What's New About Osmotic Regulation of Glycerophosphocholine

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Abstract

Glycerophosphocholine is an abundant renal medullary organic osmolyte that protects renal medullary cells from the high interstitial concentrations of NaCl and urea to which they are normally exposed. We consider the metabolism of glycerophosphocholine, its osmotic regulation, and the recently discovered molecular identity of the enzymes that osmoregulate its abundance.

Accumulation of glycerophosphocholine (GPC) within renal medullary cells helps protect them from the high interstitial concentrations of NaCl and urea to which they normally are exposed during operation of the renal concentrating mechanism (6,11). Its presence was discovered more than 50 years ago, and it was found to increase during antidiuresis in response to elevation of renal medullary interstitial NaCl and urea concentration (22,25). However, only recently were the enzymes identified that regulate its abundance in response to changing levels of NaCl and urea.

Renal Expression of Glycerophosphocholine

Karl Ullrich discovered large amounts of GPC in canine renal medullas and found that the amount of GPC increases during antidiuresis (22,25). He recognized that the intracellular GPC helps balance the osmotic pressure of the high NaCl concentration in medullary interstitial fluid. However, his discovery received little attention until an additional critical insight came much later. Then, its presence was confirmed by ^{14}N-NMR in rat and rabbit renal medullas (3) by investigators who, in the light of a recently published synthesis (26), recognized that the GPC, being a trimethylamine, might serve as a compatible organic osmolyte to protect against the high concentration of NaCl and as a counteracting organic osmolyte to protect against the high concentration of urea.

Osmoprotection by Organic Osmolytes

Intracellular organic osmolytes are accumulated by virtually all water-stressed organisms, with halobacteria being an apparent exception (5,26). The major systems of organic osmolytes include polyhydric alcohols, free amino acids and their derivatives, and combinations of urea and methylamines (26). The predominant ones in renal medullary cells are sorbitol, *myo*-inositol, glycine betaine and GPC (2). We have known for some time that sorbitol is synthesized in renal medullary cells from glucose, catalyzed by aldose reductase, that *myo*-inositol and glycine betaine are transported into the cells by SMIT and BGT1, respectively, and that the abundance of aldose reductase, SMIT, and BGT1 is regulated by the transcription factor TonEBP/OREBP (5). However, the molecular basis for osmoregulation of GPC emerged much later, as will be discussed below. Two complementary mechanisms are recognized for the protective effects of organic osmolytes in cells exposed to hyperosmolality (6,26). The first mechanism is "perturbing" vs. "compatible" solutes. Although many biochemical functions require specific inorganic ions, increasing the concentrations of these ions above those typically found in cells perturbs protein function. In contrast, organic osmolytes have much less effect,

i.e., are compatible. Acute hypertonicity, as results from high NaCl, causes osmotic flux of water out of cells, elevating the concentration of all cellular constituents, including inorganic salts. Although cells can rapidly restore their volume ("regulatory volume increase") by influx of inorganic salts followed by osmotic uptake of water, the intracellular inorganic ion concentration remains high. Organic osmolytes, such as GPC, accumulate later, associated with a decreasing concentration of intracellular inorganic salts. Thus perturbing inorganic ions are replaced by compatible organic osmolytes. The second mechanism is stabilization of native protein structure (1). High concentrations of protective organic osmolytes stabilize protein structure. The mechanism of stabilization involves strong exclusion of the protective osmolytes from the surface of proteins. In contrast, denaturing osmolytes, such as urea, accumulate at the surface of proteins. Protective osmolytes push the equilibrium of protein folding toward the native form, whereas denaturing osmolytes push it toward the unfolded form. The configuration of the protein backbone is the most important determinant of stabilization or denaturation (23). When a protective organic osmolyte opposes the perturbing effect of urea, it is said to "counteract" the urea, and high levels of trimethylamines are often found in cells exposed to high urea (26). The combination of trimethylamines and urea provides a compatible mixture of organic osmolytes. Non-methylated organic osmolytes can also stabilize native protein structure and counteract urea but are less effective apparently than trimethylamines (21). However, we can find only this one report directly comparing counteracting efficacy of trimethylamines to that of non-methylated organic osmolytes, so it is difficult to generalize. GPC is a trimethylamine, and, like other trimethylamines, it counteracts the perturbing effects of urea on proteins (7,8).

Distribution of GPC

The realization that GPC is a compatible and counteracting organic osmolyte led to numerous studies of it in renal medullary cells of various species, in other tissues, and in cultured cells (references in Ref. 11). GPC is present in the renal medullas of all species examined, including rats, rabbits, dogs, sheep, pocket mice, deer mice, and voles. The localization of GPC, NaCl, and urea within kidneys was determined by slicing them along their corticomedullary axes and measuring the solutes in extracts from different levels. There is little urea in the cortex but considerably more sodium. Both rise monotonically from their levels in the cortex to a peak at the tip of the inner medulla. Similarly, there is a gradient of GPC from very low levels in the cortex to a maximum at the tip of the papilla. The similarity of gradients is consistent with an osmo-protective role of GPC. Within the rat renal medulla, cells in the collecting ducts and the thin limbs of Henle's loop contain high levels of GPC, which increases near the tip of the papilla. Neither glomeruli nor any of the kinds of tubules in the rat renal cortex contain much GPC. Among nonrenal tissues, GPC is also present in rat brain cells, and its level there increases with salt loading (13).

Osmotic Regulation of GPC

GPC rises in renal medullary cells in vivo when the medullary interstitial levels of salt and urea to which they are exposed become elevated (references in Ref. 11). The original studies of GPC in the 1950s demonstrated that the level of GPC is higher in the inner medullas of dogs and rats when the animals are water deprived than when they are given excess water. This was later confirmed during the antidiuresis produced by water deprivation in normal rats and by administration of vasopressin to Brattleboro rats. In other experiments, renal medullary interstitial levels of NaCl and urea were varied independently by manipulating intake of salt and protein. The sum of intracellular sorbitol, GPC, betaine, and inositol correlates highly with medullary sodium, consistent with the concept that sorbitol, GPC, betaine, and inositol act in combination as compatible osmolytes by accumulating to a total concentration sufficient to balance the osmolality of the external NaCl. GPC is unique in that it alone correlates highly

with medullary urea, consistent with this trimethylamine serving to counteract urea in kidney medullary cells. Betaine is also a trimethylamine. It counteracts the perturbing effects of urea on proteins in vitro as effectively as GPC (7,8). Nevertheless, although the level of GPC in renal medullas correlates with the level of urea, betaine does not. Furthermore, GPC is present in MDCK, PAP-HT25, and mIMCD3 cells, and elevating NaCl and/or urea increases its level. In contrast, high urea decreases betaine in those tissue cultures. Thus, although betaine and GPC both effectively counteract the effects of urea, renal cells apparently prefer GPC for this purpose. The rationale for this preference has remained conjectural. Large amounts of cellular GPC are also present in tubule suspensions prepared by collagenase digestion of renal inner medullas from antidiuretic rats, provided that the solutions bathing the tissue during preparation contain high NaCl. The suspensions of renal medullary tubules and the tissue cultures have provided convenient models for understanding the osmoregulation of GPC.

GPC Metabolism

The GPC that accumulates in cells when they are exposed to high NaCl and/or urea is synthesized within the cells (references in Refs. 5,11). GPC is synthesized from phosphatidylcholine (PC) and broken down into choline and α-glycerophosphate (FIGURE 1). PC is a major lipid constituent in both the cortex and medulla of the mammalian kidney. PC synthesis in mammalian kidney cells occurs through the Kennedy pathway with choline as one of the starting materials. The synthesis of GPC from PC entails the removal of fatty acids by phospholipase activity. The synthesis of GPC could involve sequential activity of a phopholipase A and a lysophospholipase or, alternatively, activity of a single phopholipase B (FIGURE 1). The GPC that results is water soluble. It is degraded by hydrolysis to glycerol-3-phosphate (G-3-P) and choline, catalyzed by GPC:choline phosphodiesterase (GPC-PDE) activity. The choline that results can be used for resynthesis of PC.

Osmoregulation of GPC was initially studied in MDCK cells (20). Tissue culture medium ordinarily contains choline. If the choline is removed at the same time that the osmolality is increased, cell GPC rises slightly by the second day but then falls back to the baseline level. In contrast, when choline is present, the level of GPC continues to rise. Thus choline taken up from the medium contributes to synthesis of GPC, and, even when there is no choline available in the medium, there is enough of some intermediate (presumably PC) already formed in the cells to provide a transient increase in GPC. Choline is taken up into MDCK cells and isolated rat inner medullary collecting duct cells by sodium-independent transport, but that transport is not affected by osmolality. Thus osmoregulated GPC accumulation does not involve changes in choline transport capacity.

Osmoregulation of GPC Synthesis

Further studies with MDCK cells (16) showed that high NaCl increases the rate of synthesis of GPC from PC, but high urea does not. Synthesis of GPC from PC involves phospholipase-catalyzed removal of the fatty acid groups at the sn1 and sn2 positions of PC (FIGURE 1). Interestingly, although PC is consumed during synthesis of GPC, the amount of PC in MDCK cells does not decrease following high NaCl-induced increase in GPC. High NaCl increases phospholipase activity, but high urea does not. In principle, a phospholipase A could catalyze the removal of the fatty acid group at the sn1 position of PC, and a lysophospholipase, the fatty acid at sn2. Alternatively, a single phospholipase B could remove both fatty acid groups. The latter possibility turned out in fact to be the case. The phospholipase B is neuropathy target esterase (NTE) (10). NTE was originally identified as a target of organophosphates that is involved in induction by them of peripheral neuropathy in humans (14). NTE is an endoplasmic reticulum-associated protein, mostly exposed on the cytoplasmic face of the membranes (18). The mechanism of organophosphate neurotoxicity is that certain organophosphates act as

pseudosubstrates for NTE, and the covalent organophosphorylated intermediate formed by this reaction is hydrolyzed extremely slowly, thereby inhibiting the enzyme. Loss of activity disrupts phosphatidylcholine (PC) homeostasis, resulting in neuronal and glial death (19). The normal role of NTE in nerves is not completely understood. NTE is also expressed outside of the nervous system in numerous organs, including the kidney (12,15), but its role in those tissues also has not been well understood. A possible role in osmoregulating GPC became apparent when NTE was shown to be a phospholipase B that deacylates PC sequentially at positions sn1 and sn2 in *Saccharomyces cerevisiae*, yielding GPC. Yeast mutants that lack NTE activity are not able to produce intracellular GPC (29). Following up on this clue, we found that NTE in fact catalyzes osmoregulated production of GPC from PC in mIMCD3 cells (10). In these cells, high NaCl increases NTE mRNA, beginning within 8 h, and NTE protein within 16 h. Di-isopropyl fluorophosphate (DFP), a known inhibitor of NTE esterase activity, reduces the GPC accumulation, as does a siRNA that specifically decreases NTE protein abundance. In vivo, NTE protein expression is higher in the renal inner medulla than in the cortex. Also, the lower renal inner medullary interstitial NaCl concentration, which occurs chronically in ClCK1$^{-/-}$ mice and acutely in normal mice given furosemide, is associated with lower NTE mRNA and protein. Furthermore, phospholipase activity increases in inner medullas of rats when thirsting results in increased urinary (and presumably renal inner medullary) osmolality (27). TonEBP/OREBP is a transcription factor previously known to increase the expression of osmoprotective proteins, including aldose reductase, BGT1, SMIT, and HSP70 in response to high NaCl (6). Knockdown of TonEBP/OREBP by a specific siRNA inhibits the high NaCl-induced increase of NTE mRNA, indicating that high NaCl elevates NTE mRNA through TONEBP/OREBP-mediated increase in its transcription, similar to other osmoprotective genes. Transcriptional activity of TonEBP/OREBP requires binding to a specific DNA element, ORE/TonE, whose consensus sequence is NGGAAAWDHMC(N). Between bp −900 and −1 in the 5′-flanking region of NTE, there are four DNA elements that fit this consensus. Thus high NaCl increases transcription of NTE, mediated by TonEBP/ OREBP, and the resultant increase of NTE abundance and activity contributes to increased production and accumulation of GPC in mammalian renal cells in tissue culture and in vivo.

Osmoregulation of GPC Degradation

GPC is metabolized to choline, and G-3-P, catalyzed by GPC-PDE (FIGURE 1). Involvement of this enzyme in osmoregulation of GPC was originally proposed by Ullrich (24). He reasoned from two observations: *1)* GPC diesterase activity is higher in the renal cortex than in the medulla, consistent with the higher level of GPC in the medulla; and *2)* addition of NaCl and urea to homogenates of cortical tissue inhibits GPC diesterase activity. Thus the high level of urea and NaCl in the renal medulla during antidiuresis would inhibit GPC-PDE and thereby elevate GPC. This conclusion was later strengthened by observations that GPC-PDE activity is reduced by high extracellular NaCl and/or urea in MDCK (20,28) and isolated rat inner medullary collecting duct (4) cells. Further study of MDCK cells (17) elaborated on these results, showing that exposure to high NaCl or urea decreases the rate of cellular GPC degradation by approximately one-half within 20 h, accompanied by commensurate decreases in the activity of GPC-PDE. The inhibition of GPC-PDE activity is rapid. It falls more than 50% within 2 h, and with high urea alone the inhibition lasts for at least 7 days. In contrast, with high NaCl alone, GPC:PDE activity reverts to control values by 7 days, by which time increased synthesis of GPC accounts for sustained GPC accumulation.

The GPD-PDE that is involved is GDPD5 (9), a homolog of bacterial glycerol diesterases. Raising NaCl or urea decreases GDPD5 enzymatic activity. High NaCl, but not high urea, reduces *GDPD5* mRNA by destabilizing it. The level of *GDPD5* mRNA apparently is also osmotically regulated in the renal medulla in vivo. Furosemide, which lowers medullary interstitial NaCl, increases *GDPD5* mRNA, suggesting that the mRNA is normally lowered

by the high level of NaCl in the medulla. Elevating either NaCl or urea reduces GPC-PDE activity in MDCK and mIMCD3 cells. The mechanism apparently is not direct inhibition of GDPD5 activity because adding 200 mosmol/kg of NaCl, KCl, or urea to the assay buffer does not change GPC-PDE activity of GDPD5, whereas adding that much NaCl or urea to the cell culture medium does. It is possible that the higher interstitial levels of NaCl and urea in renal medullas in vivo might directly inhibit GDPD5, but that remains to be tested. Rather, when cells are exposed to high NaCl or urea, the resultant decrease in GDPD5 enzymatic activity is maintained in recombinant GDPD5 immuno-precipitated from the cells and assayed under fixed conditions in vitro. Thus hyperosmolality apparently causes a posttranslational modification in GDPD5 that inhibits its activity. We do not yet know what that post-translational modification is. A possible clue is that GDPD5 contains many potential sites for phosphorylation. Several of them are possible targets for kinases whose activity is modulated by high NaCl or urea, including Erk, ATM, and PKA. Furthermore, our recent unpublished data show that migration of recombinant GDPD5 in SDS-PAGE is noticeably retarded at 300 mosmol/kg and that this gel retardation is reduced following elevation of extracellular NaCl or urea. We hypothesize that NaCl and urea may dephosphorylate GDPD5 and thus inhibit its activity, but that remains to be proven. Another possibility is that high NaCl or urea might alter association of some other protein with GDPD5 or modify the associated protein.

GDPD5 and NTE both occur in the endoplasmic reticulum. This arrangement makes their role in metabolism of PC and GPC more efficient. Thus NTE uses PC in the endoplasmic reticululum membrane to synthesize GPC, and the choline that results when GDPD5 eventually catalyzes hydrolysis of GPC is available within the endoplasmic reticulum to replenish the PC there. Thus GDPD5, by degrading the GPC produced by NTE activity, provides a source of choline within the endoplasmic reticulum for resynthesizing PC via the Kennedy pathway.

## Conclusions and Perspectives

Although GPC was the first of the predominant osmo-protective organic osmolytes to be identified in renal medullas, understanding the molecular basis of its regulation has lagged behind understanding of the others. Part of the difficulty is that its osmoregulation involves phospholipid metabolism, which can be difficult to investigate. Also, its regulation involves both synthesis and degradation catalyzed by enzymes whose functions were either only recently discovered (NTE) or previously unknown (GDPD5). High NaCl increases synthesis of GPC by activating the transcription factor TonEBP/OREBP, which in turn increases the abundance of the phospholipase B, NTE. Much is already known about TonEBP/OREBP, and it is currently being studied intensively by several groups. On the other hand, GDPD5 is little studied, although it poses several remaining challenges relevant to GPC. For example, we do not yet understand how high NaCl destabilizes *GDPD5* mRNA. Also, the nature of the posttranslational modification by which high NaCl or high urea inhibits GDPD5 activity remains uncertain, and we do not know the agents that cause those modifications or how those agents are controlled.

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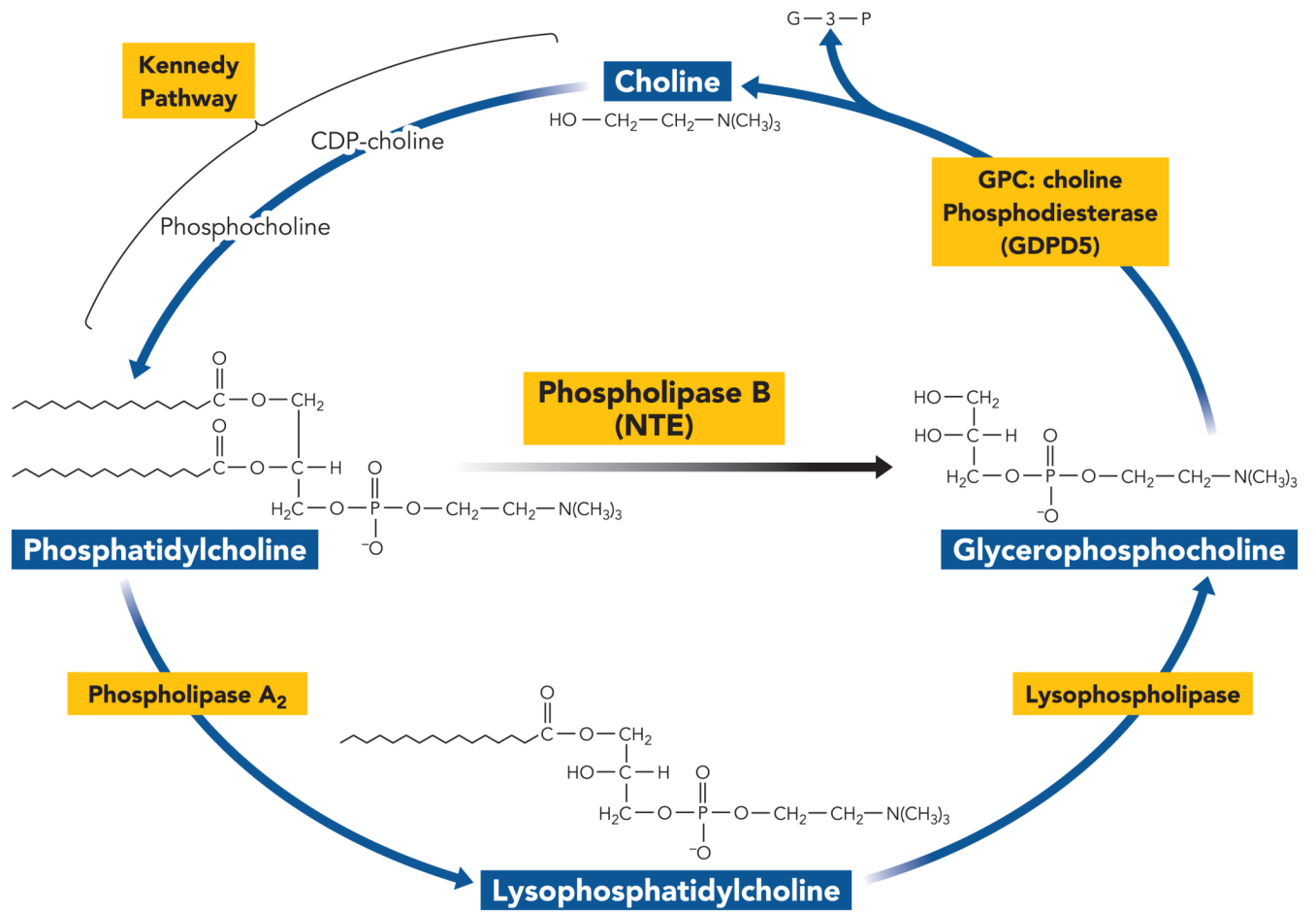
FIGURE 1. Metabolism of glycerophosphocholine
Both NTE and GDPD5 are osmotically regulated.