ONLINE SUPPLEMENT

THYMOSIN β4 DEFICIENCY EXACERBATES RENAL AND CARDIAC INJURY IN ANGIOTENSIN-II-INDUCED HYPERTENSION

Nitin Kumar[1], Tang-Dong Liao[1], Cesar A. Romero[1], Mani Maheshwari[1], Edward L. Peterson[2] and Oscar A. Carretero[1].

[1]Hypertension and Vascular Research Division, Dept. of Internal Medicine, Henry Ford Hospital, Detroit, MI 48202, [2]Department of Public Health Sciences, Henry Ford Hospital, Detroit, MI 48202

Short title: Tβ4 and end-organ damage

*Correspondence to: Oscar A. Carretero, M.D.
Hypertension and Vascular Research Division
Henry Ford Hospital
2799 West Grand Boulevard
Detroit MI 48202-2689
Phone: (313) 916-2103
Fax: (313) 916-1479
Email: ocarret1@hfhs.org

Methods

Experimental animals

Male C57BL/6J rats (Jackson Laboratories, Sacramento, CA) and male Tβ4 KO-mice (on C57BL/6 background) at 8-10 weeks old were housed at bio-resource facility at Henry Ford Hospital. Tβ4 KO-mice were provided as a kind gift from Dr. Ju Chen from the University of California at San Diego. Animals were kept at room temperature in vented cages with 12:12 light/dark cycle and fed rodent chow *ad libitum*. Animals were acclimatized to the new environment for two weeks before performing the experiments. All the surgical procedures were conducted under thiobutabarbital (125 mg/kg b.w, i.p.) anesthesia. All the experimental protocols were approved by Institutional Animal Care and Use Committee (IACUC) of Henry Ford Hospital and were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Experimental protocols

C57BL/6 and Tβ4 KO mice (n=6-14) at 8-10 weeks-old were randomly divided and subcutaneously infused with either vehicle (0.01 N acetic acid in saline solution) or Ang-II in vehicle (980 ng/kg/min, Bachem, Bubendorf, Switzerland) for 6 weeks using osmotic minipump (Alzet, Cupertino, CA, USA). Two-weeks before the minipump implantation, mice were trained for 3 times/week for blood-pressure measurement *via* non-invasive tail-cuff method. After the minipumps implantation, systolic blood pressure was measured weekly. Before urine collection, animals were acclimatized for 2-3 days in metabolic cages. At six-weeks, 24 hours urine was collected in metabolic cages for urinary albumin excretion. At the end of the study at six weeks, animals were sacrificed and tissues were weighed and collected for biochemical, western blot and histological analysis.

Systolic blood-pressure, body weight and organ weight

Systolic Blood Pressure (SBP) was measured in conscious mice by computerized tailcuff system (MC4000, Hatteras Instruments Inc, Cary, North Carolina, USA), as described elsewhere [1]. At the end of the experiment, body weight was measured, and mice were euthanized. The heart and kidneys were excised, and weighed. Heart and kidney weight to tibia length ratio were determined. The heart and kidney was then sectioned transversely into 4 sections. One section was fixed in 4% paraformaldehyde and paraffin embedded, another section was embedded in frozen tissue specimen compound (Tissue-Tek O.C.T. Compound, Sakura Finetek Inc., CA, USA), immersed in cold isopentane (VWR) and snap-frozen in liquid nitrogen and stored at -80°C. A third section from the renal cortex and heart apex was used for hydroxyproline assay. The remaining section was frozen in liquid nitrogen and stored at -80°C for western blot analysis.

Echocardiography

Echocardiography was performed at 6 weeks in non-anesthetized mice as described previously [2]. Briefly, an Acuson Sequoia 512 system (Mountain View, California, USA) equipped with a 15-MHz linear transducer was used. Left ventricle remodeling was

assessed by measuring left ventricle systolic and diastolic dimension areas (LVAs and LVAd), left ventricle dimensions (LVDs and LVDd), and posterior wall thickness (PWT). Systolic performance was assessed by measuring ejection fraction and shortening fraction.

Renal and cardiac macrophage infiltration

Kidney and heart frozen sections (6 μm) were fixed in cold acetone for 20 minutes and the endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for additional 20 minutes. Nonspecific binding was blocked with 1% bovine serum albumin. Primary monoclonal antibody, rat anti-mouse CD68 antigen, which is a marker for macrophages (clone: FA-11, 1:100 dilution, Bio-Rad, Hercules, CA, USA), was applied, and samples were incubated overnight at 4°C. PBS buffer alone and a nonspecific purified rat anti-mouse IgG were used as a negative control. The next day, sections were incubated with a secondary biotinylated antibody, goat anti-rat IgG (1:200 dilution). Immunoreactivity was detected with ABC peroxidase kit (Vectastain Elite, Vector Laboratories, Burlingame, CA, USA), and visualized with 3-amino-9-ethylcarbazole (Zymed Laboratories). Reddish-brown staining was considered positive. Sections were counterstained with hematoxylin to visualize cell nucleus. Twenty regions of each section were examined under 20x objective microscope (Nikon's Eclipse E600), photographed with a digital camera (DS-Ri1, Nikons Instrument), and evaluated by a computerized image analysis system (Microsuite Biological Imaging, Olympus America). All of the images captured and analyzed in this study were obtained using the same system unless otherwise specified. Positive cells in high-power fields were counted for each section and expressed as cells per millimeter square.

Renal and cardiac fibrosis

Picrosirius red staining (PSR) was used to quantify renal and cardiac interstitial collagen deposition, a marker of fibrosis, as previously reported [3]. Sequential 4 μm paraffin-embedded sections were stained. Briefly, tissues were post-fixed in Bouin's fluid and stained with 0.1% picrosirius red for 1 hour. Samples were then washed twice in 0.5% acetic acid. Nuclei were counterstained with hematoxylin. For renal and cardiac interstitial collagen fraction (ICF), 30 images were taken with 20x objective of Nikon's Eclipse E600 microscope with Nikon's DS-Ri1 digital camera (Nikon Instruments Inc.). The ICF was analyzed by computerized image analysis (Microsuite Biological imaging software, Olympus America, Center Valley, PA), and expressed as the ratio of the collagen positive area to the entire area of the captured field. All of the images shown in this study were captured and analyzed using the same imaging system, unless otherwise specified.

Renal and cardiac collagen content by hydroxyproline assay

Total collagen content of the renal cortex and cardiac apex was determined by hydroxyproline assay, as described previously [4]. Briefly, samples were dried, homogenized, and hydrolyzed with 6 N HCI for 16 hours at 110°C. A standard curve of 0 to 5 μg of hydroxyproline was obtained. Data were expressed as μg of collagen per mg of dry weight, assuming that collagen contains an average of 13.5% hydroxyproline.

Assay for Ac-SDKP

Ac-SDKP was measured using a commercially available, highly specific competitive enzyme-linked immunosorbent assay (ELISA) kit as described previously [5]. To harvest heart and kidneys, WT and Tβ4 KO mice were anesthetized and then transcardially perfused with 0.9% NaCl for 5-6 minutes to remove blood from the organs. The heart and kidneys were excised, individually weighed, snap-frozen in liquid nitrogen and stored at -80°C until homogenized. Heart and kidneys were homogenized in PBS (pH 7.4) containing 10 μmol/L captopril using an IKA Ultra-turax homogenizer, followed by sonication. Captopril was added to prevent Ac-SDKP degradation during the sample processing. Samples were centrifuged at 10,000 x *g* for 15 minutes at 4˚C to remove pellet (cell debris), and supernatants (heart and kidney homogenates) were collected. The total protein concentrations were determined by Bradford assay [6]. Aliquots of supernatants were stored at -80°C until further use. 50 μg of heart and kidney homogenate was used for Ac-SDKP determination by ELISA.

Statistical analysis

A nonparametric two-sample Wilcoxon test with a Fliqner-Policello correction for unequal variances was used to compare contrasts of interest in all the data. Significance was determined using Hochberg's method to adjust for multiple testing. The adjustment was made on groups of similar tests. A *P*-value less than 0.01 was considered as evidence of significant differences. Analysis was generated using SAS/STAT software, Version 9.3 of the SAS System for Windows (Copyright SAS Institute Inc, Cary, NC)

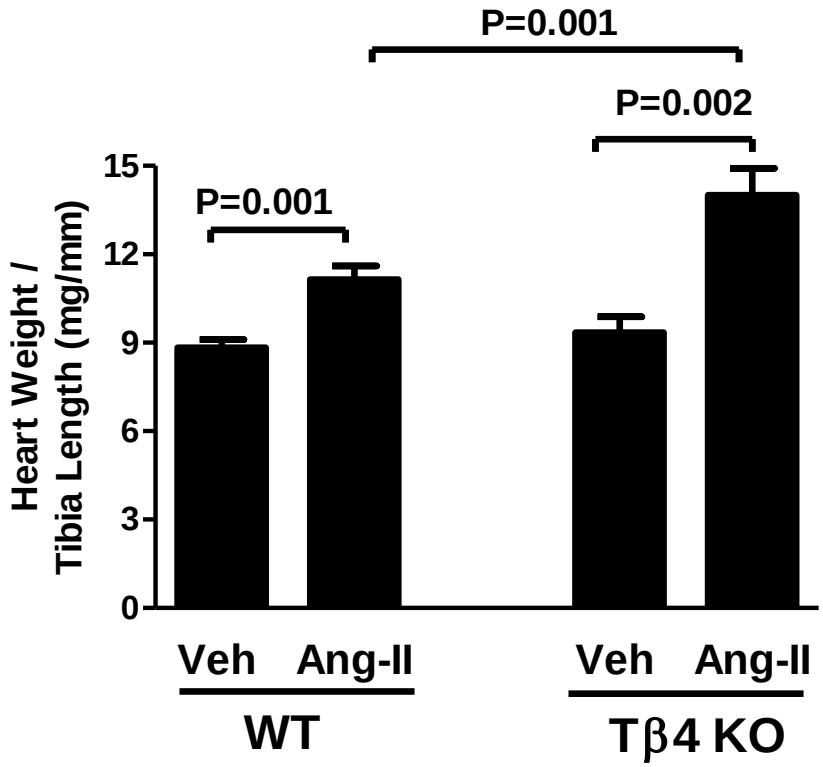
References

1. Feng M, DiPetrillo K. Non-invasive blood pressure measurement in mice. *Methods Mol Biol*. 2009;573:45-55
2. Yang XP, Liu YH, Rhaleb NE, Kurihara N, Kim HE, Carretero OA. Echocardiographic assessment of cardiac function in conscious and anesthetized mice. *Am J Physiol*. 1999;277:H1967-1974
3. Liao TD, Yang XP, D'Ambrosio M, Zhang Y, Rhaleb NE, Carretero OA. N-acetyl-seryl-aspartyl-lysyl-proline attenuates renal injury and dysfunction in hypertensive rats with reduced renal mass: Council for high blood pressure research. *Hypertension*. 2010;55:459-467
4. Cleutjens JP, Verluyten MJ, Smiths JF, Daemen MJ. Collagen remodeling after myocardial infarction in the rat heart. *Am J Pathol*. 1995;147:325-338
5. Cavasin MA, Rhaleb NE, Yang XP, Carretero OA. Prolyl oligopeptidase is involved in release of the antifibrotic peptide ac-sdkp. *Hypertension*. 2004;43:1140-1145
6. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976;72:248-254

Table S1. Body weight, heart rate and kidney weight at six weeks of Ang-II infusion in Tβ4 KO mice.

Parameters	WT-Veh	Tβ4 KO Veh	WT Ang-II	Tβ4 KO Ang-II
Body weight (g)	30±0.88	30.58±1.39	27.37±0.74	25.72±0.76*
Heart rate (bpm)	624±28	671±9	650±9	630±20
Tibia length (mm)	17.71±0.14	17.96±0.16	17.68±0.12	17.65±0.15
Kidney weight / tibia length (mg/mm)	20.23±0.93	20.33±0.96	17.89±0.79	21.04±0.72†

Beats per minute (bpm) * *P*<0.01 Tβ4 KO Veh vs Tβ4 KO Ang-II, † *P*<0.01 Tβ4 KO Ang-II vs WT Ang-II. All data are expressed as mean ± SEM; n = 6-10 in each group.



200

201 **Figure S1.** Effect of Ang-II infusion on cardiac hypertrophy in Tβ4 KO mice. Ang-II
202 infusion led to an increase in cardiac hypertrophy in WT-mice, but this was exacerbated
203 in Tβ4 KO mice.

204

205

206

207

208

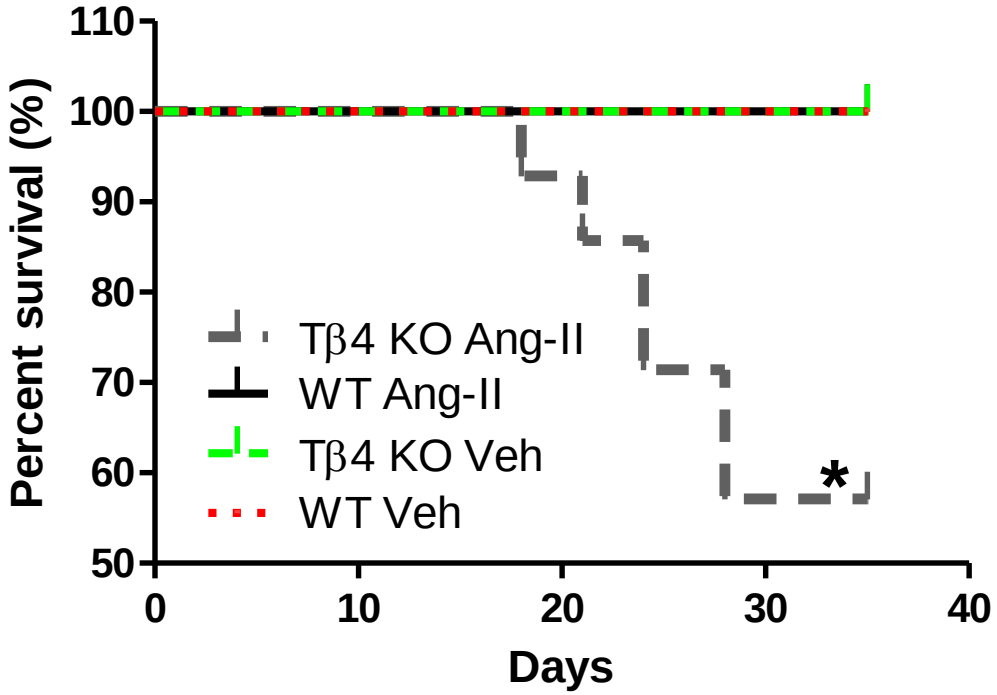


Figure S2. Effect of Ang-II infusion on mortality rate in Tβ4 KO mice. In vehicle treatment, no mortality was observed in WT as well as Tβ4 KO mice. In Ang-II infusion, Tβ4 KO mice showed markedly high mortality, compared to no mortality observed in WT. * P<0.01 Tβ4 KO Ang-II vs WT-Ang-II. All data are expressed as mean ± SEM; n = 6-14 in each group.

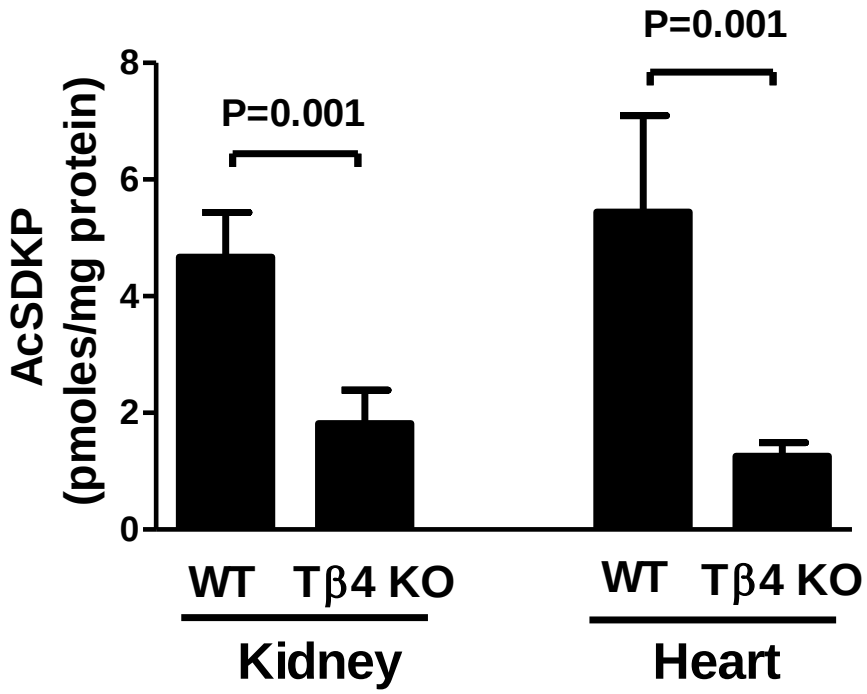


Figure S3. N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) content in the kidney and heart of Tβ4 KO mice. In normal condition, Ac-SDKP content was significantly reduced in kidney as well as in heart of Tβ4 KO mice, compared to the WT. All data are expressed as mean ± SEM; n = 6 in each group.