Deranged Platelet Calcium Homeostasis in Diabetic Patients With End-Stage Renal Failure

A possible link to increased cardiovascular mortality?

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OBJECTIVE — Platelet hyperfunction is a typical feature of the prothrombotic state that frequently complicates the natural history of diabetes. In uremia, a bleeding diathesis is present, which principally involves the primary phase of hemostasis. Thus, in patients with uremia of diabetic origin, the infrequent coexistence of two opposite alterations of hemostasis takes place. In patients with uremia, an increased incidence of cardiovascular events and related mortality is observed. This phenomenon is greatly amplified in uremia of diabetic origin. Calcium homeostasis is a critical aspect of platelet function, which has recently become available in human diseases. The aim of this study was to evaluate calcium homeostasis in platelets from patients with uremia of diabetic and nondiabetic origin.

RESEARCH DESIGN AND METHODS — We evaluated, by means of Fura 2, the intracellular concentration of ionized calcium ($[Ca^{2+}]_i$) in platelets from 18 patients with uremia of diabetic origin, 12 patients with uremia of nondiabetic origin, and 16 healthy control subjects. $[Ca^{2+}]_i$ was evaluated in resting conditions and after stimulation with 0.05, 0.1, 0.5 U/ml thrombin.

RESULTS — Platelets from uremic patients with diabetes had higher resting $[Ca^{2+}]_i$ than both control subjects ($P = 0.01$) and uremic patients without diabetes ($P = 0.001$). Similarly, after stimulation with thrombin, the absolute increase of $[Ca^{2+}]_i$ was higher ($P < 0.05$) in platelets from uremic patients with diabetes compared with both control subjects and uremic patients without diabetes. The relative increase of $[Ca^{2+}]_i$ was higher ($P < 0.05$) than normal in platelets from uremic patients after weak or intermediate strength thrombin. No correlation were present between $[Ca^{2+}]_i$ values and other clinical and laboratory variables potentially associated with platelet hyperfunction.

CONCLUSIONS — Diabetes and uremia in combination further deteriorate the abnormal platelet calcium homeostasis observed in uremia.

Diabetes is a metabolic disorder characterized by both specific (microangiopathic) and nonspecific (macroangiopathic) vascular complications (1). An imbalance of the hemostatic system toward a prothrombotic state is frequently observed in diabetic patients even in the early phases of the disease (2) and becomes more evident during its course (3). Platelets are peculiarly involved: increased adhesiveness (4), hyperaggregability (5), enhanced release reaction (6), and increased turnover (7) have been described.

Although significantly mitigated by dialytic procedures, a bleeding diathesis still represents a major problem in uremic patients (8). Primary hemostasis, i.e., the interaction between platelets and endothelium, is typically involved: enhanced production of prostacyclin (9) and nitric oxide (10) by endothelial cells, reduced platelet adhesiveness, mediated by low levels of von Willebrand factor (11), hypoaggregability (12), and defective production of thromboxane A2 (13) have been observed in uremic patients. Not surprisingly, the skin bleeding time, a bedside test that explores the primary phase of hemostasis, is frequently prolonged in uremic patients (14). The factor(s) responsible for these alterations are still a matter of debate: a role for anemia (15), parathyroid hormone (16), and for uremic toxins (17) has been proposed, among many others.

Nephropathy is the diabetes-specific complication associated with the greatest mortality in both IDDM and NIDDM (1). This disease occurs in ~30–40% of diabetic patients after 10–15 years from the diagnosis, almost invariably progressing to overt renal failure (18). Recent studies indicate that ~600 cases of end-stage renal failure occur every year in the U.K. (19). In 1985, about one-third of all patients beginning renal replacement therapy in the U.S. had diabetes (20).

From a pathophysiological point of view, the development of a disorder associated with a bleeding tendency (uremia) in patients with a prothrombotic condition such as diabetes poses the interesting question of what the net result might be of two opposite forces acting on the hemostatic system.

We considered this still unchallenged question by studying a new and peculiar aspect of platelet physiology, i.e., ionized calcium homeostasis in two groups of patients with end-stage renal failure of diabetic and nondiabetic origin.

Intracellular ionized calcium is involved in the mechanisms of signal transduction that lead to platelet activation (21). It has recently become possible to measure the intracellular concentration of ionized calcium ($[Ca^{2+}]_i$) by means of fluorescent

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Received for publication 29 June 1995 and accepted in revised form 8 February 1996.
$[Ca^{2+}]_i$, intercellular concentration of ionized calcium.

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probes, which can also detect variations in $[Ca^{2+}]_i$ such as those taking place during agonist-induced activation (22).

RESEARCH DESIGN AND METHODS — A total of 30 patients were enrolled in the study after giving their informed consent. All of them had end-stage renal failure and had undergone hemodialysis three times weekly for at least 6 months. A total of 18 patients were affected by diabetic nephropathy. The diagnosis was based on the following criteria: diabetes clinically detected from at least 15 years; diabetic microangiopathy (i.e., retinopathy and/or neuropathy); latency between the first discovery of proteinuria and uremia. Twelve patients had nondiabetic uremia. In eight of these last ones, the etiology of renal failure was known: nephrovascular disease in five, and tuberculosis, focal glomerulosclerosis, rheumatoid arthritis in one patient each. The existence of clinically relevant arteriosclerosis was established by anamnestic and/or instrumental evidence of major atherothrombotic events in the cerebral, myocardial, and lower limb districts.

Patients were being treated with a variety of drugs, namely insulin, oral hypoglycemic agents, ACE inhibitors, clonidine, furosemide, digoxin, H_2 receptor inhibitors, vitamin D, calcium salts. Patients affected by neoplasia or by other advanced organ insufficiencies, and those being treated with calcium channel blockers and with drugs known to interfere with platelet function were excluded.

Sixteen age- and sex-matched healthy subjects were chosen as control subjects. All the subjects were studied in the early morning hours after an overnight fast. All the patients were at the end of a short interdialytic period (i.e., they had received hemodialysis 2 days before).

Blood was drawn by clear puncture of an antecubital vein into plastic tubes containing 1 ml of 0.065 mol citric acid + 0.085 mol sodium citrate + 2% glucose monohydrate (ACD) solution, and platelets were processed and loaded with Fura-2 acetoxymethyl ester (Calbiochem, La Jolla, CA) as previously described (23). After the platelet washing procedures, the extracellular ionized calcium concentration was ~1 μmol/l. The fluorescence of the platelet suspension was recorded in a 37°C-thermostatted cuvette by means of a Perkin-Elmer LS-B5 fluorimeter (Perkin-Elmer Ltd., Beaconsfield, U.K.) at 340 and 380 nm excitation and 495 nm emission wavelengths. Fluorescence values were converted to $[Ca^{2+}]_i$ values (nmol/l) by means of a computer on line with the fluorimeter. The following conversion formula was adopted

$$[Ca^{2+}]_i = K_d \times \frac{(R - F_{min})}{(F_{max} - R)} \times 3.97$$

where:

K_d = dissociation constant for the dye at 37°C (= 224)

R = ratio of the fluorescence of the sample at 340 and 380 nm

F_{max} = R at the maximal fluorescence with CaCl 10 mmol/l in 10% Triton X-100 lysed cells

F_{min} = R at the minimal fluorescence with EGTA 10 mmol/l in 10% Triton X-100 lysed cells

Platelet $[Ca^{2+}]_i$ was evaluated in two conditions: at rest and after the addition of 0.05, 0.1, and 0.5 U/ml thrombin (final concentrations). All the determinations were performed within 1 h of the end of the platelet-processing phase. In fact, we have observed that during this time interval platelet $[Ca^{2+}]_i$ undergoes a progressive but not significant increase of ~10%. To further reduce this small error, the sequence of the three thrombin stimulations was randomized in each subject.

Platelet $[Ca^{2+}]_i$ was evaluated and expressed as follows: resting (nmol/l), absolute increase, after stimulation with thrombin (nmol/l), and relative increase, after stimulation with thrombin (ratio stimulated $[Ca^{2+}]_i$/resting $[Ca^{2+}]_i$).

Glycosylated hemoglobin was determined by high-performance liquid chromatography. In our laboratory, the normal reference interval is 4–6%.

Human thrombin was from Sigma (St. Louis, MO). All the other chemicals were reagent grade.

Statistical analysis was performed using the Mann-Whitney *U*, Spearman correlation, and χ^2 tests, as requested. Data are means ± SE.

Table 1—*Clinical details of the study population*

Clinical and laboratory data	Diabetic uremic patients	Nondiabetic uremic patients	Control subjects
n	18	12	16
Age (years)	49.9 ± 2.4	56 ± 4.9	48.9 ± 3.6
Sex (M/F)	9/9	7/5	9/7
Diabetes type (I/II)	10/8	—	—
Arteriosclerosis	16 (88)	5 (42)*	—
Dialysis time (months)	23.1 ± 5.5	18.8 ± 3.5	—
Mean blood pressure (mmHg)	127.3 ± 3.2	126.1 ± 3.0	103.2 ± 2.8†
Glycosylated hemoglobin (%)	8.1 ± 0.3‡	4.7 ± 0.2	5.0 ± 0.07
Hematocrit (%)	27.3 ± 0.7	24.4 ± 0.9	41.5 ± 0.57†
Plasma creatinine (μmol/l)	813.28 ± 35.36	910.52 ± 61.88	80.44 ± 6.18†
Plasma cholesterol (mmol/l)	4.76 ± 0.25	4.90 ± 0.47	5.27 ± 0.15
Plasma triglycerides (mmol/l)	2.13 ± 0.26	2.02 ± 0.4	1.23 ± 0.1†
Bleeding time (min)	6.3 ± 0.6	8.4 ± 1.3	4.4 ± 0.2†

Data are *n*, *n* (%), or means ± SE. *$P < 0.01$ vs. diabetic uremic patients. †$P < 0.001$ vs. diabetic uremic patients and nondiabetic uremic patients. ‡$P < 0.001$ vs. nondiabetic uremic patients and control subjects.

RESULTS — The clinical and laboratory characteristics of the study population are shown in Table 1.

The typical behavior of the platelet $[Ca^{2+}]_i$ curve in resting conditions and after the addition of 0.05 U/ml thrombin (final concentration) in subjects belonging to the three groups is shown in Fig. 1.

Platelet $[Ca^{2+}]_i$ was evaluated three times in the resting state (i.e., before the addition of thrombin in increasing concentrations) in each subject. The three sets of values were very similar ($P > 0.5$), so that each individual value is expressed as the mean of three determinations. Results are shown in Table 2.

Resting platelet $[Ca^{2+}]_i$ was significantly higher in diabetic uremic patients compared with both nondiabetic uremic patients and control subjects. No significant differences were observed between control subjects and nondiabetic uremic patients.

After the addition of thrombin, a sharp rise of $[Ca^{2+}]_i$ was observed in the three groups, which was proportional to the concentration of thrombin used.

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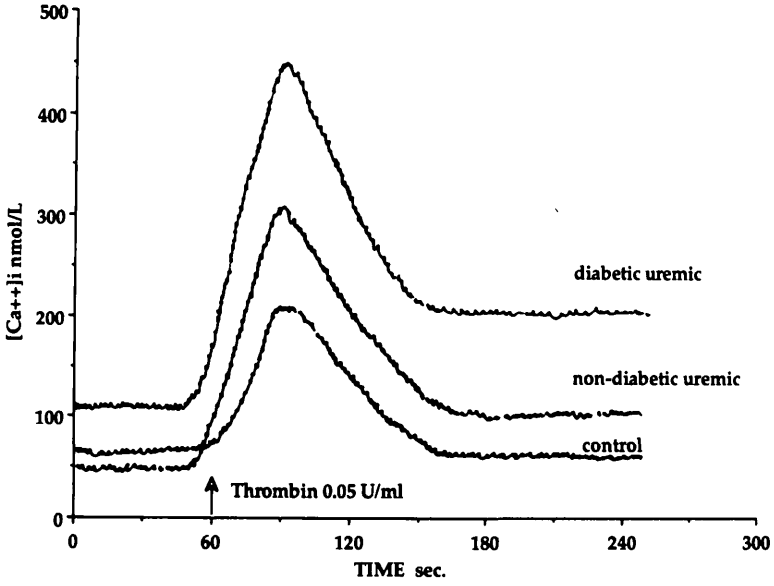

Figure 1—*Representative behavior of the platelet $[Ca^{2+}]_i$ curve at rest and after stimulation in the three study populations.*

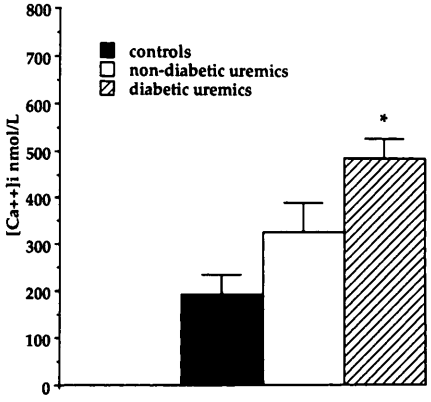

Figure 2—*Absolute increase of platelet $[Ca^{2+}]_i$ after stimulation with 0.05 U/ml thrombin. Data are means ± SE. *P < 0.01 vs. nondiabetic uremic patients and P < 0.001 vs. control subjects.*

The absolute increase of platelet $[Ca^{2+}]_i$ at each thrombin concentration was significantly higher in the diabetic uremic group compared with the nondiabetic uremic and control groups. A trend toward higher stimulated $[Ca^{2+}]_i$ was observed in nondiabetic uremic patients compared with control subjects, never reaching statistical significance. Figure 2 illustrates the absolute increase of $[Ca^{2+}]_i$ after 0.05 U/ml thrombin.

The relative increase of platelet $[Ca^{2+}]_i$ was higher in uremic patients, with and without diabetes, than in control subjects, after 0.05 U/ml thrombin (Fig. 3). After 0.1 U/ml thrombin, only nondiabetic uremic patients showed a higher relative increase of platelet $[Ca^{2+}]_i$, compared with control subjects. No differences were observed with 0.5 U/ml thrombin.

No correlations were found in the three groups between resting or stimulated $[Ca^{2+}]_i$ and the following: anagraphic age, dialytic age, mean arterial pressure, vascular status, bleeding time, glycosylated hemoglobin, hematocrit, plasma hemoglobin, creatininemia, cholesterolemia, triglyceridemia.

CONCLUSIONS — As in other cellular systems capable of graded reactions to exogenous stimuli, variations in the concentration of cytosolic calcium ions represent an important feature of platelet physiology. In resting platelets, $[Ca^{2+}]_i$ is maintained within constant levels by membrane-associated ionic pumps (24). When an agonist interacts with its specific receptor(s) on the platelet plasma membrane, the system of G proteins transduces the signal to the cytoplasmic side of the membrane (25), and a massive influx of calcium ions from both the surrounding medium and the intracellular storage organelles takes place, leading to a sharp, transient increase of $[Ca^{2+}]_i$ (24). This is pivotal to many aspects of the platelet response, leading to biochemical phenomena such as protein kinase C activation (26), myosin light chain phosphorylation (27), phospholipase A2 activation (28), and to ultrastructural modifications such as shape change (29), centralization and release of cytoplasmic granules (30), and aggregation (31).

In the present study, Fura-2 was chosen among fluorescent probes because it is probably the most suitable agent for the study of $[Ca^{2+}]_i$ in single cells (22). Thrombin was chosen because of its recognized role of physiological platelet agonist, the most frequently used in platelet calcium handling studies (32).

The present study was undertaken to evaluate ionized calcium homeostasis in a peculiar disease, end-stage renal failure of diabetic origin, where conditions capable of both activating and depressing platelet function coexist. However, some interesting information about platelet calcium homeostasis in patients with end-stage renal failure of nondiabetic origin was also obtained.

Platelet calcium homeostasis has consistently been found deranged beyond the upper limits of normality in diabetic

Table 2—*Platelet $[Ca^{2+}]_i$ in resting conditions and after thrombin*

	n	Resting	After thrombin 0.05 U/ml	After thrombin 0.1 U/ml	After thrombin 0.5 U/ml
Diabetic uremic patients	18	106 ± 10*	486 ± 41† (4.9 ± 0.7)‡	577 ± 56† (5.4 ± 0.8)	835 ± 144† (7.9 ± 1.3)
Control subjects	16	71 ± 3	192 ± 44 (1.8 ± 0.2)	256 ± 50 (2.6 ± 0.2)	420 ± 26 (5.3 ± 0.7)
Nondiabetic uremic patients	12	57 ± 6	327 ± 65 (5.7 ± 1.1)‡	403 ± 81 (6.5 ± 1.2)‡	530 ± 88 (8.3 ± 1.3)

Data are means ± SE. Results are expressed as nmol/l or ratio (in parentheses). *$P < 0.01$ vs. control subjects and $P < 0.001$ vs. nondiabetic uremic patients. †$P < 0.001$ vs. control subjects and $P < 0.05$ vs. nondiabetic uremic patients. ‡$P < 0.05$ vs. control subjects.

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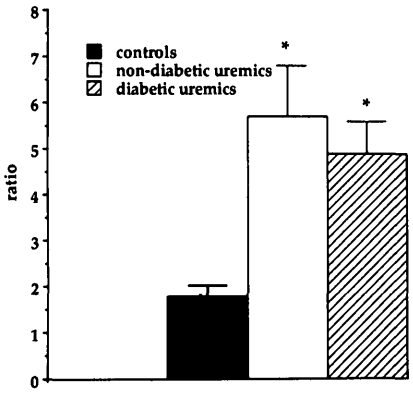

Figure 3—*Relative increase of platelet $[Ca^{2+}]_i$ after stimulation with 0.05 U/ml thrombin. Data are means ± SE. *P < 0.05 vs. control subjects.*

patients. High $[Ca^{2+}]_i$ in platelets from both type I and II diabetic patients was demonstrated in resting conditions and after challenge with various agonists (23,33–36), such a finding being attributed to platelet hyperfunction.

We have previously reported that platelets from diabetic patients with an unsatisfactory metabolic control have higher resting $[Ca^{2+}]_i$ than platelets from both healthy subjects and from satisfactorily controlled diabetic patients (23). The present results are in line with this observation: in fact, patients with renal failure of diabetic origin had a mean glycosylated hemoglobin of 8.1%, this featuring an unsatisfactory metabolic control.

Our two patient populations were remarkably similar, apart from two important differences, namely a higher glycosylated hemoglobin and a higher prevalence of arteriosclerosis in the diabetic group. We nevertheless believe that such differences are almost intrinsic to the study inclusion criteria. In fact, recent data from the EDTA Registry have highlighted the markedly increased relative risk of ischemic heart disease in diabetic patients on renal replacement regimens, the rate of death from myocardial infarction being 10 times higher in diabetic than in nondiabetic patients (37).

Various signs of platelet hyperfunction have been reported in patients with arteriosclerosis, namely reduced half-life (7), in vitro hyperaggregability (38), pathologic release of α-granule contents (39), and excessive synthesis of thromboxane A2 (40). Recently, we and others have observed that calcium homeostasis is upregulated in platelets from patients with severe arteriosclerosis and other thrombotic disorders (41–43). Indeed, arteriosclerotic vascular disease was clinically detectable in a consistent number of patients participating in the present study. This fact is not surprising considering that accelerated arteriosclerosis is a recognized feature of complicated diabetes (44). Cardiovascular and cerebrovascular events account for the majority of the excess deaths seen in diabetic patients on hemodialysis. These two causes account for over half the deaths and most occur within 2 years of the beginning of dialysis (45,46). Also in chronic renal failure of nondiabetic origin, especially when maintenance hemodialysis is given, arteriosclerosis is frequently present (47). Thus, we are induced to conclude that diabetes and its macrovascular complications together are responsible for the difference between the two uremic populations in calcium homeostasis. The relative contributions of these two pathological variables are therefore not easily assessable.

Only longitudinal studies covering the natural history of diabetes from the uncomplicated period through the early nephropathic stage to end-stage renal failure could clarify the interrelationship between diabetes, arteriosclerosis, and uremia in terms of platelet calcium homeostasis.

Diabetic nephropathy develops in a definite subset of patients, and in this subgroup of patients increased lithium-sodium countertransport and microalbuminuria are the hallmarks of incipient end-stage renal failure (48,49). Thus, it cannot be excluded that, together with unsatisfactory metabolic control, a genetically determined component might play a role in the deranged calcium homeostasis observed in diabetic patients with end-stage renal disease. It is tempting to hypothesize that, besides the well-known alteration of the lithium-sodium countertransport system, a more general defect in the cellular mechanisms controlling transmembrane ion homeostasis is a characteristic of diabetes complicated by end-stage renal failure.

In the present study, in platelets from patients with end-stage renal failure of nondiabetic origin, a higher than normal relative increase of $[Ca^{2+}]_i$ was observed, after weak (0.05 U/ml) and intermediate (0.1 U/ml) thrombin stimulation. In this respect, platelets from patients with end-stage renal failure of nondiabetic origin behaved almost in the same way as those from their diabetic counterparts. We have previously reported that thrombin induces a lower than normal relative increase of $[Ca^{2+}]_i$ in platelets from patients with severe arteriosclerosis (43), while in platelets from diabetic patients it was generally unaltered (23). So, when analyzed from this different point of view, our data bring us to the conclusion that uremia per se is able to modify platelet calcium homeostasis by mechanisms different from those observed in other pathological conditions. Enhanced synthesis of nitric oxide has been described in uremia, and this could explain the state of platelet hypofunction observed in this syndrome (10). The increase of $[Ca^{2+}]_i$ normally induced by agonists in platelets is inhibited in vitro by nitric oxide donors, which antagonize calcium influx from both intra- and extra-cellular compartments, by activating cyclic guanosine monophosphate (cGMP)-dependent protein kinase (50) and inducing reversible inactivation of protein kinase C activity (51). Considering the very short half-life of nitric oxide (52), one can speculate that platelets taken from uremic patients, whose environment rapidly changes from high levels to absence of nitric oxide, undergo a rebound phenomenon, responsible for their enhanced response to thrombin.

Acknowledgments— We wish to thank Prof. Jacopo Meldolesi for making available to us the fluorimeter, and Prof. Guido Pozza for continuous support.

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