Amino Acids (2001) 21: 151–160

Amino
Acids

Regulation of taurine transport in murine macrophages

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Accepted December 6, 2000

Summary. We studied the regulation of taurine transport in ANA1 murine macrophage cell line. Taurine uptake was upregulated by hypertonicity and downregulated by bacterial lypopolysaccharide (LPS) and other stimuli leading to macrophage activation. However combined stimulation with LPS plus hypertonic shock evoked an increase of taurine uptake that was even higher than with hypertonic shock alone. Taurine transport was not modified by LPS in GG2EE macrophages derived from C3H/Hej mouse strain, which harbour a mutated Toll-like receptor 4 (TLR4) and thus are not activated by LPS. The extracellular signal-regulated kinase (ERK) inhibitor PD98059 abrogates the effect of both LPS and hyperosmotic shock on ANA1 taurine uptake, while the p38 inhibitor SB203580 reduces the taurine uptake in control conditions and impairs only the response to hypertonicity. These results suggest that the effect of LPS on taurine transport depends on ERK pathway and can be influenced by environmental conditions.

Keywords: Amino acids – Cell volume regulation – Hypertonic shock – Bacterial lypolysaccharide – Organic osmolytes – Endotoxin resistance

Introduction

Organic osmolytes such as taurine, betaine, and myo-inositol are used by various cells to regulate their own volume (Kwon and Handler, 1995). Indeed cell exposure to hyperosmotic or hypoosmotic extracellular solutions results in cell shrinkage or swelling, respectively, which is counteracted by accumulation or release of organic osmolytes. The uptake of such compounds is carried out by specific Na^+-dependent transporters, which are upregulated at the transcriptional level following a hyperosmotic shock (Kwon and Handler, 1995). This phenomenon is critical in renal medullary cells which face drastic changes of interstitial osmolarity during transition from diuresis to antidiuresis and viceversa. Osmolyte transport is not only essential as a protective mechanism against dramatic osmotic challenges, but it may be also important to finely control cell volume in isosmotic conditions. In this regard

it is worth to note that several indications suggest that cell volume changes are a signal capable to modify cell functions such as protein turnover, metabolism, cell cycle control and signal transduction through mitogen-activated protein kinases (Bussolati et al., 1996; Häussinger, 1996). It has been recently shown that betaine, taurine and myo-inositol are actively accumulated in rodent macrophages and that hyperosmolarity increases the expression of the corresponding transporters (Warskulat et al., 1995, 1997a; Zhang et al., 1996b; Warskulat, 1997b). These organic osmolytes have a modulatory role in cell-volume sensitive processes such as phagocitosis and lypopolisaccharide (LPS)-induced release of proinflammatory mediators, tumor necrosis factor-α (TNF-α), eicosanoids and nitric oxide (NO) (Seabra, 1998; Warskulat, 1996; Zhang, 1996a; Warskulat, 1998). However it is not known if osmolyte transport is in turn regulated by activating stimuli. Therefore we investigated the consequences of macrophage activation on taurine uptake.

We have found that activation of the macrophagic cell line ANA1 (Blasi et al., 1987) in isotonic conditions leads to taurine uptake inhibition. This phenomenon seems to be mediated by activation of the MEK/ERK pathway.

Methods

Cell culture

ANA-1, GG2EE, and INF3A cells were cultured in suspension at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) Fetal Clone II serum (HyClone), 2 mM L-Glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cell density was routinely mantained between $0.75 \cdot 10^6$ cells/ml and $4 \cdot 10^6$ cell/ml.

Taurine uptake

To ensure that at the time of experiments cells were in the exponential growth phase, their density was adjusted to $0.75 \cdot 10^6$/ml 24 hours before. Cells were then centrifuged and resuspended at a density of $2 \cdot 10^6$/ml in a physiological saline solution (PSS) containing (in mM): 130 NaCl, 2 KCl, 1 KH_2PO_4, 2 $CaCl_2$, 2 $MgCl_2$, 10 glucose, 10 Na-Hepes (pH 7.3). When required, this solution was made hypertonic by addition of raffinose.

Uptake started by mixing one ml of cell suspension with one ml of a loading solution consisting in iso- or hypertonic PSS with twice the desired concentration of cold and radioactive taurine. Final radioactivity was 0.2 μCi/ml. All solutions were kept at 37°C. Taurine uptake was stopped at the desired time by placing 1 ml of the loading medium with cells in eppendorf tubes containing 200 μl dibutylphtalate and by rapidly spinning the tubes for 20 seconds in a centrifuge. After discarding the aqueous phase, the eppendorf tube was washed gently with 1 ml of PSS to remove the residual radioactivity. The dibutylphtalate was then discarded and the cell pellet was lysed overnight with 200 μl of NaOH 0.25 N.

The radioactivity contained in the cells, measured by liquid scintillation was used to calculate the amount of incorporated taurine.

Nitrite determination

The concentration of nitrite, a stable product of nitric oxide, in the medium of cell cultures was determined with the Griess reaction (Green et al., 1982).

Inhibitors

Cells were treated with SB203580 (Calbiochem) 20 μM, or PD98059 (Calbiochem) 10 μM for 24 hours. Incubation with inhibitors was simultaneous with LPS or hypertonicity treatments.

Statistics

Data are presented as mean ± SEM. Statistical significance was assessed by Student's t-test for unpaired data.

Results

ANA-1 cells were exposed at different taurine concentrations, and the amount of taurine uptake was determined at 2.5, 5, 10, and 20 minutes. The time-dependence of taurine accumulation was linear up to 10 minutes for concentrations as high as 50 μM. Accordingly, the uptake at five minutes was taken to calculate the kinetic parameters of taurine transport. The dependence of the uptake on taurine concentration could be fitted with a Michaelis-Menten equation. Data obtained from eight indipendent experiments in isosmotic medium gave a mean K_m = 15.3 ± 1.5 μM and a V_{max} of 1.43 ± 0.15 nmol/10^6 cells (Fig. 1A, solid circles). The Na^+-dependence of taurine transport was assessed by using a solution containing N-methyl-D-glucamine as the main cation. Under Na^+-free conditions, taurine uptake was inhibited by 97.7 ± 0.6% (n = 3, not shown).

Cell preincubation for 24 hours with the 400 mOsm/kg medium determined an increase of taurine accumulation (Fig. 1A). This treatment caused a significant increase of the V_{max} to 2.55 ± 0.31 nmol/10^6 cells ($p < 0.01$; n = 3) whereas the K_m was not statistically different (13.1 ± 0.8 μM).

LPS was used to determine whether taurine transport is influenced by macrophage activation. When ANA-1 cells were stimulated with LPS 1 μg/ml for 24 hours taurine uptake showed a marked decrease in V_{max} (from 1.43 ± 0.15 to 0.52 ± 0.05 nmol/10^6 cells, $p < 0.01$; n = 3) but not in K_m (16.3 ± 2.5 versus 15.3 ± 1.5 μM for treated and untreated cells, respectively; Fig. 1A). Since hypertonicity and LPS have opposite effects on taurine transporter activity, we applied both stimuli to assess the net result. After treating for 24 hours ANA-1 cells with LPS in hypertonic medium, we observed that taurine uptake was even higher than that elicited by hypertonicity alone (3.96 ± 0.47 nmol/10^6 cells) (Fig. 1B).

Inhibition of taurine uptake was also obtained by treatment with other macrophage activators as IFN-γ in combination with picolinic acid (0.59 ± 0.09 nmol/10^6 cells) or LPS (0.75 ± 0.02 nmol/10^6 cells), but not with IFNγ alone (1.28 ± 0.20 nmol/10^6 cells) (Fig. 2). Cell activation was assessed measuring NO release in culture medium.

It has been demonstrated that LPS-induced activation requires involvement of TLR4, a recently cloned transmembrane protein (Poltorak et al., 1998), member of the IL-1/Toll receptor family. To assess whether LPS

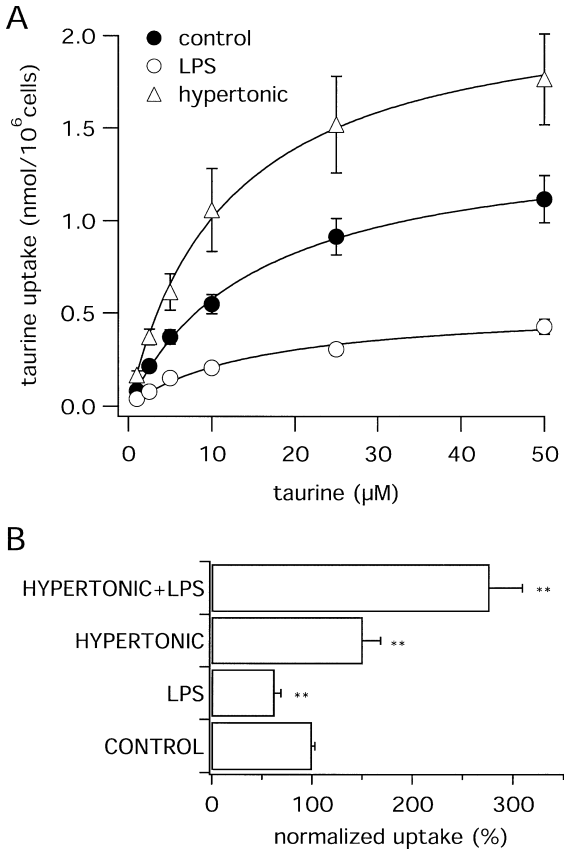

Fig. 1. Taurine uptake is stimulated in hypertonic conditions and inhibited by LPS. **A** The graphics depict the kinetics of taurine uptake in ANA-1 cells held in isotonic medium (filled circles) or in a medium made hypertonic to 400 mOsm/kg with raffinose (open triangles) or upon stimulation for 24 hours with 1 μg/ml LPS (open circles). Each point is the mean of 3–8 experiments. Data are fitted with Michaelis-Menten equation. **B** Mean values of 5 minutes taurine uptake in the presence of 25 μM taurine in control conditions, in LPS, in hypertonic medium, and in LPS plus hypertonic medium. Asterisks indicate a significant difference ($p < 0.01$) from control. Treatment with LPS or hypertonic medium reduced the cell number to about 50% of controls at 24 h. Cell viability was instead not modified

effect on taurine transporter is mediated by TLR4 we performed uptake experiments on GG2EE cells (Blasi et al., 1988) deriving from the C3H/HeJ mouse strain, which harbours a missense mutation in TLR4 and is therefore LPS unresponsive (Sultzer et al., 1993; Tominaga et al., 1997). In GG2EE cells, taurine transport was lower than in ANA1 (0.21 ± 0.019 nmol/10^6 cells), but unaffected by LPS (Fig. 2). However, stimulation with IFNγ resulted in a significant inhibition (0.13 ± 0.01 nmol/10^6 cells) (Fig. 2). With the aim of controlling whether this result is a consequence of the mouse strain from which these cells were obtained, we also studied INF3A cells, which have the same genetic background as GG2EE, but their receptor is not mutated. LPS decreased taurine uptake in INF3A cells to the same extent found in ANA-1, from 0.24 ± 0.013 to 0.114 ± 0.01 nmol/10^6 cells (Fig. 2).

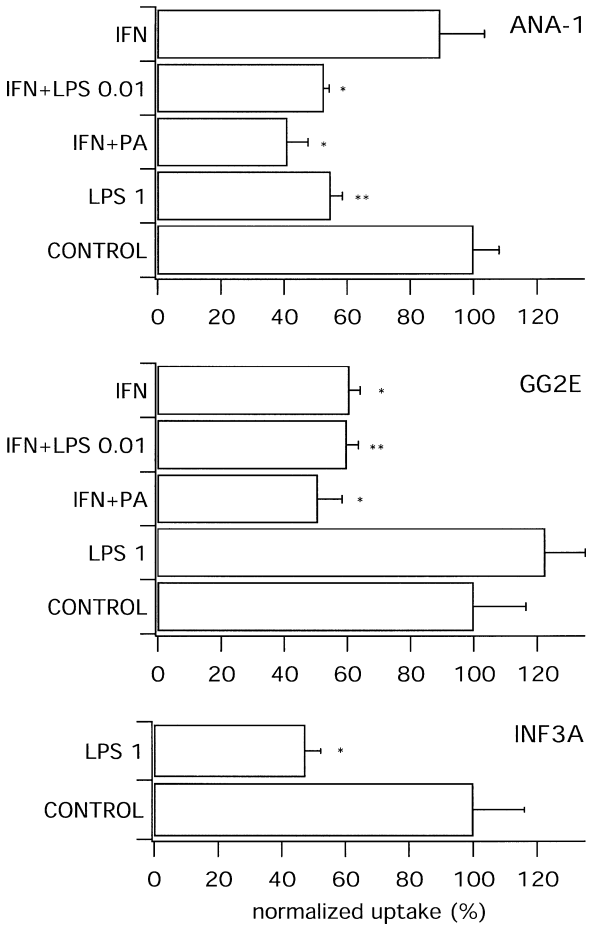

Fig. 2. Taurine uptake in activated macrophages. Cells were treated for 24 hours with different combinations of LPS (1 and 0.01 μg/ml), interferon-γ (IFN, 100 U/ml), and picolinic acid (PA, 4 mM). Data for each cell line (ANA-1, GG2EE, INF3A) are normalized to the uptake measured in resting conditions. Each bar is the mean of 4–19 experiments. Asterisks indicate a significant inhibition of taurine uptake with respect to unstimulated cells (*, $p < 0.05$; **, $p < 0.01$)

We performed a series of experiments to try to understand the mechanism through which the different stimuli affect taurine transport. One of the main murine macrophage responses to LPS is the NO synthesis, due to enhanced expression of the inducible form of nitric oxide synthase (iNOS). As expected, LPS stimulation evoked the appearance of nitrite in the culture medium of ANA-1 cells (26.3 $\pm$ 2.2 μM, n = 3). L-NAME, an inhibitor of NOS, at the concentration of 100 μM, inhibited the nitrite production by 76% (6.2 $\pm$ 2.0 μM, n = 3) but did not alter the LPS-dependent taurine uptake (not shown). Another mediator produced by activated macrophages is TNFα. However, treatment of ANA1 and INF3A with TNFα (10 ng/ml) for 24 hours did not change taurine uptake (not shown).

To try to identify the pathway involved in taurine transport modulation in ANA-1 cells we used two potent inhibitors, namely PD98059 and SB203580,

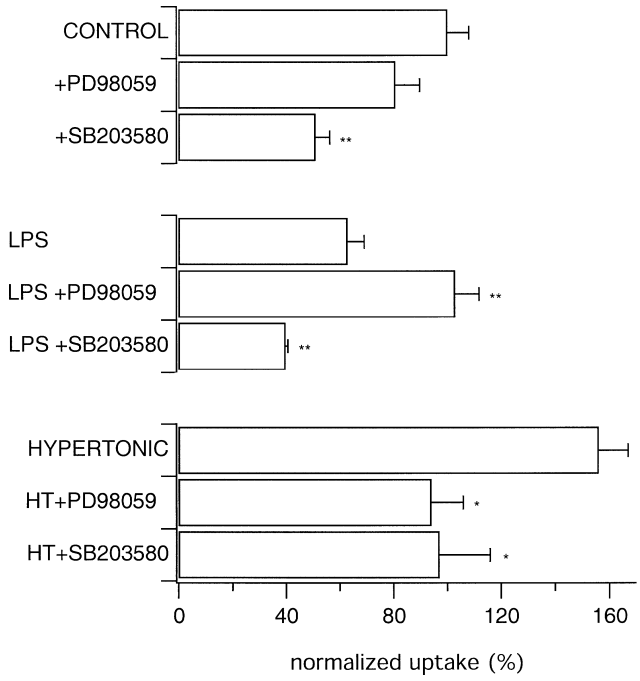

Fig. 3. Effect of PD98059 and SB203580 on taurine uptake. Control cells and LPS (1 μg/ml) or hypertonic treated cells (400 mOsm/Kg) were incubated for 24 hours with 10 μM PD98059 or 20 μM SB203580. Data are normalized to the uptake measured in resting conditions. Each bar is the mean of 4–5 experiments. Asterisks indicate a significant difference of taurine uptake with respect to unstimulated (*, $p < 0.05$; **, $p < 0.01$). Treatment with PD98059 for 24 h did not modify the number of cells nor viability. SB203580 instead reduced cell number to about 50% of controls and reduced viability. Values displayed above were obtained considering only viable cells

which block ERK and p38 kinases respectively. PD98059 10 μM alone did not significally affect taurine transport in unstimulated cells while it completely antagonised LPS effect (from 0.20 ± 0.02 to 0.35 ± 0.03 nmol/10^6 cells). Indeed taurine uptake in presence of LPS and PD98059 was essentialy the same as in untreated cells. Interestingly, PD98059 also contrasted the stimulatory effect of hypertonic shock (from 0.53 ± 0.037 to 0.32 ± 0.04 nmol/10^6 cells) (Fig. 3). SB203580 20 μM in presence of LPS do not restore taurine uptake to control values, but on the contrary taurine uptake was further reduced (from 0.20 ± 0.02 to 0.135 ± 0.003 nmol/10^6 cells). SB203580 was able to decrease taurine uptake by 50% also in control conditions. The uptake in hypertonic condition was also decreased by SB203580 from 0.53 ± 0.037 to 0.33 ± 0.064 nmol/10^6 cells (Fig. 3).

Discussion

Previous data indicate that taurine, betaine and myo-inositol behave as organic osmolytes in rodent macrophages (Warskulat et al., 1995, 1997a; Zhang

et al., 1996b). They are accumulated in cells by a Na^+-dependent mechanism and transporter activity is increased by cell preincubation with an hypertonic medium. We focused our interest on taurine transport. Taurine uptake experiments on ANA1 show that taurine transporter is dependent on extracellular Na^+ and has apparent K_m of about 15μM in both isotonic and hypertonic conditions. This relatively high affinity is comparable to that described for taurine transport in other cells (Ramamoorthy et al., 1994; Uchida et al., 1992). A comparison with previous studies on rodent macrophages is not possible since kinetic studies were not performed. Taurine uptake was upregulated by 24 hours incubation with the 400 mOsm/kg culture medium, upregulation seems to result from a higher number of active transporters as suggested by the V_{max} increase.

We asked whether taurine transport is influenced by macrophage activation. Cell incubation with LPS, which is a potent stimulus for ANA-1 cells, reduced taurine uptake by almost 50%. A similar effect was also obtained by treatment with interferon-γ in combination with picolinic acid or LPS. Considering that LPS and IFNγ activate two different signal transduction cascades the correlation between activation and taurine uptake in ANA1 and GG2EE suggests that downregulation of taurine transport is a consequence of activation of the macrophage independently from the type of activating stimulus.

The mechanism of taurine transport downregulation is unknown; we hipothezised that some mediator released from the activated cells could act in an autocrine way on taurine transporter. Two molecules were considered, namely NO and TNFα which have been reported to modulate activity of Na-dependent transporters. Indeed, it has been recently shown that NO regulates GABA transporters (Cupello et al., 1997) and that TNFα downregulates myo-inositol transport in different cell lines (Yorek et al., 1998a, 1998b). We found that ANA-1 cells produce NO upon cell activation, and that L-NAME, an inhibitor of the NO synthase, dramatically reduced NO synthesis. However, L-NAME was unable to contrast the downregulation of taurine uptake. Similarly, TNFα did not modified the taurine uptake induced by cell activation. These results rule out the involvement of NO and TNFα in taurine transport regulation.

Cell stimulation with LPS and hypertonic stress have both been linked to the activation of several kinases cascades. LPS induces activation of ERK 1/2 (Kirakis and Avruch, 1996), p38 (Weinstein et al., 1992), JNK (Sanghera et al., 1996) and IL1 signaling molecules (Zhang et al., 1999), which are part of different pathways; they all activate NFkB that is required for release of proinflammatory cytokines. ERK 1/2 (Itoh et al., 1994), p38 (Han et al., 1994), JNK (Moriguchi et al., 1995) are also involved in signal transduction evoked by hypertonic stress, but differences has been observed between cell lines and also using different osmotic agents (Duzgun et al., 2000). Indeed p38 is involved in myo-inositol and betaine transporter upregulation in human monocyte and macrophages (Denkert et al., 1998), while in murine macrophages RAW 274.6 taurine transport appears independent of the ERK pathway and p38 (Warskulat et al., 1998). Our results indicate that LPS effect on

taurine transport in ANA-1 cells is mediated by the interaction of LPS with TLR4, as indicated by the lack of response on TLR4 defective macrophages. Moreover, experiments performed with kinase inhibitors pointed to the involvement of MEK/ERK pathway. These data are consistent with recent evidences of ERK involvement in TLR4 signal transduction pathway (Yang et al., 2000). Hypertonicity instead seems to affect the integration of at least two classes of MAP kinases, the MEK/ERK and the p38. This last pathway seems also implicated in taurine uptake in control conditions.

In conclusion, our results suggest the existence of different regulatory signals affecting the activity of the taurine transporter in macrophages. A downregulation is observed in isotonic conditions upon activating the cells with LPS or other stimuli. The physiological meaning of this inhibition, with the consequent decrease of intracellular taurine concentration is unclear. Besides being an osmolyte, taurine has other functions as evident by its capacity to protect from oxidative stress (Raschke et al., 1995; Schuller-Levis et al., 1995; Trachtman et al., 1994), to modulate intracellular Ca^{2+} concentration (Bkaily et al., 1997), and to affect K^+ channel activity (Han et al., 1996). It has been shown in Kupffer cells that taurine reduces the LPS induced production of TNF-α (Seabra et al., 1998) and cox2 (cycloxigenase 2) protein levels (Warskulat et al., 1997a). Therefore, given its inhibitory effects the reduction of taurine uptake could be instrumental to the process of macrophage activation. Under hypertonic conditions, at which the osmoprotective role of taurine is probably more important, LPS instead activates taurine transport. It is possible therefore to speculate that a switch controls the direction of LPS effect, but the complexity of the signalling pathways makes it very difficult, at the actual state of knowledge, to identify it. The macrophage response to LPS in hypertonic conditions cannot be explained in a mechanistic way but underlines the importance of non receptorial environmental signals in modulating receptor-mediated responses. Indeed, the interaction between environmental and receptor-mediated signals has also been observed in hypoxia and interferon-γ stimulated macrophages (Mellilo et al., 1996).

Acknowledgements

This work was supported with grants from Ministero della Sanità (98.S12.C3).

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Received September 1, 2000