# Reduced Membrane Fluidity in Platelets From Diabetic Patients

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**Platelets from diabetic patients are hypersensitive to agonists in vitro. Membrane fluidity modulates cell function, and reduced membrane fluidity in cholesterol-enriched platelets is associated with platelet hypersensitivity to agonists, including thrombin. Decreased membrane fluidity of these platelets is attributed to an increased cholesterol-phospholipid molar ratio in platelet membranes. We examined the response of platelets from diabetic subjects to thrombin, platelet membrane fluidity, and platelet cholesterol-phospholipid molar ratio. Twelve poorly controlled diabetic subjects were compared with 12 age- and sex-matched control subjects. In response to a low concentration of thrombin, mean values for release of [$^{14}$C]serotonin from washed prelabeled platelets were not significantly different between diabetic and control subjects, but in 8 of 12 diabetic subjects, the release response was greater than in their paired control subjects. Mean steady-state fluorescence polarization values in 1,6-diphenyl-1,3,5-hexatriene–labeled platelets prepared from diabetic subjects were significantly greater than in control subjects; this indicates a decreased membrane fluidity in platelets from diabetic subjects. Total or very-low-density (VLDL), low-density (LDL), or high-density ($HDL_2$, $HDL_3$) lipoprotein cholesterol concentrations in plasma were not significantly different between groups; however, the ratio of VLDL + LDL to $HDL_2 + HDL_3$ was significantly greater in diabetic than in control subjects. There was no difference in the total platelet cholesterol-phospholipid molar ratio between groups. Thus, reduced membrane fluidity of platelets from diabetic patients could account for their increased sensitivity to agonists; reduced membrane fluidity does not appear to result from a change in the plasma or platelet cholesterol content but is associated with an increase in the ratio of plasma VLDL + LDL to $HDL_2 + HDL_3$. *Diabetes* 39:241–44, 1990**

Platelets from diabetic patients and from diabetic animals are often hypersensitive to agonists in vitro (1–4). Membrane fluidity modulates cell function (5), and reduced membrane fluidity of platelets, e.g., after their incubation with cholesterol-rich liposomes in vitro (6), has been associated with enhanced responsiveness to agonists in vitro (7); reduced platelet membrane fluidity under these circumstances probably results from an increased molar ratio of cholesterol to phospholipid in the platelet membranes (6–8). Platelets from patients with type IIA hyperlipoproteinemia also have an increased cholesterol-phospholipid molar ratio and an increased sensitivity to agonists in vitro (9,10). Because altered plasma lipoproteins and increased total plasma cholesterol concentrations have been reported in diabetic patients (2), these changes could result in reduced membrane fluidity of platelets from diabetic patients, leading to the increased sensitivity of these platelets to agonists in vitro. Likewise, erythrocytes from diabetic patients have been shown to have an increased membrane cholesterol-phospholipid molar ratio, reduced membrane fluidity (11,12), and reduced deformability (13), although the latter characteristic may be related to changes in membrane or cytoskeletal proteins rather than in membrane lipids. Alternatively, increased nonenzymatic glycosylation of erythrocyte membrane proteins from diabetic patients or in vitro incubation of erythrocytes from nondiabetic humans in mediums containing high glucose concentrations have also been associated with reduced erythrocyte membrane fluidity (14,15). Although there are numerous studies of erythrocyte membrane fluidity from diabetic patients, there are no reports about membrane fluidity of platelets in this condition.

We examined membrane fluidity in platelets; platelet cholesterol-phospholipid molar ratios; cholesterol concentra-

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tions of plasma and individual plasma very-low-density (VLDL), low-density (LDL), and high-density ($HDL_2$, $HDL_3$) lipoproteins; plasma glucose concentrations; and glycosylated hemoglobin ($HbA_{1c}$) in a group of poorly controlled diabetic patients and in control subjects.

## RESEARCH DESIGN AND METHODS

**Patients.** Twelve nonfasting diabetic patients (10 men: 6 type I [insulin dependent]; 4 type II [non–insulin dependent]; 2 women, both type II) with a mean ± SE age of 41 ± 4 yr (range 22–58 yr) and 12 age- (41 ± 3 yr, range 23–57 yr) and sex-matched control subjects were studied. Mean body weights were not significantly different between control (76.4 ± 3.6 kg) and diabetic (86.8 ± 5.5 kg) subjects. Mean duration of diabetes was 8 ± 2 yr. Of the diabetic subjects, 9 had been treated with insulin alone from the onset of their diabetes, 2 had been treated with insulin and diet, and 1 had been treated with diet and an oral hypoglycemic agent. Three diabetic subjects had vascular complications including retinopathy, coronary artery disease, and/or peripheral vascular disease. Two other diabetic subjects had evidence of neuropathy. Platelets from each diabetic subject and his or her paired control subject were studied on the same day. These studies were approved by the Committee on the Ethics of Research in Human Experimentation at McMaster University.

**Blood collection and preparation of platelet suspensions.** Blood (100 ml) was collected from a forearm vein via plastic syringes; 14 ml was added to EDTA-containing Vacutainer tubes (Becton Dickinson, Mississauga, Ontario, Canada) and used for determination of $HbA_{1c}$, plasma total and lipoprotein cholesterol, and glucose. The remaining blood was anticoagulated with acid-citrate-dextrose solution (1 ACD:6 blood, vol/vol), and washed platelets were finally resuspended at $4 \times 10^8$/ml in Tyrode's solution (pH 7.35) containing 0.35% bovine serum albumin and apyrase (16). Portions of these suspensions were used to determine membrane fluidity, release reaction, and the platelet analyses described below. Platelets used for determination of the release reaction were prelabeled in the first washing solution for 15 min with 5-hydroxytryptamine-3′-creatinine sulfate-[$^{14}$C] ([$^{14}$C]serotonin; Amersham, Oakville, Ontario, Canada; 0.6 μCi/suspension). Individuals known to have ingested drugs that affect platelet function within the last 10 days were excluded from the study.

**Platelet release reaction.** Release of $^{14}$C from [$^{14}$C]serotonin-prelabeled platelets stimulated with thrombin (Miles, Elkhart, IN) was measured as previously described (16). Imipramine (2 μM) was used to inhibit the reuptake of released serotonin.

**Fluidity measurements.** Platelet membrane fluidity was determined via fluorescence polarization (4,15) with the hydrophobic probe 1,6-diphenyl-1,3,5-hexatriene (DPH; Molecular Probes, Eugene, OR; 1 mM in tetrahydrofuran). DPH was diluted 1:100 in a platelet-suspending medium with vigorous mixing immediately before use. One volume of this diluted DPH dispersion was added to 1 vol of the platelet suspension containing $10^8$ platelets, and the mixture was incubated at 37°C for 35 min. The mixture was centrifuged at 12,000 × *g* for 10 s, the supernatant was removed, and the platelets were washed twice and resuspended in the platelet-suspending medium (16). The final platelet concentration was $5 \times 10^6$/ml.

Steady-state fluorescence polarization was measured at 37°C with a Perkin-Elmer spectrofluorometer equipped with polarizers in the excitation and emission beams. The excitation and emission wavelengths were 360 and 430 nm, respectively. Fluorescence polarization was determined from emission intensities that were polarized parallel and perpendicular to the polarized excitation with a standard formula (17). The grating transmission factor of Chen and Bowman (18) was used to correct for the depolarization effect of grating monochromators (19). A fluorescence-intensity value for a nonlabeled blank was subtracted as a correction for scattered light. Fluorescence polarization values mainly reflect lipid structural order (20), and lipid fluidity has been defined as the reciprocal of the lipid structural order parameter (21).

**Platelet analyses.** Samples of platelet suspensions (1.5 ml) were diluted with phosphate-free Tyrode's solution (3.5 ml, pH 7.35) and centrifuged at 1800 × *g* for 8 min, and platelets were washed twice in phosphate-free Tyrode's solution (5 ml). The initial diluting fluid and first washing solution also contained prostaglandin $E_1$ ($PGE_1$; 10 μM final concn). Platelets were resuspended in phosphate-free Tyrode's solution (1 ml), and the platelet count was determined with a particle counter (model $ZB_1$, Coulter, Oakville, Ontario, Canada) with a 70-μm-diameter orifice. Platelet suspensions were frozen at −20°C until analyzed. Protein was determined in 40 μl of thawed platelet suspension in duplicate (22). Lipids were extracted from a sample of platelet suspension (0.5 ml) as previously described (23). Platelet cholesterol and phosphorus contents were measured in duplicate on samples of lipid extract (160 and 20 μl, respectively; 23,24).

**Determination of $HbA_{1c}$, plasma glucose, and total and lipoprotein cholesterol concentrations.** $HbA_{1c}$ was determined by the glycoaffinity column method (25). The normal range of $HbA_{1c}$ is 4.5–7.5%. Plasma glucose was measured by the glucose oxidase method with an Ektachem (Eastman-Kodak, Rochester, NY) (26). VLDL, LDL, $HDL_2$, and $HDL_3$ were separated by the density gradient ultracentrifugation described by Hatch and Lees (27). Plasma and individual lipoprotein cholesterol concentrations were measured with a high-performance CHOD-PAP kit (cholesterol oxidase, peroxidase, 4-aminophenazone; Boehringer Mannheim, Dorval, Quebec, Canada).

**Analysis of data.** Paired or unpaired *t* tests (2 tailed) were used to determine significance of differences as indicated.

## RESULTS

Mean plasma glucose and $HbA_{1c}$ were significantly greater in diabetic subjects (271 ± 41 mg/dl and 11.9 ± 0.7%, respectively) than in control subjects (90 ± 4 mg/dl, $P < 0.001$; and 5.7 ± 0.1%, $P < 0.001$, respectively).

Mean percentage release of [$^{14}$C]serotonin from prelabeled platelets from diabetic subjects (35.2 ± 7.4%) was not significantly different from platelets from control subjects (21.4 ± 5.5%) in response to a low concentration of thrombin (0.019 ± 0.001 U/ml final concn). However, in 8 of 12 paired comparisons, platelets from diabetic subjects gave a stronger release response than platelets from control subjects. At higher concentrations of thrombin, no differences in platelet responses to thrombin were detected.

TABLE 1
Steady-state fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene–labeled platelets from diabetic and control subjects

| Fluorescence polarization | | |
|---|---|---|
| Control | Diabetic | Difference |
| 0.152 | 0.195 | +0.043 |
| 0.179 | 0.217 | +0.038 |
| 0.164 | 0.196 | +0.032 |
| 0.215 | 0.243 | +0.028 |
| 0.167 | 0.222 | +0.055 |
| 0.179 | 0.219 | +0.040 |
| 0.212 | 0.191 | −0.021 |
| 0.227 | 0.248 | +0.021 |
| 0.220 | 0.233 | +0.013 |
| 0.205 | 0.240 | +0.035 |
| 0.151 | 0.153 | +0.002 |
| 0.226 | 0.238 | +0.012 |
| 0.191 ± 0.008 | 0.216 ± 0.008 | 0.025 ± 0.006* |

Final values in each column are means ± SE. *$P < 0.002$ for paired differences.

TABLE 2
Protein, cholesterol, and phospholipid contents of platelets from diabetic and control subjects

| | Diabetic | Control |
|---|---|---|
| Protein (mg/$10^9$ platelets) | 2.6 ± 0.2 | 2.6 ± 0.2 |
| Cholesterol (nmol/$10^9$ platelets) | 326 ± 16 | 354 ± 17 |
| Phospholipid (nmol/$10^9$ platelets) | 472 ± 35 | 488 ± 27 |
| Cholesterol/protein (nmol/mg) | 129 ± 8 | 137 ± 9 |
| Phospholipid/protein (nmol/mg) | 186 ± 13 | 187 ± 10 |
| Cholesterol/phospholipid (molar ratio) | 0.71 ± 0.04 | 0.73 ± 0.03 |

Values are means ± SE for 9 subjects in each group. Paired differences were not significant.

Steady-state fluorescence polarization of platelets from diabetic subjects (0.216 ± 0.008) was significantly greater than in platelets from control subjects (0.191 ± 0.008, $P < 0.002$; Table 1).

The total protein, cholesterol, and phospholipid contents of platelets from diabetic and control subjects were not significantly different (Table 2). The mean cholesterol-phospholipid molar ratio was not significantly different between groups. No significant differences were observed between groups in total cholesterol and VLDL, LDL, $HDL_2$, and $HDL_3$ cholesterol concentrations in plasma from diabetic and control subjects (Table 3). However, the ratio of the sum of VLDL and LDL cholesterol to the sum of $HDL_2$ and $HDL_3$ cholesterol was significantly greater in diabetic subjects than in control subjects ($P < 0.05$).

## DISCUSSION

There are numerous reports in which platelets from diabetic patients were found to be hypersensitive to thrombin (1–4). Although in this experiment there was no significant difference in the mean value for the amount of thrombin-induced release from platelets between diabetic and control subjects, in 8 of 12 comparisons, platelets from diabetic subjects released more [$^{14}$C]serotonin than their paired control subjects. Increased thrombin-induced responses of platelets from diabetic subjects are also consistent with our observations in rats with diabetes that was induced by streptozocin or that occurred spontaneously (28,29).

Platelets from poorly controlled diabetic subjects exhibited greater steady-state fluorescence polarization values than platelets from control subjects. Because fluorescence polarization values mainly reflect lipid structural order (20) and lipid fluidity has been defined as the reciprocal of the lipid structural order parameter (21), the higher values of fluorescence polarization that we observed in platelets from diabetic subjects indicate increased lipid structural order and decreased platelet membrane fluidity compared with control subjects. This finding of reduced platelet membrane fluidity is consistent with reduced erythrocyte membrane fluidity that has been reported in diabetic subjects (11,12).

Decreases in membrane fluidity of platelets incubated with cholesterol-rich liposomes in vitro are associated with platelet hypersensitivity to agonists (6,7). Decreased platelet membrane fluidity under these circumstances probably results from an increased cholesterol-phospholipid molar ratio in the platelet membrane (8). Because platelets from diabetic subjects are frequently found to be hypersensitive to agonists (1–4) and diabetes is often associated with altered plasma lipids and hypercholesterolemia (2), we determined whether the cholesterol-phospholipid molar ratio was altered in the platelets from diabetic subjects. We were unable to detect differences between groups in the total cholesterol-phospholipid molar ratio of platelets. Plasma lipoprotein-cholesterol concentrations were not different between groups, although the ratio of the sum of VLDL and LDL cholesterol to the sum of $HDL_2$ and $HDL_3$ cholesterol was greater in diabetic subjects.

An alternative explanation for the reduction in membrane fluidity in platelets from diabetic patients may relate to in-

TABLE 3
Total and lipoprotein cholesterol concentrations in plasma from diabetic and control subjects

| | Total cholesterol (mM) | Lipoprotein cholesterol (mM) | | | | |
|---|---|---|---|---|---|---|
| | | VLDL | LDL | $HDL_2$ | $HDL_3$ | $\frac{VLDL+LDL}{HDL_2+HDL_3}$ |
| Diabetic | 5.16 ± 0.28 | 0.50 ± 0.09 | 3.03 ± 0.24 | 0.62 ± 0.10 | 0.31 ± 0.02 | 4.23 ± 0.48* |
| Control | 5.20 ± 0.29 | 0.36 ± 0.04 | 2.82 ± 0.15 | 0.74 ± 0.08 | 0.35 ± 0.02 | 3.06 ± 0.24 |

Values are means ± SE for 12 samples in each group. VLDL, very-low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein.
*$P < 0.05$ vs. control.

creased nonenzymatic glycosylation of platelet membrane proteins. An association has been demonstrated between an increase in glycosylation of erythrocyte membrane proteins and a decrease in their membrane fluidity (14,15). Platelet membrane proteins have been reported to be nonenzymatically glycosylated to a greater extent in diabetic subjects compared with control subjects (30,31); this may lead to a reduction in the membrane fluidity of platelets from diabetic patients. Yatscoff et al. (31) reported that the increased nonenzymatic glycosylation of platelet membrane proteins in diabetic patients was not associated with increased platelet aggregation in response to agonists, but responses to thrombin were not tested. In this study, plasma glucose concentrations and percentages of glycosylated hemoglobin were higher in diabetic subjects than in control subjects. Although the time during which platelets circulate is short compared with that of erythrocytes, nonenzymatic glycosylation of platelet membranes may account for the reduction in membrane fluidity of platelets from diabetic subjects in this study.

Reduced membrane fluidity of platelets from diabetic patients could account for the increased sensitivity to agonists that has been frequently observed with platelets from diabetic patients. In our diabetic subjects, the reduced platelet membrane fluidity did not appear to be related to altered plasma or platelet cholesterol content, although it was associated with an alteration in the ratio of plasma lipoprotein-cholesterol concentrations.

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