Impact of early life ovariectomy on blood pressure and body composition in a female mouse model of systemic lupus erythematosus

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Submitted 28 January 2014; accepted in final form 15 August 2014

Gilbert EL, Ryan MJ. Impact of early life ovariectomy on blood pressure and body composition in a female mouse model of systemic lupus erythematosus. Am J Physiol Regul Integr Comp Physiol 307: R990–R997, 2014. First published August 20, 2014; doi:10.1152/ajpregu.00038.2014.—Because of the preponderance of women affected by the chronic autoimmune disease systemic lupus erythematosus (SLE), estrogen is thought to contribute to SLE disease progression. This is supported by evidence from experimental animal models of SLE showing that removal of estrogen in young female mice delays autoantibody production and renal injury and lengthens survival. Blood pressure and changes in body composition are important cardiovascular risk factors that can be regulated by estrogens. Because cardiovascular disease is the leading cause of death in patients with SLE, we used an established female mouse model of SLE (NZBWF1) to test whether early life removal of estrogen impacts the development of hypertension and changes in body composition commonly associated with SLE. Eight-week-old female SLE and control mice (NZW/LacJ) underwent either a sham operation or ovariectomy. Body weight, body composition (fat and lean masses), and renal injury (albuminuria) were monitored until mice reached 34 wk of age, at which time mean arterial pressure was assessed in conscious animals by a carotid catheter. Early life removal of the ovaries delayed the onset of autoantibody production and albuminuria while causing an increase in body weight and fat mass. Blood pressure in the adult was not altered by early life removal of the ovaries. These data suggest that estrogens may have a permissive role for the development of SLE while helping to maintain normal body weight and composition, which is associated with reduced cardiovascular risk.

Estrogens are known immunomodulators that can promote humoral (antibody mediated) immunity (21, 32) and, therefore, contribute to SLE disease activity. In addition, the loss of estrogen or its receptors early in the life of experimental mouse models of SLE has provided convincing evidence showing that estrogens have an important role in the development and progression of the disease (5, 35, 43). Surprisingly, the contribution of estrogens to SLE disease progression in humans remains unclear, and understanding their role in the cardiovascular risk is complicated by the large body of literature pointing to cardioprotective actions of estrogens in women (22, 47) as well as their relatively safe use in women with SLE (3, 7, 8, 20, 26, 31, 40). Using an established experimental model of SLE (female NZBWFI mice), we (12) recently reported that loss of estradiol in adulthood exacerbates the hypertension and renal injury, which, when considering that loss of estrogens early in life delays disease onset, suggests that there are distinct temporal effects of estrogens on SLE disease progression and its consequences. The major goal of the present study was to extend the findings of our recent work (12) by testing whether early life removal of estrogens in female NZBWFI mice delays the onset of SLE and attenuates the hypertension in adulthood. Because loss of estrogens can have a profound impact on body composition, another cardiovascular risk factor, the second goal of the study was to assess the impact of early life ovariectomy (OVX) on body weight and body composition.

MATERIALS AND METHODS

Animals

Female NZBWFI (SLE) and NZW/LacJ [control (Ctrl)] (Jackson Laboratories, Bar Harbor, ME) mice were obtained at 3–5 wk of age. Mice were maintained on a 12:12-h light-dark cycle in temperature-controlled rooms with access to chow and water ad libitum. All experiments were performed with the approval of the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

OVX

When SLE and Ctrl mice were 8 wk old, an OVX was performed through a dorsal midline incision, as previously described (12). The OVX was performed at this age to mimic the early life removal of estrogens or its actions, as previously described by others (5, 35, 43). To confirm the efficacy of OVX, the uterus was collected and weighed at the conclusion of the study.

Blood Pressure Measurements in Adulthood

At the conclusion of the study (34 wk of age), mean arterial pressure (MAP; in mmHg) was recorded via indwelling carotid artery catheters in conscious mice, as previously described by our laboratory (37).
Autoantibody Production

Plasma anti-double-stranded (ds)DNA antibodies, a hallmark of SLE disease, were measured by commercial ELISA (Alpha Diagnostic, San Antonio, TX), as previously described (46).

Albuminuria

Urinary albumin was monitored weekly by dipstick analysis (Albustix, Tarrytown, NY) of overnight urine samples. Animals were considered to be positive for albuminuria at ≥100 mg/dl, as previously described (46). Urinary albumin was confirmed by ELISA (Alpha Diagnostic) in urine samples collected at the end of the study, as previously published by our laboratory (46).

Body Weight and Composition

Changes in body composition of the mice were monitored using MRI (Echo MRI-900TM, Echo Medical System, Houston, TX) (9). MRI measurements were performed in conscious mice placed in a thin-walled plastic cylinder with a cylindrical plastic insert added to limit movement of the mice. Mice were briefly submitted to a low-intensity electromagnetic field to measure total body fat mass, lean mass, free water, and total water. Each mouse was weighed before placement in the Echo MRI. The Echo MRI analysis was run 2 times/mouse at each time point. Values were then averaged and normalized to the body weight of the mouse.

Insulin

Insulin was assessed by commercial ELISA (catalog no. EZMRIA-13K, EMD Millipore, St. Charles, MO) in plasma samples collected from fasted mice (6-h fast), as previously described (37).

Food Intake

Food intake was assessed by individually housing mice in shoebox cages and weighing the food each day for 8 days, as previously described (37).

Protocols

Protocol 1: impact of early life OVX on SLE progression and blood pressure in the adult. SLE and Ctrl mice underwent either OVX or sham operation (sham) at 8 wk of age. Urine was collected every week and assessed for the presence of albumin, as previously described (36). When mice reached 34 wk of age, MAP was measured in conscious animals, and tissues were collected at the time of death.

Protocol 2: impact of early life on body weight and composition during SLE. The body weight of each mouse was measured weekly beginning at 8 wk of age and continuing to the end of the study at 34 wk of age. Body composition by Echo MRI was monitored every 3 wk beginning at 9 wk of age. When mice reached 20 wk of age, body composition was analyzed every other week until the conclusion of the study at 34 wk.

Statistical Analysis

Data are presented as means ± SE. Statistical analyses were performed using Graph Pad Prism 6 software. Two-factor ANOVA was used to test for the main effects of group and treatment and their interaction among uterine weight, MAP, and urine albumin excretion. When ANOVA indicated significance, Tukey’s post hoc test was used to determine individual differences. P values of <0.05 were considered statistically significant.

RESULTS

Uterine Weight

To confirm the efficacy of the OVX operation, uterine weight was measured at the time of tissue collection. Uterine weight was significantly reduced in both Ctrl and SLE mice subjected to OVX compared with sham (Ctrl sham mice: 0.10 ± 0.015 g, Ctrl OVX mice: 0.049 ± 0.004 g, SLE sham mice: 0.10 ± 0.011 g, and SLE OVX mice: 0.049 ± 0.009 g, P < 0.05, sham vs. OVX; Fig. 1).

Anti-dsDNA Antibodies

Circulating anti-dsDNA (IgG) autoantibodies were measured in plasma samples collected throughout the study. Figure 2 shows the increasing levels of autoantibodies over time in SLE sham and SLE OVX animals. The production of autoantibodies was delayed in OVX animals, significantly diverging at 28 wk of age. Compared with Ctrl sham mice, autoantibodies were significantly higher beginning at 24 wk of age (Ctrl sham mice: 24 ± 4 kU/ml and SLE sham mice: 206 ± 46, P < 0.05). Autoantibodies were similar between Ctrl and SLE mice at 8 wk of age (Ctrl sham mice: 17 ± 4 kU/ml, Ctrl OVX mice: 15 ± 3 kU/ml, SLE sham mice: 25 ± 13 kU/ml, and SLE OVX mice: 28 ± 12 kU/ml) and remained low in Ctrl sham mice even at 34 wk (61 ± 12 kU/ml). OVX in Ctrl mice did not alter autoantibody levels compared with Ctrl sham mice.

MAP

We recently reported that OVX during adulthood (at 30 wk of age) exacerbates the hypertension associated with SLE (12). In the present study, we tested whether blood pressure in adulthood during SLE is impacted by OVX in young animals (8 wk of age). Consistent with our previous results, MAP was significantly higher in SLE sham mice compared with Ctrl sham mice (Ctrl sham mice: 119 ± 5 mmHg and SLE sham mice: 138 ± 5 mmHg, P < 0.05; Fig. 3). When OVX was performed in young Ctrl and SLE mice, MAP was not altered...
in the adult (Ctrl OVX mice: 125 ± 2 mmHg and SLE OVX mice: 138 ± 3 mmHg).

Albuminuria

Over the course of the study, 42% of SLE sham mice developed albuminuria (Fig. 4A), whereas only 20% of SLE OVX mice developed albuminuria. No Ctrl mice developed albuminuria. Urinary albumin, as measured by ELISA in samples collected at 34 wk of age, was significantly increased in SLE sham mice compared with Ctrl sham mice (3.57 ± 1.9 vs. 0.03 ± 0.006 mg/day in Ctrl sham mice, \( P < 0.05 \); Fig. 4B). OVX in young female SLE mice significantly reduced urinary albumin (0.8 ± 0.3 mg/day, \( P < 0.05 \) vs. SLE sham mice). OVX in Ctrl mice did not alter urinary albumin (0.06 ± 0.05 mg/day).

Body Weight

At the beginning of the study, body weight was similar between groups (Ctrl sham mice: 23.41 ± 0.36 g, Ctrl OVX mice: 24.54 ± 0.65 g, SLE sham mice: 25.18 ± 0.50 g, and SLE OVX mice: 28.72 ± 1.1 g; Fig. 5A). At 9 wk of age, body weight was significantly greater in OVX SLE mice compared with Ctrl sham mice and remained significantly elevated to the conclusion of the study (9 wk: 32.46 ± 0.83 vs. 25.17 ± 0.41 g in Ctrl sham mice, \( P < 0.05 \); 34 wk: 46.10 ± 1.6 vs. 34.03 ± 0.43 g in Ctrl sham mice, \( P < 0.05 \)). Body weight was significantly elevated after OVX in SLE mice compared with SLE sham mice beginning at 14 wk of age and continuing to the conclusion of the study (14 wk: 38.24 ± 1.4 vs. 33.62 ±
1.2 g in SLE sham mice, \( P < 0.05; 34 \text{ wk}: 46.10 \pm 1.6 \text{ g in SLE sham mice, } P < 0.05 \). OVX did not alter body weight in Ctrl mice compared with Ctrl sham mice.

The percent increase in body weight from baseline was also significantly greater in SLE OVX mice compared with their intact counterparts (Fig. 5B).

**Body Composition**

Fat mass as a percentage of body weight was similar between groups at 9 and 12 wk (9 wk: Ctrl sham mice, 17.3 \pm 0.5%; Ctrl OVX mice, 15.0 \pm 0.8%; SLE sham mice, 15.3 \pm 0.7%; and SLE OVX mice, 16.6 \pm 0.7%; Fig. 6A). Removal of the ovaries accelerated the total body gain in fat mass in SLE mice but not in Ctrl mice. From 18 to 26 wk, fat mass was significantly higher in SLE OVX mice compared with all other groups. Fat mass in SLE OVX mice remained significantly increased compared with SLE sham mice to the conclusion of the study at 34 wk (31.6 \pm 1.7% vs. 20.4 \pm 2.2% in SLE sham mice, \( P < 0.05 \)). OVX did not alter fat mass in Ctrl mice compared with Ctrl sham mice.

Lean mass as a percentage of body weight was similar between all groups at 9 and 12 wk (9 wk: Ctrl sham mice, 75.0 \pm 0.5%; Ctrl OVX mice, 78.2 \pm 0.8%; SLE sham mice, 77.0 \pm 0.7%; and SLE OVX mice, 76.0 \pm 1.0%; Fig. 6B). OVX caused a reduction in lean mass in SLE mice beginning at 15 wk of age that paralleled the increase in total fat mass. Lean mass was significantly reduced in SLE OVX mice compared with SLE sham mice (64.3 \pm 1.2% vs. 69.8 \pm 1.5% in SLE sham mice, \( P < 0.05 \)) and remained so until the conclusion of the study at 34 wk (60.6 \pm 1.6% vs. 68.3 \pm 2.3% in SLE sham mice, \( P < 0.05 \)). Lean mass was significantly lower in SLE OVX mice compared with Ctrl sham mice from 18 to 30 wk.

Fig. 5. A: body weight (BW) in Ctrl and SLE mice subjected to either OVX or sham operation. Two-way ANOVA with repeated measure was used with factors of group and time included. Tukey’s post hoc test was used when ANOVA indicated significance. *\( P < 0.05 \), SLE OVX vs. Ctrl mice across the weeks denoted by the horizontal bar; #\( P < 0.05 \), SLE OVX vs. SLE sham mice across the weeks denoted by the horizontal bar. B: BW data expressed as percent increases. Two-way ANOVA with repeated measure was used with factors of group and time included. Tukey’s post hoc test was used when ANOVA indicated significance. *\( P < 0.05 \), SLE OVX vs. Ctrl mice across the weeks denoted by the horizontal bar; #\( P < 0.05 \), SLE OVX vs. SLE sham mice across the weeks denoted by the horizontal bar.

Fig. 6. Body composition in Ctrl and SLE mice subjected to either OVX or sham operation. A: fat mass as a percentage of BW. Two-way ANOVA with repeated measure was used with factors of group and time included. Tukey’s post hoc test was used when ANOVA indicated significance. *\( P < 0.05 \), SLE OVX vs. Ctrl mice across the weeks denoted by the horizontal bar; #\( P < 0.05 \), SLE OVX vs. SLE sham mice across the weeks denoted by the horizontal bar. B: lean mass as a percentage of BW. Two-way ANOVA with repeated measure was used with factors of group and time included. Tukey’s post hoc test was used when ANOVA indicated significance. *\( P < 0.05 \), SLE OVX vs. Ctrl sham mice; #\( P < 0.05 \), SLE OVX vs. SLE sham mice.

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AJP-Regul Integr Comp Physiol • doi:10.1152/ajpregu.00038.2014 • www.ajpregu.org
OVX in Ctrl mice did not alter lean mass compared with Ctrl sham mice.

**Food Intake**

OVX in young mice did not alter food intake at 29 and 30 wk of age (Ctrl sham mice: 3.2 ± 0.2 g/day, n = 4; Ctrl OVX mice: 3.4 ± 0.1 g/day, n = 3; SLE sham mice: 3.1 ± 0.3 g/day, n = 9; and SLE OVX mice: 3.2 ± 0.1 g/day, n = 5; Fig. 7).

**Plasma Insulin**

Plasma insulin was significantly increased in female SLE mice compared with Ctrl mice (1.13 ± 0.3 ng/ml, n = 14, vs. 0.61 ± 0.05 ng/ml, n = 9, P < 0.05; Fig. 8). OVX in young female SLE mice did not alter plasma insulin compared with intact female SLE mice (1.38 ± 0.3 ng/ml, n = 7, vs. 1.13 ± 0.3 ng/ml, n = 14). Plasma insulin was not different between Ctrl OVX mice and Ctrl sham mice (0.65 ± 0.05 ng/ml, n = 6, vs. 0.61 ± 0.05 ng/ml, n = 9).

**DISCUSSION**

The role of estrogens in human SLE is surprisingly unclear. In experimental animal models of SLE, evidence has convincingly shown that the absence of estrogens, or its receptors, early in life delays the onset and progression of SLE. However, recent data from our laboratory have shown that loss of estrogens in adulthood does not affect antibody production and exacerbates both the renal injury and hypertension associated with SLE. Taken together, these data suggest that there may be distinct temporal roles for estrogens during SLE. The major goal of the present study was to explore further this temporal role by examining the long-term impact of estrogens on blood pressure and renal injury during SLE. In addition, we sought to determine whether loss of estrogens contributes to body composition changes that would be consistent with increasing cardiovascular risk. The major new findings of this study are as follows: 1) blood pressure was not different between intact and SLE OVX mice during adulthood after early life OVX, 2) SLE mice experienced a greater weight gain than Ctrl mice after early life OVX, and 3) the increased body weight caused by early life OVX was associated with an increase in total body fat mass with a corresponding decrease in lean mass.

**Impact of Early Life OVX on SLE**

Because of the preponderance of females affected by SLE, estrogen is commonly perceived as a contributor to disease progression. Some of the strongest evidence to support this comes from studies using experimental animal models of SLE, including female NZBWF1 mice. For example, estrogen receptor-α knockout NZBWF1 mice (5) not only survived longer but also had reduced levels of pathogenic inflammatory antidsDNA autoantibodies, a hallmark of SLE disease, and reduced renal injury. This important work complemented an early study (35) in which female NZBWF1 mice ovariectomized at 2 wk of age were given supplemental estradiol and experienced a worsened disease course with increased renal injury and mortality. Consistent with these previous studies, we report here that early life OVX delays both the development of autoantibodies and onset of albuminuria, thus providing verification of the role that estrogen that plays in early life as a contributor to SLE disease development.

In humans, SLE typically impacts women beginning in the third or fourth decade of life, and the role of estrogens in human SLE is not as clear. For example, oral contraceptive use, ovulation induction therapy, and hormone therapy treatment traditionally invoke fear of lupus flare. However, there is growing evidence that oral contraceptive use and hormone therapy are safe in women with SLE and do not significantly exacerbate disease activity (3, 8, 20, 40), suggesting a more complex role for estrogens than perhaps previously assumed. Moreover, multiple studies have reported no additional risk of SLE flare and support the utility of hormone therapy in SLE (4, 7, 26, 31, 39), especially to alleviate menopausal vasomotor symptoms (7). Given the uncertainty surrounding the role of estrogen in human SLE, the published work on the protective effect of early life removal of estrogen during SLE in mice (supported by results of the present study), and our recent study showing that removal of estrogens in adult mice does not attenuate SLE disease, our work supports the concept that there are distinct temporal actions of estrogen during SLE. Importantly, female NZBWF1 mice represent a widely used and established experimental model with which to carefully examine these actions.
Impact of Early Life OVX on Blood Pressure in Adulthood During SLE

In contrast to the increased blood pressure that results from removal of estrogens in the adult SLE mouse (12), blood pressure was not different between adult SLE OVX and SLE sham mice when the procedure was performed early in life. One interpretation of this data could be that early life exposure to estrogens does not have an important physiological role in the pathogenesis of hypertension, especially given that blood pressure is significantly elevated over Ctrl animals. A second interpretation is that the removal of estrogens by OVX early in life was protective against the development of hypertension much in the same way that early life removal of estrogens delays the onset of autoantibodies and albuminuria. This could be reasoned from data in rodents showing that long-term removal of the ovaries is expected to exacerbate hypertension (15, 16, 18), whereas there is no such effect in mice with SLE. In addition, it is important to note that hypertension in the adult was not increased even in the face of the increased body weight and increased fat mass. While we favor the latter interpretation, confirmation will require future experiments to assess blood pressure longitudinally from the time of OVX until adulthood. Without longitudinal assessment of blood pressure, the question of whether the increase in blood pressure precedes the development of albuminuria arises. However, based on our previously published work showing that blood pressure and albuminuria are not necessarily associated (12, 29), we are confident this is not the case.

Studies assessing the effect of hormone therapy on cardiovascular disease and risk factors such as hypertension in women with SLE are limited. Hochman et al. (17) examined the effects of hormone therapy and risk of cardiovascular disease in postmenopausal women with SLE. This study suggested that hormone therapy does not predispose to coronary artery disease in postmenopausal women with SLE. Although the prevalence of hypertension among women with lupus was high in this study, the effect of hormone therapy on blood pressure was not reported. The Lupus in Minorities Nature Versus Nurture study in a multiethnic United States cohort showed that use of hormone therapy by postmenopausal women with SLE was not associated with vascular arterial events (11). Therefore, while experiments have not been designed to test directly the impact of estrogen on blood pressure in SLE, these studies suggested that women with SLE were able to use hormone therapy without an enhanced risk of adverse cardiovascular events.

Estrogens and Body Composition During SLE

Obesity and increased fat mass are known cardiovascular risk factors and are also associated with increased blood pressure. Body composition changes are typical of women with SLE, and SLE disease itself has independent effects on body composition (24). A comparative study of women with SLE, women with rheumatoid arthritis, and female control subjects found that abnormal body composition was more prevalent in the inflammatory autoimmune conditions of SLE and rheumatoid arthritis (41). In a longitudinal study (23), 28 premenopausal women with SLE were tracked for 3 yr to assess alterations in body composition. Both body mass index and fat mass increased over the 3-yr followup period (23). A study of childhood-onset SLE reported that SLE disease was associated with increased fat mass and decreased lean mass compared with age-matched healthy control subjects (27). Similarly, women with juvenile-onset SLE had overall decreased lean mass and a higher percentage of fat than age- and weight-matched control subjects (34).

We (37) have previously demonstrated that the female NZBWF1 mouse model mirrors the body composition changes observed in human SLE with increased adipose depots and central adiposity. In the present study, removal of estrogen significantly increased body weight, with SLE OVX mice gaining more weight both in terms of absolute number of grams and percent increase in body weight. In addition, fat mass increased in female SLE OVX mice, and lean mass decreased over the course of the study, a result that is consistent with the effect of OVX in young C57BL6/J mice (44). While SLE OVX mice exhibited significant changes in body weight and fat mass, the weight gain in Ctrl OVX mice was not as pronounced. The reason for this moderate weight gain relative to the study in C57BL6/J mice (44) is not clear; however, it may relate to the genetic background of the mice or differences in the diets used.

The increased body weight and fat mass observed in SLE OVX mice most likely does not result from hyperphagia as food intake was similar between all groups. Importantly, this finding is consistent with evidence from estrogen receptor-α- and aromatase-deficient mice in which no hyperphagia was observed (14, 19). Therefore, the mechanisms involved in the increased body weight and fat mass in SLE OVX mice may be due to reductions in spontaneous activity or from reductions in metabolism after estrogen deficiency. In women under the age of 40 yr, similar alterations have been reported after bilateral oophorectomy, including higher mean percent body fat and BMI (30). Another small study (25) of young women (age: 18–30 yr) demonstrated that young women with primary amenorrhea had large amounts of fat tissue in a predominantly android pattern. Hormonal status affects body composition in women with SLE as well. For example, Kipen et al. (24) demonstrated that increases in fat mass were significantly associated with loss of ovarian hormones. Although it was not examined in women with SLE, the effect of hormone therapy on body composition was investigated in the estrogen plus progesterin randomized controlled clinical trial of the Women’s Health Initiative (6). Women who received estrogen plus progesterin treatment for 3 yr lost less lean soft tissue mass and had reduced redistribution of fat to a central location. Clearly, estrogen affects body composition and may reduce fat tissue mass while preserving lean tissue mass. Body composition changes may be one component that contributes to the overall cardiovascular disease risk in SLE beyond classic risk factors, which alone do not completely account for the higher cardiovascular disease risk in SLE (10).

Perspectives and Significance

Much of the work conducted using experimental animal models of SLE, including the present study, has shown that early life removal of estrogens, or the absence of estrogen receptors, delays the onset of SLE. These studies have clearly demonstrated the impact that estrogens have on the development of SLE. However, the onset of SLE in humans typically...
occurs in adulthood, reaching a peak between ages of 29 and 36 yr (28), and the role of estrogens in human SLE is far less certain. Interestingly, removal of estrogens in adult mice with SLE does not offer any protection and actually exacerbates the hypertension and renal injury associated with SLE (12). Therefore, early in life, estrogens may have an important permissive role for the development of SLE, whereas their role in adulthood remains unclear and may even be cardioprotective. The purpose of the present study was to build on our recent work and advance our understanding of the different temporal effects of estrogens on cardiovascular risk factors. In light of the fact that cardiovascular disease is the leading cause of death in patients with SLE, it may be prudent to carefully consider the time course of estrogen removal in the design of future studies in experimental animal models of lupus.

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E.L.G. drafted manuscript; E.L.G. and M.J.R. edited and revised manuscript; E.L.G. performed experiments; E.L.G. and M.J.R. analyzed data; E.L.G. and M.J.R. supported by National Heart, Lung, and Blood Institute (NHLBI) Grant HL-085907, AHA Grant 12GRNT12060203, a University of Mississippi Southeast Affiliate Predoctoral Fellowship 12PRE12050150. This work was supported by National Heart, Lung, and Blood Institute (NHBLI) Grant HL-085907, AHA Grant 12GRNT12060203, a University of Mississippi Medical Center (UMMC) Intramural Research Support grant (to M.J. Ryan), and NHLBI Grant HL-051791 (to UMMC Physiology).

DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: E.L.G. and M.J.R. conception and design of research; E.L.G. performed experiments; E.L.G. and M.J.R. analyzed data; E.L.G. and M.J.R. interpreted results of experiments; E.L.G. and M.J.R. prepared figures; E.L.G. drafted manuscript; E.L.G. and M.J.R. edited and revised manuscript; E.L.G. and M.J.R. approved final version of manuscript.

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