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EFFECTS OF 4-AMINOPYRAZOLOPYRIMIDINE ON RABBIT PLASMA CHOLESTEROL, PLATELET 3-HYDROXY-3-METHYL-GLUTARYL-COENZYME A REDUCTASE AND PLATELET AGGREGATION

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Abstract—Administration of 4-aminopyrazolopyrimidine (4-APP) to rabbits resulted in a 63 per cent decrease in plasma cholesterol concentration and a 9-fold increase in platelet hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase activity. In individual rabbits, the degree of stimulation of HMG-CoA reductase activity was strongly correlated with the extent of reduction of plasma cholesterol (r 0.987). Reduction of plasma and platelet cholesterol concentrations by 4-APP treatment was associated with reduced sensitivity of platelets to aggregation by adenosine diphosphate and collagen.

In view of the possible interrelationships between plasma cholesterol and platelets in atherogenesis, several investigators have studied platelet function in hypercholesterolemic patients [1, 2]. Carvalho *et al.* [2] demonstrated that platelets from patients with type IIa hyperlipoproteinemia aggregate in response to lower concentrations of epinephrine and adenosine diphosphate than do platelets from normolipemic individuals. Later Shattil *et al.* [3] demonstrated that platelets incubated with cholesterol-rich liposomes acquired cholesterol from these vesicles and also became more sensitive to epinephrine and ADP-induced aggregation. Platelets incubated with cholesterol-poor liposomes lost cholesterol to the medium and became less sensitive to the aggregating agents. The effect of *in vivo* reduction of plasma cholesterol on platelet function has not been well investigated. In the present study, rabbits were injected with 4-aminopyrazolopyrimidine (4-APP) to reduce their plasma cholesterol levels. Balasubramaniam *et al.* [4, 5] have shown that 4-APP treatment of rats results in a several-fold increase of hydroxymethylglutaryl-coenzyme A reductase (HMG-CoA reductase, EC 1.1.1.34) activity in lung, kidney and adrenal glands as a consequence of the blocking by 4-APP of release of lipoproteins by liver into plasma. HMG-CoA reductase activity has not been investigated in platelets, but Derksen and Cohen [6] were unable to show conversion of radioactive acetate into mevalonate by human platelets. Our present work demonstrates the inducibility of HMG-CoA reductase activity in rabbit platelets and the effects of 4-APP-induced reduction in cholesterol concentration on platelet function.

MATERIALS AND METHODS

DL-[3-^{14}C]-HMG-CoA (sp. act. 18.5 μCi/μmole) was purchased from New England Nuclear. Unlabeled HMG-CoA (from PL Biochemicals, Milwaukee, WI) was added to the radioactive substrate to give a specific activity of 0.8 μCi/μmole, and this substrate was used in all the experiments. DL-[4-^{3}H]mevalonic acid was purchased from Amersham/Searle, Arlington Heights, IL. Unlabeled mevalonic acid was obtained from the Sigma Chemical Co. (St. Louis, MO) and 4-aminopyrazolopyrimidine (4-APP) from the Aldrich Chemical Co., Milwaukee, WI. New Zealand white female rabbits weighing about 5 lb were used. Experimental animals were injected intraperitoneally daily for 5 days with 20 mg of 4-APP in 1.0 ml of normal saline at pH 2.5; control animals were injected with normal saline on the same schedule. Rabbits were anesthetized at 10:00 a.m. with sodium pentobarbital, 10 mg/lb body weight. Control rabbits were processed simultaneously with the 4-APP-treated rabbits. Blood was drawn from the abdominal aorta by catheterization into a 20-ml syringe with 2 ml of 0.11 M sodium citrate.

Platelet-rich plasma (PRP) was prepared by centrifuging the citrated blood at 150 g for 15 min. The supernatant fluid was removed with a plastic pipette and then re-centrifuged at room temperature for 15 min at 150 g to remove any possible redisual non-platelet cells. Microscopic examination confirmed the presence of less than one red cell or leucocyte per 10^4 platelets. Part of the PRP was used for the aggregation studies, and the remainder was centrifuged at 4000 g for 10 min to pellet the platelets. The platelet pellet was washed with 0.04 M phosphate buffer, pH 7.2, containing 0.1 M sucrose, 0.05 M KCl, 0.03 M EDTA and 10 mM dithioerythritol. The washed platelets were suspended in the same buffer and sonicated for three 1-min intervals on ice. Then these homogenates were centrifuged at 4000 g for 10 min to remove particulate matter, and the supernatant fractions used for the enzyme assays.

For HMG-CoA assay, duplicate aliquots of 50–200 μl of the platelet homogenates were preincubated with 2.8 mM NADPH at 37° for 20 min to stabilize HMG-CoA reductase against natural inhibitors and further assayed according to a procedure described by Tormanen *et al.* [7]. Other duplicate aliquots of homogenates were inactivated by heating at 100° for

Table 1. HMG-CoA reductase activity in rabbit platelets

Experiment	Specific activity (pmoles/min/mg protein)	Plasma cholesterol (mg/dl)
Control (N = 3)	63 (31–97)	54.4 (42.4–66.1)
4-APP-treated (N = 5)	550 (209–1157)	20.7 (9.2–26.3)

5 min for use as blank controls. Then DL-[3-^{14}C]-HMG-CoA (132,500 dis./min) was added to only one set of the duplicates to give a final concentration of 300 μM. After 20 min of incubation at 37° the reaction was stopped by adding 1.0 ml of 2 N HCl to all the incubation tubes. DL-[4-^{3}H]mevalonic acid (0.04 μCi) was added only to the tubes without [^{14}C]-HMG-CoA, so as to calculate recoveries. Samples were processed then as described previously [7]. Specific activity of HMG-CoA reductase is defined as the moles of mevalonic acid formed/min/mg of protein. Protein was determined by the method of Lowry *et al.* [8] after precipitation of the protein with 5% trichloroacetic acid and redissolving the precipitate in 1 N NaOH.

Platelet aggregation studies. The PRP to be used for aggregation was adjusted to give a platelet count of 400,000/μl by diluting the PRP with platelet-poor plasma obtained by a second centrifugation at 1000 *g* for 15 min. All aggregation studies were performed in a Payton aggregometer utilizing 0.5 ml PRP. Stock reagents of 100 μM ADP, 1.0 mg/ml of collagen and 100 μM epinephrine were prepared.

Cholesterol determination. Total cholesterol in the platelet-free plasma was determined in an Abbott ABA 100 bichromatic analyzer using an enzymatic method [9]. Platelets were homogenized by sonication in 10X their volume of chloroform–methanol (2:1), and their cholesterol concentration was determined by gas chromatography in a Barber-Colman model 5,000 chromatograph using OV 17 on Gaschrom Q, 100–120 mesh at 260° and stigmasterol as the internal standard [9].

RESULTS

Plasma cholesterol in the untreated control rabbits ranged from 42 to 66 mg/dl. Five-day treatment of the experimental rabbits with 20 mg/day of 4-APP reduced the cholesterol to 9–26 mg/dl (Table 1). Treatment with 4-APP did not alter significantly the red cell or platelet count. The livers of the treated rabbits appeared pale and fatty. HMG-CoA reductase activity of the liver, which was measured at 8:00, 9:00, 10:00 and 12:00 a.m. and at 2:00 p.m., reached its highest level between 9:00 and 10:00 a.m. The specific activity of platelet HMG-CoA reductase activity in normal untreated rabbits ranged from 31 to 97 (Table 1), with a mean of 63. The specific activity in the 4-APP-treated rabbits increased about 9-fold to 550 units. The increases in the HMG-CoA reductase activity were correlated inversely with the reductions of cholesterol concentration in the plasma (Fig. 1). (The correlation coefficient was 0.987.)

As seen in Table 2, the proportion of platelets aggregated by 9.0 μM ADP was significantly lower in 4-APP-treated rabbits compared to normal controls

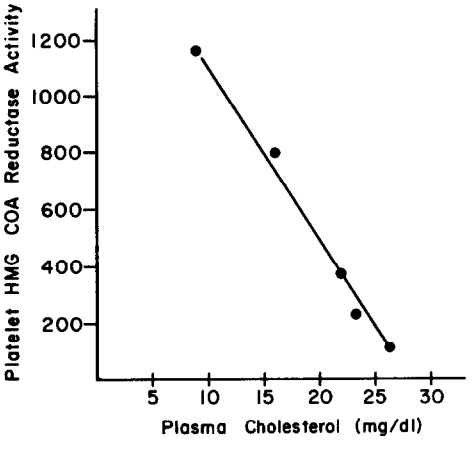

Fig. 1. Relationship between platelet HMG-CoA reductase activity (pmoles/min/mg of protein) and plasma cholesterol concentration in 4-APP-treated rabbits.

Table 2. Aggregation of platelets from control and 4-APP-treated rabbits *

Compound	Extent of aggregation Control	Extent of aggregation 4-APP	Rate of aggregation Control	Rate of aggregation 4-APP
ADP	51.8 ± 8.4	21.2 ± 13.6	125 ± 11	50 ± 13
ADP + epi	64.3 ± 3.1	30.0 ± 2.6	71 ± 23	57 ± 7
Collagen	58.8 ± 6.3	35.6 ± 10.2	106 ± 25	57 ± 16
Collagen + epi	62.5 ± 1.5	58.6 ± 2.5	85 ± 10	74 ± 16

* Results are means of four control rabbits and five 4-APP-treated rabbits ± S. D. The extent of aggregation is expressed as the percentage increase in light transmittance. The rate of aggregation is expressed as mm/min change during the steepest slope of the light transmittance recording [10]. Abbreviations: ADP = adenosine diphosphate; epi = epinephrine. Final concentrations were ADP 9.0 μM, collagen 10 μg/ml and epinephrine 8.0 μM.

($P < 0.01$). In addition, the rate of aggregation, as determined from the slope, was slower in 4-APP-treated rabbits than in controls ($P < 0.01$). Collagen-induced platelet aggregation showed similar differences between the two groups. Both the extent and the rate of aggregation decreased significantly in the 4-APP-treated animals ($P < 0.01$ for both). Epinephrine by itself did not induce aggregation of the rabbit platelets. Epinephrine at a concentration of 8.0 μM, followed by ADP at 9.0 μM or collagen at 10 μg/ml, increased the maximum aggregation induced by either ADP or collagen alone, both in control and 4-APP-treated rabbits; the latter group was still significantly lower than the control ($P < 0.01$). Following aggregation with either ADP or collagen, with or without epinephrine, platelets from all the 4-APP-treated rabbits were observed to de-aggregate to a maximum of 90 per cent over a period of 7–15 min. No de-aggregation was observed in any of the control platelets over a 15-min period. Addition of 4-APP *in vitro* at concentrations of 10^{-5} to 10^{-3} M had no significant effects on aggregation.

Platelet cholesterol concentration was reduced by 24 per cent in the drug-treated group (control 108.2 $\pm$ 2.5 μg/10^9 cells, 4-APP group 82.5 $\pm$ 5.6 μg/10^9 cells).

DISCUSSION

The capacity of platelets to synthesize cholesterol or its intermediates has not been investigated thoroughly. Derksen and Cohen [6] studied the incorporation of [^{14}C]mevalonic acid into sterols by non-human primate platelets and found most of the radioactivity in lanosterol but none in cholesterol. They were also unable to demonstrate [6] incorporation of radioactive acetate into sterols. Dietschy and Siperstein [11] have shown that most mammalian tissues are capable of synthesizing cholesterol. Extensive studies by Balasubramaniam *et al.* [4, 5] have shown that the low rate of cholesterol synthesis in tissues other than liver and intestine is due to feedback suppression of HMG-CoA reductase mediated by the low density lipoproteins secreted by the liver. Recent work [4, 5] from their laboratory showed that after 4-aminopyrazolopyrimidine treatment of rats, HMG-CoA reductase levels in lung, kidney and adrenal glands increased several-fold. Our study shows a similar increase in platelet HMG-CoA reductase, the enzyme which may have a major regulatory role in cholesterol biosynthesis by platelets. The observed stimulation of platelet HMG-CoA reductase activity was strongly correlated with the degree of reduction of plasma cholesterol resulting from treatment with 4-APP. Although most plasma lipid and lipoprotein fractions decrease following 4-APP treatment, there is no decrease in the protein concentration of the $d > 1.21$ fraction [12]. The latter fraction has been shown to be very potent in removing cholesterol from cells in tissue culture, and to cause a resulting increase in HMG-CoA reductase activity [13, 14]. A similar mechanism may have resulted in the decreased platelet cholesterol content and increased HMG-CoA reductase activities following 4-APP administration in the present experiments.

In a previous study, *in vitro* incubation of human platelets with cholesterol-poor liposomes resulted in a marked decrease in sensitivity to epinephrine-induced aggregation, although aggregation was normal in response to ADP [3]. In the present experiments, *in vivo* reduction of rabbit plasma cholesterol was associated with decreased sensitivity to both ADP and collagen-induced aggregation, with or without epinephrine enhancement. Aggregating capacity decreased with decreases in cholesterol concentrations in both the plasma and the platelets. Shattil and Cooper [15] demonstrated that the microviscosity of the platelet membrane, as determined by the mobility of a hydrophobic fluorescent probe, increased with increasing cholesterol content. They postulated that this increased microviscosity might influence the position or mobility of aggregation receptor components on the platelet membrane. Conversely, it is possible that the mechanism of the effects of the reduced cholesterol concentrations on aggregation in the present experiments might be attributable to a resulting decrease in microviscosity, with decreased accessibility of receptor sites.

Platelet aggregation has been implicated as one of the processes contributing to atheroma formation [16]. The present study suggests that *in vivo* pharmacologic reduction of plasma cholesterol can reduce platelet sensitivity to several physiologic aggregating agents; these results, therefore, may have implications regarding future experiments to inhibit development of atherosclerosis.

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