Dietary Vitamin D Restriction in Pregnant Female Mice Is Associated With Maternal Hypertension and Altered Placental and Fetal Development

Nancy Q. Liu, Yi Ouyang, Yasemin Bulut, Venu Lagishetty, Shiao Y. Chan, Bruce W. Hollis, Carol Wagner, Ozlem Equils, and Martin Hewison

Orthopaedic Hospital Research Center (N.Q.L., Y.B., V.L., M.H.) and Molecular Biology Institute (M.H.), University of California, Los Angeles, Los Angeles, California 90095; Department of Pathology (Y.O.), Veterans Affairs Medical Center, Long Beach, California 90822; School of Clinical and Experimental Medicine (S.Y.C.), University of Birmingham, Birmingham B15 2TT, United Kingdom; Departments of Pediatrics, Biochemistry, and Molecular Biology (B.W.H., C.W.), Medical University of South Carolina, Charleston, South Carolina 29425; and Medical Division (O.E.), Pfizer Inc, New York, New York 10017

Epidemiology has linked vitamin D deficiency with preeclampsia in humans. We hypothesized that low vitamin D status in pregnant mice may lead to symptoms of preeclampsia. Female BL6 mice were raised on vitamin D-sufficient or -deficient diets from weeks 4 of age and then mated with vitamin D-sufficient BL6 males at week 8. The resulting pregnant mice were either allowed to deliver pups and monitored for blood pressure (BP) and weight of offspring or euthanized at day 14 or 18 of gestation (E14 or E18) for analysis of serum, placental/kidney tissues, and fetuses. At E14 serum concentrations of 25-hydroxyvitamin D (30.1 $\pm$ 5.0 vs 1.8 $\pm$ 0.6 ng/mL, $P < .001$) and 1,25-dihydroxyvitamin D (119.5 $\pm$ 18.7 vs 37.4 $\pm$ 5.1 pg/mL, $P < .01$) were higher in sufficient vs deficient pregnant mice. At E14 BP was significantly elevated in vitamin D-deficient pregnant mice relative to vitamin D-sufficient mice for both systolic BP (124.89 $\pm$ 2.28 vs 105.34 $\pm$ 3.61 mm Hg, $P < .001$) and mean arterial pressure (115.33 $\pm$ 1.93 vs 89.33 $\pm$ 5.02 mm Hg, $P < .001$). This elevation continued through pregnancy until 7 days postpartum (PP7) but returned to baseline by PP14. Analysis of maternal kidneys showed increased expression of mRNA for renin and the angiotensin II receptor (3- and 4-fold, respectively) in vitamin D-deficient vs -sufficient mice at E14. Histological analysis of E14 placentas from vitamin D-deficient mice showed decreased vascular diameter within the labyrinth region. E14 and E18 fetuses from vitamin D-deficient mice were larger than those from vitamin D-sufficient mothers. However, by PP14 pups from vitamin D-deficient mothers weighed significantly less than those from vitamin D-sufficient mothers. Resupplementation of vitamin D periconceptually partially reversed the effects of vitamin D deficiency. These data provide further evidence that low vitamin D status may predispose pregnant women to dysregulated placental development and elevated blood pressure. **(*Endocrinology* 154: 2270–2280, 2013)**

Preeclampsia, typically characterized by maternal hypertension, proteinuria, and a variation of other signs and symptoms, is a leading cause of preterm delivery. Preeclampsia complicates up to 10% of all pregnancies, 3% severely with potential life-threatening consequences (1). Impaired vitamin D status appears to be prevalent among pregnant women in general (2), and epidemiology suggests that there is a link between vitamin D insufficiency and preeclampsia (3–5). A longitudinal study of 697 pregnant women showed an increased incidence of preeclampsia with lower maternal vitamin D levels measured at 24–26 weeks' gestation (6). Likewise, a retrospective analysis of

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

Received December 27, 2012. Accepted April 30, 2013.
First Published Online May 15, 2013

Abbreviations: Agtr1a, angiotensin II receptor; BP, blood pressure; Ct, cycle threshold; 25D, 25-hydroxyvitamin D; 1,25D, 1,25-dihydroxyvitamin D; Def, vitamin D-deficient diet; Def+, vitamin D resupplemented diet; E, embryonic day; H&E, hematoxylin and eosin; MAP, mean arterial pressure; PP, days postpartum; RAAS, renin-angiotensin-aldosterone system; Ren1, renin; SBP, systolic BP; Suff, normal, vitamin D-sufficient diet; UCLA, University of California, Los Angeles; Vdr, vitamin D receptor.

nulliparous pregnant women taking part in the Norwegian Mother and Child Cohort Study found that vitamin D supplementation during pregnancy was associated with a 27% reduction in the risk of preeclampsia compared with those who were not on supplements (7). Vitamin D insufficiency is defined by serum levels of 25-hydroxyvitamin D (25D), the inactive form of the vitamin that is metabolized to active 1,25-dihydroxyvitamin D (1,25D) by the enzyme *CYP27B1* (8). Synthesis of 1,25D occurs primarily in the kidneys, but expression of *CYP27B1* has been described for several extrarenal tissues including the placenta, suggesting a more localized function for 25D metabolism at this site.

The precise mechanism by which variations in circulating 25D may be able to influence the pathophysiology of preeclampsia is unclear. In studies using trophoblast cells from the placentas of preeclampsia pregnancies, decreased expression of *CYP27B1* has been reported relative to normal placentas, suggesting that preeclampsia is associated with aberrant localized synthesis of active 1,25D from precursor 25D (9). Synthesis of 1,25D is also dependent on the availability of 25D as substrate for *CYP27B1* so that the placental production of active vitamin D may be further compromised by low levels of maternal 25D in preeclampsia. Maternal vitamin D status may influence preeclampsia through a variety of mechanisms including immunomodulatory, inflammatory, and angiogenic responses (10, 11). Although the immunomodulatory properties of 25D and 1,25D have been postulated as one of the main factors mediating the link between vitamin D and preeclampsia (12), there have been few functional studies to test this hypothesis.

Vitamin D may also influence preeclampsia through regulation of the renin-angiotensin-aldosterone system (RAAS), the regulatory cascade that plays a key role in regulating blood pressure (BP), electrolyte balance, and body fluid homeostasis (13, 14). Tight regulation of renin synthesis and secretion is essential for effective regulation of BP, and inappropriate stimulation of the RAAS has been associated with hypertension, myocardial infarction, and stroke outside pregnancy (15). The enzyme renin cleaves angiotensinogen to angiotensin I, which is then further cleaved by the enzyme angiotensin-converting enzyme to biologically active angiotensin II (13). Several recent studies have shown that serum levels of 1,25D are inversely associated with BP or plasma renin activity (16–21), with vitamin D acting as a negative regulator of the RAAS by direct transcriptional suppression of renin gene expression (21). Studies in vivo have shown that ablation of the murine vitamin D receptor (*Vdr*) gene activates the RAAS and leads to accumulation of angiotensin II (22). Based on these observations, it has been postulated that the vitamin D system plays a key role in preventing hypertension (23). However, the functional impact of vitamin D status on maternal BP during pregnancy has yet to be studied in detail, and the aim of the current study was to address this using novel mouse models.

Materials and Methods

Animals

All the animals used in these studies were subject to recommendations for animal use and welfare outlined by the University of California, Los Angeles (UCLA) Division of Laboratory Animal Medicine as well as guidelines from the National Institutes of Health. The Animal Research Committee at the University of California, Los Angeles, approved the protocol (number 2008-132-11) for the use of mice in our study. C57BL/6J female mice (Jackson Laboratories, Sacramento, California) were raised on a normal, vitamin D-sufficient diet (Suff) or a vitamin D-deficient diet (Def) from week 4 of age. The diets used in the current study were developed in conjunction with Research Diets Inc (New Brunswick, New Jersey) to provide all the required nutrients for pregnancy but with specific variations in vitamin D. The Suff diet, also known as the control diet, contained vitamin D3 at 1000 IU/kg feed. This is relatively low compared with standard NIH-31 open formula mouse diet (which contains 4200 IU/kg feed) but still maintained an adequate intake of vitamin D per kilogram of body weight. All the other nutrients in this diet met the National Research Council requirements for pregnant mice (24).

At weeks 8 of age, female mice were mated with male mice raised on the Suff diet using timed pregnancy protocols as described previously (25). During mating and subsequent pregnancy, all female mice continued to be fed the same diet as before mating. Another group of female mice were raised on a vitamin D-deficient diet from 4 weeks but then switched to a vitamin D-sufficient diet periconceptually at week 8 of age (Def+). For each dietary group (Suff, Def, and Def+), 3 separate experiments were carried out with each using 6 mice per dietary group. The organization of diets, matings, and pregnancy of these mice is shown in Supplemental Figure 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org.

Tissue collection and analysis

Mice were euthanized at either embryonic day (E) 14 or E18 to enable the collection of placenta, kidney, and fetal tissues. Placentas were weighed and diameter determined by measuring the maximum width of the chorionic surface. Placenta and kidney tissues were also used for isolation of RNA and for histological analyses. Fetuses were used for gross and histological analyses. Blood was also collected at E14 by cardiac puncture, and the resulting serum was used to analyze circulating concentrations of 25D and 1,25D. For serum 25D, a rapid, direct RIA developed in the laboratory of author B.W.H. and manufactured by Diasorin Corporation (Stillwater, Minnesota) was used (26) An RIA manufactured by Diasorin Corporation was used to measure total circulating 1,25D concentrations (27). This assay uses a ^{125}I-labeled tracer, and samples are processed using ace-

tonitrile followed by solid-phase extraction and quantitation. Serum chemistry analyses including calcium, blood urea nitrogen, creatinine, and glucose were measured using angiotensin-converting enzyme Alera Clinical Chemistry Systems automated assays (Alfa Wassermann Diagnostic Technologies, Inc, West Caldwell, New Jersey). These assays were carried out as part of core automated assay services by DLAM/Pathology and Laboratory Medicine Services at David Geffen School of Medicine at UCLA. Urine was collected at 3 time points: the day before mating, E14, and E18 using metabolic cages to collect urine for 24 hours (Harvard Apparatus, Holliston, Massachusetts). A urine analysis to assess proteinuria was carried out by the Pathology and Laboratory Medicine Services, David Geffen School of Medicine at UCLA, using quantitative urinalysis kits (Ani Lytics, Inc, Gaithersburg, Maryland).

Blood pressure measurement by tail-cuff method

BP was measured twice before breeding (baseline), once at E14 gestation, and once on E18, postpartum day (PP) 7, and PP14 using tail-cuff plethysmography (model 229; IITC, Woodland Hills, California) in the UCLA Mouse Physiology Core Laboratory (Department of Physiology, David Geffen School of Medicine at the University of California, Los Angeles). BP measurements were made at the same time of day (afternoons) in accordance with the manufacturer's guidelines. Briefly, mice were inserted in the IITC plexiglas restrainer with the tail cuff device snugly fitted to the base of their tails. The mice were kept in a warm environment (30–32°C) for no more than 25 minutes. After a 10-minute period of equilibration, at least 3 cuff inflation readings were obtained at 2- to 5-minute intervals. The recordings were later analyzed to determine systolic BP (SBP) and mean arterial pressure (MAP). The 3 or more pressure readings were averaged from each mouse recording session to obtain the average SBP and MAP values for that time point.

Histological and immunohistochemical analyses

Sections of paraffin-embedded tissue from maternal kidneys and placenta were used first for routine histological analyses using hematoxylin and eosin (H&E) and periodic acid Schiff staining. Immunohistochemical analysis of CD31 protein expression to stain for endothelial cells and analyze placental capillary diameter was carried out using paraffin-embedded tissue sections as described previously (25). In brief, sections were deparaffinized and hydrated through a series of xylene and graded ethanol washes, and antigen retrieval was carried out in 0.01 M sodium citrate buffer in a pressure cooker at 103 kPa for 2 minutes. Slides were then incubated in 3% methanol-hydrogen peroxide for 15 minutes to quench endogenous peroxidase activity and washed in PBS (pH 7.4). Nonspecific binding was blocked with 1.5% of normal blocking serum/PBS for 1 hour at room temperature, and slides were then incubated with rabbit antimouse CD31 polyclonal antibody (1:50 dilution; Abcam, Cambridge, Massachusetts) at 4°C overnight. After a 15-minute PBS wash, biotinylated goat antirabbit IgG (1:200 diluted in 1.5% normal blocking serum/PBS; Vector Laboratories, Burlingame, California) was added to sections for 1 hour. Slides were then washed for 15 minutes in PBS and then incubated for 30 minutes with avidin and biotinylated horseradish peroxidase macromolecular complex (Vector Laboratories). Diaminobenzidine tetrahydrochloride was used to visualize protein expression, and hematoxylin (Modified Mayer's Formula; Vector Laboratories) was used to counterstain sections. Finally, the slides were dehydrated and mounted with glycerol mount medium.

The diameter of fetal capillaries was defined using CD31 to stain for endothelial cells in the placental labyrinth. The diameter of CD31-stained placental capillaries was measured twice for the widest and narrowest diameter using Nikon NIS-Elements AR3.0 software (Nikon Instruments Inc, Melville, New York) and mean values recorded. Within each field 35 capillary diameter measurements were performed on blood vessels with a clear border. At ×400 magnification, 10–15 fields (depending on individual placental sample variations) were analyzed per placenta section encompassing 5 different regions of the placental labyrinth area (ie, center, right middle, right edge, left middle, and left edge). For each dietary group, placentas from 4 different pregnancies were analyzed, with tissue from 3 placentas per pregnancy being used to generate sections.

Quantitative real-time RT-PCR

RNA was extracted as described above. cDNA was synthesized by SuperScript III reverse transcriptase and random primers (Invitrogen, Carlsbad, California) according to the manufacturer's protocol. Quantitative real-time RT-PCR (qPCR) analysis was performed using a Stratagene Mx3005P quantitative real-time RT-PCR system (La Jolla, California), using TaqMan probes and primers from Applied Biosystems (Foster City, California). Expressions of mRNAs for specific mouse target genes were assessed using the following FAM-labeled TaqMan gene expression assay probe/primer sets (from Applied Biosystems): *Cyp27b1* (Mm01165922_g1), *Ren1* (Mm02342889_g1), and *Agtr1a* (Mm00616371_m1) in conjunction with VIC/MGB probe/primer for 18S rRNA (endogenous control) (part 4319413E). All cDNAs were amplified under the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. All reactions were performed in triplicate, and each target gene expression was normalized to 18S mRNA expression. The relative amount of target gene in each sample was reported using fold changes calculated by $2^{-\Delta\Delta Ct}$ values ($\Delta\Delta$Ct value for vehicle treated control − ΔCt for treated sample) ΔCt, defined as cycle threshold (Ct) of gene of interest − Ct of 18S as described previously (25).

Statistics

All statistical analyses were carried out using Sigmaplot 9.0 software (Systat Inc, San Jose, California). Experimental means were compared statistically using an unpaired Student *t* test. Where indicated, multifactorial data were compared using 1-way ANOVA, with the Holm-Sidak method used as a post hoc multiple-comparison procedure. Statistical analyses were carried out using raw ΔCt values.

Results

Low serum concentrations of 25D and 1,25D in pregnant mice raised on a vitamin D-deficient diet

At E14, serum levels of 25D were higher in pregnant mice raised on a vitamin D-sufficient diet (Suff) vs those

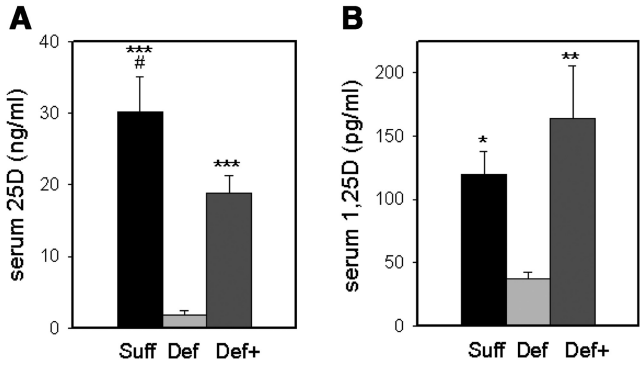

Figure 1. Effect of maternal dietary vitamin D restriction on circulating levels of vitamin D metabolites in pregnant mice. Sera were collected on E14 from 3 groups of pregnant mice: 1) mice raised from weaning (week 4) through E14 on a vitamin D-sufficient diet (Suff); 2) mice raised from week 4 through E14 on a vitamin D-deficient diet (Def); and 3) mice raised on a vitamin D-deficient diet from week 4 to week 8 and then transferred to a vitamin D-sufficient diet through E14 (vitamin D replacement; Def+). Serum concentrations of 25D (A) and 1,25D (B) were measured by RIA. Values shown are mean ± SEM for 5 separate pregnant mice. *, Statistically different from Def mice ($P < .05$); **, $P < .01$, and ***, $P < .001$; #, statistically different from Def+ mice ($P < .05$).

raised on a vitamin D-deficient diet (Def) [30.1 (mean) ± 5.0 (SEM] vs 1.8 ± 0.6 ng/mL, $P < .001$, n = 5) (Figure 1). Serum levels of 1,25D were also higher in Suff vs Def mice (119.5 ± 18.7 vs 37.4 ± 5.1 pg/mL, $P < .05$, n = 5). Resupplementation of Def mice from week 8 of age elevated serum levels of both 25D (18.9 ± 2.5 ng/mL) and 1,25D (163.7 ± 41.7 ng/mL). In the resupplemented (Def+) mice serum levels of 25D and 1,25D were significantly higher than Def mice ($P < .001$ and $P < .01$ respectively, n = 6 animals). However, serum levels of 25D in Def+ mice remained statistically lower than the Suff mice ($P < .05$, n = 6 animals) (Figure 1). The vitamin D-deficient diet had no effect on serum levels of glucose, calcium, blood urea nitrogen, and creatinine in mice at E14. Urinary protein and glucose levels were in normal ranges at all the time points in the experiments (ie, before mating, E14, and E18) in both Suff and Def mice, and there were no significant differences between the 2 groups of mice (data not shown).

Vitamin D deficiency during pregnancy is associated with elevated BP

Tail-cuff analysis of mice showed no significant difference in BP between Suff and Def mice at baseline (immediately prior to mating) (Figure 2). However, at E14, BP was significantly elevated in Def mice compared with Suff mice for both SBP (124.89 ± 2.48 vs 105.34 ± 3.61 mm Hg, $P < .001$, n = 5) and MAP (115.33 ± 1.93 vs 89.33 ± 5.02 mm Hg, $P < .001$, n = 5). This elevation continued through the remainder of pregnancy and PP7. At E18, SBP for Def vs Suff mice was 121.33 ± 3.88 vs 99.78 ± 4.12 mm Hg ($P < .01$, n = 5), and MAP was 109.33 ± 3.69 vs 88 ± 5.39 mm Hg ($P < .05$, n = 5). At PP7, the SBP for Def mice was still significantly elevated relative to Suff mice (121.30 ± 0.5 vs 112.0 ± 0.6 mm Hg, $P < .05$, n = 5), but there was no significant difference between the 2 types of mice for MAP. SBP in Def mice returned to baseline levels comparable with Suff mice by PP14 (Figure 2), consistent with changes in BP observed for humans with preeclampsia postpartum. In Def+ mice at E14 SBP and MAP were significantly decreased (111.75 ± 4.05 mm Hg for SBP and 92.71 ± 1.31 mm Hg for MAP) compared with Def mice ($P < .05$, n = 6). The SBP in Def+ at E14 showed no significant difference compared with Suff mice

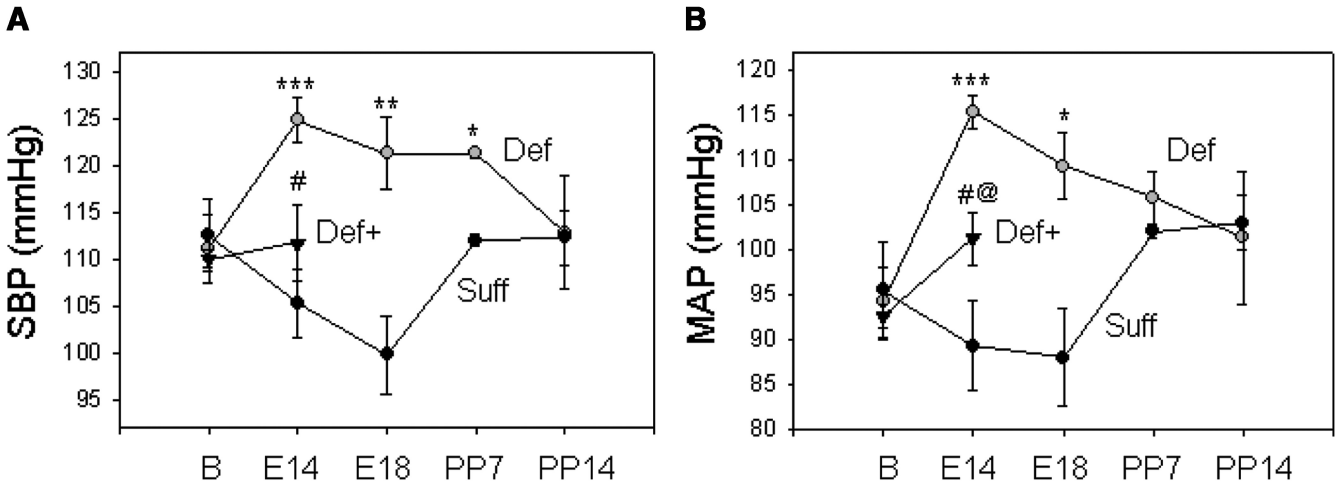

Figure 2. Effect of maternal dietary vitamin D restriction on BP in pregnant mice. SBP (A) and MAP (B) were measured before mating [baseline (B)], on E14, E18, and at PP7 and PP14 in vitamin D-sufficient (Suff) and vitamin D-deficient (Def) mice. SBP and MAP were also measured in vitamin D-replacement (Def+) mice at B and E14. Values shown are mean ± SEM for 6 separate mice. *, Statistically different from Suff mice ($P < .05$); **, $P < .01$; ***, $P < .001$.

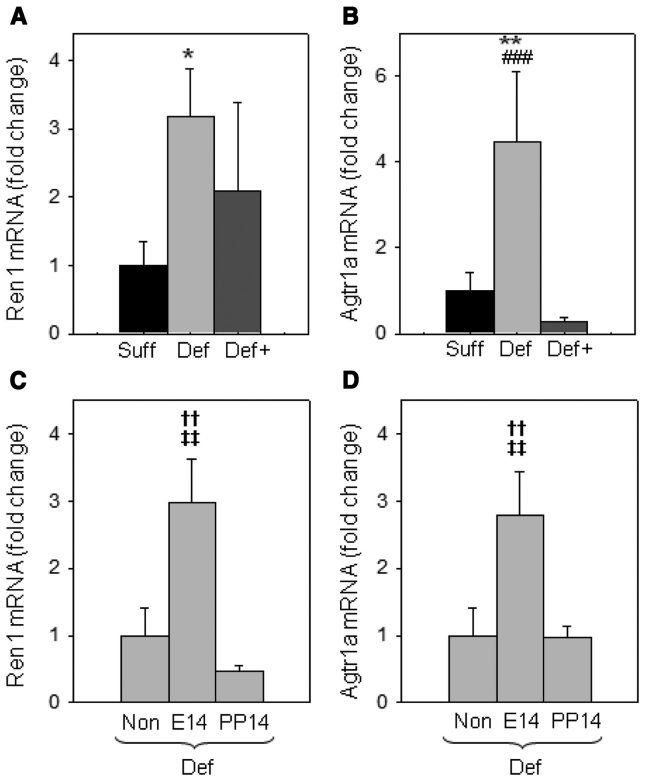

Figure 3. Effect of maternal dietary vitamin D restriction on the renal renin-angiotensin system in pregnant mice. Expression of mRNA for renin (*Ren1*) (A) and angiotensin II receptor (*Agtr1a*) (B) in kidneys from pregnant vitamin D-sufficient (Suff), vitamin D-deficient (Def), and vitamin D-replacement (Def+) mice on E14. Levels of mRNA encoding *Ren1* (C) and *Agtr1a* (D) were also assessed in nonpregnant, E14, and PP14 vitamin D-deficient mice. Data shown are fold change in mRNA relative to Suff mice (*) or Def+ mice (#) in A and B and either nonpregnant mice (*) or PP14 mice (#) in C and D. Values shown are mean ± SEM for 5 separate mice. *, Statistically different from Suff mice ($P < .05$); **, $P < .01$; ##, statistically different from Def+ mice ($P < .01$); ‡‡, statistically different from nonpregnant mice ($P < .01$); ††, statistically different from PP14 mice ($P < .01$).

at E14, but the MAP in E14 Def+ mice remained higher than the MAP in Suff mice ($P < .05$).

Effects of vitamin D status on the renal RAAS in pregnant mice

Analysis of kidney mRNA expression by real-time RT-PCR showed that expression of renin (*Ren1*) and the angiotensin II receptor (*Agtr1a*) were significantly elevated in Def vs Suff mice at E14 (3.2 ± 0.7-fold for *Ren1*, $P < .05$; 4.5 ± 1.6 for *Agtr1a*, $P < .01$, n = 5) (Figure 3, A and B). In Def+ mice expression of renal *Agtr1a* mRNA expression was decreased relative to Def mice (0.28 ± 0.1-fold, $P < .001$, n = 5), but this effect was not observed for *Ren1*. In Def mice elevated expression of *Ren1* and *Agtr1a* was evident only during pregnancy and returned to baseline levels at PP2 (Figure 3, C and D). Further RT-PCR analyses showed that expression of mRNA for mouse *CYP27B1* (*Cyp27b1*) was increased in kidneys from E14 Def mice relative to Suff mice (55.2 ± 4.1-fold higher, $P < .001$, n = 5). By contrast, in E14 placentas *Cyp27b1* showed a trend for lower mRNA in Def vs Suff mice (0.6 ± 0.1-fold, n = 5 mice). To determine whether changes in kidney expression of *Ren1* and *Agtr1a* were associated with endothelial pathology such as glomeruloendotheliosis, renal tissue sections from Suff and Def mice were analyzed histologically (Supplemental Figure 2). However, staining using H&E and periodic acid Schiff showed no evidence of aberrant glomerular histology such as swelling of endocapillary cell cytoplasm.

Effects of vitamin D status on mouse placentas

At E14 and E18, Suff and Def mice showed no significant variations in placental weight (Figure 4A). There was also no significant difference in placental diameter between Suff and Def mice at E14 (Figure 4B). However, at E18, Def mice showed lower placental diameter compared to Suff mice (8.5 ± 0.2 mm vs 9.4 ± 0.1 mm, $P < .01$). In Def+ mice, placental diameter and weight at E14 or E18 was not significantly different to Suff or Def mouse placentas (data not shown).

Histological analyses demonstrated dysregulated vascularization within the labyrinth of placentas from Def mice (Figure 5). Immunohistochemical analysis of the endothelial cell maker CD31 in E14 placentas from Def mice (Figure 5, D–I) indicated that the diameter of the lumen of fetal blood vessels in the labyrinth of placentas from Def mice (Figure 5, E and H) was narrower relative to vessels from Suff placentas (Figure 5, D and G) throughout the labyrinth. In Def+ mice, there was a partial reversal of the vascular narrowing observed in placentas from Def mice (Figure 5, F and I). Quantification of vascular diameter after CD31 staining showed that this decreased from 9.36 ± 1.32 μm (SD) in Suff mice to 5.03 ± 0.88 μm in Def mice ($P < .001$) and 6.14 ± 1.32 μm in Def+ mice ($P < .001$) (Figure 6). There was no statistical difference in vascular diameter between Def and Def+ mice.

Impaired fetal development and delivery in vitamin D-deficient mice

Analysis of fetal growth showed that E14 and E18 fetuses from Def mice were larger (length and weight) than fetuses from Suff mice (Figure 7, A and B). At E14 these differences were not ameliorated in Def+ mice. Despite the differences in fetal size and weight between the 2 groups of mice, there was no statistical difference in litter sizes between Suff (7.29 ± 0.47) and Def (7.25 ± 0.37) mice (Figure 7C). However, analysis of offspring weight postpartum showed that pups from Def mothers weighed significantly less than those from Suff mothers at PP14 to PP28 (Figure 7D). The increase in fetal weights were ob-

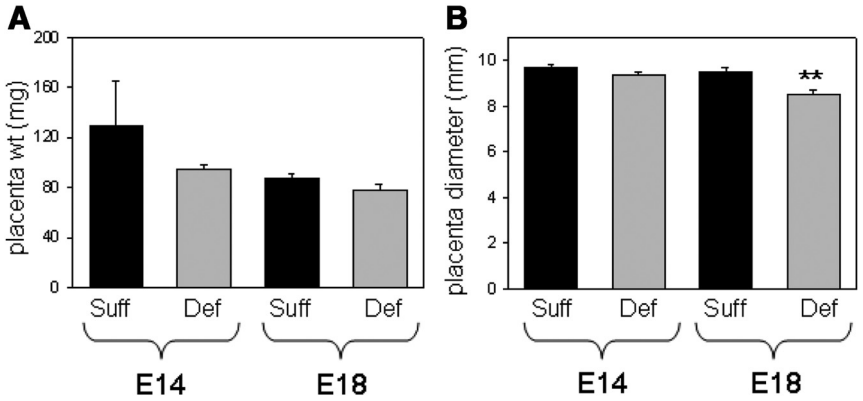

Figure 4. Effect of maternal dietary vitamin D restriction on placental diameter and weight. Placentas were collected at E14 and E18 from vitamin D-sufficient (Suff) and vitamin D-deficient (Def) pregnant mice. A, Comparison of placenta weights (milligrams). B, Comparison of placenta diameter (millimeters). Values shown are mean ± SEM for 5 separate mice. **, Statistically different from the E18 Suff mice ($P < .01$).

served despite more Def pregnancies delivering before E18 (Figure 7E).

Discussion

In previous association studies from human pregnancies, vitamin D deficiency has been linked to preeclampsia, a key adverse event for both mother and fetus (3, 5). We therefore hypothesized that vitamin D-deficient pregnant mice would demonstrate symptoms associated with preeclampsia including elevated BP and proteinuria. The data presented in this study show that mice raised from weaning through pregnancy on a vitamin D-deficient diet had elevated BP compared with their counterparts raised on a vitamin D-sufficient diet, but these mice did not demonstrate other symptoms associated with preeclampsia in humans such as proteinuria.

Sustained elevation of either SBP or MAP may be evidence for hypertension in pregnancy, and in the current study, both parameters were abnormally high in vitamin D-deficient mice. The elevated SBP observed in Def mice was not as pronounced as that reported for other models of hypertension in pregnancy, notably renin and angiotensinogen transgenic mice (29), and mice with gene ablation of corin, a cardiac transmembrane serine protease that can convert proatrial natriuretic peptide to atrial natriuretic peptide (30). However, the BP values obtained for the Def mice in this study are comparable with other mouse models of pregnancy hypertension such as the catechol-O-methyltransferase knockout mouse, which lacks the ability to generate 2-methoxyoestradiol, a potent vascular inhibitor (31). The MAP was also elevated in the pregnant Def mice, consistent with other studies such as the complement component C1q knockout mouse, which showed a maximal increase of 20 mm Hg relative to wild-type mice just prior to parturition (32). Thus, although the elevated SBP in Def mice was not entirely consistent with parameters used to define hypertension in humans, these measures are nevertheless consistent with the dysregulated BP observed for published mouse models of preeclampsia.

Significantly, elevated SBP and MAP were observed only in Def mice during pregnancy. This contrasts studies of *Vdr* knockout ($Vdr^{-/-}$) mice that present with constitutively elevated BP (33), although it is interesting to note that the difference in SBP between $Vdr^{-/-}$ and wild-type mice (20 mm Hg) was similar to that observed between the pregnant Def and Suff mice. In $Vdr^{-/-}$ mice, elevated BP was associated with a 4-fold increase in kidney Ren1 mRNA expression (33), similar to that observed in the kidneys of pregnant Def mice (see Figure 3). Active 1,25D suppresses the transcription of the *Ren1* gene via a vitamin D receptor-dependent mechanism that blocks a cAMP response element within the *Ren1* gene promoter (34). It would therefore appear that the decreased renal production of 1,25D in 25D-depleted Def mice results in dysregulation of Ren1 similar to that observed in $Vdr^{-/-}$ mice. However, elevated renal *Ren1* and *Agtr1a* were observed only in pregnant Def mice, despite the fact that nonpregnant Def mice exhibited similar low levels of serum 25D. Indeed, although serum levels of 1,25D in pregnant Def mice are lower than Suff mice, they were not significantly different from circulating 1,25D concentrations we have reported previously for nonpregnant female BL6 mice (35). This suggests that physiological changes in pregnancy can unmask the effects of 25D deficiency on the renal RAAS.

Maternal serum concentrations of 1,25D rise rapidly during early gestation, although the underlying mechanism for this is unclear (36). The immediate physiological benefit of enhanced serum 1,25D is also unclear but may contribute to the maintenance of maternal calcium homeostasis (37). In view of the data presented in this study, we can perhaps speculate that the gestational rise in maternal 1,25D is also linked to the RAAS and regulation of maternal BP. In a recent randomized supplementation trial involving pregnant women, circulating levels of 25D were shown to be directly related to circulating 1,25D levels (38), suggesting that vitamin D (25D)-deficient pregnant women may also have relatively low levels of 1,25D. This,

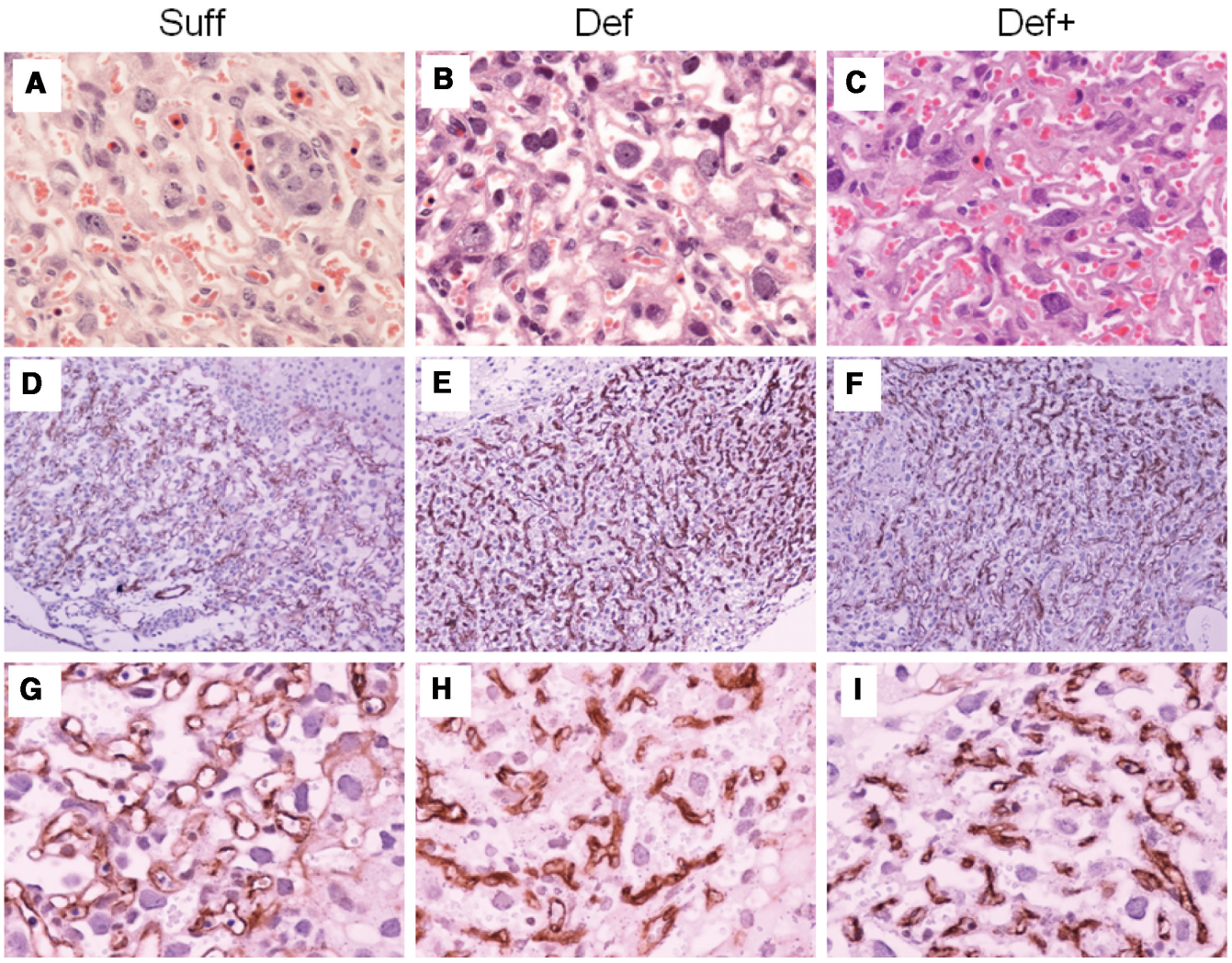

Figure 5. Effect of maternal dietary vitamin D restriction on placental histology. A–C, H&E staining of placenta tissue at E14 from the following: mice raised from weaning (week 4) through E14 on a vitamin D-sufficient diet (Suff, A); mice raised from week 4 through E14 on a vitamin D-deficient diet (Def; B); mice raised on a vitamin D-deficient diet from week 4 to week 8 and then transferred to a vitamin D-sufficient diet through E14 [vitamin D replacement (Def+; C)]. All ×400 magnification. D–I, Immunohistochemical analysis of the endothelial cell marker CD31 in placental tissue from E14 pregnant mice from Suff (D and G), Def (E and H), and Def+ (F and I) groups. Original magnification. ×X100 for Figure D–F and ×200 for G–I.

in turn, may provide a mechanism linking low serum 25D with the hypertension associated with preeclampsia (3–5, 39), and it is notable that low serum 1,25D has also been reported for women with preeclampsia (40). It is also important to recognize that although increased plasma renin and angiotensin levels occur as part of normal human pregnancy physiology, there is relative insensitivity to the RAAS system in pregnant women (13). By contrast, in preeclampsia pregnancies renin levels are only mildly elevated, (41) so that the elevated BP associated with preeclampsia appears to be due primarily to enhanced angiotensin receptor activation (13). It was therefore notable in the current study that *Agtr1a* mRNA expression was elevated in kidneys from vitamin D-deficient mice, suggesting that this may also be a key factor in vitamin D deficiency-associated dysregulation of BP during pregnancy.

Increased synthesis of 1,25D during pregnancy is thought to be due primarily to activity of *CYP27B1* in maternal kidneys. The placenta is also a major extrarenal site for synthesis of 1,25D (42), and, like the kidney, this may also be affected by decreased serum 25D under conditions of vitamin D deficiency. However, in contrast to the endocrine function of the renal vitamin D system, synthesis of 1,25D by the placenta may fulfill a more localized intracrine or paracrine role that directly affects placental function (43). In the current study, one of these functions appears to be the maintenance of normal placental angiogenesis. Previous reports have described decreased expression of CD31 in an angiotensin receptor autoantibody mouse model of preeclampsia (44). In vitamin D-deficient pregnant mice, CD31 was expressed normally but blood vessels were severely constricted in diameter. The precise

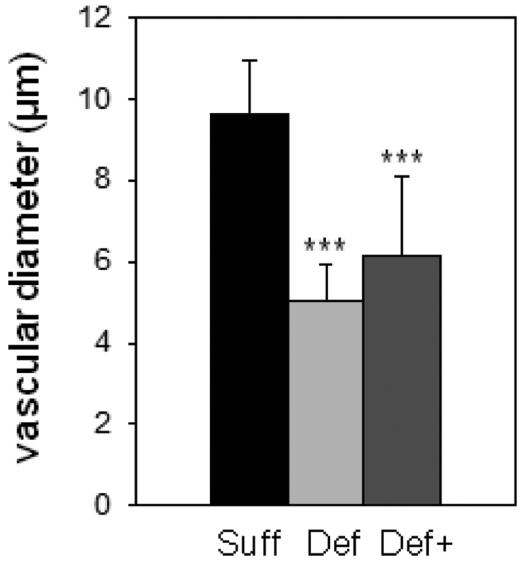

Figure 6. Effect of maternal dietary vitamin D restriction on placental vascular diameter. Quantification of vascular diameter (micrometers) based on CD31 staining in E14 placentas from vitamin D-sufficient (Suff), vitamin D-deficient (Def), and vitamin D-replacement (Def+) mice. Values shown are mean ± SD for 10–15 fields of vision at 5 different locations (center, right middle, right edge, left middle, and left edge) of placentas from 4 different pregnant mice. ***, Statistically different from Suff mice ($P < .001$).

mechanism by which vitamin D-deficiency contributes to this mechanism remains unclear, but it will be interesting in future studies to assess the effects of vitamin D metabolites on vascular function in the placenta. Interestingly, vitamin D deficiency during pregnancy produced divergent effects on renal and placental express of *Cyp27b1*, which was induced and suppressed respectively in the 2 tissues. The lower level of *Cyp27b1* mRNA in placentas from vitamin D-deficient mice is similar to that previously reported for human trophoblastic cells from preeclampsia pregnancies (9). By contrast, immunohistochemical analysis of protein for human *CYP27B1* protein has reported elevated levels of the enzyme in placenta tissue from preeclampsia pregnancies (45).

In addition to determining the impact of vitamin D-deficiency on the renal RAAS and placental function, additional experiments were carried out to assess responses to resupplementation with vitamin D. In Def mice that were transferred periconceptionally to a vitamin D-sufficient diet (Def+), serum levels of 1,25D were similar to the elevated levels observed in Suff mice. However, although serum levels of 25D rose in the Def+ mice (18.9 ng/mL, 47.3 nM) relative to Def (1.8 ng/mL, 4.5 nM), they remained significantly lower than Suff mice (30.1 ng/mL, 75.3 nM). It was therefore interesting to note that the elevated BP in E14 Def mice was only partly corrected in G14 Def+ mice (see Figure 2). In a similar fashion, analysis of renal *Ren1* and *Agtr1a* and placental morphology showed partial amelioration of the effects of vitamin D deficiency upon restoration of a vitamin D-sufficient diet at the start of pregnancy. There is ongoing debate over what constitutes normal or optimal serum 25D status (46, 47) in pregnancy. In mice, it is possible to speculate that maternal levels of 25D at 50 nM are insufficient to prevent renal and placental abnormalities that contribute to dysregulation of BP. However, this may not be true in humans. Recent studies in human pregnancies have shown that maternal supplementation with doses of vitamin D that increase maternal serum 25D above 100 nM in late gestation may decrease the risk of developing preeclampsia (48). Thus, it is possible that the serum 25D requirements for optimal human placental and pregnancy health are higher than those proposed for normal calcium homeostasis and bone metabolism.

Although Def mice showed several important features of preeclampsia, not all observations supported this. We did not observe the proteinuria that is classically associated with human preeclampsia and some mouse models of the disorder. Likewise, the Def mice showed no evidence of another characteristic lesion of human preeclampsia, glomeruloendotheliosis. It was also notable that fetuses from Def mice were larger than those from Suff mice, and this effect did not appear to be due to variations in litter size. By contrast, preeclampsia is conventionally associated with small-for-gestational-age fetuses. Large-for-gestation-age fetuses (macrosomia) may be due to several factors including generalized fetal edema (49), although we did not observe any evidence for this the current study. Maternal hyperinsulinism may be another explanation, although data from human studies have to date failed to show a link between low vitamin D status, gestational diabetes, and macrosomia (50).

Despite the unexpected data for fetal weight and size, postpartum growth was significantly impaired in offspring from Def mice, suggesting the vitamin D status in utero is an important determinant of subsequent growth and development, possibly through fetal programming. In future studies it will be interesting to maintain mice born to vitamin D-deficient mothers for extended periods of time to determine the impact of this on adult health. Other studies using mice have shown that gestational vitamin D deficiency is associated with aberrant expression and function of immune cells in the offspring (51). Finally, data showed that births in Def mice were more likely to occur preterm (<E18) compared with Suff mice. The percentage of births less than E18 in vitamin D-deficient mothers was similar to that reported for biglycan and decorin knockout mouse models of spontaneous preterm birth (52). Epidemiological studies have yet to demonstrate a clear association between vitamin D deficiency and spontaneous pre-

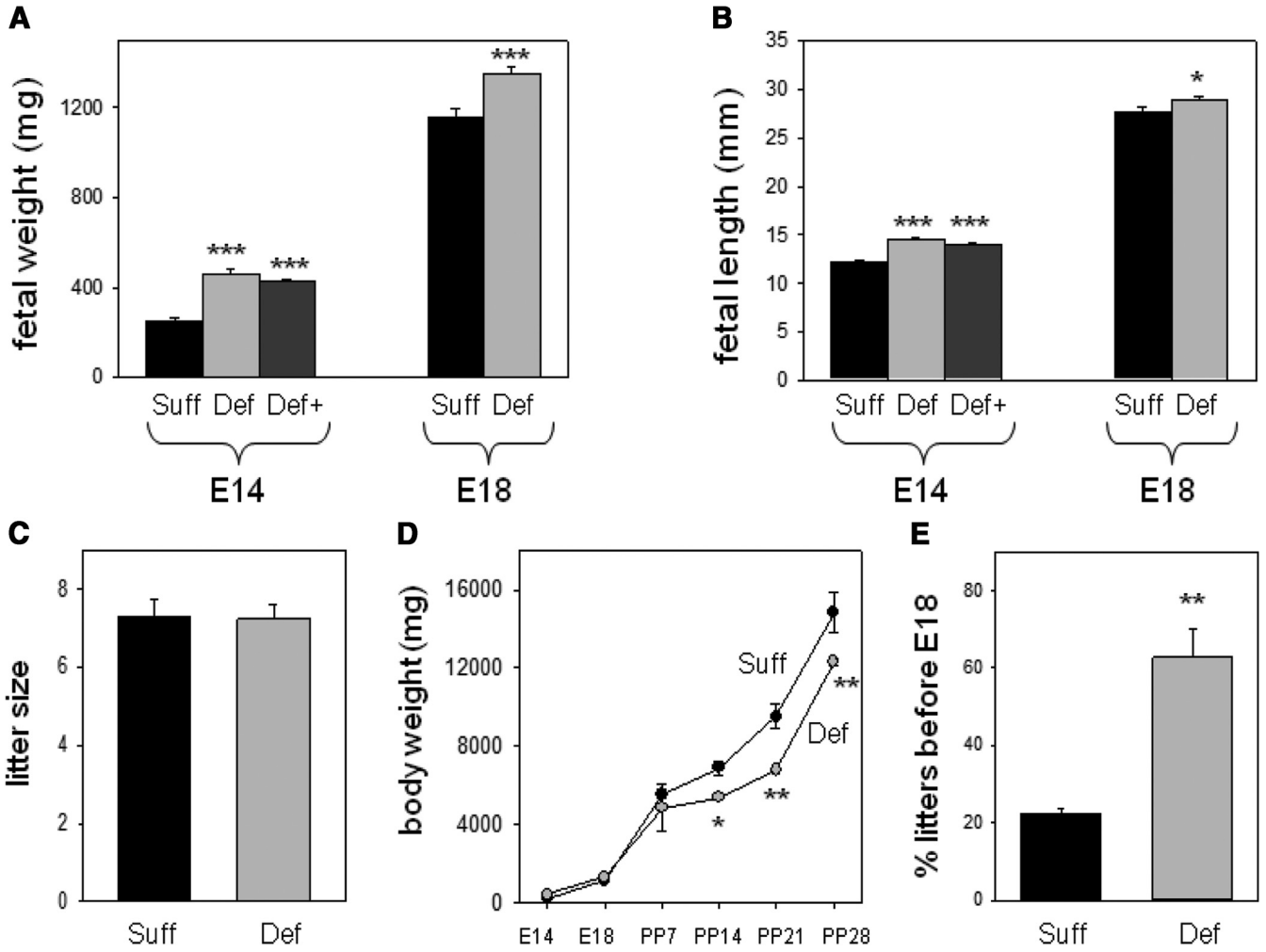

Figure 7. Effect of maternal dietary vitamin D restriction on fetal development and parturition weights (A) and size of fetuses (B) at E14 and E18 from Suff, Def, and Def+ mice (n = 16 fetuses for each group). C, Litter size for Suff and Def mice. D, Growth of fetuses and pups with time as assessed by body weights (grams) (n = 16 animals per group). In the Def group, the diet of the mother was changed to vitamin D-sufficient diet immediately after delivery. After weaning, infants raised from both groups were fed with a vitamin D-sufficient diet. E, Values shown are mean ± SEM for 12 per litter for both groups of mice. Effect of the vitamin D-deficient diet shows on birth of litters before E18. *, Statistically different from Suff mice ($P < .05$); **, $P < .01$; ***, $P < .001$.

term birth in humans (28), and at present there are no data for iatrogenic (clinically indicated) preterm births secondary to disorders such as preeclampsia in relation to maternal vitamin D status.

Data presented here suggest that low serum levels of 25D can affect both the kidney renin-angiotensin system and placental vascular development in mouse pregnancies. Vitamin D deficiency may lead to a decreased supply of 25D for the activation to 1,25D by both the kidney and placenta, with concomitant consequences for localized and systemic vitamin D actions. Restoring dietary vitamin D to normal in the periconceptual period may ameliorate these detrimental effects of vitamin D deficiency in pregnancy despite only a partial restoration of serum levels of 25D to less than 75 nM. Despite these observations, the current study does not clarify the extent to which vitamin D deficiency is a contributing factor to preeclampsia as a specific adverse event of pregnancy. Vitamin D-deficient pregnant mice did not present with significant proteinuria or evidence of glomeruloendotheliosis, 2 well-established characteristics of preeclampsia. In addition to effects on maternal health, vitamin D deficiency during pregnancy may also influence fetal and neonatal development. Further studies are required to more comprehensively define the maternal and fetal benefits of optimizing vitamin D status in utero. However, our current data strongly suggest that vitamin D deficiency during pregnancy may have detrimental effects that extend beyond the classical actions of vitamin D on skeletal homeostasis.

Acknowledgments

Address all correspondence and requests for reprints to: Martin Hewison, PhD, Room 410D, Orthopaedic Hospital Research

Center, University of California, Los Angeles, Los Angeles, California 90095. E-mail: mhewison@mednet.ucla.edu.

This work was supported by March of Dimes Grant FY10-171 (to M.H. and O.E.) and W. K. Kellogg Foundation Funding (to C.W. and M.H.).

Disclosure Summary: The authors have no disclosures to declare.

References

1. **Steegers EA, von Dadelszen P, Duvekot JJ, Pijnenborg R.** Pre-eclampsia. *Lancet*. 2010;376:631–644.
2. **Johnson DD, Wagner CL, Hulsey TC, McNeil RB, Ebeling M, Hollis BW.** Vitamin D deficiency and insufficiency is common during pregnancy. *Am J Perinatol*. 2011;28:7–12.
3. **Bodnar LM, Catov JM, Simhan HN, Holick MF, Powers RW, Roberts JM.** Maternal vitamin D deficiency increases the risk of preeclampsia. *J Clin Endocrinol Metab*. 2007;92:3517–3522.
4. **Robinson CJ, Wagner CL, Hollis BW, Baatz JE, Johnson DD.** 2011 Maternal vitamin D and fetal growth in early-onset severe preeclampsia. *Am J Obstet Gynecol*. 204:556 e551–e554.
5. **Baker AM, Haeri S, Camargo CA Jr, Espinola JA, Stuebe AM.** A nested case-control study of midgestation vitamin D deficiency and risk of severe preeclampsia. *J Clin Endocrinol Metab*. 2010;95:5105–5109.
6. **Wei SQ, Audibert F, Hidiroglou N, et al.** Longitudinal vitamin D status in pregnancy and the risk of pre-eclampsia. *BJOG*. 2012;119:832–839.
7. **Haugen M, Brantsaeter AL, Trogstad L, et al.** Vitamin D supplementation and reduced risk of preeclampsia in nulliparous women. *Epidemiology*. 2009;20:720–726.
8. **Schuster I.** Cytochromes P450 are essential players in the vitamin D signaling system. *Biochim Biophys Acta*. 2011;1814:186–199.
9. **Diaz L, Arranz C, Avila E, Halhali A, Vilchis F, Larrea F.** Expression and activity of 25-hydroxyvitamin D-1α-hydroxylase are restricted in cultures of human syncytiotrophoblast cells from preeclamptic pregnancies. *J Clin Endocrinol Metab*. 2002;87:3876–3882.
10. **Lapillonne A.** Vitamin D deficiency during pregnancy may impair maternal and fetal outcomes. Med Hypotheses 2010;74:71–75.
11. **LaMarca BD, Gilbert J, Granger JP.** Recent progress toward the understanding of the pathophysiology of hypertension during preeclampsia. *Hypertension*. 2008;51:982–988.
12. **Hypponen E.** Vitamin D for the prevention of preeclampsia? A hypothesis. *Nutr Rev*. 2005;63:225–232.
13. **Irani RA, Xia Y.** Renin angiotensin signaling in normal pregnancy and preeclampsia. *Semin Nephrol*. 2011;31:47–58.
14. **Dechend R, Gratze P, Wallukat G, et al.** Agonistic autoantibodies to the AT1 receptor in a transgenic rat model of preeclampsia. *Hypertension*. 2005;45:742–746.
15. **Ma TK, Kam KK, Yan BP, Lam YY.** Renin-angiotensin-aldosterone system blockade for cardiovascular diseases: current status. *Br J Pharmacology*. 2010;160:1273–1292.
16. **Wessling-Resnick M.** Iron homeostasis and the inflammatory response. *Annu Rev Nutr*. 2010;30:105–122.
17. **Li J, Byrne ME, Chang E, et al.** 1α,25-Dihydroxyvitamin D hydroxylase in adipocytes. *J Steroid Biochem Mol Biol*. 2008;112:122–126.
18. **Imaoka M, Morimoto S, Kitano S, Fukuo F, Ogihara T.** Calcium metabolism in elderly hypertensive patients: possible participation of exaggerated sodium, calcium and phosphate excretion. *Clin Exp Pharmacol Physiol*. 1991;18:631–641.
19. **Lind L, Hanni A, Lithell H, Hvarfner A, Sorensen OH, Ljunghall S.** Vitamin D is related to blood pressure and other cardiovascular risk factors in middle-aged men. *Am J Hypertens*. 1995;8:894–901.
20. **Kristal-Boneh E, Froom P, Harari G, Ribak J.** Association of calcitriol and blood pressure in normotensive men. *Hypertension*. 1997;30:1289–1294.
21. **Li YC.** Vitamin D regulation of the renin-angiotensin system. *J Cell Biochem*. 2003;88:327–331.
22. **Zhang Y, Kong J, Deb DK, Chang A, Li YC.** Vitamin D receptor attenuates renal fibrosis by suppressing the renin-angiotensin system. *J Am Soc Nephrol*. 2010;21:966–973.
23. **Li YC, Qiao G, Uskokovic M, Xiang W, Zheng W, Kong J.** Vitamin D: a negative endocrine regulator of the renin-angiotensin system and blood pressure. *J Steroid Biochem Mol Biol*. 2004;89–90:387–392.
24. *Nutrient Requirements of Laboratory Animals*, 4th revised ed. Washington DC: The National Academies Press; 1995.
25. **Liu NQ, Kaplan AT, Lagishetty V, et al.** Vitamin D and the regulation of placental inflammation. *J Immunol*. 2011;186:5968–5974.
26. **Hollis BW, Kamerud JQ, Selvaag SR, Lorenz JD, Napoli JL.** Determination of vitamin D status by radioimmunoassay with an 125I-labeled tracer. *Clin Chem*. 1993;39:529–533.
27. **Hollis BW, Kamerud JQ, Kurkowski A, Beaulieu J, Napoli JL.** Quantification of circulating 1,25-dihydroxyvitamin D by radioimmunoassay with 125I-labeled tracer. *Clin Chem*. 1996;42:586–592.
28. **Baker AM, Haeri S, Camargo CA Jr, Stuebe AM, Boggess KA.** A nested case-control study of first-trimester maternal vitamin D status and risk for spontaneous preterm birth. *Am J Perinatol*. 2011;28:667–672.
29. **Takimoto E, Ishida J, Sugiyama F, Horiguchi H, Murakami K, Fukamizu A.** Hypertension induced in pregnant mice by placental renin and maternal angiotensinogen. *Science*. 1996;274:995–998.
30. **Chan JC, Knudson O, Wu F, Morser J, Dole WP, Wu Q.** Hypertension in mice lacking the proatrial natriuretic peptide convertase corin. *Proc Natl Acad Sci USA*. 2005;102:785–790.
31. **Kanasaki K, Palmsten K, Sugimoto H, et al.** Deficiency in catechol-O-methyltransferase and 2-methoxyoestradiol is associated with pre-eclampsia. *Nature*. 2008;453:1117–1121.
32. **Singh J, Ahmed A, Girardi G.** Role of complement component C1q in the onset of preeclampsia in mice. *Hypertension*. 2011;58:716–724.
33. **Li YC, Kong J, Wei M, Chen ZF, Liu SQ, Cao LP.** 1,25-Dihydroxyvitamin D(3) is a negative endocrine regulator of the renin-angiotensin system. *J Clin Invest*. 2002;110:229–238.
34. **Yuan W, Pan W, Kong J, et al.** 1,25-Dihydroxyvitamin D3 suppresses renin gene transcription by blocking the activity of the cyclic AMP response element in the renin gene promoter. *J Biol Chem*. 2007;282:29821–29830.
35. **Liu N, Nguyen L, Chun RF, et al.** Altered endocrine and autocrine metabolism of vitamin D in a mouse model of gastrointestinal inflammation. *Endocrinology*. 2008;149:4799–4808.
36. **Kumar R, Cohen WR, Silva P, Epstein FH.** Elevated 1,25-dihydroxyvitamin D plasma levels in normal human pregnancy and lactation. *J Clin Invest*. 1979;63:342–344.
37. **Gertner JM, Coustan DR, Kliger AS, Mallette LE, Ravin N, Broadus AE.** Pregnancy as state of physiologic absorptive hypercalciuria. *Am J Med*. 1986;81:451–456.
38. **Hollis BW, Johnson D, Hulsey TC, Ebeling M, Wagner CL.** Vitamin D supplementation during pregnancy: double-blind, randomized clinical trial of safety and effectiveness. *J Bone Miner Res*. 2011;26:2341–2357.
39. **Robinson CJ, Alanis MC, Wagner CL, Hollis BW, Johnson DD** Plasma 25-hydroxyvitamin D levels in early-onset severe preeclampsia. *Am J Obstet Gynecol* 2010;203:366 e361–e366.
40. **Halhali A, Tovar AR, Torres N, Bourges H, Garabedian M, Larrea F.** Preeclampsia is associated with low circulating levels of insulin-like growth factor I and 1,25-dihydroxyvitamin D in maternal and

umbilical cord compartments. *J Clin Endocrinol Metab*. 2000;85:1828–1833.
41. **Langer B, Grima M, Coquard C, Bader AM, Schlaeder G, Imbs JL.** Plasma active renin, angiotensin I, and angiotensin II during pregnancy and in preeclampsia. *Obstet Gynecol*. 1998;91:196–202.
42. **Zehnder D, Evans KN, Kilby MD, et al.** The ontogeny of 25-hydroxyvitamin D(3) 1α-hydroxylase expression in human placenta and decidua. *Am J Pathol*. 2002;161:105–114.
43. **Liu NQ, Hewison M.** Vitamin D, the placenta and pregnancy. *Arch Biochem Biophys*. 2012;523:37–47.
44. **Zhou CC, Irani RA, Zhang Y, et al.** Angiotensin receptor agonistic autoantibody-mediated tumor necrosis factor-α induction contributes to increased soluble endoglin production in preeclampsia. *Circulation*. 2010;121:436–444.
45. **Ma R, Gu Y, Zhao S, Sun J, Groome LJ, Wang Y.** Expressions of vitamin D metabolic components VDBP, *CYP2R1*, *CYP27B1*, *CYP24A1*, and VDR in placentas from normal and preeclamptic pregnancies. *Am J Physiol Endocrinol Metab*. 2012;303:E928–E935.
46. **Ross AC, Manson JE, Abrams SA, et al.** The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know. *J Clin Endocrinol Metab*. 2011;96:53–58.
47. **Holick MF, Binkley NC, Bischoff-Ferrari HA, et al.** Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab*. 2011;96:1911–1930.
48. **Hollis BW, Wagner CL.** Vitamin D and pregnancy: skeletal effects, nonskeletal effects, and birth outcomes. *Calcif Tiss Int*. 2013;92:128–139.
49. **Dackor RT, Fritz-Six K, Dunworth WP, Gibbons CL, Smithies O, Caron KM.** Hydrops fetalis, cardiovascular defects, and embryonic lethality in mice lacking the calcitonin receptor-like receptor gene. *Mol Cell Biol*. 2006;26:2511–2518.
50. **Savvidou MD, Akolekar R, Samaha RB, Masconi AP, Nicolaides KH.** Maternal serum 25-hydroxyvitamin D levels at 11(+0) -13(+6) weeks in pregnant women with diabetes mellitus and in those with macrosomic neonates. *BJOG*. 2011;118:951–955.
51. **Yu S, Cantorna MT.** Epigenetic reduction in invariant NKT cells following in utero vitamin D deficiency in mice. *J Immunol*. 2011;186:1384–1390.
52. **Calmus ML, Macksoud EE, Tucker R, Iozzo RV, Lechner BE.** A mouse model of spontaneous preterm birth based on the genetic ablation of biglycan and decorin. *Reproduction*. 2011;142:183–194.