Nuclear Factor Kappa B and Matrix Metalloproteinase Induced Receptor Cleavage in the Spontaneously Hypertensive Rat

Kwan-I Sharon Wu, Geert W. Schmid-Schönbein

Abstract—Recent evidence suggests that inflammation in the spontaneously hypertensive rat (SHR) is associated with an uncontrolled matrix metalloproteinase (MMP) activity. We hypothesize that the transcription factor nuclear factor kappa B (NFκB) is overexpressed in the SHR, enhancing its MMP activity and enzymatic cleavage of the β2 adrenergic receptor (β2AR), thereby diminishing catecholamine-mediated arteriolar vasodilation. NFκB expression level and translocation were compared between Wistar Kyoto rat and SHR kidney, heart, and brain. The animals were treated with NFκB inhibitor, pyrrolidine dithiocarbamate, for 10 weeks and correlations between NFκB and MMP activity were determined. Immunohistochemistry showed that NFκB expression is increased in untreated SHR kidney (~14%) and brain hypothalamus (~22%) compared to that in Wistar Kyoto rats (P<0.05), but not in myocardium and cerebral cortex. After pyrrolidine dithiocarbamate treatment, the SHR systolic blood pressure was reduced to close to Wistar Kyoto rat levels. NFκB expression level in treated SHR was also decreased in kidney and hypothalamus compared to nontreated animals (P<0.05). Furthermore, MMP-2 and MMP-9 activities in SHR plasma were significantly reduced (~41%) by pyrrolidine dithiocarbamate treatment. Additionally, zymographic analyses and in situ zymography showed decreased MMP-2 activity in kidney homogenates and decreased MMP-1 and MMP-9 activities in brain. The level of the β2AR extracellular, but not intracellular, domain density was found to be reduced in kidney, showing a receptor cleavage process that can be blocked by pyrrolidine dithiocarbamate treatment. These results suggest NFκB is an important transcription factor in the SHR and may be involved in the enhanced MMP activity and, consequently, receptor cleavage. (Hypertension. 2011;57:261-268.)

Key Words: adrenergic receptor ■ matrix metalloproteinases ■ microcirculation ■ receptor cleavage ■ pyrrolidine dithiocarbamate

Chronic hypertension is associated with enhanced risk for cardiovascular disease, including atherosclerosis, stroke, and renal failure.1–4 There is increasing evidence suggesting a strong association between hypertension and inflammation, as well as end-organ damage, eg, expression of inducible nitric oxide synthase and inflammatory markers, elevated levels of activated leukocytes in the circulation, enhanced leukocytes cytotoxicity, oxidative stress, and apoptosis.5–12

We recently obtained evidence that members of the matrix metalloproteinase (MMP) family,13 known to degrade the extracellular matrix and connective tissue proteins in different physiological and pathological conditions,14–16 have elevated levels in hypertension. In the spontaneously hypertensive rat (SHR), elevated levels of MMP-2, MMP-9, and MMP-7 cause direct damage to cells by cleavage of the extracellular domain of several key receptors, which results in diverse cell dysfunctions.12 For example, proteolytic cleavage of the vasodilatory β2 adrenergic receptor (β2AR) causes arteriolar constriction and blood pressure elevation,17 cleavage of the extracellular domain of the insulin receptor produces insulin resistance,18 and cleavage of the vascular endothelial growth factor receptor-2 leads to endothelial cell apoptosis and capillary rarefaction in the SHR.19

β2AR is a cell surface receptor associated with sympathetic nervous system pathways and is involved in mediating smooth muscle vasodilation to balance vascular tone and blood pressure homeostasis.20,21 Overexpression of the β2AR gene in the endothelium of the carotid artery serves to restore vasorelaxation in SHR.22 Several studies also have shown a genetic susceptibility of β2AR in hypertension, especially the Arg16 allele polymorphism.23–26

This evidence raises the question, what induces the expression of MMP in the SHR? MMP can be influenced by a variety of agents and cytokines, such as IL-1,27,28 and transcription factors, like activating protein-1, signal transducer and activator of transcription, and nuclear factor kappa B (NFκB), that bind to specific elements on MMP gene promoters.13 There is evidence that NFκB activation is...
prominent in damaged kidneys or dysfunctional hearts with upregulation of p65 mRNA, increased NFκB binding activity, and elevated inhibitor of kappa kinase activity.4,29,30 NFκB is a transcription protein with regions of DNA-binding and dimerization domains, nuclear translocation signal, and binding site for the inhibitor of κB,31 which is involved in regulating genes of the inflammatory cascade.32,33 On activation by extracellular stimuli, NFκB translocates from the cytoplasm into the nucleus and regulates specific gene expression.34 The SHR has elevated levels of NFκB expression18 that possibly may be attributable to elevated free radical production induced by excess nitric oxide production and oxidative stress, which have been suggested to have associations with hypertension.9,10 Thus, we hypothesize, that NFκB induces MMP expression in the SHR, which in turn causes receptor cleavage induced by MMP, such as β2AR and consequently elevated arteriolar contraction and arterial blood pressure.

In the present study, we explored the possibility of chronic NFκB inhibition on MMP expression and receptor cleavage in the SHR as compared to its normotensive control, the Wistar Kyoto rat (WKY). Among several NFκB inhibitors, we used pyrrolidine dithiocarbamate (PDTC) as an effective inhibitor that can be administered over several weeks.29,32,35 PDTC attenuates hypertrophy and end-organ damage, and it suppresses inducible nitric oxide synthase as well as superoxide anion formation in the SHR.7,29,36–39 Even though PDTC may have several biological effects that are not fully understood, it most likely stabilizes inhibitor of κB to prevent the release of NFκB rather than acting at the level of the cytoplasmic NFκB/inhibitor of κB complex or interfering with DNA binding.29,32 We determine NFκB levels in SHR kidney, heart, and brain by immunohistochemistry, MMP-2 and MMP-9 activities in plasma, and MMP-2, MMP-1, and MMP-9 activities in heart, kidney, and brain by zymography together with the level of β2AR proteolytic cleavage as a mechanism that is involved in the arterial pressure elevation.

Materials and Methods
Male SHR at age 13 to 15 weeks and their normotensive controls, the WKY, were treated with the NFκB inhibitor PDTC (150 mg/kg per day) in drinking water for a period of 10 weeks. Untreated controls received standard chow and water ad libitum. The systolic blood pressure of 2 animals in each group was measured with the tail-cuff method every week by the same person and at the same time of day. At the end of the treatment period, the rats were administered general anesthesia (pentobarbital, 50 mg/kg). Selected tissues (brain, heart, and kidney) were removed and frozen sections (10-μm thickness for brain and 5-μm thickness for kidney and heart) were fixed and labeled with anti-NFκB p65 rabbit polyclonal antibody (sc-109; Santa Cruz Biotechnology). The antibody recognizes both the inactive form of p65 subunit, bound to p50 and inhibitor of κB in the cytoplasm, and the active monomeric form in the nucleus.

To evaluate potential enzymatic cleavage of the β2AR, purified rabbit polyclonal antibodies against the extracellular domain of the β2AR (against a peptide at the N-terminus, NLS2662; Novus Biologicals) and antibodies against the intracellular domain of the β2AR (against a peptide at the C-terminus, M-20, sc-570; Santa Cruz Biotechnology) were used on sequential sections. The primary antibodies were visualized by binding of secondary antibody conjugated to peroxidase activity with diaminobenzidine substrate. Buffer alone or nonspecific purified rabbit immunoglobulin G served as controls. Selected tissue sections were counterstained with diamino-2-phenylindole (DAPI) for location of cell nuclei.

Gelatin zymography with sodium dodecyl sulfate polyacrylamide gels was used to determine plasma protease activity. MMP-2 activity was determined in homogenized tissues using a MMP-2-specific fluorescently quenched substrate. MMP-1 and MMP-9 activities on frozen tissue sections were determined by in situ zymography using a substrate that is cleaved by both MMPs. Lysis of the substrate was assessed by examination under a fluorescence microscope. Immunolabeling intensities and fluorescence light intensity in the zymogra-
expression levels were not significantly elevated (Figure 2B). There were no differences of NFκB density in cytoplasm between WKY and SHR (Figure 1B, F) and in hypothalamus region of brain by 22% (Figure 1D, H) compared with WKY (P<0.05). The NFκB expression levels were not significantly elevated (P>0.05) in SHR myocardium (Figure 1A, E) and cerebral cortex (Figure 1C, G).

Translocation of NFκB
NFκB label density was measured in the nucleus and the cell cytoplasm. In renal tubular cells, SHR has a significantly higher NFκB label density in the nucleus, and there were no differences in cytoplasm between WKY and SHR (Figure 2B). There were no differences of NFκB density in cytoplasm between WKY and SHR in cells of renal blood vessel wall. However, SHR had significantly elevated label density in the nucleus than in cytoplasm (Figure 3B), whereas WKY and SHR had similar levels of label density in cytoplasm. No differences were found between either WKY and SHR or nucleus and cytoplasm in blood vessels of myocardium (Figure 3A). In the SHR brain, significantly higher expression in the nucleus was detected in hypothalamus and cells of its blood vessel walls but not in cerebral cortex (Figure 4).

Suppression of Blood Pressure by PDTC Treatment
Systolic blood pressure in SHR started to decrease in the second week of PDTC treatment compared with nontreated SHR (153±7 and 163±5 mm Hg, respectively, mean±SD). The time course of blood pressures in SHR during the treatment also showed a difference compared to the nontreated SHR (Figure 5). The mean±SD of the systolic blood pressure at the end of the treatment was 120±3 mm Hg in PDTC-treated SHR and was 188±4 mm Hg in nontreated SHR, whereas the mean±SD of the systolic blood pressure of the PDTC-treated and nontreated WKY rats were 115±4 mm Hg and 114±2 mm Hg, respectively.

NFκB Expression Level After PDTC Treatment
The NFκB expression level after PDTC treatment was significantly decreased by 12% compared with nontreated SHR.
in renal glomerular and tubulointerstitial areas (Figure 1B, F) and brain hypothalamus (Figure 1D, H), whereas no significant difference were detected between PDTC-treated WKY and nontreated WKY.

Receptor Cleavage of β2AR in the SHR
Cleavage of the extracellular domain of β2AR was detected by using different antibodies against its intracellular and its extracellular domains. In microvessels of the kidney, SHR had a significantly reduced expression of the extracellular domain of the β2AR (P<0.01; Figure 6 A, B). A similar trend was found in renal glomerular and tubulointerstitial areas (Figure 6 C, D). Additionally, the vessels in the myocardium of SHR had lower expression levels of the extracellular domain of β2AR (P<0.05; Supplemental Figure S1, please see http://hyper.ahajournals.org). The expression levels of the extracellular domain in the brain were not significantly different in SHR compared to WKY, including hypothalamus (Supplemental Figure S2, please see http://hyper.ahajournals.org), cerebral cortex (data not shown), and blood vessels (Supplemental Figure S2).

Attenuation of MMP Activity in Plasma by PDTC Treatment
Among the MMPs, we measured the MMP-2, MMP-1, and MMP-9 activities after PDTC treatment based on previous evidence showing increased MMP-2 and MMP-9 activities in SHR.18,19 The MMP-2 activity in plasma determined by gelatin gel zymography was not different between WKY and SHR. However, the MMP-2 activity was suppressed in both WKY and SHR after PDTC treatment by 14% and 8%, respectively. The pro-MMP-2 activity was suppressed by PDTC only in SHR and not in WKY (Supplemental Figure S3, please see http://hyper.ahajournals.org).

The SHR has elevated MMP-9 activity compared with WKY. During PDTC treatment, the MMP-9 activity was found to be suppressed in SHR but not in WKY, and the activity was also significantly lower than treated WKY (Figure S3). As control, both MMP-2 and MMP-9 activities were blocked in vitro by metal chelation with EDTA.

Tissue MMP Activity
In kidney homogenates, no difference in MMP-2 activity, determined with a specific fluorescent substrate, was found between WKY and SHR. However, after treatment both WKY and SHR showed decreased MMP-2 activity by 41% (Supplemental Figure S4A, please see http://hyper.ahajournals.org). The MMP-2 activity was not different between WKY and SHR in both heart and brain homogenates and the PDTC treatment had no effect on MMP-2 activity in either organ (Supplemental Figure S4B, C, please see http://hyper.ahajournals.org). In an alternative approach, the MMP-2 activities in brain, heart, and kidney homogenates were confirmed by gel zymology and similar results were found (Supplemental Figure S4D, E, please see http://hyper.ahajournals.org). The MMP-2 activity is blocked in vitro by EDTA (data not shown).

In the kidney, no difference in MMP-1 and MMP-9 activities, measured by in situ zymography, was found.
between WKY and SHR (Figure 7A). In contrast, SHR heart and brain exhibited significantly higher activities than WKY ($P<0.05$; Figure 7B, C). The brain of SHR with PDTC treatment and the kidney of WKY with PDTC have decreased MMP-1 and MMP-9 activities. However, the PDTC treatment did not have an effect on MMP-1 and MMP-9 activities in the heart (Figure 7B).

**Chronic NFκB Inhibition Attenuated β2AR Cleavage in the SHR**

After inhibiting NFκB with PDTC treatment, the extracellular domain density of the β2 AR was increased by 20% in renal glomerular and tubulointerstitial areas and by 39% in kidney vessels in SHR (Figure 6). In the cerebral cortex (data not shown) and hypothalamus, SHR with PDTC treatment also
exhibit significantly elevated β2AR levels (Supplemental Figure S2). No significant differences between these labels before and after PDTC treatment were found in blood vessels of the myocardium (P<0.05; Supplemental Figure S1).

**Discussion**

The current observations indicate an increased expression of the nuclear transcription factor NFκB in SHR (Figure 1F, H). Blockade of the transcription factor with PDTC serves to reduce the expression levels and the translocation into the cell nucleus with reduced MMP activity in kidney and brain (Supplemental Figure S4) and a reduction of blood pressure (Figure 5) and cleavage of the extracellular domain of the β2AR (Figure 6 and Supplemental Figures S1, S2).

The expression level of NFκB in this study was examined by immunohistochemistry. This approach permits detection of the transcription factor in different tissue regions (eg, endothelial cells vs mast cells or parenchymal cells), which in alternative approaches (eg, Western analysis) may not be possible at the microvascular level because of the need to homogenize the tissue. Separate measurements of the immunolabel density in the nucleus and in the cytoplasm also can be used to quantify nuclear translocation.

The nuclear translocation was prominent in the vessel walls of kidney and brain (Figures 2, 4), showing a possibility that NFκB activation in vessels in these tissues may be associated with endothelial dysfunction and further organ damage, such as renal failure and stroke. The endothelium of SHR microvessels exhibits enhanced apoptosis together with enhanced oxygen free radical formation and enzymes that synthesize them, together with enhanced MMP expression.

The elevated NFκB expression and activation found in glomerular and tubulointerstitial areas of the SHR are in agreement with previous studies in a renal model of hypertension. The activation of NFκB in myocardium (Figure 3B) also supports the association between myocardial infarction, hypertrophy, and hypertension. However, these results may have to be looked at from multiple perspectives, especially if apoptosis is present, as has been observed in several models of hypertension. The lack of a higher NFκB expression level in myocardium (Figure 1A, E), as seen by immunohistochemistry, may be the consequence of extensive apoptosis. Cells that undergo apoptosis lose the ability to synthesize new proteins; therefore, the group of cells that are being analyzed in our experiments consists merely of those cells still capable of protein synthesis. Significant NFκB expression in the SHR hypothalamus region but not in the cerebral cortex (Figure 1C, D, G, H) may suggest that hypertensive pathology is related to dysfunction of the hypothalamus because the output from the hypothalamus influences the nervous system and endocrine system, which in turn is associated with blood pressure control.

Blockade of NFκB by PDTC treatment facilitates a number of improvements in different hypertensive models, including reduction of blood pressure, cardiac hypertrophy, and inflammatory cell infiltration into heart and kidney tissue. In the current study, the systolic blood pressure was already lower in PDTC-treated SHR compared to nontreated SHR after 2 weeks of treatment (Figure 5). The decreased blood pressure by PDTC inhibition in this study is also consistent with the fact that suppression of acute inflammation may lower the blood pressure in the Lyon hypertensive model.

**Figure 7.** Micrographs of in situ zymography sections (top panels) using matrix metalloproteinase MMP-1 and MMP-9 substrate, and measurements of fluorescence density (bottom bar graphs) in kidney (A), heart (B), and brain (C). **P<0.01 compared to Wistar Kyoto rats (WKY). †P<0.05 compared to WKY in Student t test. N=4 for each group. Scale bar=100 μm.
The dose we used in this study (150 mg/day) has been applied in other studies without indication for toxic effect.20

PDTC treatment also suppressed NFκB expression level (Figure 1) and serves to reduce the cleavage of β2AR in the SHR (Figure 6). In our previous study, we identified a role of MMP cleaving β2AR in SHR and its attenuation after MMP inhibition.17 Therefore, we hypothesize that PDTC may have a similar effect on suppression of receptor cleavage attributable to inhibition of NFκB-induced MMP expression. In the current study, the enhanced MMP-2 and MMP-9 activity in the SHR plasma was significantly decreased after the 10-week PDTC treatment (Supplemental Figure S3A, B). This evidence is in line with the fact that blockade of NFκB almost completely inhibits expression of MMP-9, and with the presence of NFκB binding sites in the promoter region of MMP-9.45 Furthermore, among different organs we found a mixed picture of NFκB expression. For example, we did not see significantly decreased NFκB expression in the myocardium after PDTC treatment. PDTC possibly may affect other transcription factors that can suppress blood pressure without changing the total NFκB protein level.

Other off-target effects in addition to NFκB expression of PDTC also may be involved in suppression of the receptor cleavage. Administration of PDTC in different rat models reduces angiotensin II-induced inflammation and inducible nitric oxide synthase expression.37,46 The antioxidant properties of PDTC have been shown to play an important role in several biological reactions.32 Barki-Harrington et al47 provided evidence for a direct physiological interaction between angiotensin and β-adrenergic receptors, both of which are G-protein-coupled receptors; therefore, it is possible that the inhibition of the angiotensin receptor signaling pathway may have an effect on β-adrenergic receptors as a potential feedback reaction. Because PDTC may have multiple activities, more studies using other inhibitors are required to distinguish its off-target effects from its ability to inhibit NFκB.

Compared to WKY, SHR have elevated levels of MMP-2, MMP-1, and MMP-9 mRNA protein levels,18,48,49 which is in line with the current results. Furthermore, our evidence suggests that MMP may cause cleavage of the extracellular domain of β2AR in the kidney and the heart, which is consistent with previous studies showing reduced expression of this receptor as well as enhanced agonist-mediated desensitization in hypertension.50,51 Even though we did not observe significantly higher MMP-2 or MMP-1 and MMP-9 in the kidney, it is possible that other MMP activities could cause β2AR cleavage and requires further examination.

One of the important issues associated with the proteolytic activity in the SHR is that it may cause not only cleavage of the extracellular domain of the β2AR but also a variety of other surface receptors. Cleavage of the insulin receptor compromises the ability of insulin to signal intracellular glucose transport (insulin resistance18), cleavage of the vascular endothelial receptor causes endothelial apoptosis and capillary rarefaction,19 and cleavage of CD18 is associated with a reduced ability to mediate leukocyte adhesion to the endothelium in the SHR (immune suppression18). Therefore, treatment targeting the proteolytic activity, be it by interven-


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

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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 Diagnostics, 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 dithiocarbamate (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 β2AR**

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 β2AR (against a peptide at the N-terminus, NLS2662, Novus Biologicals®, Littleton, CO, USA), and antibodies against the intracellular domain of the β2AR (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 nuclei (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-NH2] (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 µm 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:

\[ I'_i = \frac{(I_i - I_D)}{(I_B - I_D)} \times 255 \]

- **\( I'_i \)** = 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:

\[ I_{\text{Threshold}} = \frac{255 \sum I_{\text{tissue}} + \sum I_{\text{empty}}}{\sum A_{\text{tissue}} + \sum A_{\text{empty}}} \]

\[ I_{\text{gray tissue}} = \frac{\sum I_{\text{tissue}} + \sum I_{\text{empty}}}{\sum A_{\text{tissue}} + \sum A_{\text{empty}}} \]

- **\( \sum A_{\text{tissue}} \)**: total tissue area
- **\( \sum A_{\text{empty}} \)**: total area without tissue
- **\( \sum I_{\text{tissue}} \)**: total pixel intensity of tissue
- **\( \sum I_{\text{empty}} \)**: total pixel intensity of area without tissue

\( I_{\text{Threshold}} \) and \( I_{\text{gray tissue}} \) 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.
(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 β2AR 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 β2AR 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
(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.
(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.