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Pancreatic Source of Protease Activity in the Spontaneously Hypertensive Rat and its Reduction During Temporary Food Restriction

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Abstract

The mechanisms underlying cell and organ dysfunctions in hypertension are uncertain. The spontaneously hypertensive rat (SHR) has elevated levels of unchecked degrading proteases compared to the control Wistar Kyoto (WKY) rat. The extracellular proteases destroy membrane receptors leading to cell dysfunctions, including arteriolar constriction and elevated blood pressure. Our goal was to identify potential sources of the uncontrolled enzymatic activity. Zymographic measurements in SHR pancreas and intestine were obtained as part of the digestive system with high levels of degrading enzymes. The results showed that SHRs have significantly higher protease activity than WKY in pancreas (22.04 ±9.01 vs 13.02 ±3.92 casein fluorescence intensity unit; $p<0.05$) and pancreatic venules (0.011 ±0.003 vs 0.005 ± 0.003 trypsin absorbance; $p<0.05$) as well as in venous blood (71.07 ±13.92 vs 36.44 ± 16.59 casein fluorescence intensity unit; $p<0.05$). The enzymatic activity is contributed by trypsin and chymotrypsin. Furthermore, a decrease of these enzyme activity levels achieved during a short-term fasting period is associated with a reduction in systolic blood pressure in SHR (135±8 mmHg vs 124±7 mmHg; $p<0.05$). The results suggest the pancreas of the SHR is a potential source for serine proteases leaking into the circulation and contributing to its protease activity.

Objective: The mechanisms underlying cell and organ dysfunctions in hypertension are uncertain. The spontaneously hypertensive rat (SHR) has elevated levels of unchecked degrading proteases compared to the control Wistar Kyoto (WKY) rat. The extracellular proteases destroy membrane receptors leading to cell dysfunctions, including arteriolar constriction and elevated blood pressure. Our goal was to identify potential sources of the uncontrolled enzymatic activity.

Methods: Zymographic and digital immunohistochemical measurements in SHR pancreas and intestine were obtained as part of the digestive system with high levels of degrading enzymes.

Results: The results showed that SHRs have significantly higher protease activity than WKY in pancreas (22.04 ±9.01 vs 13.02 ±3.92 casein fluorescence intensity unit; $p<0.05$) and pancreatic venules (0.011 ±0.003 vs 0.005 ± 0.003 trypsin absorbance; $p<0.05$) as well as in venous blood (71.07 ±13.92 vs 36.44 ± 16.59 casein fluorescence intensity unit; $p<0.05$). The enzymatic activity is contributed by trypsin and chymotrypsin. Furthermore, a decrease of these enzyme activity levels achieved during a short-term fasting period is associated with a reduction in systolic blood pressure in SHR (135±8 mmHg vs 124±7 mmHg; $p<0.05$).

Conclusions: The results suggest the pancreas of the SHR is a potential source for serine proteases leaking into the circulation and contributing to its protease activity.

Abbreviations used: SHR spontaneously hypertensive rat, WKY Wistar Kyoto rat, VEGFR2 vascular endothelial growth factor receptor 2, FPR f-Met-Leucine-Phenyalanine receptor, MMP matrix metalloproteinase, EDTA ,

Introduction

The genesis for end-organ damage that accompanies an elevated arterial blood pressure in hypertensives remains largely unknown. We proposed that the variety of cell and tissue dysfunctions present in hypertension [34] may at least in part be due to degrading enzymes by cleavage of key receptors responsible for specific cell functions [27]. Specifically, in the spontaneously hypertensive rats (SHR), increased levels of unchecked proteases in plasma and endothelium compared to the normotensive Wistar-Kyoto (WKY) and Wistar rats cause extracellular cleavage of membrane receptors and loss of associated cell functions with far reaching consequences and co-morbidities.

For example, proteolytic cleavage of β_2-adrenergic receptor results in the SHR's characteristic arteriolar constriction and elevated arterial blood pressure [36]. Cleavage of its insulin receptors leads to the insulin resistance in the SHR [9]. The ectodomain of the insulin-like growth factor-1 is clipped in the SHR [8]. Endothelial apoptosis and the resulting capillary rarefaction in the SHR is due to degradation of the vascular endothelial growth factor receptor (VEGFR2) [44,46] and the deficient leukocyte adhesion in the SHR is due to cleavage of membrane adhesion molecules [7,9]. Ectodomain cleavage of the FMLP receptor (FPR) attenuates the fluid shear stress response in the SHR [6] and contributes to its elevated blood pressure [18]. Proteolytic receptor cleavage is also detected on SHR macrophages [39] and red cells [33]. The evidence brings to light that protease activity is at the center of the SHR's complications.

The uncheck proteases in the SHR includes matrix metalloproteinases (MMP) [9,36,40,44,46]. Chronic MMP inhibition restores the membrane receptor densities and alleviates the cell and microvascular deficiencies in the SHR. But further analysis of protease

activity of SHR plasma incubated with casein substrate, which detects serine proteases along with metallo-, acid- and thiol-proteases, is only partially inhibited with EDTA (~29%), an inhibitor of MMPs [9]. We hypothesize that serine protease activity may be elevated in the bloodstream of SHR compared to WKY, which in turn may autoactivate MMPs [24,25,37].

Thus, the current study was designed to investigate new sources for serine protease activity in the SHR. As part of the digestive system, there are two organs with larger pools of serine proteases, the pancreas and the small intestine. These two sources in the SHR are currently unexplored and therefore the target of this investigation.

It has been shown that fasting or caloric restriction can significantly reduce blood pressure in SHRs without a similar reduction in WKY rats [29,32,35,38,49,50]. A number of hypotheses have been proposed to explain this phenomenon in the SHR. For instance, the reduction in blood pressure may be due to the loss of body weight. However, the hypotensive effect is observed after only 24 hours of fasting, at which point no significant weight loss has yet occurred. Another hypothesis is that the reduction in blood pressure may be due to suppression of sympathetic nervous system activity during fasting [13,21,38,50]. However, such suppression is observed in both fasted strains, SHR and WKY. Therefore, it is unlikely only dependent on sympathetic nervous system activity [13]. Blood pressure reduction could be due to decreased sodium intake during fasting, but this could not be confirmed with a sodium-deficient diet [14]. Therefore, the mechanism by which fasting reduces systemic blood pressure remains uncertain [47].

The current study was designed to identify selected pancreatic digestive enzymes levels in SHR plasma and potential sources of such elevated enzymatic activity by determining their level in pancreas and intestine. Rats were subjected to a short 18-hour period of fasting without

significant weight loss and minimal compensatory mechanisms to determine the correlation between enzymatic activity and blood pressure.

Materials and Methods

Animals

The experimental protocol was reviewed and approved by the University of California, San Diego Animal Subjects Committee. Male WKY and SHR (28-32 weeks of age, Charles River Laboratories, Wilmington, MA, USA) were used for the experiments. One control SHR animal was excluded from the study due to its normotensive blood pressure measured after femoral artery cannulation.

Fasting Treatment

Subgroups of WKY and SHRs were fasted overnight for 18 hours with water provided *ad libitum*. Control groups received standard chow and water.

Saphenous Vein Blood Collection and Glucose Measurements

The animals were placed in a restrainer with the right leg extended, the saphenous vein bifurcation site was sterilized with 100% alcohol and the right saphenous vein was punctured for blood collection with a pipette. Plasma glucose measurements were obtained with a glucose meter (Contour, Bayer, Tarrytown, NY). All plasma collections were carried out in the morning.

Anesthesia and Tissue Collection

Animals were tranquilized with xylazine (20 mg/ml, 200 μL/kg bodyweight, i.m., MWI, Nampa, ID) and administered general anesthesia (sodium pentobarbital, 50 mg/ml, 1ml/kg body weight, i.m., Ovation Pharmaceuticals, Inc., Deerfield, IL). Supplemental dose of anesthesia (5 – 10 μL sodium pentobarbital) was administered as indicated by tail pinch reflex.

Animals were kept on a water-heated pad (37^0C). A femoral artery catheter was placed to measure the central blood pressure. Blood pressure values are reported during general anesthesia. Blood was collected (~3 ml) from the femoral artery.

An abdominal midline incision was made with precaution to prevent injury to any internal organs. Phosphate buffered saline (PBS, 1ml) was injected into the peritoneal space with gentle skin massage to evenly distribute the PBS. The PBS containing peritoneal fluid was immediately collected. In addition, blood was collected from the vena cava (~3ml). After euthanasia (Fatal-Plus solution, Vortech Pharmaceuticals, Ltd., Dearborn, MI; i.v.), the intestine, and pancreas were promptly harvested and placed in PBS for immediate tissue processing.

Tissue Embedding and Sectioning

The fresh organs were cut with a scalpel into smaller blocks and embedded (Tissue-Tek Optimal Cutting Temperature, O.C.T. Compound, Sakura Finetek, Torrance, CA) on ice. The embedded tissues were then quickly frozen in liquid nitrogen and stored (-80^0C) in order to maximally preserve enzymatic activity. The organs were prepared as follows:

1. Pancreas: it was detached from the stomach and duodenum with surrounding adipose tissue removed, cut into 0.5 cm segments and embedded.
2. Intestine: Flushed and non-flushed specimens were collected to examine the presence of material in the lumen of the intestine. 1 cm segments were cut from the center (jejunum) and 4 to 5 cm away from the duodenum and the cecum and embedded longitudinally. For tissue homogenates, 2 cm portions were removed from 8 different segments along the small intestine. Each cut portion was weighed and stored at -80^oC until homogenization.

The tissue samples were sectioned (Leica CM 3500 cryostat, Bannockburn, IL) and the sections (5 μm thickness) were placed onto microscope slides (Fisherbrand Superfrost Plus Microscope Slides, Fisher Scientific, Pittsburgh, PA). Each slide contains one SHR and one WKY tissue section and labeled identically for direct comparison. Slides were stored (-20^{0}C) until processed for *in situ* zymography (carried out on the same day) and immunohistochemistry.

In-situ Zymography

Before incubation with casein substrate, tissue slides were placed on inverted Petri dishes resting in a water bath (37^{0}C for 5 minutes) to thaw and remove visible residual moisture. Upon drying, 10 μL of casein substrate (EnzChek Protease Assay Kit, E6639, Invitrogen, Eugene, OR), N_α-Benzoyl-L-arginine-7-amido-4-methylcoumarin hydrochloride (trypsin substrate, B7260, Sigma-Aldrich, St. Louis, MO) or Glutaryl-L-phenylalanine 7-amido-4-methylcoumarin (chymotrypsin substrate, 49737, Sigma) was applied onto the slides. For control, the digestion buffer from the kit or substrate buffer (50 mM Tris-HCl, 20 mM calcium chloride, pH 8) was utilized to test for autofluorescence of the tissue sections. The slides were coverslipped, coated with nail polish to prevent movement of the coverslips and evaporation of the substrate, placed in a black slide box to prevent fluorescence quenching, and incubated in a water bath (37^{0}C for 60 minutes).

After incubation, slides were viewed under an inverted fluorescence microscope with appropriate filter set (589/617 nm for casein, 366/460 nm for Nα-Benzoyl-L-arginine-7-amido-4-methylcoumarin hydrochloride, 320/370 nm for glutaryl-L-phenylalanine 7-amido-4-methylcoumarin). Fluorescent images of the tissue sections were recorded digitally. Control

sections without casein substrate were recorded in order to determine the level of tissue auto-fluorescence.

Immunohistochemical Labeling of Trypsin

Slides were air dried for 10 minutes before incubation with 100% acetone at -20^{0}C for 5 minutes. Hydrophobic circles were drawn around each tissue section on the slides before blocking of endogenous peroxidase with Peroxo-Block solution (00-2015, Invitrogen) for 30 seconds. After several quick rinses with PBS, slides were incubated with normal horse serum for 1 hour at room temperature to block non-specific binding. Trypsin antibody, which recognizes both active and inactive forms of trypsin, (SC67388, Santa Cruz) was added to the slides for overnight incubation (4^{0}C). After rinsing with PBS (3 times for 10 minutes each), the slides were incubated with rabbit ImmPRESS peroxidase polymer reagent (Vector Laboratories, Inc., Burlingame, CA) for 30 minutes followed by the addition of 3,3'-diaminobenzidinesubstrate (DAB, SK4100, Vector Laboratories) for detection of secondary antibody.

Image Analysis

The images were digitally analyzed (Image J, NIH, http://rsbweb, nih.gov/ij/). Background was eliminated by using the following equation:

$$In = (Ii - Id) / (Ib - Id) * 255$$

where *In* represents the pixel intensity of the modified image without background, *Ii* is the pixel intensity on the original image, *Ib* the pixel intensity of background without tissue section, and *Id* is the pixel intensity with the microscope light turned off.

For *in situ* zymography, the fluorescent images were split into RGB layers and only the red layer was analyzed with the casein substrate and the blue layer for the trypsin and chymotrypsin substrates. The light intensity was measured only in the tissue areas of the image. Measurement of control sections were calculated and used to determine the autofluorescence levels in the tissue. The presence of a fluorescent signal was determined by comparing the levels of autofluorescence on control sections and the signal on sample sections.

For immunohistochemical image analysis, the light microscopy images were inverted and converted into grayscale. The signal intensity on non-specific control slides was selected as lower threshold. Area (A_t) and intensity (I_t) covered by light intensity above threshold was quantified and divided by total tissue area (A_o, excluding empty spaces around the tissue). The light absorbance was calculated as $(A_t)(I_t) / (A_o)$.

Tissue Homogenization

Cold PBS was added to frozen intestinal segments (5 ml per gram of tissue) in tubes covered with ice and homogenized (Kinematica Polytron PT 1200C homogenizer, Brinkmann; Westbury, NY). Each intestinal segment was homogenized for at least 30 seconds or until disappearance of tissue chunks visible by unaided eye. The homogenates were transferred to 1.5 ml tubes and spun at 13,000g's for 15 minutes at 4^0C. The supernatants were transferred to new 1.5ml tubes and spun at 13,000g for 10 minutes at 4^0C. The supernatants were then collected and divided into aliquots and stored (-80^0C) until use.

Intestinal Protease Activity Measurements

The intestinal homogenates, along with purified proteases, were tested for general proteolytic activities (metallo-, serine, acid and sulfhydryl proteases) using casein derivative substrate (Enzchek Protease Assay Kit, E6639, Molecular Probes-Invitrogen, Carlsbad, CA).

The substrate in the Enzchek Protease Assay Kit, casein, is derivatized with pH-insensitive fluorophores. When undigested, the fluorophores are quenched and the molecule does not fluoresce. Upon cleavage by proteases, the peptides with these fluorophores will fluoresce. The level of measured fluorescence increases with the amount of peptide present. The fluorescence of the intestinal homogenates incubated with this substrate was measured in duplicates on a 96-well plate by a spectrophotometer (Spectromax Gemini XS) using the Softmax Pro software (Molecular Devices Corp., Sunnvale, CA) and expressed in relative fluorescent units (RFUs). The level of fluorescence increases with the number of sites within the casein molecule cleaved by the enzymes in the sample.

In each well of a multiwell plate, 5 µl of intestinal sample, 95 µl of digestion buffer and 100 µl of casein substrate were mixed. The plate was covered with aluminum foil to prevent evaporation, incubated at 37ºC in a spectrophotometer (protected from light), and kinetic measurements were made over a period of 60 minutes.

Plasma Protease Activity Measurements

Plasma samples were tested for trypsin activity using Nα-benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA, B3133, Sigma) substrate and chymotrypsin activity using N-succinyl-ala-ala-pro-phe-p-nitroanilide (SPNA, S7388, Sigma) substrate.

The BAPNA substrate is a chromogenic substrate for detection of trypsin-like enzymes. P-nitroaniline is released upon hydrolysis of BAPNA and detected at 405 nm. For each well, 20

µl of plasma sample was incubated with 180 µl of BAPNA substrate (0.5 mM) for 90 minutes with absorbance values taken every 15 minutes at 405 nm. For analysis, the absorbance reading at time 0 was subtracted from absorbance reading at each subsequent time point and designated as corrected absorbance.

In a similar fashion, the SPNA substrate yields 4-nitroaniline as a yellow color upon cleavage under alkaline conditions that are detected at 405 nm. 20 µl of plasma sample was incubated with 180 µl of substrate (5 mM) for 90 minutes with an additional reading at 24 hours. To eliminate the effects of autofluorescence, the absorbance at time 0 was subtracted from subsequent absorbance values.

Statistical Analysis

All measurements were summarized as mean ± standard deviation. Two-tailed student's t-test was used for comparison between groups. A probability of $p<0.05$ was considered significant.

Results

Blood pressure

The SHR group had an elevated systolic blood pressure compared to WKY rats (138±8 vs 104±4 mmHg respectively, $p<0.05$, measured under general anesthesia). Systolic blood pressure in the SHR was significantly lowered to 124±7 mmHg after 18 hours of fasting ($p=0.012$ vs no fasting), while the WKY group exhibited no significant reduction (not shown).

Enzymes in Plasma

Fresh venous plasma samples exhibited elevated trypsin activity in SHR ($p<0.05$; Figure 1a). Chymotrypsin activity in plasma was on average also higher in the SHR group, but not statistically significant (not shown).

Sources of Enzymatic Activity in Plasma

In search for a potential source of the serine protease activity in the plasma of the SHR we investigated the pancreas and small intestine, both exposed to digestive proteases.

Pancreas

In situ zymography with fluorescent casein substrate indicate that pancreatic sections of the SHR exhibited higher enzymatic activity than WKY sections (Figure 1b). This pattern was also seen on venular crossections inside the pancreas with casein substrate as well as with chymotrypsin specific substrate (Figure 1b). Immunohistochemical labeling of trypsin revealed there is an elevated level of trypsin density (active and inactive forms) in the pancreas of the

SHR (Figure 1c). Pancreatic sections incubated with trypsin substrates had on average higher levels (63%), but the difference was not significant. Both a punctuated immunohistochemical label pattern for trypsin (Figure 1c, arrowheads) is seen in the pancreatic intercalated ducts, and a lighter and more evenly distributed pattern throughout the pancreatic tissue.

Intestine

In situ zymography with fluorescent casein substrate in the intestine (on sections collected along its full length) showed no significant differences between WKY and SHR (results not shown) even though average values were higher in the SHR.

Possible Leakage Mechanisms of Enzymatic Activity into the Circulation

Two possible protease leakage mechanisms were examined. The enzymes could escape into the peritoneal space from the pancreas, which in turn is taken up by the surrounding vessels, or the enzymes could leak directly into the circulation via pancreatic venules.

No significant difference was found between the level of enzymatic activity in the peritoneal fluid of WKY and SHR (not shown). Pancreatic venules were identified by the location of venular-arteriolar pairs and their typical thin wall morphology. Fluorescent measurements in the venular walls showed that compared to WKY, SHR pancreatic venules have significantly higher proteolytic activity, including chymotrypsin activity and trypsin protein density (Figure 1b, c).

Effects of Fasting

Weight

No significant shift in body weight was found between control and fasted animals during the 18 hour solid food restriction.

Glucose Values

No difference in the blood glucose values was found before and after fasting among the groups of animals (results not shown).

Enzymatic Activity in Plasma

The elevated trypsin activity in SHR venous plasma (Figure 1a) was reduced after fasting (Figure 2a) while its chymotrypsin activity remained unchanged (Figure 2b). The chymotrypsin activity in the WKY plasma fell after fasting to values significantly below the SHR values.

Serine Protease and Activity in Pancreatic Venules

After fasting, SHR pancreatic tissue and venules had a significant decrease in trypsin label density, a feature not found in WKY rats (Figure 2c, d). The trend is in line with the associated reduction in arterial blood pressure in these two groups after food restriction. In addition, the chymotrypsin activity in the SHR pancreas fell after fasting (Figure 2e).

Discussion

The current results indicate that compared to the WKY rats, SHRs have elevated serine protease activities in pancreatic tissue, pancreatic venules and in plasma of the central circulation. The venules in the SHR pancreas also exhibited enhanced chymotrypsin activity. Even a relatively short (18 hours) fasting period serves to reduce systemic blood pressure as well as pancreatic enzyme levels and activity in plasma, pancreatic tissue and venules. The combination of active serine proteases (e.g. trypsin, chymotrypsin) may serve to activate MMPs and sets up a condition that can lead to a plethora of cellular dysfunction with blood pressure elevation as well as generation of co-morbidities by receptor cleavage [27](Figure 3).

The trend for an elevated serine protease activity in plasma of the SHR is in agreement with previous measurements [10]. Blockade of active serine protease activity in plasma requires endogenous protease blocking capacity, which may be reduced in the SHR, and could be saturated in the presence of a chronic protease leakage out of the pancreas. The serpin and specifically the anti-trypsin levels in SHRs remain undetermined.

Diverse lines of evidence suggest that proteolytic activities, such as derived from serine proteases and MMPs, are elevated in hypertensives [2,4,5,9,11,15,16,19,22,23,48]. The current evidence suggests the possibility that in addition leaking pancreatic serine proteases with elevated plasma activity may contribute to activation of proMMPs [24,25,37,42]. Even a low level chronic leakage and elevation of protease activity may compromise normal cell functions, which may constitute an initial stage in the progression of the tissue and organ damage in the SHR [26].

Chronic inhibition of MMPs in the SHR serves to attenuate cleavage of extracellular receptors domains, restore cell dysfunctions and alleviates symptoms. For example, chronic

MMP inhibition prevents cleavage of the β_2-adrenergic receptor, arteriolar constriction and elevated blood pressure [36], cleavage of the insulin receptor and insulin resistance [10]. Similarly MMP blockade preserves the VEGFR2, prevents endothelial apoptosis with capillary rarefaction [45], leukocyte/endothelial adhesion defects by cleaved P-selectin, PSGL-1, CD18, ICAM-1 [7,9,43], and restores the leukocyte fluid shear stress response by preservation of the FPR [6].

A focus of the current study was to explore potential sources for elevated enzymatic activity in the SHR. As organs with inherently high levels of degrading enzyme activities, the intestine and pancreas were examined with a general protease activity substrate. In the intestine, no significant differences between SHR and WKY were detected. This result has to be interpreted within the limitation of the zymographic techniques (detection limits of 1.3×10^{-2} enzyme units for trypsin and 5.0×10^{-5} enzyme units for chymotrypsin for the E6639 EnzChek Protease Kit). The overall activity in the intestinal epithelium was higher than that in the interstitial space but without differences between the two rat strains. In contrast, in the pancreas the SHRs exhibited significantly elevated trypsin activity and protein density. This evidence suggests a link between trypsin levels and pancreatic tissue damage in SHRs [3,30,31]. Defects in the SHR pancreas include morphological changes in young and older SHRs, inflammatory cell infiltration, stromal fibrosis, acinar atrophy, hemorrhage, degeneration and ductular proliferation. These conditions worsen with age, suggesting that SHRs may have a form of spontaneous pancreatitis [31]. The abnormalities in the SHR pancreas are also detectable in the morphological structure of pancreatic ducts, which exhibit ductular proliferation, tortuous ductal channels, crater-like depressions of ductal inner surfaces and sporadic presence of long cilia of

ductal cells in the arterioles, similar to those seen in WBN/Kob rats, a disease model for pancreatitis [3].

The elevated enzymatic activity in the pancreatic venules indicates that activated digestive enzymes leak into the bloodstream from the SHR pancreas through an autodigestion mechanism, similar to the one suggested in pancreatitis [20,41]. In severe acute pancreatitis, trypsin activity destroys the pancreatic structure itself leading to bleeding and necrosis. Transport of active trypsin through the venous system into the circulation promotes endothelial apoptosis via receptor cleavage, elevated permeability and potential bleeding.

The behavior and morphology of pancreatic acinar cells in SHR may be abnormal. Uncontrolled release of enzymes may be associated with elevation of chromogranin A in hypertension [40]. This acid-soluble protein plays an important role in the release of granules, such as catecholamines. Thus it is possible that polymorphisms in chromogranin A of SHR [17] may be associated with an uncontrolled leakage of proteases from acinar cells in the pancreas, an issue that requires further studies.

In this investigation, a short fasting period was sufficient to significantly reduce the systolic blood pressure of the SHR with a similar reduction in enzymatic activity in the plasma and pancreas. Fasting may minimize the release of pancreatic enzymes and attenuate autodigestion. It is possible that this decrease in blood pressure involves reconstitution of cleaved membrane receptors such as β_2-adrenergic receptors required for blood pressure regulation [36]. Previous fasting studies for 24+ hours have also achieved a significant arterial pressure reduction in the SHR [14,21]. Fasting is used to treat pancreatitis patients [1,28] and caloric restrictions are known to reduce blood pressure [12].

The result of the current fasting study suggests pancreatic enzymes may play a role in the mechanism of essential hypertension that should be explored in clinical studies.

Perspectives

This study provides for the first time evidence that elevated enzymatic activity in the SHR plasma is associated with leakage of activated trypsin and chymotrypsin from the pancreas and also possibly associated with an early stage of an autodigestion process [27]. Further studies are required to determine in acinar cells the molecular mechanism responsible for the release of digestive enzymes into the venous system of the pancreas of SHR. Treatment with serine protease inhibitors in SHR may help clarify the mechanism and determine whether cleavage of receptors such as insulin receptors and β_2-adrenergic receptors is attenuated in fasted animals. A more complete understanding of the mechanism of protease-derived cell dysfunction in hypertension in patients may serve as the basis for improved therapeutic interventions that address not only the elevated blood pressure by also the co-morbidities.

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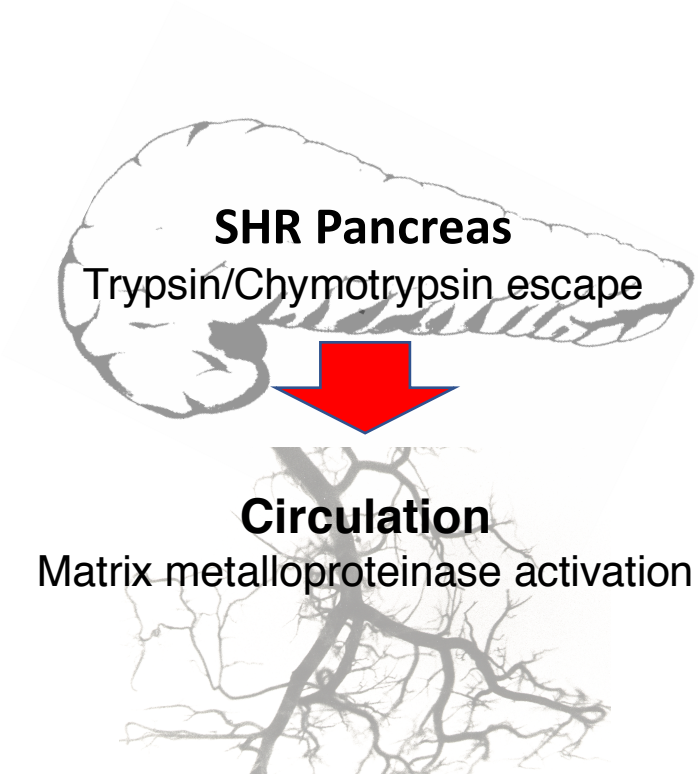
Figure Legends

Figure 1. **Pancreatic trypsin and chymotrypsin leaking into the circulation**. (**a**) Trypsin activity in venous plasma measured by specific substrate. *p<0.05 vs WKY. (**b**) *In situ* zymography micrographs (top images) of pancreatic sections in WKY and SHR incubated without and with casein substrate (first, second column from left), and pancreatic tissue sections with casein (third column) and chymotrypsin substrates (fourth column) showing arteriolar (A) and venular (V) crossections. Corresponding fluorescence values are shown in digital intensity units (lower bar graphs). *p<0.05 vs WKY. Proteolytic and chymotrypsin activities are significantly elevated in SHR venules compared to WKY rats. (**c)** Micrographs of frozen immunohistochemical sections with primary antibody against trypsin as seen with DAB substrate (top panels). Control images are without or with irrelevant primary antibody (left column). Scale bars = 100 μm. Light absorption measurements due to trypsin labeling normalized by its average value in the WKY section (right histogram) and absorption along the venules expressed as digital units (right histogram). *p<0.05 compared to WKY. n = number of rats/group. SHR shows higher density of trypsin protein labeling.

Figure 2. **Pancreatic digestive activity in fed and fasted state.** (**a)** Trypsin and (**b**) chymotrypsin activity levels in venous plasma. SHR venous plasma trypsin and WKY chymotrypsin activities are significantly reduced after fasting. *p<0.05 SHR control compared to WKY control. +p<0.05 WKY compared to WKY fasted. **p<0.05 WKY fasted compared to SHR fasted. Micrographs of immunohistochemical sections of (**c**) pancreatic tissue, and (**d**) pancreatic venules with primary antibody against trypsin as seen by DAB substrate during feeding ad libitum (Control) and after 18 hours of food restriction (Fasted). Histograms (below the micrographs) show mean ± SD of digital light absorption measurement (see Methods). *p<0.05 SHR compared to WKY. **p<0.05 WKY Fasted compared to SHR Fasted. (**e**) *In situ* zymography micrographs for chymotrypsin of pancreatic venules before and after 18 hours of fasting. (Right) Fluorescence intensity measurements (see Methods) shown as mean ± standard deviation. Average chymotrypsin activity is significantly elevated in SHR venules compared to that of WKY venules and is reduced by fasting. ++p<0.05. n = number of rats/group. Scale bars = 100 μm.

Figure 3. Schematic of serine protease release in the SHR pancreas into the circulation, MMP activation and consequently proteolytic receptor cleavage and multiple cell dysfunction leading to typical co-morbidities in the SHR.



Proteolytic Receptor Cleavage	Cell and Systems Dysfunctions
- Beta-2 adrenergic receptor	Arteriolar constriction, arterial pressure elevation
- Insulin receptor, IGF-1	Hyperglycemia, insulin resistance
- Insulin growth factor-1 receptor	Growth, differentiation, blood pressure,
- Vascular Endothelial Growth Factor Receptor -2 (VEGFR-2)	Endothelial apoptosis and capillary rarefaction
- Leukocyte adhesion receptor (P-selectin/PSGL-1/CD18/ICAM-1)	Leukocyte adhesion deficiency
- formyl peptide receptor (fPR)	Leukocyte mechanotransduction defect, capillary resistance elevation

Figure 3. Schematic of serine protease release in the SHR pancreas into the circulation, MMP activation and consequently proteolytic receptor cleavage and multiple cell dysfunction leading to typical co-morbidities in the SHR.

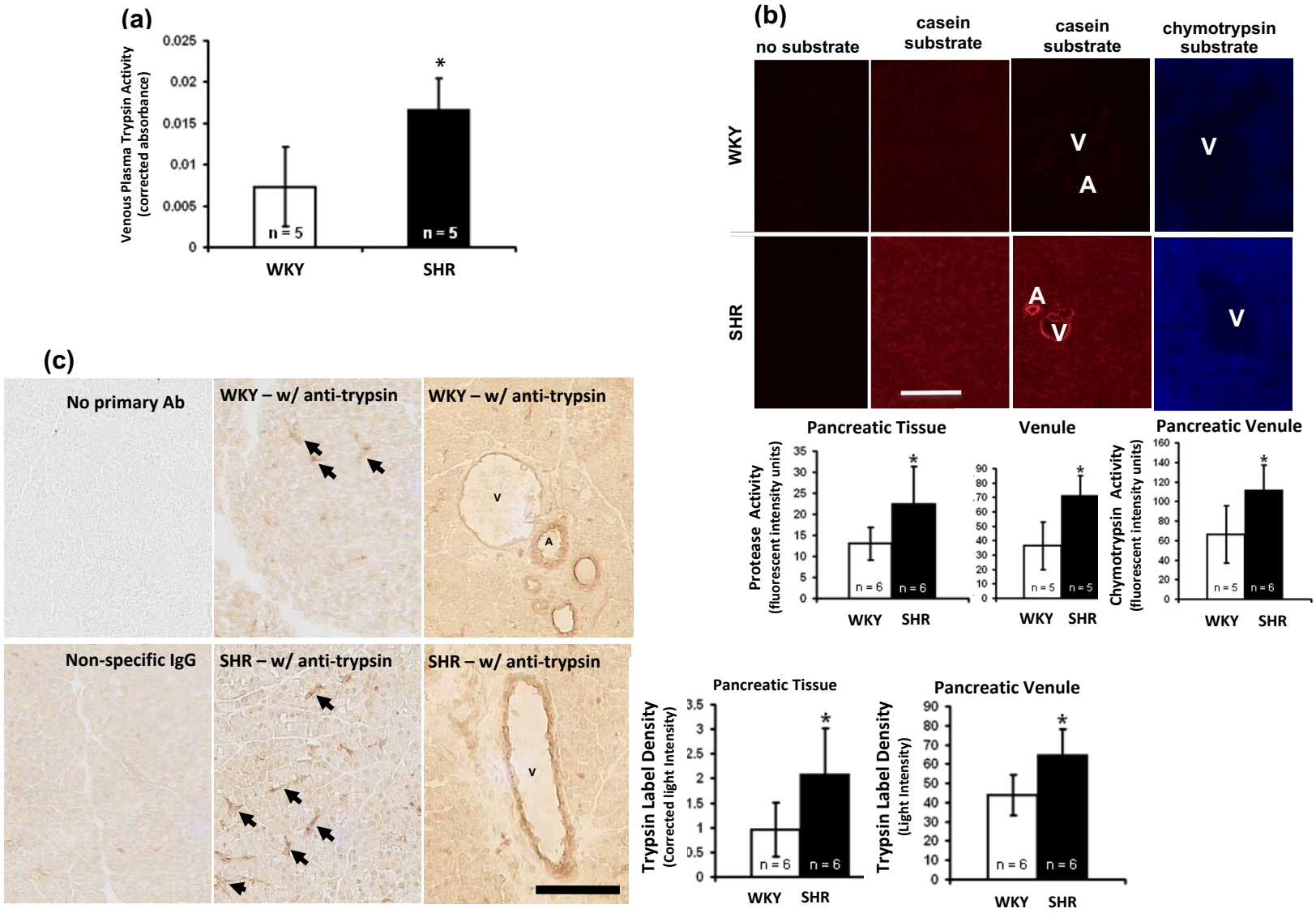

(a)
Venous Plasma Trypsin Activity (corrected absorbance)
0.025
0.02
0.015
0.01
0.005
0
n = 5
n = 5
*
WKY
SHR
(b)
no substrate
casein substrate
casein substrate
chymotrypsin substrate
WKY
SHR
V
A
V
A
V
V
Pancreatic Tissue
Venule
Pancreatic Venule
Protease Activity (fluorescent intensity units)
Chymotrypsin Activity (fluorescent intensity units)
n = 6
n = 6
n = 5
n = 5
n = 5
n = 6
WKY
SHR
(c)
No primary Ab
WKY – w/ anti-trypsin
WKY – w/ anti-trypsin
V
A
Non-specific IgG
SHR – w/ anti-trypsin
SHR – w/ anti-trypsin
V
Pancreatic Tissue
Pancreatic Venule
Trypsin Label Density (Corrected light Intensity)
Trypsin Label Density (Light Intensity)
n = 6
n = 6
n = 6
n = 6
WKY
SHR

Figure 1

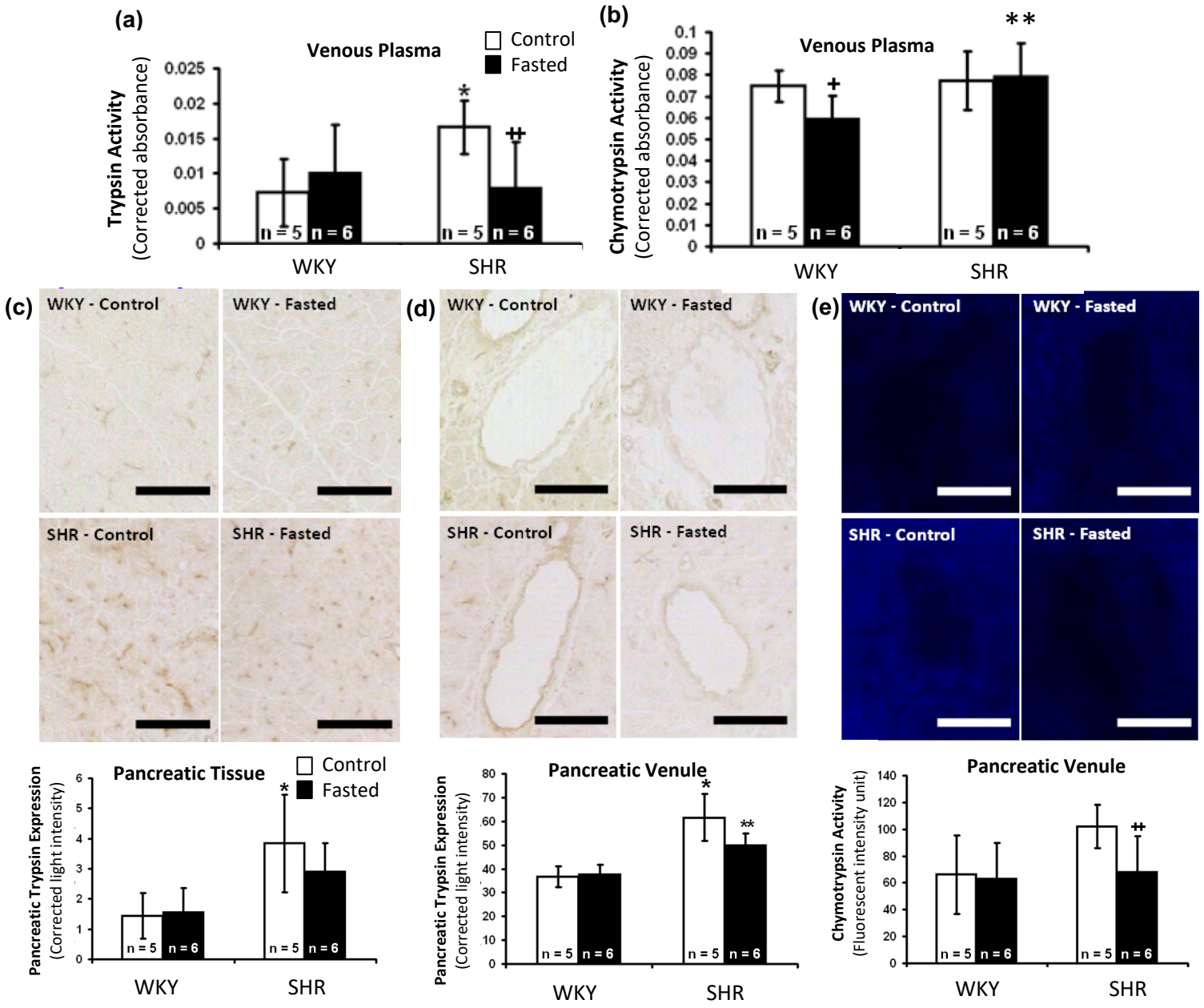

(a)
Venous Plasma
Control
Fasted
Trypsin Activity
(Corrected absorbance)
0.025
0.02
0.015
0.01
0.005
0
*
++
n = 5
n = 6
n = 5
n = 6
WKY
SHR
(b)
Venous Plasma
Chymotrypsin Activity
(Corrected absorbance)
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
**
+
n = 5
n = 6
n = 5
n = 6
WKY
SHR
(c)
WKY - Control
WKY - Fasted
SHR - Control
SHR - Fasted
(d)
WKY - Control
WKY - Fasted
SHR - Control
SHR - Fasted
(e)
WKY - Control
WKY - Fasted
SHR - Control
SHR - Fasted
Pancreatic Tissue
Control
Fasted
Pancreatic Trypsin Expression
(Corrected light intensity)
6
5
4
3
2
1
0
*
n = 5
n = 6
n = 5
n = 6
WKY
SHR
Pancreatic Venule
Pancreatic Trypsin Expression
(Corrected light intensity)
80
70
60
50
40
30
20
10
0
*
**
n = 5
n = 6
n = 5
n = 6
WKY
SHR
Pancreatic Venule
Chymotrypsin Activity
(Fluorescent intensity unit)
140
120
100
80
60
40
20
0
++
n = 5
n = 6
n = 5
n = 6
WKY
SHR

Figure 2