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Blunting of estrogen modulation of cardiac cellular chymase/RAS activity and function in SHR

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Abstract

The relatively low efficacy of ACE-inhibitors in the treatment of heart failure in women after estrogen loss may be due to their inability to reach the intracellular sites at which angiotensin (Ang) II is generated and/or the existence of cell-specific mechanisms in which ACE is not the essential processing pathway for Ang II formation. We compared the metabolic pathway for Ang II formation in freshly isolated myocytes (CMs) and non-myocytes (NCMs) in cardiac membranes extracted from hearts of gonadal-intact and ovariectomized (OVX) adult WKY and SHR rats. Plasma Ang II levels were higher in WKY vs. SHR (strain effect: WKY: 62 ± 6 pg/mL vs. SHR: 42 ± 9 pg/mL; $P < 0.01$), independent of OVX. The enzymatic activities of chymase, ACE, and ACE2 were higher in NCMs vs. CMs, irrespective of whether assays were performed in cardiac membranes from WKY or SHR or in the presence or absence of OVX. E2 depletion increased chymase activity, but not ACE activity, in both CMs and NCMs. Moreover, cardiac myocyte chymase activity associated with diastolic function in WKYs and cardiac structure in SHRs while no relevant functional and structural relationships between the classic enzymatic pathway of Ang II formation by ACE or the counter-regulatory Ang-(1–7) forming path from Ang II via ACE2 were apparent. The significance of these novel findings is that targeted cell-specific chymase rather than ACE inhibition may have a greater benefit in the management of HF in women after menopause.

Keywords

chymase; diastolic function; estrogen; intracrine; myocyte; non-myocyte; ovariectomy; paracrine; renin angiotensin system

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Introduction

The renin angiotensin system (RAS) is important in both normal cardiovascular physiology and disease states such as hypertension, left ventricular hypertrophy, and heart failure. It is beginning to be recognized that the presence of RAS genes and products in tissues reflect local actions in which the angiotensins through paracrine and autocrine mechanisms regulate cellular function and cell-to-cell communication. The local production of angiotensins, specifically heart angiotensin II (Ang II), act on surrounding cells or exert actions on the originating cell itself in response to pressure or volume overload stress (Ferrario et al., 2016). Local Ang II generation stimulates migration, proliferation, hypertrophy, and or synthetic capacity of vascular smooth muscle cells, cardiac myocytes, and or fibroblasts (Bouzeghrane and Thibault, 2002; Kim and Iwao, 2000; Viereck et al., 2014). Cardiac Ang II can also increase production of growth factors such as TGF β and extracellular matrix proteins, such as collagen and fibronectin, ultimately affecting left ventricle (LV) structure and function (Lee et al., 1995; Rosenkranz, 2004; Schultz Jel et al., 2002; Viereck et al., 2014). Indeed, RAS activation, particularly at the tissue level seems to be the instigator of LV remodeling (Hayden et al., 2011), and is a potent mediator of oxidative stress damage in addition to exacerbating adverse effects of inflammation (Hoogwerf, 2010; Mehta and Griendling, 2007; Suzuki et al., 2003; Welch, 2008). Angiotensins act intracellularly either following internalization of peptides through a receptor mediated mechanism or generation of peptides within the intracellular compartment (De Mello and Danser, 2000; Ferrario et al., 2016; Kumar et al., 2009). RAS components have been identified in cardiac cells including fibroblasts, endothelial cell, myocytes, and macrophages, as well as on the cell membrane. Intracellular detection of gene transcripts for renin, angiotensinogen, angiotensin converting enzyme (ACE), chymase, angiotensin converting enzyme 2 (ACE2), and angiotensin type 1 (AT₁), type 2 (AT₂), and mas receptors back the presence of a functional intracrine RAS. This intracrine system is independent from the circulating RAS, as shown in the presence of ACE inhibition and AT₁R blockade (Baker and Kumar, 2006; Ferrario et al., 2005; Singh et al., 2007), and is capable of cell-contained production of Ang II, which can then interact with intracellular AT₁R to exert biological actions (Reyes et al., 2017).

Interactions between estrogens and the RAS have been implicated in having a prominent role in mediating sex differences in cardiovascular diseases. RAS activity is higher in cardiovascular tissues from males than in the same tissues from females (Fischer et al., 2002; Komukai et al., 2010; Pendergrass et al., 2008). Experimental and clinical evidence further indicates that estrogen regulates angiotensinogen, renin, tissue AT₁R and AT₂R, and aldosterone production (Dean et al., 2005; Hoshi-Fukushima et al., 2008; Macova et al., 2008; Nickenig et al., 1998; Schunkert et al., 1997; Tsuda et al., 2005). For example, in the heart, activation of the Ang II-AT₁R axis after estrogen loss can affect diastolic function by altering the relaxation properties of cardiomyocytes and by changing the composition of the extracellular matrix, specifically collagen content and type, which, in turn, reduces LV distensibility (Zhao et al., 2014). We have further shown that 17 β -estradiol treatment attenuates OVX-associated increases in cardiac Ang II and diastolic dysfunction in congenic hypertensive rats expressing the renin gene (mRen2.Lewis) (Jessup et al., 2013), in part by inhibiting cardiac mast cell chymase but not cardiac ACE expression (Wang et al., 2013).

Interestingly, in women with hypertension or heart failure, pharmacological approaches aimed at the inhibition of ACE have met with minimal success (Garg and Yusuf, 1995; Ghali et al., 2003; Shekelle et al., 2003; Wing et al., 2003). Whether increased vulnerability to LV remodeling and diastolic dysfunction after ovarian estrogen loss involves the noncanonical pathway by which chymase (produced within cardiomyocytes or brought into these cells from activated mast cells) generates Ang II and the intracellular effects of Ang II are not effectively blocked by ARBs remains unclear (Ferrario et al., 2014).

For these reasons, activation of a cardiac RAS by estrogen loss and the significance of changes with respect to cell type and individual RAS components may be important for pathophysiological aspects of age and female sex-specific cardiac disease, as well as provide the impetus for more targeted therapies, particularly in women. Therefore, the aims of the present study were to determine: 1) the influence of endogenous estrogens on the angiotensin-forming pathways in cardiac myocytes (CMs) and non-myocyte (NCMs) cell membranes from normotensive and hypertensive hearts, and 2) assess the relationships between intracellular RAS enzymatic activities and cardiac function and structure. Given that diastolic dysfunction can manifest in postmenopausal women without overt elevations in blood pressure (BP) (Kane et al., 2011; Lee et al., 2016), we used ovariectomized and sham-operated adult WKY and SHR to determine the modulatory role of estrogens on intracellular Ang II and angiotensin-1–7 (Ang-1–7) forming enzymatic activity and on gene expression of RAS-related components in cardiac cells.

Methods

Animals

Ten-week-old female SHR (n=12) and WKY (n= 12) rats were purchased from Charles River. Animals were maintained on a 12-hour light/dark cycle with ad libitum access to soy-free rat chow (Teklad Global 16% Protein Rodent Diet, Envigo, Madison, Wisconsin) and tap water, and housed in pairs. All procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the Institutional Animal Care and Use Committee of Wake Forest School of Medicine (protocol # A14-064). All possible steps were taken to avoid animal suffering at each stage of the experiment.

Experimental protocol

At 12 weeks of age, SHR and WKY rats were randomly assigned to undergo either bilateral ovariectomy (SHR-OVX: n=6; WKY-OVX n=6) or sham surgery, referred as gonadal-intact (SHR-intact: n=6; WKY-intact: n=6). Bilateral ovariectomy was performed under 2% isoflurane anesthesia, as previously described (Jessup et al., 2010). The absence of ovaries and decrease in uterine weight upon postmortem examination were used to confirm the efficacy of gonadectomy (Kim et al., 2005). Systolic blood pressure (SBP) was measured prior to surgery (experimental week 0; Fig. 1) and then weekly, using the tail-cuff method. Seven weeks after OVX or sham surgery, rats underwent transthoracic echocardiograms under anesthesia using an isoflurane 1.5%/oxygen mixture. One week later, rats were anesthetized with sodium pentobarbital (100 mg/kg, IP), and the heart was removed with the

animals under deep anesthesia. Trunk blood, obtained at the time of sacrifice, was used for the measurement of plasma angiotensinogen and Ang II levels.

Blood pressure (BP) measurement

Systolic BP was measured noninvasively using a volume-pressure recording tail-cuff method on conscious, restrained rats using the CODA 6 system (Kent Scientific Corp, Torrington, CT). Briefly, rats were gently placed in the restrainer and allowed to rest quietly for 10 min at 30°C prior to obtaining 5 acclimation cycles followed by 10 measurement cycles. Three or more systolic BP measurements were collected and averaged. All measurements were performed between 9:00 – 11:00 a.m. for all groups, to account for any diurnal variations.

Cardiac structure and function measurements

LV structure and function were determined using a commercially available echocardiograph equipped with both PureWave 12–4 MHz sector and 15–7 MHz linear transducers (CX50 CompactXtreme System; Philips Medical Systems, Amsterdam, The Netherlands) by the same investigator (LG), who was blinded to the experimental groups as previously described (Jessup et al., 2010). In brief, rats were anesthetized with an isoflurane (1.5%) oxygen mixture by nose cone and secured in the supine position to a warm (37.5°C) imaging platform. A commercially available paw sensor was applied for continuous pulse oximetry and heart rate (PhysioSuite Monitor, Kent Scientific Corp, Torrington, CT) monitoring to gauge the depth of anesthesia and to maintain the animal's safety throughout the procedure. A 12 MHz phased array transducer was used to obtain 2D-guided, LV M-mode images in the short-axis parasternal view using LV end diastolic and end systolic dimensions (LVEDD and LVESD) and posterior and anterior wall thicknesses (PWTed and AWTed) at end diastole. The apical 4-chamber view was used for transmitral inflow Doppler (early transmitral filling or maximum *E*-wave velocity) and septal tissue Doppler measurements (early mitral annular descent or *e'*) of diastolic function. Heart rate was determined from five consecutive RR intervals from pulsed-Doppler inflow tracings. The fractional shortening (FS) of the LV was expressed as $\%FS = (LVEDD - LVESD) / LVEDD \times 100$. The relative wall thickness (RWT) was calculated as $(PWTed + AWTed) / LVEDD$. Echocardiographic LV mass (LVM) was obtained by the standard cube function formula using the formula, $LVM (mg) = 1.04 \times [(LVEDD + PWT + AWT)^3 - LVEDD^3]$ where 1.04 is the specific gravity of myocardium.

Cell isolation procedure

Rats were anesthetized with pentobarbital (Akorn Inc., Lake Forest, IL) 100 mg/kg body weight by intraperitoneal injection. Upon verification of deep anesthesia by the absence of response to tail/toe pinches, the heart was quickly removed and trimmed in an ice-cold, calcium-free perfusion buffer (126 mM NaCl, 4.4 mM KCl, 1 mM MgCl₂, 4 mM NaHCO₃, 10 mM HEPES, 11 mM glucose, 30 mM 2,3-butanedione monoxime (Sigma, St. Louis, MO), 5 mM taurine, pH 7.35). The heart was then cannulated through the aorta on an Easycell System for Cardiomyocyte Isolation (Harvard Apparatus, Holliston, MA) and perfused at 37°C with calcium-free perfusion buffer at a flow rate of 4 ml/min for 5 min until the effluent became clear. The heart was switched to digestion buffer (perfusion buffer plus 50 μM CaCl₂ and 1 mg/ml collagenase II; Worthington Biochemical Corp., Lakewood, NJ) and

perfused for 20–25 min at a flow rate of 4 ml/min until the heart was pale and flaccid. The heart was pulled from the cannula and the ventricles were transferred to a 60-mm sterile dish containing 11 ml of transfer buffer (perfusion buffer plus 0.1 mM CaCl₂ and 2% bovine serum albumin; Sigma St. Louis, MO) and cut into small pieces. The minced tissue was incubated in a 37°C water bath for 10 min. The cell suspension was filtered through a 100-µm mesh cell strainer (BD Biosciences, San Jose, CA) to remove tissue debris and spun at 420 rpm at room temperature for 2 min. The supernatant was used for non-CM processing, while the pellet portion underwent further processing for CM RAS activity and for CM mRNA expression of RAS components.

Briefly, the supernatant, containing non-myocytes, was collected in a 15 mL tube and centrifuged at 1500 rpm at 4°C for 3 min. The resulting pellet of non-CMs was washed (1 mL PBS), and then transferred into a 2 mL tube for gently mixing, and centrifugation at 300 g, 4°C for 3 min. This washing, mixing, and centrifugation process was replicated once before the non-CM pellet was stored at –80°C until measurement of chymase, ACE, and ACE2 activities. One original CM pellet was washed with PBS and then divided equally into two tubes (1 mL/tube). Each tube was gently mixed and centrifuged at 300 g, 4°C for 3 min. Supernatants were discarded and the washing, mixing and centrifugation cycle was repeated once before pellets were stored at –80°C for later measurement of rat chymase 1 (CAM 1), ACE, and ACE2 membrane activities. The second CM pellet was suspended in 1mL of QIAzol (Qiagen Inc., Valencia, CA), mixed, and homogenized prior to storing at –80°C for subsequent RNA isolation and relative mRNA expression measurements.

Measurements of ACE, ACE2 and chymase enzymatic activities in myocyte and non-myocyte membranes

Cell membrane activities were determined by HPLC, as previously described (Ahmad et al., 2016). Because cardiac chymase adheres to proteoglycans within the cell membrane, a 1M NaCL buffer solution was substituted for the typical normal salt (125 mM NaCL) buffer for the determination of chymase activity. Radiolabeled (¹²⁵I) rat angiotensin-1–12 [Ang-(1–12)] was used as a substrate for the determination of chymase activity. ¹²⁵I-Ang I was employed as the substrate for ACE activity and ¹²⁵I-Ang II was used as the substrate for the measurement of ACE2 activity.

RNA isolation and RAS mRNA quantification of cardiomyocytes

Total RNA was isolated from cardiomyocytes using the RNeasy Lipit Tissue Mini Kit (Qiagen Inc, Germantown, MD) and further purified using RNeasy MinElute Cleanup Kit (Qiagen Inc, Germantown, MD) followed by quality assessment on an Agilent 2100 bioanalyzer, as previously described (Wang et al., 2016b). Relative quantification of mRNA levels by real-time qPCR for angiotensinogen, renin, ACE, ACE2, chymase, AT1R, AT2R, and Mas was performed using a SYBR Green PCR kit (Qiagen Inc, Germantown, MD), as previously described (Wang et al., 2013). Amplification and detection were performed with the ABI7500 Sequence Detection System (Applied Biosystems, Foster City, CA). Sequence-specific oligonucleotide primers were designed according to published GenBank sequences and confirmed with OligoAnalyzer 3.0. The primer sequences are listed in the Supplemental

Table. The relative target mRNA levels in each sample were normalized to GAPDH. Expression levels are reported relative to the geometric mean of the control group.

Measurement of plasma angiotensinogen and angiotensin II

Plasma was stored at -80°C for measurements of circulating angiotensinogen and Ang II by radioimmunoassay (RIA) as described previously (Ferrario et al., 2005; Ishiyama et al., 2004; Iyer et al., 1998). For the measurement of angiotensin peptides, a cocktail of protease inhibitors was used for collecting blood to prevent peptide degradation and generation (Kohara et al., 1991).

Measurement of cardiac angiotensin II

Immunohistochemistry staining of heart sections (4 µm thick) for cardiac Ang II was performed in LV tissue from OVX SHR (n = 5) and WKY (n = 4) and sham-operated (SHR-sham: n = 4; WKY-sham: n = 5) controls. Briefly, tissue sections were incubated with rabbit polyclonal antibodies against angiotensin II (1:500, Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA) overnight at 4°C, rinsed with PBS, and incubated with 1:500 diluted biotinylated secondary IgG (Vector Laboratories, Burlingame, CA) for 1 hour at room temperature. Antibody binding was detected with the Vectastain ABC Elite avidin/biotin and ImmPACT DAB peroxidase substrate kits (Vector Laboratories, Burlingame, CA). Images (200x) were taken from 5–10 random fields under a Nikon Eclipse TE300 inverted microscope (Spach Optics Inc., Pittsford, NY) and NIH ImageJ software (<https://imagej.nih.gov/ij/>) was used to quantify the immunoreactive Ang II staining in the heart.

Reagents and Antibodies

Rat Ang-(1–12) (>99% purity) was purchased from GenScript USA Inc. (Piscataway, NJ). Radioactive ¹²⁵I was purchased from PerkinElmer Life and Analytical Sciences, Inc. (Waltham, Massachusetts). All other chemicals used in this study were of analytical grade and were obtained from Sigma (St. Louis, MO) and Fisher Scientific (Atlanta, GA).

Data analysis

All values are expressed as mean ± SEM. Two-way ANOVA was used to determine the effect of strain (SHR vs. WKY) and estrogen status (OVX vs. intact). A two-way repeated measures ANOVA was used to determine the effect of time and estrogen status on systolic blood and post-hoc comparisons were evaluated using Bonferroni's test of multiple comparisons. Simple linear regression analysis was used to determine the relationships between RAS enzymatic activities within the membranes from CMs and non-CMs and echocardiographic-derived indices of LV mass and filling pressures. Data were analyzed using the software GraphPad Prism Version 6 (GraphPad Software, Inc., La Jolla, CA). A p < 0.05 was considered statistically significant.

Results

Estrogen loss led to hypertrophic changes in both strains and worsening diastolic function in WKYs

As previously reported (Maris et al., 2005), systolic BP was significantly higher in awake SHR compared with WKYs (Fig. 1). Echocardiographic assessment of LV dimensions and contractility documented a slight increase in LVEDD in WKY but no difference in % FS between strains (Table 1).

Ovariectomy induced increases in relative wall thickness and LV mass in both WKY and SHR. Transmitral early filling velocity was not affected by strain or estrogen status, while a decrease in early mitral annular velocity, or e' , was found in WKYs, particularly with OVX. Correspondingly, increases in E/e' were observed specifically in WKYs following estrogen loss, indicative of worsening diastolic function (Table 1). Altogether, the hemodynamic and echocardiographic alterations induced by OVX demonstrate a more marked effect of estrogen loss in cardiac function worsening in normotensive WKY rats, an effect that was present in the absence of afterload changes.

Effect of Estrogen Loss on Renin Angiotensin System Components

Figure 2 shows that compared to WKY, hypertension in SHR is associated with lower plasma angiotensinogen and Ang II levels and that removal of the ovaries causes a small but significant increase in plasma angiotensinogen only in WKY. Whole tissue cardiac Ang II levels, as determined by immunohistochemistry, were significantly increased after estrogen loss ($F(1, 14) = 10.30$, $P = 0.006$), particularly in WKYs, despite no overall strain difference (Figure 3).

Insights into the effects of estrogen loss on cardiac myocyte-specific RAS components are documented in Table 2 and Fig. 4. In general, gene transcript values for AGT, chymase, ACE, ACE2, AT₁ and mas receptors are significantly lower in SHR as compared with WKY rats. While OVX has no effect on RAS mRNA levels in SHR, it leads to robust increases in chymase (Fig. 4), ACE2, AT₁, and renin gene expression in WKY cardiomyocytes.

Because previous work performed in hearts from male rats suggested a differential expression of angiotensins and Ang II-forming enzymes in cardiac myocytes (CM) compared to non-cardiac myocytes (NCM) (unpublished data and Fu L., et al. 2016), we evaluated these two cardiac cellular components in the absence and in the presence of OVX. In general, NCM enzymatic activities are several-fold higher than the corresponding activities in CMs (Fig. 5). Chymase activity was nearly 40-fold higher in NCMs compared to CMs, and for the NCMs, activities were highest in cells from WKY vs. SHR and OVX vs. intact rats ($P < 0.05$ strain and E2 effects, respectively). In CMs, chymase activity increased in both SHR and WKY after OVX (Estrogen effect: $P < 0.05$) (Fig. 5A). Neither strain nor gonad status influenced ACE activity within membranes of NCMs and CMs (Fig. 5B). ACE2 activity was higher in CM membranes from WKY compared to SHR ($P < 0.05$ strain effect), irrespective of OVX, while in NCMs, strain accounted for about 16% of the total variance (strain effect: $P = 0.07$) (Fig. 5C). In addition, cardiac chymase activity in CMs correlated with increases in E/e' in WKY females ($r = 0.719$, $P = 0.019$) whereas in SHR,

it was linked to hypertrophic changes, defined by increases LV mass ($r = 0.640$, $P = 0.046$ (Fig. 6).

Discussion

As an extension of our recent work pointing to the pathophysiological significance of an alternative Ang II-forming pathway within the estrogen deficient female heart (Wang et al., 2013), we now show that: 1) the enzymatic activities of chymase, ACE, and ACE2 are much higher in NCMs irrespective of whether the assays are performed in cardiac membranes from WKY or SHR or in the presence or absence of surgically-induced menopause; and 2) estrogen deficiency increases chymase activity, but not ACE activity, in both CM and NCM membranes. Moreover, cardiac myocyte chymase activity associates with diastolic function in WKYs and LV structure in SHRs while no relevant cardiac functional and structural relationships between the classic enzymatic pathway of Ang II formation by ACE or the counter-regulatory Ang-(1–7) forming path from Ang II via ACE2 are apparent. In parallel with the heightened Ang II-forming chymase activity in CM of WKY, increases in chymase mRNA expression levels, particularly after estrogen loss, exist.

While chymase mediates the majority of Ang II production in the rodent and human heart (Ahmad et al., 2016; Ferrario et al., 2014), the process appears to be highly compartmentalized, involving extracellular, intravascular and intracellular locations (Dell'Italia et al., 1997; Urata et al., 1993). Chymase is an intracellular enzyme found in mast cells, endothelial cells, cardiac fibroblasts, and mesenchymal interstitial cells of the heart (Fu et al., 2016; Urata et al., 1993) that is responsive to stretch, ischemia reperfusion injury, and pressure overload (Jin et al., 2001; Li et al., 2015; Pat et al., 2010). Chymase activity has also been identified in neonatal cardiac myocytes (Ahmad et al., 2011). Chymase inhibition by chymostatin prevented the formation of intracellular Ang II formation in cultured rat myocytes exposed to high glucose (Singh et al., 2007). Moreover, intracellular administration of chymostatin also abolished the effect of intracellular angiotensin-(1–12) [Ang-(1–12)] via Ang II on cardiomyocyte electrical properties, and specifically, potassium currents (De Mello et al., 2016). Our findings of heightened Ang II production from Ang-(1–12) in myocyte membranes of WKY hearts in response to estrogen deficiency further confirm the potential for a functional intracellular chymase. Activation of an intracellular Ang-(1–12)/Ang II/chymase system can have important physiologic consequences given that worsening diastolic function in WKYs and increasing LV mass in SHRs were directly related to increasing myocyte chymase activity in this study.

What remains unanswered is whether chymase, released from mast cells or other non-myocyte cells, is taken up by myocytes or directly synthesized within these cells to promote the intracellular formation of Ang II. Our finding of a significant increase in myocyte chymase mRNA expression following OVX, particularly in isolated myocytes from WKYs, favors intracellular synthesis of chymase. In addition, CMs and cardiac fibroblasts (CF) are spatially intermingled in the myocardium, with virtually every CM bordering one or more CF (Kohl et al., 2005), making CM-to-CF crosstalk a possibility (Matsusaka et al., 1999; Takeda and Manabe, 2011; Viereck et al., 2014; Zhang et al., 2012)). Recent work from Fu et al. (Fu et al., 2016) demonstrates that fibroblasts are an abundant source of chymase in the

heart, particularly during stress states of volume overload. Moreover, Ang II-induced exosome release from CFs has recently been shown to serve as a crucial paracrine mechanism by which Ang II intensifies its own signaling in cardiomyocytes (Lyu et al., 2015).

To understand the contribution of local RAS to phenotypic changes in the heart following estrogen loss, it is important to eliminate estrogenic effects that might influence the development of hypertension. Indeed, hypertension is a major risk for diastolic dysfunction and left ventricular hypertrophy and it is a common correlate to heart failure with preserved ejection (Kane et al., 2011; Lalande and Johnson, 2008), particularly among aging women (Katholi and Couri, 2011; Redfield et al., 2005). Importantly, and consistent with others, BPs over the course of the study in both strains were not affected by OVX (Martin et al., 2008; Reckelhoff, 2001). Consistent with our previous study, worsening of diastolic function, defined by reductions in e' and increases E/e' , was observed in WKY rats while LV hypertrophy (Wang et al., 2016a), expressed by increases in LV mass, was apparent in both rat strains after OVX. Taken together, it is unlikely that the intracellular RAS and diastolic function were influenced by BP.

Our findings support the presence of an intracellular chymase-mediated Ang II-forming system that is responsive to estrogens. Despite a lack of studies on the modulatory role of estrogens on Ang II formation within cardiac cells, our findings agree with experimental studies involving the whole heart. Specifically, the linkage between increased cardiac chymase expression and activity to cardiac Ang II in ovariectomized rats was mitigated by estrogen replacement (Wang et al., 2013; Zhao et al., 2014). Relating to this concept, we showed that chronic activation of the novel estrogen receptor GPER by its agonist G1 limited the adverse effects of estrogen loss on cardiac structure and function in hypertensive OVX mRen2 rats (Wang et al., 2012), in a manner presumed to be partially due to anti-proliferative actions of GPER on cardiac mast cells (Zhao et al., 2015). Although an association with the cardiac RAS was not determined, Li et al. also showed that estrogen inhibited mast cell chymase release to prevent pressure overload-induced adverse cardiac remodeling by TAC in ovariectomized rats (Li et al., 2015).

Hemodynamic overload induces cardiac hypertrophy, in part by activating the local RAS (Baker, et al., 1992; Dostal and Baker, 1999), which include intracellular RAS in both CMs and NCMs. The present study shows chymase activity in NCMs is much higher than that in CMs, suggesting that NCMs might have more important roles in the development of cardiac hypertrophy and remodeling than CMs. In support of this notion, *in vitro* findings showed that exogenously administered Ang II to adult CMs required co-culture with a small amount of NCMs in order to stimulate myocyte growth and elevations in atrial natriuretic peptide and brain natriuretic peptide expression (Harada et al., 1997; Kim et al., 1995; Gray et al., 1998). Also, NCMs can induce myocyte hypertrophy even in the absence of Ang II (Harada, et al., 1997; Nishida, et al., 1993) or biomechanical stress *in vitro* (van Wamel, et al., 2001; van Wamel et al., 2002), suggesting that autocrine/paracrine factors produced by NCMs might have a key role in the connection between cardiac cells. While the mechanisms of a cell-to-cell connection of cardiac chymase was not defined in this study, our findings extend

the physiological relevance of including NCMs in future studies focused on roles of local RAS in the development of female sex-specific cardiac pathology.

Limitations

Even though this is the first study comparing the metabolic pathway for Ang II formation in freshly isolated CMs and NCMs in cardiac plasma membranes extracted from hearts of gonadal-intact and ovariectomized hypertensive and normotensive rats, it remains to be determined whether the same differential results may be obtained in older animals. On the other hand, the clinical significance of the current findings is underscored by recent reports documenting that early onset menopause is, indeed, linked to incident HF later in life (Appiah D, et al., 2016; Ebong IA, et al., 2014). In addition, there is a strong body of evidence implicating a significant contribution of local tissue-derived cardiac and vascular RAS in age-related remodeling (Strait and Lakatta, 2012). To understand the full extent of intracellular chymase/Ang II in the development of female specific heart disease, additional studies are needed that focus on the effects of estrogen loss on intracellular RAS metabolism in young, middle, and old-aged animals.

We further show indirect evidence that besides extracellular chymase from mast cells (Wang et al., 2013), chymase internalized in both CMs and NCMs after degranulation from mast cells might also play a role in intracellular Ang II production and the pathogenesis of diastolic dysfunction. To provide direct evidence for the link between intracellular chymase in cardiac cells and the development of diastolic dysfunction, the generation of cell-type specific chymase knockout mice, e.g., cardiomyocyte specific chymase knockout, might be needed. Finally, intracellular levels of Ang II in CMs and NCMs were not determined in this study due to a limited amount of sample from the freshly isolated cells and the lack of a highly sensitive assay procedure for detecting Ang II concentrations in cells. Nonetheless, in previous work using the mRen2.Lewis hypertensive rat, we showed increased cardiac Ang II levels after estrogen loss and this was directly related to cardiac chymase expression (Wang et al., 2013). Moreover, Ribeiro et al. (Ribeiro et al., 2012) reported that AT1 receptor blockade prevented ovariectomy-induced myocardial contractile dysfunction in normotensive Wistar rats. The increased cardiac Ang II immunoreactivity found in LV tissue of OVX vs. gonadal-intact WKY and SHR rats suggest that intracellular Ang II production from chymase, within cardiomyocytes, is likely partially involved in the anatomic and functional derangements of the LV after estrogen loss. Even so, a more specific cardiomyocyte chymase knockdown model will be needed in future studies to determine cause/effect of this phenomenon.

In summary, a well-established experimental model of genetic hypertension reveals that estrogen has more of a tonic role on cardiac structure modulation than function that is superimposed on a differential expression of cardiac tissue RAS gene transcripts and blood levels of AGT and Ang II when compared to normotensive WKY rats. While estrogen depletion causes no changes in BP in either strain it does have an important influence on cardiac mechanics expressed through increases in cardiac mass and worsening of filling pressures, particularly in the WKY strain. Once again, the impact of estrogen removal on diastolic function appears to be more significant in the normotensive rat, theoretically

explained by the possibility that the long-standing hypertrophy in SHR allows their overloaded ventricles to be better prepared to face impending stress. Affirming previous conclusions, OVX causes marked changes in biomarkers of cardiac tissue RAS activity that are neither similar directionally or magnitude-wise comparable in both rat strains. Gene transcript changes due to OVX in SHR for chymase, ACE, ACE2, AGT, AT₁ and AT₂ receptors are very modest in magnitude and directionally the opposite of those demonstrated in WKY rats. These significant differences suggest, but obviously do not prove, that estrogen may have a remarkable non-genomic activity in nuclear mechanisms associated with or accounting for transcriptional events. The rendering of this interpretation at the level of CM versus NCM demonstrates, however, that these transcriptional differences are limited to chymase, and even more specifically to the chymase activities of both CM and NCM membranes obtained from SHR and WKY rats. OVX has no effect on ACE and ACE2 activities in the CM and NCM of both strains. While uncoupling of gene transcripts and enzymatic activity has been reported in many other experimental situations unrelated to these studies, we are the first to document these changes in membranes of cardiac contractile and non-contractile components. Moreover, the observation that chymase activity correlates with worsening of diastolic function buttresses the importance of chymase rather than ACE as a critical tissue Ang II forming enzyme and its role in modulating cardiac function. That the observations gathered in this study pave the road for a further understanding of the cardioprotective actions of estrogen adds weight to an urgent need to address how sex-related impact on cellular physiology may drastically affect the effectiveness of current therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Weekly tail-cuff systolic blood pressure in conscious, intact (sham-operated) and OVX female WKY and SHR. SHR had significantly higher blood pressures compared to WKY over the 6-week study period (strain effect: $P < 0.0001$) without effects from OVX. Values are expressed as mean ($\pm$ SEM); $N=6$ per group. OVX, ovariectomy.

Figure 2.

Plasma angiotensinogen (AGT) and plasma angiotensin II (Ang II) in SHR and WKY female rats. Data indicate differences in (A) plasma AGT and (B) plasma Ang II with respect to estrogen status (intact versus OVX). Values are reported as mean $\pm$ SEM; SHR, $N=6$ per group. WKY rats, $N=6$ per group. Black bars represent gonadal intact rats; hatched bars represent OVX rats. SHR rats had lower AGT and Ang II levels compared with WKY, and OVX increased plasma AGT in WKYs only (2-way ANOVA). #### $P<0.0001$; ## $P<0.01$ (strain effect); * $P<0.05$ (estrogen $\times$ strain effect); ANOVA = analysis of variance; SEM = standard error of the mean.

Fig 3 A

Figure 3.

Cardiac Ang II levels in the left ventricles of sham-operated and OVX SHR and WKY rats. (A) Representative images showing Ang II staining in the left ventricles. (B) Cardiac Ang II intensity quantified by ImageJ. Black bars represent gonadal intact rats; hatched bars represent OVX rats. Data indicate that estrogen loss significantly increased Ang II levels in LV tissue, despite no overall strain difference (2-way ANOVA). Values are reported as mean $\pm$ SEM; SHR-intact: $N=4$, SHR-OVX: $N=5$, WKY-intact: $N=5$; WKY-OVX: $N=4$. $**P < 0.01$ (estrogen effect); ANOVA = analysis of variance; SEM = standard error of the mean.

Figure 4.

(A) Chymase mRNA expression levels in cardiomyocytes of SHR and WKY female rats. (B) PCR product for CM chymase on agarose gel electrophoresis. Data indicate SHR rats had lower chymase mRNA compared with WKY, and OVX increased gene expression in WKYs only (2-way ANOVA). (B) A single band for chymase and no band (Negative) for RNA sample without reverse transcription, confirming specificity for chymase. Values are reported as mean $\pm$ SEM; SHR, $N=6$ per group. WKY rats, $N=6$ per group. Black bars represent gonadal intact rats; hatched bars represent OVX rats. ### $P < 0.001$ (strain effect); ** $P < 0.01$ (estrogen $\times$ strain effect); ANOVA = analysis of variance; SEM = standard error of the mean.

Figure 5.

Angiotensin-forming enzyme activity in cardiomyocyte (CM) and non-cardiomyocyte (NCM) from SHR and WKY female rats. Data indicate differences in (A) chymase activity in CMs (left) and NCMs (right) with respect to estrogen status (intact versus OVX); in NCMs, WKY chymase activity exceeds that of SHR (strain effect). (B) ACE activity is not different between strains nor is it affected by estrogen status. (C) ACE2 activity in CMs is higher in WKY compared to SHR, irrespective of estrogen status; NCMs showed no estrogen or strain effects (2-way ANOVA). Values are reported as mean $\pm$ SEM; SHR, $N=6$ per group. WKY rats, $N=6$ per group. Black bars represent gonadal intact rats; hatched bars represent OVX rats. ## $P < 0.01$ (strain effect); * $P < 0.05$, ** $P < 0.01$ (estrogen effect); ANOVA = analysis of variance; SEM = standard error of the mean.

A Cardiomyocytes - WKY

B Cardiomyocytes - SHR

Figure 6.

Relationship between cardiac myocyte chymase activity and diastolic function, defined by E/e', and left ventricular hypertrophy, defined by LV mass. Linear relationships (solid line) and 95% confidence intervals (dotted line) between (A) CM chymase activity and E/e' in WKY rats, and (B) CM chymase activity and LV mass in SHRs. Worsening diastolic function, indicated by elevations in E/e', was related to chymase activity (top) in WKYs, while in SHRs, it was related to LV mass.

Table 1

Echocardiographic indices of LV morphology and function

	SHR Mean $\pm$ SEM	WKY Mean $\pm$ SEM	Strain P-value	E2 P-value	Strain $\times$ E2 P-value
Left Ventricular Dimensions					
LVEDD (cm)					
Intact	0.613 $\pm$ 0.016	0.656 $\pm$ 0.018	0.045	0.422	0.777
OVX	0.625 $\pm$ 0.012	0.682 $\pm$ 0.037			
LVESD (cm)					
Intact	0.348 $\pm$ 0.018	0.352 $\pm$ 0.028	0.465	0.787	0.58
OVX	0.330 $\pm$ 0.015	0.358 $\pm$ 0.026			
PWTed (cm)					
Intact	0.171 $\pm$ 0.006	0.161 $\pm$ 0.014	0.688	0.0008	0.544
OVX	0.202 $\pm$ 0.001	0.206 $\pm$ 0.008			
AWTcd (cm)					
Intact	0.165 $\pm$ 0.006	0.164 $\pm$ 0.008	0.799	0.0001	0.718
OVX	0.206 $\pm$ 0.008	0.211 $\pm$ 0.010			
RWT					
Intact	0.553 $\pm$ 0.025	0.492 $\pm$ 0.039	0.2	0.010	0.754
OVX	0.648 $\pm$ 0.041	0.610 $\pm$ 0.038			
LV Mass					
Intact	1.075 $\pm$ 0.061	1.058 $\pm$ 0.059	0.374	0.009	0.264
OVX	1.201 $\pm$ 0.020	1.349 $\pm$ 0.110			
Left Ventricular Function and Heart Rate					
FS%					
Intact	43 $\pm$ 2	47 $\pm$ 2	0.481	0.244	0.619
OVX	46 $\pm$ 4	48 $\pm$ 1			

	SHR Mean $\pm$ SEM	WKY Mean $\pm$ SEM	Strain P-value	E2 P-value	Strain $\times$ E2 P-value
E _{max} (cm/s)					
Intact	104 $\pm$ 2	110 $\pm$ 5	0.5	0.085	0.432
OVX	96 $\pm$ 4	110 $\pm$ 8			
e' (cm/s)					
Intact	5.1 $\pm$ 0.2	4.7 $\pm$ 0.3	0.001	0.071	0.046
OVX	5.1 $\pm$ 0.05	3.9 $\pm$ 0.1			
E/e'					
Intact	20.6 $\pm$ 0.7	21.1 $\pm$ 0.7	0.003	0.001	0.007
OVX	21.4 $\pm$ 0.8	27.9 $\pm$ 1.4			
Heart rate (bpm)					
Intact	360 $\pm$ 8	370 $\pm$ 14	0.033	0.434	0.09
OVX	356 $\pm$ 8	330 $\pm$ 7			

SHR, spontaneously hypertensive rats; WKY, Wistar Kyoto rats; OVX, ovariectomy; LV, left ventricular; LVEDD, left ventricular end diastolic dimension; LVESD, left ventricular end systolic dimension; PW/Ted, posterior wall thickness end diastole; AW/Ted, anterior wall thickness end diastole; RWT, relative wall thickness; LV mass, left ventricular mass; FS, fractional shortening; E_{max}, Early transmitral filling velocity; e', early mitral annular velocity; E/e', Early transmitral filling-to-mitral annular velocity ratio. The groups consisted of intact-SHR (N=6), OVX-SHR (N=6), intact-WKY (N=6), OVX-WKY (N=6). Using a two-way ANOVA, significant differences in structure and function, with respect to strain, estrogen (E2) status (intact vs. OVX), and strain $\times$ E2 status, were determined. P-values are presented on the right of the table. For convenience, significant differences are indicated in bold.

Table 2

RAS-related gene expression levels in cardiomyocytes

Genes	SHR Mean $\pm$ SEM	WKY Mean $\pm$ SEM	Strain P-value	E2 P-value	Strain $\times$ E2 P-value
Chymase					
Intact	0.41 $\pm$ 0.05	0.92 $\pm$ 0.06	0.0001	0.001	0.0004
OVX	0.35 $\pm$ 0.04	2.04 $\pm$ 0.23			
ACE					
Intact	1.11 $\pm$ 0.10	1.85 $\pm$ 0.26	0.0001	0.965	0.165
OVX	0.80 $\pm$ 0.07	2.18 $\pm$ 0.33			
ACE2					
Intact	1.21 $\pm$ 0.21	1.32 $\pm$ 0.15	0.0120	0.203	0.030
OVX	0.94 $\pm$ 0.11	2.28 $\pm$ 0.39			
Angiotensinogen					
Intact	1.04 $\pm$ 0.06	2.06 $\pm$ 0.35	0.0001	0.346	0.43
OVX	0.71 $\pm$ 0.05	2.03 $\pm$ 0.20			
AT ₁ R					
Intact	1.14 $\pm$ 0.10	1.34 $\pm$ 0.18	0.004	0.099	0.020
OVX	0.92 $\pm$ 0.10	2.56 $\pm$ 0.57			
Renin					
Intact	1.70 $\pm$ 0.19	1.49 $\pm$ 0.16	0.419	0.133	0.039
OVX	1.60 $\pm$ 0.99	2.04 $\pm$ 0.11			
Mas receptor					
Intact	0.92 $\pm$ 0.05	1.70 $\pm$ 0.25	0.0001	0.696	0.268
OVX	0.81 $\pm$ 0.08	1.93 $\pm$ 0.20			

SHR, spontaneously hypertensive rats; WKY, Wistar Kyoto rats; ovariectomy, OVX; ACE, angiotensin converting enzyme; ACE2, angiotensin converting enzyme 2; AT₁R, angiotensin receptor 1. Gene transcripts were measured in SHR and WKY cardiomyocytes that were extracted from hearts 8 weeks after OVX or sham procedures (gonads intact). The groups consisted of intact-SHR (N=6), OVX-SHR

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(N=6), intact-WKY (N=6), OVX-WKY (N=6). Using a two-way ANOVA, significant differences in mRNA expression levels, with respect to strain, estrogen (E2) status (intact vs. OVX), and strain × E2 status, were determined. P-values are presented on the right of the table. For convenience, significant differences are indicated in bold.