PLATELET HYPERACTIVATION AND PERIPHERAL ADRENERGIC/ SEROTONERGIC IMPAIRMENT IN TYPE 2 DIABETES MELLITUS PATIENTS – IMPLICATIONS FOR CARDIOVASCULAR RISK

Flávio Reis[1,2], *Maria Vítor Campos*[1,3], *Luísa Ruas*[3], *Filipe Palavra*[1], *Daniela Marado*[1], *Elisa Ribeiro*[1], *Luís Almeida*[1], *Teresa Alcobia*[1], *Margarida Lourenço*[4], *C.A. Ferrer-Antunes*[4], *Aida Palmeiro*[4], *Manuela Carvalheiro*[3], *Frederico Teixeira*[1,2]

[1] Therapeutics Unit, Institute of Pharmacology and Experimental Therapeutics, Medicine Faculty
[2] Institute of Climatology and Hydrology, University of Coimbra, 3004-504 Coimbra, Portugal
[3] Endocrinology, Diabetes and Metabolism Unit
[4] Laboratory of Haematology, Coimbra University Hospital, Coimbra, Portugal

Abstract

Type 2 diabetes mellitus is a pathological condition often associated with an increased cardiovascular risk, which could result both from central and/or peripheral disturbances whose causes remains to be fully elucidated. The purpose of this study was to evaluate some of the main clinical laboratorial CV parameters, the platelet activation, as well as the peripheral sympathetic and serotonergic nervous systems.

A group of type 2 diabetes mellitus patients and a sex- and age-matched control group of healthy volunteers were compared. A complete clinical laboratorial evaluation was performed (blood pressures; haematology and coagulation; lipid profile; electrolytes, protein and renal and liver function parameters) using standard laboratory methods. Plasma NA and AD and platelet NA, AD, 5-HT and 5-HIAA contents were assessed by HPLC-ECD. Collagen and ADP-evoked whole blood platelet aggregation was measured through the impedance method. Thrombin-evoked platelet $[Ca^{2+}]i$, with and without Ca^{2+}_{ext} and staurosporin, a PKC inhibitor, was measured by using fura-2 fluorescence.

The diabetic group showed minor differences regarding the classical lipid profile parameters when compared with the control group. Concerning the other laboratorial examinations, the main changes were obtained in the platelet parameters: lower values of PTC and PCT and higher of PDW. The diabetes patients had lower plasma NA (423 ± 49 pg/ml; $P<0.05$) and

AD (347 ± 62 pg/ml) and also platelet NA (226 ± 76 pg/ml; $P<0.05$) and AD (837 ± 50 pg/ml) contents *vs* the control individuals (plasma: NA – 858 ± 103, AD – 574 ± 154; platelet: NA – 457 ± 34, AD – 1119 ± 132 pg/ml). Platelet 5-HT concentration was also lower in the group of patients (5.0 ± 0.7 ng/ml; $P<0.01$) *vs* control (32.1 ± 6.5 ng/ml), whereas 5-HIAA values were identical. ADP-induced platelet aggregation was significantly higher in the diabetic group (16.2 ± 2.9 ohms; $P<0.05$) than in the control (8.4 ± 1.7 ohms), in agreement with the lower PTC and PCT values, indicatives of microaggregates formation, and with higher PDW, suggestive of platelet activation. Thrombin-evoked intracellular calcium release (156.7 ± 8.0 Δnmol/l; $P<0.05$) and transmembrane influx (192.3 ± 10.2 Δnmol/l; $P<0.05$) showed a higher increase in the group of diabetic patients then in the control individuals (123.5 ± 32.6 Δnmol/l and 147.8 ± 4.1 Δnmol/l, respectively). Staurosporin-evoked PKC inhibition did not significantly influence those $[Ca^{2+}]i$ variations.

In conclusion, the type 2 diabetes group demonstrated platelet aggregation and $[Ca^{2+}]i$ contents augmentation together with 5-HT concentration decrease, which might contribute to the structural and/or functional vascular impairment associated with the increased risk of thromboembolic events and CV complications. The platelet hyperactivated state encountered even in the absence of clear-cut variations of the classic CV risk parameters suggests that platelet activation might be a useful predictor/marker of CV risk. Furthermore, since platelets might be viewed as models, in several features, for VSMC and for the monoaminergic neurons, the relevant changes on $[Ca^{2+}]i$ contents and on adrenergic and serotonergic measures here demonstrated, reinforces the usefulness of platelets as tools for the study of CV disturbances in type 2 diabetic patients.

INTRODUCTION

Type 2 diabetes mellitus is associated with variable levels of insulin resistance and/or deficiency and often times also linked with increased risk of cardiovascular (CV) complications, namely hypertension, atherosclerosis, thromboembolic events and microangiopathies (1-5), which could result both from central and/or peripheral CV dysfunctions, whose underlying mechanisms remain to be fully elucidated.

Chronic activation of the sympathetic nervous system (SNS) might be a pathogenic mechanism by which diabetes, and particularly hyperinsulinemia, induces cardiovascular disturbances (6-8). Plasma catecholamines (CAs) are usually measured to estimate sympathetic activation but, due to its transient character, there is controversy about its feasibility in accessing *per se* SNS activity (9,10). Therefore, as previously suggested, concomitant evaluation of plasma and platelet CA contents might better evaluate peripheral SNS activity (11). Serotonin (5-HT: 5-hydroxitryptophan), is another biological natural amina of great physiological and pathophysiological relevance, particularly concerning the CV system. Serotonin is stored peripherally in the platelet dense granules and has been reported to play an important role in thrombotic complications and increases in blood pressure (12). When activated,

platelets adhere and aggregate to the damaged endothelium, releasing CA and 5-HT (among other compounds), which may act as vasoconstrictors and also extend aggregation to other agonists, thus promoting thrombus formation.

Impaired platelet function in diabetic patients have been described (13,14), but some of the underlying mechanisms remains uncertain, such as the intracellular signals that regulates intracellular calcium concentration ($[Ca^{2+}]i$) homeostasis. Impaired calcium regulation might be of significance for the impaired insulin secretion and action in type 2 diabetes (15), as well as for the development of CV complications, being a common link between these two important features of the disease. Therefore, intracellular Ca^{2+} is an essential second messenger in platelet responses and is the major determinant of vascular smooth muscle cells (VSMC) contractility (16,17), leading to increased peripheral resistance that determines vascular impairment. Understanding the general principles of cellular Ca^{2+} regulation is crucial to the pathophysiology of several cardiovascular diseases, such as it is in hypertension (18). Changes in cellular calcium metabolism have been also reported in diabetic patients and animal models of type 2 diabetes (15). However, whereas the relationship between cytosolic Ca^{2+} and vasoconstriction has been clearly established, the molecular basis of high Ca^{2+} levels in diabetes remains unclear. One factor that regulates VSMC and platelet activity is the protein kinase C (PKC), particularly because of its role on protein phosphorylation that precedes activation (19). Furthermore, PKC also has a role on Ca^{2+} movements (intracellular Ca^{2+} release from dense tubular system or transmembrane influx) that determines its haemostasis, but its role on the type 2 diabetes mellitus $[Ca^{2+}]i$ augment is less studied.

As vectors of the vascular tone, mediators of thrombotic complications and promoters of arteriosclerosis (20,21), platelets might be of great relevance for the study CV pathophysiology. These corollaries, as well as their easy clinical accessibility and the features they have in common with monoaminergic neurons and VSMC (22,23), make them useful tools in the study of pathophysiological abnormalities related with catecholamines and 5-HT turnover and to calcium movements, respectively.

The purpose of this study was, thus, to evaluate some of the main clinical CV parameters (blood pressures, haematology, coagulation, lipid profile), to assess the peripheral sympathetic and serotonergic nervous systems (by simultaneously measuring plasma and platelet CAs and platelet 5-HT) and platelet activation (through whole-blood aggregation and $[Ca^{2+}]i$ testing, and the influence of PKC modulation). The platelets usefulness as predictors/markers of CV risk and as mirrors for the VSMC and monoaminergic neurons changes in type 2 diabetes mellitus was also under debate.

MATERIALS AND METHODS

Subject selection and characterization

The study was performed using a group of 12 type 2 diabetic patients (diabetic group) and 8 sex- and age (± 5 yrs)-matched healthy volunteers (control group). Subject selection and

Table 1 – General characterization of groups: anthropometric data, blood pressures and diabetes indexes

Parameter	Control Group	Diabetic Group
Age (yrs)	58.4 ± 5.4	61.3 ± 3.3
BMI (kg/m^2)	29.0 ± 0.1	29.9 ± 1.7
SBP (mmHg)	113.0 ± 5.4	143.8 ± 4.8
DBP (mmHg)	74.0 ± 3.7	77.5 ± 2.8
Glucose (mg/dl)	87.2 ± 7.0	182.6 ± 16.5**
HbA1c (%)	5.5 ± 0.2	8.1 ± 1.2*

Each bar represents the mean of 12 patients and 8 controls ± SEM. Significant differences between the groups are expressed: **P<0.01. BMI: body-mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; HbA1c: glucosylated haemoglobin.

characterization was done in the Endocrinology, Diabetes and Metabolism Unit of the Coimbra University Hospital. The study was conducted according to the principles established in the Helsinki Declaration. All the subjects (diabetic patients and control healthy volunteers) were informed and have given written consent. Glucose and glucosylated haemoglobin (HbA1c) values were employed to characterize the diabetes state (Table 1). All the diabetic patients were under oral anti-diabetic therapy and with no further medication. No differences were observed in anthropometric evaluation or fat distribution. Body weight was measured in the morning, after the subjects had voided, to the nearest 0.1 kg. Body height was measured barefoot and to the nearest 0.5 cm. No differences were observed in body mass index (BMI) between the 2 groups (Table 1). The systolic and diastolic blood pressure (SBP and DBP) values were the mean of two measures on the right arm in a random zero sphygmomanometer, with a cuff size corresponding to the size of the right arm. No statistical differences were observed in the SBP and DBP values between the 2 groups (Table 1), but the high value of SBP in the diabetic group should be reported.

Blood collection and samples separation

Blood was collected to tubes containing the appropriate anticoagulant solution (in accordance with the protocol) and then centrifuged (160 ×g for 10 min. at 20ºC) to obtain platelet-rich plasma (PRP). The platelet pellet and the platelet-poor plasma (PPP) were then recovered by a new centrifugation (900 ×g, 10 min., 20ºC), in order to be used in the different experimental protocols.

Clinical laboratorial examinations

After one night fasting, blood samples were drawn to appropriated tubes provided with anticoagulant solution for the determination of haematological and biochemical parameters. Several laboratory examinations related with hematology (blood cell counts and coagulation: Table 2), lipid profile (cholesterol lipids, triglycerides and apolipoproteins: Table 3) and electrolytes, protein and renal and liver function (Table 4) were made in the diabetic patients and in the control volunteers, using standard laboratory techniques for human clinical practice.

Plasma and platelet catecholamines contents

Plasma and platelet catecholamines (NA and AD) contents were measured

as previously described (24). In brief, to 2 ml of plasma or platelet suspension, obtained as above-mentioned, 100 ng/ml of the internal standard 3,4-dihydroxybenzylamine (DHBA), 50 mg of alumina (Bioanalytical Systems Inc., West Lafayette, ID, U.S.A.) and 1 ml of Tris buffer (1 mol/l, pH 8.6) were added. The catecholamines in the samples, extracted by using the method of alumina adsorption and appropriate microfilter systems, were finally re-suspended in perchloric acid (100 mmol/l) in order to be further quantified by HPLC with electrochemical detection (HPLC-ECD). In a Gilson Applied Chromatographic System, a Biophase ODS RP18 analytical column (250 × 4.6, ∅ = 5 micron; Bioanalytical Systems Inc., U.S.A.) was used and separation was achieved by using an isocratic solvent system consisting of an acetate-citrate buffer (sodium acetate 0.1 mol/l, citric acid 0.1 mol/l), containing sodium octane sulphonate (0.5 mmol/l), EDTA (0.15 mmol/l), dibutylamine (1 mmol/l) and 10% methanol. A flow rate of 1 ml/min was maintained and detection of the chromatographed CAs achieved by using a 141 Gilson electrochemical detector model (650 mV). In order to correct the possible CAs loss during alumina extraction, NA and AD concentrations were calculated by using the authentic standards of each and the internal standard DHBA (Sigma Chemicals, St. Louis, MO, U.S.A.). The NA and AD values were expressed in pg/ml.

Platelet 5-HT and 5-HIAA contents

Platelet serotonergic measurements were performed as previously described (25). In brief, the platelet pellet, collected as previously described, was re-suspended in 1 ml of a buffer solution (pH 7.4) containing (in mmol/l): NaCl (145), KCl (5), $MgSO_4$ (1), $CaCl_2$ (1), D-glucose (10), and 120 µl of perchloric acid (70 %) was added. Following 15 min. at ice temperature, the suspension was finally centrifuged at 730 ×g for 10 min. at 20ºC and the supernatant containing the released 5-HT and 5-HIAA was collected for quantification. Platelet 5-HT and 5-HIAA contents were determined by HPLC-ECD, using the above-described conditions and equipment. The concentrations in samples, calculated by using authentic standards as reference (Sigma Chemicals, St. Louis, MO, U.S.A.), were expressed in ng/ml.

Whole blood platelet aggregation

Whole blood platelet aggregation was measured by assessing the electric impedance (26), using a Chronolog aggregometer (Chrono Log Corp., Havertown, Penn., USA.). This technique is based on the detection of changes in electrical resistance between two electrodes submerged in the sample. Half a milliliter of fresh heparinized whole blood and 0.5 ml of 0.9% NaCl were mixed using a magnetic stirrer and allowed to balance at 37ºC for 5 min. before adding the agonists (collagen – equine type I: 10 µg/ml; ADP: 2 µmol/l; from (Chrono Log Corp., Havertown, Penn., USA.).

Platelet intracellular free calcium concentration

Preparation of fura-2-loaded platelets – The platelet pellet, obtained

as previously described, containing 100 μmol/l of acethyl-salicylic acid (ASA), was re-suspended and incubated for 10 min. in a physiological saline solution containing (in mmol/l) NaCl (145), KCl (5), $MgSO_4.7H_2O$ (1), Hepes (10), D-glucose (10) and 20 μg/ml apyrase (to prevent activation by residual traces of ADP). After having been washed and re-suspended in the same buffer without apyrase, the platelets were then loaded with 5 μmol/l fura-2/AM (Molecular Probes Inc., Eugene, OR, U.S.A.) and incubated for 45 min. at 37ºC. After adding an anticoagulant and washing once before final suspension in the same solution, the platelet suspension was then centrifuged (900 ×g 10 min. 20ºC).

Fluorescence measurements – Fluorescence was measured at the emission wavelength of 510 nm, with the excitation wavelength continuously switched between 340 and 380 nm (FluoroMax spectrofluorometer, SPEX Industries Inc., Edison, U.S.A.). Fluorescence measurements were carried out at 37ºC with continual agitation. The ratio of the fluorescence intensities at the two excitation wavelengths was used to determine $[Ca^{2+}]_i$, based on the following equation: $[Ca^{2+}]_i = Kd\ [(R-Rmin)/(Rmax-R)]\times\beta$ (27). $[Ca^{2+}]_i$ calibration was achieved by lysing the cells with 50 μmol/l digitonin in the presence of 1 mmol/l $CaCl_2$ or 10 mmol/l EGTA (pH 9.0).

Experimental conditions of stimulation – $[Ca^{2+}]_i$ was tested at basal conditions and after addition of 0.5 U/ml thrombin (bovine, lyophilised, from DiaMed AG, Cressier, Switzerland). Thrombin stimulation was tested in the presence (after 1 mmol/l $CaCl_2$ addition) and absence of extracellular calcium (after 1 μmol/l EDTA addition) in order to evaluate intracellular release and transmembranar influx and in the presence and absence of a PKC inhibitor (staurosporin – 0.1 μmol/l).

Statistical analysis

Results are expressed as means ± standard error of the mean (SEM) of n individuals for each group (as indicated). Groups were tested for differences by performing the analysis of variance (ANOVA) and Fisher's PLSD (*least protected significant difference*) test, using the Statview 4.53 software from Abacus Concepts Inc. (Berkeley, CA, USA). Differences were considered statistically significant at a P value < 0.05.

RESULTS

Clinical laboratorial examinations

Concerning the blood cell counts and the coagulation parameters, the platelets values must be emphasized. Therefore, platelet count (PTC) and plateletocrit (PCT) in the diabetic group were significantly lower (P<0.05) than in the control (Table 2), which might indicate the formation of micro-aggregates resulting from increased platelet activation (confirm the results on whole blood platelet aggregation further described). Furthermore, a higher platelet deviation width (PDW) value was encountered, which is an additional indicative of increased activation, reinforcing thus the existence of a platelet hyperaggregation state. No significant differences were obtained in white blood cells (WBC), red blood cells (RBC) and coagulation parameters (Table 2).

Table 2 – Blood cell counts and coagulation parameters

Parameter	Control Group	Diabetic Group
WBC (10^9/l)	4.42 ± 0.60	5.77 ± 0.78
NE (%)	55.90 ± 3.36	55.06 ± 4.39
LY (%)	32.82 ± 3.32	33.60 ± 1.96
MO (%)	7.72 ± 0.62	5.56 ± 1.48
EO (%)	3.12 ± 0.38	2.44 ± 0.57
BA (%)	0.44 ± 0.08	0.43 ± 0.11
RBC (10^{12}/l)	4.13 ± 0.19	4.01 ± 0.10
HGB (g/dl)	12.63 ± 0.46	11.95 ± 0.38
HCT (%)	37.37 ± 1.67	35.36 ± 0.92
MCV (fl)	90.51 ± 0.99	88.15 ± 1.16
MCH (pg)	30.64 ± 0.42	29.80 ± 0.50
MCHC (g/dl)	33.86 ± 0.50	33.79 ± 0.38
RDW (%)	22.30 ± 6.00	13.37 ± 0.18
PLT (10^{12}/l)	217.71 ± 13.18	124.60 ± 31.98*
MPV (fl)	8.53 ± 0.44	8.48 ± 0.52
PCT (%)	0.18 ± 0.01	0.10 ± 0.02*
PDW (%)	15.83 ± 1.05	31.35 ± 9.63*
Coagulation		
Prothrombin Time (s)	13.43 ± 0.41	12.65 ± 0.34
Prothrombinaemia (%)	103.00 ± 5.69	102.17 ± 4.58
APTT (s)	28.10 ± 0.72	26.93 ± 1.64
Fibrinogen (g/l)	2.76 ± 0.21	3.63 ± 0.41
FVIII AG (%)	148.35 ± 21.45	150.40 ± 23.08

Each bar represents the mean of 12 patients and 8 controls ± SEM. Significant differences between the groups are expressed: *P<0.05. WBC: white-blood-cells; NE: neutrophils; LY: lymphocytes; MO: monocytes; EO: eosynophils; BA: basophyls; RBC: red-blood-cells; HGB: haemoglobin; HCT: haematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCH: mean corpuscular haemoglobin concentration; RDW: red distribution width; PLT: platelet count; MPV: mean platelet volume; PCT: plateletocrit; PDW: platelet distribution width; APTT: activated partial thromboplastin time; FVIII AG: Factor VIII-antigen.

Regarding the lipid profile parameters, despite numerical changes between the two groups in some of the values evaluated, these variations did not reach statistical significance: increased total cholesterol (T-Chol), low-density lipoprotein cholesterol (LDL-Chol), triglycerides (TGs), apolipoprotein B (ApoB) and decreased high-density lipoprotein cholesterol (HDL-Chol) and ApoA1 (Table 3). The small number of significant differences encountered were an increased ApoB/ApoA1 ratio (P<0.05) and a decreased level of alpha-electrophoresis band (P<0.01), when compared with the control (Table 3).

The laboratorial analysis for electrolytes, protein and renal and liver function evaluation demonstrated a single statistical significant difference: the reduction of albumin content (P<0.05) in the diabetic group when compared with the control (Table 4).

Plasma and platelet catecholamines contents

Plasma NA concentration was significant lower in the diabetic group (423 ± 49 pg/ml; P<0.01) then in the control (858 ± 103 pg/ml). The plasma AD content, despite lower, did not significantly differ from the control (Fig. 1). In agreement with the plasma CAs concentrations, platelet

Table 3 – Lipid profile parameters

Parameter	Control Group	Diabetic Group
T-Chol (mg/dl)	189.2 ±14.2	199.2 ± 9.8
HDL-Chol (mg/dl)	49.6 ± 3.7	44.1 ± 2.0
LDL-Chol (mg/dl)	98.6 ± 6.3	117.8 ± 8.8
TGs (mg/dl)	71.0 ± 6.8	123.9 ± 14.3
Apo A1 (mg/dl)	177.6 ± 12.5	161.3 ± 8.6
Apo B (mg/dl)	95.4 ± 7.4	117.4 ± 8.2
Apo B/Apo A1	0.50 ± 0.02	0.7 ± 0.06*
Alpha (%)	30.2 ± 1.6	23.8 ± 1.1**
Prebeta (%)	13.6 ± 3.0	18.3 ± 3.6
Beta (%)	56.2 ± 2.5	53.4 ± 5.4

Each bar represents the mean of 12 patients and 8 controls ± SEM. Significant differences between the groups are expressed: *P<0.05 and **P<0.01. T-Chol: total-cholesterol; HDL-Chol: high-density lipoprotein cholesterol; LDL-Chol: low-density lipoprotein cholesterol; TGs: triglycerides; Apo: apolipoprotein.

Table 4 – Parameters of electrolytes, protein and renal and liver function evaluation

Parameter	Control Group	Diabetic Group
Calcium (mg/dl)	9.14 ± 0.19	9.37 ± 0.16
Sodium (mmol/l)	138.60 ± 0.51	137.46 ± 0.62
Potassium (mmol/l)	4.18 ± 0.05	4.36 ± 0.10
Chloride (mmol/l)	104.60 ± 1.21	104.73 ± 2.73
Osmolarity (mosm/kg)	277.60 ± 0.93	281.64 ± 1.41
BUN (mg/dl)	17.00 ± 0.63	19.27 ± 2.05
Creatinine (mg/dl)	0.82 ± 0.04	0.91 ± 0.05
Uric Acid (mg/dl)	4.28 ± 0.84	4.54 ± 0.20
Protein, Total (g/dl)	6.92 ± 0.08	7.26 ± 0.17
Albumin (g/dl)	4.34 ± 0.07	4.04 ± 0.05**
SGPT (ALT) (U/l)	26.00 ± 5.32	29.36 ± 3.85
SGOT (AST) (U/l)	25.00 ± 3.32	22.18 ± 2.19
Alkaline Phosphatase (U/l)	67.20 ± 8.27	81.36 ± 10.11
GGT (U/l)	34.20 ± 9.81	34.64 ± 9.46
Bilirubin, Total (mg/dl)	0.58 ±0.06	0.73 ± 0.08
LDH (U/l)	323.00 ± 5.11	335.73 ± 14.43

Each bar represents the mean of 12 patients and 8 controls ± SEM. Significant differences between the groups are expressed: **P<0.01. BUN: blood urea nitrogen; GGT: Gamma-glutamyltranspeptidase; SGPT (ALT): Glutamic-Pyruvic Transaminase; SGOT (AST): Glutamic-Oxalocetic Transaminase; LDH: Lactic Acid Dehydrogenase.

NA content was significantly lower in the diabetic group (457 ± 34 pg/ml; P<0.05) than in the control one (226 ± 76 pg/ml), without significant difference in the AD levels, even considering the lower numerical value, but that did not reach statistical significance (Fig. 1).

Platelet 5-HT and 5-HIAA contents

Platelet 5-HT concentration was significantly lower in the diabetic patients (4.96 ± 0.72 ng/ml; P<0.01) when compared with the control volunteers (32.11 ± 6.50 ng/ml). No differences in the platelet 5-HIAA contents were reported between the diabetic and the control group (Fig. 2).

Whole blood platelet aggregation

Collagen-induced whole blood platelet aggregation was higher in the diabetic patients (18.78 ± 3.55 ohms) when compared with the control individuals (16.00 ± 2.32 ohms), but with-

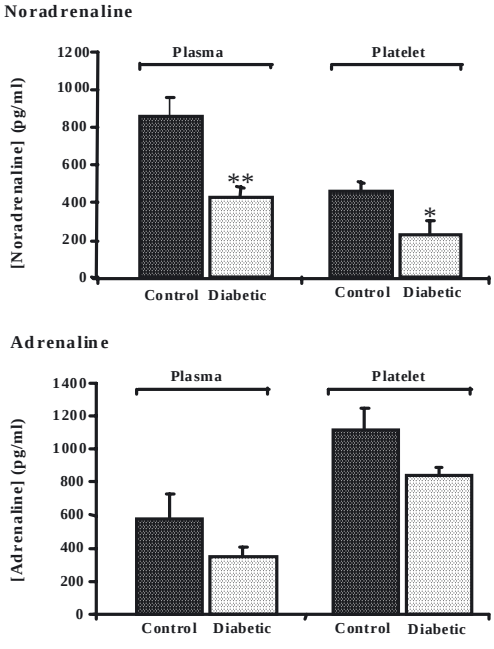

Fig. 1 – Plasma and platelet noradrenaline and adrenaline contents in the type 2 diabetic patients group and in the control group of healthy volunteers. Each bar represents the mean of 12 patients and 8 controls ± SEM. Significant differences between the groups are expressed: **P*<0.05 and ***P*<0.01.

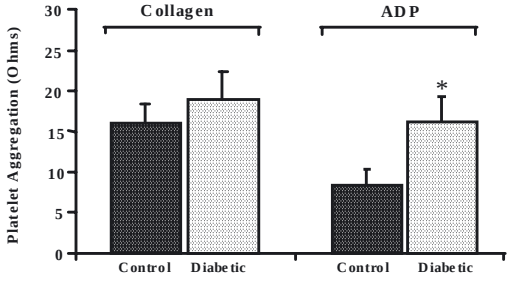

Fig. 3 – Collagen (2 µg/ml) and ADP (10 µmol/l)-evoked whole blood platelet aggregation in the type 2 diabetic patients group and in the control group of healthy volunteers. Each bar represents the mean of 12 patients and 8 controls ± SEM. Significant differences between the groups are expressed: **P*<0.05.

out reaching statistical significance (Fig. 3). However, ADP-induced whole blood platelet aggregation was significantly higher in the diabetic group (16.17 ± 2.9 ohms; *P*<0.05) than in the control (8.43 ± 1.66 ohms) (Fig. 3).

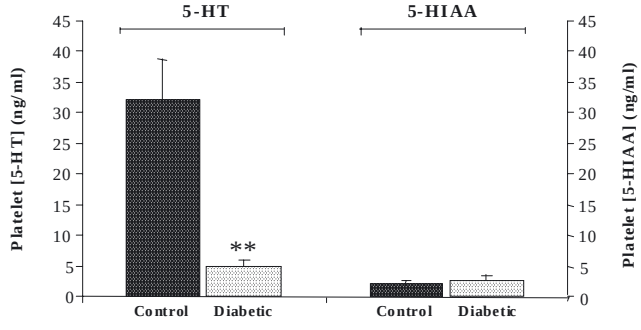

Fig. 2 – Platelet 5-HT and 5-HIAA contents in the type 2 diabetic patients group and in the control group of healthy volunteers. Each bar represents the mean of 12 patients and 8 controls ± SEM. Significant differences between the groups are expressed: ***P*<0.01.

Platelet intracellular free calcium concentration

In the presence of EGTA to produce extracelullar calcium quelation, $[Ca^{2+}]i$ is governed by intracellular mobilization. In the diabetic group there was a higher variation of $Ca^{2+}i$ mobilization (156.7 ± 8.0 Δnmol/l; *P*<0.05) than in the control group (123.5 ± 32.6 Δnmol/l). When the extracellular calcium was present (in the absence of EGTA), transmembrane calcium influx variation was also higher in the diabetic patients (192.3 ± 10.2 Δnmol/l; *P*<0.05) than in the control volunteers (147.8 ± 4.1 Δnmol/l) (Fig. 4).

The presence of a PKC inhibitor, staurosporin, originated only slight variations against the opposed situation (staurosporin absence). In the diabetic group the variations of $Ca^{2+}i$ mobilization and transmembrane influx were 151.0 ± 7.4 Δnmol/l and 206.3 ± 9.2 Δnmol/l, respectively, while in the control group the values were 128.5 ± 3.6

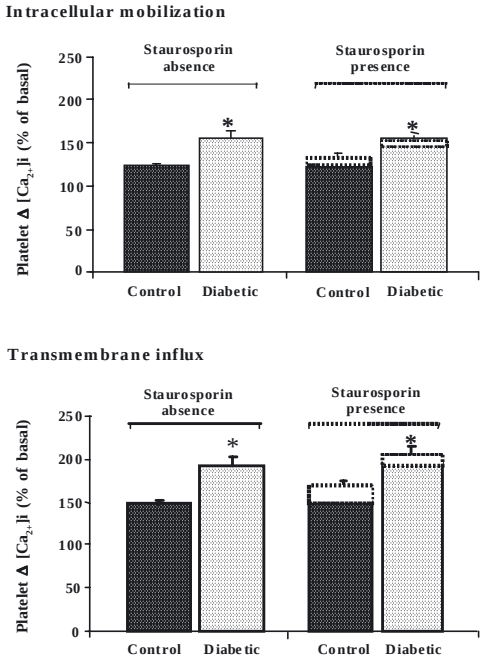

Fig. 4 – Platelet intracellular free calcium concentration variations in the type 2 diabetic patients group and in the control group of healthy volunteers. Platelets were incubated with fura-2/AM (5 μmol/l) for 45 min. and further stimulated with thrombin (0.5 U/ml) in the presence of 1 mmol/l EGTA to quelate calcium in order to assess intracellular calcium mobilization and in the presence of 1 mmol/l of $CaCl_2$ in order to restore the external $[Ca^{2+}]$ and assess transmembrane calcium influx. The values obtained are the variations of peak $[Ca^{2+}]i$ (achieved 1 min. after the addition of the agonist) in relation to the basal values. Each bar represents the mean of 12 patients and 8 controls ± SEM. Significant differences between the groups are expressed: **P*<0.05.

Λnmol/l and 169.1 ± 6.7 Λnmol/l, respectively (Fig. 4). None of these variations reached statistical significance when compared with the situation of staurosporin absence, indicating no relevant role for PKC in the above-described differences.

DISCUSSION

Type 2 diabetes mellitus is commonly associated with an increased risk of CV complications, namely hypertension, atherosclerosis, thromboembolic events and microangiopathies. The dyslipidaemia profile, related with both the cholesterol lipoproteins and with the TGs levels, is one of the best characterized disturbances underlying the increased CV risk (28,29). In our study, despite some tendentious indications of lipid metabolism impairment, only the ApoB/ApoA1 ratio and the alpha values in the diabetic patients were significantly different from those obtained in the control subjects, which seems to indicate an ordinary lipid profile. The laboratorial examinations that illustrated pronounced changes were those related with the platelets: PTC and PCT values decreased in the diabetic group, indicative of micro-aggregates formation resultant from increased platelet aggregation, and the PDW value increased, suggestive of increased activation. These data reinforces the existence of a platelet hyperaggregation state in the diabetic patients. All the other classical clinical laboratorial examinations related with the CV risk (BMI, blood pressures, cell counts and coagulation), as well as the laboratorial analysis for electrolytes, protein and renal and liver function evaluation, revealed identical values to those obtained in the control healthy volunteers. Consequently, even though the well-defined diabetes pattern given by the glucose and HbA1c values, the classic CV risk factors demonstrated an ordinary state in the group of 12 patients under investigation, which might be attributed to the homogeneity of the diabetic group: all the 12 patients

were under oral anti-diabetic therapy and with no further medication.

Hyperinsulinemia and increased sympathetic activity are usually viewed as mediators of the relation between obesity and CV disturbances (6-8). Insulin seems to play a role in the sympatho-adrenal turnover of catecholamines (10), and, when using catecholamines levels to assess cardiovascular risk, these multiple interactions should be considered. Both central and peripheral CAs changes might be utilized to estimate sympatho-adrenal activity. Noradrenaline, the main neurotransmitter from the sympathetic nerves, is released as the result of sympathetic nerve activation. In enervated peripheral tissues, NE is stored in vesicles from adrenergic nerve terminals. Adrenaline, which functions as a circulating hormone, is mainly derived from the adrenal medulla, from which is released to the circulation upon sympathetic stimulation and then taken by peripheral tissues. Both the NA and the AD circulates in its free form only in small amount and for short periods, which makes them less reliable tools to evaluate SNS activity in non-standard situations. Thus, a more consistent evaluation of basal SNS tone is more difficult to accomplish by measuring only plasma CAs levels. In order to overcome this problem, some authors have suggested the parallel study of platelet CAs contents (11,30). Due to its embryo-genetic, biochemical and morphofunctional features, platelets are usually seen as peripheral models for the monoaminergic neuron (31). Platelets selectively uptake catecholamines from the plasma, from many areas of the body, and store them for relatively long periods, thus overcoming possible regional differences, which is the main problem of plasma CAs analysis. Since platelets are incapable of synthesizing the monoamines, their content derives entirely from those released into the bloodstream by the adrenals or the SNS terminals and can be considered a reliable and stable long-term index of SNS activity (11,30). Accordingly, the simultaneous measurement of NA and AD contents in plasma and in platelets might be a better index of peripheral SNS activity in diabetes.

In our study, plasma NA content significantly decreased in the diabetic patients. The plasma and platelet AD contents in these subjects, even numerically lower than in the healthy individuals, did not reach statistical significance. The plasma NA decrease was accompanied by a parallel platelet variation, which seems to indicate an identical pattern in the two compartments, thus confirming that platelet catecholamines content does not merely reflect the circulating plasma catecholamines level, but it is rather the result of a dynamic balance between these two compartments, as was previously suggested both in physiological and pathological situations (24,32,33). Altered SNS activity might be viewed as a mechanism underlying the increased CV disturbances in diabetic patients (6-8). The decreased levels of the free aminas obtained do not excludes that hypothesis, because other relevant pathways related with the CAs life in circulation (such as metabolism and/or conjugation) or with the peripheral tissues CAs turnover (such as nerve terminals release and uptake) were not addressed in this study. Therefore, there are reports from other studies of insulin-induced increased NA uptake in peripheral tissues, thus contributing to decreased free circulating content (6,34), which would agree with our values in plasma and

platelets. In any case, taking in consideration the simultaneous plasma and platelet variations obtained, our results are indicatives of important peripheral adrenergic nervous system impairment in diabetic patients; deserving further studies in order to elucidate the mechanisms underlying the variations observed.

Serotonin, due to its pro-aggregatory and vasoconstrictor properties, might play a key role in the increased risk of thromboembolic events and CV complications in diabetes. Platelets are the main storage site of 5-HT and changes in their contents may reflect important physiopathological disturbances. In the current study there was a platelet 5-HT content decrease in the diabetic group. Low platelet 5-HT concentration may be due to decreased uptake, increased release or decreased degradation in its metabolite 5-HIAA. However, similar 5-HIAA levels were obtained in the diabetic and in the control group, suggesting no significant influence of monoamine-oxidase (MAO) activity for the decreased 5-HT content. 5-HT uptake was not evaluated in this study, but an increased 5-HT release from the platelets is expected according to the whole blood platelet aggregation results, which demonstrated a hyper-aggregation state. Therefore, activated platelets release higher amounts of some vasoconstriction agents, such as 5-HT. If serotonin is abnormally released by platelets, an increase in the plasma concentration of this amine could be expected (35,36), and consequently an increased deposition on collagen fibers of the vessel wall, thus promoting direct vasoconstriction and platelet aggregation amplification (35,37,38).

In harmony with the platelet hyperactivated state in the diabetic group, platelet $[Ca^{2+}]i$ was also elevated when compared with the control subjects. Therefore, thrombin-evoked intracellular calcium release and transmembrane influx were higher in the diabetic patients. Intracellular calcium is a key factor for platelet responses and VSMC contractile activity. However, whereas the relationship between cytosolic calcium and vasoconstriction has been clearly established, the molecular basis of high calcium levels in type 2 diabetes mellitus remains to be fully elucidated. It is known that intracellular calcium have an important influence on both the glucose and the insulin mechanisms (15), thus reinforcing its probable importance for the vascular and platelet hyperactivation associated with diabetes. In our study, the increased $[Ca^{2+}]i$ previously reported by other authors was confirmed, both as result of intracellular calcium release from the platelet dense tubular system and from the transmembrane influx. PKC activity is associated with key steps of platelet and VSMC activation, particularly protein phosphorylation, and is also pivotal in the Ca^{2+} movements that determine increased $[Ca^{2+}]i$. However, the involvement of PKC activity in the increased $[Ca^{2+}]i$ in type 2 diabetes mellitus is not yet elucidated. PKC inhibition by staurosporin did not have a significant influence on the above described $[Ca^{2+}]i$ variations between the two groups, excluding, thus, its contribution as a relevant step for the observed calcium haemostasis impairment. Since platelets are seen as models for VSMC in several features, particularly in intracellular calcium regulation, and are more easily obtained from humans, the changes here reported confirms not only the exist-

ence of a platelet hyperactivated state in the diabetic patients but also the relevance of platelet studies for the knowledge of changes on other critical cells for CV risk development, such as the VSMCs.

In conclusion, the type 2 diabetes group demonstrated the absence of a clear-cut dyslipidaemia pattern, since only minor alterations of the lipid profile parameters were observed. The peripheral catecholamines contents changes suggest an SNS impairment that could be followed by analysing the platelet contents, which reflects the plasma variations. The increased platelet aggregation and $[Ca^{2+}]i$ contents in the diabetic group, together with decreased 5-HT concentration, might facilitate/contribute to the vascular dysfunction associated with the increases risk of thromboembolic events and CV complications in type 2 diabetes mellitus. Thus, the current study suggests that platelet activation might be a useful tool as predictor/marker of CV risk, even in the absence of well-defined impairment of the classical laboratorial CV parameters. Furthermore, since platelets might be viewed as models for VSMC and for the monoaminergic neurons in several features, and since relevant changes on $[Ca^{2+}]i$ contents and on adrenergic and serotonergic measures were encountered, platelets might be useful for the study of CV disturbances in diabetic type 2 patients.

Aknowledgements

We would like to thank all the subjects who kindly accepted to participate in the study, as well as the nursing staff of the Endocrinology, Diabetes and Metabolism Unit of the Coimbra University Hospital, for their helpful. Our special thanks also to Luisa Ponte, Luis Borges and Liliana Pires, which gave their contribution during part of the experimental work.

Corresponding author:
Prof. Frederico Teixeira
Instituto de Farmacologia e Terapêutica Experimental
Faculdade de Medicina – Universidade de Coimbra
3004–504 Coimbra Portugal
Tel.: +351 239 857777
Fax.: +351 239 836200
E-mail: fredjt@ci.uc.pt or flavio@ci.uc.pt

REFERENCES

1. Lebovitz HE. Pathogenesis of type 2 diabetes. *Drug Benefit Trends* 2000; 12(suppl A): 8-16.
2. Calles-Escandon J, Garcia-Rubi E, Mirza S, Mortensen A. Type 2 diabetes: one disease, multiple cardiovascular risk factors. *Coronary Artery Dis* 1999; 10: 23-30.
3. Sowers JR, Epstein M. Diabetes mellitus and associated hypertension, vascular disease and nephropathy. *Hypertension* 1995; 26: 869-878.
4. Lopes-Virella MF, Virella G. Cytokines, modified lipoproteins and atherosclerosis in diabetes. *Diabetes* 1996; 45(supl. 3): S40-S44.
5. Sobel BE. Insulin resistance and thrombosis: a cardiologist's view. *Am J Cardiol* 1999; 84: 37-41.
6. Moreau P, Lamarche L, Laflamme AK, Calderone A, Yamaguchi N, Champlain J. Chronic hyperinsulinaemia and hypertension: the role of the sympathetic nervous system. *J Hypertens* 1995; 13: 333-340.
7. Reaven GM, Lithell H, Landsberg L. Hypertension and associated metabolic abnormalities: the role of insulin resistance and the sympathoadrenal system. *N Engl J Med* 1996; 334: 374-381.
8. Masuo K, Mikami H, Ogihara T, Tuck ML. Familial hypertension, insulin, sympathetic activity and blood pressure elevation. *Hypertension* 1998; 32: 96-100.
9. Esler M, Jennings G, Lambert G, Meredith I, Horne M, Eisenhofer G. Overflow of catecholamine neurotransmitters to the circulation: source, fate and functions. *Physiol Rev* 1990; 70: 963-985.
10. Paramore DS, Fanelli CG, Shah SD, Cryer PE. Forearm norepinephrine spillover during standing, hyperinsulinemia, and hypoglycaemia. *Am J Physiol* 1998; 275(38): E872-E881.
11. Chanberlain KG, Pestell RG, Best JD. Platelet catecholamine contents are cumulative

indexes of sympathoadrenal activity. *Am J Physiol* 1990; 259: 141-147.
12. Vanhoutte PM. Serotonin, hypertension and vascular diseases. *Neth J Med* 1991; 38: 35-42.
13. Oskarsson HJ, Hofmeyer TG. Platelets from patients with diabetes mellitus have impaired ability to mediate vasodilation. *J Am Coll Cardiol* 1996; 27: 1464-1470.
14. Nenci GG, Paliani U. Diabetes, coagulation and vascular events. *Recenti Prog Med* 2000; 91(2): 86-90.
15. Levy J. Abnormal cell calcium homeostasis in type 2 diabetes mellitus. *Endocrine* 1999; 10(1): 1-6.
16. Siess W. Molecular mechanisms of platelet activation. *Physiol Rev* 1989; 69: 58-178.
17. Van Breemen C, Saida K. Cellular mechanisms regulating [Ca^{2+}]i in smooth muscle. *Annu Rev Physiol* 1989; 51: 315-329.
18. Dominiczak AF, Bohr DF. Cell membrane abnormalities and the regulation of intracellular calcium concentration in hypertension. *Clin Sci* 1990; 79: 415-423.
19. Pollock WK, Sage SO, Rink TJ. Stimulation of Ca^{2+} efflux from fura-2-loaded platelets activated by thrombin or phorbol myristate acetate. *FEBS Lett*. 1987; 210: 132-136.
20. Body SC. Platelet activation and interactions with the microvasculature. *J Cardiovasc Pharmacol* 1996; 27(suppl 1): S13-S25.
21. White JG. Platelets and atheriosclerosis. *Eur J Clin Invest* 1994; 24: 25-29.
22. Pletscher. Platelets: use and limitations. *Experientia* 1988; 44: 152-155.
23. Born GVR. Platelets and neurobiological research. *Experientia* 1988; 44: 113-126.
24. Reis, F., Tavares, P., Teixeira, F. The distribution of catecholamines between plasma and platelets in cyclosporin A-induced hypertensive rats. *Pharmacol Res* 2000; 41: 129-135.
25. Reis, F., Tavares, P., Rito, L.C., Teixeira, H.M., Santos-Dias, J.D., Ferrer-Antunes, C.A., Mesquita, J.F., Teixeira, F. Platelet activation is increased in cyclosporin A-induced hypertensive rats. *J Cardiovasc Pharmacol* 2000; 36: 56-64.
26. Cardinal DC, Flower RJ. The electronic aggregometer: a novel device for assessing platelet behaviour in blood. *J Pharmacol Methods* 1980; 3: 135-158.
27. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 1985; 260:3440-3450.
28. Henry RR. Preventing cardiovascular complications of type 2 diabetes: focus on lipid management. *Clin Diabetes* 2001; 19(3): 113-120.
29. Bloomgarden ZT. Obesity, hypertension, and insulin resistance. *Diabetes Care* 2002; 25(11): 2088-2097.
30. Ueda E, Kuchii M, Hano T, Ueno Y, Jo Y, Shima H, Nishio I, Masuyama Y. Platelet noradrenaline content as an integrated measure of variations in plasma noradrenaline. *Jpn Circ J* 1990; 54: 383-390.
31. Stahl SM. Platelets as pharmacologic models for the receptors and biochemistry of monoaminergic neurons. In: Longenecker GL, eds. *The platelets: physiology and pharmacology*. London: Academic Press, 1985: 307-340.
32. Blandini F, Martignoni E, Sances E, Bono G, Nappi G. Combined response of plasma and platelet catecholamines to different short-term stress. *Life Sci* 1995; 56: 1113-1120.
33. Smith CCT, Curtis LD, Delamonthe AP, Prichard BNC, Betteridge DJ. The distribution of catecholamines between platelets and plasma in normal human subjects. *Clin Sci* 1985; 69: 1-6.
34. Bhagat B, Burke WJ, Dhalla NS. Insulin-induced enhancement of uptake of noradrenaline in atrial strips. *Br J Pharmacol* 1981; 74: 325-332.
35. Malyszko J, Urano T, Knofler R, Taminato A, Yoshimi T, Takada Y, Takada A. Daily variations of platelet aggregation in relation to blood and plasma serotonin in diabetes. *Thromb Res* 1994; 75(5): 569-576.
36. Barradas MA, Gill DS, Fonseca VA, Mihhailidis DP, Dandona P. Intraplatelet serotonin in patients with diabetes mellitus and peripheral vascular disease. *J Clin Investig* 1988; 18: 399-404.
37. Miranda JF, Alabadi JA, Lloréns S, Apodaca RFR, Centero JM, Alborch E. Experimental diabetes induced hyperreactivity of rabit renal artery to 5-hydroxytryptamine. *Eur J Pharmacol* 2002; 439: 121-127.
38. Pietraszek MH, Takada Y, Takada A, Fujita M, Watanabe I, Taminato A, Yoshimi T. Blood serotonergic mechanisms in type 2 (non-insulino-dependent) diabetes mellitus. *Thromb Res* 1992; 66: 765-774.

Títulos dos Autores:
Flávio Reis: Mestre em Biologia Celular e Doutorando em Ciências Biomédicas, Faculdade de Medicina da Universidade de Coimbra.
Maria Vítor Campos, Luísa Ruas: Especialistas em Endocrinologia, Hospitais da Universidade de Coimbra.
Filipe Palavra, Daniela Marado, Elisa Ribeiro: Estudantes da Licenciatura em Medicina, Faculdade de Medicina da Universidade de Coimbra.
Luís Almeida: Prof. Auxiliar da Faculdade Medicina da Universidade Coimbra.
Teresa Alcobia: Mestre em Saúde Pública, Faculdade Medicina Universidade Coimbra.
Margarida Lourenço, Carlos-Alberto Ferrer-Antunes, Aida Palmeiro: Médicos especialistas em Hematologia, Hospitais da Universidade de Coimbra.
Manuela Carvalheiro, Frederico Teixeira: Professores Catedráticos, Faculdade Medicina da Universidade Coimbra.