Platelet Function in Watanabe Heritable Hyperlipidemic Rabbits
Decreased Sensitivity to Thromboxane A$_2$

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The characteristics of platelets from seven 5-7-month-old homozygous Watanabe heritable hyperlipidemic (WHHL) rabbits (plasma cholesterol, 13.9±1.7 mM, mean±SEM) were compared with those of platelets from normocholesterolemic age/weight- and sex-matched control rabbits (plasma cholesterol, 2.2±0.3 mM). Whole-blood platelet count and platelet size and protein content were not different in the two groups of rabbits, and the platelets from the WHHL rabbits were not enriched in cholesterol as indicated by identical mean cholesterol:phospholipid molar ratios (C/P). Responses of washed platelets stimulated with various agonists were studied to determine the effects of the genetically determined hypercholesterolemia on the various pathways of platelet aggregation in the absence of plasma components. In platelets from WHHL rabbits compared with controls, aggregation induced by ADP (0.5–5 μM) did not differ; collagen-induced (0.25–1.5 μg/ml) responses (aggregation, secretion of carbon-14-labeled serotonin from the amine storage granules of prelabeled platelets, and thromboxane A$_2$ [TxA$_2$] formation) were significantly less extensive; with aspirin-treated platelets, aggregation and secretion of granule contents induced by the TxA$_2$ mimetic U46619 (0.25–1 μM) were significantly less extensive; and thrombin-induced (0.005–0.1 unit/ml) responses of untreated platelets (aggregation, secretion of granule contents, and TxA$_2$ formation) or aspirin-treated platelets (aggregation and secretion of granule contents) did not differ. These observations are in direct contrast with previous studies of platelets from rabbits with diet-induced hypercholesterolemia, in which responses to TxA$_2$ and thrombin were enhanced. Platelets from WHHL rabbits are hyposensitive to aggregation induced by TxA$_2$.

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Hypercholesterolemia is recognized as a major risk factor for atherosclerosis; however, its effects on platelets, which are involved in the development, progression, and thromboembolic complications of atherosclerosis, have not yet been thoroughly established. In patients with familial hypercholesterolemia (type II hyperbetalipoproteinemia), platelet aggregation, secretion of granule contents, and arachidonic acid metabolism via the cyclooxygenase pathway have been reported to be enhanced in some experiments, but enhancement was not evident in others. Platelet hypersensitivity has also been reported to be associated with diet-induced hypercholesterolemia in experimental animals, and we have recently observed that platelets from cholesterol-fed rabbits are hypersensitive to aggregation induced in at least two ways: by thromboxane A$_2$ (TxA$_2$) and by a thrombin-induced mechanism independent of TxA$_2$.

The Watanabe heritable hyperlipidemic (WHHL) rabbit is an animal model of familial hypercholesterolemia (for review, see Reference 22) in which atherosclerotic lesions commonly develop in the aorta by at least 3 months of age. A recent abstract indicates that platelets from WHHL rabbits are hyperreactive in terms of secretion of carbon-14-labeled serotonin from prelabeled platelets stimulated with a low concentration of collagen. However, a detailed examination of the functions of platelets from WHHL rabbits compared with those of platelets from normocholesterolemic control rabbits without atherosclerosis has not been reported previously. With platelets from WHHL rabbits, we were able to study the effects of hypercholesterol-
emia on platelets without the complicating effects of dietary cholesterol. Using washed platelets from WHHL rabbits and controls, we investigated responses of platelets stimulated with ADP, collagen, the TxA2 mimetic U46619, or thrombin, so that the effects of genetically determined hypercholesterolemia on several pathways of platelet aggregation could be determined. Other platelet characteristics that have been reported to be altered in hypercholesterolemia, including whole-blood platelet count, platelet size, and the free cholesterol:phospholipid molar ratio (C/P) were also measured to determine whether these characteristics were abnormal in the WHHL rabbit.

Methods

Animals

Homozygous female WHHL rabbits (2.52 ±0.21 kg, mean±SD, n=7) were obtained from the WHHL rabbit colony at the National Institutes of Health, Bethesda, Md. Age/weight- and sex-matched New Zealand White rabbits were used as controls. The rabbits received a regular chow diet that contained 0.006% cholesterol (Purina Rabbit Chow Checkers Performance Blend,Ralston Purina Canada Inc., Longueuil, Canada) and water ad libitum.

Measurement of Plasma Cholesterol and Platelet Characteristics

One day before exsanguination, 8.5-ml samples of blood were taken from rabbit ear arteries into tubes containing acid-citrate-dextrose. The whole-blood platelet count was determined by phase-contrast microscopy. Platelets were isolated by differential centrifugation, and total cholesterol concentration in the platelet-free plasma was measured. The platelets were resuspended in a calcium-free Tyrode's solution (without albumin, pH 6.5). The platelets were resuspended in a calcium-free Tyrode's solution containing 0.35% albumin and 0.02% ethylene glycol-bis(β-aminoethy1 ether)-N,N,N',N'-tetraacetic acid, pH 6.5, and platelet size was determined as described previously.

Any contaminating red blood cells were removed by centrifugation (2,300g for 10 minutes) of the platelet suspension through isosmolar Percoll solution (d=1.343 g/cm3, pH 6.5; Pharmacia LKB Biotechnology, Uppsala, Sweden); the platelets remained at the Tyrode's-Percoll interface and were removed, diluted, and washed with calcium-free Tyrode's solution (without albumin, pH 6.5). The platelets were resuspended in a calcium-free modified Tyrode's solution (without phosphate or albumin, pH 6.5) and were counted using a Coulter counter (Coulter Electronics, Hialeah, Fla.). Total platelet protein was measured by the method of Lowry et al. Platelet lipids were extracted by a modified method of Bligh and Dyer; phosphate and free cholesterol were determined, and the C/Ps were calculated.

Platelet Aggregation Studies

The rabbits, 5–7 months of age, were anesthetized by intravenous injection of a solution of sodium pentobarbital and then exsanguinated via a polyethylene catheter inserted into the carotid artery. The anticoagulant used was the acid-citrate-dextrose solution of Aster and Jandl. Suspensions of washed platelets were prepared as described elsewhere. In the first washing fluid, the contents of the amine storage granules of the platelets were labeled with [14C]serotonin (as 5-hydroxy-3'-14C-tryptamine creatinine sulfate, 60 mCi/mmol, Amersham Corp., Oakville, Canada; 0.05 μCi/ml platelet suspension). The extent of uptake of the radioisotope (80%) was similar in both groups of rabbits. For the preparation of aspirin-treated platelets, 500 μM aspirin (Sigma Chemical Co., St. Louis, Mo.) was included in the first washing fluid. (Aspirin-treated platelets did not aggregate in response to 1 μg/ml collagen.) Platelet suspensions (0.5×10^9/ml in Tyrode's solution containing 0.35% albumin, 5 μM imipramine [GEIGY Canada, Dorval, Canada], and apyrase, pH 7.35) were incubated at 37°C for at least 20 minutes before testing. Platelet aggregation was induced by the following agonists: ADP (Sigma); acid-soluble collagen prepared from bovine tendon collagen (Sigma); U46619 (a generous gift of The Upjohn Co., Kalamazoo, Mich.); and thrombin (bovine thrombin [topical], Parke-Davis, Scarborough, Canada). Aggregation was studied at 37°C in an aggregometer (Payton Associates, Scarborough, Canada). For all the experiments with U46619 and some of the experiments with thrombin, the platelets were pretreated with aspirin as described above. The extent of aggregation was calculated as previously described, with 100% aggregation as the maximum possible increase in light transmission with the platelet-suspending medium. Three minutes after the addition of ADP or U46619 or 5 minutes after the addition of collagen or thrombin, supernatant samples were prepared by centrifugation of the platelet suspension for 1 minute at 12,000g in an Eppendorf centrifuge. These samples were used to measure secretion of [14C]serotonin from prelabeled platelets and formation of thromboxane B2 (TxB2) (by radioimmunoassay; NEK-007, NEN Canada, Lachine, Canada).

Analysis of Aortas

After exsanguination, the rabbit aortas were dissected free, cleaned of excess adventitial tissue, and fixed in neutral-buffered formalin. After at least 72 hours, the fixed aortas were opened longitudinally and stained with Oil Red O; the luminal surface was examined for lipid staining.

Statistical Analyses

Values are reported as mean±SEM, with the number of experiments indicated. Student's t tests were used to analyze differences between values for mean plasma cholesterol and characteristics of plate-
**Table 1.** Plasma cholesterol and platelet count, volume, protein, and cholesterol:phospholipid molar ratio in control and Watanabe heritable hyperlipidemic rabbits

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean±SEM*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma cholesterol (mM)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.22±0.25</td>
</tr>
<tr>
<td>WHHL</td>
<td>13.88±1.67†</td>
</tr>
<tr>
<td>Whole-blood platelet count (×10⁹/ml)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.43±0.03</td>
</tr>
<tr>
<td>WHHL</td>
<td>0.50±0.04</td>
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<tr>
<td>Median platelet volume (μm³)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.78±0.26</td>
</tr>
<tr>
<td>WHHL</td>
<td>4.32±0.14</td>
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<tr>
<td>Platelet protein (mg/10⁹ platelets)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.49±0.08</td>
</tr>
<tr>
<td>WHHL</td>
<td>1.40±0.03</td>
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<tr>
<td>Platelet cholesterol (nmol/10⁹ platelets)</td>
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</tr>
<tr>
<td>Control</td>
<td>128±7</td>
</tr>
<tr>
<td>WHHL</td>
<td>115±9</td>
</tr>
<tr>
<td>Platelet phospholipid (nmol/10⁹ platelets)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>183±16</td>
</tr>
<tr>
<td>WHHL</td>
<td>166±15</td>
</tr>
<tr>
<td>Cholesterol:phospholipid molar ratio</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.73±0.06</td>
</tr>
<tr>
<td>WHHL</td>
<td>0.72±0.07</td>
</tr>
</tbody>
</table>

WHHL, Watanabe heritable hyperlipidemic.  
* n=7.  † p<0.001, control vs. WHHL.

lets from WHHL and control rabbits. Differences in responses of the platelets to the various agonists were determined by use of a nonorthogonal two-way analysis of variance, with an assumption of an interaction between agonist concentration and the rabbit strain. Differences were deemed to be statistically significant when p<0.05.

**Results**

Lipid-rich areas that stained with Oil Red O were visible only in the aortas from the WHHL rabbits. The lesions occurred adjacent to the ostia of the intercostal arteries and the vessels arising from the aortic arch as has been observed previously by Buja et al. 24

The mean plasma cholesterol level of the WHHL rabbits was significantly higher than that of the control rabbits (Table 1). However, no significant differences were found between WHHL and control rabbits in whole-blood platelet count, platelet size, platelet protein content, or the C/P of the platelets (Table 1).

**Platelet Responses**

The extent of aggregation induced by ADP (0.5, 1, and 5 μM) was not significantly different with washed platelets from WHHL rabbits compared with those from control rabbits (Figure 1). As has been reported previously for rabbit platelets, 38,47 ADP induced only the primary phase of aggregation, and this response was not associated with appreciable secretion of [¹⁴C]serotonin or formation of TxB₂.

In response to collagen (0.25–1.5 μg/ml), aggregation and secretion of dense granule contents (from platelets prelabeled with [¹⁴C]serotonin) were significantly less extensive with platelets from WHHL rabbits than with platelets from control rabbits (Figures 2A and 2B). However, the difference between TxB₂ formation by collagen-stimulated platelets from WHHL rabbits and control rabbits did not reach statistical significance (Figure 2C). The decreased collagen-induced responses of platelets from WHHL rabbits were also observed in one experiment in which platelet-rich plasma from blood anticoagulated with sodium citrate (one part 3.8% citrate to nine parts blood), instead of suspensions of washed platelets, was used.

In response to the stable TxA₂ mimetic U46619 (0.25, 0.5, and 1 μM), aspirin-pretreated platelets from WHHL rabbits also aggregated less extensively and secreted less [¹⁴C]serotonin than did similarly treated platelets from control rabbits (Figures 3A and 3B).

Neither aggregation (Figure 4A), secretion of [¹⁴C]serotonin (p>0.4, n=4), nor TxB₂ formation (p>0.15, n=4) stimulated by low concentrations of thrombin (0.005–0.1 unit/ml) was significantly different in platelets from WHHL rabbits compared with platelets from control rabbits. Thrombin-induced aggregation (Figure 4B) and secretion (p>0.15, n=4) by aspirin-pretreated platelets from WHHL and control rabbits were also similar.

**Discussion**

In the present study, we examined the functions of platelets from WHHL rabbits (mean plasma cholesterol, 13.9 mM) compared with those of platelets from normocholesterolemic New Zealand White control rabbits. We observed that collagen- or U46619-induced responses of platelets from WHHL

![Figure 1](https://example.com/figure1.png)  

**Figure 1.** Line plot of effect of ADP (0.5, 1, and 5 μM) on aggregation (%) of washed platelets from Watanabe heritable hyperlipidemic rabbits (●) or controls (○). Values are mean±SEM, n=3; p>0.1, two-way analysis of variance.
rabbits were decreased compared with control platelets, but ADP-induced and thrombin-induced responses were not significantly different. The genetically determined hypercholesterolemia was not associated with an increased cholesterol content of the platelets.

With the exception of ADP, agonists that induce platelet aggregation also cause the formation of TxA2 and secretion of granule contents, and these amplify the platelet responses.26 To consider the effect of the genetically determined hypercholesterolemia on the various reactions that cause and/or amplify responses of platelets, we investigated platelet responses to ADP (primary aggregation), collagen, or thrombin (aggregation, secretion of granule contents, and formation of TxA2), and the responses of platelets (aggregation and secretion of granule contents) to the TxA2 mimetic U46619 or thrombin when TxA2 formation was inhibited by aspirin. The studies were done with suspensions of washed platelets instead of platelet-rich plasma, so that the effects of the genetically determined hypercholesterolemia on the platelets could be determined in the absence of hypercholesterolemic plasma components.

Previously, enhanced platelet reactivities have been associated with hypercholesterolemia, whether genetically determined in patients (who also have some cholesterol in their diets)1-8 or experimentally induced in animals by including cholesterol in the diet.11-20 In recent studies, we21 investigated the pathways of aggregation26 of platelets from rabbits fed a diet enriched with a low amount (0.25%, wt/wt) of cholesterol for 8 weeks; in these animals, mean plasma cholesterol levels were 15 mM. Platelets from the cholesterol-fed rabbits were hypersensitive to aggregation induced by TxA2 and to aggregation by a thrombin-induced mechanism independent of TxA2. The platelets became enriched in cholesterol as indicated by a significant increase in the C/P.21 Thus, our findings in the present experiments, that platelets from WHHL rabbits are hyposensitive to collagen and are not different in their responses to thrombin, were completely unexpected. It should be noted that even though the mean plasma cholesterol levels were similar in WHHL rabbits and cholesterol-fed rabbits, the lipoprotein profiles would have been very different; the hypercholesterolemia in the WHHL rabbit is mainly the result of elevated levels of low density lipoprotein,22 whereas in the cholesterol-fed rabbit, it is the result of elevated levels of very low density lipoprotein.48

Rabbit platelets, like human platelets suspended in a medium containing a physiological concentration of Ca2+, undergo a primary aggregation response when stimulated with ADP and do not form TxA2 or secrete the contents of their storage granules to an
appreciable extent.\textsuperscript{26,38,47-49} In the present study as in our previous study with rabbits with diet-induced hypercholesterolemia, we found that platelets from WHHL rabbits do not respond differently to ADP than do platelets from control rabbits.

Collagen-induced responses are mediated largely through the TxA\textsubscript{2} that is formed and the ADP that is secreted when platelets adhere to collagen\textsuperscript{26-50}; collagen-induced aggregation and secretion of granule contents were lower in WHHL rabbits compared with those of control rabbits, whereas thromboxane formation was the same. The lower responses to collagen of platelets from WHHL rabbits are due, at least in part, to a decreased response to the TxA\textsubscript{2} that they form as demonstrated by hyposensitivity of platelets from WHHL rabbits to the stable TxA\textsubscript{2} mimetic U46619, when feedback amplification was blocked with aspirin. This indicates that after stimulation with collagen, platelets from WHHL rabbits are forming TxA\textsubscript{2}, but they are not as sensitive to it. In contrast, in rabbits with diet-induced hypercholesterolemia, platelet hypersensitivity to collagen is due to both increased formation of TxA\textsubscript{2} and increased responsiveness to TxA\textsubscript{2}.\textsuperscript{21}

In cholesterol-fed rabbits, thrombin-induced platelet responses, including those that are independent of TxA\textsubscript{2} formation, are also hypersensitive\textsuperscript{21} at low concentrations of thrombin (0.0013–0.01 unit/ml) but not at 0.1 unit/ml. When platelets (untreated or aspirin treated) from WHHL rabbits were stimulated with low concentrations of thrombin, no significant difference from thrombin-induced responses of platelets from control rabbits was demonstrable.

Recently, responses of platelets from rats with genetically determined hypercholesterolemia (RICO) have been examined; hypersensitivity to thrombin-induced aggregation was independent of arachidonic acid metabolism, but responses to ADP
or collagen were not different from the responses of platelets from control rats. The type of hypercholesterolemia that occurs in these RICO rats (elevated low density lipoprotein and high density lipoprotein cholesterol) is very different from that which occurs in WHHL rabbits. The results from the studies with RICO rats, cholesterol-fed rabbits, and WHHL rabbits indicate that the responsiveness of the various pathways of platelet aggregation depend not on the presence of hypercholesterolemia per se but on the lipoprotein profiles in the hypercholesterolemia.

Other characteristics of platelets that have been reported previously to be altered in hypercholesterolemia are whole-blood platelet count and platelet size, protein content, and C/P. These were also not significantly different in WHHL rabbits than in control rabbits. It should be noted that in an attempt to reduce technical variability in the C/Ps, duplicate samples for assays of free cholesterol and lipid-phosphorus were taken from the platelet lipid extracts at the same time, and assays were done on the same day. In a previous study in which male rabbits were used, the C/P of their platelets before cholesterol feeding was 0.59±0.04 (n = 10), but this value was not significantly different from the value of 0.73±0.06 (n = 7) obtained with female control rabbits in the present study.

The nearly identical C/Ps of platelets from control and WHHL rabbits indicate that the circulation of platelets in a hypercholesterolemic plasma with a mean plasma cholesterol concentration of 14 mM does not result in an enrichment of the platelets in cholesterol. Others have found that the cholesterol content of platelets from normal subjects cannot be increased by incubation in vitro with plasma from patients with type II hyperbetalipoproteinemia or with plasma from cholesterol-fed guinea pigs. However, in patients with genetically determined hypercholesterolemia or in rabbits with diet-induced hypercholesterolemia, the C/P of platelets has been reported to be increased. It may be that in these experimental animals and patients (who are ingesting cholesterol in their diets), cholesterol-enriched platelets originate from cholesterol-enriched megakaryocytes; evidence from Schick and Schick indicates that, at least in the cholesterol-fed guinea pig, the megakaryocyte may be the primary determinant of the cholesterol content of platelets. Dietary cholesterol could reach the bone marrow via clearance of chylo micron-cholesterol remnants. The dietary intake of cholesterol by WHHL rabbits eating the normal chow diet is very low, so very little cholesterol would reach the bone marrow via chylomicron-cholesterol remnants.

The most unexpected observation in these studies was that collagen- or U46619-induced responses of platelets from WHHL rabbits were decreased compared with those of platelets from control rabbits. Previously, in a study of human patients with familial hypercholesterolemia (who would have had cholesterol in their diets), platelet aggregation in response to collagen was reported to be hypersensitive compared with that of control subjects; U46619-induced aggregation was reported not to be altered. As noted above, the decreased responses to collagen of platelets from WHHL rabbits are due, at least in part, to decreased responses to the TxA2 that they form, but it is not known whether other aspects of platelet reactivity to collagen, for example, the initial adherence of platelets to collagen, are also reduced in platelets from WHHL rabbits. The reduced responses to U46619 of platelets from WHHL rabbits must be due to decreased binding of the mimetic to the TxA2 receptor, that is, a decreased number of TxA2 receptors and/or a decreased receptor affinity, or an altered signal transduction mechanism, or both. Whether this result from the type of hypercholesterolemia in the WHHL rabbits or a genetic defect in addition to the mutation in the gene for the low density lipoprotein receptor has yet to be determined.

References


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