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SELF-INDUCTION BY TRIACETYLOLEANDOMYCIN OF ITS OWN TRANSFORMATION INTO A METABOLITE FORMING A STABLE 456 nm-ABSORBING COMPLEX WITH CYTOCHROME P-450

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Abstract—Triacetyloleandomycin (TAO), a macrolide antibiotic containing a tertiary amine function, $-N(CH_3)_2$, gave a small type I binding spectrum with, and was slightly demethylated by, microsomes from control rats. No detectable 456 nm-absorbing complex was formed *in vitro* upon incubation of control microsomes with TAO and NADPH; no complex formed *in vivo* could be detected in microsomes from rats killed 1 hr after a single dose of TAO, 1 mmole/kg i.p. Repeated administration of TAO, 1 mmole/kg i.p. daily for 4 days increased the liver weight/body weight ratio, hepatic microsomal protein concentration, NADPH–cytochrome *c* reductase activity, and the amplitude of the TAO type I binding spectrum but did not change the CO-binding spectrum of dithionite-reduced microsomes or the activity of TAO demethylase. Microsomes isolated from rats treated with repeated doses of TAO exhibited an enormous absorption peak at 456 nm; the absorption at 456 nm was slightly increased upon incubation with TAO and NADPH; the absorption peak at 456 nm disappeared upon treatment of the microsomes with 50 μM potassium ferricyanide. After disruption of the complex by potassium ferricyanide, cytochrome P-450 in TAO-treated rats was increased by 260% above that in control microsomes; the amplitude of the TAO-type I binding spectrum was increased by 4500% and TAO-demethylase activity was increased by 670%. It is concluded that TAO induces its own transformation into a metabolite which forms a stable complex with the iron (II) of reduced cytochrome P-450.

Triacetyloleandomycin (TAO) is commonly used as an antibiotic in humans. Both in humans [1] and in rats [2] administration of TAO produces ultrastructural changes of the liver suggesting microsomal enzyme induction. However, several drug interactions observed in humans [3–7] suggest that TAO administration decreases the hepatic metabolism of other drugs.

The chemical structure of TAO involves a tertiary amine function [8]. Several secondary or tertiary amines [9–19] are dealkylated and oxidized by cytochrome P-450 into metabolites, probably nitrosoalkanes [20–23], which form stable complexes with the iron (II) of reduced cytochrome P-450. These stable complexes are formed *in vitro* in the presence of the parent amines, NADPH, and oxygen, but may also be formed *in vivo* [14]. Once formed, nitrogenous complexes may hold cytochrome P-450 in the ferrous state; nitrogenous complexes formed *in vivo* may persist in isolated microsomes where they can be directly demonstrated without prior addition of dithionite [14]. The formation of these stable complexes has 3 main consequences [14]: (a) it produces a Soret peak around 455 nm; (b) it prevents the fixation of CO on cytochrome P-450 and therefore reduces the CO-difference spectrum of dithionite-reduced microsomes; (c) it also prevents the fixation of O_2 and blocks cytochrome P-450 activity. Nitrogenous complexes are unstable in the ferric state; treatment of the microsomes with potassium ferricyanide oxidizes the iron to the ferric state, displaces the metabolite from its complex, regenerates native cytochrome P-450 and restores its CO-binding capacity and activity [14]. The formation of stable complexes is increased by phenobarbital pretreatment [14]. Compounds transformed into complex-forming metabolites, such as SKF 525-A, may induce microsomal enzymes [10] and might increase their own transformation into complex-forming metabolites.

In this communication, we present evidence that TAO induces its own transformation into a metabolite which forms a stable complex with reduced cytochrome P-450. In another communication [24], we report that TAO administration decreases cytochrome P-450 activity with other substrates.

MATERIALS AND METHODS

Animals, chemicals and treatments. Male Sprague–Dawley rats, weighing 180–200 g, were purchased from Charles River France (St-Aubin les Elbeuf, France). Animals were allowed food (Autoclavé 113, UAR, France) and water *ad lib.* TAO

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was a generous gift from Pfizer France, Orsay, France. TAO, 0.062–1 mmole/kg was administered i.p. or p.o. in 5 ml/kg of HCl (final pH, 2.35); control rats received HCl i.p. or p.o., pH 2.35. Rats were used 1 hr or 24 hr after a single dose and 24 hr after the last of 4 daily doses.

Drug-metabolizing enzymes. Rats were stunned and the liver was removed. Liver fragments were minced and homogenized in 3 vol. 0.154 M KCl, 0.01 M sodium–potassium phosphate buffer, pH 7.4. The liver homogenate was centrifuged at 10,000 *g* and the 10,000 *g* supernatant was centrifuged at 100,000 *g*. Microsomal pellets were resuspended in the same buffer and centrifuged again at 100,000 *g*. Washed hepatic microsomes were used immediately after their preparation.

Hepatic microsomal protein concentration and NADPH–cytochrome *c* reductase activity were measured as previously reported [25]. Hepatic microsomal cytochrome P-450 concentration was measured as the CO-difference spectrum of dithionite-reduced microsomes as described by Omura and Sato [26]; the same technique was repeated after first adding 50 μM potassium ferricyanide to another batch of microsomes.

Metabolism of TAO. The binding spectrum of TAO with hepatic cytochrome P-450 was measured with a microsomal suspension adjusted to 2 mg microsomal protein/ml. TAO dissolved in methanol (25 nmoles/μl) was added to 2.5 ml of the microsomal suspension in the test cuvette while the same volume of methanol was added to the reference cuvette. The binding spectrum was recorded from 330 to 510 nm on an Aminco DW_2 spectrophotometer. The same technique was repeated after first adding 50 μM potassium ferricyanide to another batch of microsomes.

The demethylation of TAO was measured by a technique identical to that used for measuring the demethylation of ethylmorphine [25] but with 0.3 mM TAO as the substrate; TAO was added in 25 μl of ethanol to the test flasks while 25 μl of ethanol were added to the blank flasks; blank and test flasks were incubated for 15 min and the amount of formaldehyde formed was measured with the Nash reagent; this technique was performed both with untreated microsomes and with microsomes treated with 50 μM potassium ferricyanide.

The *in vivo* formation of complexes absorbing around 455 nm was measured with microsomal suspensions adjusted to 2 mg microsomal protein/ml; microsomes from control rats were placed in the reference cuvette while microsomes from TAO-treated rats were placed in the test cuvette; the difference spectrum was recorded from 380 nm to 500 nm in an Aminco DW_2 spectrophotometer.

The *in vitro* formation of complexes absorbing around 455 nm was measured with a microsomal suspension containing 2 mg microsomal protein/ml. The same microsomal suspension was placed in both cuvettes, 0.3 mM TAO was added to the test cuvette, and 1 mM NADPH was added to both cuvettes. The difference spectrum was recorded from 380 nm to 500 nm in an Aminco DW_2 spectrophotometer.

In rats killed 1 hr or 24 hr after a single i.p. dose of HCl, or 24 hr after the last of 4 daily i.p. or p.o.

Table 1. Effect of TAO administration on hepatic drug-metabolizing enzymes*

	Liver weight/ body weight (%)	Microsomal proteins (mg/g liver)	NADPH–cytochrome *c* reductase activity (nmoles/min/mg microsomal protein)	Cytochrome P-450	
				without potassium ferricyanide (nmoles/mg microsomal protein)	with
Control	3.8 ± 0.3	40 ± 7	75 ± 15	0.7 ± 0.2	0.7 ± 0.1
1 hr after TAO, 1 mmole/kg i.p.	3.9 ± 0.3	43 ± 7	82 ± 19	0.7 ± 0.1	0.7 ± 0.1
24 hr after TAO, 1 mmole/kg i.p.	3.8 ± 0.4	44 ± 7	75 ± 17	0.7 ± 0.1	0.9 ± 0.2†
TAO, 1 mmole/kg i.p. daily for 4 days	4.2 ± 0.8†	54 ± 9†	113 ± 18†	0.7 ± 0.2	2.5 ± 0.4†
TAO, 1 mmole/kg p.o. daily for 4 days	4.5 ± 0.2†	61 ± 10†	92 ± 4†	0.6 ± 0.1	2.4 ± 0.2†
TAO, 0.25 mmole/kg i.p. daily for 4 days	4.0 ± 0.5	42 ± 4	84 ± 16	0.7 ± 0.1	1.0 ± 0.1†
TAO, 0.062 mmole/kg i.p. daily for 4 days	3.7 ± 0.5	39 ± 4	78 ± 15	0.6 ± 0.1	0.8 ± 0.1

* Rats were killed 1 hr or 24 hr after a single dose of TAO, 1 mmole/kg i.p. or 24 hr after the last of 4 daily doses of TAO i.p. or p.o. Cytochrome P-450 was measured as the CO-difference spectrum of dithionite-reduced microsomes both with untreated microsomes and with microsomes to which 50 μM potassium ferricyanide had been added. Results are means ± S.D. for at least 8 rats.

† Significantly different from that in control rats, $P < 0.05$.

Table 2. Effect of TAO administration on TAO metabolism*

	Amplitude of type I binding spectrum with 0.3 mM TAO		Formation of formaldehyde with 0.3 mM TAO		Difference spectrum at 456 nm between microsomes from TAO-treated rats and microsomes from control rats ($O.D._{456-490} \times 10^3$/mg microsomal protein/ml)
	without potassium ferricyanide ($O.D._{388-420} \times 10^3$/mg microsomal protein/ml)	with potassium ferricyanide ($O.D._{388-420} \times 10^3$/mg microsomal protein/ml)	without potassium ferricyanide (nmoles/min/mg microsomal protein)	with potassium ferricyanide (nmoles/min/mg microsomal protein)	
Control	1.5 ± 0.5	1.5 ± 0.4	0.22 ± 0.07	0.25 ± 0.10	
1 hr after TAO, 1 mmole/kg i.p.	1.2 ± 0.6	1.4 ± 0.5	0.19 ± 0.04	0.19 ± 0.07	not detected
24 hr after TAO, 1 mmole/kg i.p.	6 ± 2†	7 ± 5†	0.36 ± 0.25†	0.50 ± 0.49†	12 ± 2
TAO, 1 mmole/kg i.p. daily for 4 days	15 ± 7†	69 ± 22†	0.25 ± 0.13	1.92 ± 0.42†	103 ± 18

* Rats were killed 1 hr or 24 hr after a single dose of TAO, 1 mmole/kg i.p. or 24 hr after the last of 4 daily doses of TAO, 1 mmole/kg i.p. daily for 4 days. The type I binding spectrum of TAO was recorded with untreated microsomes and with microsomes previously treated with 50 μM potassium ferricyanide; the concentration of microsomal proteins was 2 mg/ml microsomal suspension. TAO demethylase activity was measured as the formation of formaldehyde by untreated microsomes or microsomes previously treated with 50 μM potassium ferricyanide. The *in vivo* formation of complexes absorbing light at 456 nm was recorded as the difference spectrum between microsomes from TAO-treated rats and microsomes from control rats. Results are means ± S.D. for at least 8 rats.

† Significantly different from that in control rats, $P < 0.05$.

doses of HCl, hepatic drug-metabolizing enzymes, TAO binding spectrum, and TAO demethylation were not different from those in microsomes from untreated control rats.

RESULTS

Hepatic drug-metabolizing enzymes. Repeated administration of high doses of TAO (1 mmole/kg i.p. daily for 4 days) increased the liver weight/body weight ratio, hepatic microsomal protein concentration and NADPH–cytochrome *c* reductase activity (Table 1). The CO-difference spectrum of dithionite-reduced microsomes measured without prior treatment of the microsomes with potassium ferricyanide was similar in microsomes from TAO-treated rats and in microsomes from control rats (Table 1). Treatment of the microsomes with 50 μM potassium ferricyanide did not increase the CO-binding spectrum in microsomes from control rats or in microsomes from rats killed 1 hr after a single dose of TAO, but increased it in microsomes from other TAO-treated rats (Table 1). After treatment of the microsomes with potassium ferricyanide, microsomal cytochrome P-450 concentration was similar in control rats and in rats killed 1 hr after a single dose, but was increased by 30 per cent in rats killed 24 hr after a single dose, and by 260 per cent in rats killed after 4 daily doses of TAO, 1 mmole/kg i.p. daily (Table 1). Lower repeated doses led to lesser increases (Table 1).

TAO type I binding spectrum. TAO, 0.3 mM, gave a barely detectable type I binding spectrum with hepatic microsomes from control rats and from rats killed 1 hr after a single dose of TAO (Table 2); the K_s could not be determined accurately. The amplitude of the type I binding spectrum was much higher with microsomes from rats killed 24 hr after a single dose of TAO and was still higher in microsomes from rats killed after 4 daily doses of TAO (Table 2, Fig. 1); in the latter rats, the K_s was 0.036 mM. Treatment of the microsomes with 50 μM potassium ferricyanide did not change the amplitude of the TAO type I binding spectrum in microsomes from control rats or from rats killed 1 hr after a single dose of TAO, but further increased it in microsomes from other TAO-treated rats (Table 2). After treatment of the microsomes with potassium ferricyanide, the amplitude of the TAO type I binding spectrum was similar in control rats and in rats killed 1 hr after a single dose, but was increased by 370 per cent in rats killed 24 hr after a single dose, and by 4500 per cent in rats killed after 4 daily doses of TAO, 1 mmole/kg i.p. daily (Table 2).

TAO demethylase activity. TAO, 0.3 mM, was similarly demethylated by hepatic microsomes from control rats and from rats killed 1 hr after a single dose of TAO (Table 2). TAO demethylase activity was increased in microsomes from rats killed 24 hr after a single dose but was not increased in microsomes from rats killed after 4 daily doses of TAO (Table 2). Treatment of the microsomes with 50 μM potassium ferricyanide further increased TAO demethylase activity in microsomes from rats killed 24 hr after a single dose and markedly increased it in rats killed after 4 daily doses (Table 2). After treatment of the microsomes with potassium ferricyanide, TAO demethylase activity was similar in control rats and in rats killed 1 hr after a single dose, but was increased by 100 per cent in rats killed 24 hr after a single dose, and by 670 per cent in rats killed after 4 daily doses of TAO, 1 mmole/kg i.p. daily (Table 2).

In vivo *and* in vitro *formation of a complex absorb-*

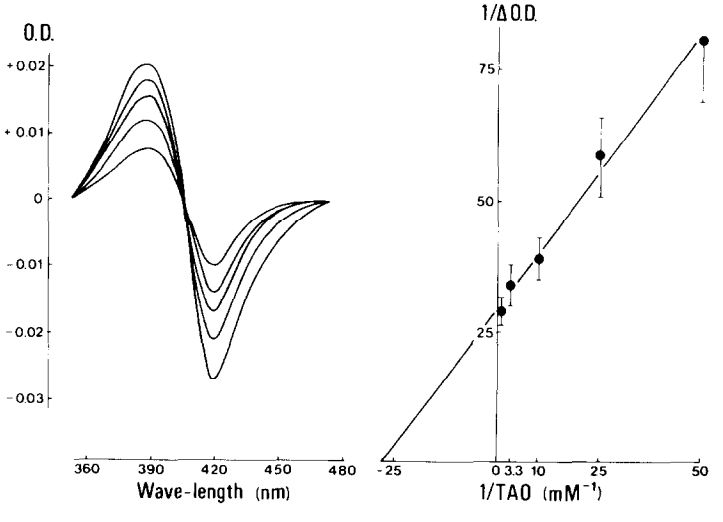

Fig. 1. Binding spectrum of TAO with microsomes from rats treated with repeated doses of TAO. Rats were killed 24 hr after the last of 4 daily doses of TAO, 1 mmole/kg i.p. daily for 4 days. Washed hepatic microsomes were prepared; microsomes were not treated with potassium ferricyanide. A microsomal suspension adjusted to 2 mg microsomal protein/ml was placed in the test and reference cuvettes; TAO in various concentrations (0.02 mM, 0.04 mM, 0.1 mM, 0.3 mM and 1 mM) was added in the test cuvette. The type I binding spectra from a single experiment are shown on the left part of the figure while a double reciprocal plot from data of 7 experiments is shown on the right part of the figure. The K_s for TAO was 0.036 mM.

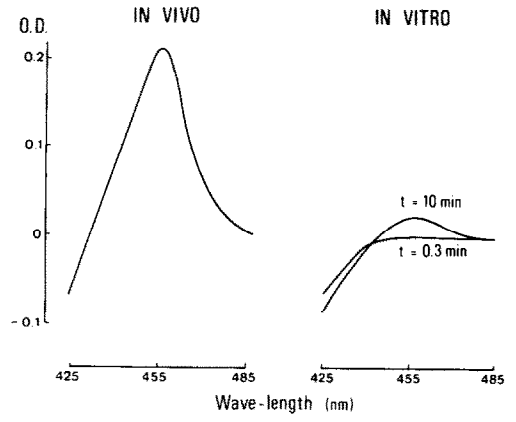

Fig. 2. Formation of complexes absorbing at 456 nm. The left part of the figure shows the difference spectrum obtained by placing microsomes from a control rat in the reference cuvette and microsomes from a rat killed 24 hr after 4 daily doses of TAO, 1 mmole/kg i.p. daily, in the test cuvette; microsomal suspensions were adjusted to 2 mg microsomal protein/ml. The right part of the figure shows the difference spectra obtained by placing microsomes from a rat killed 24 hr after the last of 4 daily doses of TAO, 1 mmole/kg i.p. in both cuvettes, adding 0.3 mM TAO in the test cuvette and 1 mM NADPH in both cuvettes and recording after 0.3 and 10 min.

ing at 456 nm. When microsomes from control rats were placed in the reference cuvette and microsomes from rats killed after 4 daily doses of TAO were placed in the test cuvette, there was an enormous absorption peak at 456 nm (Table 2, Fig. 2); this peak was not detected in microsomes from rats killed 1 hr after a single dose of TAO and was moderate in microsomes from rats killed 24 hr after a single dose of TAO (Table 2). The absorption peak at 456 nm was not modified by addition of dithionite; it disappeared after addition of 50 μM potassium ferricyanide to the microsomes.

In vitro, when microsomes from rats treated with 4 daily doses of TAO were placed in both cuvettes, and 0.3 mM TAO was added to the test cuvette, and 1 mM NADPH was added to both cuvettes, a small absorption peak appeared at 456 nm (Fig. 2). The absorption peak at 456 nm was intermediate at 5 min and fully developed at 10 min; the absorption peak at 456 nm disappeared after addition of 50 μM potassium ferricyanide. No peak at 456 nm could be detected with microsomes from control rats.

DISCUSSION

Induction by TAO of TAO metabolism. Repeated administration of TAO, 1 mmole/kg i.p. daily, increased the liver weight/body weight ratio, hepatic microsomal protein concentration, NADPH–cytochrome *c* reductase activity and cytochrome P-450 concentration measured in the presence of potassium ferricyanide (Table 1). The increase in total cytochrome P-450 concentration was progressive and dose-dependent (Table 1). These findings suggest that TAO administration induces hepatic drug-metabolizing enzymes.

TAO is known to be deacetylated to oleandomycin [27], but, to our knowledge, has not been reported to be metabolized by hepatic microsomes. TAO, a lipophilic drug that contains a tertiary amine function, $—N(CH_3)_2$, could bind to, and be demethylated by, cytochrome P-450. Indeed, TAO gave a small type I binding spectrum with hepatic cytochrome P-450, and was slightly demethylated by hepatic microsomes in control rats (Table 2). In microsomes from rats killed after repeated doses of TAO, 1 mmole/kg i.p. daily, the amplitude of the TAO type I binding spectrum and the activity of TAO demethylase, measured after addition of potassium ferricyanide, were increased by 4500 and 670 per cent, respectively, above that in control microsomes, although cytochrome P-450 was increased by only 260 per cent. These observations suggest that TAO administration induces species of cytochrome P-450 that have a high binding capacity and a high demethylating activity for TAO.

Formation of a stable complex with induced cytochrome P-450. Several secondary or tertiary amines [9–19] are dealkylated and oxidized by cytochrome P-450 into metabolites which form stable complexes with reduced cytochrome P-450 (see Introduction). Complexed cytochrome P-450 (a) exhibits a Soret peak around 455 nm, (b) is unable to bind CO, and (c) is unable to oxidize substrates. Treatment of the microsomes with potassium ferricyanide regenerates native cytochrome P-450 and reverses the above-mentioned phenomena. None of these various phenomena was detected 1 hr after the administration of a single dose of TAO (Table 1 and Table 2). However, after repeated doses of TAO, (a) there was an enormous absorption peak at 456 nm (Fig. 2) and this peak disappeared after treatment of the microsomes with potassium ferricyanide, (b) the CO-binding capacity of dithionite-reduced microsomes was normal in untreated microsomes but high in potassium ferricyanide-treated microsomes (Table 1), and (c) TAO demethylase activity was normal in untreated microsomes but high in potassium ferricyanide-treated microsomes (Table 2). These observations suggest that in TAO-induced rats, TAO is transformed into a metabolite which forms a stable complex with the iron (II) of reduced cytochrome P-450, results in the appearance of a Soret peak at 456 nm, prevents the fixation of CO, and blocks the activity of cytochrome P-450 with TAO (Table 2) and other substrates [24].

Masking of induction by the formation of a stable complex. The property of potassium ferricyanide to restore the CO-binding capacity of previously complexed cytochrome P-450 allows estimation of total cytochrome P-450, uncomplexed cytochrome P-450, and complexed cytochrome P-450 (measured as the difference between total and uncomplexed cytochrome P-450); the amount of complexed cytochrome P-450 calculated in this way was in close agreement with that calculated from the optical absorption at 456 nm by using molar extinction coefficients previously reported for similar complexes [13, 23]. It is noteworthy that the increase in total cytochrome P-450 concentration in TAO-treated rats was related to the presence of complexed cytochrome P-450 whereas the concentration of uncomplexed

Table 3. Characteristics of uncomplexed and previously complexed cytochrome P-450 of TAO-treated rats*

	Amplitude of TAO type I binding spectrum ($O.D._{388-420} \times 10^3$/nmole cytochrome P-450/ml)	TAO demethylase activity (nmoles formaldehyde/min/nmole cytochrome P-450)
Cytochrome P-450 of control rats	2	0.31
Uncomplexed cytochrome P-450 of TAO-treated rats	21	0.36
Previously complexed cytochrome P-450 of TAO-treated rats†	30	0.93

* Values are calculated from the data presented in Table 1 and Table 2 in control rats or in rats treated with TAO, 1 mmole/kg i.p. daily for 4 days.

† Values for previously complexed cytochrome P-450 are not directly observed but are calculated from the differences observed between microsomes treated with 50 μM potassium ferricyanide (total cytochrome P-450) and untreated microsomes (uncomplexed cytochrome P-450) of TAO-treated rats.

cytochrome P-450 always remained constant and equal to that in control microsomes (Table 1).

Significance of remaining uncomplexed cytochrome P-450. The amplitude of the TAO type I binding spectrum calculated per nmole of cytochrome P-450 (Table 3) was increased both in uncomplexed cytochrome P-450 of TAO-treated rats and in previously complexed cytochrome P-450 of TAO-treated rats. This observation indicates that the remaining uncomplexed cytochrome P-450 is not made up merely of normal species of cytochrome P-450 but is at least partly made up of induced species (with a high binding capacity for TAO). Whereas both previously complexed, and uncomplexed, cytochrome P-450 of TAO-treated rats exhibited a high TAO-binding capacity, only previously complexed cytochrome P-450 exhibited a high TAO demethylase activity (Table 3). Dealkylation of tertiary amines is a prerequisite for the formation of complex-forming metabolites [14]. These observations may indicate that TAO induces species of cytochrome P-450 with a high TAO demethylase activity and/or a high TAO-binding capacity; that or those species with a high TAO demethylase activity may be selectively complexed *in vivo* (Table 3), whereas species with an unchanged TAO demethylase activity may remain uncomplexed *in vivo* (Table 3) and be barely complexed *in vitro* (Fig. 2).

It is concluded that TAO induces its own transformation into a metabolite which forms a stable 456 nm-absorbing complex with reduced cytochrome P-450. We are currently testing the possibility that some other macrolides may have similar effects.

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