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In Vivo Inhibition of Cholesterol Uptake in Rabbit Aortas by 7-Ketocholesterol[1] (39197)

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Previously we have presented evidence of *in vitro* inhibition of cholesterol uptake by coronary arteries of man, pig, and rabbit aortas after addition of 7-ketocholesterol to the perfusion fluid (1). Kandutsch and Chen, and Brown and Goldstein have shown that 7-ketocholesterol in cell linings from man and mice inhibits cholesterol synthesis through inhibition of the enzyme HMG-CoA reductase (2, 3).

Our published data suggested that in the perfused artery, inhibition of cholesterol uptake occurs by a competitive process (1). No data are available in the literature concerning inhibition of cholesterol uptake by the vascular wall *in vivo*. The subsequent experiments are concerned with results of the inhibitory effect of 7-ketocholesterol on cholesterol uptake of aortas of rabbits *in vivo*.

Methods. Twenty-four female New Zealand rabbits were used. The inhibitory steroid, 7-ketocholesterol, was administered by iv injection, using a bile salt (sodium glycocholate) as the solubilizing agent. The control animals received only the bile salt. In order to measure cholesterol uptake by the arterial wall, about 5 ml of the animal's own plasma was sonicated with 250 μCi of [^{3}H]-1,2-cholesterol which was injected into the rabbit through an ear vein. The rabbit was sacrificed 1 1/2 hr later, and the aorta (between left carotid artery branch point and diaphragm) was dissected, cleaned, and washed. A blood sample was collected just prior to killing the animal. The aorta and plasma were analyzed as described below. A water soluble complex of sodium glycocholate and 7-ketocholesterol (molecular ratios 2:1) was prepared which remained stable for about 3 min. Twenty-five milligrams of 7-ketocholesterol and 50 mg of bile salt were dissolved in about 1.5 ml of 2:1 chloroform methanol mixture. The solution was then evaporated at a fast rate. Five milliliters of sterile saline were then added. The mixture dissolved in saline, forming a clear solution which was immediately injected into the animal. If the saline solution was left standing, 7-ketocholesterol separated in about 3 min. If this solution was injected into the rabbits, the animal died within a few minutes. The animals received four or six injections in equal intervals during a 1 1/2-hr period.

When 7-ketocholesterol was administered by stomach tube, 200–400 mg of 7-ketocholesterol along with an equal amount of sodium glycocholate (Steraloids, Inc., Pawling, New York) were dissolved in about 15 ml of 2:1 chloroform methanol mixture, and the solvent was evaporated *in vacuo*. The resultant solid mixture was suspended in 20 ml of saline by means of a glass homogenizer and fed to the rabbit through a No. 7 Foley catheter introduced into the stomach. The balloon of the Foley catheter was partially filled with a contrast material (gastrographin) to verify its position below the diaphragm under a fluoroscope. For feeding purposes, the ratio of bile salt to the steroid was reduced to 1:1 because of the laxative effect of the bile salt.

To make [^{3}H]cholesterol soluble for iv injection, 250 μCi of [^{3}H]cholesterol dissolved in benzene were placed into a test tube and dried under a stream of dry N_2. About 5 ml of freshly prepared plasma were added to this tube, placed in an ice bath, and twice subjected to sonication (at 20 kHz, 20

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W) for 30 sec each, with an interval of 1 min. Lipoprotein denaturation was prevented by maintaining low temperature and ultrasonic power output (4). The electrophoretic patterns of sonicated and unsonicated rabbit plasma were identical. The radioactivity in the sonicated plasma was concentrated mainly in the pre-β- and β-lipoprotein fractions, and to a lesser extent in the chylomicrons. This plasma was then brought to room temperature and injected into the rabbit from which the plasma had been obtained. The aorta was opened longitudinally, the adhering tissue and most of the adventitia were scraped with a razor blade (5), and the remaining fibrous tissue was carefully dissected in a saline trough. The tissue was then washed with several exchanges of saline, blotted, weighed, and crushed into a fine powder under liquid nitrogen. The lipids from this crushed tissue were extracted using the procedure of Folch *et al.* (6). Separation of lipids was carried out by means of thin-layer chromatography on silica gel (F-254) according to the method of Freeman and West (7). 7-Ketocholesterol fractions and cholesterol were identified by means of authentic samples developed simultaneously. The free cholesterol fraction was scraped directly into a counting vial and the ^{3}H-radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer in the presence of a toluene–dioxane scintillator (8). The 7-ketocholesterol fraction was eluted from the silica gel using Folch mixture and was quantitatively estimated in a Packard 7400 dual-flow gas chromatograph (9). The plasma lipids were extracted by the method of Folch *et al.* (6) using 5 ml of plasma and 100 ml of Folch mixture. The total and free cholesterol contents of the lipid extract were measured by the method of Zak *et al.* (10). The thin-layer and gas-chromatographic, as well as liquid-scintillation, counting procedures were identical to those used for the aorta. The cholesterol uptake by the aorta was calculated using the following formula:

$$\text{Cholesterol uptake (nmole/g)} = \frac{[^3\text{H}]\text{DPM/g aorta} \times \text{free cholesterol content (nmole/ml) of plasma}}{[^3\text{H}]\text{DPM/ml plasma}},$$

where DPM represents the disintegrations per minute.

Results. Table I illustrates that the aortic uptake of cholesterol was less in the presence of 7-ketocholesterol in plasma (mean 19.5 ± 2.1, as compared to 31.8 ± 6.1). This difference was statistically significant ($P < 0.05$). Animals that received six injections of 7-ketocholesterol had, in general, higher plasma and aortic concentrations of this steroid (Expts. 8–13). Table II summarizes the results of gastric feeding of 7-ketocholesterol on cholesterol uptake by the aorta. It is noted that the 7-ketocholesterol in plasma was low (mean 2.2 ± 0.4 nmole/ml plasma) as compared to animals receiving iv injection of the steroid. In animals fed 7-ketocholesterol, no inhibition of aortic cholesterol uptake could be detected (Table II). This was probably the result of the low plasma concentration of 7-ketocholesterol in these experiments.

Discussion. The *in vivo* experiments reported here on cholesterol uptake by rabbit aortas after iv injection of 7-ketocholesterol confirm previous findings in which it could be shown *in vitro* that the presence of 7-ketocholesterol inhibits cholesterol uptake by coronary arteries or aortas of humans, pigs, and rabbits (1). Rabbits were employed in this series, since the inhibition of aortic cholesterol uptake by 7-ketocholesterol had been previously demonstrated *in vitro*. Future *in vivo* experiments will be extended to other species. Apparently the inhibitory effects of 7-ketocholesterol are present in a wide variety of species as demonstrated *in vitro* (1). Table I illustrates that *in vivo* inhibition depends on a certain threshold plasma level of 7-ketocholesterol. Only after multiple (four to six) iv injections, each containing 25 mg (group A, Table I) over a period of 1 1/2 hr with bile salts, was it possible to demonstrate an inhibitory effect. The difference in cholesterol uptake between this group and the control (animals injected with bile salts only) was significant ($P < 0.05$; Table I). The plasma concentrations of the steroid after gastric feeding were insufficient to inhibit aortic cholesterol uptake ($P > 0.1$; Table II). Parl and Gutstein have demonstrated that in rats after 24 hr, elevated blood levels of bile salts, resulting from biliary obstruction, and epithelial cells

TABLE I. EFFECT OF 7-KETOCHOLESTEROL INJECTIONS ON AORTIC CHOLESTEROL UPTAKE.

Rabbit number	Plasma concentrations (nmole/ml)			Aortic uptake (nmole/ml)	
	7-Ketocholesterol	Free cholesterol	Total cholesterol	Free cholesterol	7-Ketocholesterol
Group A					
A1[a]	3.5	109	312	8.1	6.0
A2[a]	4.2	140	491	26.0	7.2
A3[a]	5.7	200	673	28.1	6.7
A4[a]	7.7	312	923	25.2	3.7
A5[a]	8.5	190	523	30.2	13.2
A6[a]	8.7	491	1347	10.1	6.2
A7[a]	10.7	452	1287	29.1	8.5
A8[b]	10.5	182	624	19.0	5.2
A9[b]	20.0	340	1034	19.0	9.5
A10[b]	20.2	510	1130	18.6	17.3
A11[b]	31.7	400	1000	16.0	25.6
A12[b]	16.0	350	970	13.0	19.4
A13[b]	20.5	360	900	11.5	15.0
				19.5 ± 2.1	
Group B					
B1[a]		421	1186	32.2	
B2[a]		291	663	37.2	
B3[a]		151	562	81.4	
B4[a]		473	1698	35.1	
B5[a]		855	2563	34.1	
B6[b]		312	814	15.1	
B7[b]		242	663	26.0	
B8[b]		320	680	20.7	
B9[b]		430	860	22.0	
B10[b]		290	770	19.6	
				31.8 ± 6.1	

[a] Four injections.
[b] Six injections.

TABLE II. EFFECT OF FEEDING OF 7-KETOCHOLESTEROL IN BILE SALT.

Experiment number	Amount of 7-ketocholesterol fed (mg)	Bile salts added (mg)	Number of feedings[a]	Plasma concentrations (nmole/ml)			Uptake by aorta (nmole/g)	
				7-Ketocholesterol	Total cholesterol	Free cholesterol	Cholesterol	7-Ketocholesterol
D1	200	500	4	2.0	1030	541	34.9	14.7
D2	200	200	13	1.3	1030	370	40.6	14.0
D3	200	200	13	3.0	820	349	31.6	16.7
D4	400	400	8	2.3	1140	401	30.3	23.0
				2.2 ± 0.4			34.4 ± 2.3	17.1 ± 2.0
E1	–	500	4	–	1759	569	19.9	–
E2	–	200	13	–	2481	880	33.9	–
E3	–	200	13	–	1940	611	33.1	–
E4	–	400	8	–	911	339	24.1	–

[a] Feedings were carried out twice daily.

of various target organs, including coronary arteries, are damaged (11). However, in control experiments, when bile salts were administered no change in cholesterol uptake was found.

The results show the importance of adequate plasma concentrations of 7-ketocholesterol in the inhibition of aortic cholesterol uptake *in vivo*. Although a statistically significant inhibition of cholesterol uptake by 7-ketocholesterol was present after iv injection (Table I), this inhibition was not as marked or as uniform as that previously demonstrated in *in vitro* experiments (1).

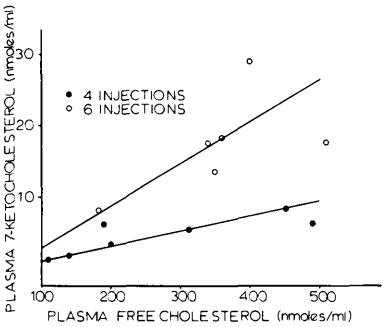

FIG. 1. Plasma cholesterol vs 7-ketocholesterol concentrations in rabbits after multiple iv injections of 7-ketocholesterol with bile salt.

The difference appears to be due to lower plasma concentrations of 7-ketocholesterol in injected animals (8.8 ± 1.6 nmole/ml *in vivo*, as compared to from 50–1000 nmole/ml *in vitro*). The relationship between plasma concentrations of 7-ketocholesterol and cholesterol following iv injection of the former are illustrated in Fig. 1. A direct relationship is demonstrated, suggesting that the two steroids bind to plasma lipoproteins in a similar manner. At higher plasma levels of 7-ketocholesterol (Expts. A8–A13, Table I), the slope increases (Fig. 1), indicating that lipoprotein binding sites in plasma are still not saturated. Uptake of 7-ketocholesterol by the aorta could be confirmed in the experiments of this series. We have previously demonstrated that human coronary arteries do not synthesize cholesterol *in vitro*, suggesting a lack of HMG-CoA reductase activity. However, there was significant inhibition of cholesterol uptake by these vessels in the presence of 7-ketocholesterol (12). Therefore, it is unlikely that inhibition of the enzyme HMG-CoA reductase plays a significant role in the inhibition of cholesterol uptake by aortas or coronary arteries. It appears that competitive inhibition may be responsible for the effect of 7-ketocholesterol on cholesterol uptake. This is suggested by the observation of a direct relationship between the plasma concentrations of the two steroids, but definite evidence for this is still lacking.

Summary. The iv injection of 7-ketocholesterol into rabbits, made soluble by combining with bile salts, inhibited cholesterol uptake by the aorta. However, the inhibition was not as marked or as uniform as previously demonstrated in *in vitro* experiments. This difference may have been the result of lower plasma concentrations of 7-ketocholesterol in the injected animals. Gastric feeding of 7-ketocholesterol failed to inhibit aortic cholesterol uptake, probably because of inadequate plasma concentrations of the inhibitory steroid. The results suggest that the mechanism of 7-ketocholesterol on aortic cholesterol uptake is through competitive inhibition.

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