ONLINE SUPPLEMENT

NF kappa B and Matrix Metalloproteinase induced Receptor Cleavage in the Spontaneously Hypertensive Rat

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Short Title: NF kappa B Upregulation in Hypertension

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DETAILED MATERIALS AND METHODS

Animals

The experimental protocol was reviewed and approved by the University of California, San Diego Animal Subjects Committee. Male SHR at 13-15 weeks of age and their normotensive controls, the Wistar Kyoto (WKY) (Charles River Laboratories, Wilmington, MA, USA) of comparable age were first tranquilized with Xylazine (20 mg/ml, 200 µl / kg bodyweight i.m) (MWI, Nampa, ID). After 15 minutes, general anesthesia was administered (Nembutal, 50 mg/ml, 1 ml/ kg bodyweight, i.m.) (Pentobarbital Sodium Injection, Ovation Pharmaceuticals, Inc.,Deerfield, IL). After 15 minutes, reflex level was tested with a tail pinch to assure a surgical level of anesthesia. Polyethylene(PE) catheters (PE50, I.D. 0.5mm/ O.D. 0.956 mm, Becton Dickinson Primary Care Diagonistics, Sparks, MD) were placed into the femoral artery and femoral vein prior to start of surgery. The systolic blood pressure was recorded by a laboratory computer (Power Macintosh G3 with MacLab, Apple Computer Company, Cupertino, CA). Supplemental doses of anesthesia were administered intravenously at a dose of 5 mg/kg as needed after reflex testing. Body temperature was maintained at 37°C by a water-heated animal stage. At the end of study, the animals were euthanized (sodium pentobarbital 120 mg/kg body weight, i.v.).

Experimental Protocol

Subgroups of the WKYs and SHR rats were treated with NF-κB inhibitor, pyrrolidine dithiocarbarmate (PDTC, 150 mg/kg/day ; Sigma-Aldrich, St.Louis, MO). The drug was given in drinking water for a period of 10 weeks. Untreated group received standard chow and water ad libitum. PDTC has a molecular weight of 164 Da and under chronic conditions likely passes the blood brain barrier.

Determination of Systolic Blood Pressure

Two animals from each group were measured by the tail-cuff method. The blood pressure was measured every week by the same person and at the same time of day. Cannulation of each rats under anesthesia were also performed to confirm the measurements obtained from tail-cuff method.

Tissue Preparation

After the tissues (brain, heart and kidney) were removed from the animals, they were cut and embedded in Tissue-Tek O.C.T. (Optimal Cutting Temperature) Compound (Sakura Finetek, Torrance, CA). Each tissue sample was sectioned using a Leica CM 3500 cryostat onto Fisherbrand Superfrost Plus Microscope Slides (Fisher Scientific, Pittsburgh, PA). Section thickness was fixed at 10 µm for brain, and 5µm for kidney and heart.

Immunohistochemical Labeling of NF-κB

Frozen sections were fixed with methanol or acetone at -20°C for 5 minutes. Endogenous peroxidase was quenched by peroxidase blocking solution (Peroxo-Block; invitrogen, Carlsbad, CA). Non-specific immune-adsorption was blocked by incubation with 5 % goat serum in PBS-T (0.1% Triton X-100 in PBS) for 1 hour. The sections were then labeled with anti - NFκB p65 rabbit polyclonal antibody (sc-109, Santa Cruz Biotechnology, Santa Cruz, CA) (1:50 in 5% goat serum in PBS-T, v/v). This antibody recognized both the inactive form of p65 subunit, bound to p50 and IκB in the cytoplasm, and the active monomeric form in the nucleus. Buffer alone

without primary antibody or nonspecific purified rabbit immunoglobulin G (IgG) served as controls. Sections were washed three times in PBS-T before ImmPRESS peroxidase reagent (ImmPRESS anti-rabbit Ig peroxidase kit; Vector Laboratories Inc., Burlingame, CA) was used as secondary antibody. Peroxidase activity was visualized with diaminobenzidine (DAB) substrate (Vector Laboratories Inc.). Selected tissue sections were counterstained for location of cell nuclei (VECTASHIELD mounting medium with DAPI, Vector Laboratories Inc.)

Immunohistochemical Labeling of β_2AR

Frozen sections were fixed with acetone at -20°C for 5 minutes. Endogenous peroxidase was quenched by peroxidase blocking solution (Peroxo-Block; invitrogen, Carlsbad, CA). Non-specific immune-adsorption was blocked by incubation with 5 % goat serum in PBS-T (0.1% Triton X-100 in PBS) for 1 hour. The sections were then labeled with purified rabbit polyclonal antibodies against the extracellular domain of the β_2AR (against a peptide at the N-terminus, NLS2662, Novus Biologicals®, Littleton, CO, USA), and antibodies against the intracellular domain of the β_2AR (against a peptide at the C-terminus, M-20, sc-570, Santa Cruz Biotechnology®, San Diego, CA, USA). Buffer alone and nonspecific purified rabbit immunoglobulin G (IgG) served as controls. Sections were washed three times in PBS-T before ImmPRESS peroxidase reagent (ImmPRESS anti-rabbit Ig peroxidase kit; Vector Laboratories Inc., Burlingame, CA) was used as secondary antibody. Peroxidase activity was visualized with diaminobenzidine (DAB) substrate (Vector Laboratories Inc.). Selected tissue sections were counterstained for location of cell nucrlei (VECTASHIELD mounting medium with DAPI, Vector Laboratories Inc.).

Gelatin Gel Zymography Protocol

Gelatin zymography was carried out with 0.6 µl of animal plasma. SDS gels (10% degassed Acrylamide/ Bis) with gelatin (0.8 mg/ml) were loaded with plasma samples and run (~120V, constant voltage) until bromophenol blue tracking dye reaches the bottom of the gel. The gels were incubated in the renaturing buffer (2.5% v/v triton x-100) during gentle agitation for 60 minutes at room temperature. The gels were then incubated in the developing buffer under 37°C overnight for maximum sensitivity. The gels were stained with Coomassie Blue R-250 and then de-stained (destaining buffer with Methanol : Acetic acid : Water, 50 : 10 : 40 ratio) until areas of gelatinolytic activity appear as clear sharp bands (where the protease had digested the gelatin) over the blue background.

MMP-2 activity assay

Tissue were homogenized using PBS. Supernatant were collected after centrifuge 10,000 g for 10 mins. Same volume of supernatant was added to 5 µM MMP-2 substrate [MCA-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH_2] (Calbiochem, La Jolla, CA) and PBS. Reactions were incubate at room temperature for 20 mins and evaluate with a Luminonmeter (SpectraMax Gemini XS, Molecular Devices, Sunnyvale, CA) with 325 nm excitation and 393 nm emission filters.

***In situ* zymography**

Frozen sections were warm up at 37°C humidified chamber for 10 mins prior to assay. 100 µm MMP-1 and 9 substrate [Dnp-Pro-Cha-Gly-Cys(Me)-His-Ala-Lys (Nma)-NH2] (American Peptide Company, APC, Sunnyvale, CA) were mixed well with 0.1% agarose solution. Sections were incubated with the substrate in a

humidified chamber at 37°C for 1 hour. Lysis of the substrate was assessed by examination under a light microscope.

Image Analysis

The images of the sections were digitized and processed with Image J (NIH, http://rsbweb.nih.gov/ij/). In order to eliminate the background and generate new images for light absorption analysis, the following equation is used for each pixel:$I_I' = (I_I - I_D) / (I_B - I_D) * 255$

I_I'= new image

I_I = pixel intensity on the image

I_B = pixel intensity without section

I_D = pixel intensity when no microscope light is on

Then the new images were inverted and converted into grayscale for further analysis. Since only tissue area was used for light absorption measurement, areas on the sections without tissue were set to an intensity of zero as a lower threshold.

In order to get average value of light intensity over the tissue and exclude the areas without tissue, the following equations are used:

$$I_{Threshold} = \frac{255 \sum A\ tissue + 0 \sum A\ empty}{\sum A\ tissue + \sum A\ empty}$$

$$I_{gray\ tissue} = \frac{\sum I\ tissue + \sum I\ empty}{\sum A\ tissue + \sum A\ empty}$$

ΣA tissue: total tissue area

ΣA empty: total area without tissue

ΣI tissue: total pixel intensity of tissue

ΣI empty: total pixel intensity of area without tissue

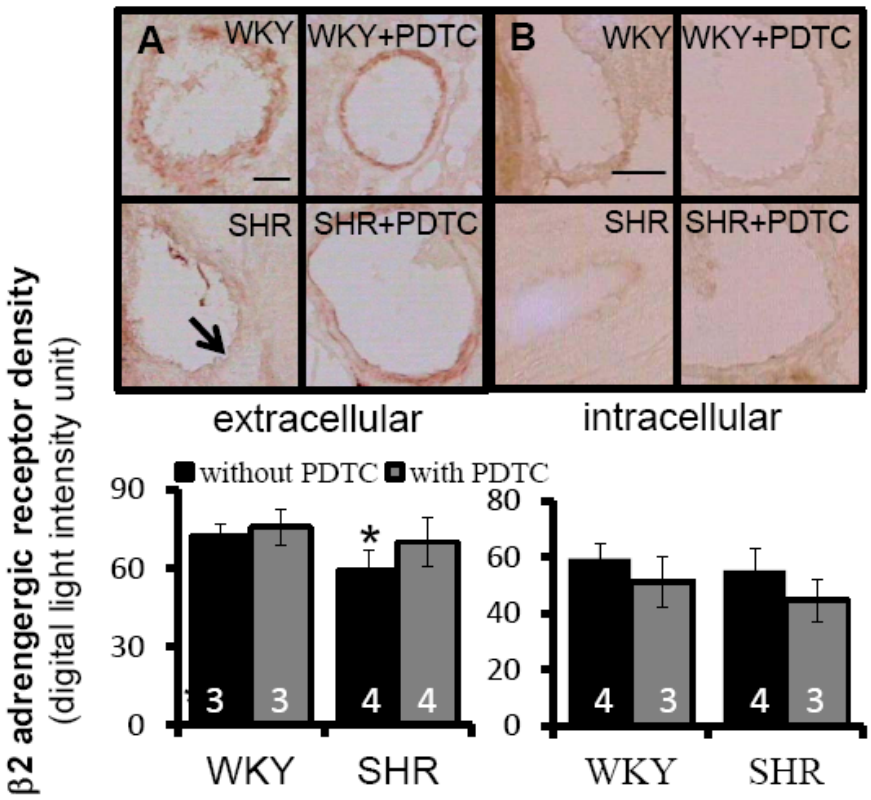
$I_{Threshold}$ and $I_{gray\ tissue}$ were measured with the program and then the total tissue pixel intensity and the total tissue area can be calculated from these two equations respectively. The immunohistochemical label intensity is derived by taking the total tissue pixel intensity divided by total tissue area.

In order to investigate the translocation of NF-κB, the NF-κB label intensity were measured separately in nucleus and cytoplasm. Location of nucleus was identified by DAPI co-staining. If the NF-κB expression level shows significant increase in nucleus compared to cytoplasm, it is considered to have NF-κB activation.

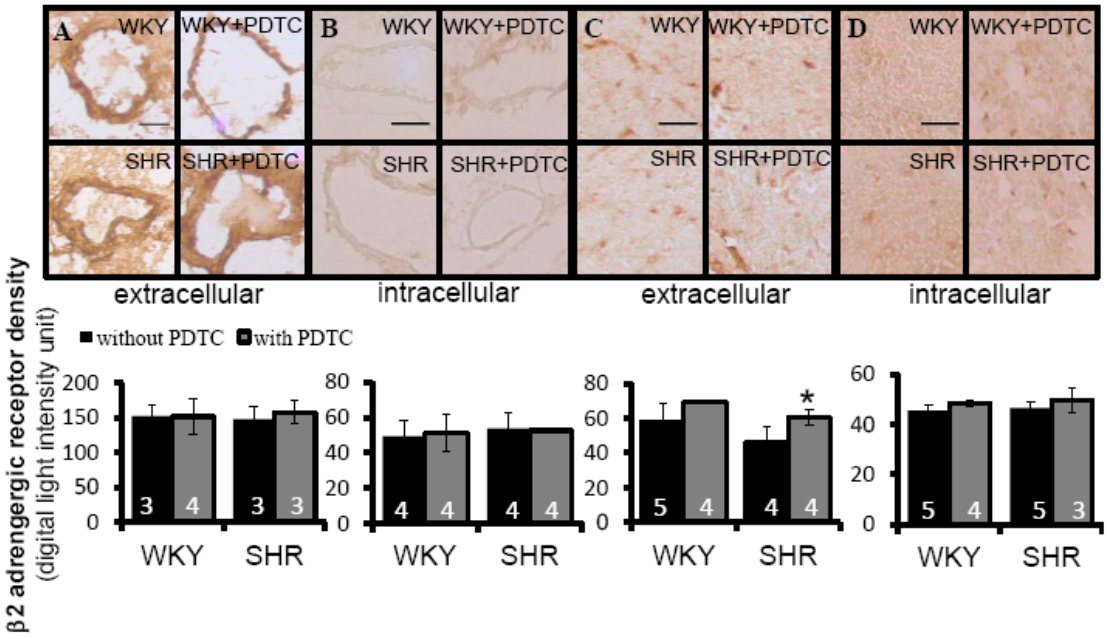
Statistical Analysis

All measurements are presented as mean ± standard deviation. Comparisons of mean values between animal groups were carried out by two-tailed student's t-test. $p < 0.05$ was considered statistically significant.

S1

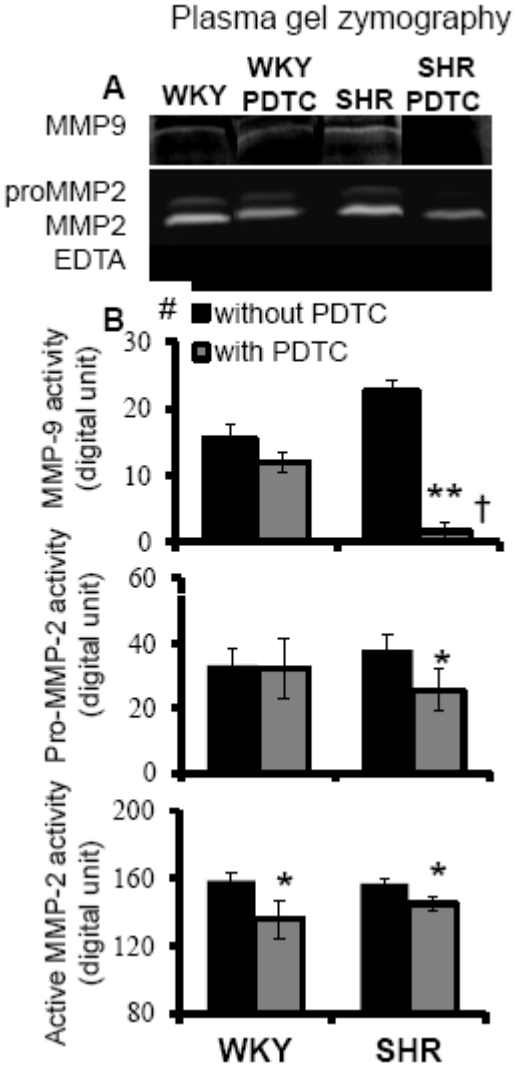


(Top panels) Micrographs of immunohistochemical sections and in vertical alignment (bottom bar graphs) optical density measurements of the extracellular **(A)** and intracellular **(B)** domains of the β_2AR in the vessels of the heart. Note the reduced labeling on SHR endothelium (arrow). *$p<0.05$ compared to WKY in student's t-test. Number of rats is indicated in the bar. Scale bar= 100 μm



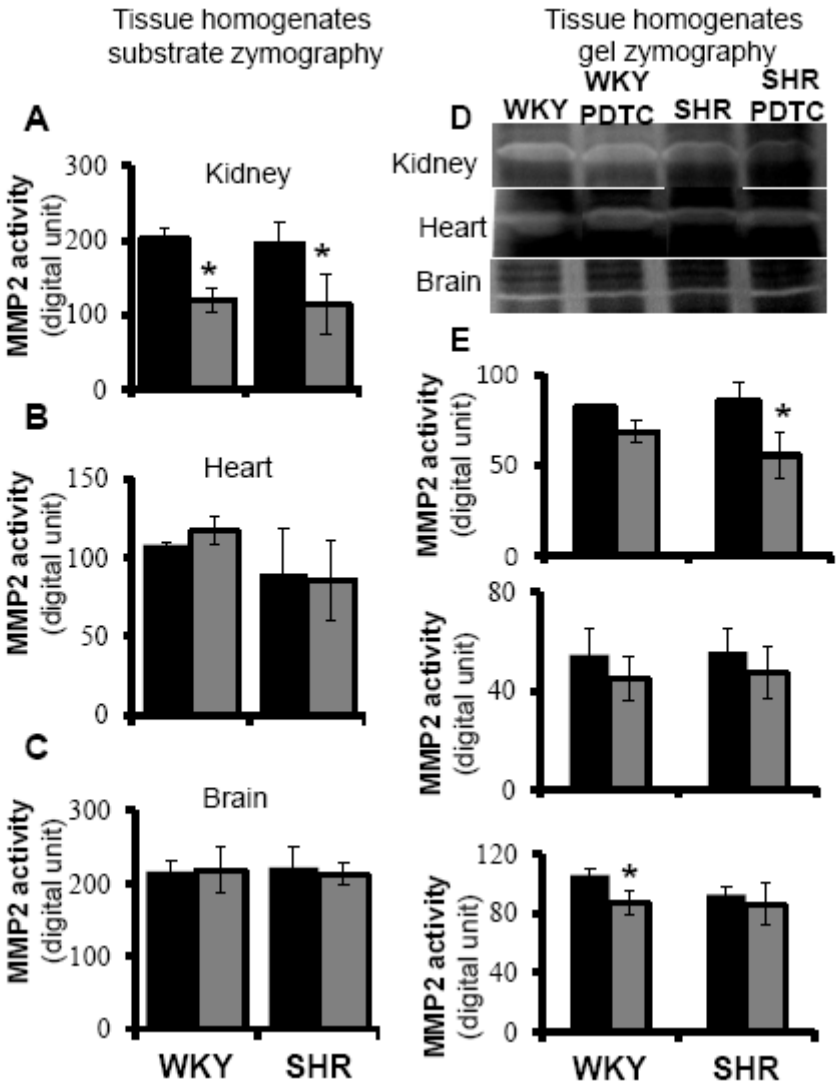
(Top panels) Micrographs of immunohistochemical sections and in vertical alignment (bottom bar graphs) optical density measurements of the extracellular **(A)** and intracellular **(B)** domains of the β_2AR in the vessels of the brain and the extracellular **(C)** and intracellular **(D)** domains in hypothalamus. *p<0.05 compared to non-treated SHR in student's t-test. Number of rats is indicated in the bar. Scale bar= 100 μm

S3



(A) MMP activity in WKY and SHR plasma without and with PDTC treatment measured by gelatine zymography. The protease activity in plasma was confirmed with gel zymography using molecular weight standards. Images were derived from same gels but different locations. **(B)** Measurements of each specific MMP activity. *p<0.05 compared to non-treated group, †p<0.05 compared to treated WKY, **p<0.01 compared to non-treated SHR, #p<0.05 compared to non-treated WKY in student's t-test. N=4 rats in each group.

S4



(Top to bottom) MMP-2 activity in kidney homogenates **(A)**, heart homogenates **(B)**, and brain homogenates **(C)** were determined by specific fluorescently quenched substrates. (**D**) MMP-2 activity in tissue homogenates by gel zymography. **(E)** Measurements of MMP-2 activity in kidney, heart and brain. *$p<0.05$ compared to non-treated group. N=4 rats in each group.