Original Paper

Enhanced Store-Operated Calcium Entry in Platelets is Associated with Peripheral Artery Disease in Type 2 Diabetes

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Key Words

Platelets • Store-operated calcium entry • Peripheral artery disease • Type 2 diabetes

Abstract

Background/Aims: Platelet dysfunction plays an important role in thrombosis in diabetes with peripheral artery disease (PAD). Store-operated calcium entry (SOCE) and stromal interaction molecule 1 (STIM1) regulate platelet activity by modulating calcium influx. We hypothesized that enhanced SOCE in platelets is associated with diabetes with PAD. ***Methods:*** We studied the activity of platelets from healthy participants and from type 2 diabetic patients. Platelet calcium influx and protein expression of STIM1 and sarcoendoplasmic reticulum Ca^{2+}-ATPase 3 (SERCA3) were investigated. ***Results:*** Compared with platelets from diabetic patients without PAD, platelets from diabetic patients with PAD exhibited significantly increased SOCE . Menthol administration completely inhibited calcium influx in platelets from diabetic patients without PAD, but this effect was blunted in those from diabetic patients with PAD. Furthermore, the increase in SOCE was correlated with the ankle brachial index (ABI) in diabetic patients. High glucose significantly up-regulated STIM1 and SERCA3 protein expression and induced the phosphorylation of phospholipase C (PLC) in platelets from healthy participants. This effect was attenuated in the presence of menthol or U73122, an inhibitor of PLC. Similarly, significant increases in STIM1 and SERCA3 protein expression were found in platelets from diabetic patients compared to those from healthy participants. ***Conclusion:*** Platelets from diabetic patients with PAD exhibited enhanced Store-operated calcium influx, which was associated with elevated STIM1/SERCA3 expression via a PLC-dependent pathway and was inhibited by menthol.

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Introduction

Type 2 diabetes mellitus (DM) frequently leads to cardiovascular complications, including peripheral artery disease (PAD). Platelet dysfunction is closely associated with thrombosis in type 2 diabetes with PAD. The ankle brachial index (ABI) is a noninvasive index used to evaluate PAD. Platelets from diabetic patients are known to exhibit hyperaggregability and hyperactivity, which is closely related to thrombosis [1, 2]. Platelets are non-electrically excitable cells, and store-operated calcium entry (SOCE) is a major mechanism for calcium influx in platelets. Ca^{2+} mobilization, especially Ca^{2+} entry, is a crucial platelet activation-associated event in diabetic patients [3-6]. Stromal interaction molecule 1 (STIM1) has been identified as a Ca^{2+} sensor and is located near Ca^{2+} stores. STIM1 is a transmembrane protein that communicates information about the amount of stored Ca^{2+} to plasma membrane channels and regulates platelet function [7]. Sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA) is a membrane protein that functions to pump cytosolic Ca^{2+} into intracellular stores against a steep concentration gradient. Human platelets express several SERCA protein isoforms [8]. Platelet activation depends on phospholipase C (PLC) activity that is triggered by the stimulation of G protein-coupled-receptor-mediated calcium release from the endoplasmic reticulum (ER). Platelet activation has been shown to contribute to the development of thrombosis [9-11], and several drugs that antagonize platelet hyperactivity and hyperaggregability are currently used in clinic. Skepinone-L, a novel and highly selective inhibitor of p38 mitogen-activated protein kinase (p38 MAPK), impairs platelet activation and thrombus formation [12]. Balhimycin, the most widely used glycopeptide antibiotic, induces platelet apoptosis by increasing cytosolic Ca^{2+} levels [13], and vancomycin exposure leads to platelet activation and apoptosis [14]. It is intriguing to explore whether functional dietary factors can improve platelet dysfunction in diabetes with PAD. Menthol, a natural compound, is responsible for the minty flavor and smell of the mint plant and is widely used in food and drugs. TRPM8 is a cold-sensitive channel that senses cool temperatures (<28°C) and is activated upon menthol application [15]. A recent study showed that menthol, independent of TRPM8 activity, modifies PLC signaling, which then leads to changes in cellular functions [16]. We also showed that high glucose levels are associated with increased platelet TRPC6 channel function and that this association is mediated by a phosphatidylinositol 3-kinase (PI3K)-dependent pathway [2]. However, the relationship between the functional status of patients with PAD and platelet dysfunction as well as whether menthol can improve platelet activation in diabetes remains elusive. In this study, we tested the hypothesis that enhanced store-operated calcium influx is associated with a STIM1/PLC pathway in platelets from diabetic patients with PAD and that platelet activation can be improved by menthol.

Materials and Methods

Study participants

Adult participants (45-75 years old) were recruited to our center in Daping Hospital in Chongqing, China. Healthy volunteers were screened with qualified medical examinations in our hospital. The participants were not taking any medications during this period and had normal blood pressure (BP) and fasting blood glucose (FBG). Blood samples were collected from the participants in the morning by venipuncture. Blood glucose and lipid parameters were measured in the Chongqing Institute of Hypertension. BP was obtained on three occasions while the participants were in a sitting position and following a rest of 10 min. Hypertension was defined according to the guidelines of WHO/ISH32. A diagnosis of hypertension was based on systolic BP of at least 140 mmHg or a diastolic (Korotkoff phase V) BP of at least 90 mmHg, as obtained by conventional sphygmomanometer methods [17]. Type 2 DM was defined as FBG ≥ 126 mg/dl or HbA1c > 6.5% or ongoing treatment for DM. The participants were not pregnant and had no other major defects in other systems or organs. All subjects provided written informed consent, and this clinical study was also conducted in accordance with the NIH Human Subjects Policies and Guidance. The research protocol was approved by the ethics committee of the university hospital.

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Measurement of ankle brachial index (ABI) and toe brachial index (TBI)

ABI and TBI are commonly used to evaluate the functional status of the peripheral arteries in the clinic. PAD was defined as an ABI of less than 0.90. The absence of PAD was defined as an ABI between 0.90 and 1.30 [18]. All measurements were performed after at least 30 min of acclimatization in a room that was maintained at a temperature of 23–25°C, with the subject in a supine position. The ABI was determined as the ratio of ankle systolic BP to brachial systolic BP, with both BPs determined using an automatic Omron VP-2000/1000 device (Omron Healthcare, Kyoto, Japan).

To measure toe pressure, a 25-mm digital cuff was placed around the base of the great toe, and a photo-transducer was taped to the pulp of the toe. The cuff was inflated to a maximum of 200 mmHg and then slowly deflated. Using a plethysmograph (EC-5R plethysmograph; DE Hokanson, Bellevue, WA), the point at which the arterial wave form reappeared was defined as the toe systolic BP. The toe pressure was reported as the mean of three measurements. Similarly to the ABI, the TBI was calculated as the ratio of toe systolic BP to brachial systolic BP. The higher of the left and right pressures was used as the brachial pressure when calculating the ABI and TBI. All measurements were performed by an appropriately trained physician [19].

Platelet preparation and fluorescence measurements

Platelets were obtained from normotensive (NT) control subjects or patients with type 2 DM. Fresh blood was centrifuged at 1300 rpm for 10 min to produce PRP. The PRP was then centrifuged at 3000 rpm for 15 min, washed twice and resuspended in Hanks balanced salt solution containing (in mmol/L) 136 NaCl, 5.40 KCl, 1 $CaCl_2$, 0.44 KH_2PO_4, 0.34 Na_2HPO_4, and 10 HEPES, pH 7.4, and incubated with 1 µmol/L of the fluorescent dye 1-[2-(5-Carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methylphenoxy)-ethane-N,N,N'N'-tetraacetic acid acetoxymethylester (fura2-AM; Merck Biosciences) for 60 min. After the solution was centrifuged at 3000 rpm for 15 min to remove extraneous dye, the platelet pellet was again resuspended in Hanks balanced salt solution. The platelet counts were adjusted to 1×10^8 platelets/mL [2].

Fluorescence measurements were performed as previously described by our group using a 96-well fluorescence plate reader (Fluoroskan Ascent Fluorometer, Thermo LabSystems Oy, Helsinki, Finland) with an emission wavelength of 510 nm and excitation wavelengths of 340 nm and 380 nm. The 340 nm/380 nm fluorescence ratio was then calculated [2].

Statistics

All data are presented as the mean ± SEM of at least 15 to 20 independent experiments and were compared using two-tailed Student's t-test or ANOVA as appropriate. Relations between variables were investigated using Spearman correlation. Two-sided P values below 0.05 were considered to indicate statistical significance.

Results

Patient characteristics

The clinical and biochemical characteristics of the NT participants, the patients with type 2 DM and the hypertensive diabetic (HDM) patients are presented in Table 1. There were no significant differences in the mean age or gender ratio between the three groups (P > 0.05). However, the BP levels, FBG, postprandial blood glucose (PBG), high-density lipoprotein (HDL), ABI, TBI, waist circumference (WC) and body mass index (BMI) were significantly higher in the diabetic patients compared to the NT participants (P < 0.05 - 0.01).

Store-operated calcium influx was increased in platelets from diabetic patients

Compared with that in platelets from NT participants, calcium influx was significantly increased in platelets from diabetic patients with or without hypertension in the absence of extracellular calcium after the discharge of intracellular Ca^{2+} stores was induced by thapsigargin (TG 1 µM) [increased fluorescence ratio, 0.43 ± 0.03 in the DM group (n = 20) and 0.63 ± 0.05 in the HDM group (n = 17) versus 0.32 ± 0.02 in the NT group (n = 23); P < 0.05 and P < 0.001, respectively; Fig. 1A-1D]. Compared to that in platelets from the NT

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Table 1. Baseline characteristics of the enrolled participants

Characteristic	NT (n=25)	DM (n=28)	HDM (n=25)
Age (Years±SD)	67.25±8.62	69.60±9.87	68.33±11.50
Male (n, %)	41.26%	40.88%	39.58%
Blood Pressure (mmHg)±SD			
SBP	114±9	114±12	147±23**
DBP	72±7	77±14	79±16
Plasma Glucose (mmol/L)±SD			
Fast	5.20±0.52	6.53±1.73**	7.69±3.11**
Postprandial	6.90±1.53	10.30±4.08*	17.95±6.61**
Triglycerides (mmol/L)	1.24±0.89	1.55±1.21	1.50±1.11
Cholesterol (mmol/L)±SD			
Total	4.56±1.05	4.42±1.24	4.65±1.52
HDL-C	1.42±0.19	1.08±0.33**	1.05±0.28**
LDL-C	2.53±0.76	2.96±0.88	2.91±0.68
Hepatic enzymes (IU/l)±SD			
Aspartate aminotransferase	23.26±14.12	22.65±15.06	19.92±8.10
Alanine aminotransferase	19.72±10.31	21.35±7.43	20.22±7.21
Uric acid (μmol/L)±SD	316.5±102.4	319.6±91.42	313.5±101.7
Blood urea nitrogen (mmol/L) ±SD	5.32±1.92	6.45±1.89	6.75±3.60
Serum creatine (μmol/L)±SD	75.86±6.67	86.15±16.65	90.49±10.91
Obesity-related parameters±SD			
BMI (kg/m²)	24±3.47	24±3.53	26±2.97**
Waist circumference (cm)	80±10	80±8	84±12*#
Vacular functions±SD			
ABI	1.13±0.08	0.93±0.12	0.82±0.26**##
TBI	0.95±0.14	0.74±0.39	0.62±0.27**##

*$P< 0.05$, or **$P< 0.01$, Vs NT; #$P< 0.05$, or ##$P<0.01$, Vs DM.

participants, the store-operated calcium influx was increased by 34% in the platelets from the DM patients and was increased by 97% in those from the HDM patients.

Menthol inhibited store-operated calcium influx in platelets from diabetic patients
Menthol administration significantly reduced store-operated calcium influx in platelets [increased fluorescence ratio, 0.35 ± 0.04 in the DM platelets (n = 20); 0.38 ± 0.06 in the HDM platelets (n = 17)]. However, the store-operated calcium influx were no significantly difference in the HDM patients or DM patients compared with NT participants (Fig. 1E).

Effect of menthol on elevated store-operated calcium influx in platelets from diabetic patients
To determine whether store-operated calcium influx in platelets was associated with the functional status of the diabetic patients with PAD, we divided the diabetic patients into two subgroups according to their ABI and TBI values. The increased store-operated calcium influx in the platelets from the diabetic patients with ABI < 0.9 was significantly greater than that in those from the diabetic patients with ABI ≥ 0.9. The store-operated calcium influx in the platelets from the diabetic patients with an ABI ≥ 0.9 was only 43% greater than that in those from the NT participants (Fig. 2A), but the store-operated calcium influx was 112% greater in platelets from the diabetic patients with an ABI < 0.9 (Fig. 2B). Similarly, the store-operated calcium influx was increased by 34% in the platelets from the diabetic patients with a TBI ≥ 0.7 (Fig. 2C) and by 104% in those from the diabetic patients with a TBI < 0.7
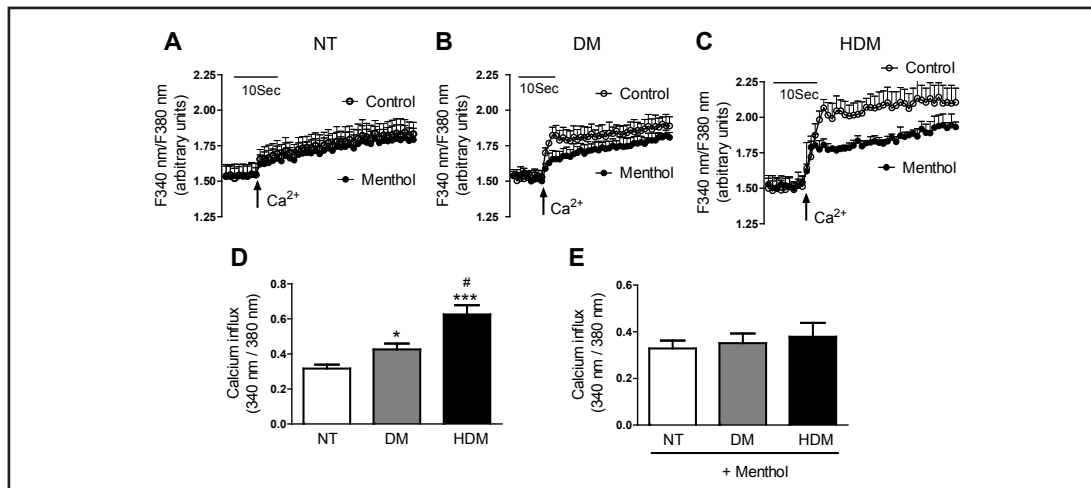
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Fig. 1. Increased store-operated calcium influx in platelets from patients with type 2 diabetes mellitus. Store-operated calcium influx in platelets from (A) healthy control individuals (NT), (B) patients with type 2 diabetes mellitus (DM) and (C) diabetic patients with hypertension (HDM) in the absence of extracellular calcium after the induction of the discharge of intracellular Ca^{2+} stores by thapsigargin (TG 1 μM). Platelets were treated with menthol (300 μM, filled circles or bars) or remained under control conditions (open circles or bars). (D, E) Summary data for the NT, DM and HDM groups. *P < 0.05 or ***P< 0.001 compared to the NT group. # P < 0.05 compared to the DM group.
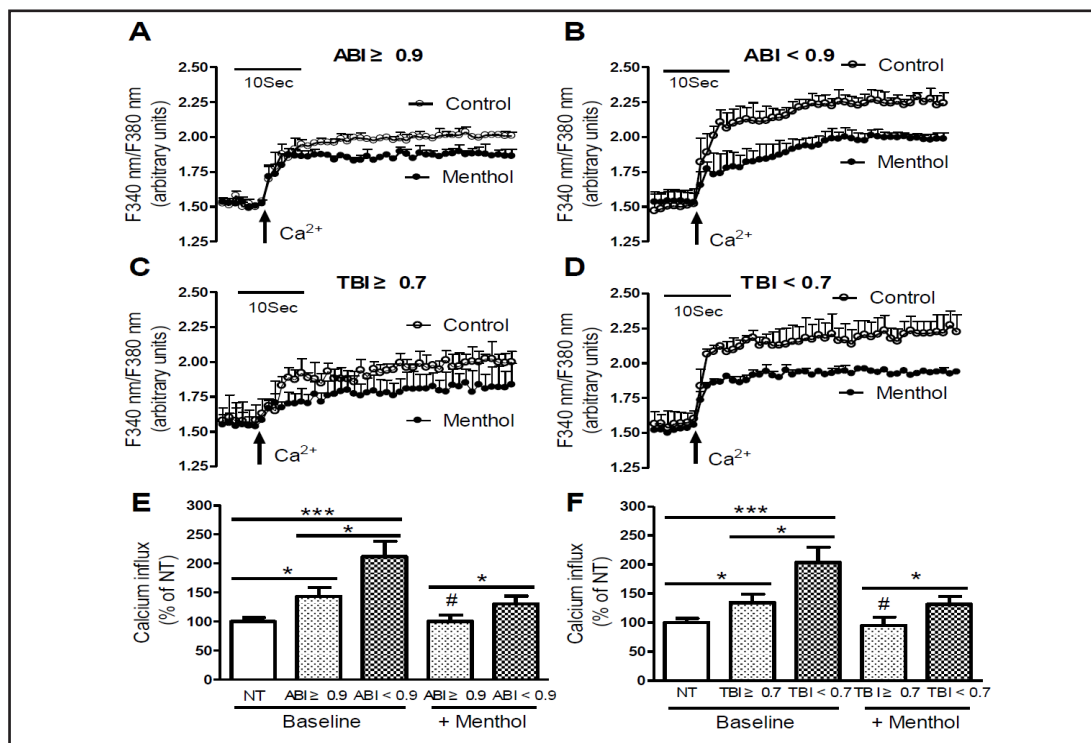

Fig. 2. Menthol inhibition of elevated store-operated calcium influx in platelets from diabetic patients. Store-operated calcium influx in platelets from patients with type 2 diabetes mellitus (DM) and from diabetic patients with hypertension (HDM) with an ABI ≥ 0.9 (A), an ABI < 0.9 (B), a TBI ≥ 0.7 (C), or a TBI < 0.7 (D) in the absence of extracellular calcium after the induction of the discharge of intracellular Ca^{2+} stores by thapsigargin (TG 1 μM). Platelets were treated with menthol (300 μM, filled circles or bars) or remained under control conditions (open circles or bars). (E, F) Summary data. *P < 0.05 or ***P < 0.001 compared to the indicated groups. # P > 0.05 compared to the baseline of the NT group.
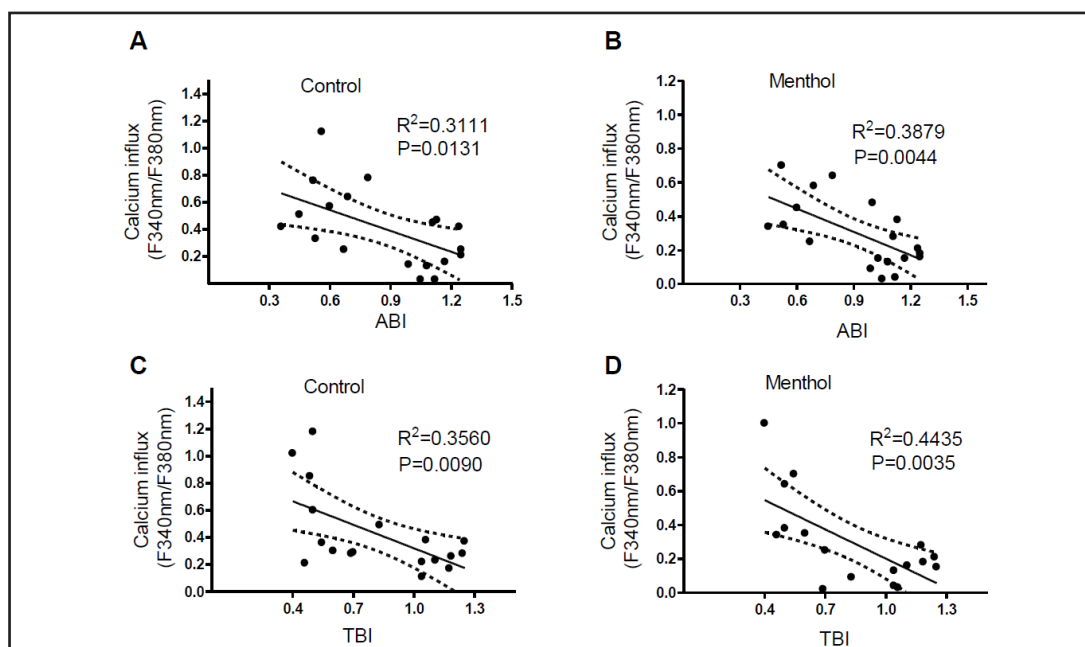
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Fig. 3. Thapsigargin (TG)-induced store-operated calcium influx in platelets is closely correlated with ABI and TBI values in diabetic patients. Relationships between increased store-operated calcium influx and ABI (A, B) and between increased store-operated calcium influx and TBI (C, D) under control conditions (left panels) or in the presence of menthol (right panels). Correlations with significant difference as $P < 0.05$.

(Fig. 2D). Furthermore, menthol completely inhibited calcium influx in the platelets from the diabetic patients with an ABI ≥ 0.9 or TBI ≥ 0.7 but only partially inhibited the influx in those from the diabetic patients with an ABI < 0.9 or TBI < 0.7 (Fig. 2E, 2F).

Correlation between the increase in store-operated calcium influx in platelets and the functional status of diabetic patients with PAD

Importantly, the increased store-operated calcium influx in platelets was significantly negatively correlated with both ABI and TBI values in the diabetic patients with or without hypertension [ABI: $r^2 = 0.3111$, P = 0.0131, Fig. 3A; TBI: $r^2 = 0.3560$, P = 0.009; Fig. 3C]. Even in the presence of menthol, this correlation remained [ABI: $r^2 = 0.3879$, P = 0.0044, Fig. 3B; TBI: $r^2 = 0.4435$, P = 0.0035; Fig. 3D].

Menthol inhibited high glucose-mediated calcium entry in platelets from diabetic patients through a STIM1/SERCA3/PLC-dependent pathway

We next studied which mechanism was responsible for the effects of menthol on calcium entry in the platelets from the diabetic patients. Following the administration of high levels of glucose, the increases in STIM1 and SERCA3 protein expression were greater in the platelets from the diabetic patients compared with those from the NT participants (Fig. 4A, 4B). In the platelets from the NT participants, the administration of high glucose not only elevated STIM1 and SERCA3 expression but also increased the phosphorylation of PLC after the discharge of intracellular Ca^{2+} store was induced by thapsigargin, and this effect of glucose was both dose- and time-dependent (Fig. 4C, 4D). Furthermore, the administration of menthol or U73122, an inhibitor of PLC, reversed these effects of high glucose in platelets (Fig. 4E). In the platelets from the diabetic patients, the protein expression of STIM1 and SERCA3 and the phosphorylation of PLC were significantly higher than the corresponding measures in the control platelets. Menthol or U73122 administration completely inhibited the enhanced phosphorylation of PLC in the platelets from the diabetic patients (Fig. 4F).
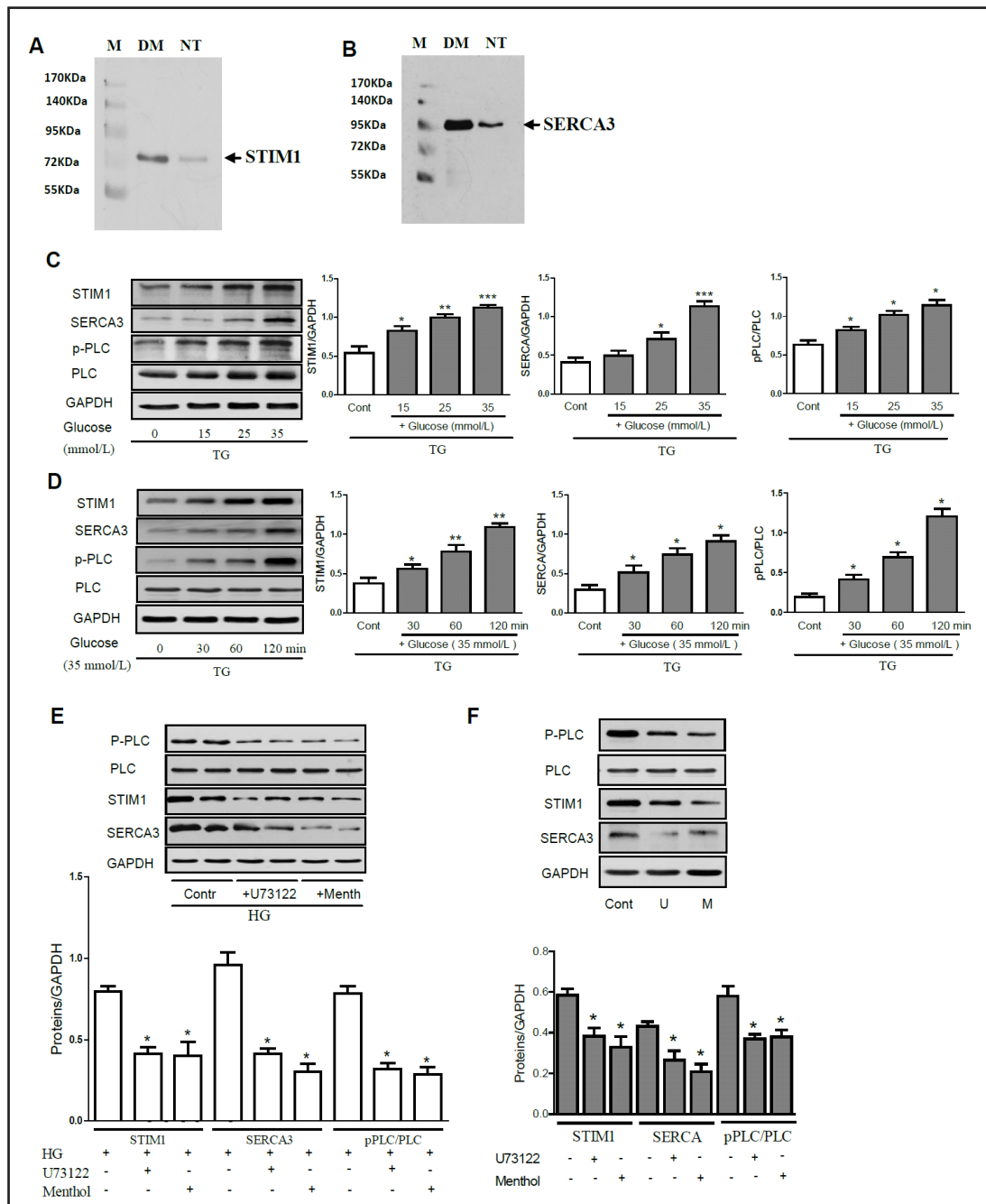
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Fig. 4. Menthol inhibition of the high glucose-induced increase in STIM1 and SERCA3 expression in platelets is mediated by a PLC-dependent pathway. (A) STIM1 and (B) SERCA3 protein levels were detected in platelets from healthy control individuals (NT) or patients with type 2 diabetes mellitus (DM) by immunoblotting. M, marker. (C) Dose-dependent and (D) time-dependent effects of high glucose on STIM1 and SERCA3 protein expression and on the phosphorylation of PLC in platelets from healthy controls in the presence of thapsigargin (TG 1 µM). Summary data show *P < 0.05, ** P < 0.01, or *** P < 0.001 compared to the corresponding control. (E) STIM1 and SERCA3 protein expression and PLC phosphorylation were detected by immunoblotting in platelets from healthy controls following pretreatment with high glucose (HG, 35 mmol/L) and in the presence of thapsigargin (TG 1 µM) and menthol (300 µM) or U73122 (10 µM). (F) STIM1 and SERCA3 protein expression and PLC phosphorylation were detected by immunoblotting in platelets from patients with type 2 DM in the presence of thapsigargin (TG 1 µM) and menthol (300 µM; M) or U73122 (10 µM; U). Data are presented as the mean ± SEM. *P < 0.05 compared to the corresponding control.

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Discussion

Platelet hyperactivity and hyperaggregability play a crucial role in thrombosis in type 2 DM. Altered platelet Ca^{2+} homeostasis is associated with thrombosis, but the underlying mechanisms remain elusive. In this study, we demonstrated that store-operated calcium influx was elevated in platelets from diabetic patients with PAD. Furthermore, the increased store-operated calcium influx in the platelets was correlated with the functional status of the diabetic patients with PAD. High glucose increased STIM1 and SERCA3 protein expression, but the administration of U73122 or menthol reversed the inhibitory effect of high glucose on the phosphorylation of PLC. Our study showed that enhanced store-operated calcium influx in platelets is associated with a STIM1/SERCA3/PLC–dependent pathway and can be inhibited by menthol in patients with type 2 DM.

Ca^{2+} release from intracellular stores and/or the induction of Ca^{2+} entry increases the levels of free calcium in platelets, which leads to platelet hyperactivity and hyperaggregability. Previous studies have shown that $[Ca^{2+}]i$ is elevated in platelets from diabetic patients, but the underlying mechanisms have not yet been clearly identified, and some conflicting results have been reported. Platelets easily adhere to the vascular endothelium and aggregate more readily in diabetic patients than in healthy subjects. Hyperglycemia can alter intracellular calcium homeostasis and increase SOCE, which is associated with platelet hyperactivity in diabetic patients [20]. In platelets from diabetic patients, SOCE is more sensitive to extracellular glucose concentrations [21]. In diabetic patients, pretreatment of platelets with catalase, an analog of vitamin E, has been shown to reverse the enhanced Ca^{2+} entry that is evoked by the administration of thapsigargin in conjunction with ionomycin or thrombin [22]. The activation of platelets stimulates the translation of Orai1, which augments platelet Ca^{2+} signaling [23]. Thrombin-mediated platelet activation is associated with rapid depolymerization of F-actin prior to the release of Ca^{2+} from intracellular stores and the induction of Ca^{2+} entry [24]. Platelet activation and migration as well as thrombus formation are related to PI3K/Akt signaling [25-27]. We and others have reported that PI3K-mediated stimulation of platelets through the TRPC6 channel is involved in platelet activation in diabetic patients [2, 28]. Plasma membrane-localized STIM1 plays a role in the regulation of SOCE by changing the extracellular calcium level. In human platelets, the activity of both the plasma membrane-associated Ca^{2+}-ATPase and SERCA depends on the initial rate of calcium influx [29]. Treatment of platelets with the SERCA inhibitor thapsigargin induced Mn^{2+} entry that was inhibited by extracellular Ca^{2+} in a concentration-dependent manner via a process that likely involved Orai1 [30]. Continuous increases in vitamin D_3 plasma levels have also been shown to decrease NF-κB-dependent STIM1/Orai1 expression and to influence platelet cytosolic Ca^{2+} concentrations, platelet activation, and thrombus formation [31]. Furthermore, the activation of cation channels such as the Ca^{2+} channel Orai1 and Ca^{2+}-activated K^+ channels plays a role in the regulation of platelet function [32, 33]. Thapsigargin-induced Ca^{2+} entry results from the activation of the STIM1/Orai1, showing an involvement of PLC/PKC pathway in SOCE in human endothelial cells [34]. A recent study demonstrated that impaired ER Ca^{2+} refilling in coronary endothelial cells (ECs) from diabetic mice was related to decreased STIM1 protein expression and attenuated endothelium-dependent relaxation in diabetic coronary arteries [35]. In addition, TRPC family members are also involved in SOCE, although their role remains controversial.

PAD is a major complication in diabetic patients [1, 2]. ABI and TBI are commonly used to measure functional abnormalities in small and large arteries in diabetes. Decreases in ABI and TBI in patients indicate abnormal blood flow in larger and smaller arteries, which has been shown to predict cardiovascular morbidity [36]. Few studies have investigated the interaction between platelet Ca^{2+} mobilization and the functional status of the peripheral arteries. In this study, we found a significant negative correlation between increased store-operated calcium influx in platelets and reduced ABI or TBI in diabetic patients. Although drug-based antiplatelet therapy is important to treat thrombotic diseases, effective dietary factors would be an optimal way to prevent thrombosis in the general population that is

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exposed to a high risk of thrombosis [37, 38]. Menthol, a compound produced by the peppermint herb, is widely used in the food and pharmaceutical industries as a cooling/ soothing agent and odorant. The cold-sensitive channel TRPM8 senses cool temperatures (<28°C) and is specifically activated by menthol [2, 3]. Menthol induces Ca^{2+} release from intracellular stores in some cell lines, such as HEK293 cells, lymph node carcinoma of the prostate (LNCaP) cells, and Chinese hamster ovary (CHO) cells. Several lines of evidence indicate that the menthol-mediated pathway is TRPM8-independent [15]. Our previous study showed that chronic dietary menthol confers vascular benefits and reduces high BP as well as preventing the development of obesity via TRPM8 activation [39, 40]. This study demonstrated that the inhibitory effect of menthol on store-operated calcium influx in platelets was impaired in diabetic patients with PAD. We further demonstrated that menthol reduced the enhanced store-operated calcium influx by inhibiting the phosphorylation of PLC in platelets from diabetic patients based on the following evidence: (1) platelets that were pretreated with menthol or U73122, a PLC inhibitor, reduced the increase in store-operated calcium influx in platelets from diabetic patients; (2) high glucose enhanced STIM1/SERCA3 protein expression and induced the phosphorylation of PLC in platelets from healthy subjects in a dose- and time-dependent manner, and these effects could be reversed by menthol; (3) menthol administration inhibited the enhanced STIM1/SERCA3 protein expression that was associated with the PLC pathway in platelets from diabetic patients. These results provide insight into the beneficial effects of menthol in diabetic patients with PAD. Improving platelet dysfunction through chronic menthol treatment may represent a promising way to prevent thrombotic disease in diabetes.

In summary, this study demonstrated that in platelets from diabetic patients with PAD, the STIM1/SERCA3-mediated increase in store-operated calcium influx is associated with activation of the PLC pathway. We also showed that menthol administration can antagonize platelet activation in diabetic patients.

Acknowledgements

Disclosure Statement

No potential conflicts of interest were disclosed.

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