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TRAF3IP2 mediates atherosclerotic plaque development and vulnerability in *ApoE*$^{-/-}$ mice

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ABSTRACT

Background and aims: Atherosclerosis is a major cause of heart attack and stroke. Inflammation plays a critical role in the development of atherosclerosis. Since the cytoplasmic adaptor molecule TRAF3IP2 (TRAF3-Interacting Protein 2) plays a causal role in various autoimmune and inflammatory diseases, we hypothesized that TRAF3IP2 mediates atherosclerotic plaque development.
Methods: TRAF3IP2/ApoE double knockout (*DKO*) mice were generated by crossing *TRAF3IP2*$^{-/-}$ and *ApoE*$^{-/-}$ mice. *ApoE*$^{-/-}$ mice served as controls. Both *DKO* and control mice were fed a high-fat diet for 12 weeks. Plasma lipids were measured by ELISA, atherosclerosis by *en face* analysis of aorta and plaque cross-section measurements at the aortic valve region, plaque necrotic core area, collagen and smooth muscle cell (SMC) content by histomorphometry, and aortic gene expression by RT-qPCR.
Results: The plasma lipoprotein profile was not altered by *TRAF3IP2* gene deletion in *ApoE*$^{-/-}$ mice. While total aortic plaque area was decreased in *DKO* female, but not male mice, the plaque necrotic area was significantly decreased in *DKO* mice of both genders. Plaque collagen and SMC contents were increased significantly in both female and male *DKO* mice compared to respective controls. Aortic expression of proinflammatory cytokine (Tumor necrosis factor α, TNFα), chemokine (Chemokine (C-X-C motif) Ligand 1, CXCL1) and adhesion molecule (Vascular cell adhesion molecule 1, VCAM1; and Intercellular adhesion molecule 1, ICAM1) gene expression were decreased in both male and female *DKO* mice. In addition, the male *DKO* mice expressed markedly reduced levels of extracellular matrix (ECM)-related genes, including TIMP1 (Tissue inhibitor of metalloproteinase 1), RECK (Reversion-Inducing-Cysteine-Rich Protein with Kazal Motifs) and ADAM17 (A Disintegrin And Metalloproteinase 17).
Conclusions: TRAF3IP2 plays a causal role in atherosclerotic plaque development and vulnerability, possibly by inducing the expression of multiple proinflammatory mediators. TRAF3IP2 could be a potential therapeutic target in atherosclerotic vascular diseases.

1. Introduction

Atherosclerosis is an important contributing factor for cardiovascular diseases such as heart attack and stroke [1]. It is one of the major leading causes of death in Western countries and its incidence is increasing alarmingly in developing countries [1]. Atherosclerosis results in gradual narrowing of arterial lumen due to lipid accumulation and infiltration of immune cells into the subintimal space, and involves complex cellular and molecular interactions, leading to plaque development, rupture, and thrombosis [3]. Although the major stages involved in atherosclerotic plaque

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development and stability have been well described, the molecular mechanisms underlying its pathogenesis are not fully understood. Identifying newer molecules and determining their role in atherosclerosis development and progression will help us better understand the disease process and the development of novel therapeutic strategies.

TRAF3-Interacting Protein 2 (TRAF3IP2; also known as CIKS [Connection To IKK And SAPK/JNK] or Act1 [NF-Kappa-B Activator 1]) is a cytoplasmic adaptor molecule and activator of the transcription factors nuclear factor κB (NF-κB) and activator protein 1 (AP-1) in an I kappa B kinase (IKK)- and c-Jun N-terminal kinase (JNK)-dependent manner [20,21]. NF-κB and AP-1 regulate the expression of various pro-atherogenic mediators, including proinflammatory cytokines, chemokines, adhesion molecules and ECM degrading MMPs, that play critical roles in atherosclerotic plaque formation and progression [30].

The major cell types involved in atherosclerotic plaque formation (*e.g.*, endothelial cells, SMCs, and macrophages) express TRAF3IP2 [39–44]. High glucose and ox-LDL induce TRAF3IP2 expression and endothelial dysfunction *in vitro* [40,44]. Angiotensin II (AngII), aldosterone, interleukin-18 (IL-18), and advanced oxidation protein products (AOPPs) that play a role in atherogenesis induce TRAF3IP2 expression *in vitro* [34,39,41–43], suggesting that TRAF3IP2 could play a role in atherosclerosis. Therefore, we hypothesized that TRAF3IP2 mediates atherosclerotic plaque development and progression.

2. Materials and methods

2.1. Generation of TRAF3IP2/ApoE double knockout (DKO) mice

All animal studies were approved by the Institutional Animal Care and Use Committee at Tulane University in New Orleans, LA, and conformed to the Guide for the Care and Use of Laboratory Animals published by the NIH. *TRAF3IP2*$^{-/-}$ mice (C57Bl/6 background) were previously described [8]. *ApoE*$^{-/-}$ mice (C57Bl/6 background) were purchased from The Jackson Laboratory (Ann Harbor, ME). *TRAF3IP2* and *ApoE* heterozygous mice (*TRAF3IP2*$^{+/-}$/*ApoE*$^{+/-}$) were generated by crossing *TRAF3IP2*$^{-/-}$ and *ApoE*$^{-/-}$ mice, and the double knockout mice (*TRAF3IP2*$^{-/-}$/*ApoE*$^{-/-}$; *DKO*) by intercrossing *TRAF3IP2*$^{+/-}$/*ApoE*$^{+/-}$ mice. Lack of *TRAF3IP2* expression in aortas of *DKO* mice was confirmed by RT-qPCR (Table 2).

2.2. Genotyping

A genomic polymerase chain reaction (PCR) was used to identify the wild-type (WT) and mutant alleles of *TRAF3IP2* (WT product: 250bp; mutant product: 453bp) and *ApoE* (WT product: 145bp; mutant product: 250bp) (Supplementary data, upper panels). *TRAF3IP2*: primer 1–5′-CTGGCATGTTTTCTCTTGTTTC-3’; primer 2: 5′-GCCTTCTAAAGAAACTGGCTTC-3’; primer 3: 5′-CAATCATGTGTTCAGTCAGC-3’; primer 4: 5′-GCTCTATGGCTTCTGAGG-3’. The PCR conditions were as follows: denaturation at 95 °C for 30 s, annealing at 55 °C for 45 s, and extension at 72 °C for 30 s. *ApoE*: primer 1: 5′-GCCTAGCCGAGGGAGAGCCG-3’; primer 2: 5′-TGTGACTTGGGAGCTCTGCAGC-3’; primer 3: 5′-GCCGCCCCGACTGCATCT-3’. PCR conditions were as follows: denaturation at 94 °C for 30 s, annealing at 68 °C for 40 s, and extension at 72 °C for 60 s.

2.3. Diet

Eight week-old male and female *DKO* and *ApoE*$^{-/-}$ mice (n = 10–15/group) were fed a high fat (Western) diet (42% of total calories from fat; 0.15% cholesterol; #TD88137, Harlan-Teklad) for twelve weeks. Body weights were recorded weekly and blood was collected by cardiac puncture prior to euthanasia.

2.4. Atherosclerosis quantification

Mice were anesthetized and perfused initially with saline and then with 4% paraformaldehyde plus 5% sucrose. Heart and entire aorta were dissected and fixed overnight in 4% paraformaldehyde plus 5% sucrose. Atherosclerosis was quantified by *en face* analysis of aorta and determination of plaque cross-sectional area at the aortic root. For *en face* analysis, adventitial fat was removed from the aorta and stained with Oil Red O and opened longitudinally, pinned *en face* and photographed. The total arterial surface area and total plaque were determined by Image-Pro PLUS v 6.0 software (Media Cybernetics Inc.). The extent of lesion development was determined as a percentage of the total area of the aorta that was occupied by Oil Red O-positive atherosclerotic lesions. For determination of plaque cross-sectional area, serial 6 μm-thick sections from the entire aortic root area were stained with hematoxylin and eosin (H&E). Plaque cross-sectional area was determined by quantifying the plaque area in images (DP70 digital camera) using Image-Pro PLUS software. The mean value of plaque cross-sectional areas from 3 sections was used to estimate the extent of atherosclerosis in each animal.

2.5. Features of plaque stability

The necrotic core area was determined in aortic valve plaques by quantifying acellular area (H&E negative), which also contains cholesterol crystals. Aortic valve sections were stained with Masson’s trichrome, photographed and collagen positive area was quantified. Smooth muscle cell content in plaques was determined by immunohistochemistry of aortic valve sections. Sections were incubated with mouse alpha-smooth muscle actin (αSMA) antibody (Millipore) or isotype-matched mouse IgG (Abcam), followed by secondary antibody and Avidin, Alexa Flour® 488 conjugate (ThermoFisher). Nuclei were stained with DAPI. Images were taken using a fluorescent microscope and αSMA-positive area in the plaques was quantified.

2.6. Quantitative real-time RT-qPCR

Mice were anesthetized and perfused with saline. The whole aorta was dissected and cleaned in RNA*later*® (ThermoFisher) and stored at −80 °C until further use. Total RNA was isolated using Trizol reagent (Sigma). Total RNA (0.5 μg) was reverse transcribed into cDNA using a reverse transcription kit. Collagen, type I, α1 (ColIα1; Assay ID: Mm00801666), Collagen, type III, α1 (ColIIIα1; Assay ID: Mm1254476), Matrix metalloproteinase 2 (MMP2; Assay ID: Mm01253621), MMP9 (Assay ID: mm00600163), MMP14 (Assay ID: Mm01318969), Tissue inhibitor of metalloproteinase 1 (TIMP1, Assay ID: Mm00441818), Reversion-Inducing-Cysteine-Rich Protein with Kazal Motifs (RECK, Assay ID: Mm01342144), A disintegrin and metalloproteinase domain 10 (ADAM10, Assay ID: Mm00545742), ADAM17 (Assay ID: Mm00456428), Tumor necrosis factor α (TNFα, Assay ID: Mm00443258), Interleukin-6 (IL-6, Assay ID: Mm00446191), IL-18 (Assay ID: Mm00434226), Intercellular adhesion molecule (ICAM1, Assay ID: Mm01175876), Vascular cell adhesion molecule 1 (VCAM1, Assay ID: Mm00449197), Monocyte chemoattractant protein 1 (MCP 1, Assay ID: Mm00441242), Chemokine (C-X-C) ligand 1 (CXCL1, Assay ID: Mm00433859), Angiotensin II receptor, type 1 (AGTR1A, Assay ID: Mm01957722) and Angiotensin II receptor, Type 2 (AGTR2; Assay ID: Mm01341373) mRNA levels were determined by RT-qPCR using TaqMan® probes (Applied Biosystems). Data were analyzed using the $2^{-\Delta\Delta Ct}$ method.