Effect of Lactation on Postpartum Cardiac Function of Pregnancy-Associated Hypertensive Mice

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Preeclampsia is a serious complication during pregnancy, and recent epidemiological studies indicate the association between preeclampsia and cardiac morbidity and mortality during the postpartum period. Although the risk of cardiovascular diseases in the postpartum period is affected by lactation, its role in maternal heart with a history of preeclampsia remains unclear. In this study, we investigated postpartum change in cardiac remodeling and function of pregnancy-associated hypertensive (PAH) mice with and without lactation. The systolic blood pressure was increased in PAH mice at day 19 of gestation (E19) and was reduced to normal levels in both lactating and nonlactating (NL) groups in the postpartum period. Histological analyses revealed that cardiac hypertrophy and macrophage infiltration in PAH mice at E19 were improved in both lactating and NL groups at 4 weeks postpartum (4W-PP), while marked fibrosis remained. Increased mRNA expression of profibrotic genes and proinflammatory cytokines in PAH mice at E19 was significantly reduced in both lactating and NL groups at 4W-PP. Echocardiographic analysis found no significant differences in fractional shortening between PAH mice and C57BL/6J mice at E19. On the other hand, at 4W-PP, NL PAH mice showed normal fractional shortening, but lactating PAH mice exhibited significant decreases in cardiac contractility compared with NL PAH mice. These results show that cardiac remodeling induced by hypertension during pregnancy are improved in the postpartum period except fibrosis, whereas lactation induces cardiac contractile dysfunction in mice with a history of pregnancy-associated hypertension.

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Preeclampsia is a life-threatening pregnancy disorder characterized by maternal hypertension and proteinuria after 20 weeks of gestation that occurs in approximately 2%–8% of pregnancies (1). Preeclampsia is associated with cardiovascular dysfunction, as represented by endothelial dysfunction, left ventricular (LV) hypertrophy, increased biomarkers for cardiac damage, and cardiac diastolic dysfunction. Most pathological conditions related to preeclampsia, such as hypertension and LV hypertrophy, are improved after parturition. However, recent clinical studies have revealed that previously preeclamptic women have an increased risk of cardiovascular disease (2, 3). Although hypertensive disorders during pregnancy, including preeclampsia, have been thought to be a risk factor of peripartum cardiomyopathy (PPCM), characterized by acute heart failure between 1 month before and 5 months after delivery (4), alterations in preeclampsia-related cardiac pathology during the postpartum period are poorly understood.

Lactation is essential for the reproduction of mammals. Although lactation is widely acknowledged to benefit infant conditions, lactation also affects maternal health. Because lactation is an energetically expensive process, maternal metabolic changes occur during lactation in peripheral tissues, including mammary gland, adipose tissue, liver, skeletal muscle, and heart (5-8). Recent epide-
miological data indicate that increased duration of lactation has been related to reduced cardiovascular disease risk in postmenopausal women (9). Moreover, it is suggested that breastfeeding is associated with recovery of LV systolic function in PPCM patients (10). On the other hand, Hilfiker-Kleiner et al (11) reported the association between onset of PPCM and the lactation hormone prolactin. Therefore, the effect of lactation on postpartum cardiac function remains controversial. Furthermore, both preeclampsia and lactation may be involved in the onset of PPCM, but the effect of lactation on cardiac remodeling and function in postpartum women with a history of hypertensive disorders during pregnancy is not elucidated.

We previously generated pregnancy-associated hypertensive (PAH) mice, by mating female mice expressing human angiotensinogen (hAGT) with male mice expressing human renin (12-14). PAH mice develop preeclamptic features, such as maternal hypertension and proteinuria in late pregnancy, and their fetuses show intrauterine growth retardation (IUGR). We also reported that PAH mice exhibit cardiac hypertrophy, fibrosis, apoptosis, and increased plasma levels of cardiac biomarkers, and these cardiac injuries were ameliorated by treatment with selective angiotensin II type 1 (AT1) receptor blocker (Olmecartan) (15). In this study, we investigated the change in cardiac remodeling and function of PAH mice with and without lactation in the postpartum period.

**Materials and Methods**

**Animals**

PAH mice were generated by mating females expressing hAGT with males expressing human renin as described previously (12, 15). At day 0 post-partum (day 0 postpartum = day of parturition), PAH mice and age-matched C57BL/6J control (wild-type [WT]) mice divided into nonlactating (NL) and lactating (Lac) groups. The WT-NL and PAH-NL groups of dams had their litters removed from their cages. In the WT-Lac and PAH-Lac groups, the dams had their litters removed and were allowed to nurse 8 foster pups from ICR mice delivered on the same day. C57BL/6J and ICR mice were purchased from CLEA Japan Inc (Tokyo, Japan). Animal experiments were performed in a humane manner and approved by the Institutional Animal Experiment Committee of the University of Tsukuba. Experiments were conducted in accordance with the Regulation of Animal Experiments of the University of Tsukuba and the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

**Physiological analysis**

Systolic blood pressure (BP) was measured in conscious mice by the tail-cuff method (BP-98a; Softron, Tokyo, Japan) as described previously (12-15). Echocardiography was performed with a Vevo 2100 High-Resolution Imaging System (Visual Sonics Inc, Toronto, Ontario, Canada) equipped with a 40-MHz transducer. Mice were anesthetized with 4% isoflurane, body fur was removed from the left anterior thorax, and ultrasound gel was applied. During imaging, mice were maintained under 2% isoflurane using a nose cone. Short-axis M-mode images were recorded at the papillary muscle level. Fractional shortening (FS) was calculated as follows: $FS (%) = \frac{[LV\ interior\ diameter\ diastole - LV\ interior\ diameter\ systole]}{LV\ interior\ diameter\ diastole} \times 100$. The calculations were performed on three different points in time of each mouse.

**Histological analysis**

For histological analysis, mice were anesthetized with isoflurane, and hearts were removed immediately, weighed, placed in 50mM KCl/PBS to induce diastolic arrest, fixed with 4% paraformaldehyde for 24 hours at 4°C, and embedded in paraffin. Sections were cut to 3-μm thickness using a rotary microtome (HM340E; Microm International GmbH, Walldorf, Germany).

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*Figure 1. Validation of lactation ability in PAH mice. A, Observation of milk bands in pups raised on milk from both WT and PAH mice at day 3 post-partum. Milk bands are marked by arrowheads. B, Average weights of 8 foster pups until weaning. The data are presented as mean ± SEM; n = 3-4 litters per group. #P < .05. C, Maternal systolic BP in nonpregnant (NP), pregnant, and postpartum mice. The data are presented as mean ± SEM; n = 3-4 mice per group. *P < .001 vs WT-NL group.*
After deparaffinization, the 3-μm-thick slices were stained with Masson’s trichrome reagent. Images were obtained using a BX53 microscope and DP21 digital camera (OLYMPUS, Tokyo, Japan). Cardiac fibrosis was quantified as percentage of blue area by using ImageJ software (National Institutes of Health, Bethesda, MD) in four randomly selected fields (magnification, ×200).

**Immunohistochemistry**

To assess macrophage infiltration, deparaffinized heart sections were stained with rat anti-F4/80 antibody (1:50; AbD Serotec, Oxford, UK) using biotinylated antirat IgG (Vector Laboratories, Burlingame, CA) and tyramide signal amplification (TSA) system (PerkinElmer, Waltham, MA) for detection and counterstained with Alexa Fluor 594-conjugated wheat germ agglutinin (Invitrogen, Carlsbad, CA) and Hoechst 33258. Fluorescence images were obtained using a BIOREVO BZ-9000 fluorescence microscope (Keyence, Osaka, Japan). The numbers of F4/80-positive cells in 10 different randomly chosen areas were determined using a BZ-II analyzer (Keyence) and ImageJ software (National Institutes of Health).

**Quantitative real-time PCR analysis**

Total RNA was extracted from frozen heart tissues using ISOGEN (NIPPON GENE Co, Tokyo, Japan). The cDNA was synthesized from 5 μg of total RNA using ReverTra Ace (Toyobo, Osaka, Japan). Gene expression was assessed using real-time PCR with SYBR-Green PCR master mix and the Thermal Cycler Dice Real Time System (TAKARA BIO Inc, Shiga, Japan). Transcript levels for TGF-β1, collagen I, TNF-α, IL-6, and monocyte chemoattractant protein-1 (MCP-1) were determined as the number of transcripts relative to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primer sequences are available in Supplemental Table 1 (published on The Endocrine Society’s Journals Online website at http://endo.endojournals.org).

**Statistical analysis**

Statistical comparison was performed using GraphPad Prism version 5 for Macintosh (GraphPad Software, San Diego, CA). Data were analyzed with Student’s t test, one-way ANOVA followed by Tukey’s post hoc test, and two-way ANOVA followed by Bonferroni’s post hoc test. Significant differences were defined as \( P < .05 \).

**Results**

**PAH mice have the ability to breastfeed their foster pups**

We previously showed that the fetuses from PAH mice exhibit severe IUGR at day 19 of gestation (E19) (13). To eliminate the influence of IUGR on lactation in PAH mice, WT and PAH mice were allowed to nurse foster pups from ICR mice. At day 3 postpartum, we observed milk in the stomachs of the pups reared by both WT and PAH mice (Figure 1A). The body weight gain was observed in the pups raised on milk from both WT and PAH mice, but the pups nursed by PAH dams were significantly smaller than pups nursed by WT dams (Figure 1B). The systolic BP was increased in PAH mice at E19 and was reduced to normal levels in both PAH-NL and PAH-Lac groups in the postpartum period (Figure 1C). These data indicate that PAH mice were able to breastfeed foster pups and that lactation does not affect BP in the postpartum period.

**Figure 2.** Effect of lactation on cardiac fibrosis in PAH mice. A, Representative Masson’s trichrome staining in heart sections. Magnification, ×200; scale bar, 100 μm. B, Bar graph summarizes quantification of fibrosis. The data are presented as mean ± SEM; \( n = 4-5 \) per group. \( * P < .05 \) vs WT-E19. C, Quantitative real-time PCR analysis of the TGF-β1 and collagen I genes in heart. The data are presented as mean ± SEM; \( n = 3-5 \) per group. \( # P < .001 \) vs WT-E19; \( \dagger P < .05 \) vs PAH-E19. n.s., not significant.
Lactation does not affect the alterations in cardiac hypertrophy and fibrosis in postpartum PAH mice

To examine the effect of lactation on cardiac remodeling, we assessed the heart weight and the degree of cardiac fibrosis during pregnancy and in the postpartum period. PAH mice exhibited increased heart weight and heart weight to body weight ratio compared with WT mice at E19, whereas reduction in heart weight was observed in both PAH-NL and PAH-Lac mice at 4W-PP compared with PAH mice at E19 (Supplemental Figure 1A). PAH mice showed marked fibrosis compared with WT mice at E19, and similar levels of fibrosis were observed in the hearts from PAH-NL and PAH-Lac mice at 4W-PP (Figure 2, A and B). No fibrosis was detected in nonpregnant WT and PAH (hAGT), WT-NL, and WT-Lac mice (Supplemental Figure 1B). At E19, TGF-β1 and collagen I mRNA expression levels in PAH mice were significantly higher than in WT mice. In contrast, elevated TGF-β1 and collagen I mRNA expression were decreased to normal levels in both PAH-NL and PAH-Lac mice at 4W-PP (Figure 2C). These data suggest that the cardiac fibrosis appearing during pregnancy is sustained until 4W-PP with or without lactation, although the collagen synthesis does not occur in the postpartum period.

Lactation does not affect the alterations in cardiac inflammation in postpartum PAH mice

Excessive activation of AT1 receptor signaling pathways plays a critical role in cardiac remodeling in PAH mice (15). Several proinflammatory cytokines play an important role in the onset and progression of angiotensin II-induced cardiovascular remodeling (16-19). The elevation of TNF-α, IL-6, and MCP-1 mRNA expression was observed in PAH heart compared with that in WT mice at E19. These increased levels of mRNA expression were significantly reduced in both PAH-NL and PAH-Lac groups at 4W-PP (Figure 3A). Consistent with change in the mRNA expression levels of MCP-1, a chemotactic cytokine to recruit macrophage into organs, immunohistochemical analysis revealed that elevated macrophage infiltration into cardiac tissues in PAH mice at E19 were improved in both PAH-NL and PAH-Lac groups at 4W-PP (Figure 3, B and C).

Lactation induces cardiac contractile dysfunction in lactating PAH mice

To investigate whether lactation affects postpartum cardiac function in PAH mice, we carried out echocardiographic analysis during pregnancy and in the postpartum period. There were no significant differences in FS between PAH mice and WT mice at E19. At 4W-PP, WT-NL, WT-Lac, and PAH-NL mice showed normal FS, but PAH-Lac mice exhibited significant decreases in cardiac contractility compared with PAH-NL mice (Figure 4).

Discussion

In the present study, we investigated the effect of lactation on cardiac remodeling and function in postpartum PAH mice. We found that PAH mice are able to breastfeed and that lactation plays an important role for the onset of
cardiac contractile dysfunction, whereas lactation does not affect the alterations in cardiac remodeling in postpartum PAH mice.

Because foster pups nursed by PAH dams showed continuous growth, PAH dams have the ability of breastfeeding by using foster pups. Therefore, we thought that effect of lactation on postpartum PAH dams is assessable by comparison between lactating and NL groups. However, significant reduction in body weight of foster pups was observed in contrast with pups nursed by WT dams. Because foster pups nursed by WT and PAH dams were born from ICR mice in this study, the difference in foster pups’ growth between both groups might be due to the amount of milk ejection, nutrient quality of milk, and/or frequency of breastfeeding. Although it has been reported that angiotensin II-AT1 receptor signaling plays a critical role in mouse postlactational mammary gland involution (20), the role of activated AT1 receptor signaling during pregnancy in the development of mammary gland and lactation behavior remains unclear. Additional research will be required for determining the cause of suppressed growth of foster pups in the PAH group.

Because lactation is closely involved with endocrine alterations (21, 22), the change in the balance of circulating hormones might influence cardiac function. Recent studies indicate the beneficial effects of lactation on cardiovascular diseases in postpartum women (9, 10), whereas prolactin, which is necessary for maternal behavior and milk production, is cleaved by cathepsin D under excessive oxidative stress in the postpartum heart and its proteolytic product 16-kDa prolactin induces PPCM (11). Thus, the effect of lactation on postpartum cardiac function remains a matter of debate. In this study, we found that lactating PAH mice exhibited reduced FS, whereas lactating WT and NL PAH mice did not. This finding clearly shows that lactation has adverse effects on cardiac function in PAH mice. Because hypertension and cardiac inflammatory response are improved after delivery in PAH mice, other factors induced by hypertension during pregnancy can change the effect of lactation to adverse action on cardiac function. Therefore, persistent fibrosis in the postpartum period might be involved in the reduction of cardiac contractility depending on lactation in PAH mice, although other possibilities are not excluded.

Associations between hypertension during pregnancy and PPCM have been well described by many studies (4, 10, 23, 24). For instance, hypertensive disorders during pregnancy, in particular preeclampsia, have been reported in a higher frequency (approximately 40%) of women with PPCM in the United States and Japan (4, 25). Soluble fms-like tyrosine kinase 1 (sFlt1) is an anti-angiogenic protein released from placenta, and elevated circulating levels of sFlt1 have been observed in women with preeclampsia (26, 27). Interestingly, Patten et al (28) reported that sFlt1 causes cardiomyopathy, and plasma sFlt1 levels are high in women with PPCM at 4-6 weeks postpartum. These indicate that elevated sFlt1 levels in preeclampsia contribute to at least the PPCM that is associated with preeclampsia. Thus, up-regulation of sFlt1 induced by preeclampsia is associated with postpartum cardiac function. We previously reported that plasma sFlt1 levels at E19 are significantly higher in PAH dams than those in WT dams (29), but the role of sFlt1 in lactating PAH mice is still unknown. Further investigation is necessary to reveal the mechanisms underlying the induction of contractile dysfunction by lactation in PAH mice.

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