 0030-0950/85/0003-0097 $2.00/0

THE EFFECTS OF CROCETIN ON PLASMA LIPIDS IN RATS[1]

JENNIFER CAPPEL COUSINS[2] and THEODORE L. MILLER,[3] Department of Chemistry, Ohio Wesleyan University, Delaware, OH 43015

ABSTRACT. The caroteroid compound, crocetin, has been shown to lower plasma lipid levels in previous animal studies. In this investigation the total cholesterol, HDL cholesterol and total triglyceride levels were monitored in rats fed two different hyperlipemic diets for either 22 or 168 d depending on the diet. Injections of crocetin via subcutaneous and intraperitoneal routes were compared. No significant hypolipemic action was observed for any of the groups receiving crocetin injections in this study. The results clearly show that subcutaneous injections of crocetin in rats do not lower plasma lipid levels. Since crocetin has been shown to lower plasma lipid levels in rats given intraperitoneal injections of crocetin, hepatic activation or higher blood concentrations of the drug is apparently required in rats. The results also suggest that the mechanism responsible for crocetin's physiological activity is different in rats and rabbits.

OHIO J. SCI. 85 (3): 97-101, 1985

INTRODUCTION

Crocetin (8,8′-diapo-ψ,ψ′-carotenedioic acid) has been used in the treatment of experimental atherosclerosis in rabbits and rats (Gainer and Chisolm 1974, Gainer and Jones 1975, Pool et al. 1976). These studies along with other investigations (Gainer et al. 1976, Wilkins et al. 1977, Chisolm et al. 1973, Wilkins and Gainer 1979) were based on the drug's ability to increase oxygen diffusivity in plasma (Chisolm et al. 1972, Chisolm and Gainer 1973). More recently, pharmacokinetic parameters (J. L. Gainer, pers. comm. 1983) have been determined.

The physiological mechanism of crocetin activity is not understood, but there is evidence that it might involve the prevention of hypoxia (Chilsolm et al. 1972, 1973, Gainer and Chisolm 1974, Pool et al. 1976). The striking reduction in the severity of experimental atherosclerosis in rabbits stimulates us to investigate the mechanism of crocetin activity. Results of absorption and fluorescence studies indicate that crocetin binds strongly to albumin (Miller et al. 1982). Thus, the mechanism by which crocetin reduces the effects of experimental atherosclerosis and increases oxygen diffusivity must reflect strong plasma albumin binding.

The initial goal of this study was to determine if using crocetin in rats with diet-induced hyperlipemia caused a change in the relative distribution of plasma lipoproteins. The results for several groups of rats fed two different hyperlipemic diets are outlined.

METHODS AND MATERIALS

Sprague-Dawley rats weighing about 200 g each were used. Only male rats were studied, and they were allowed to eat and drink *ad libitum*. All groups were fed either a hypercholesterolemic (Schurr et al. 1976) or high butterfat (Pool et al. 1976) diet. Different groups of rats were used in each study.

The crocetin used in this investigation was extracted from saffron, a spice containing several carotenoids, in the laboratory of John L. Gainer, University of Virginia. The purity of samples is routinely determined spectroscopically (λ_{max} = 420 nm) and by melting point determinations. The purity was confirmed by HPLC studies in our laboratory.

Solid crocetin was dissolved in a solution of 0.16 M NaCl that was slightly basic, pH 7.7. One group of animals was given the drug in 0.01 M phosphate buffer at pH 7.7, with 0.16 M NaCl.

[1]Manuscript received 7 December 1984 and in revised form 21 February 1985 (#84-56).

[2]Present Address: Department of Chemistry, University of Illinois at Urbana-Champaign, Urbana, IL 61801

[3]Author to whom correspondence should be sent.

Concentrations of crocetin solutions used in the injection program were determined spectroscopically in terms of SM units. One SM unit is the amount of crocetin per ml of solution which results in an absorbance of 1.00 at 445 nm (J. L. Gainer, pers. comm. 1983). Each crocetin treated group received subcutaneous or intraperitoneal injections twice a day, 0.15 units per ml total blood volume. Dosage was determined by estimating the total blood volume from body weight. The control animals received injections of isotonic saline in comparable volumes on the same schedule as the treated groups.

Blood samples were taken with heparinized capillary tubes from the medial orbital of the eye with the animals lightly etherized during the procedure. The animals were fasted for 16 h before the blood was collected. Serum samples were stored at 0 to 5°C, and all analyses were completed within 28 h. Duplicate serum samples were analyzed for all rats, and the average value is reported for each group.

Total serum cholesterol and triglyceride levels were determined by the enzymatic method using a manual procedure (A-Gent reagents by Abbott Laboratories). The high density lipoprotein (HDL) cholesterol levels were determined by a precipitation procedure coupled with the enzymatic method. Lower density lipoproteins were removed from the serum by precipitation with a sulfated polysacharride-divalent metal cation reagent (Steel et al. 1976, Warnick and Albers 1978). Pooled serum standards (Sigma Chemical Co.) were analyzed to evaluate the reproducibility of the manual enzymatic methods. The overall relative standard deviation for all of the results was about seven percent.

RESULTS AND DISCUSSION

Crocetin exhibits unusual chemistry when dissolved in phosphate buffer (Miller et al. 1982). Consequently, the first study was designed to evaluate the effect of crocetin treatment when the drug is administered in phosphate buffer. Three groups of six animals were used in this study: a control group, a group treated with crocetin in NaCl solution and a group treated with crocetin in NaCl solution buffered with phosphate. All of the animals were fed a hypercholesterolemic diet (Schurr et al. 1976), and the injection program was initiated on the day the special diet was introduced. All injections were administered subcutaneously.

The results of the serum analyses for the three groups of rats are compiled in table 1. Over the 168 d of the investigation no significant difference in the total cholesterol levels was observed. In fact, the values for the treated groups were slightly higher than the control group over most of the test period. The same trend was observed for the total serum triglyceride levels. However, the triglyceride levels were not substantially elevated. HDL cholesterol levels remained essentially constant during the test period indicating that crocetin doesn't appear to facilitate the formation of high density lipoproteins.

Although crocetin has been shown to be effective in lowering lipid levels in rats (Pool et al. 1976), no reduction was observed under the experimental conditions used in study one. However, two important parameters were varied in this investigation. First, the diet contained a much lower level of cholesterol and fat. The second difference was the injection mode; the drug was injected subcutaneously in this study but by intraperitoneal injections in the previous rat study (Pool et al. 1976). A second study was undertaken to examine these differences.

The high butterfat diet (Pool et al. 1976) was used in the second study. This diet contains 5.4% cholesterol and 40% butterfat so the plasma lipid levels are elevated much more. The animals quickly become lethargic, dehydrated and contracted severe diarrhea on this diet. After seven days on the diet, 24 rats were divided into three groups, and the crocetin injections were started. Crocetin in 0.16 M NaCl solution was administered by either subcutaneous or intraperitoneal injections.

The results of the serum analyses for the second study are shown in table 2. After 29 d on the diet and 22 d of treatment, a substantial increase in total serum cholesterol levels was observed. The results for the intraperitoneal group is similar to the earlier study (Pool et al. 1976, J. L. Gainer, pers. comm. 1983). They observed initially higher levels of serum cholesterol in the treated group but after 24 d of treatment, all of the treated groups had lower cholesterol levels than the control group. Although not statistically significant, the trend of lower values after 22 d of

TABLE 1

Serum lipid levels for study one.

Day of Test	Group*	Total Cholesterol (mg/dL)	HDL Cholesterol (mg/dL)	Total Triglycerides (mg/dL)
1	Control (6)	120 ± 8	37 ± 6	124 ± 16
	Water (6)	118 ± 17	38 ± 6	149 ± 17
	Phosphate (6)	113 ± 10	40 ± 8	121 ± 20
15	Control (6)	239 ± 79	32 ± 8	101 ± 8
	Water (6)	223 ± 57	36 ± 5	117 ± 20
	Phosphate (6)	266 ± 39	30 ± 6	120 ± 17
38	Control (6)	343 ± 86	45 ± 8	138 ± 27
	Water (6)	409 ± 91	35 ± 5	124 ± 21
	Phosphate (6)	509 ± 129	29 ± 5	107 ± 25
59	Control (6)	476 ± 113	38 ± 8	113 ± 10
	Water (6)	457 ± 125	38 ± 11	112 ± 9
	Phosphate (4)	518 ± 99	39 ± 5	122 ± 18
80	Control (6)	384 ± 53	34 ± 9	106 ± 10
	Water (6)	403 ± 134	33 ± 8	127 ± 30
	Phosphate (4)	390 ± 123	27 ± 7	125 ± 18
119	Control (6)	337 ± 78	37 ± 7	129 ± 27
	Water (6)	411 ± 180	38 ± 10	112 ± 15
	Phosphate (4)	503 ± 59	39 ± 11	140 ± 38
168	Control (6)	372 ± 113	55 ± 8	141 ± 40
	Water (6)	449 ± 178	54 ± 3	168 ± 64
	Phosphate (4)	440 ± 98	34 ± 18	149 ± 29

*Groups: Control — control group receiving injections of saline. Water — treated group receiving injections of crocetin in 0.16 M NaCl solution. Phosphate — treated group receiving injections of crocetin in NaCl solution buffered with phosphate. The number of animals in the group on a given analysis day is shown in parentheses.

treatment with intraperitoneal injections began to emerge in our data, but mortality among this group was high making interpretation difficult. However, the cholesterol level for the group receiving subcutaneous injections was higher than the control group. There were no significant differences among the groups for either the total triglyceride or HDL cholesterol levels.

Finally, a third study was performed to clearly establish the trends of serum lipid levels in animals receiving subcutaneous injections of crocetin. The conditions outlined for study two were employed, but the drug was only injected subcutaneously. The results of the serum analyses for the third study are presented in table 2. It now appears clear that crocetin administered by subcutaneous injections is ineffective in producing the lipid lowering effects observed with intraperitoneal injections.

There are at least two possible explanations for this effect: (1) intraperitoneal injection provides a bolus of material that is absorbed fairly efficiently and results in higher crocetin concentrations in the blood, or (2) microsomal activity of the liver may be responsible for the lipid lowering capacity of crocetin in rats. However, the mechanism for the effect of crocetin on lipid levels in rabbits (Gainer and Chisolm 1974, Gainer and Jones 1975, Pool et al. 1976) must be different or the drug must be effective at much lower levels because the drug was introduced by intramuscular injections in those studies. Although crocetin appears to aid in the prevention of atherosclerosis in rabbits and to reduce the serum lipid levels in rats, additional in-

TABLE 2
Serum lipid levels for study two and three.

Study	Day of Treatment*	Group**	Total Cholesterol (mg/dL)	HDL Cholesterol (mg/dL)	Total Triglycerides (mg/dL)
Two	0	Control (8)	227 ± 72	38 ± 7	193 ± 59
		Subcu (8)	229 ± 48	37 ± 8	183 ± 37
		IP (8)	226 ± 57	45 ± 14	171 ± 51
	9	Control (8)	540 ± 83	47 ± 22	308 ± 89
		Subcu (7)	518 ± 91	56 ± 16	289 ± 48
		IP (6)	411 ± 134	61 ± 26	289 ± 52
	14	Control (7)	431 ± 103	40 ± 6	356 ± 108
		Subcu (6)	480 ± 120	27 ± 4	333 ± 54
		IP (6)	552 ± 78	31 ± 4	383 ± 43
	22	Control (7)	574 ± 90	34 ± 2	242 ± 114
		Subcu (6)	699 ± 114	33 ± 6	291 ± 120
		IP (4)	543 ± 102	31 ± 6	297 ± 112
Three	0	Control (8)	228 ± 55	NA#	167 ± 55
		Subcu (8)	227 ± 54	NA	222 ± 79
	7	Control (8)	525 ± 251	NA	219 ± 38
		Subcu (8)	427 ± 117	NA	241 ± 57
	14	Control (8)	573 ± 196	NA	177 ± 41
		Subcu (8)	496 ± 212	NA	188 ± 67
	21	Control (8)	543 ± 150	NA	245 ± 41
		Subcu (8)	684 ± 322	NA	241 ± 76

*Treatment started after seven days on the high butterfat diet.
**Groups: Control — control group receiving injections of saline, subcutaneous once a day and intraperitoneal once a day. Subcu — treated group receiving subcutaneous injections of crocetin. IP — treated group receiving intraperitoneal injections of crocetin. The number of animals in the group on a given analysis day is shown in parentheses.
#NA — No analysis.

vestigations are required to elucidate the mechanism responsible for its activity.

ACKNOWLEDGMENTS. The authors gratefully acknowledge the support of this work by the General Electric Foundation. They also thank Howard A. I. Newman and Suman T. Patel, Department of Pathology, The Ohio State University, for assistance during the initial stage of the study and Diane Gerkin, Department of Pharmacology, The Ohio State University, for helpful suggestions.

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