Regulation of Murine Fetal–Placental Calcium Metabolism by the Calcium-sensing Receptor

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Abstract

The calcium-sensing receptor (CaSR) regulates PTH secretion to control the extracellular calcium concentration in adults, but its role in fetal life is unknown. We used CaSR gene knockout mice to investigate the role of the CaSR in regulating fetal calcium metabolism.

The normal calcium concentration in fetal blood is raised above the maternal level, an increase that depends upon PTH–related peptide (PTHrP). Heterozygous (+/−) and homozygous (−/−) disruption of the CaSR caused a further increase in the fetal calcium level. This increase was modestly blunted by concomitant disruption of the PTHrP gene and completely reversed by disruption of the PTH/PTHrP receptor gene. Serum levels of PTH and 1,25-dihydroxyvitamin D were substantially increased above the normal low fetal levels by disruption of the CaSR. The free deoxypyridinoline level was increased in the amniotic fluid (urine) of $CaSR^{-/-}$ fetuses; this result suggests that fetal bone resorption is increased. Placental calcium transfer was reduced, and renal calcium excretion was increased, by disruption of the CaSR.

These studies indicate that the CaSR normally suppresses PTH secretion in the presence of the normal raised (and PTHrP-dependent) fetal calcium level. Disruption of the CaSR causes fetal hyperparathyroidism and hypercalcemia, with additional effects on placental calcium transfer. (*J. Clin. Invest.* 1998. 101:2812–2820.) Key words: placenta • PTH • parathyroid glands • 1,25-dihydroxyvitamin D • calcitonin

Introduction

The extracellular calcium level is tightly controlled during adult life through the actions of the recently described calcium-sensing receptor (CaSR)[1] (1, 2). The CaSR is a G protein–coupled cell surface receptor with seven transmembrane-spanning regions (1–3). Its mRNA is most abundant in the parathyroids and kidneys of the adult (3, 4); it is also present in the C cells of the thyroid (5), the CNS (6), and other tissues (2). The parathyroid CaSR responds to increases in the extracellular calcium by inhibiting PTH synthesis and secretion (1, 2); a fall in the extracellular calcium stimulates synthesis and secretion of PTH. PTH, in turn, regulates the extracellular calcium by stimulating osteoclast-mediated skeletal resorption, increasing renal tubular reabsorption of calcium and decreasing renal tubular reabsorption of phosphate, and stimulating renal synthesis of 1,25-dihydroxyvitamin D (7). In renal tubules, activation of the CaSR inhibits reabsorption of calcium; the presence of the CaSR in C cells of the thyroid suggests a role for the CaSR in regulating calcitonin as well (1, 2).

Inactivating mutations in the CaSR gene increase PTH release from the parathyroids, decrease renal calcium clearance, and increase the blood calcium level (1, 2). In the heterozygous state, such mutations have been shown to cause generally modest elevations in serum calcium and PTH, and inappropriately low urinary calcium excretion (familial hypocalciuric hypercalcemia [FHH]) (2, 8). Homozygous inactivating mutations of the CaSR have been identified in some infants with the potentially lethal syndrome of neonatal severe primary hyperparathyroidism (2, 8). This syndrome is characterized by marked hypercalcemia, elevated PTH blood levels, subperiosteal bone resorption, and demineralization of the skeleton with fractures. In contrast, activating or gain-of-function mutations of the CaSR increase the inhibition of PTH secretion and increase the renal clearance of calcium, thereby lowering the blood calcium (9, 10).

The phenotype of patients with activating and inactivating mutations of the CaSR have thus demonstrated the central role of the CaSR in regulating calcium homeostasis in postnatal life. In contrast, the role of the CaSR in fetal calcium homeostasis has been largely unexplored. Calcium homeostasis of human and other mammalian fetuses is characterized, at

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1. *Abbreviations used in this paper:* CaSR, calcium-sensing receptor; IRMA, immunoradiometric assay; PTHrP, PTH–related peptide; WT, wild-type; +/−, heterozygous; −/−, homozygous.

least in late gestation, by a serum calcium that is raised above the ambient maternal serum calcium level, and very low PTH and 1,25-dihydroxyvitamin D levels compared to the normal adult levels (11, 12). The fetal parathyroids are necessary for normal calcium homeostasis, since parathyroidectomy of fetal lambs has been shown to reduce both the fetal blood calcium below the maternal level and the transfer of calcium across the perfused placentas of these same lambs (13, 14). These studies in fetal lambs found that PTH–related peptide (PTHrP), but not PTH, stimulated placental calcium transfer (15, 16). We have recently reported (17) that PTHrP-null fetal mice have a blood calcium level that is reduced to the maternal level. Placental calcium transfer is also reduced, and can be increased by PTHrP but not PTH administration (17). Human data on the relative roles of PTHrP and PTH in fetal life are lacking, although studies of human cord blood have established that PTHrP concentrations are up to 15-fold higher than simultaneously measured PTH levels (18–20).

Since fetal blood calcium is maintained at a level higher than the maternal one and fetal PTH levels are suppressed, CaSR may accomplish this suppression in fetal life. The expression of CaSR mRNA in the third branchial pouches of mice (Ho-Pao, C.L., J.G. Seidman, and C.E. Seidman, unpublished data) suggests a possible role for the CaSR in regulating fetal parathyroid gland activity. In contrast, the kidneys express very low levels of CaSR mRNA until the first postnatal day in rats (21); therefore, the CaSR might not play a major role in regulating renal tubular calcium handling by the fetal kidneys.

To explore the potential role of the CaSR in fetal life and the mechanisms of the normal fetal hypercalcemia, we utilized the CaSR gene-knockout model previously described (22). In this model, heterozygous ($CaSR^{+/-}$) pups develop normally after birth but have increased PTH and serum calcium levels, and reduced urinary calcium excretion, similar to humans with FHH. Homozygous ($CaSR^{-/-}$) pups have more severe hypercalcemia, parathyroid gland hyperplasia, and osteopenia; they die within 3 wk of birth. The homozygous condition is, therefore, similar to neonatal severe primary hyperparathyroidism in humans. Our studies indicate that the fetal parathyroid gland CaSR suppresses PTH secretion in response to the normal high circulating Ca^{2+} levels. In turn, disruption of the CaSR gene disturbs fetal calcium homeostasis largely by causing fetal hyperparathyroidism and by affecting PTHrP-stimulated placental calcium transport.

Methods

Materials. ^{45}Ca and [^{51}Cr]EDTA were obtained from New England Nuclear/Dupont Corp. (Boston, MA). Taq DNA Polymerase and proteinase K were obtained from Fisher Scientific Corp. (Pittsburgh, PA).

Knockout mice. CaSR receptor, PTHrP, and PTH/PTHrP receptor gene knockout mice were obtained by targeted disruption of the murine genes in embryonic stem cells, as previously described (22–24). Each knockout line was back-crossed for several generations into the outbred Black Swiss strain to provide a comparable genetic background among the three different colonies. Subsequently, $CaSR^{+/-}$ mice were genetically crossed with mice that were either $PTHrP^{+/-}$ or PTH/PTHrP $receptor^{+/-}$. In this manner, mice were obtained that were both $CaSR^{+/-}$ and $PTHrP^{+/-}$, or both $CaSR^{+/-}$ and PTH/PTHrP $receptor^{+/-}$. These doubly heterozygous mice were utilized in specific experiments delineated below.

Male and female mice were mated overnight; the presence of a vaginal mucus plug on the morning after mating marked gestational day 0.5. Normal gestation in these mice is 19 d. All mice were given a standard chow diet and water. All studies were performed with the prior approval of the Institutional Animal Care and Use Committee of the Massachusetts General Hospital.

Whole blood and amniotic fluid collection. In general, fetal blood was drawn on day 18.5 of gestation to maximize the volume of serum or whole blood obtained. Pregnant dams were killed by cervical dislocation, and a cesarean section was quickly performed to remove the uterus intact. In turn, each individual fetus was removed from its amniotic sac, and its neck was incised to transect the left carotid and jugular vessels. Whole blood was collected into heparinized capillary tubes for ionized calcium measurements, and plain capillary tubes (without anticoagulant) for serum collection. The volume of fetal blood collected measured 40–75 μl from each fetus. Heparinized samples were kept on ice until used. All other samples were separated by centrifugation; sera were then stored at −20°C until used.

Amniotic fluid was collected from fetuses on day 17.5 of gestation, since the amniotic fluid was generally too scanty and viscous on day 18.5. Cesarean sections were similarly done to remove the uterus intact. The individual gestational sacs were lanced with a 21-gauge needle, and amniotic fluid was collected into heparinized 100-μl capillary tubes. Approximately 75 μl of clear, colorless fluid was obtained from each gestational sac and kept on ice until used for ionized calcium measurements, or stored at −20°C until used in an RIA.

Maternal blood was collected into capillary tubes from the tail vein, immediately before a cesarean section on day 18±0.5 of pregnancy.

Placental calcium transport. This procedure has been described in detail elsewhere (17). Briefly, pregnant dams on day 17±0.5 of gestation were lightly anaesthetized and given an intracardiac injection of 50 μCi [^{45}Ca] and 50 μCi [^{51}Cr]EDTA in 0.9% saline (total injection volume, 100 μl). 5 min later, the dam was killed, and each fetus was asphyxiated. ^{51}Cr and ^{45}Ca activity was determined for each whole fetus using a γ-counter (Packard Instrument Co., Meriden, CT), and a liquid scintillation counter (Beckman, Inc., Fullerton, CA), respectively. The ratio of ^{45}Ca:^{51}Cr activity was then calculated for each fetus. In the experiments utilizing the CaSR knockout mice, the data were normalized to the mean $^{45}Ca/^{51}Cr$ activity ratio of the $CaSR^{+/-}$ fetuses in each litter, so the results from different litters could be compared. In experiments involving the double-knockout of PTHrP and the CaSR, normalization was more complicated due to the potential for up to nine different fetal genotypes in each litter. We have previously established that heterozygosity for PTHrP did not significantly affect placental calcium transport (17); therefore, the data were normalized to a mean calculated from fetuses in each litter that were both wild type (WT) for the CaSR and either PTHrP-WT or $PTHrP^{+/-}$.

Chemical assays. Ionized calcium (whole blood and amniotic fluid) was measured on a Ca^{2+}/pH analyzer (model 634; Ciba-Corning, Corning, NY). Total calcium and magnesium were determined using photometric assays (Sigma Chemical Co., St. Louis, MO). Serum PTH was measured using a rodent PTH 1–34 immunoradiometric assay (IRMA) kit (Nichols Institute, San Juan Capistrano, CA). Serum 1,25-dihydroxyvitamin D was measured using an RIA kit that requires a sample size of only 125 μl (Immunodiagnostic Systems Ltd., Boldon, Tyne, and Ware, UK). Calcitonin was measured with an IRMA specific for rat calcitonin (Immutopics, San Clemente, CA) (25). In lieu of mouse calcitonin standards, values were expressed as picogram-equivalents rat calcitonin per milliliter. Free deoxypyridinoline was measured in amniotic fluid using a human ELISA kit (Pyrilinks-D, Metra Biosystems, Mountain View, CA). Creatinine was measured photometrically using the creatinine/PAP assay (Boehringer Mannheim, Indianapolis, IN). All assays were performed as described in each instruction manual. For the PTH and calcitonin assays, three or four samples of mouse serum were pooled together to obtain 100-μl volumes. These pooled samples were then diluted with 100 μl of the zero standard to bring the volume up to the specified

sample size of 200 μl. For the 1,25-dihydroxyvitamin D assay, four or five samples of mouse serum were pooled together to obtain the required sample size of 125 μl. In the case of the PTH IRMA, results that fell below the detection limit of an assay (specifically, < 2.0 pg/ml) were assigned a value equal to the detection limit for the statistical analysis.

PCR. The tail of each fetus was clipped and placed in lysis buffer (100 mM Tris-HCl, pH 8.0; 500 mM EDTA, pH 8.0; 0.2% SDS; 200 mM NaCl, and 100 μg/ml proteinase K), and digested at 55°C for 12–24 h. Genomic DNA was extracted and purified using phenol-chloroform, precipitated with a 25:1 solution of ethanol and 3 M sodium acetate (pH 5.2), and resuspended in TE (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA, pH 8.0). Oligonucleotide primers were designed that were specific for the native CaSR gene sequence and the inserted neomycin gene sequence; the three sequences were TCT GTT CTC TTT AGG TCC TGA AAC A, TCA TTG ATG AAC AGT CTT TCT CCC T, and TCT TGA TTC CCA CTT TGT GGT TCT A. The genomic DNA was then amplified in a single-tube, 36-cycle PCR reaction using a thermal cycler (PTC-200 Peltier; MJ Research, Cambridge, MA). The validity of the PCR genotyping was confirmed in preliminary experiments by Southern blotting of the genomic DNA with independent probes that were described previously (22).

For the double-knockout mice, genotyping of the CaSR alleles was first accomplished by PCR, using the oligonucleotide primers for the CaSR described above. The PTHrP or PTH/PTHrP receptor genotype was subsequently determined by Southern blotting, using probes that have been described elsewhere (23, 24).

Statistical analysis. Data were analyzed using SYSTAT 5.2.1 for Macintosh (SYSTAT Inc., Evanston, IL). ANOVA was used for the initial analysis; Tukey's test was used in the posthoc analysis to determine which pairs of means differed significantly from each other. Two-tailed probabilities are reported, and all data are presented as mean±SE.

Results

Fetal blood ionized calcium in CaSR knockout mice. Within litters resulting from mating $CaSR^{+/-}$ females to $CaSR^{+/-}$ males, a normal blood ionized calcium level (1.69±0.03 mmol/liter) was observed in WT fetuses. The ionized calcium level was modestly increased in $CaSR^{+/-}$ and $CaSR^{-/-}$ fetuses ($P <$ 0.001 vs. WT) (Fig. 1 *a*). The similar calcium level maintained in $CaSR^{+/-}$ and $CaSR^{-/-}$ fetuses is in sharp contrast to the differences found in early postnatal life, when severe hypercalcemia is found in the $CaSR^{-/-}$ pups (22). This similarity suggests that some aspect of the intrauterine environment prevents the $CaSR^{-/-}$ fetus from achieving a higher blood calcium level.

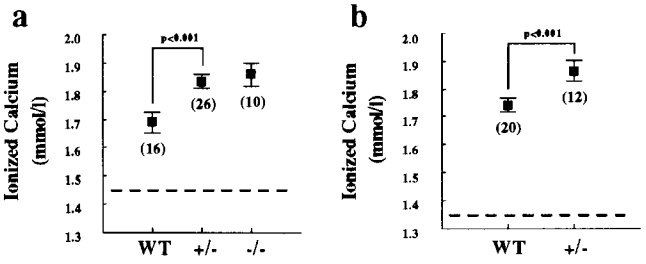

Figure 1. Whole blood ionized calcium. Ionized calcium in fetuses (gestational day 18.5) obtained from (*a*) $CaSR^{+/-}$ female × $CaSR^{+/-}$ male and (*b*) WT female × $CaSR^{+/-}$ male matings. Dashed line in each graph indicates the respective mean maternal ionized calcium for $CaSR^{+/-}$ and WT females. The number of observations for each genotype is indicated within parentheses.

$CaSR^{+/-}$ adult mice have a modestly increased ionized calcium compared with normal (22), and it is conceivable that hypercalcemia of $CaSR^{+/-}$ pregnant mice might affect the fetus. To assess effects of the maternal calcium level on the fetal calcium level, the ionized calcium was also measured in fetuses obtained from mating WT females to $CaSR^{+/-}$ males. The ionized calcium levels in WT and $CaSR^{+/-}$ fetuses obtained from WT, normocalcemic females (Fig. 1 *b*) did not differ from the respective calcium levels in litters obtained from $CaSR^{+/-}$, hypercalcemic females (Fig. 1 *a*). Therefore, the fetal ionized calcium level is set by the CaSR independently of the ambient maternal calcium concentration. Furthermore, since the ionized calcium was higher in $CaSR^{+/-}$ dams vs. WT dams ($P <$ 0.01) (*dashed lines*, Fig. 1, *a* and *b*), the maternal–fetal calcium gradients (fetal minus maternal calcium concentration) were lower for fetuses of $CaSR^{+/-}$ dams compared with their respective counterparts in litters of WT dams ($P < 0.002$). This indicates that a specific maternal–fetal calcium gradient is not directly maintained by the fetus, but is a consequence of the higher fetal calcium level being set independently of the ambient maternal calcium concentration.

Placental calcium transport in CaSR knockout mice. The higher fetal blood calcium resulting from disruption of the CaSR could be maintained by such diverse mechanisms as increased placental calcium transfer, increased skeletal calcium resorption, decreased mineral accretion by the skeleton, and increased renal tubular reabsorption of calcium. We first examined the contribution of placental calcium transfer by methods that we have established previously, in which pregnant mice were given an intracardiac injection of ^{45}Ca and [^{51}Cr]EDTA (17). [^{51}Cr]EDTA remains in the extracellular fluid and has been used in placental perfusion studies in rodents to control for blood flow differences between placentas (26). In this manner, the transfer of ^{45}Ca across the placenta could be compared among the pups of a given litter.

In the offspring from $CaSR^{+/-}$ females mated to $CaSR^{+/-}$ males, the relative transfer of ^{45}Ca was modestly reduced in $CaSR^{+/-}$ fetuses compared with WT, but was substantially reduced to ~ 60% of the WT value in $CaSR^{-/-}$ fetuses (Fig. 2 *a*). In offspring from WT females mated to $CaSR^{+/-}$ males, the relative transfer of ^{45}Ca was significantly reduced in $CaSR^{+/-}$ fetuses compared with their WT littermates (Fig. 2 *b*). Since the $CaSR^{+/-}$ and $CaSR^{-/-}$ fetuses had similarly elevated blood calciums, the decrease in ^{45}Ca transport in the $CaSR^{-/-}$ fetuses cannot simply be explained by an altered fetal–maternal calcium gradient. That placental calcium transfer was decreased by disruption of the CaSR was an unexpected finding. Placental calcium transfer cannot explain, therefore, how the $CaSR^{+/-}$ and $CaSR^{-/-}$ fetuses maintain an increased level of ionized calcium in the blood compared with WT. This implies that skeletal and/or renal tubular handling of calcium must be altered in $CaSR^{+/-}$ and $CaSR^{-/-}$ fetuses to maintain the increased ionized calcium level. The markedly reduced placental calcium transfer in $CaSR^{-/-}$ fetuses may, at least in part, explain why these fetuses are unable to achieve a higher level of ionized calcium compared with their $CaSR^{+/-}$ littermates.

The higher ionized calcium level in $CaSR^{+/-}$ and $CaSR^{-/-}$ fetuses might increase the rate at which calcium returns to the mother from the fetal circulation. Although such "backflux" of calcium across the placenta has been observed to be at least < 10% and in many studies to be < 1% of the forward flow in normal fetal lambs and rodents (27, 28), an increased rate of

backflux in $CaSR^{+/-}$ and $CaSR^{-/-}$ fetuses could at least partly explain why less ^{45}Ca remains in these fetuses at the end of the placental calcium transfer experiments. Fetal–maternal backflux of calcium is technically difficult to assess, however, so we cannot evaluate this possibility.

Preliminary experiments in these mice suggest that the rate of calcium backflux is still very low compared with maternal–fetal flux, so that backflux does not interfere with the measurement of maternal–fetal calcium flux. Further, this possibility was minimized by the short time point used in the ^{45}Ca protocol (5 min after the maternal injection), in which time it would seem unlikely that ^{45}Ca could have crossed the placenta, been diluted in the fetal circulation, and returned back to the maternal circulation in sufficient amounts to account for the 40% decrease in placental calcium transfer observed in $CaSR^{-/-}$ fetuses. Indeed, when fetuses were recovered 30 min after the maternal injection, which should allow for backflux of isotope to become more apparent, the decreases in placental calcium transfer of $CaSR^{+/-}$ and $CaSR^{-/-}$ fetuses were of similar magnitude to the decreases occurring at 5 min (data not shown). Thus, backflux of calcium, even in the $CaSR^{-/-}$ fetuses, is not sufficiently high to importantly lower the apparent forward flow of ^{45}Ca from mother to fetus.

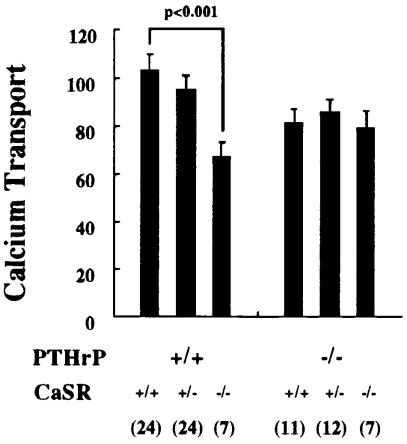

Figure 3. Placental calcium transport in PTHrP × CaSR double-knockout mice. Placental calcium transfer measured at 5 min in fetuses (gestational day 17.5) obtained from mating $CaSR^{+/-}$ $PTHrP^{+/-}$ (double-heterozygous) females and males. The data were normalized to the mean $^{45}Ca/^{51}Cr$ ratio of fetuses that were CaSR-WT, and either PTHrP-WT or $PTHrP^{+/-}$. Data for $PTHrP^{+/-}$ fetuses have been omitted for clarity. The number of observations for each genotype is indicated within parentheses.

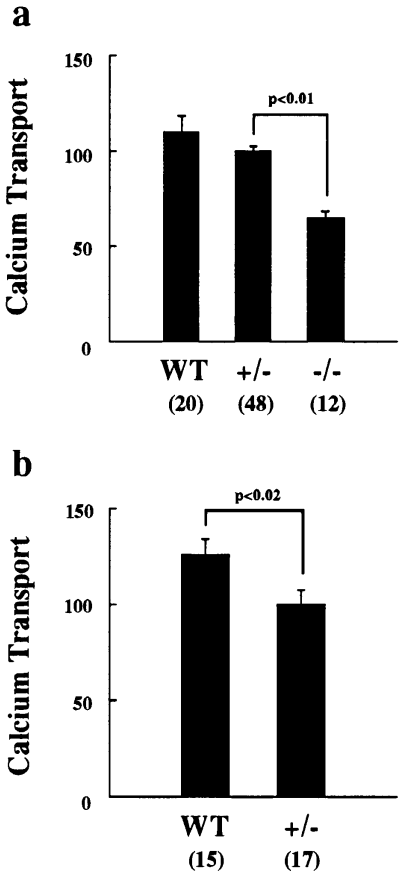

Figure 2. Placental calcium transport in CaSR knockout mice. Placental calcium transfer measured at 5 min in fetuses (gestational day 17.5) obtained from (*a*) $CaSR^{+/-}$ female × $CaSR^{+/-}$ male and (*b*) WT female × $CaSR^{+/-}$ male matings. Data were normalized to the mean $^{45}Ca/^{51}Cr$ ratio of $CaSR^{+/-}$ fetuses within each litter. The number of observations for each genotype is indicated within parentheses.

Effect of CaSR disruption on PTHrP-stimulated placental calcium transfer. The mechanisms whereby calcium transfer across the placenta is regulated are not well understood, but PTHrP has been shown to stimulate placental calcium transfer in fetal lambs (15, 16) and $PTHrP^{-/-}$ fetal mice (17). Thus, suppression of PTHrP-stimulated placental calcium transfer could be one mechanism whereby disruption of the CaSR reduces placental transport. To assess this, we used the PTHrP knockout model, in which we have previously established that placental calcium transfer is reduced in $PTHrP^{-/-}$ fetal mice (17). Upon mating double-heterozygous ($PTHrP^{+/-}$, $CaSR^{+/-}$) males and females, nine different fetal genotypes were produced, including $PTHrP^{-/-}$ fetuses that had none, one, or both copies of the CaSR allele disrupted.

Pregnant double-heterozygous ($PTHrP^{+/-}$, $CaSR^{+/-}$) females were given an intracardiac injection of ^{45}Ca and [^{51}Cr]EDTA, and the fetuses were recovered after 5 min. $PTHrP^{-/-}$ fetuses with two normal CaSR alleles had reduced placental calcium transfer compared with WT fetuses, as previously reported (Fig. 3) (17). When these fetuses were compared to $PTHrP^{-/-}$ fetuses that also had one or both copies of the CaSR allele disrupted, no further reduction in placental calcium transfer was observed (Fig. 3). Thus, the reduction in placental calcium transfer caused by disruption of the CaSR may be at least partly due to suppression of PTHrP-stimulated placental calcium transfer.

Deoxypyridinoline excretion indicates increased skeletal resorption. Although skeletal abnormalities of $CaSR^{-/-}$ mice are not morphologically apparent until early neonatal life (Ho-Pao, C.L., K. Lee, D.A. Conner, B.R. Olsen, G.V. Segre, J.G. Seidman, and C.E. Seidman, manuscript submitted for publication), decreased flux of calcium into fetal bone or increased resorption of calcium from fetal bone could contribute to the increased level of calcium in the blood of $CaSR^{+/-}$ and $CaSR^{-/-}$ fetuses. To assess the rate of skeletal resorption, free deoxypyridinoline was measured in amniotic fluid collected from fe-

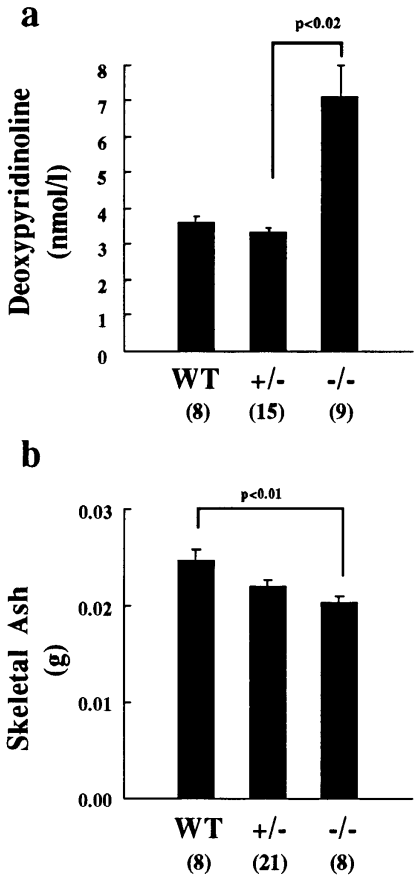

Figure 4. Amniotic fluid deoxypyridinoline and ash weight of skeleton. (*a*) Free deoxypyridinoline concentration in amniotic fluid (gestational day 17.5). (*b*) Ash weight of fetal skeletons (gestational day 18.5). The number of observations for each genotype is indicated within parentheses.

tuses on day 17.5 of gestation. Deoxypyridinoline functions as a crosslink of mature collagen in bone and other tissues, and is excreted intact into the urine when such collagen is degraded (29). Changes in urinary excretion rates of deoxypyridinoline may, therefore, reflect changes in the rate of bone resorption. Indeed, the free deoxypyridinoline concentration in amniotic fluid was significantly higher from $CaSR^{-/-}$ fetuses, as compared with WT and $CaSR^{+/-}$ littermates (Fig. 4 *a*). This is consistent with increased resorption of collagen from the fetal skeleton, and suggests that skeletal calcium resorption is increased in the homozygotes. Increased bone resorption may not explain fully the increase in blood calcium, since the level of deoxypyridinoline was not increased in $CaSR^{+/-}$ fetuses.

As predicted by the increase in bone resorption in $CaSR^{-/-}$ fetuses, the ash weight of the skeletons of $CaSR^{-/-}$ fetuses is decreased (Fig. 4 *b*). This decrease may also result from a change in the rate of bone mineralization, which has not been measured in this study.

Renal calcium excretion. Observations from adult $CaSR^{+/-}$ mice (22) and from humans with FHH indicate that renal tubular reabsorption of calcium is increased by inactivating mutations of the CaSR, such that renal calcium clearance is reduced. A similar effect in fetal kidneys could partly explain how the higher blood calcium in $CaSR^{-/-}$ and $CaSR^{+/-}$ fetuses is maintained. However, CaSR mRNA is expressed at very low levels in the kidneys until the first day after birth (21); consequently, disruption of the CaSR might have minimal effects on renal tubular calcium handling. As a rough index of renal calcium excretion, the total and ionized calcium concentration was measured in amniotic fluid from fetuses on day 17.5 of gestation. Amniotic fluid is largely composed of urine, in addition to secretions from the extraembryonic membranes and fetal lungs (30). The amniotic fluid calcium concentration (total and ionized) was increased equally in $CaSR^{+/-}$ and $CaSR^{-/-}$ fetuses compared with WT (Fig. 5). This is consistent with increased excretion of calcium by the fetal kidneys in proportion to raised blood calcium level and consequent increased renal filtered load of calcium. Since the blood calcium levels (and probably the filtered loads of calcium) are similar in $CaSR^{+/-}$ and $CaSR^{-/-}$ fetal mice, the similar amniotic fluid calciums suggest that fetal tubular handling of calcium is not affected by disruption of the CaSR gene. Although this does not exclude the possibility that renal tubular calcium reabsorption is modestly increased by disruption of the CaSR, it does suggest that renal tubular handling of calcium is also not a major mechanism whereby the higher blood calcium level is maintained.

Hyperparathyroidism due to CaSR disruption. The evidence presented thus far indicates that skeletal resorption is increased by homozygous disruption of the CaSR, while placental calcium transport is decreased. This pattern might be expected to result from increased secretion of PTH from the parathyroids consequent to disruption of the CaSR, since PTH stimulates bone resorption via the PTH/PTHrP receptor, but PTH does not share the ability of PTHrP to stimulate placental calcium transport (15–17). To test this hypothesis, the serum PTH level was measured in fetuses obtained from $CaSR^{+/-}$ females mated to $CaSR^{+/-}$ males. The serum PTH level was undetectable (< 2.0 pg/ml) in WT fetuses, modestly increased in $CaSR^{+/-}$ fetuses, and substantially increased in $CaSR^{-/-}$ fetuses (Fig. 6 *a*). Sera were also pooled from fetuses obtained from mating WT females to $CaSR^{+/-}$ males. Again, the PTH levels of $CaSR^{+/-}$ fetuses were significantly higher than the PTH levels of WT fetuses (Fig. 6 *b*). In comparing the results of Fig. 6, *a* and *b*, it is apparent that the PTH levels in fetuses

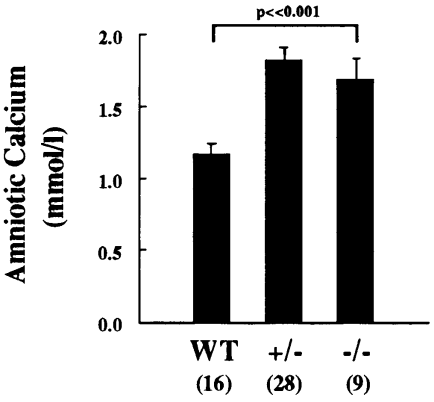

Figure 5. Amniotic fluid total calcium concentrations. Total calcium concentrations are shown from amniotic fluid (gestational day 17.5). The number of observations for each genotype is indicated within parentheses.

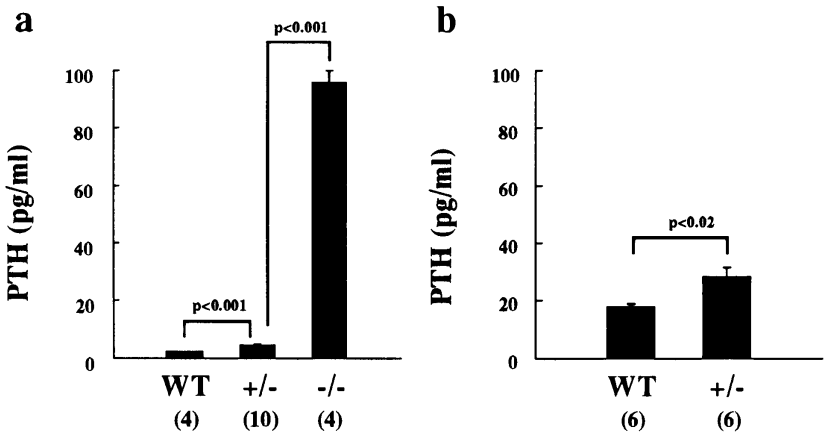

Figure 6. Serum PTH 1-34 levels. Serum PTH in fetuses (gestational day 18.5) obtained from (*a*) $CaSR^{+/-}$ female × $CaSR^{+/-}$ male and (*b*) WT female × $CaSR^{+/-}$ male matings. The number of samples for each genotype is indicated within parentheses. Each sample represents pooled sera from three to four fetuses.

of WT mothers were fivefold higher than their counterparts in litters obtained from $CaSR^{+/-}$ mothers. Maternal hypercalcemia in the $CaSR^{+/-}$ mothers may have partly suppressed the fetal parathyroids, accounting for the corresponding lower fetal PTH levels.

To further assess the physiological impact of the increase in serum PTH caused by heterozygous or homozygous ablation of the CaSR, the serum 1,25-dihydroxyvitamin D level was determined on pooled fetal sera. The increased serum PTH was accompanied by a stepwise increase in the serum 1,25-dihydroxyvitamin D levels of $CaSR^{+/-}$ and $CaSR^{-/-}$ fetal mice, as compared with WT ($P < 0.001$) (Fig. 7 *a*). Although significantly increased compared with WT, the serum 1,25-dihydroxyvitamin D level of $CaSR^{-/-}$ fetuses (146±11 pmol/liter) was still substantially lower than the normal level found in WT adult mice, which ranges from 300 to 500 pmol/liter (data not shown).

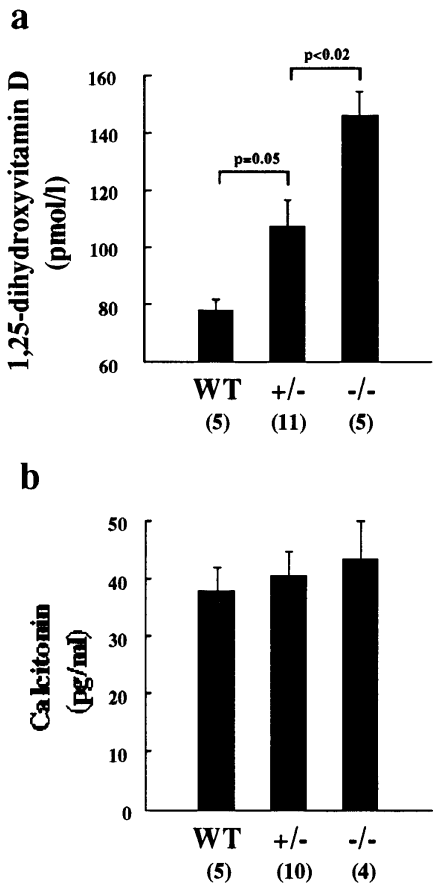

Figure 7. Serum 1,25-dihydroxyvitamin D and calcitonin levels. (*a*) Serum 1,25-dihydroxyvitamin D levels; (*b*) serum calcitonin levels. The number of samples for each genotype is indicated within parentheses. Each sample represents pooled sera from four to five fetuses.

In contrast to the marked effect of CaSR disruption on serum PTH and 1,25-dihydroxyvitamin D levels, serum calcitonin levels were not significantly affected by the disrupted CaSR and raised fetal serum calcium (Fig. 7 *b*). This indicates that, despite the presence of the CaSR on calcitonin-producing thyroidal C cells, the fetal C cells may be relatively unresponsive to further increases in the serum calcium level.

PTH/PTHrP receptor is required for the hypercalcemic effects of CaSR disruption. Finally, we wished to show that worsening hyperparathyroidism (suggested by the increased PTH, 1,25-dihydroxyvitamin D, and deoxypyridinoline levels) was indeed required for maintaining the increased ionized calcium level in blood caused by disruption of the CaSR. This might be best established by studying the effects of CaSR ablation in double-knockout fetuses that also lack the PTH gene. In the absence of PTH, CaSR ablation might then have no effect. Since a PTH gene knockout was not yet available for study, we chose to compare the effects of CaSR ablation in double-knockout fetuses that also lack either PTHrP, or the PTH/PTHrP receptor. The latter mice are expected to be unresponsive to the actions of both PTH and PTHrP that are mediated by the PTH/PTHrP receptor.

We have previously reported that $PTHrP^{-/-}$ fetuses have a blood ionized calcium that is reduced to the maternal level, i.e., the fetal–maternal calcium gradient is obliterated (17). This occurs despite the presence of two normal PTH alleles in the $PTHrP^{-/-}$ fetuses. In contrast, PTH/PTHrP $receptor^{-/-}$ fetuses, which are unable to respond to amino-terminal forms of PTH or PTHrP, have a blood ionized calcium that is lower than the maternal calcium level (17).

Double-knockout matings were performed as described in Methods. In $PTHrP^{-/-}$ fetal mice that were also WT for the CaSR, the ionized calcium was reduced compared with WT (Fig. 8). In $PTHrP^{-/-}$ fetuses that had one or two copies of the

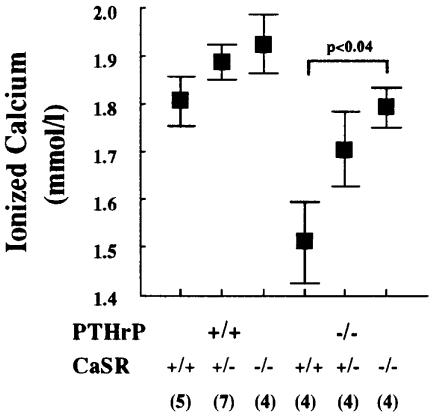

Figure 8. Blood ionized calcium in PTHrP × CaSR double-knockout mice. Data for PTHrP$^{+/-}$ fetuses have been omitted for clarity. The number of observations for each genotype is indicated within parentheses.

disrupted CaSR allele, the ionized calcium was raised to a level equal to WT, but lower than that of WT fetuses having one or two copies of the disrupted CaSR allele (Fig. 8). That this increase is due to the actions of PTH was confirmed by comparing these results to those of the double-knockout involving the PTH/PTHrP receptor and the CaSR (Fig. 9). PTH/PTHrP receptor$^{-/-}$ fetuses with two normal CaSR alleles had a low ionized calcium compared with WT. When PTH/PTHrP receptor$^{-/-}$ fetuses also had one or two copies of the disrupted CaSR allele, no change in the ionized calcium level resulted. Thus, the elevation of blood calcium in CaSR$^{+/-}$ and CaSR$^{-/-}$ fetuses requires activation of the PTH/PTHrP receptor, presumably through binding of PTH and/or PTHrP.

Discussion

We have shown that heterozygous or homozygous ablation of the CaSR gene results in a further increase in the fetal ionized calcium above the maternal level. Ablation of the CaSR is also associated with a stepwise increase in the fetal serum PTH and 1,25-dihydroxyvitamin D levels, and increased excretion of calcium and free deoxypyridinoline into the amniotic fluid. Calcitonin levels are not significantly altered by ablation of the CaSR. Activation of the PTH/PTHrP receptor is required for the hypercalcemic effect of CaSR disruption to be seen, but PTHrP is not needed. PTHrP$^{-/-}$ fetuses had a further increase in their ionized calcium in response to CaSR disruption, while PTH/PTHrP receptor$^{-/-}$ fetuses responded to CaSR disruption with no increase in the ionized calcium level. Increased skeletal calcium resorption, probably mediated by the increase in PTH and 1,25-dihydroxyvitamin D levels, at least partly explains how the fetuses maintain a higher ionized calcium than normal after CaSR disruption. The possibility that the rate of skeletal accretion of calcium might be altered by ablation of the CaSR, and help determine the level of ionized calcium, could not be assessed in these experiments. Finally, placental calcium transport is downregulated by mechanisms that require the presence of PTHrP.

These findings in fetal mice show some striking differences from what has been observed postnatally in the CaSR knockout mice, and postnatally in humans with FHH and neonatal severe primary hyperparathyroidism. These differences can be explained partly by differences in CaSR expression in utero as compared with postnatal expression (for example, postnatal increase in the renal expression of CaSR mRNA [21]), and by the strong influence the placenta has, compared with that of the intestines and kidneys, on regulating water and solute composition in the fetus. The ionized calcium level of CaSR$^{-/-}$ fetuses was no higher than that of the CaSR$^{+/-}$ littermates, in contrast to the striking elevation seen in CaSR$^{-/-}$ pups, and in human neonates with neonatal severe primary hyperparathyroidism. The CaSR$^{-/-}$ fetus may be unable to maintain an even higher ionized calcium because placental calcium transport is substantially decreased in CaSR$^{-/-}$ fetuses. (It is also possible that backflux of calcium at an increased rate from the CaSR$^{-/-}$ fetus minimizes the increase in blood calcium in these mice; we were unable to test this hypothesis.) Further, the severe hypercalcemia in neonates is aggravated by volume depletion and hemoconcentration, but the placenta prevents such volume depletion, such that the hematocrits of WT, CaSR$^{+/-}$, and CaSR$^{-/-}$ fetuses did not differ (data not shown).

The increased maternal ionized calcium level of CaSR$^{+/-}$ mice could influence fetal calcium homeostasis by affecting how readily calcium is transferred across the placenta, and by influencing the level at which the fetal blood calcium is set. The PTH levels of WT fetuses taken from CaSR$^{+/-}$ dams were lower than the PTH levels observed in WT fetuses of WT dams, indicating that maternal hypercalcemia may have suppressed the fetal parathyroids. Conversely, a CaSR$^{+/-}$ fetus from a CaSR$^{+/-}$ mother might be aided by maternal hypercalcemia in achieving its higher ionized calcium level, whereas a CaSR$^{+/-}$ fetus from a WT mother might have to increase its parathyroid activity even further to achieve the same higher ionized calcium level. This is consistent with observations in humans: some children who are CaSR$^{+/-}$ with normal mothers had more severe hypercalcemia and did more poorly postnatally compared with CaSR$^{+/-}$ children of CaSR$^{+/-}$ mothers (2). We also observed that CaSR$^{+/-}$ and WT fetuses had ionized calcium levels that were not affected by whether the mother was WT (normocalcemic) or CaSR$^{+/-}$ (hypercalcemic), al-

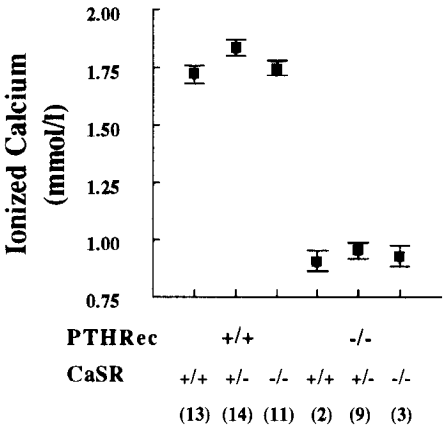

Figure 9. Blood ionized calcium in PTH/PTHrP receptor × CaSR double-knockout mice. Data for PTH/PTHrP receptor$^{+/-}$ fetuses have been omitted for clarity. *PTHRec*, PTH/PTHrP receptor. The number of observations for each genotype is indicated within parentheses.

though the maternal–fetal gradients were reduced by maternal hypercalcemia. This indicates that the fetus normally sets its ionized calcium level independently of the ambient maternal calcium concentration, and does not set a specific calcium gradient with respect to the mother. This finding is consistent with observations previously made in fetuses of vitamin D–deficient rats (31). These fetuses attained normal fetal serum calcium levels despite maternal hypocalcemia, and the respective maternal–fetal calcium gradients were increased.

The observed decrease in placental calcium transport in $CaSR^{-/-}$ and $CaSR^{+/-}$ fetuses is incompletely understood. A portion of the decrease in placental calcium transport probably results from the steeper gradient against which calcium must be actively transported, caused by the elevation of the fetal blood calcium. However, since the ionized calcium level of $CaSR^{+/-}$ and $CaSR^{-/-}$ fetuses is identical, this cannot account for the more substantial decrease in placental calcium transport that is observed in the $CaSR^{-/-}$ fetus. The measurements of ^{45}Ca transport in mice homozygous for disruption of the PTHrP gene and heterozygous or homozygous for disruption of the CaSR gene showed that the CaSR does not influence placental calcium transport in the absence of PTHrP. Thus, the CaSR requires the presence of PTHrP to influence placental calcium transport.

In late gestation, during the time of rapid transfer of calcium across the placenta and accretion of this mineral by the developing skeleton, circulating PTH and 1,25-dihydroxyvitamin D levels are normally low. Both the PTH/PTHrP receptor, which does not appear to mediate placental calcium transport (15–17), and the vitamin D receptor are expressed in the placenta. The increased PTH and 1,25-dihydroxyvitamin D levels caused by disruption of the CaSR might in turn act, directly or indirectly, to suppress PTHrP levels in blood or locally in the placenta, and/or PTHrP's effect on placental calcium transfer. Also, the increased ionized calcium might directly inhibit PTHrP secretion by placental trophoblasts, as has been observed in cultured human cytotrophoblasts (32). Since the blood calcitonin levels are unchanged in CaSR mutant mice, it is unlikely that calcitonin mediates the actions of the CaSR on placental calcium transport.

PTHrP is produced by many diverse tissues in fetal life, many of which, including the parathyroids and placenta, might contribute to the circulating PTHrP level and the regulation of placental calcium transport. The finding that placental calcium transport was reduced by parathyroidectomy of fetal sheep raised the possibility that PTHrP of parathyroid origin is largely responsible for maintaining placental calcium transport. On the other hand, Tucci et al. (33) noted only very small amounts of PTHrP mRNA in murine fetal parathyroids, which contained abundant PTH mRNA. The hypothesis that the fetal parathyroid secretes physiologically relevant amounts of PTHrP has not been tested by measurements of PTHrP in fetal blood after parathyroidectomy. Thus, further experiments are needed to identify the tissue source(s) of PTHrP responsible for controlling placental calcium transport.

Similarly, since the CaSR is expressed in different tissues of the normal fetus, and is eliminated from all tissues of the CaSR-null fetus, it is difficult to be certain which tissue(s) are most responsible for the observed phenotype of the CaSR-null fetus. In preliminary experiments, we have localized CaSR mRNA expression to the intraplacental yolk sac by in situ hybridization; it remains to be determined what role, if any, this site of expression might have in regulating placental calcium transport, or determining the phenotype of the CaSR-null fetus.

These experiments have shown that disruption of the CaSR gene in fetal life perturbs what may normally be a largely PTHrP-regulated fetal environment. The role of the normal CaSR may be to keep PTH suppressed, and through it, 1,25-dihydroxyvitamin D as well, in response to the normal high, PTHrP-dependent, fetal blood calcium. In the absence of PTHrP, as in the $PTHrP^{-/-}$ fetus, the fetal blood calcium falls to a level that triggers the CaSR to permit increased PTH secretion. The $PTHrP^{-/-}$ fetus then maintains a blood calcium at the adult level, which is also equal to the ambient maternal calcium level (thus, the maternal–fetal gradient is obliterated), perhaps because the fetal parathyroid CaSR responds in the same manner as it does in the adult parathyroids.

In summary, this study has shown that heterozygous or homozygous ablation of the CaSR increases the fetal ionized calcium level by causing a stepwise worsening of fetal hyperparathyroidism, and also causes a stepwise downregulation of placental calcium transport. CaSR disruption may, directly or indirectly, downregulate PTHrP or its effect on the placenta. The normal CaSR in fetal life keeps PTH suppressed (and through it, 1,25-dihydroxyvitamin D). After the loss of placental calcium transport at birth, the resulting lowering of the blood calcium would then be expected to trigger PTH secretion.

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