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Postnatal establishment of allelic Gαs silencing as a plausible explanation for delayed onset of parathyroid hormone-resistance due to heterozygous Gαs disruption

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Abstract

Pseudohypoparathyroidism type-Ia (PHP-Ia), characterized by renal proximal tubular resistance to parathyroid hormone (PTH), results from maternal mutations of *GNAS* that lead to loss of Gαs activity. Gαs expression is paternally silenced in the renal proximal tubule, and this genomic event is critical for the development of PTH-resistance, as patients display impaired hormone action only if the mutation is inherited maternally. The primary clinical finding of PHP-Ia is hypocalcemia, which can lead to various neuromuscular defects including seizures. PHP-Ia patients frequently do not present with hypocalcemia until after infancy, but it has remained uncertain whether PTH-resistance occurs in a delayed fashion. Analyzing reported cases of PHP-Ia with documented *GNAS* mutations and mice heterozygous for disruption of *Gnas*, we herein determined that the manifestation of PTH-resistance caused by the maternal loss of Gαs, i.e.

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Supplemental Information is included.

Disclosures

All authors state that they have no conflicts of interest.

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hypocalcemia and elevated serum PTH, occurs after early postnatal life. To investigate whether this delay could reflect gradual development of paternal Gαs silencing, we then analyzed renal proximal tubules isolated by laser capture microdissection from mice with either maternal or paternal disruption of *Gnas*. Our results revealed that, whereas expression of Gαs mRNA in this tissue is predominantly from the maternal *Gnas* allele at weaning (three-weeks postnatal) and in adulthood, the contributions of the maternal and paternal *Gnas* alleles to Gαs mRNA expression are equal at postnatal day 3. In contrast, we found that paternal Gαs expression is already markedly repressed in brown adipose tissue at birth. Thus, the mechanisms silencing the paternal Gαs allele in renal proximal tubules are not operational during early postnatal development, and this finding correlates well with the latency of PTH-resistance in patients with PHP-Ia.

Keywords

pseudohypoparathyroidism; parathyroid hormone; stimulatory G protein; *GNAS*; imprinting; renal proximal tubule; cyclic AMP

Introduction

Certain genes are expressed in a parent-of-origin specific manner from a single allele, and genetic aberrations disrupting this monoallelic expression are the cause of a variety of human diseases and tumors (1,2). *GNAS* is a complex gene leading to several transcripts that show monoallelic expression (3,4). One of the major gene products of *GNAS* is the α-subunit of the stimulatory G protein (Gαs), a ubiquitously expressed signaling protein that mediates the actions of many hormones, neurotransmitters, and autocrine/paracrine factors via the generation of cAMP. More recently described products from *GNAS* include the maternally expressed transcript encoding NESP55 and paternally expressed transcripts A/B (also referred to as 1A), extra-large Gαs (XLαs), and *GNAS* antisense (5–8). Promoters of these latter gene products are differentially methylated. In contrast, the promoter of Gαs lacks methylation, and in most tissues, Gαs expression is biallelic (7,9). However, despite the lack of differential methylation at its promoter, Gαs is expressed predominantly from the maternal allele in a small number of tissues including pituitary, thyroid, and renal proximal tubules (10–13).

Mutations of *GNAS* cause several human diseases, and monoallelic maternal expression of Gαs contributes to the pathogenesis of most of these diseases. For example, somatic mutations leading to constitutive Gαs activity (*gsp* oncogene) are found in growth hormone-secreting pituitary adenomas (14). However, these somatotroph tumors develop only if the mutations are located on the maternal *GNAS* allele (10). Moreover, paternal Gαs expression is frequently derepressed in these tumors, regardless of the presence of activating Gαs mutations (10), presumably contributing to their pathogenesis. Loss-of-function mutations in or abnormal imprinting of the maternal *GNAS* allele lead to pseudohypoparathyroidism type-Ia (PHP-Ia) and PHP-Ib, respectively (15,16), which are both associated with end-organ resistance to the actions of PTH and some other hormones that signal via Gαs. In contrast, mutations on the paternal allele have no effect on hormone action as Gαs expression from

the paternal allele is normally suppressed due to imprinting in hormone target tissues (17,18).

Parathyroid hormone (PTH) binds to a Gαs-coupled receptor and acts on bone, as well as the renal proximal and distal tubules, to regulate calcium and phosphate homeostasis (19). In bone, PTH increases both bone formation and bone resorption. In renal proximal tubules, it induces the biosynthesis of 1,25 dihydroxy vitamin D ($1,25(OH)_2D$) and inhibits the tubular reabsorption of phosphate, and in renal distal tubules, it enhances the tubular reabsorption of calcium. The paternal Gαs allele is silenced in the renal proximal tubule (13,20), but not in bone or the distal part of the nephron (9,21). Accordingly, resistance to PTH in patients with PHP-Ia and PHP-Ib occurs primarily in the renal proximal tubule (22). These patients typically develop hypocalcemia and hyperphosphatemia, combined with elevated serum PTH (23). Responsiveness of bone and renal distal tubules to PTH, on the other hand, appears to be preserved, as patients with PHP can show evidence for increased bone turnover and hypocalciuria (24).

Clinical features resulting from PTH-resistance in PHP-Ia and PHP-Ib are often observed after the first year of life, and it has therefore been suggested that PTH-resistance in these PHP forms develops in a delayed fashion (24–26). On the other hand, several cases of PHP-Ia with documented *GNAS* mutations have been reported in whom PTH levels were elevated during infancy (27–32). Moreover, although the presence of *GNAS* mutations was not investigated, hypocalcemia due to PTH resistance has been reported in a substantial number of infants and newborns (33–41). Hence, it has remained unclear whether the renal proximal tubular PTH-resistance caused by maternal loss of Gαs manifests itself during early postnatal life. This is an important question, considering that the paternal Gαs silencing has an important role in the development of this biochemical defect (42). If this hormonal abnormality indeed develops in a delayed fashion, it may indicate that the silencing of the paternal Gαs allele is not established at birth but instead develops postnatally, suggesting, in turn, that the mechanisms underlying this critical, yet poorly understood, epigenetic process are subject to developmental regulation. The temporal profile of allelic Gαs silencing in the renal proximal tubule, however, has also remained *hitherto* unknown.

We herein reviewed reported PHP-Ia cases with documented *GNAS* mutations and investigated mice heterozygous for *Gnas* disruption regarding the temporal development of PTH-resistance. Our findings indicate that PTH-resistance caused by the loss of maternal *GNAS* allele occurs in a delayed fashion. Moreover, we determined that paternal Gαs silencing is established during postnatal development of mouse renal proximal tubules, thus providing a plausible explanation for the latency of PTH-resistance in PHP-Ia.

Materials and Methods

Analysis of PHP-Ia patients with documented GNAS mutation

The literature was reviewed between the years of 1998 and 2011, and reports in which the description of PHP-Ia patients included confirmed *GNAS* mutations and information on serum calcium, phosphorus, and/or PTH were selected (see Supplemental Information). Patients who were classified as PHP-Ic and carried receptor-uncoupling Gαs mutations were

also included. Analyses were conducted on those for whom serum calcium and PTH levels, as well as the presenting symptom and age at presentation, were noted. Based on these criteria, 97 of 120 reported cases were included in the analyses.

Generation and maintenance of mice

Mice with universal targeted disruption of exon 1 or exon 2 of *Gnas* were described previously (13,43). Mice heterozygous for disruption of either *Gnas* exon were maintained on FVB and CD1 backgrounds, respectively. For the generation of mice with maternal *Gnas* ablation, wild-type males were mated with female mice heterozygous for paternal *Gnas* ablation, so that the dams were not hypocalcemic. For the generation of mice with paternal *Gnas* ablation, wild-type females were mated with male mice heterozygous for paternal *Gnas* ablation. Data were obtained from both female and male mice. The genders of three- and six-day old pups could not be determined reliably, preventing us from making comparisons between males and females during early postnatal development. These studies were approved by the Institutional Subcommittee on Research Animal Care.

PTH-induced plasma cAMP increase

Evaluation of PTH responsiveness by the measurement of plasma cAMP levels has been done essentially as described (44). Mice were injected subcutaneously with 40 μg/kg [Nle8, 21, Y34]rPTH(1–34) (PTH(1–34)), which was synthesized at the Massachusetts General Hospital Biopolymer Core Facility. Blood was collected into EDTA tubes 10 min after injection. cAMP in the plasma before and after PTH(1–34) injection was quantified by a radioimmunoassay.

Calcium and PTH measurements

Blood ionized calcium levels were measured from blood samples obtained from the neck by using Siemens Rapidlab 348 analyzer. Plasma intact PTH measurements were performed as described previously (45).

Tissue isolation and qRT-PCR

Fifteen-twenty min after injection of intracardiac albumin conjugated with Alexa-Fluor®-555 (0.04 mg/kg body weight), mice were sacrificed, and kidneys were snap frozen in O.C.T embedding medium (Tissue-Tek). After sectioning, renal proximal tubules were isolated by laser capture microdissection (Massachusetts General Hospital Cell Biology/ Microscopy Core Facility), as described previously (46) (see Supplemental Figure for images showing fluorescently marked proximal tubules at different ages). Brown adipose tissue (BAT) was isolated from the interscapular area of newborn mice. RNA isolation was performed as previously described (45). Quantitative real-time RT-PCR (qRT-PCR) was performed by using the OneStep RT-PCR Kit (Qiagen) with Taqman probes (Mm00530548_m1 for Gαs and Mm00607939_s1 for beta-actin from Applied Biosystems). To amplify *Cyp27b1* and *Cyp24a1* mRNA, total RNA was transcribed by using a commercially available set of High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNA was prepared with random hexamer primers, according to the manufacturer's instructions. Real-time PCR was performed by using the SYBR Premix Ex

Taq (Tli RNase H Plus) (Takara-Clontech). Primers for *Cyp27b1* were 5′-CAA ATG GCT TTG TCC CAG AT-3′ and 5′-GGC TGT CTT CCG AAT GGT TA-3′. Primers for *Cyp24a1* were 5′-GTG CGG ATT TCC TTT GTG AT and 5′-GGG ATT CCG GGA TAG ATT GT-3′. All amplifications were carried out at least in duplicates. Dissociation curve analysis of the PCR amplicons was performed to verify single product amplification.

Statistical analyses

Statistical significance for differences between two groups ($p<0.05$) was determined by either the non-parametric Mann-Whitney U test (for the age data reported for PHP-Ia patients) or the Student's two-tail *t*-test (for biochemical and molecular data obtained from mouse models). Means of more than two groups (for PTH-induced elevation of plasma cAMP) were analyzed by using one-way analysis of variance, followed by Tukey's pair-wise comparisons. Non-parametric Kruskal-Wallis test, followed by Dunn's multiple comparisons, was used for the statistical analysis of differences among the median ages of patients with hypocalcemia, TSH elevation, obesity, or normocalcemic PTH elevation as presenting finding.

Results

The onset of PTH-resistance in patients with PHP-Ia

We identified 120 cases with PHP-Ia reported between 1998 and 2011 in whom inactivating *GNAS* mutations were found and serum calcium, phosphorus, and/or PTH values given (Supplemental Table and Supplemental References). Of those cases, 97 had information regarding the presence or absence of hypocalcemia, the presenting symptom, and the age at presentation. In this latter group, evidence for PTH-resistance was noted in 94 cases, who either had hypocalcemia (60 cases) or normocalcemia with elevated PTH levels (34 cases) (Fig. 1A, top). The remaining three cases were diagnosed with PHP-Ia because of documented *GNAS* mutations on the maternal allele and the presence of Albright's hereditary osteodystrophy. The age at which hypocalcemia was noted, either by symptoms or by clinical exam/lab, ranged from 0.3 to 60 years, with only two cases being infants, i.e. at age one year or younger (Fig. 1A, top; Supplemental Table). The age at which normocalcemic elevation of PTH was noted, i.e. by laboratory analysis, ranged from 0.2 to 22 years, and eight of the latter cases were infants (Fig. 1A; Supplemental Table). The median age of those who presented with normocalcemic PTH-resistance was significantly lower than the median age of those who presented with hypocalcemia (2.6 vs. 7.8 years) (Fig. 1A, top), consistent with the notion that the elevation of PTH precedes the hypocalcemia in PHP-Ia (47,48). Thirty-five cases of PHP-Ia showed hypocalcemia as the presenting finding (Fig. 1B, top). In comparison, elevation of thyroid stimulating hormone (TSH), which indicates TSH-resistance and mostly compensated hypothyroidism, was observed in 84 of 90 cases in whom this was examined, and 17 of those cases had TSH elevation as the presenting finding (Fig. 1B, top). Obesity, which is another maternally inherited feature of PHP-Ia (49), was noted in 65 out of 89 cases in whom body-mass index or the presence of obesity was noted. Seventeen of those cases were reported as having obesity as the presenting finding of this disease (Fig. 1B, top). The median age of patients who presented first with hypocalcemia (10 years) was significantly higher than the median

age of patients who presented first with either TSH elevation (0.1 years) or obesity (4.4 years) (Fig. 1B, top). We also compared the age at which normocalcemic PTH elevation was documented to the age at which the first presenting finding was noted as either TSH elevation or obesity. For this comparison, we excluded, from the group with normocalcemic PTH elevation, those patients whose presenting finding was either TSH elevation (9 patients) or obesity (8 patients). The median age for this re-defined group of patients (4.0 years) was still significantly higher than the median age of patients who presented first with TSH elevation, but not significantly different from the median age of those who presented first with obesity (Fig. 1B, top). We did not observe significant differences between males and females for any of these parameters, although the median age at which normocalcemic PTH elevation was documented tended to be lower in males than in females (1.7 vs 5.0 years; p=0.08) (Fig. 1A, middle and bottom). The individual gender-specific trends were similar to those observed in the entire patient group; however, statistical significance could not be reached in females with respect to the difference between the median age at which hypocalcemia was noted and the median age at which normocalcemic PTH elevation was documented (7.8 vs. 5.0 years; p=0.18) (Fig. 1A, bottom). In addition, some of the multiple comparisons performed within individual genders did not show statistical significance, likely reflecting diminished sample size (Fig. 1B, middle and bottom). Taken together, these results indicate that in patients with PHP-Ia (i) the end-organ resistance to PTH manifests itself mostly, but not always, after infancy, (ii) the elevation of PTH precedes the development of hypocalcemia, and (iii) the presentation of PTH-resistance occurs later than some other findings of PHP-Ia, including TSH-resistance.

In vivo PTH-induced cAMP response in $E2^{m-/+}$ mice

To investigate the age-dependence of PTH-resistance resulting from maternal loss of Gαs in a more controlled setting, we analyzed mice heterozygous for disruption of maternal *Gnas* exon 2 ($E2^{m-/+}$). These mice, which have been generated as a model of PHP-Ia, show hypocalcemia with elevated serum PTH levels (13). Since PHP-Ia patients have been reported to display a blunted plasma cAMP response to PTH injection (50,51), which is considered to reflect the effect of PTH on kidney (52), we first asked whether PTH-induced plasma cAMP response was blunted in $E2^{m-/+}$ mice. Subcutaneous injection of PTH(1–34) led to a marked increase (4.3-fold over baseline) in plasma cAMP levels of 5-month old wild-type mice within 10 min (Fig. 2A). A slightly lower response, albeit statistically significant, was observed in age-matched $E2^{+/p-}$ mice (3.6-fold), which are heterozygous for disruption of paternal *Gnas* exon 2 (Fig. 2A). As expected, the PTH-induced increase in plasma cAMP was markedly lower (1.3-fold) in 5-month old $E2^{m-/+}$ mice than wild-type littermates (Fig. 2A), confirming resistance to the action of PTH in adult $E2^{m-/+}$ mice. However, in three-week-old $E2^{m-/+}$ mice, although a blunted plasma cAMP response to PTH injection was observed compared to wild-type littermates, this response was significantly stronger (2.7-fold) than that observed in adult $E2^{m-/+}$ mice (Fig. 2B, C). These results thus suggested that the latency of PTH-resistance observed in PHP-Ia patients could also occur in $E2^{m-/+}$ mice.

PTH-induced cAMP and postnatal changes in serum calcium and PTH levels of $E1^{m-/+}$ and $E1^{+/p-}$ mice

Following the generation of $E2^{m-/+}$ and $E2^{+/p-}$ mice, it has become clear that *GNAS* gives rise to multiple gene products that utilize exon 2 (3,4). *GNAS* exon 1, however, is exclusively utilized by Gαs, and therefore, we continued our investigations using the more recently generated mouse model in which *Gnas* exon 1 is deleted either maternally ($E1^{m-/+}$) or paternally ($E1^{+/p-}$) (43). We first measured the plasma concentration of cAMP in three-month old $E1^{m-/+}$ mice basally and 10 min after subcutaneous injection of PTH(1–34). Whereas wild-type littermates showed a 2.7-fold increase over the baseline, $E1^{m-/+}$ mice showed only a 1.4-fold increase, with the PTH-induced cAMP level being significantly lower in the latter) (Fig. 2D). Thus, PTH-resistance could also be demonstrated in adult $E1^{m-/+}$ mice by this method. However, three-week old $E1^{m-/+}$ mice showed a PTH-induced plasma cAMP response that was only slightly lower than that obtained from wild-type littermates (1.8-fold compared to 2.1-fold over baseline), and the difference in cAMP levels after PTH injection was not statistically significant (p=0.71) (Fig. 2E). The PTH-induced plasma cAMP response was also normal in three-week old $E1^{+/p-}$ mice, which showed a 2.5-fold increase over baseline compared to the 2.2-fold increase obtained from wild-type littermates; the difference between PTH-induced cAMP levels was not statistically significant (p=0.89; Fig. 2F). These results suggested that PTH-resistance in three-week old $E1^{m-/+}$ mice is not fully established, consistent with the latency of PTH resistance.

Similar to the biochemical findings observed upon the maternal loss of *Gnas* exon 2, maternal deletion of *Gnas* exon 1 has previously been shown to cause hypocalcemia with elevated PTH levels in adult mice (20,45); note that serum $1,25(OH)_2D$ levels in $E1m^{-/+}$ mice tend to be lower but not significantly different from the levels in wild-type littermates (45). To determine the age of onset of hypocalcemia and elevated PTH, we analyzed the $E1^{m-/+}$ and $E1^{+/p-}$ mice during the early postnatal period. Analyzing both males and females together, blood ionized Ca levels in three-day, six-day, and three-week old $E1^{m-/+}$ mice did not significantly differ from those in wild-type littermates, although three-week old $E1^{m-/+}$ mice tended to have lower levels than wild-type (p=0.28) (Fig. 3A). In two-month old $E1^{m-/+}$ mice, however, ionized Ca levels were significantly lower than wild-type littermates (Fig. 3A). PTH levels in three-day and six-day old $E1^{m-/+}$ mice were similar to those in wild-type littermates, but the levels were 3.1- and 2.5-fold higher in three-week and two-month old $E1^{m-/+}$ mice than wild-type, respectively (Fig. 3B). Between female and male $E1^{m-/+}$ mice, no significant differences in serum Ca and PTH existed at age three weeks and two months, except that ionized Ca was found to be significantly lower in male than in females at age two months (Table 1). By and large, results from individual genders were similar to those obtained from the entire group. Both female and male $E1^{m-/+}$ mice had significantly lower ionized Ca levels than wild-type littermates at age two months, but hypocalcemia was not present at age three weeks. On the other hand, elevation of PTH was observed in both female and male $E1^{m-/+}$ mice at the latter age; however, the difference between the PTH levels of three-week old $E1^{m-/+}$ mice and wild-type littermates was statistically significant in females only. Thus, similar to what is observed in patients with PHP-Ia, the progression of PTH-resistance is delayed, and the increase of serum PTH precedes hypocalcemia in $E1^{m-/+}$ mice.

Unlike in $E1^{m-/+}$ mice, ionized Ca levels in $E1^{+/p-}$ mice were not significantly different from those in wild-type littermates in all age groups (Fig. 3C). PTH levels in $E1^{+/p-}$ mice were also similar to wild-type; however an unexplained elevation of 1.7-fold was detected in the PTH levels of three-week old $E1^{+/p-}$ mice compared to wild-type littermates (Fig. 3D). No differences between genders were observed in ionized Ca and PTH levels, except for a small but statistically significant difference between the ionized Ca levels of two-month old female and male $E1^{+/p-}$ mice (Table 2). In addition, the PTH level in male $E1^{+/p-}$ mice tended to be higher than in female $E1^{+/p-}$ mice at age three-weeks ($p=0.08$). In fact, the elevation of PTH in three-week old $E1^{+/p-}$ mice was observed only in males. Thus, data obtained from $E1^{+/p-}$ mice are mostly consistent with those observed in humans who have inactivating mutations in paternal *GNAS*, i.e. pseudopseudohypoparathyroidism (PPHP) (17,53).

Allelic expression of Gαs in the renal proximal tubule

Gαs is expressed predominantly from the maternal allele in the proximal tubule, while biallelic Gαs expression has been documented in other portions of the nephron, including the distal tubule (9,21). To investigate whether the latency in PTH-resistance could be due to delayed development of paternal Gαs silencing in the renal proximal tubule, we measured the levels of Gαs mRNA in this tissue in $E1^{m-/+}$ and $E1^{+/p-}$ mice, thus determining the expression from either the paternal or the maternal *Gnas* allele, respectively. We isolated the renal proximal tubules by laser capture microdissection following injection of fluorescently-labeled albumin, which was utilized to mark proximal tubules (46). Clear staining of tubules could not be obtained consistently in the neonates, as the small size of the pups and, additionally in the case of $E1^{m-/+}$ pups, the edema that existed around the upper torso (43), prevented us from performing the injections effectively. Therefore the earliest samples we obtained were from three-day old pups. qRT-PCR analysis using total RNA obtained from these renal proximal tubules showed that at postnatal day 3 the levels of Gαs mRNA in the $E1^{m-/+}$ and $E1^{+/p-}$ mice were similar and approximately half the level in wild-type littermates (Fig. 4A). The level of Gαs mRNA in three-week old $E1^{m-/+}$ mice was significantly reduced compared to the level in three-day old $E1^{m-/+}$ mice. The reduced Gαs mRNA level in three-week old $E1^{m-/+}$ mice corresponded to 40 ± 3% of that in wild-type littermates, and this was significantly lower than the Gαs mRNA level in three-week old $E1^{+/p-}$ mice, in which the level was 61 ± 5% of wild-type (Fig. 4A). Likewise, Gαs mRNA levels in two-month old $E1^{m-/+}$ and $E1^{+/p-}$ mice were 35 ± 2% or 65 ± 6% of wild-type, respectively (Fig. 4A), being significantly different from each other but not from the levels obtained from three-week old mice of the same genotype. These findings indicate that while Gαs is expressed equally from both maternal and paternal alleles at postnatal day 3, it is predominantly maternal at age three-weeks and two-months. In contrast, Gαs mRNA level in neonatal BAT of $E1^{m-/+}$ mice was markedly lower (14 ± 1%) than that in wild-type littermates (Fig. 4B), indicating that Gαs is expressed predominantly from the maternal allele in BAT at birth.

Cyp27b1 and Cyp24a1 expression in proximal tubules of $E1^{m-/+}$ and $E1^{+/p-}$ mice

To determine whether the Gαs mRNA levels correlate with the levels of *Cyp27b1* mRNA, which encodes 25-hydroxyvitamin D3 1-alpha hydroxylase and is induced by PTH via Gαs

signaling in the proximal tubule, we measured this transcript by qRT-PCR using total RNA extracted from the microdissected tubules. At postnatal days 3 and 21, *Cyp27b1* levels in $E1^{m-/+}$ mice were similar to those in wild-type littermates, whereas at age two months these levels tended to be mildly reduced in $E1^{m-/+}$ mice (Fig. 5A); however, the difference compared to wild-type littermates was not statistically significant (p=0.18). $E1^{+/p-}$ mice showed no marked differences in *Cyp27b1* levels compared to wild-type (Fig. 5B). We also measured the levels of *Cyp24a1* mRNA, which encodes vitamin D 24-hydroxylase. No significant differences were present between $E1^{m-/+}$ mice and wild-time littermates at postnatal days 3 and 21, whereas the levels tended to be lower in $E1^{m-/+}$ mice at age two-months (Fig. 5C); however, the difference was not statistically significant (p=0.10). $E1^{+/p-}$ mice did not show significant differences from wild-type littermates regarding *Cyp24a1* expression levels (Fig. 5D).

Discussion

In this study, we examined reported cases of PHP-Ia with documented *GNAS* mutations and found that elevated PTH levels with or without hypocalcemia becomes manifest mostly, but not always, after infancy. By using mouse models of this disorder we also showed that, although the Gαs mutation is present from the time of conception, hypocalcemia and elevated PTH levels develop after early postnatal life. In addition, taking advantage of mice in which maternal or paternal *Gnas* exon 1 is ablated, we examined the allelic expression of Gαs in the renal proximal tubule, thus showing that both parental alleles contribute equally to Gαs expression during the early postnatal period, with increasing relative expression from the maternal allele during development.

The results of our analyses are consistent with what have been described in several case reports (47,48,54,55), indicating that the elevation of PTH precedes the manifestation of hypocalcemia. The increase in PTH may be responsible for maintaining normal serum calcium levels for a certain period of time, due likely to the actions of PTH in bone and renal distal tubules. It is also likely that, as evidenced in $E2^{m-/+}$ and $E1^{m-/+}$ mice, the degree of end-organ resistance increases with age, preventing elevated PTH levels from being able to maintain normal serum calcium. In addition, high oral calcium intake, as in breast or formula fed infants, could perhaps help delay or mitigate the hypocalcemia. Note that the three-week old $E1^{m-/+}$ mice analyzed in our study were not yet weaned. In early postnatal life, a small number of PHP-Ia patients were reported to have hypocalcemia or normocalcemia with elevated PTH, unlike the data obtained from our $E1^{m-/+}$ mice. This finding in humans could reflect other genetic or non-genetic factors, such as insufficient vitamin D or calcium intake during infancy, or perhaps defects related to calcium metabolism in the mother who is also a carrier of the *GNAS* mutation.

According to our analyses, male and female PHP-Ia patients do not differ significantly with respect to the age at which the evidence of PTH-resistance is observed. Our results from $E1^{m-/+}$ mice also do not indicate gender-specific differences regarding the temporal development of PTH-resistance, at least based on the analysis of three-week old and two-month old mice. Using the same strain of adult $E1^{m-/+}$ mice, we have previously detected no gender differences in serum calcium, PTH, phosphorus, and $1,25(OH)_2D$ values (45).

Likewise, no gender-specific differences were observed in serum calcium, PTH, and phosphorus levels of adult $E2^{m-/+}$ mice (13). Thus, gender may not play a significant role in the onset and the progression of PTH-resistance in PHP-Ia, but this question needs to be addressed through additional studies.

PTH-induced cAMP generation has been found to be reduced in proximal tubule enriched renal cortices of adult $E2^{m-/+}$ mice (13) and in kidney membranes from another adult mouse model in which maternal *Gnas* exon 1 was ablated (20). Our findings in $E1^{m-/+}$ and $E2^{m-/+}$ mice with respect to PTH-induced changes in plasma cAMP are consistent with those previous studies, indicating resistance to PTH. Moreover, our results demonstrate a progressive increase of PTH-resistance in both of these PHP-Ia mouse models. However, three-week old $E1^{m-/+}$ mice, unlike three-week old $E2^{m-/+}$ mice, did not show a significantly blunted plasma cAMP response to PTH. This discrepancy could reflect the differences between the background strains in which these two models are maintained (FVB and CD1, respectively) or the finding that $E2^{m-/+}$ mice show retarded kidney development (L.S.W. unpublished data). Additionally, the ~80% pre-weaning mortality in $E2^{m-/+}$ mice (13) resulted essentially in the analysis of a selected group of survivors, and this selected group may have shown poor responsiveness to PTH due to some other reasons. Note that, although there are various different PTH responsive tissues, the PTH-induced cAMP elevation in the plasma appears to reflect cAMP generation in a tissue(s) in which Gαs is silenced from the paternal allele. This interpretation is based on our data from adult $E1^{m-/+}$ and $E2^{m-/+}$ mice and the finding that PTH-induced cAMP generation is markedly blunted in both urine and plasma of adult mice in which Gαs is silenced from both parental alleles due to loss of *Gnas* imprinting (model of PHP-Ib) (56). Given the data indicating that PTH-induced elevation of plasma cAMP reflects the action of this hormone in kidney (52), this tissue is likely to be the renal proximal tubule. Thus, the apparent absence of a significantly blunted plasma cAMP response in three-week old $E1^{m-/+}$ mice suggests that renal proximal tubular resistance to PTH is not yet fully established at that age.

Serum 1,25(OH)2D levels have been reported to be slightly lower in two-month old $E1^{m-/+}$ mice than wild-type littermates, although the difference was not statistically significant (45). This finding is consistent with the mild reduction of *Cyp27b1* expression in proximal tubules of two-month old $E1^{m-/+}$ mice. The concomitant increase in serum PTH in these animals thus indicates PTH-resistance in the renal proximal tubule. Similarly, proximal tubular *Cyp27b1* expression is normal despite significant elevation of serum PTH in three-week old $E1^{m-/+}$ mice, and this inappropriately normal *Cyp27b1* expression also provides evidence for PTH-resistance at this age. These findings correlate well with the marked reduction of Gαs mRNA in proximal tubules of three-week and two-month old $E1^{m-/+}$ mice. On the other hand, PTH reduces *Cyp24a1* mRNA stability (57), and thus, the mild reduction of *Cyp24a1* mRNA in proximal tubules of two-month old $E1^{m-/+}$ mice could indicate that this action of PTH is not impaired. However, renal *Cyp24a1* is under the control of various other factors (58), and the mild reduction in its expression level could be secondary to reduced serum calcium and/or 1,25(OH)2D levels. Further investigations are necessary to determine the relative roles of *Cyp27b1* and *Cyp24a1* in the development of hypocalcemia in PHP-Ia.

The finding that $E1^{+/p-}$ mice are normocalcemic correlates well with findings in patients with PPHP. However, the elevation of PTH in three-week old $E1^{+/p-}$ mice is unexpected, as patients with PPHP are described as having Albright's hereditary osteodystrophy without evidence for hormone resistance (53). Serum PTH has also been found to be modestly elevated in another paternal *Gnas* exon 1 knockout mouse model in adulthood, and like $E1^{+/p-}$ mice, the latter mice were normocalcemic (20). This discrepancy between data from humans and mice could perhaps be explained by species-specific differences. Alternatively, there might be some patients with paternal inactivating *GNAS* mutations who have high-normal or mildly elevated PTH levels in the absence of hypocalcemia. Given that Gαs is normally expressed from both parental alleles in bone and renal distal tubules (9,21), the elevation of PTH in three-week old $E1^{+/p-}$ mice may reflect a modest degree of Gαs haploinsufficiency in those tissues. In that case, however, the PTH elevation in three-week old $E1^{m-/+}$ mice would also be due, at least partly, to this putative Gαs haploinsufficiency, because the PTH levels in $E1^{m-/+}$ is only modestly, and not statistically significantly, higher than those in $E1^{+/p-}$ mice at this age (434 vs. 325 pg/ml; p=0.15) (see Fig. 3). In fact, our finding that three-week old $E1^{m-/+}$ mice show an apparently normal PTH-induced plasma cAMP response also correlates with this interpretation (see Fig. 2E). Moreover, it has been shown that derepression of paternal Gαs allele reduces, but does not normalize, the elevated serum PTH levels in another mouse model of PHP-Ia, in which an inactivating missense mutation is present within maternal *Gnas* exon 6 (42). It thus appears that factors other than paternal Gαs silencing may contribute to the PTH-resistance that results from the loss of maternal Gαs allele. Further investigations are needed to address this possibility.

Consistent with our results, Zheng et al. have shown no evidence for Gαs imprinting in human fetal renal cortices (59). Thus, the mechanisms silencing the paternal Gαs allele in the renal proximal tubule operate only after birth, perhaps reflecting the immaturity of renal tubules at birth and during early postnatal life (25). In contrast, the allelic silencing of Gαs appears to occur much earlier in various other tissues, considering the findings in mice that loss of the maternal Gαs allele leads to neonatal subcutaneous edema (13,43,60,61) and that derepression of the paternal Gαs allele leads to early postnatal growth retardation (62). Based on clinical findings in patients with PHP-Ia, allelic Gαs silencing might also be present in neonatal thyroid (63–65). Moreover, our results, consistent with evidence obtained from mice with paternal deletion of exon A/B (termed 1A in mice) (42), show that paternal Gαs expression is repressed in BAT already at birth. Interestingly, however, a previous study has shown that the paternal Gαs allele is expressed almost to the same extent as the maternal Gαs allele in adult BAT (43). Thus, the mechanisms governing this silencing event may be subject to tissue-specific temporal constraints. Of note, the postnatal decline of Gαs silencing in BAT coincides with the temporal expression of XLαs in this tissue, which is abundant at birth but reduces drastically after early postnatal development (66). It remains to be determined whether XLαs expression, which occurs exclusively from the paternal allele, is involved in the tissue-specific allelic silencing of Gαs. Similar to *GNAS*, several other genes demonstrate tissue-specific monoallelic expression (67). Some of those genes, such as *KCNQ1*, *GRB10*, and *IGF2*, are also developmentally regulated in this regard, at least in certain tissues (68–71). It is possible that analogous mechanisms regulate the allelic

silencing of these genes and *GNAS* via tissue-specific factors that are expressed at different stages of development.

Our results indicate that the contribution of the paternal allele is ~35% of the total in two-month old mouse proximal tubules, i.e. markedly more than the paternal Gαs contribution in neonatal BAT, which is ~14% (see Fig. 4A and 4B). This finding, consistent with previous observations made upon the analysis of renal cortices (20), is highly unlikely to reflect contamination from surrounding tissues, because we obtained renal proximal tubules by laser capture microdissection. By using this method in our lab we have previously isolated mouse proximal tubules and showed that the isolated tissue was positive for various proximal tubule-specific markers (e.g. aldolase, the V-ATPase E-subunit, and the Arf6-GDP/GTP exchange factor ARNO), but not distal tubule- (e.g. caveolin-1 and caveolin-2) or endothelial-specific markers (e.g. CD31 and ICAM1) (72). Therefore, it appears that the paternal silencing of Gαs expression in the renal proximal tubule is incomplete. In that case, however, Gαs mRNA levels in proximal tubules of adult patients with PHP-Ib – in whom both *GNAS* alleles, partially or entirely, show a paternal-specific imprinting profile – would be predicted to be ~70% of the levels in healthy individuals and higher than the levels in adult patients with PPHP, who would have ~65% Gαs levels compared to healthy individuals. Considering that patients with PHP-Ib, but not those with PPHP, have significant hypocalcemia, it is possible that there are species-specific differences in the extent of Gαs imprinting between humans and rodents. No data is currently available from PHP-Ia patients or healthy humans regarding the paternal silencing of Gαs in the renal proximal tubule, but a more pronounced degree of silencing may indeed exist in humans, considering that the biochemical phenotype of mice heterozygous for ablation of maternal *Gnas* exon 1 is less severe than that observed in most patients with PHP-Ia (13,20).

On the other hand, we have recently generated a mouse model of PHP-Ib, in which the Gαs silencing occurs on both parental alleles due to specific methylation changes on the maternal *Gnas* allele (61). Adult PHP-Ib mice demonstrated hypocalcemia and elevated serum PTH (56). Thus, additional factors may underlie our observation that the silencing of paternal Gαs allele in the mouse renal proximal tubule is incomplete. For example, it is conceivable that the paternal Gαs allele is more dramatically silenced but only within a small portion of the proximal tubule. This would reconcile the finding that, whereas PTH-induced plasma cAMP response is almost completely blunted, substantial Gαs mRNA expression is observed in samples obtained from the entire proximal tubule (~35% of wild-type). However, an alternative explanation could also exist, as it is conceivable that the plasma cAMP response to PTH reflects mostly the action of this hormone in a different tissue in which the paternal Gαs allele is silenced. In that case, reduction of Gαs levels in that tissue may be the primary cause of the observed blunting in the cAMP response. For example, pituitary could play a role. Gαs expression is predominantly maternal in anterior pituitary (10), and it has been shown that PTH-induced release of prolactin observed in healthy subjects is significantly blunted in patients with PHP (51).

The delayed establishment of paternal Gαs silencing in the renal proximal tubule could explain the latency of PTH-resistance in PHP-Ia patients. However, other possible mechanisms have yet to be ruled out. The paternally expressed Gαs variant XLαs, which

can mimic Gαs actions and is expressed in mouse kidney during the early postnatal period (45,73,74), remains intact due to the maternal transmission of *GNAS* mutations in PHP patients, making it possible that this protein mediates the early postnatal actions of PTH. Another possibility is that the proximal tubular actions of PTH during this period are mediated through another G protein or in a G protein-independent manner, as PTHR can couple to other G proteins and signal, at least under certain conditions, through mechanisms that are G-protein independent (75,76).

In summary, our investigation of reported PHP-Ia cases and mice with heterozygous ablation of *Gnas* showed that PTH-resistance resulting from loss of the maternal Gαs allele develops after early postnatal life. We also revealed that paternal silencing of Gαs in the renal proximal tubule is established after the early postnatal period, unlike in BAT in which the paternal allele is already repressed at birth. The delayed onset of paternal Gαs silencing in the renal proximal tubule provides a plausible explanation for the latency of PTH-resistance in PHP-Ia patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The abreviations used are

PHP	pseudohypoparathyroidism
PTH	parathyroid hormone
Gαs	the alpha-subunit of the stimulatory G protein
TSH	thyroid stimulating hormone
BAT	brown adipose tissue

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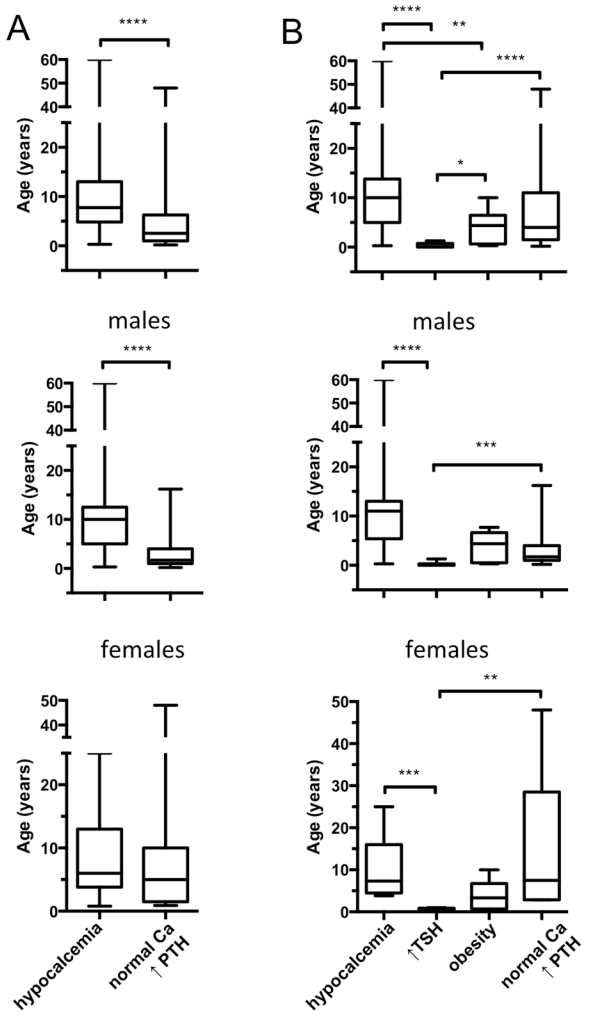

Figure 1.

Box and whisker plots showing the ages of PHP-Ia patients at which PTH-resistance was diagnosed based on either hypocalcemia (n=60) or normocalcemia with elevated (↑) PTH (n=34) (Panel A, top) or at which hypocalcemia (n=35), elevated (↑) TSH (n=17), or obesity (n=17) was noted as the first presenting symptom (Panel B, top). Patients with normocalcemic PTH elevation excluding those who first presented either with TSH elevation or obesity (n=17) are also included in the comparison (Panel B, top). Plots showing the ages of male (Panel A, middle) or female PHP-Ia (Panel A, bottom) patients at which PTH-resistance was diagnosed based on either hypocalcemia (males=33, females=27) or normocalcemia with elevated (↑) PTH (males=19, females=15). Plot showing the ages of male (Panel B, middle) and female (Panel B, bottom) PHP-Ia patients at which hypocalcemia (males=23, females=12), elevated (↑) TSH (males=11, females=6), or obesity (males=5, females=12) was noted as the first presenting symptom. Patients with normocalcemic PTH elevation excluding those who first presented either with TSH elevation or obesity (males=11, females=6) are also included in the comparisons (Panel B, middle and bottom). ****, $p<0.0001$; ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$.

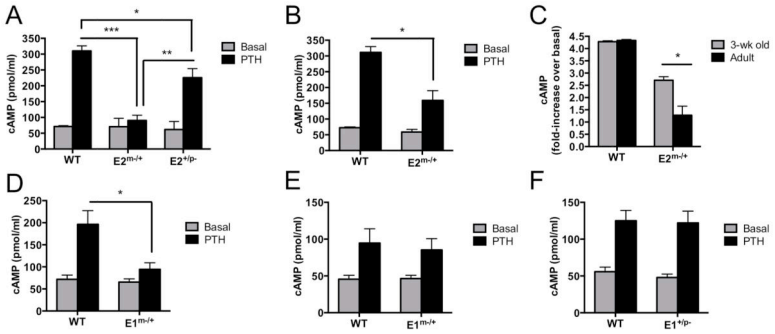

Figure 2.

PTH-induced increase in plasma cAMP is blunted in $E2^{m-/+}$ and $E1^{m-/+}$ mice. Changes in plasma cAMP levels obtained in response to subcutaneous administration of PTH(1–34) in adult $E2^{m-/+}$ and $E2^{+/p-}$ mice (Panel A), three-week old $E2^{m-/+}$ mice (Panel B), adult $E1^{m-/+}$ mice (Panel D), three-week old $E1^{m-/+}$ mice (Panel E), and three-week old $E1^{+/p-}$ mice (Panel F) compared to wild-type (WT) littermates. Panel C: Difference between three-week old and adult $E2^{m-/+}$ mice with respect to the fold-increase of plasma cAMP levels in response to PTH(1–34). Data represent mean ± S.E.M. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; Panel A, n=10, 3, 3 (basal) and 9, 3, 3 (PTH); panel B, n=9, 3 (basal) and 8, 3 (PTH); panel D, n=6, 5 (WT) and 6, 5 (PTH); panel E, n=3, 4 (WT) and 3, 4 (PTH); panel F, n=7, 9 (WT) and 7, 9 (PTH).

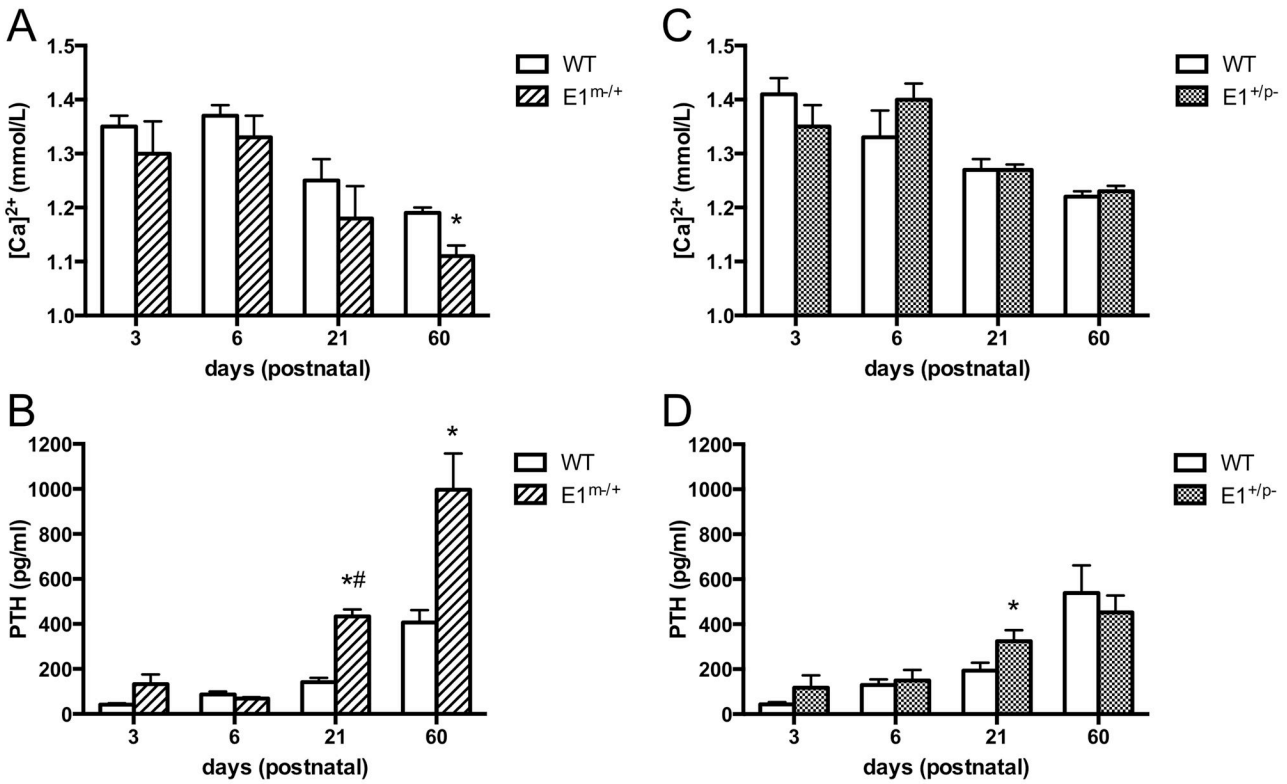

Figure 3.

Hypocalcemia and elevated serum PTH levels become manifest gradually in $E1^{m-/+}$ mice after birth. Blood ionized calcium (Panels A and C) and PTH (Panels B and D) levels of $E1^{m-/+}$ and $E1^{+/p-}$ mice were measured at indicated postnatal days. Data represent mean ± S.E.M. *, $p<0.05$ vs wild-type (WT) littermates; #, $p<0.05$ vs 60-day old $E1^{m-/+}$ mice. For calcium values, n=5, 12, 8, 9 (WT) and 5, 6, 8, 8 ($E1^{m-/+}$) or n=9, 6, 11, 10 (WT) and 6, 9, 15, 15 ($E1^{+/p-}$). For PTH values, n=5, 21, 8, 24 (WT) and 5, 8, 9, 16 ($E1^{m-/+}$) or n=9, 7, 12, 12 (WT) and 6, 9, 14, 12 ($E1^{+/p-}$).

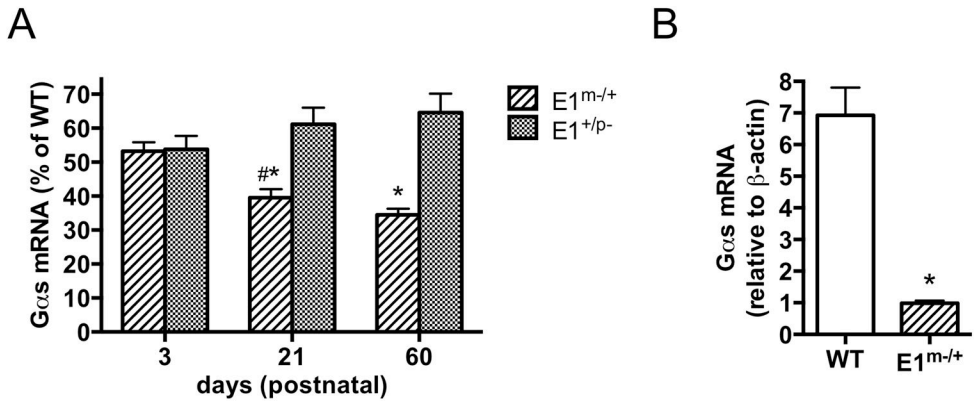

Figure 4.

Gαs mRNA expression becomes more prominent from the maternal allele in mouse renal proximal tubules postnatally (Panel A), but is maternal in BAT at birth (Panel B). Expression in $E1^{m-/+}$ and $E1^{+/p-}$ mice is shown relative to the level in wild-type (WT) littermates for each genotype at indicated postnatal days for renal proximal tubules. The levels in both renal proximal tubules and neonatal BAT (for $E1^{m-/+}$ mice) were initially determined relative to β-actin mRNA. The ratio of Gαs to β-actin mRNA in proximal tubules of three-day, 21-day, and 60-day old wild-type mice was 0.51 ± 0.03, 0.52 ± 0.02, or 0.59 ± 0.03, respectively. Data represent mean ± S.E.M. #, $p<0.05$ vs three-day old $E1^{m-/+}$ mice; *, $p<0.05$ vs age-matched $E1^{+/p-}$ mice; panel A, n=3, 6, 3 ($E1^{m-/+}$) and 4, 4, 4 ($E1^{+/p-}$); panel B, n=7 (WT) and 3 ($E1^{m-/+}$).

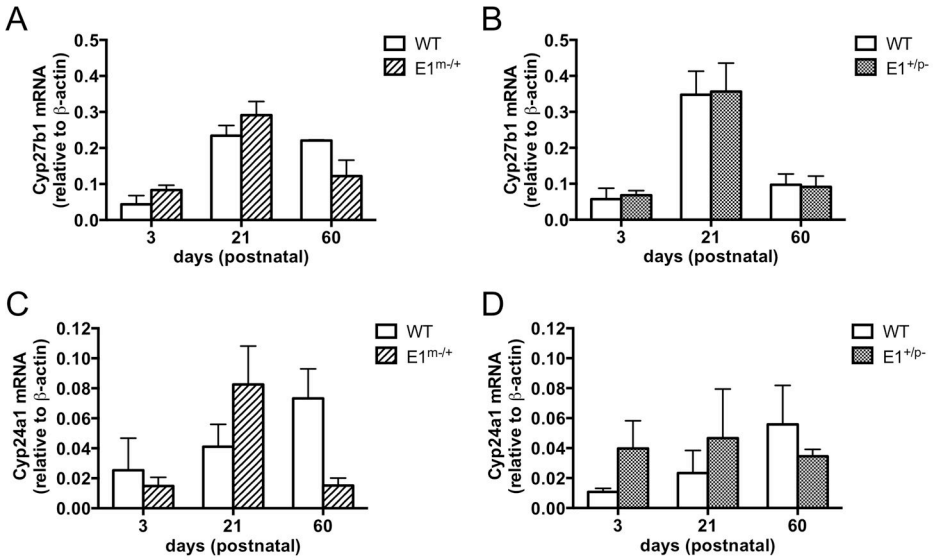

Figure 5.

Two-month old $E1^{m-/+}$ mice tend to have mildly diminished *Cyp27b1* and *Cyp24a1* expression in the renal proximal tubule compared to wild-type (WT) littermates. *Cyp27b1* (Panel A and B) and *Cyp24a1* (Panel C and D) mRNA levels were measured relative to β-actin mRNA levels. Data are mean ± S.E.M. for each genotype at indicated postnatal days. The sample numbers are as in figure 4, Panel A.

Table 1

Blood ionized Ca and plasma PTH levels of female and male $E1^{m-/+}$ mice and wild-type littermates at age three weeks and two months.

	3-week old				2-month old			
	$E1^{m-/+}$		WT		$E1^{m-/+}$		WT	
	Female	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**
Ca (mmol/L)	1.21	1.24	1.31	1.20	1.15	1.07	1.20	1.19
SEM	0.05	0.06	0.04	0.01	0.01	0.02	0.01	0.02
N	4	3	5	2	4	4	5	4
P (vs. Male)	0.70414		0.12693		0.01698		0.57573	
P (vs. WT)	0.12457	0.64855			0.03620	0.00578		
PTH (pg/ml)	428.8	409.1	133.4	166.9	877.5	1068.4	278.4	534.9
SEM	23.3	61.2	20.3	50.7	261.7	210.6	56.4	81.0
N	4	4	6	2	6	10	12	12
P (vs. Male)	0.77405		0.47650		0.58266		0.01641	
P (vs. WT)	0.00001	0.06683			0.00770	0.02001		

Table 2

Blood ionized Ca and plasma PTH levels of female and male $E1^{+/p-}$ mice and wild-type littermates at age three weeks and two months.

	3-week old				2-month old			
	$E1^{+/p-}$		WT		$E1^{+/p-}$		WT	
	Female	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**
Ca (mmol/L)	1.29	1.27	1.25	1.29	1.27	1.21	1.23	1.21
SEM	0.02	0.02	0.02	0.03	0.02	0.01	0.02	0.02
N	7	7	4	6	7	8	6	4
P (vs. Male)	0.46630		0.37697		0.03302		0.60560	
P (vs. WT)	0.29850	0.53182			0.22601	0.93380		
PTH (pg/ml)	221.4	350.4	248.2	158.6	368.9	619.8	397.0	680.9
SEM	37.9	53.1	78.5	22.6	94.5	78.1	166.0	174.0
N	6	7	5	6	8	4	6	6
P (vs. Male)	0.08196		0.26385		0.11781		0.26508	
P (vs. WT)	0.75199	0.00966			0.87844	0.79398		