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Kv1.3 potassium channel mediates macrophage migration in atherosclerosis by regulating ERK activity

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ABSTRACT

Ion channels expressed in macrophages have been tightly related to atherosclerosis by coupling cellular function. How the voltage-gated potassium channels (Kv) affect macrophage migration remain unknown. The aim of our study is to investigate whether Kv1.3-ERK signaling pathway plays an important role in the process. We explored the expression of Kv1.3 in coronary atherosclerotic heart disease and found Kv1.3 channel was increased in acute coronary syndrome patients. Treatment of RAW264.7 cells with Kv1.3 small interfering RNA, suppressed cell migration. The expression of phosphorylated ERK1/2 also decreased after knockdown of Kv1.3. On the other hand, overexpression of Kv1.3 channel promoted cell migration and ERK1/2 phosphorylation. U-0126, the mitogen-activated protein kinase inhibitors, could reverse macrophage migration induced by Kv1.3 channel overexpression. Downregulation of Kv1.3 channel by siRNA could not further inhibit cell migration when cells were treated with U-0126. It means that ERK is downstream signal of Kv1.3 channel. We concluded that Kv1.3 may stimulate macrophage migration through the activation of ERK.

1. Introduction

The migration of macrophages into the arterial intima appears at all stages in the development of the atherosclerosis. Indeed, in the early fatty lesions, macrophages dominate the primary of cellular infiltrate, and differentiation of macrophages into foam cells is considered to establish a chronic inflammatory environment within the artery [1]. The mechanisms supporting the migration of macrophages are not fully understood.

Numerous studies have indicated that ion channels expressed in macrophages play an important role in atherosclerosis. However, the function of voltage-gated K^+ channels (Kv channels) in atherosclerosis has not been directly elucidated yet. Kv1.3 is the Kv channel subtype, which has six transmembrane domains (S1–S6), including a voltage sensor (S4) and a pore-forming region [2]. The investigation of Kv1.3 channel in vascular smooth muscle cell and lymphocytes has become more popular. It has been known that Kv1.3 channel is functional in proliferating mouse and human vascular smooth muscle cells and have positive effects on cell migration [3]; in T cells, Kv1.3 is reported to be a primary regulator of cell activation and blockade of Kv1.3 attenuates the production of proinflammatory cytokines and proliferation in vitro [4–6]. Whereas, Kv1.3 channels in macrophages have drawn little attention. Since atherosclerosis and inflammation are also closely related with cellular metabolism and K^+ currents are the major component of membrane potential which could affect the flow of other ions and critical for cell function, Kv1.3 channel may represent a novel target of atherosclerosis.

Extracellular signal-regulated kinase (ERK) is one of the major classes of mitogen-activated protein kinases (MAPK), which regulate a wide array of cellular processes. The activation of ERK is a critical signal for cell activation, such as proliferation, differentiation and so on [7]. Evidence showed that activating K_{ATP} channel elicited ERK activation and cell proliferation but inhibiting K_{ATP} channel depressed ERK activation and cell proliferation in U87 cells [8]. Morever, our previous research had been shown that ERK was involved in both potassium channels opening and macrophage inflammation [9]. Thus, it has been proposed that it acts as a

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downstream trigger linking the surface receptors and the signaling pathways.

The aim of this study is to investigate the regulation mechanisms by which Kv1.3 channel controls macrophages migration. Our results demonstrate that Kv1.3 channel augments macrophages migration via activating ERK and suggest that Kv1.3 channel plays a critical role in the development of atherosclerosis.

2. Methods

2.1. Reagents and antibodies

DMEM, fetal bovine serum, and trypsin were purchased from Gibco (Grand Island, NY, USA). Trizol regent and Lipofectamine™ 2000 were purchased from Invitrogen (Carlsbad, CA, USA). Real-time RT-PCR assay kits were purchased from Takara (Dalian, China). PCR primers were synthesized by Biosun (Jinan, China). The siRNA specific for Kv1.3 was designed by GenePharma Co. Ltd (Shanghai, China). Kv1.3 plasmid was synthesized by OriGene Tec. Inc (Beijing, China). Kv1.3 antibody was purchased from Alomone (Alomone Labs, Jerusalem, Israel). GAPDH antibody was obtained from Zhongshan Biotech (Guangzhou, China). Anti-total-ERK1/2 and phosphorylated ERK1/2 antibodies were from Cell Signaling Technology, USA. U-0126 was from Sigma (St Louis, MO). Horseradish peroxidase-conjugated secondary antibody was obtained from Abcam (Cambridge, MA, USA). The chemiluminescence (ECL) kit was obtained from Amersham Pharmacia (Piscataway, NY). Other chemicals were of the highest purity grade.

2.2. Biological samples

177 subjects from the cardiology department of Qilu Hospital, Shandong University were enrolled in the study. According to the clinical diagnosis, subjects were divided into stable angina pectoris (SAP), unstable angina pectoris (UAP), acute myocardial infarction (AMI) and control groups. The SAP group consisted of 43 patients with typical effort angina or positive exercise test result but no episodes of angina at rest. The UAP group consisted of 45 patients who had two episodes of chest pain at rest or an episode of chest pain lasting over 20 min in combination with ST-elevations > 0.1 mV during pain, without elevations of creatin kinase (CK). The AMI group comprised 44 patients who were defined as typical chest pain, ST-elevation > 0.1 mV in at least two contiguous leads and CK levels of more than twice the upper limit or elevated troponin levels. The control group consisted of 45 patients with chest pain syndrom or paroxysmal supraventricular tachycardia under frequency ablation. None of them had a visible coronary artery stenosis. People with evidence of recent infectious disease, immunological disorders, fever, using of anti-inflammatory drugs, major surgery or neoplastic disease were excluded from the study. Blood samples (20 ml peripheral blood via venous puncture) were obtained once from the enrolled volunteers. Informed consent was obtained from all subjects based on a protocol approved by the Ethics Committee of Qilu Hospital of Shandong University and adhered to the principles in the Declaration of Helsinki.

2.3. Cell isolation and culture

Monocytes were isolated by Ficoll (Histopaque-1077, Sigma–Aldrich Co., Ltd., UK) gradient centrifugation from blood according to the introduction. These cells were plated in six-well culture plates in 2 ml of complete medium (RPMI-1640 supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 50 U/ml streptomycin) and stored at 37 °C for 2 h. After 2 h, non-adherent cells were removed. The adherent cells were cultured with complete medium contained 100 ng/ml recombinant human GM-CSF and 100 ng/ml IL-4 at 37 °C under 5% CO_2. After 7 days, the cultures developed an adherent monolayer and clusters of macrophages.

RAW264.7 cells were purchased from ATCC (Manassas, Va., USA) and cultured in DMEM supplemented with 10% FBS, 100 μg/ml streptomycin, 100 U/ml penicillin. For in vitro experiments, cells were made quiescent by incubation in medium without FBS for 12 h. Drug or vehicle was added to each well and the medium was added for a total volume of 100 μl. Then the cells were incubated at 37 °C with 5% CO_2 and the medium was changed every 24 h.

2.4. Electrophysiological recordings

Whole-cell currents were recorded at room temperature (21–23 °C) using the whole-cell patch-clamp technique with an HEKA EPC-10 patch clamp amplifier and controlled with Pulse software (HEKA, Lambrecht, Germany). Micropipettes were made from borosilicate glass capillary tubes with an outside diameter of 1.5 mm. After being fire-polished and filled with pipette solution, the resistance was 3–5 MΩ. Signals were sampled at 3 kHz and filtered at 1 kHz. Electrodes were filled with the following solution (in mM): 80 K-aspartate, 42 KCl, 3 phosphocreatine, 10 KH_2PO_4, 3 MgATP, 5 HEPES-K, and 5 EGTA-K (adjusted to pH 7.25 with KOH). The extracellular solution contained (in mM) 136 NaCl, 4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 HEPES-Na, and 10 D-glucose, and it was adjusted to pH 7.40 with NaOH. Macrophages were clamped to a holding potential of −80 mV. The current–voltage (IV) protocol consisted of 15 pulses ranging from −80 to +60 mV, in 10 mV steps. Each pulse was 250 ms long. Peak amplitude (pA) was normalized by capacitance values (pF). Data analysis was performed using IGOR and the Origin software.

2.5. Cell transfection

The sequences of siRNA against Kv1.3 are 5′-CCUGCACCAC-GAACAAUAATT-3' (sense) and 5′-UUAUUGUUCGUGGUGCAGGTT-3' (antisense); the sequences of non-silencing RNA are 5′-UUCUCC-GAACGUGUCACGUTT-3' (sense) and 5′-ACGUGACACGUUCGGA-GAATT-3' (antisense). The RAW264.7 cells at 1×10^5 cells per dish were transfected with siRNA or Kv1.3 plasmid using Lipofectamine™ 2000 according to the manufacturer's protocol. The efficiency of knockdown or overexpression the target protein was determined by western blot analysis.

2.6. Migration assays

Migration assays were assessed by transwell cell culture chambers with polycarbonate filters with 5 μm pores (BD Biosciences, Oxford, UK). In brief, 1×10^5 pretreated cells were suspended in 100 μl DMEM containing 0.5% FBS and added to the upper compartment of the insert. The lower chamber was filled with 100 μL DMEM containing 10% FBS. After incubation for 6 h at 37 °C in a 5% CO_2 incubator, cells were scraped from the upper surface, duplicate membranes fixed, and migrated cells stained with crystal violet. Cells were counted in 6 random fields, leading to an average number of cells.

2.7. Real-time quantitative RT-PCR

Total RNA was extracted from macrophages with trizol according to the instructions of the manufacturer. The purity of extracted RNA was measured by extinction at 260 nm and the purity was determined by the 260/280 ratio. The 20 μl reverse-transcription systems including 1 μl oligo-dT primer, 1 μl dNTP mixture, 1 μl reverse transcriptase, 4 μl buffer, 2 μg of total RNA and RNase-free