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Protein kinases as regulators of osmolyte accumulation under stress conditions: An overview

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1. ABSTRACT

Accumulation of osmolytes, during cell volume perturbations, as cell volume regulators is ensured through their de novo synthesis, decreased degradation and transport from their site of synthesis to the site of utility through various transport systems. Among these, transport system mediated accumulation has been observed to be quite significant during long term cell volume perturbation. Under stress conditions, these osmolyte transporters are regulated at transcriptional as well as translational level. At translational level, protein kinases carry out phosphorylation of osmolyte transporters and have been shown to play a crucial role in cell volume homeostasis. In fact phosphorylation of osmolyte transporters on their conserved residues regulates the uptake and efflux of osmolytes by cells. Additionally, accumulated osmolytes in turn have been shown to modulate the structure and functioning of protein kinases. The present review has tried to provide an overview about the role of protein kinases in regulation of osmolyte accumulation under stress conditions. Due to their ability of regulating osmolyte accumulation, potential of protein kinases as therapeutic targets for diseases like cancer has also been highlighted.

2. INTRODUCTION

Living cells are inundated in extracellular fluid with almost constant osmolarity. However, significant perturbations encountered during stress conditions like high salt, high temperature, high pressure and pathophysiological conditions (hypoxia, hypernatremia, cancer, uremia etc.) compromise the cell volume constancy which in turn destabilizes the structural as well as functional integrity of cellular macromolecules (1). In mammalian system, renal medulla of kidneys frequently experience osmotic stress (hyper and hypotonic), as a part of urine concentrating mechanisms or urine dilution.

Additionally organs like brain also experience variations in cellular osmolarity. Majority of the cells respond to these cell volume perturbations by activating cell volume regulatory mechanisms. As soon as cell volume discrepancies are sensed by the cells, various cellular signaling pathways are activated which regulate the cell volume changes through a number of regulatory mechanisms. In hyposmotic stress, swollen cells release KCl, cellular water and organic solutes thereby returning the cell volume to its original size through the process of regulatory cell volume decrease (RVD) (2, 3). On the other hand, shrunken cells, through the process of regulatory cell volume increase (RVI), return to their original volume by up-taking KCl and cellular water (2). Although under hyperosmotic stress, the cells in the initial phase of volume regulation utilize electrolytes for maintaining cell volume but prolonged exposure of macromolecules to these perturbing cellular electrolytes might severely damage them (3). In order to overcome long-term cell volume perturbations without any effect on cellular machinery, organisms maintain their cellular osmolarity through preferential accumulation of small molecular weight organic solutes called as osmolytes. In contrast to high electrolyte concentration, these osmolytes do not perturb cellular macromolecules and are thus preferentially accumulated over electrolytes/salts (4, 5). The biological distribution of these osmolytes and their characteristic features has been summarized in detail by Yancey and Somero (6). Generally accumulation of osmolytes under stress conditions, takes place in three ways: - (i) enhanced de novo synthesis (glycerol, sorbitol) (ii) decreased degradation (glycerophosphorylcholine) and (iii) enhanced cellular uptake (myoinositol, betaine, taurine and amino acids) by increasing the activity of their transporters.

Although a typical cell utilizes all the above mentioned mechanisms for osmolyte accumulation, osmolyte transporter mediated mechanism holds significance as it is preferably used during long term cell volume perturbations (7). Availability of these transporters is largely dependent on their enhanced expression with transcriptional regulation facilitated by tonicity responsive enhancer/tonicity responsive element/binding protein (TonE/Ton EBP) (8) (9). However, during stress conditions like heat shock, induction of osmolyte transporter genes is highly specific and are thus not abundantly expressed. In this context, regulation of osmolyte transport through post translational modification (PTM), has also been reported. Ability to regulate osmolyte transporters at post translational level has been shown to be possible due to the presence of a number of post translational regulatory sites on these transporters. Among all PTMs, phosphorylation of osmolyte transporters has been found to modulate their activity. For instance, MI and betaine co- transporter exhibit consensus sequences for phosphorylation by Protein Kinase A (PKA) and Protein Kinase C (PKC) (10, 11). Cell line studies have shown that MI and betaine co- transporter phosphorylation by these kinases results in the inhibition of respective osmolyte movement across their membranes (12). Similarly, conserved sequence for phosphorylation by PKC and other kinases like casein kinase 2 (CK2) have been observed in taurine transporter (13). PKA mediated stimulation of taurine transporter leads to taurine uptake while as PKC activation reduces its activity and rendering the cells insensitive to PKA-mediated stimulation. In addition to this, osmolytes in turn have been shown to modulate the activity of protein kinases under *in vitro* conditions.

The present review was therefore aimed to focus on the role of protein kinases in osmolyte mediated cell volume homeostasis vis-a-vis the significance and expression of osmolyte transporters as a vital cellular machinery for adaptation to long-term cell volume perturbations. Additionally, the ability of osmolytes to modulate the activity of kinases under *in vivo* and *in vitro* conditions has been discussed. Future insights regarding the role of protein kinase mediated osmolyte transport in diseases like cancer is highlighted.

3. OSMOLYTE ACCUMULATION - AN ADAPTIVE RESPONSE AGAINST STRESS

Osmolytes are small molecular weight ubiquitously occurring organic compounds, accumulated by organisms/cells under stress conditions to stabilize cellular macromolecules like DNA and proteins without perturbing their structure and function (6). Osmolytes have been grouped into

three major classes: polyhydroxy compounds (polyols), free amino acids and their derivatives and methylammonium compounds (14). Polyols, amino acids and their derivatives enhance protein stability under native conditions with little or no effect on protein function and are considered as compatible osmolytes. On the other hand, methylammonium compounds counteract the denaturing effect on protein stability and function and thus behave as counteracting osmolytes. Compatible osmolytes belonging to polyols are good at protecting against extreme temperature and dehydration stresses, while as amino acids generally protect the cells against salt stress. In contrast, methylamines protect against denaturing effects of urea, pressure and temperature (15, 6).

Intracellular osmolyte accumulation is mainly mediated by three processes. The first process involves the intracellular synthesis of osmolytes by metabolic reactions. For instance, sorbitol is primarily generated through metabolic reactions from D-glucose. Cells uptake D-glucose via uniport or symport systems and readily convert it into sorbitol by the enzyme Aldose Reductase (AR) in presence of NADPH as cofactor (16). Second method involves the decreased degradation of osmolytes e.g., glycerophosphorylcholine (GPC). GPC: choline diesterase is involved in the breakdown of GPC and has been found to be the most prevalent osmosensitive enzyme after long term exposure to hypertonic stress (17). Inhibition of this enzyme reduces the breakdown of GPC, thereby increasing its concentration. Third method involves the uptake of osmolytes across cell membrane by specific transport systems. These transport systems use energy in the form of electrochemical gradient to uptake osmolytes into the cells.

Osmolyte accumulation has been observed as a universal response against alterations in the intra and extracellular osmolarity of living cell (4, 18). These are generally found to be accumulated by organisms of diverse nature across all kingdoms. However, organisms experiencing environmental stresses face the strongest selective pressures for accumulating osmolytes (19). In fact their accumulation in specific subcellular locations of cells coordinate their movement over long and short distances thereby helping them to protect and stabilize the cellular macromolecules within the organism (20). For instance, marine organisms like elasmobranchs cope with the high osmolality of sea water by accumulating methylamine osmolytes along with urea in their tissues and extracellular fluid (4, 6). In mammalian renal medulla cells, subsequent exposure to high salt and urea during urine concentrating mechanisms has shown to accumulate high levels of osmolytes such as betaine, inositol, taurine, sorbitol and GPC (4). Except sorbitol, accumulation of other osmolytes in renal medulla cells is mediated by co-transporters. Accumulation of compatible osmolytes in rat renal epithelial cells has been shown to reduce the effects of macromolecular crowding and ionic strength. As a result of osmolyte accumulation in renal cells, mRNA stress granules formed during hypertonic stress have been shown to be disassembled by osmolytes leading to increased cell survival chances (21). Failure of taurine accumulation in cats resulted in renal malformations and progressive kidney damage (22, 23). Additionally, osmolytes such as amino acids, choline, creatinine, inositol and taurine accumulation normalizes the fluid volume in the mammalian brain. (1). Taurine accumulation has been also found to be very important in retina development as well as in regulation of transparency of eye lens (22, 23). In fact, severe and progressive retinal degeneration has been demonstrated in taurine transporter (TauT) knockout mouse (22). Furthermore, liver cells also accumulate betaine, inositol and taurine to regulate the Kupffer cell functioning specific for cell volume maintenance during phagocytosis (24, 25). In addition to this, osmolyte accumulation regulates skeletal muscle development and their deficiency results in severe structural defects (26).

Microorganisms have been also shown to accumulate osmolytes such as proline, trehalose, glutamate, glycine betaine, ectoine and carnitine as stress response (27). For instance, *E. coli* and *S. typhimurium* accumulate osmolytes available in their surroundings but synthesize and accumulate osmolytes like trehalose only if exogenous osmolytes are lacking (17). However, betaine or proline if available are preferentially accumulated through their transporters simultaneously decreasing the endogenous osmolyte synthesis. Two important

proteins, proP and proU, are used by these microorganisms for betaine accumulation (28). ProP is weakly inducible over constitutive levels by hyperosmotic and nutritional stress whereas proU is strongly inducible by only hyperosmotic stress (29, 30). Due to this, proU has been considered as a key protein for osmotic adaptation in bacteria. Moreover, based on the affinity, inducibility and rate of osmolyte transport system for accumulation of glycine betaine, high affinity and low affinity transport systems have been observed in *S. aureus* (31). Osmotic pressure has been shown to induce the low-affinity transport system while as glycine betaine, itself induces the high-affinity transport system (32). This signifies the importance of low-affinity transport system in the glycine betaine accumulation mediated osmotic adaptation. Similarly in *L. monocytogenes,* the transporters accumulate glycine betaine to confer cold tolerance during osmotic adaptation (33).

Furthermore, plants that are subjected to stress accumulate a range of organic osmolytes like betaine, ectoine, glucose, proline, isoleucine, mannitol, sucrose, pinitol and inositol (17). Functional osmolyte transporters in plants such as *Mesembryanthemum* species, uptake and accumulate osmolytes like myoinositol (MI) according to proton gradient and low salt concentrations (20). Drastic increase in the transport of MI has been observed in salt-stressed ice plants for synthesis of D-ononitol by using a stress-induced inositol methyl-transferase (34). Proline transporters (ProTs) accumulate proline in tissues and organs of plants under salt stress or during development, sustaining the redox states in water-stressed plants rather than stabilizing cellular contents (35, 36). In fact, compromised accumulation of proline in mutants resulted in increased accumulation of reactive species inducing oxidative damage. Glycine betaine is abundantly accumulated in plants exposed to dehydration, drought, salinity, heat and cold stress. In general, osmolyte accumulation has been seen as a vital adaptive mechanism for imparting drought and salt tolerance in plants. In certain cases process of osmolyte accumulation has been focused for improving the stress tolerance of plants and simultaneous enhancement of crop productivity (37). Transgenic plants are engineered for accumulation of these compatible solutes under stress-inducible as well as tissue-specific regulation. For this, osmolyte transporter or synthesizing genes are targeted in transgenic plants for imparting resistance and stress tolerance (38, 39).

Transporter mediated osmolyte accumulation has been favored during long term stress management in the cells (2, 7). Importance of these transporters in maintaining the cell volume homeostasis during stress has been well demonstrated in kidney, liver and other organs. Till now, three important osmolyte transporters namely, taurine transporter (TauT), myoinositol transporter (SMIT) and betaine transporter (BGT1) involved in the transport of taurine, myoinositol and betaine have been thoroughly studied (40-42). All of them are coupled transport systems and mediate the transport of their respective osmolytes through secondary active transport, where Na^+ ions are required to generate the electrochemical gradient for the net osmolyte movement (40). Taurine transporter (TauT) and the betaine transporter (BGT1 for betaine and GABA transport) are members of Na^+ and Cl^- coupled transporter gene family. The coupling of one Cl^- and two Na^+ to each molecule of osmolyte (taurine/betaine) provides the electrochemical gradient for accumulation of very high levels of taurine and betaine during hypertonic stress. Myoinositol transporter is Na^+ dependent transporter (SMIT) where two Na^+ ions are exchanged for one molecule of myoinositol (40). SMIT have a structural similarity with glucose transporters characterized by a large number of transmembrane domains and some inverted repeat domains (43). In view of the importance of osmolyte accumulation (OA) through osmolyte transporters cells exhibit certain controls at transcriptional and translational levels of transporter expression and simultaneously regulate and maintain cell volume homeostasis.

4. REGULATION OF OSMOLYTE ACCUMULATION

4.1. At transcriptional level: - expression of osmolyte transporter

Generally the accumulation of osmolytes is controlled at transcriptional level for the expression of osmolyte synthesizing as well as osmolyte

transporting genes (2). During stress, cells respond to stimulus by transcribing osmolyte transporter genes and thus expressing osmolyte transporter. Although the osmosensing mechanism of cells is largely unknown, various signaling pathways have been considered to be involved such as Mitogen activated protein kinase (MAPK), Janus kinase (JNK), Myosin light chain kinase (MLCK) etc. As soon as the cells sense osmotic stress, cytosolic transcription regulator Tonicity enhancer binding protein (TonEBP) is phosphorylated and activated (44). In fact, a recent publication suggests the role played by DNA damage inducible kinase-ATM in the TonE/TonEBP mediated transcription (45). Activated TonEBP complex is translocated to the nucleus for binding with Tonicity enhancer element (TonE) at the promoter region of osmolyte transporter gene. Binding leads to transcription of osmolyte transporter genes and subsequent accumulation of respective osmolytes (46). In fact, TonEBP has been established as a central regulator during hypertonic stress response. In addition to this, deletion in the TonEBP gene has been observed to completely block the expression of genes for betaine-gamma amino butyric acid (BGT1) and sodium myoinositol (SMIT) osmolyte transporters (47). TonEBP deficient mice have been found to exhibit renal medulla atrophy, and high apoptotic rates (47). Interestingly, osmolyte transporter gene regulation for various non-neuronal cells in brain such as astrocytes, microglial, ependymocytes, oligodendrocytes and endothelial cells neither express TonEBP at basal conditions nor at prolonged hypertonicity (48, 49). In spite of the absence of TonEBP, most of osmolyte transporter genes have been found to be up regulated and highly expressed in these cells, which indicate that the regulation of transporter stimulation might also be facilitated by some other mechanisms. Figure 1 illustrates various steps involved in transporter mediated osmolyte accumulation.

Transcription of osmolyte transporter genes such as myoinositol (SMIT), betaine (BGT1) (50) and taurine (TAUT) (51) genes in mammalian kidneys serve as a critical event in response to hypertonicity. In fact, abundant expression of osmolyte transporter mRNA has been observed in hypertonic Madin-Darby Canine Kidney (MDCK) cells (52, 10). Hypertonicity markedly induces the transcription of SMIT and BGT1 genes resulting in increased abundance of their mRNAs (53). In fact, BGT1 mRNA abundance is enhanced more than 10-fold, thereby increasing the transport capacity by 5–10 fold (54, 55, 10). On the other hand, liver macrophages (Kupffer cells) have also been shown to express increased BGTl and TAUT mRNA levels during hypertonic stress (25). Conversely, decreased expression of BGTl and TAUT mRNA levels have been observed during hyposmotic exposure (25). The mRNA expression levels of sodium-dependent myoinositol co-transporter (SMIT) have been observed to be strongest in cultured astrocytes in rats. Additionally TAUT mRNA levels have also been highly expressed in cultured neurons and substantially expressed in cultured astrocytes, microglia or fibroblasts in rats. However, enhanced BGT1 mRNA expression have been observed in microglia only (56). Such studies do infer that expression of cell type-specific osmolyte transporters strongly correlate with mRNA and protein expression levels in rats. A diverse set of functionally related transporters are also encoded by bacterial species probably for long-term adaptation to their environments. For instance, enhanced transcription of osmolyte transporters genes in *E.coli*, have been found especially ProU for proline as well as glycine betaine transport inside the cell (28). It should be noted that responses to hyperosmotic stress have been comprehensively studied and recognized in renal and non-renal tissues under pathophysiological conditions like hypernatremia, hyperglycemia. In fact, Protein kinase mediated osmolyte regulation has been specifically observed in hypertonic stress. Moreover, hyperosmotic stress has been shown to exhibit a strong association with inflammation and ultimately leads to other types of stresses in the cell such as oxidative stress. Therefore, the involvement of osmolytes in maintaining cell volume homeostasis during hyperosmotic stress is significant.

Even though the transcription is the foremost step towards osmolyte accumulation by osmolyte transporters during stress, complex regulatory mechanisms involving a number of factors such as signaling pathways and type of stress regulate this process. Signaling pathways involving protein kinases regulate the expression levels of osmolyte transporters through TonEBP in

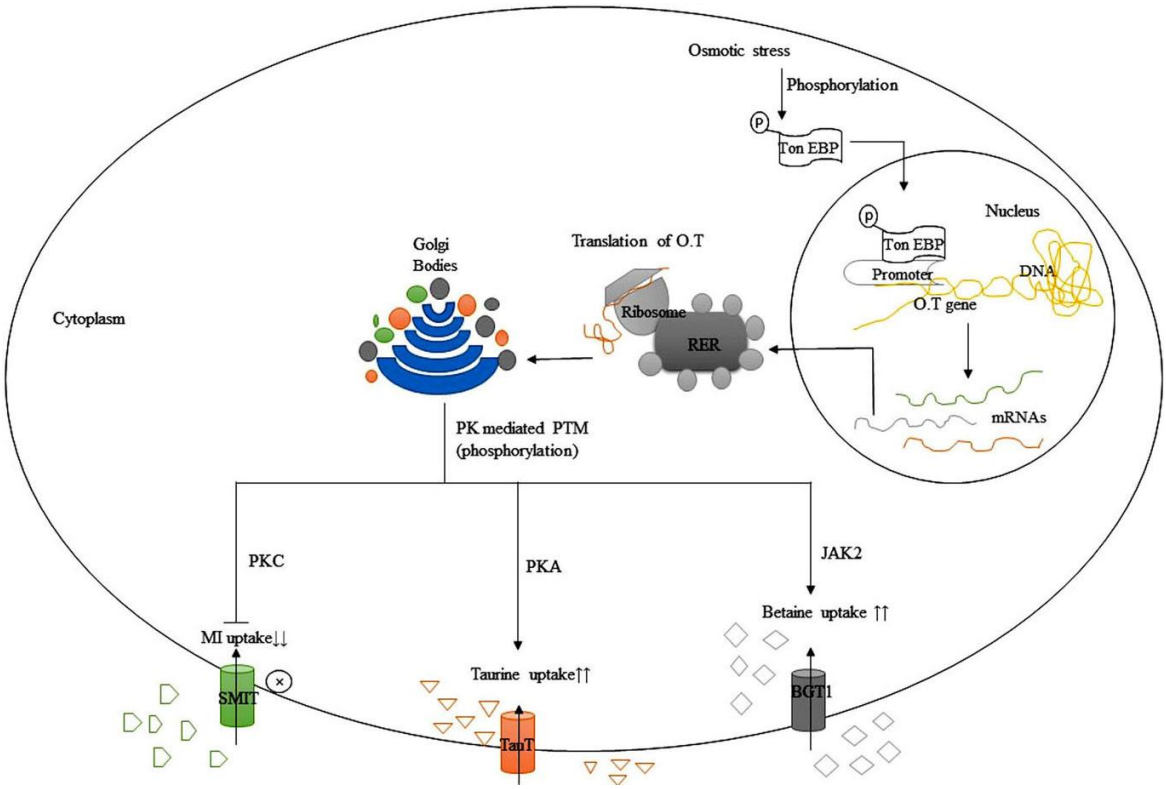

Figure 1. Schematic diagram depicting expression of osmolyte transporter and protein kinase mediated phosphorylation under stress conditions: - osmotic stress sensed by cell results in the phosphorylation of TonEBP in the cytosol and its subsequent translocation to nucleus where it binds the TonE on the promoter region of osmolyte transporter gene. This stimulates the transcription of genes and production of mRNAs that are translocated back to cytosol where they are translated into osmolyte transporter proteins. These transporters undergo PTMs including protein kinase mediated phosphorylation to catalyze the transport of osmolytes. Protein kinases are known to either stimulate or inhibit osmolyte transporters. O.T gene: Osmolyte transporter gene, RER: Rough endoplasmic reticulum, PTM: Post translational modifications, PKC: Protein kinase C, PKA: Protein kinase A, JAK2: Janus kinase 2, SMIT: myoinositol transporter, TauT: Taurine transporter, BGT1: Betaine transporter

mammalian models e.g., serum and glucocorticoid inducible kinase 1 (SGK1) (57). Hypertonic stress is mostly found to induce increased transcription of osmolyte transporter genes. However, under certain stress conditions such as heat shock, the transcriptional control for osmolyte accumulation is not sufficient. Especially BGT1 expression has been found to be unaffected by the elevated temperatures clearly indicating, that heat shock stress does not induce the transcription of betaine transporter gene (58). In addition to this, binding sites for transcription factors such as p53 and NF-kβ exist on TAUT gene that also mediate their regulation (59). In order to express the functional protein on the plasma membrane, gene transcription is followed by the translation to regulate osmolyte accumulation. Interestingly, protein kinases have been shown to be rapidly activated during hypertonic stress and facilitating the post translational modification of these transporters and affecting the osmolyte accumulation during cell volume homeostasis. (60, 61).

4.2. At translational level - Role of protein kinases

Soon after the translation of mRNA transcripts into functional osmolyte transporters, the transporter proteins undergo post translational modifications. Post translational modifications of these proteins occur in trans-Golgi network and subsequently are localized to plasma membrane. At post translational level, osmolyte transporters are

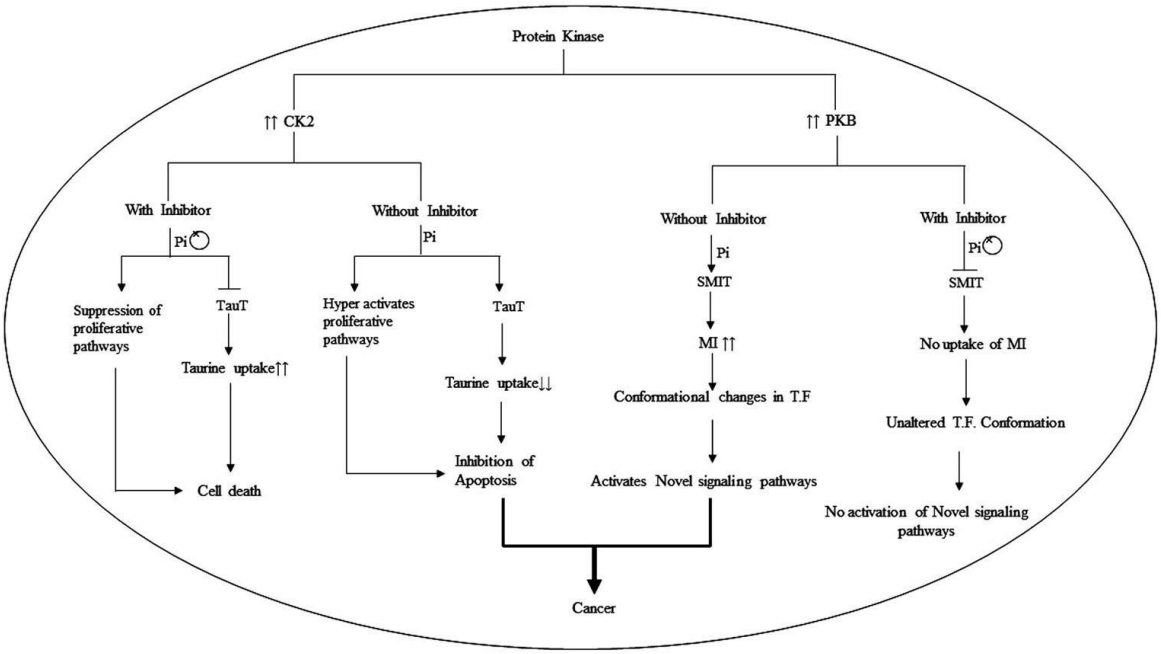

Figure 2. Protein kinase mediated osmolyte accumulation and their effects on cell physiology under disease conditions. Protein kinase mediated phosphorylation exerts different effect on osmolyte transporters. PK either inhibit or activate osmolyte transporters osmolyte accumulation as per the requirement of cell to maintain homeostasis. In certain cancers, protein kinase mediated uptake of osmolytes such as taurine can lead to cell death whereas preventing myoinositol accumulation through its transporter in prostate cancer can help to reduce the metastatic potential. CK2: Casein Kinase 2, PKB: Protein kinase B, T.F: Transcriptional factors

regulated by protein kinases for their expression and activity (62, 63). During these modifications, osmolyte transporters like SMIT, BGT1 and TauT undergo phosphorylation due to the presence of conserved sequences in their structures (62, 63). Phosphorylation of osmolyte transporters have important implications on their transport mechanism wherein their up-regulation or down-regulation takes place and thus affects the osmolyte accumulation. Although this modification regulates osmolyte transporters acutely but it is of immense value during stress conditions for transport of osmolytes across the cell membrane. Various activators or deactivators of protein kinases mediate either phosphorylation or inhibit phosphorylation with rapid effects on subsequent enhancement or diminution of transporter protein function. Activation of MAPKs and MLCK cascades regulate osmolyte accumulation during hypertonic stress-induced responses by phosphorylating the upstream factors in the expression of osmolyte transporters (42, 64, 65). Figure 2 represents the role of protein kinases in osmolyte accumulation during normal and stress conditions.

So far protein kinases have been shown to play a key role in the functioning and activation of three osmolyte transporters including- SMIT, BGT1 and TauT as described below.

4.2.1. Taurine transporter

One of the important osmolytes for the maintenance of cell volume is the highly abundant semi-essential beta-amino sulfonic acid, taurine. Physiochemical properties of taurine allow its movement across plasma membrane of a cell and regulate the cell volume (66, 67). Taurine mediated osmoregulation is essential for normal development and functioning of brain and thus plays a critical role in various abnormal states like migraines, epilepsy and ischemia (68, 69). Furthermore, cell volume regulatory response of taurine has been described in detail by Lambert (59). Knock out studies in mouse photoreceptor cells have also shown that the concomitant failure to accumulate cellular taurine causes programmed

cell death either due to impairment of taurine retinoid transport between pigment epithelium and retina or due to decreased antioxidative capacity (70, 22, 71). Apart from its role in osmoregulatory pathways taurine is known to be involved in a number of physiological processes like antioxidative defense, neuromodulation, cholesterol regulation, insulin and calcium signaling (72, 70, 73-76).

Taurine transporter (TauT) belongs to an important family of transport protein solute carrier 6 (SLC6) which are Na^+/Cl^- dependent transporters consisting of about 16 highly homologous members including neurotransmitter transporters (77). TauT is approximately 70 KDa protein comprising of 620 amino acids. A number of distinct TauT isoforms exist based on their molecular weight (78-80), affinity towards taurine (79) and stoichiometry (81). TauT contains 12 transmembrane domains typical of the Na^+Cl^- coupled SLC6 transporter family and the leucine transporter from *Aquifex aeolicus* (LeuTAa) (82, 83). Amino acid residues on its transmembrane domains (TM1, TM3, TM6 and TM8) coordinate Na^+ and taurine binding pockets and hence reflect the variation in Na^+ binding sites or stoichiometry (83). TauT transports taurine into the cells by secondary active transport in which Na^+ movement coupled with Cl^- provides the electrochemical gradient for taurine transport. The movement of Na^+ is maintained by ATP hydrolysis on the basolateral Na^+/K^+-ATPase within the cells (84). Taurine is released passively via a volume-sensitive leak pathway which is permeable to a number of osmolytes (59, 85, 86). These channels maintain a balance of cellular taurine content. Binding of Na^+ to the intra-membrane domains of TauT leads to tertiary structure changes which in turn facilitates the binding of taurine (59). In addition to this, 2.5:1:1 Na^+ Cl^- taurine stoichiometry is required to achieve maximum transport rate (80). However, the availability of TauT isoforms or their differential regulation, this ratio have been found to be prone to alteration among various cell types (59).

Post translational modifications, especially phosphorylation, regulate TauT functioning due to the presence of several putative consensus phosphorylation sites on intracellular domains of TauT for serine/threonine kinases like PKA, PKC and casein kinase 2 (CK2) (78). Fourth intracellular segment (S_4) of TauT, comprising of several conserved charged residues, also display a number of potential phosphorylation sites (87). In fact, site directed mutagenesis studies have shown that the highly conserved Ser-322 plays an important role in the functioning of TauT by PKC phosphorylation (88). PKC mediated phosphorylation of Ser-322 leads to inactivation of TauT, which in turn dramatically decreases the taurine transport. This putative PKC phosphorylation site when masked by polyclonal peptide antibody has been shown to stimulate taurine influx in the *Xenopus laevis* oocyte (89). Additionally, PKC mediated phosphorylation of Ser-322 controlled the steady-state of tauT activity as Ser-322 mutation unaffected K_m but changed V_{max} of TauT (88). However, other mutations like K319Q and D325Y have been found to result in enhanced taurine uptake indicating a significant role of these amino acids in the binding site of TauT (90). Furthermore, Lys-319 and Asp-325 have been shown to play important role in the gating function of the TauT (90). Moreover, Arg-324 also functions as a part of binding site for taurine as R324G mutation has been observed to increase the K_m of the transporter i.e., decreasing its affinity and thus reducing cellular transport of taurine. Ser-322 phosphorylation by PKC has been found to modulate the three dimensional structure of the gate and blocking the binding of taurine to Arg-324. Altogether, these studies infer that Arg-324 is important for the ionic binding to TauT and Ser-322 is the strategic part of the gate controlled by PKC phosphorylation (90).

It has been found that activators and inhibitors of PKC affect the phosphorylation state of TauT in X. *leavis* oocytes (87). Activators like phorbol esters activate PKC which in turn decreases the transport of taurine. Involvement of PKC in the down regulation of transport has been further ascertained by calphostin C, a PKC inhibitor that blocks the phorbol ester mediated down regulation of taurine transport and enhancing taurine uptake by 20%. Another PKC inhibitor, staurosporine, has been shown to enhance taurine uptake by 400% (87). PKC mediated phosphorylation also renders the TauT insensitive to stimulation by cyclic adenosine monophosphate (cAMP) in ELA cells. PKC mediated phosphorylation of TauT affects not only the binding

of substrate/co-substrate to the transporter but its availability to other kinases as well (78). Similarly, activated PKA has been shown to stimulate taurine uptake in Ehrlich Lettré ascites tumor (ELA) cells with cellular taurine concentration reaching upto 50 mM (91). However, PKC activation has been shown to decrease taurine uptake and render these cells impervious to PKA stimulation (92). Results altogether indicate that dephosphorylated state of TauT is important for the influx of taurine while as inhibition of taurine influx requires the phosphorylated state. It has been also observed that free radical exposure, such as peroxide to mouse fibroblast NIH3T3 cells, reduces their taurine uptake by inhibiting phosphatases and maintaining tyrosine residues in phosphorylated state similar to that of serine/threonine phosphorylated state of TauT (86).

Casein Kinase 2 (CK2), a serine/threonine kinase, has been also considered as a regulator for active uptake and passive release of taurine (78, 81). CK2 is constitutively active and up-regulated in growth stimulated cells as well as in most types of the cancers for their proliferation and inhibition of apoptosis (93, 94). CK2 mediated phosphorylation of TauT has been found to reduce its affinity towards Na^+ and decreases the transport rate which in turn has been shown to decrease taurine levels in cancer cells (78). Pharmacological inhibition of CK2 has been found to sensitize cancerous cells for apoptosis via caspase signaling pathways and at the same time potentiate the uptake of taurine (95, 96). It has been observed through motif scanning that Thr-28 present in the TM1 at the cytosolic side of TauT, is the putative target for CK2 mediated phosphorylation (78). Specific CK2 inhibitors like 4,5,6,7-tetrabromo-2-azabenzimidazole (TBB) and 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT) elicit a conformational change in TauT and leading to its increased affinity towards taurine and enhancing transport capacity while as reducing the Na^+:taurine stoichiometry (78). Moreover, similar effect of CK2 inhibition on taurine transport has been found in low and high CK2 expressing mouse NIH3T3 fibroblasts and ELA cells. Such studies indicate that CK2 can suppress taurine accumulation regardless of its expression levels in the cells. CK2 inhibition thus points out the role of taurine for cells which otherwise is down-regulated during cancer. Altogether results do infer that protein kinases such as PKA, PKC and CK2 regulate the taurine transport through phosphorylation of TauT at their consensus sites which in turn modulate the functioning of the cells under stress.

4.2.2. Myoinositol transporter

Myoinositol is the most noticeable form of inositol ($C_6H_{12}O_6$) that occurs naturally as a vital cellular constituent. MI and its derivatives, are implicated in numerous important processes of cell, such as signaling, vesicular trafficking and cell adhesion (97, 98). MI along with other osmolytes function in the cell volume regulation and maintenance of cellular ionic composition. (99, 100). It acts as the second messenger of hormones to regulate Ca^{2+} levels in the cytosol as well as in phosphate inositol-3-kinase (PI3K) pathway (101). MI is particularly important in brain where its decreased levels are associated with brain edema and myelinolysis during hypernatremia (102). Dysfunctional SMIT and misregulated MI levels have been associated with several neurological diseases including Alzheimer's diseases, Down's syndrome, multiple sclerosis and bipolar disorder (103, 104). Although renal cells synthesize inositol but, its accumulation in cells exposed to hypertonic conditions, is mediated by its transporter rather than de novo synthesis. Cloning and expression of canine SMIT cDNA in *Xenopus* oocytes has shown to encode 718-amino acid single polypeptide of 79500 daltons, with 12 hydrophobic membrane spanning domains similar to those found in sodium glucose transporter (SGLT) family Following sixth and eleventh transmembrane domains long charged loops, different from sodium glucose transporter have also been found. Potential PKA and PKC phosphorylation sites have been found in the cytoplasmic domains of the transporter for intracellular kinases to regulate the transporter functionality (105).

SMIT with respect to its amino acid sequence belongs to Na^+ dependent transporters (106). Being an integral membrane protein, SMIT transports one molecule of MI coupled with efflux of two Na^+ molecules (106). Increased V_{max} of SMIT has been observed in different cell types such as MDCK cells and brain derived C6 glioma cells wherein MI

accumulation increases over 1,000 times (>l00 mM intracellular concentration) during hypertonicity (11). Till date three different MI cotransporters have been recognized in mammalian tissues namely SMIT1, SMIT2 and SMIT3 with SMIT1 being the major transporter (106). These multiple SMIT isoforms arise due to the presence of multiple alternate splicing and polyadenylation sites in SMIT gene which in turn contribute to heterogeneity in the regulation of these transporters (107). In fact, the last putative transmembrane domain in one of the SMIT isoform has been found to be replaced with intracellular carboxy termini exhibiting a novel PKA phosphorylation site and multiple PKC phosphorylation sites. This accounts for the heterogeneity in the structure and regulation of myoinositol transporters. SMIT activity is widely distributed in neuronal cells, retinal and lens epithelial cells, renal cells and pulmonary artery endothelial cells. SMIT mediates the absorption of MI in renal tubular cells and generates positive cerebrospinal fluid MI levels relative to blood in choroid plexus (107).

Mammalian SMIT has been also shown to exhibit rather different affinities for MI and differential responses to protein kinase activation in a tissue specific manner regardless of the presence of conserved phosphorylation sites across species (107). For example, PKA activators have been found to acutely reduce SMIT activity in MDCK cells while as increasing its activity in human Retinal pigment epithelium (RPE) cells. Additionally, increased SMIT activity has been found during diabetic state in renal glomerular cells and cultured mesangial cells while as decreased activity in endoneurial preparations from streptozotocin diabetic rats and cultured bovine lens epithelial cells. Similar to TauT, SMIT has also been regulated through kinase mediated phosphorylation. PKA and PKC phosphorylates SMIT on their consensus sequences which leads to inhibition of the transport. About 30% of the SMIT activity is inhibited due to the activation of PKA or PKC and the effects of these two kinases are non – additive as they inhibit the transporter via a common pathway (108). However, continued exposure to PKC activators (active phorbol esters) has shown PKC down-regulation resulting in 150% increase in the SMIT activity.

AMP-dependent kinase (AMPK) has been shown to down-regulate the SMIT resulting in the prevention of MI uptake during energy depletion, that would otherwise have driven the uptake of osmotically obliged water and thus cell swelling. SMIT1 has also been shown to be activated by the serum and glucocorticoid-inducible kinase (SGK1, 2, 3) and the closely related protein kinase B (PKB) (44). In fact, SMIT1 and SGK1 are co-localized in various organs like kidney (109, 110, 11) and brain (111, 11), indicating close interaction of SGK1 with SMIT. SGK1 mediated activation of SMIT1 is important since the catalytically inactive kinase isoforms of SGK1 have been found to be unable to stimulate the MI movement and its uptake across the membrane. Molecular mechanism of SMIT1 up-regulation have been proposed to involve direct phosphorylation of SMIT or indirect phosphorylation on an intermediate protein. In fact, SGK1 has been found to stabilize SMIT1 in the plasma membrane by phosphorylating ubiquitin ligase Nedd4-2 and thereby inactivating it (44). SGK1 mediated phosphorylation abolishes Nedd4-2 interaction with its target on plasma membrane and enhances SMIT activity. Additionally, Janus activated kinase2 (JAK2) has been found to act as a powerful regulator of the SMIT. JAK2 down-regulates the transporter and minimizes MI uptake by cells. Exposure of JAK2 inhibitor such as AG490 has been shown to offset the effects of this kinase slowly indicating indirect regulation of SMIT. In fact down-regulation of SMIT by JAK2 has been suggested to be appropriate for the effects of JAK2 activating hormones and cytokines, such as leptin, growth hormone, and erythropoietin (112).

In addition to Sodium myoinositol transporter, a novel type of H^+ dependent MI transporter (HMIT) has been reported in mammals, which couples H^+ gradient to cotransport MI. HMIT has been shown to possess 12 transmembrane domains with similar membrane topology as that of glucose transporters GLUT1–5. Although the important motifs (residues in transmembrane domains) are conserved for the transporter activity (113). Surprisingly, both HMIT and SMIT transport inositol with no amino acid sequence homology. HMIT is highly enriched in brain, especially astrocytes and some neurons, and is highly active in

acidic pH whereas SMIT is inactive in acidic pH which suggests that the activation of HMIT depends on acidification of extracellular milieu (113). Interestingly, depending on the pH and osmolarity, HMIT and SMIT are partially co-localized in the brain which suggests that the uptake of MI is differentially regulated in different tissues (113).

4.2.3. Betaine transporter

Glycine betaine/Betaine is one of the most important osmolyte accumulated by most of the tissues for cell volume regulation (114). Betaine has been shown to act as a counteracting osmolyte for protecting macromolecules like proteins against denaturing effects of urea. In addition to this, betaine serves as the source of methyl groups for homocysteine to form methionine (41). Accumulation of betaine has been shown to counterbalance a significant proportion of osmolarity gap in the rat kupffer cells during osmotic stress (115). Betaine accumulation is primarily due to its transporter BGT1 as originally observed in MDCK cells (116), and subsequently found in other mammalian tissues such as the central nervous system, liver etc., (117-120). Betaine accumulation is very low in mammalian brain wherein the role of BGT1 for cell volume regulation has been found to be insignificant (41). Low levels of betaine have been found in outer medulla while higher levels have been detected in renal papilla. BGT1 has been found to be specifically up regulated by nitric oxide produced in renal medulla due to hypertonic NaCl. BGT1, being a member of Na^+Cl^- dependent coupled transporters, is involved in transport of neurotransmitters like GABA, amino acids (creatine) and osmolytes. BGT1 mediates the uptake of GABA to reduce the extracellular concentration of GABA and thereby enhancing neuronal excitation. Structurally BGT1 has been shown to encompass 614 amino acids comprising of 12 putative hydrophobic transmembrane α-helices, with amino and carboxyl termini facing cytosol (41). The carboxyl terminus has a sorting signal and a structural domain of 80-90-amino acid residues called as PDZ association motif that binds to the LIN7 PDZ-domain containing protein. LIN7, LIN2 and LIN10 form a tripartite complex through their PDZ domains, and are used in the assembly of junctional components in neurons, epithelia, and glial cells (121). Binding of single LIN7 PDZ domain (a type I PDZ domain) to carboxyl terminus of BGT1 is selective in nature and favours the retention of BGT1 in the plasma membrane (122). In fact, their association has been shown to avert BGT1 internalization and it's targeting to the basolateral surface of cell clearly mediating transporter accumulation on the lateral junctional surface of cells (123). Prevention of internalization acts as the main regulatory mechanism for rapidly modulating the BGT1 surface expression. Transporter functioning requires substrate recognition mostly by amino group of BGT1, additionally one or two Cl^- ions are transported for each GABA/betaine molecule. The transport has been found to be governed by a coupling ratio of 1:1:3 for GABA, betaine and Na^+ ions per transport cycle. Hence, the binding of betaine to extracellular side of the transporter is followed by Cl^- and finally by Na^+. Cl^- has been found to assist the translocation of betaine but a significant transport rate exists even if extracellular Cl^- is not available (121).

Numerous consensus sites for protein kinases have been reported in the intracellular domains of BGT1 indicating post translational regulation through phosphorylation. For instance, PKC mediated phosphorylation at Thr-612 of BGT1 interrupts the PDZ interaction with LIN7, prompting a clathrin mediated relocalization of the transporter to an intracellular recycling compartment with a corresponding decline in its activity (121). On the other hand, PKA mediated phosphorylation behave differently with almost no effect on activity. However, Thr-40 at amino terminus is considered to act as a hot spot for normal movement and insertion of BGT1 in the plasma membrane. In fact, mutation of Thr to Ala has been found to result in trapping of the transporter intracellularly within the trans-Golgi network consequently decreasing BGT1 activity and inhibiting betaine uptake. However, mutant forms of BGT1 (T40E and T40D) have been found to circulate normally to the membrane during hypertonic stress (41). JAK2, another novel regulator of the BGT1, has been shown to increase

Table 1. Effect of protein kinases on osmolyte transporters

Transporter/ Osmolyte accumulated	Protein kinases involved	Mode of phosphorylation	Effect on transporter	References
TauT/ Taurine	PKA[1] PKC[2], CK2[3]	Direct Direct	Stimulates uptake Suppresses uptake	(91)(92) (81)
SMIT/ Myoinositol	PKA, PKC, AMPK[4] and JAK2[5] SGK1[6], PKB[7]	Direct Indirect Indirect, Direct	Decreases uptake - Increases uptake	(108) (112)(44)
BGT1/ Betaine	PKC, AMPK JAK2 PKA	Direct Indirect Direct	Decreases uptake Increases uptake No effect	(121) (126)(125)(108)
[1]Protein kinase A, [2]Protein kinase C, [3]Casein kinase 2, [4]AMPK Adenosine monophosphate activated protein kinase, [5]Janus activated kinase, [6]Serum -glucocorticoid inducible kinase, [7]Protein kinase B				

the transport of GABA/betaine considerably by boosting the transport rate without significantly affecting the substrate binding affinity (124). It has been found to enhance the insertion of BGT1 in the plasma membrane. In fact, recent studies have shown that JAK2 can up-regulate other transporters such as glucose transporters (SGLT1), the glutamate transporters (EAAT1–4) and the Na^+ coupled neutral amino acid transporter ($B^{(0)}AT$) (125). Moreover, AMPK has been shown to down-regulate BGT1 during energy depletion and inhibiting uptake of betaine that would otherwise drive the uptake of osmotically obliged water resulting in swelling (126). A specific PKC inhibitor, GF109203X, has been shown to largely prevent the inhibitory effects of PKC phosphorylation on BGT1 although not completely. Similarly JAK2 inhibitor AG490 has been shown to down regulate BGT1 by inhibiting the insertion of transporter into the membrane. Varied effects of different protein kinases on the three osmolyte transporters is summarized in Table 1.

5. PROTEIN KINASES AS THERAPEUTIC TARGETS

Protein kinase mediated phosphorylation of osmolyte transporters has been shown to alter the uptake of osmolytes which in turn affect several important physiological processes in the cell. Protein kinases like CK2 is not only involved in cell growth and proliferation but also equally suppresses the apoptosis (127). CK2 has been shown to influence the activity of different pathways and components of apoptotic regulation such as PI3K/Akt signaling pathways, caspases, inhibitors of apoptotic proteins, and reactive oxygen species networks (128). It up regulates PI3K/PTEN/Akt/mTOR, JAK/STAT and Ras/MEK/ERK signaling modules by phosphorylation controlling cell survival and proliferation (127). CK2 also phosphorylates many components of NF-Kβ resulting in the dissociation of its inhibitor (iKβ) and increasing the transcriptional activity of NF-Kβ for cell survival. Additionally, it directly inhibits many components of intrinsic and extrinsic apoptotic pathways, by targeting caspase signaling. CK2 has been shown to suppress tumor suppressor genes like p53. CK2 hyper activation clearly indicate its involvement in cancer. In fact, increased expression of CK2 has been observed in several cancers such as acute and chronic lymphocytic leukemia, chronic myelogenous leukemia, adenocarcinoma of breast, kidney and prostate etc., (129). CK2 has been also implicated in various neurodegenerative diseases such as Parkinson's disease where it has been found to phosphorylate α -synuclein and synphilin-1 in the lewy bodies (129). In fact, CK2 has been suggested to play crucial role in the formation of intracytoplasmic inclusions in alpha synucleinopathies. Memory impairments, brain ischemia and other rare neurodegenerative diseases have also been associated with elevated CK2 expression. Furthermore, aberrant role of CK2 in the pathophysiology of other non-cancerous diseases has been plausibly discussed by Guerra and Issinger (94). CK2 therefore seems to be indispensable for cell survival and its impact

on apoptosis makes it an ideal candidate for targeting cancer.

Osmolytes like taurine have been shown to induce apoptosis in colon cancer cells (130). In fact, the chlorinated taurine (TauCl) in the cells have been found to induce mitochondrial damage and eventually resulting in programmed cell death (131). In contrast, the presence of several osmolytes such as amino acids and threitol have been observed in oral cancer cells with increased metastatic potential (132). Interestingly, it has been recently suggested that osmolyte induced conformational changes in the transcriptional factors can alter their functional plasticity in the cells. The accumulated osmolytes modify the intrinsically disordered domains (IDRs) of transcription factors conferring non-canonical functions to them and inducing oncogenesis and metastasis (132). For instance, the metabolomic analysis of prostate cancer cells depicted inositol as the main predictor of metastasis. Inositol has been suggested to modify the transcriptional factors and promoting the formation of novel signaling pathways that would push the cells towards metastasis (132). Similarly, MI transport has also been shown to be dysregulated in pathogenesis of many clinical disorders such as hyponatremia, myelinolysis with decreased MI concentration (102). Studies do clearly indicate the role of kinases and osmolytes in diseases like cancer. Inhibition of CK2 mediated phosphorylation of TauT might help in the uptake of taurine that will eventually help in the apoptosis of cancerous cells. Similarly, protein kinase mediated phosphorylation of SMIT in prostate cancer cells would limit the inositol uptake and reduce the metastatic chances of these cancer cells. Inhibition of protein kinase that govern phosphorylation on SMIT would allow the uptake of MI and correction of clinical condition. A strong correlation of protein kinase expression and osmolyte accumulation via transporters may thus provide some interesting insights. Protein kinases vis- a- vis osmolyte accumulation might serve as attractive therapeutic targets as modulation of protein kinase mediated uptake of osmolytes might lead to either decreased metastatic potential or induce apoptosis in cancer cell.

6. REGULATION OF PROTEIN KINASES BY OSMOLYTES UNDER *IN VITRO CONDITIONS*

Osmolytes being universal protein stabilizers under stress conditions have been shown to regulate the structure and activity of a number of cellular protein systems *in vitro* as well as *in vivo* such as molecular chaperones and antioxidant protein systems (17, 133). Regulation of important protein systems of cells by osmolytes indirectly regulate protein homeostasis in the cells. Similarly, several *in vitro* studies have shown that osmolytes can regulate the structure and functional activity of protein kinases under different conditions. Osmolytes like glycerol and trehalose have been shown to impart protection against thermal denaturation to enzyme phosphofructokinase (PFK), an important regulatory protein kinase of glycolysis (134). Osmolyte induced stabilization of PFK led to enhanced catalytic activity of this enzyme by the shifting of different complex oligomeric configurations within PFK (134). In addition to this, glycerol has been shown to prevent aggregation and thermal inactivation of creatine kinase (CK) in a concentration dependent manner (135). The protective effect of glycerol on CK has been suggested to be due to increased stability of native CK in presence of glycerol. By comparing the denaturation rate of this enzyme, glycerol has been found to enhance the structural stability of holoenzyme rather than its flexible active site (135). Osmolytes have also been shown to regulate arginine kinase (AK) by preventing its guanidinium hydrochloride induced unfolding and inactivation (136). Osmolytes were found to reduce the inactivation rate constant while as increasing midpoint of denaturation and transition free energy changes. In addition to this, osmolytes remarkably barred AK aggregation in a concentration-dependent manner during refolding (136). Halophilic nucleoside diphosphate kinase has also been shown to effectively refold in presence of Trimethylamine-N-oxide (TMAO) under high salt concentration (137). A recent study on the refolding of recombinant Pelodiscus *sinensis* brain type creatine kinase P-CKB has shown that glycine prevented its aggregation

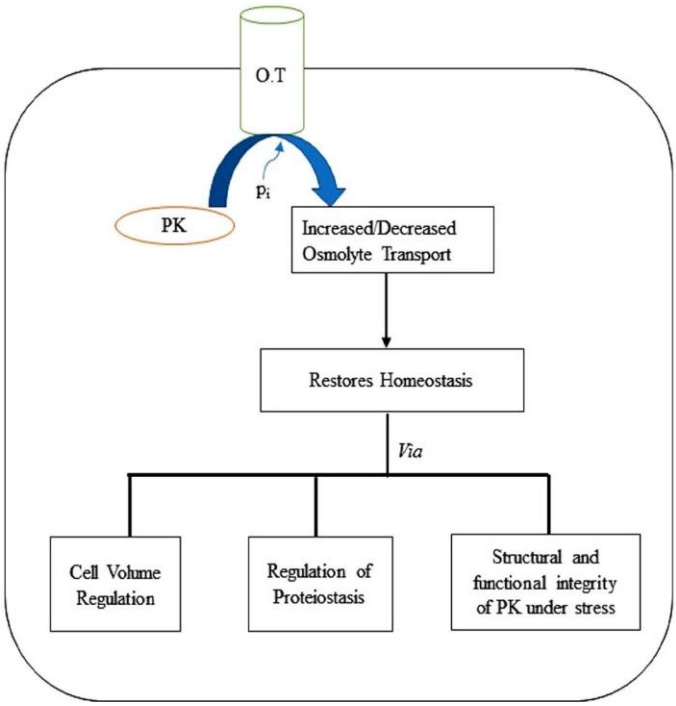

Figure 3. Interplay between protein kinases and osmolyte accumulation for cell volume homeostasis.

during refolding (138). Altogether, results observed do suggest that the complex interaction between osmolytes and protein kinases at least under *in vitro* conditions, might potentiate the ability of cells to control cell volume homeostasis by controlling protein stability, aggregation and refolding during stress conditions.

7. CONCLUSION

Among the different ways of osmolyte accumulation, osmolyte transporter mediated accumulation is preferred during long term stress regulation probably due to the fact that coupled transport mechanisms for accumulation of osmolytes is potent enough to generate more than 500-fold concentration gradient across the plasma membrane. It is quite evident that hypertonic stress induces osmolyte transport genes for rapid uptake of osmolytes through transporters. Accumulation of osmolytes through transporters is regulated at translational level by protein kinases due to the presence of evolutionary conserved protein kinase phosphorylation sites in osmolyte transporters. It seems that nature, throughout the evolutionary period, has somehow conserved these sequences for regulation of osmolyte accumulation by kinases in highly evolved organisms like humans. An interplay between protein kinases and osmolyte accumulation for cell volume homeostasis and proteiostasis signifies the importance of protein kinase mediated regulation of osmolyte accumulation (Figure 3). For instance, sustained accumulation of taurine potentiates the cells ability to maintain cell volume homeostasis by regulating protein kinases as well as proteiostasis machinery (59). More importantly, the ability of osmolytes to regulate protein kinases, at least under *in vitro* conditions, infers that these osmolytes might be maintaining the structural and functional integrity of protein kinases in cells under *in vivo* conditions.

8. FUTURE PERSPECTIVES

Strong correlation of protein kinase expression and transporter mediated osmolyte accumulation suggested that targeting osmolyte

transporters via protein kinase activators and inhibitors may regulate the accumulation of osmolytes under several diseased conditions like cancer, neurodegenerative etc. For instance, accumulation of inositol in prostate cancer cells can be prevented by targeting the transporters like SMIT through protein kinase activators which in turn suppress inositol accumulation and hence reduce the metastatic risk of prostate cancer. Moreover, CK2 inhibition has the potential to sensitize cancer cells for apoptosis by enhancing the protein kinase mediated taurine uptake into these cells. More studies need to be carried out in this direction. Additionally, *in vivo* systematic investigations regarding the ability of osmolytes to regulate structural and functional integrity of protein kinases are highly warranted.

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Abbreviations: TonEBP, Tonicity enhancer binding protein; TonE, Tonicity enhancer; RER, Rough endoplasmic reticulum; PKC, Protein kinase C; PKA, Protein kinase A; JAK2, Janus kinase 2; SMIT, Sodium myoinositol transporter; TauT, Taurine transporter; BGT1, Betaine transporter; RPE, Retinal pigment epithelium; HMIT, H^+ Myoinositol transporter; NF-kB, Nuclear factor kappa-light-chain-enhancer of activated B cells; TMAO, trimethylamine -N-oxide; CK, creatine kinase; AK, arginine kinase, TM, transmembrane; CK2, casein kinase 2; MDCK, Madin-Darby canine kidney; PDZ, post synaptic density protein(PSD95), drosophila disc large tumor suppressor(Dlg1) and zonula occludens-1 protein(Zo-1)

Key Words: Osmolyte, Protein kinase, Cell volume regulation, Osmolyte transporter, Cancer, Phosphorylation, betaine transporter, taurine transporter, myoinositol transporter

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