Implication of CD38 Gene in Autophagic Degradation of Collagen I in Mouse Coronary Arterial Myocytes

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1. **Animal procedure**

Both $CD38^{-/-}$ and wild-type ($CD38^{+/+}$) mice on C57BL/6 background were purchased from the Jackson Laboratory. $CD38^{+/+}$ and $CD38^{-/-}$ mice (8 weeks of age; male) were fed 10-week normal diet or Western diet containing (g%): protein 20, carbohydrate 50, and fat 21 (Dytes, Bethlehem, PA, USA), as described previously (1). Mice were sacrificed by cervical dislocation under ether anesthesia. The hearts with coronary artery were obtained for coronary dissection or frozen in liquid nitrogen for preparation of frozen section slides. All experimental protocols were reviewed and approved by the Animal Care Committee of Virginia Commonwealth University. All animals were provided standard rodent chow and water ad libitum in a temperature-controlled room.

2. Masson's trichrome staining protocol for collagen fiber

Deparaffinize and rehydrate the paraffin section and then wash it in distill water. Then the section was stained successively in hematoxylin working solution and Biebrich scarlet-acid fuchsin solution which was followed by differentiation in phosphomolybdic- phosphotungstic acid solution. Transfer section directly to aniline blue solution stained for 5-10 minutes. After rinsing briefly in distilled water, the section was differentiated in 1% acetic acid solution for 2-5 minutes followed by dehydration very quickly through 95% and 100% ethyl alcohol. Through the protocol above, the collagen fibers will be stained blue, the nuclei will be stained black and the background is stained red.

3. Primary cell culture of mouse CAMs

CAMs were isolated from $CD38^{+/+}$ or $CD38^{-/-}$ mice as previously described (2). In brief, mice were deeply anesthetized with an intraperitoneal injection of pentobarbital sodium (25 mg/kg). The heart was excised with an intact aortic arch and immersed in a petri dish filled with ice-cold Krebs-Henseleit solution. A 25-gauge needle filled with Hanks' buffered saline solution was inserted into the aortic lumen opening while the whole heart remained in the ice-cold buffer solution. The opening of the needle was inserted deep into the heart close to the aortic valve. The needle was tied in place with the needle tip as close to the base of the heart as possible. The

infusion pump was started with a 20 ml syringe containing warm HBSS through an intravenous extension set at a rate of 0.1 ml/min for 15 min. HBSS was replaced with warm enzyme solution (1 mg/ml collagenase type I, 0.5 mg/ml soybean trypsin inhibitor, 3% bovine serum albumin (BSA), and 2% antibiotic), which was flushed through the heart at a rate of 0.1 ml/min. Perfusion fluid was collected at 30-, 60-, and 90-min intervals. At 90 min, the heart was cut with scissors, and the apex was opened to flush out the cells that collected inside the ventricle. The fluid was centrifuged at 1000 rpm for 10 min, the cell-rich pellets were mixed with one of the media described below, and the cells were plated on 2% gelatin-coated six-well plates and incubated in 5% CO_2 at 37 °C. Advanced Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 10% mouse serum, and 2% antibiotics was used for isolated smooth muscle cells. The identification of CAMs was based on positive staining by anti-α-actin antibody and the SMC morphology. The medium was replaced 3 days after cell isolation and then once or twice each week until the cells grew to confluence. All studies were performed with cells of passage of 3-5.

4. Western blot analysis

Western blot analysis was performed as described previously (3). In brief, proteins from the CAMs or dissected coronary arteries were extracted using sucrose buffer (20 mM HEPES, 1 mM EDTA, 255 mM sucrose, cocktail of protease inhibitors (Roche), pH 7.4). After boiling for 5 min at 95 °C in 5× loading buffer, 30 μg total protein was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins of these samples were then electrophoretically transferred at 100 V for 1 hour onto a PVDF membrane (Millipore, USA). The membrane was blocked with 5% nonfat milk in Tris-buffered saline-Tween 20, and after washing, the membrane was probed with primary antibodies overnight at 4 °C, followed by incubation with horseradish peroxidase-labeled IgG (1:5000). Primary antibodies used are rabbit anti-collagen 1 (dilution 1:1000, Cell Signaling Technology), rabbit anti-LC3B (dilution 1:1000, Abcam), rabbit anti-MMP-9 (dilution 1:1000, Abcam), rabbit anti-TIMP1 (dilution 1:1000, Abcam) or goat anti-β-actin (1:500, Santa Cruz). The immunoreactive bands

were detected by chemiluminescence methods and visualized on Kodak Omat X-ray films. Densitometric analysis of the images obtained from X-ray films was performed using the Image J software (NIH).

5. Confocal Microscopic Analysis

For confocal analysis, cultured CAMs were grown on glass coverslips, stimulated or unstimulated, fixed in 4% paraformaldehyde in phosphate-buffer saline (PFA/PBS) for 15 min. After being permeablized with 0.1% Triton X-100/PBS and rinsed with PBS, the cells were incubated overnight at 4 °C with indicated primary antibodies: mouse anti-collagen 1 and rabbit anti-lamp 1 or rabbit anti-LC3B (1:200, Cell Signaling). After washing, these slides probed with primary antibodies were incubated with Alexa-488- or Alexa-555-labeled secondary antibodies for 1 h at room temperature. The slides were mounted and subjected to examinations using sequentially scanning on a laser scanning confocal microscope (Fluoview FV1000, Olympus, Japan), with photos being taken and the co-localization analyzed by the Image Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA). The summarized co-localization efficiency data was expressed as Pearson correlation coefficient (PCC) as described previously (4).

REFERENCES

1. M. Bjursell, A. K. Gerdin, C. J. Lelliott, E. Egecioglu, A. Elmgren, J. Tornell, J. Oscarsson and Y. M. Bohlooly: Acutely reduced locomotor activity is a major contributor to Western diet-induced obesity in mice. *Am J Physiol Endocrinol Metab*, 294(2), E251-60 (2008) doi:10.1152/ajpendo.00401.2007
2. M. Xu, Y. Zhang, M. Xia, X. X. Li, J. K. Ritter, F. Zhang and P. L. Li: NAD(P)H oxidase-dependent intracellular and extracellular O2*- production in coronary arterial myocytes from CD38 knockout mice. *Free Radic Biol Med*, 52(2), 357-65 (2012) doi:10.1016/j.freeradbiomed.2011.10.485
3. Y. Zhang, M. Xu, M. Xia, X. Li, K. M. Boini, M. Wang, E. Gulbins, P. H. Ratz and P. L. Li: Defective autophagosome trafficking contributes to impaired autophagic flux in coronary arterial myocytes lacking CD38 gene. *Cardiovasc Res*, 102(1), 68-78 (2014) doi:10.1093/cvr/cvu011
4. M. Xia, C. Zhang, K. M. Boini, A. M. Thacker and P. L. Li: Membrane raft-lysosome redox signalling platforms in coronary endothelial dysfunction induced by adipokine visfatin. *Cardiovasc Res*, 89(2), 401-9 (2011) doi:10.1093/cvr/cvq286