et al.*[5] specified cytochrome P-450 as a bioactivation step by examining the covalent binding of ^{14}C-metabolites of $^{14}CCl_4$ to microsomal proteins as a measure of the conversion of CCl_4 to reactive intermediates.

This study was begun to determine which step of the electron transport pathway is concerned with the activation of CCl_4, by using sonicated microsomes and a reconstituted microsomal enzyme system. Lipid peroxidation was measured as an indicator of CCl_4 bioactivation.

Materials and methods

Seven- to eight-week-old female Wistar strain rats were used. Male rats of the same strain and age were used for purification of the enzymes.

Microsomes were prepared from livers well-perfused with a cold 0.15 M KCl solution. The livers were homogenized with 4 vol. of 0.15 M KCl–10 mM EDTA (pH 7.5) and centrifuged at 15,000 *g* for 15 min. The supernatant fraction was then centrifuged at 125,000 *g* for 30 min, and the precipitated microsomes, after washing with 0.1 M potassium phosphate buffer (pH 7.5), were suspended in the same buffer (40–50 mg protein/ml).

Cytochrome P-450 and NADPH–cytochrome *c* reductase were purified from liver microsomes isolated from rats treated with phenobarbital (80 mg/kg/day, i.p., 5 days) according to the methods described by Imai and Sato[6] and Imai[7]. Our final cytochrome P-450 preparation (7.9 nmoles/mg of protein) was only partially purified, if judged from the reported value of 16.8 nmoles/mg of protein[6], but no NADPH–cytochrome *c* reductase was detected. The specific activity of the NADPH–cytochrome *c* reductase preparation was 1.3 μmoles cytochrome *c* reduced/mg of protein/min (6.52 μmoles cytochrome *c* reduced/ml/min) at 25°, using horse heart cytochrome *c* as an electron acceptor.

Microsomal lipids were extracted by the method described by Folch *et al.*[8] and lipid phosphorous was determined by the method of Bartlett[9]. Liposomes (20 μmoles P_i/ml) were prepared just before use by sonicating the solvent-free lipids in 0.1 M potassium phosphate buffer (pH 7.5) in an ice bath using a Branson micro-tip for a total of 40 sec, with several interruptions, with a power of 40 W. The microsomal suspension (20 mg protein/ml) was sonicated similarly.

The degree of lipid peroxidation was assessed by measuring malondialdehyde (MDA) in the reaction mixture. The regular method is as follows: sonicated microsomes (1 mg protein/50 μl) or liposomes (1 μmole P_i/50 μl) were mixed with NADPH–cytochrome *c* reductase (activity: 0.33 μmoles cytochrome *c* reduced/min/50 μl, which was omitted in the case of sonicated microsomes, and cytochrome P-450 (2 nmoles/50 μl) in a final volume of 0.15 ml. After a preincubation period of 20 min at 37°, the mixture was cooled in an ice bath and brought to 1.0 ml with 0.1 M potassium phosphate buffer (pH 7.5), and then NADPH (400 μM) and 0.5 μl CCl_4 were added (complete system). The test tube was capped, mixed vigorously for about 10 sec using a mixer, and incubated for 20 min at 37°. The MDA produced in the reaction mixture was determined by a thiobarbituric acid method[10] using the extinction coefficient $1.56 \times 10^5\ cm^{-1}\ M^{-1}$ for the colored product. Endogenous MDA content was subtracted from the value obtained. Any variations of the procedure are given in the legends.

Protein was determined by the method of Lowry *et al.*[11].

Results and discussion

Initially, the effect of added cytochrome P-450 on microsomal lipid peroxidation was examined. Sonicated microsomes were mixed with cytochrome P-450 in a small volume and preincubated in order to enhance the incorporation of the hemoprotein into microsomal vesicles[12], and were then peroxidized after dilution. As shown in Fig. 1, MDA production in the complete system

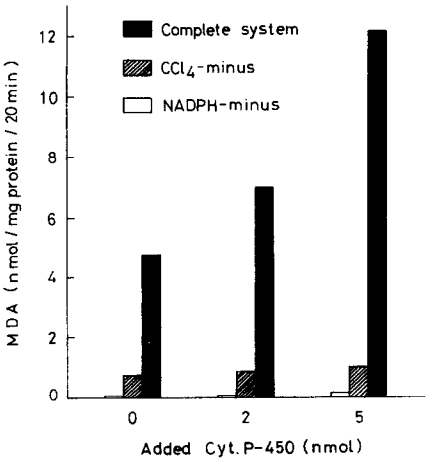

Fig. 1. Enhancement of CCl_4-induced malondialdehyde (MDA) production by exogenously added cytochrome P-450 in sonicated microsomes. Experimental details are given in Materials and Methods. Each value is the average of duplicate assays.

(containing both NADPH and CCl_4) was enhanced by preincubation with exogenously added cytochrome P-450. Stimulation of lipid peroxidation by CCl_4 in the absence of added cytochrome P-450 may be due to the presence of endogenous cytochrome P-450 in the sonicated microsomes. On the other hand, MDA production in the CCl_4-minus system (containing NADPH only) and in the NADPH-minus system (containing CCl_4 only) was only minimally affected. These observations suggest the involvement of cytochrome P-450 in an activation step of CCl_4.

This point was examined further by using a reconstituted microsomal NADPH-dependent enzyme system (Fig. 2) in which liposomes prepared from microsomal lipids were preincubated with NADPH–cytochrome *c* reductase and cytochrome P-450 at 37° for incorporation of the enzymes into lipid vesicles. No activators such as ADP- or pyrophosphate-iron[10] was added. As shown in Fig. 2A, when liposomes were preincubated in the presence of both of the enzymes, stimulation of MDA production by CCl_4 (complete system) increased with preincubation time, reaching the maximum at 10–20 min, while MDA production in the CCl_4-minus system was rather independent of preincubation time. Liposomes preincubated with either the reductase or cytochrome P-450 alone did not show any significant MDA production even in the complete system. These observations indicate that, in addition to the reductase, cytochrome P-450 is required and must be incorporated into lipid membranes for the stimulation of lipid peroxidation by CCl_4. The hemoprotein requirement was subsequently confirmed by testing the effects of varying amounts of cytochrome P-450 on MDA production (Fig. 2B). Enhancement of MDA production by CCl_4, i.e. the difference between MDA production in both the complete and CCl_4-minus systems, was evident with increasing amounts of the hemoprotein. In order to verify the reconstituted system, the stimulatory actions of $CHCl_3$, CCl_4 and $CBrCl_3$ were compared (Fig. 2C). A tendency similar to that observed with microsomes[13, 14] was obtained and $CBrCl_3$ was seen to be more powerful than CCl_4, but $CHCl_3$ had no stimulatory action.

Thus, we conclude that, in addition to NADPH–cytochrome *c* reductase, cytochrome P-450 is essential for stimulation of microsomal lipid peroxidation by CCl_4, probably as an activation step for CCl_4 metabolism to the more toxic free radicals.

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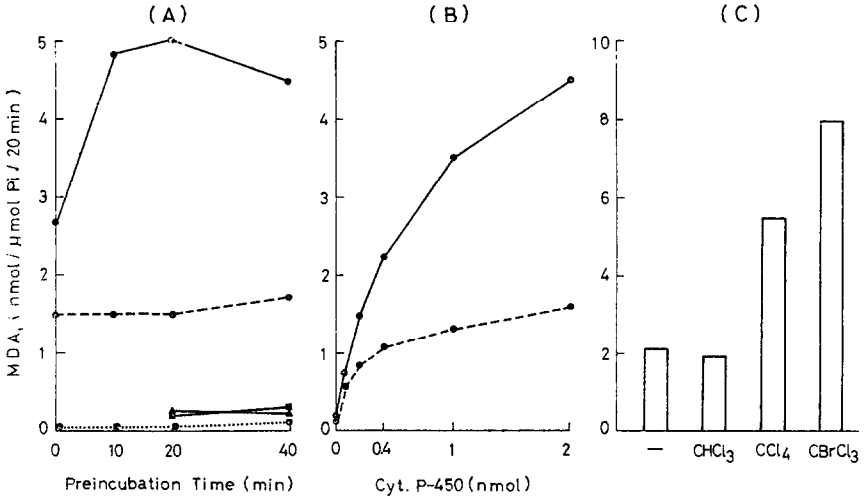

Fig. 2. Stimulation of malondialdehyde (MDA) production by CCl_4 in the reconstituted microsomal enzyme system. The regular method is given in Materials and Methods. Each point is the average of duplicate assays. Panel A: requirement of both NADPH–cytochrome *c* reductase and cytochrome P-450. Liposomes, after preincubation with NADPH–cytochrome *c* reductase and cytochrome P-450 for 0, 10, 20 and 40 min, were peroxidized in the complete (NADPH plus CCl_4) (●—●), CCl_4-minus (●– –●), or NADPH-minus (●·····●) system. As controls, liposomes were preincubated with reductase (▲—▲) or cytochrome P-450 (■—■) alone, and peroxidized in the complete system. Panel B: effect of the amount of added cytochrome P-450. Liposomes were preincubated with various amounts of cytochrome P-450 in the presence of the reductase, and then peroxidized in the complete (●—●) and CCl_4-minus (●– –●) systems. Panel C: comparison with other halogenated methanes. The regular method was used with 0.5 μl of halogenated methanes.

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Biochemical Pharmacology, Vol. 27, pp. 1985–1986

0006-2952/78/0801-1985$02.00/0

Effect of reserpine on the monoamine oxidase (MAO) activity in rat liver and brain

(*Received* 12 *December* 1977; *accepted* 28 *February* 1978)

A variety of drugs affect the concentration of serotonin within the nervous system; prominent among these is reserpine. Reserpine depletes tissues of serotonin and other amines. Kimbal and Vallejo [1] found that reserpine induced headache in migraine sufferers. Experiments performed in this direction showed that a subcutaneous (s.c.) injection of reserpine produced typical unilateral headache 6 hr after injection [2–5]. Reserpine releases serotonin from its major stores, i.e. brain, intestine and platelets. Further studies have shown that reserpine and also other related Rauwolfia alkaloids deplete stores of catecholamines in addition to serotonin in the brain and other organs. Most of the pharmacological effects of these alkaloids have been attributed to this action. The depletion and restoration rates are different in different organs. In view of these observations it was thought worthwhile to study the metabolism of neurotransmitter monoamines. Since these are the substrates for the enzyme monoamine oxidase (MAO), (monoamine: 0_2 oxidoreductase; E.C. 1.4.3.4), experiments were undertaken to study the *in vivo* effect of reserpine on the activity of MAO.

Norwegian rats weighing between 200–250 g. were given an intramuscular (i.m.) injection of a single dose of 0.2 mg reserpine/100 g wt of the rat. The drug Reserpine used in the experiments was a preparation of CIBA–Geigy, India, bearing the trade name Serpasil. The rats were sacrificed by stunning and decapitation, 24, 48, 72, 96 and 120 hr after injection. The liver and brain were removed immediately and homogenized in freshly prepared 0.5 M phosphate buffer pH 7.4. The crude homogenates were then centrifuged in an International Refrigerated Centrifuge at 0–4° at 2500 rpm for 15 min. The pellet consisting of nucleus and other cell debris was discarded. The supernatant was made up in volume with 0.5 M phosphate buffer pH 7.4 to 20 ml/g wt of liver and 40 ml/g wt of brain and this was used as the enzyme preparation.

The MAO activity in the different enzyme preparations was determined by using serotonin as well as tyramine as substrates in the different incubation mixtures. The incubation mixture contained 1 ml enzyme preparation, 0.4 ml of 0.5 M phosphate buffer pH 7.4 and 1 ml of substrate (600 μg/ml serotonin or 260 μg/ml of tyramine). The incubation was terminated at the end of 1 hr by the addition of 0.6 ml of 25% trichloro acetic acid (TCA) and immediate chilling. Control tubes were prepared by pretreating with TCA before the addition of the enzyme preparation, in order to ascertain the enzyme activity at zero hr. Serotonin was estimated in the supernatant by the Method of Udenfriend, Weissbach and Clark [6] and tyramine by the method of Udenfriend and Cooper [7]. The protein (of the enzyme) precipitated as the result of addition of TCA at the termination of incubation, was dissolved in 0.1 N NaOH and estimated by the method of Sutherland *et al.* [8]. Normal rats were used as controls and were decapitated immediately after an i.m. injection of reserpine.

The effect of reserpine on the MAO activity in rat liver and brain is shown in Figs. 1 and 2 respectively. The MAO activity was expressed in terms of sp. act. i.e. μg of substrate utilized/mg of protein. Administration of a single dose of reserpine produced an increase in the MAO activity in the rat liver with both serotonin and tyramine as substrates, reaching its maximum on day 3 (72 hr). The activity declined on the subsequent days and reached the normal level (control) on day 5 (120 hr). While the MAO activity showed a more than 2-fold increase with serotonin, the increase with tyramine was slightly less than 2-fold. The MAO activity in brain also showed an increase, reaching its maximum in 3 days (72 hr) with serotonin and in 4 days (96 hr) with tyramine. In both the cases the normal level was reached on day 5 (120 hr). The activity with serotonin showed a 2-fold increase and that with tyramine was less than 2-fold. In both the tissue preparations, the sp. act. with serotonin as substrate was higher than tyramine, throughout.

DISCUSSION

Results clearly indicate that administration of reserpine brings about considerable increase in the activity of MAO, thereby altering the metabolism of serotonin and tyramine in the liver and brain of rats. Increase in the activity of the enzyme will lead to higher rate of degradation or utilization of these amines. Eventually this will bring about a depletion of their stores in the liver and brain. It is widely believed that alteration in the neurotransmitter responses is involved in the precipitation of migraine attacks. Biochemical agents which alter the metabolism of these neurotransmitter amines include reserpine [9]. Some workers have observed that i.m. injection of reserpine in migrainous and normal