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EFFECT OF TETRACYCLINE ON THE METABOLISM OF [1-^{14}C]OLEATE BY THE LIVER*

KERRY J. BREEN†, STEVEN SCHENKER and MURRAY HEIMBERG‡
Departments of Pharmacology and Medicine, Vanderbilt University, School of Medicine, and the Veterans Administration Hospital, Nashville, TN, U.S.A.

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Abstract—The effects of tetracycline on the synthesis, outward transport, and accumulation of triglycerides from [1-^{14}C]oleate were investigated using the isolated perfused rat liver. The effects of the antibiotic on production of CO_2 and ketone bodies were studied also. Tetracycline either was added to the medium perfusing livers from normal fed male rats, or alternatively, was administered to rats intravenously (pure tetracycline HCl, or Achromycin, 100 mg/kg body weight) 3 hr prior to removal of the livers for perfusion. The quantity of oleate infused was sufficient to yield approximately half-maximal rates of output of triglycerides. Under these conditions, on the basis of the isotopic data, the hepatic accumulation of triglycerides derived from the exogenous sources (perfusate ^{14}C free fatty acid) was balanced by a decrease in outward transport triglycerides, whether tetracycline was added directly to the medium perfusing the livers *in vitro* or was administered to the animals *in vivo*. Decreases in hepatic oxidation of oleate induced by tetracycline did not appear to be an important mechanism for induction of steatosis. These data suggest that the fatty liver resulting from intoxication by tetracycline results primarily from inhibition of outward transport of triglycerides.

Tetracycline administered in excessive doses can produce acute fatty liver in humans [1]. This antibiotic has been shown to produce a dose-dependent accumulation of triglycerides in the isolated perfused rat liver, an effect accompanied by a dose-dependent reduction in secretion of triglycerides by the liver [2]. In those studies [2], impaired release of triglycerides by the perfused liver accounted for only 30–50 per cent of the observed accumulation of triglycerides, in contrast to predictions based on experiments with the intact rat [3]. In the perfusion studies [2], the balance of triglycerides was estimated chemically, and the perfusate concentration of free fatty acid was maintained at concentrations known to produce approximately maximal rates of output of triglycerides from livers of male rats [4]. To explore this discrepancy in accountability, we studied the effects of tetracycline on the disposition of [1-^{14}C]oleic acid by the isolated perfused rat liver. The data indicate that, although additional mechanism(s) other than inhibition of output of triglycerides may exist in this experimental model for production of fatty liver, with the smaller quantity of free fatty acids made available to the liver, decreased output of [^{14}C]triglyceride fatty acid was equal to that which accumulated in the tissue in the presence of tetracycline.

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†Present address: Department of Medicine, St. Vincent's Hospital, University of Melbourne, Melbourne, Australia.

‡Present address: Department of Pharmacology, University of Missouri-Columbia, School of Medicine, Columbia, MO 65212. Please send requests for reprints to this address.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats (200–275 g) obtained from the Holtzman Co., Madison, WI, were used in all experiments. Animals were allowed free access to Purina laboratory chow and water at all times.

Administration of tetracycline. Several routes and forms of administration of tetracycline were chosen, together with appropriate control treatments. In certain experiments, 10 or 20 mg of pure tetracycline hydrochloride was added to the medium perfusing livers removed from normal rats; no additions were required for the controls. In other experiments, intact rats were treated with Achromycin or with pure tetracycline hydrochloride, administered as a slow infusion of 100 mg/kg, as described previously [2, 5]. Control rats received infusions of either ascorbic acid (250 mg/kg) or 0.9% NaCl [5]. Three hr after treatment, the livers were removed from the animals and were perfused *in vitro*.

Conditions of perfusion. Livers taken from rats pretreated with tetracycline *in vivo*, and livers taken from untreated rats, were perfused under identical conditions using procedures and equipment [2, 5, 6] described previously. The perfusate consisted of 47 ml of defibrinated blood (obtained from normal fed male rats) and 23 ml of Krebs–Henseleit bicarbonate buffer, pH 7.4 [7]. A complex of oleic acid and bovine serum albumin [8], containing 283.2 μmoles fatty acid, 8.9 μCi of [1-^{14}C]oleic acid and 5 g albumin/50 ml complex, was infused at the rate of 66.4 μmoles/hr for 4 hr following a preliminary 20-min period of equilibration. In various experiments as indicated, tetracycline HCl (10 or 20 mg in 1.0 ml of 0.9% NaCl) was added to the perfusate reservoir immediately preceding the infusion of fatty acid.

Production of $^{14}CO_2$. Throughout the perfusion period, the 95% O_2:5% CO_2 gas mixture used to oxygenate the perfusate was drawn continuously under negative pressure (approximately -7 cm H_2O) through three gas-dispersing traps in series, each containing 150 ml of 10% KOH, and a fourth trap containing CaO solution. The radioactive $^{14}CO_2$ was counted as reported previously [9]. Recovery of $^{14}CO_2$ collected from $Ba^{14}CO_3$ placed in the perfusion apparatus was essentially complete.

Methods for chemical and isotopic analyses. Samples of perfusate were withdrawn for analysis of lipids and determination of radioactivity at 0, 1, 2, 3 and 4 hr in each experiment. Aliquots of erythrocyte-free perfusate were extracted with chloroform–methanol (2:1, v/v) [8] and assayed for triglycerides [10,11] and free fatty acids [12]. Output of ketone bodies was determined by assay of the water-soluble, protein-free extract of the perfusate [13]. At the end of the experiments, lipids were extracted from the liver [14] and analyzed for triglycerides as described for perfusate extracts. Radioactivity in water-soluble and lipid-soluble extracts of perfusate, and in lipid extracts of liver was measured in each experiment with a Beckman CPM-liquid scintillation counter. Aliquots of the water-soluble extracts were counted using a scintillation fluid containing 9 vol. toluene (diphenyloxazole, 6 g/liter) to 1.2 vol hydroxide of hyamine (1 M in CH_3OH). Radioactivity was measured in various lipid fractions isolated from perfusate or liver; these fractions were separated either by thin-layer chromatography [15] or by column procedures into total neutral lipids and total phospholipids [15]. The scintillation fluid for counting lipid radioactivity consisted of toluene to which diphenyloxazole (4 g/liter) and *p*-bis-1,2-(phenyloxazolyl)-1-benzene (100 mg/liter) had been added.

Chemicals. All chemicals used were reagent grade. Solvents were redistilled from glass before use. Oleic acid (99 per cent purity) was purchased from Applied Science Laboratories, State College, PA, and from Supelco, Bellefonte, PA. Bovine serum albumin (Fraction V powder) was obtained from Pentex, Inc., Kankakee, IL, and was purified before use [16]. Achromycin (100 mg tetracycline HCl buffered with 250 mg ascorbic acid) and pure tetracycline HCl were gifts of the Lederle Co., Pearl River, NY. [1-^{14}C]oleic acid (sp. act. 57.7 mCi/m-mole) was purchased from the Radiochemical Center, Amersham.

Expression of data and statistical analysis. Hepatic production of various metabolites was calculated as described previously [4] and expressed per g liver wet wt. Per cent incorporation of radioactivity into CO_2, ketone bodies, and hepatic and perfusate lipid fractions was calculated as follows:

$$\text{Per cent incorporation} = \frac{100\ (\text{dis./min recovered in fraction})}{(\mu\text{Ci [}^{14}\text{C]oleic acid infused})\ (2.22 \times 10^6)}$$

Statistical significance of differences between groups was evaluated by calculation of Student's values for *t*.

RESULTS

The effects of tetracycline on the chemically measured hepatic secretion of triglycerides, accumulation of triglycerides in the liver and hepatic ketogenesis are shown in Table 1. Tetracycline added to the perfusate in doses of 10 and 20 mg caused a 53.8 and 97.4 per cent decrease, respectively, in output of triglycerides. Tetracycline administered to the animals

Table 1. Effects of tetracycline on accumulation of triglycerides in the perfused liver, and output of triglycerides and ketone bodies, estimated chemically*

Treatment group	Liver wt (g)	Hepatic TG secretion (μmoles/g/4 hr)	Hepatic TG at 4 hr (μmoles/g)	Ketone body production (μmoles/g/4 hr)
Addition of tetracycline to the perfusate				
1. Controls (4)	9.0 ± 0.5	3.05 ± 0.30	10.5 ± 0.4	19.2 ± 4.7
2. Tetracycline 10 mg (5)	9.4 ± 0.4	1.41 ± 0.09†	10.3 ± 0.6	13.9 ± 2.4†
3. Tetracycline, 20 mg (7)	9.7 ± 0.4	0.08 ± 0.08†	13.6 ± 0.7†	13.0 ± 1.5†
Treatment of animals with tetracycline				
4. Ascorbate controls (4)	9.8 ± 0.4	2.93 ± 0.37	6.1 ± 0.9	22.9 ± 2.5
5. Achromycin, 100 mg/kg (4)	9.9 ± 0.6	1.51 ± 0.34†	12.7 ± 1.0†	19.8 ± 2.8
6. Tetracycline HCl, 100 mg/kg (4)	10.4 ± 0.3	0.99 ± 0.28†	15.6 ± 0.9†	21.0 ± 1.3

*Data are expressed as means ± S. E., after 4 hr of perfusion. See text for explanation of treatment details. Tetracycline refers to pure tetracycline HCl without ascorbic acid. Ascorbate was administered (250 mg/kg) to control animals when indicated since the commercial preparation of Achromycin was buffered with 250 mg ascorbic acid/100 mg of antibiotic. The figures in parentheses refer to the number of perfusions in each group. Although calculated as triglycerides, analysis actually was for total neutral glycerides in these experiments, since triglyceride was not isolated in pure form by thin-layer chromatography. Hepatic triglycerides in livers prior to perfusion, measured in other animals treated similarly, were 6.5 ± 0.8, 6.7 ± 0.7, 11.1 ± 1.0 and 9.2 ± 0.5 μmoles/g for livers from normal controls, ascorbate controls, Achromycin-treated rats, and tetracycline (pure) -treated animals respectively. Uptake of free fatty acid was not measured in these experiments, but had been shown previously to be unaffected by treatment with tetracycline [2]. TG = triglyceride.

†Significantly different from controls, $P < 0.05$.

Table 2. Effects of tetracycline on incorporation of [1-^{14}C]oleic acid into perfusate and hepatic triglycerides, perfusate ketone bodies and respired $^{14}CO_2$*.

Treatment group	Perfusate triglyceride†	Hepatic triglyceride‡	Ketone bodies§	$^{14}CO_2$	Total recovery‖
Addition of tetracycline to the perfusate					
1. Controls (7)	15.3 ± 2.3	28.9 ± 3.2	6.5 ± 1.6	10.8 ± 1.4	92.1 ± 3.4
2. Tetracycline, 10 mg (5)	11.0 ± 1.4¶	33.6 ± 1.8¶	5.0 ± 1.2	7.4 ± 0.5¶	87.1 ± 3.4
3. Tetracycline, 20 mg (7)	5.9 ± 0.7¶	49.9 ± 2.9¶	3.0 ± 0.9¶	7.3 ± 1.1¶	90.0 ± 2.3
Treatment of animals with tetracycline					
4. Ascorbate controls (5)	19.1 ± 1.5	22.7 ± 4.2	7.4 ± 1.1	10.8 ± 1.9	87.6 ± 4.4
5. Achromycin, 100 mg/kg (4)	11.4 ± 2.2¶	38.4 ± 2.9¶	5.7 ± 1.8	12.5 ± 1.4	93.7 ± 1.9
6. Tetracycline, 100 mg/kg (4)	10.1 ± 1.0¶	38.4 ± 1.7¶	5.7 ± 0.3	11.8 ± 0.5	86.4 ± 1.7

*Data are expressed as means ± S. E., after 4 hr of perfusion, and are the per cent of infused ^{14}C incorporated into various fractions.

†Perfusate triglycerides, isolated by thin-layer chromatography.

‡Hepatic triglycerides (total neutral glycerides).

§Radioactivity was determined in the water-soluble fraction of the perfusate and assumed to represent ketone equivalent.

‖Total recovery was based on sums of radioactivity in the perfusate and hepatic lipid-soluble extracts, respired $^{14}CO_2$ and water-soluble perfusate fractions.

¶Significantly different from controls, $P < 0.05$.

(100 mg/kg) either as Achromycin or pure tetracycline also reduced the output of triglycerides. Tetracycline increased hepatic triglyceride concentration, except at the 10 mg dose, when added to the medium. Ketone body production was reduced by treatment with tetracycline when it was added to the perfusate, but was not reduced in livers from treated rats. Uptake of free fatty acid by the liver was unaffected by the tetracycline.

Incorporation of [^{14}C]oleic acid into perfusate and liver triglycerides, ketone bodies and CO_2 is shown in Table 2. Recovery of isotope averaged about 90 per cent and did not differ between treatment groups. Addition of tetracycline to the medium, or treatment of the animal with tetracycline reduced the incorporation of [^{14}C]oleic acid into perfusate triglycerides in a dose-related manner, the maximum reduction observed being 61.4 per cent with the 20 mg dose of tetracycline *in vitro*. These effects were associated with increases in incorporation of oleate into triglycerides, again in a hepatic dose-related manner. Incorporation of ^{14}C into water-soluble perfusate fractions (ketone bodies) was reduced significantly only by the addition of 20 mg tetracycline to the perfusate *in vitro* ($P < 0.005$), an effect observed throughout the 4 hr of the experiment. Incorporation of ^{14}C into the water-soluble fraction was not reduced significantly by treatment of the animal with pure tetracycline or Achromycin. In controls from either experimental series, 10.8 per cent of the ^{14}C

Table 3. Effects of tetracycline on incorporation of [1-^{14}C]oleic acid into hepatic and perfusate triglycerides*

Treatment group	Hepatic TG conc† (A)	Hepatic TG radioactivity‡ (B)	Specific activity§ (B/A)	RSA‖ (C)	[^{14}C]oleate converted to: Hepatic TG¶ (D)	Perfusate TG¶ (E)	Sum (D + E)
Addition of tetracycline to the perfusate							
1. Controls (4)	8.93 ± 0.75	5.65 ± 0.44	6.54 ± 1.03	1.02 ± 0.15	8.95 ± 1.31	6.07 ± 0.51	15.02
2. Tetracycline, 20 mg (4)	12.42 ± 0.78**	9.66 ± 0.59**	7.90 ± 0.71	1.14 ± 0.11	13.91 ± 0.77**	1.52 ± 0.24**	15.43
Treatment of the animals with tetracycline							
3. Ascorbic acid controls (4)	4.65 ± 1.55	3.22 ± 1.34	6.20 ± 0.79	0.96 ± 0.06	4.57 ± 1.73	5.78 ± 0.40	10.35
4. Achromycin, 100 mg/kg (4)	11.49 ± 1.62**	6.26 ± 0.74**	5.56 ± 0.41	0.79 ± 0.05	8.87 ± 0.98	3.04 ± 0.53**	11.91
5. Saline (0.9% NaCl) controls (2)	8.20	4.40	5.36	0.79	6.51	4.10	10.61
6. Tetracycline (pure), 100 mg/kg (4)	14.83 ± 0.85	5.96 ± 0.13	4.05 ± 0.20	0.58 ± 0.02	8.49 ± 0.20	2.59 ± 0.28	11.08

*All data are expressed as means ± S. E. after 4 hr of perfusion. Figures in parentheses indicate number of experiments. Triglyceride (TG) in all cases was isolated by thin-layer chromatography.

†Hepatic triglyceride concentration measured chemically, μ/moles/g.

‡Radioactivity in liver triglycerides, dis./min × 10^{-5}/g.

§Specific activity of hepatic triglycerides, dis./min × 10^{-4}/μmole of triglyceride.

‖Relative specific activity = $\frac{\text{dis/min/}\mu\text{mole TG}}{\text{dis./min/}\mu\text{mole oleic acid infused}}$.

¶Expressed as μmoles [^{14}C]oleic acid converted/g of liver.

**Significantly different from controls, $P < 0.05$.

appeared in respired CO_2 over the 4 hr. Incorporation was decreased modestly with either the 10 or 20 mg dose of tetracycline added to the perfusate *in vitro* ($P < 0.005$), but a dose–response relationship was not apparent. No effect on $^{14}CO_2$ production was observed with livers from animals treated with tetracycline *in vivo*.

It is probable that treatment with tetracycline did not alter the conversion of [1-^{14}C]oleic acid into the sum total of hepatic and perfusate triglycerides (Table 3). Reduction of incorporation of oleate into perfusate triglycerides, however, appeared to be balanced by an equivalent increase in incorporation into hepatic triglycerides. Conversion of oleic acid to triglyceride was higher in the experiments in which antibiotic was added *in vitro*, both in the control and tetracycline-treated, than in the experiments using livers from tetracycline-treated animals. The lower relative specific activity values for hepatic triglycerides in the animals treated with tetracycline *in vivo* probably results from accumulation of hepatic triglycerides prior to removal for perfusion.

DISCUSSION

These studies confirm and extend the predictable dose-dependent effects of tetracycline on impairment of triglyceride secretion and accumulation of triglycerides in the liver. In the current experiments, less oleic acid (266 μmoles/4 hr) was infused than previously [2]. This lesser quantity of free fatty acid has been shown to produce approximately half-maximal rates of secretion of triglycerides, which ordinarily would not be expected to produce any significant accumulation of triglycerides in the liver [4]. It is probable that the effects of tetracycline on fatty acid oxidation did not contribute in any important way to hepatic accumulation of triglycerides. Tetracycline diminished the oxidation of oleic acid to CO_2 and ketone bodies only when added to the medium *in vitro*, and at high concentrations of the antibiotic. It seems likely that the exogenous free fatty acid is the major source of hepatic triglycerides as reflected by the appropriate reduction in release of newly synthesized triglyceride, best demonstrated in the experiments in which tetracycline was added *in vitro*.

Certain differences were noted in perfused livers from rats treated with tetracycline *in vivo*. The total amount of [^{14}C]oleic acid esterified to triglyceride (columns D and E, Table 3) was diminished by some 30 per cent in both control and treated rats, in comparison to the experiments in which tetracycline was added *in vitro*. This decreased esterification of [^{14}C]oleic acid was appropriately accompanied by a decrease in relative specific activity of hepatic triglyceride compared with experiments in which antibiotic was added to the medium, and may result from an increase in hepatic triglyceride during the period in which the animals were treated with an infusion of drug or ascorbate alone. This effect may be related to the stress of the treatment period prior to removal of the liver for perfusion; this effect has been observed previously in relation to the stress of fasting [4].

As shown in Table 3, the sums of hepatic and perfusate triglycerides synthesized from [1-^{14}C]oleate are constant in both series of experiments. The conclusion is, therefore, justified, on the basis of the isotopic evidence, that the accumulation of hepatic triglycerides by livers from normal fed male rats perfused under the conditions of these experiments resulted from inhibition of outward transport of triglycerides derived from exogenous free fatty acid. This mechanism, further, probably is quantitatively of major importance in the pathogenesis of a fatty liver resulting from intoxication with tetracycline.

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