Low intracellular magnesium levels promote platelet-dependent thrombosis in patients with coronary artery disease

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Background Although reduced intracellular levels of magnesium have been described in patients with acute myocardial infarction, its significance as a regulator of thrombosis remains unknown.

Methods and Results To determine whether reduced intracellular levels of magnesium enhance platelet-dependent thrombosis, we evaluated 42 patients with coronary artery disease (CAD) by exposing porcine aortic media to their flowing unanticoagulated venous blood for 5 minutes by using an ex vivo perfusion (Badimon) chamber. Baseline analysis demonstrated significant associations between intracellular levels of magnesium, platelet-dependent thrombosis ($P = .02$), and platelet P-selectin (CD62P) expression ($P < .05$). Patients were divided into 2 groups: below (n = 22) and above (n = 20) the median intracellular levels of magnesium (1.12 μg/mg protein). There were no significant differences in age, body mass index, serum lipids, fibrinogen, platelet count, or serum magnesium levels between the two groups. Platelet-dependent thrombosis was significantly higher in patients with intracellular levels of magnesium below compared with above median (150 ± 128 vs 45 ± 28 $\mu m^2/mm$, $P < .004$). Neither platelet aggregation nor CD62P expression was significantly different between the two groups.

Conclusions Platelet-dependent thrombosis was significantly increased in patients with stable CAD with low intracellular levels of magnesium, suggesting a potential role for magnesium supplementation in CAD. (Am Heart J 2000;140:212-8.)

Platelet activation is a key element in acute vascular thrombosis, which is important in the pathogenesis of acute myocardial infarction and complications of coronary balloon angioplasty. Studies have demonstrated that magnesium can suppress platelet activation by either inhibiting platelet-stimulating factors such as thromboxane A_2 or by stimulating synthesis of platelet-inhibitory factors such as prostacyclin (prostaglandin I_2).[1-3] These observations together with our finding of reduced mortality rates in thrombolysis-ineligible patients with acute myocardial infarction treated with intravenous magnesium[4-6] have led us to hypothesize that magnesium may modulate platelet-dependent thrombosis. In this study, we used an ex vivo perfusion chamber (Badimon) model of acute platelet-dependent thrombosis in patients with stable coronary artery disease (CAD) to determine whether platelet-dependent thrombosis was related to intracellular magnesium levels.

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Methods

Study population

Patients were recruited from a supervised cardiac exercise and rehabilitation program at Cedars-Sinai Medical Center. Inclusion criteria included men and women >20 years of age, with CAD documented by prior myocardial infarction, coronary artery bypass grafting operation, or coronary angiography or angioplasty. Exclusion criteria included unstable angina, congestive heart failure New York Heart Association class >IV, chronic diarrhea, kidney failure (serum creatinine >3 mg/dL), acute myocardial infarction <3 months, hyperthyroidism/hypothyroidism, type I (insulin-dependent) diabetes mellitus, peripheral vascular disease, history of drug or alcohol abuse, chronic liver disease, or refusal to sign the informed consent form. The study was approved by the institutional review board, and witnessed informed consent was obtained from each patient.

Platelet-dependent thrombosis measurement

After an overnight fast, a 19-gauge butterfly catheter was inserted atraumatically without a tourniquet into an antecubital vein. Flowing unanticoagulated venous blood from the patient was drawn over a segment of prepared porcine aortic media held in a tubular superfusion flow chamber by a peristaltic pump placed distal to the chamber. The chamber was designed to mimic the cylindrical shape of blood vessels and contained a window that permitted direct exposure of the aor-

tic media to the flowing venous blood.[7,8] A perfusion chamber with an internal diameter of 1.0 mm was selected to generate shear rates of 800 s^{-1}, at a flow rate of 5 mL/min for 5 minutes. This shear rate corresponds to that encountered in mild to moderately stenosed arteries. The aortic media used in the superfusion chamber was obtained from normal pigs by opening the aorta longitudinally and peeling off and discarding the intima and a thin portion of the subjacent media. The remaining aortic media was then divided into 35 × 15-mm segments to be placed inside the superfusion flow chamber to be exposed to flowing blood in the chamber. Exposure of the arterial media simulates a deep arterial wall injury with a thrombogenic response similar to that of a plaque rupture.

After the perfusion, the aortic media strips were removed from the chambers, fixed in 2% glutaraldehyde in 2 mol/L sodium cacodylate, and processed for morphologic analysis. The tissues were stained with hematoxylin-phloxine-safranin. The stained histologic tissue was then analyzed under a light microscope, and platelet thrombus formation on the aortic media was quantified morphometrically by computer-assisted morphometry, with the use of image analysis software (Bioscan, Optimas, Wash). All measurements were made in a blinded fashion by 2 different observers who were blinded to the patient's intracellular magnesium levels, and intra-assay variability was 5.5% ± 5.6%. Thrombus size measurements were expressed as the average of 6 analyzed sections per tissue (2 in the proximal, 2 in the mid, and 2 in the distal section), expressed as the surface area in square micrometers and normalized to the cross-sectional diameter of the exposed media (in millimeters). This morphometric method has been previously validated and shows a strong correlation ($r = 0.84$, $P = .0001$) between the amount of indium-labeled platelets deposited on the media and the morphometrically assessed thrombus size.[9]

Platelet aggregation and P-selectin flow cytometry

Citrated whole blood samples (4.5 mL) were taken from the patients before starting the ex vivo thrombosis experiment, and this was diluted with an equal volume of isotonic saline. Collagen-induced (2 μg/mL) whole blood platelet aggregation was measured by impedance aggregometry.[10] Platelet aggregation was measured as the maximal change in impedance produced 6 minutes after the addition of collagen and expressed in ohms and the rate (slope) expressed in ohms per minute.

Platelet α-granule release, detected by expression of P-selectin (the CD62P antigen or GMP-140), was measured by whole blood flow cytometry. Blood samples were prepared for flow cytometry analysis with the use of the whole blood method described by Janes et al.[11] Five μL of citrated blood was added to tubes containing 50 μL of HEPES-buffered saline (NaCl 0.145 mol/L; KCl 5 mmol/L; $MgSO_4$ 1 mmol/L; HEPES 10 mmol/L; pH 7.4) plus 5 μL of FITC-conjugated anti-human monoclonal antibodies (Becton-Dickinson, San Jose, Calif). After gentle mixing, the samples were incubated for 20 minutes, then diluted with 0.5 mL of 0.2% formyl saline to inhibit further activation. A sample incubated with FITC-conjugated irrelevant mouse immunoglobulin G_1 at identical concentration served as negative isotype control. Incubations were carried out at room temperature (22° to 26° C). All samples were analyzed within 1 hour of collection in a FACScan flow cytometer (Becton-Dickinson Immunocytometry Systems) with LYSIS II software. The platelet population was identified on the basis of their forward-scatter (size) and side-scatter (granularity) characteristics and the expression of glycoprotein IIIa (FITC-CD61 profile), a membrane protein present exclusively on all resting and activated platelets. Activated platelets were identified on the basis of the expression of P-selectin (PE-CD62 profile), a platelet α-granule protein that is only expressed on the platelet surface after activation and degranulation. Platelet CD62P expression was measured at baseline and after stimulation with ADP (5 μm/L) (Chronolog Corp, Havertown, Pa). For each sample, 5000 platelets were gated, and platelet activation was expressed as the number of CD-62+ve platelets as a percentage of CD-61+ve platelets after adjusting for nonspecific fluorescence.

Intracellular magnesium concentration

Mononuclear (lymphocytes) cells were isolated from the whole blood by modified Elin's method.[12] At room temperature, heparinized blood (10 mL) was mixed with an equal volume of buffered saline and glucose solution (BSG) at pH 7.4, containing NaCl 8.1 g/L (0.14 mol/L), Na_2HPO_4 1.22 g/L, and $NaH_2PO_4 \cdot H_2O$ 0.194 g/L. Twenty milliliters of Ficoll-paque (Amersham-Pharmacia Biotech, Uppsala, Sweden) was then layered below the blood and BSG with the use of a clean pipette and was then centrifuged at 400*g* for 35 minutes. The mononuclear cell layer was collected from the Ficoll-plasma interface, washed with 10 mL of BSG, and centrifuged at 600*g* for 10 minutes. The supernatant was then discarded and the pellet was washed in another 10 mL of BSG and centrifuged at 2000*g* for 10 minutes. The pellet was then brought up in 2 mL of distilled water and frozen until time of assay. Before assay, the cells were thawed and then lysed by sonication. The intracellular levels of magnesium in isolated mononuclear cells was measured by atomic absorption spectrophotometry (normal value 1.23 ± 0.02 μg/mg protein).[13] Intra-assay variability for intracellular magnesium was 5.1% ± 7.2% and interassay variability was 7% ± 5%.

Statistical analysis

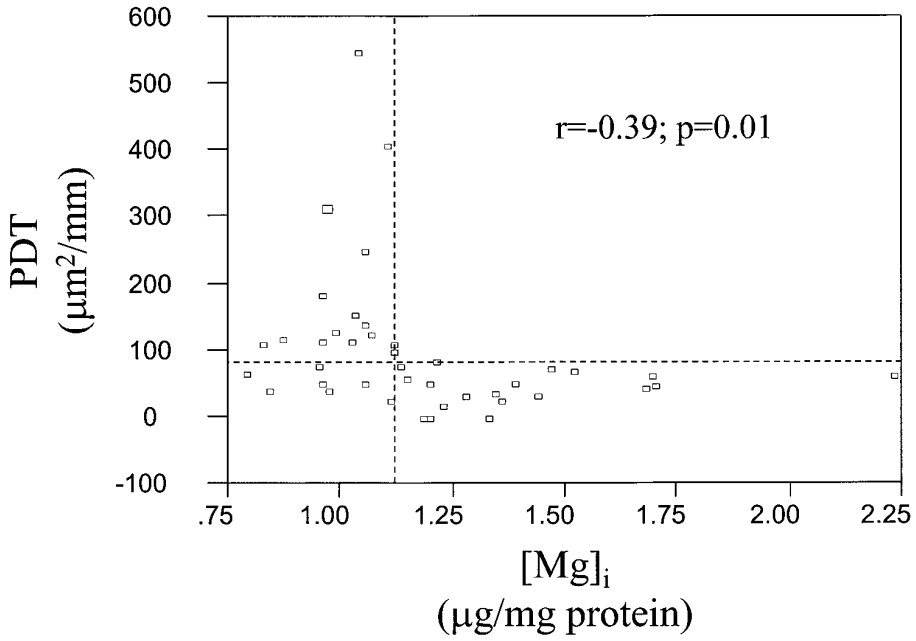
Group data are expressed as mean ± SD. Comparison of biochemical measurements was performed with the use of the unpaired Student's *t* test and Wilcoxon signed-rank test. The relation between baseline characteristics and intracellular magnesium concentration with PDT was performed by means of the Pearson correlation, as appropriate. Log transformations were used to normalize data for regression analysis. Predictors of PDT were determined by means of linear regression and multiple stepwise regression analysis. A value of $P < .05$ was required to reject the null hypothesis.

Results

Our study population comprised 42 stable coronary patients (37 men and 5 women), with a mean age of 68 ± 9 years (range 48 to 83 years). All patients had stable CAD as evidenced by a previous myocardial infarction (n = 23), coronary artery bypass grafting (n = 26), or coronary angioplasty (n = 23).

Platelet-dependent thrombosis was significantly correlated inversely with intracellular magnesium level ($r = -0.39$, $P = .01$) and positively with resting systolic blood pressure ($r = 0.34$, $P = .03$) and total serum cholesterol

Figure 1



Correlation of intracellular magnesium concentration *([Mg]$_i$)* and platelet-dependent thrombosis *(PDT)*. *Dashed lines* demonstrate median intracellular magnesium level of 1.12 μg/mg protein and median thrombus volume of 64 μm²/mm.

Table I. Baseline characteristics of study population

	[Mg]$_i$ (μg/mg protein)		
Variable	**≤1.12 (n = 22)**	**>1.12 (n = 20)**	***P* value**
Men (%)	86	90	1.00
Age (y ± SD)	68 ± 9	69 ± 10	.92
BMI (kg/m2)	26	26	.93
Prior MI (%)	63	45	.35
Prior PTCA (%)	55	55	.77
Prior CABG (%)	64	60	1.00

BMI, Body mass index; *[Mg]$_i$,* intracellular magnesium level; *MI,* myocardial infarction; *PTCA,* percutaneous coronary angioplasty; *CABG,* coronary artery bypass grafting.

level (r = 0.39, P = .02). After adjustment in a multivariate stepwise regression model, intracellular magnesium level remained the only independent and significant predictor of platelet-dependent thrombosis (P = .02).

Correlation analysis of intracellular magnesium and platelet-dependent thrombosis (Figure 1) demonstrated that patients with intracellular magnesium concentrations above the median of 1.12 μg/mg protein had the lowest thrombus volume when compared with patients with concentrations equal to or below the median magnesium level (r = –0.39, P = .01). We therefore divided our patients into two groups: below or equal to (n = 22) and above (n = 20) the median intracellular magnesium level of 1.12 μg/mg protein. The two groups were similar with respect to male sex, age, body mass index, clinical features (Table I), and the use of cardiac medications, including diuretics (Table II). All patients received aspirin and two thirds received lipid-lowering medications. The two groups were also similar with regard to the baseline lipid values of total cholesterol, low-density lipoprotein, high-density lipoprotein, triglycerides, apolipoprotein A-I, apolipoprotein B, and fibrinogen, platelet count, and serum magnesium levels (Table III).

There was no difference in the mean serum magnesium level between the two groups (2.10 ± 0.18 vs 2.02 ± 0.21 mg/dL, P = .17), although the mean intracellular magnesium level was significantly higher in the group above compared with the group below or equal to the median intracellular magnesium level (1.37 ± 0.28 vs 0.98 ± 0.09 μg/mg protein, P < .0001).

Correlation analysis between P-selectin expression (CD62P antigen) of unstimulated platelets and intracellular magnesium levels was statistically significant (r = –0.31, P < .05) and demonstrated that the lower the intracellular magnesium levels, the higher the CD62P expression, a marker of platelet activation.

Table II. Concomitant medication

Variable	$[Mg]_i$ (μg/mg protein) ≤1.12 (n = 22)	>1.12 (n = 20)	*P* value
β-Blockers (%)	27	45	.34
Calcium antagonists (%)	32	35	1.00
Digoxin (%)	9	10	1.00
Lasix (%)	14	10	1.00
Aspirin (%)	100	100	1.00
Long-acting nitrates (%)	5	15	.33
ACE inhibitors (%)	36	20	.32
Lipid-lowering agents (%)	55	75	.21

ACE, Angiotensin-converting enzyme; *$[Mg]_i$,* intracellular magnesium level.

Table III. Lipids, platelets, and serum magnesium

Variable	$[Mg]_i$ (μg/mg protein) ≤1.12	>1.12	*P* value
Total cholesterol (mg/dL)	175 ± 23	167 ± 30	.30
LDL-C (mg/dL)	96 ± 26	99 ± 26	.58
HDL-C (mg/dL)	42 ± 10	40 ± 11	.82
Triglyceride (mg/dL)	156 ± 132	139 ± 73	.29
Apo A-I (mg/dL)	127 ± 15	119 ± 17	.90
Apo B (mg/dL)	89 ± 20	86 ± 27	.80
Fibrinogen (mg/dL)	301 ± 73	289 ± 43	.48
Platelet count ($\times 10^3$)	197 ± 51	197 ± 48	.39
Serum [Mg] (mg/dL)	2.02 ± 0.21	2.10 ± 0.18	.17

Apo, Apolipoprotein; *LDL-C,* low-density lipoprotein cholesterol; *HDL-C,* high-density lipoprotein cholesterol; *[Mg],* magnesium level; *$[Mg]_i$,* intracellular magnesium level. Values are expressed as mean ± SD.

Platelet-dependent thrombosis was significantly higher in patients with intracellular magnesium levels below than above median levels (150 ± 128 vs 45 ± 28 $\mu m^2/mm$, $P < .005$) (Figures 2 and 3). Neither platelet aggregation (10.0 ± 5.4 vs 8.4 ± 5.2 Ω, $P = .37$), nor non-stimulated platelet P-selectin expression (25.6% ± 20.8% vs 16.7% ± 18.1% gated, $P = .14$) (Figure 3) were significantly different between the two groups.

Discussion

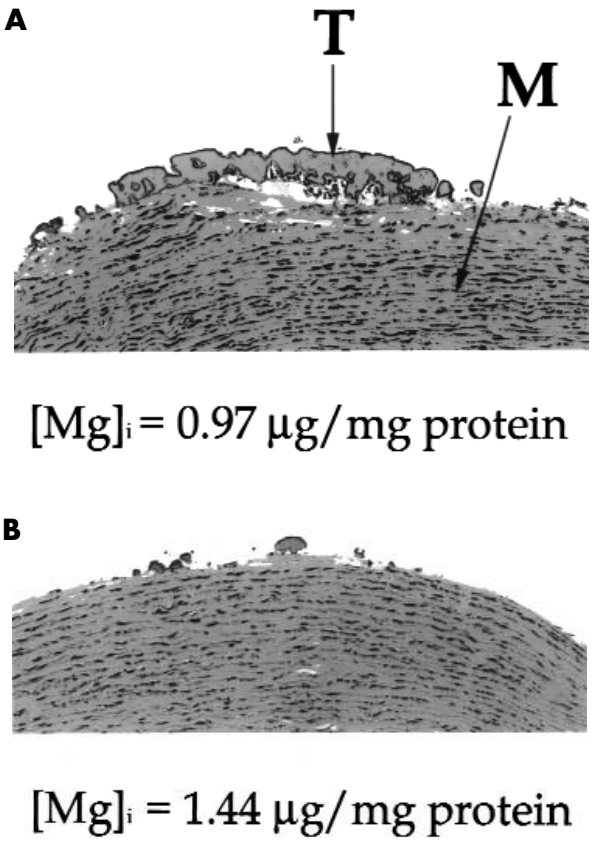
Our study demonstrated that platelet-dependent thrombosis is significantly increased in patients with stable CAD with low intracellular levels of magnesium.

Serum magnesium, like serum potassium, is often normal despite depletion of total body magnesium.[13] Intracellular levels of magnesium are more accurate measures[14]; however, there is often poor correlation of intracellular red blood cell and intracellular mononuclear cell magnesium,[15] and these may correlate poorly with the magnesium content of muscle in different disease states.[16] Of the two, intracellular mononuclear cell magnesium is a better indicator of the magnesium status of the heart.[13,14] For example, in a recent study of patients in the coronary care unit, only 7.7% had low serum magnesium levels, but 53% showed low levels of mononuclear cell magnesium.[13]

In this study, we have demonstrated for the first time that intracellular magnesium is an independent and significant predictor of platelet-dependent thrombosis. The ex vivo model of thrombogenesis used in this study has been previously validated and has been shown to correlate well with ^{111}In-labeled platelet deposition.[9] The exact mechanism by which magnesium influences the thrombus volume is not clear. Some experimental work in animal models[17,18] has shown hypercoagulability and increased platelet aggregation during hypomagnesemia, which might contribute to thrombus formation. Extracellular magnesium level is inversely related to platelet aggregation and ATP release[19] and can inhibit a wide variety of agonists of platelet aggregation, such as thromboxane A_2, and stimulate prostacyclin (prostaglandin I_2) synthesis.[20] Recent work has demonstrated that administration of magnesium reduces platelet aggregability in healthy volunteers.[2] It also inhibits fibrinogen-mediated platelet aggregation, which plays a crucial role in acute thrombotic events.[3] Gawaz et al[3] have demonstrated that platelet aggregation, fibrinogen binding, and expression of P-selectin on the platelet surface are all effectively inhibited by intravenous magnesium supplementation. Because glycoprotein IIb/IIIa is the only glycoprotein on the platelet surface that binds fibrinogen, Gawaz et al[3] speculated that magnesium supplementation directly impairs fibrinogen interaction with the glycoprotein IIb/IIIa complex. Because fibrinogen binding to the platelet membrane and surface expression of P-selectin requires previous cellular activation,[21] the inhibitory effect of magnesium might be a consequence of direct interference of the cation with the agonist-receptor interaction or with intracellular signal transduction event. Fibrinogen–glycoprotein IIb-IIIa interaction is regulated by divalent cations, and at pharmacologic levels magnesium may inhibit binding of fibrinogen to glycoprotein IIb/IIIa by altering receptor conformation. This might be caused by competition of magnesium with calcium ions for calcium-binding sites in the glycoprotein IIb subunit.[21]

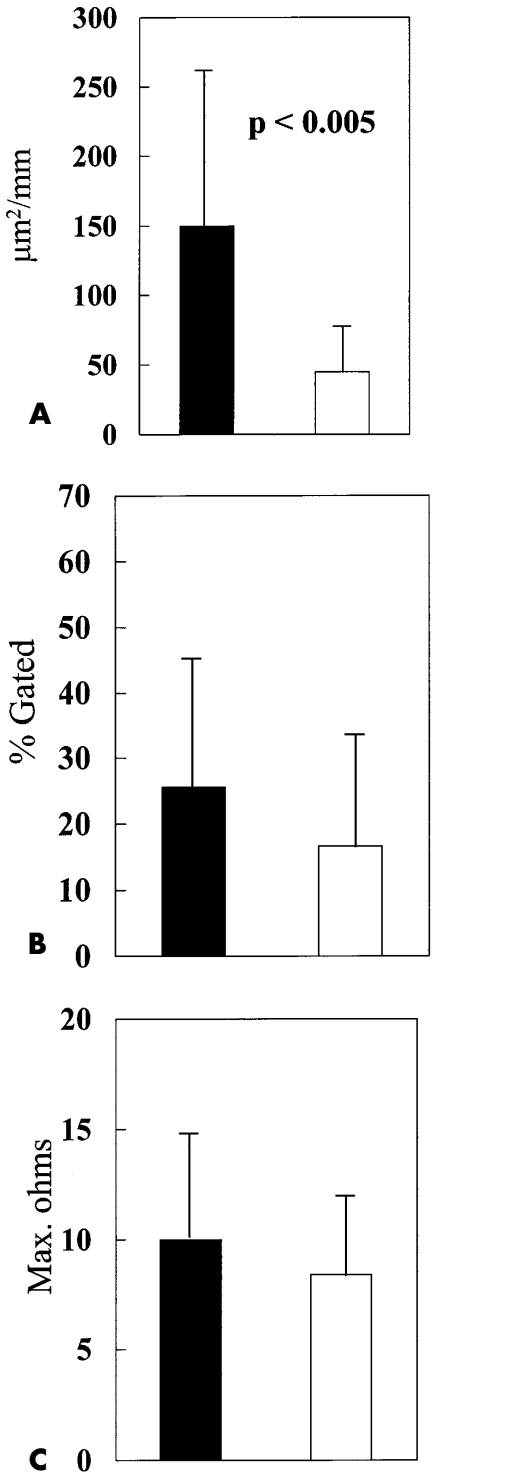
The expected normal platelet aggregation response in a normal donor population without aspirin[22] is 16 ± 5 Ω; however, in our study population the mean platelet aggregation was 9.8 ± 5.9 Ω. Aspirin treatment has a partial inhibitory effect on collagen-induced platelet aggregation, which may explain the lower values in our patients. In the current study we demonstrated a similar inverse correlation of intracellular magnesium levels with thrombus formation and platelet P-selectin (CD62P) expression but not with platelet aggregation. This discrepancy between the relation of magnesium with platelet activa-

Figure 2



Representative histologic sections (hematoxylin-phloxine-safranin stain) showing platelet-dependent thrombosis deposited *(T)* on porcine aortic media *(M)* taken from 2 patients: **A**, Patient with intracellular magnesium below median, with large thrombus area, and **B**, patient with intracellular magnesium above median and smaller thrombus area. *$[Mg]_i$*, Intracellular magnesium concentration.

Figure 3



A, Platelet-dependent thrombosis. **B**, P-selectin (CD62P antigen) expression. **C**, Platelet aggregation in patients with less than or equal to *(closed bars)* compared with more than *(open bars)* median (1.12 µg/mg protein) intracellular magnesium levels. Data are mean ± SD.

tion (CD62P expression) and aggregation may potentially be related to the fact that all patients were taking aspirin, an agent that inhibits platelet aggregation but not platelet activation.[23] Therefore the enhanced platelet-dependent thrombosis demonstrated in patients with low intracellular magnesium levels in our study can be partially explained as a result of enhanced platelet adhesion irrespective of platelet aggregability. The relation between intracellular magnesium levels and platelet CD62P expression may provide additional insights into the pathogenesis of thrombotic disease states. Gawaz et al[24] recently demonstrated that spontaneous (and ADP-induced) P-selectin surface expression on platelets and platelet-leukocyte adhesion was increased in patients with symptomatic CAD compared with normal control patients, and intravenous magnesium administration sig-

nificantly reduced both platelet surface expression of P-selectin and platelet-leukocyte adhesion ex vivo.

Epidemiologic evidence linking magnesium deficiency to CAD and sudden death has been investigated for more than 3 decades.[25-29] In the Atherosclerosis Risk in Communities (ARIC) Study,[30] the relation of serum and dietary magnesium and CAD incidence over 4 to 7 years of follow-up was examined in a sample of 13,922 middle-aged adults free of baseline CAD from 4 US communities. After adjustment for traditional risk factors, the relative risk of CAD across quartiles of serum magnesium was 1.00, 0.92, 0.48, and 0.44 (P for trend = .009). These findings suggest that low magnesium may contribute to the pathogenesis of coronary atherosclerosis or acute thrombosis. Nadler et al[31] have demonstrated that magnesium deficiency produces insulin resistance and increased thromboxane synthesis. These changes would lead to an increase in platelet aggregation and release in growth factors as well as to direct vasoconstriction.

Intracellular measurement of magnesium may predict the potential of thrombus formation in patients with CAD and help physicians to decide on supplementing their patients with magnesium. The lymphocyte magnesium measurement used in the current study is a difficult and time-consuming technique. Recently a much simpler technique, measuring magnesium in sublingual cells scraped from mucosa adjacent to the frenulum by means of dispertive radiographic analysis, has been described and correlated well with human cardiac myocyte magnesium level.[32]

In a randomized, prospective, double-blind, crossover, placebo-controlled study, our group has recently demonstrated that magnesium oxide tablets (800 to 1200 mg/d) supplemented to patients with CAD for 3 months inhibited platelet-dependent thrombus formation significantly by 35% compared with placebo[33]; this reinforces our current results of the importance of intracellular magnesium measurement as predictor of platelet-thrombus formation in patients with CAD.

In conclusion, our study demonstrated that platelet-dependent thrombosis is significantly increased in patients with stable CAD with low intracellular levels of magnesium. These results may suggest a potential protective role for magnesium supplementation in CAD.

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