Altered Myosin Light-Chain Phosphorylation in Resting Platelets From Premenopausal Women With Diabetes

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Gender-related differences in the rate of coronary heart disease (CHD) between premenopausal women and men are greatly diminished in women with diabetes mellitus (DM). This may be related, in part, to altered platelet function in premenopausal diabetic women. Hyperglycemia may contribute to increase platelet aggregation through enhancement of oxidative stress, increased nitric oxide (NO) destruction, and increased myosin light-chain (MLC) phosphorylation (MLC-P). Accordingly, we investigated functional and biochemical parameters of platelet function in 32 women (14 premenopausal and postmenopausal controls and 18 age-matched patients with DM); platelet MLC-P and cyclic guanosine monophosphate ([cGMP] reflecting NO) were assessed. Other parameters including age, body mass index (BMI), waist to hip ratio, total cholesterol, and platelet count were not different in the control and diabetic groups. In the premenopausal women, baseline MLC-P was lower in women with DM versus the control group ($P = .02$). GMP levels were similar in the two groups at baseline (22.7 ± 3 fmol/mL in controls *v* 23.1 ± 3 fmol/mL in diabetic subjects) and 3 minutes after insulin exposure. The platelet content of ascorbic acid (AA), an endogenous antioxidant compound, was elevated in premenopausal women with DM ($P = .02$) compared with the controls. Despite similar estradiol (βE_2) levels, platelets of premenopausal women with DM exhibited reduced MLC-P. This paradoxic difference may be accounted for by an increase in platelet AA, as this suggests decreased platelet oxidative stress in this patient population. These observations indicate that an altered redox state and associated MLC-P of platelets does not contribute to enhanced platelet aggregation and CHD in premenopausal women with DM.
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WHILE CORONARY HEART DISEASE (CHD) has been generally considered to affect men, the World Health Organization in 1991[1] reported that it is the leading cause of death for women of all ages in the United States. This disease accounts for nearly 30% of all deaths among women.[2,3] Diabetes mellitus (DM) seems to eliminate the normal gender-related differences in the prevalence of CHD between premenopausal women and men.[4-6] The risk of coronary events in diabetic women is double that of nondiabetic women, even when corrected for diabetes-associated hypertension, dyslipidemia, and obesity.[6] Thus, the protective effects of endogenous estrogens normally observed in premenopausal nondiabetic women appear to be blunted in diabetic women[4]; however, the mechanisms by which diabetes obviates the cardiovascular protective effects of female sex hormones are not well understood.

The increased cardiovascular disease prevalence in individuals with DM appears to be multifactorial. These pathologic factors include enhanced platelet aggregation, relatively greater coagulation and decreased fibrinolytic activity, lipoprotein abnormalities, endothelial dysfunction, and enhanced oxidative stress.[7-10] There is increasing evidence that hyperglycemia contributes to alterations in coronary and systemic blood flow and platelet function and to increased vascular permeability through enhancement of oxidative stress and production of free radicals.[11-16] The cellular handling of this stress requires a balance between the generation of free radicals (superoxide anions) and antioxidants.[12] An increase of oxygen free radicals in diabetes has been implicated in the reduction of cellular antioxidants, as well as a decrease in the function of antioxidant enzymes and an increase in nitric oxide (NO) degradation. Furthermore, hyperglycemia in diabetic women decreases estradiol (βE_2)-mediated NO production.[17] Although several studies have indicated that levels of ascorbic acid (AA) and altered redox status[11-16] are decreased in plasma and tissues from diabetics compared with controls, platelet AA levels are augmented in diabetics compared with controls.[18] Plasma ascorbate depletion does not necessarily reflect the level of AA, and probably metabolic control, as well as intracellular lipoperoxides, modulate platelet AA.[18]

Abnormalities of vasomotion, platelet aggregation, and endothelial permeability may be attributable to altered activation/phosphorylation of myosin light chain (MLC), a protein known to play a key role in the contractile activation of platelets and endothelial and vascular smooth muscle cells.[19-22] Fukuda et al[23] have reported that spontaneous platelet aggregation is enhanced in type 2 diabetic patients. In that study, myosin regulatory light-chain phosphorylation (MLC-P), a requisite step in platelet aggregation,[23] was greater in type 2 diabetic patients than in control subjects irrespective of age. Spontaneous platelet aggregation was positively associated with basal MLC-P, leading to the suggestion that increased basal MLC-P in platelets contributed to platelet hyperaggregability in type 2 diabetic patients.[23] However, the study did not examine the impact of gender and/or menopausal state. To further under-

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stand the gender-related differences observed in diabetes,[17] we sought to determine if the negative effect of diabetes on the cardiovascular protective effects of endogenous estrogens might be due in part to alterations in platelet function via changes in MLC-P or hyperglycemia-induced oxidative stress. Since NO and its second messenger cyclic guanosine monophosphate (cGMP) inhibit MLC-P via increased MLC phosphatase activity,[24] we hypothesized that increased oxidative stress in the platelets of diabetic patients may negate estrogen effects through increased NO destruction and lead to an increase of MLC-P.[25]

SUBJECTS AND METHODS

Subjects

We studied 32 women: 14 premenopausal (pre-M) and postmenopausal (post-M) controls aged 18 to 70 years and matched DM patients. In the control group, there were 9 pre-M and 5 post-M women, while in the DM group, there were 10 pre-M and 8 post-M women with clinical DM randomly selected for the present study. The division of the groups into pre-M and post-M was based on estrogen levels.

Control or DM subjects using antiplatelet agents (ie, aspirin) were excluded or asked to return after stopping the medication for at least 1 week. Specific testing to ensure that patients were not taking aspirin or antiplatelet medications was not performed. Patients with severe hyperlipidemia, uncontrolled hypertension, and serum creatinine above 1.4 mg/dL were excluded. The duration of diabetes, body mass index (BMI), waist to hip ratio, diabetes treatment, blood pressure, and race were ascertained. The fasting blood glucose, glycated hemoglobin, creatinine, and lipid profile were measured as part of the routine clinic visit. Estrogen and progesterone (*P*) levels, the redox status, and platelet MLC-P and cGMP were analyzed on blood samples obtained by routine venipuncture.

Hormone Assessment

βE_2 and P levels were measured by radioimmunoassay (Coat-a-Count; Diagnostic Products, Los Angeles, CA). The detection limit (or minimal detectable dose) of the assay defined as the concentration of 95% B/B_o was approximately 6 pg/mL for βE_2 and 0.02 ng/mL for P. The intraassay coefficient of variation was between 3.0% and 5.0%.

Oxidative Stress

AA, dihydro AA (DHAA), and other redox products were quantified by high-performance liquid chromatography with electrochemical detection as described by Schell and Bode.[26] Briefly, samples were homogenized in ice-cold 0.3% metaphosphoric acid, 0.1 mmol/L EDTA, and 1 mmol/L thiourea to stabilize AA and DHAA and centrifuged at 19,000 $\times$ *g* for 20 minutes. The supernatant fraction was analyzed directly or reduced for 10 minutes at room temperature with 10 mmol/L 2-mercaptoethanol. Samples were injected onto a C18 reverse-phase Waters (Boston, MA) cartridge-type column, and AA/DHAA were detected with an ESA (Boston, MA) model 5100 A Coulochem Detector.[12] Glutathione (GSH) and glutathione disulfide (GSSG) samples were prepared as for AA/DHAA measurement and analyzed according to the method of Griffith.[27]

Platelet Preparation

One volume of 3.8% sodium citrate was immediately added to 9 vol blood, and the mixture was centrifuged at 150 $\times$ *g* for 10 minutes at room temperature.[8] The supernatant platelet-rich plasma (PRP) was aspirated and recentrifuged at 1,500 $\times$ *g* for 10 minutes to obtain platelet-poor plasma. The PRP was then suspended at a mean platelet count of approximately 2.5×10^8/mL with Tyrode buffer.[8,23]

Quantitative Analysis of MLC-P

MLC-P in platelets (at baseline and 3 minutes after thrombin 10 U final concentration) was assessed on urea-glycerol gels using chicken gizzard MLC (Sigma, St Louis, MO) as a standard.[28,29] PRP (100 μL) was mixed with 500 μL lysis buffer (25 mmol/L Tris, pH 8.0, 100 mmol/L Na^+ -pyrophosphate, 100 mmol/L NaF, 250 mmol/L NaCl, 0.5% 3-[(3-cholamidopropyl)dimethyl ammonio]-1-propanesulfonate (CHAPS), 1% Triton X-100, 10 mmol/L EGTA, 5 mmol/L EDTA, 500 μmol/L 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), 1 μmol/L E-64, 1 μmol/L leupeptin, and 1 μg/mL aprotinin). After centrifugation at 4°C and 14,000 $\times$ *g* for 15 minutes, trichloroacetic acid ([TCA] 20%, 200 μL) was added to the supernatant (200 μL) and the preparation was then cooled on ice for 30 minutes. The PRP-TCA solution was centrifuged at 4°C and 10,000 $\times$ *g* for 20 minutes. Sediments were washed 3 times with diethyl ether, dried, and solubilized in urea glycerol gel sample buffer (6.7 mol/L urea, 10 mmol/L dithiothreitol, 18 mmol/L Tris, pH 8.6, 20 mmol/L glycine, 5% saturated sucrose, and 0.004% bromophenol blue). Urea glycerol gels were used as described by Persechini et al[30] with the Mini-Protean II vertical electrophoresis system (Bio-Rad, Hercules, CA) with 0.75-mm gel spacers. After preelectrophoresis at 200 V for 120 minutes at 25°C, samples (20 μL) were electrophoresed for 120 minutes at 250 V. Electrophoretic transfer of proteins from urea glycerol gels onto polyvinylidene difluoride (PVDF) was followed by immunodetection.[27,28] The PVDF membranes were incubated for 30 minutes at 37°C with 4 μg/mL monoclonal anti-MLC 20K (Sigma Chemical). Subsequently, the PVDF membranes were incubated for 30 minutes at room temperature with 2 μg/mL anti-mouse IgG antibody conjugated with horseradish peroxidase (ICN Biomedicals, Costa Mesa, CA) and exposed to ECL Western blotting detection reagents (Amersham, Life Science, Arlington Heights, IL). Densitometry of immunoblots was performed using a Laser Densitometer equipped with a recording integrator (Molecular Dynamics, Sunnyvale, CA).

For protein quantitation, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels were analyzed with a protein standard of appropriate size (trypsin inhibitor; Sigma) at different known concentrations. SDS-PAGE gels were stained with Gelcode blue stain reagent (Pierce, Rockford, IL) and dried overnight onto cellophane membranes, and a standard curve was obtained by laser densitometry. The protein concentrations of the subjects' samples were then calculated from the arbitrary density units of each sample as compared with the standards.

cGMP Assay

Platelets were exposed to insulin 100 nmol/L[31] for 3 minutes and changes in basal and/or stimulated cGMP levels were used as a surrogate measure of NO production. Platelet cGMP was extracted with alcohol and ether and analyzed by radioimmunoassay using an acetylation protocol (Amersham Pharmacia Biotech, Piscataway, NJ).

Statistical Analysis

The data are expressed as the mean $\pm$ SE. Both Student's *t* test and the Mann-Whitney 2-sample test were used to calculate differences between the groups. A *P* value less than .05 was considered statistically significant.

RESULTS

Patient Characteristics

The age, race, BMI, waist to hip ratio, cholesterol level, and platelet count were not statistically different between the controls and diabetics overall and between the pre-M diabetic and control women. The mean glycated hemoglobin in the DM population was 10.5%. As a hormonal assessment, βE_2 and P levels were obtained to ascertain the impact of circulating

Table 1. Profile of the Control Subjects and DM Women (mean ± SE)

Parameter	Control		DM	
	All (n = 14)	Pre-M (n = 9)	All (n = 18)	Pre-M (n = 10)
Age (yr)	38.8 ± 3.5	29.5 ± 1.4	43.4 ± 3.6	32.3 ± 3.3
BMI (kg/m^2)	24.9 ± 1.0	24.2 ± 1.6	28.1 ± 1.3	26.3 ± 1.4
Waist to hip ratio	0.79 ± 3.3	0.82 ± 4.1	0.87 ± 0.0	0.84 ± 3.6
Total cholesterol (mg/dL)	210 ± 10	206 ± 13	198 ± 7	195 ± 10
Glycated hemoglobin (normal, 4%-8%)			10.6 ± 2.4	10 ± 2.4
Fasting serum glucose (mg/dL)	75.7 ± 10	74.5 ± 1.4*	153 ± 57	160 ± 74*
Platelet count ($\times 10^3/\mu$L)	186.7 ± 20.4	161.4 ± 21.4	204.5 ± 22.0	230.1 ± 30.1

* Paired *t* test, $P = .01$.

hormones on platelet MLC-P. βE_2 levels were slightly higher in the pre-M control group (128 ± 35 *v* 81 ± 23 pg/mL in DM pre-M women) and P levels were slightly higher in pre-M DM women (1.9 ± 0.8 ng/mL in C *v* 4.0 ± 2.1 ng/mL in DM), but these differences were not significant. Table 1 summarizes the characteristics of these two well-matched populations.

Quantitative Measurement of MLC-P

Figure 1 shows a representative MLC-P immunoblot. Samples obtained from control and diabetic women were electrophoresed along with standard protein and immunoblotted with the corresponding antibody. The amount of protein in the sample was estimated based on a standard calibration curve obtained from trypsin inhibitor as a protein standard, which was linear within the range of 0 to 25 μg of sample loaded. Thrombin stimulation caused a shift in the phosphorylation state from the baseline, as shown in the two bands on the gel.

Figure 2 presents the mean values for patient MLC-P in platelets at baseline, estimated by densitometry and obtained from pre-M control (n = 9), post-M control (n = 5), pre-M DM (n = 10), and post-M DM (n = 8) groups. Although baseline MLC was marginally different ($P = .06$) between the control and DM patients overall, in pre-M diabetics, it was significantly lower ($P = .02$) versus the pre-M controls. These differences are not the result of unequal protein loading, as the measurement of the protein concentration showed similar amounts in the pre-M controls (14.9 ± 1.2 μg/20 μL) and pre-M DM group (14.2 ± 0.7 μg/20 μL, $P = .5$). Thrombin-stimulated MLC-P, although lower in the pre-M DM group, was not different between the groups (data not shown). We then determined whether the cGMP level, as a surrogate for NO, was different at baseline in platelets of diabetic women compared with controls and whether the response to insulin was impaired.

NO Assessment

cGMP values were similar between the groups at baseline (22.7 ± 3 fmol/mL in control *v* 23.1 ± 3 fmol/mL in DM) and after 3 minutes of exposure to insulin (70.1 ± 26 in control *v* 64.5 ± 19 in DM). This was true in platelets obtained from the pre-M control and pre-M DM groups as well (Fig 3), suggesting that differences in NO levels do not contribute to the lower MLC-P.

Oxidative Stress

AA, GSH, and GSSG in platelets and plasma from control and DM groups were quantified. Table 2 lists the results of some oxidative stress parameters in platelets and plasma from pre-M control and pre-M DM subjects. AA detected in platelets was significantly higher in pre-M DM women ($P = .02$) versus the pre-M control group. No other significant differences were obtained for other measures of platelet redox status among the groups. No differences were found in plasma. Since plasma measures of AA levels were identical between the groups, the increase in the platelets of diabetic women probably represents a specific response to the diabetic state.

DISCUSSION

In this investigation, we observed that platelet MLC-P at baseline was lower in pre-M diabetic women than in controls. GMP levels were not different between these two groups of age-matched women, but intraplatelet AA concentrations were significantly elevated in pre-M DM women, perhaps indicative of a compensatory scavenger mechanism for the increase in oxidative stress.[12] Our finding of increased AA in platelets in this diabetic population is similar to the findings of Seghieri et al[18] in a group of patients with type 1 diabetes. Platelet aggre-

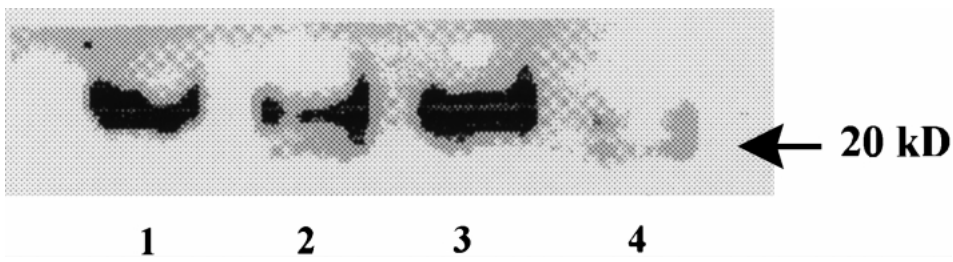

Fig 1. Representative immunoblot of platelet proteins separated on a urea-glycerol gel and detected with a specific MLC antibody. Lanes 1 and 3, baseline samples; lanes 2 and 4, same patient after treatment with 10 U thrombin; enhanced phosphorylation is shown by the downward shift.

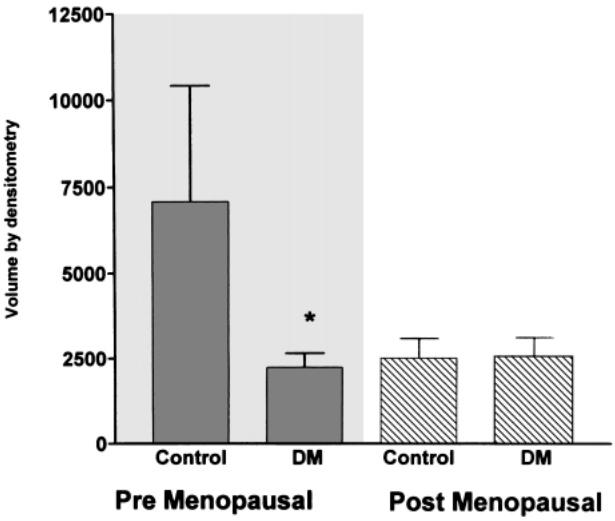

Fig 2. Densitometric analysis of MLC gels presented as the mean ± SE of arbitrary volumes in control and DM women. *$P = .02$, control *v* DM. No differences were detected in MLC-P at baseline in the post-M women.

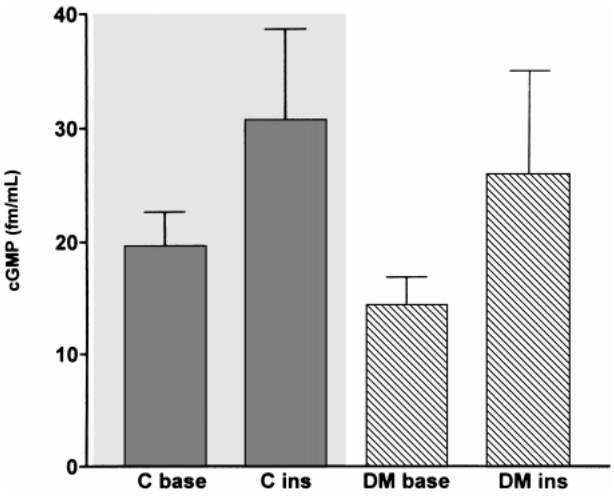

Fig 3. Radioimmunoassay of cGMP in platelets of pre-M women at baseline and after 3 minutes of exposure to insulin. Although insulin significantly enhanced cGMP levels, there were no differences between the 2 groups.

gation and adhesion are characteristically accentuated in persons with diabetes.[8,32,33] These functional abnormalities appear to relate to platelet intracellular calcium mobilization and phosphoinositide turnover.[32-34] In such platelets, increases in spontaneous aggregation correlate with increased MLC-P on serine 19, the site phosphorylated by MLC kinase.[23] This leads to actomyosin contraction and platelet aggregation.[22,35,36] Dephosphorylation of MLC by MLC phosphatase decreases the activity of actomyosin ATPase.[22,35] However, the pre-M DM women in this study had decreased basal MLC-P. Recent studies indicate that diabetes is associated with decreased platelet NO production.[37,38] NO inhibits platelet aggregation and adhesion by increasing intracellular cGMP levels and decreasing calcium through activation of soluble guanylate cyclase.[39,40] One hypothetic explanation for the increase of MLC-P in DM reported by Fukuda et al[23] is that reduced platelet NO in diabetic persons may also contribute to enhanced MLC-P and consequent aggregation/adhesion, as NO-induced increases in cGMP activate MLC phosphatase and cause Ca^{2+} desensitization. This speculation is not supported by our study as we did not observe evidence of decreased NO, since the levels of cGMP at baseline and after 3 minutes of exposure to insulin were similar among the groups.

The increased AA levels in pre-M diabetic subjects may reflect a compensatory response to increased oxidative stress, eg, peroxynitrite production. AA has the potential to protect both the cytosolic and membrane components of cells from oxidative damage.[41] Recent evidence suggests that platelets of diabetic subjects contain inducible NO synthase (iNOS), one of the three isoforms of the enzyme generating NO from L-arginine.[42] Of the three NOS isoenzymes, iNOS produces 10- to 50-fold more NO than the constitutive NOS isoform. In addition, iNOS under conditions of limiting L-Arg can produce superoxide (O_2^-), as well as NO.[43] These two radicals react at diffusion-controlled rates to yield peroxynitrite ($ONOO^-$).[44] iNOS also has been reported in megakaryocytes of subjects with CHD.[45] It is therefore conceivable that the high levels of $ONOO^-$ production observed in vitro[42] may result from the induction of iNOS by diabetes-related factors. The presence of iNOS in diabetic platelets combined with the in vitro demonstration of increased peroxynitrite production may explain our failure to document decreased NO production between the control and DM women. The increased peroxynitrite level[41]

Table 2. Profile of Oxidative Stress in Pre-M Control and DM Women (mean ± SE)

Parameter	Pre-M Control		Pre-M DM	
	Platelets	Plasma	Platelets	Plasma
Protein (mg/mL)	14.9 ± 1.1		14.2 ± 0.7	
AA				
pmol/1,000 platelets	24.8 ± 2.8*		35.6 ± 3.0*	
nmol/mL plasma		17.6 ± 5.7		28.8 ± 5.6
GSH				
Total/1,000 platelets	10.6 ± 2.5		16.9 ± 4.3	
nmol/mL plasma		3.1 ± 0.5		2.7 ± 0.4
GSSG (total/1,000 platelets)	3.19 ± 0.5	Not detected	2.72 ± 0.4	Not detected

* $P < .05$.

may serve as a stimulus for the high platelet AA concentration and scavenging capacity found in the pre-M DM women.

In conclusion, neither βE_2 nor baseline platelet NO concentrations were lower in the pre-M diabetic women and therefore cannot be implicated in the lack of cardiovascular protection usually afforded by the pre-M state. However, the scavenging action of an apparently compensatory increase in platelet AA, perhaps in response to enhanced iNOS, may have maintained "normal" NO levels and thus decreased basal MLC-P in the pre-M diabetic individuals. Finally, these results do not substantiate a prior report in Japanese diabetic patients of increased MLC-P in resting platelets. Our observations underscore the importance of considering gender and menopausal status when assessing platelet function in diabetic persons. Since this study suggests a link between iNOS and oxidative stress, further studies should be undertaken to determine if iNOS-dependent peroxynitrite is responsible for platelet dysfunction in vivo and to identify the diabetes-derived agent(s) that induces iNOS expression in platelets,[42] as well as the possible correlation with MLC-P and AA levels.

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