



## 7-Ketocholesterol induces the reduction of KCNMB1 in atherosclerotic blood vessels



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### ABSTRACT

Hypertension is a high-risk symptom in atherosclerotic patients, and vascular rigidity is one of the main factors leading to hypertension.  $\beta$ 1-Subunit of BKCa channel (KCNMB1; MaxiK $\beta$ 1) has been reported as a modulator of vascular flexibility. To determine the relationship between atherosclerosis and KCNMB1, we studied some atherogenic factors affecting vascular tone. Blood of atherosclerotic patients shows increased concentration of 7-ketocholesterol (7K), which has been studied as a harmful lipid to blood vessels. Our data showed that KCNMB1 was significantly down-regulated in the presence of 7K, in a dose-/time-dependent manner in vascular smooth muscle cells (VSMCs). And, the reduction of KCNMB1 was confirmed in cell images of 7K-stimulated VSMCs and in vessel tissue images of *ApoE* knock-out mice. To determine whether aryl hydrocarbon receptor (AhR) was involved in the reduction of KCNMB1 by 7K-stimulation, protein level of AhR was analyzed by Western blot. Our data showed that the reduction of KCNMB1 was modulated through the AhR pathway. In conclusion, results of our study suggest that 7K induces the reduction of KCNMB1 through the AhR pathway.

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### 1. Introduction

Atherosclerosis is a chronic, inflammatory disease characterized by accumulation of lipids (such as cholesterol crystals, oxysterols, modified fatty acids, aldehydes, and lysophospholipids) and fibrous elements in the coronary artery [1,2]. In atherosclerotic patients, flexibility of the inflammatory blood vessel is decreased, and blood pressure is elevated. The relationship of hyperlipemia and atherosclerosis has been studied, however, the mechanisms are not well known.

Oxysterols are derived non-enzymatically, either from the diet or from *in vivo* oxidation, or are formed enzymatically during cholesterol catabolism. The most abundant oxysterol in human blood is 27-

hydroxycholesterol (27OH-chol). Other abundant oxysterols are 7 $\alpha$ -hydroxycholesterol (7 $\alpha$ OH-chol), 7-ketocholesterol (7K), and 7 $\beta$ -hydroxycholesterol (7 $\beta$ OH-chol). However, the major types of oxysterols in atherosclerotic plaques are 7K and lower levels of 7 $\alpha$ OH-chol, 7 $\beta$ OH-chol, and 27OH-chol [3,4]. Compared with normal tissues or blood, a much higher 7K: cholesterol ratio has been reported for atherosclerotic plaques [5]. 7K predisposes human aorta smooth muscle cells (SMCs) to Fas-mediated cell death [6], and induces interleukin-6 (IL-6), an inflammatory and atherogenic cytokine, via the p38 MAPK pathway in human vascular smooth muscle cells (hVSMCs) [7]. Many studies have reported the toxicity of 7K, however, a relationship of 7K and hypertension is unclear.

Chronic hypertension is known to be associated with increased morbidity and mortality from stroke, coronary artery disease, congestive heart failure, renal disease, and so on [8,9]. Arterial rigidity is persistently increased as a result of malfunction of vessel relaxation in chronic hypertension [9]. Arterial rigidity is regulated by functional balance of the ion channels responsible for cellular

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depolarization and hyperpolarization. The increased arterial rigidity is mainly related to depolarization of smooth muscle, which may have resulted from dysfunction of ion channels responsible for cell membrane hyperpolarization [10]. The membrane depolarization activates voltage-dependent L-type  $\text{Ca}^{2+}$  channels, induces an increase in  $\text{Ca}^{2+}$  influx and global intracellular  $\text{Ca}^{2+}$  level, and causes vessel constriction [11,12]. Large-conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  (BKCa or MaxiK) channels are believed to play an important role in hyperpolarization of vascular smooth muscle cells (VSMCs) [13]. BKCa channels are comprised of an alpha gene (*kcnma1*) encoding a pore-forming subunit and one of four beta genes (e.g., *kcnmb1*) encoding cell-restricted modulatory subunits [14–16]. KCNMB1 confers heightened channel sensitivity to calcium and voltage in VSMCs, which provides for efficient fine-tuning of vascular rigidity [17,18]. The functionality of KCNMB1 has been studied extensively, however, much less is known regarding its transcriptional regulation. In a recent study, expression of the calcium channel protein is influenced by hypoxia-induced factor-1 (HIF-1) and histone deacetylase [19].

Hypertension and atherosclerosis have been studied by many researchers, and KCNMB1 have also been well studied. However, the involvements among the factors are unclear. In this study, we show that hypertension caused by atherosclerosis is related to a down-regulation of KCNMB1 by oxysterol.

## 2. Materials and methods

### 2.1. Cell culture

Human aortic vascular smooth muscle cells (HA-VSMCs) were purchased from American Type Culture Collection (ATCC; Manassas, VA), and were grown in Kaighn's modification of Ham's F-12 (F-12K) medium (ATCC) containing 15% fetal bovine serum (FBS). The cells were used at passage 7–9. The cells were maintained in a humidified incubator at 37 °C under 5%  $\text{CO}_2$  atmosphere.

### 2.2. Reagents

Cholesterol and 7-ketocholesterol (7K) were purchased from Sigma–Aldrich, and 7 $\alpha$ -hydroxy-cholesterol (7 $\alpha$ OH-cho) and 7 $\beta$ -hydroxy-cholesterol (7 $\beta$ OH-cho) were purchased from Steraloid (Newport, RI). 27-hydroxy-cholesterol (27OH-cho) was purchased from Santa-Cruz (Delaware Avenue, CA). The lipids were dissolved in absolute ethanol. Primary antibody against human MaxiK $\beta$  (KCNMB1) was purchased from Santa-Cruz (Delaware Avenue, CA). Antibody against AhR was purchased from Abcam (Cambridge, CB4 0FL, UK). Antibody against  $\beta$ -actin was purchased from Calbiochem (La Jolla, CA). Horseradish peroxidase-conjugated secondary antibodies against goat, rabbit, and mouse IgG were purchased from Santa-Cruz, and AlexaFluo 488-conjugated secondary antibody was purchased from Invitrogen (Carlsbad, CA).

### 2.3. Western blot analysis

For whole cell lysates, cells were collected after treatment with or without oxysterols. The cells were lysed with lysis buffer (1% SDS, 1 mM  $\text{NaVO}_3$ , 10 mM Tris–HCl, pH 7.4) containing protease inhibitor cocktail (Sigma–Aldrich). The protein content of each sample was determined using the bicinchoninic acid method (BCA, Pierce, Rockford, IL). The samples were separated by SDS-PAGE, followed by transfer to PVDF membranes (Millipore Corp., Bedford, MA). After incubation for one hour with 5% skim milk in 0.1% Tween 20/TBS to block non-specific binding sites of the primary antibody, the membrane was probed with indicated primary antibodies at 4 °C overnight. After washing three times with 0.1%

Tween 20/TBS for 15 min each, the membrane was incubated with HRP-conjugated secondary antibodies for one hour at room temperature. After washing three times with the washing buffer for 15 min each, bands were detected using the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham Pharmacia Biotech, Piscataway, NJ).

### 2.4. Immunocytofluorescence

Cells cultured on a coverslip placed in a 6-well plate were washed with PBS, and fixed for 20 min with 1% paraformaldehyde at room temperature. After incubation with blocking solution (5% skim milk in PBS) for one hour to reduce non-specific binding, the cells were incubated at 4 °C overnight with antibody against KCNMB1 diluted in blocking solution (1:100). After washing three times with PBS for 15 min each, the cells were incubated for one hour with indicated secondary antibody diluted in PBS (1:250) at room temperature in darkness. After washing three times with PBS for 15 min each, the slides were mounted in Vectashield™ (Vector Laboratories, Burlingame, CA) and visualized using a confocal microscope.

### 2.5. Immunohistofluorescence

Mouse slide samples were kindly gifted from Professor Koanhoi Kim. The tissues were washed with PBS, and fixed for 20 min with 4% paraformaldehyde at room temperature; after incubation with blocking solution (3% BSA in PBS) for one hour to reduce non-specific binding, they were incubated at 4 °C overnight with antibody against KCNMB1 diluted in blocking solution (1:50). After washing three times with PBS for 15 min each, the slides were incubated for one hour with indicated secondary antibody diluted in PBS (1:100) at room temperature in darkness. After washing three times with PBS for 15 min each, the slides were mounted in Vectashield™ (Vector Laboratories, Burlingame, CA) and visualized using a confocal microscope.

### 2.6. Statistical analysis

Statistical analysis (one-way ANOVA) was performed using PRISM version 5.0 (GraphPad software, San Diego, CA).  $P < 0.05$  was considered statistically significant.

## 3. Results

### 3.1. Relationship between KCNMB1 and oxysterols

To determine which oxysterols influenced KCNMB1 in blood of atherosclerotic patients, HA-VSMCs were stimulated for 48 h with 5  $\mu\text{g}/\text{ml}$  cholesterol, 7K, 7 $\alpha$ OH-cho, 7 $\beta$ OH-cho, and 27OH-cho, and proteins extracted from the cells were analyzed by Western blotting.

KCNMB1 was significantly decreased in the presence of 7K, but not by cholesterol or other oxysterols (Fig. 1A). To confirm this result, we investigated the reduction of KCNMB1 with 7K. The cells were treated for indicated times with indicated concentration of 7K. As shown in Fig. 1B, KCNMB1 of HA-VSMCs was significantly reduced by treatment with 5 and 10  $\mu\text{g}/\text{ml}$  of 7K. However, an increase in dying cells was observed in cells treated with 10  $\mu\text{g}/\text{ml}$  of 7K. KCNMB1 was also affected by 7K-treatment time. In Fig. 1C, the channel protein was decreased at 48 h after treatment with 5  $\mu\text{g}/\text{ml}$  of 7K. These results demonstrated that 7K affected the protein level of KCNMB1.

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