INFLUENCE OF GENDER ON OXIDATIVE STRESS, LIPID PEROXIDATION, PROTEIN DAMAGE AND APOPTOSIS IN HEARTS AND BRAINS FROM SPONTANEOUSLY HYPERTENSIVE RATS

Jun Ren

Division of Pharmaceutical Sciences & Center for Cardiovascular Research and Alternative Medicine, University of Wyoming, Laramie; Faculty of Basic Medicine, Peking Union Medical College, Beijing, China

SUMMARY

1. Hypertension leads to oxidative stress, lipid and protein damage, apoptosis and impaired cardiac contractile function. However, impact of gender on these hypertension-associated abnormalities has not been elucidated.

2. The present study evaluated the oxidative stress, lipid/protein damage, apoptosis and contractile function were assessed by glutathione (GSH): reduced glutathione (GSSG) ratio, malondialdehyde (MDA) levels, protein carbonyl levels and caspase-3 activity, respectively. Cardiomyocyte contractile function was examined including peak shortening (PS), time-to-PS (TPS), time-to-90% relengthening (TR90) and maximal velocity of shortening/relengthening (±dL/dt). The SHR cardiomyocytes displayed prolonged TR90 compared with age-matched WKY counterparts. Male but not female SHR cardiomyocytes possessed longer resting cell length, normal TPS and prolonged TR90. All mechanical parameters were comparable between male and female WKY rats with the exception of a higher TR90 in females. Hypertension did not significantly affect the GSH:GSSG ratio in the heart and brain tissues of either gender. Brain from female WKY rats displayed a reduced GSH:GSSG ratio. The MDA levels were unchanged and elevated, respectively, in SHR heart and SHR brain tissues from both genders. Protein carbonyl formation and caspase-3 activity were unaffected by hypertension or gender.

3. In summary, these results suggest that gender affects hypertension-associated oxidative stress, lipid and protein damage, apoptosis in heart and brain tissues and cardiomyocyte contractile function.

Key words: brain, gender, heart, hypertension, myocyte shortening, tissue damage.

INTRODUCTION

Gender difference in the pathogenesis, progression and manifestation of a large variety of cardio- and cerebrovascular diseases has been well documented. As a major risk factor for stroke and myocardial infarction, hypertension differs dramatically between men and women with respect to its prevalence, impact and clinical management. Premenopausal women have a relatively lower arterial blood pressure compared to the age-matched men and postmenopausal women, suggesting that ovarian hormones, especially oestrogen and progesterone, play a role in blood pressure regulation. Gender differences in vasculature and neuroendocrine systems are also present, which affect haemostasis, ventricular pumping, vascular reactivity and vascular tone. Whether gender plays an essential role in the onset of hypertensive complications and organ damage continues to be an issue of intense debate. Gender difference in hypertension and related complications may be visualized through somewhat gender-specific antihypertensive treatment. Men and women seem to require distinct antihypertensive regimens owing to overt differences in the progression and presentation of hypertension and its complication. For example, hypertension often leads to hypertrophic cardiomyopathy, a genetic disease that is mainly characterized by cardiac hypertrophy and ventricular contractile dysfunction owing to genomic changes in response to elevated ventricular volume- or pressure-overload. Intriguingly, gender plays a significant role in the determination of intrinsic myocardial contractile function and, possibly, manifestation of cardiac contractile property in hypertension. Nonetheless, the impact of gender on hypertrophic cardiomyopathy, especially at the level of cardiomyocytes, has not been elucidated.

The spontaneously hypertensive rat (SHR), a widely used rodent model for human essential hypertension, develops left ventricular hypertrophy, and cardiac mechanical and electrical abnormalities in response to high blood pressure and total peripheral resistance. In addition, SHR, especially the stroke-prone strain (SHRSP), elaborate brain injury that is characterized by neuronal death, inflammation and altered permeability of the blood–brain barrier (BBB). The aim of the present study was to examine the impact of gender on hypertension-associated oxidative stress, lipid and protein damage, apoptosis in heart and brain tissues, as well as cardiomyocyte...
contractile function in SHR and their age-matched normotensive Wistar-Kyoto (WKY) rats.

METHODS

Experimental animals

The experimental procedures were approved by the Institutional Animal Care and Use Committees at the University of North Dakota (Grand Forks, ND, USA) and the University of Wyoming (Laramie, WY, USA). All animal procedures were in accordance with the NIH animal care standards. Age-matched adult male and female WKY and SHR (~12 months of age) were obtained from Taconic Company (Germantown, NY, USA) and were maintained on a 12/12 h light/dark illumination cycle and allowed food and water ad libitum. Systolic blood pressure and heart rate were measured in conscious animals using a semi-automated, amplified tail-cuff device (Model ITT-31; IITC Inc., Woodland Hills, CA, USA). Before the experimental period, the rats were conditioned to the restraining cylinders and blood pressure measurement. Rats were prewarmed at 29°C for 8–10 min to facilitate tail blood flow before their blood pressure was measured. The mean of three tail-cuff readings was used as the systolic and diastolic blood pressure value. Bodyweights were measured with a standard laboratory scale.7

Cell isolation

Left ventricular myocytes were isolated as described elsewhere.7 In brief, animals were killed and their hearts were rapidly removed and perfused (at 37°C) with Krebs’-Henseleit bicarbonate (KHB) buffer containing the following composition (in mmol/L): NaCl 118; KCl 4.7; CaCl2 1.25; MgSO4 1.2; KH₂PO₄ 1.2; NaHCO₃ 25; N-[2-hydro-ethyl]-piperazine-N‘-[2-ethanesulfonic acid] (HEPES) 10; glucose 11.1 (pH 7.4). Hearts were subsequently perfused with Ca²⁺-free KHB containing 223 U/mL collagenase II (Worthington Biochemical Corp., Freehold, NJ, USA). After perfusion, the left ventricle was removed, minced and the cells were filtered through a nylon mesh (300 µm). Myocytes were resuspended and washed with Ca²⁺-free KHB buffer to remove any remnant enzyme, and extracellular Ca²⁺ was added incrementally and slowly back to a concentration 1.25 mmol/L. The cell yield was approximately 60–70% in all four rat groups tested. Mechanical properties of myocytes remained relatively stable for 12–24 h. Cells were not used if they had obvious sarcomemmal blebs or spontaneous contractions. Only rod-shaped myocytes with clear edges were selected for study.

Cell shortening/relengthening

Mechanical properties of ventricular myocytes were assessed using a SoftEdge MyoCam® system (IonOptix Corporation, Milton, MA, USA). In brief, left ventricular myocytes were placed in a Warner chamber mounted on the stage of an inverted microscope (Olympus IX-70; Olympus, Tokyo, Japan) and superfused (~1 mL/min at 25°C) with a buffer containing (in mmol/L): NaCl 131; KCl 4; CaCl₂ 1; MgCl₂ 1; glucose 10; HEPES 10 (pH 7.4). The cells were field stimulated with a suprathreshold voltage at a frequency of 0.5 Hz for 3 ms, using a pair of platinum wires placed on opposite sides of the chamber and connected to an electrical stimulator (FHC Inc.; Brunswick, NE, USA). The myocyte being studied was displayed on a computer monitor using an IonOptix MyoCam camera. IonOptix SoftEdge software was used to capture changes in cell length during shortening and relengthening. Cell shortening and relengthening were assessed using the following indices: peak shortening amplitude (PS), which indicated peak ventricular contractility; time-to-peak shortening (TPS), which indicated systolic duration; time-to-90% relengthening (TR₀.9), which indicated diastolic duration; and maximal velocities of shortening (+dL/dt) and relengthening (–dL/dt), which indicated maximal velocities of ventricular pressure rise/fall. In the case of altering stimulus frequency (i.e. 0.1, 0.5, 1.0, 3.0 and 5.0 Hz), a steady state contraction of myocyte was achieved (usually after the first 5–6 beats) prior to the recording of PS.

Glutathione and glutathione disulphide assay

Ventricular and brain tissues were homogenized in four volumes (w/v) of 1% picric acid. Acid homogenates were centrifuged at 16 000 g for 30 min and supernatant fractions were collected. Supernatant fractions were assayed for total glutathione (GSH) and reduced glutathione (GSSG) by the standard recycling method. The procedure consisted of using one-half of each sample for GSSG determination and the other half for GSH. Samples for GSSG determination were incubated at room temperature with 2 µL of 4-vinylpyridine (4-VP) per 100 µL sample for 1 h after vigorous vortexing. Incubation with 4-VP conjugates any GSH present in the sample so that only GSSG is recycled to GSH without interference by GSH. The GSSG (as GSH·2H₂O) was then subtracted from the total GSH to determine the actual GSH level and GSH : GSSG ratio, which is used as an indicator for oxidative stress.11 A reduced GSH : GSSG ratio usually indicates an enhanced oxidative stress status.

Protein carbonyl assay and lipid peroxidation

The carbonyl content of protein was determined as described elsewhere.12 Briefly, proteins were extracted and minced to prevent proteolytic degradation. Nucleic acids were eliminated by treating the samples with 1% streptomycin sulphate for 15 min, followed by a 10 min centrifugation (11 000 g). Protein was precipitated by adding an equal volume of 20% trichloroacetic acid (TCA) to protein (0.5 mg) and centrifuged for 1 min. The TCA solution was removed and the sample resuspended in 10 mmol/L 2,4-dinitrophenylhydrazine (2,4-DNPH) solution. Samples were incubated at room temperature for 15–30 min. After adding 500 µL of 20% TCA, samples were centrifuged for 3 min. The supernatant was discarded, the pellet washed in ethanol : ethyl acetate and allowed to incubate at room temperature for 10 min. The samples were centrifuged again for 3 min and the ethanol : ethyl acetate steps repeated twice or more times. The precipitate was resuspended in 6 mol/L guanidine solution, centrifuged for 3 min and any insoluble debris removed. The maximum absorbance (360–390 nm) of the supernatant was read against appropriate blanks (water, 2 mol/L HCI) and the carbonyl content was calculated using the molar absorption coefficient of 22 000 mol/L cm⁻¹. Lipid peroxidation was assessed by measuring tissue malondialdehyde (MDA) levels using high-performance liquid chromatography (HPLC). The MDA peak eluted at 8 min and was detected using a Waters 474 scanning fluorescence detector with excitation at 532 nm and emission at 553 nm.13

Caspase-3 assay

Caspase-3 is an enzyme activated during the induction of apoptosis. Caspase-3 activity was determined according to a method published previously.13 Briefly, 1 mL of phosphate-buffered saline (PBS) was added to ventricular or brain tissues. Tissues were homogenized and centrifuged at 10 000 g at 4°C for 10 min. The supernatant was discarded and pellets were lysed in 100 µL of ice-cold lysis buffer [50 mmol/L HEPES, pH 7.4; 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS); 1 mmol/L dithiothreitol (DTT); 0.1 mmol/L ethylenediaminetetraacetic acid (EDTA); 0.1% nonidet® P-40 (NP40)]. The assay for caspase-3 activity was carried out in a 96-well plate. Each well contained 30 µL of lystate, 70 µL of assay buffer (50 mmol/L HEPES, pH 7.4; 0.1% CHAPS; 100 mmol/L NaCl; 10 mmol/L DTT; 1 mmol/L EDTA) and 20 µL of caspase-3 colorimetric substrate Ac-DEVD-pNA (Sigma Chemicals; St. Louis, MO, USA). The 96-well plate was incubated at 37°C for 2 h, during which time caspase in the sample was allowed to cleave the chromophore pNA from the substrate molecule. Absorbance readings were obtained at 405 nm with the caspase-3 activity being directly proportional to the colorimetric reaction. Protein content was determined using the Bradford method.14

Data analysis

Data are given as mean±SEM. Differences were evaluated by analysis of variance (ANOVA) using Tukey’s test as a post hoc test. A P-value less than 0.05 was deemed statistically significant.
Effect of increasing stimulus frequency on peak shortening in myocytes from male and female WKY rats and SHR

To investigate the possible derangement of cardiac excitation–contraction coupling in WKY and SHR cardiomyocytes at higher frequencies, the electrical stimulus frequency was enhanced incrementally to 5.0 Hz (300 b.p.m.) and the steady-state peak shortening was recorded. Cells were initially stimulated to contract at 0.5 Hz for 5 min to ensure a steady state before commencing the frequency study. All recordings were normalized to the PS obtained at 0.1 Hz from the same myocyte. Figure 2a shows resting cell length with increasing stimulus frequency in WKY and SHR myocytes of both genders. Male but not female SHR cardiomyocytes exhibited a significant longer resting cell length at all frequencies tested. Figure 2b shows negative staircases in PS, with increasing stimulus frequency in all four myocyte groups tested. The threshold of significant reduction in PS (compared with PS obtained at 0.1 Hz) was between 0.5 and 1.0 Hz in the male WKY group. Interestingly, hypertension and female gender independently triggered a leftward shift in the threshold of significant reduction in PS between 0.5 and 0.7 Hz for the male SHR and female WKY groups. Somewhat surprisingly, the combination of hypertension and female gender failed to maintain this leftward shift in the threshold of PS reduction with increased stimulus frequency.

Effect of gender on oxidative stress, lipid/protein damage and apoptosis in hearts and brains

It has been speculated that oxidative stress, lipid/protein damage and apoptosis play a role in compromised cardiac and cerebral function under hypertension. Cardiomyocytes that were isolated from WKY males exhibited significantly longer duration of relengthening (TR90) when compared with the gender-matched WKY rats. Interestingly, TR90 was significantly longer in female WKY rats compared with their male counterparts (Fig. 1f). However, the organ size was not different between WKY rats of both genders, with the exception of a reduced liver size in females. Male but not female SHR cardiomyocytes exhibited a considerably larger resting cell length compared to those of the gender-matched WKY group. This was not seen in cardiomyocytes isolated from female hearts. Gender alone had little effect on resting cell length (Fig. 1a). Myocytes from SHR of both genders displayed significantly reduced ability by SHR myocytes to shorten was associated with decreased maximal velocities of shortening and relengthening (±dL/dt) in both genders. Similar to its effect on PS, gender itself did not affect ±dL/dt in WKY rats (Fig. 1b). The reduced ability by SHR myocytes to shorten was associated with decreased maximal velocities of shortening and relengthening (±dL/dt) in both genders. Similar to its effect on PS, gender itself did not affect ±dL/dt in WKY rats (Fig. 1c,d). The duration of shortening (TPS) was unaffected by either hypertension or gender (Fig. 1e).

Mechanical properties of cardiomyocytes in male and female WKY rats and SHR

Sustained hypertension often results in cardiac hypertrophy. Cardiomyocytes that were isolated from male hearts exhibited considerably larger resting cell length compared to those of the gender-matched WKY group. This was not seen in cardiomyocytes isolated from female hearts. Gender alone had little effect on resting cell length (Fig. 1a). Myocytes from SHR of both genders displayed significantly reduced ability by SHR myocytes to shorten was associated with decreased maximal velocities of shortening and relengthening (±dL/dt) in both genders. Similar to its effect on PS, gender itself did not affect ±dL/dt in WKY rats (Fig. 1c,d). The duration of shortening (TPS) was unaffected by either hypertension or gender (Fig. 1e).

Myocytes from male but not female SHR exhibited a prolonged duration of relengthening (TR90) when compared with the gender-matched WKY rats. Interestingly, TR90 was significantly longer in female WKY rats compared with their male counterparts (Fig. 1f).

**RESULTS**

**General features of Wistar-Kyoto and spontaneously hypertensive rats (SHR)**

The impacts of gender and hypertension on blood pressure, heart rate, body, liver, kidney and brain weight (as well as organ size; i.e. organ weight normalized to bodyweight) are shown in Table 1. As expected, SHR exhibited a significantly elevated systolic blood pressure; reduced bodyweight; reduced weight of liver, kidney and brain; and enlarged heart weight and size compared to their gender-matched WKY counterparts. Diastolic blood pressure, heart rate and size of the liver, kidney and brain were not different between gender-matched WKY rats and SHR. Female gender displayed significantly reduced body and organ weight as well as reduced diastolic blood pressure. However, the organ size was not different between WKY rats of both genders, with the exception of a reduced liver size in females.

<table>
<thead>
<tr>
<th></th>
<th>WKY male (n = 7)</th>
<th>SHR male (n = 8)</th>
<th>WKY female (n = 10)</th>
<th>SHR female (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bodyweight (g)</td>
<td>475.3 ± 15.8</td>
<td>369.9 ± 13.0*</td>
<td>378.3 ± 14.6†</td>
<td>231.9 ± 6.5*</td>
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<td>Heart weight (g)</td>
<td>1.57 ± 0.05</td>
<td>1.93 ± 0.06*</td>
<td>1.22 ± 0.05†</td>
<td>1.32 ± 0.11</td>
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<td>HW/BW (mg/g)</td>
<td>3.32 ± 0.15</td>
<td>5.26 ± 0.29*</td>
<td>3.24 ± 0.15</td>
<td>5.67 ± 0.39*</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>18.82 ± 1.18</td>
<td>15.57 ± 0.62*</td>
<td>11.05 ± 0.54†</td>
<td>8.21 ± 0.46†</td>
</tr>
<tr>
<td>LW/BW (mg/g)</td>
<td>39.63 ± 2.26</td>
<td>42.16 ± 1.19</td>
<td>29.56 ± 1.74†</td>
<td>35.59 ± 2.09</td>
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<tr>
<td>Kidney weight (g)</td>
<td>8.34 ± 0.37</td>
<td>9.36 ± 0.30</td>
<td>6.82 ± 0.27</td>
<td>8.30 ± 0.15</td>
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<tr>
<td>Brain weight (g)</td>
<td>2.28 ± 0.02</td>
<td>1.87 ± 0.03*</td>
<td>1.87 ± 0.04†</td>
<td>1.18 ± 0.06†</td>
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<tr>
<td>Br/BW (mg/g)</td>
<td>4.84 ± 0.18</td>
<td>5.10 ± 0.24</td>
<td>4.98 ± 0.15</td>
<td>5.07 ± 0.18</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>120.7 ± 5.8</td>
<td>157.0 ± 8.0*</td>
<td>122.1 ± 4.4</td>
<td>158.5 ± 4.1*</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>100.5 ± 3.4</td>
<td>108.6 ± 6.8</td>
<td>74.7 ± 10.8†</td>
<td>118.1 ± 7.6</td>
</tr>
<tr>
<td>Heart rate (b.p.m.)</td>
<td>421.8 ± 13.0</td>
<td>438.0 ± 21.1</td>
<td>437.0 ± 18.2</td>
<td>465.3 ± 14.1</td>
</tr>
</tbody>
</table>

Values are the mean ± SEM.

*P < 0.05 compared with respective WKY group; †P < 0.05 compared with male counterpart.

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Impact of gender on hypertensive heart function

...suggesting a high oxidative stress condition in female WKY rats. Hypertension did not significantly affect the GSH : GSSG ratio in either gender (Fig. 3b). In addition, lipid peroxidation, which was evaluated as levels of MDA, was essentially unchanged in SHR heart tissues compared with the gender-matched WKY group. However, MDA levels were significantly elevated, in a comparable manner, in the SHR brains of both genders. There was no gender difference in MDA levels in either heart or brain tissues (Fig. 4a,b). Protein carbonyl formation, which was used as an indicator of protein damage, was enhanced in male SHR hearts, but not in female SHR hearts or in the SHR brain tissues of either gender. There was no gender difference in protein carbonyl levels (Fig. 4c,d). Consistent with the protein carbonyl data, caspase-3 activity, which was used as an index for apoptosis, was significantly elevated in male SHR hearts, but not in female SHR hearts or in the SHR brain tissues of either gender. Finally, there was no gender difference in heart or brain caspase-3 activity (Fig. 4e,f).

**DISCUSSION**

Data from the study herein revealed some disparate observations between male and female SHR cardiomyocytes, heart and brain tissues. Cardiomyocytes from SHR displayed mechanical abnormalities that were somewhat similar to those reported previously for hypertrophic cardiomyopathy.\(^7,18,19\) Cardiomyocytes from both male and female SHR displayed reduced PS and ±dL/dt in a similar manner, compared with their gender-matched WKY counterparts. Intriguingly, only male but not female SHR cardiomyocytes possessed longer resting cell length, normal TPS and prolonged TR\(_{90}\), indicating a gender difference in the phenotype of cardiomyopathy.
The data herein also observed subtle but significant gender difference in cardiomyocyte contractile property under normotensive conditions; that is, prolonged duration of relengthening (TR90) in females. Although there was little indication of oxidative stress (GSH : GSSG ratio) in the hypertensive heart and brain tissues of either gender, the GSH : GSSG ratio was reduced in female WKY brains, indicating a higher oxidative stress in normotensive female brains. In addition, MDA levels were not altered in the hypertensive heart (either gender) whereas MDA levels were significantly higher in SHR brain tissues of either gender. Protein carbonyl and caspase-3 activity were both elevated in male but not female SHR hearts. Nonetheless, brain protein carbonyl and caspase-3 activity were unaffected by hypertension or gender. In summary, these data suggest that gender difference exists in hypertension-associated oxidative stress, lipid and protein damage, apoptosis in heart and brain tissues, as well as in ‘hypertrophic’ cardiomyocyte contractile dysfunction. In conjunction with the notion of prolonged TR90 duration in female WKY rat cardiomyocytes, it is not difficult to rationale that the gender difference in hypertrophic cardiomyopathy in these cardiomyocytes is simply owing to the intrinsic difference in TR90 rather than hypertension. The observation of longer resting cell length in the male SHR group depicts a possible relationship between the female gender (e.g. the female reproductive hormones oestrogen and progesterone) and the cardiac remodelling process. This finding is consistent with the notion that heart failure is less common and less severe in females probably owing to a more favourable cardiac remodelling. Several hypotheses have been postulated for gender difference in cardiac remodelling, including oestrogen, the β-adrenergic system, the renin–angiotensin system and protection against cardiomyocyte apoptosis in female hearts. This seems to be supported by protection against protein carbonyl formation and caspase-3 activation in female SHR observed in the present study. Nonetheless, data from the study herein did not favour lipid peroxidation (as indicated by MDA levels) as a contributing factor for gender difference in cardiomyocyte contractile dysfunction under hypertension. The ‘female advantage’ in cardiac remodelling should have a significant clinical implication in that it may prompt the use of a female myocardial progenitor or stem cells for tissue repair and regeneration.
engineering in ‘male’ cardiac failure, on the premise of giving better protection against myocardial apoptosis and unfavourable ventricular remodelling.20

Data from the current study did not reveal any significant gender difference in brain oxidative stress, protein damage and apoptosis under hypertension. The only notable gender difference was a reduced GSH : GSSG ratio in female WKY brains. Hypertension enhanced brain lipid peroxidation in a similar manner between genders. Collectively, these data do not favour oxidative stress, protein damage and apoptosis as likely roles in gender difference in hypertensive brain injury that is characterized by neuronal death, inflammation and altered permeability of the BBB.9,10 In fact, little evidence has been documented describing a unique gender disparity in hypertension-triggered brain injury. Up-to-date, hypertension-related gender difference in brains is largely restricted to neuroendocrine and cerebrovascular response to psychological stress. It is believed that the gender-specific brain structures and cognitive processes may be responsible for sexually dimorphic stress responses associated with the hypothalamic–pituitary–adrenal (HPA) axis.21 Finally, it is worth noting that the gender difference that exists in other organ systems may indirectly contribute to the gender disparity found in hearts and brains. For example, a gender dimorphism has been shown with regard to levels of advanced glycation endproducts, oxidative stress markers and nitric oxide synthase (NOS) in hypertensive kidneys.22 Given the essential roles of these molecules in the regulation of cardiac and cerebral structure as well as function, gender disparity observed in hearts and brains may be contributed, at least in part, by altered circulating mediators that originate from the kidney and other organ systems under hypertensive state.

In summary, findings from the present study revealed that the existence of gender difference in hypertension-induced cardiomyocyte contractile dysfunction occurs through a mechanism(s) that is possibly related to the protection of female hypertensive hearts against protein damage and apoptosis. Unlike hearts, little gender
difference has been noted in the brain tissues from WKY rats and SHR. Given what we know about the gender disparity or dimorphism in cardio- and cerebrovascular function, the in-depth mechanism and effect of sex hormones on the pathogenesis of hypertrophic cardiomyopathy and on hypertension associated with other organ damage warrants further investigation.

ACKNOWLEDGEMENTS

The author greatly appreciates the technical assistance of Dr Bruce Culver, Dr Xiaochun Zhang, Miss Jennifer M Nunn, Ms Bonnie H Zhao and Miss Lindsay Hueckstaedt from the University of Wyoming and Dr Jack Saari from Human Nutrition Research Center (Grand Forks, ND, USA).

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