

Platelet Sarcoplasmic Endoplasmic Reticulum Ca^{2+} -ATPase and μ -Calpain Activity Are Altered in Type 2 Diabetes Mellitus and Restored by Rosiglitazone

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Background—Platelets from patients with type 2 diabetes mellitus display hyperaggregability and increased thrombogenic potential.

Methods and Results—In platelets from patients with type 2 diabetes mellitus, we found enhanced tyrosine nitration and inactivation of the sarcoplasmic endoplasmic reticulum Ca^{2+} -ATPase (SERCA-2), elevated platelet $[\text{Ca}^{2+}]_i$, and activation of μ -calpain. The tyrosine nitration of SERCA-2 and the activation of μ -calpain in vitro in platelets from healthy volunteers could be evoked in vitro by peroxynitrite. Platelet endothelial cell adhesion molecule-1 was identified as a μ -calpain substrate; its in vitro degradation was stimulated by peroxynitrite and prevented by calpain inhibitors. Calpain activation also was linked to hyperresponsiveness to thrombin and the loss of platelet sensitivity to nitric oxide synthase inhibitors. Platelets from patients with type 2 diabetes mellitus (hemoglobin $\text{A}_{1c} > 6.6\%$) contained little or no intact platelet endothelial cell adhesion molecule-1, whereas degradation products were detectable. The peroxisome proliferator-activated receptor- γ agonist rosiglitazone increased SERCA-2 expression in megakaryocytes, and treating patients with type 2 diabetes mellitus with rosiglitazone for 12 weeks increased platelet SERCA-2 expression and Ca^{2+} -ATPase activity, decreased SERCA-2 tyrosine nitration, and normalized platelet $[\text{Ca}^{2+}]_i$. Rosiglitazone also reduced μ -calpain activity, normalized platelet endothelial cell adhesion molecule-1 levels, and partially restored platelet sensitivity to nitric oxide synthase inhibition.

Conclusion—These data identify megakaryocytes/platelets as additional cellular targets for peroxisome proliferator-activated receptor- γ agonists and highlight potential benefits of rosiglitazone therapy in cardiovascular diseases. (*Circulation*. 2008;117:52-60.)

Key Words: CD31 antigens ■ platelets ■ PPAR gamma ■ sarcoplasmic reticulum
■ sarcoplasmic reticulum calcium-transporting ATPases

Diabetes mellitus, a major risk factor for vascular diseases, is associated with accelerated atherosclerosis and a high rate of arterial thrombotic complications. A number of studies support the concept that platelets contribute to the pathogenesis and progression of the vascular complications of diabetes mellitus.¹ Moreover, platelets obtained from patients with type 1 or 2 diabetes mellitus are hyperreactive and demonstrate increased adhesiveness and exaggerated aggregation and thrombus generation.² Different mechanisms have been suggested to be responsible for this enhanced activation such as abnormal Ca^{2+} -ATPase activity^{3,4} and Ca^{2+}

homeostasis,⁵ altered surface expression of glycoprotein receptors and adhesive proteins on the platelet surface, increased binding of fibrinogen, and impairment in platelet signaling such as nitric oxide (NO) production.⁶

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Thiazolidinediones such as troglitazone and rosiglitazone are ligands of peroxisome proliferator-activated receptor- γ (PPAR- γ) that effectively decrease blood glucose by improving sensitivity to insulin (for review, see elsewhere⁷). PPAR- γ also is expressed in human megakaryocytes and

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platelets,⁸ and rosiglitazone has been reported to exert anti-platelet effects *in vitro*⁸ and to reduce the activity of circulating platelets in patients with coronary artery disease.⁹ The aim of the present investigation was to identify the molecular mechanism(s) underlying the increase in platelet sensitivity associated with type 2 diabetes mellitus. Moreover, given the evidence that PPAR- γ agonists do more than sensitize cells to the actions of insulin and are reported to improve endothelial function in diabetic patients,¹⁰ we also assessed the consequences of rosiglitazone therapy on platelet Ca^{2+} signaling and sensitivity to NO synthase (NOS) inhibition.

Methods

For detailed descriptions of materials and methods, please see the online-only Data Supplement.

Study Population

A total of 50 patients (20 women, 30 men; mean age, 47 ± 5.4 years; age range, 30 to 70 years) with type 2 diabetes mellitus attending the clinic for routine control visits were included in the study and were divided into 3 groups: hemoglobin (Hb) A_{1c} , 6% to 6.5% (fasting plasma glucose, 7.5 ± 0.9 mmol/L; $n=18$), 6.6% to 7.4% (fasting plasma glucose, 8.2 ± 0.7 mmol/L; $n=15$), and $\geq 7.5\%$ (fasting plasma glucose, 10.3 ± 0.7 mmol/L; $n=17$). All patients were treated with insulin alone or in combination with metformin. Nondiabetic, age-matched subjects (12 women, 8 men; mean age, 42.4 ± 4.5 years; age range, 25 to 65 years; HbA_{1c}, $5.2 \pm 0.6\%$; fasting plasma glucose, 5.1 ± 0.2 mmol/L) who had not taken any medication known to interfere with platelet aggregation for at least 10 days before the experiment served as the control group.

Rosiglitazone Treatment

Thirteen type 2 diabetic patients (4 women, 9 men; age range, 48 to 68 years) not included in the population described above were included in this open study. Patients had been treated with diet alone ($n=8$), sulfonylurea alone ($n=2$), metformin alone ($n=2$), or a combination of metformin and sulfonylurea ($n=1$). At entry, patients had an HbA_{1c} $< 7.5\%$, and showed no evidence of macrovascular complications. Medication for concomitant disorders (hypertension, $n=9$; hypercholesterolemia, $n=6$) was maintained throughout the study. The study protocol was approved by the ethics committee of the Technical University of Dresden (No. EK156082004), and all of the participants gave written informed consent. After baseline measurements of platelet function and metabolic parameters, patients received rosiglitazone 4 mg BID (Avandia, GlaxoSmithKline, Munich, Germany) for 12 weeks. In some cases, plasma nitrotyrosine levels were assessed by ELISA (Hycult Biotechnology, Uden, the Netherlands) according to the manufacturer's instructions.

Platelet Isolation and Aggregation

Human platelets were isolated and either resuspended for Ca^{2+} measurement and aggregation studies or lysed for Western blotting as described.^{11,12}

Immunoblotting

Washed human platelets were solubilized, and Triton X-100-soluble proteins were separated by SDS-PAGE (8%) as described.¹² To assess the tyrosine nitration of the Ca^{2+} -ATPase, sarcoplasmic endoplasmic reticulum Ca^{2+} -ATPase (SERCA-2) was immunoprecipitated using the conditions described, except that samples were not boiled in SDS-PAGE sample buffer.

Calpain Activity Assay

Calpain activity was assessed by monitoring the formation of the fluorescent metabolite 7-amino-4-methylcoumarin (AMC) from N-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin.

Assay of Ca^{2+} -ATPase Activity

SERCA-2 activity was measured with an enzyme-coupled spectrophotometric assay in which hydrolysis of ATP is coupled to the oxidation of NADH as described.¹³

Megakaryocytes

CD34⁺ bone marrow stem cells were purchased from Cambrix (Verviers, Belgium) and cultured in Iscove's modified Dulbecco's medium (Cambrix) containing 10% FCS, 40 ng/mL thrombopoietin, 50 ng/mL stem cell factor, 5 ng/mL interleukin-3, and 20 ng/mL interleukin-6 to stimulate megakaryocyte differentiation as described.¹⁴

Statistical Analysis

Data are expressed as mean \pm SEM. Statistical evaluation was performed with Student *t* test for paired and unpaired data or 1-way ANOVA followed by a Bonferroni *t* test when appropriate. Values of $P < 0.05$ were considered statistically significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Activation of μ -Calpain in Platelets From Patients With Type 2 Diabetes Mellitus

The aggregation induced by a range of thrombin concentrations was consistently greater in platelets from individuals with diabetes mellitus than in platelets from healthy donors (Figure 1A). Platelet hyperaggregability was correlated with a significant increase in basal $[\text{Ca}^{2+}]_i$ and an exaggerated Ca^{2+} response to thrombin (Figure 1B). Platelets from diabetic subjects contained lower levels of latent, N-terminus domain-intact, Ca^{2+} -activated protease μ -calpain than platelets from nondiabetic individuals (Figure 1C). Total μ -calpain levels were not significantly different in platelets from the 2 groups, but a shift existed in the μ -calpain doublet (75 to 80 kDa) that was consistent with an increase in the relative amount of the N-terminus domain-truncated (active) protein in platelets from patients with diabetes mellitus (Figure 1C). These findings correlated with a marked increase in platelet calpain activity (Figure 1D). No difference in these results was observed when platelets were isolated as described or when the calpain inhibitor calpastatin was present throughout, indicating that the platelet isolation procedure per se was not responsible for the activation of the protease (data not shown).

Platelet Endothelial Cell Adhesion Molecule-1 Is Degraded in Platelets From Patients With Type 2 Diabetes Mellitus

Although full-length platelet endothelial cell adhesion molecule-1 (PECAM-1; 130 kDa) was readily detectable in platelets from nondiabetic/healthy subjects using an antibody directed against the C terminus of the protein, little or no PECAM-1 was detected in platelets from subjects who had type 2 diabetes mellitus (HbA_{1c} $> 6.6\%$). Using a second PECAM-1 antibody, we observed that diabetes mellitus was not associated with the loss of PECAM-1 protein but rather with its C-terminal cleavage and that 2 degradation products (≈ 124 and 118 kDa) were detectable in platelets from diabetic individuals (Figure 2).

To determine whether an elevation in platelet $[\text{Ca}^{2+}]_i$ could elicit the degradation of PECAM-1 by calpain, Ca^{2+} levels

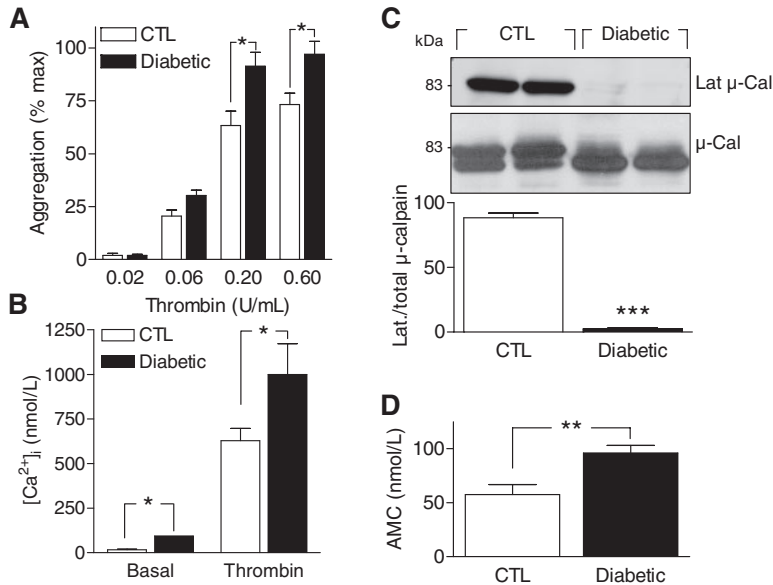


Figure 1. Hyperaggregability to thrombin of platelets from patients with type 2 diabetes mellitus (HbA_{1c}, 6.6% to 7.4%) is related to increased [Ca²⁺]_i and calpain activity. A, Thrombin (0.02 to 0.6 U/mL)-induced aggregation was assessed in washed human platelets from healthy donors (CTL) and patients with type 2 diabetes mellitus. B, Basal [Ca²⁺]_i and the thrombin (0.2 U/mL)-induced Ca²⁺ peak in washed human platelets from healthy donors and patients with type 2 diabetes mellitus. C, Consequences of diabetes mellitus on total and latent μ -calpain (Lat μ -Cal) levels (C) and calpain activity in platelet lysates (D). Bar graphs summarize data obtained using platelets from 5 to 10 individuals in each group. **P*<0.05, ***P*<0.01, ****P*<0.001 vs control.

were altered in platelets from healthy volunteers by incubating intact platelets with high concentrations of extracellular Ca²⁺. Under the conditions used, an extracellular concentration of 1, 3, or 5 mmol/L Ca²⁺ (30 minutes) increased platelet Ca²⁺ from 64±10 nmol/L (Ca²⁺-free buffer) to 150±12, 292±18, and 370±8 nmol/L, respectively (*P*<0.01; n=4). This procedure resulted in the progressive loss of the 130-kDa PECAM-1 and the appearance of degradation products and was attributed to the activation of the Ca²⁺-sensing receptor because the antagonist NPS 2390 prevented the Ca²⁺-induced activation of μ -calpain (see Figure I in the online-only Data Supplement). The Ca²⁺-induced degradation of PECAM-1 was prevented in intact platelets by the calpain inhibitor PD 105606 and in platelet lysates by calpastatin (Figure 2B), but not by the proteasome inhibitor lactacystin (not shown). An augmented thrombin-induced

platelet aggregation that was not influenced by NOS inhibition (Figure 2C) also was observed in human platelets exposed to extracellular Ca²⁺ to activate μ -calpain and to cleave PECAM-1 before assessing aggregation. This effect was prevented by coincubation with PD 105606 (data not shown).

Tyrosine Nitration and Inactivation of SERCA-2 in Platelets From Patients With Type 2 Diabetes Mellitus

The patient collective was expanded (total number screened, 50) and divided into 3 groups to reflect the effectiveness of glycemic control: HbA_{1c} of 6% to 6.5%, 6.6% to 7.4%, and ≥7.5%. A gradual loss of full-length PECAM-1 occurred, so levels were reduced in platelets from patients with HbA_{1c} of 6.6% to 7.4%, whereas mainly degradation products were

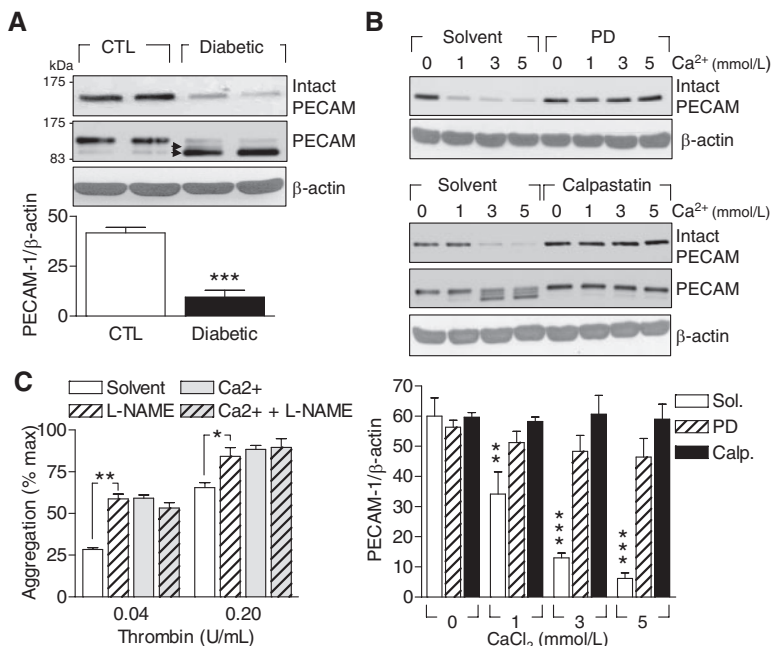


Figure 2. Effect of diabetes mellitus and elevated calpain activity on platelet PECAM-1 levels and responsiveness to NOS inhibition. A, Representative blots comparing the expression of PECAM-1 in platelets from healthy donors (CTL) and patients with type 2 diabetes mellitus. To demonstrate the equal loading of each lane, the blots were reprobbed with antibodies recognizing β -actin. Arrowheads highlight the 124- and 118-kDa PECAM-1 degradation products. B, The effect of Ca²⁺-induced calpain activation on PECAM-1 levels in platelets from healthy donors. Experiments were performed in the presence of solvent (Sol; 0.1% dimethyl sulfoxide), PD105606 (PD; 200 μ mol/L), or calpastatin (Calp; 0.2 U/mL). C, Comparison of thrombin (0.04 to 0.2 U/mL)-induced platelet aggregation in human platelets maintained under control conditions or incubated with extracellular Ca²⁺ (5 mmol/L for 30 minutes). Experiments were performed in the absence and presence of L-NAME (300 μ mol/L). Bar graphs summarize data obtained using platelets from 5 to 10 individuals. **P*<0.05, ***P*<0.01, ****P*<0.001 vs control, solvent, or the effects observed in the absence of Ca²⁺.

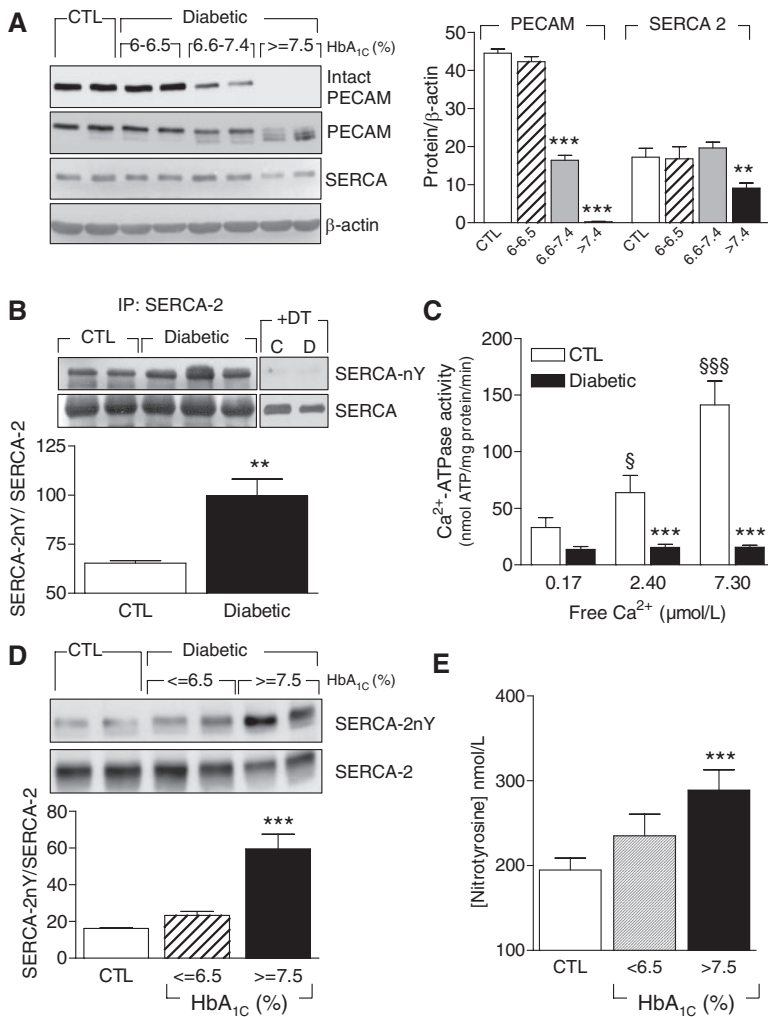


Figure 3. Effect of diabetes mellitus on the expression, tyrosine nitration, and activity of SERCA-2. **A**, Expression of PECAM-1 and SERCA-2 in platelets from healthy donors (CTL) and patients with type 2 diabetes mellitus (HbA_{1c}, 6% to 6.5%, 6.6% to 7.4%, and ≥7.5%). **B**, The tyrosine nitration of SERCA-2 (SERCA-2 nY) in platelets from healthy donors and diabetic patients (HbA_{1c}, 6.6% to 7.4%). To demonstrate the specificity of the antibody used, membranes were treated with sodium dithionite (+DT; 100 mmol/L for 5 minutes); to demonstrate the equal loading of each lane, the blots were reprobed with antibodies recognizing the total SERCA-2 protein. **C**, Ca²⁺-ATPase activity was assessed in the same platelet preparations shown in **B** over a range of different concentrations of free Ca²⁺. **D**, SERCA-2 nY in platelets from healthy donors and diabetic patients (HbA_{1c} <6.5% and >7.5%). **E**, Nitrotyrosine levels in plasma from healthy donors and diabetic patients (HbA_{1c} <6.5% and >7.5%). Bar graphs summarize data obtained using platelets from 4 to 8 individuals in each group. **P*<0.05, ***P*<0.01, ****P*<0.001 vs control; §*P*<0.05, §§§*P*<0.001 vs Ca²⁺-ATPase activity at 0.17 μmol/L Ca²⁺.

detected in individuals with HbA_{1c} ≥7.5% (Figure 3A). SERCA-2 levels were decreased only in patients with HbA_{1c} ≥7.5%, whereas no significant changes in the expression of SERCA-3 were detected (not shown).

Given that evidence exists of enhanced oxidative stress in platelets from patients with diabetes mellitus,¹⁵ we assessed SERCA-2 tyrosine nitration in platelets from subjects with HbA_{1c} of 6.6% to 7.4% (ie, conditions in which cleavage of platelet PECAM-1 occurred but SERCA-2 protein levels were normal). Using an antibody that recognizes 2 adjacent tyrosine nitration sites (nTyr 294,295),¹⁶ we found that the SERCA-2 immunoprecipitated from platelets from nondiabetic subjects was partially tyrosine nitrated. No tyrosine nitration of SERCA-2 was detected in membranes treated with sodium dithionite, indicating the specificity of the signal, whereas the binding of the SERCA antibody was unaffected. The tyrosine nitration of SERCA-2 was significantly enhanced in platelets from diabetic individuals (Figure 3B), and measurement of Ca²⁺-ATPase activity in the same platelet preparations revealed a significant loss of function (Figure 3C). The tyrosine nitration of SERCA-2 (Figure 3D) and the nitrotyrosine concentration detected in plasma (Figure 3E) also increased with HbA_{1c} levels.

To demonstrate a link between oxidative stress and changes in SERCA-2, we next assessed the effects of authentic

ONOO⁻ or the ONOO⁻ donor SIN-1. Incubation of platelets from nondiabetic individuals with either source of ONOO⁻ increased the tyrosine nitration of SERCA-2 (Figure 4A). A biphasic effect of ONOO⁻ was present on Ca²⁺-ATPase activity; lower concentrations (10 to 100 μmol/L) increased and higher concentrations (0.5 to 1 mmol/L) significantly decreased platelet Ca²⁺-ATPase activity (Figure 4B). Correspondingly, PECAM-1 levels were preserved in platelet lysates treated with a low concentration (50 μmol/L) of ONOO⁻, whereas in platelets treated with higher concentrations of ONOO⁻ (1 mmol/L), PECAM-1 was degraded. The latter effects could be prevented by the calpain inhibitor calpastatin, indicating a direct link between redox state, Ca²⁺-ATPase activity, and calpain activation (Figure 4C).

Rosiglitazone Treatment Enhances SERCA-2 Expression and Activity in Platelets From Patients With Diabetes Mellitus

The PPAR-γ agonist rosiglitazone increases SERCA-2 gene expression in cardiac myocytes.¹⁷ Therefore, to assess whether the PPAR-γ agonist could affect long-term platelet SERCA-2 expression, we treated bone marrow–derived CD34⁺ stem cells, cultured with a megakaryocyte-differentiation cocktail, with rosiglitazone. This led to a

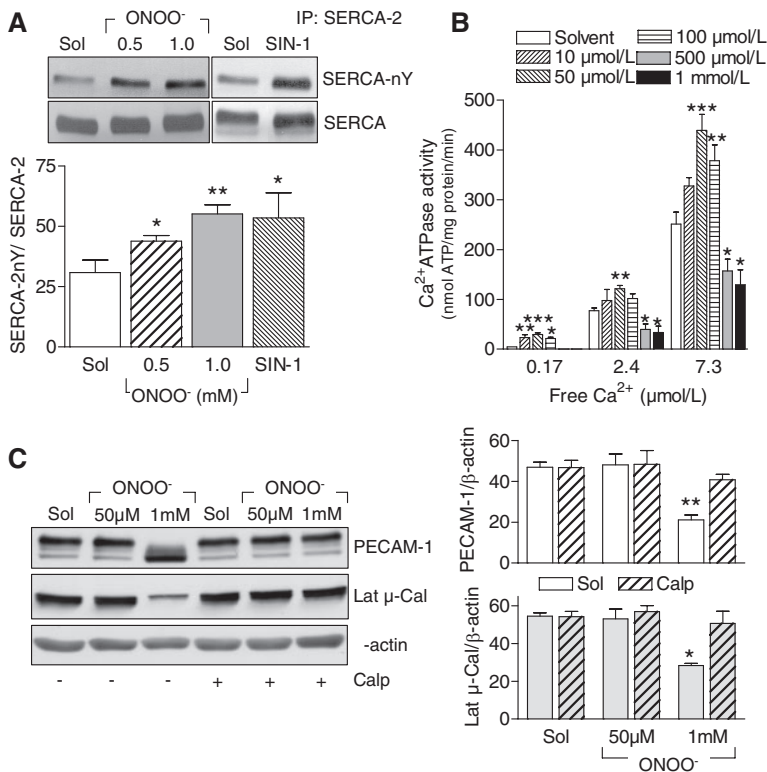


Figure 4. Effect of ONOO⁻ and SIN-1 on the tyrosine nitration of SERCA-2 and Ca²⁺-ATPase and calpain activity in platelets from healthy donors. **A**, Effect of ONOO⁻ (0.5 and 1 mmol/L for 5 minutes) and SIN-1 (5 mmol/L for 5 minutes) on the tyrosine nitration of SERCA-2 immunoprecipitated from platelets from healthy donors. **B**, Comparison of the effects of solvent (Sol; 0.05% NaOH or Na acetate) and ONOO⁻ (10 μmol/L to 1 mmol/L for 5 minutes) on Ca²⁺-ATPase activity in platelet preparations from healthy subjects. **C**, Effect of calpastatin (Calp; 0.2 U/mL) on the ONOO⁻ (50 μmol/L or 1 mmol/L)-induced changes in latent μ-calpain (Lat μ-Cal) and PECAM levels. Bar graphs summarize data obtained using platelets from 4 to 6 individuals in each group. **P*<0.05, ***P*<0.01, ****P*<0.001 vs control or solvent.

>10-fold increase in SERCA-2 expression (Figure 5A). We found no direct effect of rosiglitazone (10 μmol/L for 24 hours) on SERCA-2 levels in washed human platelets and no effect of the PPAR-γ agonist on PECAM-1 or μ-calpain levels in megakaryocytes, in the human megakaryocyte cell line Meg-01, or in human endothelial cells (not shown).

We then determined whether the treatment of diabetic individuals with rosiglitazone affected platelet SERCA-2

expression and/or Ca²⁺-ATPase activity. In the type 2 diabetes population studied, rosiglitazone treatment (8 mg/d for 12 weeks) significantly reduced fasting plasma glucose and HbA_{1c} levels and increased sensitivity to insulin (the Table). Platelet SERCA-2 levels were reduced in the study population, but rosiglitazone therapy led to an increase in SERCA-2 (Figure 5B) and to a decrease in plasma nitrotyrosine levels and the tyrosine nitration of SERCA-2 (Figure 5C and 5D).

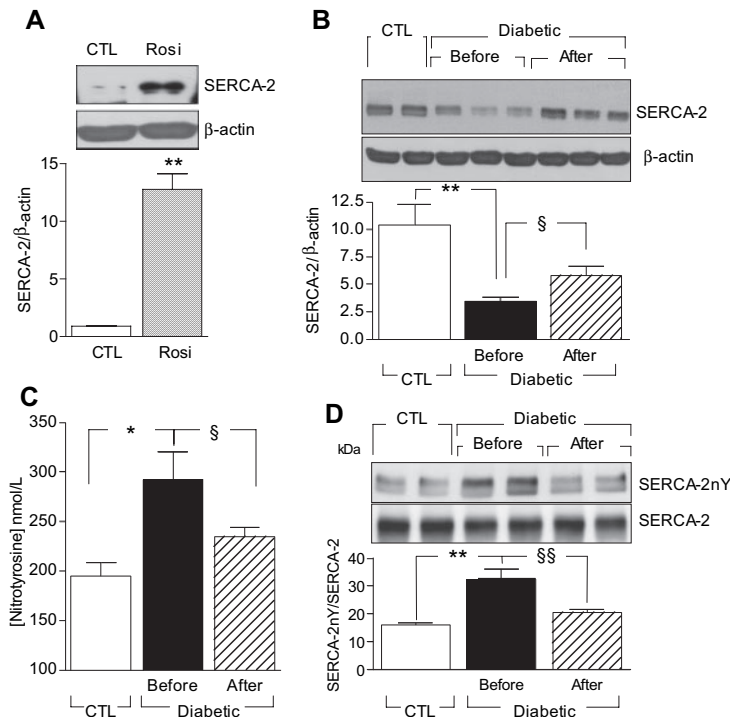


Figure 5. Effect of rosiglitazone on the expression and tyrosine nitration of SERCA-2. **A**, Effect of rosiglitazone (Rosi; 1 μmol/L for 96 hours) on the expression of SERCA-2 in human hematopoietic stem cells after differentiation toward megakaryocytes. Bar graph summarizes data obtained using 3 separate cell preparations. **B**, SERCA-2 expression in platelets from healthy donors (CTL) and from patients with type 2 diabetes mellitus before and after treatment with rosiglitazone (8 mg/d) for 12 weeks. **C** and **D**, Nitrotyrosine levels in plasma (**C**) and the tyrosine nitration of platelet SERCA-2 (**D**) in healthy donors and diabetic patients before and after treatment with rosiglitazone. Bar graphs summarize data obtained in samples from 4 to 9 healthy and 5 to 13 diabetic individuals. **P*<0.05, ***P*<0.01 vs control; §*P*<0.05, §§*P*<0.01 vs before treatment.

Table. Metabolic Parameters of Patients (n=13) at Baseline and After 12 weeks of Treatment With Rosiglitazone (8 mg/d)

	Baseline	After Rosiglitazone
BMI, kg/m ²	28.8±1.3	29.1±1.4
Weight, kg	83.2±4.3	83.9±4.2
FPG, mmol/L	7.8±0.3	6.5±0.4*
HbA _{1c} , %	6.8±0.2	6.5±0.2*
M _c , mg·kg ⁻¹ ·min ⁻¹	1.9±0.3	3.2±0.2†
TG, mmol/L	1.6±0.2	1.7±0.2
LDL, mmol/L	3.2±0.2	3.8±0.2
HDL, mmol/L	1.4±0.1	1.4±0.1
Systolic BP, mm Hg	131.4±3.0	129.1±3.4
Diastolic BP, mm Hg	80.3±2.0	77.2±3.3

BMI indicates body mass index; FPG, fasting plasma glucose; M_c, insulin sensitivity index; TG, triglycerides, LDL, low-density lipoprotein; HDL, high-density lipoprotein; and BP, blood pressure.

*P<0.05, †P<0.01 vs patients at baseline.

These effects were accompanied by increased platelet Ca²⁺-ATPase activity (Figure 6A), a decrease in basal platelet [Ca²⁺]_i, and the normalization of the Ca²⁺ response to thrombin (Figure 6B).

Rosiglitazone Treatment Decreases μ-Calpain Activity and Restores PECAM-1

Given that an improvement in Ca²⁺ homeostasis would be expected to result in a decrease in calpain activity, we assessed the effects of rosiglitazone therapy on μ-calpain. As before, diabetes mellitus was not associated with a global alteration in μ-calpain expression but with an increase in the relative amount of the active 76-kDa protein (not shown). Although μ-calpain expression was unaffected by rosiglitazone, therapy was associated with the reappearance of the ≈80-kDa latent form of the protein (Figure 7A) and decreased calpain activity to values seen in nondiabetic individuals (Figure 7B). Intact PECAM-1 (130 kDa), which was not detectable at all in platelets sampled before the start of therapy, was detected after treatment with rosiglitazone, and the levels present were not significantly different from those found in platelets from nondiabetic individuals (Figure 7C). These changes were accompanied by an attenuation of the thrombin-induced aggregation so that responses recorded using platelets from patients treated with rosiglitazone were not significantly different from those of platelets from healthy volunteers (Figure 7D). Moreover, rosiglitazone therapy partially restored the sensitivity of platelets to NOS inhibition (Figure 7E).

Discussion

The major finding of the present study is that type 2 diabetes mellitus is associated with the cleavage of platelet PECAM-1 through a mechanism involving the tyrosine nitration of SERCA-2, an increase in [Ca²⁺]_i, and the activation of the Ca²⁺-dependent protease μ-calpain. Moreover, treating subjects with type 2 diabetes mellitus with the PPAR-γ agonist rosiglitazone successfully reversed many of these changes and restored platelet [Ca²⁺]_i, calpain activity, and PECAM-1

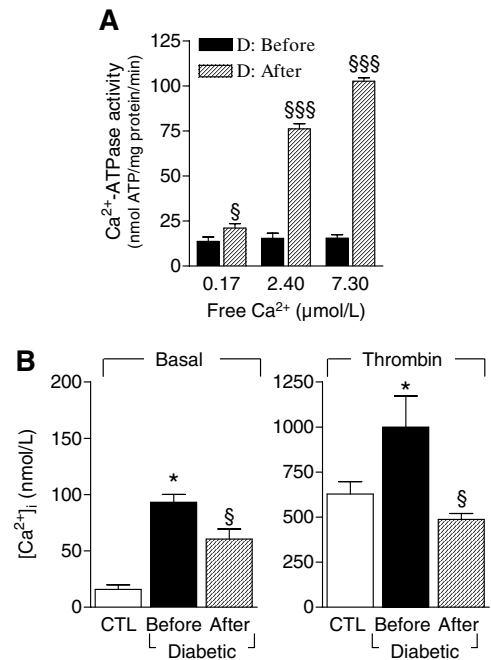


Figure 6. Effect of rosiglitazone on the activity of SERCA-2 and platelet [Ca²⁺]_i. A, Ca²⁺-ATPase activity in platelets from healthy donors and diabetic patients before and after treatment with rosiglitazone. B, Basal [Ca²⁺]_i and the thrombin (0.2 U/mL)-induced Ca²⁺ peak in washed platelets from diabetic patients measured before and after rosiglitazone therapy. Bar graphs summarize data obtained in samples from 9 healthy and 13 diabetic individuals. *P<0.05 vs control; §P<0.05, \$\$\$P<0.001 vs before treatment.

to levels comparable to those detected in nondiabetic subjects. From these results, it is clear that megakaryocytes/platelets are an additional cellular target for PPAR-γ agonists.

Intracellular Ca²⁺ homeostasis in platelets from patients with type 2 diabetes mellitus is reported to be altered, leading to an increased adhesiveness and spontaneous aggregation. One factor that contributes to the disturbed platelet [Ca²⁺]_i in diabetic subjects is a marked reduction in Ca²⁺-ATPase activity.^{3,4} Although human platelets coexpress multiple Ca²⁺-ATPases,¹⁸ we concentrated on SERCA-2 because its activity is reported to be regulated by tyrosine nitration¹⁹. Indeed, we observed that the decrease in Ca²⁺-ATPase activity in platelets from diabetic patients with normal SERCA-2 expression can be accounted for by the tyrosine nitration of the protein. In vitro, the effects of ONOO⁻ on SERCA activity appear to be biphasic because the short-term application of low concentrations of ONOO⁻ increased platelet Ca²⁺-ATPase activity. These observations are in line with a previous report in native vascular smooth muscle cells in which ONOO⁻ (10 to 50 μmol/L) increased SERCA activity via the S-glutathiolation of critical cysteine residues.²⁰ Higher concentrations of ONOO⁻, on the other hand, enhanced the tyrosine nitration of SERCA-2 in platelets from nondiabetic individuals and decreased platelet Ca²⁺-ATPase activity, findings that are consistent with reports in overexpressing systems²¹ and in skeletal and smooth muscle.^{22,23} At this point, we can only speculate about the in vivo source of the ONOO⁻ that targets platelets and other plasma proteins and

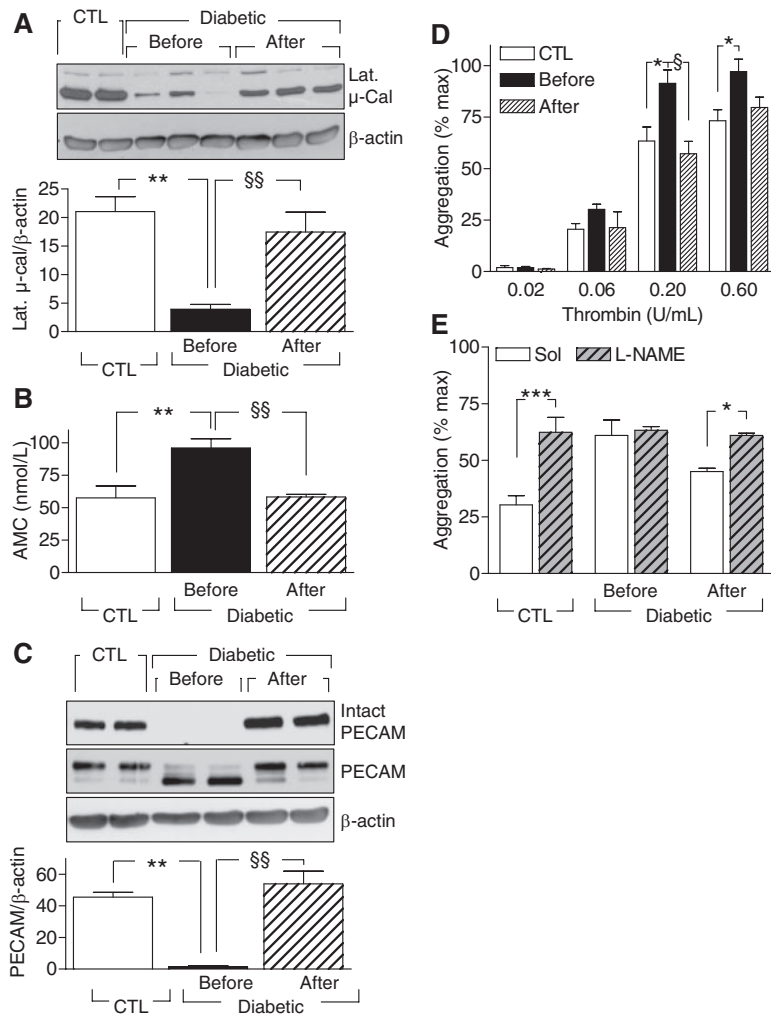


Figure 7. Rosiglitazone attenuates μ -calpain activity, restores intact PECAM-1, and improves platelet responsiveness to thrombin in platelets from patients with type 2 diabetes mellitus. **A**, Latent μ -calpain (Lat μ -Cal) levels. **B**, Calpain activity assessed by the formation of AMC. **C**, PECAM-1 levels. **D** and **E**, Thrombin-induced aggregation assessed in platelets from healthy donors (CTL) and patients with type 2 diabetes mellitus isolated before and after treatment with rosiglitazone (8 mg/d) for 12 weeks. **E**, Aggregation experiments performed in the absence and presence of L-NAME (300 μ mol/L) using 0.1 U/mL thrombin. Bar graphs summarize data obtained in samples from 9 healthy and 13 diabetic individuals. * P <0.05, ** P <0.01, *** P <0.001 vs control; § P <0.05, §§ P <0.01 vs before treatment.

results in their increased tyrosine nitration,²⁴ but it is certainly possible that it is derived from an uncoupled NOS in either the vascular wall or other circulating cells.²⁵

Over the last few years, several reports have highlighted the importance of calpain in platelet granule secretion and aggregation.²⁶ Indeed, μ -calpain-deficient murine platelets exhibit an impaired tyrosine phosphorylation of several proteins and reduced aggregation and clot retraction.²⁷ A number of calpain substrates have already been identified in platelets and include actin binding protein, protein kinase C, Src, the cytosolic domain of β_3 integrin, SNAP-23 (for review, see elsewhere²⁸), and PECAM-1.²⁹ PECAM-1 is of particular interest given its role in the negative regulation of platelet function.^{30,31} Platelets from mice deficient in PECAM-1 are hyperresponsive to stimulation with collagen and thrombin and demonstrate enhanced aggregation, secretion, and adhesion to these agonists.^{32,33} Our results indicate that the enhanced production of an oxidant such as ONOO⁻ is responsible for the cleavage of PECAM-1 in platelets from diabetic individuals because it was possible to reproduce the tyrosine nitration of SERCA-2, the inhibition of Ca²⁺-ATPase activity, the N-terminus truncation of latent μ -calpain, and the degradation of PECAM-1 using authentic ONOO⁻. In addition, the ONOO⁻-induced activation of calpain and

degradation of PECAM-1 in platelets from nondiabetic individuals were prevented by calpain inhibitors. Moreover, rosiglitazone therapy, which led to an attenuation of calpain activity in platelets from subjects with diabetes mellitus, resulted in the restoration of 130-kDa PECAM-1 levels and an attenuation of thrombin-induced aggregation. Given the role of PECAM-1 in regulating platelet responsiveness, it is tempting to attribute the hyperresponsiveness observed in platelets from diabetic individuals to the degradation of this adhesion molecule. However, this is most probably an oversimplification of the events occurring given the spectrum of platelet proteins that are likely to be affected by the activation of μ -calpain.

Platelet-derived NO can modulate platelet aggregation,³⁴ platelet recruitment, and bleeding time.³⁵ Given the link between PECAM-1 and NO in endothelial cells³⁶ and the fact that NOS inhibition failed to increase platelet aggregation in Ca²⁺-activated human platelets, it seemed logical to assess the effects of diabetes mellitus on platelet endothelial NOS. Indeed, NOS inhibition increased the aggregation of platelets from healthy volunteers but did not affect the thrombin-induced aggregation of platelets from diabetic individuals, whereas rosiglitazone therapy partially restored the sensitivity of platelets to N^G-nitro-L-arginine methyl ester (L-

NAME). However, we were unable to unequivocally detect the endothelial NOS protein in the patient platelet samples available.

Thiazolidinediones such as rosiglitazone do more than sensitize cells to the actions of insulin. They are reported to reduce oxidative stress,³⁷ to improve endothelial function,¹⁰ and to reduce carotid intima-media thickness³⁸ in diabetic patients. A number of studies also have demonstrated that these compounds possess so-called pleiotropic effects that directly influence atherogenesis. Indeed, rosiglitazone decreases markers of inflammation and endothelial cell activation in nondiabetic patients with coronary artery disease.³⁹ However, relatively little is known about the direct effects of thiazolidinediones on platelet function other than that pioglitazone attenuates platelet hyperaggregability in rats⁴⁰ and protects against thrombosis in a mouse model of obesity and insulin resistance.⁴¹ In humans with type 2 diabetes mellitus, troglitazone reduces platelet-dependent thrombus formation,⁴² and rosiglitazone decreases the percentage of P-selectin-positive platelets in nondiabetic patients with coronary artery disease.⁹ Given that rosiglitazone has been reported to increase SERCA-2 gene expression in cardiac myocytes,¹⁷ we determined its effect on platelet SERCA-2 expression. We found that rosiglitazone increased SERCA-2 expression in CD34⁺ bone marrow cells cultured with a megakaryocyte differentiation cocktail and led to an increase in SERCA-2 expression in platelets from diabetic individuals. At the same time, rosiglitazone decreased plasma nitrotyrosine levels and the tyrosine nitration of SERCA-2, indicating that the compound may act at the level of Ca²⁺-ATPase expression and by reducing oxidative stress. It is currently unclear, however, whether these effects are mediated by the activation of PPARs or by other intracellular mechanisms.

Given the role of PECAM-1 in limiting platelet aggregation and dense granule secretion,³² it is conceivable that the maintenance of platelet PECAM-1 levels and that of other μ -calpain substrates by rosiglitazone may significantly dampen the contribution of platelets and platelet-derived products to the development of vascular diseases associated with type 2 diabetes mellitus and represent a novel facet of the pleiotropic effects of the thiazolidinediones. Although it is tempting to attribute the rosiglitazone-induced increase in platelet Ca²⁺-ATPase activity, decrease in [Ca²⁺]_i, and normalization of μ -calpain activity and platelet PECAM-1 levels to the effects of the compound on SERCA-2 expression, the actions of rosiglitazone are most probably a combination of its effects on plasma glucose levels, reactive oxygen species production, and platelet Ca²⁺ signaling.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Diabetes mellitus, a major risk factor for vascular diseases, is associated with accelerated atherosclerosis and a high rate of arterial thrombotic complications. We found that platelets from patients with type 2 diabetes mellitus that display hyperaggregability to thrombin also manifest enhanced tyrosine nitration and inactivation of the sarcoplasmic endoplasmic reticulum Ca^{2+} -ATPase, elevated platelet $[\text{Ca}^{2+}]_i$, and activation of μ -calpain. One consequence of this cascade of events was that platelet endothelial cell adhesion molecule-1 was identified as a μ -calpain substrate, and its in vitro degradation was stimulated by peroxynitrite and prevented by calpain inhibitors. Calpain activation also was linked to hyperresponsiveness to thrombin and the loss of platelet sensitivity to nitric oxide synthase inhibitors. Moreover, platelets from patients with type 2 diabetes mellitus (hemoglobin $\text{A}_{1c} > 6.6\%$) contained little or no intact platelet endothelial cell adhesion molecule-1, whereas degradation products were detectable. These changes could be largely reversed by treating diabetic patients with the peroxisome proliferator-activated receptor- γ agonist rosiglitazone (8 mg/d for 12 weeks). Although heated debate currently exists with respect to rosiglitazone and the treatment of cardiovascular disease, it seems that megakaryocytes/platelets are additional cellular targets for peroxisome proliferator-activated receptor- γ agonists and that there may be a beneficial effect of rosiglitazone therapy on platelet function.

Platelet Sarcoplasmic Endoplasmic Reticulum Ca^{2+} -ATPase and μ -Calpain Activity Are Altered in Type 2 Diabetes Mellitus and Restored by Rosiglitazone

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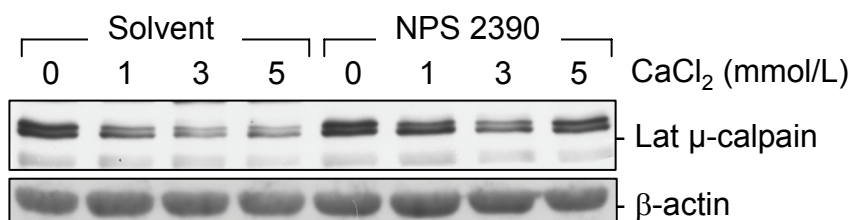


Figure I: Representative blots showing the effect of the calcium-sensing receptor antagonist NPS 2390 (100 μmol/L, 30 min) on the activation of μ-calpain by high concentrations of extracellular Ca²⁺ activation in platelets from healthy donors. Similar results were obtained in 3 additional experiments.

Methods

Materials. Calpastatin and PD150606 were from Calbiochem (Darmstadt, Germany), and SIN-1 from Alexis (Grünberg, Germany). Two different PECAM-1 antibodies were used; a polyclonal anti-PECAM-1 (C-20; Santa Cruz Biotechnology Inc., Heidelberg, Germany) that recognized only the full length, C-terminal intact 130 kDa protein and a monoclonal anti-PECAM-1, clone JC/70 A (DAKO A/S, Glostrup, Denmark) which recognized full length PECAM-1 as well as the 124 and 118 kDa degradation products. The antibodies against N-terminal (domain I) and against domain IV of μ -calpain were from Sigma (Steinheim, Germany). The SERCA-2 antibody was from Affinity Bioreagents (Grünberg, Germany), and the horse radish peroxidase-conjugated antibody recognizing two adjacent tyrosine nitration sites (nTyr 294,295) in the sarcoplasmic endoplasmic reticulum calcium ATPase (SERCA-2-nY) was generated and characterized as described.¹ All other drugs and antibodies were from Sigma.

Biochemical analyses. Cholesterol and triglyceride were measured using the CHOD-PAP and GOD-PAP test kits (Roche). Plasma insulin was measured by EIA (Bio-Source Fleurus) and HbA_{1c} was measured by high-pressure liquid chromatography assay using the Diamat analyzing system (BioRad laboratories, Munich, Germany). Insulin sensitivity (M_c) was determined using the euglycemic hyperinsulinemic clamp and calculated as the quantity of glucose metabolized divided by the measured plasma insulin concentration, normalized for body weight, as described.²

Platelet isolation and aggregation. Human platelets were isolated as previously described.³ Plasma-free platelet suspensions were obtained by centrifugation (900 g, 7 min) of the platelet-rich plasma and the resulting pellet was washed (centrifugation at 900 g for 4 min) in Ca²⁺-free HEPES platelet buffer (mmol/L: NaCl, 136; KCl, 2.6; MgCl₂, 0.93; NaH₂PO₄, 3.26; glucose, 5.5; HEPES, 3.7 pH 7.4 at 37°C). The resulting pellet was either resuspended for Ca²⁺ measurement and for aggregation studies as previously described,⁴ or lysed for Western blotting or for the measurement of enzyme activity.

Immunoblotting. Washed human platelets were solubilised and Triton X-100-soluble proteins were separated by SDS-PAGE (8%) as described.³ Membranes were blotted with appropriate antibodies and visualized by enhanced chemiluminescence using a commercially available kit (Amersham, Freiburg, Germany). To assess the tyrosine nitration of the Ca²⁺ATPase, SERCA-2 was immunoprecipitated using the conditions described, with the exception that samples were not boiled in SDS-PAGE sample buffer.

Calpain activity assay. The calpain substrate N-succinyl-Leu-Leu-Val-Tyr7-amido-4-methylcoumarin (200 μ mol/L) was dissolved in calpain reaction buffer (mmol/L): HEPES 50; CaCl₂ 5; DTT 1; in μ mol/L: lactacystin 10; PMSF 100 and containing aprotinin 2 μ g/mL and chymostatin, leupeptin, antipeptin and pepstatin (1 μ g/mL each, pH 7.4 37°C). Platelet lysates (100 μ g protein) were added and the formation of the fluorescent metabolite 7-amino-4-methylcoumarin (AMC) was monitored using a spectrofluorimeter (excitation λ 360 nm, emission λ 460 nm).

Assay of Ca²⁺-ATPase activity. Washed human platelets were suspended in buffer (mmol/L): Tris-HCl: 50, EDTA 1, EGTA 1, DTT 0.1, glucose 5, KCl 154, and containing okadaic acid 10 nmol/L, leupeptin 100 μ g/mL, aprotinin 100 μ g/mL and PMSF 100 μ mol/L. Cells were lysed by ultrasound (4°C, 10 seconds) and crude membranes were purified from the homogenates by one 8000g centrifugation step. The resulting supernatant was centrifuged at 100,000g (60 minutes). Thereafter, the pellet corresponding to the membrane fraction was resuspended and frozen in liquid nitrogen until use. SERCA 2 activity was measured using an enzyme-coupled spectrophotometric assay in which hydrolysis of ATP is coupled to the oxidation of NADH, as described.⁵

Megakaryocytes. CD34⁺ bone marrow stem cells were purchased from Cambrix (Verviers, Belgium) and cultured in Iscove's modified Dulbecco's medium (Cambrix) containing 10% fetal calf serum, thrombopoietin 40 ng/mL, stem cell factor 50 ng/mL, interleukin-3 5 ng/mL and interleukin-6 20 ng/mL, to stimulate megakaryocyte differentiation as described.⁶

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