Atherosclerosis 241 (2015) 313–321

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Txnip ablation reduces vascular smooth muscle cell inflammation and ameliorates atherosclerosis in apolipoprotein E knockout mice

Chang Hyun Byon, Tieyan Han, Judy Wu, Simon T. Hui*

Division of Cardiology, Department of Medicine, University of California at Los Angeles, Los Angeles, CA 90095, USA

ARTICLE INFO

Article history:
Received 10 September 2014
Received in revised form
9 May 2015
Accepted 17 May 2015
Available online 3 June 2015

Keywords:
Inflammation
Atherosclerosis
Oxidative stress

ABSTRACT

Objective: Inflammation of vascular smooth muscle cells (VSMC) is intimately linked to atherosclerosis and other vascular inflammatory disease. Thioredoxin interacting protein (Txnip) is a key regulator of cellular sulfhydryl redox and a mediator of inflammasome activation. The goals of the present study were to examine the impact of Txnip ablation on inflammatory response to oxidative stress in VSMC and to determine the effect of Txnip ablation on atherosclerosis *in vivo*.

Methods and results: Using cultured VSMC, we showed that ablation of Txnip reduced cellular oxidative stress and increased protection from oxidative stress when challenged with oxidized phospholipids and hydrogen peroxide. Correspondingly, expression of inflammatory markers and adhesion molecules were diminished in both VSMC and macrophages from Txnip knockout mice. The blunted inflammatory response was associated with a decrease in NF-κB nuclear translocation. Loss of Txnip in VSMC also led to a dramatic reduction in macrophage adhesion to VSMC. *In vivo* data from Txnip-ApoE double knockout mice showed that Txnip ablation led to 49% reduction in atherosclerotic lesion in the aortic root and 71% reduction in the abdominal aorta, compared to control ApoE knockout mice.

Conclusion: Our data show that Txnip plays an important role in oxidative inflammatory response and atherosclerotic lesion development in mice. The atheroprotective effect of Txnip ablation implicates that modulation of Txnip expression may serve as a potential target for intervention of atherosclerosis and inflammatory vascular disease.

1. Introduction

Vascular smooth muscle cells (VSMC) are vital to the maintenance of vascular tone and play a critical role in many vascular diseases, including atherosclerosis, hypertension, in-stent stenosis and aneurysms [1]. In response to vascular injury or oxidative stimuli, VSMC undergo a phenotypic change to a "proliferative, migrating and synthetic" state, characterized by excess extracellular matrix and inflammatory cytokine production with expression of adhesion molecules such as intercellular ICAM-1 and VCAM-1 [2]. Secretion of paracrine signals by activated VSMC promotes leukocyte infiltration into the damaged vessel wall (reviewed in [3]). These changes in VSMC contribute to atherosclerotic plaque growth and the formation of fibrotic cap, which stabilizes and prevents plaque rupture. Oxidation products of phospholipid 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine (oxPAPC) mimic the *in vivo* effects of minimally modified LDL [4]. In addition to inducing multiple proatherogenic changes in endothelial cells and macrophages [5], oxPAPC stimulates VSMC differentiation and proliferation [6–8]. These findings highlight the role of oxidative stress and reactive oxygen species (ROS) in atherogenesis.

Thioredoxin interacting protein (Txnip) was originally identified by yeast two-hybrid analysis as a negative regulator of thioredoxin-1 (Trx1) [9], a key determinant of cellular sulfhydryl redox homeostasis. We and others have demonstrated that Txnip modulates cellular glucose utilization and mitochondrial oxidation of metabolic fuels [10–14]. Txnip-null mice cannot survive prolong fasting and exhibit hypoglycemia, hyperketonemia and

Abbreviations: Txnip, thioredoxin interacting protein; WT, wild type; VSMC, vascular smooth muscle cells; TKO, Txnip knockout; Trx, thioredoxin; DHE, dihydroethidium; E_h, redox potential; E'_0, midpoint potential; ROS, reactive oxygen species; oxPAPC, oxidized 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine; Prdx, peroxiredoxin; GPx, glutathione peroxidase; MCP-1, monocyte chemotactic protein-1; HO-1, heme oxygenase-1; PAI-1, plasminogen activator inhibitor-1; CD36, cluster of differentiation 36; SMA, smooth muscle actin.

* Corresponding author. Division of Cardiology, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095-1679, USA.
E-mail address: sthui@mednet.ucla.edu (S.T. Hui).

http://dx.doi.org/10.1016/j.atherosclerosis.2015.05.020
0021-9150/

hypertrilgyceridemia [13]. Besides its involvement in cellular redox and energy metabolism, there is increasing evidence that indicates the importance of Txnip in vascular function and inflammation process. Genetic association studies showed that polymorphism affecting Txnip expression is linked to hypertension and arterial stiffness [15,16]. Studies in endothelial cells showed that Txnip promotes inflammatory response in response to disturbed flow [17]. In addition, Txnip is required for NLRP3 inflammasome activation and IL-1β production in cultured THP-1 cells [18]. However, the role of Txnip in VSMC inflammation is not well understood. Given the critical role of Txnip in redox homeostasis and inflammation, we hypothesize that ablation of Txnip expression would protect VSMC from oxidative stress and reduce inflammation. In the present study, we used VSMC isolated from TKO mice to investigate the effects of Txnip ablation on cellular redox status and inflammatory response. In addition, we also assessed the impact of Txnip on atherosclerosis *in vivo*. Our data shows that Txnip plays an important role in both VSMC inflammation and atherosclerosis.

2. Materials and methods

2.1. Animal studies

Generation and characterization of Txnip knockout (TKO) mice were described previously [19]. TKO mice were bred with ApoE knockout mice (in C57BL/6 background) to generate heterozygous Txnip-ApoE double knockout ($Txnip^{+/-}/ApoE^{+/-}$) mice. Heterozygous mice were then inter-crossed to generate homozygous Txnip-ApoE double knockout ($Txnip^{-/-}/ApoE^{-/-}$) mice. Circulating monocytes profiling was carried out using the Heska (Loveland, CO) HemaTrue™ Veterinary Hematology Analyzer. Blood was collected in 20-μl heparin-coated glass capillaries and processed using standard procedures as per instructions from Heska. All procedures described were approved by the Institutional Animal Care and Use Committee of the University of California at Los Angeles.

2.2. Cell culture

Primary VSMC were isolated by enzymatic dissociation from the aortas of TKO and C57BL/6 mice and cultured in growth media as described previously [20]. The purity and identity of SMC were determined by immunohistochemical staining with smooth muscle–specific α-actin antibody (Sigma Chemical Co., St. Louis, Missouri, USA). All cells were positive for smooth muscle α-actin (SMA). In addition, expression levels of endothelial cell markers and smooth muscle markers were measured by quantitative real-time PCR to ensure the identity of SMC lineage. VSMC were grown to 100% confluence and then switched to serum-free media for 1 h. Cells were treated with OxPAPC (gift from Judith Berliner, 40 μg/ml) or H_2O_2 (0.4 mM) in DMEM containing 1% FBS for 4 h. All experiments were performed with VSMC at passages 5 to 9. Bone marrow-derived macrophages were isolated from the femur and tibia of C57BL/6 mice and cultured as described previously [21].

2.3. Measurement of cellular ROS levels

Cells were stained with 5 μM dihydroethidium (DHE) (Molecular Probes, Eugene, OR, U.S.A.) for 30 min at 37 °C. Fluorescence of DHE was captured with a fluorescence microscope (excitation wavelength at 488 nm and emission wavelength at 585 nm) and quantified using ImageJ software (U.S. National Institutes of Health, Bethesda, MD) and normalized to cell area.

2.4. Redox Western blot

Redox Western analysis of Trx-1 was performed as previously described [22]. Briefly, cells were lysed in cold G-lysis buffer (50 mM Tris, 6 M guanidine HCl, 3 mM EDTA, 0.5% Triton X-100, pH 8.30) containing 50 mM iodoacetic acid [23]. Derivatized proteins were desalted and separated by native PAGE and Western blotted with anti-mouse Trx1 antibody (Cell Signaling). The intensity of bands corresponding to oxidized (Trx_{ox}) and reduced Trx1 (Trx_{red}) was quantified using ImageJ software (NIH Bethesda, MD). Trx1 redox potential (E_h) was calculated from the ratio of oxidized to reduced Trx1 by using the Nernst equation with E_o (midpoint potential) = −254 mV at pH 7.4 and 25 °C [23–25]:

$$E_h = E_o + (RT/2F)\ x\ \ln([Trx_{ox}]/[Trx_{red}])$$

where R = universal gas constant, T = absolute temperature and F = Faraday constant

2.5. Extraction of total RNA and quantitative real-time PCR

Total RNA was isolated from VSMC using Trizol (Invitrogen) and reverse transcribed into cDNA using ABI MultiScribe Reverse Transcription Kit (Applied Biosystems) as manufacturer's instruction. SYBR Green-based quantitative real-time PCR was performed using SYBR Premix Ex Taq (TaKaRa) on LightCycler® 480 Instrument II (Roche). The primers used were listed in Supplementary Table S1.

2.6. Macrophage adhesion assay

Macrophage-to-VSMC adhesion studies were performed as described previously with modifications [26]. Briefly, macrophages were labeled by incubating with BCECF-AM (1.6 μM) in PBS at 37 °C for 30 min. Fluorescently labeled cells were washed twice with medium containing 1% FBS/DMEM. Macrophages (4×10^4 cells/well) were added to 24 wells containing confluent VSMC monolayers that were serum-starved for 1 h and then treated with OxPAPC (40 μg/ml) or H_2O_2 (0.4 mM) for 4 h. After incubation for 15 min at 37 °C, medium was removed and VSMC layers with attached macrophages were gently washed twice with PBS. BCECF AM-labeled macrophages were observed under fluorescence microscope (Leica) and imaged by cold CCD camera after fixed with 4% paraformaldehyde. Fluorescently labeled macrophages were analyzed and quantified using ImageJ software (NIH Bethesda, MD).

2.7. Subcellular fractionation of NFκB

Cytosolic and nuclear fractions were prepared using the NE-PER Nuclear Protein Extraction kit (Pierce) according to manufacturer's instruction. Protein concentration was determined by BCA Protein Assay kit (Pierce). Equal amounts of protein were loaded in each well and resolved by SDS-PAGE, followed by Western blot analysis using specific antibody to NFκB (p65). Amount of protein in each sample was quantified by densitometry and normalized to that of lamin A/C (nuclear) or tubulin (cytosolic).

2.8. Histological analyses and quantification of atherosclerosis lesion

Hearts from 28 weeks old male mice were perfused with saline. Following cryo-embedding in OCT, serial cryosections were prepared through the ventricle until the aortic valves appeared. From then on, every fifth 10-μm section was collected on poly-d-