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OXIDATIVE ACTIVATION OF THE THIOPHENE RING BY HEPATIC ENZYMES

HYDROXYLATION AND FORMATION OF ELECTROPHILIC METABOLITES DURING METABOLISM OF TIENILIC ACID AND ITS ISOMER BY RAT LIVER MICROSOMES

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Abstract—Tienilic acid (TA) is metabolized by liver microsomes from phenobarbital-treated rats in the presence of NADPH with the major formation of 5-hydroxytienilic acid (5-OHTA) which is derived from the regioselective hydroxylation of the thiophene ring of TA. During this *in vitro* metabolism of TA, reactive electrophilic intermediates which bind irreversibly to microsomal proteins are formed. 5-Hydroxylation of TA and activation of TA to reactive metabolites which covalently bind to proteins both required intact microsomes, NADPH and O_2 and are inhibited by metyrapone and SKF 525A, indicating that they are dependent on monooxygenases using cytochromes P-450. Microsomal oxidation of an isomer of tienilic acid (TAI) bearing the aroyl substituent on position 3 (instead of 2) of the thiophene ring also leads to reactive intermediates able to bind covalently to microsomal proteins. Covalent binding of TAI, as that of TA, depends on cytochrome P-450-dependent monooxygenases and is almost completely inhibited in the presence of sulfur containing nucleophiles such as glutathione, cysteine or cyteamine. These results show that 5-OHTA, which has been reported as the major metabolite of TA *in vivo* in humans, is formed by liver microsomes by a cytochrome P-450-dependent reaction. They also show that two thiophene derivatives, TA and TAI, bind to microsomal proteins after *in vitro* metabolic activation, TAI giving a much higher level of covalent binding than TA (about 5-fold higher) and a much higher covalent binding: stable metabolites ratio (4 instead of 0.5).

The metabolic activation of benzenic rings by cytochrome P-450-dependent monooxygenases is now well documented [1]. In many cases, their hydroxylation occurs with the intermediate formation of an electrophilic arene oxide metabolite which is able to covalently bind to cell macromolecules. Similar mechanisms occur during metabolic activation of the furan ring [2]. However, very little is known on the metabolism and metabolic activation of thiophene derivatives [3]. Thiophene metabolites only identified so far are thienyl mercapturic acids deriving possibly from reaction of glutathione with an intermediate thiophene 2,3-oxide [4]. As far as drugs containing thiophene rings are concerned, only data on their *in vivo* metabolism have been reported so far, and the formation of metabolites deriving from the hydroxylation of tienilic acid [5], suprofen [6], morantel [7], tiquizine [8], tenoxicam [9] and ticlopidine [10] has been described.

In order to determine the metabolic pathways responsible for the oxidation of thiophene rings and to know whether this oxidation occurs with formation of reactive metabolites, we have studied the oxidation of thiophene derivatives by liver microsomes. This paper reports data on the metabolism by rat liver microsomes of two thiophene compounds, tienilic acid (TA†), a natriuretic and uricosuric drug [11, 12] and its isomer, TAI, with the aroyl substituent in position 3 (instead of 2 in TA) of the thiophene ring. It shows that the 5-hydroxylation of TA found *in vivo* [5] also occurs in rat liver microsomes and is cytochrome P-450 dependent, it describes a simple assay to measure this activity, and reports that reactive metabolites derived from TA and its isomer bind irreversibly to microsomal proteins.

MATERIALS AND METHODS

Chemicals. All the chemical reagents were of the highest quality commercially available. Tienilic acid, [^{14}C]tienilic acid, 5-hydroxy-tienilic acid, the isomer of tienilic acid (TAI) and [^{14}C]TAI were given to us by laboratories Anphar-Rolland (Chilly-Mazarin, France). 5-Hydroxytienilic acid was prepared as described [5] and [^{14}C]TA and TAI (label in the keto group, 25 Ci/mol) were respectively prepared by CEA Saclay (France) [13] and by Amersham (Bucks, U.K.). HPLC analysis (see below) of [^{14}C]TA and [^{14}C]TAI showed that their radioactive purity was higher than 98%. SKF 525A (diethylaminoethyl 2,2-

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† Abbreviations: TA, tienilic acid; TAI, tienilic acid isomer; 5-OHTA, 5-hydroxy tienilic acid; SKF 525A, diethylaminoethyl 2,2-diphenyl valerate; TCPO, 3,3,3-trichloropropene oxide; GC-MS, gas chromatography coupled to mass spectrometry.

diphenylvalerate) was a gift of Dr Fort (SKF Laboratories, France).

Rat liver microsomes. Male Sprague–Dawley rats (Iffa Credo, France) were treated by phenobarbital (1 g/L in drinking water) for 5 days, fasted overnight and killed by cervical dislocation. Livers were removed, perfused with saline and microsomes prepared as described previously [14]. Their cytochrome P-450 content was determined by the method of Omura and Sato [15] and protein concentrations were measured by the method of Lowry *et al.* [16].

Microsomal oxidation of tienilic acid (or its isomer TAI). Incubations were made in glass tubes in a final volume of 150 μL except for measurements as a function of time where the final volume was 1 mL.

Microsomes (0.05–0.2 mg protein) were suspended in 100 μL sodium phosphate buffer 0.1 M, pH 7.4. Incubation was started by adding 50 μL of the same buffer containing a NADPH generating system (0.15 μmol of NADP, 1.5 μmol of glucose-6-phosphate and 0.2 units of glucose-6-phosphate dehydrogenase) and the radioactive substrate (15 nmol of TA or TAI, 5–25 Ci/mol). After 5–30 min incubation at 37°, a 50-μL aliquot was taken for covalent binding measurements. Then 150 μL of methanol containing 3 nmol of unlabeled 5-OHTA was added to the incubation mixture to stop the reaction. After centrifugation at 2000 *g* for 5 min, the supernatant was analysed by HPLC.

HPLC analysis of TA metabolites. Formation of 5-hydroxytienilic acid was quantified by reverse-phase HPLC on an Altex Ultrasphere ODS column (150 × 4.6 mm) eluted at 1 mL/min by a gradient from 0.1 M ammonium acetate buffer (pH 4.6) to 50% CH_3CN in H_2O in 20 min with UV detection at 270 or 310 nm. Fractions were collected in 3-mL propylene tubes every 0.5 min and radioactivity was quantified after addition of 2 mL of Picofluor 30 (Packard) in a Packard Tricarb 300 scintillation counter with external standard quench correction. The amount of 5-hydroxytienilic acid was computed by summing the radioactivity under the UV peak.

Identification of the major metabolite of TA. In order to confirm the 5-OHTA structure of the major metabolite of TA, supernatants from microsomal incubates, to which unlabeled 5-OHTA was not added, were studied by HPLC by using three other columns and different solvent systems: a μ Bondapak C_{18} column (Waters, 300 × 3.9 mm) eluted by a gradient using sodium phosphate 0.1 M pH 7 and methanol (from 0 to 60% in 30 min), a C_8MOS Hypersil (250 × 4.6 mm) column and a Spherisorb ODS2 (150 × 4.6 mm) column with gradients using 0.1 M ammonium acetate pH 4.6 and 50% CH_3CN in H_2O (with various profiles). In every case, the major metabolite (UV and radioactivity detection) comigrated with authentic 5-OHTA.

Identification of the major metabolite of TA was confirmed by GC-MS according to a previously described procedure [5]. This metabolite was separated by HPLC from a 3 mL incubation experiment, extracted by ether after acidification of the medium and immediately treated by diazomethane in ether and subjected to a gas chromatography-mass spectrometry analysis using a Nermag R10-10 apparatus and a CPSil 5CB column (25 m × 0.32 mm) at 280°. The GC peak retention time and mass spectrum (both in electronic impact and chemical ionization (NH_3) modes) of the methylated metabolite were identical to those of methylated 5-OHTA [5].

In vitro *covalent binding studies.* These measurements were done according to previously described procedures [17, 18].

The proteins of aliquots of incubation mixtures (50 μL) were precipitated on glass fiber filter disks (Whatman GF/B) by dipping them in 10% trichloroacetic acid. Proteins entrapped in the filter paper disk were washed twice by methanol and then by ethylacetate. We checked that further washings with methanol and ethyl acetate did not modify the radioactivity level of the disk.

After solvent evaporation at 60°, proteins entrapped in the disk were put in scintillation vials, 2 mL of "toluene scintillator" (Packard) were added and radioactivity bound to proteins was counted in a Packard Tricarb-300 scintillation counter.

RESULTS

Microsomal oxidation of TA: formation of 5-hydroxy-TA as a major metabolite and covalent binding of TA metabolites to microsomal proteins

Incubation of radioactive TA (0.1 mM, ^{14}C on the keto group) with liver microsomes from phenobarbital-pretreated rats in the presence of NADPH (or a NADPH-generating system) led to the formation of 5-OHTA as a major metabolite as well as of more polar metabolites in lower amounts. This was shown after HPLC analysis of the crude reaction mixture using either a UV-visible or a radioactivity detection (Fig. 1). Identification of 5-OHTA as a metabolite was done by comparison with an authentic sample using four different HPLC columns and several different solvent systems (see Materials and Methods). It was confirmed by isolation of the metabolite after separation by HPLC, methylation by diazomethane and analysis by GC-MS according to a previously described procedure [5]. As it has previously been found that 5-OHTA was relatively unstable under acidic conditions and reacted easily with aldehydes or ketones [5], a reverse phase HPLC technique with elution by a weak acid after dilution of radiolabeled 5-OHTA with carrier non-radiolabeled 5-OHTA was used to study microsomal oxidation of TA. HPLC analysis must be done as soon as possible after the incubation, as the recovery of 5-OHTA was found to decrease when the analysis was done after several days of storage of the incubates even at −20° (50% decrease after 4 days). As shown in Fig. 2A, formation of 5-OHTA was only linear during the first 5 min of the incubation and did not increase after 20 min. Formation of 5-OHTA increased linearly when the amount of microsomal proteins increased from 0.6 to 2 mg protein per mL incubate (Fig. 2B). This formation increased with TA concentration and reached a maximum value around 80 μM (Fig. 2C).

During incubations of TA with rat liver microsomes and NADPH, irreversible binding of reactive metabolites of TA to microsomal proteins occurred. It linearly increased as a function of time for about

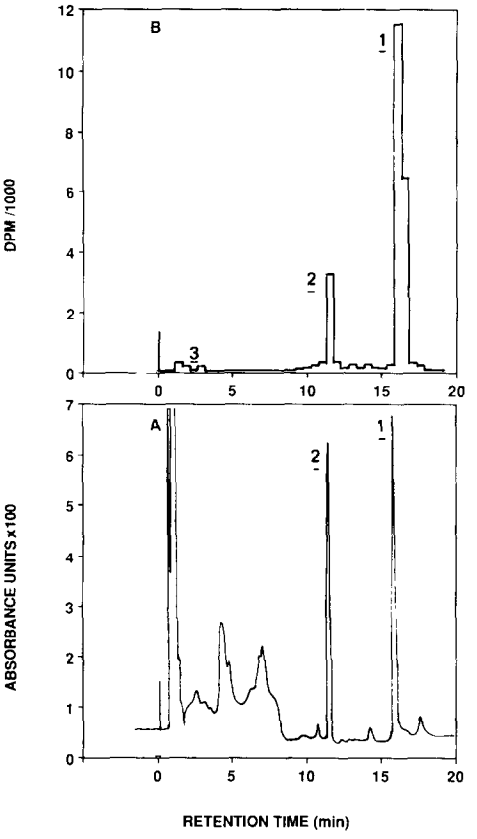

Fig. 1. Reverse-phase HPLC profile of microsomal metabolites of radiocative tienilic acid. (A) UV detection at 270 nm. (B) Radioactivity detection (dpm per fraction of 0.5 min). Analysis done after 20 min incubation of 10 μM [^{14}C]TA with 0.7 mg microsomal liver protein (from phenobarbital pretreated rats) per mL. (1) TA, (2) 5-OHTA, (3) polar metabolites (with a retention time corresponding to the diacid metabolite of TA detected *in vivo* [5]). The compounds with the retention times between 2 and 8 min and detected at 270 nm were also observed in incubations without TA.

10 min and as a function of protein concentration between 0.6 and 3 mg protein per mL (Fig. 3).

Radioactive measurements showed that the sum of unchanged TA and its metabolites either detected by HPLC or bound to microsomal proteins corresponded to 95 ± 5% of starting TA.

Effects of various factors on the formation of 5-OHTA and on the irreversible binding of TA metabolites to microsomal proteins

As shown in Table 1, the formation of 5-OHTA as well as the activation of TA to metabolites able to bind irreversibly to microsomal proteins were both enzymatic and dependent on NADPH and O_2 as cofactors since both activities almost disappeared when boiled microsomes, no NADPH or anaerobic conditions were used. Classical inhibitors of microsomal cytochromes P-450 such as metyrapone and SKF 525A led to a 50% inhibition of both phenomena already at a 10 μM concentration. α-Naphtoflavone was almost without effect at this concentration but led to a 50% inhibition of 5-OHTA formation and of covalent binding of TA metabolites to microsomal proteins at a 100 μM concentration. Addition of NADH to the complete incubation system containing NADPH led to a 50% increase of the hydroxylating activity as previously found for many other microsomal monooxygenase activities.

On the contrary, heating of microsomes for 5 min at 45° which was reported to inactivate microsomal flavine monooxygenases [19] was without effect on TA oxidation. 3,3,3-Trichloropropene oxide (TCPO, 0.5 mM), a classical inhibitor of microsomal epoxide hydrolase, exhibited no inhibitory effect.

The presence of strong nucleophiles such as glutathione, cysteine or cysteamine in the incubation medium led to a dramatic decrease of the irreversible binding of TA metabolites to proteins (Table 1). In particular, this irreversible binding was almost totally abolished in the presence of 6 mM cysteamine or cysteine. However, these sulfur nucleophiles did not inhibit 5-OHTA formation, and, on the contrary, a clear increase of this activity was observed in the presence of glutathione.

Irreversible binding of reactive metabolites of an isomer of TA to microsomal proteins

The ability of the isomer of tienilic acid (TAI) with the keto group on position 3 of the thiophene ring (Fig. 4) to form reactive metabolites upon oxidation by rat liver microsomes was also studied. Incubation of radioactive TAI (^{14}C on the Keto group) with liver microsomes in the presence of NADPH and O_2 was performed under conditions identical to those used previously in the case of TA. As shown in Fig. 5, microsomal oxidation of TAI led to a much higher level of covalent binding to proteins than the oxidation of TA (12 nmol bound per nmol P-450 after 20 min instead of about 2.5 in the case of TA). Covalent binding of TAI metabolites exhibited characteristics similar to that of TA metabolites (Fig. 6). It was almost abolished in the absence of NADPH or when using boiled microsomes, did not seem to depend on a flavine monooxygenase as it was not decreased after heating microsomes at 45° for 5 min, but was clearly decreased in the presence of classical inhibitors of cytochrome P-450-dependent monooxygenases, such as metyrapone and SKF 525A. This covalent binding was greatly reduced if the incubations were done in the presence of good sulfur-containing nucleophiles such as glutathione, cysteine and cysteamine (Fig. 6). Inhibition of covalent binding by glutathione was maximum already at 0.5 mM, and the best inhibitory effect was obtained with 6 mM cysteine.

Comparison of the metabolic activations of TA and its isomer by rat liver microsomes

Table 2 compares the fates of TA and TAI in rat liver microsomes that were deduced from the aforementioned experiments and in particular from the radioactivity balance. 5-Hydroxylation of TA represented about 65% of its total biotransformation. Its activation into reactive metabolites responsible for covalent binding to proteins accounted for nearly 30%. Its oxidation into polar metabolites (see Fig. 1B) such as the diacid [4-carboxy-2,3-

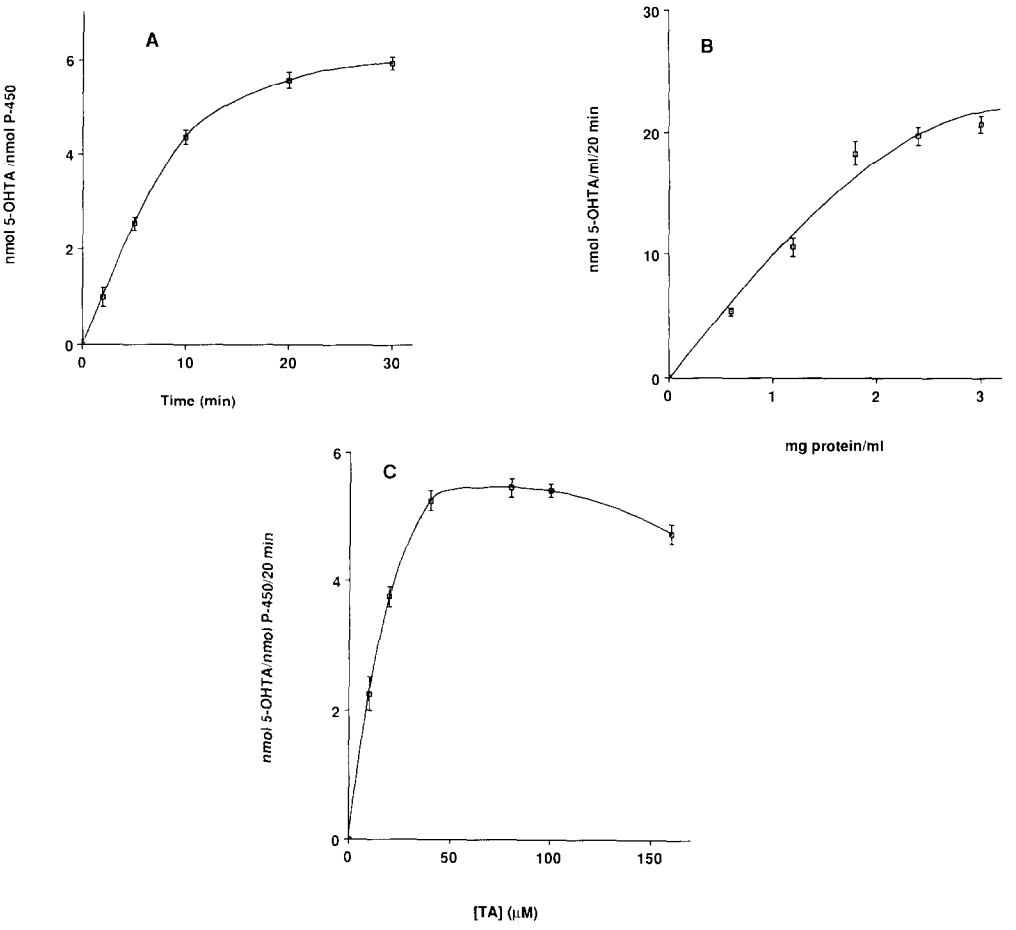

Fig. 2. Formation of 5-hydroxytienilic acid by liver microsomes from phenobarbital treated rats, as a function of time (A), protein concentration (B) and tienilic acid concentration (C). (A) Time course of 5-OH TA formation with 100 μM TA and 0.7 mg protein per mL (2.5 nmol P-450 per mg protein). (B) 5-OH TA formation as a function of protein concentration (1.8 nmol P-450 per mg protein). (C) 5-OH TA Formation as a function of TA concentration (0.7 mg protein per mL: 1.95 nmol P-450 per mg protein). Each point of the curves was the mean value from two experiments.

dichlorophenoxy]acetic acid, which was detected *in vivo* in rat and man urine [5, 12], was a minor pathway (3%). In the case of TAI, covalent binding became a major reaction accounting for about 80% of the total metabolism of this compound. TAI also led to several stable metabolites which were observed in HPLC under conditions identical to those used for the study of microsomal oxidation of TA (Fig. 1). These TAI metabolites had retention times similar to 5-OHTA (data not shown) but have not been characterized so far. These metabolites only represented 18% of the total fate of TAI (Table 2).

DISCUSSION

The aforementioned results show that 5-hydroxylation of the thiophene ring of tienilic acid is by far the major route of metabolism of TA in rat liver microsomes, as it was the case *in vivo* in rat and man [5]. Thus, rat liver microsomes appear as a satisfactory model to investigate the mechanisms of metabolic oxidation of TA at the molecular level.

The absolute requirement of undenaturated microsomes and of NADPH and O_2 for 5-hydroxylation to occur indicate that this activity is dependent on a monooxygenase. The lack of effect of heating microsomes at 45° for 5 min under conditions which have been reported to inactivate microsomal flavoprotein-dependent monoxygenases, and the clear inhibitory effects of classical inhibitors of cytochrome P-450-dependent monooxygenases such as metyrapone or SKF-525A (Table 1), show that 5-hydroxylation of TA is catalysed by the latter enzymes.

During its metabolic oxidation, TA is also transformed into reactive metabolites which irreversibly bind to microsomal proteins. Under the conditions employed, the ratio between activity followed by covalent binding and 5-hydroxylation is about 0.5 (Table 2). These two reactions exhibit very similar characteristics, since activation of TA into metabolites able to bind to microsomal proteins is also dependent on NADPH and O_2, not affected by heating microsomes at 45° and markedly inhibited by metyrapone or SKF 525A, in a manner very similar to 5-hydroxylation of TA.

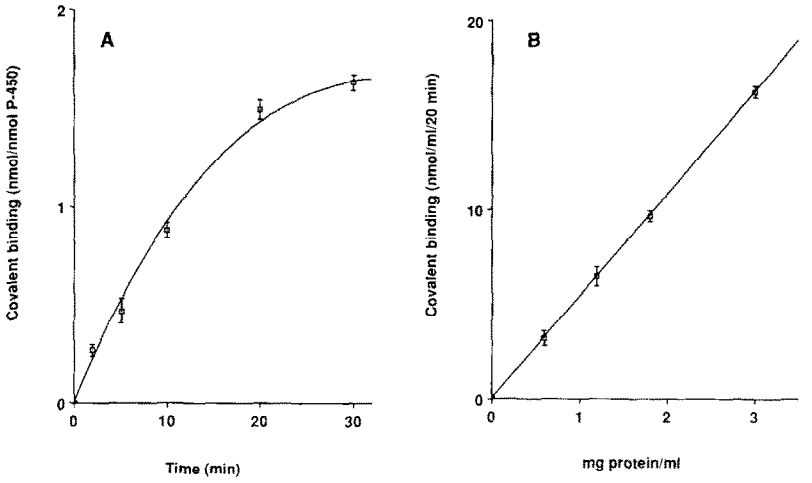

Fig. 3. Covalent binding of TA metabolites to microsomal proteins as a function of the incubation time (A) and protein concentration (B). Covalent binding was measured as indicated in Materials and Methods, from the experiments described in Fig. 2A and B. Non-specific covalent binding was measured on identical incubations performed with boiled microsomes between 0 and 20 min. It did not vary significantly with time and corresponded to about 4% of the total covalent binding (specific + non-specific) measured at 20 min. The values shown on the curves were those corresponding to the specific covalent binding.

Table 1. Effects of various factors on the metabolism of TA by liver microsomes from phenobarbital treated rats

	5-OHTA formation (% of control)	Covalent binding
Complete system*	100%	100%
Boiled microsomes†	0 ± 2	1 ± 2
Microsomes heated 5 min at 45°	102 ± 6	104 ± 5
−NADPH‡	2 ± 2	3 ± 2
+NADH (1 mM)	140 ± 20	150 ± 10
$-O_2$	3 ± 2	
+Metyrapone (0.01 mM)	46 ± 6	50 ± 6
+Metyrapone (0.1 mM)	45 ± 6	43 ± 7
+SKF 525A (0.01 mM)	56 ± 5	57 ± 6
+SKF 525A (0.1 mM)	54 ± 4	19 ± 8
+α-Naphthoflavone (0.01 mM)	88 ± 5	87 ± 8
+α-Naphthoflavone (0.1 mM)	42 ± 5	48 ± 7
+TCPO (0.5 mM)§	98 ± 6	104 ± 5
+Glutathione 6 mM‖	177 ± 30	17 ± 5
+Cysteine 6 mM‖	80 ± 5	7 ± 2
+Cysteamine 6 mM‖	115 ± 10	5 ± 2

* Control 20 min incubations were performed with a complete system using 100 μM TA, 0.7 mg microsomal protein per mL (3.3 nmol P-450 per mg protein) and a NADPH generating system as described in Materials and Methods. One hundred per cent control value for 5-hydroxylation of TA was 5.9 nmol 5-OHTA formed per nmol P-450 per 20 min. One hundred per cent control value for covalent binding of TA metabolites to microsomal proteins was 2.5 nmol bound per nmol P-450 per 20 min.

† Microsomes heated 5 min at 100°.

‡ Complete system without the NADPH-generating system.

§ TCPO = 3,3,3-trichloro-propeneoxide.

‖ One hundred per cent control values for the last three experiments were 4.4 nmol 5-OHTA formed per nmol P-450 per 20 min and 1.5 nmol covalently bound per nmol P-450 per 20 min (microsomes containing 2.6 nmol P-450 per mg protein were used). Results are means ± SE from four determinations.

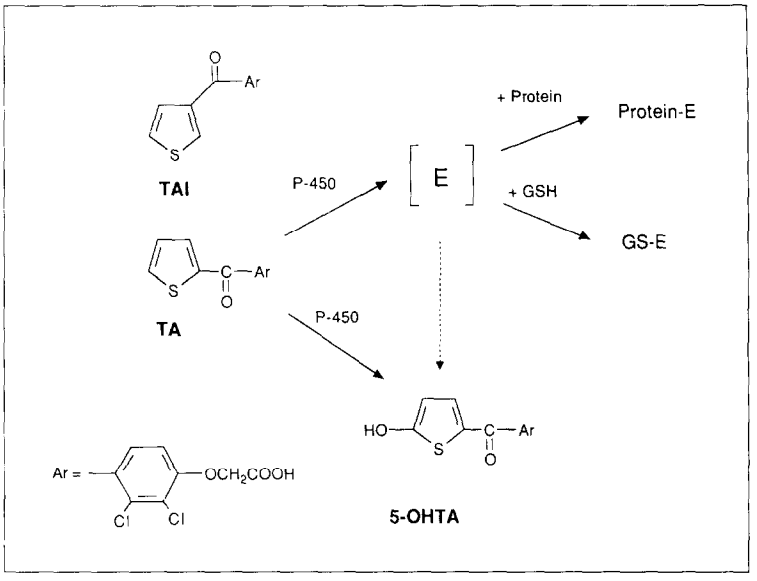

Fig. 4. Schematic view of the metabolic activation of tienilic acid.

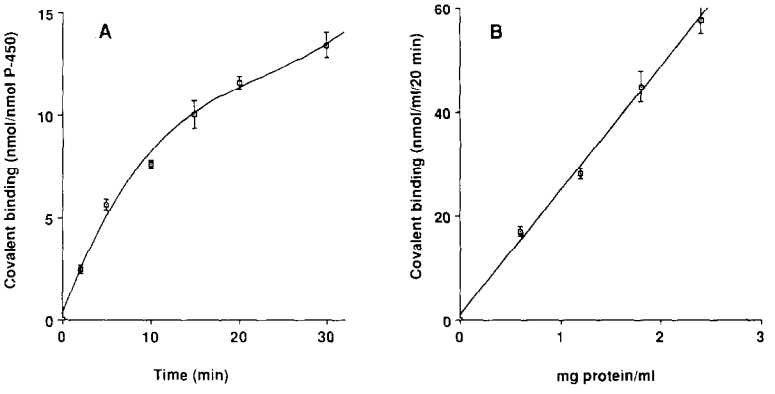

Fig. 5. Covalent binding of TAI metabolites to microsomal proteins as a function of the incubation time (A) and protein concentration (B). Conditions identical to those of Fig. 3 but with TAI instead of TA. Percentage of the non-specific covalent binding, not varying with incubation time (see Fig. 3), was about 5%.

This indicates that 5-OHTA and reactive metabolites of TA able to bind irreversibly to microsomal proteins are all formed by cytochrome P-450-catalysed oxidation of TA. 5-OHTA and reactive metabolites could either be formed by distinct reactions or derive from a common reactive intermediate (Fig. 4). More work is needed to differentiate between these mechanistic pathways. These reactive metabolites (or intermediates) must be electrophilic in nature since they are efficiently trapped by nucleophiles added to the incubation medium and containing a sulfur atom, such as glutathione, cysteine or cysteamine (Table 1). The mechanistic scheme of Fig. 4 could explain the different effects of 100 μM SKF 525A on the hydroxylation of TA (50% inhibition) and on the covalent binding of TA metabolites (80% inhibition, Table 1) since SKF 525A contains a tertiary amine function and could act not only as an inhibitor of cytochromes P-450 but also as a nucleophile able to trap the electrophilic metabolites. It could also explain the lack of linearity of 5-OHTA formation as a function of time already after 5 min (Fig. 2), since irreversible reactions of electrophilic metabolites of TA with components of the monooxygenase system involved in TA oxidation could result in their partial inactivation. Accordingly, microsomal incubations of TA in the presence of glutathione lead to a formation of 5-OHTA which remains linear for longer times (at least 10 min, data not shown) and to higher final amounts of 5-OHTA (Table 1).

The isomer of TA bearing the keto group on position 3 of the thiophene ring is also oxidized by liver microsomes with formation of electrophilic metabolites which bind to microsomal proteins. Metabolic oxidation of TAI gives a much higher level

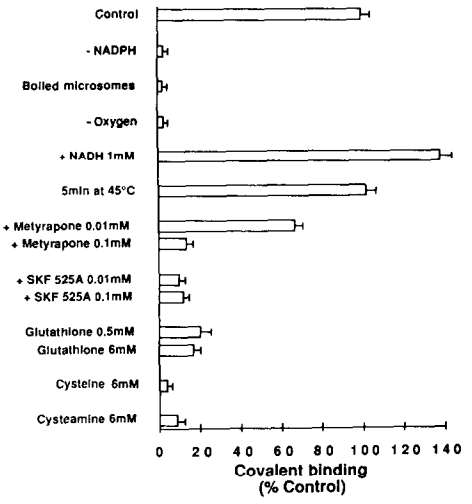

Fig. 6. Effects of various factors on the covalent binding of TAI metabolites on liver microsomal proteins from phenobarbital-treated rats. Control (20 min) incubations were performed with the complete system described in Materials and Methods. Microsomes contained 2.5 nmol P-450 per mg protein. Level of covalent binding for control incubations: 11.9 nmol covalently bound per nmol P-450 per 20 min. Mean values ± SE from four experiments.

Table 2. Fate of radioactive TA and TAI upon oxidative metabolism by rat liver microsomes

	Fate of TA (nmol/nmol P-450/20 min)	Fate of TAI
5-OHTA (or stable metabolites of TAI)	4.9 ± 1.5	2.7 ± 1.2
Polar metabolites	0.26 ± 0.07	0.3 ± 0.05
Covalent binding	2.5 ± 0.8	11.9 ± 1.5

Values are ± SE from seven experiments in duplicate using five different preparations of microsomes from phenobarbital-treated rats.

Conditions as in Materials and Methods using 100 μM TA or TAI and 0.7 mg protein per mL (1.8 to 2.4 nmol P-450 per mg protein).

of covalent binding than that of TA itself (5-fold higher). Contrary to TA, TAI does not give a major stable metabolite amounting for at least 60% of its total biotransformation. Finally, the ratio between the amounts of stable metabolites and of reactive metabolites leading to an irreversible binding to proteins observed with TAI (0.25) is very different from that observed with TA [2] (Table 2).

Metabolic activation of TAI leading to its irreversible binding to microsomal proteins exhibits qualitative characteristics very similar to those observed in the case of TA. It seems also derived from electrophilic metabolites formed by cytochromes P-450-dependent monooxygenases.

This is the third example of thiophene derivatives (with TA and thiophene itself [4]) which is found to be metabolically oxidized with formation of electrophilic metabolites. More work is needed to determine the nature of these electrophilic reactive intermediates (or metabolites) and to understand their reactions with microsomal proteins.

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