MOLECULAR PHARMACOLOGY, 17, 395–399

Effect of Interferon Inducing Agents (Polyriboinosinic Acid·Polyribocytidylic Acid and Tilorone) on the Heme Turnover of Hepatic Cytochrome *P*-450

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Received January 2, 1979; Accepted November 19, 1979

SUMMARY

EL AZHARY, R., K. W. RENTON AND G. J. MANNERING. Effect of interferon inducing agents (polyriboinosinic acid·polyribocytidylic acid and tilorone) on the heme turnover of hepatic cytochrome *P*-450. *Mol. Pharmacol.* 17: 395–399 (1980).

The cytochrome *P*-450 and heme contents of microsomes are lowered markedly in livers of rats treated with the interferon inducing agents, tilorone and poly(rI·rC) (polyriboinosinic acid·polyribocytidylic acid). The turnover of cytochrome *P*-450 heme was studied to determine whether the loss of cytochrome *P*-450 and heme was due to a decreased synthesis or to an increased degradation of the hemoprotein. Two populations of cytochrome *P*-450 exist in hepatic microsomes, one with a relatively short and one with a long half-life. The half-lives of cytochrome *P*-450 heme in control animals were determined to be about 8 and 40 h. After the administration of tilorone or poly(rI·rC), the half-life of the short-lived cytochrome *P*-450 was lowered to about 5 h but that of the long-lived cytochrome *P*-450 was not changed. These interferon inducing agents produced no appreciable decrease in the incorporation of the radioactivity of administered ALA-3,5-^{3}H or glycine-2-^{14}C into the heme of cytochrome *P*-420 derived from cytochrome *P*-450. However, a rise in specific activity was observed 10 and 24 h after the administration of poly(rI·rC) and tilorone, respectively. This increase in specific activity is interpreted to mean that interferon inducing agents increase the rate of breakdown of preformed cytochrome *P*-450 heme and that some of this is replaced by newly synthesized labeled heme. It is concluded from these results that poly(rI·rC) and tilorone lower the concentration of hepatic hemoproteins by increasing their degradation rather than by depressing their synthesis.

INTRODUCTION

In a recent communication (1) we showed that the administration of the interferon inducing agents; polyriboinosinic acid·polyribocytidylic acid (poly(rI·rC)) and tilorone, caused a depression of hepatic hemoproteins (cytochrome *P*-450, cytochrome b_5, catalase, and tryptophan 2,3 dioxygenase). δ-Aminolevulinic acid synthetase (ALA-S) activity was also depressed, and heme oxygenase activity was induced. These changes, which reflect perturbations of heme biosynthesis and degradation, would be expected to alter the rate of incorporation of radioactive precursors of cytochrome *P*-450 heme. Activity of the heme biosynthetic pathway can be evaluated by measuring the rate of incorporation of ALA-3,5-^{3}H and glycine-2-^{14}C into cytochrome *P*-450 heme.

Levin and Kuntzman (2, 3) used ALA-3,5-^{3}H to label cytochrome *P*-450 heme *in vivo* and determined the turnover rate of the heme moiety. They observed a biphasic breakdown of cytochrome *P*-450 heme. The fast- and slow-phase components of heme breakdown had half-lives of 7–8 and 46–48 h, respectively. The current investigation was undertaken to determine what effect poly(rI·rC) and tilorone might have on each of the two half-lives of cytochrome *P*-450.

MATERIALS AND METHODS

Materials. Poly(rI·rC), δ-aminolevulinic acid (ALA), glycine, crude pancreatic lipase (steapsin), and hemoglobin were purchased from Sigma Chemical Company, St. Louis, Missouri. δ-Aminolevulinic acid-3,5-^{3}H (ALA-3,5-^{3}H) and glycine-2-^{14}C were purchased from New England Nuclear Corp., Boston, Massachusetts. Tilorone HCl was a gift from Richardson–Merrell, Inc., Cincinnati, Ohio.

This work was supported by Grant GM 15477 from the National Institutes of Health.

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0026-895X/80/030395-05$02.00/0

Treatment of animals. Unless indicated otherwise, male Sprague–Dawley strain rats (90–100 g) were used. In studies of the turnover of cytochrome *P*-450 heme, rats were given daily doses of poly(rI·rC) (10 mg in 0.9% saline/kg, ip) or tilorone HCl (50 mg in 0.9% saline/kg, po) for 4 days. Control rats received saline (0.5 ml, ip, or 1.0 ml, po). Twenty-four hours after the fourth dose of either agent, 0.255 mg of ALA-3,5-^{3}H (494 mCi/mmol) in saline/kg was injected into the tail vein and the animals were killed 5, 8, 10, 16, 24, 40, 48, 72, or 96 h later. Rats killed at 40 or 48 h received a fifth dose of the interferon inducing agent; those killed at 72 h, a sixth dose; and those killed at 96 h, a seventh dose.

In studies of the uptake of ALA-3,5-^{3}H into cytochrome *P*-450 heme, animals were treated with either a single dose or four daily doses of poly(rI·rC) or tilorone. ALA-3,5-^{3}H was injected into the tail vein 2 h after the single dose or 24 h after the last of the multiple doses before killing the animals. Doses of poly(rI·rC), tilorone, and ALA-3,5-^{3}H were the same as those used in the turnover studies.

In studies of the incorporation of glycine-2-^{14}C into cytochrome *P*-450 heme, rats were given 10 mg of poly(rI·rC) 3, 6, 10, or 16 h before injecting 30 μCi of glycine-2-^{14}C (49 mCi/mmol)/rat, ip. When tilorone was used, it was given 12, 16, or 24 h before the injection of labeled glycine. Animals were killed 60 min after the injection of glycine-2-^{14}C.

Microsomal preparations. Microsomes were prepared from male Sprague–Dawley strain rats (90–100 g) as described previously (4). Microsomes treated with steapsin as described by Omura and Sato (5, 6) were used when ALA-3,5-^{3}H or glycine-2-^{14}C was used to radiolabel cytochrome *P*-450 heme. This treatment removes cytochrome b_5 from the microsomes and converts cytochrome *P*-450 to cytochrome *P*-420, thereby rendering the microsomes free of all heme other than that derived from cytochrome *P*-450. Calculations were based on an 85–90% recovery of cytochrome *P*-450 heme as cytochrome *P*-420 heme.

Determination of ALA-3,5-^{3}H incorporation into cytochrome P-450 heme. ALA is a precursor of heme which is committed exclusively to heme synthesis. The method of Omura and Sato (5, 6) was used to determine the cytochrome *P*-450 content of microsomes before steapsin treatment. The same procedure was used to determine the cytochrome *P*-420 content of steapsin-treated microsomes except that an extinction coefficient of 111 rather than 91 mM^{-1} cm^{-1} was used (6). Suspensions of the steapsin-treated microsomes (about 0.5 mg of protein) were counted in 15 ml of Aquasol in a liquid scintillation spectrometer.

Determination of the incorporation of glycine-2-^{14}C into cytochrome P-450 heme. The incorporation of glycine-2-^{14}C into cytochrome *P*-450 heme was determined by measuring the radioactivity of heme isolated from steapsin-treated microsomes by the method of Fox and Thomson (7) as described by Maines and Anders (8).

Determination of the heme content of microsomes. The heme content of microsomes was measured using the pyridine hemochromogen method of Omura and Sato (5, 6).

TABLE 1

Effects of poly(rI·rC) and tilorone on the heme, cytochrome P-450, and cytochrome b_5 contents of hepatic microsomes

Rats (150–200 g) received 10 mg poly(rI·rC)/kg, ip, or 50 mg tilorone HCl/kg, po, daily for 4 days. Control animals received 0.5 ml saline, ip, or 1 ml saline, po. The animals were killed 24 h after the last administration. Values represent mean ± SE. Values in parentheses = number of animals.

	Microsomal heme	Cytochrome *P*-450	Cytochrome b_5
	nmol/mg protein	*nmol/mg protein*	*nmol/mg protein*
Control	1.26 ± 0.0 (10)	0.88 ± 0.03 (10)	0.42 ± 0.02 (10)
Poly(rI·rC)	0.88 ± 0.1 (10)	0.50 ± 0.04* (10)	0.32 ± 0.02* (10)
% of control	69%	57%	76%
Control	1.44 ± 0.1 (4)	1.12 ± 0.07 (4)	0.49 ± 0.01 (4)
Tilorone	0.83 ± 0.0* (4)	0.48 ± 0.01* (4)	0.41 ± 0.01 (4)
% of control	58%	43%	82%

* Significantly different from controls ($P < 0.05$).

RESULTS

Effects of poly(rI·rC) and tilorone on the cytochrome P-450, cytochrome b_5, and heme contents of hepatic microsomes. The effects of the administration of poly(rI·rC) or tilorone for 4 days on the microsomal cytochrome *P*-450, cytochrome b_5, and heme contents are summarized in Table 1. Poly(rI·rC) caused losses of cytochrome

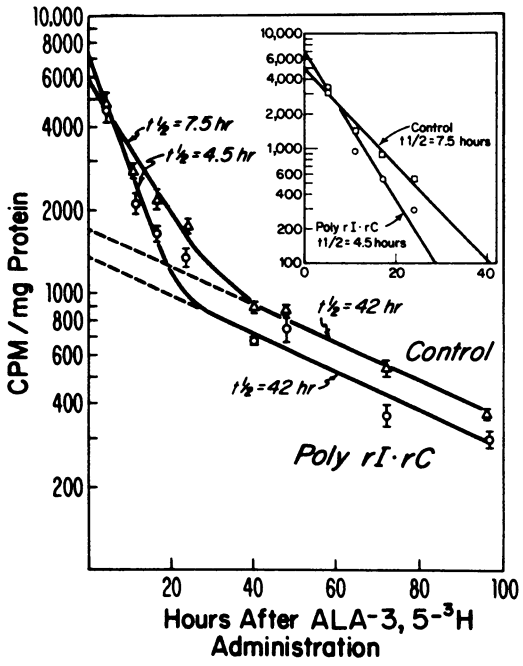

FIG. 1. *Effect of poly(rI·rC) on the half-lives of cytochrome P-450 heme*

Rats were injected (ip) for 4 days with 10 mg of poly (rI·rC). Twenty-four hours after the last dose, the animals were injected (iv) with 0.255 mg of ALA-3,5-^{3}H (sp act, 494 mCi/mmol)/kg and killed at indicated times thereafter. Microsomes isolated from their livers were treated with steapsin to remove cytochrome b_5 and convert cytochrome *P*-450 to cytochrome *P*-420. The radioactivity of these microsomes was determined. Each value represents the mean ± SE obtained from three rats. Corrected half-lives were obtained by extrapolating the slow phase to 0 time and subtracting the extrapolated values from the uncorrected fast-phase portion of the curve (inset).

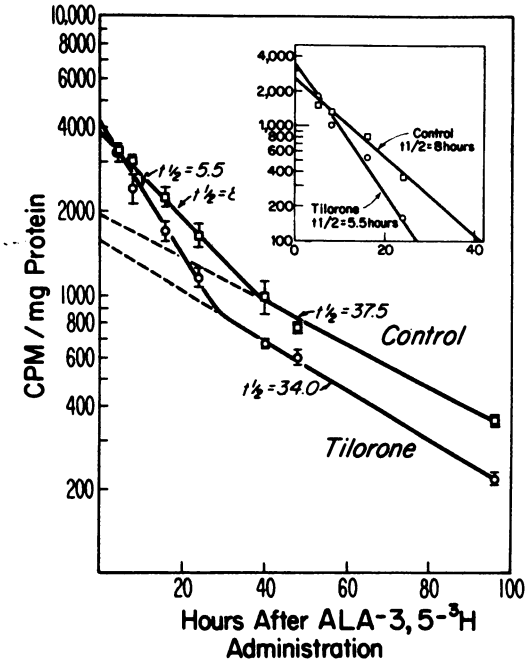

FIG. 2. *Effect of tilorone on the half-lives of cytochrome P-450 heme* Experimental conditions were the same as those given for Fig. 1 except that tilorone (50 mg/kg, po) was substituted for poly(rI·rC).

P-450 and cytochrome b_5 of 43 and 24%, respectively. About 30% of the microsomal heme was lost; this closely approximates the loss of heme predicted from the losses of cytochrome *P*-450 and b_5. The effects of tilorone were similar to those produced by poly(rI·rC).

Effects of poly(rI·rC) and tilorone on the half-lives of cytochrome P-450 heme. When control rats were injected with ALA-3,5-^{3}H and killed at selected time intervals, the incorporation of radiolabel into heme was shown to be biphasic (Figs. 1 and 2), as was first reported by Levin and Kuntzman (2, 3). The half-life of heme during the first (fast) phase was about 8 h; that of the second (slow) phase, about 40 h. The administration of poly(rI·rC) to rats for 4 days lowered the half-life of the first phase from 7.5 to 4.5 h, but no change was seen in the half-life of the second phase (Fig. 1). Tilorone had similar effects on the half-life of the first phase, which was lowered from 8 to 5.5 h, and on the half-life of the second phase, which was not altered appreciably.

TABLE 2

Effects of interferon inducing agents on the incorporation of δ-aminolevulinic acid-3,5-^{3}H into cytochrome P-450

Poly(rI·rC), 10 mg/kg (ip), or tilorone HCl, 50 mg/kg (po), was administered to rats daily for 4 days or as a single dose. ALA-3,5-^{3}H (0.255 mg/kg, sp act 494 mCi/mmol) in saline was injected via the tail vein 24 h after the last of the multiple doses or 2 h after the single dose. The rats were killed 1 h after the injection of ALA-3,5-^{3}H. Radioactivity incorporated into microsomal *P*-450 after its conversion to *P*-420 was determined. Results represent mean ± SE. Values in parentheses = number of animals.

	Cytochrome *P*-450	cpm/nmol *P*-450
	nmol/mg protein	
Control	0.61 ± 0.03 (5)	16,319 ± 1,401 (5)
Tilorone (3 h)	0.61 ± 0.03 (5)	16,754 ± 1,308 (5)
% of control	100%	103%
Tilorone (4 days)	0.42 ± 0.02* (5)	26,257 ± 2,647* (5)
% of control	69%	161%
Control	0.64 ± 0.05 (3)	13,343 ± 332 (3)
Poly(rI·rC) (3 h)	0.49 ± 0.03* (3)	17,286 ± 1,086* (3)
% of control	77%	130%
Poly(rI·rC) (4 days)	0.39 ± 0.01* (3)	23,574 ± 75* (3)
% of control	63%	177%

* Significantly different from controls ($P < 0.05$).

It can be calculated (2, 3) from Fig. 1 that the proportion of the fast-phase component to the slow-phase component in microsomes from untreated rats was 2.3:1 and, in microsomes from poly(rI·rC)-treated rats, 3.8:1. Corresponding ratios of 1.3:1 and 1.8:1 were obtained in the tilorone experiment (Fig. 2).

Effects of poly(rI·rC) and tilorone on the incorporation of ALA-3,5-^{3}H into cytochrome P-450. Data presented in Table 2 show that tilorone produced no change in the incorporation of ALA-3,5-^{3}H into cytochrome *P*-450 heme when administered 3 h before the animals were killed, but poly(rI·rC) caused a 30% increase in incorporation. When poly(rI·rC) or tilorone was administered for 4 days, the specific activity, calculated as cpm/nmol *P*-450, was increased 177 and 161%, respectively.

Effects of poly(rI·rC) and tilorone on the incorporation of glycine-2-^{14}C into cytochrome P-450 heme. The effects of poly(rI·rC) and tilorone on the incorporation of labeled glycine into cytochrome *P*-450 heme are shown in Tables 3 and 4. Three and six hours after poly(rI·rC)

TABLE 3

Effect of poly(rI·rC) on the incorporation of glycine-2-^{14}C into cytochrome P-450 heme

Rats received a single injection of 10 mg poly(rI·rC/kg) (ip). Control animals received 0.5 ml saline (ip). Animals were killed at the indicated times after poly(rI·rC) administration. Thirty microcuries of glycine-2-^{14}C was injected (ip) 1 h before the animals were killed. Values represent mean ± SE. Values in parentheses = number of animals.

	Control (no poly(rI·rC))	Hours after poly(rI·rC) administration			
		3	6	10	16
Cytochrome *P*-450 (nmol/mg)	1.66 ± 0.08 (12)	1.79 ± 0.04 (4)	1.66 ± 0.17 (4)	1.44 ± 0.08 (4)	0.97 ± 0.09* (3)
% of control		108%	100%	87%	58%
cpm/nmol *P*-420	440 ± 47 (12)	335 ± 20 (4)	357 ± 14 (4)	957 ± 137* (4)	1051 ± 63* (3)
% of control		76%	80%	217%	239%

* Significantly different from controls ($P < 0.05$).

TABLE 4

Effect of tilorone on the incorporation of glycine-2-^{14}C into cytochrome P-450 heme

Experimental conditions were the same as those given in Table 3 except that tilorone (50 mg/kg, po) was substituted for poly(rI·rC) and microsomes were collected 12, 16, and 24 h after its administration rather than 3, 6, 10, and 16 h, as was the case when poly(rI·rC) was administered; the longer time intervals were used because a longer time is required for tilorone than for poly(rI·rC) to depress cytochrome *P*-450 (1).

	Control (no tilorone)	Hours after tilorone administration		
		12	16	24
Cytochrome *P*-450 (nmol/mg protein)	1.15 ± 0.08 (4)	1.10 ± 0.04 (4)	1.29 ± 0.01 (4)	0.79 ± 0.14* (4)
% of control		96%	113%	69%
cpm/nmol *P*-420	559 ± 62 (4)	491 ± 8 (4)	599 ± 31 (4)	969 ± 112* (4)
% of control		88%	107%	174%

* Significantly different from controls ($P < 0.05$).

administration, the incorporation of radioactivity into cytochrome *P*-450 heme was depressed to 76 and 80% of the control level, respectively. After 10 and 16 h, the specific activity had increased to 217 and 239% of the control values and the cytochrome *P*-450 (i.e., *P*-420) level was lowered to 87 and 58% of the control values, respectively.

Twelve and sixteen hours after tilorone administration, the specific activity of cytochrome *P*-450 heme was not significantly different from that of controls, but after 24 h it was 74% higher (Table 3). At that time the cytochrome *P*-450 level had been lowered by about 30%.

DISCUSSION

Previous studies in this laboratory have demonstrated that the interferon inducing agents, tilorone and poly(rI·rC) (polyriboinosinic acid·polyribocytidylic acid), decrease the hepatic cytochrome *P*-450 content and several other hemoproteins in the liver (1, 9). A loss of microsomal heme content equivalent to the loss of cytochromes *P*-450 and b_5 is reported in this communication. These decreases in cytochrome *P*-450 and heme may be due either to an increase in breakdown or to a decrease in the synthesis of the enzyme or its heme prosthetic group. Levin and Kuntzman (2, 3) showed that the breakdown of cytochrome *P*-450 is comprised of two components, a fast phase with a half-life of 7 to 9 h and a slow phase with a half-life of 46 to 48 h. These half-lives of cytochrome *P*-450 heme do not necessarily reflect the turnover of cytochrome *P*-450; the protein and heme portions of the cytochrome may be turning over asynchronously, as is the case with cytochrome b_5 (10). It is quite conceivable that the two phases may represent two populations of cytochrome *P*-450 with different constants for the dissociation of protein and heme. The administration of poly(rI·rC) and tilorone caused a decrease in the half-life of the first phase without altering that of the second phase appreciably. These agents also caused an increase in the ratio of the fast- to slow-phase components. These results would be expected if the interferon inducing agents increased the breakdown of cytochrome *P*-450 heme, possibly by decreasing the affinity of the heme for the protein moiety of the cytochrome *P*-450 represented by the fast phase. The increase in heme breakdown by interferon inducing agents is suggested by previous studies which showed that heme oxygenase activity was induced by the administration of tilorone or poly(rI·rC) (1).

The decreases in the cytochrome *P*-450 and heme content of microsomes might also be due to a decrease in the rate of heme synthesis. The interferon inducing agent did not decrease the incorporation of glycine-2-^{14}C or ALA-3,5-^{3}H at any of the time intervals studied. However, an increase in the specific activity of the heme was observed 10 and 24 h after the administration of poly(rI·rC) and tilorone, respectively. This might appear paradoxical in view of our previous observation (1) that a decrease in ALA-S activity occurs a few hours after the administration of either agent. It is suggested that the amount of labeled heme incorporation into cytochrome *P*-450 is a function of the specific radioactivity of the hepatic heme pool. This, in turn, is dependent on the biosynthetic and degradative pathways. An increase in heme oxygenase activity and the consequent breakdown of heme with no change in ALA-S activity would increase the rate of breakdown of the preformed heme, which would be replaced by newly synthesized, labeled heme. This would explain the delayed rise in the specific activity of the heme. It is to be noted that this increase in specific activity was observed at a time when ALA-S activity would have returned to its normal level (1).

It is concluded from these studies that interferon inducing agents lower the concentrations of hepatic hemoproteins by increasing their degradation rather than by depressing their synthesis. It remains to be determined whether the primary target for increased degradation is the protein or the heme moiety of the hemoprotein.

REFERENCES

1. el Azhary, R., and G. J. Mannering. Effects of interferon agents (polyriboinosinic acid·polyribocytidylic acid, tilorone) on hepatic hemoproteins (cytochrome *P*-450, catalase, tryptophan 2,3-dioxygenase, mitochondrial cytochromes), heme metabolism, and cytochrome *P*-450-linked monooxygenase systems. *Mol. Pharmacol.* **15**: 698–707 (1979).
2. Levin, W., and R. Kuntzman. Biphasic decrease of radioactive hemoprotein from liver microsomal carbon monoxide-binding particles. Effect of 3-methylcholanthrene. *J. Biol. Chem.* **244**: 3671–3676 (1969).
3. Levin, W., and R. Kuntzman. Biphasic decrease of radioactive hemoprotein from liver microsomal carbon monoxide-binding particles. Effect of phenobarbital and chlordane. *Mol. Pharmacol.* **5**: 499–506 (1969).
4. el Masry, S., G. M. Cohen and G. J. Mannering. Sex-dependent differences in drug metabolism in the rat. 1. Temporal changes in the microsomal drug-

metabolizing system of the liver during sexual maturation. *Drug Metab. Dispos.* **2**: 267–298 (1974).
5. Omura, T., and R. Sato. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.* **239**: 2370–2378 (1964).
6. Omura, T., and R. Sato. The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purification and properties. *J. Biol. Chem.* **239**: 2379–2385 (1964).
7. Fox, J. B., and J. S. Thomson. The formation of green heme pigments from metmyoglobin and methemoglobin by the action of nitrite. *Biochemistry* **3**: 1323–1328 (1964).
8. Maines, M. D., and M. W. Anders. Characterisation of the heme of cytochrome *P*-450 using gas chromatography/mass spectrometry. *Arch. Biochem. Biophys.* **159**: 201–205 (1973).
9. Renton, K. W., and G. J. Mannering. Depression of hepatic cytochrome *P*-450-dependent monooxygenase systems with administered interferon inducing agents. *Biochem. Biophys. Res. Commun.* **73**: 343–348 (1976).
10. Bock, K. W., and P. Siekevitz. Turnover of heme and protein moieties of rat liver microsomal cytochrome b_5. *Biochem. Biophys. Res. Commun.* **41**: 374–380 (1970).

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