Calcineurin activity is required for cardiac remodelling in pregnancy

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**Aims** Calcium fluctuations and cardiac hypertrophy occur during pregnancy, but the role of the well-studied calcium-activated phosphatase, calcineurin, has not been studied in this setting. The purpose of this study was to determine whether calcineurin signalling is required for cardiac remodelling during pregnancy in mice.

**Methods and results** We first examined calcineurin expression in the heart of mice during pregnancy. We found both calcineurin levels and activity were significantly increased in early-pregnancy and decreased in late-pregnancy. Since progesterone levels start to rise in early-pregnancy, we investigated whether progesterone alone was sufficient to modulate calcineurin levels in vivo. After implantation of progesterone pellets in non-pregnant female mice, cardiac mass increased, whereas cardiac function was maintained. In addition, calcineurin levels increased, which is also consistent with early-pregnancy. To determine whether these effects were occurring in the cardiac myocytes, we treated neonatal rat ventricular myocytes (NRVMs) with pregnancy-associated sex hormones. We found that progesterone treatment, but not oestradiol, increased calcineurin levels. To obtain a functional read-out of increased calcineurin activity, we measured the activity of the transcription factor NFAT, a downstream target of calcineurin. Progesterone treatment significantly increased NFAT activity in NRVMs, and this was blocked by the calcineurin inhibitor cyclosporine A (CsA), showing that the progesterone-mediated increase in NFAT activity requires calcineurin activity. Importantly, CsA treatment of mice completely blocked pregnancy-induced cardiac hypertrophy.

**Conclusion** Our results show that calcineurin is required for pregnancy-induced cardiac hypertrophy, and that calcineurin activity in early-pregnancy is due at least in part to increased progesterone.

**Keywords** Calcineurin • Cardiac hypertrophy • Pregnancy • Progesterone • NFAT

1. Introduction

Serum calcium levels vary during pregnancy. For example, they are significantly higher in the first and second trimester, but are significantly lower in the third trimester, compared with non-pregnant (NP) controls. Epidemiological studies suggest that calcium supplementation during pregnancy is highly correlated with a reduced risk of pregnancy-induced hypertension and pre-eclampsia, which may be due to modifying intracellular calcium concentrations.

Calcium activates calcineurin, which dephosphorylates cytoplasmic nuclear factor of activated T cells (NFAT), inducing translocation of NFAT to the nucleus. In the heart, NFAT activates pro-hypertrophic target genes. In particular, calcineurin has been implicated as an important player in pathological hypertrophy and heart disease. As shown in numerous studies, calcineurin activity is elevated in the hearts of patients with cardiac hypertrophy and heart failure, and inhibition of this pathway delays the progression to pathological heart failure in animal models. In contrast, its role in physiological cardiac hypertrophy has not been clearly established. For example, cardiac calcineurin activity as assessed in cardiac NFAT reporter mice does not change after exercise. Consistent with a lack of calcineurin activation in exercise, calcineurin protein levels are reduced after voluntary wheel running training in mice. One study reported that CsA does not block exercise-induced cardiac hypertrophy. In contrast, Eto et al. reported that calcineurin activity is significantly increased in rats after voluntary wheel running training and treatment with CsA completely blocks exercise-induced cardiac hypertrophy. These results are inconclusive as to the role of calcineurin in exercise-induced cardiac remodelling. The role of calcineurin in cardiac remodelling over the time course of pregnancy has not been addressed until the current study.

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While progesterone is a critical hormone for establishing and maintaining pregnancy, it can also induce protein synthesis in cardiac muscle and hypertrophy in isolated cardiac myocytes. Moreover, it has been shown that progesterone modifies intracellular calcium concentrations in a cell type-dependent manner. For example, progesterone stimulates a rapid influx of extracellular calcium and thus increases intracellular-free calcium concentrations in T cells. In contrast, progesterone significantly attenuates intracellular calcium concentrations in myometrial cells and vascular smooth muscle cells.

We have recently established that although exercise and pregnancy are both considered physiological stimuli for the heart, they have distinct transcriptional profiles. Since there are known alterations in serum calcium during pregnancy and calcineurin plays a critical role in other forms of cardiac hypertrophy, we investigated the role of calcineurin in the response of the heart to pregnancy in this study. Furthermore, because progesterone has been shown to modulate intracellular calcium concentrations, we examined the role of progesterone in calcineurin signalling by implanting time-release progesterone pellets in normally cycling virgin female mice. Finally, we assessed the role of progesterone in regulating endogenous calcineurin activity in the cardiac myocytes. Most importantly, inhibition of calcineurin by CsA during pregnancy blocked pregnancy-induced cardiac hypertrophy. Taken together, these results show that activation of calcineurin in early-pregnancy, which may be mediated partially by the progesterone surges in early-pregnancy, is required for pregnancy-mediated cardiac hypertrophy.

2. Methods

2.1 Animals and procedures

We used 3- to 4-month-old female C57Bl/6 mice. We previously described our processes of mating and pregnancy. We studied pregnant mice at 7 days of gestation (early pregnancy, EP), 11 days of gestation (mid pregnancy, MP), and 18–19 days of gestation (late pregnancy, LP). Virgin female mice in diestus served as NP controls.

Ten to fifty mg/kg/day of CsA attenuates or blocks various pathological stimuli and transgenic mouse models of hypertrophic cardiomyopathy. Groth et al. showed that 30 mg/kg/day of CsA results in resorptions of ~50% of foetuses. Thus, we subjected mice to daily subcutaneous injections of CsA (25 mg/kg/day; 10 days for MP or 18 days for LP) beginning on the first day of pregnancy. Since 25 mg/kg/day of CsA caused a large number of pregnant mice to reabsorb their pups, we injected mice with 10 mg/kg/day of CsA. Low doses of CsA did not affect pregnancy or cardiac hypertrophy. Further, 25 mg/kg/day of CsA injected for 10 days between Days 9 and 18 of gestation also did not block pregnancy-induced cardiac hypertrophy. Thus, we report the results of 25 mg/kg/day CsA administration, which was injected from the first day of pregnancy.

We randomly divided 3-month-old normally cycling virgin female C57Bl/6 mice into two groups. We implanted one group of mice with time-release progesterone pellets (Innovative Research of America, Sarasota, FL, USA), and the other group with placebo pellets. Each pellet contained 25 mg of either progesterone or placebo. The pellets were designed to release their contents over a 21-day period (25 mg/21-day release pellet), and mice were exposed to pellets for 19 days.

We housed animals in a temperature- and light-controlled room with food and water available ad libitum. At a given time point, we weighed the mice and then sacrificed them by cervical dislocation after inhalation of isoflurane. We handled and euthanized all of the animals according to the guidelines of the University of Colorado Animal Use and Care Committee, consistent with regulations for vertebrate animal research outlined by the National Institutes of Health. The University of Colorado at Boulder Animal Care and Use committee approved protocol #1002.08.

2.2 Cardiac tissue collection and western blot analysis

We prepared and homogenized left ventricular (LV) tissue as described previously. The calcineurin antibody was from BD Transduction laboratories (cat# 610 260), and GAPDH was from cell signalling (cat# 2118). We used four to six animals per group each with three technical replicates per animal, and a representative blot is shown. Calcineurin activity was measured with a colorimetric assay (Enzo: BML-AK816) according to the manufacturer’s instructions. Phosphatase activity was measured by detection of free phosphate released from the calcineurin-specific Rl phosphatopeptide using the BIOMOL green reagent, and normalized to protein content. We performed all assays in triplicate in three independent experiments.

2.3 Quantitative real-time PCR (qRT–PCR) and assessment of LV functions by echocardiography

See Supplementary material online, methods.

2.4 Measurements of serum progesterone and oestradiol levels

We measured serum oestradiol and progesterone levels with a Beckman Coulter Access II (UCH-CTRC Lab, Denver, CO, USA) as described previously.

2.5 Treatment of neonatal rat ventricular myocytes

Detailed methods have been described previously. In brief, we serum starved cells for 24 h before progesterone (94.34 ng/mL) and/or oestradiol (136.19 pg/mL) treatment. To determine endogenous NFAT activity in neonatal rat ventricular myocytes (NRVMs), we co-infected with adenoviruses (multiplicity of infection of 100) encoding the NFAT-luciferase reporter gene and cytomegalovirus promoter (CMV)/β-galactosidase (a control for infection efficiency). The next day, we removed the viruses by washing the cells with serum-free media, and followed this with different treatments, including oestradiol, progesterone, the combination of oestradiol and progesterone, CsA (50 ng/μL), and the combination of CsA and progesterone. We lysed the cells after 48 h of treatment and collected them for luciferase and β-galactosidase assays. We calculated relative luciferase activities by normalizing luciferase activities to internal control CMV/β-galactosidase activities. All infection data are the means ± standard error of mean (SEM) of three independent experiments each performed in triplicate.

2.6 Data and statistical analysis

All results are expressed as mean ± SEM. We tested statistical significance with either Student’s t-test or analysis of variance followed by Fisher’s least significant difference (LSD) for multiple group comparisons. P < 0.05 was regarded as significant among groups.

3. Results

3.1 Calcineurin activity increases in EP

In agreement with previous studies, pregnancy is associated with significant cardiac hypertrophy during MP and LP (Figure 1A and Supplementary material online, Table S1). While the calcium-activated phosphatase, calcineurin, has been strongly implicated in mediating pathological cardiac hypertrophy, it is not known whether calcineurin is involved in pregnancy-induced cardiac hypertrophy. It is logical to assess calcineurin activity during pregnancy since serum calcium levels...
are high in EP and MP compared with NP. Furthermore, progesterone modifies intracellular calcium concentrations13 and progesterone levels start to rise in EP.19,20 For example, progesterone levels begin to rise and reach a plateau in EP (34–54 ng/mL),19,20 rise again and peak at Day 15–16 (82–113 ng/mL),12,20 and gradually decrease to a low value (<0.8 ng/mL) at immediate postpartum.20 We first measured the levels of calcineurin protein by western blot analysis. The levels of calcineurin were significantly increased in EP, but returned to NP levels by MP, and fell significantly below NP levels during LP (Figure 1B). Calcineurin phosphatase activity paralleled the changes observed in protein levels (Figure 1C). As a downstream target of calcineurin, the transcription factor NFAT is dephosphorylated and thus activated by calcineurin. Once activated, NFAT up-regulates the expression of numerous target genes, including the modulatory calcineurin-interaction protein (MCIP)1,4.21 Thus, as a read-out of NFAT activity, we measured MCIP1.4 mRNA levels by qRT–PCR in hearts at various stages of pregnancy. MCIP1.4 levels were significantly increased in EP, but significantly decreased in LP (Figure 1D), which corresponded to the levels and activity of calcineurin shown in Figure 1B and C. Taken together, our data show that calcineurin levels and activity are dynamically regulated during pregnancy.

3.2 Progesterone pellet implantation increases serum progesterone levels in normally cycling mice

Given its role in modulating intracellular calcium concentrations in cells, we tested whether progesterone could activate calcineurin and its downstream targets, in the absence of pregnancy by implanting time-release progesterone pellets in normally cycling virgin female C57BL/6 mice. Nineteen days of progesterone pellet implantation significantly increased body weight (BW) compared with the placebo control mice (Figure 2A and Supplementary material online, Table S2). This increase in BW may be partially due to an increase in plasma volume.11 Uterine weight was not significantly different in the progesterone group compared with the placebo control group (Figure 2B). Next, we measured serum progesterone and oestradiol levels. Serum progesterone levels were significantly higher in the progesterone group compared with the placebo control group (Figure 2C), whereas serum oestradiol levels were not altered (Figure 2D). However, progesterone levels achieved with pellet implantation (13.12 ± 0.12 ng/mL) were not as high as EP (≏34–54 ng/mL).20

3.3 Progesterone induces physiological cardiac hypertrophy with increases in calcineurin levels

The per cent increase in LV mass normalized to tibial length was significantly greater (12%) in the progesterone group compared with the placebo control group. By comparison, pregnancy-induced cardiac hypertrophy was 20% in MP and 26% in LP (Figure 3A). We measured the activation states of several signalling pathways that have been implicated in pregnancy-induced cardiac hypertrophy, including Akt, GSK3β, ERK1/2, and p38.12 We found that progesterone treatment of mice did not alter the activation states of cardiac Akt, GSK3β, ERK1/2, and p38...
Calcineurin in the pregnant heart

Figure 2 The effects of progesterone treatment on physical characteristics in normally cycling virgin female mice. (A) BW was significantly increased with progesterone treatment. Filled square, progesterone group; open square, placebo group. (B) Uterine weight was not changed with progesterone pellet implantation compared with placebo. (C) Progesterone pellet implantation increased serum progesterone levels, (D) without altering serum oestradiol levels. n = 5 for placebo and n = 6 for progesterone. Values are mean ± SEM. * P < 0.05, significantly different from the placebo group.

(data not shown), but significantly increased calcineurin levels compared with the placebo control group (Figure 3B). However, these changes in the levels of calcineurin protein did not result in significant changes in calcineurin activity due to very large variability (Figure 3C). Interestingly, in contrast to pregnancy, MCIP 1.4 levels were significantly decreased in the progesterone-treated group (Figure 3D). Thus, progesterone significantly increased cardiac mass and increased calcineurin levels but did not recapitulate the entire pregnancy response of the heart.

Calcineurin signalling has been implicated as an important player in pathological cardiac hypertrophy. Since decreased cardiac function and re-expression of foetal genes are typically associated with pathological cardiac hypertrophy, we evaluated cardiac function by echocardiography (Supplementary material online, Figure S1 and Supplementary material online, Table S2) in placebo and progesterone-treated mice. All functional parameters related to M-mode echocardiography were not significantly different at pre-implantation. However, progesterone-treated mice had increased wall thickness after 19 days of progesterone treatment. Increased wall thickness was not accompanied by cardiac dysfunction as assessed by the per cent fractional shortening (Supplementary material online, Figure S1 and Supplementary material online, Table S2), suggesting that progesterone did not change haemodynamic function. Next, we profiled the expression of a number of genes that are typically associated with pathological cardiac hypertrophy. We and others have reported that atrial natriuretic peptide, brain natriuretic peptide, α-myosin heavy chain (MyHC), and β-MyHC are not altered in pregnancy. In agreement with the pregnancy profiles of cardiac gene expression, these genes were not altered by progesterone treatment (Supplementary material online, Figure S2).

Increased fibrosis and an imbalance between angiogenesis and cardiac muscle mass have been suggested to contribute to the progression to heart failure from adaptive cardiac hypertrophy. Previously, we and other group showed that there is no induction of fibrosis in the hearts of pregnant mice. To further characterize progesterone-induced cardiac hypertrophy, we measured collagen and angiogenic factor mRNA levels. mRNA levels of collagens including Col1a1, Col3a1, and Col8a1 were not altered, whereas connective tissue growth factor (CTGF) mRNA levels were significantly decreased by progesterone treatment (Supplementary material online, Figure S3). Thus, our data are consistent with our hypothesis that progesterone-induced cardiac hypertrophy is not of a pathological nature since it appears not to be associated with activation of pro-fibrotic gene expression.

Next, we measured mRNA levels of angiogenic factors, including PGC-1α (Pgc1a), vascular endothelial growth factor a (Vegfa), angioptelin-1 (Angpt1), Angpt2, fibroblast growth factor 2 (Fgf2), and platelet-derived growth factor alpha (Pdgfa). Previous studies showed that myocardial angiogenesis, as assessed by increases in capillary density per cardiomyocyte and Vegfa, is significantly increased during pregnancy. On the other hand, anti-angiogenic signalling is induced in LP and a greater increase in anti-angiogenic genes is highly associated with peripartum cardiomyopathy. In addition, it has been shown that progesterone induces angiogenesis by up-regulation of Vegfa in myometrial cells. In agreement with previous studies, Pgc1a, Vegfa, Angpt1, and Fgf2 mRNA levels were significantly increased in either EP or MP and returned to the NP level in LP (Supplementary material online, Figure S4). Unexpectedly, Pgc1a and Vegfa were significantly down-regulated by progesterone treatment.

3.4 Calcineurin-NFAT signalling is up-regulated by progesterone treatment in NRVMs

It has been shown that progesterone modifies intracellular calcium concentrations, but in a tissue-specific manner. However, there has been relatively little work done to evaluate progesterone-mediated
signalling pathways in the context of cardiac myocytes. Thus, we sought to determine the role of sex hormones (oestradiol and/or progesterone) in the regulation of the calcineurin signalling pathway in NRVMs. We found that calcineurin levels were significantly increased in NRVMs treated with 94.34 ng/mL of progesterone, which is a level that can be achieved by mice during pregnancy (Figure 4A). We assessed endogenous calcineurin activity using an NFAT-luciferase reporter as a read-out. NRVMs were infected with an adenovirus expressing an NFAT-luciferase reporter gene (AdNFAT4-Luc) and subsequently treated with oestradiol, progesterone, and the combination of progesterone and oestradiol. NRVMs treated with progesterone and the combination of progesterone and oestradiol had significantly elevated NFAT-luciferase activity when compared with the vehicle-treated group (≥ 2 fold) (Figure 4B). We next tested whether the progesterone-mediated increase in NFAT activity required calcineurin by treating NRVMs with CsA. As indicated in Figure 4B, CsA completely blocked the progesterone-mediated increase in NFAT activity. However, similar to the in vivo progesterone implant study (Figure 3D), progesterone significantly decreased MCIP1.4 mRNA levels (Figure 4C). Taken together, our results provide evidence that progesterone activates calcineurin signalling in cardiac myocytes, culminating in activation of NFAT.

### 3.5 Calcineurin inhibition blocks pregnancy-induced cardiac hypertrophy

Since our data showed that progesterone increased NFAT activity through calcineurin signalling in cardiac myocytes, we sought to determine whether calcineurin activity is required for pregnancy-induced cardiac hypertrophy. To address this, we administered CsA (25 mg/kg/day) daily, beginning on the first day of pregnancy, and sacrificed mice at MP and LP because those time points were associated with significant cardiac hypertrophy in untreated mice (Figure 1A and Supplementary material online, Table S1). We began the experiments on 15 mice per group, but only 7 of the 15 mice were able to sustain pregnancy. This agrees with a previous study demonstrating that CsA (20–30 mg/kg/day) injected during pregnancy in mice correlated with reduced implantation frequency and increased the rate of foetal death. Although we had a small number of mice per group, our results demonstrated that inhibiting calcineurin activity (Figure 5A) at the beginning of pregnancy completely blocked cardiac hypertrophy (Figure 5B). Next, we measured whether inhibition of calcineurin blocked the activation states of cardiac ERK1/2, Akt, and p38 that have been shown to be altered during pregnancy. For example, we demonstrated that phosphorylation levels of pro-survival molecules ERK1/2 and Akt are significantly increased, while the levels of stress-kinase p38 are significantly decreased during pregnancy. Here, we found that calcineurin inhibition blocked pregnancy-associated increase in ERK1/2 in MP and fell significantly below NP levels in LP (Figure 5C). In addition, calcineurin inhibition blocked pregnancy-associated increase in phosphorylation levels of Akt (Figure 5D). In contrast, calcineurin inhibition did not de-repress p38 during pregnancy (Figure 5E). Taken together, our results show that calcineurin is required for cardiac hypertrophy in the early stages of pregnancy and that ERK and Akt may be important mediators in this process.
4. Discussion

The calcineurin signalling pathway has been implicated as an important player in pathological cardiac hypertrophy,7 while its role in exercise-induced cardiac hypertrophy remains controversial.7,8,10 A sustained increase in intracellular calcium activates calcineurin, a phosphatase that dephosphorylates the important transcription factor, NFAT, and thereby induces hypertrophic genes.27 To our knowledge, this is the first study to explore the role of calcineurin signalling over the time course of pregnancy. As summarized in Figure 6, calcineurin levels and activity are increased in EP and that increase in calcineurin is required for pregnancy-mediated cardiac hypertrophy by the rise in progesterone in early pregnancy.19,20 To test this hypothesis, progesterone pellets were implanted in normally cycling virgin female mice, and we found that progesterone induces cardiac hypertrophy and increases calcineurin levels. Finally, pregnancy-induced cardiac hypertrophy is completely blocked with CsA treatment. Further, calcineurin inhibition also blocks ERK1/2 and Akt activation. These results imply that calcineurin activation transiently initiates the pathways responsible for the development of physiological hypertrophy in pregnancy.

While most studies conclude that sustained calcineurin activation plays a key role in the transition from hypertrophy to heart failure,27 Rimbaud et al.28 conclude that calcineurin is not involved in pregnancy-induced cardiac hypertrophy by showing a three-fold decrease in MCIP1 expression in LP.28 Their result agrees with our results in LP. However, we have also demonstrated that calcineurin is dynamically regulated during pregnancy, since, in contrast to LP, it is up-regulated in EP. Given the potent negative impact of constitutively active calcineurin activity on the heart, continuous activation of calcineurin would most likely lead to deleterious effects on the hearts of pregnant mice. Pregnancy-induced cardiac hypertrophy displays many of the hallmarks of physiological hypertrophy,12 and this seems likely to be achieved at least in part by the biphasic regulation of calcineurin during pregnancy. It has been shown that treatment of mice with oestradiol significantly attenuates pathological cardiac hypertrophy by degradation of calcineurin.29,30 Here, we show that calcineurin activity is significantly decreased in LP (below NP levels), at the time that oestradiol levels are highest.12 Thus, a significant decrease in calcineurin levels and activity in LP may be due to the surge of oestradiol.12 The regression of pregnancy-induced cardiac hypertrophy occurs by 7 days postpartum,31 and calcineurin levels and activity are at NP levels by MP and significantly below NP in LP. Thus, we can speculate that the signalling cascades governing cardiac regression are already activated in LP. Further efforts to elucidate the role of oestradiol in cardiac regression could provide additional insights into the signalling pathways governing cardiac regression following parturition.

Interestingly, our results in NRVMs showed that oestradiol did not block the progesterone-mediated increase in calcineurin activity (Figure 4A and B, combination). The fact that oestradiol did not affect the progesterone-mediated increase in calcineurin activity in NRVMs shown in Figure 4A and B is contradictory to the situation in LP in which calcineurin activity is lowest (Figure 4B–D). Although we...
treated NRVMs with 136.19 pg/mL (500 pM) oestradiol (the serum level in mice in LP12), it may be that different concentrations are needed for studies in NRVMs. For example, 10 nM oestradiol blocks phenylephrine-induced cardiac hypertrophy in NRVMs29 and induces the MCIP1.4 gene.32

Our in vivo experiments using progesterone pellet implantation provide further insight into a novel mechanism by which progesterone could activate calcineurin signalling. Increased calcineurin expression levels after progesterone treatment as shown in Figure 3B lend support to our hypothesis that the up-regulation of calcineurin in EP is mediated by increased progesterone levels. Unexpectedly, unlike in pregnancy (Figure 1C and D), calcineurin activity and MCIP 1.4 mRNA levels in progesterone-treated hearts (Figure 3C and D) and NRVMs (Figure 3C) were not consistent with calcineurin protein expression levels. As shown in Figure 3C, calcineurin activity was not different between the placebo and progesterone groups, mainly due to the large variability in the progesterone group. Progesterone treatment significantly decreased MCIP 1.4 mRNA levels (Figure 3D), which contradicts what we saw in hearts of pregnant mice (Figure 1D). Further, Pgc1a and Vegfa were significantly down-regulated in the progesterone

**Figure 5** CsA blocks pregnancy-induced cardiac hypertrophy with blocking the induction of ERK and AKT. (A) The per cent increase in calcineurin activity was significantly higher in EP and lower in LP (-CsA). CsA blocked calcineurin activity (+CsA) in MP. (B) The per cent increase in LV/TL was significantly higher in MP and LP compared with NP. The CsA treated group (+CsA) did not exhibit pregnancy-induced cardiac hypertrophy. (C) ERK1/2 phosphorylation was significantly decreased in LP, but (D) Akt phosphorylation was not changed during pregnancy with CsA treatment. (E) p38 phosphorylation was significantly decreased in MP with CsA treatment. Values are means ± SEM. *P < 0.05, significantly different from NP.
group, while these were significantly increased in EP and MP (Supplementary material online, Figure S4). These differing results may be due to the fact that progesterone levels during pregnancy are bi-phasic [significantly up in EP with a significant drop before they reach peak levels in between MP and LP (15–16 days of gestation)20], while progesterone levels from pellet-treated mice or NRVMs are constant. Supporting our assumption, a previous study demonstrated that long-term progesterone exposure suppresses VEGF/FGF2-driven endothelial cell proliferation, while the progesterone surge in EP up-regulates VEGF in myometrial cells.26

Although MCIP1.4 is often used as an indicator of NFAT activation by calcineurin, it is apparent that the regulation of calcineurin activity and MCIP is complex. Donaldson et al.29 demonstrated that increased MCIP1.4 mRNA levels are correlated with increased LV mass and calcineurin protein expression in mice following pressure overload, which agrees with our pregnancy data (Figure 1). Conversely, Pedram et al.32 demonstrated that endothelin-1-induced hypertrophy is accompanied by increased calcineurin activity and increased NFAT-luciferase activity but by decreased MCIP 1.4 levels in NRVMs. These MCIP1.4 results agree with our data from progesterone-treated hearts (Figure 3D). In addition, they showed that 10 nM of oestradiol induces MCIP1.4 genes.32 In fact, our NRVMs treated with 500 pM of oestradiol leads to a trend in increased MCIP1.4 levels (Figure 4C). Thus, a previous study32 and our result from NRVMs suggest that progesterone is actually blocking the effects of oestrogen on MCIP 1.4 expression.

We recently demonstrated that progesterone induces cellular hypertrophy through the activation of ERK1/2.12 In the current study, we also showed that progesterone activates calcineurin in cardiomyocytes (Figure 4A). Studies from gain- and loss-of-function in genetically altered mice and in isolated cardiomyocytes have demonstrated that calcineurin and ERK1/2 signalling pathways are inter-dependent such that activation of calcineurin in myocytes leads to up-regulation in ERK1/2 signalling.34 Isoproterenol-induced activation of ERK1/2 is significantly attenuated by CsA treatment, and the hearts of mice that overexpress the dominant negative form of calcineurin cannot activate ERK1/2 in response to isoproterenol. In addition, sustained Akt activation is inhibited by CsA in mesangial cells.35 We showed here calcineurin inhibition by CsA treatment during pregnancy blocked ERK1/2 (Figure 5C) and Akt activation (Figure 5D). Thus, our results from NRVMs in the current study (Figure 4A), in a previous study,12 and CsA treatment during pregnancy support the previous notion that calcineurin, ERK1/2, and Akt are inter dependent.

CsA has been used as a powerful immunosuppressive agent for organ transplantation and autoimmune disease treatments.36 While the number of pregnancies in patients undergoing CsA therapy has grown with a positive outcome,37,38 these pregnancies are often associated with increased risks of pregnancy-induced hypertension, pre-eclampsia, miscarriage, and preterm delivery.39 The present findings may have important clinical implications because blocking calcineurin signalling in EP prevents the initiation of the pathways responsible for the development of physiological hypertrophy; thus, the maternal heart cannot deal with the stress of pregnancy.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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**Figure 6** A model summarizing calcineurin activation during pregnancy-induced cardiac hypertrophy. See detailed information in Section 4.
injections in mice. We also thank Dr Kristen K. Barthel for critical reading of the manuscript.

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References
10. Chung E, Heimiller J, Leinwand LA. Distinct cardiac transcriptional profiles defining pregnancy-induced hypertrophy in mice. We also thank Dr Kristen K. Barthel for critical reading of the manuscript.