

Effect of homocysteine on calcium mobilization and platelet function in type 2 diabetes mellitus

N. Alexandru^a, I. Jardín^b, D. Popov^a, M. Simionescu^a, J. García-Estañ^c,
G. M. Salido^b, J. A. Rosado^b

^a Institute of Cellular Biology and Pathology 'N. Simionescu', Bucharest, Romania

^b Department of Physiology (Cell Physiology Research Group), University of Extremadura, Cáceres, Spain

^c Department of Physiology, Faculty of Medicine, University of Murcia, Murcia, Spain

Received: September 4, 2007; Accepted: December 9, 2007

Abstract

Type 2 diabetes mellitus induces a characteristic platelet hyperactivity that might be due to several factors including oxidative stress and abnormal intracellular Ca^{2+} homeostasis. Hyperhomocysteinaemia is considered a risk factor in the development of thrombosis although its effect on platelet function and the mechanisms involved are still poorly understood. Here we show that homocysteine (Hcy) induce a concentration-dependent increase in endogenous production of reactive oxygen species (ROS), which was significantly greater in platelets from diabetic patients than in controls. Platelet treatment with Hcy resulted in Ca^{2+} release from the dense tubular system and the acidic stores. Ca^{2+} mobilisation-induced by Hcy consisted in two components, an initial slow increase in intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and a rapid and marked increase in $[\text{Ca}^{2+}]_i$, the second leading to the activation of platelet aggregation. As well as ROS generation, Ca^{2+} mobilization and platelet aggregation were significantly greater in platelets from diabetic donors than in controls, which indicate that platelets from diabetic donors are more sensitive to Hcy. These findings, together with the hyperhomocysteinaemia reported in diabetic patients, strongly suggest that Hcy might be considered a risk factor in the development of cardiovascular complications associated to type 2 diabetes mellitus.

Keywords: thrombin • homocysteine • aggregation • platelets • calcium signalling • ROS production

Introduction

Homocysteine (Hcy) is a sulphur-containing amino acid produced by conversion of methionine, an essential amino acid that is regularly consumed within the diet. Hcy is metabolized by re-methylation or trans-sulphuration, processes that require either vitamin B12 or B6, respectively, as co-factors [1]. In normal conditions, the plasma levels of Hcy in human beings are low (5–15 μM); however, genetic defects in the enzymes metabolizing Hcy or environmental factors markedly increase homocysteinaemia. Mutations in cystathione- β -synthase or 5,10-methyltetrahydrofolate reductase are associated with excess levels

of Hcy (> 50 μM and up to 400 μM) [1–4]. Environmental factors include reduced folate as well as vitamins B12 and B6 intake, increasing methionine intake, smoking, coffee drinking, and the administration of certain drugs, such as steroids or cyclosporine [1].

Hyperhomocysteinaemia is associated with increased risk of atherosclerosis and the development of arterial and venous thrombosis [5]. Studies in animal models have revealed that hyperhomocysteinaemia result in increased oxidant stress [6], impaired endothelial function [7,8] and increased thrombogenicity [8]. Hyperhomocysteinaemia has also been associated with an increase in platelet activation [9]. Leoncini *et al.* [10] have reported that Hcy increase platelet responsiveness to thrombin in a concentration-dependent manner. In addition, acute Hcy increases in rats have been shown to be associated to an increase in platelet aggregation [11]. Some of the effects of Hcy on human platelet function have been explained by a reduction in nitric oxide formation or bioavailability [10, 12]. In addition, platelet hyperactivity induced by Hcy

*Correspondence to: Dr. J. A. ROSADO,
Department of Physiology, University of Extremadura,
Av. Universidad s/n, Cáceres 10071, Spain.
Tel.: +34 927 257154
Fax: +34 927 257110
E-mail: jarosado@unex.es

has been shown to involve activation of the p38 MAPK/cPLA₂ pathway, leading to the synthesis of thromboxane A₂ and the activation of integrin $\alpha_{IIb}\beta_3$ [13]. Homocysteine-induced platelet hyperactivity also involves activation of PLC γ_2 , which requires signals through oxygen free radicals [14].

Oxidative stress is a phenomenon involved in platelet hyperactivity in patients with type 2 diabetes mellitus [19]. In this type of diabetes mellitus, oxidative stress impairs glucose uptake in muscle cells and adipocytes leading to chronic hyperglycaemia, which, in turn, causes glucotoxic alterations in many cell types, such as pancreatic β cells, resulting in a decrease in insulin content, secretion and activity [20, 21]. In addition, diabetes mellitus leads to several cardiovascular disorders, including platelet dysfunction associated to an abnormal intracellular Ca²⁺ homeostasis, which are the major cause of morbidity and mortality in type 2 diabetes mellitus [22–24]. Type 2 diabetic individuals have been reported to show 40% greater plasma levels of Hcy than healthy individuals [25]; therefore, as Hcy stimulates oxidative stress and inhibits nitric oxide synthesis, hyperhomocysteinaemia has been presented as a candidate for the generation of oxidative damage in type 2 diabetes.

Thus, in an attempt to elucidate the intracellular mechanisms involved in Hcy-induced effects in diabetic patients, we have investigated aggregation, intracellular Ca²⁺ mobilisation and endogenous reactive oxygen species (ROS) generation induced by Hcy in platelets from type 2 diabetics and healthy individuals.

Materials and methods

Materials

Apyrase (grade VII), EGTA, aspirin, bovine serum albumin (BSA), thrombin, ADP, thapsigargin (TG), 2,5 di-(tertbutyl)-1,4-hydroquinone (TBHQ) and Hcy were from Sigma (Madrid, Spain). Fura-2 acetoxymethyl ester (fura-2/AM), 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA) and calcein AM were from Molecular Probes (Leiden, The Netherlands). All other reagents were of analytical grade and purchased from Panreac (Barcelona, Spain).

Participants

The studied population consisted of 28 type 2 diabetic and 26 age- and gender-matched healthy (control) donors. The clinical and metabolic characteristics of type 2 diabetic patients and healthy individuals are summarized in Table 1. Clinical laboratory parameters were measured by routine laboratory methods. Blood glucose concentration in diabetic patients was in the range of 180–240 mg/dl. The glycosylated Hb levels (HbA1c) were used as an index of metabolic control. Only blood of diabetic patients with a level of HbA1c > 6 % was selected for experiments. The control individuals were age- and gender-matched healthy people that had HbA1c levels in the normal range (3.5–5 %).

Table 1 Clinical and metabolic characteristics of type 2 diabetic patients and healthy individuals

	Healthy individuals	Type 2 diabetics
Number	26	28
Age (years)	57±8	58 ± 9
BMI	26.1±2.9	27.7 ± 2.5
Diabetes duration (years)	-	7.8±2.8
Fasting glycemia (mg/dl)	92±5	190±12
HbA _{1c} (%)	4.2±0.5	8.5± 0.8*
Albumin (g/l)	4.3±0.2	4.3 ± 0.4
Cholesterol (mmol/l)		
Total	5.9±0.5	6.0 ± 0.9
HDL	1.5±0.4	1.5 ± 0.6
VLDL	0.6±0.1	0.6 ± 0.3
LDL	4.0±0.8	4.0 ± 0.9
Triglycerides (mmol/l)	1.2±0.5	1.3 ± 0.4
Fibrinogen (mg/dl)	330±28	324 ± 35
Creatinine (mg/dl)	1.0±0.2	1.0 ± 0.3
Blood pressure (mmHg)		
Systolic	124±8	127 ± 7
Diastolic	82±5	83 ± 5

Values given are presented as mean ± S.E.M. **P*<0.05 compared to healthy donors.

Platelet preparation

Blood was obtained from type 2 diabetic patients and healthy volunteers in the Clinical Analysis Laboratory, Cáceres, Spain, as approved by Local Ethical Committees and in accordance to the Declaration of Helsinki. Blood was collected at 9:00 a.m. Blood was mixed with one-sixth volume of acid/citrate dextrose anticoagulant containing (in mM): 85 sodium citrate, 78 citric acid and 111 D-glucose. Platelet-rich plasma was then prepared by centrifugation for 5 min. at 700 *g* and, except for aggregation studies, aspirin (100 μ M) and apyrase (40 μ g/ml) were added. Cells were then collected by centrifugation at 350 *g* for 20 min. and re-suspended in HEPES-buffered saline (HBS) containing (in mM): 145 NaCl, 10 HEPES, 10 D-glucose, 5 KCl, 1 MgSO₄, pH 7.45 and supplemented with 0.1% w/v BSA and 40 μ g/ml apyrase.

Platelets viability

This was assessed by calcein loading of the cells and trypan blue exclusion technique. For calcein loading, platelets were incubated for 30 min. with 5 μ M calcein-AM at 37°C, centrifuged at 350 *g* for 20 min. and the pellet suspended in HBS. Cells were treated with the agents used in this study for the times indicated, centrifuged again and re-suspended in HBS. Two milliliter aliquots were used for fluorescence recording (spectrophotometer Varian Ltd., Madrid, Spain) at λ ex: 494 nm and λ em: 535 nm. In this assay ~95% of cells were viable in our platelet suspensions, a result further confirmed by the trypan blue exclusion technique. No significant effect on cell viability was detected after treatment with Hcy or after incubation in HBS for 6000 sec., the longest period investigated for platelet aggregation.

Platelet aggregation

Platelet aggregation of washed platelets was monitored in a Chronolog (Havertown, PA, U.S.A.) aggregometer. Platelets (300 μ l) prepared as described above were suspended in HBS and stimulated in the aggregometer cuvettes at 37°C under stirring at 1200 rpm [19]. The time required to reach the maximum aggregation rate was then determined from the traces recorded.

Intracellular ROS Production through the Oxidation of CM-H₂DCFDA

CM-H₂DCFDA is a ROS-sensitive probe that can be used to detect ROS production in living cells. It passively diffuses into cells, where its acetate groups are cleaved by intracellular esterases, releasing the corresponding dichlorodihydrofluorescein derivative. CM-H₂DCFDA oxidation yields a fluorescent adduct (DCF) that is trapped inside the cell. Platelet-rich plasma was incubated at 37°C with 10 μ M CM-H₂DCFDA acetyl ester for 30 min., platelets were then centrifuged and the pellet was re-suspended in HBS. Fluorescence was recorded from 1 ml aliquots using a Shimadzu Spectrophotometer (Shimadzu, Japan) at λ ex: 488 nm and λ em: 530 nm. Traces were fitted to the equation: $y = A + Bx$ where B is the slope.

Measurement of intracellular free calcium concentration ([Ca²⁺]_i)

Cells were incubated at 37°C with 2 μ M fura-2 AM for 45 min., collected by centrifugation at 350 *g* for 20 min. and re-suspended in HBS. Fluorescence was recorded from 1 ml aliquots of magnetically stirred platelet suspensions (108 cells/ml) at 37°C using the fluorescence Spectrophotometer (Varian Ltd., Madrid, Spain) with λ ex: 340 and 380 nm and λ em: 505 nm. Changes in [Ca²⁺]_i were monitored using the fura-2 340/380 fluorescence ratio and calibrated according to the method of Grynkiewicz *et al.* [26]. Ca²⁺ release and influx was estimated using the integral of the rise in [Ca²⁺]_i for 2.5 min. after addition of agonists or CaCl₂, respectively [27].

Statistical analysis

Data are shown as mean \pm S.E.M. analysis of statistical significance was performed using Student's *t*-test. $P < 0.05$ was considered to be significant for a difference. Correlations were calculated with the use of GraphPad Prism software (version 2.0, Graph Pad Software for Science, San Diego, USA).

Results

ROS generation induced by homocysteine in platelets from healthy and type 2 diabetic patients

Intracellular ROS production was estimated using CM-H₂DCFDA. As shown in Figure 1A–C, treatment of human platelets from

healthy donors with Hcy resulted in a slow, but sustained increase in the DCF fluorescence. The results of linear regression show that Hcy evoked endogenous ROS generation in a concentration-dependent manner with slopes of $30 \pm 1 \times 10^{-5}$, $51 \pm 1 \times 10^{-5}$ and $67 \pm 1 \times 10^{-5}$ at the concentrations 50, 100 and 200 μ M, respectively.

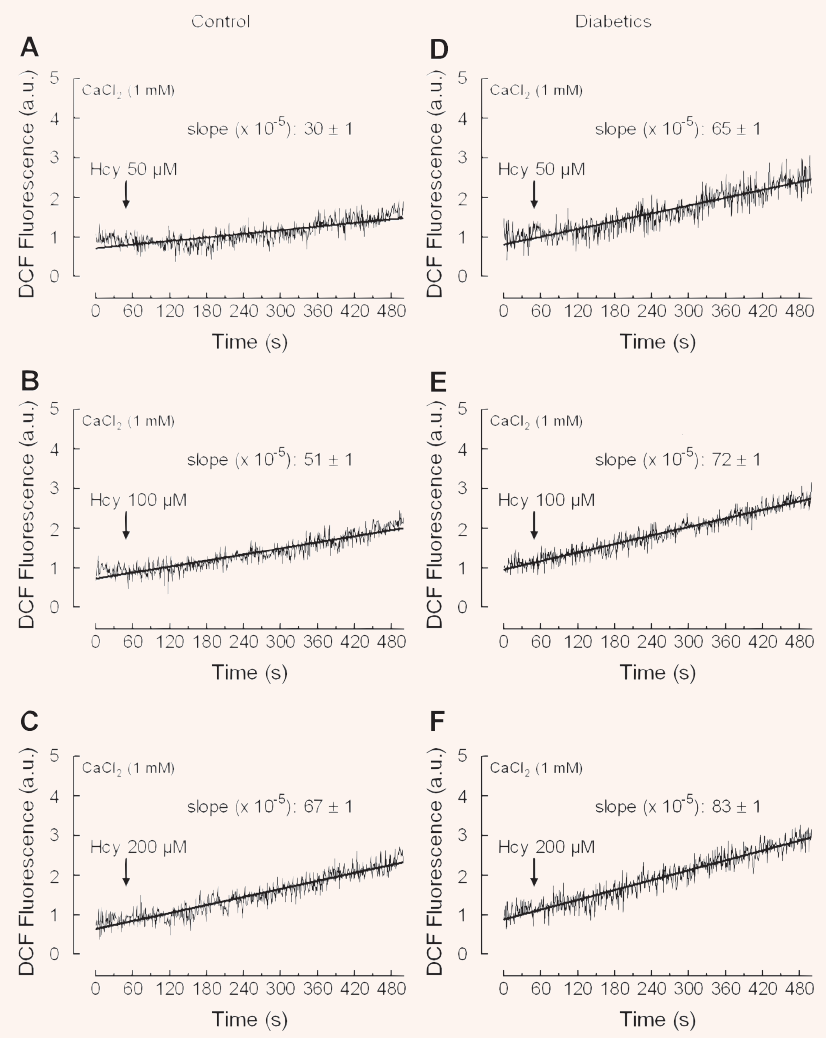
Treatment of platelets from diabetic donors with increasing concentrations of Hcy resulted also in a concentration-dependent increase in DCF fluorescence (Fig. 1D–F). However, in diabetic platelets the linear regression slopes were $65 \pm 1 \times 10^{-5}$, $72 \pm 1 \times 10^{-5}$ and $83 \pm 1 \times 10^{-5}$ at the Hcy concentrations of 50, 100 and 200 μ M, respectively; ($P < 0.05$). This indicates that in diabetes the rate of endogenous ROS generation induced by Hcy in platelets was significantly greater compared to that observed in platelets from healthy donors. A statistically significant correlation was observed between Hcy concentration and endogenous ROS production both in healthy individuals ($y = 0.071x - 2.142$, $R^2 = 0.998$, $P < 0.05$) and diabetic donors ($y = 0.125x - 0.301$, $R^2 = 0.994$, $P < 0.05$), which is consistent with previous studies reporting correlation between plasma Hcy concentration and platelet ROS levels [25].

Homocysteine induces Ca²⁺ mobilization in platelets from healthy and type 2 diabetic patients

The possibility that Hcy-induced endogenous ROS production might be associated to calcium mobilisation, as previously reported [19, 28, 29], was tested by examining the effect of Hcy on Ca²⁺ release from the intracellular stores and Ca²⁺ entry through the plasma membrane. In human platelets, two Ca²⁺ stores have been described: the TBHQ-insensitive dense tubular system (DTS) and the TBHQ-sensitive acidic stores based on immunolocalization studies [30], the different sensitivity to TG and TBHQ [31, 32] and the distinct sensitivity to agonists [33, 34]. We have investigated the effect of Hcy on Ca²⁺ release from the DTS, by previous depletion of the acidic stores using TBHQ. In a Ca²⁺-free medium, treatment of platelets from healthy donors with 20 μ M TBHQ induced a sustained increase in [Ca²⁺]_i; subsequent addition of Hcy induced a concentration-dependent increase in [Ca²⁺]_i indicative of Ca²⁺ release from the DTS (Figs. 2A, C, E and G). In platelets from type 2 diabetic individuals Hcy evoked a concentration-dependent Ca²⁺ release from the DTS, which was found to be significantly higher at 50, 100 and 200 μ M than those observed in control platelets (Figs. 2B, D, F and G; $P < 0.05$).

In order to investigate Ca²⁺ release from the acidic stores induced by Hcy we have depleted the DTS, by using a combination of ADP (10 μ M) and TG (10 nM), which selectively inhibits SERCA2b present in the DTS [35], prior to the addition of Hcy. Treatment of platelets with ADP + TG resulted in a transient increase in [Ca²⁺]_i indicative of Ca²⁺ release from the DTS (Figs. 3A–F). Subsequent addition of Hcy induced a sustained increase in [Ca²⁺]_i that was found to be significantly greater in platelets

Fig. 1 Homocysteine induces reactive oxygen species (ROS) generation in platelets from healthy and diabetic donors. Human platelets from healthy (A–C) or diabetic donors (E–F) were loaded with CM-H₂DCFDA and then treated with 50 μ M (A), 100 μ M (B) or 200 μ M (C) homocysteine (Hcy) in a medium containing 1 mM Ca²⁺. Traces are the average of 10 independent experiments. Kinetic parameters (slopes) were determined by linear regression, which is depicted as the line drawn through the points. Slope values are mean \pm S.E.M.



from diabetics than in controls (Fig. 3G; $P < 0.05$). Hcy-evoked Ca²⁺ release from the acidic stores was not found to be concentration dependent. Hcy concentration and Ca²⁺ release from the DTS were significantly correlated in healthy individuals ($y = 4.688x + 728$, $R^2 = 0.995$, $P < 0.05$) and diabetic donors ($y = 2.897x + 1141$, $R^2 = 0.994$, $P < 0.05$). However, no correlation was found between Hcy concentration and Ca²⁺ release from the acidic stores in healthy ($y = 0.717x + 1272$, $R^2 = 0.448$, $P = 0.53$) and diabetic donors ($y = -1.127x + 2212$, $R^2 = 0.615$, $P = 0.42$).

We have further investigated the ability of Hcy to induce Ca²⁺ entry. As shown in Figures 4A and B, treatment of platelets with increasing concentrations of Hcy (50–200 μ M) in a medium containing 1 mM Ca²⁺ resulted in a rapid, concentration dependent and sustained increase in [Ca²⁺]_i that was found to be significantly higher to that observed in the absence of extracellular Ca²⁺ (data not shown). The rise in [Ca²⁺]_i evoked by Hcy was found to be sig-

nificantly increased in diabetic patients compared to controls (Fig. 4A and B; $P < 0.05$).

Subsequently, we questioned whether Hcy impairs Ca²⁺ mobilization induced by agonists. Treatment of platelets with 0.1 U/ml thrombin resulted in a transient increase of [Ca²⁺]_i. The initial peak [Ca²⁺]_i elevation above basal after thrombin was 297 \pm 37 and 392 \pm 56 nM in platelets from healthy and diabetic donors (Fig. 4C and D; $P < 0.05$), which is consistent with previous studies reporting abnormal Ca²⁺ mobilization in diabetic platelets [23, 24, 36]. The subsequent addition of Ca²⁺ (300 μ M) to the external medium induced a sustained elevation in [Ca²⁺]_i indicative of Ca²⁺ entry. As shown in Figures 4C and D, Ca²⁺ entry was significantly higher in platelets from diabetic patients (the integrals of the rise in [Ca²⁺]_i for 2.5 min after addition of CaCl₂ were 14,111 \pm 2334 and 52,108 \pm 7560 nM/s in healthy and diabetic patients, respectively; $P < 0.05$). In the presence of 50 μ M Hcy

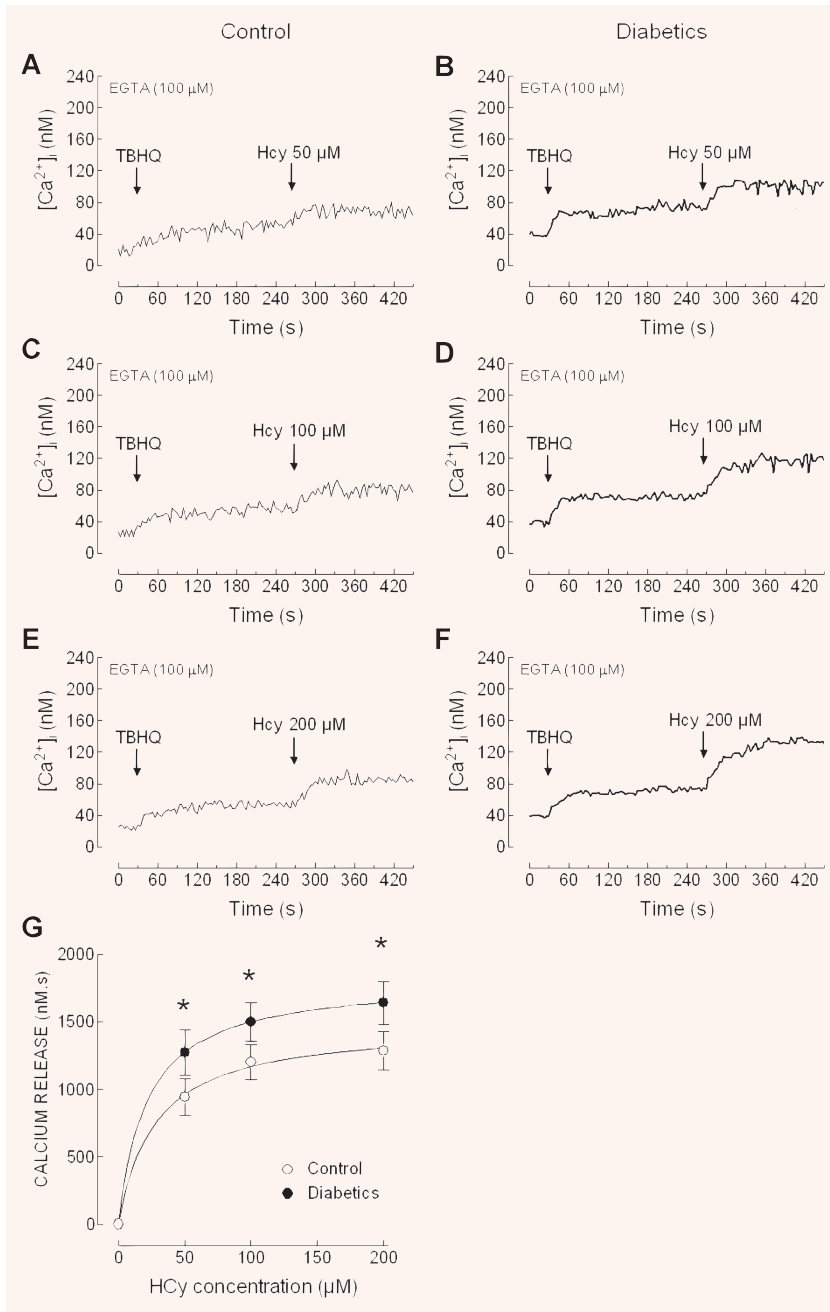
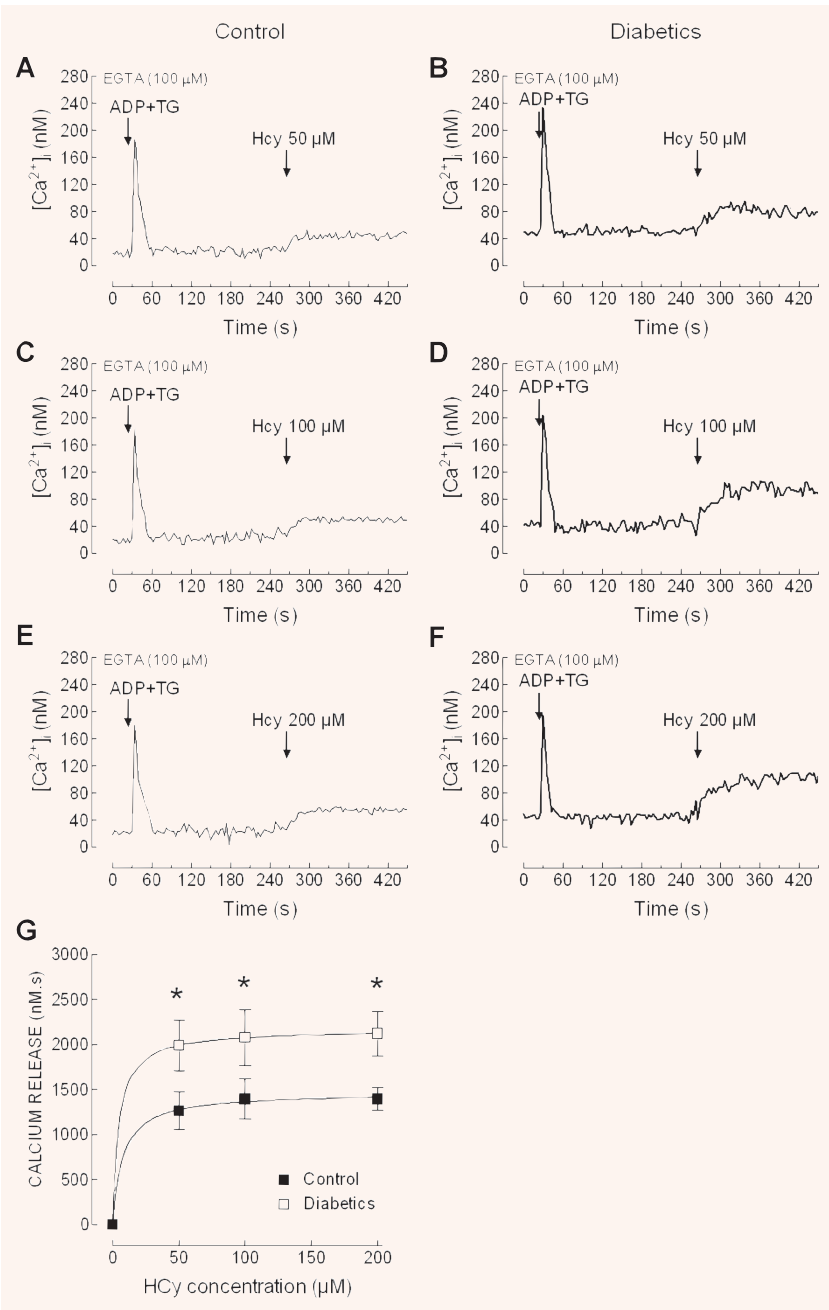


Fig. 2 Homocysteine releases Ca^{2+} from TBHQ-insensitive stores in platelets from healthy and diabetic donors. Human platelets from healthy (**A**, **C** and **E**) and diabetic subjects participants (**B**, **D** and **F**) were suspended in a Ca^{2+} -free medium (100 μM EGTA was added). Cells were treated with 20 μM TBHQ and 4 min. later homocysteine (Hcy; 50, 100 or 200 μM) was added, as indicated. Traces are representative of 10–21 independent experiments. (**G**). Values represent Hcy-induced Ca^{2+} release estimated as described in Material and methods. $P < 0.05$ compared to Hcy-evoked Ca^{2+} release in platelets from healthy donors.

thrombin-evoked Ca^{2+} release was significantly reduced (the initial peak $[\text{Ca}^{2+}]_i$ elevation above basal after thrombin was 71 ± 6 and 139 ± 10 nM in platelets from healthy and diabetic donors, respectively, and similar results were obtained with 100 and 200 μM Hcy; Fig. 4C and D; $P < 0.05$). Hcy also impaired thrombin-evoked Ca^{2+} entry in healthy and diabetic individuals (the integrals of the rise in $[\text{Ca}^{2+}]_i$ for 2.5 min. after addition of CaCl_2 were

5850 ± 283 , 5389 ± 352 and 5203 ± 383 nM/s in platelets from healthy individuals and 8755 ± 996 , 8578 ± 235 and 8324 ± 124 nM/s in platelets from diabetic donors, treated in the presence of 50, 100 and 200 μM Hcy; Fig. 4C and D; $P < 0.05$). Similarly, Hcy significantly reduced Ca^{2+} mobilization evoked by 10 μM ADP, including both, Ca^{2+} release from the intracellular stores (the initial peak $[\text{Ca}^{2+}]_i$ elevation above basal after ADP was 160 ± 7 and

Fig. 3 Homocysteine releases Ca^{2+} from TBHQ-sensitive stores in platelets from healthy and diabetic donors. Human platelets from healthy (A, C and E) and diabetic individuals (B, D and F) were suspended in a Ca^{2+} -free medium (100 μM EGTA was added). Cells were treated with 10 μM ADP in combination with 10 nM TG and 4 min later homocysteine (Hcy; 50, 100 or 200 μM) was added, as indicated. Traces are representative of 10–14 independent experiments. (G), Values represent Hcy-induced Ca^{2+} release estimated as described in Material and methods. $P < 0.05$ compared to Hcy-evoked Ca^{2+} release in platelets from healthy donors.



218±11 nM in platelets from healthy and diabetic donors in the absence of Hcy and 82±5 and 129±12 nM in platelets from healthy and diabetic individuals in the presence of 50 μM Hcy; Figs 4E and F; $P < 0.05$), and Ca^{2+} entry (the integrals of the rise in $[\text{Ca}^{2+}]_i$ for 2.5 min after addition of CaCl_2 were 5341±470 and 7210 ± 611 nM in platelets from healthy and diabetic donors in the absence of Hcy and 2892±352 and 4125±512 nM in platelets from healthy and diabetic individuals in the presence of 50 μM Hcy; Figs. 4E and F; $P < 0.05$).

Effect of homocysteine on platelet aggregation

As shown in Figures 5 and 6(A–C) and Table 2, treatment of platelets suspended in a medium containing 1 mM Ca^{2+} with Hcy was without effect for several minutes, then induced a slow shape change indicated by a decrease in light transmission, followed by a large increase in light transmission as platelets aggregated. The percentage and rate of aggregation was found to be greater in platelets from

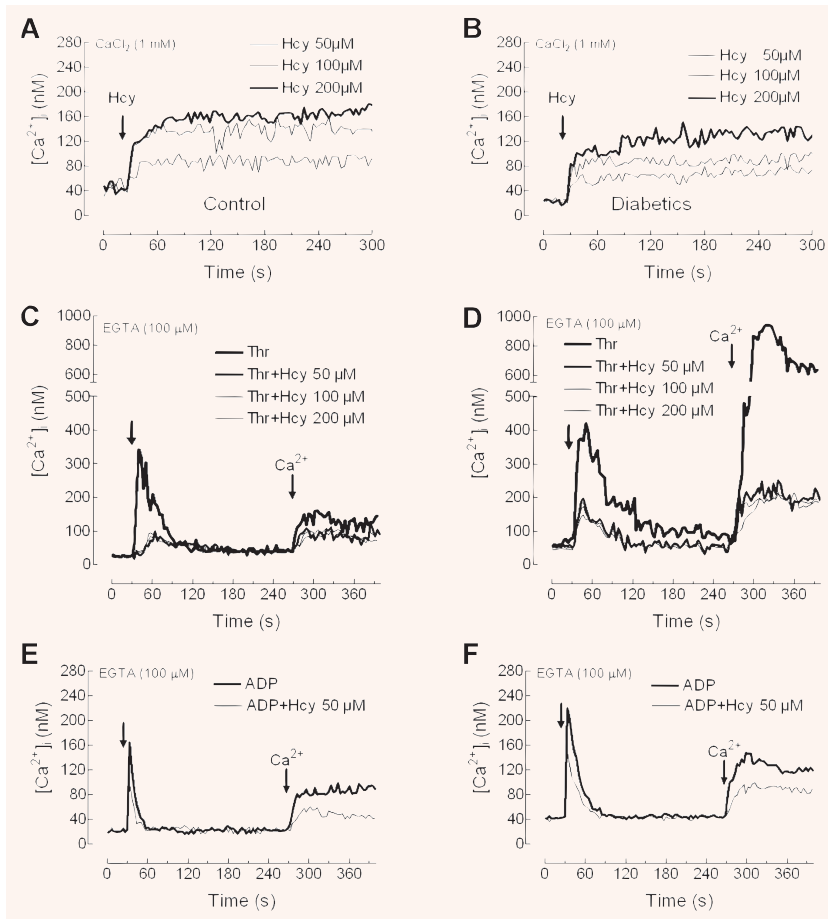


Fig. 4 Homocysteine stimulates Ca^{2+} entry and impairs Ca^{2+} mobilization by thrombin in platelets from healthy and diabetic donors. (**A** and **B**) Human platelets from healthy (**A**) and diabetic individuals (**B**) were suspended in HBS containing 1 mM Ca^{2+} and then exposed to increasing concentrations of homocysteine (Hcy; 50–200 μM). (**C–F**) Human platelets from healthy (**C** and **E**) and diabetic individuals (**D** and **F**) were suspended in a Ca^{2+} -free medium (100 μM EGTA was added). Cells were treated (thick arrow) with either 0.1 U/ml thrombin (Thr; **C** and **D**) or with 10 μM ADP (**E** and **F**), in the absence or presence of 50, 100 or 200 μM Hcy, as indicated, and 4 min. later CaCl_2 (300 μM) was added to the medium to initiate Ca^{2+} entry. Traces are representative of 8–12 independent experiments.

diabetic donors compared to controls (Table 2), which is consistent with the greater effect of Hcy in ROS production and Ca^{2+} mobilization in diabetics shown above. In contrast, the lag time was significantly extended in platelets from patients with type 2 diabetes mellitus at all the concentrations of Hcy used (Table 2).

Further examination of Ca^{2+} mobilization by Hcy in the same cell preparation used to determine platelet aggregation reported an initial slow and sustained increase in $[\text{Ca}^{2+}]_i$, followed by a rise in the rate of increase of $[\text{Ca}^{2+}]_i$ indicative of Ca^{2+} influx (Figs 5 and 6D–F). The change in the rate of Ca^{2+} entry coincides with the initiation of shape change during platelet aggregation, which suggests that the rise in $[\text{Ca}^{2+}]_i$ might be responsible for Hcy-evoked platelet aggregation.

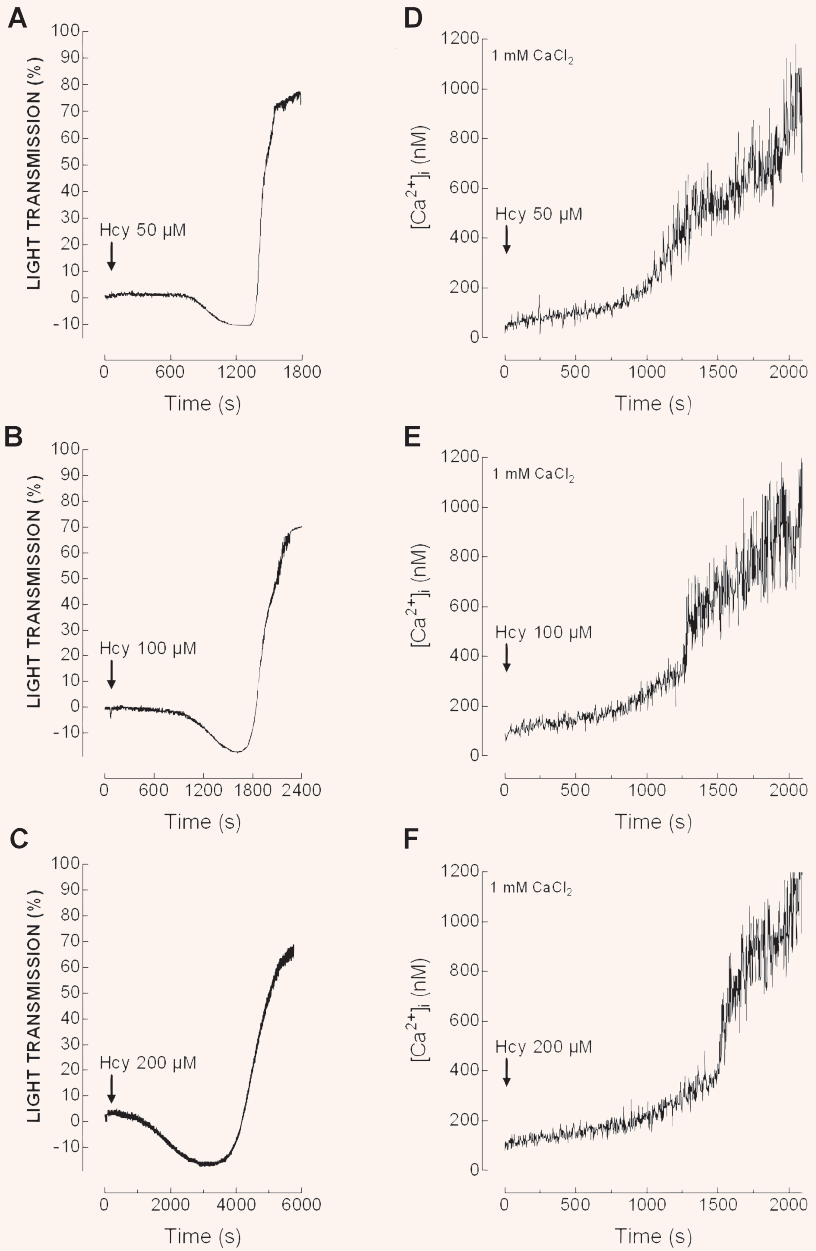
Despite we found that Hcy induces platelet aggregation and Ca^{2+} mobilization, we did not find a satisfactory correlation between Hcy concentrations and the percentage of platelet aggregation either in healthy ($y = -0.125x + 83$, $R^2 = 0.909$, $P = 0.19$) or diabetic donors ($y = -0.091x + 89$, $R^2 = 0.802$, $P = 0.28$), as well as between Hcy concentration and Ca^{2+} entry in healthy ($y = 2320x + 2,20,800$, $R^2 = 0.729$, $P = 0.34$) or diabetic donors ($y = 3676x + 5,24,500$, $R^2 = 0.949$, $P = 0.14$).

Discussion

Circulating platelets are activated in response to a variety of physiological agonists, such as thrombin that become available after the vascular injury. Platelet responsiveness might be altered by a number of pathological situations, including hypertension [37], myeloproliferative disorders [38] or diabetes mellitus [23, 36]. Oxidative stress has been shown to be involved in the development of abnormal signalling pathways leading to platelet hyperaggregability [39, 40].

Hyperhomocysteinaemia is considered an independent risk factor for vascular thrombosis; however the mechanisms underlying these alterations remains poorly understood. The role of oxidative stress in hyperhomocysteinaemia-induced cardiovascular injury is plausible since Hcy promotes oxidant injury to vascular and blood cells [13, 14, 25]. Platelets from type 2 diabetic patients are chronically exposed to high plasma levels of Hcy [25], which might be involved in the development of cardiovascular complications in these patients. Using preparations of washed platelets, we have found here that Hcy was able to induce endogenous

Fig. 5 Homocysteine induces shape change and platelet aggregation. Human platelets from healthy donors were suspended in HBS containing 1 mM Ca^{2+} and then treated with increasing concentrations of homocysteine (Hcy; 50–200 μM), as indicated. Aggregation of human platelets was induced at a shear rate of 1200 rpm at 37°C in a Chronolog aggregometer as described in Material and methods. Elevations in $[\text{Ca}^{2+}]_i$ were monitored using the 340/380 nm ratio and traces were calibrated in terms of $[\text{Ca}^{2+}]_i$. Traces shown are representative of 11 separate experiments.



generation of ROS that was markedly greater in cells from diabetic individuals. This fact, together with the continuous exposure to hyperhomocysteinaemia [25], could increase the risk of thrombosis. The greater endogenous ROS production in platelets from diabetic donors compared to controls is consistent with previous observations using platelet agonists [40–42].

Several mechanisms have been reported to underlie the effect of Hcy on platelet function, including activation of cPLA₂ and

PLC- γ 2 [12, 13]. A recent study has reported that Hcy induces Ca^{2+} release from intracellular pools in washed platelets [13], although the authors did not discriminate the identity of the Ca^{2+} stores. Human platelets present two separate Ca^{2+} stores differentiated by the distinct sensitivity to TG and TBHQ. The main store, expressing SERCA2b, which is inhibited by low concentrations of TG and is insensitive to TBHQ [32], has long been identified as the DTS, and the TBHQ-sensitive store has recently been identified as an

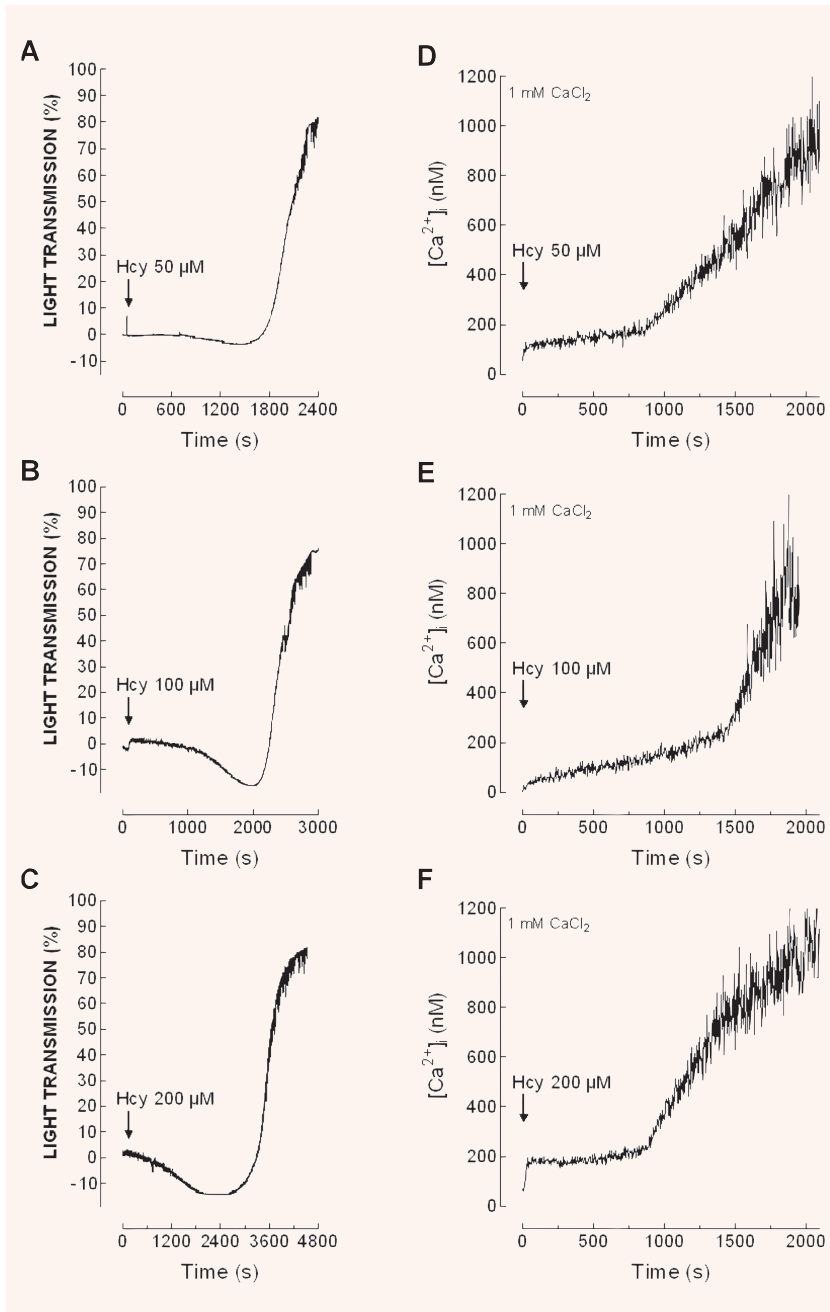


Fig. 6 Shape change and aggregation induced by homocysteine in platelet from diabetic patients. Human platelets from type 2 diabetics were suspended in HBS containing 1 mM Ca²⁺ and then treated with increasing concentrations of homocysteine (Hcy; 50–200 μM), as indicated. Aggregation of human platelets was induced at a shear rate of 1200 rpm at 37°C in a Chronolog aggregometer as described in Material and methods. Elevations in [Ca²⁺]_i were monitored using the 340/380 nm ratio and traces were calibrated in terms of [Ca²⁺]_i. Traces shown are representative of 11 separate experiments.

acidic organelle [33] expressing SERCA3 [30]. Here we show that Hcy releases Ca²⁺ from both stores. Our results indicate that depletion of the DTS by Hcy occurs in a concentration-dependent fashion while that of the acidic stores was not found to be concentration dependent; the latter result is consistent with our previous observations using the physiological agonist thrombin [43]. We have found that Hcy, at concentrations between 50 and 200 μM,

impairs thrombin-evoked Ca²⁺ release and Ca²⁺ entry, and Ca²⁺ mobilization by ADP was also found to be reduced by 50 μM Hcy. Similar findings have been reported in a human umbilical cord endothelial cell line [44]. The effect of Hcy inducing Ca²⁺ mobilization per se but impairing agonist-induced Ca²⁺ mobilization resembles that previously reported for H₂O₂ in platelets, which plays a dual role in Ca²⁺ signalling, both stimulating Ca²⁺ mobilization

by itself but also impairing agonist-evoked Ca^{2+} fluxes [28]. The inhibitory effect of H_2O_2 on agonist-evoked Ca^{2+} mobilization has been attributed to the impairment of the dynamics of actin cytoskeleton polymerization–depolymerization induced by agonists [45, 46]. Actin re-organization, as well as microtubular remodelling, has been widely demonstrated to be required for the activation of store-operated Ca^{2+} entry and secretion in platelets and other non-excitabile cells [47–50]. In umbilical endothelial cells, the inhibitory effect of Hcy on store-operated Ca^{2+} entry has been reported to be mediated through inhibition of actin polymerization [44], which, as for H_2O_2 , might be responsible for the effect of Hcy on agonist-evoked Ca^{2+} mobilization in human platelets.

Store-operated Ca^{2+} entry is a mechanism regulated by the filling state of the intracellular Ca^{2+} stores that has been presented as a major Ca^{2+} entry mechanism involved in the activation of a number of cellular processes and is required for full platelet activation [51–53]. The results in this study show that Hcy per se activates Ca^{2+} entry which consists of two phases: an initial stage consisting in a small but sustained increase in $[\text{Ca}^{2+}]_i$ followed by a rapid increase in $[\text{Ca}^{2+}]_i$. Interestingly, the pattern of Ca^{2+} mobilization parallels that of platelet aggregation by Hcy. During the initial phase of Ca^{2+} mobilization we did not detect activation of platelet aggregation or cell shape change, probably due to the low $[\text{Ca}^{2+}]_i$ reached at this stage, but the initiation of the second phase, which induces a remarkable increase in $[\text{Ca}^{2+}]_i$ seems to induce the beginning of platelets shape change and aggregation. Although speculative, the rapid Ca^{2+} mobilization observed during the second phase might be mediated by direct effects of Hcy on the Ca^{2+} handling mechanisms, perhaps by ROS production [28, 54, 55], or through the activation of secretory pathways leading to the release of platelet agonists. Taking together, the findings in this study suggest that Hcy-induced Ca^{2+} mobilization is involved in the activation of platelet aggregation.

No significant correlation was found between Hcy concentration and either the second phase of Ca^{2+} entry or the percentage of aggregation. Despite we have suggested that ROS production might be involved in Hcy-induced Ca^{2+} mobilisation, the oxidative stress induced by high concentrations of Hcy (ROS production is enhanced as cells are exposed to greater concentrations of Hcy (see Fig. 1)) might exert inhibitory effects on Ca^{2+} mobilization as previously reported [28, 56], thus preventing a satisfactory correlation between Hcy concentrations and Ca^{2+} mobilization.

In summary, our results demonstrate that Hcy induces endogenous ROS generation in human platelets, which might lead to Ca^{2+}

Table 2 Homocysteine induces platelet aggregation

Hcy concentration (μM)	Lag-time (s)	% rate	% aggregation
Platelets from healthy donors			
50	689.8±141.5	52.6±4.0	79.0±3.6
100	715.1±116.2	62.0±6.9	67.2±4.1
200	990.2±168.1	41.0±1.9	60.2±5.1
Platelets from type 2 diabetic patients			
50	1054.9± 220.7*	48.0±3.5	86.8±1.5*
100	1316.3±223.9*	51.5±7.6	76.4±3.7*
200	1593.8± 307.4*	45.8±2.4*	71.8±4.8*

Human platelets were suspended in a HBS containing 1 mM Ca^{2+} and then were treated at 37°C with increasing concentrations of homocysteine and platelet aggregation was induced at a shear rate of 1200 rpm at 37°C. Values given are presented as mean ± S.E.M. of 11 separate determinations. * $P < 0.05$ compared to the response in platelets from healthy donors.

mobilization, as previously reported [54, 55], and, subsequently, to platelet aggregation. All the parameters tested in the present study, ROS generation, Ca^{2+} mobilization and platelet aggregation are more elevated in platelets from diabetic donors than in controls, even though they were exposed to the same concentrations of Hcy, which indicates that platelets from diabetic donors are more sensitive to plasma Hcy levels. These findings, together with the reported hyperhomocysteinaemia in patients with type 2 diabetes mellitus [25, 57], which has been associated with the development of atherosclerosis, thrombosis and other cardiovascular disorders [5, 9, 58], strongly suggest that Hcy might be considered a risk factor in the development of cardiovascular disorders associated to type 2 diabetes mellitus.

Acknowledgements

This work was supported by M.E.C.–FEDER (BFU2007-60104/BFI), Junta de Extremadura–Consejería de Educación, Ciencia y Tecnología & FEDER (2PR04A009) and Consejería de Sanidad y Consumo. N. Alexandru was the recipient of 3 month training at University Extremadura, Spain financially supported by a grant from the FP6 European Community SSA-SERA Project 016873. We thank Dr. Hernandez-Cruz for providing blood samples and Mercedes Gómez Blázquez for her technical assistance.

References

- Undas A, Brozek J, Szczeklik A. Homocysteine and thrombosis: from basic science to clinical evidence. *Thromb Haemost.* 2005; 94: 907–15.
- Frantzen F, Faaren AL, Alfheim I, Nordhei AK. Enzyme conversion immunoassay for determining total homocysteine in plasma or serum. *Clin Chem.* 1998; 44: 311–16.
- Selhub J. Homocysteine metabolism. *Ann Rev Nutr.* 1999; 19: 217–46.
- Wierzbicki AS. Homocysteine and cardiovascular disease: a review of the evidence. *Diab Vasc Dis Res.* 2007; 4: 143–50.
- Cattaneo M. Hyperhomocysteinemia, atherosclerosis and thrombosis. *Thromb. Haemost.* 1999; 81: 165–76.
- Kanani PM, Sinkey CA, Browning RL, Allaman M, Knapp HR, Haynes WG. Role of oxidant stress in endothelial dysfunction produced by experimental hyperhomocysteinemia in humans. *Circulation.* 1999; 100: 1161–8.
- Stamler JS, Osborne JA, Jaraki O, Rabbani LE, Mullins M, Singel D,

- Loscalzo J.** Adverse vascular effects of homocysteine are modulated by endothelium-derived relaxing factor and related oxides of nitrogen. *J Clin Invest.* 1993; 91: 308–18.
8. **Lentz SR.** Mechanisms of homocysteine-induced atherothrombosis. *J Thromb Haemost.* 2005; 3: 1646–54.
9. **Coppola A, Davi G, De Stefano V, Mancini FP, Cerbone AM, Di Minno G.** Homocysteine, coagulation, platelet function, and thrombosis. *Semin Thromb Hemost.* 2000; 26: 243–54.
10. **Leoncini G, Pascale R, Signorello MG.** Effects of homocysteine on L-arginine transport and nitric oxide formation in human platelets. *Eur J Clin Invest.* 2003; 33: 713–9.
11. **Durand P, Lussier-Cacan S, Blache D.** Acute methionine load-induced hyperhomocysteinemia enhances platelet aggregation, thromboxane biosynthesis, and macrophage-derived tissue factor activity in rats. *FASEB J.* 1997; 11: 1157–68.
12. **Upchurch GR Jr, Welch GN, Fabian AJ, Freedman JE, Johnson JL, Keaney JF Jr, Loscalzo J.** Homocyst(e)ine decreases bioavailable nitric oxide by a mechanism involving glutathione peroxidase. *J Biol Chem.* 1997; 272: 17012–7.
13. **Leoncini G, Bruzzese D, Signorello MG.** Activation of p38 MAPKinase/cPLA2 pathway in homocysteine-treated platelets. *J Thromb Haemost.* 2006; 4: 209–16.
14. **Leoncini G, Bruzzese D, Signorello MG.** A role for PLCgamma2 in platelet activation by homocysteine. *J Cell Biochem.* 2007; 100: 1255–65.
18. **Jardin I, Ben Amor N, Bartegi A, Pariente JA, Salido GM, Rosado JA.** Differential involvement of thrombin receptors in Ca²⁺ release from two different intracellular stores in human platelets. *Biochem J.* 2007; 401: 167–74.
19. **Redondo PC, Jardin I, Hernandez-Cruz JM, Pariente JA, Salido GM, Rosado JA.** Hydrogen peroxide and peroxynitrite enhance Ca²⁺ mobilization and aggregation in platelets from type 2 diabetic patients. *Biochem Biophys Res Commun.* 2005; 333: 794–802.
20. **Rudich A, Tirosh A, Potashnik R, Hemi R, Kanety H, Bashan N.** Prolonged oxidative stress impairs insulin-induced GLUT4 translocation in 3T3-L1 adipocytes. *Diabetes.* 1998; 47: 1562–9.
21. **Cooksey RC, Jouihan HA, Ajioka RS, Hazel MW, Jones DL, Kushner JP, McClain DA.** Oxidative stress, beta-cell apoptosis, and decreased insulin secretory capacity in mouse models of hemochromatosis. *Endocrinology.* 2004; 145: 5305–12.
22. **Cimminiello C, Milani M.** Diabetes mellitus and peripheral vascular disease: is aspirin effective in preventing vascular events? *Diabetologia.* 1996; 39: 1402–4.
23. **Saavedra FR, Redondo PC, Hernandez-Cruz JM, Salido GM, Pariente JA, Rosado JA.** Store-operated Ca²⁺ entry and tyrosine kinase pp60^{src} hyperactivity are modulated by hyperglycemia in platelets from patients with non insulin-dependent diabetes mellitus. *Arch Biochem Biophys.* 2004; 432: 261–8.
24. **Rosado JA, Saavedra FR, Redondo PC, Hernandez-Cruz JM, Salido GM, Pariente JA.** Reduced plasma membrane Ca²⁺-ATPase function in platelets from patients with non-insulin-dependent diabetes mellitus. *Haematologica.* 2004; 89: 1142–4.
25. **Signorello MG, Viviani GL, Armani U, Cerone R, Minniti G, Piana A, Leoncini G.** Homocysteine, reactive oxygen species and nitric oxide in type 2 diabetes mellitus. *Thromb Res.* 2007; 120: 607–13.
26. **Grynkiewicz G, Poenie M, Tsien RY.** A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J Biol Chem.* 1985; 260: 3440–50.
27. **Rosado JA, Sage SO.** Farnesylcysteine analogues inhibit store-regulated Ca²⁺ entry in human platelets: evidence for involvement of small GTP-binding proteins and actin cytoskeleton. *Biochem J.* 2000; 347: 183–92.
28. **Redondo PC, Salido GM, Pariente JA, Rosado JA.** Dual effect of hydrogen peroxide on store-mediated calcium entry in human platelets. *Biochem Pharmacol.* 2004; 67: 1065–76.
29. **Tintinger GR, Theron AJ, Potjo M, Anderson R.** Reactive oxidants regulate membrane repolarization and store-operated uptake of calcium by formyl peptide-activated human neutrophils. *Free Radic Biol Med.* 2007; 42: 1851–7.
30. **Kovacs T, Berger G, Corvazier E, Paszty K, Brown A, Bobe R, Papp B, Wuytack F, Cramer EM, Enouf J.** Immunolocalization of the multi-sarco/endoplasmic reticulum Ca²⁺ ATPase system in human platelets. *Br J Haematol.* 1997; 97: 192–203.
31. **Bobe R, Bredoux R, Wuytack F, Quarck R, Kovacs T, Papp B, Corvazier E, Magnier C, Enouf J.** The rat platelet 97-kDa Ca²⁺ ATPase isoform is the sarcoendoplasmic reticulum Ca²⁺ ATPase 3 protein. *J Biol Chem.* 1994; 269: 1417–24.
32. **Enyedi A, Paszty K, Kovacs T, Sarkadi B, Gardos G, Magnier C, Wuytack F, Enouf J.** Simultaneous presence of two distinct endoplasmic-reticulum-type calcium-pump isoforms in human cells. Characterization by radio-immunoblotting and inhibition by 2,5-di-(t-butyl)-1,4-benzohydroquinone. *Biochem J.* 1992; 288: 297–302.
33. **Lopez JJ, Camello-Almaraz C, Pariente JA, Salido GM, Rosado JA.** Ca²⁺ accumulation into acidic organelles mediated by Ca²⁺- and vacuolar H⁺-ATPases in human platelets. *Biochem J.* 2005; 390: 243–52.
34. **Lopez JJ, Redondo PC, Salido GM, Pariente JA, Rosado JA.** Two distinct Ca²⁺ compartments show differential sensitivity to thrombin, ADP and vasopressin in human platelets. *Cell Signal.* 2006; 18: 373–81.
35. **Jardin I, Redondo PC, Salido GM, Rosado JA.** Phosphatidylinositol 4,5-bisphosphate enhances store-operated calcium entry through hTRPC6 channel in human platelets. *Biochim Biophys Acta.* 2007 doi:10.1016/j.bbamcr.2007.07.007.
36. **Chaabane C, Dally S, Corvazier E, Bredoux R, Bobe R, Ftouhi B, Raies A, Enouf J.** Platelet PMCA- and SERCA-type Ca²⁺ ATPase expression in diabetes: a novel signature of abnormal megakaryocytopoiesis. *J Thromb Haemost.* 2007; 5: 2127–35.
37. **Blankenship KA, Dawson CB, Aronoff GR, Dean WL.** Tyrosine phosphorylation of human platelet plasma membrane Ca²⁺-ATPase in hypertension. *Hypertension.* 2000; 35: 103–7.
38. **Avram S, Lupu A, Angelescu S, Olteanu N, Mut-Popescu D.** Abnormalities of platelet aggregation in chronic myeloproliferative disorders. *J Cell Mol Med.* 2001; 5: 79–87.
39. **Jardin I, Redondo PC, Salido GM, Pariente JA, Rosado JA.** Endogenously generated reactive oxygen species reduce PMCA activity in platelets from patients with non-insulin-dependent diabetes mellitus. *Platelets.* 2006; 17: 283–8.
40. **Leoncini G, Signorello MG, Piana A, Carrubba M, Armani U.** Hyperactivity and increased hydrogen peroxide formation in platelets of NIDDM patients. *Thromb Res.* 1997; 86: 153–60.
41. **Schaeffer G, Wascher TC, Kostner GM, Graier WF.** Alterations in platelet Ca²⁺ signalling in diabetic patients is due to increased formation of superoxide anions and reduced nitric oxide production. *Diabetologia.* 1999; 42: 167–76.
42. **Bouaziz A, Salido S, Linares-Palomino PJ, Sanchez A, Altarejos J, Bartegi A,**

- Salido GM, Rosado JA.** Cinnamtannin B-1 from bay wood reduces abnormal intracellular Ca^{2+} homeostasis and platelet hyperaggregability in type 2 diabetes mellitus patients. *Arch Biochem Biophys.* 2007; 457: 235–42.
43. **Jardin I, Ben Amor N, Bartegi A, Pariente JA, Salido GM, Rosado JA.** Differential involvement of thrombin receptors in Ca^{2+} release from two different intracellular stores in human platelets. *Biochem J.* 2007; 401: 167–74.
44. **Zhang HS, Xiao JH, Cao EH, Qin JF.** Homocysteine inhibits store-mediated calcium entry in human endothelial cells: evidence for involvement of membrane potential and actin cytoskeleton. *Mol Cell Biochem.* 2005; 269: 37–47.
45. **Rosado JA, González A, Salido GM, Pariente JA.** Effects of reactive oxygen species on actin filament polymerisation and amylase secretion in mouse pancreatic acinar cells. *Cell Signal.* 2002; 14: 547–56.
46. **Zhu D, Tan KS, Zhang X, Sun AY, Sun GY, Lee JC.** Hydrogen peroxide alters membrane and cytoskeleton properties and increases intercellular connections in astrocytes. *J Cell Sci.* 2005; 118: 3695–703.
47. **Rosado JA, Salido GM, Jensen RT, Garcia LJ.** Are tyrosine phosphorylation of p125(FAK) and paxillin or the small GTP binding protein, rho, needed for CCK-stimulated pancreatic amylase secretion? *Biochim Biophys Acta.* 1998; 1404: 412–26.
48. **Patterson RL, van Rossum DB, Gill DL.** Store-operated Ca^{2+} entry: evidence for a secretion-like coupling model. *Cell.* 1999; 98: 487–99.
49. **Rosado JA, Sage SO.** A role for the actin cytoskeleton in the initiation and maintenance of store-mediated calcium entry in human platelets. *Trends Cardiovasc Med.* 2000; 10: 327–32.
50. **Bouaziz A, Amor NB, Woodard GE, Zibidi H, Lopez JJ, Bartegi A, Salido GM, Rosado JA.** Tyrosine phosphorylation / dephosphorylation balance is involved in thrombin-evoked microtubular reorganisation in human platelets. *Thromb Haemost.* 2007; 98: 375–84.
51. **Parekh AB.** Store-operated Ca^{2+} entry: Dynamic interplay between endoplasmic reticulum, mitochondria and plasma membrane. *J Physiol.* 2003; 547: 333–48.
52. **Putney JW Jr.** Store-operated calcium channels: How do we measure them, and why do we care? *Sci STKE.* 2004; 243: 1–3.
53. **Rosado JA, Redondo PC, Sage SO, Pariente JA, Salido GM.** Store-operated Ca^{2+} entry: vesicle fusion or reversible trafficking and *de novo* conformational coupling? *J Cell Physiol.* 2005; 205: 262–9.
54. **Ho PI, Collins SC, Dhitavat S, Ortiz D, Ashline D, Rogers E, Shea TB.** Homocysteine potentiates beta-amyloid neurotoxicity: role of oxidative stress. *J Neurochem.* 2001; 78: 249–53.
55. **Redondo PC, Salido GM, Rosado JA, Pariente JA.** Effect of hydrogen peroxide on Ca^{2+} mobilisation in human platelets through sulphhydryl oxidation dependent and independent mechanisms. *Biochem Pharmacol.* 2004; 67: 491–502.
56. **Elliott SJ, Schilling WP.** Oxidative stress inhibits bradykinin-stimulated $45Ca^{2+}$ flux in pulmonary vascular endothelial cells. *Am J Physiol.* 1991; 260: H549–56.
57. **Koubaa N, Nakbi A, Smaoui M, Abid N, Chaaba R, Abid M, Hammami M.** Hyperhomocysteinemia and elevated ox-LDL in Tunisian type 2 diabetic patients: Role of genetic and dietary factors. *Clin Biochem.* 2007; 40: 1007–14.
58. **Rasouli ML, Nasir K, Blumenthal RS, Park R, Aziz DC, Budo-ff MJ.** Plasma homocysteine predicts progression of atherosclerosis. *Atherosclerosis.* 2005; 181: 159–65.