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# Higher mitochondrial potential and elevated mitochondrial respiration are associated with excessive activation of blood platelets in diabetic rats

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ABSTRACT

*Aims:* The high glucose concentration observed in diabetic patients is a recognized factor of mitochondrial damage in various cell types. Its impact on mitochondrial bioenergetics in blood platelets remains largely vague. The aim of the study was to determine how the metabolism of carbohydrates, which has been impaired by streptozotocin-induced diabetes may affect the functioning of platelet mitochondria.
*Materials and methods:* Diabetes was induced in Sprague Dawley rats by intraperitoneal injection of streptozotocin. Platelet mitochondrial respiratory capacity was monitored as oxygen consumption (high-resolution respirometry). Mitochondrial membrane potential was assessed using a fluorescent probe, JC-1. Activation of circulating platelets was monitored by flow cytometry measuring of the expressions of CD61 and CD62P on a blood platelet surface. To determine mitochondrial protein density in platelets, Western Blot technique was used.
*Key findings:* The results indicate significantly elevated mitochondria mass, increased mitochondrial membrane potential ($\Delta\Psi m$) and enhanced respiration in STZ-diabetic animals, although the respiration control ratios appear to remain unchanged. Higher $\Delta\Psi m$ and elevated mitochondrial respiration were closely related to the excessive activation of circulating platelets in diabetic animals.
*Significance:* Long-term diabetes can result in increased mitochondrial mass and may lead to hyperpolarization of blood platelet mitochondrial membrane. These alterations may be a potential underlying cause of abnormal platelet functioning in diabetes mellitus and hence, a potential target for antiplatelet therapies in diabetes.


## 1. Introduction

Atherosclerosis is the most common complication in both type 1 [1] and type 2 diabetes mellitus [2]. It has been well established that blood platelets contribute to the progression of these pathological states [2]. The circulating platelets in diabetic patients often exhibit increased hypersensitivity, which indicates increased priming, as well as the enhanced reactivity in response to agonists [3, 4]. This may contribute to atheroma formation, resulting in micro- and macrovascular diseases, including coronary and cerebral arteries [5]. However, blood platelets are now known to be not only the mediators of thrombosis, but also to play a key role as immune cells [6] and can initiate and accelerate numerous vascular inflammatory conditions [7], including the early steps of atherosclerosis development [8]. A number of studies have demonstrated that platelet-linked inflammation seems directly related to the ability of platelets to secrete cytokines, chemokines, and immunoglobulins upon their stimulation [7]. Therefore, due to their dual role in the aetiology of atherosclerosis, blood platelets may be regarded as important targets, with potent diagnostic and therapeutic applications [8].

Despite the fact that high incidence of blood platelet dysfunctions is a well-known phenomenon in patients with diabetes mellitus [4], the list of possible mechanisms underlying these impairments is still far from complete, and the participation of mitochondria in this process remains clearly underestimated. This is a particularly important point, as the energy produced in mitochondria is required for platelet activation and aggregation [9–11], and hence, any changes in bioenergetics may lead to alterations in the functioning of whole cells [12].

It is commonly acknowledged that high glucose concentrations in diabetes constitute a recognized factor of mitochondrial damage in numerous cell types, such as skeletal muscle and heart muscle fibers, kidney and brain tissue, in both insulin-deficient and insulin-resistant states [13]. Therefore, the exposure of blood platelets to elevated amounts of glucose delivered to platelets may plausibly lead to changes in the functioning of platelet mitochondria, especially considering the fact that glucose is the main energy substrate for platelets, and about 50% of ATP is produced in platelets by metabolising pyruvate-dependent acetyl-coenzyme A in the tricarboxylic acid cycle [14]. Moreover, the insulin-independent type 3 glucose transporter, regarded as the main glucose transporter in blood platelets, has a relatively high Michaelis–Menten constant of 10 mM [15]. The combination of these phenomena, together with the increased activity of certain enzymes

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intimately associated with glycolysis and energy production commonly encountered in diabetes [16–18], can lead to elevated synthesis and accelerated metabolism of acetyl-CoA inside platelet mitochondria [16, 17, 19] and to increased production of adenine nucleotides under conditions of hyperglycaemia [19].

It is important to note that adenine nucleotides play an essential role in platelet functioning, including the maintenance of cell shape, release and secretion phenomena, platelet adhesion and aggregation [19, 20]. Elevated concentrations of intracellular ADP and ATP can also regulate cytosolic levels of free $Ca^{2+}$ [19, 21], which itself plays an essential role in blood platelet activation [22]. Importantly, accelerated substrate oxidation may also result in increased mitochondrial membrane potential and the overproduction of reactive oxygen species [23], known to be the by-products of increased activity of mitochondrial oxidative chain. These, in turn, may play the role of second messengers, stimulating phospholipase C activity and enhancing the arachidonic acid metabolism, thus further contributing to the overproduction of thromboxane $A_2$ and the more intense aggregation of hypersensitive platelets [24].

Despite widespread awareness of such a plethora of intra- and pericellular phenomena crucial for appropriate blood platelet functioning, there is still a paucity of validated evidence which would allow an evaluation of the possible associations between the energetic state of mitochondria and platelet activation, especially under diabetic conditions. Furthermore, the knowledge of platelet mitochondrial function in diabetes is extremely limited and the literature evidence concerning this topic is scarce. Therefore, the principal aim of the study was to determine the impact of the impaired carbohydrate metabolism, present in animal model of long-term experimental streptozotocin-induced diabetes on the functioning of blood platelet mitochondria. The monitoring of several parameters of mitochondrial functioning on one hand, and several hallmarks of blood platelet activation and reactivity on the other, elucidated the possible associations between mitochondrial bioenergetics and the functioning of platelets in diabetic animals.

## 2. Materials and methods

### 2.1. Chemicals

Reagents were purchased as indicated or were from standard sources. Streptozotocin, required to induce diabetes, as well as rotenone, oligomycin, FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone), antimycin A, protease inhibitor cocktail (P-8340) and the primers for qRT-PCR were purchased from Sigma-Aldrich (Steinheim, Germany). PE-labelled anti-CD61 gating antibodies were from Becton Dickinson (Erembodegem, Belgium) and FITC-labelled anti-CD62P antibodies was from eBioscience (San Diego, CA, USA). Immunoenzymatic kits for determining rat ATP synthase, $H^+$ transporting, mitochondrial $F_0$ complex (subunit F6), as well as rat soluble CD40L, were purchased from Cusabio (Wuhan, P.R. China). Anti-cytochrome *c* oxidase subunit VIb monoclonal antibodies for Western Blot were purchased from Abcam (Cambridge, United Kingdom). Antibodies against c-actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Peroxidase substrate for chemiluminescence enhancer was bought from Merck Millipore (Darmstadt, Germany). Blue X-Ray camera film for Kodak was from Carestream Health, Inc. (Rochester, NY, USA). JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide), the potentiometric dye for the determination of mitochondrial membrane potential was purchased from Molecular Probes (Invitrogen, Carlsbad, California, USA). Actrapid®, a short-acting human insulin solution for injections was from Novo Nordisk (Bangalore, India). Thromboxane $B_2$ enzyme-linked immunoassay kit was from Cayman Chemicals (Ann Arbor, MI, USA). Ketamine and xylazine for animal anaesthesia were purchased from Biowet-Pulawy (Pulawy, Poland). All other reagents and solvents used in this study were of the highest analytical reagent grade. Water used for the solution preparation and glassware washing was passed through an Easy Pure UF water purification unit (Thermolyne Barnstead, Dubuque, IO).

### 2.2. Animals

One hundred and fifteen two-month-old Sprague Dawley rats (males, 180–300 g) were randomly, non-proportionally allocated into two groups: control non-diabetic animals (45 rats) and diabetic animals (70 rats). In order to induce diabetes, the animals were intraperitoneally injected with streptozotocin (STZ) (dissolved in 0.1 M citrate buffer, pH 4.5) at the dose of 65 mg/kg b.w. (in 500 μl bolus) [25]. The control group (non-diabetic rats) received a vehicle (citrate buffer). The development of diabetes was diagnosed on the basis of non-fasting blood glucose concentration. The animals in which blood glucose level exceeded 300 mg/dl on the $7^{th}$ day following the STZ injection were considered STZ-diabetic. They were included in the study and their blood glucose was monitored roughly once a month.

Prior to the experiment, the animals were housed under standard environmental conditions (25 °C, with a light/dark cycle of 12 h/12 h) with continuous access to food (standard chow) and water. After 5 months of confirmed diabetes, the surviving individuals from both groups were anaesthetised with an intramuscular injection of ketamine (100 mg/kg b.w.) and xylazine (23.32 mg/kg b.w.) and their blood was collected for further analyses. In addition, a new group of 30 seven-month-old control (healthy, non-diabetic) rats were sacrificed to assess the impact of glucose and insulin on blood platelets from non-diabetic animals.

The experiments were conducted in accordance with the US National Institute of Health and the EU Directive 2010/63/EU for animal experiments.

### 2.3. Blood collection and preparation

Blood was collected from the abdominal aorta into a tube containing 3.2% sodium citrate (the final citrate:blood ratio 1:9 vol/vol). Blood plasma for measuring the generation of thromboxane $B_2$ ($TXB_2$) and the level of soluble CD40L (sCD40L, a marker of past platelet activation when circulating in the blood [26]) was obtained by blood centrifugation at 1000*g* for 15 min at 4 °C. In the oxygraphic part of the study, a suspension of intact platelets was used. Blood was centrifuged (190*g*, 12 min, 37 °C, in the presence 62.5 ng/ml prostaglandin E1) to obtain platelet-rich plasma (PRP). PRP was then centrifuged (2000*g*, 15 min, 37 °C) to isolate blood platelets; the platelet pellet was suspended in either Tyrode's 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) or platelet poor plasma (PPP, 2000*g*, 15 min, 37 °C) to achieve a suspension of $1 \times 10^8$ platelets/ml.

The platelet lysate was prepared for the quantitation of F0 subunit 6 of ATP synthase as follows. The platelet suspension ($4 \times 10^8$ cells/ml) was first centrifuged (2000*g*, 15 min, 37 °C). The pellet was resuspended in lysis buffer (100 mM Tris, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, pH 7.4 with 1% Triton X-100 and 0.5% sodium deoxycholate) and then incubated for 30 min on ice in the presence of the protease inhibitor cocktail (104 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), 80 μM aprotinin, 4 mM bestatin, 1.4 mM E-64, 2 mM leupeptin and 1.5 mM pepstatin A), which was added to the lysis buffer at a dilution of 1:100.

### 2.4. Biochemical measurements

Blood glucose was assessed at the termination of the experiment in all animals using standard diagnostic procedures, and the content of glycated haemoglobin (GHb) was measured using an Olympus AV 640 biochemical analyser. The concentration of sCD40L was measured in plasma using an immunoenzyme kit. To evaluate $TXB_2$ generation in plasma, the aliquot of anticoagulated whole blood was mixed for 1 h using the rotary coagulation mixer, and then supplemented with 0.5 mM acetylsalicylic acid to avoid non-specific artefactual $TXB_2$