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Osmotic Regulation of ATA2 mRNA Expression and Amino Acid Transport System A Activity

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When porcine endothelial cells were exposed to hypertonicity, both the level of ATA2 (amino acid transporter 2) mRNA and activity of amino acid transport System A increased transiently, peaking after about 6 and 9 h, respectively. Cycloheximide, like actinomycin D, prevented both responses, showing that an earlier step also involves protein synthesis. Withdrawal of hypertonicity after 6 h increased the rate of down regulation. These findings confirm that ATA2 is a major isoform of System A and show that changes in the expression of ATA2 mRNA precede both the induction and subsequent down regulation of transport activity.

Key Words: **ATA2 mRNA; System A; amino acid transport; hyperosmolarity; endothelial cell.**

The amino acid transport known as "System A" was discovered several decades ago (1), soon shown to be widespread, and since then has been studied extensively. Its susceptibility to various different kinds of regulation has also been known for a long time (2), but the underlying mechanisms involved have remained as elusive as the mechanism of the transport process itself. One form of regulation is the transient increase of the activity of System A in cells exposed to hypertonicity, which has been demonstrated to occur in a similar manner in a variety of different cells (3–13). Inhibitors of transcription or translation prevent this increase of activity in response to hypertonicity, but exactly which message (or messages) and protein (or proteins) are involved is unresolved. For example, experiments with NBL-1 cells were interpreted in terms of an osmotically-sensitive gene coding for a protein that activates system A, with no change in the number of the transporters (14), whereas studies with MDCK cells led to the conclusion that "up regulation of System A" was "likely due to an increase in transporter number" (10). The latter study also concluded that the subsequent down regulation of System A activity might be caused by "an intracellular factor produced during prolonged hypertonic stress."

The main reason for this slow and uncertain progress has undoubtedly been the difficulty of identifying and characterising the transporter at the molecular level, because this meant that no useful probes, such as antibodies or cDNA, have been available for the appropriate experimental study of regulation. Now, however, this unsatisfactory situation has been changed by the recent cloning of at least three different transporters with some properties characteristic of the "classical" System A (15–21), which obviously must be considered as a group of similar transporters. One of these, designated ATA2 (16, 20) or SAT2 (19) has been described as "an amino acid transporter with functional characteristics and tissue expression pattern identical to that of System A" (16). We have used its cDNA as a probe to investigate the induction of System A activity in some porcine endothelial cells during their exposure to hypertonicity. The results presented below serve to confirm the identity of ATA2 as a major isoform of System A, and reveal that changes in the cellular content of ATA2 mRNA are associated with both the induction and the down regulation of the transporter's activity.

MATERIALS AND METHODS

Materials. A plasmid containing the human ATA2 cDNA was kindly provided by Dr. Vadivel Ganapathy (Department of Biochemistry and Molecular Biology, Medical College of Georgia, GA) and a probe for 28S rRNA was obtained from Dr. Lorenza Tacchini (University of Milan, Italy). Endothelial cells, obtained and cultured from pig pulmonary arteries, and all other materials were exactly as

Abbreviations used: ActD, actinomycin D; ATA, amino acid transporter; ChX, cycloheximide; MeAIB, *N*-methylaminoisobutyric acid.

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described in detail previously (13). The osmolality of normal growth medium was about 0.3 osmol/kg. Hypertonic growth medium contained an additional 200 mM sucrose, giving a final osmolality of about 0.5 osmol/kg.

Methods. Total RNA was extracted from cultured cells using the Ultraspec RNA Isolation System from Biotecx (22). RNA samples (30 μg) were transferred to nylon filters. The quality and quantity of RNA blotted on the membranes were checked by ultraviolet absorption. Human ATA2 cDNA (20) and 28S rRNA were nick-translated (Amersham kit) with [α-^{32}P]dCTP (3000 Ci/mmol). Hybridization, washing, and autoradiography were carried out as described previously (23). The rate of uptake of MeAIB (used as the characteristic substrate of amino acid transport System A) by endothelial cells was measured exactly as explained previously (13), after the cells had been incubated in isotonic or hypertonic medium for the desired time.

RESULTS

The usual stimulation of System A activity in response to hypertonicity (Fig. 1A) was preceded by the detection of ATA2 mRNA in the cells (Figs. 1B and 1C). The amount of the mRNA was already high after 3 h of hypertonic incubation, when induced transport activity was only just detectable, and it had decreased markedly by 9 h, when transport activity peaked. Thereafter the transport activity also declined quite quickly. ATA2 mRNA has since been detected in cells exposed to hypertonicity for only 0.5 h, before a significant increase of amino acid transport could be measured (results not shown). This pattern is clearly consistent with the mRNA being a precursor to the induced activity of System A.

The effects of inhibitors of transcription (actinomycin D) and translation (cycloheximide) on these cellular responses to hypertonicity are illustrated in Fig. 2. Each inhibitor abolished the increase not only of System A activity (Fig. 2A) but also of the amount of ATA2 mRNA (Figs. 2B and 2C). Hence, although transcription is necessary for the induction of System A activity, and the latter is accompanied by an increase in the amount of ATA2 mRNA, the inhibition by cycloheximide shown in Fig. 2B indicates that the translation of some protein other than the transporter itself must be necessary first.

The data in Fig. 3 show that withdrawal of hypertonicity after 6 h caused a rapid loss of ATA2 mRNA, quickly followed by a loss of the induced transport activity of System A. The ATA2 mRNA decayed with a half-life ($t_{1/2}$) of about 0.5 h (compared with about 3 h under hypertonic conditions), and System A activity decreased with $t_{1/2} \approx 2.5$ h (compared with 6–7 h under hypertonic conditions).

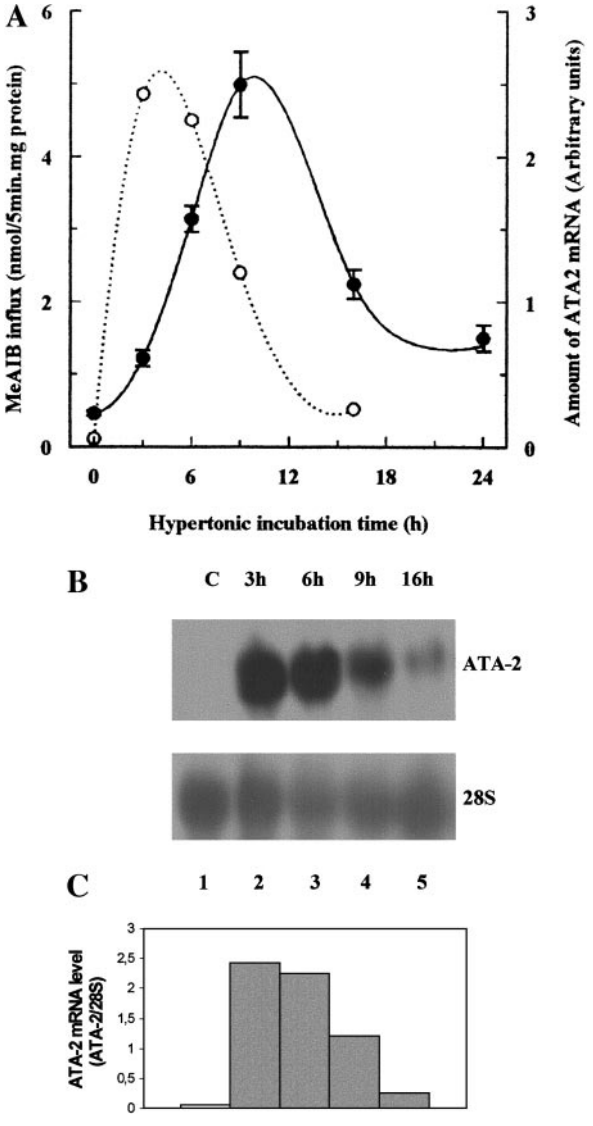

FIG. 1. Time course of the effect of hypertonicity on MeAIB uptake and the expression of ATA2 mRNA. Endothelial cells were incubated for the indicated times in hypertonic (0.5 osmol/kg) medium, sucrose being used as the extra osmolyte. Control cells were incubated in isotonic (0.3 osmol/kg) medium. (A) Solid symbols: initial rates of uptake of MeAIB by endothelial cells were measured as described in the text. Values are the means (± SEM) of 6 measurements. Open symbols: values of the ratio ([ATA2 mRNA]/[28S rRNA]) from C. (B) Total cellular RNA was extracted and analysed for ATA2 mRNA by Northern blotting as described in the text, with 28S rRNA used for standardisation. C, control cells. (C) Densitometric quantification of the blots in B, normalised to the level of 28S rRNA.

DISCUSSION

The results presented above show that hypertonic stimulation of System A activity is preceded by an increase in the cellular content of ATA2 mRNA, which suggests that increased transcription of the gene underlies the increase in transport activity. Since cycloheximide prevents this increase in ATA2 mRNA, however, it in turn must depend upon, and be preceded by, the production or increased production of at least one protein. The latter could be (i) a component of any part

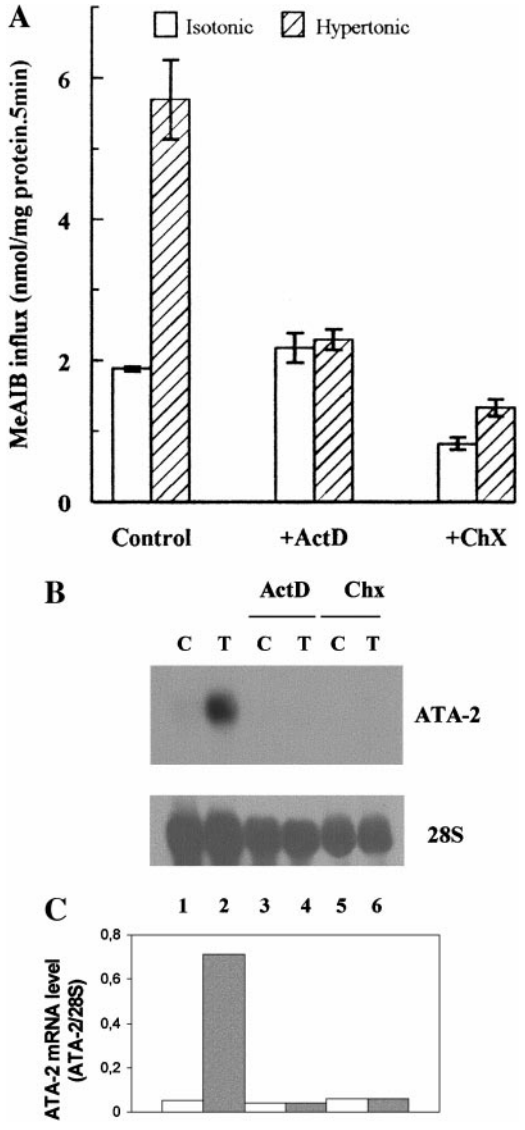

FIG. 2. Inhibition of responses to hypertonicity by actinomycin D and cycloheximide. Endothelial cells were incubated for 6 h in either isotonic (0.3 osmol/kg) or hypertonic (0.5 osmol/kg) medium, in the absence or presence of 800 nM actinomycin D (ActD) or 35 μM cycloheximide (ChX). (A) Initial rates of uptake of MeAIB by endothelial cells were then measured as described in the text. Open bars, isotonic medium; shaded bars, hypertonic medium. Values are the means (± SEM) of 4 measurements. (B) Total cellular RNA was extracted and analysed for ATA2 mRNA by Northern blotting as described in the text, with 28S rRNA used for standardisation. C, control cells incubated in isotonic medium. T, test cells incubated in hypertonic medium. (C) Densitometric quantification of the blots in B, normalised to the level of 28S rRNA.

of the cellular pathway from the hypertonic signal to the transcription process; (ii) a transcription factor necessary for transcription of the ATA2 gene; (iii) a derepressor of that transcription; or (iv) an inhibitor of the normal degradation of ATA2 mRNA. The transcription of the protein's own gene might also be necessary.

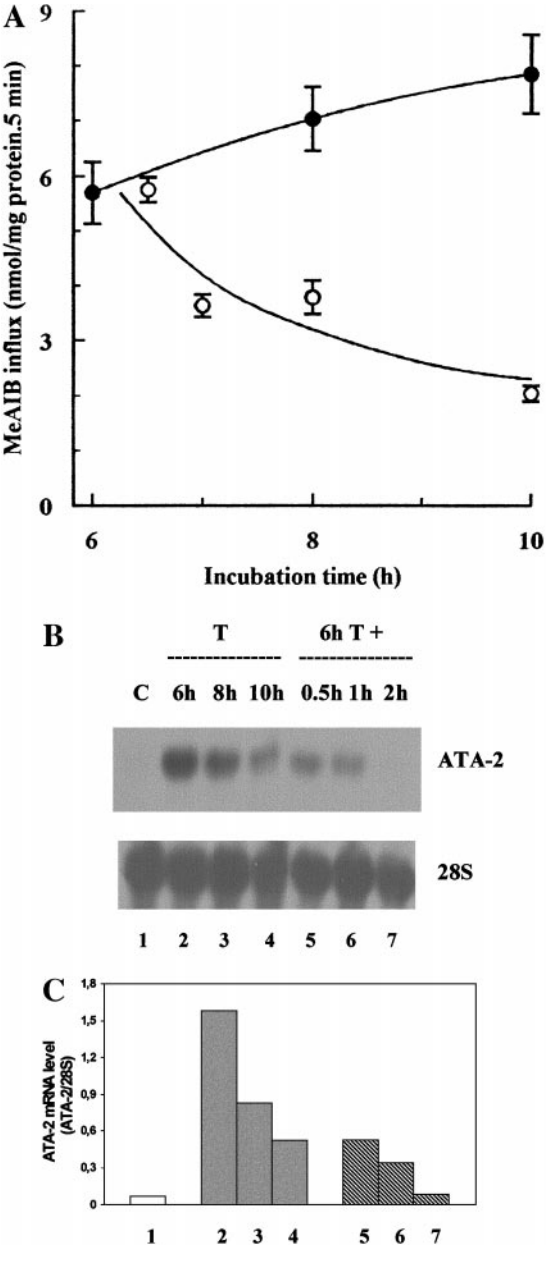

FIG. 3. Effects of withdrawal of hypertonicity. Samples from one set of endothelial cells were incubated for 6, 8, or 10 h in hypertonic (0.5 osmol/kg) medium. Samples from another set were incubated identically for the first for 6 h but were then rinsed and incubated in isotonic (0.3 osmol/kg) medium for a further 0.5 to 4 h. (A) Initial rates of uptake of MeAIB by the endothelial cells were measured as described in the text. Solid symbols: samples from hypertonic conditions throughout. Open symbols: samples from cells transferred to isotonic conditions a after initial 6 h. Values are the means (± SEM) of 4 measurements. (B) Total cellular RNA was extracted and analysed for ATA2 mRNA by Northern blotting as described in the text, with 28S rRNA used for standardisation. C (lane 1), control cells incubated in isotonic medium. T (lanes 2–4), test cells, incubated in hypertonic medium. T^+ (lanes 5–7), cells transferred to isotonic medium after 6 h. (C) Densitometric quantification of the blots in B, normalised to the level of 28S rRNA.

Although it is impossible to distinguish among these various different explanations at present, each would fit with the interpretation that hypertonic stimulation of System A activity results from an increased synthesis of the transporter, not from an increase in the catalytic activity of the transporters already in the plasma membrane. Hence our findings do not agree with the suggested explanation of the similar hypertonic stimulation of System A activity in NBL-1 cells (14).

The subsequent down-regulation of System A activity, whether under conditions of prolonged hypertonicity or its withdrawal, is similarly preceded by a decrease in the cellular content of ATA2 mRNA. This suggests that a decrease in the net synthesis of ATA2 is responsible for the down-regulation of transport activity, rather than an inhibition of transporters already present in the plasma membrane. Hence the transporters themselves, or at least the newly synthesised ones, must turn over quite rapidly. If this situation proves to be the same in MDCK cells, which in every other respect behave almost identically to these endothelial cells under hypertonic conditions, it will not support Kempson's suggestion of down regulation being caused by an intracellular inhibitor of System A activity produced during prolonged hypertonic stress (10). On the other hand, it would support his alternative interpretation that the persistence of System A activity in membrane vesicles isolated from the cells might have been due to contamination by cytoplasmic membranes containing System A, formed by endocytosis of the plasma membrane as part of the normal turnover of the transporter (10).

Whatever suppresses the initial stimulation of transcription under prolonged hypertonic conditions is obviously less effective than the complete removal of the hypertonic signal, because the rate of down regulation was considerably increased when hypertonicity was withdrawn after the 6 h (Fig. 3). This observation is similar to that made, over a longer time period, in the study of the betaine/GABA transporter, BGT1 (13). Hence it's likely that the mechanisms of both the induction of ATA2 activity and its subsequent down regulation are similar to those for the induction and down regulation of BGT1.

Looked at another way, these findings provide confirmation that ATA2 is indeed a major isoform of the virtually ubiquitous amino acid transport System A. ATA2 was first cloned from rat skeletal muscle (16) and our results with porcine endothelial cells have been obtained with the use of human ATA2 cDNA as a probe (20), so there is no indication of either species or tissue specificity. In contrast, ATA3 (21) has a very limited tissue distribution and ATA1 (15, 18) does not exhibit all the characteristics of the classically defined System A.

It is interesting to note that partial hepatectomy causes a similar increase in System A activity that is not accompanied by an increase in expression of ATA2 mRNA, indicating that, unless another system A isoform is involved, post-transcriptional regulation mediates this response in regenerating liver (24).

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REFERENCES

1. Oxender, D. L., and Christensen, H. N. (1963) Distinct mediating systems for the transport of neutral amino acids by the Ehrlich cell. *J. Biol. Chem.* **238,** 3686–3699.
2. Guidotti, G. G., Borghetti, A. F., and Gazzola, G. C. (1978) The regulation of amino acid transport in animal cells. *Biochim. Biophys. Acta* **515,** 329–366.
3. Tramacere, M., Petronini, P. G., Severini, A., and Borghetti, A. F. (1984) Osmoregulation of amino acid transport activity in cultured fibroblasts. *Exp. Cell Res.* **151,** 70–79.
4. Petronini, P. G., Tramacere, M., Mazzini, A., Piedimonte, G., Silvotti, L., and Borghetti, A. F. (1987) Hyperosmolarity-induced stress proteins in chick embryo fibroblasts. *Exp. Cell Res.* **172,** 450–462.
5. Chen, J. G., Klus, L. R., Steenbergen, D. K., and Kempson, S. A. (1994) Hypertonic upregulation of amino acid transport system A in vascular smooth muscle cells. *Am. J. Physiol.* **267,** C529–C536.
6. Dall'Asta, V., Rossi, P. A., Bussolati, O., and Gazzola, G. C. (1994) Response of human fibroblasts to hypertonic stress. *J. Biol. Chem.* **269,** 10485–10491.
7. Petronini, P. G., De Angelis, E., Borghetti, A. F., and Wheeler, K. P. (1994) Osmotically inducible uptake of betaine via amino acid transport system A in SV-3T3 cells. *Biochem. J.* **300,** 45–50.
8. Ruiz-Montasell, B., Gomez-Angelats, M., Casado, F. L., Felipe, A., McGivan, J. D., and Pastor-Anglada, M. (1994) Evidence for a regulatory protein involved in the increased activity of system A for neutral amino acid transport in osmotically stressed cells. *Proc. Natl. Acad. Sci. USA* **91,** 9569–9573.
9. Kempson, S. A., Hoshaw, J. M., Hinesley, R. S., and McAteer, J. A. (1997) Hyperosmotic stress up-regulates amino acid transport in vascular endothelial cells. *Kidney Int.* **52,** 1332–1339.
10. Kempson, S. A. (1998) Differential activation of system A and betaine/GABA transport in MDCK cell membranes by hypertonic stress. *Biochim. Biophys. Acta* **1372,** 117–123.
11. Dall'Asta, V., Bussolati, O., Sala, R., Parolari, A., Alamanni, F., Biglioli, P., and Gazzola, G. C. (1999) Amino acids are compatible osmolytes for volume recovery after hypertonic shrinkage in vascular endothelial cells. *Am. J. Physiol.* **276,** C865–C872.
12. De Angelis, E., Petronini, P. G., Borghetti, P., Borghetti, A. F., and Wheeler, K. P. (1999) Induction of betaine-γ-aminobutyric acid transport activity in porcine chondrocytes exposed to hypertonicity. *J. Physiol. (Lond.)* **518,** 187–194.
13. Petronini, P. G., Alfieri, R. R., Losio, M. N., Caccamo, A. E., Cavazzoni, A., Bonelli, M. A., Borghetti, A. F., and Wheeler, K. P. (2000) Induction of BGT-1 and amino acid System A transport

activities in endothelial cells exposed to hyperosmolarity. *Am. J. Physiol.* **279,** R1580–R1589.

14. McGivan, J. D., and Pastor-Anglada, M. (1994) Regulatory and molecular aspects of mammalian amino acid transport. *Biochem. J.* **299,** 321–334.
15. Varoqui, H., Zhu, H., Yao, D., Ming, H., and Erickson, J. D. (2000) Cloning and functional identification of a neuronal glutamine transporter. *J. Biol. Chem.* **275,** 4049–4054.
16. Sugawara, M., Nakanishi, T., Fei, Y-J., Huang, W., Ganapathy, M. E., Leibach, F. H., and Ganapathy, V. (2000) Cloning of an amino acid transporter with functional characteristics and tissue expression pattern identical to that of system A. *J. Biol. Chem.* **275,** 16473–16477.
17. Reimer, R. J., Chaudhry, F. A., Gray, A. T., and Edwards, R. H. (2000) Amino acid transport System A resembles System N in sequence but differs in mechanism. *Proc. Natl. Acad. Sci. USA* **97,** 7715–7720.
18. Wang, H. P., Huang, W., Sugawara, M., Devoe, L. D., Leibach, F. H., Prasad, P. D., and Ganapathy, V. (2000) Cloning and functional expression of ATA1, a subtype of amino acid transporter A, from human placenta. *Biochem. Biophys. Res. Commun.* **273,** 1175–1179.
19. Yao, D., Mackenzie, B., Ming, H., Varoqui, H., Zhu, H., Hediger, M. A., and Erickson, J. D. (2000) A novel System A isoform mediating Na^+/neutral amino acid cotransport. *J. Biol. Chem.* **275,** 22790–22797.
20. Hatanaka, T., Huang, W., Wang, H. R., Sugawara, M., Prasad, P. D., Leibach, F. H., and Ganapathy, V. (2000) Primary structure, functional characteristics and tissue expression pattern of human ATA2, a subtype of amino acid transport system A. *Biochim. Biophys. Acta* **1467,** 1–6.
21. Sugawara, M., Nakanishi, T., Fei, Y-J., Martindale, R. G., Ganapathy, M. E., Leibach, F. H., and Ganapathy, V. (2000) Structure and functions of ATA3, a new subtype of amino acid transport system A, primarily expressed in the liver and skeletal muscle. *Biochim. Biophys. Acta* **1509,** 7–13.
22. Chomczynski, P., and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162,** 156–159.
23. Petronini, P. G., Alfieri, R. R., De Angelis, E., Campanini, C., Borghetti, A. F., and Wheeler, K. P. (1993) Different HSP70 expression and cell survival during adaptive responses of 3T3 and transformed 3T3 cells to osmotic stress. *Br. J. Cancer* **67,** 493–499.
24. Freeman, T. L., and Mailliard, M. E. (2000) Postranscriptional regulation of ATA2 transport during liver regeneration. *Biochem. Biophys. Res. Commun.* **278,** 729–732.