Role of estrogen in diastolic dysfunction

Zhao Z, Wang H, Jessup JA, Lindsey SH, Chappell MC, Groban L. Role of estrogen in diastolic dysfunction. Am J Physiol Heart Circ Physiol 306: H628–H640, 2014. First published January 10, 2014; doi:10.1152/ajpheart.00859.2013.—The prevalence of left ventricular diastolic dysfunction (LVDD) sharply increases in women after menopause and may lead to heart failure. While evidence suggests that estrogens protect the premenopausal heart from hypertension and ventricular remodeling, the specific mechanisms involved remain elusive. Moreover, whether there is a protective role of estrogens against cardiovascular disease, and specifically LVDD, continues to be controversial. Clinical and basic science have implicated activation of the renin-angiotensin-aldosterone system (RAAS), linked to the loss of ovarian estrogens, in the pathogenesis of postmenopausal diastolic dysfunction. As a consequence of increased tissue ANG II and low estrogen, a maladaptive nitric oxide synthase (NOS) system produces ROS that contribute to female sex-specific hypertensive heart disease. Recent insights from rodent models that mimic the cardiac phenotype of an estrogen-insufficient or -deficient woman (e.g., premature ovarian failure or postmenopausal), including the ovariectomized congenic mRen2.Lewis female rat, provide evidence showing that estrogen modulates the tissue RAAS and NOS system and related intracellular signaling pathways, in part via the membrane G protein-coupled receptor 30 (GPR30; also called G protein-coupled estrogen receptor 1). Complementing the cardiovascular research in this field, the echocardiographic correlates of LVDD as well as inherent limitations to its use in preclinical rodent studies will be briefly presented. Understanding the roles of estrogen and GPR30, their interactions with the local RAAS and NOS system, and the relationship of each of these to LVDD is necessary to identify new therapeutic targets and alternative treatments for diastolic heart failure that achieve the cardiovascular benefits of estrogen replacement without its side effects and contraindications.

diastolic dysfunction; Doppler echocardiography; estrogen; G protein-coupled receptor 30; mRen2; sex differences

Heart failure (HF) is a serious, potentially life-threatening condition that affects 5.3 million Americans and is one of the leading hospital discharge diagnoses (1, 72, 142). The prevalence of HF increases with age for both sexes, and, given the aging population, the number of patients with HF is expected to steadily increase. Approximately 2.5 million women in the United States have HF (85), and HF accounts for one-third of all disease-related mortality in American women (11). Nevertheless, HF in women remains a poorly understood syndrome and has not received the same level of public awareness as coronary heart disease (CHD). Although women with HF survive longer than men with HF, they remain symptomatic [e.g., have shortness of breath and difficulty exercising (30), have a lower quality of life (31, 35, 52), and have a significantly higher annual percent increase in hospitalization rates (30, 67, 103, 117, 135)] compared with men with HF. Understanding the sex-based differences in HF will have significant clinical implications, informing both risk factor screening and the development of effective interventions for this vulnerable yet expanding population.
Cardiac dysfunction, with or without systolic dysfunction, is associated with left ventricular (LV) diastolic dysfunction (LVDD). Diastolic dysfunction refers to mechanical and functional abnormalities present during relaxation and filling of the ventricle. It is a preclinical state in which the heart adapts to changes that cause abnormal relaxation or increased LV stiffness by increasing left atrial pressure so that the LV continues to be loaded with the appropriate volume for contraction. Patients with asymptomatic LVDD are included in American College of Cardiology/American Heart Association guidelines as having either stage A or B preclinical HF (66). With LVDD, any abnormal increase in diastolic filling pressure corresponds to a less distensible or less compliant ventricle during the filling phase of the cardiac cycle. Consequently, a “stiff” ventricle is less able to increase its stroke volume without a further elevation of left atrial pressure. Pressure reflected backward through the open mitral valve into the atrium and pulmonary veins can cause shortness of breath, elevation of pulmonary venous pressure, and decreased exercise capacity (5, 73). Thus, the distinction between diastolic HF or HF with preserved ejection fraction (HFpEF) and LVDD is merely the presence of congestive heart failure symptoms. One in six patients with asymptomatic LVDD will develop overt HF symptoms within 5 yr of diagnosis (4, 5).

LVDD, as the precursor to diastolic HF, is seen in both men and women, but it is more prevalent in postmenopausal women, suggesting a link between LVDD and estrogen deficiency. The marked increase in HF incidence in women after 55 yr of age also supports the idea that estrogen confers a protective effect that is lost after menopause (103, 114). However, negative results in clinical trials of hormone replacement therapy (HRT) have led to the speculation that the late initiation of estrogen replacement might not reverse cardiovascular damage or prevent further disease progression (60, 118). While recently completed and ongoing clinical trials continue to explore the “timing hypothesis” (see Ref. 48; http://clinicaltrials.gov/ct2/show/NCT00114517)–that estrogen replacement might have different cardiovascular protective capacity depending on how soon it is given after menopause–reverse translational research is critically important to understand the mechanistic actions of estrogen that are relevant to the maintenance of cardiac diastolic function and structure.

Overwhelming evidence from clinical and basic research has implicated an activated circulating and tissue renin-angiotensin-aldosterone system (RAAS) in the pathogenesis of diastolic dysfunction that occurs after the loss of ovarian estrogens (3, 162). Furthermore, chronic activation of the RAAS increases oxidative stress and reduces nitric oxide (NO) bioavailability in estrogen-sensitive tissues, leading to endothelial dysfunction (164), inflammation (74), and immune dysfunction (43). Each of these processes is associated with obesity, diabetes, renal disease, and hypertension, diseases characterized by reductions in myocardial relaxation and LV compliance. To investigate the relationships between estrogen loss, an activated RAAS, and/or a reduced or maladaptive local cardiac NO synthase (NOS) system in the pathogenesis of diastolic dysfunction, we have used the congenic mRen2.Lewis rat, a monogenetic hypertensive model that overexpresses the mouse renin gene. The female mRen2.Lewis rat is particularly well suited for mechanistic studies of diastolic dysfunction because early bilateral ovariectomy (OVX) in the mRen2.Lewis rat consistently exacerbates increases in systolic blood pressure, LV remodeling (including collagen deposition and myocyte hypertrophy), and diastolic functional impairment (14–16, 41, 63–65, 149, 150), key features emulating the cardiac phenotype of an estrogen-insufficient or -deficient woman (e.g., premature ovarian failure or postmenopausal). Using this model, we have shown that low-dose estrogen replacement (17β-estradiol) or activation of the novel membrane estrogen receptor (ER) G protein-coupled receptor 30 [GPR30; also called G protein-coupled receptor 30 (GPER)1], with its specific agonist BH2, dihydrobipterin, exacerbates increases in systolic blood pressure, LV remodeling and diastolic dysfunction (including collagen deposition and myocyte hypertrophy), and diastolic functional impairment (14–16, 41, 63–65, 149, 150), key features emulating the cardiac phenotype of an estrogen-insufficient or -deficient woman (e.g., premature ovarian failure or postmenopausal). Using this model, we have shown that low-dose estrogen replacement (17β-estradiol) or activation of the novel membrane estrogen receptor (ER) G protein-coupled receptor 30 [GPR30; also called G protein-coupled estrogen receptor (GPER)1], with its specific agonist G-1, limits the adverse effects of ovarian hormone loss on blood pressure (81), diastolic function, and cardiac fibrosis, even in the absence of overt alterations in blood pressure (149), in part through deactivation of the circulating and tissue RAAS (150). Additionally, ovarian estrogen loss in this rat model increases renal and cardiac neuronal NOS (nNOS), which exacerbates the effects of salt on renal damage and proteinuria.
(159, 161), impairs myocardial relaxation, and increases perivascular fibrosis (63); these effects were reversed by treatment with a specific nNOS inhibitor, L-VNIO. Figure 1 shows the potential mechanisms by which estrogen-sensitive hypertension and/or an overactive RAAS modulate cardiac NOS, leading to the formation of ROS and subsequent diastolic dysfunction and LV remodeling.

In this review, we briefly discuss the physiological and pathophysiological determinants and Doppler echocardiographic correlates of LVDD relevant to preclinical models of sex-specific hypertensive heart disease and describe recent advances in our understanding of the roles of estrogen loss in the development of diastolic dysfunction and LV remodeling, focusing on the contributions of the cardiac RAAS and NOS system.

Physiological, Pathophysiological, and Echocardiographic Correlates of Diastolic Dysfunction

Hypertension is the primary risk factor for diastolic dysfunction and is the leading cause of diastolic HF in postmenopausal women (56, 122). In the general population, hypertension affects ~15% of women and 20% of men; however, the prevalence of hypertension increases sharply after menopause, and more women than men become hypertensive as they age (49, 139). Moreover, steep increases in systolic blood pressure and pulse pressure occur after menopause, which have important implications for the maintenance of the diastolic and systolic reserve in the elderly (18, 71, 113). Hypertension in postmenopausal women also results in LV hypertrophy (LVH). LV hypertrophy is a major causative factor in reduced myocardial relaxation and diastolic compliance, which are key cardiac components of diastolic dysfunction. In addition to hypertension, obesity and obesity-related diseases, including insulin resistance and type II diabetes, are associated with the development of LVDD in women (2, 27, 109, 110). Obesity is also one of the strongest risk factors for HFPEF (39, 46, 102).

Recent experimental and clinical evidence also suggest an intriguing link between the kidney and heart that should be considered in the development of LVDD in postmenopausal women. Data from healthy community dwellers show that mild chronic renal insufficiency is a risk factor for diastolic dysfunction (24, 146). Also, patients with diastolic dysfunction who develop clinically significant HF are likely to have renal insufficiency, irrespective of their age, sex, and comorbid conditions (145, 146). Taken together with the risk excess for cardiovascular events that has been demonstrated among postmenopausal women with mild renal dysfunction (e.g., calculated glomerular filtration rate < 70 ml/min) (100, 108), any indication of chronic kidney disease should activate practitioners to screen for early changes in cardiac structure and diastolic function in this population. Although the precise mechanisms underlying the cardiorenal connection to female sex-specific LVDD are not known, findings from the uninephrectomized male rat model suggest that mild renal insufficiency may mediate early cardiac apoptosis and fibrosis (91).

Diastolic dysfunction is associated with abnormalities of active relaxation and passive stiffness of the LV. Active relaxation represents the speed of transition from the contracted state, or systole, to the relaxed state, or diastole, and is related to the reuptake of Ca²⁺ into the sarcoplasmic reticulum of the contracted myocyte. Passive stiffness is primarily linked to myocardial compliance and the effects of tissue fibrosis. With the advent of Doppler echocardiography, assessment of diastolic function has become increasingly more practical in both the clinical and reverse translational cardiovascular research arenas (112, 124). Several of the more commonly used Doppler echocardiographic parameters of diastolic dysfunction for basic research are shown in Table 1. However, it is important to understand the limitations of this noninvasive tool (92), which primarily reflect on the dynamic nature of the ever-changing diastolic function. For instance, Doppler transmirtal velocity early deceleration time and tissue Doppler early diastolic mitral annular velocity (e') are frequently used measures of myocardial relaxation. Early deceleration time changes with loading conditions, whereas e' is relatively load independent. Similarly, the transmitral early-to-late filling ratio (E/A) fluctuates with ventricular pressures (5), and e' is used to distinguish pseudonormal (E/A > 1) from normal filling patterns.

As with active relaxation analysis, accurate Doppler echocardiographic measures of intrinsic passive diastolic properties, such as stiffness, are difficult (92). Given that diastolic filling pressure reflects the load-dependent changes in chamber stiffness, the ratio of early transmitral inflow velocity to early mitral annular velocity (E/e') is used as a biomarker of increased filling pressure (92). As the left atrial pressure increases, early filling (E) increases, reflecting the increased pressure gradient between the atrium and ventricle. Since mitral annular velocity (e') is unaffected by loading conditions, E/e' is considered a dynamic index of filling pressure. For instance, E/e' might be normal at rest but abnormally elevated with exertion, clinically presenting as breathlessness and reduced exercise capacity due to the sudden increase in left atrial pressure (73). Likewise, rats with relatively high E/e' at rest may exhibit shorter treadmill exercise tolerance times, suggesting a more severe diastolic dysfunction phenotype (42).

Animal Models of Postmenopausal LV Diastolic Function

The OVX rodent model is commonly used to study the role of estrogen in the maintenance of cardiac structure and function as it closely recapitulates the sex hormone milieu of surgical menopause and natural menopause in humans (87). However, despite its frequent use, there are some important limitations of this animal model that are worth mentioning. First, the abrupt loss of ovarian hormones does not model the hormonal transition of perimenopause to menopause (36, 87). Second, the removal of ovaries is performed at different life stages. For example, rodents may undergo OVX at 2–6 mo (regular estrous cycles), 11 mo (the beginning of acyclicity), or 18 mo (the beginning of constant estrous) to include specific age-related factors in the model (87, 132). Young and old rats respond differently to OVX and to exogenous hormone replacement, making it difficult to compare findings across studies (17). Even so, we can glean important insights into the mechanistic functions of ovarian hormones, and particularly estrogen, on the preservation of the female cardiac phenotype. Here we review a few commonly used models that have been helpful for the study of diastolic dysfunction in the context of estrogen loss.

AJP-Heart Circ Physiol • doi:10.1152/ajpheart.00859.2013 • www.ajpheart.org


Table 1. Classification of different stages of diastolic dysfunction relative to control animals with mitral flow and mitral annular velocity by conventional and tissue Doppler imaging and limitations with measures

<table>
<thead>
<tr>
<th>Mitral Flow and Tissue Doppler Imaging Parameters</th>
<th>Abbreviation, Units</th>
<th>Impaired Relaxation With Normal Filling Pressure</th>
<th>Impaired Relaxation With Elevated Filling Pressure</th>
<th>Pseudonormal</th>
<th>Restrictive Filling</th>
<th>Limitations</th>
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<tr>
<td>Early filling velocity</td>
<td>$E_1$, cm/s</td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td>Same as control</td>
<td>$\uparrow\uparrow$</td>
<td>Loading conditions (e.g., decreased preload induces a decrease in $E_1$; increased preload produces an increase in $E_1$)</td>
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<tr>
<td>Ratio of early to late filling velocity</td>
<td>$E/A$</td>
<td>$&lt;1$</td>
<td>$&lt;1$</td>
<td>$\leq 1$</td>
<td>$&gt;2$</td>
<td>Loading conditions (e.g., heart rate (e.g., fused $E$ and $A$ waves with tachycardia); atrial function (e.g., no $A$ wave with atrial fibrillation))</td>
</tr>
<tr>
<td>Deceleration time of early filing</td>
<td>DT, ms</td>
<td>$\uparrow$</td>
<td>$\uparrow$</td>
<td>Same as control</td>
<td>$\downarrow$</td>
<td>Loading conditions (e.g., decreased preload produces an increase in DT; increased preload shortens DT)</td>
</tr>
<tr>
<td>Early mitral annular velocity</td>
<td>$e'$, cm/s</td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td>Represents regional function; myocardial tethering (e.g., fibrotic myocardium adjacent to normal tissue may show normal $e'$); septal $e'$ more load dependent than lateral $e'$; tachycardia in rodents induces increased $e'$</td>
</tr>
<tr>
<td>Ratio of early diastolic filling velocity to early mitral annular velocity or filling pressure</td>
<td>$E/e'$</td>
<td>Same as control</td>
<td>$\uparrow$</td>
<td>Same or slightly increased</td>
<td>$\uparrow\uparrow$</td>
<td>Loading conditions</td>
</tr>
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$\uparrow$, increase; $\downarrow$, decrease.

OVX mRen2.Lewis rats. The mRen2.Lewis strain, which expresses the mouse renin 2 (mRen2) gene, is a congenic model of ANG II-dependent hypertension that is generated by the successive backcross of the (mRen2)27 transgenic rat onto the Lewis background (14). This congenic strain exhibits marked sex differences in the extent of hypertension, with male rats exhibiting higher systolic blood pressures and a greater extent of oxidative stress than female rats of similar age or the background Lewis strain (14, 61, 106, 107). The characterization of this genetic model has further demonstrated that male mRen2.Lewis rats have higher circulating levels of ANG II, angiotensinogen, plasma renin, and serum angiotensin-converting enzyme (ACE) activity compared with female rats, which might explain the observed sex-related differences in cardiovascular pathology and disease progression (107).

In female mRen2.Lewis rats, the loss of estrogen by OVX (performed at 4–5 wk of age) exacerbates hypertension, increases Doppler-derived LV filling pressures, and decreases $e'$ or myocardial relaxation. These changes can be prevented by the treatment with 17β-estradiol or G-1, a selective agonist of GPR30 (14, 65, 149). Further characterization of this animal model found that estrogen loss by OVX-induced cardiomyocyte hypertrophy and cardiac fibrosis, important processes that contribute to diastolic dysfunction. Moreover, prolonged intake of a high-salt diet by mRen2.Lewis female rats resulted in reduced LV diastolic compliance and increased relative wall thickness, cardiomyocyte size, and midmyocardial interstitial and perivascular fibrosis, which were attenuated by treatment with G-1 (62). This model is also unique in that estrogen depletion by OVX markedly exacerbates the development and maintenance of hypertension that appears to be dependent on an activated RAAS (14–16, 150). Thus, the female mRen2.Lewis rat has helped us understand the mechanisms that drive diastolic dysfunction in postmenopausal women and has provided us with a potential animal model for evaluating preventive and therapeutic interventions for diastolic dysfunction.

Although estrogen loss is initiated at a set age in the OVX mRen2.Lewis rat (~5 wk) that is far earlier than the age at which menopause begins in middle-aged women, mRen2. Lewis female congenics consistently exhibit accelerated increases in systolic blood pressure and LV hypertrophy after the depletion of ovarian hormones by bilateral OVX, characteristics that commonly occur in women after the transition to menopause. It remains unclear to what extent changes in diastolic function and the consequent risk of diastolic HF after menopause are consequences of hormonal changes versus advancing age. The mRen2.Lewis model allows us to examine the role of ovarian estrogens, independent of age-related cardiovascular changes, in the pathogenesis of diastolic heart disease. It is important to note that the mRen2.Lewis female rat is a renin-overexpressing animal model, which might not accurately emulate RAAS-related changes in women after menopause, as the Ren2 gene is expressed in cardiac myocytes.

Other models. LVDD is related to reduced ventricular relaxation (primarily affecting early diastole) and increased myocardial and chamber stiffness (primarily affecting late diastole) (8, 70, 140, 167). Various rodent models have been developed to mimic the abnormalities in ventricular relaxation and/or LV compliance that occur after estrogen loss; these models may be useful for the study of female sex-specific diastolic dysfunction.
Rat pressure overload (PO) models are commonly used to understand the mechanisms that underlie the progression of diastolic dysfunction to HF (104). To this end, one of the earliest studies that shed light on the potential cardioprotective effects of estrogen in hypertensive heart disease was reported by Douglas et al. (28) using Wistar rats subjected to chronic PO induced by transverse aortic constriction (TAC). These investigators showed that at 6 wk after aortic banding in weanling rats, LV remodeling, ventricular function, and the extent of hypertrophy appeared similar in male and female rats. However, at 20 wk after TAC, only male rats showed an early transition to HF, with an onset of cavity dilatation, loss of concentric remodeling, elevated wall stress, and diastolic dysfunction. Correspondingly, in female rats subjected to 4 wk of suprarenal aortic constriction, estrogen loss by OVX increased myocardial fibrosis, elevated LV end-diastolic pressure, and decreased the transmitral Doppler early-to-late filling velocity ratio compared with ovary-intact rats subjected to the same afterload insult (98).

In addition to estrogen’s role in the regulation of extracellular remodeling and presumably LV compliance, its beneficial effects on limiting diastolic dysfunction in the PO rat might also involve cardiac Ca\(^{2+}\) regulation. Weinberg et al. (153) showed that cardiac sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA)2a gene expression was unchanged in hearts from estrogen-intact female Wistar rats subjected to TAC but was reduced in hearts from their male counterparts subjected to the same PO perturbation, revealing another possible explanation for the male sex-specific reduction in the functional reserve after TAC (153). For relaxation to occur, intracellular Ca\(^{2+}\) must decline, thereby causing the dissociation of Ca\(^{2+}\) from troponin C. The four mechanisms of Ca\(^{2+}\) removal from the cytosol include activation of SERCA, sarcomemal Na\(^+/\)Ca\(^{2+}\) exchanger, sarcomemal Ca\(^{2+}\) ATPase, and mitochondrial Ca\(^{2+}\) uniport. A defect in any of these proteins could result in the impairment of myocardial relaxation. Alterations in phospholamban, a protein that regulates SERCA2 and the sarcomemal Na\(^+/\)Ca\(^{2+}\) exchanger, has also been implicated in diastolic dysfunction (70, 76). In addition, Ca\(^{2+}\) leak from the sarcoplasmic reticulum via ryanodine receptors may contribute to diastolic dysfunction (120). Taken together, estrogen’s protective effects in the PO heart that might limit the progression of diastolic dysfunction include effects on interstitial remodeling and possibly intracellular Ca\(^{2+}\) through its regulation of SERCA2a and the Na\(^+/\)Ca\(^{2+}\) exchanger (75).

In addition to surgically induced PO models, spontaneously hypertensive rats (SHRs) are commonly used to study LV remodeling and cardiac dysfunction secondary to essential hypertension (96). SHRs with OVX have been used to understand the relationship between estrogen and the development of cardiac fibrosis (90). Although diastolic function per se has not been evaluated in this model, estrogen loss accelerates cardiac fibrosis, a key contributor to myocardial and chamber stiffness (9, 151).

The inbred Brown Norway rat and the hybrid Brown Norway × Fischer 344 rat are considered to be ideal models for the study of normal cardiac aging because their cardiovascular phenotype is not confounded by concomitant obesity and renal dysfunction (84). Although there are no reports using these rats to study diastolic dysfunction as it relates to aging women, Knowlton and colleagues found that at 9 wk after OVX (performed at 18–22 mo) of female Brown Norway rats, extracellular matrix-related gene expression was significantly increased in the heart; this effect could be attenuated by 17β-estradiol treatment (105, 136). While functional and structural phenotypic changes with respect to estrogen status were not reported, their findings suggest that the aged OVX Brown Norway female rat might be a suitable model to study diastolic dysfunction. In fact, in a preliminary study of middle-aged Brown Norway × Fischer 344 female rats (18 mo of age), we observed subtle but significant increases in systolic blood pressure and reductions in tissue Doppler-derived measures of diastolic function 8 wk after OVX compared with age-matched, sham-operated female rats (unpublished observations). Additional work is underway to determine the potential of this normative aging model in the study of diastolic dysfunction as it relates to the postmenopausal woman.

To begin to understand the roles of estrogen in the pathogenesis of obesity- and/or insulin-resistance-induced LVDD, two reverse translational animal models may be considered. Manrique et al. (89) recently showed that young female mice fed a Western diet high in fat and fructose corn syrup abrogated the protective effects of estrogen on whole body insulin sensitivity. Eight weeks of the Western diet also preferentially promoted the development of diastolic dysfunction in female mice as opposed to male mice, and this cardiac phenotype was associated with increased myocardial oxidative stress, increased collagen type 1 expression (a marker of stiffness), altered Ca\(^{2+}\) handling, and a pronounced decrease in Akt/ endothelial NOS (eNOS) activation. Similarly, Murase et al. (99) showed that estrogen replacement exacerbated LVDD and cardiac fibrosis and further increased oxidative stress and inflammation in a new rat model of metabolic syndrome (OVX-Dahlsalt-sensitive/obese rats). In contrast to the aforementioned models of hypertension- or PO-induced LVDD, these metabolic-related models of LVDD suggest that the cardioprotective effects of estrogen may be lost under conditions of diet-induced insulin resistance, obesity, and diabetes. The structural and functional cardiac implications of insulin resistance and the diabetic state, in the context of estrogen, have been extensively reviewed by Reichelt et al. (115).

**Mechanisms of Estrogen’s Protective Effects on Diastolic Function**

Despite representing a disease continuum of great clinical importance and urgency, diastolic dysfunction in postmenopausal women is not well understood, and the mechanisms involved are not yet clear. Here, we review recent work from our group and others that has revealed potential mechanisms by which loss of estrogen induces diastolic dysfunction.

**Estrogen, NOS, and diastolic dysfunction.** The molecular mechanisms associated with estrogenic modulation of hypertensive heart disease, and specifically LVDD, are complex, but reduced NO availability and altered NOS system component expression and activity have been implicated as potential contributors. Briefly, NO activity is the net result of a balance between its production by NOS and its inactivation by ROS (i.e., free radicals), such as superoxide (119). Under various pathological states, both NO and superoxide are increased by NOS with a net balance being a decrease in NO activity. This concept of “uncoupling” of NOS has been implicated as a...
major factor responsible for endothelial dysfunction, diastolic dysfunction, and LV remodeling (97, 131, 138, 141).

While all three NOS isoforms (eNOS, inducible NOS, and nNOS) have been identified in the heart, only nNOS and eNOS are considered to constitute isoforms whose activity in the formation of NO is dependent on Ca^{2+}/calmodulin and other cofactors, including tetrahydrobiopterin (BH₄), flavin mononucleotide, flavin adenine dinucleotide, and reduced nicotine adenine dinucleotide phosphate. Because BH₄ is influenced by the estrogenic milieu (126), it may be involved in the cardiac phenotype of women after menopause. BH₄ appears to act as an allosteric modulator of the NOS complex, contributing to the dimerization of NOS monomers (156, 157), which is necessary for the generation of NO (144, 147). Under conditions of suboptimal BH₄ concentration or the oxidation of BH₄ necessary for the generation of NO (148, 149), BH₄ is converted to dihydrobiopterin, both of which are involved in NO production, the former in the preferential production of ROS rather than NO. GTP cyclohydrolase has been implicated in BH₄ biosynthesis, and it is regulated by estrogen (95, 126, 128). Animal studies have shown decreased BH₄ bioavailability in the aorta of female rats after OVX (78) and that 17β-estradiol therapy normalizes BH₄ levels and suppresses ROS generation in aortic tissue. Moreover, in cultured endothelial cells, estrogens increase BH₄ levels through upregulation of GTP cyclohydrolase mRNA and activity (95). Yamaleyeva et al. (159, 161) showed that the adverse effects of estrogen loss on kidney structure and function in the salt-sensitive, hypertensive mRen2.Lewis female rat, including increased renal nNOS expression, could be reversed by a specific nNOS inhibitor. Taken together, it seems plausible that estrogens may be involved in the maintenance of a “favorable” cardiac NOS complex and likely the preferential production of NO over superoxide.

To test this possibility, we studied the roles of NOS and BH₄ in the maintenance of cardiac structure and diastolic function in the OVX mRen2.Lewis female rat (Table 2). Estrogen deprivation resulted in a relative deficiency in cardiac BH₄, which was associated with an increase in cardiac superoxide production, a reduction in cardiac NO release (nitrate), and adverse LV remodeling and diastolic dysfunction. Importantly, chronic exogenous BH₄ supplementation for 4 wk after the onset of hypertension (64, 149) reversed the unfavorable effects of estrogen loss on diastolic function, superoxide production, and cardiac collagen deposition (64). Moreover, chronic treatment with the specific nNOS inhibitor L-VNIO limited the adverse effects of a presumed uncoupled or maladaptive NOS on cardiac structure and diastolic function (63).

While we can only speculate on the contribution of decreased NO and/or increased ROS toward OVX-elicited cardiac remodeling and diastolic derangement, we do know from data in the mRen2.Lewis parent strain, the (mRen2)27 rat, that blockade of mineralocorticoid receptors diminishes oxidative stress, which, in turn, attenuates collagen deposition in the heart associated with diastolic dysfunction in male rats (47). Similarly, cardiac oxidative stress due to ANG II stimulation from the activation of the RAAS in the (mRen2)27 rat induces structural and functional changes within the heart (47, 154). We do not yet know if these observations hold true in the mRen2.Lewis rat. Aside from the potential consequences of an activated RAAS, which is known to at least uncouple eNOS within the vasculature (123), it is possible that in our studies the diminished NO availability provoked further collagen deposition. NO has been shown to regulate matrix metalloproteinases (MMPs) in the heart (54, 86), which are responsible for the intricate balance of collagen turnover. NO appears to modulate this activity through activation of ERK and Akt pathways (7, 12, 26, 32). In contrast to the direct actions of NO, ROS has been implicated in collagen synthesis by mediating MMP induction or stimulation, decreasing tissue inhibitors of metalloproteinase levels, and stimulating collagen synthesis (147). Certainly, MMPs are increased in age-related diastolic dysfunction (20, 59). Since decreased NO and increased oxidative stress augment MMP release (23, 50, 143), these findings point to an additional mechanism that may account for the development of cardiac fibrosis in the OVX mRen2.Lewis model.

**Estrogen and the RAAS in diastolic dysfunction.** Activation of the RAAS is associated with hypertension, cardiac hypertrophy, impaired cardiomyocyte relaxation, and cardiac fibrosis, which contribute to the impairment of diastolic function. Evidence suggests that the RAAS is involved in the sex-related differences and cardiac dissimilarities that occur during the pre- to postmenopausal transition. ACE activity is higher in men than in women among healthy young adults (164), whereas in postmenopausal women, plasma ACE activity is similar to that in men of the same age (25, 94, 125). Estrogen replacement has been shown to reduce ACE activity in postmenopausal women (111, 125). In hypertensive rats, ACE activity is higher in male rats than in female rats (107), and estrogen treatment decreases ACE activity in the plasma, kidney, and aorta of OVX female rats (10, 14). Mounting evidence indicates that estrogen also regulates other components of the RAAS, such as angiotensinogen, renin, tissue ANG II type 1 and 2 receptors (AT₁R and AT₂R, respectively), and aldosterone production (6, 57, 101, 116, 139). In the heart, activation of the ANG II-AT₁R axis can affect diastolic function by altering the relaxation properties of cardiomyocytes (19) and by changing the composition of the extracellular matrix, specifically collagen content and type, which, in turn, reduces LV distensibility (166, 167).

In mRen2.Lewis female rats, the local and circulating RAAS are also regulated by estrogen, which has subsequent effects on blood pressure and diastolic function. OVX mRen2.Lewis rats treated with the AT₁R blocker olmesartan exhibit a reduction in blood pressure similar to that seen with 17β-estradiol replacement (14). Estrogen replacement in the mRen2.Lewis rat

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<th>Table 2. Summary of key cardiovascular end points in mRen2.Lewis rats after OVX and OVX + 4 wk of nNOS inhibition or BH₄ treatment</th>
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<tr>
<td><strong>Hypertension</strong></td>
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<tr>
<td>BH₄:BH₂</td>
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<tr>
<td>Cardiac ROS</td>
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<tr>
<td>Cardiac NO</td>
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<tr>
<td>Fibrosis</td>
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<td>Diastolic function</td>
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<tr>
<td>Treatment was initiated at 11 wk of age after the establishment of overt hypertension (63, 64), OVX, ovarectomy; nNOS, neuronal nitric oxide (NO) synthase; BH₄, tetrahydrobiopterin; BH₂, dihydrobiopterin.</td>
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</table>
also corrects the increase in circulating activities of renin and ACE as well as reduces plasma levels of ANG II and increased circulating ANG-(1–7) (14). Moreover, 17β-estradiol treatment attenuates OVX-associated increases in cardiac ANG II and diastolic dysfunction (150). The regulation of cardiac ANG II might be due to the inhibition of chymase expression by estrogen (Table 3). Chymase is an alternative pathway that generates ANG II. Interestingly, in women with HF, pharmacological approaches aimed at the inhibition of ACE have met with minimal success (37, 38, 130, 155). Further studies are needed to determine whether the development of diastolic dysfunction after ovarian hormone loss involves the noncanonical pathway by which chymase (produced within cardiomyocytes or brought into these cells from activated mast cells) (79, 150) generates ANG II.

Several lines of evidence suggest that aldosterone excess might play a key role in diastolic dysfunction associated with ovarian estrogen deprivation. Aldosterone is mainly synthesized and released from the adrenal gland (158), and estrogen may regulate aldosterone synthesis and secretion via downregulation of AT1Rs in the adrenal gland (158). In OVX mRen2.Lewis rats, plasma aldosterone tended to be higher compared with sham-operated rats; aldosterone decreased significantly after estrogen treatment of OVX rats compared with vehicle-treated OVX rats (Table 3). Adverse effects of aldosterone on cardiac remodeling and diastolic dysfunction have been reported in both animal models and clinical studies (29, 47, 129). Compared with age-matched Sprague-Dawley rats, male (mRen2)27 rats at 8–9 wk of age display higher systolic blood pressure, plasma aldosterone levels, cardiac hypertrophy and fibrosis, and cardiac oxidative stress as well as impaired LV diastolic relaxation without changes in systolic function. Treatment with the specific aldosterone antagonist spironolactone for 3 wk improved diastolic dysfunction and reduced cardiac fibrosis and oxidative stress independent of changes in systolic blood pressure (47). These findings are consistent with data from recent randomized, controlled clinical trials of long-term aldosterone receptor blockade, which showed improved LV diastolic function in both male and female patients with diastolic HF who received spironolactone (29). These data suggest that aldosterone might play a role in postmenopausal diastolic dysfunction and that aldosterone antagonism may be a plausible treatment option. In contrast, recent data from the Treatment of Preserved Cardiac Function Heart Failure with an Aldosterone Antagonist trial showed that HFPEF patients randomized to spironolactone treatment did not outperform placebo-treated patients on the primary composite outcome of cardiovascular death, heart failure hospitalization, or aborted cardiac arrest. Even so, spironolactone-treated patients had significantly fewer hospitalizations for HF than control patients, supporting its therapeutic potential in reducing the burden of disease (137).

### Table 3. Chymase/ANG II/aldosterone and diastolic function in mRen2.Lewis rats after OVX and OVX + 4 wk of estrogen treatment

<table>
<thead>
<tr>
<th></th>
<th>Plasma ANG II, pg/ml</th>
<th>Cardiac ANG II Staining Intensity</th>
<th>Cardiac Chymase Protein/GAPDH</th>
<th>Plasma Aldosterone, pg/ml</th>
<th>e'- cm/s</th>
<th>E/e'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated rats</td>
<td>17.4 ± 2.1</td>
<td>129 ± 16</td>
<td>0.53 ± 0.09</td>
<td>23.4 ± 5.8</td>
<td>3.38 ± 0.16</td>
<td>19.2 ± 0.8</td>
</tr>
<tr>
<td>OVX rats</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle treatment</td>
<td>31.2 ± 10.2</td>
<td>173 ± 9*</td>
<td>1.14 ± 0.17*</td>
<td>36.6 ± 7.5</td>
<td>2.87 ± 0.18</td>
<td>23.0 ± 1.1*</td>
</tr>
<tr>
<td>17β-Estradiol treatment</td>
<td>8.8 ± 1.4</td>
<td>141 ± 12</td>
<td>0.59 ± 0.13</td>
<td>12.1 ± 0.8†</td>
<td>3.76 ± 0.19†</td>
<td>17.0 ± 0.9†</td>
</tr>
</tbody>
</table>

n = 7–10 rats/group. *P < 0.05 vs. sham-operated rats; †P < 0.05 vs. vehicle-treated OVX rats.

**Estrogen and natriuretic peptides in diastolic dysfunction.** The cardiac natriuretic peptides (NPs), including atrial NP, brain NP, and their related peptides, may also have an important role in the pathogenesis of LVDD after the menopausal transition. A positive relationship between female sex steroids and the production/secretion of NPs has been demonstrated in experimental (22, 58) and clinical (69, 77) studies. In addition to possessing diuretic, natriuretic, and vasodilatory properties, NPs have emerged as potent anti-inflammatory and antihypertrophic humoral factors in the heart, either by directly inhibiting the sympathetic RAAS or through the release or action of endothelin (22, 148). Findings from the Prospective Comparison of Angiotensin Receptor Neprilysin Inhibitor and Angiotensin Receptor Blocker on Management of Heart Failure with Preserved Ejection Fraction trial further underscore the therapeutic potential of augmenting the NP system via inhibition of nephrilysin (the key enzyme responsible for NP breakdown) in combination with an angiotensin receptor blocker in the management of diastolic HF or HFPEF (133, 148). Therefore, it is plausible that a relative deficiency of NPs in the female heart after the loss of ovarian estrogens contributes to the cardiac fibrotic processes that underlie the postmenopausal diastolic dysfunction phenotype.

**A Novel ER, GPR30, and Diastolic Function**

Although preclinical studies have reported the presence of ERα and ERβ in cardiomyocytes and cardiofibroblasts (44), the role of estrogen in the maintenance of diastolic function remains poorly understood. An orphan GPER (GPR30) was discovered in triple-negative breast cancer cells that binds estrogen at high affinity and improves cardiac function and structure in an ER-independent manner; this discovery has revealed new insights into the cardioprotective effects of estrogen, beyond the genomic and nongenomic mechanisms of classic ERs (8, 13, 26, 32–34, 68, 152). GPR30-associated actions in the heart were first described in a myocardial ischemia-reperfusion injury animal model (26). RT-PCR and immunoblot analysis further confirmed the expression of GPR30 in both porcine and human coronary artery vascular smooth muscle cells (VSMCs). GPR30 activation by the selective agonist G-1 relaxes porcine aortic rings, rat mesenteric resistance vessels, and human coronary arteries in an endothelium-independent manner (81, 163). Moreover, a GPR30 activation-induced vasodilatory response was found to be abrogated in carotid arteries in GPR30 knockout mice compared...
with control mice as well as in the intact mRen2.Lewis rat maintained on a high-salt diet (45, 81). In the mesenteric vessels, both endothelial denudation and the NOS inhibitor N-nitro-L-arginine methyl ester achieve a similar extent of inhibition of estradiol- and G-1-induced vasorelaxation; however, there remains an endothelium-independent component that uses cAMP as a signaling molecule (83). Interestingly, the nonendothelial vasorelaxation response is reduced in the mesentery of older female Lewis rats and associated with reduced expression of GPR30 (82). In this regard, Yu et al. (163) demonstrated that GPR30 specifically activates the large-conductance Ca^2+ and voltage-activated K^+ channel in coronary artery VSMCs.

Consistent with in vitro and ex vivo findings, in vivo studies have demonstrated that GPR30 activation by G-1 reduces infarct size in the isolated perfused male mouse heart (7). The cardioprotective effects of GPR30 might be mediated by the ERK pathway, as the effects of G-1 are abolished by the ERK kinase inhibitor PD-98059. GPR30 has also been shown to be involved in systemic blood pressure regulation. Chronic G-1 treatment decreases blood pressure in O VX hypertensive mRen2.Lewis rats in a dose-dependent manner but does not influence blood pressure in intact female or male congenic rats (Table 4) (80, 81, 149). In intact female rats, we assume that endogenous estrogens are producing a maximal effect on blood pressure that cannot be enhanced by the additional agonist. The lack of an effect in male hypertensive mRen2.Lewis rats may result from a blunted vasodilatory response to G-1 in resistance arteries from these animals and decreased expression of vascular GPR30 (82). These results indicate that GPR30 activation by G-1 at doses of >100 μg·kg⁻¹·day⁻¹ exerts a tonic vasodilatory influence to lower blood pressure.

The activation of GPR30 with G-1 has also been found to preserve diastolic function and structure in O VX mRen2.Lewis female rats relative to vehicle-treated O VX littermates (149). Low doses of G-1 (50–100 μg·kg⁻¹·day⁻¹) limit the O VX-related increase in LV filling pressure, LV mass, wall thickness, cardiomyocyte size, and cardiac fibrosis. The mechanisms involved were further determined in in vitro studies focusing on the effects of GPR30 on cardiomyocyte hypertrophy and cardiac fibroblast proliferation. We found that G-1 treatment attenuates ANG II-induced hypertrophy in H9c2 cardiomyocytes and that the GPR30 antagonist G-15 inhibits the effects of both 17β-estradiol and G-1. Moreover, G-1 also inhibits the proliferation of cardiac fibroblasts derived from adult Sprague-Dawley rats. These studies revealed the importance of GPR30 in the maintenance of female sex-specific cardiac structure and function, which likely involve effects on both cardiomyocytes and cardiac fibroblasts. The RAAS also appears to modulate the protective effects of G-1 on diastolic function. Our preliminary data showed a significant increase in plasma ANG I and a trend toward increased plasma ANG II and, curiously, ANG-(1–12) in O VX mRen2.Lewis rats compared with sham control rats; G-1 treatment reversed these changes, suggesting that the regulation of RAAS by GPR30 may be involved in preserving diastolic function (Table 5).

In high-salt diet-induced diastolic dysfunction in female mRen2.Lewis rats, activation of GPR30 by G-1 also increases LV lusitropy (e') and improves the e'-to-a' ratio, as determined by tissue Doppler, and is associated with attenuation of wall thickness, myocyte hypertrophy, and cardiac fibrosis (62). Studies using different animal models have concluded that the activation of GPR30 preserves diastolic function and heart structure. Further studies are needed to determine if the protective effects of GPR30 are mediated through similar mechanisms as estrogen, e.g., by regulating the cardiac RAAS and NOS system.

### Clinical Perspective of HRT

Several large, randomized, controlled clinical trials of postmenopausal HRT have been undertaken since the 1990s and have examined a number of end points, including cardiovascular health. The Women’s Health Initiative conducted the first randomized, placebo-controlled primary prevention trial of estrogen plus progestin in 16,608 postmenopausal women who were followed for an average of 5.2 yr to assess the incidence of CHD as well as the overall risks and benefits (40, 118). Women in the HRT arm received no cardiovascular benefit of CHD as well as the overall risks and benefits (40, 118). Women in the HRT arm received no cardiovascular benefit of CHD as well as the overall risks and benefits (40, 118). Women in the HRT arm received no cardiovascular benefit of CHD as well as the overall risks and benefits (40, 118). Women in the HRT arm received no cardiovascular benefit of CHD as well as the overall risks and benefits (40, 118). Women in the HRT arm received no cardiovascular benefit of CHD as well as the overall risks and benefits (40, 118). Women in the HRT arm received no cardiovascular benefit of CHD as well as the overall risks and benefits (40, 118).

### Table 4. Dose-dependent systolic blood pressure effects of G-1 in O VX m Ren2.Lewis rats

<table>
<thead>
<tr>
<th>No. of rats</th>
<th>Vehicle</th>
<th>50 μg·kg⁻¹·day⁻¹ G-1</th>
<th>100 μg·kg⁻¹·day⁻¹ G-1</th>
<th>400 μg·kg⁻¹·day⁻¹ G-1</th>
<th>800 μg·kg⁻¹·day⁻¹ G-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>4</td>
<td>6</td>
<td>5</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Day 14</td>
<td>3</td>
<td>147 ± 3</td>
<td>140 ± 4</td>
<td>173 ± 6</td>
<td>169 ± 7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>5</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>167 ± 7*</td>
<td>163 ± 4*</td>
<td>161 ± 6*</td>
<td>150 ± 7†</td>
</tr>
</tbody>
</table>

Values are in mmHg. *P < 0.05 vs. sham-vehicle-treated rats; †P < 0.05 vs. day 0

### Table 5. Plasma angiotensins and diastolic function in O VX m Ren2.Lewis rats treated with vehicle or G-1 for 2 wk

<table>
<thead>
<tr>
<th>Plasma ANG I, pg/ml</th>
<th>Plasma ANG II, pg/ml</th>
<th>Plasma ANG-(1–12), pg/ml</th>
<th>e', cm/s</th>
<th>E/e'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated</td>
<td>66 ± 7</td>
<td>17 ± 3</td>
<td>143 ± 29</td>
<td>3.4 ± 0.2</td>
</tr>
<tr>
<td>O VX rats</td>
<td>94 ± 8*</td>
<td>25 ± 2</td>
<td>270 ± 59</td>
<td>2.6 ± 0.1*</td>
</tr>
<tr>
<td>Vehicle treatment</td>
<td>53 ± 4†</td>
<td>16 ± 3</td>
<td>145 ± 38</td>
<td>3.6 ± 0.2†</td>
</tr>
</tbody>
</table>

n = 7–10 rats/group. *P < 0.05 vs. sham-operated rats; †P < 0.05 vs. vehicle-treated O VX rats.
was no significant decrease in the rates of primary CHD events or secondary cardiovascular events among women in the HRT group compared with the placebo group (40). However, further analysis of these carefully conducted trials showed that there was, on average, a 10-yr delay between the onset of menopause and the initiation of estrogen therapy. Interestingly, a more recent study showed a lower rate of CHD events and total mortality when HRT was initiated in younger women (<60 yr) in close proximity to the onset of menopause (<10 yr) and no effect or a possible adverse effect on these end points when HRT was initiated in older women (>60 yr) more than 20 yr after the onset of menopause (54). It was hypothesized that the vessels and myocardium of older postmenopausal women undergo significant age-related remodeling and development of pathology, including systolic hypertension, so that the “late” initiation of estrogen replacement (10 yr or more after the loss of ovarian hormone production) may not reverse cardiovascular damage or prevent further disease progression (74). The apparent timing-related benefit of HRT on CHD has been reported in a large meta-analysis of 23 randomized controlled clinical trials enrolling ~39,000 women, which revealed a 32% reduction in CHD incidence in women starting HRT before 60 yr of age or <10 yr after menopause (121). This risk reduction was lost in women older than 60 yr of age or >10 yr after menopause (121). These results, along with animal studies in nonhuman primates, support the timing hypothesis, which posits that women respond differentially with respect to CHD based on the timing of HRT initiation relative to age and/or time since menopause (21).

Further underscoring the plasticity of the ovarian hormonal response in cardiovascular tissue with respect to age and possibly salt status, we showed that late OVX (15 wk of age) of mRen2.2Leis rats conveyed renal protective effects from a high-salt diet compared with age-matched, ovary-intact hypertensive littersmates (160). The magnitude of the CHD reduction for women <60 yr of age or <10 yr since menopause when randomized to HRT was similar to that seen in observational studies of women who initiated HRT at the time of menopause (54, 55). The Kronos Early Estrogen Prevention Study was recently completed and found several favorable effects of HRT in newly menopausal women, including improved vasomotor symptoms, bone mineral density, and mood outcomes (88). Although none of these large trials examined the influence of HRT per se on diastolic function, its involvement is nearly certain; LVDD is associated with ischemia, and it precedes both diastolic and systolic HF. Clearly, further clinical and basic studies are needed to determine the basis for these clinical trial findings and the relationship among estrogen, preclinical LVDD, and overt heart disease, including the direct effects of estrogen on cardiomyocytes and fibrosis and the molecular mechanisms involved.

Summary

Basic research is essential for understanding the cellular and molecular mechanisms underlying diastolic dysfunction. Estrogen appears to be an important factor that protects against cardiac remodeling and diastolic dysfunction in women. The protective effects of estrogen involve the regulation of the cardiac RAAS and NOS system as well as the newly characterized estrogen receptor GPR30. The exact roles of estrogen and GPR30 on cardiomyocyte relaxation, cardiac fibroblasts, and collagen production as well as the related mechanisms leading to postmenopausal LVDD are the focus of current research.

GRANTS

This work was funded in whole or part by National Institutes of Health Grants AG-042758 (to L. Groban), AG-033727 (to L. Groban), HL-56793 (to M. C. Chappell), and HL-103974 (to S. H. Lindsey), Doctoral Research Grant of Shandong Province BS2010Y1005, and National Natural Science Foundation of China Grant 81270175 (to Z. Zhao).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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