Transient receptor potential channel M2 contributes to neointimal hyperplasia in vascular walls

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1. Introduction

Atherosclerosis is a common medical problem in industrialized countries and has major impact on survival and quality of life. A hallmark of atherosclerosis is vascular wall thickening in the intimal layer, namely neointimal hyperplasia. Neointimal hyperplasia is an early event in the development of atherosclerotic lesions. Neointimal hyperplasia narrows vascular lumen, resulting in occlusive vascular disease, such as coronary artery disease, myocardial infarction and stroke [1]. Angioplasty with stent placement and bypass surgery are primary treatment options. They are effective in relieving symptoms (e.g. angina, dyspnea, and fatigue) and reducing mortality. However, long-term benefits are limited by the formation of neointima in the stents and vein grafts, leading to in-stent restenosis and vein graft failure [2–4]. Thus, exploring the molecular mechanism of neointimal hyperplasia and developing novel therapeutic strategies are of significance.

Vascular smooth muscle cells (VSMCs) are the major cellular component of neointima. At the sites of vessel injury, VSMCs in the media undergo phenotypic switch: dedifferentiate from a quiescent, contractile phenotype to a proliferative, synthetic phenotype [5]. These VSMCs migrate from the media layer to the intima layer and proliferate, with increased secretion of extracellular matrix, contributing to the formation of neointima [5,6]. A key event in atherosclerotic development is over-production of reactive oxygen species (ROS). ROS are released from macrophages, neutrophils and vascular cells [7]. ROS over-production promotes the progression of neointimal hyperplasia by stimulating multiple pathological events, including VSMC migration and proliferation, monocyte/macrophage and neutrophil infiltration, inflammation, extracellular matrix deposit, and vascular cell death [8–11]. Ca2+ signaling is another important player in neointimal...
formation and vascular remodeling [12,13]. As a versatile second messenger, Ca\(^{2+}\) plays an essential role in proliferation and migration of VSMCs [12,13]. Injured vascular walls often show an alteration of Ca\(^{2+}\) signaling pathways in VSMCs, including down-regulation of L-type Ca\(^{2+}\) channels and up-regulation of T-type Ca\(^{2+}\) channels and TRPC channels [12].

Transient receptor potential channel M2 (TRPM2) is a Ca\(^{2+}\)-permeable cation channel activated by H\(_2\)O\(_2\), adenosine 5′-diphosphoribose (ADP-ribose) and Ca\(^{2+}\). The channel is expressed in neurons, VSMCs, vascular endothelial cells and inflammatory cells [14–16]. TRPM2 is suggested to be a cellular sensor for oxidative stress [17–19]. It has been reported that H\(_2\)O\(_2\), as a ROS, activates TRPM2 to induce excessive Ca\(^{2+}\) influx, resulting in Ca\(^{2+}\) overload and consequent cell death in neurons, hematopoietic cells and vascular endothelial cells [18,19]. TRPM2 is also involved in inflammation. TRPM2-mediated Ca\(^{2+}\) entry stimulates cytokine production in monocytes, resulting in an aggravated inflammatory response in colon colitis [16].

Based on the crucial role of ROS and Ca\(^{2+}\) signaling in neointimal hyperplasia, and the property of TRPM2 as a ROS-sensitive Ca\(^{2+}\) entry channel, in the present study we explored the possible role of TRPM2 in neointimal hyperplasia of blood vessels. In rodent models, neointimal hyperplasia was induced by placing a perivascular cuff (or collar) around rat/mouse arteries. The results from this study demonstrated TRPM2 as a key mediator in neointimal growth.

![Figure 1](image-url)
Fig. 2. TRPM2 up-regulation in the neointimal region of cuff-injured rat femoral arteries. (A) Cross sections of artery stained with anti-TRPM2 antibody showing numerous TRPM2-positive cells in the neointima and the adjacent media region. n = 4. (B) Western blot of adventitial-removed artery segments probed with anti-TRPM2 antibody and anti-β-tubulin antibody. Data are expressed as mean ± SEM. n = 4. ** p < 0.01 vs. sham. (C) Double immunofluorescent labeling of the artery with anti-α-smooth muscle actin (α-SMA) antibody (red) and anti-TRPM2 antibody (green). Arrowhead indicates the internal elastic lamina. n = 5. (D) Representative traces of [Ca^{2+}]_i (left) and summary of peak [Ca^{2+}]_i (right) rises in response to HX–XO (100 μM HX + 20 mU/ml XO) in freshly prepared SMCs. (E) Representative traces of the [Ca^{2+}]_i (left) and summary of peak [Ca^{2+}]_i (right) showing effect of TM2E3 (10 μg/ml) or pre-immune IgG (10 μg/ml, as control) on HX–XO-induced [Ca^{2+}]_i rise. In bar charts, data are expressed as mean ± SEM. n = 4 (D) and 5 (E) independent experiments (15 to 30 cells per experiment). ** p < 0.01. Cuff, neointimal SMCs from cuff-injured arteries; Sham, normal medial SMCs from sham-operated arteries.
2. Methods

2.1. Chemicals and antibodies

Unless stated, all chemicals were purchased from Sigma. Akt inhibitor IV, Akt inhibitor X, mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) antibody, goat polyclonal anti-Axl antibody, rabbit polyclonal anti-β-tubulin antibody, mouse monoclonal anti-β-actin antibody, and horseradish peroxidase-conjugated donkey anti-goat IgG were obtained from Santa Cruz Biotechnology. R428 was obtained from Selleck Chemicals. Mouse monoclonal anti-α-smooth muscle actin (α-SMA) antibody was obtained from Dako. Rabbit monoclonal anti-α-SMA antibody, biotinylated goat anti-rabbit IgG and biotinylated goat anti-mouse IgG were obtained from Abcam. Rabbit monoclonal anti-phospho-Axl antibody, rabbit polyclonal anti-Akt antibody and rabbit polyclonal anti-phospho-Akt antibody were obtained from Cell Signaling Technology. Alexa Fluor 555 donkey anti-mouse IgG and Alexa Fluor 488 goat anti-rabbit IgG were obtained from Invitrogen. Horseradish peroxidase-conjugated anti-rabbit IgG and horseradish peroxidase-conjugated anti-mouse IgG were obtained from Amersham Pharmacia Biotech. Antibodies against TRPM2 were the home-made rabbit polyclonal anti-TRPM2 antibody and TM2E3 antibody. Anti-TRPM2 antibody targets to an epitope in the N terminus of TRPM2 (ILKELSKEEEDTDSSEEMLA) [20]. TM2E3 antibody targets to amino acid residues in E3 region of TRPM2 [21]. Pre-immune IgG was also home-made.

2.2. Animals

Male Sprague–Dawley (SD) rats were obtained from the Laboratory Animal Services Center, the Chinese University of Hong Kong, Hong Kong, China. In Trpm2 knockout mice, the Trpm2 gene was disrupted by deleting the exon that contributes to the putative pore region of the TRPM2 [16]. Animals were kept in specific pathogen-free environment where lighting was controlled (12:12-hour light–dark cycle) and temperature was maintained at 22–23 °C, with free access to tap water and standard rodent diet. All animal experiments were conducted in conformity with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health, and the procedures were approved by the Animal Experimentation Ethics Committee, the Chinese University of Hong Kong.

![Fig. 3. Reduced neointimal hyperplasia in cuff-injured carotid artery of Trpm2 knockout mice.](image-url)
2.3. Vascular injury induced by cuff placement

The surgical procedures of cuff-induced artery injury were performed according to previously described methods [22,23] with slight modifications. Briefly, for rat femoral artery cuff placement, SD rats (8 week old) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). The femoral artery of rat was isolated from the surrounding tissues under a dissection microscope. A polyethylene tube (length, 5 mm, inner diameter, 0.86 mm; outer diameter, 1.27 mm; Becton Dickinson and Company) was opened up longitudinally and loosely placed around the artery. The contralateral femoral artery of the same rat was used as control, undergoing isolation but without cuff placement (sham operation). Then the surgical site was sutured with a 6-0 polypropylene suture. 2 weeks after the surgery, the rats were sacrificed by CO2-asphyxia and the femoral arteries were harvested for study. For mouse carotid artery cuff placement, the Trpm2 knockout mice (12 week old) and their wild-type counterparts were anesthetized with ketamine/xylazine (80/10 mg/kg, i.p.). The carotid artery was isolated and a smaller polyethylene tube (length, 2–3 mm, inner diameter, 0.58 mm; outer diameter, 0.965 mm; Becton Dickinson and Company) was used as the cuff. 3 weeks after the surgery, the mice were sacrificed by CO2-asphyxia and the carotid arteries were harvested.

2.4. Generation of TRPM2-specific blocking antibody TM2E3 and preimmune IgG

TM2E3 antibody was raised in rabbit using the strategy developed by Xu et al. [21]. Briefly, a peptide corresponding to the TRPM2 putative pore region was synthesized and conjugated to a keyhole limpet hemocyanin (KLH) at the Alpha Diagnostic International. The coupled TM2E3 peptide (0.5 mg) was injected subcutaneously in the back of a rabbit followed by two boost doses. TM2E3 antiserum was collected 4 weeks after the second boost. IgG was purified from the TM2E3 antiserum using a HiTrap Protein G column (GE Healthcare). For control, pre-immune IgG was obtained from pre-immune serum with HiTrap Protein G column.

2.5. Histological analysis and immunohistochemistry

The harvested rat femoral arteries and mouse carotid arteries were fixed in 4% paraformaldehyde and embedded in paraffin. 5 μm transverse sections were cut and hematoxylin and eosin (HE) staining was performed. The areas of intima and media were measured using ImageJ software and the ratio of the intimal area to the medial area (I/M ratio) was calculated.

To explore the cell cycle activity and expression level of TRPM2 in neointima, immunohistochemical labeling of PCNA and TRPM2 was performed. 5 μm paraffin-embedded rat femoral artery sections were incubated with anti-TRPM2 antibody (10 μg/ml) or anti-PCNA antibody (1:100) overnight at 4 °C. Sections were then incubated with biotinylated secondary antibodies, and finally horseradish peroxidase-labeled streptavidin. Signals were visualized by peroxidase–diaminobenzidine reaction. The involvement of VSMCs was assessed by double immunofluorescent labeling of TRPM2 and α-SMA in sections of rat arteries. Anti-TRPM2 antibody (10 μg/ml) and mouse anti-α-SMA antibody (1:100) were applied to 5 μm paraffin-embedded sections overnight.

Fig. 4. Role of TRPM2 in H2O2-stimulated migration of rodent aortic SMCs. (A) Immunofluorescent labeling with anti-α-SMA antibody (green), verifying the identity of the primary cultured rat and mouse aortic SMCs. Nuclei were stained with DAPI (blue). (B) and (C) Scratch wound healing assay showing inhibitory effect of TM2E3 (10 μg/ml) on H2O2 (10 μM)-induced migration of rat aortic SMCs (B) and effect of Trpm2 knockout on H2O2 (10 μM)-induced migration of mouse aortic SMCs (C). Dashed lines denote the wound edge. Cell migration was expressed as the percentage of closed wound area after incubation for 48 h. Data are expressed as mean ± SEM. n = 6 (B) and 4 (C) independent experiments. * p < 0.05, ** p < 0.01. WT, wild-type; Trpm2 KO, Trpm2 knockout.

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at 4 °C. Bound antibodies were detected with Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 555 donkey anti-mouse IgG. Fluorescence was monitored using a laser scanning confocal microscope (FV1000, Olympus). For these immunostaining experiments, specificity of signals was confirmed by replacing primary antibodies with the matched pre-immune IgG.

2.6. Western blotting

Tissue lysates of intimal and medial layers of rodent arteries and whole-cell lysates of rodent aortic smooth muscle cells (SMCs) were extracted with protein extraction buffer. Protein concentrations were determined by Bradford assay. Proteins were separated on 7.5% SDS-PAGE gels and transferred to PVDF membranes, and probed with anti-TRPM2 antibody (2 μg/ml), anti-PCNA antibody (1:500), anti-Axl antibody (1:1000), anti-phospho-Axl antibody (1:1000), anti-Akt antibody (1:1000) or anti-phospho-Akt antibody (1:1000) followed by the appropriate horseradish peroxidase-conjugated secondary antibodies. Immunolabeling was visualized by ECL plus Western blotting detection reagents (Amersham Pharmacia Biotech). Immunoblots with anti-β-tubulin antibody (1:500) or anti-β-actin antibody (1:500) were performed to get internal controls.

Fig. 5. Role of TRPM2 in H2O2-stimulated proliferation of rodent aortic SMCs. (A) and (B) EdU incorporation assay showing inhibitory effect of TM2E3 (10 μg/ml) and Trpm2 knockout on H2O2 (10 μM)-induced proliferation of rat (A) and mouse (B) aortic SMCs. Nuclei were stained with Hoechst 33342 (blue). Cell proliferation was expressed as the percentage of EdU-positive cells (red). Data are expressed as mean ± SEM. n = 7 (A) and 9 (B) independent experiments. ** p < 0.01. (C) and (D) MTT assay showing inhibitory effect of TM2E3 (10 μg/ml) and Trpm2 knockout on H2O2 (10 μM)-induced proliferation of rat (C) and mouse (D) aortic SMCs. In C, pre-immune IgG group in the absence of H2O2 was normalized to 100%. Data are expressed as mean ± SEM. n = 4 independent experiments. ** p < 0.01. WT, wild-type; Trpm2 KO, Trpm2 knockout.

(E) Western blot of aortic SMCs probed with anti-PCNA antibody and anti-β-tubulin antibody. Data are expressed as mean ± SEM. n = 3. ** p < 0.01, WT, wild-type; Trpm2 KO, Trpm2 knockout.
2.7. Detection of ROS production by dihydroethidium (DHE) fluorescence

Fresh frozen sections (10 μm) of rat femoral arteries were incubated with DHE (5 μM) for 10 min at 37 °C. Fluorescence was monitored using FV1000 laser scanning confocal imaging system (Olympus) at excitation/emission of 488/605 nm. Fluorescent intensity in the arterial intimal and medial area was analyzed by the Fluoview software (Olympus).

2.8. Isolation of smooth muscle cells from neointimal and medial layers

Cuff-injured rat femoral arteries and their sham-operated controls were excised. Under an operating microscope, the arteries were cleaned from connective tissue and cut longitudinally. The endothelium was gently removed mechanically. Then neointima of cuff-injured arteries was scraped. The tissues were digested with 0.2% collagenase type IA, 0.9% papain, 0.5% BSA and 10 mM dithiothreitol in Ca2+-free PBS at 37 °C. The isolated VSMCs were seeded onto round cover slips in 12-well plates and cultured with DMEM supplemented with 1% fetal bovine serum (FBS), penicillin and streptomycin (100 U/ml and 100 μg/ml, respectively), and 1% L-glutamine for 8 h before [Ca2+]i measurement. For sham-operated arteries, the VSMCs were isolated from medial layer as control in [Ca2+]i study.

2.9. Culture of rodent aortic smooth muscle cells

Rodent aortic SMCs were obtained using explant method as described elsewhere [24] with slight modifications. Briefly, SD rat (6 week old), Trpm2 knockout mice (4 week old) and wild-type mice (4 week old) were sacrificed by CO2-asphyxia. The aortic arteries were excised under sterile conditions. Vessel lumens were opened longitudinally and the endothelium was removed mechanically. The arteries were cut into small pieces (2 mm × 2 mm), and placed in 35 mm culture dishes with lumen side down and with a distance of 5 mm from each other. The explants were allowed to air dry to adhere and then cultured with DMEM supplemented with 10% FBS, penicillin and streptomycin (100 U/ml and 100 μg/ml, respectively), and 1% L-glutamine. The culture medium was replaced every 3–4 days. Aortic SMCs grew out from the explants and achieved confluence after approximately 7 days. Cells were harvested with trypsin and used for experiments at passage 2–4. Cell identity was verified by immunofluorescent staining of α-SMA using the rabbit anti-α-SMA antibody (1:100).

2.10. [Ca2+]i measurement

SMCs were loaded with a membrane permeant fluorescence dye Fluo-4/AM (Molecular Probes) to measure [Ca2+]i responses to hypoxanthine–xanthine oxidase (HX–XO) or H2O2. Briefly, the cells were seeded on round cover slips and were incubated with 5 μM Fluo-4/AM and 0.02% Pluronic acid F-127 in normal physiological saline solution (NPSS) for 1 h at room temperature. NPSS contained in mM: 1 CaCl2, 140 NaCl, 1 KCl, 1 MgCl2, 10 glucose, and 5 HEPES, pH 7.4. If needed, the cells were pre-treated with TM2E3 (10 μg/ml) or preimmune IgG as control (10 μg/ml) at 37 °C for 2 h. The cover slips were then pinned in a specially designed chamber and bathed in NPSS. HX–XO (100 μM HX + 20 mU/ml XO) or H2O2 (500 μM) were applied to the bath along the side of the chamber. The Fluo-4 fluorescence was recorded using a FV1000 laser scanning confocal imaging system at excitation/emission of 488/515 nm at room temperature. The [Ca2+]i response was expressed as a ratio of real-time fluorescence intensity relative to the basal intensity before HX–XO or H2O2 was applied (F1/F0).

Fig. 6. TRPM2 mediates H2O2-induced [Ca2+]i rise in rodent aortic SMCs. Representative traces of [Ca2+]i (left) and summary of peak [Ca2+]i (right) showing that H2O2 (500 μM)-induced [Ca2+]i rise was inhibited by TM2E3 (10 μg/ml) in rat aortic SMCs (A) and was reduced in Trpm2 knockout mouse aortic SMCs (B). Data are expressed as mean ± SEM. n = 6 (A) and 5 (B) independent experiments (15 to 30 cells per experiment). ** p < 0.01. WT, wild-type; Trpm2 KO, Trpm2 knockout.
2.11. Cell migration assay

Cell migration was evaluated using scratch wound healing assay. Rodent aortic SMCs were seeded in a 6-well plate and cultured to >95% confluence. Cells were synchronized with 0.5% FBS DMEM for 24 h and then treated with H$_2$O$_2$ (10 μM) and EdU (10 μM) in 0.5% FBS DMEM, with/without the present of TM2E3 (10 μg/ml), Akt inhibitor IV (5 μM) or Akt inhibitor X (10 μM). After incubation for 48 h, for each well, three fields with the wound area were chosen and photographed using a microscope coupled to a digital camera. Percentage of the closed wound area which indicates the cell migration was analyzed using ImageJ software.

2.12. Cell proliferation assay

Cell proliferation was determined with 5-ethynyl-2′-deoxyuridine (EdU) incorporation assay. Rodent aortic SMCs were seeded in a 24-well plate. After achieving 50% confluency, cells were synchronized with 0.5% FBS DMEM for 24 h and then treated with H$_2$O$_2$ (10 μM) and EdU (10 μM) in 0.5% FBS DMEM, with/without the present of TM2E3 (10 μg/ml). After incubation for 48 h, cells were fixed, permeabilized, and incubated with reaction cocktail of Click-it Plus EdU Imaging Kits (Life Technologies) for 30 min. For nuclear staining, the cells were incubated with Hoechst 33342 (5 μg/ml) for 30 min. The cells were imaged by a laser scanning confocal imaging system (Olympus FV1000). Cell proliferation was expressed as the percentage of EdU-positive cells.

In another set of experiment, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was also used to determine cell proliferation. Rodent aortic SMCs were seeded in a 96-well plate. After achieving 50% confluency, cells were synchronized with 0.5% FBS DMEM for 24 h and then treated with H$_2$O$_2$ (10 μM) and EdU (0.5 μg/ml), with/without the present of TM2E3 (10 μg/ml). After incubation for 48 h, for each well, three fields with the wound area were chosen and photographed using a digital camera. Percentage of the closed wound area which indicates the cell migration was analyzed using ImageJ software.

2.13. Statistical analysis

All values are expressed as mean ± SEM. The data for each condition were from at least three independent experiments. Statistical differences were determined using paired or unpaired Student’s t-test, or one-way ANOVA followed by the Newman–Keuls test. Differences were considered significant at p < 0.05.

3. Results

3.1. ROS over-production and TRPM2 up-regulation in neointima of cuff-injured rat femoral arteries

To determine whether TRPM2 is potentially involved in neointimal hyperplasia, we first studied a rat model of cuff-induced intimal thickening. A perivascular cuff was placed around rat femoral arteries. 2 weeks after cuff placement, distinct neointimal formation was observed (Fig. 1A), with markedly increased I/M ratio (0.35 ± 0.04 in cuff-injured arteries vs. 0.04 ± 0.01 in contralateral sham-operated arteries, p < 0.01, n = 5). Immunostaining showed a great number of PCNA-positive cells in the neointimal and its adjacent media region (Fig. 1B). Immunoblots of adventitial-removed arteries confirmed the up-regulation of PCNA protein expression in cuffed rat femoral arteries (Fig. 1C), which indicated the enhanced cell cycle activity in cuff-injured arteries. The in situ ROS production in arteries was determined with the fluorescent probe DHE, which intercalates to DNA and emits red fluorescence when oxidized by superoxide anion [26]. DHE signal was markedly increased in the neointima and media of cuff-injured arteries (Fig. 1D). DHE signal was markedly increased in the neointima and media of cuff-injured arteries (Fig. 1D).
of cuff-injured arteries (Fig. 1D), suggesting a ROS over-production during hyperplasia.

TRPM2 expression was examined. Numerous TRPM2-positive cells were found in the neointimal and the adjacent medial regions in cuff-injured arteries (Fig. 2A). Immunoblots of adventitial-removed arteries confirmed an up-regulation of TRPM2 protein expression in intimal and medial layers of cuff-injured arteries (Fig. 2B). Alpha-SMA was used as a marker of SMCs. Double immunofluorescent labeling showed considerable overlap of TRPM2 and α-SMA signals (Fig. 2C), indicating that these TRPM2-positive cells are predominantly SMCs. These data demonstrate that up-regulated expression of TRPM2 in SMCs is an accompaniment of neointimal formation.

We further measured the ROS-induced [Ca^{2+}]i rises in freshly isolated neointimal SMCs and normal medial SMCs. ROS were generated by application of HX–XO system [7]. Compared with the normal SMCs from sham-operated artery, neointimal SMCs from cuff-injured artery showed a larger [Ca^{2+}]i rise in response to HX–XO (Fig. 2D). TM2E3 is a home-made blocking antibody against TRPM2 [21,27]. Previously, we have shown that TM2E3 is specific to TRPM2 and can effectively inhibit the activity of TRPM2 channel [27]. Pre-treatment with TM2E3 (10 μg/ml) for 2 h markedly suppressed the HX–XO-induced [Ca^{2+}]i rise in SMCs derived from neointimal regions, indicating abundant presence of functional TRPM2 channels in these cells. TM2E3 also caused a small inhibition on HX–XO-induced [Ca^{2+}]i rise in SMCs derived from sham-operated arteries, suggesting the existence of TRPM2 channels in normal VSMCs. Notably, when TRPM2 was blocked by TM2E3, no difference was showed in the amplitude of [Ca^{2+}]i responses between the neointimal SMCs and the normal SMCs, which indicates that the up-regulated TRPM2 in neointimal SMCs is largely responsible for the enhanced [Ca^{2+}]i response to ROS (Fig. 2E).

The specificity of two antibodies, the anti-TRPM2 antibody used in immunoblots and in tissue section immunostaining and TM2E3 used in functional blockage, were further confirmed. In immunoblots, both antibodies could recognize the targeted band with expected molecular weight of ~170 kDa in the aortic SMCs from wild-type mice but not in those from Trpm2 knockout mice (Supplementary Fig. 1A). In addition, the anti-TRPM2 antibody could stain the aortic SMCs from wild-type mice but not those from Trpm2 knockout mice (Supplementary Fig. 1B).
3.2. Cuff-induced neointimal hyperplasia was reduced in Trpm2 knockout mice

A mouse model of cuff-induced neointimal hyperplasia was also established in carotid arteries of Trpm2 knockout mice and their wild-type counterparts. There was no visual difference between Trpm2 knockout mice and wild-type mice in general appearance or behavior. Clear neointimal formation was observed in carotid arteries 3 weeks after cuff placement in wild-type mice (Fig. 3A). Immunostaining with anti-alpha-smooth muscle actin (α-SMA) antibody verified that >95% of the cells were SMCs (Fig. 4A).

In scratch wound healing assay, H2O2 treatment (10 μM, 48 h) enhanced the migration of rat and mouse aortic SMCs, as indicated by an increase of closed wound area. TM2E3 (10 μg/ml) inhibited the [Ca2+]i rise in response to H2O2 (Fig. 6A). Similarly, aortic SMCs from Trpm2 knockout mice showed a much smaller [Ca2+]i response to H2O2, compared with those from wild-type mice (Fig. 6B).

In order to determine whether the Ca2+ increases actually mediate the proliferation and migration of VSMCs, we applied EGTA (3 mM) into the culture medium of rodent aortic SMCs and reduced the extracellular Ca2+ concentration from ~1.8 mM to ~80 nM (close to the resting cytosolic Ca2+ level). In scratch wound healing assay, both the basal and H2O2-induced cell migration were dramatically inhibited by EGTA (Fig. 7A). In MTT assay, application of EGTA abolished the H2O2-stimulated cell proliferation (Fig. 7B).

These data indicate that TRPM2 is functionally involved in the migration and proliferation of cultured VSMCs and reinforce the forenamed results that Trpm2 disruption reduced neointimal hyperplasia.

3.4. TRPM2 mediates ROS-induced activation of Axl/Akt pathway in VSMCs

We further explored the signaling pathway downstream of TRPM2. It has been previously reported that H2O2 activates Axl (a redox-sensitive receptor tyrosine kinase) and its downstream Akt pathway to induce VSMC migration and proliferation [28,29]. Here, we blocked Akt with either Akt inhibitor IV that targets the ATP-binding site of a kinase upstream of Akt, or Akt inhibitor X that suppresses the kinase reactivity of Akt by inhibiting its phosphorylation. It was found that...
both Akt inhibitor IV and Akt inhibitor X substantially reduced the H$_2$O$_2$-stimulated migration and proliferation of rat aortic SMCs (Fig. 8A, B). H$_2$O$_2$ rapidly induced Axl and Akt phosphorylation in rat aortic SMCs in a time-dependent manner. The phosphorylation reached the maximum in 10 min and afterwards decayed. H$_2$O$_2$ treatment did not affect the total protein levels of Axl and Akt (Fig. 8C). More importantly, inhibiting TRPM2 by pre-treating the rat aortic SMCs with TM2E3 (10 μg/ml) for 2 h dramatically suppressed the H$_2$O$_2$-induced activation of Axl and Akt (Fig. 8D). The activation of Axl and Akt was abolished by chelating Ca$^{2+}$ in culture medium with EGTA (3 mM) (Fig. 8D). In addition, R428 (5 μM), a selective small-molecule inhibitor of Axl tyrosine kinase activity, reduced the activation of Akt (Fig. 8D). These results suggest that TRPM2 mediates the H$_2$O$_2$-induced VSMC migration and proliferation via the downstream Axl/Akt signaling pathway.

4. Discussion

The major findings of the present study are as follows: 1) Cuff injury of rat femoral arteries induced neointimal hyperplasia, accompanied by an up-regulation of TRPM2 protein expression in neointimal SMCs. Furthermore, ROS were over-produced and PCNA-positive proliferating cells were numerous in the neointimal region. The neointimal SMCs isolated from the cuff-injured arteries displayed a greater TRPM2-mediated [Ca$^{2+}$]$\text{c}$ response to ROS challenge than the normal SMCs from non-cuffed arteries. 2) In cuff injury model, neointimal hyperplasia was markedly reduced in Trpm2 knockout mice compared with wild-type mice. PCNA expression was also reduced in Trpm2 knockout mice. 3) Inhibiting TRPM2 by TM2E3 attenuated the H$_2$O$_2$-induced migration and proliferation of rat aortic SMCs. The migration and proliferation of aortic SMCs were also reduced in Trpm2 knockout mice. 4) TM2E3 substantially inhibited the H$_2$O$_2$-induced activation of Axl/Akt pathway in rat aortic SMCs. Taken together, these data strongly suggest that TRPM2 channels contribute to the progression of neointimal growth and neointimal hyperplasia of blood vessels.

Rodent model of cuff-induced neointimal hyperplasia was employed. Cuff placement first causes inflammatory responses that result in leukocyte infiltration and endothelial injury. The subsequent processes are the migration of VSMCs from media layer to intima layer where they proliferate, together with deposition of extracellular matrix, to form the main elements of neointima [30,31]. These steps well imitate the development of human atherosclerosis [32]. In addition, this model, to certain degree, mimics the mechanical damage resulted from human angioplasty and bypass surgery [9]. In clinical practice, although angioplasty and bypass surgery usually recovers the coronary blood flow, many patients experience vascular restenosis (reformation of neointima) afterward, mainly due to surgery-related mechanical damage [2]. Based on these reasons, animal models of cuff-injury have been developed to explore the mechanisms of neointimal formation and its treatment options [9,23]. This model is widely used and has been proven to be reproducible and easily quantifiable [9,23]. In experiments, we found that ROS were over-produced and TRPM2 expression was up-regulated in the SMCs in neointimal and adjacent medial regions of cuff-injured arteries. Because TRPM2 is a cellular sensor for ROS, it is envisaged that ROS act on TRPM2 to induce excessive Ca$^{2+}$ influx, triggering pathological responses and resulting in neointimal growth. To its support, we found that Trpm2 knockout mice displayed much reduced neointimal hyperplasia. These results strongly suggest that TRPM2 is an important mediator in neointimal growth and that inhibiting TRPM2 could be a means to prevent neointimal growth.

The mechanism of TRPM2 involvement in neointimal hyperplasia was explored. A major causative factor for neointimal growth is excessive production of ROS. At sites of vessel injury, ROS are over-produced by inflammatory cells and VSMCs. The accumulated ROS stimulate migration of VSMCs from media layer to the neointimal region, and enhance VSMC proliferation [5,6,10,33]. These events promote neointimal growth and facilitate the progression of atherosclerotic lesions [5,10,33]. TRPM2 is a ROS-sensitive Ca$^{2+}$-permeable channel [17,19]. Thus it is reasonable to suggest that ROS may activate TRPM2 in VSMCs to cause a cytosolic Ca$^{2+}$ rise, which then serves as a second messenger to stimulate VSMC migration and proliferation. In experiments, we found that TRPM2 was predominantly expressed in SMCs in neointimal regions in rodent model of neointimal hyperplasia. Furthermore, TRPM2-mediated Ca$^{2+}$ response to ROS was much larger in neointimal SMCs derived from cuff-injured arteries than in normal SMCs from non-cuffed arteries. In scratch wound healing assay, H$_2$O$_2$ treatment enhanced the migration of rodent aortic SMCs. In EdU incorporation assay and MTT assay, H$_2$O$_2$ treatment stimulated SMC proliferation. Importantly, blocking TRPM2 by TM2E3 or Trpm2 knockout markedly reduced the migration and proliferation of the SMCs. These data support the notion that ROS act through TRPM2 to stimulate the migration and proliferation of VSMCs as a possible underlying mechanism for neointimal growth and atherosclerotic progression. Previous studies have shown that several other Ca$^{2+}$-permeable channels, including TRPC4, TRPC5, TRPC6 and Orai1, may also be involved in the migration and proliferation of VSMCs [34–37]. However, there is lack of evidence as to whether these other channels play a role in neointimal hyperplasia of blood vessels.

Axl is a redox-sensitive receptor tyrosine kinase. It has been reported that H$_2$O$_2$ can activate Axl to facilitate VSMC migration via an Akt-dependent pathway [28,29]. In addition, neointima formation in response to cuff injury was reduced in Axl knockout mice compared with Axl wild-type mice [28]. Here we found that H$_2$O$_2$ induced Ca$^{2+}$ influx via TRPM2 channel in rodent aortic SMCs, and that inhibiting TRPM2 or chelating extracellular Ca$^{2+}$ almost abolished the H$_2$O$_2$-induced phosphorylation of Axl and Akt. These data indicate that Axl/Akt may be the signaling pathway downstream of TRPM2 in VSMC activation and neointimal hyperplasia. However, it remains unclear how TRPM2 activation induces Axl/Akt phosphorylation. Reports show that Axl is activated by its endogenous ligand Gas6, a growth factor that belongs to the vitamin K-dependent protein family [28,38]. Thus, it is possible that Ca$^{2+}$ influx via TRPM2 channels might induce the production of Gas6, which acts on Axl via an autocrine/paracrine mechanism and results in the phosphorylation of Axl and Akt. However, further studies are needed to clarify this issue.

Previously, another TRP isoform, TRPC1, has been suggested to be involved in neointimal growth [22]. In that study, inhibiting TRPC1 with a TRPC1-specific blocking antibody was found to reduce neointimal hyperplasia of in vitro cultured human saphenous vein [22]. However, the role of TRPC1 in neointimal growth is controversial. A later study reported no difference in neointimal formation between wild-type and Trpc1 knockout mice [39]. Here, with the use of Trpm2 knockout mice, we provided convincing evidence for a critical role of TRPM2 in neointimal growth. The results also highlight an exciting possibility of targeting TRPM2 as a possible therapeutic option for the treatment of neointimal hyperplasia and/or restenosis. Up to the present, almost no effective therapeutic option is available for the treatment or prevention of atherosclerotic neointimal formation, except for lipid-lowering drugs [40,41]. In this regard, TRPM2 is a membrane channel protein, which can be easily accessed by chemical and immunological agents. TM2E3 is a blocking antibody highly specific to TRPM2 [27], therefore it offers an attractive option to reduce neointimal growth. However, further study is needed to determine the efficacy and potency of TM2E3 to TRPM2 as well as its toxicity before the strategy can be further considered.

5. Conclusion

We demonstrated that TRPM2 plays an important role in neointimal growth of blood vessels. Its role in neointimal hyperplasia is related, at least partly, to its modulating action on VSMC migration and proliferation via Axl/Akt signaling pathway (Fig. 9). As a ROS-sensitive...
Ca^{2+}-permeable channel, the identification of TRPM2 links excessive ROS to Ca^{2+} entry and VSMC activation in neointimal hyperplasia, and makes TRPM2 a highly promising drug target for therapy of occlusive vascular diseases. Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jbbadis.2015.03.014.

Conflict of interest

The authors report no relationships that could be construed as a conflict of interest.

Transparency Document

The Transparency document associated with this article can be found, in the online version.

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