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Alterations in platelet Ca^{2+} signalling in diabetic patients is due to increased formation of superoxide anions and reduced nitric oxide production

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Summary Increased aggregation of platelets might contribute to the development of vascular complication in diabetes mellitus. In this study release of superoxide anions, intracellular Ca^{2+} signalling and nitric oxide formation stimulated by the receptor-dependent agonist adenosine 5′-diphosphate (ADP) and the receptor-independent stimulus thapsigargin, were compared in platelets isolated from patients with Type II (non-insulin-dependent) diabetes mellitus and healthy control subjects. Diabetes augmented intracellular Ca^{2+} release and Ca^{2+} entry to ADP by 40 and 44 % (control subjects: $n = 11$; diabetic: $n = 6$), while the median effective concentration (EC_{50}) of ADP to initiate Ca^{2+} signalling was similar in both groups. The effect of thapsigargin on Ca^{2+} concentration was increased by 69 % in diabetic patients (control subjects: $n = 22$; diabetic patients: $n = 9$). In addition, release of superoxide anions was 70 % greater in diabetic patients (control subjects: $n = 9$; diabetic patients: $n = 6$). Treatment of platelets from control subjects with the superoxide anion-generating mixture xanthine oxidase and hypoxanthine or buthioninesulphoximine (BSO) mimicked the effect of diabetes on platelet Ca^{2+} signalling. The antioxidant glutathione normalized enhanced Ca^{2+} response in the diabetic group (control subjects: $n = 5$; diabetic patients: $n = 6$). Basal and thapsigargin-evoked nitric oxide synthase activity was reduced in the diabetic group by 85 and 64 %, respectively (control subjects: $n = 13$; diabetic subjects: $n = 13$). The nitric oxide-donor 2-(N,N-diethylamino)-diazenolate-2-oxide sodium (DEA/NO) normalized enhanced Ca^{2+} signalling in platelets preincubated with xanthine oxidase and hypoxanthine ($n = 12$) and in those from diabetics (control subjects: $n = 6$; diabetic patients: $n = 6$). Inhibition of nitric oxide synthase by N-nitro-L-arginine (L-NA) augmented thapsigargin-induced Ca^{2+} signalling by 51 % ($n = 8$). These data indicate that in diabetes platelet Ca^{2+} signalling might be enhanced by excessive superoxide production and an attenuated negative direct or indirect feedback control by nitric oxide, due to its reduced production. [Diabetologia (1999) 42: 167–176]

Keywords Platelet, Ca^{2+} signalling, nitric oxide, superoxide anions, ADP, thapsigargin.

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Abbreviations: ADP, Adenosine 5′-diphosphate; BSO, buthioninesulphoximine; $[Ca^{2+}]$; free intracellular Ca^{2+} concentration; DEA/NO, 2-(N,N-diethylamino)-diazenolate-2-oxide sodium; EDTA, ethylenediamine-tetraacetic acid; EGTA, ethylene glycol-bis-(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid; L-NA, ^{w}N-nitro-L-arginine; HEPES, N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]; SOD, superoxide dismutase; Tris, tris(hydroxymethyl)aminomethane; CR, column recovery; IP_3, inositol 1,4,5-triphosphate; (C*), total [3H]-L-citrulline synthesis.

Diabetes mellitus is associated with an increased risk of thrombosis, circulatory dysfunction and atherosclerosis. Attenuated endothelium-dependent relaxation [1], altered smooth muscle contractility [2] and enhanced reagibility of platelets [3, 4, 5] are thought to constitute the main promoters for vascular complications and atherogenesis in diabetes mellitus. Although increased aggregation of platelets isolated from diabetic patients to autacoids, such as ADP or thrombin, has been clearly shown [6], the molecular mechanism of this hyperreactivity of the thrombocytes is still not clear.

Interaction of autacoids with their specific receptors on the platelets' cell membrane results in activa-

tion of phospholipase C-mediated synthesis of diacylglycerol and inositol 1,4,5-trisphosphate (IP_3). The latter evokes intracellular Ca^{2+} release from the endoplasmic reticulum Ca^{2+} stores [7]. In addition, the depletion of intracellular Ca^{2+} stores initiates activation of a so called capacitative Ca^{2+} entry [7, 8]. Both, intracellular Ca^{2+} release and capacitative Ca^{2+} entry increase free intracellular Ca^{2+} concentration [Ca^{2+}]; and induce platelet aggregation by stimulation of the thromboxane A_2 biosynthesis [9]. In platelets from diabetic patients, increased intracellular Ca^{2+} response was reported [10]. So far no detailed analysis of diabetes-mediated changes in platelet Ca^{2+} signalling was conducted and the biochemical mechanisms of this increased platelet Ca^{2+} mobilization are not clear. On the other hand, an increase in [Ca^{2+}]; might favor the biosynthesis of nitric oxide by the Ca^{2+}-sensitive, constitutive platelet nitric oxide synthase [11]. Nitric oxide has been shown to increase platelet cGMP concentrations by stimulation of soluble guanylyl cyclase [12, 13] resulting in several intracellular reactions such as activation of protein kinases and lowering of basal intracellular Ca^{2+} [14] and in an attenuation of platelet aggregation [15]. Remarkably, basal nitric oxide synthase activity in homogenates of platelets obtained from diabetic patients is reduced [16] while differences in the stimulation of nitric oxide synthase activity upon autacoid stimulation has not been investigated so far. Besides its effect on the platelet signalling pathways mentioned above, diabetes is associated with increased hydrogen peroxide production in platelets from diabetic patients [17, 18]. Recently, increased production of superoxide anions has been described to contribute to changes in endothelial function under hyperglycaemic conditions [19, 20]. So far limited information is provided about increasing formation of superoxide anions in platelets in diabetes and, if so, to which extent the increased production of superoxide anions contributes to the observed changes of platelet functions.

Thus, there are many studies reporting diabetes-induced changes in platelet function but the intracellular mechanisms and the role of reactive oxygen species in the reported changes of platelet function in diabetes are still unclear. The aim of our study was to assess the effect of diabetes on platelet signal transduction in more detail and to correlate the observed alterations with increased free radical formation. To investigate the effect of diabetes on receptor-mediated Ca^{2+} signalling, the effects of the receptor-dependent agonist ADP were tested. In addition, the effect of diabetes on platelet capacitative Ca^{2+} entry was exclusively tested using the Ca^{2+} ATPase inhibitor which activates Ca^{2+} entry by Ca^{2+} store depletion independently of any receptors or second messengers. We also investigated the effect of glutathione, a powerful antioxidant on diabetes-induced changes of platelet Ca^{2+} signalling to verify whether prevention of free radical release might have an impact on diabetes-modified signal transduction in platelets.

Table 1. Clinical characteristics of the two groups of subjects

	Control subjects (n = 33)	Diabetic subjects (n = 30)
Sex (men/women)	18/15	18/12
Age (years)	48.4 ± 13.9	51.3 ± 9.9
BMI (kg/m^2)	24.8 ± 4.5	28.7 ± 6.4
Duration of diabetes (years)	–	6 ± 2
HbA_{1c} (%)	5.4 ± 0.4	8.3 ± 1.3*
D-glucose (mmol/l)	5.2 ± 0.7	9.9 ± 2.4*
Cholesterol (mmol/l)	6.6 ± 1.5	6.1 ± 0.9
LDL-cholesterol (mmol/l)	4.2 ± 1.1	3.8 ± 0.8
HDL-cholesterol (mmol/l)	1.6 ± 0.4	1.4 ± 0.4
Triglycerides (mmol/l)	3.1 ± 4.7	2.5 ± 1.5
Blood pressure		
systolic (mm Hg)	131 ± 4	135 ± 3
diastolic (mm Hg)	78 ± 3	81 ± 4
Retinopathy	–	3
Microalbuminuria	–	4
Diabetes therapy		
Diet alone	–	12
Insulin	–	18

Data are means ± SD
Statistical significant differences in parameters between the groups are marked with "*" and indicate higher values in the patient group evaluated by one-way ANOVA following a post hoc analysis with Scheffe's F-test ($p < 0.05$)

Materials and methods

Subjects. In this study, cell activity to autacoid stimulation was assessed in platelets isolated from 33 healthy subjects and 30 Type II (non-insulin-dependent) diabetic patients recruited from a diabetes outpatient clinic. Type II diabetes was diagnosed in all patients according the clinical NDDG (national diabetes data group) criteria [21]. All subjects entered the study voluntarily and gave informed consent. To be eligible, diabetic patients had to be normotensive and free of clinical overt macrovascular disease. Retinopathy exceeding microaneurysms (severity level 21) as well as macroalbuminuria were also exclusion criteria. Antihyperglycaemic treatment in the diabetic group was either by diet alone or by premixed insulin twice daily and patients did not receive any other long-term drug treatment. As shown in Table 1, groups differed only in terms of blood D-glucose concentration and HbA_{1c}. Blood was collected from all subjects in the morning after an overnight fast. Clinical laboratory parameters were measured by routine laboratory methods.

Materials. Fura-2 acetoxymethylester was purchased from Lambda Fluorescence Technologies (Graz, Austria). 2-(N,N-diethylamino)-diazenolate-2-oxide sodium (DEA/NO) was purchased from Alexis (Läufelfingen, Switzerland). Buffer salts were obtained from Merck (Darmstadt, Germany). The

radioactive compounds were bought from New England Nuclear (Vienna, Austria). All other materials were supplied by Sigma Chemicals (Vienna, Austria).

Platelet isolation. Blood was collected after an overnight fast from healthy control subjects and patients with Type II diabetes. A complete blood profile was done for each subject (Table 1). Isolation of platelets was carried out according to Hashimoto et al. [22]. Briefly, the blood sample (20 ml) was mixed with 2.2 ml of an anticoagulant solution containing in mmol/: 85 trisodium citrate, 111 D-glucose, 71 citric acid, pH 4.5. After the cells were centrifuged at $200 \times g$ for 30 min at room temperature (i.e. 22 °C) the upper phase (i.e. platelet rich plasma) was transferred into another 15 ml tube and centrifuged with $600 \times g$ for 5 min. The platelet pellet was washed twice with buffer B containing in mmol/l: 134 NaCl, 5 D-glucose, 1 ethylenediamine-tetraacetic (EDTA) and 15 Tris (pH 6.3). The platelets were resuspended in buffer A (in mmol/l: 129 NaCl, 2.8 KCl, 0.8 KH_2PO_4, 8.9 $NaHCO_3$, 0.8 $MgCl_2$, 5.6 D-glucose, 10 Hepes, pH 7.4). Density of platelet suspension was determined by cell counting with pulse area analysis (resistance measurement) using Casy-1 (Schaerfe, Reutlingen, Germany). Cell density was adjusted at $1.3–1.7 \times 10^8$ platelets/ml by an adequate addition of buffer A.

Ca^{2+} measurements. Intracellular Ca^{2+} concentration was measured using conventional fluorescence spectroscopy with fura-2 acetoxymethylester as described previously [23]. Briefly, the platelets suspended in buffer A were incubated for 45 min at room temperature with 2 mmol/l fura-2 acetoxymethylester, washed twice and resuspended in buffer A. Intracellular free Ca^{2+} concentration was monitored in a stirred spectrofluorometer by measuring fluorescence at 340 and 380 nm excitation and 510 nm emission. Due to the reported errors in the calibration of the intracellular Ca^{2+} concentration, free intracellular Ca^{2+} concentration is expressed as ratio units of 340/380 nm excitation and 510 nm emission. To demonstrate the accuracy of the presentation of intracellular Ca^{2+} concentration as ratio units (F_{340}/F_{380}), Ca^{2+} signalling was regularly calibrated using the technique as described by Grynkiewicz et al. [24].

Measurement of platelet nitric oxide synthase activity. Activity of platelet nitric oxide synthase was determined by measuring the conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline similar to the procedure described previously in endothelial cells [19]. To 990 µl platelet suspension ($1.3–1.7 \times 10^8$/µl) containing 2×10^6 dpm [^{3}H]-L-arginine in buffer A, 10 ml buffer A (i.e. control) or 10 µl agonist (i.e. ADP, thapsigargin) were added. To verify nitric oxide synthase-dependent conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline, experiments were carried out in the presence of 1 mmol/l ^{w}N-nitro-L-arginine (L-NA). Conversion of [^{3}H]-L-arginine to [^{3}H]-L- citrulline in the presence of L-NA did not exceed 5 % of basal conversion in the absence of L-NA ($A_{L\text{-}NA}$), while in the presence of L-NA we did not detect any stimulatory effect of thapsigargin/ADP on the conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline. After 15 min incubation under constant stirring at room temperature, the reaction was stopped by addition of 1 ml chilled buffer A, washed and centrifuged with $900 \times g$ for 5 min at 4 °C. The platelet pellet was lysed with 1.00 ml 0.01 mol/l HCl. After 30 min at 4 °C, radioactivity of a 100 µl aliquot was determined using liquid scintillation counting for calculation of the incorporated radioactivity (R*) during experimental conditions (R* = dpm × 10). To the remaining sample, 100 µl of a 200 mmol/l sodium acetate buffer (pH 13.0) containing 10 mmol/l L-citrulline was added. To separate the amino acids, 900 µl of this solution (final pH 5.0–5.2) was applied to ion exchange resin columns (7 ml; AG 50W-X8, 400 mesh, H^+ form). The [^{3}H]-L-citrulline was eluted with 1 ml water and radioactivity was measured. From each column, column recovery (CR) was measured by applying known amounts of [^{14}C]-L-citrulline to each column and measuring the recovery in the eluate within 1 ml water (CR = dpm applied/dpm found). Total [^{3}H]-L-citrulline synthesis (C*) was calculated using dilution correction (100/81) and CR according to the following equation: C* = (dpm measured × CR) × 100/81. Activity of platelet nitric oxide synthase activity (A_{NOS}) was calculated as the percentage of [^{3}H]-L-citrulline radioactivity of the incorporated radioactivity subtracted by the L-NA-resistant [^{3}H]-L-citrulline [$A_{NOS} = (C^* \times 100/R^*)\text{-}A_{L\text{-}NA}$] and is expressed as fold increase ($A_{NOS/stimulated}/A_{NOS/basal}$) in the conversion of incorporated [^{3}H]-L-arginine to [^{3}H]-L-citrulline to the addition of the stimulants.

Determination of superoxide anion release. Release of superoxide anions was determined photometrically as the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome c as previously described [19, 20]. Briefly, platelets were adjusted in buffer A to 10^8 cells/ml in the absence or presence of 200 U/ml SOD and transferred into a stirred 6-fold holder (3 for each with and without SOD) in a photometer, Hitachi U-2000 (Inula, Vienna, Austria). After addition of 10 mmol/l ferricytochrome c release of superoxide anions were monitored at 550 nm for 60 min. The difference of extinction between samples in the absence and presence of SOD indicate superoxide anion-mediated reduction of ferricytochrome c. Concentration of superoxide anions was calculated using the molar extinction coefficient of reduced form of ferricytochrome c (e = 21.000; [25]).

Experimental generation of superoxide anions. For the exposure of platelets to experimentally produced superoxide anions, platelets were treated for 1 h in buffer A containing 300 µU/ml xanthine oxidase and 1 mmol/l hypoxanthine at room temperature as described previously [26].

Statistical analysis. Experiments for each subject were done in triplicate and given "*n*" values representing the number of patients or healthy control subjects of this study. Data points represent the means ± SEM. The EC_{50} values are expressed as the mean with 95 % confidential intervals in parenthesis. Analysis of variance (ANOVA) was used for data evaluation and statistical significance of differences between means was estimated by Scheffe's *F*-test. The level of significance was defined as $p < 0.05$.

Results

Comparison of cell size and volume of platelets. Distribution of cell size, mean diameter and cell volume of the platelets was measured in diabetic patients and healthy control subjects. There was no difference in cell size (control subjects: 2.77 ± 0.23 µm, $n = 10$; diabetic patients: 2.78 ± 0.22 µm, $n = 10$) and cell volume (control subjects: 13.86 ± 0.70 fl, $n = 10$; diabetic patients: 13.49 ± 0.81 fl, $n = 10$) between both groups.

Diabetes-related differences in platelet Ca^{2+} signalling to autacoid stimulation. Intracellular Ca^{2+} response to ADP stimulation was investigated in fura-2 loaded

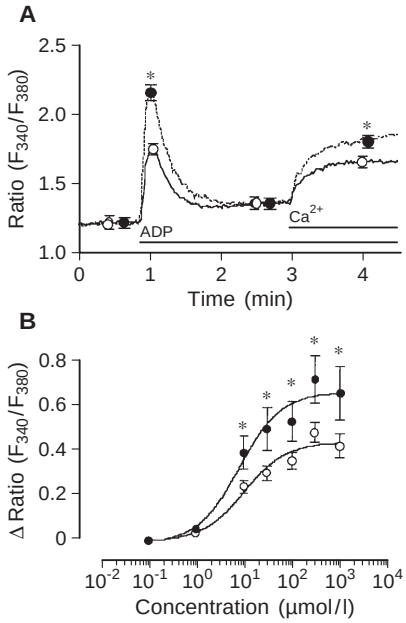

Fig. 1 A, B. Diabetes mellitus induces changes in ADP-evoked Ca^{2+} signalling in platelets. Freshly isolated washed platelets were loaded with fura-2/am for 45 min as described under Methods. After loading procedure cells were washed twice and resuspended in a concentration of 10^8/ml in buffer A. **A** Representative tracings of the platelet Ca^{2+} signalling evoked by 100 μmol/l ADP in the nominal absence of extracellular Ca^{2+} followed by the addition of 2.5 mmol/l Ca^{2+}. Points indicate the means ± SEM (control subjects: ○, $n = 11$; diabetic subjects: ●, $n = 6$). *$p < 0.05$ vs effect in platelets from healthy control subjects. **B** Intracellular rises in free Ca^{2+} concentration in the nominal absence of extracellular Ca^{2+} concentration (i.e. intracellular Ca^{2+} release) to the ADP concentrations indicated. Identical results were obtained for the increases in the intracellular Ca^{2+} concentration to the addition of extracellular Ca^{2+} to ADP stimulated cells. Data represent the means ± SEM (control subjects: ○, $n = 11$; diabetic subjects: ●, $n = 6$). *$p < 0.05$ vs effect in platelets from healthy control subjects

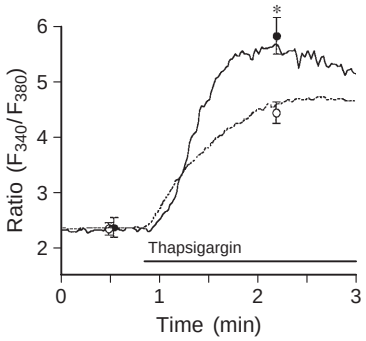

Fig. 2. Diabetes mellitus induces changes in thapsigargin-evoked Ca^{2+} signalling in platelets. Representative tracings of the platelet Ca^{2+} signalling evoked by 1 μmol/l thapsigargin in the presence of 2.5 mmol/l extracellular Ca^{2+} in freshly isolated washed platelets were loaded with fura-2/am for 45 min. Points indicate the means ± SEM (control subjects: ○, $n = 22$; diabetic subjects: ●, $n = 9$). *$p < 0.05$ vs effect in platelets from healthy control subjects

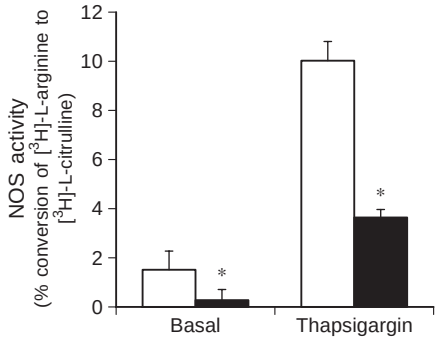

Fig. 3. Diabetes mellitus effects nitric oxide synthase activity in human platelets. In freshly isolated and washed platelets from diabetic and healthy subjects, activity of nitric oxide synthase under basal condition and during stimulation with 1 μmol/l thapsigargin was measured. Each column represents the mean ± SEM (control subjects: open columns, $n = 13$; diabetic patients: filled columns, $n = 13$). *$p < 0.05$ vs effect in platelets from healthy control subjects

platelets isolated from diabetic and healthy subjects. Platelets were stimulated in nominal Ca^{2+} free solution (no Ca^{2+} added) with the ADP concentration indicated, followed by the addition of 2.5 mmol/l Ca^{2+}. The intracellular Ca^{2+} release upon 100 μmol/l ADP was increased by 44 % in platelets isolated from diabetic patients compared with that in platelets from control subjects (Fig. 1A; control subjects: $n = 11$; diabetic patients: $n = 6$). Hence, the increase of intracellular Ca^{2+} concentration to the addition of 2.5 mmol/l Ca^{2+} to ADP stimulated cells was 40 % greater than in control platelets (Fig. 1A). Figure 1B summarizes the effect of various ADP concentrations on intracellular Ca^{2+} concentration in platelets from diabetic and control subjects (control: $n = 11$; diabetic: $n = 6$). While increases in intracellular Ca^{2+} concentration in response to ADP higher than 10 μmol/l were greater in platelets of diabetic patients compared with those measured in platelets from healthy subjects, EC_{50} values for ADP remained unchanged in both groups (control group: 10.32 (5.3–20.1) μmol/l; diabetic group: 8.02 (2.7–23.9) μmol/l/).

In addition to the enhanced Ca^{2+} signalling in response to the IP_3-generating autacoid ADP, increases in free intracellular Ca^{2+} concentration to the ATPase inhibitor thapsigargin were also found to be enhanced by 67 % in platelets derived from diabetic patients compared with those obtained in platelets from the control group (Fig. 2; control subjects: $n = 22$; diabetic group: $n = 9$).

Nitric oxide synthase activity. To elucidate platelet nitric oxide synthase activity the conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline was monitored in platelets isolated from diabetic and non-diabetic subjects. Nitric oxide synthase activity was measured under

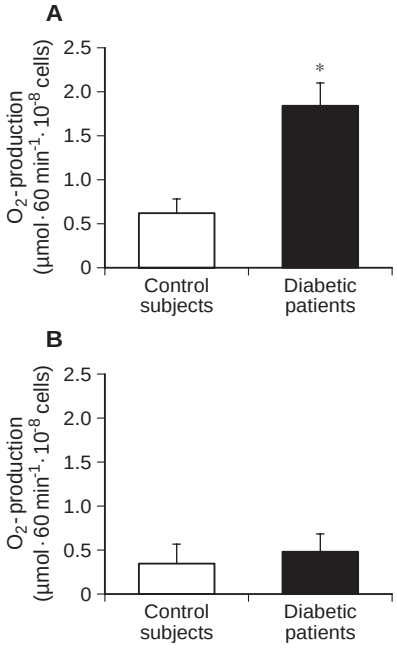

Fig. 4 A, B. Diabetes mellitus increases release of superoxide anions from human platelets. Release of superoxide anions from freshly isolated washed platelets was measured over 1 h by the SOD-sensitive reduction of ferricytochrome c photometrically monitored at 550 nm as described under Methods. **A** Release of superoxide anions in platelets freshly isolated from control and diabetic subjects. Each column represents the means ± SEM (control subjects: open columns, $n = 9$; diabetic subjects: filled columns, $n = 9$). $^*p < 0.05$ vs effect in platelets from healthy control subjects. **B** Release of superoxide anions after an incubation with 10 mmol/l glutathione at room temperature for 45 min. Each column represents the means ± SEM (control subjects: open columns, $n = 5$; patients diabetic: filled columns, $n = 6$)

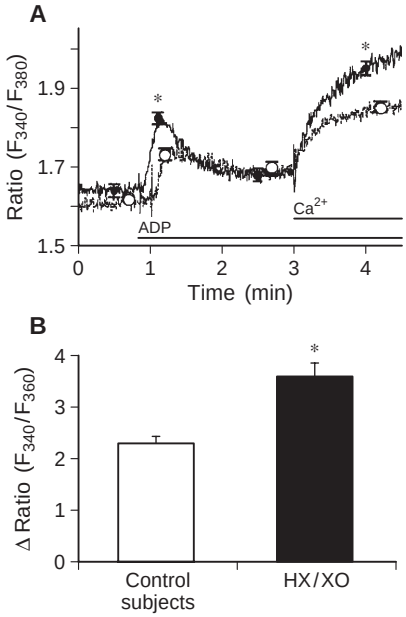

Fig. 5 A, B. Treatment of platelets with the superoxide anion generating mixture xanthine oxidase/hypoxanthine mimicked the effect of diabetes on platelet Ca^{2+} signalling induced by ADP (**A**) and thapsigargin (**B**). Freshly isolated washed platelets from healthy persons were incubated in buffer A for 1 h without (○, open columns) and with 300 μU/ml xanthine oxidase in the presence of 1 mmol/l hypoxanthine (●, filled columns). **A** As bars indicate platelets were stimulated in the nominal absence of extracellular Ca^{2+} with 100 μmol/l ADP followed by the addition of 2.5 mmol/l Ca^{2+}. Points represent the means ± SEM. $^*p < 0.05$ vs the effect in non-treated platelets ($n = 5$ for each group). **B** Platelets were stimulated in the presence of 2.5 mmol/l extracellular Ca^{2+} with 1 μmol/l thapsigargin for 3 min. Columns represent the maximum increase in intracellular Ca^{2+} concentration in response to thapsigargin. $^*p < 0.05$ vs the effect in non-treated platelets ($n = 9$ for each group)

basal conditions and on stimulation with autacoids. In platelets from diabetic patients, basal nitric oxide synthase activity was 15% of that measured in the control subjects (Fig. 3; control: $n = 13$; diabetic $n = 13$). In addition, the effect of 1 μmol/l thapsigargin nitric oxide synthase activity was reduced by 64% in platelets from diabetic patients compared with that measured in platelets of non-diabetic subjects (Fig. 3; control: $n = 13$; diabetic: $n = 13$).

In contrast to the large difference in nitric oxide synthase activity in both basal and stimulated conditions, there was no difference in the uptake of the nitric oxide synthase substrate L-arginine between platelets isolated from diabetic patients or healthy control subjects (control: 15.79 ± 1.81 dpm $\times 10^3/10^8$ cells $\times$ 15 min, $n = 13$; diabetic: 19.6 ± 0.99 dpm $\times 10^3/10^8$ cells $\times$ 15 min, $n = 13$, NS compared with control subjects, control: $n = 13$; diabetic: $n = 13$).

Superoxide anion release. To verify whether under diabetic conditions, increased superoxide anion formation occurs in human platelets, the release of superoxide anions from platelets donated from diabetic and healthy subjects was compared. In platelets from diabetic subjects, release of superoxide anions was increased by 70% compared with that measured in platelets from healthy subjects (Fig. 4A; control: $n = 9$; diabetic: $n = 9$). Incubation of platelets with 10 mmol/l glutathione for 45 min at room temperature strongly reduced superoxide anion release in both groups, while no further difference between platelets derived from diabetic and non-diabetic subjects was found (Fig. 4B; control: $n = 5$; diabetic: $n = 6$).

Effect of superoxide production on platelet Ca^{2+} signalling. To elucidate the contribution of the increased superoxide anion production and release found in platelets from diabetic patients to the observed hyperreactivity of the Ca^{2+} signalling to agonists, platelets isolated from healthy subjects were incubated for 60 min in buffer A containing 300 μU/ml xanthine oxidase and 1 mmol/l hypoxanthine. Treatment of platelets with the superoxide anion-generating sys-

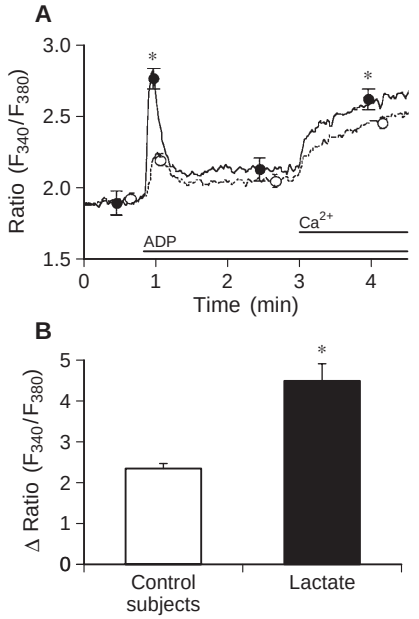

Fig. 6 A, B. Treatment of platelets with L-lactate mimicked the effect of diabetes on platelet Ca^{2+} signalling induced by ADP (**A**) and thapsigargin (**B**). Freshly isolated washed platelets from healthy individuals were incubated in buffer A for 1 h without (○, open columns) and with 10 mmol/l L-lactate (●, filled columns). **A** As bars indicate platelets were stimulated in the nominal absence of extracellular Ca^{2+} with 100 μmol/l ADP followed by the addition of 2.5 mmol/l Ca^{2+}. Points represent the mean ± SEM. *$p < 0.05$ vs the effect in non-treated platelets (n = 5 for each group). **B** Platelets were stimulated in the presence of 2.5 mmol/l extracellular Ca^{2+} with 1 μmol/l thapsigargin for 3 min. Columns represent the maximum increase in intracellular Ca^{2+} concentration in response to thapsigargin. *$p < 0.05$ vs the effect in non-treated platelets ($n = 7$ for each group)

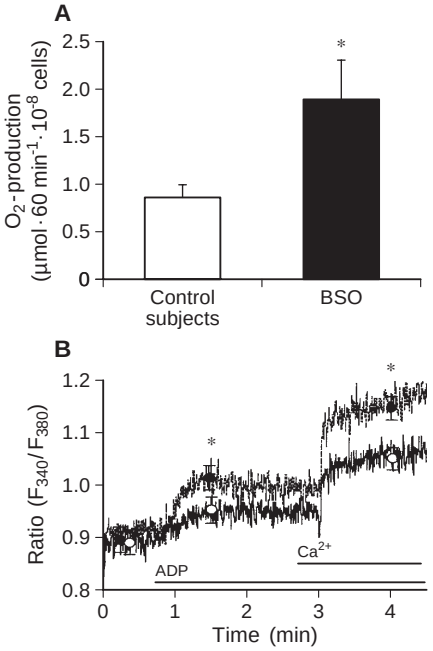

Fig. 7 A, B. Effect of BSO on O_2^- release (**A**) and ADP-induced Ca^{2+} signalling (**B**) in platelets. Freshly isolated washed platelets from healthy subjects were incubated for 2 h in buffer A without or with 5 mmol/l BSO. **A** Release of superoxide anions from isolated washed platelets incubated without (open columns, $n = 6$) or with BSO (filled columns, $n = 6$). *$p < 0.05$ vs control subject. **B** As bars indicate platelets which were treated in buffer A without (○, $n = 17$) or with BSO (●, $n = 18$) were stimulated in the nominal absence of extracellular Ca^{2+} with 100 μmol/l ADP followed by the addition of 2.5 mmol/l Ca^{2+}. Points represent the mean ± SEM. *$p < 0.05$ vs the effect in non-treated platelets

tem increased ADP-induced Ca^{2+} response by 55 and 73 % (intracellular Ca^{2+} release and Ca^{2+} entry, respectively (Fig. 5A; $n = 5$)). In agreement with these findings treatment of platelets with 300 μU/ml xanthine oxidase in the presence of 1 mmol/l hypoxanthine increased thapsigargin induced elevation of intracellular Ca^{2+} concentration in Ca^{2+} containing solution by 55 % (Fig. 5B; $n = 9$).

Preincubation of platelets isolated from healthy subjects with 10 mmol/l L-lactate for 2 h at room temperature mimicked the effect of xanthine oxidase and diabetes on platelet Ca^{2+} signalling (Fig. 6A; $n = 6$). Like the effect of ADP, thapsigargin-evoked (1 μmol/l) increase in free intracellular Ca^{2+} concentration in the presence of 2.5 mmol/l extracellular Ca^{2+} was increased by 90 % in cells pretreated with 10 mmol/l L-lactate (Fig. 6B; $n = 7$).

Lowering glutathione content on the platelets by incubation with 5 mmol/l buthioninesulphoximine (BSO) for 2 h increased the release of superoxide anions from platelets by 137 % (Fig. 7A; $n = 6$) and mimicked the effect of diabetes on platelet Ca^{2+} signalling in response to 100 μmol/l ADP (Fig. 7B; $n = 6$).

Effect of glutathione on diabetes-induced changes in platelet Ca^{2+} signalling. Preincubation of platelets from diabetic patients with 10 mmol/l glutathione for 1 h before the experiment normalized platelet Ca^{2+} signalling to ADP, while treatment of platelets of healthy subjects with glutathione did not influence the ADP-induced Ca^{2+} signalling (Fig. 8A; control subjects: $n = 6$; diabetic subjects: $n = 6$). Figure 8B summarizes the effect of the pretreatment with glutathione on the effectiveness of various ADP concentrations to initiate changes in intracellular Ca^{2+} concentration in platelets from diabetic and healthy persons. The glutathione treatment normalized Ca^{2+} signalling in platelets from diabetic patients in all concentrations, while EC_{50} values for ADP remained unchanged in both groups after treatment with glutathione (control subjects: 3.3 (0.8–14.0) μmol/l; diabetic subjects: 3.8 (1.0–14.7) μmol/l). In agreement with these findings glutathione normalized enhanced thapsigargin-induced Ca^{2+} increases in platelets isolated from diabetic persons (Fig. 8C; control: $n = 10$; diabetic: $n = 10$).

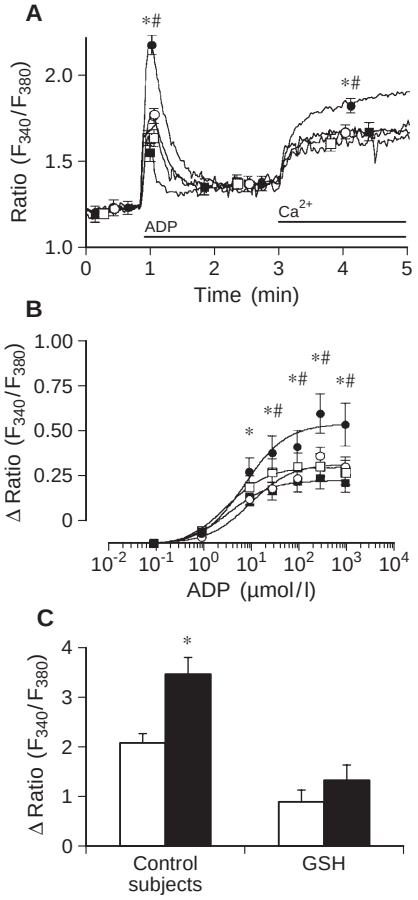

Fig. 8 A–C. Glutathione normalized autacoid-induced Ca^{2+} signalling in platelets isolated from diabetic patients. Freshly isolated washed platelets from healthy subjects (○, □) diabetic patients (●, ■) were preincubated for 45 min in the absence (○, ●) or presence (□, ■) of 10 mmol/l glutathione at room temperature. **A** Representative tracings of the effect of 100 μmol/l ADP on Ca^{2+} concentration in platelets isolated from diabetic and non-diabetic subjects. As bars indicate cells were stimulated in the absence of extracellular Ca^{2+} with 100 μmol/l ADP followed by the addition of 2.5 mmol/l Ca^{2+}. Points indicate the means ± SEM. $*p < 0.05$ vs non-diabetic control subjects and $^{\#}p < 0.05$ vs the response without treatment with glutathione (control subjects: $n = 5$; diabetic subjects: $n = 6$). **B** Intracellular rises in free Ca^{2+} concentration in the nominal absence of extracellular Ca^{2+} concentration to the ADP concentrations indicated in platelets from control subjects and diabetic patients without and with an incubation for 45 min with 10 mmol/l glutathione at room temperature. $*p < 0.05$ vs non-diabetic control subjects and $^{\#}p < 0.05$ vs the response without treatment with glutathione (control subjects: $n = 5$; diabetic patients: $n = 6$). **C** Platelets were stimulated in the presence of 2.5 mmol/l extracellular Ca^{2+} with 1 μmol/l thapsigargin for 3 min. Columns represent the maximal increase in intracellular Ca^{2+} concentration in response to thapsigargin in platelets from control subjects (open columns) and diabetic patients (filled columns). $*p < 0.05$ vs control subjects ($n = 10$ for each group)

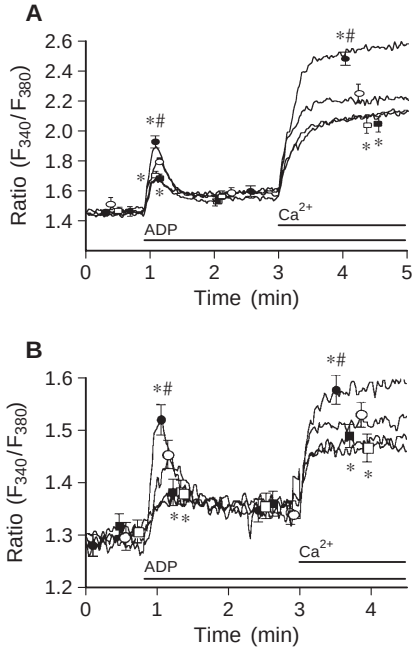

Fig. 9 A, B. Effect of nitric oxide on ADP-induced Ca^{2+} signalling in platelets without and with preincubation with xanthine oxidase/hypoxanthine (**A**) and on platelets isolated from control and diabetic patients (**B**). **A** Freshly isolated washed platelets from healthy individuals were incubated for 1 h in buffer A without (○, □) and with (●, ■) 300 μU/ml xanthine oxidase and 1 mmol/l hypoxanthine. Before the experiments, cells were washed twice and incubated for 5 min without (○, ●) or with (□, ■) 1 μmol/l DEA/NO, washed and resuspended in nominal Ca^{2+} free buffer A. As bars indicate platelets were stimulated in the nominal absence of extracellular Ca^{2+} with 100 μmol/l ADP followed by the addition of 2.5 mmol/l Ca^{2+}. Points represent the mean ± SEM ($n = 12$ each group). $*p < 0.05$ vs control subjects and $^{\#}p < 0.05$ vs HX/XO + NO. **B** Freshly isolated platelets from control subjects (○, □) and diabetic subjects (●, ■) were incubated in buffer A for 5 min without (○, ●) or with (□, ■) 1 μmol/l DEA/NO, washed and resuspended in nominal Ca^{2+} free buffer A. As bars indicate platelets were stimulated in the nominal absence of extracellular Ca^{2+} with 100 μmol/l ADP followed by the addition of 2.5 mmol/l Ca^{2+}. Points represent the means ± SEM ($n = 6$ each group). $*p < 0.05$ vs control subjects and $^{\#}p < 0.05$ vs diabetic group

Effect of nitric oxide on superoxide anion-evoked changes in platelet Ca^{2+} signalling. Preincubation of platelets with the nitric oxide donor DEA/NO (1 μmol/l; [27]) for 5 min before stimulation with 100 μmol/l ADP diminished intracellular Ca^{2+} release and capacitative Ca^{2+} entry by about 20 and 28 %, respectively (Fig. 9A). Moreover, a 5 min incubation period with the NO donor DEA/NO (1 μmol/l) normalized ADP-induced Ca^{2+} signalling in platelets pretreated for 1 h with 300 μU/ml xanthine oxidase in the presence of 1 mmol/l hypoxanthine (Fig. 9A; $n = 12$). Also a 5 min incubation with the NO donor DEA/NO (1 μmol/l) normalized ADP-induced Ca^{2+} signalling in platelets isolated from diabetic patients (Fig. 9B; $n = 6$).

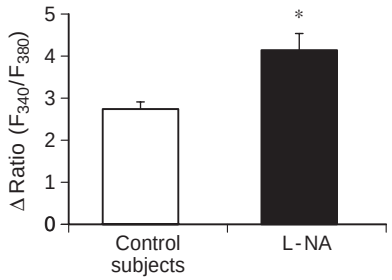

Fig. 10. Effect of an inhibition of nitric oxide synthase with L-NA on thapsigargin-induced platelet Ca^{2+} signalling. In the presence of 2.5 mmol/l extracellular Ca^{2+} freshly isolated washed platelets from healthy subjects were stimulated with 1 μmol/l thapsigargin for 3 min in the absence (open column, $n = 8$) or presence of 1 mmol/l L-NA (filled column, $n = 8$). Columns represent the maximum increase in intracellular Ca^{2+} concentration in response to thapsigargin. $*p < 0.05$ vs control subjects

Inhibition of platelet nitric oxide synthase by 1 mmol/l L-NA augmented platelet Ca^{2+} signalling in response to 1 μmol/l thapsigargin (Fig. 10; control: $n = 8$ in each group), while the effect of ADP (100 μmol/l) was not changed in the presence of L-NA (data not shown).

Discussion

We studied diabetes-induced changes in autacoid-induced Ca^{2+} signalling and nitric oxide biosynthesis in human platelets. In addition release of superoxide anions from platelets isolated from diabetic patients was compared with that measured in platelets from healthy subjects. We provided evidence that in diabetes increased superoxide anion production in platelets occurs, resulting in an increase in agonist-induced intracellular Ca^{2+} signalling. Moreover, basal and thapsigargin-stimulated nitric oxide synthase was greatly reduced in platelets from diabetic patients. This might result in an attenuation of the nitric oxide-mediated direct or indirect negative feedback for Ca^{2+} signalling. Our data suggest that platelet hyperreactivity in diabetes is, at least in part, due to an increased autacoid-induced intracellular Ca^{2+} signalling because of increased superoxide anion formation and reduced nitric oxide synthase activity.

Increased platelet aggregation in diabetes is thought to be due to increased cellular responsiveness of thrombocyte signal transduction for aggregation, associated with attenuated compensatory mechanisms (e. g. nitric oxide formation). In this study, intracellular Ca^{2+} signalling as main regulator to autacoid-induced aggregation is shown to be increased in platelets from diabetic patients. These findings are consistent with a report that showed enhanced autacoid-induced Ca^{2+} responses in platelets from diabetic subjects [10]. Our data clearly indicate enhanced intracellular Ca^{2+} release to the IP_3-generating agonist ADP. There is an enhancement of inositolphosphate formation to thrombin in platelets from diabetic patients [28]. In spite of the enhanced intracellular Ca^{2+} signalling in response to ADP, the EC_{50} values in the diabetic and the control group were similar. These data suggest that the observed enhancement in platelet Ca^{2+} signalling in response to ADP in diabetic patients is not caused by an increased sensitivity of the P2Y1 receptor to ADP [29]. In platelets, Ca^{2+} entry follows activation of a so-called capacitative Ca^{2+} entry. The activity of this Ca^{2+} influx pathway correlates with the depletion of intracellular Ca^{2+} stores by way of IP_3 [7]. The increase in Ca^{2+} entry in platelets of diabetic patients might be caused by intracellular Ca^{2+} store depletion in response to a given ADP concentration and is more pronounced in platelets from diabetic persons. Since Ca^{2+} entry activated by thapsigargin, which depletes all Ca^{2+} stores by inhibition of endoplasmic reticulum Ca^{2+} ATPases [30], was also enhanced in platelets from diabetic patients, a direct effect of diabetes on Ca^{2+} entry pathway is suggested.

The effect of diabetes on the intracellular Ca^{2+} response to ADP was mimicked by preincubation of platelets with the superoxide anions-generating mixture xanthine oxidase/hypoxanthine and an induction of intracellular superoxide anion production by an inhibition of intracellular glutathione synthesis with BSO [19]. Since we could show that in platelets from diabetic patients, superoxide anion release is greater than that obtained in platelets from healthy subjects, these data might indicate that increased Ca^{2+} signalling of platelets in diabetes is caused by the enhanced production and release of superoxide anions. This hypothesis is also confirmed by our findings that preincubation of platelets with L-lactate, which increased platelet intrinsic superoxide anion formation, mimicked the effect of diabetes on platelets Ca^{2+} signalling pathways. L-lactate has been demonstrated to mimic the effect of diabetes on blood flow by inducing radical changes of $NAD^+/NADH^+$ ratio, which might result in an overproduction of superoxide anions [31]. Hence, in endothelial cells hyperglycaemia-induced changes in Ca^{2+} signal transduction pathways, which are quite similar to those in platelets [7], were recently shown to be caused by enhanced superoxide anion production by D-glucose autoxidation [19]. Consistent with the similarities between the changes of endothelial and platelet responsiveness and the potential role of superoxide anions to constitute the trigger for the alterations in the Ca^{2+} signalling, the antioxidant glutathione normalized the enhanced Ca^{2+} signalling in platelets isolated from diabetic patients (this study) and that of endothelial cells during hyperglycaemia [32].

These data clearly indicate similarities in changes in platelet and endothelial cell Ca^{2+} signalling during diabetes and hyperglycaemia, which might be due to the great similarities in the Ca^{2+} controlling mechanisms in both cell types. In platelets, however, basal and autacoid-stimulated formation of nitric oxide was markedly reduced by diabetes while in endothelial cells controversial findings have been made [33, 34]. The reduced basal nitric oxide formation in intact platelets during diabetes found in our study are consistent with previous findings of attenuated nitric oxide production in homogenates of platelets isolated from diabetic patients [16, 35]. In addition, our data show, for the first time, activation of platelet nitric oxide synthase by an increase of platelet free Ca^{2+} concentration. We failed to obtain activation of nitric oxide synthase by ADP, a finding also reported previously [36]. In contrast to ADP, the Ca^{2+} ATPase inhibitors thapsigargin (this study) and 2,5-di-(t-butyl)-1,4-benzohydroquinone (BHQ; [11]) induce activation of nitric oxide synthase in human platelets. The difference between ADP and thapsigargin in terms of their properties to stimulate platelet nitric oxide synthase reported here are unclear and might be caused by thapsigargin having a greater effect on intracellular Ca^{2+} concentration than ADP. Because of the reduced basal nitric oxide activity, thapsigargin-stimulated nitric oxide activity was strongly reduced in platelets from diabetic patients, despite the increased Ca^{2+} signalling occurring to this agonist in this group. In arteries of diabetic patients reduced endothelium-dependent relaxation was shown to improve by co-incubation with L-arginine [37, 38]. In platelets, L-arginine was essential for nitric oxide biosynthesis in both groups (data not shown), but there was no reduced L-arginine uptake in the platelets from diabetic patients which would explain the blunted nitric oxide synthase activity. In spite of these findings, there was evidence in alloxan diabetic rats of an increased arginase activity [39] which might result in a diversion of L-arginine to other end-points than NO.

It has been reported that the lack of cofactors essential for nitric oxide synthase activity (i.e. NADPH, tetrahydrobiopterine) might be responsible for an attenuation of endothelial dependent relation in diabetes [40] and hypercholesterolaemia [41]; whether this lack of cofactor(s) is responsible for the reduced nitric oxide synthase activity in diabetes or not needs further investigation. Alternatively, diabetic conditions might already affect expression of nitric oxide synthase in the megacaryocytes, resulting in a reduced appearance of this enzyme in the platelets in diabetes.

Beside the reduced nitric oxide biosynthesis reported here, it might be expected that the increased production of superoxide anions favors the scavenging of nitric oxide by this type of oxygen radical, a phenomenon recently reported in endothelial cells under hyperglycaemic conditions [20]. Thus, in diabetes the concentration of nitric oxide is limited by its reduced biosynthesis and an enhanced degradation. This reduction of nitric oxide might put platelet cells in a more sensitive state, due to reduced pacemaker potential of the nitric oxide on platelet aggregation. Since nitric oxide attenuates platelet Ca^{2+} signalling to ADP (this study), thrombin and 2,5-di-(t-butyl)-1,4-benzohydroquinone, another Ca^{2+} ATPase inhibitor [11], and therefore, constitutes an important negative feedback mechanism during platelet activation by autacoids, the reduced nitric oxide production (and enhanced nitric oxide degradation by superoxide anions) in platelets during diabetes might favor autacoid Ca^{2+} signalling and, thus, aggregation. This hypothesis is further supported by our findings that the nitric oxide donor DEA/NO [27], t = 2.1 min at 37 °C and pH 7.4) normalized upregulated Ca^{2+} signalling to ADP in platelets isolated from diabetic patients and after preincubation with the superoxide anion-generating system xanthine oxidase/hypoxanthine. These findings agree with a report which showed that the precursor of nitric oxide, L-arginine, inhibited increased platelet activity in alloxan diabetic rats [39]. Since L-arginine as well as nitric oxide are known to interact with superoxide anions [42], however, it remains to be investigated whether or not the beneficial effect of L-arginine and nitric oxide to normalize platelet function and Ca^{2+} signalling is due to scavenging properties or a direct effect on Ca^{2+} signalling mechanisms.

We think that diabetes is accompanied with an increased release of superoxide anions in platelets, resulting in an increased intracellular Ca^{2+} signalling to stimulation with autacoids. At the same time biosynthesis of nitric oxide, a compensatory mechanism to prevent hyperreactivity of platelets, is strongly attenuated in diabetes. Overall these phenomena might contribute to the enhanced platelet aggregation found in diabetic patients. Due to the potency of glutathione to normalize platelet Ca^{2+} signalling in platelets from diabetic patients, this study might guide us to a therapeutic prevention against diabetes-mediated hyperreactivity of platelets and additional studies are urgently needed.

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References

1. Cohen R (1993) Dysfunction of vascular endothelium in diabetes mellitus. Circulation 87 [Suppl V]:V67–V76
2. Hattori Y, Kawasaki H, Kanno M, Ganmdo S, Fukao M (1994) Attenuated contractile response of diabetic rat aorta to caffeine but not to noradrenaline in Ca^{2+}-free medium. Eur J Pharmacol 256: 215–219
3. Levy SB (1984) Platelets and diabetes mellitus. N Engl J Med 311: 665–667
4. Bastyr EJ, Kadrofske MM, Dershimer C, Vinik AI (1989) Decreased platelet phosphoinositide turnover and enhanced platelet activation in IDDM. Diabetes 38: 1097–1102
5. Shukla SD, Paul A, Klachko DM (1992) Hypersensitivity of diabetic human platelets to platelet activating factor. Throm Res 66: 239–246
6. van Zile J, Klipatrick M, Laimins M, Sagel J, Virella G (1981) Platelet aggregation and release of ATP after incubation with soluble immune complex purified from the serum of diabetic patients. Diabetes 30: 575–579
7. Heemskerk JWM, Sage SO (1994) Calcium signalling in platelets and other cells. Platelets 5: 295–316
8. Sage SO, Merritt JE, Hallam TJ, Rink TJ (1989) Receptor-mediated calcium entry in fura-2-loaded human platelets with ADP and thrombin. Biochem J 258: 923–926
9. Rho M-C, Nakahata N, Nakamura H, Murai A, Ohizumi Y (1995) Activation of rabbit platelets by Ca^{2+} influx and thromboxane A_2 release in an external Ca^{2+}-dependent manner by zooxanthellatoxin-A, a novel polyol. Br J Pharmacol 115: 433–440
10. Mazzanti L, Rabini RA, Faloia E, Fumelli P, Bertoli E, Pirro RD (1990) Altered cellular Ca^{2+} and Na^{+} transport in diabetes mellitus. Diabetes 39: 850–854
11. Sang K-HLQ, Lantoine F, Devynck M-A (1996) Influence of authentic nitric oxide on basal cytosolic $(Ca^{2+})_i$ and Ca^{2+} release from internal stores in human platelets. Br J Pharmacol 119: 1361–1366
12. Trovati M, Massucco P, Mattiello L et al. (1996) The insulin-induced increase of guanosine-3,5-cyclic monophosphate in human platelets is mediated by nitric oxide. Diabetes 45: 768–770
13. Trovati M, Anfossi G, Massucco P et al. (1997) Insulin stimulates nitric oxide synthesis in human platelets and, through nitric oxide, increases platelet concentrations of both guanosine-3,5-cyclic monophosphate and adenosine-3,5-cyclic monophosphate. Diabetes 46: 742–749
14. Nakashima S, Tohmatsu T, Hattori H, Okano Y, Nozawa Y (1986) Inhibitory action of cGMP on secretion, phosphoinositide hydrolysis and calcium mobilization in thrombin-stimulated human platelets. Biochem Biophys Res Commun 135: 1099–1104
15. Moncada S, Palmer RMJ, Higgs EA (1991) Nitric Oxide: Physiology, Pathophysiology, and Pharmacology. Pharmacol Rev 43: 109–142
16. Rabini RA, Staffolani R, Fumelli P, Mutus B, Curatola G, Mazzanti L (1998) Decreased nitric oxide synthase activity in platelets from IDDM and NIDDM. Diabetologia 41: 101–104
17. Catella-Lawson F, FitzGerald GA (1996) Oxidative stress and platelet activation in diabetes mellitus. Diabetes Res Clin Pract 30 [Suppl]:S13–S18
18. Leoncini G, Signorello MG, Piana A, Carrubba M, Armani U (1997) Hyperactivity and increased hydrogen peroxide formation in platelets of NIDDM patients. Throm Res 86: 153–160
19. Graier WF, Simecek S, Kukovetz WR, Kostner GM (1996) High-D-glucose-induced changes in endothelial Ca^{2+}/EDRF signalling is due to generation of superoxide anions. Diabetes 45: 1386–1395
20. Graier WF, Posch K, Wascher TC, Kukovetz WR, Kostner GM (1997) Role of superoxide anions in changes of endothelial vasoactive response during acute hyperglycaemia. Horm Metab Res 29: 622–629
21. Service FJ, Rizza RA, Zimmerman BR, Dyck PJ, O'Brien PC, Melton LJ (1997) The classification of diabetes by clinical and C-peptide acriteria. A prospective population-based study. Diabetes Care 20: 198–201
22. Hashimoto Y, Watanabe T, Kinoshita M (1993) Ca^{2+} entry pathways activated by the tumor promoter thapsigargin in human platelets. Biochim Biophys Acta 1220: 37–41
23. Graier WF, Wascher TC, Lackner L, Toplak H, Krejs GJ, Kukovetz WR (1993) Exposure to elevated D-glucose concentrations modulates vascular endothelial cell vasodilatory response. Diabetes 42: 1497–1505
24. Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 260: 3440–3450
25. Steinbrecher UP (1988) Role of superoxide in endothelial-cell modification of low-density lipoproteins. Biochim Biophys Acta 959: 20–30
26. Graier WF, Hoebel BG, Paltauf-Doburzynska J, Kostner GM (1998) Effects of superoxide anions on endothelial Ca^{2+} signalling pathways. Arterioscler Thromb Vasc Biol 18: 1470–1479
27. Morley D, Maragos CM, Zhang XY, Boignon M, Wink DA, Keefer LK (1993) Mechanism of vascular relaxation induced by the nitric oxide (NO)/nucleophile complexes, a new class of NO-based vasodilators. J Cardiovasc Pharmacol 21: 670–676
28. Ishii H, Umeda F, Hashimoto T, Nawata H (1990) Changes in phosphoinositide turnover, Ca^{2+} mobilization, and protein phosphorylation in platelets from NIDDM patients. Diabetes 39: 1561–1568
29. Savi P, Beauverger P, Labouret C et al. (1998) Role of P2Y1 purinoceptor in ADP-induced platelet activation. FEBS Lett 422: 291–295
30. Thastrup O, Dawson AP, Scharff O et al. (1989) Thapsigargin, a novel molecular probe for studying intracellular calcium release and storage. Agents and Actions 27: 17–23
31. Williamson JR, Chang K, Frangos M et al. (1993) Hyperglycemic pseudohypoxia and diabetic complications. Diabetes 42: 801–813
32. Graier WF, Simecek S, Hoebel B, Wascher TC, Dittrich P, Kostner GM (1997) Antioxidants prevent high D-glucose-enhanced endothelial Ca^{2+}/cGMP response by scavenging superoxide anions. Eur J Pharmacol 322: 113–122
33. Wu G, Meininger CJ (1995) Impaired arginine metabolism and NO synthesis in coronary endothelial cells of the spontaneously diabetic BB rat. Am J Physiol 269:H1312–H1318
34. Cosentino F, Hishikawa K, Katusic ZS, Lüscher TF (1997) High glucose increases nitric oxide synthase expression and superoxide anion generation in human aortic endothelial cells. Circulation 96: 28–28
35. Martina V, Bruno GA, Trucco F et al. (1998) Platelet cNOS activity is reduced in patients with IDDM and NIDDM. Thromb Haemost 79: 520–522
36. Lantoine F, Brunet A, Bedioui F, Devynck J, Devynck M-A (1995) Direct measurement of nitric oxide production in platelets: Relationship with cytosolic Ca^{2+} concentration. Biochem Biophys Res Commun 215: 842–848
37. Pieper GM, Siebeneich W, Dondlinger LA (1996) Short-term oral administration of L-arginine reverses defective endothelium-dependent relaxation and cGMP generation in diabetes. Eur J Pharmacol 317: 317–320
38. Pieper GM, Siebeneich W, Moore-Hilton G, Roza AM (1997) Reversal by L-arginine of a dysfunctional arginine/nitric oxide pathway in the endothelium of the genetic diabetic BB rat. Diabetologia 40: 910–915
39. Mendez JD, Zarzoza E (1997) Inhibition of platelet aggregation by L-arginine and polyamines in alloxan treated rats. Biochem Mol Biol Int 43: 311–318
40. Pieper GM (1997) Acute amelioration of diabetic endothelial dysfunction with a derivative of the nitric oxide synthase cofactor, tetrahydrobiopterin. J Cardiovasc Pharmacol 29: 8–15
41. Stroes E, Kastelein J, Cosentino F et al. (1997) Tetrahydrobiopterin restores endothelial function in hypercholesterolemia. J Clin Invest 99: 41–46
42. Wascher TC, Posch K, Wallner S, Hermetter A, Kostner GM, Graier WF (1997) Vascular effects of L-arginine: anything beyond a substrate for the NO-synthase? Biochem Biophys Res Commun 234: 35–38