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Interactions of Dihydralazine with Cytochromes P4501A: A Possible Explanation for the Appearance of Anti-Cytochrome P4501A2 Autoantibodies

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SUMMARY

The antihypertensive drug dihydralazine may, on rare occasions, cause immunoallergic hepatitis characterized by anti-cytochrome P450 (P450)1A2 autoantibodies. To understand the first steps leading to this immune reaction, we studied the covalent binding of dihydralazine metabolites to microsomes from rat and human livers. Upon incubation with NADPH and microsomes, dihydralazine formed metabolites that reacted with heme (as evidenced by destruction of heme, formation of 445-nm light-absorbing complexes, and covalent binding of heme to P450 apoprotein) and covalently bound to microsomal proteins. Formation of these metabolites was shown (by NADPH dependence, induction by β-naphthoflavone, and immunoinhibition by anti-P4501A antibodies) to be mediated by P4501A. Finally, these metabolites appeared to bind to P4501A2, which produced them. These results support the following scheme for the first steps of this autoimmune reaction: P4501A2 metabolizes dihydralazine into reactive metabolites that then bind to it, forming a neoantigen that triggers an immune response characterized by autoantibodies against P4501A2.

A frequent target of drug-induced toxicity is the liver, due to the active metabolism of drugs in this organ. Two mechanisms may be responsible for drug-induced hepatitis, i.e., a direct one in which reactive metabolites are formed and directly damage critical cell targets (1, 2) and an indirect one in which one or more reactive metabolites covalently bind to proteins, which then behave as neoantigens and trigger an abnormal immunological response, leading to the disease. An example of the latter is hepatitis induced by halothane (3, 4), tienilic acid (5–7), or dihydralazine (8–11). The liver diseases induced by these three drugs exhibit similar clinical characteristics. First, the onset of hepatitis is delayed. Also, immunoallergic manifestations such as fever or eosinophilia may be present, and after rechallenge the disease appears more rapidly and may be more severe. Finally, the sera of these patients contain antimicrosome autoantibodies (3, 5, 6, 9–12).

We have previously shown that the anti-LM autoantibodies present in dihydralazine-induced hepatitis are directed against P4501A2 (11, 12), a P450 (13) that is induced by dihydralazine (12). Fig. 1 summarizes the hypothesis that was suggested (11) to explain the triggering of this disease. In the present study we investigated steps 1 and 2 of this scheme. We have clearly shown that, indeed, one or more reactive metabolites are produced by liver microsomes from rats and humans. These metabolites can bind to the P450 protein and cause covalent binding of heme to the protein. This binding appears to be essentially restricted to the P450 that generates the reactive metabolites, namely P4501A2.

Materials and Methods

Chemicals. Electrophoresis reagents were from Serva Fine Biochemicals (Heidelberg, Germany). Nitrocellulose sheets were from Bio-Rad Laboratories (Richmond, CA). Peroxidase-conjugated immunoglobulins were obtained from Dako-patts (Copenhagen, Denmark), resorufin from Aldrich Chimie (Strasbourg, France), NADPH, 7-ethoxyresorufin, and 7-pentoxyresorufin from Boehringer (Mannheim, Germany), and Protein A-Sepharose from Pharmacia (Uppsala, Sweden). [^{14}C]Dihydralazine (6.67 μCi/μmol) was a generous gift from Ciba-Geigy (Basel, Switzerland). Other reagents were of the highest quality available and were purchased either from Prolabo (Paris, France) or from Sigma Chemical Co. (St. Louis, MO).

Animals and treatments. Male Sprague-Dawley rats (150–200 g; Iffa-Credo, les Oncins, France) had free access to water and to a standard diet (UAR, Villemoisson, France). Some rats were treated with β-naphthoflavone (40 mg/kg dissolved in corn oil, intraperitoneally, once daily for 3 days) and killed 24 hr after the last dose. Dihydralazine sulfate (100 mg/kg in 0.154 M NaCl, intraperitoneally,

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ABBREVIATIONS: anti-LM, anti-liver microsomes; P450, cytochrome P450.

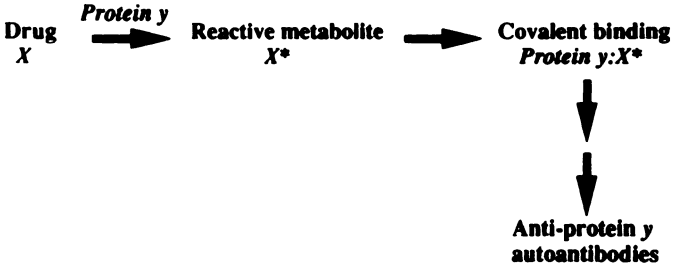

Fig. 1. Postulated initial events in dihydralazine-induced autoimmune hepatitis.

once) was administered either to untreated rats or to rats 24 hr after the last dose of β-naphthoflavone, and animals were killed 24 hr later.

Human livers. Human livers were obtained from donors for kidney transplantation. Livers were removed within 30 min after circulatory arrest and were frozen at −80° as small cubes. Collection of human samples was done in compliance with French regulations. Microsomes and homogenates were prepared as described previously (14).

Assays. Immunoblotting analysis was performed as described previously (15, 16), using 4-chloro-1-naphthol for development. Protein concentrations were measured by the method of Lowry *et al.* (17), using bovine serum albumin as the standard. P450 was measured according to the method of Omura and Sato (18).

IgG preparation. Sera containing anti-LM antibodies were obtained from patients suffering from dihydralazine-induced hepatitis. Preparation of rabbit anti-rat P4501A1/2 has been described (15), and anti-human P4502C was obtained against P450MP as described (19). IgG fractions were prepared by Protein A-Sepharose chromatography according to the manufacturer's protocol and were dialyzed against 0.154 M NaCl.

Binding spectra of dihydralazine with P450-Fe^{3+}. A technique using two tandem cuvettes was used to investigate the binding spectrum of dihydralazine with P450-Fe^{3+}. Two cuvettes were placed on each side, one containing the buffer (0.154 M KCl, 0.01 M sodium-potassium phosphate buffer, pH 7.4) and the other containing the microsomal suspension (2 mg of protein/ml) in the same buffer. After the base-line was recorded, dihydralazine (0.01–0.3 mM) was added both to the microsomal suspension on the sample side and to the buffer solution on the reference side, whereas the same volume of 0.154 M NaCl was added to the other two cuvettes. The difference spectrum was recorded from 360 to 510 nm with an SLM-Aminco DW-2C spectrophotometer.

P450 and heme after administration of dihydralazine. Microsomal heme was measured according to the method of Omura and Sato (18). Uncomplexed, complexed, and total P450 levels were determined as reported (20). P450 was measured with a first batch of microsomes according to the method of Omura and Sato (18). The presence of P450-Fe^{2+}-drug (metabolite) complex(es) absorbing around 445 nm was investigated with a second batch. Potassium ferricyanide (50 μM) was added to the reference cuvette and incubated at 37° for 5 min to destroy the complex, if any, in this cuvette. The difference spectrum around 445 nm measured the Soret peak of the complex in the sample cuvette. A molar extinction coefficient of 75 mM^{-1} cm^{-1} was used (21). To determine total P450, potassium ferricyanide was also added to the sample cuvette, so that the complex, if any, was destroyed in both cuvettes. The procedure of Omura and Sato (18) was then repeated. Because complexed P450, when present, had been converted into uncomplexed P450, this technique then measured total P450.

***In vitro* formation of P450-Fe^{3+}-NH_2—NH—R or P450-Fe^{3+}-NH—N—R complexes.** *In vitro* formation of P450-drug (metabolite) complexes was determined as previously described by Pessayre *et al.* (20). The microsomal suspension (2 mg of protein/ml) was distributed into two cuvettes maintained at 37°. In some experiments, α-naphthoflavone (100 μM) or methoxsalen (250 μM) was added to both cuvettes. Dihydralazine (0.1 mM) was added in 20 μl of 0.154 M NaCl to the sample cuvette, whereas 20 μl of 0.154 M NaCl were added to the reference cuvette. The base-line was recorded with an SLM-Aminco DW-2C spectrophotometer. The reaction was initiated by addition of NADPH (1 mM) to both cuvettes. The successive spectra were recorded from 360 to 660 nm. Absorption at 445 nm reached a plateau at 5 min.

***In vitro* destruction of P450.** The *in vitro* destruction of P450 was investigated as previously reported by Fouin-Fortunet *et al.* (22). Microsomal suspensions were prepared that contained dihydralazine (1 mM), microsomes (2 mg of protein/ml), NADP (0.5 mM), glucose-6-phosphate (10 mM), glucose-6-phosphate dehydrogenase (0.3 unit/ml), and EDTA (1.5 mM), in 0.15 M sodium-potassium phosphate buffer, pH 7.4. Half of the flasks were kept in ice throughout and served as time 0 samples. The other flasks were incubated at 37° for 20 min and then placed once again on ice. The microsomal suspension was then divided between two cuvettes, and P450 was determined as the CO-difference spectrum of dithionite-reduced microsomes. In some flasks, the NADPH-generating system was omitted. In other flasks, α-naphthoflavone (100 μM) was added.

Covalent binding of ^{14}C-labeled heme to microsomal proteins. Microsomal heme was labeled as described by Davies *et al.* (23). β-Naphthoflavone-treated and untreated rats were fasted for 16 hr and injected with δ-amino[4-^{14}C]levulinic acid (104 μCi/kg, 195 μmol/kg in 250 μl of water, intraperitoneally). Four hours later, animals were sacrificed and hepatic microsomes were prepared. An incubation mixture (3 ml) was prepared containing 12 mg of microsomal protein, in 0.154 M KCl, 1.5 mM EDTA, 0.05 M sodium-potassium phosphate buffer, pH 7.4. In some flasks, dihydralazine (1 mM) and α-naphthoflavone (10 μM) were added. The reaction was initiated by addition of NADPH (2 mM). After 20 min of incubation at 37° the tubes were placed on ice. The irreversible binding of radiolabeled heme to microsomal proteins was determined as described by Davies *et al.* (23). Samples (0.7 ml) were taken from the reaction mixture and proteins were precipitated with 10 volumes of methanol containing 5% (v/v) H_2SO_4. Pellets were rewashed twice with the methanol/H_2SO_4 mixture. Protein pellets were dried and dissolved in 0.6 ml of 1 N NaOH. One

TABLE 1

Effects of dihydralazine administration on hepatic microsomal P450 and heme

Some rats were pretreated for 3 days with β-naphthoflavone (β-NF) (100 mg/kg daily). Twenty-four hours after the last dose, rats received one dose of dihydralazine (DHZ) (100 mg/kg intraperitoneally), and they were killed 24 hr later. The amounts of uncomplexed P450 were determined from the CO-binding spectrum of dithionite-reduced microsomes. Total P450 was similarly determined after preincubation of microsomes for 5 min with 50 μM potassium ferricyanide. The amounts of complexed P450 were determined from its Soret peak at 445 nm, with a molar extinction coefficient of 75 mM^{-1} cm^{-1} (21). Results are means ± standard errors for eight rats.

Treatment	Microsomal protein	Uncomplexed P450	Complexed P450	Total P450	Total heme
	mg/g of liver	*nmol/mg of protein*	*nmol/mg of protein*	*nmol/mg of protein*	*nmol/mg of protein*
Control	39 ± 1	0.66 ± 0.03	<0.05	0.69 ± 0.03	1.51 ± 0.15
Control + DHZ	42 ± 2	0.48 ± 0.05[a]	<0.05	0.49 ± 0.05	1.33 ± 0.08
β-NF	32 ± 1	1.28 ± 0.08	<0.05	1.24 ± 0.07	2.07 ± 0.07
β-NF + DHZ	31 ± 1	0.95 ± 0.07[a]	0.31 ± 0.10	1.22 ± 0.08[b]	1.60 ± 0.08[a]

[a] Significantly different from rats not treated with dihydralazine (*t* test for independent data), $p < 0.01$.
[b] Significantly different from uncomplexed P450 (*t* test for dependent data), $p < 0.001$.

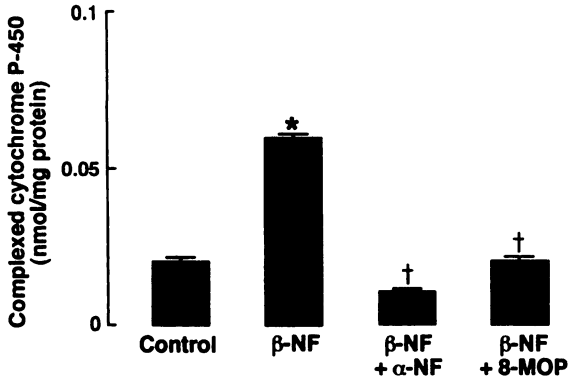

Fig. 2. *In vitro* formation of P450-Fe^{2+}-NH_2—NH—R or P450-Fe^{2+}-NH=N—R complexes (with R being hydralazine). Microsomes from control rats and *β-naphthoflavone-treated (β-NF)* rats (2 mg of protein/ml) were distributed in two cuvettes maintained at 37°. In some experiments, *α-naphthoflavone (α-NF)* (100 μM) or methoxsalen (*8-MOP*) (250 μM) was added to both cuvettes. Dihydralazine (0.1 mM) was added to the sample cuvette. The base-line was recorded. The reaction was initiated by addition of NADPH (1 mM) to both cuvettes, and successive spectra were recorded from 360 to 660 nm. Complexed P450 formed after 5 min of incubation was calculated using an absorption coefficient of 75 mM^{-1} cm^{-1} (21). Results are means ± standard errors for 10 experiments. *, Significantly different from microsomes from control rats, $p < 0.01$. †, Significantly different from microsomes from *β-naphthoflavone-treated* rats incubated without P450 inhibitors, $p < 0.01$.

aliquot (20 μl) was used to determine proteins, whereas another aliquot (0.4 ml) was acidified with 12 N HCl and counted for ^{14}C radioactivity.

Microsomal oxidation of dihydralazine and covalent binding studies. Incubations were made in glass tubes at a final volume of 150 μl, except for measurements of localization of covalently bound radioactivity by immunoblotting, where the final volume was 0.5 ml. Microsomes (0.3–0.5 mg of protein) were suspended in 100 μl of 0.1 M sodium phosphate buffer, pH 7.4. Incubation was started by addition of 50 μl of the same buffer containing an NADPH-generating system (0.15 μmol of NADP, 1.5 μmol of glucose-6-phosphate, and 0.2 unit of glucose-6-phosphate dehydrogenase) and [^{14}C]dihydralazine (0.1 μCi, 100 μM, or 0.3 μCi, 300 μM). After a 30-min incubation at 37°, a 50-μl aliquot was taken for covalent binding measurements. When IgGs were used for immunoinhibition of covalent binding, IgGs (from anti-LM human serum, anti-rat P4501A1/2 rabbit serum, and anti-rat P4503A rabbit serum) were preincubated for 2 min with microsomes in 100 μl of sodium phosphate buffer at 4°, and the incubation was started as indicated above. Proteins from aliquots of incubation mixtures (50 μl) were precipitated on glass fiber filter disks (Whatman GF/B) that had been presoaked in 10% trichloroacetic acid. Proteins trapped on the filter disk were washed twice with 5% trichloroacetic acid, methanol, and ethyl acetate. We checked that additional washings with methanol and then with ethyl acetate did not modify the radioactivity of the disk. After solvent evaporation at 60°, radioactivity of proteins trapped on the disk was counted in a Packard Tri-Carb 300 scintillation counter.

Immunoblots of P450 and localization of covalently bound radioactivity. In these experiments, hepatic microsomes were incubated as described above and microsomal proteins were treated as described. The proteins were dissolved overnight at room temperature in 10 mM Tris buffer, pH 7.4, with 1% sodium dodecyl sulfate and were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described by Laemmli (24). Usually, microsomal proteins (50–100 μg) were loaded into wells (four wells loaded for each incubation) and other wells were loaded with nonincubated microsomes. After electrophoretic separation, proteins were electrotransferred to nitrocellulose. The different lanes were cut out and incubated first with the primary antibodies and then with peroxidase-conjugated anti-rabbit immunoglobulins; peroxidase was stained with 4-chloro-1-naphthol. The lanes were then cut into successive 1.5- or 2-mm strips perpendicular to the migration direction, to localize radioactivity. Each strip was placed in ACS II scintillation fluid and counted for ^{14}C radioactivity.

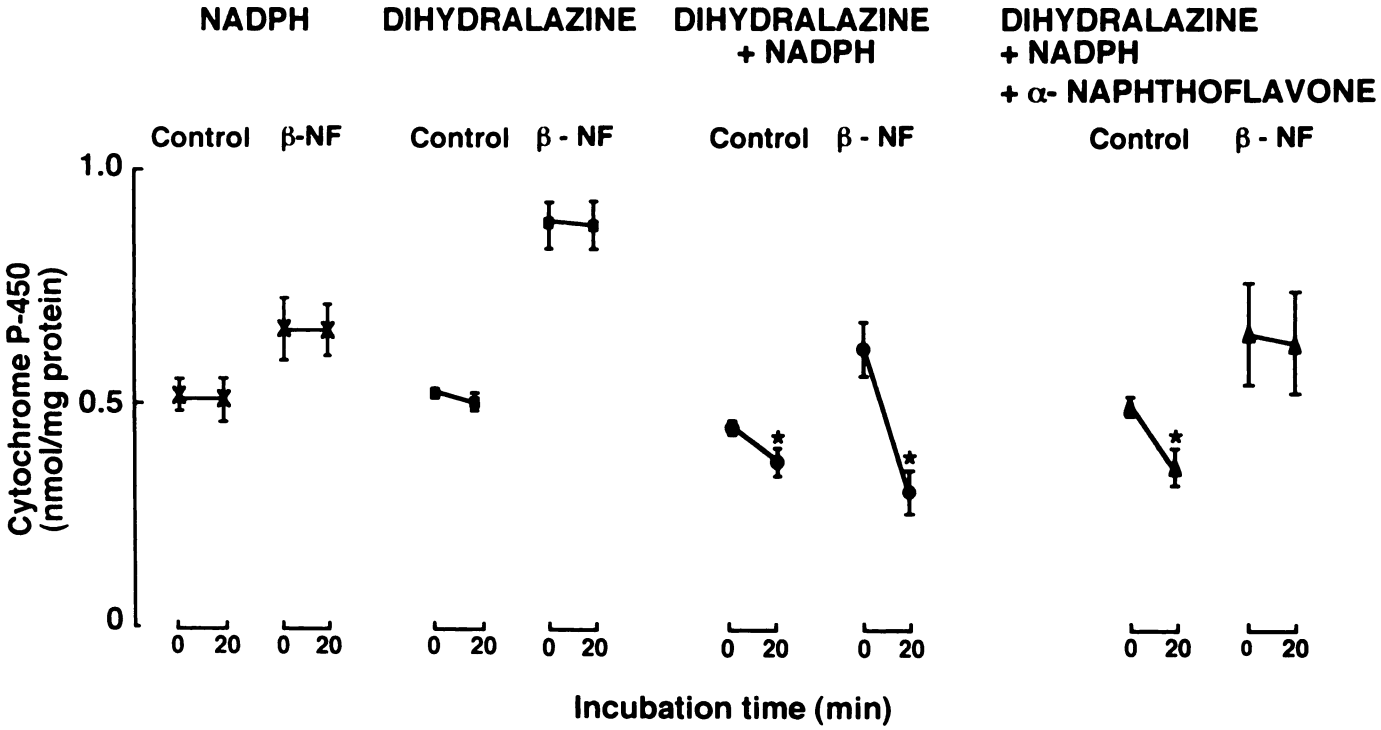

Fig. 3. *In vitro* loss of CO-binding P450 after incubation with dihydralazine and an NADPH-generating system. Microsomes from control rats and *β-naphthoflavone-treated (β-NF)* rats (2 mg of protein/ml) were incubated for 20 min at 37°, with or without dihydralazine (1 mM), an NADPH-generating system, and *α-naphthoflavone* (100 μM). Results are means ± standard errors for four experiments. *, Significantly different from the corresponding nonincubated samples, $p < 0.05$.

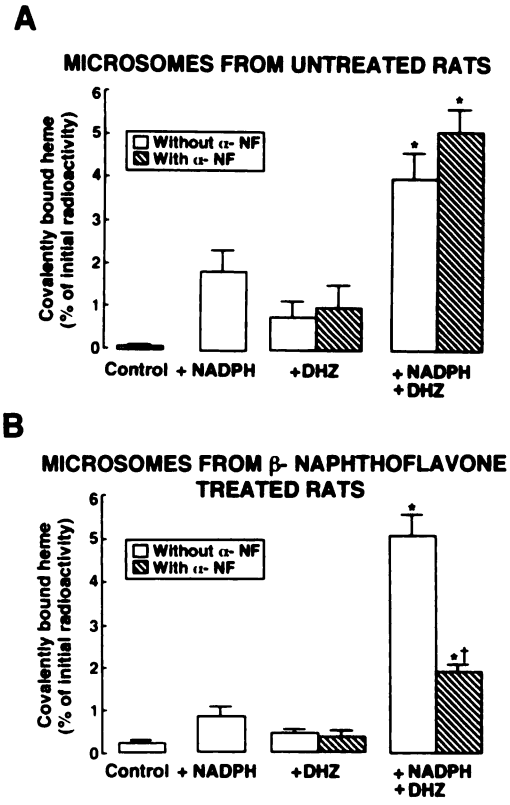

Fig. 4. *In vitro* covalent binding of ^{14}C-labeled heme to microsomal proteins. Untreated rats (A) or β-naphthoflavone-treated rats (B) received δ-amino[4-^{14}C]levulinic acid. Four hours later, animals were sacrificed and hepatic microsomes were prepared. The incubation mixture (3 ml) contained 12 mg of microsomal protein and 1.5 mM EDTA. In some flasks, dihydralazine (*DHZ*) (1 mM) and/or α-naphthoflavone (*α-NF*) (10 μM) was added. The reaction was initiated by the addition of NADPH (2 mM) and was carried out at 37° for 20 min. The radioactivity of ^{14}C-labeled heme covalently bound to microsomal proteins was expressed as the percentage of the total radioactivity initially present in the incubate. Results are means ± standard errors for five experiments. *, Significantly different from incubations with either NADPH alone or DHZ alone, $p < 0.01$. †, Significantly different from the incubation without α-NF, $p < 0.01$.

TABLE 2

Effect of pretreatment with β-naphthoflavone on the NADPH-independent and NADPH-dependent covalent binding of dihydralazine to rat liver microsomal proteins

[^{14}C]Dihydralazine (0.1 μCi, 100 μM) was incubated for 30 min with untreated or β-naphthoflavone-treated rat liver microsomes, and covalent binding to microsomal proteins was determined. Each value is the mean ± standard error for three incubations.

Incubation	Covalent binding
	pmol/min/mg of protein
Microsomes from untreated rats	
Without NADPH	37 ± 4
With NADPH	58 ± 2[a]
NADPH-dependent binding	21 ± 3
Microsomes from β-naphthoflavone-treated rats	
Without NADPH	39 ± 1
With NADPH	149 ± 4[b]
NADPH-dependent binding	110 ± 5

[a] Significantly different from incubation without NADPH, $p < 0.05$.
[b] Significantly different from incubation without NADPH, $p < 0.01$.

TABLE 3

Effect of various factors on the NADPH-dependent covalent binding of dihydralazine metabolites to hepatic microsomal proteins from β-naphthoflavone-treated rats

In the standard system, [^{14}C]dihydralazine (0.1 μCi, 100 μM) was incubated for 30 min with rat liver microsomes from β-naphthoflavone-treated rats, with or without an NADPH-generating system. The NADPH-dependent covalent binding to microsomal proteins was determined. In other flasks, either boiled microsomes were used or superoxide dismutase (SOD), catalase, or glutathione was added. Each value is the mean ± standard error of two or three incubations (*n*).

Incubation	NADPH-dependent covalent binding	
	pmol/min/mg of protein	
Standard system	110 ± 5	(*n* = 3)
With boiled microsomes	4 ± 3	(*n* = 3)
Standard system + SOD (0.3 mg/ml)	109; 113	(*n* = 2)
Standard system + catalase (0.3 mg/ml)	118; 120	(*n* = 2)
Standard system + glutathione (5 mM)	51; 47	(*n* = 2)

TABLE 4

Effect of various factors on the NADPH-dependent covalent binding of dihydralazine metabolites to human liver microsomal proteins

In the standard system, [^{14}C]dihydralazine (0.1 μCi, 100 μM) was incubated for 30 min with human liver microsomes (HL1004), with or without an NADPH-generating system. The NADPH-dependent covalent binding to microsomal proteins was determined. In other flasks, either boiled microsomes were used or superoxide dismutase (SOD), catalase, or glutathione was added. Values are means ± standard errors for two or three incubations (*n*).

Incubation	NADPH-dependent covalent binding	
	pmol/min/mg of protein	
Standard system	12 ± 1	(*n* = 3)
With boiled microsomes	<1	(*n* = 2)
Standard system + SOD (0.3 mg/ml)	12 ± 1	(*n* = 3)
Standard system + catalase (0.3 mg/ml)	11 ± 3	(*n* = 3)
Standard system + glutathione (5 mM)	6.7 ± 1[a]	(*n* = 3)

[a] Significantly different from the standard system, $p < 0.05$.

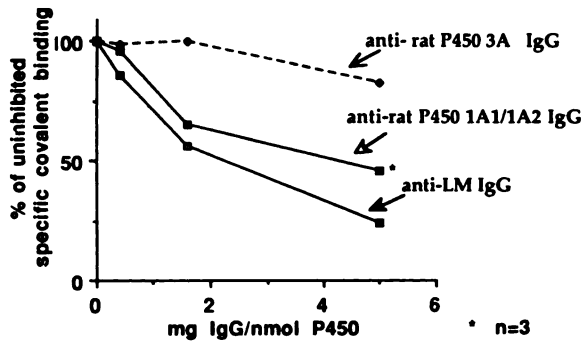

Fig. 5. Immunoinhibition of the NADPH-dependent covalent binding of [^{14}C]dihydralazine to microsomal proteins. Covalent binding to liver microsomes from β-naphthoflavone-treated rats was studied in the presence of [^{14}C]dihydralazine (100 μM), the NADPH-generating system, and anti-LM IgG (⊡), anti-rat P4501A1/1A2 IgG (■), or anti-rat P4503A IgG (◆). Control activity was 113 pmol of dihydralazine bound/min/mg of protein. Each value is the mean of two incubations except for one value (*), which is the mean of three incubations.

Results

Effects of dihydralazine on P450 and heme *in vivo*. Twenty-four hours after administration of a single dose of dihydralazine (100 mg/kg, intraperitoneally), there was a slight decrease in uncomplexed P450 (Table 1). The dihydralazine-induced loss of uncomplexed P450 was 0.18 nmol/mg of protein

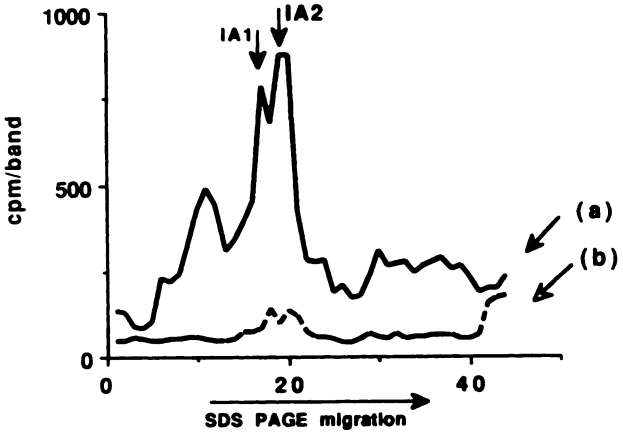

Fig. 6. Covalently bound radioactivity and immunoblot of microsomes from β-naphthoflavone-treated rats. Microsomal proteins (1 mg) were incubated with [^{14}C]dihydralazine (0.3 μCi, 0.3 mM) in the presence (*trace a*) or absence (*trace b*) of an NADPH-generating system. The final volume was 0.5 ml. Microsomal proteins from each incubation were loaded in four wells. After electrophoretic separation, proteins were electrotransferred to nitrocellulose and exposed to antibodies as indicated in Materials and Methods. A sheet corresponding to four lanes was cut every 1–1.5 mm and counted. *SDS PAGE*, sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Arrow IA2*, comigration with rat P4501A2. *Arrow IA1*, comigration with rat P4501A1.

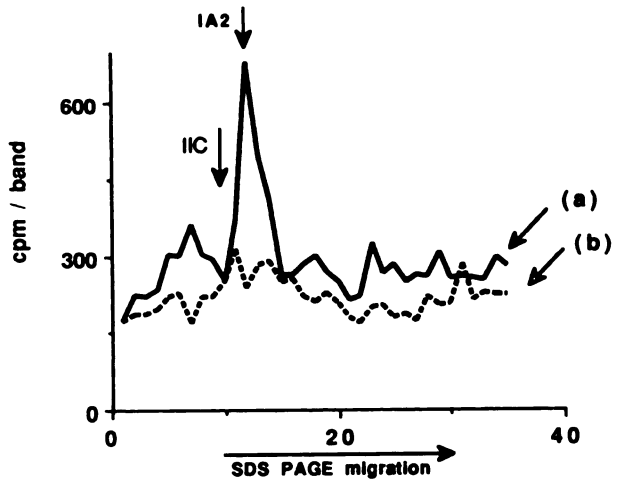

Fig. 7. Covalently bound radioactivity and immunoblot of human liver microsomes (HL1004). Microsomal proteins (1.5 mg) were incubated with [^{14}C]dihydralazine (0.3 μCi, 0.3 mM) in the presence (*trace a*) or absence (*trace b*) of an NADPH-generating system. The final volume was 0.5 ml. Microsomal proteins from each incubation were loaded into four wells. After electrophoretic separation, proteins were electrotransferred to nitrocellulose and exposed to antibodies as indicated in Materials and Methods. A sheet corresponding to four lanes was cut every 1–1.5 mm and counted. *SDS PAGE*, sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Arrow IA2*, comigration with human P4501A2. *Arrow IIC*, location of human P4502C.

in nonpretreated rats and 0.33 nmol/mg of protein in rats treated with β-naphthoflavone (Table 1), an inducer of P4501A (15). Different mechanisms appear to be responsible for the decrease in P450 in various groups of animals. In β-naphthoflavone-pretreated rats, the dihydralazine-induced loss in uncomplexed P450 was mostly due to the presence of a substantial amount (0.31 nmol/mg of protein) of 445-nm light-absorbing P450-Fe^{2+}-dihydralazine (metabolite) complexes that could not bind carbon monoxide (Table 1). Indeed, disruption of these complexes after exposure for 5 min to potassium ferricyanide significantly increased the CO-binding capacity of dithionite-reduced microsomes in rats treated with β-naphthoflavone and dihydralazine (Table 1). After addition of potassium ferricyanide, "total" P450 was then similar in microsomes from rats treated with both β-naphthoflavone and dihydralazine and in microsomes from rats treated with only β-naphthoflavone (Table 1). In nonpretreated rats receiving dihydralazine, no P450-Fe^{2+}-xenobiotic complexes were detected. All of these results indicated that P4501A participated in the formation of the P450-Fe^{2+}-dihydralazine (metabolite) complexes (Table 1). Dihydralazine administration did not significantly decrease microsomal heme in nonpretreated rats but significantly decreased it in β-naphthoflavone-pretreated rats (Table 1).

Binding spectra of dihydralazine with P450-Fe^{3+}. Dihydralazine (0.3 mM) gave a small type II binding spectrum with P450-Fe^{3+} from control rats. The amplitude of the binding spectrum was larger in microsomes from rats treated with β-naphthoflavone. This pretreatment increased the maximal amplitude (ΔA_{max} of 0.006 and 0.01 for untreated and β-naphthoflavone-treated rats, respectively), without modifying the apparent K_S for the binding of dihydralazine (0.02 and 0.03 mM for untreated and β-naphthoflavone-treated rats, respectively). These results also support the involvement of P4501A in the binding of dihydralazine, because pretreatment with β-naphthoflavone increased the amplitude of the binding spectrum.

***In vitro* formation of 445-nm light-absorbing P450-Fe^{2+}-NH_2—NH—R or P450-Fe^{2+}-NH=N—R complexes (with R being hydralazine) in the presence of dihydralazine and NADPH.** A 445-nm light-absorbing complex progressively developed upon incubation of microsomes with dihydralazine and NADPH (Fig. 2). The amplitude was usually maximal after 5 min of incubation. Whereas microsomes from untreated rats were virtually unable to form the 445-nm light-absorbing complexes, a marked formation of 445-nm light-absorbing complexes was observed with microsomes from β-naphthoflavone-treated rats, indicating the involvement of P4501A (Fig. 2). The *in vitro* formation of these complexes by microsomes from β-naphthoflavone-treated rats (Fig. 2) was reduced by the addition of α-naphthoflavone (100 μM), an inhibitor of P450s of the 1A subfamily (25), or by the addition of methoxsalen (8-methoxypsoralen) (250 μM), a suicide inhibitor of several P450s, including P4501A2 (26).

To test the effect of potassium ferricyanide on the stability of these complexes, the complexes were formed for 5 min as described above. A NADPH-consuming system of glutathione disulfide (1 mM) and glutathione reductase (3 units/ml) was then added. After 3 min, potassium ferricyanide (50 μM) was added. Five minutes later, recording of the 445-nm light-absorbing species showed a marked decrease in the complexes (data not shown).

***In vitro* loss of CO-binding capacity in microsomes incubated with dihydralazine and an NADPH-generating system.** The CO-binding capacity of P450 was not modified when microsomes were incubated with dihydralazine alone or with the NADPH-generating system alone (Fig. 3). However, when microsomes were incubated with both dihydralazine (1 mM) and the NADPH-generating system, a marked decrease in CO-binding occurred (Fig. 3). The loss of CO-binding P450 was about 21% in microsomes from control rats and 45% in microsomes from β-naphthoflavone-pretreated rats (Fig. 3). Addition of α-naphthoflavone (100 μM), a specific inhibitor of

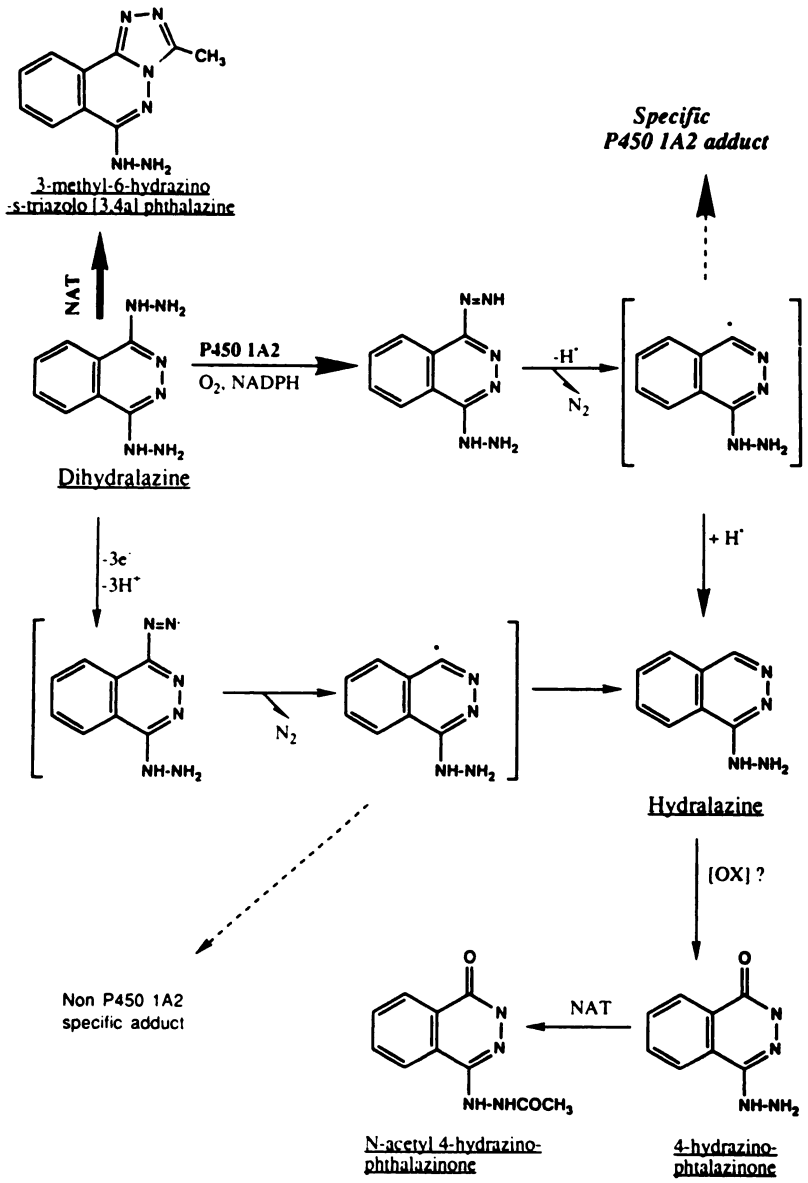

Fig. 8. Postulated metabolism of dihydralazine. All *underlined* metabolites were found in human urine samples (42, 43).

the P4501A subfamily, suppressed the loss of CO-binding P450 in microsomes from β-naphthoflavone-treated rats but not in microsomes from control rats (Fig. 3). Thus, the loss of CO binding also seems to be linked, at least in part, to P4501A.

***In vitro* irreversible binding of heme to microsomal proteins.** Rats received δ-amino[4-^{14}C]levulinic acid to label microsomal heme and were used to prepare hepatic microsomes. Little covalent binding of [^{14}C]heme to microsomal proteins occurred when microsomes were incubated with NADPH alone or dihydralazine alone (Fig. 4). After incubation with both NADPH and dihydralazine, however, about 4% of microsomal heme covalently bound to microsomal proteins in microsomes from untreated rats and 5% bound to microsomes from β-naphthoflavone-treated rats (Fig. 4). For comparison, the percentage of heme covalently bound to microsomal proteins in the presence of CCl_4 (5 mM) was 8.5 ± 1.0% and 3.3 ± 0.3% (mean ± standard error for six experiments) in microsomes from control and β-naphthoflavone-treated rats, respectively. Addition of α-naphthoflavone (10 μM) to the reaction mixture did not modify the covalent binding of heme induced by dihydralazine in microsomes from untreated-rats but decreased it markedly in microsomes from β-naphthoflavone-treated rats (Fig. 4). Thus, in β-naphthoflavone-treated rats, covalent binding of heme seemed to involve P4501A.

***In vitro* covalent binding of dihydralazine metabolites to liver microsomes.** When [^{14}C]dihydralazine was incubated with untreated rat liver microsomes and a NADPH-generating system, radioactivity covalently bound to microsomal proteins (Table 2). The amount of binding was significantly decreased in the absence of the NADPH-generating system, although the residual background binding was high (Table 2). This background binding was similar in microsomes from β-naphthoflavone-treated animals (Table 2). The NADPH-dependent covalent binding was increased 5-fold by pretreatment of rats with β-naphthoflavone (Table 2). This NADPH-dependent binding was nearly abolished when incubation was carried out with boiled microsomes, indicating a requirement for enzyme activity (Table 3). Addition of superoxide dismutase or catalase

had no effect (Table 3). Addition of 5 mM glutathione decreased the NADPH-dependent binding by >50% (Table 3).

Similarly, NADPH-dependent covalent binding of [^{14}C]dihydralazine (100 μM) occurred in all human liver microsomes tested; the mean ± standard error of covalent binding for six human livers was 14 ± 1 pmol/min/mg of protein (range, 11–20 pmol/min/mg of protein). This binding was abolished with boiled microsomes (Table 4). It was unchanged upon addition of either superoxide dismutase or catalase and was decreased 44% by 5 mM glutathione (Table 4).

Form of P450 involved in metabolic activation. The NADPH-dependent covalent binding of dihydralazine to β-naphthoflavone-treated rat liver microsomes was markedly inhibited by rabbit anti-rat P4501A1/2 IgG; at 5 mg of IgG/nmol of P450, immunoinhibition reached up to 50% (Fig. 5). Anti-LM IgG from a patient with dihydralazine-induced hepatitis inhibited this binding by >70% (Fig. 5). Anti-P4503A IgG, used as a control, was not inhibitory (Fig. 5). These immunoinhibition experiments were not carried out with human liver microsomes because the activity was too low to accurately detect inhibition.

Effect of *N*-acetyltransferase on NADPH-dependent covalent binding. The NADPH-dependent covalent binding of [^{14}C]dihydralazine to hepatic proteins of a rat liver homogenate from β-naphthoflavone-treated rats decreased 70% (two experiments) in the presence of 2 mM acetyl-CoA, the necessary cofactor for *N*-acetyltransferase.

Immunoblots of P450 and localization of covalently bound radioactivity. After incubation of microsomes from β-naphthoflavone-treated rats with [^{14}C]dihydralazine and NADPH, localization of the covalently bound radioactivity showed a peak in the region of P4501A2 and another peak in the region of P4501A1 (Fig. 6). No peak was found without the NADPH-generating system, and the background radioactivity was low (Fig. 6). When the investigation was performed with human liver microsomes, a very dominant peak of covalently bound radioactivity comigrated with P4501A2 (Fig. 7). No peak was observed in the region of P4502C (Fig. 7). No significant peak was observed when the incubation was carried out without the NADPH-generating system (Fig. 7), indicating that this specific covalent binding was NADPH dependent.

Discussion

The purpose of this study was to investigate the production and fate of dihydralazine-derived reactive metabolites and to interpret the role of the metabolites in the triggering of immunoallergic hepatitis caused by this drug. Results clearly demonstrated an interaction of dihydralazine or its metabolites with P450 heme. The first type of interaction with heme was the formation of a 445-nm light-absorbing P450-Fe^{2+}-drug (metabolite) complex. Indeed, administration of dihydralazine to β-naphthoflavone-treated rats decreased uncomplexed P450 and resulted in the *in vivo* formation of a 445-nm light-absorbing complex (Table 1). This complex was destroyed upon addition of potassium ferricyanide (Table 1), indicating a ferrous hydrazine (P450-Fe^{2+}-NH_2—NH—R) or ferrous diazene (P450-Fe^{2+}-NH═N—R) complex (27). Because P450-Fe^{2+}-NH_2—NH—R and P450-Fe^{2+}-NH═N—R complexes exhibit similar absorption spectra, with a λ_{max} located around 445 nm (27), they could not be distinguished from one another in the present results. Formation of a 445-nm light-absorbing P450-Fe^{2+}-drug (metabolite) complex also occurred *in vitro* upon incubation of rat liver microsomes with both dihydralazine and NADPH (Fig. 2). No 486-nm light-absorbing P450-Fe^{3+}—R complex was observed, possibly because of the instability of these complexes in the presence of O_2 (27). A second type of interaction with P450 heme involved the covalent binding of heme to microsomal proteins when rat liver microsomes were incubated with both dihydralazine and NADPH (Fig. 4). A similar effect was observed with various chemicals forming free radicals (28–32). The metabolism-based covalent binding of the heme prosthetic group to the apoprotein during the reductive debromination of $BrCCl_3$ by myoglobin has been attributed to the addition of the trichloromethyl free radical to a vinyl group of heme, followed by the reaction of the resulting radical heme species with an amino acid of the protein (31).

In the present study, the formation of P450-Fe^{2+}-dihydralazine (metabolite) complexes and the covalent binding of heme to the apoprotein caused a loss of CO-binding capacity when rat hepatic microsomes were incubated with both NADPH and dihydralazine (Fig. 3). All of these effects partly involved P4501A, because they were increased by *in vivo* pretreatment with β-naphthoflavone, a classical inducer of the P4501A subfamily in rats (15), and were decreased *in vitro* upon addition of α-naphthoflavone, a classical inhibitor of P4501A forms (25) (Figs. 1–3; Table 1).

The results also showed that the NADPH-dependent metabolism of dihydralazine yielded reactive metabolites that covalently bound to liver microsomal proteins. These reactive metabolites were produced by P450, because (a) covalent binding was NADPH dependent (Table 2), (b) the NADPH-dependent covalent binding was abolished by microsome boiling (Tables 3 and 4), (c) the NADPH-dependent binding was not dependent on reactive oxygen species, because catalase and superoxide dismutase had no effect (Tables 3 and 4), (d) the NADPH-dependent covalent binding was increased by induction by β-naphthoflavone, a P4501A inducer (Table 2), and (e) the binding was specifically inhibited by antibodies against rat P4501A or human (anti-LM) P4501A2 (Fig. 5). The latter observations further indicate a role of the P4501A subfamily in the production of the reactive metabolites. These metabolites appear to be electrophilic, because covalent binding was decreased 50% in the presence of 5 mM GSH (Tables 3 and 4). It is noteworthy that the NADPH-independent background level of covalent binding was quite high, compared with the NADPH-dependent binding. This was probably due to the capacity of hydrazine compounds to auto-oxidize and to spontaneously generate reactive metabolites in the presence of oxygen and metal ions (33, 34). It is interesting to note that this background did not increase when dihydralazine was incubated with microsomes from β-naphthoflavone-treated rats. These results support the existence of two oxidative pathways leading to reactive intermediates, one that is P450 and NADPH dependent and another that is P450 and NADPH independent and occurs spontaneously (in the presence of O_2 and metal ions). Human liver microsomes also produced reactive metabolites when incubated with both dihydralazine and NADPH, although at a lower level than did microsomes from untreated rats.

This study also showed that the NADPH-dependent reactive metabolites of dihydralazine mainly bound to proteins comigrating with P4501A (Figs. 6 and 7). Although exhaustion of the available sample of [^{14}C]dihydralazine precluded immuno-

precipitation experiments, these observations, taken together with other data, strongly suggested that the reactive metabolite bound mainly to the enzyme that formed it. (a) Induction and immunoinhibition experiments clearly showed that P4501A was mainly involved in the metabolic activation of dihydralazine. (b) In rats, which expressed both P4501A1 and P4501A2, two peaks of covalently bound radioactivity comigrating with P4501A1 and -1A2 were observed (Fig. 6). (c) In humans, who expressed only P4501A2, only one peak comigrating with P4501A2 was detected (Fig. 7). (d) Autoantibodies found in the sera of patients with dihydralazine-induced hepatitis recognized only one protein, namely human P4501A2, and not P4501A1 (12). Taken together, these observations support our initial hypothesis (Fig. 1) that the reactive metabolite is so highly reactive that it binds immediately within the active site of the enzyme that generated it (P4501A2 in humans) and this modified protein then serves as a neoantigen and triggers an abnormal immune response, leading to autoantibodies and immunoallergic hepatitis. Similarly, tienilic acid formed intermediates that bound selectively to the P4502C9 protein (35) and triggered autoantibodies directed against P4502C9 (5, 6).

The mechanism whereby the covalent modification of P450 may lead to anti-P450 autoantibodies remains unknown. As a hypothetical mechanism, it has been suggested that normally quiescent autoreactive B lymphocytes expressing a membrane immunoglobulin directed against a normal epitope of P450 may, after the death of an hepatocyte, recognize and internalize this P450 and present peptides derived from it on their HLA class II molecules (36, 37). P450 peptides modified by the covalent binding of the reactive metabolite ("modified self") might then be recognized by certain helper T cells, leading to the clonal expansion and maturation of the B lymphocyte and the secretion of an immunoglobulin with the same specificity as the initial membrane immunoglobulin, i.e., directed against a normal epitope of P450 (36, 37).

Because only a few subjects, however, develop autoantibodies and the disease, it is clear that some forms of individual susceptibility must also be involved. In the present study, all human microsomes tested generated reactive metabolites, suggesting that the idiosyncratic occurrence of dihydralazine-induced hepatitis cannot be explained on the basis of metabolic activation performed by only a few subjects. Dihydralazine-induced hepatitis occurs mainly in slow acetylators (38, 39), and in the present study covalent binding to the proteins of a whole homogenate decreased 70% when *N*-acetyltransferase was activated by the addition of its cofactor, acetyl-CoA. Thus, the unique susceptibility of slow acetylators might be explained by higher covalent binding in these subjects. However, because about 50% of Caucasians are slow acetylators, it is likely that other, still unknown, genetic factors, possibly involving the susceptibility to develop immunity, e.g., HLA molecules able to present a particular alkylated peptide (40), may also be required to explain the development of anti-P450 autoantibodies and hepatitis in a few subjects treated with dihydralazine.

In addition to dihydralazine and tienilic acid, several other drugs including chloramphenicol (41) and methoxsalen (26) covalently bind to the apoprotein of P450. It remains unknown whether these drugs can lead to anti-P450 autoantibodies in a few patients. It is likely, however, that both the nature of the haptenic group and the particular peptides on which the metabolite binds may modulate immunogenicity.

In conclusion, three pathways may be distinguished in the metabolism of dihydralazine (Fig. 8). The *N*-acetylation pathway diverts the drug away from two oxidative pathways, both of which form reactive metabolites as well as the stable metabolites hydralazine and 4-hydrazinophthalazinone (42, 43). One of these oxidative pathways is P450 independent and leads to nonspecific covalent binding. The second oxidative pathway is P4501A2 dependent and produces reactive metabolites that may bind either to the apoprotein or to the heme of P4501A2. P4501A2 modified by the covalent binding of the reactive metabolite (and/or heme) may serve as a neoantigen, triggering the immune response and the appearance of anti-LM autoantibodies in a few subjects. Because anti-LM autoantibodies recognize P4501A2 epitopes expressed on the outer surface of human hepatocyte plasma membrane (44), they may contribute to the immunological destruction of hepatocytes in this disease. These results are in good agreement with the hypothesis (11) proposed in Fig. 1.

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