Enhancement of Platelet Aggregation by Low-Density Lipoproteins From IDDM Patients

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Low-density lipoprotein (LDL) is known to enhance platelet sensitivity to some aggregating agents. In this study, we observed that LDL isolated from patients with insulin-dependent diabetes mellitus (IDDM) enhanced thrombin-induced platelet aggregation to a greater extent than LDL isolated from matched controls ($P < .01$). Thromboxane B_2 production during aggregation was also significantly more enhanced by LDL isolated from IDDM than by control LDL ($P < .01$). There was no difference in the lipid composition (free and esterified cholesterol, total phospholipids, and triglycerides) of LDL isolated from diabetic and control subjects. In contrast, the extent of glycosylation of LDL isolated from diabetic patients was significantly greater than that observed in LDL from normal subjects ($P < .01$), and a positive correlation ($r = .605$, $P < .01$) between the degree of LDL glycosylation and the rate of platelet aggregation was observed. LDL glycosylated in vitro enhanced thrombin-, collagen-, and adenosine 5′-diphosphate–induced platelet aggregation to a greater extent than control LDL ($P < .01$). Although LDL glycosylated in vitro was taken up by platelets to a greater extent than control LDL ($P < .05$), the lipid composition (free cholesterol and phospholipid) of platelets was not significantly changed. We postulate that an increased degree of glycosylation of LDL may enhance its uptake by platelets and lead to increased platelet reactivity to aggregating agents, probably by altering the structure of the platelet membrane. The enhancement of platelet aggregation by LDL may contribute to the accelerated development of atherosclerosis in diabetes mellitus.
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Both platelets and low-density lipoprotein (LDL) are known to play an important individual role in the development of atherosclerosis (1); however, the contribution of their combined interaction to atherogenesis has not been fully elucidated. Recently, LDL has been shown to enhance platelet sensitivity to aggregating agents (2,3), which may explain why platelets obtained from patients with familial hypercholesterolemia are more sensitive to aggregating agents (4,5). Platelets obtained from diabetic patients have also been shown to exhibit increased sensitivity to aggregating agents (6–9). It is not known whether platelet-LDL interactions also contribute to the increased sensitivity of platelets to aggregating agents observed in diabetic patients. Recently, our group demonstrated that the plasma level of LDL cholesterol was inversely correlated with the ED_{50} of platelet aggregation for arachidonic acid (8). No studies have been conducted previously to examine the effect of lipoproteins isolated from diabetic patients on platelet function.

To clarify the cause of hypersensitivity to aggregating agents of platelets obtained from diabetic patients, we compared the effect of LDL isolated from patients with insulin-dependent diabetes mellitus (IDDM) and matched controls on thrombin-induced platelet aggregation. We also compared the effect of LDL glycosylated in vitro and control LDL on platelet aggregation induced by thrombin, collagen, and adenosine 5′-diphosphate (ADP).

MATERIALS AND METHODS

Subjects. For isolation of LDL, 10 patients (2 men, 8 women) with IDDM were recruited from the Pediatric Metabolic Clinic and the Private Diagnostic Clinic at the Medical University of South Carolina, Charleston. IDDM was diagnosed according to the criteria established by the National Diabetes Data Group (10). Sex- and age-matched normal subjects (1 man, 4 women) were also recruited simultaneously with each

TABLE 1
Glycemic parameters and plasma lipid profile of diabetic and control subjects

	Fasting plasma glucose (mM)	HbA_{1c} (%)	Cholesterol (mM)	VLDL cholesterol (mM)	LDL cholesterol (mM)	HDL cholesterol (mM)	Triglyceride (mM)
IDDM	10.6 ± 2.2*	8.1 ± 0.7*	4.76 ± 0.39	0.23 ± 0.05	3.39 ± 0.39	1.16 ± 0.05	0.87 ± 0.08
Controls	4.6 ± 0.1	6.4 ± 0.2	4.65 ± 0.34	0.31 ± 0.08	3.18 ± 0.34	1.16 ± 0.18	0.97 ± 0.09

Values are means ± SE. VLDL, very-low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein.
*$P < .05$.

patient. Some of the control subjects were, however, recruited more than once. The patients' ages ranged from 13 to 26 yr (mean 18.5 yr). The controls' ages ranged from 15 to 27 yr (mean 19.0 yr). The body mass indexes of normal subjects ranged from 18.6 to 25.5 (mean 21.3 kg/m^2), and those of patients with IDDM ranged from 15.8 to 34.6 (mean 24.3 kg/m^2). One patient had retinopathy and another had trace proteinuria. None of the patients were receiving drug therapy other than insulin. Their doses of insulin averaged 66.3 ± 9.7 U/day. The control subjects took no medication. Informed consent was obtained from all participants in the study. Parental consent was obtained for minors.

Lipid and lipoprotein levels as well as fasting plasma glucose and HbA_{1c} levels of the diabetic and control subjects studied are shown in Table 1. The patients with IDDM had increased levels of fasting plasma glucose and HbA_{1c} compared with control subjects. However, there were no differences in their lipid/lipoprotein profiles. For isolation of platelets and LDL for in vitro glycosylation, normal controls were recruited. All of the subjects denied taking drugs that may affect platelet function during a period of ≥2 wk before the blood collection.

Isolation of lipoproteins. Venous blood was collected after an overnight fast and was immediately mixed with EDTA-Na^+ (6.7 mM, pH 7.4) after collection. LDL ($1.019 < \text{density} < 1.063$) was isolated by sequential ultracentrifugation in a preparative ultracentrifuge after appropriate adjustment of density with solid KBr (11). The isolated LDL fraction was washed and concentrated by centrifugation at the appropriate density. The LDL preparation was dialyzed against 154 mM NaCl with 0.3 mM EDTA-Na^+ (pH 7.4) and filtered through a 0.22-μm membrane. The protein content of LDL was determined by the method of Lowry et al. (12).

Platelet aggregation and thromboxane B_2 assay. Platelets were isolated from citrated blood obtained from the normal volunteers by the method described by Vargas et al. (13). The isolated, washed platelet preparation was suspended in Tyrode's buffer and was counted (Coulter counter model ZF) and adjusted to 4×10^8/ml. The platelets were then incubated at 22°C with LDL (1 mg protein/ml) for 30 min. In preliminary experiments, this concentration of LDL and time of incubation were found to provide optimal stimulation of platelet aggregation. The incubation of LDL with platelets was always started 1 h after the final resuspension of platelets. After the incubation with LDL, thrombin (0.5 U/ml, Parke Davis, Detroit, MI) was added to the platelets. Platelet aggregation was performed by the turbidimetric method of Born (14) by use of an aggregometer (model 400, Chronolog, Broomall, PA). Platelet aggregation rate was determined 1 min after the addition of thrombin. Light transmittance was continuously monitored by a recorder (Chronolog). The blank samples included Tyrode's buffer with or without isolated LDL. The percentage of transmittance of the isolated platelets with or without LDL was recorded as 0% and that of the appropriate blank as 100%.

To determine whether the addition of LDL from normal and diabetic subjects had a similar effect on platelet reactivity regardless of the batch of platelets used, the effect of LDL isolated from three diabetic subjects and their matched controls on platelet aggregation was assessed with two different batches of platelets. The difference in the aggregation rate of platelets incubated with LDL isolated from normal and diabetic subjects was similar in the two batches of platelets studied.

Thromboxane B_2 was assayed by radioimmunoassay as previously described (8). To measure thromboxane B_2, 50-μl aliquots of the platelet suspension were taken exactly 1 min after the addition of thrombin and frozen at −70°C.

The aggregation rate and thromboxane B_2 production from platelets incubated with LDL were expressed as a percentage of the level determined from platelets incubated without LDL, which was considered as the 100% level.

Measurement of glycosylation of LDL. LDL was diluted to 1 mg/ml and stored at −20°C until glycosylation was measured. LDL glycosylation was measured by affinity chromatography as we have previously described (15). Briefly, apoprotein of LDL was reduced by tritiated sodium borohydride (360 mCi/mmol, New England Nuclear, Boston, MA) so that tritium was incorporated into the ketoamine group

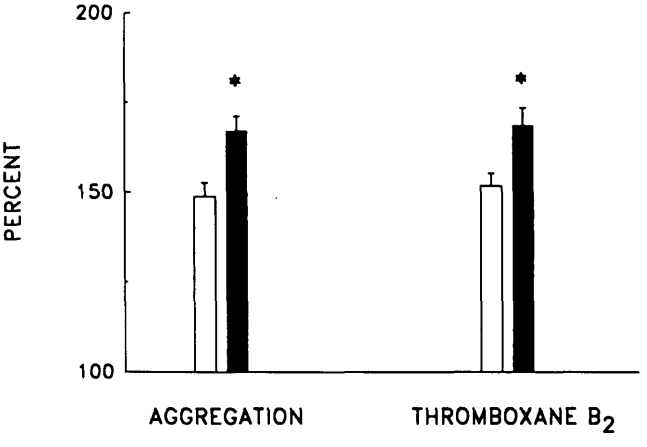

FIG. 1. Aggregation and thromboxane B_2 production from platelets incubated with low-density lipoprotein (LDL) isolated from control (*open bars*) and diabetic (*solid bars*) subjects. Values (means ± SE) are expressed as percentages, assuming that aggregation rate and thromboxane B_2 production from platelets incubated without LDL is 100%. *$P < .01$ compared with control LDL by signed-rank test.

TABLE 2
Glycosylation and lipid composition of low-density lipoprotein isolated from diabetic and control subjects

	Glycosylation (cpm/μg)*	Phospholipids	Free cholesterol	Cholesterol esters	Triglycerides
IDDM	56.5 ± 1.8†	1.50 ± 0.07	1.23 ± 0.06	3.52 ± 0.15	0.26 ± 0.04
Controls	43.5 ± 1.0	1.41 ± 0.08	1.09 ± 0.04	3.20 ± 0.10	0.26 ± 0.03

Values are means ± SE. Lipid values are expressed as micromoles per milligram protein of low-density lipoprotein.
*Measures of [^{3}H]hexitol amino acid.
†$P < .01$.

formed by the attachment of glucose to protein. After acid hydrolysis, the resultant tritiated glycosylated amino acids ([^{3}H]hexitol amino acids) were eluted through affinity chromatography, and radioactivity was counted. The amount of hydrolyzed apoprotein applied to chromatography was measured and expressed in micrograms. The extent of glycosylation was expressed as counts per minute per microgram of hydrolyzed protein.

Aggregation of platelets incubated with LDL glycosylated in vitro. Normal LDL were incubated with various concentrations (10, 20, 80, and 150 mM) of glucose for 7 days at 37°C. Control LDL was incubated under the same conditions but without addition of glucose. No reducing agent was added. After the incubation, extensive dialysis was performed, and the LDL preparations were incubated with washed platelets to examine their effect on platelet aggregation and thromboxane B_2 production. The methodology and conditions previously described were used, except that collagen and ADP were also included as aggregating agents. The final concentrations were 2 μg/ml and 5 μM for collagen and ADP, respectively.

Accumulation of LDL glycosylated in vitro in platelets. LDL glycosylated in vitro with 150 mM glucose and control LDL were iodinated by the method of McFarlane as modified by Bratzler et al. (16). Normal washed platelets (10^9/ml) were incubated in duplicate for 5 h at 37°C with labeled LDL in the presence or absence of 25-fold excess of unlabeled LDL. After washing, radioactivity of platelet pellets was counted.

Lipid composition of platelets incubated with LDL glycosylated in vitro. Washed platelets (10^9/ml) were incubated with LDL glycosylated in vitro (1 mg protein/ml) for 30 min at 37°C. After the incubation, the platelets were washed, and a lipid extraction was performed by the method of Folch et al. (17). The phospholipid and free-cholesterol content of the platelet lipid extract was measured.

Other methods. Plasma glucose was assayed by the glucose oxidase method with a Beckman glucose analyzer (18). Hemoglobin A_{1c} was measured by an isoelectric focusing method as described by Spicer et al. (19). Cholesterol and triglyceride levels were measured by the semiautomated method standardized by the Lipid Research Clinics Program (20). Free and total cholesterol in lipid extracts were measured by gas chromatography as previously described (21) after hydrolysis by Ishikawa's method (22). The phosphorus content of phospholipids was assayed by the method of Bartlett (23).

Statistical analysis was performed with the Wilcoxon's signed-rank test. Correlation coefficient (r) was determined by Spearman's rank correlation. Statistical significance of the correlation coefficient was determined by the method of Fisher and Yates.

RESULTS

As shown in Fig. 1, the reactivity to thrombin of platelets incubated with LDL isolated from IDDM (167.0 ± 4.1%) was significantly higher than that of platelets incubated with LDL isolated from matched controls (148.8 ± 3.8%). To determine whether thromboxane B_2 production was also enhanced, we examined its level 1 min after the addition of thrombin. Thromboxane B_2 production during aggregation of platelets incubated with LDL isolated from IDDM (168.7 ± 4.7%) was

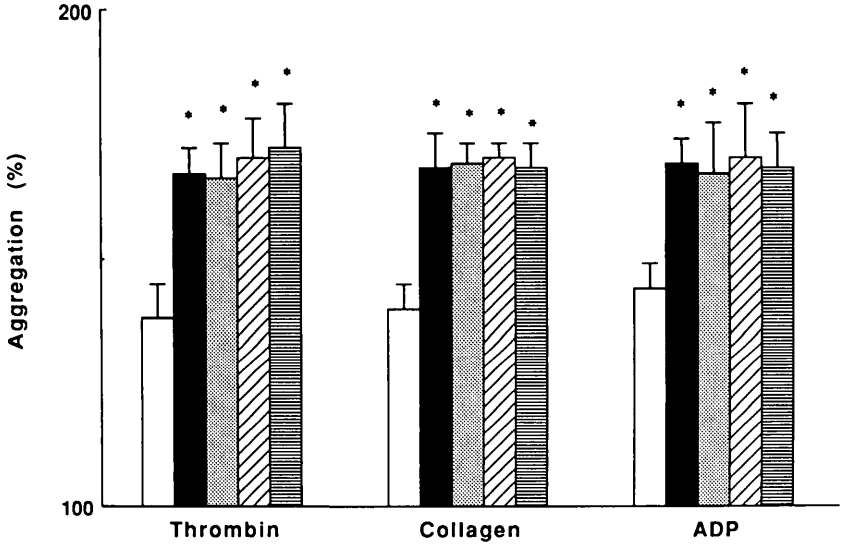

FIG. 2. Aggregation of platelets incubated with control low-density lipoprotein (LDL; *open bars*) and LDL glycosylated in vitro by incubating with 10 (*solid bars*), 20 (*stippled bars*), 80 (*hatched bars*), and 150 (*horizontally shaded bars*) mM glucose. Values (means ± SE of 4 experiments) are expressed as percentages, assuming that aggregation rate of platelets incubated without LDL is 100%. Levels of glycosylation of LDL glycosylated in vitro with 10, 20, 80, and 150 mM glucose were 68.4, 80.1, 152.9, and 260.9 cpm/μg, respectively. *$P < .01$ compared with control LDL by signed-rank test.

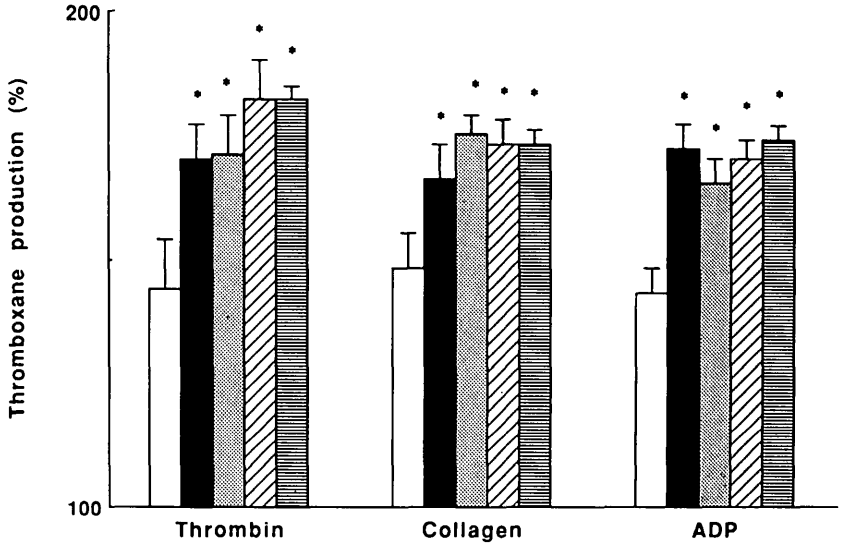

FIG. 3. Thromboxane B_2 production during aggregation of platelets incubated with control low-density lipoprotein (LDL; *open bars*) and LDL glycosylated in vitro by incubating with 10 (*solid bars*), 20 (*stippled bars*), 80 (*hatched bars*), and 150 (*horizontally shaded bars*) mM glucose. Values (means ± SE of 4 experiments) are expressed as percentages, assuming that thromboxane B_2 production from platelets incubated without LDL is 100%. *$P < .01$ compared with control LDL by signed-rank test.

significantly higher than that observed in platelets incubated with control LDL (151.7 ± 3.5%) (Fig. 1).

To investigate whether differences in the extent of glycosylation or in the lipid composition of LDL isolated from diabetic and normal subjects could explain the differences observed in the aggregation of platelets, we determined LDL glycosylation and LDL lipid composition. The lipid composition (free cholesterol, cholesteryl esters, triglycerides, and total phospholipids) of LDL isolated from diabetic and control subjects was not significantly different, and no correlation with platelet aggregation was seen. In contrast, as shown in Table 2, the glycosylation of LDL isolated from diabetic patients was significantly higher than that of LDL isolated from controls. A positive correlation between the degree of glycosylation of LDL and platelet aggregation was found in control and diabetic subjects ($r = .605$, $P < .01$).

To examine whether the glycosylation of LDL plays a key role in enhancing platelet aggregation, we measured the effect of LDL with various degrees of glycosylation on thrombin-, collagen-, and ADP-induced platelet aggregation and thromboxane B_2 production. The incubation of platelets with LDL glycosylated in vitro enhanced the reactivity of the platelets to each of the three aggregating agents and the production of thromboxane B_2 to a greater extent than the incubation with control LDL, as shown in Figs. 2 and 3. However, both the reactivity of platelets to the aggregating agents and the production of thromboxane B_2 were similar for the various LDL preparations, although their degree of glycosylation varied according to the concentration of glucose in the incubation media.

To determine whether the increased reactivity of platelets when incubated with glycosylated LDL was due to an increased uptake of glycosylated LDL, we measured the accumulation in platelets of LDL glycosylated in vitro. Glycosylated LDL was accumulated in platelets to a greater extent than control LDL, as shown in Table 3.

To determine whether the increased uptake of LDL glycosylated in vitro induced a change in the lipid composition of the platelet membrane, we measured the free-cholesterol and phospholipid content of platelets after the incubation with LDL glycosylated in vitro. There was no significant increase in their free-cholesterol and phospholipid contents. No change of the molar ratio of free cholesterol and phospholipids was observed in platelets incubated with any of the LDL preparations glycosylated in vitro (Table 4).

DISCUSSION

It has been postulated that abnormal platelet function may be one of the factors contributing to the increased incidence of macrovascular disease in diabetic patients (6–9). Numerous mechanisms have been proposed to explain the role of platelets in atherogenesis. Most of these center around the observation that platelets adhere to and aggregate at sites of endothelial injury and release numerous biologically active compounds to the environment (1).

In diabetic patients, increased sensitivity of platelets to aggregating agents has been well documented by our group and by others (6,7). Recently, we demonstrated that the plasma level of LDL cholesterol in IDDM patients was significantly correlated with the sensitivity of platelets to arachidonic acid (8). Increased platelet aggregation has been shown, however, in diabetic patients even when their plasma lipid and lipoprotein levels are normal. Thus, although plasma lipoprotein levels may play a role in enhancing platelet aggregation in diabetes, other mechanisms may also be present. We postulated that in diabetes an abnormal interaction between platelets and lipoproteins may be present, leading to an enhancement of platelet reactivity to aggregating agents.

The data presented in this article seem to confirm this postulate. We have shown that platelets incubated with LDL

TABLE 3
Accumulation of glycosylated and control low-density lipoprotein (LDL) by normal platelets

Amount of labeled LDL	Glycosylated LDL	Control LDL	*P*
10 μg/ml medium	198.0 ± 17.8	133.3 ± 3.5	<.05
60 μg/ml medium	1044.6 ± 73.2	627.3 ± 71.2	<.01

Values (ng LDL/10^9 platelets) are means ± SE of 4 different experiments.

TABLE 4
Lipid composition of platelets incubated with control and glycosylated low-density lipoprotein (LDL)

	Phospholipids (μmol/10^9 platelets)	Free cholesterol (μmol/10^9 platelets)	Free cholesterol to phospholipids (molar ratio)
Control LDL	0.320 ± 0.010	0.196 ± 0.019	0.577 ± 0.032
Glycosylated LDL			
10 mM glucose	0.320 ± 0.011	0.194 ± 0.014	0.580 ± 0.029
20 mM glucose	0.318 ± 0.013	0.186 ± 0.005	0.584 ± 0.025
80 mM glucose	0.315 ± 0.010	0.193 ± 0.011	0.595 ± 0.031
150 mM glucose	0.312 ± 0.012	0.189 ± 0.011	0.587 ± 0.025
No LDL	0.316 ± 0.012	0.188 ± 0.011	0.577 ± 0.029

Values are means ± SE of 4 different experiments. LDL was glycosylated in vitro by incubating with 10, 20, 80, or 150 mM glucose.

isolated from patients with IDDM aggregated to a greater extent when exposed to thrombin than those incubated with LDL from normal controls. Because the same batch of platelets and equal LDL concentrations were used, the differences observed in platelet reactivity resulted from an abnormal interaction between the LDL isolated from diabetic patients and the platelets. Maximal enhancement in platelet aggregation was observed at 30 min after the addition of LDL to the platelets. This suggests that binding of LDL to platelets is responsible for the increase in platelet reactivity.

Two different mechanisms by which binding of LDL to platelets may alter their reactivity to thrombin may be operating in diabetes. On one hand, the binding of LDL to platelets may be altered due to the change either in the protein or lipid moiety of the LDL particles. On the other hand, the binding of LDL to platelets may be normal, but alterations in the lipid composition of LDL may induce changes in the platelet membrane by promoting an exchange between the lipid constituents of the membrane and those of LDL.

A possible modification in the protein moiety of LDL isolated from diabetic patients that may alter the binding of LDL to platelets is nonenzymatic glycosylation. In this study, we demonstrated a positive correlation between the degree of glycosylation of LDL and the enhancement of platelet aggregation. We also demonstrated that LDL glycosylated in vitro had a similar effect and increased the reactivity of platelets to various aggregating agents. Maximal enhancement in platelet aggregation seems to be obtained with a relatively modest degree of LDL glycosylation.

Increased binding of glycosylated LDL to the platelets is probably the mechanism responsible for the enhancement of platelet aggregation observed in the presence of glycosylated LDL. We were able to demonstrate that LDL glycosylated in vitro was taken up to a greater extent (1.5-fold) by platelets than control LDL. This increase in the uptake by platelets of glycosylated LDL is not surprising. Contrary to what happens to most other cells, scavenger cells, e.g., platelets and monocytes, seem to recognize glycosylated LDL preferentially. Recently, we demonstrated that human monocytes recognize glycosylated LDL to a greater extent than normal LDL and that the uptake of glycosylated LDL seems to be mediated by a pathway independent of both the classic LDL and scavenger receptor pathways (21).

Another mechanism that may play a role in the enhancement of platelet aggregation by LDL isolated from diabetic patients is the oxidation of the lipid moiety of the LDL and formation of oxidized LDL. Oxidation of LDL could enhance platelet aggregation by damaging the platelet membrane (24). Sato et al. (25) observed a significantly higher plasma level of lipid peroxides in diabetic patients with angiopathy.

Finally, changes in the lipid composition of LDL may also alter platelet function. It is known that the lipid composition of the platelet membrane, especially the free-cholesterol-to-phospholipid ratio, plays an important role in platelet reactivity to aggregating agents (26). In this study, we found no difference in the absolute levels of the major lipid constituents of LDL or their relative proportions between the LDL isolated from normal and diabetic subjects. Because the fatty acid composition of the LDL phospholipids was not determined, we cannot rule out that a more subtle difference in the LDL lipid composition may have been involved in the enhancement of the reactivity of platelets incubated with LDL from diabetic patients. However, it is not very likely because we were able to alter the reactivity of platelets to aggregating agents by using LDL glycosylated in vitro in a manner similar to that observed with LDL isolated from diabetic patients. The lipid composition of the control and glycosylated LDL in the experiments with LDL glycosylated in vitro was identical because the same LDL pool was used.

Because it is known that the lipid composition of platelet membrane is important in determining platelet reactivity to aggregating agents (27), we measured free cholesterol and phospholipid and their molar ratio in platelets incubated with LDL glycosylated in vitro and normal LDL to determine whether the increased uptake of glycosylated LDL could induce a change in the lipid composition of the platelet membrane. Although the binding of LDL glycosylated in vitro by platelets was increased, little change in free cholesterol and phospholipid was found by incubating platelets with any of the LDL preparations. It has been shown, however, that a small uptake of cholesterol into the platelet membrane that may be sufficient to alter the platelet function may not result in a significant increase in the cholesterol content of platelets (3,28). Although we cannot rule out the possibility of losing the LDL bound to the platelet membrane during the washing and extraction procedures, we suggest that the increased binding of glycosylated LDL may alter the structure of platelet membrane leading to an increased sensitivity of the platelets to aggregating agents without significantly altering the levels of lipid constituents.

Increased thromboxane B_2 production has been shown

during aggregation of platelets isolated from diabetic patients (7,8). To determine whether an increase in thromboxane B_2 production could be induced by exposure of platelets to LDL isolated from diabetic patients, we compared the production of thromboxane B_2 during aggregation of platelets incubated with LDL from normal and diabetic subjects. A significant increase in thromboxane B_2 production was observed during the aggregation of platelets incubated with LDL from diabetic patients. We also found an increased thromboxane B_2 production from platelets incubated with LDL glycosylated in vitro. Our data suggest that glycosylated LDL contributes to the hyperaggregability of platelets in diabetes mellitus, not only by enhancing the reactivity of platelets to aggregating agents but also by increasing the production of thromboxane B_2, a well-known proaggregatory substance.

In conclusion, our study shows another mechanism by which lipoprotein abnormalities may contribute to the acceleration of atherosclerosis in diabetes mellitus.

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