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METABOLIC EFFECTS OF CLOFIBRATE AND OF CHOLESTYRAMINE ADMINISTRATION TO DOGS*

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Abstract—Dogs were given clofibrate, 50 mg/kg of body weight/day for 6 days or 50 mg/kg of body weight/day for 2 days, followed by 40 mg/kg of body weight every 30–36 hr over an additional 9-day period. Cholestyramine, 0·7 g/kg of body weight/day, was administered to an additional group of dogs over an 11-day period. Plasma cholesterol concentrations in dogs treated with clofibrate for either 6 or 11 days decreased 24 per cent and decreased 16 per cent in dogs given cholestyramine. ^{14}C incorporation into bicarbonate from 3-^{14}C-pyruvate, from [U-^{14}C]-L-alanine and from [1-^{14}C]glucose was significantly increased by liver slices from clofibrate-treated dogs when compared with tissue from control animals, but clofibrate did not result in any changes in [^{14}C]cholesterol or [^{14}C]fatty acid formation from either [3-^{14}C]pyruvate or from [U-^{14}C]alanine. [^{14}C]Cholesterol formation from 6-[^{14}C]glucose was virtually abolished in liver slices taken from dogs treated with clofibrate for 11 days. Liver slices from dogs treated with cholestyramine showed a 3·5- to 4·0-fold increase in ^{14}C incorporation into cholesterol from both [3-^{14}C]pyruvate and [6-^{14}C] glucose, and a significant increase in ^{14}C incorporation from [1-^{14}C]glucose into bicarbonate. Clofibrate administration resulted in decreased liver glycogen concentrations and decreased ^{14}C incorporation into glycogen from [^{14}C]glucose, but ^{14}C incorporation into glycogen from [U-^{14}C]L-alanine was unaffected. If the hypocholesterolemic effect of clofibrate in dogs results from decreased cholesterol synthesis, changes in glucose metabolism may form the metabolic basis rather than alterations in pyruvate (or acetyl CoA) metabolism.

CLOFIBRATE is one of the more frequently prescribed drugs in current use for the management of hyperlipidemia of various origins. However, extensive clinical and experimental studies have not clearly defined the metabolic changes produced by clofibrate which are responsible for its hypotriglyceridemic and hypocholesterolemic effects. Included among the proposed mechanisms of action of clofibrate are a decrease in cholesterol synthesis,[1–3] decrease in hepatic lipoprotein secretion,[4] decrease in plasma unesterified fatty acid concentration,[5,6] decrease in triglyceride synthesis with or without accelerated removal of triglyceride from plasma,[7] and alterations in thyroid hormone distribution.[8,9]

The experiments reported here were planned to determine the metabolic consequences of clofibrate administration to dogs, which might be related to the hypocholesterolemic effects of the drug, and to compare the metabolic effects of clofibrate administration to the effects seen in dogs given cholestyramine. Dogs treated with cholestyramine were included as standards for comparison, since the pharmacodynamics of cholestyramine are well known.[10–12]

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METHODS

The experimental subjects were 34 mongrel dogs, 20 females and 14 males. All dogs were given unrestricted access to water and to a dry dog food diet (Purina Dog Chow, Ralston-Purina Company, St. Louis, Mo.) for 3 weeks prior to and throughout the experimental periods.

In an initial series of experiments, each of seven dogs, five females and two males, was given clofibrate, 50 mg/kg of body weight per day orally, for a period of 6 days. Seven additional dogs, five females and two males, served as controls. Blood samples were taken from each dog for determination plasma cholesterol concentrations at the start and at the end of the experimental period.

At the conclusion of the experimental period, each dog was anesthetized with pentobarbital sodium, 30 mg/kg, and was rapidly exsanguinated. Segments from three lobes of the liver were removed and placed in iced beakers. Liver slices were cut with a Stadie–Riggs tissue slicer, and slices weighing 500 $\pm$ 25 mg were incubated in bicarbonate buffer solution according to methods described by Gans and Shaefer.[13] The incubation solution was prepared containing one of the following substrates: (1) sodium pyruvate, 5 mM, to which [3-^{14}C]pyruvate was added to provide 0·5 μc ^{14}C/6 ml; and (2) L-alanine, 7·5 mM, to which was added [U-^{14}C]L-alanine* to provide 1·0 μc/6 ml. Liver slices from each dog were incubated in triplicate with each of the substrates.

The second series of experiments utilized 20 dogs, six as control, six treated with cholestyramine, and eight treated with clofibrate, the dogs in each treatment group having been divided equally between the sexes. The duration of treatment was 11 days. The dose of clofibrate in this series of experiments was 50 mg/kg body weight/day for the initial 2 days and was then reduced to 40 mg/kg body weight given at 30- to 36-hr intervals. This lower dose of clofibrate was selected for the 11-day experimental period, since Platt and Thorp[14] reported that the half-life of clofibrate in dogs is 40 hr. Cholestyramine was packed into gelatin capsules, and 0·7 g/kg body weight/day in two divided doses was administered to each dog.

Each dog was anesthetized with pentobarbital sodium, 30 mg/kg body weight, and the left kidney was retracted into an incision made in the left flank area. The hilus of the kidney was clamped, the kidney was removed, quickly drained of blood and placed in an iced beaker. Slices of cortex and medulla were cut with a Stadie-Riggs tissue slicer and 500 $\pm$ 25 mg quantities were incubated in solution containing [3-^{14}C]-pyruvate as outlined for liver slices. Through a midline incision, the cecum and ileum were brought into view. A segment of ileum measuring 15 cm from the cecum was isolated and clamps were placed on the intestine and on the blood vessels supplying this terminal portion of the ileum. The ileal segment was removed, flushed through twice with iced 0·154 M NaCl solution, cut longitudinally, and dropped into an iced electrolyte solution (0·154 M NaCl, 5·5 mM KCl) containing 5 mM glucose. The mucosal surface was washed with the iced solution and strips of ileal mucosa were cut with sharp scissors. Mucosal segments weighing 500 $\pm$ 25 mg were incubated in

* Obtained from New England Nuclear Corp., Boston, Mass. The specific radioactivity of [3-^{14}C]-pyruvate was 5·44 mc/m-mole and, when diluted with unlabeled pyruvate in the incubation solution, provided an initial specific radioactivity of 2·2 $\times$ 10^4 counts/min/μmole. The specific radioactivity of [U-^{14}C]-L-alanine was 123 mc/m-mole and, when diluted with unlabeled L-alanine in the incubation solution, provided an initial specific radioactivity of 2·78 $\times$ 10^4 counts/min/μmole.

solution containing [3-^{14}C]pyruvate according to the methods described. The dog was then exsanguinated, segments of the liver were removed, and liver slices were prepared and incubated with [3-^{14}C]pyruvate as described above. Additional 500 mg samples of liver slices were incubated in duplicate in: (1) a solution containing 5 mM glucose to which was added [1-^{14}C]glucose, and (2) a solution containing 5 mM glucose to which was added [6-^{14}C]glucose* to provide 1·0 μc ^{14}C/6 ml of solution.

At the conclusion of the incubation period, 1-ml aliquots of incubation medium from each flask were placed into diffusion flasks, 1 ml 5 N H_2SO_4 was added to each diffusion flask, the evolved $^{14}CO_2$ was trapped in 0·5 ml Hyamine, and the Hyamine was transferred to counting vials for radioassay.

Radioassays of [^{14}C]cholesterol and [^{14}C]fatty acids in the tissue slices were performed according to methods outlined by Gans and Shaefer.[13] Digitonin complexed with cholesterol does not interfere with the ferric chloride reagent, and this reagent was used to determine the cholesterol concentrations and subsequently the specific radioactivity and ^{14}C incorporation into cholesterol.

The fluor mixture consisted of 0·5% 2,5-diphenyloxazole and 0·1% 1,4 bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene in toluene. Ten ml of the fluor mixture and 2 ml ethanol were added to each counting vial. Radioassays were performed using a Packard Tri-Carb liquid scintillation spectrometer.

Tissues incubated with L-alanine or with glucose were transferred to centrifuge tubes containing 1 ml of 30% KOH and the centrifuge tubes were placed in a boiling water bath until complete dissolution of the tissue had occurred. Glycogen was precipitated by the addition of alcohol.[15] The glycogen pellet was redissolved in 1 ml water and again precipitated with alcohol. The supernatant fluid from each wash of samples of tissues incubated with L-alanine and with [6-^{14}C]glucose were combined and used for the estimation of ^{14}C incorporation into cholesterol and fatty acids as described.

The glycogen pellet was then dissolved in 1 ml of iced 10% trichloroacetic acid and centrifuged to remove any precipitate. The supernatant fluid was decanted and glycogen was precipitated by the addition of alcohol. Glycogen was then washed twice by dissolving in 1 ml water followed by the addition of alcohol. Each glycogen sample was dissolved in 1 ml water, aliquots were taken for appropriate dilution and estimation of glycogen concentration,[16] and 0·1-ml aliquots were added to counting vials and dispersed in 12 ml of the alcoholic fluor mixture containing 2·5% thixotropic gel (Cab-O-Sil).

Plasma and tissue cholesterol concentrations were determined according to methods previously described.[16] Liver glycogen concentrations were determined as outlined.

RESULTS

Plasma cholesterol concentrations. It was not possible to obtain dogs which were homogeneous with regard to plasma cholesterol concentrations. In order to provide an equal distribution among the various groups, we included within each group of dogs a large range of plasma cholesterol concentrations. The magnitude of the variations precluded the production by either clofibrate or cholestyramine of groups of

* Obtained from New England Nuclear Corp., Boston, Mass. The specific radioactivity of [1-^{14}C]-glucose was 8·85 mc/m-mole and of [6-^{14}C]glucose, 4·6 mc/m-mole. When diluted with unlabeled glucose in the incubation solution, the following initial specific radioactivities were obtained: [1-^{14}C]-glucose, 3·8 $\times$ 10^4 counts/min/μmole; [6-^{14}C]glucose, 2·7 $\times$ 10^4 counts/min/μmole.

TABLE 1. PLASMA CHOLESTEROL CONCENTRATIONS IN CONTROL DOGS AND IN DOGS TREATED WITH CLOFIBRATE OR WITH CHOLESTYRAMINE*

Treatment	N	Plasma cholesterol concentrations (mg/100 ml)			
		Start	6 days	11 days	Δ
First series, clofibrate for 6 days					
Control	7	163 ± 14·3	169 ± 13·2		+ 5·4 ± 5·3
Clofibrate	7	168 ± 34·4	128 ± 26·8 (> 0·05)		−40·3 ± 8·5 (< 0·01)
Second series, clofibrate or cholestyramine for 11 days					
Control	6	171 ± 14·0		171 ± 22·1	0 ± 9·2
Clofibrate	8	171 ± 14·2		129 ± 5·4 (< 0·05)	−42 ± 18·8 (> 0·05)
Cholestyramine	6	175 ± 16·0		147 ± 20·6 (> 0·05)	−20 ± 8·1 (< 0·05)

* All data in this and subsequent tables are expressed as the mean ± 1 standard error of the mean. Figures in parentheses indicate the level of significance of the difference between control and treatment groups.

dogs whose plasma cholesterol concentrations would be significantly different from those of the control groups (Table 1). The results, therefore, were compared statistically on the basis of both the absolute values and the mean changes in plasma cholesterol concentrations (Table 1).

Clofibrate in a dose of 50 mg/kg body weight/day for 6 days produced a mean decrease in plasma cholesterol concentrations of 40 mg/100 ml (Table 1). A decrease in plasma cholesterol concentrations of comparable magnitude was observed in dogs given clofibrate for 11 days at the reduced dose level (Table 1). In this second series, however, variability was greater because dogs with higher cholesterol concentrations (170 mg/100 ml or greater) showed large decreases in plasma cholesterol concentration in response to clofibrate, while in two dogs with relatively low plasma cholesterol concentrations (121 and 126 mg/100 ml), no changes accompanied clofibrate treatment. Cholestyramine administration resulted in a mean decrease in plasma cholesterol concentrations of 28 mg/100 ml (Table 1).

Liver cholesterol and glycogen concentrations. Cholesterol concentrations in the livers of control dogs were the same in both series of experiments, although greater variation was present in the first series (Table 2). Treatment with clofibrate did not significantly alter liver cholesterol concentrations in the first series of experiments (Table 2). In the second series of experiments, liver tissue from clofibrate-treated dogs, but not from dogs treated with cholestyramine, contained significantly greater concentrations of cholesterol (Table 2). In both series of experiments, glycogen concentrations were significantly reduced in livers from clofibrate-treated dogs (Table 2).

[3-^{14}C]-pyruvate metabolism. There was a significant increase in ^{14}C incorporation into bicarbonate medium by liver slices from clofibrate-treated dogs (Table 3). ^{14}C incorporation into cholesterol and into fatty acids was not changed by clofibrate administration (Table 3).

Prior to beginning the second series of experiments, we performed a group of experiments to determine the relative rates of [^{14}C]cholesterol formation from [3-^{14}C]-pyruvate by mucosal strips from dog terminal ileum and jejunum. The mucosa of the terminal ileum was much more active than that of the jejunum, indicating a basic similarity in regional intestinal cholesterogenesis when the dog is compared with the

TABLE 2. LIVER CHOLESTEROL AND GLYCOGEN CONCENTRATIONS IN DOGS TREATED WITH CLOFIBRATE OR CHOLESTYRAMINE

Treatment	N	Cholesterol (mg/g wet wt.)	Glycogen* (mg/g wet wt.)
First series of experiments			
Control	7	2·4 ± 0·12	63·2 ± 6·3
Clofibrate	7	2·7 ± 0·13	16·9 ± 5·8 (<0·01)
Second series of experiments			
Control	6	2·4 ± 0·06	47·2 ± 5·6
Clofibrate	8	3·0 ± 0·14 (<0·01)	12·5 ± 3·0 (<0·01)
Cholestyramine	6	2·5 ± 0·10	44·5 ± 12·0

* Expressed as milligrams of glucose. Figures in parentheses indicate significance of difference between respective groups.

TABLE 3. METABOLISM *in vitro* OF [3-^{14}C]PYRUVATE BY LIVER SLICES FROM CONTROL DOGS AND FROM DOGS TREATED WITH CLOFIBRATE OR WITH CHOLESTYRAMINE

Treatment	N	% Incorporation added ^{14}C/500 mg tissue/3 hr into		
		Bicarbonate	Cholesterol	Fatty acids
First series of experiments, treatment period 6 days				
Control	7	7·3 ± 0·94	1·04 ± 0·21	3·55 ± 0·97
Clofibrate	7	14·3 ± 0·53 (<0·01)	1·20 ± 0·23	3·43 ± 0·50
Second series of experiments, treatment period 11 days				
Control	6	8·6 ± 0·7	1·14 ± 0·13	2·20 ± 0·24
Clofibrate	8	14·8 ± 0·7 (<0·001)	0·98 ± 0·18	2·60 ± 0·69
Cholestyramine	6	10·4 ± 1·4	4·12 ± 0·25 (<0·001)	4·32 ± 1·38

rat and the squirrel monkey.[17,18] Accordingly, ileal mucosa was used in the second series of experiments.

^{14}C incorporation into liver cholesterol from [3-^{14}C]pyruvate was increased by a factor of 3·5 in liver slices taken from cholestyramine-treated dogs (Table 3). Neither cholestyramine nor clofibrate treatment resulted in changes in [^{14}C]cholesterol formation or in ^{14}C incorporation into fatty acids and bicarbonate by ileal mucosa or by kidney slices. The tabulation of these negative results has been omitted.

Metabolism of L-*alanine.* ^{14}C incorporation from [U-^{14}C]L-alanine into bicarbonate was greater with liver slices from clofibrate-treated dogs than with liver slices of control dogs (Table 4). The formation of ^{14}C-labeled cholesterol, fatty acids and glycogen was comparable in liver slices from clofibrate-treated and from control dogs.

^{14}C-glucose metabolism. ^{14}C incorporation into bicarbonate from [1-^{14}C]glucose was greater than that from [6-^{14}C]glucose in control tissue, giving a C_1/C_6 ratio of 1·2 ± 0·06 (Table 4). A significant increase in ^{14}C incorporation into bicarbonate from [1-^{14}C]glucose was recorded in liver tissue from dogs treated with cholestyramine and from dogs treated with clofibrate; ^{14}C incorporation into bicarbonate from [6-^{14}C]glucose was not changed and the C_1/C_6 ratio in both treatment groups was increased. Limited formation of [^{14}C]cholesterol from [6-^{14}C]glucose occurred; the incorporation of [^{14}C] from [6-^{14}C]glucose into cholesterol was increased in liver tissue from cholestyramine-treated dogs, but was virtually undetectable in liver tissue from clofibrate-treated dogs (Table 4). ^{14}C Incorporation from [6-^{14}C]glucose into fatty acids was not significantly changed by either treatment (Table 4).

^{14}C Incorporation into glycogen of liver slices from control dogs was similar whether the source was [1-^{14}C] or [6-^{14}C]glucose and was unchanged in tissue from cholestyramine-treated dogs (Table 4). Treatment with clofibrate, however, resulted in a decrease in ^{14}C incorporation from [^{14}C]glucose into glycogen (Table 4).

Neither the administration of clofibrate nor of cholestyramine was accompanied by any changes in body weight.

DISCUSSION

Cholestyramine binds bile salts and accelerates the fecal excretion of these acid steroid metabolites of cholesterol.[10–12] The large increase in [^{14}C]cholesterol formation

TABLE 4. METABOLISM OF [^{14}C]ALANINE AND OF [^{14}C]GLUCOSE BY DOG LIVER SLICES *in vitro* FROM CONTROL DOGS AND FROM DOGS TREATED WITH CLOFIBRATE OR WITH CHOLESTYRAMINE

Group	N	% Incorporation added ^{14}C/500 mg tissue/3 hr into				
		Bicarbonate	C_1/C_6	Cholesterol	Fatty acids	Glycogen
From [U-^{14}C]L-alanine						
Control	7	9·6 ± 1·20		0·30 ± 0·05	1·03 ± 0·28	0·72 ± 0·13
Clofibrate	7	13·6 ± 1·00 (<0·05)		0·36 ± 0·07	1·23 ± 0·22	0·77 ± 0·30*
From [1-^{14}C]glucose						
Control	5	1·4 ± 0·17	1·2 ± 0·06			2·0 ± 0·67
Clofibrate	7	2·1 ± 0·12 (<0·01)	1·6 ± 0·12 (<0·02)			0·4 ± 0·12 (<0·05)
Cholestyramine	6	3·4 ± 0·64 (<0·05)	2·1 ± 0·27 (<0·02)			1·7 ± 0·60
From [6-^{14}C]glucose						
Control	5	1·2 ± 0·16		0·07 ± 0·02	0·11 ± 0·02	2·0 ± 0·60
Clofibrate	7	1·4 ± 0·13		Trace†	0·08 ± 0·04	0·5 ± 0·16 (<0·05)
Cholestyramine	6	1·6 ± 0·17		0·29 ± 0·06 (<0·02)	0·32 ± 0·12	2·0 ± 0·60

* Average of samples from four experiments. Liver slices from three experiments contained no glycogen after incubation.
† Less than 0·05% incorporation added ^{14}C.

from [^{14}C]pyruvate and from [^{14}C]glucose by liver slices from cholestyramine-treated dogs was anticipated, since the entire process of liver cholesterol synthesis and bile salt formation should be accelerated as the result of enhanced bile salt excretion.[12] Changes in glucose oxidation also may have been related to the effects of cholestyramine. The initial oxidative step in the pentose phosphate pathway involves the formation of NADPH, which is an essential cofactor in the hydroxylation of cholesterol.[19,20] Thus an increase in oxidation of [1-^{14}C]glucose by the liver of cholestyramine-treated dogs indicates enhanced glucose oxidation via the pentose phosphate pathway, thereby providing the additional NADPH required for more rapid formation and excretion of bile salts.

Clofibrate administration for 6 or 11 days did not alter cholesterogenesis *in vitro* from pyruvate by dog liver, intestinal mucosa or kidney. These results indicate a species difference between the dog and the rat, since hepatic cholesterol synthesis from acetate in the rat is markedly diminished by clofibrate treatment.[1,2] [^{14}C]Cholesterol formation from [6-^{14}C]glucose, however, was virtually abolished in liver slices from dogs treated with clofibrate for 11 days. If the hypocholesterolemic effect of clofibrate does result from decreased cholesterol synthesis in the dog, as apparently it does in the rat[1,2] and in man,[3] the metabolic control sites influenced by clofibrate would appear to involve glucose rather than pyruvate (or acetyl CoA) metabolism.

The results of experiments on liver glycogen constitute additional evidence for a prominent effect of clofibrate on glucose metabolism. Decreases in liver glycogen concentrations and in the incorporation *in vitro* of [^{14}C]glucose into liver glycogen have been observed in rats treated with clofibrate[21] and occurred also in clofibrate-treated dogs. The decrease in [^{14}C]glucose incorporation into glycogen by liver slices occurred to the same extent as the decrease in glycogen concentrations in liver tissue from dogs treated with clofibrate, the specific radioactivity of the glycogen being the same in liver slices from control as from clofibrate-treated dogs. These results suggest that clofibrate administration is accompanied by a decrease in glycogen synthesis from glucose, although effects of the drug on glycogenolysis cannot be excluded. Moreover, there was no decrease in gluconeogenesis in liver from clofibrate-treated dogs, since ^{14}C incorporation into glycogen from [^{14}C]alanine was the same in tissue from clofibrate-treated as from control dogs.

In summary, a comparison of the metabolic effects of clofibrate and cholestyramine administration to dogs suggests the following conclusions: (1) Changes in hepatic pyruvate and glucose metabolism in cholestyramine-treated dogs are directly related to the increased excretion of bile salts with consequent enhancement of cholesterol (and presumably bile salt) synthesis. (2) Clofibrate, however, appears to alter hepatic glucose metabolism, and changes in glucose metabolism, rather than in pyruvate (or acetyl CoA) metabolism, may form the basis for the hypocholesterolemic effects of clofibrate in the dog.

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