Persistence of 1,25D-induced hypercalciuria in alendronate-treated genetic hypercalciuric stone-forming rats fed a low-calcium diet

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Frick KK, Asplin JR, Culbertson CD, Granja I, Krieger NS, Bushinsky DA. Persistence of 1,25D-induced hypercalciuria in alendronate-treated genetic hypercalciuric stone-forming rats fed a low-calcium diet. Am J Physiol Renal Physiol 306: F1081–F1087, 2014. First published February 26, 2014; doi:10.1152/ajprenal.00680.2013.—Genetic hypercalciuric stone-forming (GHS) rats demonstrate increased intestinal Ca absorption, increased bone resorption, and reduced renal tubular Ca reabsorption leading to hypercalciuria and all form kidney stones. GHS have increased vitamin D receptors (VDR) at these sites of Ca transport. Injection of 1,25(OH)2D3 (1,25D) leads to a greater increase in urine (u)Ca in GHS than in control Sprague-Dawley (SD), possibly due to the additional VDR. In GHS the increased uCa persists on a low-Ca diet (LCD) suggesting enhanced bone resorption. We tested the hypothesis that LCD, coupled to inhibition of bone resorption by alendronate (alen), would eliminate the enhanced 1,25D-induced hypercalciuria in GHS. SD and GHS were fed LCD and half were injected daily with 1,25D. After 8 days all were also given alen until euthanasia at day 16. At 8 days, 1,25D increased uCa in SD and to a greater extent in GHS. At 16 days, alen eliminated the 1,25D-induced increase in uCa in SD. However, in GHS alen decreased, but did not eliminate, the 1,25D-induced hypercalciuria, suggesting maximal alen cannot completely prevent the 1,25D-induced bone resorption in GHS, perhaps due to increased VDR. There was no consistent effect on mRNA expression of renal transcellular or paracellular Ca transporters. Urine CaP and CaOx saturation (SS) increased with 1,25D alone in both SD and GHS. Alen eliminated the increase in CaP SS in SD but not in GHS. If these results are confirmed in humans with IH, the use of bisphosphonates, such as alen, may not prevent the decreased bone density observed in these patients.

We have established a strain of hypercalciuric rats by selectively inbreeding Sprague-Dawley (SD) rats for increased uCa excretion (2, 3, 5, 11–13, 15–22, 24, 25, 29, 33, 36, 37, 40–42, 44, 45, 52, 62, 66, 67). Our hypercalciuric rat colony has been maintained by continual selection and inbreeding for over 90 generations; each rat now consistently excretes approximately 8 to 10-fold more uCa than SD controls when fed an ample Ca diet (2, 3, 5, 11–13, 15–22, 24, 25, 29, 33, 36, 37, 40–42, 44, 45, 52, 62, 66, 67). These rats all develop kidney stones (2, 17, 18) and have been named genetic hypercalciuric stone-forming (GHS) rats (2, 3, 5, 11–13, 15–20, 22, 24, 25, 29, 33, 36, 37, 40–42, 44, 45, 52, 62, 66, 67).

Like patients with IH, GHS rats exhibit normal sCa (15), increased intestinal Ca absorption (44) and bone resorption (42), decreased renal tubule Ca reabsorption (62), and normal s1,25D levels (40, 41, 44, 66, 67) in addition to decreased bone mineral density (24, 33). Hypercalciuria is also a polygenic trait in GHS rats (36). GHS rats have elevated levels of vitamin D receptor (VDR) protein in Ca-transporting organs including the kidney, intestine, and bone (40, 42, 44).

Administration of 1,25D to normal subjects leads to hypercalciuria with changes in intestine, kidney, and bone Ca transport typical of those observed in IH (1, 46). This increased uCa indicates that the action of 1,25D in increasing intestinal Ca absorption (35) and bone Ca resorption (1, 46) exceeds any 1,25D-mediated change in renal tubular Ca reabsorption (8). While most IH patients have normal s1,25D levels (68), some have elevated s1,25D levels that may account for the phenotype (6, 9, 39, 59). High VDR levels have been found in male IH stone formers in at least one study (26), suggesting elevated VDR levels may play a role in hypercalciuria in some human stone formers.

Administration of 1,25D to GHS and SD rats fed a normal Ca diet (NCD) leads to a greater increment in hypercalciuria in GHS rats than in SD (29). This observation is consistent with a model in which the greater number of VDR in GHS rats is relatively undersaturated with 1,25D under basal conditions, but with addition of more 1,25D, the mass action of the hypercalciuria persisted in GHS either with or without additional 1,25D (30), suggesting that the increased uCa was derived from increased bone resorption. In this study we tested the hypothesis that in rats fed LCD, inhibition of bone resorption with a maximal dose of the bisphosphonate alendronate (alen) would result in equalization of uCa between GHS and SD rats.

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METHODS

Animals. The genetic GHS rats were derived from SD rats (Charles River Laboratories, Kingston, NY) by successively inbreeding the most hypercalciuric progeny of each generation (12, 15, 17, 25, 33, 41, 42, 44, 62). Eight-week-old male GHS rats from the 91st generation and 8-wk-old male SD rats (Charles River Laboratories, Kingston, NY) were used in this study.

Experimental conditions. At the start of the study (day 0), 16 SD and 16 GHS rats, all 25D replete, were placed in metabolic cages and fed 13 g/day low-Ca diet (LCD; 0.02% Ca, providing a maximum of 2.6 mg Ca/day; Harlan-Teklad, Indianapolis, IN) and given deionized, distilled water ad libitum. Also starting on day 0, by random allocation, eight rats in each group were injected intraperitoneally daily with 1,25D (25 ng/100 g body wt; American Regent, Shirley, NY) in saline and eight rats in each group with only saline. This dose of 1,25D elicits a maximal physiologic response (27, 28, 66). Starting on day 8, urine from each rat was collected for four 24-h periods. On days 9 and 11, urine was acidified with HCl and on days 10 and 12 urine was collected in thymol. Collections in thymol were used for pH and Cl determinations.

Urine and serum chemistry. Urine Ca, Mg, P, ammonia, and creatinine were measured spectrophotometrically using a Beckman CX5 Pro autoanalyzer (Beckman Instruments, Brea, CA). Urine K, Cl, and Na were measured by ion-specific electrodes on the Beckman CX5. Urine pH was measured using a glass electrode and citrate, oxalate, and sulfate were measured by ion chromatography using a Dionex ICS 2000 system (Dionex Corporation, Sunnyvale, CA). Serum Ca and P were determined colorimetrically (BioVision, Milpitas, CA). Serum parathyroid hormone (PTH) was determined by EIA for intact-PTH (ALPCO, Salem, NH). We have used these methods previously (2, 3, 24, 29, 30).

Urine supersaturation. The CaOx and CaHPO4 (CaP) ion activity product were calculated using the computer program EQUIL 2 (65) as we have done previously (11–13, 16, 19–22, 29, 30). Ratios of 1 denote a sample at equilibrium, >1 denotes supersaturation, and <1 denotes undersaturation. We have found excellent correspondence between calculated and experimentally measured saturation in urine and blood and in bone culture medium (2, 3, 24).

RNA purification. Kidneys were bisected and placed in 2 ml RNAlater (Ambion, Grand Island, NY) at 4°C overnight and then transferred to −70°C until purification. Each kidney was homogenized in 6 ml TriZol (Invitrogen, Grand Island, NY) using a glass homogenizer, and RNA purification was conducted according to manufacturer’s instructions. Aqueous and phenol phases were separated by centrifugation after addition of 1-bromo-3-chloropropane.

Quantitative real-time polymerase chain reaction. Kidney RNA was transcribed to cDNA using an iScript kit (Bio-Rad, Hercules, CA). Gene specific targets were amplified and analyzed by real-time polymerase chain reaction with a MyIQ cycler (Bio-Rad) and Sybr Green (IQ Supermix; Bio-Rad). To normalize gene expression, the geometric mean of expression of RNA for L13a, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activator protein, zeta polypeptide, and succinate dehydrogenase complex, subunit a, flavoprotein was calculated for each sample (63). Primer sets used were described previously (29). All expression values were calculated relative to the mean expression in SD + vehicle.

Statistics. Values were compared by ANOVA using the Bonferroni correction for multiple comparisons, with a conventional computer program (Statistica; StatSoft, Tulsa, OK). Values are expressed as means ± SE. P ≤ 0.05 considered significant.

RESULTS

Urine and serum chemistry. On LCD, the GHS rats excreted 4.3 ± 0.5 mg/day of Ca (Fig. 1, left). As total available dietary Ca with LCD was only 2.6 mg/day, the GHS were in negative total body Ca balance. There were no differences in uNa among the four groups over the two time periods (Table 1), which provides evidence for a constant amount of food intake. Injection of 1,25D increased uCa to 24.4 ± 1.6 mg/day in GHS and from 1.6 ± 0.2 to 13.7 ± 1.0 mg/day in SD indicating that this hormone increased the negative Ca balance in GHS rats and induced negative Ca balance in SD rats.

After injection of alen, uCa continued to be significantly elevated with concurrent 1,25D injection in GHS but not in SD (Fig. 1, middle) indicating that while the bisphosphonate completely eliminated the 1,25D-induced hypercalciuria in the SD rats, the GHS rats continued to be hypercalciuric.

Alen inhibited more bone resorption in GHS, as measured by a greater difference in uCa before and after alen in GHS compared with SD rats given 1,25D (Fig. 1, right). However, in the GHS rats, the increase in uCa with 1,25D was of sufficient magnitude that even with inhibition of more bone resorption in GHS than SD rats, alen was unable to completely inhibit the increase in uCa (Fig. 1, middle).

Although there was no significant difference in sCa or sPTH between the GHS and SD rats given alen without exogenous
1,25D, both values trended lower in GHS rats (Table 1). Injection of 1,25D led to a numerical increase in sCa in both GHS and SD; however, the change in sCa was only significant in GHS. There were no differences in sP among the four groups. Injection of 1,25D led to a numerical decrease in sPTH in both GHS and SD, but the change in sPTH was only significant in SD. Both the SD and GHS rats were 25D replete; however, s25D levels in either group. Expression of markers of renal calcium transport.

Expression of markers of renal calcium transport. The source of the continued hypercalciuria in the GHS rats on LCD injected with alen and receiving 1,25D must be from bone, the only significant repository of Ca in the body. This continued hypercalciuria indicates that there is residual Ca reabsorption from bone due to direct effects of 1,25D on bone and/or secondary to incomplete renal reabsorption of filtered Ca. To further define the potential mechanisms of altered renal Ca reabsorption, we determined the effects of alen and 1,25D on expression of genes related to renal Ca transport.

Active transport. There was no difference in the RNA expression of transient receptor potential vanilloid (TRPV) 5, TRPV6, plasma membrane Ca ATPase (PMCA), or Na/Ca exchanger (NCX1) between GHS and SD rats given alen without additional 1,25D (Table 2). Expression of calbindin D28k was elevated in GHS + alen rats but there was no difference in expression of klotho. With alen, 1,25D increased expression of TRPV6 only in SD rats. NCX expression was decreased in SD rats given both alen and 1,25D. Levels of TRPV5, calbindinD28k, and PMCA were not significantly altered in either SD or GHS rats with alen and 1,25D.

Paracellular Ca transport. Renal Ca reabsorption occurs in the thick ascending limb of Henle (TALH), via paracellular transport through tight junctions, which contain claudin 16 and claudin 19 (38), and permeability of cations through tight junctions is regulated by claudin 14 (32). Without 1,25D expression of claudins 16, 19, and 14 were not different between GHS and SD (Table 2). 1,25D did not significantly affect expression of claudins 16, 19, or 14. Expression of the calcium-sensing receptor (CaR), the outward modulating K channel (ROMK), and the Na/K/2Cl transporter (NKCC2) did not differ between SD and GHS with or without 1,25D.

**DISCUSSION**

The phenotype of the GHS rats is very similar to human IH (14), including increased intestinal Ca absorption (44), increased bone resorption (42), and decreased renal tubule Ca reabsorption (62). The levels of serum 1,25D are generally normal in the GHS rats (40, 41, 44, 66, 67); however, we have found that the GHS rats have an increased number of VDR in each of these Ca-transporting organs (31, 40, 42, 44). We hypothesized that in the GHS rats a portion of the increased VDR would not be occupied by endogenous 1,25D and that the addition of exogenous 1,25D would cause a greater biological response in GHS compared with SD rats.

To explore the importance of the dysregulation of Ca transport in each organ in the development of the hypercalciuria, we first placed the GHS rats on a normal Ca diet and determined that the increased number of VDR were biologically active by demonstrating a greater increase in uCa in response to exogenous 1,25D in the GHS rats compared with the control SD rats (29). We then drastically reduced dietary Ca to minimize intestinal Ca absorption and again demonstrated a greater increment of uCa in the GHS rats compared with SD indicating that there was enhanced 1,25D-induced bone resorption and/or a decrease in renal tubular Ca reabsorption (30). In this study
we not only reduced intestinal Ca absorption with LCD, but we inhibited bone resorption with a maximal dose of the bisphosphonate alendronate. In the presence of alendronate, the hypercalcemia in GHS rats fed LCD was completely inhibited without exogenous 1,25D, and was significantly reduced with administration of 1,25D.

The GHS rats fed LCD and injected with both alendronate and 1,25D continued to be hypercalciuric, with uCa exceeding available dietary Ca, indicating that they were in negative Ca balance. This elevated uCa must originate principally from bone resorption, which could not be completely inhibited by this maximal dose of alendronate.

In addition to Ca circulating in the blood and that stored in the skeleton, an additional source of Ca might be from soft tissue, particularly vascular, calcification. Price et al. has reported that bisphosphonates inhibit vascular calcification induced by toxic doses of cholecalciferol (53) or by a low protein diet in uremic rats (55). However, Price et al. also found (54) that the dose response for inhibition of vascular calcification by etidronate was identical to the dose response for inhibition of bone resorption, suggesting that the origin of the Ca in soft tissue calcification was from bone. This is consistent with the observation that osteoprotegerin null mice develop both osteoporosis and vascular calcification (10). Our data support the hypothesis that the source of the increased uCa in GHS + 1,25D must be from bone; whether there is intermediate deposition of Ca in soft tissue remains to be determined. We cannot rule out the possibility of direct effects of bisphosphonates on vascular calcification.

Nitrogen-containing bisphosphonates such as alendronate decrease bone resorption by inhibiting osteoclastic farnesyl diphosphate synthase (FPPS), an enzyme critical in the mevalonate pathway which provides the substrate for the geranylgeranylation of Rac, Rho, and cdc42, small molecules necessary for osteoclastic activity (28, 57). The IC50 for alendronate inhibition of osteoclast activity in vitro (pit formation on ivory) is 2 nM (45, 58). When comparing several bisphosphonates in vivo (28), the maximal reduction in osteoclasts positive for the mevalonate pathway was achieved with an identical dose of alendronate used in the current study. An alendronate dose of 1.9 μmol·100 g−1·day−1 caused a significant decrease in the markers of bone resorption uPyr and uD-Pyr in vivo (4). Muhlbaier et al. (51) found essentially total inhibition of arotinoid-induced bone resorption by an alendronate dose of 1.9 μmol·100 g−1·day−1 in vivo. In this study we injected the

### Table 2. Relative RNA expression of components of renal calcium transport

<table>
<thead>
<tr>
<th>Component</th>
<th>SD + Alen</th>
<th>GHS + Alen</th>
<th>SD + Alen + 1,25D</th>
<th>GHS + Alen + 1,25D</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPV5</td>
<td>0.170 ± 0.073</td>
<td>0.61 ± 0.073</td>
<td>0.50 ± 0.042</td>
<td>0.52 ± 0.042</td>
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<tr>
<td>TRPV6</td>
<td>0.160 ± 0.073</td>
<td>1.42 ± 0.073</td>
<td>2.20 ± 0.073*</td>
<td>2.00 ± 0.073</td>
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<tr>
<td>PMCA</td>
<td>0.100 ± 0.073</td>
<td>1.29 ± 0.073</td>
<td>1.01 ± 0.062</td>
<td>0.97 ± 0.062</td>
</tr>
<tr>
<td>NCX1</td>
<td>0.100 ± 0.073</td>
<td>0.68 ± 0.073</td>
<td>0.30 ± 0.062*</td>
<td>0.49 ± 0.072*</td>
</tr>
<tr>
<td>Calbindin</td>
<td>0.100 ± 0.073</td>
<td>2.27 ± 0.073</td>
<td>1.08 ± 0.081*</td>
<td>1.72 ± 0.081*</td>
</tr>
<tr>
<td>klotho</td>
<td>0.100 ± 0.073</td>
<td>1.14 ± 0.073</td>
<td>0.82 ± 0.042</td>
<td>0.89 ± 0.042</td>
</tr>
<tr>
<td>Claudin</td>
<td>0.100 ± 0.073</td>
<td>1.50 ± 0.073</td>
<td>0.68 ± 0.062*</td>
<td>0.93 ± 0.062*</td>
</tr>
<tr>
<td>Claudin</td>
<td>0.100 ± 0.073</td>
<td>1.66 ± 0.073</td>
<td>1.78 ± 0.073</td>
<td>1.62 ± 0.073</td>
</tr>
<tr>
<td>Claudin</td>
<td>0.100 ± 0.073</td>
<td>2.26 ± 0.073</td>
<td>1.04 ± 0.020</td>
<td>1.41 ± 0.020</td>
</tr>
<tr>
<td>CaR</td>
<td>0.100 ± 0.073</td>
<td>1.73 ± 0.073</td>
<td>0.86 ± 0.062*</td>
<td>1.05 ± 0.062</td>
</tr>
<tr>
<td>ROMK</td>
<td>0.100 ± 0.073</td>
<td>1.28 ± 0.073</td>
<td>0.22 ± 0.062*</td>
<td>1.42 ± 0.062*</td>
</tr>
<tr>
<td>NCCK1</td>
<td>0.100 ± 0.073</td>
<td>1.43 ± 0.073</td>
<td>0.70 ± 0.033*</td>
<td>1.15 ± 0.033*</td>
</tr>
</tbody>
</table>

Results are means ± SE. Relative RNA expression of selected components of renal calcium transport in SD and GHS rats fed LCD, without or with exogenous 1,25D. TRPV, transient receptor potential vanilloid; PMCA, plasma membrane Ca ATPase; NCX1, Na/Ca exchanger; CaR, calcium-sensing receptor; ROMK, outward modulating K channel; NCCK, Na/K/2Cl transporter. *P < 0.05, compared with SD; †P < 0.05, compared with GHS.
rats with a dose of alen (15.4 μmol·100 g⁻¹·day⁻¹) which was equal to (28) or far exceeded (4, 51) these doses indicating that the use of more alen would not have led to a greater inhibition of bone resorption.

The marked increase in uCa excretion with 1,25D leads to the increase in CaP SS in both SD and GHS rats before treatment with alen (Fig. 2, top left). That the CaP SS is not higher in the GHS than the SD rats receiving 1,25D is explained by the trend to a higher urine volume and lower urine pH in the GHS group. When treated with alen, uCa falls significantly in the rats treated with 1,25D, leading to lower CaP SS compared with pretreatment with alen.

Although there was no significant difference in uOx between GHS and SD rats, numerically uOx was higher in GHS, consistent with the observation that patients with IH may exhibit mild hyperoxaluria (7). Compared with SD, the GHS rat has increased intestinal Ca absorption (44) leading to more free oxalate in the intestine and greater availability for Ox absorption and excretion. There was no difference in CaOx SS between GHS and SD rats without exogenous 1,25D; administration of the hormone led to a significant increase in CaOx SS in both groups.

Before alen, the molar ratio of Ca to Ox in SD without exogenous 1,25D was 15 to 1, while the Ca to Ox ratio in GHS without exogenous 1,25D was similar, at 18 to 1. With exogenous 1,25D, the ratios for SD and GHS were 40 to 1 and 54 to 1, respectively. This higher ratio drives the increased CaOx SS illustrated in Fig. 2 (bottom left). However, there is such an excess of Ca to Ox that there is insufficient Ox available to drive SS despite the increased uCa (61). With alen, the molar ratio without 1,25D for SD was 10 to 1 and for GHS was 8 to 1. With both alen and 1,25D, the ratio for SD was 5 to 1 while the ratio for GHS increased to 20 to 1. This higher GHS ratio would help account for the elevated CaOx SS seen in GHS plus alen plus 1,25D (Fig. 2, bottom middle).

The enhanced uCa could originate from dysregulation of renal tubular Ca reabsorption by PTH, leading to a lower sCa which stimulated bone resorption. However, in this case sPTH should be increased in the GHS rats given 1,25D. In this study sPTH was not stimulated, consistent with our prior observations (29, 30). To explore whether 1,25D contributes to alterations in renal tubular Ca reabsorption and hypercalciuria in GHS rats, we determined the effects of alen and 1,25D on the expression of genes related to renal Ca transport. In a prior study in the GHS rats fed a NCD we found increases in RNA levels for these markers of active renal Ca transport. While small changes were found in other components of renal Ca transport, there was no consistent pattern in the comparison of GHS to SD with or without 1,25D. Since changes in RNA abundance do not necessarily reflect the magnitude of Ca reabsorption, further studies examining protein levels and transport activity will be necessary to determine which, if any, of these transporters may be altered in GHS rats. In particular, it would be interesting to directly compare rates of renal tubular Ca reabsorption from GHS and SD rats treated with 1,25D and/or alen. We have previously found that parathyroidectomized GHS rats fed a NCD or LCD had a significant decrease in renal tubular Ca reabsorption compared with SD rats (62).

Bone is a large repository of base (43, 56). Increased bone resorption induced by 1,25D will lead to the release of base into the systemic circulation when inhibition of bone resorption with alen will decrease release of base into the circulation. As the increase in uCa with 1,25D+alen results in net bone resorption, there will clearly be release of base into the systemic circulation which has the potential to increase renal tubular Ca reabsorption and thus decrease uCa excretion. However, it is unclear if the increase in bone resorption is of sufficient magnitude to alter systemic acid-base balance resulting in alterations in uCa. To accurately measure systemic acid-base parameters, we have found it necessary to place indwelling arterial catheters (23, 64), which was not done here. Thus in the current study we cannot determine if alterations in bone resorption induced by 1,25D and alen resulted in changes in systemic acid-base parameters that then affected renal tubular Ca reabsorption.

Since the GHS rats have normal levels of s1,25D, we have postulated that their elevated tissue levels of VDR in the Ca-transporting organs would be relatively undersaturated with 1,25D compared with SD rats. We previously tested the hypothesis that, while fed NCD, administration of 1,25D, which should result in greater VDR saturation, would lead to enhanced uCa excretion in GHS compared with SD rats. We found that administration of 1,25D to the GHS rats fed NCD increased the hypercalciuria to a greater extent than in SD (29), perhaps due to their increased VDR. This additional uCa must come from either enhanced intestinal Ca absorption and/or increased bone Ca resorption. We then utilized LCD to remove the contribution of intestinal Ca absorption to uCa (30). We found that administration of 1,25D to rats fed LCD increased hypercalciuria to a greater extent in GHS than in SD controls; these results provided clear support for enhanced bone resorption as the source of this additional uCa in GHS rats. In the current study we used a maximal dose of the bisphosphonate alen to inhibit bone resorption in rats fed LCD. In SD rats, alen eliminated the 1,25D-induced hypercalciuria; however, in GHS rats the increase in uCa was decreased but not eliminated. These results demonstrate that a maximal dose of alen is unable to completely prevent the 1,25D-induced bone resorption in GHS, perhaps due to the increased VDR in the bone cells of these rats (42). As elevated levels of VDR levels have been found in male IH stone-formers in at least one study (26), if these results are confirmed in similar hypercalciuric humans, they suggest that the clinical use of bisphosphonates, such as alen, may not completely prevent the hypercalciuria and decreased bone density observed in these patients.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: K.K.F., N.S.K., and D.A.B. conception and design of research; K.K.F., J.R.A., C.D.C., and N.S.K. performed experiments; K.K.F.,
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