MINIREVIEW

Osmoregulation of Neutral Amino Acid Transport (43917A)

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Abstract. **Neutral amino acids are important organic osmolytes found in most osmotically stressed cells. During prolonged hypertonic stress, accumulation of neutral amino acids contributes to the cell volume recovery and may protect cellular proteins against salt denaturation. Hypertonicity activates system A transport, a major neutral amino acid transporter in mammalian cells, by a microtubule-dependent mechanism. Similar to the regulation of amino acid efflux induced by hypotonic-swelling, influx via system A may be under the control of intracellular ionic strength. An osmotically sensitive repressor may negatively regulate the gene expression of a regulatory protein which activates the preexisting system A transporter protein. Hypertonically activated protein kinases may be involved in this osmoregulation of amino acid transport.** [P.S.E.B.M. 1995, Vol 210]

Living cells are under a constant struggle against volume perturbation because substrates accumulated by metabolism or transport tend to produce cell swelling. Polarized epithelial cells, which are designed for transcellular transport, must use volume regulatory mechanisms to prevent any potential imbalance between apical and basolateral membrane transport of solutes and ions. Cells normally living in an anisotonic environment, such as the inner medulla of the kidney, depend on volume regulatory mechanisms for their survival.

In the past decade, a great variety of volume regulatory mechanisms has been discovered in many types of mammalian cells. In response to hypotonic swelling cells can activate a regulatory volume decrease (RVD) mechanism to return the cell volume close to normal (1). On the other hand, hypertonic shrinking leads to a marked increase in intracellular K^+ concentrations and activation of a regulatory volume increase (RVI) mechanism. RVI in many eukaryotic cells is due to the activation of $Na^+/K^+/2Cl^-$ cotransporters or Na^+/H^+ and Cl^-/HCO_3^- exchangers (1). During RVI, the resultant influx of electrolytes, together with the elevated intracellular K^+, increases the intracellular ionic strength, which may lead to denaturation of the cellular proteins. As the stress is prolonged, cells begin to accumulate organic osmolytes to balance the extracellular osmotic pressure. This allows the intracellular ionic concentration to be decreased (2). Free neutral amino acids, polyhydric alcohols, and methylamines are the three types of organic osmolyte systems found in most osmotically stressed organisms (3–5). Unlike ions, these neutral organic osmolytes can be accumulated without adverse effects on intracellular proteins, substrates, or cofactors (3, 4).

Recently, it has been found that in many cells hypertonic stress activates system A transport, a metabolically important transport system for many neutral amino acids. In this review, we summarize these re-

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cent studies on the osmoregulation of amino acid transport and focus on the neutral amino acids which are commonly found in proteins. The role of other organic osmolytes, such as taurine and betaine, and their specific transport systems has been extensively studied and reviewed elsewhere (2, 6).

Nonmetabolic Functions of Intracellular Amino Acids

Given the central role of protein synthesis in cellular function, great emphasis has been put on understanding the metabolic reactions of amino acids. However, other nonmetabolic functions for amino acids are now recognized, and the following are some examples. Through amino acid-protein interactions, glutamate, aspartate, and glycine function as neurotransmitters to open the ligand-gated ion channels in the brain. Specific gene transcription may be controlled by the availability of amino acids, for example the tryptophan-activated repressor that regulates transcription of the *trp* operon in *Escherichia coli* (7). Protein degradation in liver cells may be regulated by the cell volume changes induced by amino acid flux (8).

In vitro studies showed that neutral amino acids such as proline, alanine, and serine can stabilize lysozyme structure against thermal denaturation (9). Glycine-based osmolytes also appear to have a marked protective role for proteins against thermal unfolding (10). This suggests that neutral amino acids may be involved in stress responses *in vivo*. Some indirect evidence in support of this are the findings that system A transport, a major neutral amino acid transport system, may be coordinately regulated with heat shock proteins in the mutants of Chinese hamster ovary (CHO) cells (11) and may be activated by hypertonic stress in many cells (see next section). Accumulation of organic osmolytes attenuates the expression of heat-shock proteins in response to hypertonic stress and heat-shock stress (12). A study in fibroblasts showed that inhibition of the proliferation rate by hypertonicity was more marked in transformed SV3T3 cells compared with the normal counterpart 3T3 cells. This may be partly due to the impaired ability of SV3T3 cells to increase their intracellular pool of amino acids and to activate the volume regulatory response (13). Degradation of long-lived proteins can be regulated by amino acid availability, which may be another indication of the possible role of amino acids in stress responses (8, 14). However, whether amino acid deprivation would result in the denaturation of cellular proteins and activate the expression of heat shock proteins has not been directly explored.

There are only a few studies on the possible mechanism of the compatable role and/or stabilizing effect of neutral amino acids on proteins. Alanine, proline, serine, and glycine, as well as other neutral osmolytes, usually increase the surface tension of water and exclude themselves from the water-protein interface. This exclusion is a thermodynamically unfavorable process, which will require the area of the water-protein interface to be minimized. This will stabilize the proteins in their native globular state because this conformation has a smaller surface area than the denatured state (9). On the other hand, some organic osmolytes such as betaine do not change surface tension but have a large partial specific volume that excludes them from the internal domain of a protein (15). This may create an osmotic force by which the components of the protein macromolecule may be pushed together and prevented from unfolding (16). Finally, it cannot be ruled out that there may be some interactions between organic osmolytes and ions, which might protect proteins by decreasing the ionic activity in aqueous solutions (3).

Osmoregulation of Intracellular Amino Acid Content

The free amino acid concentration inside cells may be controlled by influx, metabolism, and efflux. Influx of amino acids into mammalian cells occurs via seven major amino acid transport systems and some minor systems (17). Na^+-dependent transport of neutral amino acids via system A is a growth-regulated amino acid transport system present in many cells (18). Amino acid transport into cells is one of the essential steps for cellular protein metabolism and may be a rate-limiting step (19). For example, hepatic utilization of several amino acids for gluconeogenesis may be limited by their rate of influx (20). This indicates that metabolic control can occur at the plasma membrane level. As discussed in the previous section, amino acids may also be involved in the osmotic stress response, and it is not surprising that part of this response involves plasma membrane transport systems. Amino acids such as taurine and betaine are concentrated into renal cells via specific transport systems which are induced by hypertonic challenge (21, 22). Prolonged hypertonicity also activates system A transport (23–25), a relatively nonspecific transporter, and increases the intracellular concentration of many neutral amino acids (23, 24). This increase in the accumulation of amino acids may be due to the activation of Na^+-dependent system A transport. A preliminary study with MDCK cells showed that the increase in intracellular ninhydrin positive substances after hypertonic challenge was abolished when excess α-(methylamino)isobutyric acid (MeAIB) was included in the hypertonic incubation medium. MeAIB is a synthetic non-metabolic substrate that is transported only by system A. This finding suggests that the increase in ninhydrin positive substances is due, at least in part, to influx via system A transport (Chen J-G, Kempson

SA, unpublished). The intracellular accumulation of MeAIB and neutral amino acids was also increased in cultured fibroblasts during 3 hr of hypertonic stress (26). An additional role for system A during hypertonic challenge may include influx of betaine (27, 28), another major organic osmolyte. Although only system A is activated by hypertonicity (23, 24), the substrates accumulated by system A may rapidly exchange with other amino acids (29) so that many amino acids, in addition to system A substrates, can be accumulated during hypertonicity (24).

Metabolic changes induced by cell volume change may also contribute to regulation of the free amino acid content. Studies in hepatocytes indicated that cell volume may be a trigger for metabolic control of cell function (8). Hypotonic swelling led to an increase in anabolic metabolism, which would decrease intracellular free amino acids. In contrast, hypertonic shrinking induced catabolism, which would increase free amino acids (8). Studies in rat renal papillary slices indicated that under hypertonic conditions amino acids contribute to cell volume maintenance mainly by metabolic accumulation and by decrease in efflux (30, 31). Efflux of organic osmolytes is a rapid process compared with the accumulation by influx and may be mediated by a volume sensitive anion channel (1, 32, 33). Under hypotonic conditions mammalian cells will undergo rapid swelling followed by RVD. In cultured chick heart cells (34) and MDCK cells (32) the RVD is mediated in part by an increased efflux of amino acids including taurine, glutamate, glycine, and alanine. Amino acid efflux also contributes significantly to cell volume regulation in astrolytes exposed to hypotonicity (35). In trout erythrocytes, volume regulatory amino acid efflux was directly related to cytoplasmic ionic strength (36). There was significant loss of amino acids during isotonic swelling induced by addition of urea but not NH_4Cl. The former will decrease ionic strength inside the cells, and the latter will increase it due to chloride entry (36). This suggests that amino acid content may be controlled in part by intracellular ionic strength.

Mechanism for the Hypertonic Activation of System A

The system A transporter protein has not been identified, but its regulation has been extensively studied (18, 37). In numerous cell types it has been shown that transport of neutral amino acids via system A can be induced by incubation of cells in amino acid free medium. This starvation-induced activation (derepression) of system A may involve synthesis of new transporters. Reversal of this activation occurs upon addition of certain repressive amino acids to the growth medium. Part of this downregulation process may require gene expression and protein synthesis. Based on these adaptive studies in hepatocytes (38, 39) and in CHO mutants (40, 41), two molecular models have been put forth. Both proposed that the adaptation involves the regulation of system A gene expression (39, 40). The model of Englesberg *et al.* suggests that A system amino acids bind to an intracellular inactive repressor and convert it to an active repressor that prevents the expression of the system A gene (Fig. 1). In addition, the active repressor inactivates the existing system A transporter (40).

The hypertonic activation of system A transport involves an increase in the V_{max} without a change in K_m (23, 24). Studies on the renal NBL-1 cells and mutants of CHO cells indicated that hypertonic upregulation was additive to the derepression, and depended on the preexisting activity of system A (42, 43). Hypertonic treatment of human fibroblasts initially caused cell hyperpolarization and so increased the driving force for system A transport. Subsequently, the activation of system A was blocked by cycloheximide indicating a mechanism dependent on new protein synthesis (26). The synthesis-dependent activation of system A by hypertonicity in NBL-1 cells was insensitive to tunicamycin (42, 43), an inhibitor of protein glycosylation. This contrasts to derepression which induces the synthesis of glycoproteins. It was suggested that there were separate mechanisms for the activation of system A by hypertonicity and starvation

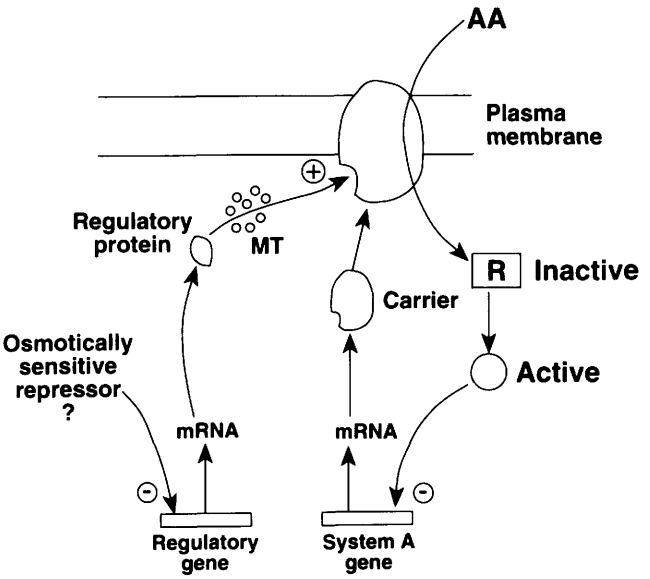

Figure 1. Model for regulation of system A amino acid transport. According to this model, modified from Ref. 42, osmoregulation of system A is regulated by a mechanism that is different from starvation-induced activation. In the absence of extracellular amino acids (AA), system A repressor (R) remains inactive and cannot downregulate the expression of system A gene (39, 41). During hypertonic stress the increase in intracellular ionic strength will denature the osmotically sensitive repressor which will relieve inhibition of expression of the regulatory gene. This will result in increased expression of the regulatory protein which, in turn, activates pre-existing system A transporters by a microtubule (MT)-dependent mechanism.

(derepression). The former may involve a regulatory protein coded by an osmotically regulated gene. When either renal epithelial MDCK cells or vascular smooth muscle A10 cells were returned to isotonic medium after hypertonic activation, the recovery (downregulation) of system A required new protein synthesis (44). This suggests indirectly that the regulatory protein may be negatively controlled by a short-lived repressor protein, although this repressor may be different from the one involved in the adaptive response to starvation (Fig. 1).

Hypertonic challenge will induce a decrease in cell volume and increase the intracellular ionic concentration (45). It has been found that hypertonic stress resulted in the activation of heat shock proteins (46), which suggests that the cellular proteins may be denatured by the increase in the intracellular ionic strength. It is conceivable that the repressor of the regulatory gene for system A may be sensitive to and denatured by the increased ionic strength (Fig. 1). In A10 cells and mouse embryo fibroblasts (23, 47), system A can be activated by ouabain treatment, which will increase the intracellular Na^+ concentration by inhibiting the Na^+/K^+-ATPase. These studies suggest that the signal for the hypertonic upregulation of system A may be the increase in the intracellular Na^+ concentration or the total ionic strength. The latter possibility is due to the fact that the Na^+/K^+-ATPase pumps $3Na^+$ out and $2K^+$ in and inhibition by ouabain would lead to more Na^+ accumulation than K^+ loss. Denaturation of an osmotically sensitive repressor may be one possible mechanism by which an increased concentration of intracellular ions may activate amino acid influx via system A (Fig. 1). Increased ionic strength was proposed previously as a signal that triggers the hypertonic activation of aldose reductase synthesis in renal epithelial cells (48). Given the additional observation of increased amino acid efflux in response to decreased intracellular ionic strength in trout erythrocytes (see previous section), we can speculate that intracellular ionic strength may be one of the determinants of intracellular amino acid content.

A volume decrease was induced in chick embryo fibroblasts when extracellular sodium chloride was replaced with isosmotic disaccharide. The reason for the volume change is unclear. However, this maneuver also decreased the intracellular Na^+ and the total intracellular ionic concentration, and led to activation of system A (49). This indicates that macromolecular crowding and confinement, instead of ionic strength, may be important for the system A activation in this cell. Molecular crowding can result in dramatic increases in the activity of the individual macromolecules, suggesting that system A may be under positive control by an activator. This will be different from negative control by an osmotically sensitive repressor (45, Fig. 1). These controversial studies suggest there may be heterogeneous mechanisms for the activation of system A for different cells. It is difficult to resolve whether crowding or ionic strength is the determinant for the hypertonic activation because hypertonicity will induce both changes.

Possible Role of Microtubules and Protein Kinases for the Osmoregulation of System A

Microtubules are required for the intracellular protein transport processes such as endocytosis (50), transcytosis (51), and exocytosis (52). Hypertonic activation of system A can be abolished by colcemid, which is an inhibitor of microtubules (43). It has been suggested (42) that the hypertonicity-activated regulatory protein for system A may be a cytoskeletal protein or that its effect requires the integrity of the microtubule network (Fig. 1). System A upregulation in many cells required intact microtubules. For example, upregulation of system A transport in skeletal muscle in response to insulin or adaptation involves synthesis of new carriers and requires the integrity of microtubule function (53). Colchicine has also been found to prevent the increased capacity for system A transport in the proliferating hepatocytes during liver regeneration (54). Although it is generally accepted that microtubules may guide intracellular trafficking of the proposed regulatory protein or the transporter itself to the plasma membrane (42, Fig. 1), it is not necessarily true for system A. For example, microtubules may be involved in some way in degradation of the system A repressor during osmotic stress. In a related study, the alkalinization of lysosomes and resultant inhibition of proteolysis, induced by hypotonic swelling, was inhibited by microtubule disruption (55). In the case of a hypertonic challenge, if the repressor was not degraded when microtubules were disrupted then system A would not be activated.

In A10 cells, the effect of colchicine on the hypertonic activation of system A transport was diminished because microtubule disruption by itself, in serum free medium, activates system A transport (56). The activation of system A by microtubule disruption required new protein synthesis and was not additive with the stimulation of system A by addition of serum (56). It has been suggested that a protein kinase may be associated with microtubules (57, 58), and microtubule disruption may activate DNA synthesis (59) and specific gene expression (60). It is possible that a similar process may account for activation of system A by microtubule disruption in A10 cells.

There are many examples of the regulation of system A amino acid transport by hormones and growth factors (18, 37). In renal epithelial LLC-PK1 cells, system A was activated in two phases by incubation with an activator of protein kinase C (61). The initial phase

of activation involved phosphorylation and the later phase required synthesis of glycoproteins, presumably transporters. It has been speculated that there may be a protein kinase that is involved in the acute volume regulatory response. This kinase stimulates shrinking-activated pathways, such as Na^+/H^+ exchange and $Na^+/K^+/2Cl^-$ cotransport, which mediate the acute response to hypertonic challenge (62). It is possible that protein kinases activated by hypertonicity may also be involved in the osmoregulation of system A transport during chronic hypertonic stress, although the prolonged osmoregulation may require gene expression and protein synthesis instead of protein phosphorylation as in the acute response. One effect of hypertonicity in MDCK cells was activation of a mitogen-activated protein (MAP) kinase and its activator by a mechanism dependent on protein kinase C (63). Recently, a newly identified MAP kinase was found to be activated by hypertonicity (64, 65). Its expression in yeast restored the capacity of mutant cells for growth in hypertonic environment (64, 65). It would be interesting to know if a similar MAP kinase is involved in the adaptive regulation of system A transport by prolonged osmotic stress.

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