Hypercalcemia induces targeted autophagic degradation of aquaporin-2 at the onset of nephrogenic diabetes insipidus

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Hypercalcemia can cause renal dysfunction such as nephrogenic diabetes insipidus (NDI), but the mechanisms underlying hypercalcemia-induced NDI are not well understood. To elucidate the early molecular changes responsible for this disorder, we employed mass spectrometry–based proteomic analysis of inner medullary collecting ducts (IMCD) isolated from parathyroid hormone–treated rats at onset of hypercalcemia-induced NDI. Forty-one proteins, including the water channel aquaporin-2, exhibited significant changes in abundance, most of which were decreased. Bioinformatic analysis revealed that many of the downregulated proteins were associated with cytoskeletal protein binding, regulation of actin filament polymerization, and cell-cell junctions. Targeted LC-MS/MS and immunoblot studies confirmed the downregulation of 16 proteins identified in the initial proteomic analysis and in additional experiments using a vitamin D treatment model of hypercalcemia-induced NDI. Evaluation of transcript levels and estimated half-life of the downregulated proteins suggested enhanced protein degradation as the possible regulatory mechanism. Electron microscopy showed defective intercellular junctions and autophagy in the IMCD cells from both vitamin D– and parathyroid hormone–treated rats. A significant increase in the number of autophagosomes was confirmed by immunofluorescence labeling of LC3. Colocalization of LC3 and Lamp1 with aquaporin-2 and other downregulated proteins was found in both models. Immunogold electron microscopy revealed aquaporin-2 in autophagosomes in IMCD cells from both hypercalcemia models. Finally, parathyroid hormone withdrawal reversed the NDI phenotype, accompanied by termination of aquaporin-2 autophagic degradation and normalization of both nonphoshorylated and S256-phosphorylated aquaporin-2 levels. Thus, enhanced autophagic degradation of proteins plays an important role in the initial mechanism of hypercalcemic-induced NDI.

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Hypercalcemia is an electrolyte imbalance often encountered in tertiary care medicine, occurring as a result of hyperparathyroidism, vitamin D overdose, and multiple malignant diseases. This potentially life-threatening condition adversely affects multiple organ systems, producing cardiac arrhythmia, seizure, and renal insufficiency accompanied by nephrogenic diabetes insipidus (NDI). The 2 common clinical features of NDI are (i) polyuria and (ii) low urine osmolality due to impaired urinary concentrating ability.[1]

The urinary concentrating mechanism plays a critical role in maintaining whole-body water balance. The ability of the kidneys to concentrate urine depends on 2 processes: (i) generation of hypertonicity in the medullary interstitium and (ii) arginine vasopressin (AVP)-mediated water and urea transport in the collecting duct via the water channel proteins or aquaporins (AQP2, AQP3, AQP4), urea transporters (UT-A1, UT-A3), and other signaling molecules.[2–4] Hypercalcemia decreases medullary interstitial hypertonicity by downregulating the bumetanide-sensitive Na^+-K^+-$2Cl^-$ cotransporter (*Slc12a1*), thereby decreasing interstitial NaCl concentration and disrupting the countercurrent multiplier system.[5,6] Regarding transport processes in the inner medullary collecting duct (IMCD) tubules, rats experiencing long-term hypercalcemia exhibited resistance to AVP-elicited water transport, but increased basal and AVP-elicited urea permeability.[7]

Several studies *in vivo* and *in vitro* have identified intracellular responses related to hypercalcemia-induced IMCD resistance to AVP. First, in rats exhibiting NDI due to long-term hypercalcemia, reductions were observed in AVP regulation of both AQP2 abundance and targeting to the apical plasma membrane of IMCD cells.[8] A study *in vitro* demonstrated that high extracellular Ca^{2+} concentrations attenuated cyclic adenosine monophosphate (cAMP) accumulation, which blocked both *AQP2* gene expression and AQP2-bearing vesicle exocytosis.[9] In other studies, hypercalcemia-induced hypercalciuria was shown to activate the calcium-sensing receptor (CaSR) expressed in apical membranes of IMCD cells[10]; *in vitro* experiments demonstrated that CaSR activated by high levels of extracellular Ca^{2+} reduced coupling efficiency between the AVP receptor (V2R) and adenylyl cyclase via a calcium/calmodulin-dependent mechanism, attenuating *AQP2* transcription by inhibiting the protein kinase A (PKA) pathway.[11]

The previous studies in animal models of the hypercalcemia-induced urinary concentrating defect were performed several days after the onset of polyuria.[5–8] Consequently, the early events and molecular mechanism(s) initiating hypercalcemia-induced NDI are unknown. Using a parathyroid hormone (PTH)–induced hypercalcemic animal model, we investigated these early changes, when the onset of NDI in hypercalcemic rats was first detectable, focusing on the IMCD. The results of this study using multiple approaches and 2 different animal models of hypercalcemia revealed that autophagic degradation of a specific set of proteins including AQP2 was observed at the early onset of NDI. These findings support our recent study indicating a major role for autophagy as an early event in the development of NDI resulting from hypokalemia, another common electrolyte imbalance.[12]

RESULTS

Hypercalcemia-induced NDI following PTH treatment

Rats were treated with PTH (15 μg/kg/day) or vehicle for up to 2 days. After 1 day, in the PTH-treated rats, a significantly higher blood calcium level developed, and they excreted more urinary calcium (Figure 1a,b). Also after 1 day, the PTH-treated rats produced significantly less concentrated urine and a higher urine volume (Figure 1c,d). The serum sodium and potassium levels and fractional excretion of sodium did not significantly differ between the 2 groups (Supplementary Figure S1). These findings indicated that water diuresis, not natriuresis, was responsible for polyuria in hypercalcemic rats with early NDI. Furthermore, after 1 day of PTH treatment, a significant decrease (65%) in phosphorylation of AQP2 at Ser256 (the vasopressin-sensitive regulatory site) was observed in the inner medulla, but the apparent decrease in total AQP2 abundance (35%, $P = 0.06$) did not reach significance for these samples (Figure 1e). The percentage of reduction in pS256-AQP2 was significantly greater than the reduction in total AQP2 (abundance of pS256-AQP2 is 59% lower than that of total AQP2, see Supplementary Data 1). Based on these results, PTH treatment for 1 day was used for subsequent analyses to study the early molecular events involved in hypercalcemia-induced NDI.

Identification of global molecular changes in PTH-treated rat IMCD by proteomics and bioinformatics

To identify changes in IMCD proteins and phosphoproteins in the early stage of hypercalcemia-induced NDI, rats were treated with PTH or vehicle for 1 day. Following liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis, a total of 5829 unique peptides were identified, corresponding to a total of 1619 proteins. A volcano plot (Figure 2a) demonstrates the average $\log_2$(protein abundance ratios) for all 551 of the proteins observed in at least 3 biological replicates versus the significance, expressed in $-\log_{10}(P$ value). Based on a dual statistical criterion (see Supplementary Methods), 36 proteins were significantly decreased in abundance, whereas only 5 proteins were significantly increased in abundance in IMCD cells from hypercalcemic rats (Supplementary Tables S1 and S2). Note that AQP2 was significantly decreased by 28% in this proteomic experiment, quantitatively in agreement with the immunoblotting result shown in Figure 1e. A total of 1779 unique phosphopeptides were identified, containing 1616 unique phosphorylation sites. Of the latter, 645 phosphorylation sites from 355 proteins passed the quality threshold established by the software analysis tools (see Supplementary Methods).[13,14] A volcano plot shown in Figure 2b illustrates the average $\log_2$(phosphopeptide ratios) for all 224 of the unambiguous phosphopeptides (161 proteins) identified in at least 3 biological replicates; of these phosphopeptides, 10 were significantly decreased, as judged by the dual statistical criterion (Supplementary Table S3). In agreement with the previous immunoblotting result (Figure 1e), pS256 of AQP2 was significantly decreased by 50%. No phosphopeptides with significantly increased abundance were observed. (Loading controls for LC-MS/MS analysis are shown in Supplementary Figure S2.)

The complete list of protein and phosphopeptide identification and quantification, along with associated gene ontology (GO) terms are provided in publicly accessible online databases at https://hpcwebapps.cit.nih.gov/ESBL/Database/HyperCa/Total_Protein.htm and https://hpcwebapps.cit.nih.gov/ESBL/Database/HyperCa/Phosphopeptide.htm. The proteins and phosphoproteins exhibiting a significant change were further investigated by the DAVID bioinformatics resources.[15] Highly represented annotations were grouped into clusters. The most significantly enriched clusters included proteins and phosphoproteins with the functional GO terms *cytoskeletal protein binding*, *regulation of actin filament polymerization*, *cell-cell junctions*, and *mitochondrial membrane* (Table 1 and Supplementary Table S4). Interestingly, mTOR (mechanistic target of rapamycin) was among the small number of proteins found to be decreased in the *mitochondrial membrane* group. Significantly, LC-MS/MS quantitation of mTOR protein was based on the detection