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Blood proteolytic activity elevation and plasma protein degradation in spontaneously hypertensive rat models

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Blood proteolytic activity elevation and plasma protein degradation in spontaneously
hypertensive rat models

A thesis submitted in partial satisfaction of the

Requirements for the degree Master of Science

in

Bioengineering

by

Jason C. Chow

Committee in Charge:

> Professor Geert W. Schmid-Schönbein, Chair
> Professor Marcos Intaglietta
> Professor Daniel T. O'Connor

2011

The Thesis of Jason C. Chow is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego
2011

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ABSTRACT OF THE THESIS

Blood proteolytic activity elevation and plasma protein degradation in spontaneously
hypertensive rat models

by

Jason C. Chow

Master of Science in Bioengineering

University of California, San Diego, 2011

Professor Geert W. Schmid-Schönbein, Chair

Hypertension is associated with elevated risk for cardiovascular diseases and renal
failure. Recent evidence suggests that there exist elevated levels of unchecked degrading
enzymatic activity in hypertensives that are not normally present in healthy controls.

These uncontrolled enzymes have been shown to cleave receptors, leading to the destruction of membrane receptors and impair cellular and bodily functions.

The objective of this study is to examine the possibility of plasma protein degradation by the enhanced enzymatic activity in hypertension. Blood and plasma proteinase levels and activity were determined and compared in the control Wistar Kyoto rat and the spontaneously hypertensive rat (SHR). Plasma proteins were analyzed for degradation, and peptide fragment levels were compared between the WKY and SHR group. A candidate protein, albumin, was examined for evidence of fragmentation in the plasma and urine.

Kinetic spectrometric analysis results showed that the SHR has elevated proteinase activity ($p < 0.05$) in both the plasma and whole blood. Gelatin gel zymography showed elevated levels of ~19.1 kDa, 67.3 kDa and 73.7 kDa gelatinase (possibly MMP7, MMP2, pro-MMP2, respectively) in the plasma of the SHR ($p < 0.05$). The SHR plasma was found to have reduced ~ 21.9 kDa, 33.3 kDa, 46.2 kDa and 226.1 kDa protein levels, increased ~2 kDa protein/peptide level and elevated plasma protein fragment levels ($p < 0.05$). A low molecular weight protein/peptide (~17.2 kDa) was elevated in the SHR urine. Albumin fragments (~17 kDa) were discovered in increased levels in the SHR urine ($p < 0.05$). Plasma incubation studies showed elevated fragment formation rate in the SHR plasma ($p < 0.05$) and the formation rate seems to be lowered by the addition of 10 mM EDTA. These results provide for the first time a kinetic characterization of the enhanced proteinase activity in the SHR plasma. The evidence suggests that the enhanced enzymatic level in the circulation of the SHR can cause not only receptor cleavage, but also plasma protein degradation, leading to elevated levels of fragment levels in the blood and urine.

Chapter 1. Introduction

1.1 Hypertension

Hypertension is defined as a condition with an arterial pressure of equal to or over 140/ 90 mmHg (8,17). It affects more than 60 million people in the United States (10,26). Resulting from the evidence for a combination of genetic, behavioral, hormonal and environmental factors, hypertension is widely regarded as a multi-factorial disease (8,41). Although chronic hypertension is associated with elevated risk for atherosclerosis, stroke, as well as renal and heart damage, its pathogenesis remains relatively unknown.

Many models of hypertension are available, such as acute, genetic and transgenic and hormonal models (18,29,44). In this study, I will be using the genetic model of hypertension, the spontaneously hypertensive rat and its normotensive control, the Wistar Kyoto (49). The SHR models develop chronic hypertension gradually over time without any intervention, making it a relatively simple model to emulate and study a model of essential hypertension in humans.

1.2 Hypertension and Inflammation

Recent evidence suggests an association between chronic hypertension and inflammation since arterial hypertension has been suggested to be associated with pro-inflammatory makers like leukocyte adhesion molecules, expression of chemokines, heat shock proteins, angiotensin etc. (41). Elevated levels of activated neutrophils, monocytes and other leukocytes has been shown in the circulation of the SHR, in addition to elevation of iNOS expression and other vascular inflammatory markers (7,30,59). SHRs

show a deficiency for leukocyte adhesion to the endothelium, resulting from lowered CD 18 and P-selectin expression (26,63). In hypertensives, leukocytes displayed increased cytotoxicity, as well as spontaneous degranulation of neutrophils (62). Macrophage and lymphocyte infiltration has been found in the SHR kidney, which can be responsible for the development of hypertension in the SHR (56). The presence of oxygen free radial production, oxidative stress and extensive apoptosis in the micro-vascular endothelium are additional markers of inflammation (19,38,42). Haptoglobin alpha-1 chain, part of an inflammation-induced plasma protein that is thought to attenuate the inflammatory process and act as a plasma antioxidant (32), has been found in essential hypertensives' plasma but not in healthy individuals (15). Furthermore, suppression of acute inflammation reduces the effect of hypertension in light of the evidence that a macrophage toxin treatment, silica, has been reported to lower blood pressure in Lyon hypertensive rats (6).

1.3 Hypertension and End Organ Damage

Epidemiological studies have shown that hypertensives are at enhanced risk for cardiovascular illness, kidney failure and stroke (41). Therefore it is important to understand the mechanism that leads to chronic hypertension. Hypertensives show signs of cardiovascular lesions and pathophysiological process, such as endothelial activation, inflammation and thrombogenesis, which can contribute to the development of hypertension but also other complications encountered in hypertensives (17,69). In a double transgenic rat model (dTGR), where rats transgenic for angiotensinogen and renin genes were crossed, hypertension developed and the rats suffered from fatal end-organ cardic and renal damage, with fibrosis and necrosis found in these organs (44).

1.4 MMP Gelatinases

MMP gelatinases belong to a MMP family of 24 structurally related zinc containing enzymes that are known to degrade the extracellular matrix proteins (24,67). MMPs have been classified into collagenases, gelatinases, stromelysins, membrane type and matrilysins, based on their substrate specificity (15). MMPs play a central role in maintaining homeostasis, including vascular modeling and cellular migration (15,25,51). MMPs are physiologically regulated by hormones, gene regulation, zymogen activators and ECM interaction, and are inhibited by tissue inhibitors of metalloproteinase (TIMP) and endogenous MMP inhibitors (MMPIs) (66,67).

MMP 2 and MMP 9 (Gelatinase A and B) belong to the gelatinase MMP subgroup that digest mainly gelatin. They have specific catalytic domains that have high affinity for gelatin, collagen and laminin (17). MMP 2 and 9 expression are regulated by various factors such as cytokines, such as IL-1, and transcription factors, as well as the downstream effect of activator protein 1 (AP1) (21,39). In this study, the kinetic proteolytic activity of the plasma MMP gelatinases will be investigated in the SHR using fluorescently quenched substrates.

1.5 The Autodigestion Hypothesis and Chronic Hypertension

1.5.1 The Autodigestion Hypothesis

The autodigestion hypothesis was recently developed by our lab, which states that in chronic hypertension, the loss of certain cellular and bodily functions could be at least in part caused by the presence of unchecked degrading enzymes cleaving the protein receptors responsible for those functions (20,60,65). The idea for autodigestion originated from studies of a severe form of inflammation, shock, in which the mucosal barrier of the

digestive tract is disrupted, leading to the escape of active digestive enzymes into the circulation and consequently multi-organ failure (14,57,58). Protease inhibitors significantly reduced multi-organ failure, supporting the autodigestion hypothesis (23,46).

1.5.2 Autodigestion in Chronic Hypertension

Recent studies on unchecked protease activity in the spontaneously hypertensive rat (SHR) provided further support for the hypothesis that unchecked enzymatic activity may be causing tissue damage. Using a fluorescently-quenched substrate Casein (cleavage by metallo, serine, acid and sulfhydryl proteases), the degrading activity in the SHR plasma was found to be significantly elevated compared to the normotensive control (65). Enhanced proteolytic activity in the microcirculation was also documented and shown to cause cleavage at the extracellular domain of the insulin receptor-α in the SHR, leading to impaired glucoses transport, high blood glucose levels and insulin resistance (20). Other receptors like the integrin CD 18 and endothelial growth factor receptor 2 (VEGFR-2) were also observed to be cleaved by the enhanced proteolytic activity (63,65).

Recent evidence suggests that autodigestion in the SHR involves proteolytic activity by matrix metalloproteinases (MMPs). Tran el al. determined elevated levels of MMP 2, 9 and 7 activities in the SHR plasma (65). MMP 7 and 9 were found to be capable of cleaving the extracellular domain of the VEGFR-2 receptor and the enhanced MMP 7 and 9 activities were shown to be at least partially responsible for the lower levels of extracellular domains of VEGFR in the cardiac microvessels of the SHR, as the administration of chronic MMP inhibitor doxycycline attenuated the receptor cleavage process (65). Renal cortex MMP 2 and 9 gelatinase levels, as well as MMP 9 and 7

levels in the medulla were found to be increased in the SHR (20), providing a degradation mechanism that may lead to glomeruli injury and the elevated urinary protein levels in the SHR (45).

The addition of EDTA, a MMP inhibitor (20), reduced plasma proteolytic activity by ~29% (65), suggesting that proteases other than the MMPs may also be involved in the autodigestion mechanism. Serine proteases may be involved in the activation of certain MMPs. (13). Serine protease activity in the plasma, especially plasma trypsin and kallikrein-1 (KLKB1) activity, has been shown to be elevated in the SHR (13). Initial evidence suggests that serine proteases may at least in part originate from the pancreas and leak into the circulation through pancreatic venules (13), possibly leading to pro-enzyme MMP activation and consequently receptor cleavage in the SHR.

1.6 Plasma Protein Abnormalities in Hypertension

In 1983, Cloix et al. discovered by using 2-dimensional gel electrophoresis two proteins (molecular weight = 16 kDa, isoelectric points of 4.7 and 5.1) in the SHR plasma that were absent in the WKY and WKY rendered hypertensive by methylprednisolone (16, 47). These proteins were considered as biochemical products of primary hypertension. A similar additional plasma protein band (13 kDa, isoelectric point of 4.5) was discovered in essential hypertensive humans, and termed "hypertension associated protein" (16). Based on molecular weight, John et al. estimated this protein as the alpha-1 chain fragment of haptoglobin (16). According to the autodigestion hypothesis, these low molecular weight proteins or peptides may be fragments or end-products of the enhanced enzymatic activity in SHR.

Serum albumin has many functions in the circulation. It is an abundant ~65 kDa plasma protein that maintains oncotic pressure of the plasma. It serves as a transporter molecule for hydrophobic molecules such as thyroid hormone T4, fatty acids and cholesterol, amino-acid tryptophan, bilirubin, as well as other fat soluble hormones like corticosterone and aldosterone (4,9,34,43,48,71). Yamada et al showed that T3 and T4 concentrations are lower in the circulation of the SHR (70). Circulating cholesterol, bilirubin, corticosterone and triacylglycerol levels are also reduced in hypertensives (33,37,68). Furthermore, the concentration of albumin-bound tryptophan in the plasma was found to be lowered in the SHR compared to WKY (27). These abnormalities can possibly be explained by defective albumin carriers in chronic hypertension due to cleavage under the autodigestion hypothesis. If the circulating albumin molecules were degraded or altered by the unchecked proteases in the SHR, it would provide a plausible explanation for defective albumin molecules leading to the documented abnormalities in the SHR plasma. Therefore, albumin is considered in this study a candidate protein for autodigestion and one objective of this study would be to demonstrate degradation and fragment formation of albumin in the SHR plasma and urine.

1.7 Goals of this Investigation

The overall aim of this thesis is to examine the elevated proteolytic activity in the SHR plasma and show proteolytic degradation of plasma proteins in the SHR circulation. Previous studies in our lab have found elevated plasma protease levels and activity (MMP 2,9,7 and trypsin) in the SHR (13,65). Another goal is to also examine the whole blood protease activity in order to include proteases that are associated with blood cells (5,22,40,50), in an effort to better address the overall enzymatic activity in the

circulation. Using kinetic studies, the biochemical properties of the proteolytic activity will be further characterized. In light of the recent evidence of enhanced plasma protease activity and receptor cleavage in the SHR, plasma proteins will be examined for degradation and fragment formation. A candidate protein, albumin, will be examined for degradation and fragments in both the plasma and the urine.

1.7.1 Objective

The objective of this study is to examine the plasma proteolytic activity and search for possible protein degradation in the circulation of the SHR.

1.7.2 Hypothesis

We hypothesize that in the SHR model, the enhanced enzymatic levels leads to degradation of major proteins in the circulation, leading to loss of bodily functions. With degradation of plasma proteins, protein cleavage fragments are present in the plasma and the urine.

1.7.3 Specific Aims

1) Determine the level and activity of proteases in the plasma of the SHR and compare to those in the WKY, as well as characterizing the enzyme kinetics of the proteolytic activity.

2) Compare the level of proteolytic activity in the whole blood of the SHR and WKY.

3) Determine the level of plasma protein degradation and fragment formation in the SHR plasma, and the type of enzymes responsible for protein degradation.

4) Determine proteolytic degradation of a candidate protein, albumin, by detecting albumin fragments in the plasma and the urine of the SHR.

Chapter 2. Materials and Methods

2.1 Animals and Tissue Collection

The experimental protocol was reviewed and approved by the University of California, San Diego Animal Subjects Committee. Male SHR at age of 17 weeks and their normotensive controls, the Wistar Kyoto (WKY) of comparable age were studied under general anesthesia (Nembutal, 50 mg/ml, 1 ml/kg bodyweight, i.m.) (Pentobarbital Sodium Injection, Ovation Pharmaceuticals, Inc., Deerfield, IL). After 25 minutes, reflex level was tested with a tail pinch to ensure a surgical level of anesthesia. All rats utilized in this study were fully anesthetized and unresponsive to the toe pinch after the waiting period.

Upon anesthesia, the animals were secured onto a cutting board with a heating pad keeping the body temperature at 37°C. The fur on the left leg was shaved and polyethylene catheters (PE 50, I.D. 0.5 mm/O.D. 0.956 mm, Becton Dickinson Primary Care Diagonistics, Sparks, MD) were placed into the left femoral artery and femoral vein. The systolic and mean arterial blood pressure was recorded continuously for 10 minutes by the laboratory computer (Power Macintosh G3 with MacLab, Apple Computer Company, Cupertino, CA). 5 mg/kg supplemental doses of anesthesia were administered intravenously after tail pinch testing.

Upon blood pressure measurement recording, 2.5 ml of blood was collected from the animals through the left femoral artery and vein catheter to pre-heparinized syringes. Lidocaine was injected and a midline incision was made with care to prevent injury and

excessive bleeding of internal organs. ~500 µl of urine was collected from the bladder and stored at -80°C until testing. At the end of the study, the animals were euthanized by an intravenous injection of Fatal Plus (130 mg/kg body weight) (Pentobarbital Sodium, Vortech Pharmaceuticals, Ltd., Dearborn, MI).

2.2 Determination of Systolic Blood Pressure

The systolic blood pressure was measured in two animals from each group using the tail-cuff method. The blood pressure was measured every other day by the same investigator and at the same time of the day.

2.3 Saphenous Vein Blood Collection

The animals were placed in a restrainer with the right leg extended outside of the restrainer. The surrounding hair was shaved and the saphenous vein bifurcation was located. A puncture was made on the right saphenous vein for whole blood collection.

2.4 Blood Plasma Collection and Urine Processing

Upon blood collection, the blood was centrifuged at 1000g for 15 minutes at 4°C. The plasma layer was then collected and checked for hemolysis. The plasma was stored in -80°C until testing. The urine was centrifuged at 10,000g for 15 minutes at 4°C. The supernatant was collected and stored at -80°C until processing.

2.5 Spectrometric Protease Activity Measurement Protocol

2.5.1 Plasma Caseinolytic Activity

Frozen plasma samples (-80°C) were unfrozen on ice and tested simultaneously for overall protease activity (Enzcheck BODIPY, casein derivative catalogue. No.E-6639; Molecular Probes, Carlsbad, Calif, USA; cleavage by metallo-, serine, acid, and sulfhydryl proteases). A final concentration of 5 µg/ml of BODIPY casein solution (with

5 mM Tris-HCl, 10nM sodium azide, pH 7.8) was used with 50 µl of plasma per sample in a 96-well plate. Negative controls with BODIPY casein alone and plasma controls of plasma alone were also included. Protease activity levels were determined from the fluorescent intensity (excitation 590nm, emission 530nm) after a 1 hour incubation at 37°C (SpectraMax Gemini XS, Molecular Devices, Sunnyvale, Calif., USA; in fluorescent units).

2.5.2 Whole Blood Caseinolytic Activity

Upon blood collection, BODIPY casein solution was added to 60 µl of whole blood to a final concentration of 5ul/ml (with 5 mM Tris-HCl, 10nM sodium azide, pH 7.8). The suspension was incubated at 37°C for one hour, followed by centrifugation (20,000g for 5 min) and extraction of the supernatant. The protease activity was determined from the fluorescent intensity of the supernatant in a 96-well plate.

2.5.3 Plasma Gelatinase Activity

Frozen plasma samples (-80°C) were unfrozen and tested simultaneously for gelatinase activity (Enzcheck DQ gelatin, fluorescein conjugate, D-12054, Molecular Probes, Carlsbad, Calif, USA). For each sample, 50 µl of plasma was used to create a final concentration of 10 µg/ml DQ gelatin, 50mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, 0.2 mM sodium azide, pH 7.6. The samples were applied onto a 96 well plate and fluorescent intensity (excitation 496 nm, emission 515 nm) was read every 2 minutes for 60 minutes to produce a kinetic reading. Gelatinase activity was determined from the florescent intensity from the DQ gelatin.

2.5.4 Whole Blood Gelatinase Activity

50 µl of whole blood was used to create a final concentration of 10 µg/ml DQ gelatin, 50mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, 0.2 mM sodium azide, pH 7.6. The suspension was incubated at 37°C for 4 minutes, followed by centrifugation (10,000g for 5 min) and extraction of the supernatant. The gelatinase activity was determined from the fluorescent intensity of the supernatant in the 96-well plate.

2.6 Gelatin Gel Zymography Protocol

Unfrozen plasma (0.8 µl) and urine (0.3 µl) samples were separated on a 10% SDS gel with porcine gelatin (0.1g/ml) at 120V (constant voltage) for 80 minutes (Mini-protean Tetra Cell, 165-800, Bio-Rad, Calif, USA). The gels were then washed four times, 15 minutes each, in renaturing buffer (2.5 % v/v triton x-100) with agitation at room temperature. Then the gels were incubated in 50 ml of developing buffer (50 mM Tris-HCL, 10 mM $CaCl_2$, 0.02% NaN_3, pH 7.5) for 18 hours at 37°C. The gels were then stained with Brilliant Coomassie Blue R250 (0.5% w/v) for 4 hours, followed by destaining by immersing in 50 ml destaining buffer (1:5:4 acetic acid:methanol:water) until areas of gelatinolytic activity appeared as sharp clear bands against the blue background. Images of the gels were scanned by a scanner (Canon LIDE 200) for band intensity analysis in ImageJ.

2.7 Protein and Peptide Count

2.7.1 Bradford Total Protein Count Protocol

The Bradford Assay (BR Protein Assay, 500-006, Bio-Rad, Calif, USA) was used to determine the total protein concentration. For each sample, 10 µl of unfrozen plasma was added to Coomassie Brilliant Blue G-450 dye solution at a ratio of 1:5 (plasma:dye) to a 96-well micro-plate. The mixture was given 6 minutes to equilibrate at room

temperature and absorbance readings at 595 nm were made by the spectrometer. The

protein concentration between the SHR group and WKY group were compared based on

their respective absorbance readings.

2.7.2 Fluoraldehyde Peptide Count Protocol

10 µl of unfrozen plasma was added to separate wells in a micro-plate. 200 µl of

0.8 mg/ml o-phthalaldehyde (OPA) (Fluoraldehyde Reagent Solution, 26025, Thermo-

scientific, Rockford, IL, USA) solution was added simultaneously and the mixture was

given 2 minutes to equilibrate on a shaker. Fluorescent intensity was measured

(excitation 360 nm, emission 430 nm). Fluorescent intensity reflects the amount of

primary amino ends/ peptides in the plasma sample.

2.8 Gel Electrophoresis

Unfrozen plasma (0.6 µl) and urine (1 µl) samples were separated on a 10% SDS gel

at 80V (constant voltage) (Mini-protean Tetra Cell, 165-800, Bio-Rad, Calif, USA) for

100 minutes or until the dye front reaches the bottom of the gel. The gels were then

incubated over night in Coomassie Blue R-250 Staining solution (0.025% w/v, 40%

Methanol), followed by immersing in destaining buffer (40% Methanol, 10% Acetic

Acid) until clear sharp protein bands were observed against the clear background.

For more sensitive staining, some gels were silver-stained (SilverQuest, LC6070,

Invitrogen, Carlsbad, Calif, USA). Following 10% SDS gel electrophoresis, the gels were

fixed in a fixative solution (40% ethanol, 10% acetic acid) for 1 hour. The gels were then

immersed in a sensitizing solution for 20 minutes, staining solution for 15 minutes and

developing solution for 8 minutes or until sharp proteins bands were observed. All the

gels were scanned by a scanner (Cannon LIDE 200) for band intensity analysis.

2.9 Western Blot and Immunodetection of Albumin

Unfrozen plasma (0.1 nl) and urine (0.01 µl) samples were separated on a 4-20%

gradient pre-casted SDS gel at 195V (constant voltage) (Mini-protean Tetra Cell, 165-

800, Bio-Rad, Calif, USA) for 40 minutes or until the dye front reaches the bottom of the

gel. The separated proteins on the gel were transferred to a supported nitrocellulous

membrane (0.2um supported Nitrocellulous, 162-0097, Bio-Rad, Calif, USA) at 110v

(constant voltage) for 45 minutes on an ice water bath to prevent overheating. The

membrane was immersed in blocking solution (3% Bovine Serum Albumin) for 16 hours.

Then the membrane was coated with 20 ml of 0.02 ng/ml horseradish peroxidase

conjugated anti-albumin sheep immunoglobulin (A110-134P, Bethyl Laboratories,

Montgomery, TX, USA) for 2 hours at 4°C. The membrane was then washed with Tris-

Buffered Saline with 0.05% Tween 20 for 1 hour. 6 ml of enhanced chemiluminescent

substrate solution (ECL kit, 32106, Thermo-Scientific, Rockford, IL, USA) and 5

minutes was given for the reaction to initiate before the chemiluminescence was recorded

on a film and developed. The developed film was scanned and the band intensity was

used to determine albumin levels.

2.10 Plasma Incubation and Controlled Degradation

Unfrozen plasma samples were placed in a micro-centrifuge tube. The tube

opening was then sealed to prevent evaporation during the incubation. The tube was then

incubated at a 37°C for 24 hours. After incubation, the tube was centrifuged for 5 minutes

at 1000g. Inhibitors were also added to separate sets of samples to observe the effects

(EDTA 10 mM, ANGD 0.4 mM, PMSF 1 mM). The plasma samples (both stored and

incubated, with and without inhibitors) were subjected to protein and peptide count and gel electrophoresis. The percentage change was analyzed.

2.11 Data Analysis

For the gel electrophoresis and western blot experiments, the digitalized images were converted to 8-bit and then subjected to automatic background subtraction in ImageJ. The intensity of the resulting bands was then reported in digital intensity units.

For the controlled degradation experiments, the percentage change from the incubation was calculated by the following equation:

$$Percentage\ Change = \frac{Signal_{After\ Incubation} - Signal_{Before\ Incubation}}{Signal_{Before\ Incubation}} \times 100\%$$

2.12 Statistical Analysis

All measurements are shown as Mean ± Standard Deviation. Two tailed Student's t-test was used for comparison between animal groups. A probability of $p < 0.05$ was considered statistically significant.

Chapter 3. Results

3.1 Blood and Plasma Protease Activity

3.1.1 Plasma Gelatinase Level

Arterial and venous plasma samples were incubated in a cross-linked gelatin SDS gel to determine the Gelatinase level. In the arterial plasma, the results showed significantly higher levels of ~19.1 kDa, 67.3 kDa and 73.7 kDa (pro-enzyme of 67.3 kDa) gelatinases in the SHR group compared to the WKY group ($p < 0.05$, $n = 4$) (Figure 1). In the venous plasma, the results showed significantly higher levels of ~19 kDa gelatinase in the SHR rats compared to the WKY rats ($p < 0.05$) (Figure 3). The same trend is observed for the ~24.3 kDa, 67.3 kDa, 73.7 kDa and 86.2 kDa lytic enzyme bands. (Figure 2, 3).

3.1.2 Blood and Plasma Gelatinolytic Activity

Whole blood and plasma samples were incubated with fluorescently quenched substrates to detect gelatinolytic activity. The results showed significantly elevated (~17%, $p < 0.05$) plasma gelatinolytic activity in the SHR group compared to the WKY group (Figure 4). Kinetic measurements were also carried out to confirm enzyme activity. In 4 out of 4 SHR rats, plasma gelatinolytic activity was detected as enzyme kinetic curves, while only 1 out of 4 WKY rats showed significant plasma gelatinolytic activity (enough to be detected by the assay) (Figure 6).

Whole blood gelatinolytic activity was found to be significantly elevated (~21%, $p < 0.05$) in the SHR rats compared to the WKY rats ($n = 4$) (Figure 5).

3.1.3 Blood and Plasma Caseinolytic Activity

Whole blood and plasma samples were incubated with fluorescently quenched casein substrates to detect general protease activity. For both the blood and plasma samples, no significant differences were found between the SHR and WKY rats in caseinolytic activity (Figure 4, 5).

3.2 Plasma Protein Analysis

3.2.1 Plasma Protein and Peptide Count

The Bradford Assay was used to determine total plasma protein concentration. O-phthalaldehyde (OPA) was used to determine the total peptide count. The results showed no significant differences in total plasma protein level between the SHR and WKY group (Figure 7). OPA assay showed significantly higher peptide levels ($p < 0.05$) in SHR rats compared to WKY rats (Figure 8).

3.2.2 Plasma Gel Electrophoresis

High density unidimensional SDS gels were used to analyze the plasma protein layout. R-250 Coomassie Blue staining (detection limit: 1µg) showed significantly decreased protein levels ($p < 0.05$, $n = 4$) at bands ~ 21.9 kDa, 33.3 kDa, 46.2 kDa and 226.1 kDa in the SHR group compared to the WKY group (Figure 9, 10). The protein band at ~25.6 kDa is absent in the SHR group but present in the WKY group. The SHR rats have an additional protein band at ~181.2 kDa compared to the WKY rats (Figure 9). Silver staining (detection limit: 2.5 ng) showed significantly higher level of the ~ 2 kDa band in the SHR group compared to the WKY group (Figure 11).

3.2.3 Plasma Albumin Detection

Western blot immunodetection against albumin was done to compare plasma albumin levels. The results showed significantly elevated plasma albumin levels in the SHR group compared to the WKY group ($p < 0.05$, n = 4) (Figure 12).

3.3 Urine Analysis

3.3.1 Urine Proteolytic Activity

Gelatin Zymography was carried out on the urine to determine protease levels. The results showed that gelatinolytic bands of ~90 kDa and ~40 kDa were present in the SHR urine but undetectable in the WKY urine (Figure 13).

3.3.2 Urine Protein Analysis

Gel electrophoresis was done with urine samples and stained with Coomassie Blue R-250. Band intensity analysis showed significantly elevated levels of ~17.2 kDa protein/peptide band in the SHR urine compared to the WKY urine ($P < 0.05$, n = 3) (Figure 14).

Western blot against albumin showed high levels of albumin fragments (~17 kDa) in the SHR urine (present in 3 out of 3 animals) compared to none to very low levels (present in 1 out of 3 animals) in the WKY urine (15).

3.4 Plasma Protein Controlled Degradation

Plasma samples were incubated in-vitro to amplify protein degradation end-products (fragments). The rate of fragment formation in the SHR plasma was shown to be significantly higher ($p < 0.05$, n = 4) than that of WKY plasma (Figure 16). When 10 mM EDTA was added, the rate of fragment formation in the SHR plasma decreased. The addition of 1 mM PMSF and 0.4 mM ANGD did not reduce the degradation rate.

Gel electrophoresis was used to determine which protein was degraded and fragment formed. The results showed that the degradation rate of a ~33.1 kDa protein is significantly higher ($p < 0.05$, $n = 4$) in the SHR plasma compared to WKY plasma (Figure 17, 18). Before incubation, the presence of a ~38.4 kDa protein band is stronger in the SHR plasma compared to the WKY plasma. Upon incubation, the ~38.4 kDa band formation is significantly faster ($p < 0.05$) in the WKY compared to the SHR, bringing both protein levels to similar levels after incubation. This could be explained by a more active enzymatic activity in the SHR, leading to higher levels of the end-product (~38.4 kDa band) initially before incubation. Upon plasma incubation, the end-product accumulated in the WKY samples, bringing in the end the two groups to similar levels.
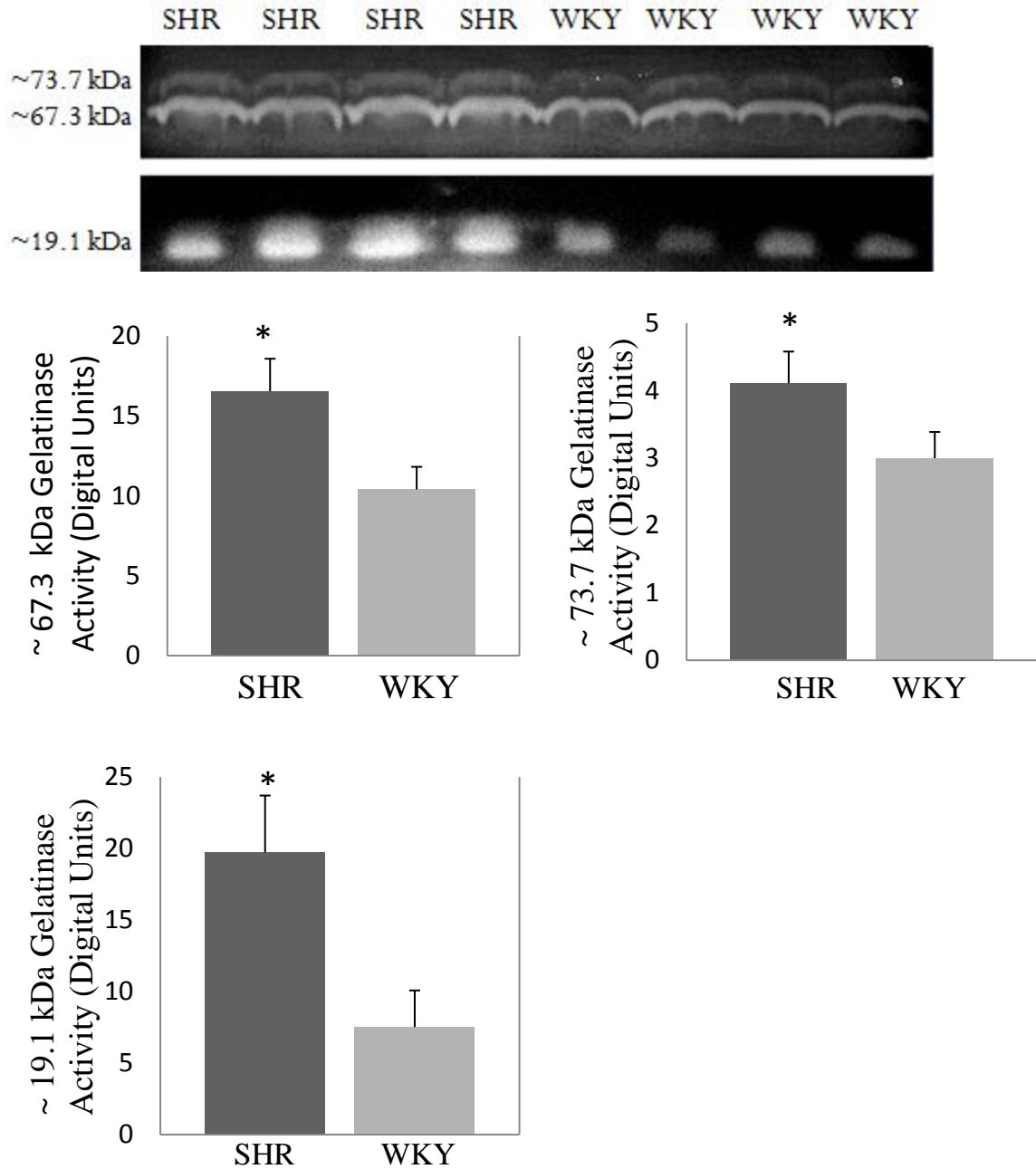
Figure 1. Arterial plasma protease activity (~67.3 kDa, 73.7 kDa, 19.1 kDa) in the SHR and WKY determined by gelatin gel zymography. Molecular weights of the bands were calculated using molecular weight standards. *$p<0.05$, n = 4 in each group.
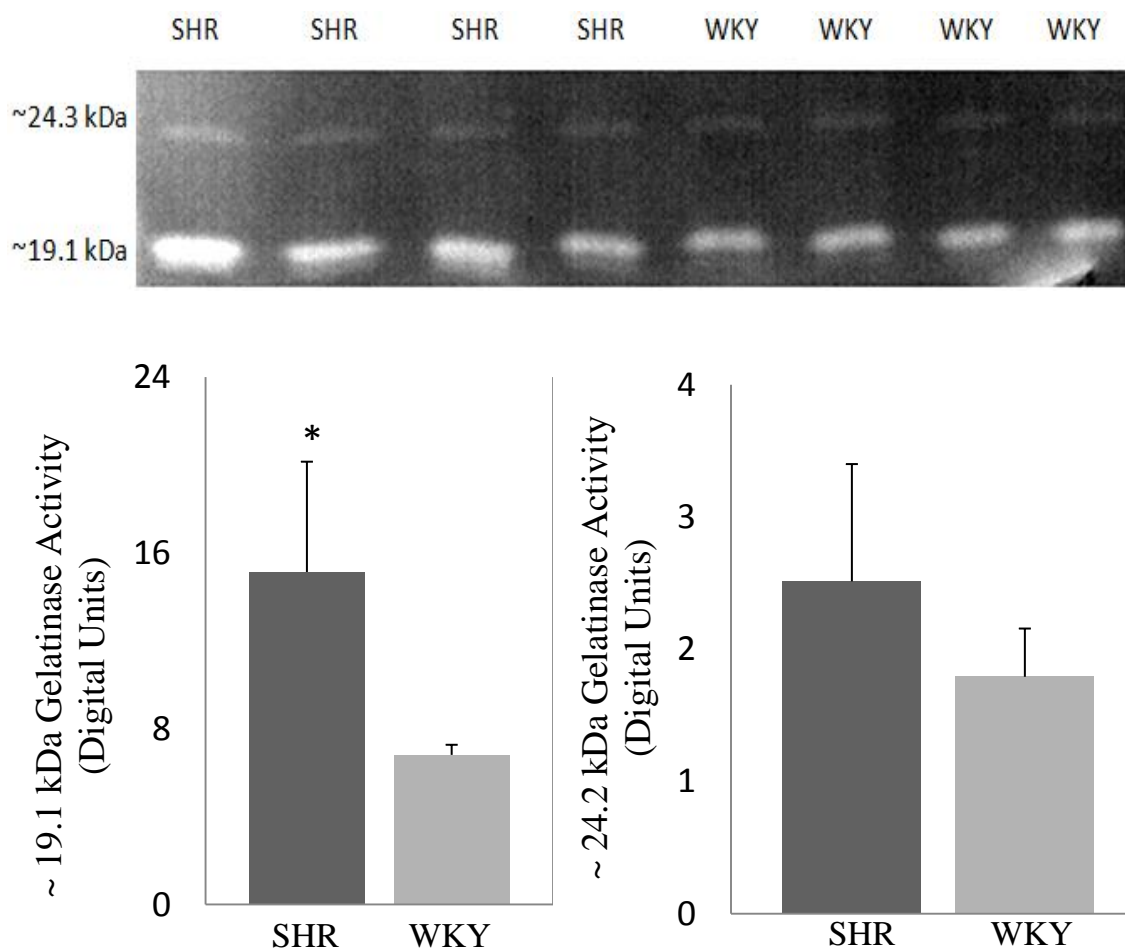
Figure 2. Venous plasma protease activity (~19.1 kDa, 24.2 kDa) in the SHR and WKY determined by gelatin gel zymography. Molecular weights of the bands were calculated using molecular weight standards. *p<0.05, n = 4 in each group.
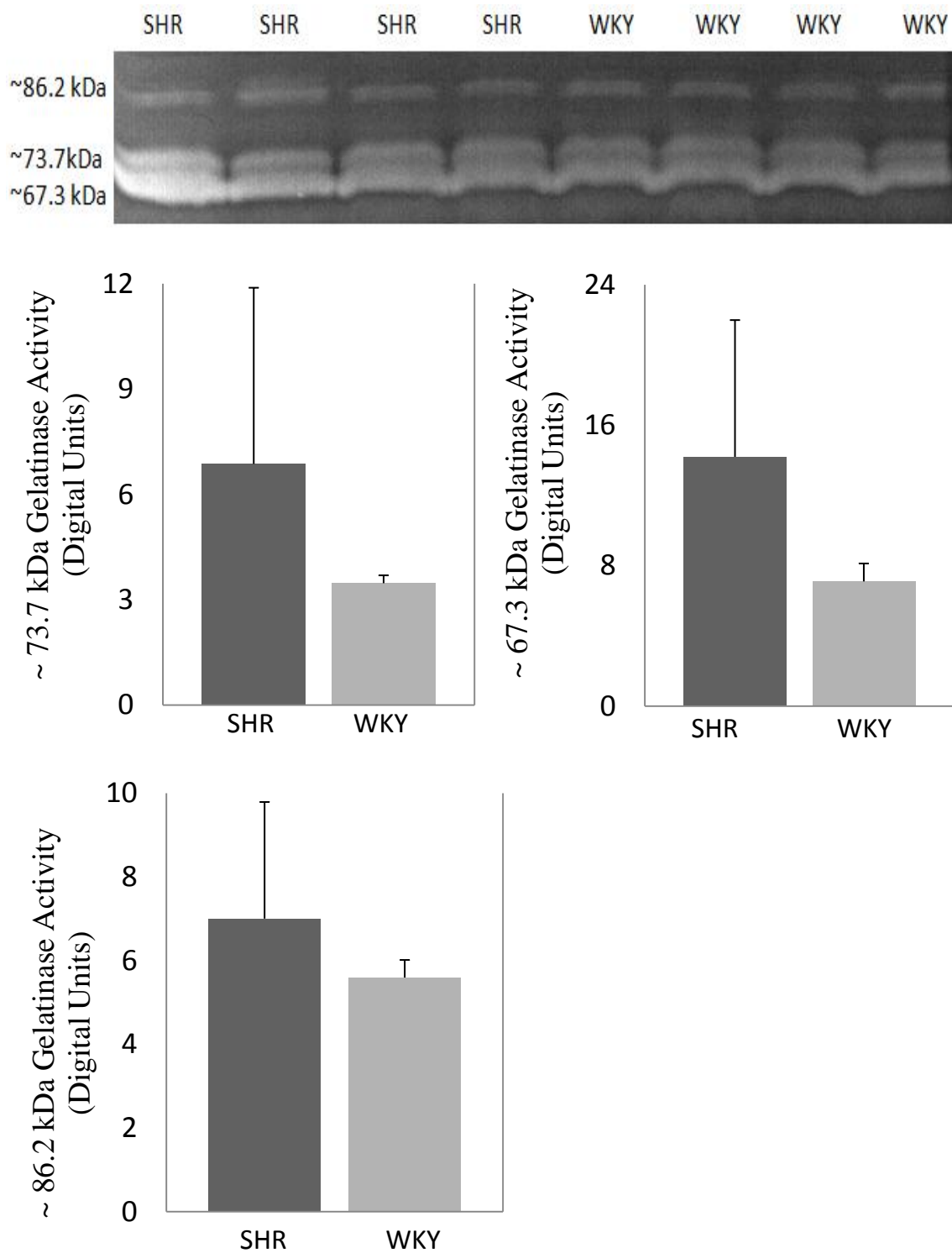
Figure 3. Venous plasma protease activity (~67.3 kDa, 73.7 kDa and 86.2 kDa) in the SHR and WKY determined by gelatin gel zymography. Molecular weights of the bands were determined with molecular weight standards. *p<0.05, n = 4 in each group.
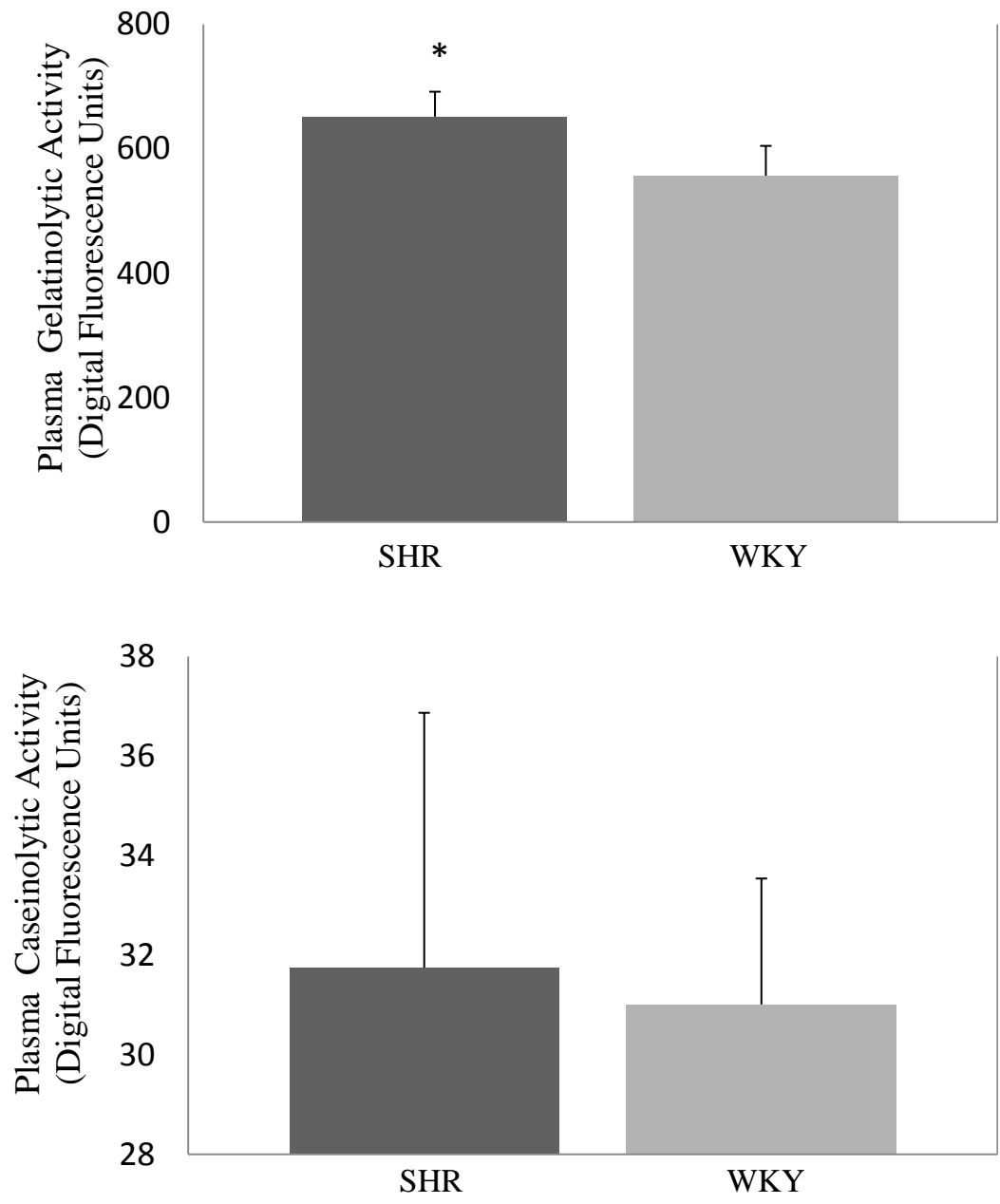
Figure 4. Measurements of plasma protease activity in the SHR and WKY. Gelatinolytic and caseinolytic activities were compared after 60 minutes of incubation with their respective fluorescently quenched substrates. * $p < 0.05$, n = 4 in each group.
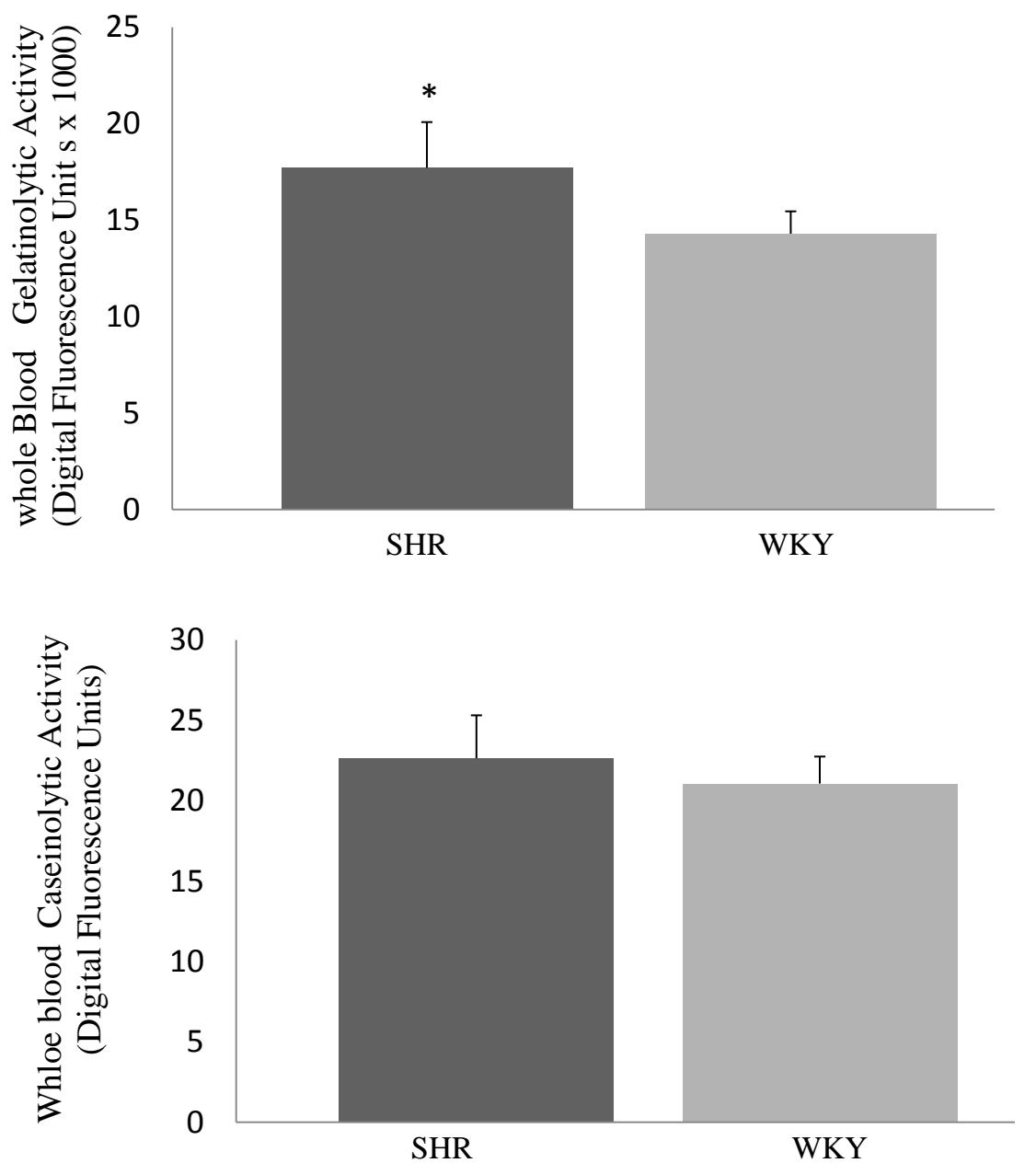
Figure 5. Measurements of whole blood protease activity in the SHR and WKY. Gelatinolytic and caseinolytic activities were compared after 5 (for gelatinase) and 60 (for caseinolytic activity) minutes of incubation with their respective fluorescent substrates. * $p < 0.05$, n = 4 in each group.
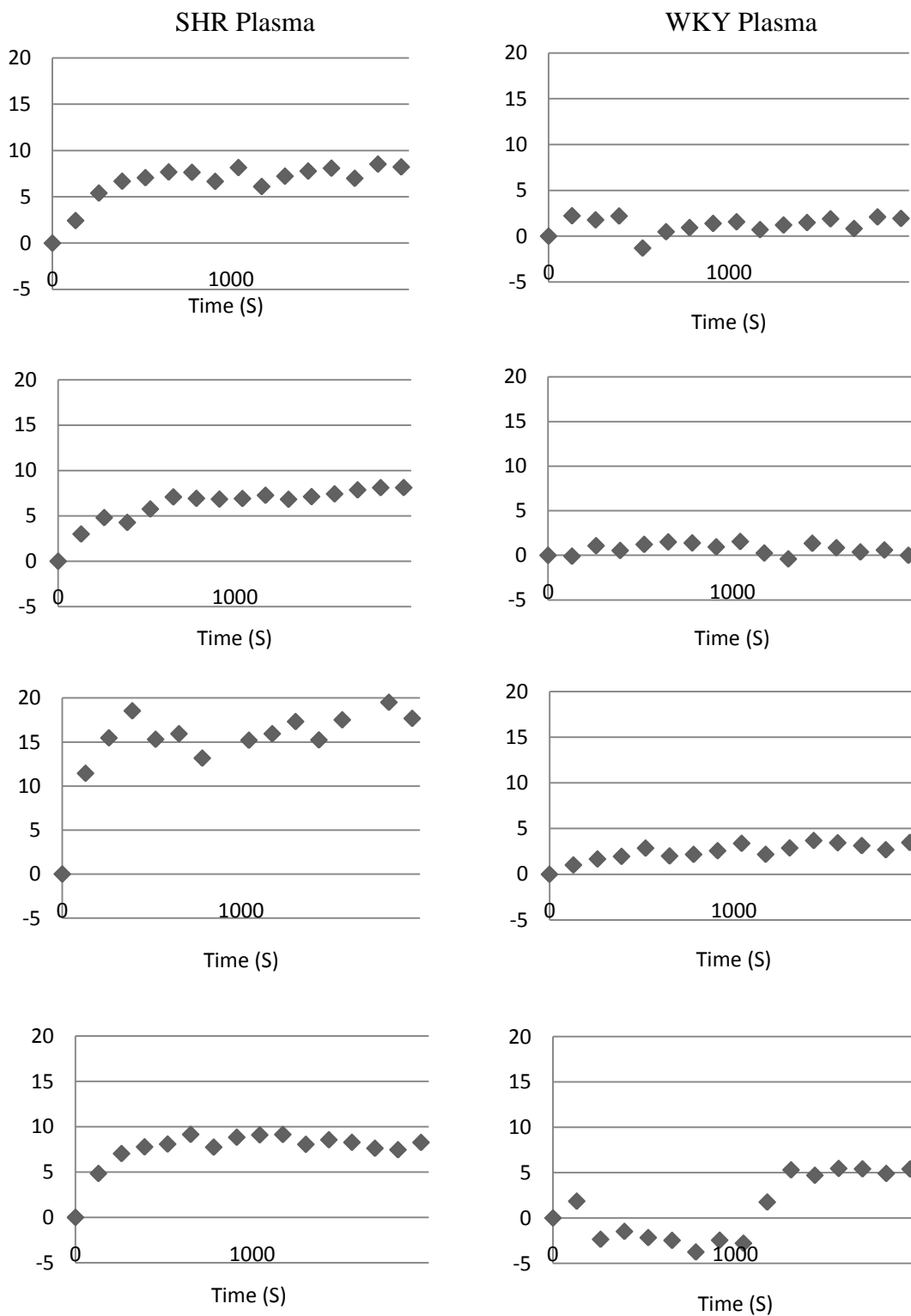
Figure 6. Kinetic measurements of plasma protease activity in the SHR and WKY. Gelatinolytic activities were recorded for ~60 minutes after the addition of fluorescent substrates. Each graph was then normalized to a single starting point. Each graph represents a single sample from each group.
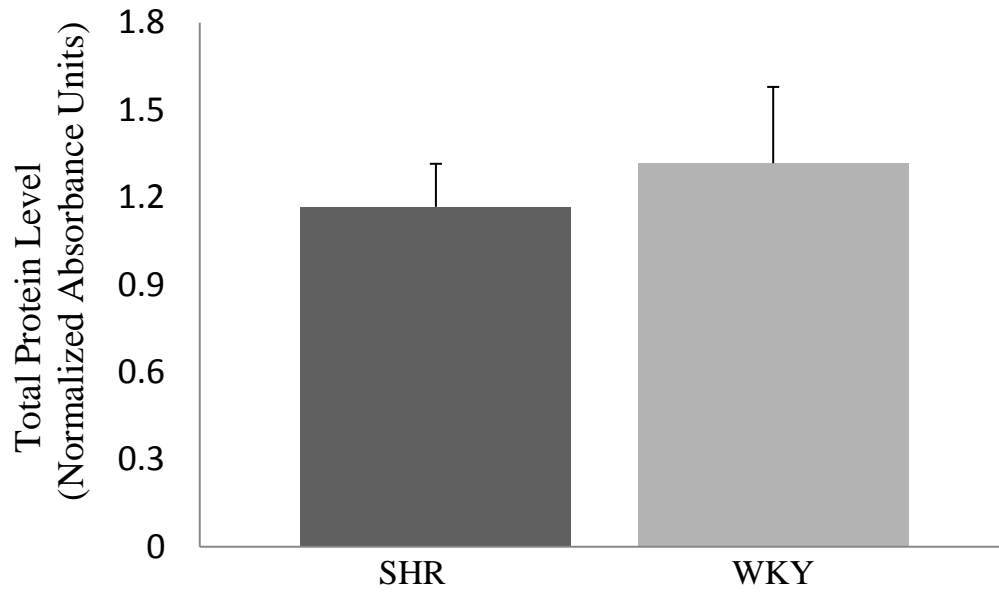
Figure 7. Measurements of total protein level in the plasma using the coomassie blue Bradford Assay. Absorbance readings were made 6 minutes following the addition of coomassie blue dye. n = 4 in each group.
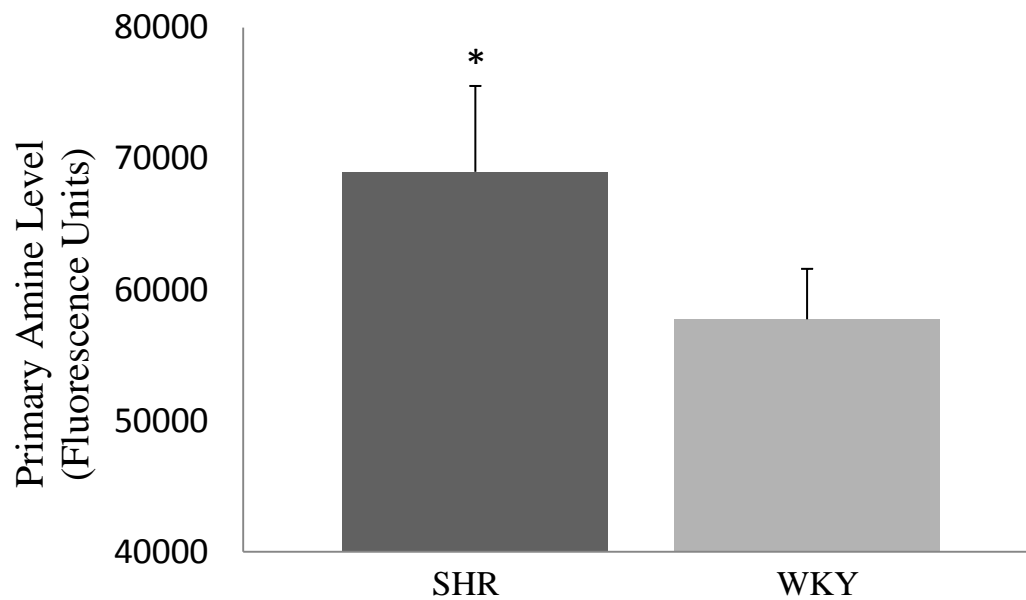
Figure 8. Measurements of primary amine level in the plasma using o-phthalaldehyde (OPA). Fluorescence readings were made 2 minutes following the addition of OPA. * $p<0.05$, n = 4 in each group.
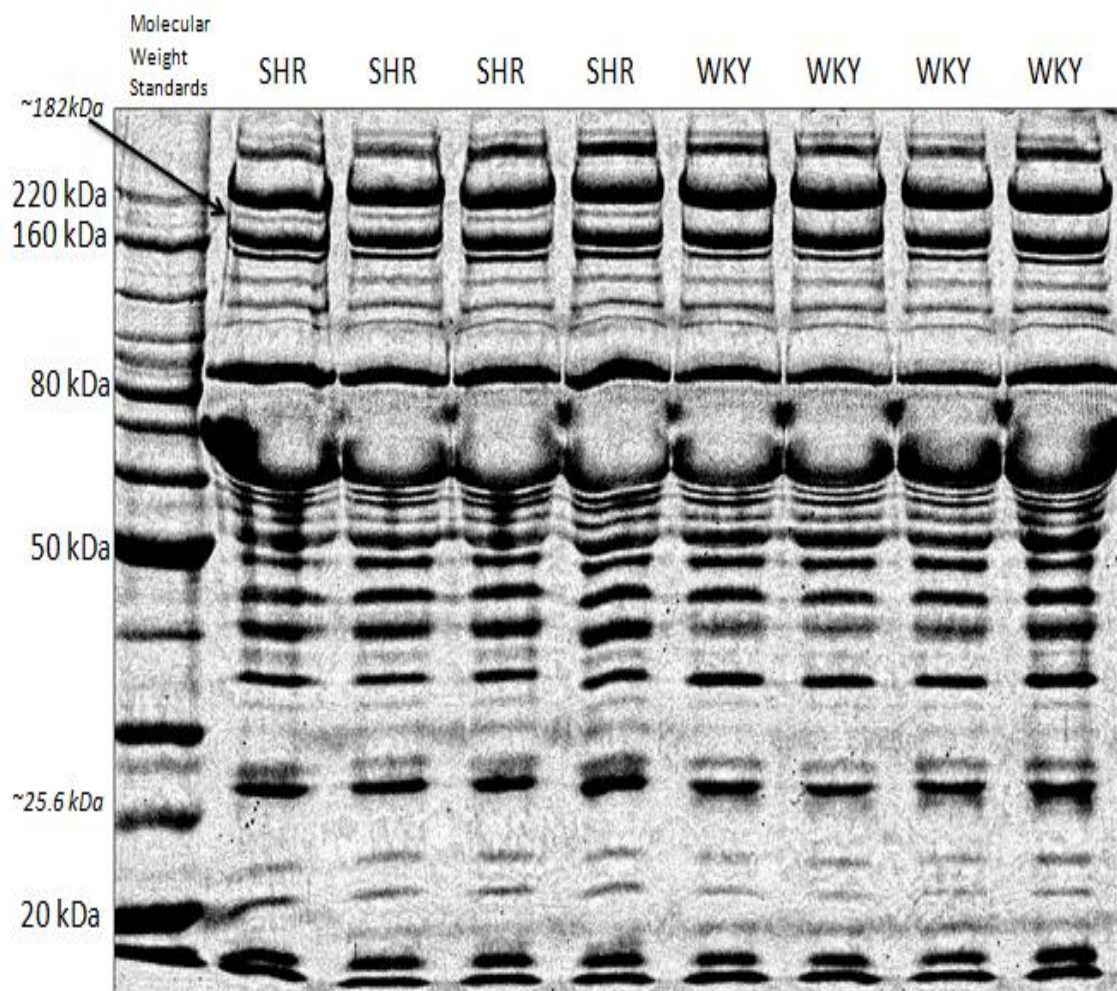
Figure 9. Plasma protein layout using gel electrophoresis (10% SDS gel) followed by coomassie blue staining. The molecular weights of the standards are labeled on the left. Note that a protein band weighted at ~25.6 kDa (labeled on the side) is absent in the SHR plasma. A ~181.2 kDa protein band (located between the 220 kDa and 160 kDa mark) is present in the SHR plasma but absent from the WKY plasma. Band intensity analysis is located in Figure 10.
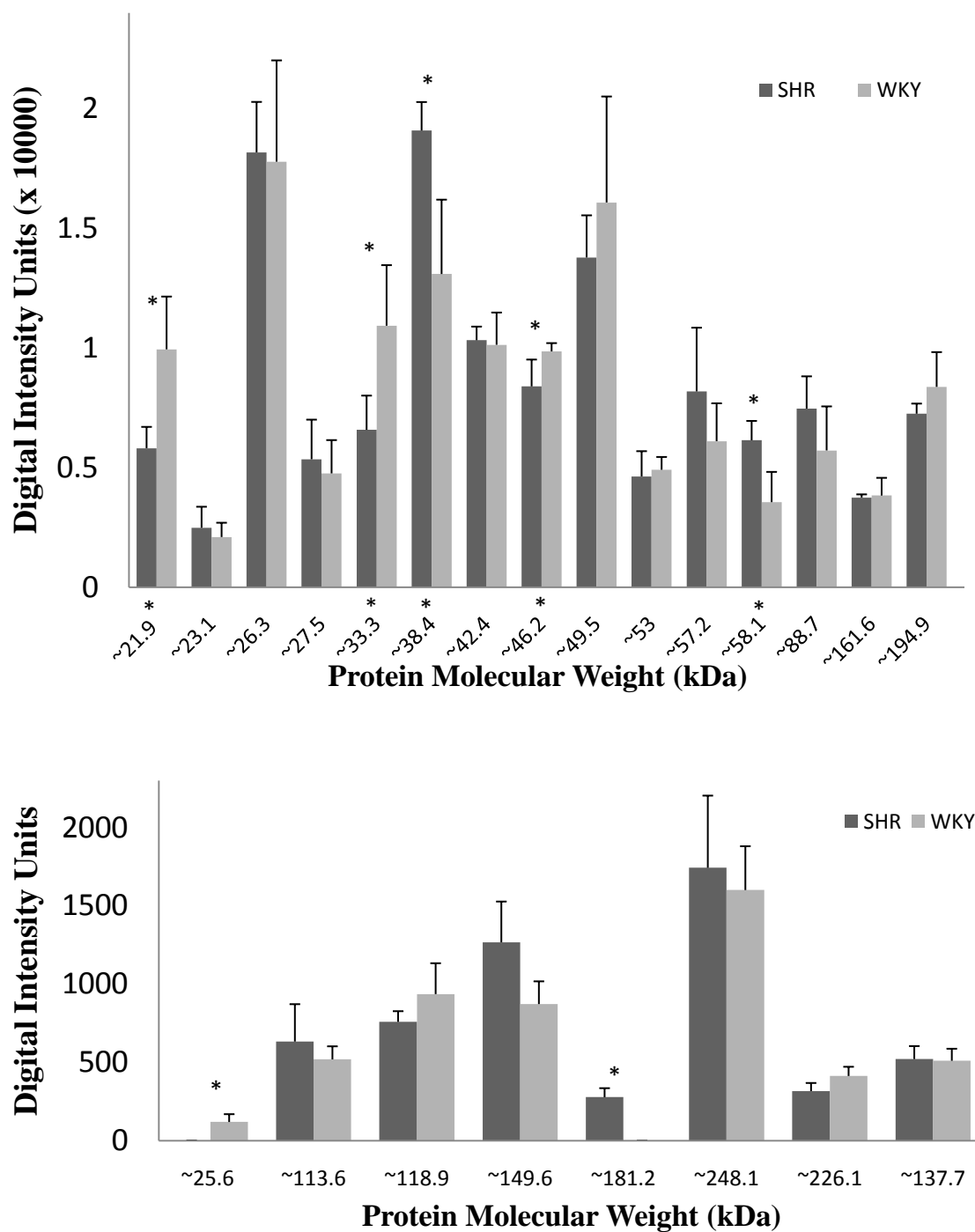
Figure 10. Measurements of high abundance (top) and low abundance (bottom) protein levels in the SHR and WKY plasma. * $p<0.05$, n = 4 in each group.
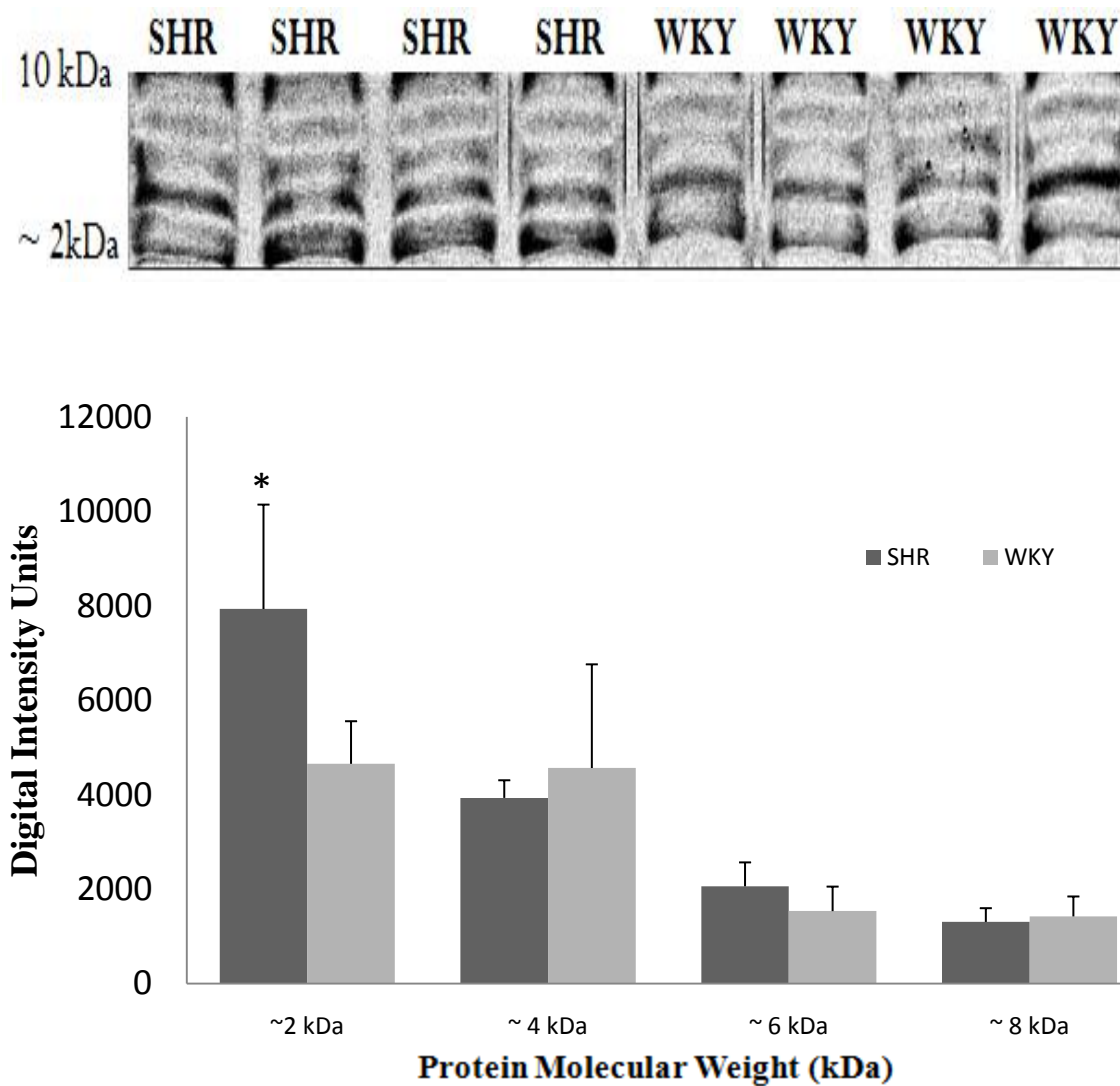
Figure 11. Measurements of low-molecular weight protein/peptide level in the plasma. Plasma samples were separated on a 4-20% SDS gel, followed by silver staining. The 10 kDa mark was provided by the standard protein ladder. * $p<0.05$, n = 4 in each group.
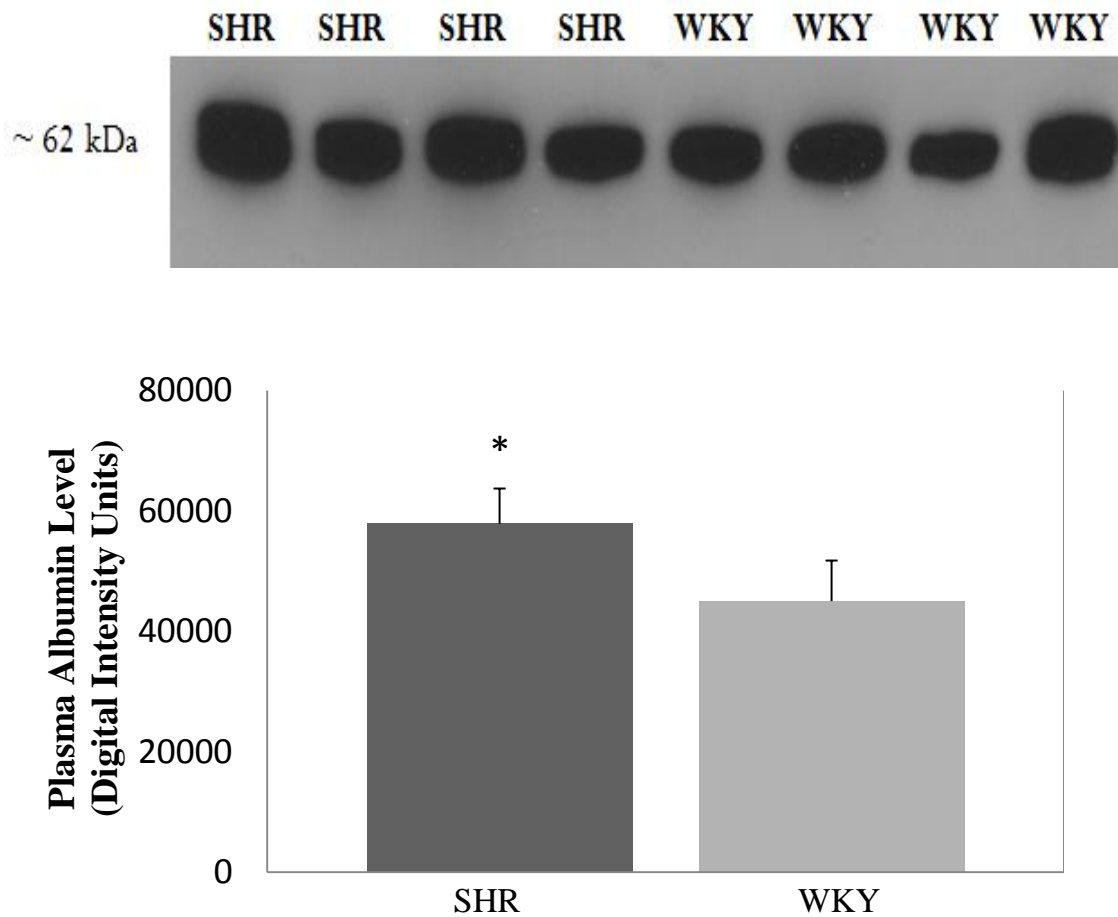
Figure 12. Measurements of plasma albumin level via western blot immunodetection.
* $p < 0.05$, n = 4 in each group.
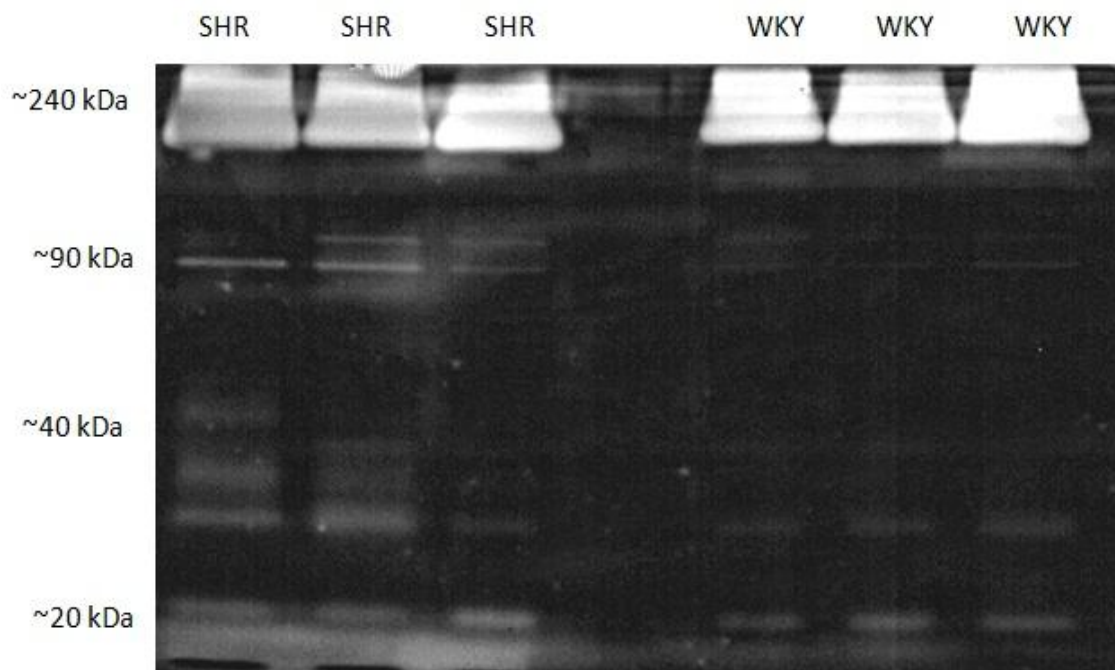
Figure 13. Detection of protease activity in the urine using gelatin zymography. Note that the SHR urine has enhanced proteolytic activity at ~90 kDa and ~40 kDa.
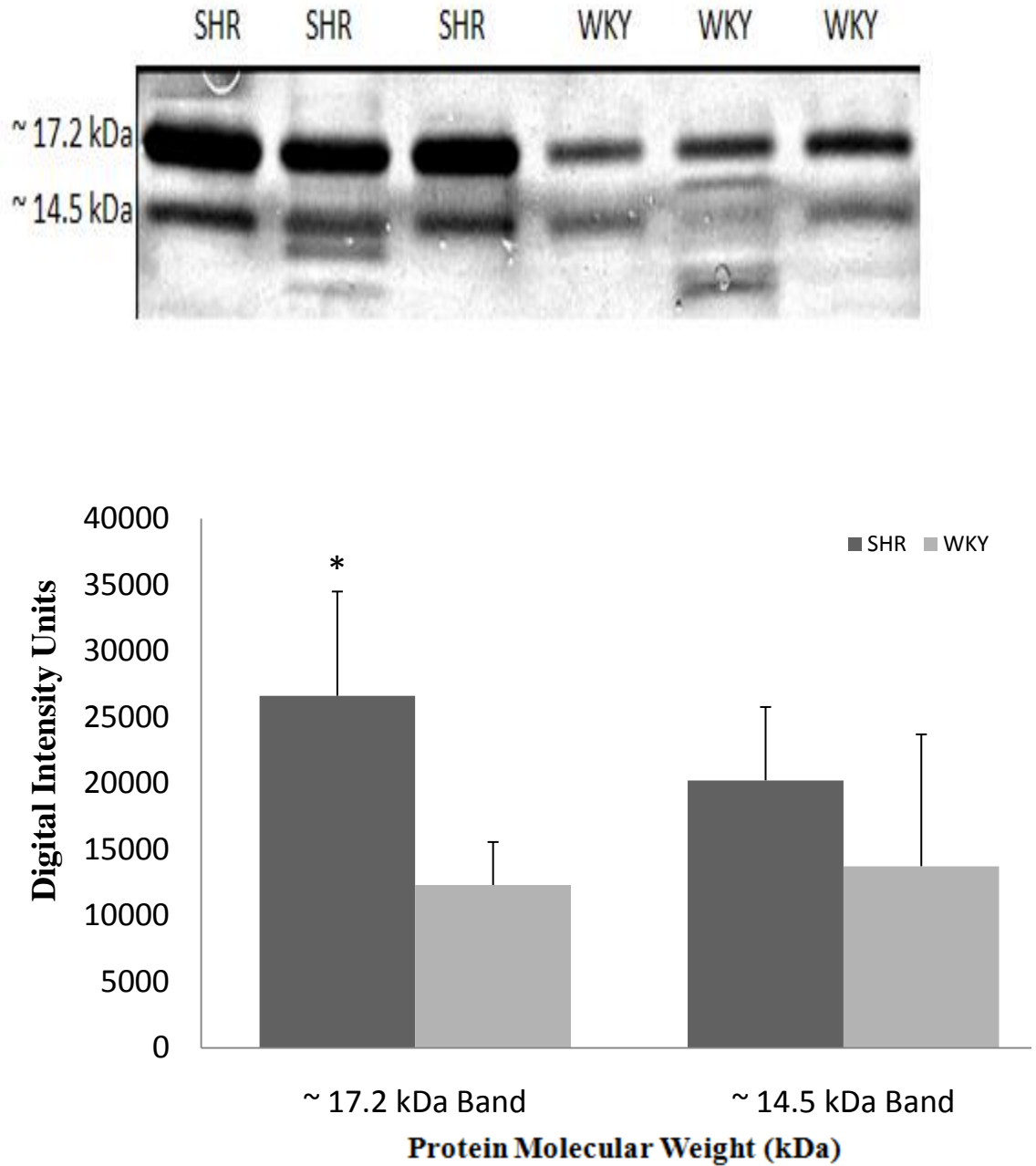
Figure 14. Urinary protein analysis using 4-20% high density SDS gel electrophoresis followed with coomassie blue staining. * p<0.05, n = 3 in each group.
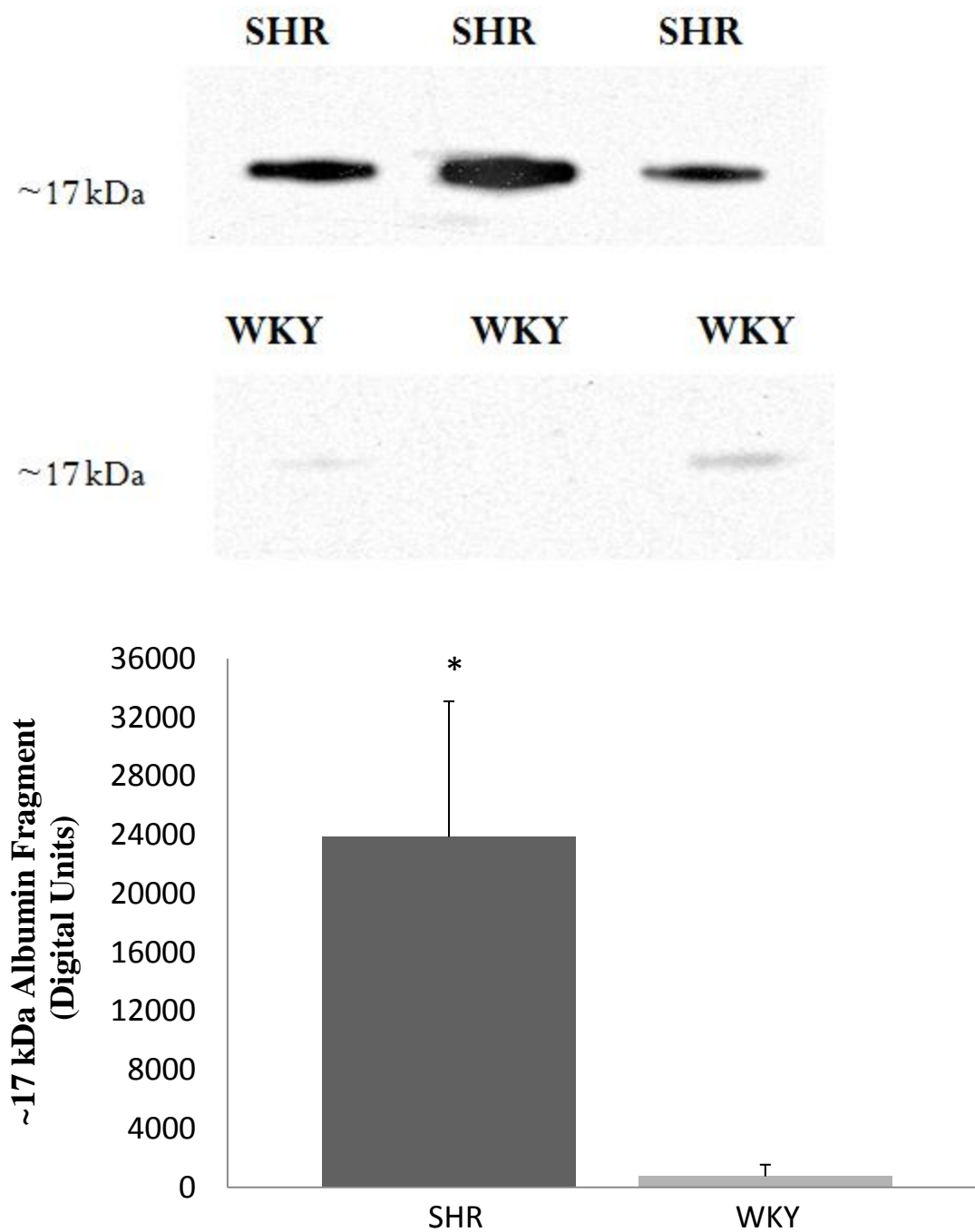
Figure 15. Western blot immunodetection of albumin in the urine. Albumin fragments of ~ 17 kDa were found at significantly elevated levels in the SHR urine and were only detected in trace amounts in the WKY. (* $p < 0.05$, n = 3)
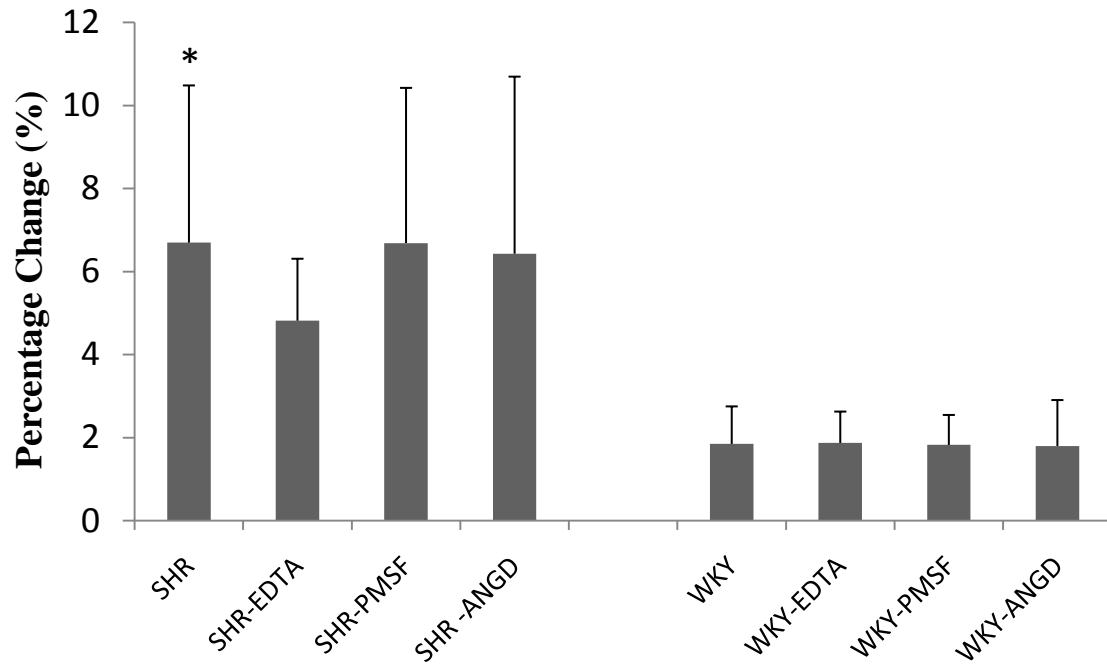
Figure 16: Fragment formation upon incubation in the SHR and WKY plasma. The plasma was incubated at 37°C for 24 hours. Treatments with 10 mM EDTA, 1 mM PMSF and 0.4 mM ANGD are also shown. * SHR vs WKY, $p < 0.05$, n = 4.
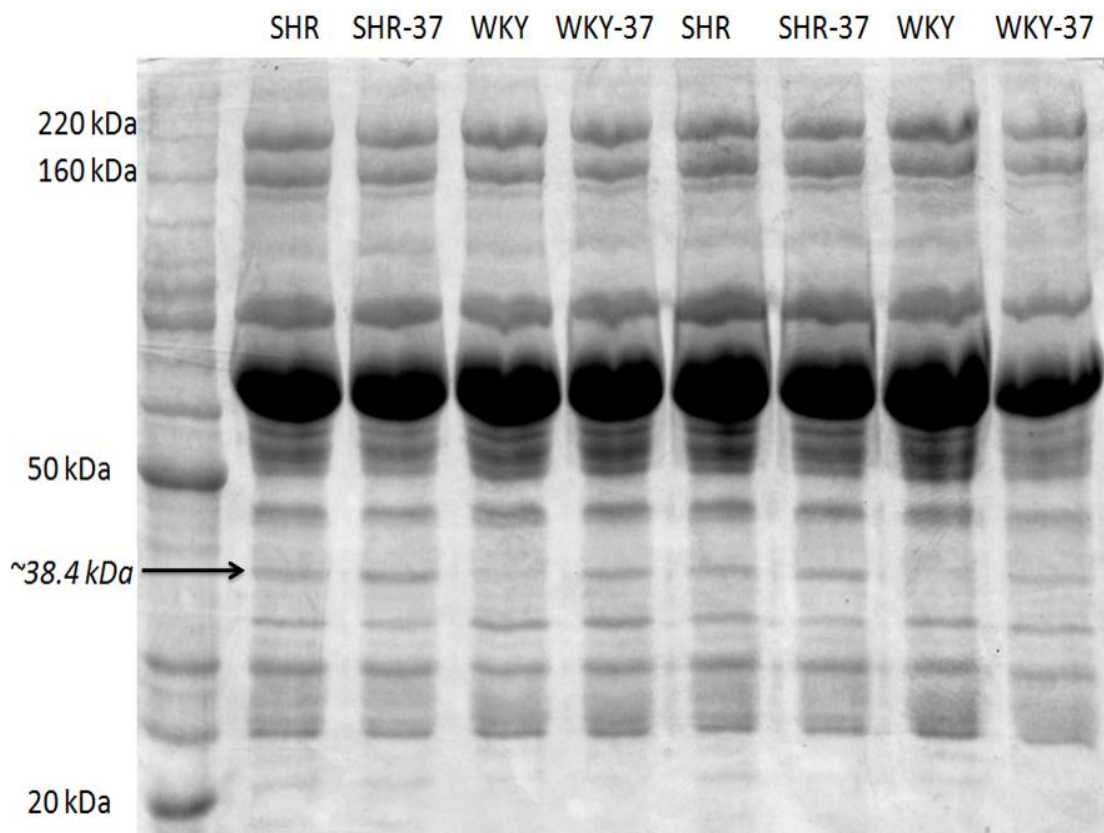
Figure 17. Gel electrophoresis analysis of the controlled in-vitro degradation experiment (see Methods P. 13). The sample that has been incubated at 37°C for 24 hours has been designated with '-37'. Note the ~38.4 kDa protein band that has initially higher levels in the SHR (arrow). Upon incubation, the presence of this band became stronger in the WKY, bringing the final protein level similar in both groups. This suggests enhanced enzymatic activity in the SHR plasma.
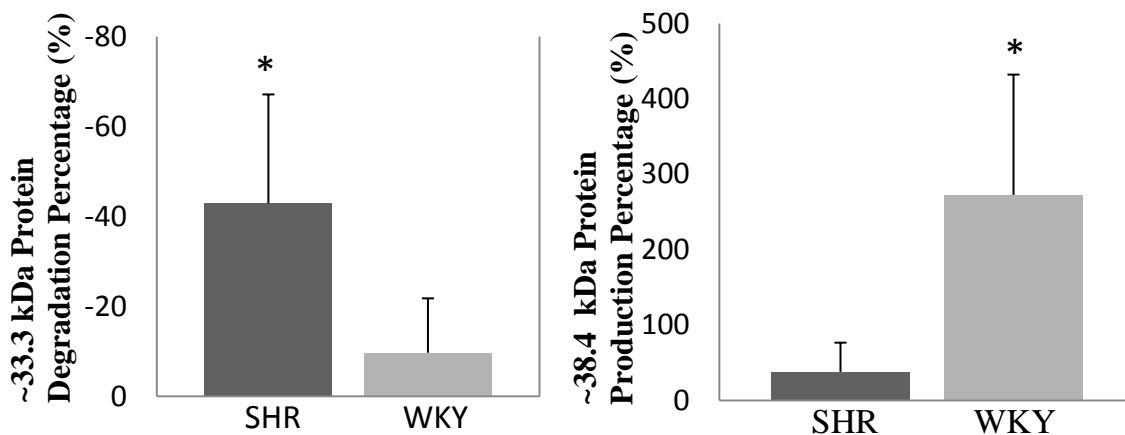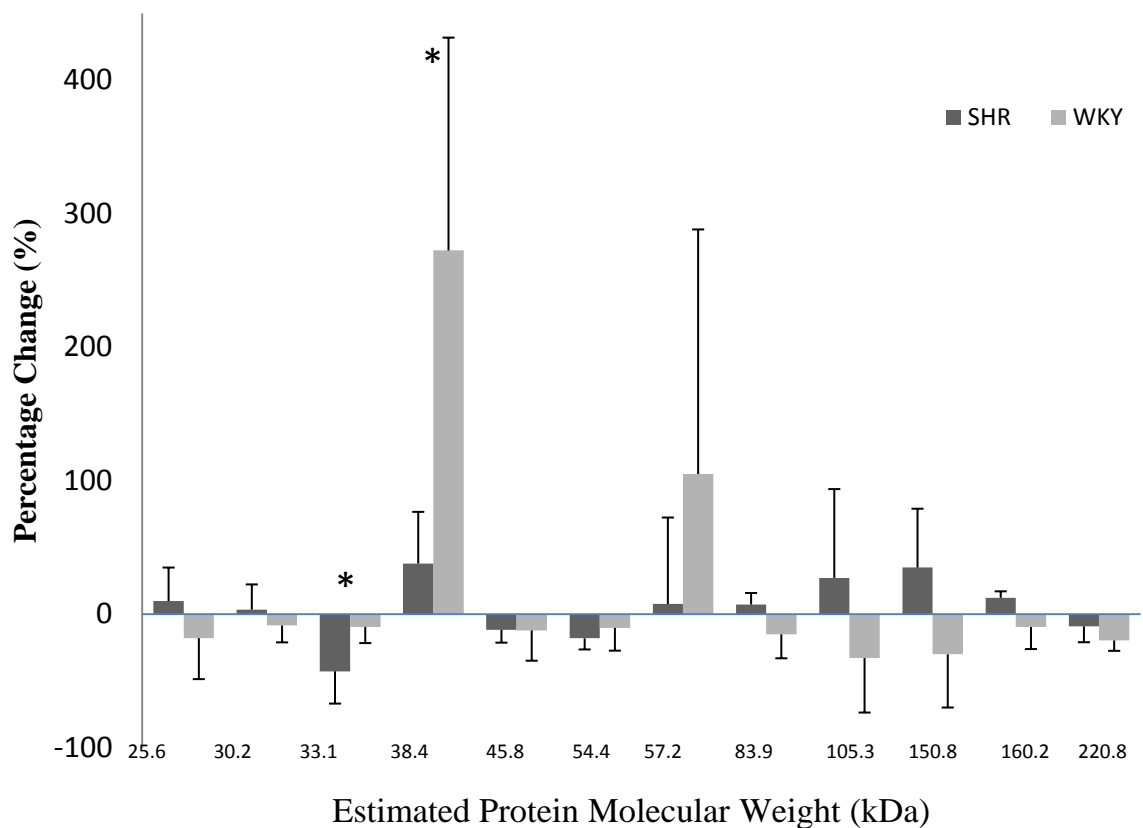
Figure 18. Controlled degradation protein degradation/production analysis. The plasma was incubated at 37°C for 24 hours .The change is calculated by take the difference between the signal before incubation and after incubation divided by the signal before incubation and shown as percentage (%).

Chapter 4. Discussion

4.1 Summary

The current results indicate that the SHR group has both elevated protease level and activity in the plasma and urine that is associated with elevation in blood pressure. The plasma peptide fragment level is also elevated in the SHR group, while the total protein level is not. Partial blockade of proteolytic activity by EDTA has been shown to suppress the protein fragment formation process in the SHR plasma. Multiple proteins (~ 21.9 kDa, 25.5 kDa, 33.3 kDa, 46.2 kDa and 226.1 kDa) are either absent or reduced in the SHR plasma. A low molecular weight protein/peptide (~ 2 kDa) has elevated level in SHR plasma. The SHR plasma has a ~181.2 kDa protein band that is absent in the WKY plasma. Albumin levels were found to be elevated in the SHR plasma. Albumin fragments (~17.1 kDa) were discovered in the SHR urine. These results support the autodigestion mechanism due to unchecked enzymatic activity.

4.2 Whole Blood, Plasma and Urine Enzymatic Activity

As shown in previous studies in our lab, protease levels are elevated in the SHR organs and such elevated levels of proteases may cause cleavage of receptors that are important for cellular functions (20,65). Delano et al. and Tran et al. uncovered for the first time elevation of caseinolytic protease activity and MMP gelatinase levels in the circulation (65). This study confirms the elevation of plasma gelatinase levels in the venous side of the SHR circulation and also found elevated levels in the arterial side. The current study also showed by the real time kinetics an elevated proteolytic activity in the SHR plasma. Kinetic studies showed plasma enzymes saturating at around 10 minutes

after the application of substrate, and the fluorophores started to lose fluorescence at as soon as the 15 minute time point. Therefore in future protease measurements, the incubation time should be limited to no more than 30 minutes for accurate activity assessment. This study also found elevated protease activity in whole blood, in order to include proteases that are associated with blood cells (on membrane surfaces) into consideration for total protease activity (5,22,40,50). It is possible that these active enzymes are responsible for receptor cleavage in various vessels and organs.

This study also uncovered elevated proteases level in the SHR urine. The observation is consistent with the elevated plasma activity results and the possibility that some of the plasma proteases may accumulate in the urine via glomerular filtration.

4.3 Plasma Protein Analysis

The objective in this study was to determine if plasma proteins are being degraded in the SHR due to the elevated proteolytic activity in the circulation. The results showed no differences between the WKY and SHR in total protein concentration, while the primary amine level is significantly elevated in the SHR plasma, suggesting that the SHR plasma contains higher levels of protein fragments. The multiple proteins that were absent or significantly reduced in the SHR plasma (21.9 kDa, 25.5 kDa, 33.3 kDa, 46.2 kDa and 226.1 kDa) serve as possible candidate proteins digested by the unchecked enzymatic activity. The low molecular weight peptide/protein (~2 kDa) found in higher levels in the SHR plasma could be a degradation product. The high density gel has a lower detection limit of 2-3 kDa, which is a major limitation in peptide fragment identification since most digested protein fragments belongs to the sub-kilodalton range

(35,52,55,31). Nonetheless, spectrometric primary amine counting provides evidence of the fragments' elevated presence in SHR plasma.

4.4 ~182 kDa Protein Band

The SHR plasma contains a ~182 kDa band that is absent from the WKY plasma. Estimating by molecular weight, this protein band is likely a cardiac isoform of alpha-2 macroglobulin (CA2M, 182 kDa) (53), which is considered a protein marker for early to moderate staged cardiac hypertrophy (54). MMPs (especially collagenases) are linked to the development of cardiac hypertrophy (64, 36), and the discovery of the additional ~182 kDa band is consistent with the elevated protease levels in the SHR plasma.

4.5 In-Vitro Controlled Plasma Protein Degradation

The plasma was incubated to amplify in-vitro the effect of fragment formation in the plasma. The results showed higher fragment formation rate in the SHR plasma. When the divalent chelating reagent EDTA was added, the fragment formation was suppressed. This suggests that EDTA-inhibited proteases, such as MMPs (20), are at least partially responsible for the fragment formation mechanism upon plasma incubation. The results are also consistent with other studies in our lab of a reduction in plasma proteolytic activity in the SHR upon the addition of EDTA (65). A ~33.1 kDa protein was found to have an elevated degradation rate in the SHR upon plasma incubation. In fact, this protein corresponds to the same protein as the ~33.3 kDa protein (protein's calculated molecular weight can fluctuate ±20% on different gels) that was reported in the past section to have significantly reduced levels in the SHR plasma (without incubation). Thus, such protein is considered as a strong candidate for autodigestion in the SHR plasma. Based on molecular weight, this protein is likely ApoE-Lipoprotein or C4 Compliment Protein (2).

4.6 Albumin: Candidate Protein for Autodigestion

Albumin was chosen as a candidate protein to search for evidence for proteolytic autodigestion. Albumin has been shown to be degraded by proteases and form protein fragments in the plasma and urine in other disease models (12,28). I found elevated plasma albumin level in the SHR. Albumin fragments (~17.1 kDa) were also present in SHR urine according to western analysis. The amount of urine used (0.01 μl) in the western blot experiment was 100 times more than the amount of plasma used (0.1 nl), and this is because the abundant proteins in the plasma causes non-specific binding of the anti-albumin antibody when > 0.1 nl plasma is used. Therefore, it is possible that if the level of albumin fragments is very low, the immunodetection method might not be sensitive enough to produce a signal when too little plasma volume is used. It is also possible that the plasma albumin fragments were small enough to pass through the glomeruli filter in the kidney and accumulate in the urine to high enough concentrations that are within the sensitivity range of the current immunodetection method.

The ~17.1 kDa albumin fragment found in this study corresponds to reported large albumin fragments (ranges from 12 kDa to 22 kDa) from the plasma and urine that were generated by cleavage following basic amino acids with trypsin-like specificity (12,28,31). Studies in our lab have recently discovered significantly elevated plasma trypsin activity in the SHR (along with trends for elevated chymotrypsin activity) (15). These results suggest a proteolytic process of albumin fragment formation in the circulation, followed by entrance of these fragments into the urine via glomerular filtration in the SHR.

Chapter 5. Conclusions

This present study for the first time provides evidence that the elevated enzymatic activity in the SHR circulation is possibly associated with proteolysis and degradation of plasma proteins, as part of the autodigestion hypothesis in chronic hypertension. It also provides a list of candidate plasma proteins that might be degraded in the SHR. To further test the autodigestion hypothesis, further work should identify the proteins that were reduced or absent, or have a higher degradation rate in the SHR. Identification of such proteins and their associated loss of functions due to proteolytic destruction might be crucial to discover the underlying mechanisms of disorders stemmed from hypertension (ex. Insulin receptor cleavage and diabetes (20)). In addition, treatment with different protease inhibitors, such as doxycycline and cyklokapron, may be tested to reduce plasma protein cleavage and fragment formation. A more complete understanding of the underlying mechanism of protease elevation in hypertension can serve as the basis for improving therapeutic solutions and prevention.

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