Trimethylamine oxide decreases stress induction of Hsp70 and organic acid transport by isolated choroid plexus of the dogfish shark, *Squalus acanthias*

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Denatured or abnormal cellular proteins (misfolded) initiate activation of the heat shock genes[1]. The increased production of heat shock proteins (Hsps) is thus associated with a variety of cellular stresses such as hyperthermia and exposure to heavy metals and can increase tolerance of subsequent additional stress. This pre-conditioning may pre-dispose the cell or organism to survive an otherwise lethal stress. Hsps are protein chaperones and as such stabilize protein structure. Many so-called osmolytes are cytoprotectants, acting to stabilize tertiary protein structure, and certain animal groups, such as marine elasmobranchs, maintain extraordinarily high concentrations of these stabilizers. Dogfish sharks utilize very high concentrations of urea (300-400 mM) to remain slightly hyperosmotic to seawater. Trimethylamine oxide (TMAO, 70 mM) serves to counteract the detrimental effects of urea on protein chemistry[4]. We hypothesized that the high levels of cytoprotectant supplant the role of Hsps and alter or dampen the functional response to stress. We had previously demonstrated active transport of the organic anion, 2,4-dichlorophenoxyacetic acid (2,4-D), by choroid plexus isolated from dogfish shark[3]. Transepithelial transport can be dramatically altered by mildly denaturing stresses[2]. In the present study, we utilized isolated dogfish choroid plexus to investigate the effects TMAO on stress-induced modulation of active transepithelial transport of 2,4-D.

Isolated choroid plexus was heat shocked by 1-hour incubation at 23.5°C, a temperature that exceeded the normothermic condition (13.5°C) by 10°C; the holding tank temperature varied from 10°C–14°C. Freshly isolated IVth plexus was divided in two roughly equal halves, and each half-plexus was placed in a 35 mm Petri dish with sterile elasmobranch Ringer (ER, in mM: 280 NaCl, 6 KCl, 4 $CaCl_2$, 3 $MgCl_2$, 1 NaH_2PO_4, 0.5 Na_2SO_4, 350 urea, 72 TMAO, 2.5 glucose and 8 $NaHCO_3$, pH 7.8). One half-plexus was then rinsed and incubated in sterile Leibovitz's (L-15) medium modified for elasmobranch tissues (L-15E) by supplementation with 142 mM $NaCl_2$, 2.75 mM $CaCl_2$, 2 mM $MgCl_2$, 350 mM urea, and 72 mM TMAO; the corresponding half-plexus was incubated in TMAO-free L-15E medium in which TMAO was replaced isosmotically with urea. Tissues were heat shocked at 23.5°C by water submersion for 1 h then incubated at 13.5°C (humidified air) for an additional 1.5 h, non-heated tissues were held at 13.5°C for 3.5 h. After treatment, each half-plexus was mounted in an Ussing chamber filled with ER (gassed with humidified 99% O_2/1% CO_2) and attached to a voltage clamp for measurement of 2,4-D transport and bioelectrical properties; chamber temperature was maintained at 13-13.5°C. Unidirectional absorptive (CSF-to-blood) flux of 10 μM ^{14}C-2,4-D was determined under short-circuit conditions. After 1 h mediated transport was completely blocked with a combination of 100 μM 2,4,5-trichlorophenoxyacetic acid and 10 mM PAH. The remaining flux was considered passive leak. For non-heated tissues, removal of TMAO from incubation medium had no effect 2,4-D transport. However, whereas the active flux of 2,4-D in tissues heat shocked without TMAO was comparable to transport in non-heated tissues, in tissues heat-shocked with TMAO, transport was markedly impaired. Immunoblot analysis indicated that diminution of transport by heat shock in the presence of TMAO coincided with attenuated induction of Hsp70. Thermal stress induced accumulation of Hsp70, regardless of the presence of TMAO; however, heat-induced accumulation of Hsp70 (normalized to actin) in TMAO-free tissues was 2-fold greater than that in tissues heat shocked

with TMAO present. Semi-quantitative RT-PCR showed that in the non-heat stressed condition, the relative level of Hsp70 mRNA in TMAO-free tissue was nearly twice that in tissue incubated with TMAO (n=3).

TMAO also altered the effect of zinc on organic anion transport and HSP70 induction. Segments of isolated choroid plexus were incubated for 6 h (13.5°C) without or with 50 μM $ZnSO_4$ in L-15E containing TMAO or TMAO-free L-15E; all tissues were then incubated for additional 1.5 h 'recovery' in fresh zinc-free L-15E with TMAO. In the zinc-free condition, 2,4-D transport in TMAO-free tissues was no different than transport in tissues incubated with TMAO. Zinc exposure in the presence of TMAO did not alter active transport. However, in the absence of TMAO, zinc exposure significantly enhanced transport of 2,4-D. Irrespective of the presence of TMAO, zinc treatment induced slight (40%) Hsp70 accumulation, but the increase in Hsp70 was roughly 250% higher in the absence of TMAO. Zinc-induced Hsp70 mRNA expression was similarly altered by TMAO. In zinc-free conditions, the relative level of hsp70 mRNA in the absence of TMAO was roughly 50% greater than that in tissue incubated with TMAO. Zinc induced moderate increases in Hsp70 mRNA in both the presence and absence of TMAO. Nevertheless, levels of Hsp70 mRNA in tissues treated with zinc without TMAO were 70% greater than those in tissues treated with zinc in the presence of TMAO (n = 2).

These data indicate that physical and chemical stressors may directly modulate transepithelial absorption of organic anions across the elasmobranch CSF-blood barrier and concurrently alter Hsp70 expression. Moreover, the nature of the observed modulation of transport in dogfish choroid plexus was dependent on the presence of TMAO, and furthermore, paralleled the tissue's capacity to up-regulate Hsp70. While TMAO may counteract the adverse affects of urea on protein biochemistry in elasmobranch tissue under otherwise non-stressful conditions, the osmolyte may dampen the cellular stress response, *e.g.*, induction of Hsp70, following perturbations of the ambient or internal milieu. Furthermore, as observed here, a potential consequence of this impaired up-regulation of Hsp70 in the stressed choroid plexus epithelium was diminished integrated tissue function, transepithelial transport of organic anions.

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