

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

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Received: October 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  $\text{Ca}^{2+}$  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 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 homocysteine resulted in  $\text{Ca}^{2+}$  release from the dense tubular system and the acidic stores.  $\text{Ca}^{2+}$  mobilization-induced by homocysteine consisted in two components, an initial slow increase in intracellular free  $\text{Ca}^{2+}$  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,  $\text{Ca}^{2+}$  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 homocysteine. These findings, together with the hyperhomocysteinaemia reported in diabetic patients, strongly suggest that homocysteine 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 is a sulphur-containing amino acid produced by conversion of methionine, an essential amino acid that is regularly consumed within the diet. Homocysteine is metabolized by re-methylation or transsulphuration, processes that require either vitamin B12 or B6, respectively, as cofactors [1]. In normal conditions, the plasma levels of homocysteine in humans are low (5–15  $\mu\text{M}$ ); however, genetic defects in the enzymes metabolizing homocysteine or environmental factors markedly increase homocysteinaemia. Mutations in cystathione- $\beta$ -synthase or 5,10-methylenetetrahydrofolate reductase are associated

with excess levels of homocysteine (>50  $\mu\text{M}$  and up to 400  $\mu\text{M}$ ) [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 homocysteine increase platelet responsiveness to thrombin in a concentration-dependent manner. In addition, acute homocysteine increases in rats have been shown to be associated to an increase in platelet aggregation [11]. Some of the effects of homocysteine on human platelet function have been explained by a

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reduction in nitric oxide formation or bioavailability [10, 12]. In addition, platelet hyperactivity induced by homocysteine has been shown to involve activation of the p38 mitogen-activated protein kinase (MAPK)/cytosolic phospholipase A (cPLA<sub>2</sub>) pathway, leading to the synthesis of thromboxane A<sub>2</sub> and the activation of integrin  $\alpha_{IIb}\beta_3$  [13]. Homocysteine-induced platelet hyperactivity also involves activation of PLC- $\gamma$ 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 [15]. 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  $\beta$  cells, resulting in a decrease in insulin content, secretion and activity [16, 17]. In addition, diabetes mellitus leads to several cardiovascular disorders, including platelet dysfunction associated to an abnormal intracellular Ca<sup>2+</sup> homeostasis, which are the major cause of morbidity and mortality in type 2 diabetes mellitus [18–20]. Type 2 diabetic subjects have been reported to show 40% greater plasma levels of homocysteine than healthy subjects [21]; therefore, as homocysteine 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 homocysteine-induced effects in diabetic patients, we have investigated aggregation, intracellular Ca<sup>2+</sup> mobilization, and endogenous reactive oxygen species (ROS) generation induced by homocysteine in platelets from type 2 diabetics and healthy subjects.

## Materials and methods

### Materials

Apyrase (grade VII), ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), aspirin, bovine serum albumin (BSA), thrombin, adenosine diphosphate (ADP), thapsigargin (TG), 2,5 di-(tertbutyl)-1,4-hydroquinone (TBHQ) and homocysteine (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

**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
B.M.I.	26.1 ± 2.9	27.7 ± 2.5
Diabetes duration (years)	-	7.8 ± 2.8
Fasting glycaemia (mg/dl)	92 ± 5	190 ± 12
HbA1C (%)	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
Low-density lipoprotein (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.

routine laboratory methods. Blood glucose concentration in diabetic patients was in the range of 180 to 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 participants were age- and gender-matched healthy individuals that had HbA1c levels in the normal range (3.5–5%).

### 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  $\mu$ m) and apyrase (40  $\mu$ 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 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), 10 D-glucose, 5 KCl, 1 MgSO<sub>4</sub>, pH 7.45 and supplemented with 0.1% w/v BSA and 40  $\mu$ 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  $\mu\text{m}$  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 millilitre aliquots were used for fluorescence recording (spectrophotometer Varian Ltd., Madrid, Spain) at  $\lambda$  ex: 494 nm and  $\lambda$  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  $\mu\text{l}$ ) prepared as described above were suspended in HBS and stimulated in the aggregometer cuvettes at 37°C under stirring at 1200 rpm [15]. The time required to reach the maximum aggregation rate was then determined from the traces recorded.

## Intracellular ROS production through the oxidation of CM-H<sub>2</sub>DCFDA

CM-H<sub>2</sub>DCFDA 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-H<sub>2</sub>DCFDA oxidation yields a fluorescent adduct (DCF) that is trapped inside the cell. Platelet-rich plasma was incubated at 37°C with 10  $\mu\text{m}$  CM-H<sub>2</sub>DCFDA 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  $\lambda$  ex: 488 nm and  $\lambda$  em: 530 nm. Traces were fitted to the equation:  $y = A + Bx$  where B is the slope.

## Measurement of intracellular free calcium concentration ([Ca<sup>2+</sup>]<sub>i</sub>)

Cells were incubated at 37°C with 2  $\mu\text{m}$  fura-2 AM for 45 min, collected by centrifugation at 350 *g* for 20 min. and resuspended 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  $\lambda$  ex: 340 and 380 nm and  $\lambda$  em: 505 nm. Changes in [Ca<sup>2+</sup>]<sub>i</sub> were monitored using the fura-2 340/380 fluorescence ratio and calibrated according to the method of Grynkiewicz *et al.* [22]. Ca<sup>2+</sup> release and influx was estimated using the integral of the rise in [Ca<sup>2+</sup>]<sub>i</sub> for 2.5 min after addition of agonists or CaCl<sub>2</sub>, respectively [23].

## 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

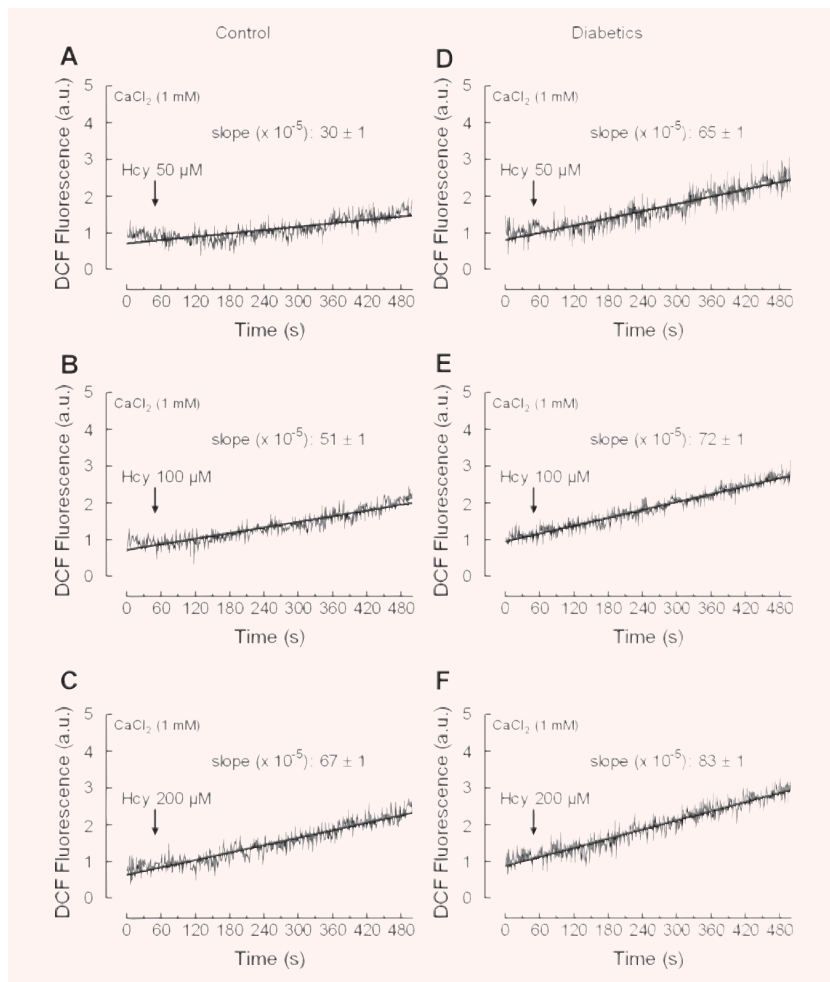
### ROS generation induced by Hcy in platelets from healthy and type 2 diabetic patients

Intracellular ROS production was estimated using CM-H<sub>2</sub>DCFDA. As shown in Figures 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  $\mu\text{m}$ , 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  $\mu\text{m}$ , 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 [21].

### induces Ca<sup>2+</sup> mobilization in platelets from healthy and type 2 diabetic patients

The possibility that Hcy-induced endogenous ROS production might be associated to calcium mobilization, as previously reported [15, 24, 25], was tested by examining the effect of Hcy on Ca<sup>2+</sup> release from the intracellular stores and Ca<sup>2+</sup> entry through the plasma membrane. In human platelets two Ca<sup>2+</sup> stores have been described: the TBHQ-insensitive dense tubular system (DTS) and the TBHQ-sensitive acidic stores based on immunolocalisation studies [26], the different sensitivity to TG and TBHQ [27, 28] and the distinct sensitivity to agonists [29, 30]. We have investigated the effect of Hcy on Ca<sup>2+</sup> release from the DTS, by previous depletion of the acidic stores using TBHQ. In a Ca<sup>2+</sup>-free medium, treatment of platelets from healthy donors with 20  $\mu\text{m}$  TBHQ induced a sustained increase in [Ca<sup>2+</sup>]<sub>i</sub>; subsequent addition of Hcy induced a concentration-dependent increase in [Ca<sup>2+</sup>]<sub>i</sub> indicative of Ca<sup>2+</sup> release from the DTS (Fig. 2A, C, E and G). In platelets from type 2 diabetic patients Hcy evoked a concentration-dependent Ca<sup>2+</sup> release from the DTS, which was found to be significantly higher at 50, 100 and 200  $\mu\text{m}$  than those observed in control platelets (Fig. 2B, D, F and G;  $P < 0.05$ ).



**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  $\mu\text{M}$  (A), 100  $\mu\text{M}$  (B) or 200  $\mu\text{M}$  (C) homocysteine (Hcy) in a medium containing 1 mM  $\text{Ca}^{2+}$ . 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.

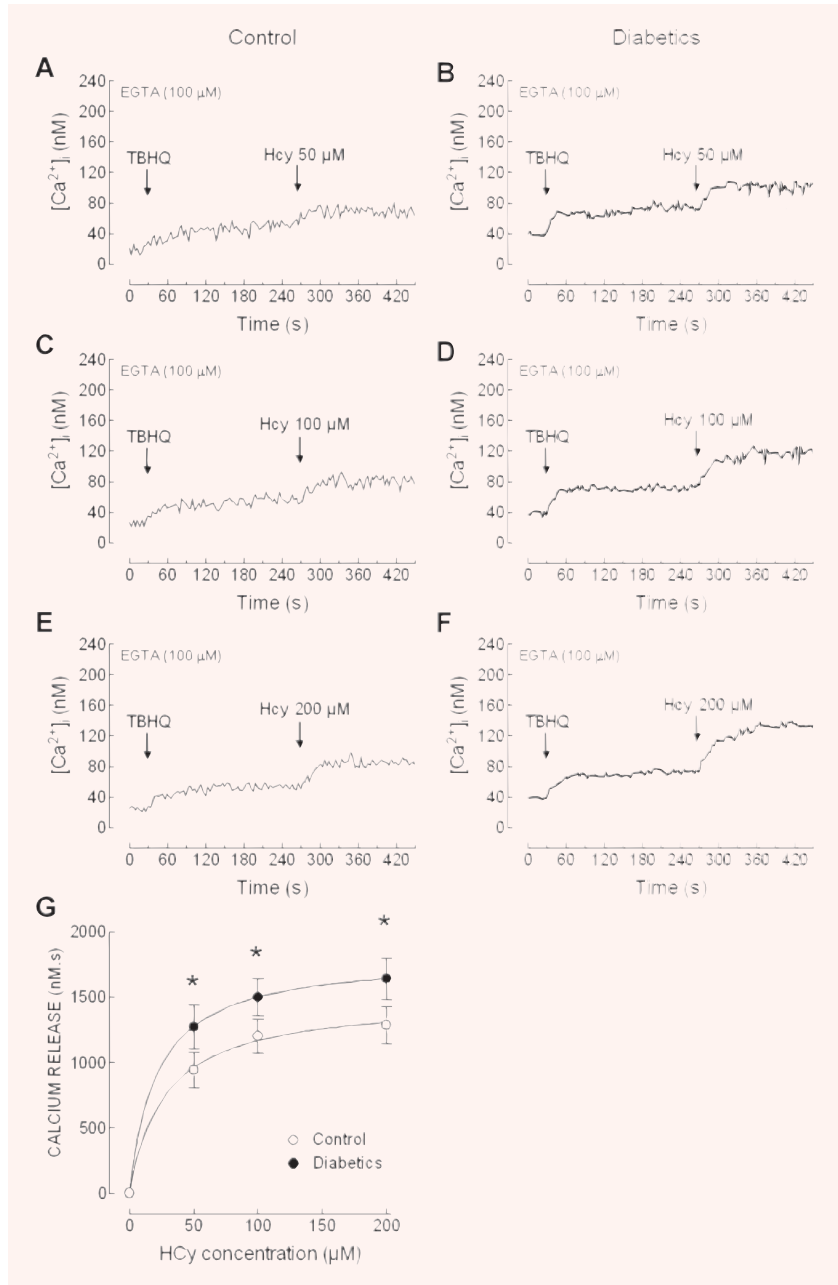
In order to investigate  $\text{Ca}^{2+}$  release from the acidic stores induced by Hcy we have depleted the DTS, by using a combination of ADP (10  $\mu\text{M}$ ) and TG (10 nM), which selectively inhibits SERCA2b present in the DTS [35], prior to the addition of Hcy. Treatment of platelets with ADP + TG resulted in a transient increase in  $[\text{Ca}^{2+}]_i$  indicative of  $\text{Ca}^{2+}$  release from the DTS (Fig. 3A–F). Subsequent addition of Hcy induced a sustained increase in  $[\text{Ca}^{2+}]_i$  that was found to be significantly greater in platelets from diabetics than in controls (Fig. 3G;  $P < 0.05$ ). Hcy-evoked  $\text{Ca}^{2+}$  release from the acidic stores was not found to be concentration-dependent. Hcy concentration and  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  entry. As shown in Figure 4A and B, treatment of platelets with

increasing concentrations of Hcy (50–200  $\mu\text{M}$ ) in a medium containing 1 mM  $\text{Ca}^{2+}$  resulted in a rapid, concentration-dependent and sustained increase in  $[\text{Ca}^{2+}]_i$  that was found to be significantly higher to that observed in the absence of extracellular  $\text{Ca}^{2+}$  (data not shown). The rise in  $[\text{Ca}^{2+}]_i$  evoked by Hcy was found to be significantly increased in diabetic patients compared to controls (Fig. 4A and B;  $P < 0.05$ ).

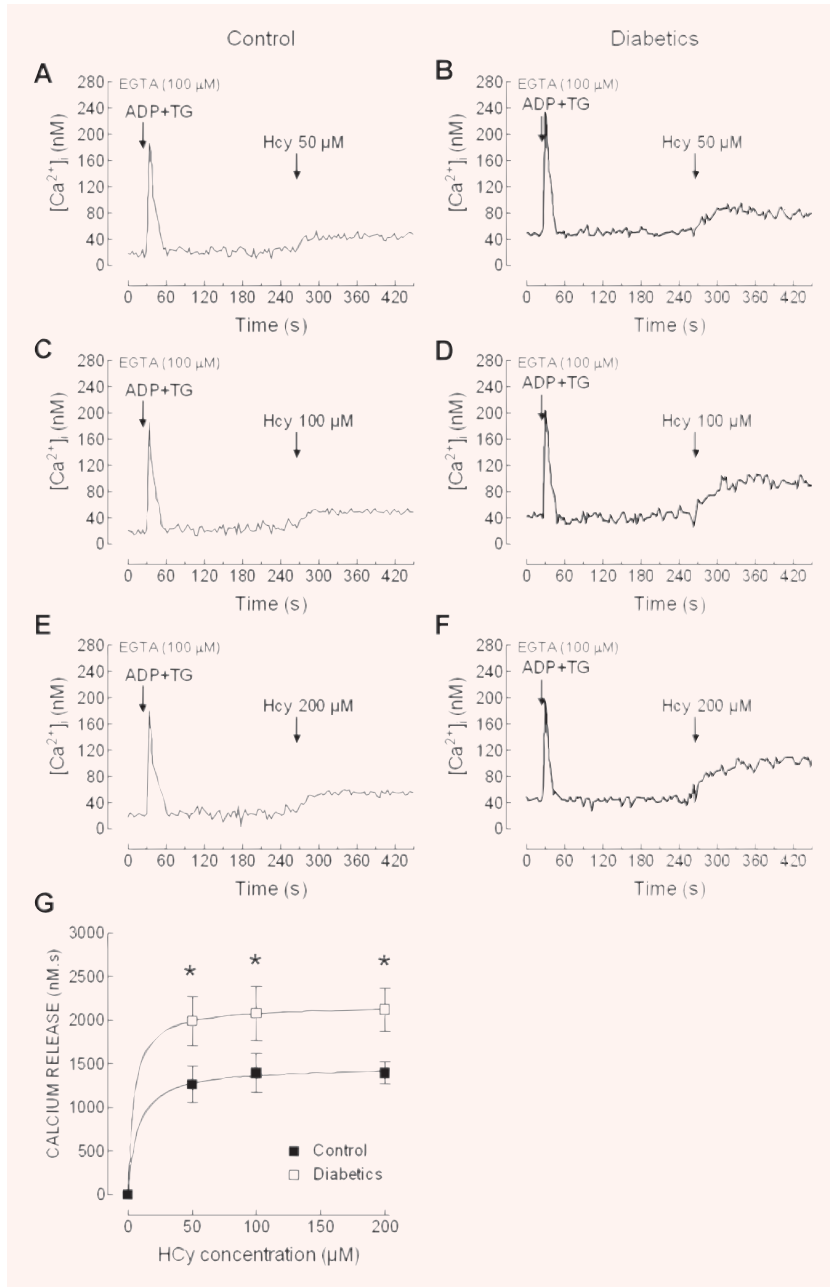
Subsequently, we questioned whether Hcy impairs  $\text{Ca}^{2+}$  mobilization induced by agonists. Treatment of platelets with 0.1 U/ml thrombin resulted in a transient increase of  $[\text{Ca}^{2+}]_i$ . The initial peak  $[\text{Ca}^{2+}]_i$  elevation above basal after thrombin was  $297 \pm 37$  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  $\text{Ca}^{2+}$  mobilization in diabetic platelets [19, 20, 32]. The subsequent addition of  $\text{Ca}^{2+}$  (300  $\mu\text{M}$ ) to the external medium induced a sustained elevation in  $[\text{Ca}^{2+}]_i$  indicative of  $\text{Ca}^{2+}$  entry. As shown in Figure 4C and D,  $\text{Ca}^{2+}$  entry was significantly higher in platelets from diabetic patients (the integrals of the rise in  $[\text{Ca}^{2+}]_i$  for 2.5 min. after addition of  $\text{CaCl}_2$  were  $14111 \pm 2334$  and  $52108 \pm 7560$  nM/s in healthy and

**Fig. 2** Homocysteine releases  $\text{Ca}^{2+}$  from 2,5 di-(tertbutyl)-1,4-hydroquinone (TBHQ)-insensitive stores in platelets from healthy and diabetic donors. Human platelets from healthy individuals (**A, C and E**) and diabetic patients (**B, D and F**) were suspended in a  $\text{Ca}^{2+}$ -free medium (100  $\mu\text{M}$  EGTA was added). Cells were treated with 20  $\mu\text{M}$  TBHQ and 4 min. later homocysteine (Hcy; 50, 100 or 200  $\mu\text{M}$ ) was added, as indicated. Traces are representative of 10–21 independent experiments. (**G**) Values represent Hcy-induced  $\text{Ca}^{2+}$  release estimated as described in Material and methods.  $P < 0.05$  compared to Hcy-evoked  $\text{Ca}^{2+}$  release in platelets from healthy donors.



diabetic patients, respectively;  $P < 0.05$ ). In the presence of 50  $\mu\text{M}$  Hcy thrombin-evoked  $\text{Ca}^{2+}$  release was significantly reduced (the initial peak  $[\text{Ca}^{2+}]_i$  elevation above basal after thrombin was  $71 \pm 6$  and  $139 \pm 10$  nM in platelets from healthy and diabetic donors, respectively, and similar results were obtained with 100 and 200  $\mu\text{M}$  Hcy; Fig. 4C and D;  $P < 0.05$ ). Hcy also impaired thrombin-evoked  $\text{Ca}^{2+}$  entry in healthy individuals and diabetic patients (the integrals of the rise in  $[\text{Ca}^{2+}]_i$  for

2.5 min. after addition of  $\text{CaCl}_2$  were  $5850 \pm 283$ ,  $5389 \pm 352$  and  $5203 \pm 383$  nM/s in platelets from healthy individuals and  $8755 \pm 996$ ,  $8578 \pm 235$  and  $8324 \pm 124$  nM/s in platelets from diabetic donors, in the presence of 50, 100 and 200  $\mu\text{M}$  Hcy; Fig. 4C and D;  $P < 0.05$ ). Similarly, Hcy significantly reduced  $\text{Ca}^{2+}$  mobilization evoked by 10  $\mu\text{M}$  ADP, including both  $\text{Ca}^{2+}$  release from the intracellular stores (the initial peak  $[\text{Ca}^{2+}]_i$  elevation above basal after ADP was  $160 \pm 7$  and  $218 \pm 11$  nM in



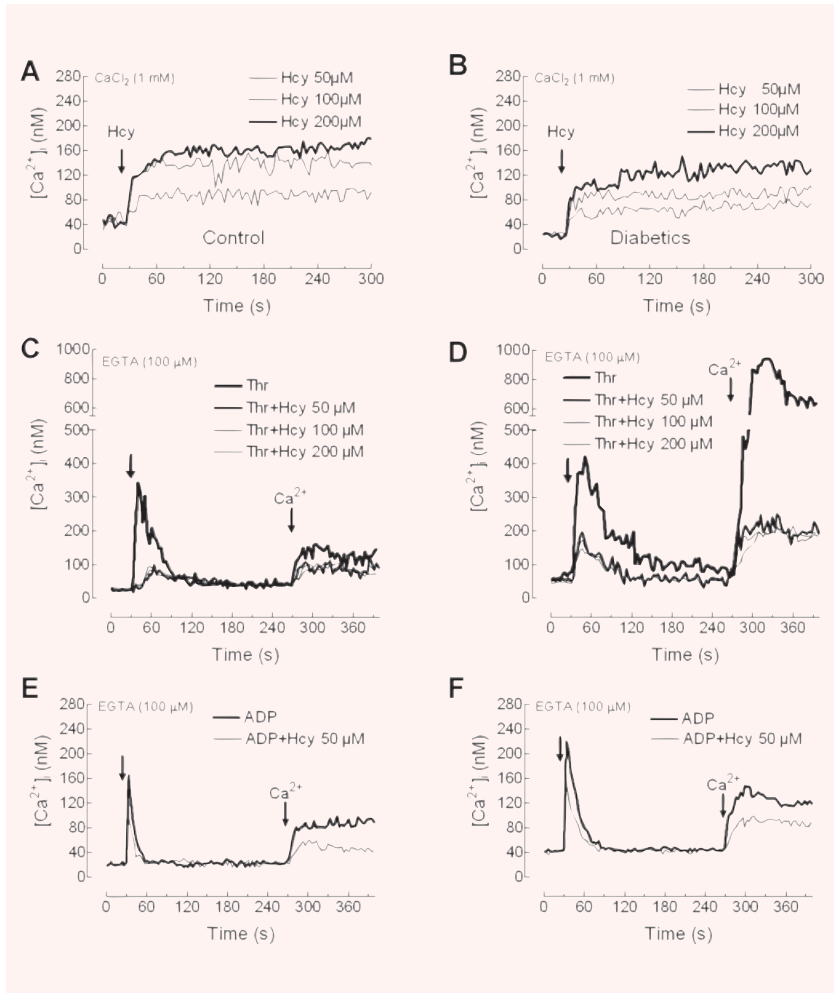
**Fig. 3** Homocysteine releases  $\text{Ca}^{2+}$  from TBHQ-sensitive stores in platelets from healthy and diabetic donors. Human platelets from healthy individuals (**A**, **C** and **E**) and diabetic patients (**B**, **D** and **F**) were suspended in a  $\text{Ca}^{2+}$ -free medium ( $100 \mu\text{M}$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

platelets from healthy and diabetic donors in the absence of Hcy and  $82 \pm 5$  and  $129 \pm 12 \text{ nM}$  in platelets from healthy individuals and diabetic patients in the presence of  $50 \mu\text{M}$  Hcy; Fig. 4E and F;  $P < 0.05$ ) and  $\text{Ca}^{2+}$  entry (the integrals of the rise in  $[\text{Ca}^{2+}]_i$  for 2.5 min. after addition of  $\text{CaCl}_2$  were  $5341 \pm 470$  and  $7210 \pm 611 \text{ nM}$  in platelets from healthy and diabetic donors in the absence of Hcy and  $2892 \pm 352$  and  $4125 \pm 512 \text{ nM}$  in platelets from healthy individuals and diabetic patients in the presence of  $50 \mu\text{M}$  Hcy; Fig. 4E and F;  $P < 0.05$ ).

### Effect of Hcy on platelet aggregation

As shown in Figures 5 and 6(A–C) and Table 2, treatment of platelets suspended in a medium containing  $1 \text{ mM}$   $\text{Ca}^{2+}$  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 diabetic donors compared to controls (Table 2), which is

**Fig. 4** Homocysteine stimulates  $\text{Ca}^{2+}$  entry and impairs  $\text{Ca}^{2+}$  mobilization by thrombin in platelets from healthy and diabetic donors. **(A and B)** Human platelets from healthy individuals **(A)** and diabetic patients **(B)** were suspended in HEPES-buffered saline (HBS) containing 1 mM  $\text{Ca}^{2+}$  and then exposed to increasing concentrations of homocysteine (Hcy; 50–200  $\mu\text{M}$ ). **(C–F)** Human platelets from healthy individuals **(C and E)** and diabetic patients **(D and F)** were suspended in a  $\text{Ca}^{2+}$ -free medium (100  $\mu\text{M}$  EGTA was added). Cells were treated (thick arrow) with either 0.1 U/ml thrombin (Thr; **C and D**) or with 10  $\mu\text{M}$  ADP **(E and F)**, in the absence or presence of 50, 100 or 200  $\mu\text{M}$  Hcy, as indicated, and 4 min. later  $\text{CaCl}_2$  (300  $\mu\text{M}$ ) was added to the medium to initiate  $\text{Ca}^{2+}$  entry. Traces are representative of 8–12 independent experiments.



consistent with the greater effect of Hcy in ROS production and  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  influx (Figs. 5 and 6D–F). The change in the rate of  $\text{Ca}^{2+}$  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.

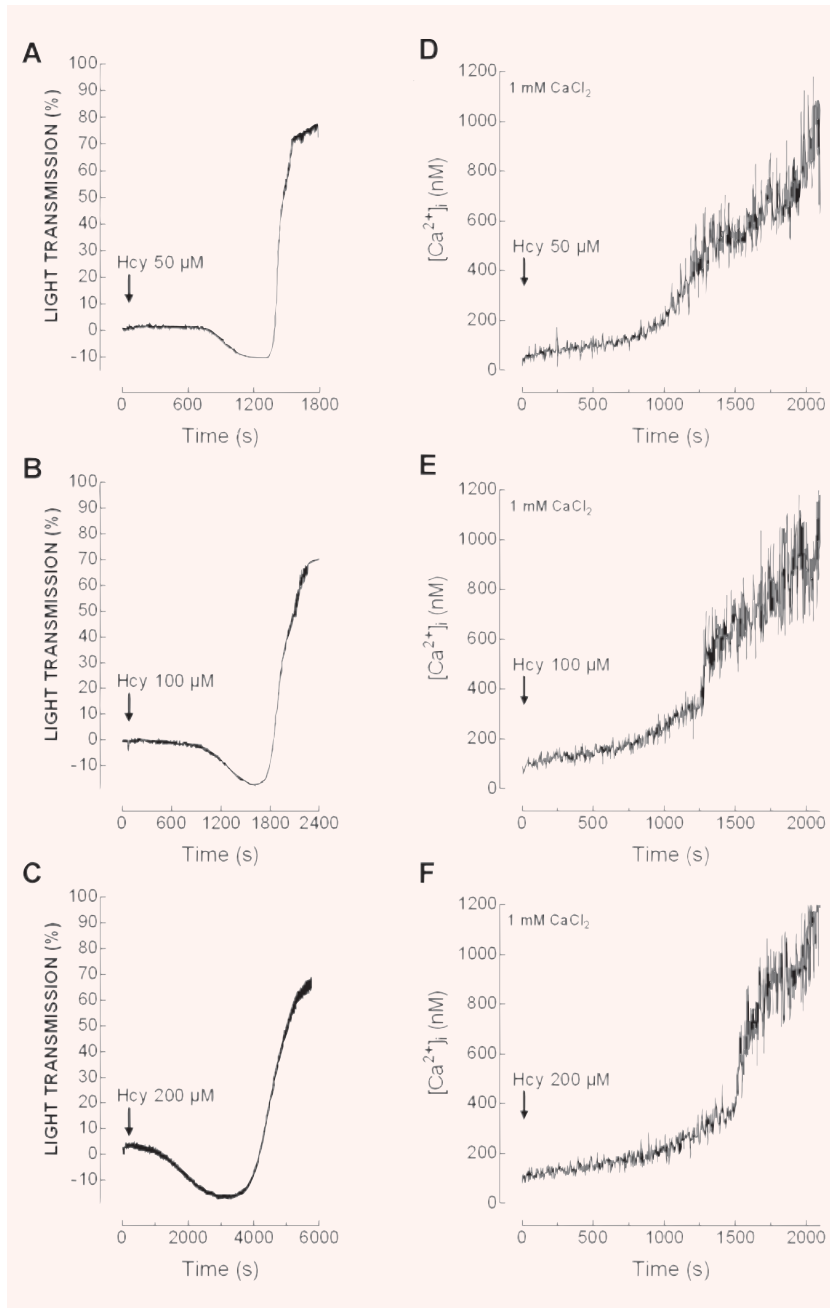
Despite we found that Hcy induces platelet aggregation and  $\text{Ca}^{2+}$  mobilization, we did not find a satisfactory correlation between Hcy concentrations and the percentage of platelet aggregation either in healthy ( $y = (0.125x + 83, R^2 = 0.909, P = 0.19)$ ) or diabetic donors ( $y = (0.091x + 89, R^2 = 0.802, P = 0.28)$ ), as well as between Hcy concentration and  $\text{Ca}^{2+}$  entry

in healthy ( $y = 2320x + 220,800, R^2 = 0.729, P = 0.34$ ) or diabetic donors ( $y = 3676x + 524500, R^2 = 0.949, P = 0.14$ ).

## 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 [33], myeloproliferative disorders [34] or diabetes mellitus [19, 32]. Oxidative stress has been shown to be involved in the development of abnormal signalling pathways leading to platelet hyperaggregability [35, 36].

Hyperhomocysteinaemia is considered an independent risk factor for vascular thrombosis; however the mechanisms underlying these alterations remains poorly understood. The role of



**Fig. 5** Homocysteine induces shape change and platelet aggregation. Human platelets from healthy donors were suspended in HBS containing 1 mM  $\text{Ca}^{2+}$  and then treated with increasing concentrations of homocysteine (Hcy; 50–200  $\mu\text{M}$ ), 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.

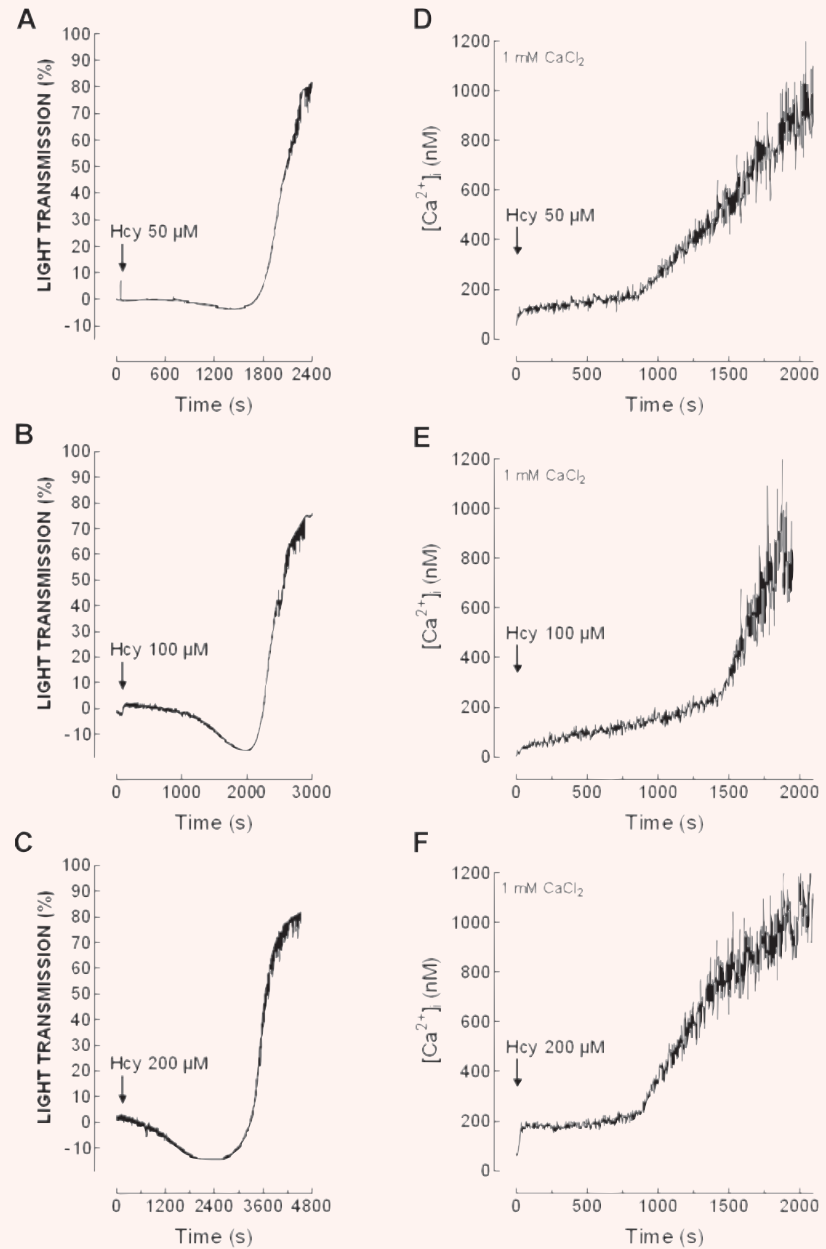
oxidative stress in hyperhomocysteinaemia-induced cardiovascular disorders is plausible since Hcy promotes oxidant injury to vascular and blood cells [13, 14, 22]. Platelets from type 2 diabetic patients are chronically exposed to high plasma levels of Hcy [21], which might be involved in the development of cardiovascular complications in these patients. Using preparations of washed platelets, we have found here that Hcy was able to induce endogenous generation of ROS that was markedly

greater in cells from diabetic patients. This fact, together with the continuous exposure to hyperhomocysteinaemia [21], 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 [36–38].

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



**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 Ca<sup>2+</sup> 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 [Ca<sup>2+</sup>]<sub>i</sub> were monitored using the 340/380 nm ratio and traces were calibrated in terms of [Ca<sup>2+</sup>]<sub>i</sub>. Traces shown are representative of 11 separate experiments.



and PLCγ2 [12, 13]. A recent study has reported that Hcy induces Ca<sup>2+</sup> release from intracellular pools in washed platelets [13], although the authors did not discriminate the identity of the Ca<sup>2+</sup> stores. Human platelets present two separate Ca<sup>2+</sup> 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 [28], has long

been identified as the DTS, and the TBHQ-sensitive store has recently been identified as an acidic organelle [29] expressing SERCA3 [26]. Here we show that Hcy releases Ca<sup>2+</sup> 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

**Table 2** Homocysteine induces platelet aggregation

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

Human platelets were suspended in a HBS containing 1 mM  $\text{Ca}^{2+}$  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  $\pm$  S.E.M. of 11 separate determinations. \* $P < 0.05$  compared to the response in platelets from healthy donors.

the physiological agonist thrombin [34]. We have found that Hcy, at concentrations between 50 and 200  $\mu\text{m}$ , impairs thrombin-evoked  $\text{Ca}^{2+}$  release and  $\text{Ca}^{2+}$  entry, and  $\text{Ca}^{2+}$  mobilization by ADP was also found to be reduced by 50  $\mu\text{m}$  Hcy. Similar findings have been reported in a human umbilical cord endothelial cell line [34]. The effect of Hcy inducing  $\text{Ca}^{2+}$  mobilization *per se* but impairing agonist-induced  $\text{Ca}^{2+}$  mobilization resembles that previously reported for  $\text{H}_2\text{O}_2$  in platelets, which plays a dual role in  $\text{Ca}^{2+}$  signalling, both stimulating  $\text{Ca}^{2+}$  mobilization by itself but also impairing agonist-evoked  $\text{Ca}^{2+}$  fluxes [24]. The inhibitory effect of  $\text{H}_2\text{O}_2$  on agonist-evoked  $\text{Ca}^{2+}$  mobilization has been attributed to the impairment of the dynamics of actin cytoskeleton polymerization–depolymerization induced by agonists [41, 42]. Actin re-organization, as well as microtubular remodelling, has been widely demonstrated to be required for the activation of store-operated  $\text{Ca}^{2+}$  entry and secretion in platelets and other non-excitable cells [43–46]. In umbilical endothelial cells, the inhibitory effect of Hcy on store-operated  $\text{Ca}^{2+}$  entry has been reported to be mediated through inhibition of actin polymerization [40], which, as for  $\text{H}_2\text{O}_2$ , might be responsible for the effect of Hcy on agonist-evoked  $\text{Ca}^{2+}$  mobilization in human platelets.

Store-operated  $\text{Ca}^{2+}$  entry is a mechanism regulated by the filling state of the intracellular  $\text{Ca}^{2+}$  stores that has been presented as a major  $\text{Ca}^{2+}$  entry mechanism involved in the activation of a number of cellular processes and is required for full platelet activation [47–49]. The results in this study show that Hcy *per se* activates  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  mobilization parallels that of platelet aggregation by Hcy. During the initial phase of  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  mobilization observed during the second phase might be mediated by direct effects of Hcy on the  $\text{Ca}^{2+}$  handling mechanisms, perhaps by ROS production [24, 50, 51], 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  $\text{Ca}^{2+}$  mobilization is involved in the activation of platelet aggregation.

No significant correlation was found between Hcy concentration and either the second phase of  $\text{Ca}^{2+}$  entry or the percentage of aggregation. Despite we have suggested that ROS production might be involved in Hcy-induced  $\text{Ca}^{2+}$  mobilization, 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  $\text{Ca}^{2+}$  mobilization as previously reported [24, 52], thus preventing a satisfactory correlation between Hcy concentrations and  $\text{Ca}^{2+}$  mobilization.

In summary, our results demonstrate that Hcy induces endogenous ROS generation in human platelets, which might lead to  $\text{Ca}^{2+}$  mobilization, as previously reported [50, 51], and, subsequently, to platelet aggregation. All the parameters tested in the present study, ROS generation,  $\text{Ca}^{2+}$  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 [21, 53], which has been associated with the development of atherosclerosis, thrombosis and other cardiovascular disorders [5, 9, 54], 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.

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