Thrombosis Research 133 (2014) 1097–1104

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

Platelet activation patterns are different in mouse models of diabetes and chronic inhibition of nitric oxide synthesis

Marcin Rozalski, Hassan Kassassir, Karolina Siewiera, Agnieszka Klepacka, Radoslaw Sychowski, Cezary Watala *

Department of Haemostasis and Haemostatic Disorders, Medical University of Lodz, Poland

ARTICLE INFO

Article history:
Received 20 December 2013
Received in revised form 26 February 2014
Accepted 26 March 2014
Available online 1 April 2014

Keywords:
Platelet
Diabetes
Db/db mice
Hypertension
Chronic inhibition of nitric oxide synthase
L-NAME

ABSTRACT

Currently, there are several animal models of diabetes mellitus and hypertension, but relatively little is known about blood platelet function in these models. The aim of this work was to characterise and compare platelet reactivity and activation in db/db mice (mouse model of diabetes) and mice receiving L-NAME (model of chronic inhibition of NO synthesis), using various platelet function assays. We found higher platelet activation (circulating resting platelets) in db/db mice than in db/+ heterozygotes, as evidenced by elevated expressions of CD62P and CD40L and a lower expression of CD42b. The expression of COX-1 was significantly increased, and the phosphorylation of vasodilator stimulated phosphoprotein (VASP) Ser^{157} significantly reduced in platelets from db/db mice. Similarly, we observed platelet hyperreactivity in db/db mice following the in vitro responses to 20 μg/ml collagen (reflected by increased expressions of CD62P and CD40L, and reduced CD42b), 20 μM ADP (reduced CD42b) and lower concentrations of thrombin (0.025 U/ml) (increased CD62P, JON/A, bound vWF, and bound fibrinogen). Otherwise, platelet hyporeactivity was revealed for higher thrombin (0.25 U/ml) (reduced CD62P and bound vWF), while hyperreactivity occurred for CD40L and bound Fg in db/db mice compared to non-diabetic control, db/+. Plasma levels of sCD40L, but not of sCD62P, were increased in db/db mice; also plasma TXB_2 concentrations were over 3.5-fold higher in this group than in the heterozygous db/+ mice ($P < 0.01$). In contrast, in the mice administered with L-NAME, no statistical differences in expressions of platelet activation markers were found between mice supplemented with L-NAME and controls. Likewise, the TXB_2 level did not differ between L-NAME mice and controls, but L-NAME mice had significantly higher plasma levels of sCD62P and sCD40L than controls. In conclusion, these two studied models differ in the overall picture of blood platelet activation and reactivity, as they demonstrated opposite time sequence patterns of platelet activation in circulating blood. More generally, our study provides another argument for the opinion that multiparametric analysis of platelet function offers a much better tool for investigation and minimizes the likelihood of artefacts.

Introduction

Many reports have described alterations in haemostatic function, including blood platelet abnormalities, in diabetes and hypertension. Both conditions are diseases of human civilization and their prevalence is rapidly increasing. In general, platelets from patients with diabetes tend to respond more frequently even to subthreshold stimuli and become exhausted, consumed and finally hyposensitive more quickly, contributing to accelerated thrombopoiesis and release of large 'fresh' and hyperreactive platelets [1 and references therein]. Several platelet abnormalities, also mainly characterised by platelet hyperreactivity [2], have been attributed to hypertension; they include increased mean platelet volume [3], decreased serotonin concentration [4], elevated fraction of platelet microparticles [5], higher expression of P-selectin [6], increased Ca^{2+} mobilization [7,8], and attenuation of NO bioavailability [9].

Animal models are useful for studying both diabetes and hypertension, including the haemostatic malfunctions associated with those disorders. Several rodent models of diabetes are available. The most commonly used are probably the models in which the disease is induced with chemotoxic agents, such as streptozotocin or alloxan. Although chemical induction is a convenient method for generating experimental diabetes, it can have unpredictable influences on the animal and directly damages other tissues [10]. One genetic knock-out model with features of human type 2 diabetes is the db/db obese mouse model. Animals with the db/db mutation have a premature stop codon inserted into the mRNA transcript of the leptin receptor, which

* Corresponding author at: Mazowiecka 6/8, 92-215 Lodz, Poland. Tel.: +48 42 2725720; fax: +48 42 2725730.
E-mail address: cezary.watala@umed.lodz.pl (C. Watala).

http://dx.doi.org/10.1016/j.thromres.2014.03.041
0049-3848/

ultimately leads to the production of an intracellular domain lacking the short form of the receptor, associated with functional changes leading to obesity, hyperinsulinemia and hyperglycaemia. Altogether this constellation of impairments leads to a chronic diabetic state resembling type 2 diabetes in humans [11,12].

Another interesting vascular model, in which platelet activation appears altered, is the model of hypertension induced pharmacologically in rats or mice by the systemic inhibition of nitric oxide synthase (NOS) [13–16]. Nitric oxide is a signalling molecule synthesized from L-arginine by NOS. Of the three known isoforms of nitric oxide synthase: endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) [16], eNOS is the predominant isoform expressed in the vascular endothelium, where it regulates platelet aggregation upon in vivo stimulation [17], although nNOS and iNOS also demonstrate anti-thrombotic effects. In the aforementioned animal model of hypertension, the enzyme (eNOS and nNOS) is chronically inhibited by administering L-NG-Nitroarginine Methyl Ester (L-NAME). This model is considered useful for understanding the role of NO and its effect on the status of blood platelet function. It can also be helpful for demonstrating differences in circulating blood platelet behaviour after stimulation *in vivo* when NO is lacking [18–21].

The aim of this work was to verify the hypothesis that circulating platelets are hyperactivated, i.e. demonstrate higher *in vivo* activation and priming and are more reactive, in both db/db mice and in the animals chronically administered with L-NAME than in the respective control animals. We analysed selected markers of platelet activation in circulating blood by flow cytometry *ex vivo* and also monitored platelet responses upon stimulation by agonists *in vitro*. We also measured three platelet markers in plasma, thromboxane B_2 (TXB_2) and the soluble forms of CD62P (sCD62P) and CD40L (sCD40L). These can be regarded as late indicators of episodes of platelet activation in the circulation [22–25].

Materials and Methods

Drugs and Chemicals

Anaesthetics: sedazin (20 mg/ml xylazine) and ketamine (100 mg/ml ketamine hydrochloride) were obtained from Biowet (Biowet, Pulawy, Poland). Low molecular weight heparin (LMWH) was from Sanofi Aventis (Paris, France). FITC- or PE-conjugated rat anti-CD41/61, PE-conjugated rat anti-CD62P, rat anti-CD42b/PE, PE-conjugated JON/A antibodies (rat anti-the active complex $\alpha_{IIb}\beta_3$), FITC-conjugated rat anti-von Willebrand factor and rat anti-fibrinogen antibodies were purchased from Emfret Analytics (Eibelstadt, Germany) and anti-CD40L antibody was purchased from BioLegend (San Diego, CA, USA). FITC-conjugated mouse monoclonal antibody against the phosphorylated VASP Ser-157 and appropriate blocking phosphopeptide (VASP assay) were purchased from NanoTools (Teningen, Germany). PE-conjugated mouse monoclonal anti-COX-1, the FITC-conjugated mouse monoclonal anti-COX-2 antibodies and the relevant blocking peptides were from *Cayman Chemicals (Ann Arbor, MI, USA)*. The Thromboxane B2 EIA Kit was from Cayman Chemicals (Ann Arbor, MI, USA). The mouse soluble P-selectin ELISA Kit and Mouse sCD40L Platinum ELISA were from Cusabio (Wuhan, P.R. China) and eBioscience (San Diego, CA, USA), respectively. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Animals

Db/db and Db/+Mice

Male C57BL/6 J strain BKS.Dock7(m)+/+Lepr(db)/J (n = 22) genetically diabetic mice (hereafter referred to as db/db and used as the animal model of type 2 diabetes) and their non-diabetic lean littermates (hereafter referred to as db/+) (n = 17), 20 weeks old at the time of blood collection/withdrawal, were provided by Charles Rivers Laboratory (Calco, Italy). The db/db mice were obese (55.6 ± 4.3 g in db/db *vs*. 28.5 ± 2.4 g in db/+) and characterized by significantly elevated markers of short- (blood glycaemia) and long-lasting hyperglycemia (see the '*Results*' section).

Mice with Inhibited NOS

Male C57BL/6 J mice (20 weeks old at the time of analysis) were purchased from The Jackson Laboratory (Lyon, France). The animals were randomly allocated to either the control or the treated group and were administered respectively with pure water or with 50 mg/kg L-NAME b.m. in the drinking water for eight weeks (200 ml water portions were changed every two days in order to ensure the stability of L-NAME solution). Several literature reports have pointed that owing to the chronic inhibition of NOS in this model, the mice develop hypertension [13,14]. The administration of L-NAME was not associated with body mass change (body mass in the range of 26–31 g, not different between groups).

Animal Anaesthesia, Blood Collection and Preparation

Animals were anaesthetized with an intramuscular injection of ketamine (100 mg/kg b.w.) and xylazine (23.32 mg/kg b.w.). Blood was collected from the inferior aorta on 10 U/ml LMWH in TBS buffer (20 mM Tris–HCl, 137 mM NaCl, pH 7.3), diluted (1:25) with modified Tyrode buffer (134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, pH 7.0, 5 mM glucose, 0.35% w/v bovine serum albumin) and centrifuged at 900 ×*g* for 5 min in 37 °C to obtain blood cells devoid of plasma. The pellet of blood cells was resuspended in 1.25 ml of modified Tyrode buffer and used in the study as 'washed blood'. Before the assay was started, $CaCl_2$ was added to the 'washed blood' to a final concentration of 1 mM. The separated plasma was used for further analysis of markers of platelet activation (soluble platelet membrane glycoproteins, thromboxane B_2). Prior to anaesthesia the animals were housed in an isolated room under a 12 h light–dark cycle and were given free access to water and standard chow for rodents (Altromin Maintenance Diet). All experiments were performed in accordance with the guidelines formulated by the European Community for the Use of Experimental Animals (L358-86/609/EEC) and the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85–23, revised 1985). All procedures used in these experiments were approved by local Ethical Committees on Animal Experiments (approval number 32/LB471/2009).

Flow Cytometry, Immunoenzymatic and Biochemical Analyses

Flow Cytometric Analysis of Platelet Surface Activation Markers

Platelets were gated on the basis of binding anti-CD41/61 antibodies. To analyse platelet activation the following platelet surface membrane markers were monitored: CD62P (P-selectin), activated GPIIb/IIIa complex, CD40L (CD154) and CD42b (GPIbα), binding of von Willebrand factor and binding of fibrinogen to platelets surface. With enhanced activation of circulating platelets and hyperreactivity we expected to reveal the increased expressions of the first three membrane antigens, reduced expression of the last one (CD42b) and elevated binding of released vWf and Fg to platelets. To ensure minimal overlap between the green and orange fluorescences, all flow cytometric measurements were fluorescence-compensated on a daily basis for each set of measured samples, based on the measurements of the reference samples stained with FITC-conjugated anti-CD41/61 or PE-conjugated anti-CD41/61 (constitutive antigens on the platelet surface). The laser parameters were set using BD Cytometer Setup and Tracking Beads before each measurement. Flow cytometric assays of platelet surface membrane antigens were performed within 30 min of staining on unfixed blood cells with light scatter (threshold 200) and FL1 fluorescence gates set on the platelet fraction. The green or orange fluorescence of the stained platelets was recorded for FITC (channel FL1 filter transmitting at 530 nm with a bandwidth of 30 nm) or PE (channel FL2 filter