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Altered platelet calsequestrin abundance, Na^+/Ca^{2+} exchange and Ca^{2+} signaling responses with the progression of diabetes mellitus

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ABSTRACT

Introduction: Downregulation of calsequestrin (CSQ), a major Ca^{2+} storage protein, may contribute significantly to the hyperactivity of internal Ca^{2+} ($[Ca^{2+}]_i$) in diabetic platelets. Here, we investigated changes in CSQ-1 abundance, Ca^{2+} signaling and aggregation responses to stimulation with the progression of diabetes, especially the mechanism(s) underlying the exaggerated Ca^{2+} influx in diabetic platelets.
Materials and methods: Type 1 diabetes was induced by streptozotocin in rats. Platelet $[Ca^{2+}]_i$ and aggregation responses upon ADP stimulation were assessed by fluorescence spectrophotometry and aggregometry, respectively. CSQ-1 expression was evaluated using western blotting.
Results: During the 12-week course of diabetes, the abundance of CSQ-1, basal $[Ca^{2+}]_i$ and ADP-induced Ca^{2+} release were progressively altered in diabetic platelets, while the elevated Ca^{2+} influx and platelet aggregation were not correlated with diabetes development. 2-Aminoethoxydiphenyl borate, the store-operated Ca^{2+} channel blocker, almost completely abolished ADP-induced Ca^{2+} influx in normal and diabetic platelets, whereas nifedipine, an inhibitor of the nicotinic acid adenine dinucleotide phosphate receptor, showed no effect. Additionally, inhibition of Na^+/Ca^{2+} exchange induced much slower Ca^{2+} extrusion and more Ca^{2+} influx in normal platelets than in diabetic platelets. Furthermore, under the condition of Ca^{2+}-ATPase inhibition, ionomycin caused greater Ca^{2+} mobilization and Ca^{2+} influx in diabetic platelets than in normal platelets.
Conclusions: These data demonstrate that platelet hyperactivity in diabetes is caused by several integrated factors. Besides the downregulation of CSQ-1 that mainly disrupts basal Ca^{2+} homeostasis, insufficient Na^+/Ca^{2+} exchange also contributes, at least in part, to the hyperactive Ca^{2+} response to stimulation in diabetic platelets.

Introduction

Platelet dysfunction characterized by enhanced Ca^{2+} signaling and aggregation in response to various stimuli is one of the critical pathological changes in diabetic patients [1–3]. As diabetes progresses, platelet hyperactivity develops and spontaneous platelet aggregation may take place in patients with an obvious metabolic disorder [4,5]. Importantly, deregulated platelet function predisposes diabetes patients to cardiovascular complications. However, the underlying mechanism(s) for the platelet hyperactivity and hyperaggregability in diabetes, as well as the identity of potential targets for its prevention are not fully understood. A number of Ca^{2+} effectors such as surface expression of store-operated Ca^{2+} channels (SOC) [6] and Ca^{2+}-ATPases in the plasma membrane (PM) and endoplasmic reticulum (ER) [3,7] have been found to be altered and therefore proposed as potential contributors to the disrupted Ca^{2+} signaling response to stimulation in diabetic platelets.

Calsequestrin (CSQ; subtypes CSQ-1 and CSQ-2), was first found in skeletal muscle in 1972 and is now well recognized as a critical Ca^{2+}-binding protein in the sarcoplasmic reticulum (SR). This Ca^{2+} storage protein is also expressed in ER, but its function in non-muscle cells is unknown [8,9]. In a previous study, we found that CSQ-1 participates in the regulation of Ca^{2+} signaling in platelets, but its expression is reduced and may contribute to the increased Ca^{2+} leak at rest and exaggerated Ca^{2+} release following stimulation in platelets from both diabetic patients and diabetic rats [9]. In the present study, we further address the issue of CSQ-1-associated disruptions in platelets during the progression of diabetes, particularly in relation to the deterioration of Ca^{2+} homeostasis that occurs during the course of diabetes. We also

Abbreviations: CSQ-1, calsequestrin-1; 2-APB, 2-aminoethoxydiphenyl borate; TG, tharpsigargin; SOC, store-operated Ca^{2+} channel; NCX, Na^+/Ca^{2+} exchanger; STZ, streptozotocin; NMDG, N-methyl-D-glucamine; KB-R7943, 2-[2-[4-(4-nitrobenzyloxy) phenyl] ethyl] isothiourea methane sulfonate; NAADP, nicotinic acid adenine dinucleotide phosphate receptor; SR, sarcoplasmic reticulum; ER, endoplasmic reticulum; PM, plasma membrane.

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sought to determine the possible mechanism(s) for the hyperactivity of Ca^{2+} influx following stimulation in diabetic platelets, which appears to be unrelated to the CSQ-1 dysfunction [9]. We used the model of streptozotocin (STZ)-induced type 1 diabetes in this study, because of its rapid diabetic progression with typical pathological changes and susceptibility to cardiovascular complications at early stages of the disease. We found that the gradual downregulation of CSQ-1 is in accordance with the progressive disruption of normal Ca^{2+} release. In addition, altered Na^{+}/Ca^{2+} exchange may also contribute to the Ca^{2+} hyperactivity and aggregation in diabetic platelets.

Materials and Methods

This study was approved by the Capital Medical University Animal Care and Use Committee, and all studies were conducted in accordance with the 'Guide for the Care and Use of Laboratory Animals' adopted by the Beijing government and 'Guide for the Care and Use of Laboratory Animals' published by the US National Institutes of Health (publication No. 85–23, revised 1996).

Materials

Fura2/AM was purchased from Molecular Probes (Invitrogen Inc, Shanghai, China). All the other chemicals, unless otherwise specified, were obtained from Sigma-Aldrich (Shanghai, China).

Induction of Diabetes

Male Sprague–Dawley rats (200 ± 10 g) were purchased from SLAC Laboratory Animal Co. Ltd. (Shanghai, China) and housed in temperature-controlled cages (22 to 24 °C) and given free-access to water and formulated food.

A single dose STZ (65 mg/kg, i.p.) regimen was used to induce pancreatic islet cells destruction and persistent hyperglycemia. In the following week after injection, rats with ≥16.67 mmol/L non-fasting blood glucose were considered diabetes and selected for this study. The body weight, blood glucose, basal blood pressure, platelet function and CSQ-1 expression of each animal from both healthy and diabetes groups were monitored until 12 weeks to assess the progression of diabetes. All the animals were randomized to be anesthetized with sodium pentobarbital (45 mg/kg, i.p.).

Platelet Preparation and Aggregation

As described previously [10], platelets were collected from each rat and the platelet pellet was prepared and washed twice with HEPES buffer saline solution (HBSS: composition in mmol/L: NaCl 145; KCl 5; $MgSO_4$ 1; HEPES 5; pH 7.4) containing 1 mmol/L EGTA and 0.1 U/mL apyrase. The platelet concentration was adjusted with HBSS to 1×10^8 cells/mL for the Ca^{2+} signaling examination and 3×10^8 cells /mL for aggregation measurement or stored at −80 °C for Western blot analysis.

For aggregation measurement, the washed platelet preparation was pre-warmed with HBSS containing 1 mmol/L Ca^{2+} at 37 °C for 5 minutes with constant stirring and agonist-induced aggregation response was measured with a four-channel PACK-4 aggregometer (Helena Laboratories) and reported as the peak height of aggregation.

Ca^{2+} Measurement

As previously described [10,11], $[Ca^{2+}]_i$ was monitored in platelets loaded with 1 μmol/L Fura-2/AM at 37 °C for 20 minutes. Changes in the Fura-2 fluorescence in the cell suspension were measured at excitation wavelengths of 340 nm and 380 nm and an emission wavelength of 510 nm using a fluorescence spectrophotometer (Hitachi, F7000) with constant stirring at 37 °C. The basal $[Ca^{2+}]_i$ was measured in resting platelets without any stimulation, while Ca^{2+} release and Ca^{2+} influx in response to ADP (0.1 mmol/L) were respectively measured in Ca^{2+}-free (0 Ca^{2+} and 0.1 mmol/L EGTA) medium for 5 minutes and then in 0.5 mmol/L Ca^{2+} HBSS medium for another 4 minutes. Changes in $[Ca^{2+}]_i$ were expressed as 340 nm/380 nm fluorescence ratio (F340 nm/380 nm).

Western Blotting

As described previously [10], aliquots of platelet suspension were lysed in RIPA buffer containing 2 mmol/L polymethylsulfonyl fluoride and 2 μg/mL protease inhibitor cocktail (Santa Cruz Biotechnology, CA) for 10 minutes on ice. Lysates were used for Western blotting analysis. Antibodies specific for CSQ-1(ABR, Thermo Fisher Scientific Inc. Rockford, IL) and β-actin (Santa Cruz Biotechnology, CA) at dilutions of 1:600 and 1:1000 were used overnight at 4 °C, respectively. The horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, CA) were diluted at 1:3,000.

Statistical Analysis

Data are means ± SEM of seven to nineteen independent measurements. Statistical comparisons between groups were carried out with a two-tailed Student *t*-test. $P < 0.05$ was considered statistically significant.

Results

Diabetes Induction

At the beginning of the experiment, animal body weight and blood pressure were comparable in all groups. Two weeks after STZ injection, most rats demonstrated hyperglycemia in both fasting and non-fasting tests, and rats with ≥16.67 mmol/L non-fasting blood glucose level were assigned to the type 1 diabetes group. As shown in Fig. 1, control rats maintained their weight and retained normal basal blood pressure throughout the 12-week experiment, while STZ-treated rats failed to gain weight normally from the 4th week after the appearance of hyperglycemia. In addition, STZ-treated rats developed hypertension from the 8th week that was worse by the 12th week (Fig. 1C, D). Cataracts and even death occurred in some of the diabetic animals (data not shown). Thus, the data demonstrated a gradual progression of type 1 diabetes during the 12-week time course in the STZ-treated rats.

Status of CSQ Expression in Platelets During Diabetes Progression

By normalization with β-actin, the relative abundance of CSQ-1 protein was determined in platelets from control and STZ rats at time points of 2, 4, 8 and 12 weeks after the occurrence of hyperglycemia. As shown in Fig. 2A, CSQ-1 expression was reduced in diabetic platelets compared with that in the age-matched controls. By normalizing with control CSQ-1, a tendency of CSQ-1 reduction was observed as the diabetes progressed (Fig. 2B, C), suggesting a progressive reduction in platelet CSQ-1 expression or a progressive increase in its degradation associated with development of type 1 diabetes. This result may indicate a potential cause for the deterioration of Ca^{2+} signaling that is observed in the development of diabetes.

Ca^{2+} Homeostasis and Aggregation Changes in Platelets During Diabetes Progression

Ca^{2+} signaling and aggregation responses were assessed and compared between normal and diabetic platelets by measurements of the basal $[Ca^{2+}]_i$, and ADP (0.1 mmol/L)-induced Ca^{2+} release, Ca^{2+} influx and aggregation (Fig. 3H). Basal $[Ca^{2+}]_i$ was measured in resting