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Biochimica et Biophysica Acta 1193 (1994) 323–329

BBA Biochimica et Biophysica Acta

Increased lipid fluidity in synaptosomes from brains of hyperosmolal rats

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Received 16 March 1994

Abstract

Taurine, a product of sulfur amino acid metabolism, is important in cerebral osmoregulation. To understand the adaptive changes in transport which accompany different hyperosmolal states, we determined lipid composition and fluorescence anisotropy of synaptosomal liposomes from rats with chronic hypernatremic dehydration (CHD), streptozocin-induced (STZ) diabetes, and insulin treated diabetes. Induction of CHD increased serum osmolality, and enhanced in vitro synaptosomal taurine uptake ($P < 0.01$, $n = 3$, vs. control). Fluorescence anisotropy studies showed that the fluidity of lipids from CHD synaptosomes was higher than control ($P < 0.05$, $n = 3$). STZ-diabetes resulted in hyperglycemia, increased serum osmolality, and stimulated synaptosomal taurine uptake ($P < 0.01$, $n = 3$, vs. control). Insulin treatment of diabetic rats restored serum osmolality and taurine transport to control values. The fluidity of diabetic rat brain synaptosomal lipids was significantly higher than control ($P < 0.05$, $n = 3$); fluidity was normalized by insulin administration to diabetic rats. Total fatty acid, cholesterol, and cholesterol/phospholipid molar ratio of CHD, STZ, and insulin treated diabetic rats were similar to control. However, the ratio of saturated to unsaturated fatty acids was decreased in hyperosmolal states. This suggests that adaptive increases in cerebral taurine transport during hyperosmolality may result from a direct effect on membrane composition that alters fluidity and permits enhanced transmembrane flux of osmoprotective molecules.

Key words: Synaptosome; Taurine transport; Hyperosmolality; Fluorescence anisotropy; Lipid fluidity

1. Introduction

Taurine (2-aminoethane sulfonic acid), long considered an inert end-product of sulfur amino acid metabolism, has been shown to be important in bile salt synthesis [1], cell proliferation [2], control of glycolysis and glycogenesis [3], modulation of cellular calcium flux [4], and cerebral osmoregulation [5,6]. Taurine is an essential amino acid in certain species, such as the cat due to limited activity of the enzyme cysteine-sulfinate decarboxylase [7]. In humans, adults are capable of synthesizing more taurine than infants, due to higher hepatic levels of cysteine-sulfinate decarboxylase; therefore taurine is considered a conditionally essential amino acid [8,9].

The concentration of taurine as a free amino acid is highest within brain cells, and it is unevenly distributed within the central nervous system [7]. Studies have shown that amino acids regulate cerebral cell volume during osmotic stress by functioning as organic osmolytes [5]. Taurine is chief among these, accounting for more than half of this solute pool. Taurine is ideally suited for this function because it is not incorporated into structural proteins.

We have recently shown in isolated rat brain synaptosomes that hyperosmolal states result in increased cerebral transmembrane transport of taurine, which is mediated by alterations of the β-amino acid carrier system [10,11]. This adaptive response serves to increase the cytoplasmic pool of this osmoprotective solute, reducing the plasma to cell osmolal gradient, and limiting cellular dehydration. In order to further understand this adaptive change which occurs during different hyperosmolal states, we determined the lipid composition and fluorescence anisotropy (reciprocal of

Abbreviations: CHD, chronic hypernatremic dehydration; STZ, streptozocin; DPH, 1,6-diphenyl-1,3,5-hexatriene.

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SSDI 0005-2736(94)00114-5

fluidity) of cerebral liposomes isolated from diabetic rats, insulin treated normoglycemic diabetic rats, and rats with chronic hypernatremic dehydration.

2. Materials and Methods

2.1. Animals

Male Sprague-Dawley rats (Taconic Farms, Germantown, NY) 200–400 g were used in all experiments. Animals were housed in a facility which was maintained at 25°C, with a 12-h light/dark cycle, were fed standard rodent chow (Ralston Purina, St. Louis, MO) containing 22.8% protein, and were given free access to water.

2.2. Experimental conditions

Chronic Hypernatremic Dehydration (CHD) was induced for 48 h according to previously established techniques [6]. Rats were deprived of water for 24 h prior to intraperitoneal injections of 1 M NaCl. The volume of hypertonic saline was calculated to raise serum Na^+ to 180 mmol/l over the following 24 h. Rats were provided with food, but no water during the 48 h period of hypernatremic dehydration. Control rats, which had free access to water and received sham injections (1 ml of normal saline), were studied in parallel with experimental animals.

Experimental animals were made diabetic, as previously described [11], by injection of a single dose of streptozocin (STZ, 60 mg/kg) infused via the tail vein. Control animals received an injection of the buffered citrate STZ diluent. To prevent acute hypoglycemia after STZ administration, experimental animals received a 10% glucose solution for 48 h. Hyperglycemia was documented 48 h after induction by STZ injection. Rats were then allowed to remain hyperglycemic for an additional 5 days before being used for preparation of synaptosomes. A group of STZ-diabetic rats was made normoglycemic by B.I.D. injections (4–6 U/day) of ultra-long acting insulin for 5 days.

After induction of either diabetes or CHD, rats were decapitated and a free-flowing, non-hemolyzed blood sample obtained for determination of serum glucose, Na^+, creatinine and taurine concentrations. The rat brains were excised and placed in ice-cold isolation media containing 320 mM sucrose, 10 mM Tris-HCl and 1 mM K^+-EDTA (pH 7.4) for preparation of synaptosomes.

2.3. Isolation of synaptosomes

Synaptosomes were prepared and isolated according to the method of Fraser et al. [12]. Brains from one or two rats (2–3 g) were minced, and washed three times to remove blood and extraneous tissue fragments, brought up to a volume of 15 ml with isolation media, and homogenized 15 strokes in a glass Dounce homogenizer (Wheaton, Millville, NJ). Cellular debris was removed by centrifugation ($1300 \times g$, 3 min). The supernatant was respun ($18\,000 \times g$, 10 min) to obtain the crude synaptosomal pellet. This pellet was resuspended in isolation media by re-homogenization, and layered onto a discontinuous Ficoll gradient composed of 11 and 7.5% layers. The gradients were then spun at $100\,000 \times g$ for 70 min and synaptosomes were isolated from the interface between the two Ficoll layers. Synaptosomes were aspirated from this area, washed in isolation media, and spun at $17\,000 \times$ g for 10 min. Purified synpatosomes were resuspended in isolation media to result in a protein concentration of ~5 mg/ml. All synaptosome preparations were stored at −70°C, and used within 3 months of preparation.

2.4. Synaptosomal taurine uptake

Taurine uptake by synaptosomes was measured in the presence of an inwardly directed 140 mM NaCl gradient, following a 15 min incubation in media containing 100 μM taurine, and trace (5 μCi) [^{3}H]taurine, according to previously published methods [6,10,11]. The synaptosomal suspension was vacuum filtered through a 0.45-μm pore size cellulose acetate membrane, and the retained radioactivity determined.

2.5. Lipid analysis

Total synaptosomal lipid composition was determined in synaptosomal lipids extracted as previously described, in which recovery was greater than 95% as determined by the co-elution of radioactively labeled lipid standards [13]. Briefly, the cell pellet (approx. 50 mg) was homogenized in 2 ml of CH_3OH, followed by addition of 2 ml of $CHCl_3$. The mixture was then centrifuged, and the pellet was reextracted with 2 ml of $CHCl_3/CH_3OH/HCl$ (100:100:1, v/v). The combined extracts were adjusted to a $CHCl_3/CH_3OH$ ratio of 2:1, and partitioned with 0.2 vol. of 0.1 M HCl. The lower phase was collected, and the upper phase was reextracted with $CHCl_3/CH_3OH$ (85:15, v/v). The combined lower phases were washed with 3 ml of 0.1 M HCl/CH_3OH (1:1, v/v) and centrifuged. The upper phase was removed, and the lower phase was washed with 3 ml of CH_3OH/H_2O (1:1, v/v). The lipid extracts were dried under nitrogen and then redissolved in the appropriate solvent for subsequent analysis.

2.6. Fatty acid analysis

Fatty acid composition of synaptosomal lipids was determined by gas-liquid chromatography (GLC), as

previously described [13]. Synaptosomal lipids were hydrolyzed and methylated with 1 ml of methanolic BF_3 (≈ 50 μg lipid/ml) at 75°C for 15 min. Fatty acid methyl esters were partitioned by addition of 2 ml of hexane and 1 ml of H_2O. This mixture was vortexed and centrifuged, and the upper layer containing the fatty acid methyl esters was removed and evaporated under N_2. Samples were redissolved in 25 μl of $CHCl_3$-containing methylheptadecanoic acid (17:0) as an internal standard. GLC analysis was performed by injection of 2 μl of sample utilizing a Shimadzu GC-9A dual-column gas chromatograph (Shimadzu, Columbia, MD) equipped with a flame ionization detector. Separations were performed with a 1.7 M column (5 mm o.d., 3 mm i.d.) packed with 5% diethylene glycol (DEGS) on a 100/120 mesh Supelcoport from Supelco (Bellefonte, PA). The temperature program was 160°C for 2 min, linearly increased to 210°C at 5C°/min, and held constant for 20 min. Nitrogen, the carrier gas, was set at 20 ml/min. Quantitation of fatty acids was by comparison to the internal standard (17:0) using a Shimadzu CR3A computing integrator.

2.7. Phospholipid analysis

Membrane phospholipid content was measured as total lipid-associated phosphate. Phosphate concentration of the synaptosomal lipid extract was quantified by phosphorous analysis as previously described [13,14]. The samples were incubated with 50 μl of $HClO_4$ at 180–200°C for 30 min followed by addition of 500 μl of Vaskovsky reagent and incubated at 100°C for 30 min. Results were obtained by comparison to a KH_2PO_4 standard curve.

2.8. Liposomes

Liposomes were prepared from extracted N_2-dried synaptosomal membrane lipids, after suspension of lipid in phosphate-buffered saline containing the lipid-soluble probe, 1,6-diphenyl-1,3,5-hexatriene (DPH). Lipid suspensions were then sonicated under N_2 for 10 min at 4°C, and the resultant liposome dispersion was centrifuged at $10\,000 \times g$ for 10 min. The supernatant was then used for fluorescence polarization studies.

2.9. Fluorescence polarization studies

For these experiments, the lipid soluble fluorescence probe 1,6-diphenyl-1,3,5-hexatriene (DPH) was used and prepared according to established techniques [15,16]. Estimates of relative membrane fluidity were calculated following fluorescence polarization measurements using a Shimadzu RF-540 spectrofluorophotometer fitted with a thermoregulated sample chamber and automatic polarizers. The term 'fluidity' is used to describe the motional freedom of DPH within a membrane bilayer. Determination of absolute fluidity is limited in an anisotropic membrane or liposome suspension (as opposed to a homogeneous, isotropic medium) because of the inability to accurately reproduce the three-dimensional structure of the hydrophobic bilayer. Therefore the steady-state fluorescence anisotropy r (the reciprocal of fluidity) is employed to estimate relative degrees of fluidity, following probe incorporation into the bilayer.

Values for r were calculated from fluorescence polarization measurements using the equation:

$$r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$$

where $I_{\parallel}$ and $I_{\perp}$ equal fluorescence intensities parallel and perpendicular, respectively, to the excitation plane (excitation wavelength = 360 nm for DPH, 365 nm for 12-AS; emission wavelength = 430 nm for both probes). Scattered light plus ambient medium fluorescence contributed $< 5\%$ to the total fluorescence intensity throughout the temperature range utilized in all studies.

Fluorescence probe excited-state lifetimes were not specifically determined in these studies; however, the relative constancy of this variable throughout these experiments was assessed by measurements of total fluorescence intensity ($F = I_{\parallel} + 2I_{\perp}$) for each synaptosomal liposome preparation. For examination of the effects of temperature, preparations were warmed to 40°C and the fluorescence polarization was measured continuously as the suspension was cooled at a rate of 0.5C°/min to 5°C.

2.10. Conjugated diene assay

The synaptosomal content of conjugated dienes was determined using the procedure of Massey and Burton [17]. Synaptosomes were rinsed twice with phosphate-buffered saline containing 0.01 M EDTA and homogenized in the same solution with added 5% TCA. Samples were centrifuged at $2000 \times g$ for 10 min, and the pellets were extracted twice with 3 ml of chloroform/methanol (2:1, v/v). Two ml of distilled water were added, samples were vortexed and the aqueous phases aspirated. The lipid extracts were dried under N_2, and redissolved in 1–2 ml acetonitrile. Absorbance was monitored at 235 nm, and the amount of conjugated dienes was expressed as arbitrary units (AU).

3. Results

Induction of CHD in rats resulted in a significant increase in serum osmolality (284 ± 4 vs. 334 ± 4 mosmol/kg, control vs. CHD respectively, $P < 0.01$, $n = 3$). Fig. 1A and B illustrates the effect of this

Table 1

	r(DPH)	
	25°C	37°C
Control	0.195 ± 0.0022	0.158 ± 0.0017
CHD	0.176 ± 0.0033 *	0.141 ± 0.0012 *
Control	0.194 ± 0.0030	0.162 ± 0.0031
STZ	0.178 ± 0.0032 *	0.142 ± 0.0035 *
STZ + insulin	0.201 ± 0.0043	0.165 ± 0.0040

* $P < 0.05$, compared to each respective control, CHD, chronic hypernatremic dehydration. STZ, streptozocin.

change on the synaptosomal uptake of taurine. Transmembrane taurine flux was significantly increased by 12.5% in CHD rats compared to normosmotic animals. Additional studies (not shown) indicate that the intrasynaptosomal volume of distribution (space into which solute is transported exclusive of binding), determined by the measurement of inulin space [10], was the same in both experimental groups.

To establish whether CHD altered physical properties of brain synaptosomes, fluorescence anisotropy studies were performed, and the results are shown in Table 1. These data show that r(DPH), the reciprocal of fluidity, was significantly lower compared to synaptosomes prepared from control (normosmotic) synaptosomes. Therefore, synaptosomal lipid fluidity was significantly higher at the two temperatures shown, and over the entire temperature range examined (5–40°C).

In order to determine whether alterations of synaptosomal taurine transport also occurred during hyperglycemia and whether they were reversible, diabetes was induced in two groups of rats, one that was untreated, and a second group in which hyperglycemia was corrected with insulin. Taurine uptake by synaptosomes from these animals was compared with uptake by synaptosomes prepared from control rats. As shown in Fig. 1C, diabetes induced by STZ resulted in a significant increase of serum osmolality compared to control, and administration of insulin reduced osmolality to control values (301 ± 2 vs. 338 ± 4 vs. 299 ± 3 mosmol/kg, control vs. STZ vs. STZ + insulin, respectively, $P < 0.01$, $n = 3$). The synaptosomal uptake of taurine by these three groups is shown in Fig. 1D. STZ-induced diabetes resulted in a significant 13.5% increase of synaptosomal solute uptake when compared to control. Taurine uptake in synaptosomes isolated from diabetic rats rendered normoglycemic with insulin was significantly lower than diabetic rats, and similar to control.

Fluorescence polarization studies were performed on synaptosomal lipid liposomes, comparing STZ-diabetes, STZ plus insulin, and control rats, and are shown in Table 1. These data show that the fluidity of synaptosomal lipid liposomes from diabetic rats is significantly higher than control and insulin treatment normalized this measurement at the two temperatures shown. A representative Arrhenius plot of a single fluorescence anisotropy study is shown in Fig. 2. This

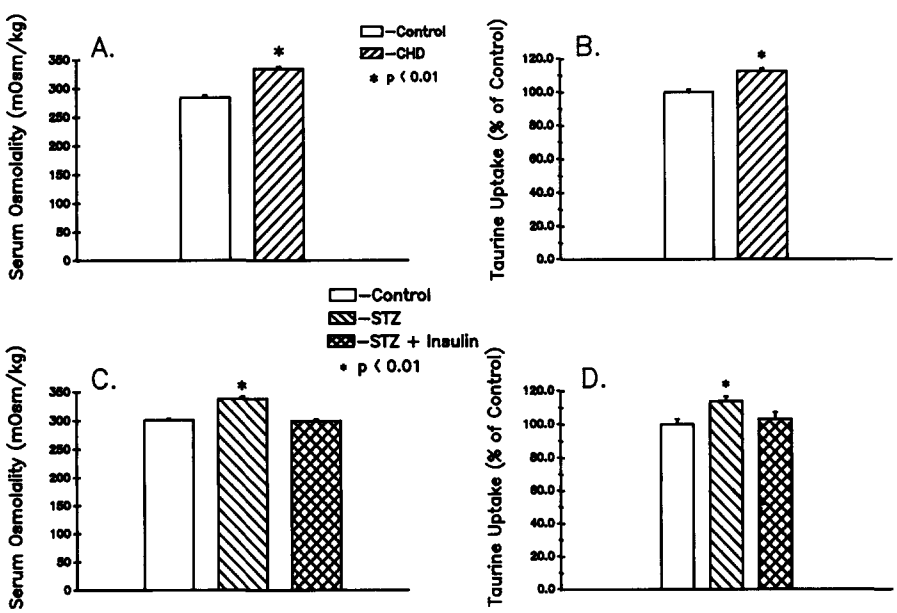

Fig. 1. Serum osmolality (mosmol/kg body weight) of control rats (□), and rats with chronic hypernatremic dehydration (CHD) (A). Uptake of 100 μM taurine by synaptosomes from control rats (□), and rats with chronic hypernatremic dehydration (CHD) (B). Serum osmolality (mosmol/kg body weight) of control rats (□), rats with streptozocin induced diabetes (STZ), and normoglycemic diabetic rats (STZ + insulin) (C). Uptake of 100 μM taurine by synaptosomes from control rats (□), rats with streptozocin induced diabetes (STZ), and normoglycemic diabetic rats (STZ + insulin) (D). Uptake is expressed as % of control uptake after a 15 min incubation. * Significantly different from corresponding control values, $P < 0.01$, $n = 3$.

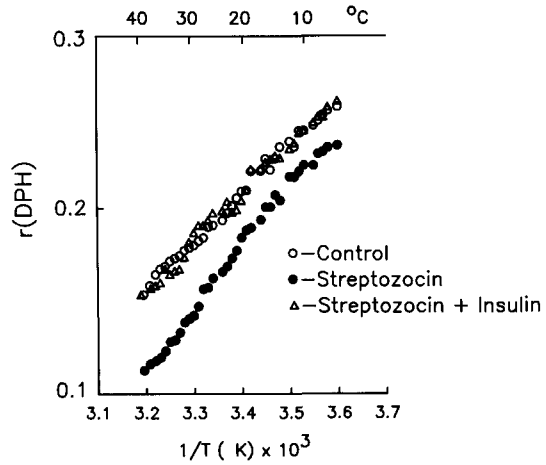

Fig. 2. Representative Arrhenius plots of the fluorescence anisotropy, r(DPH), of synaptosomal lipid liposomes from brains of control rats (○), rats with streptozocin induced diabetes (●), and normoglycemic diabetic rats (△).

demonstrates the significant ($P < 0.05$) differences indicated in Table 1, and shows that this relationship is maintained over the entire temperature range examined (5–40°C). There were no thermotropic transitions evident in any of the three groups examined.

Diabetes induced hyperglycemia has been associated with cell membrane alterations including the increased production of lipid peroxidation products. To determine whether glucose or hyperosmolality-induced lipid peroxidation was responsible for the changes in transport and membrane fluidity, the conjugated diene concentration of various synaptosomal preparations were compared. There were no differences in conjugated diene concentration among control, STZ or CHD synaptosomes, respectively (6.08 ± 0.37 vs. 5.52 ± 0.32 vs. 4.92 ± 0.45, AU/mg protein, mean ± S.E.M., $n = 3$).

To establish whether the hyperosmolality-induced changes of solute transport and fluorescence anisotropy were due to alterations of membrane composition, the lipid composition of liposomes from control, STZ, STZ + insulin and CHD rat synaptosomes was determined. Table 2 shows that there were no differences in liposomal cholesterol and total phospholipid content in the various experimental conditions. Accordingly, there were no differences in the cholesterol to phospholipid molar ratio. However, while there was no difference in the total fatty acid composition of liposomal lipids (data not shown), there was a significant decrease in the ratio of saturated to unsaturated fatty acids, in STZ versus control synaptosomes ($P < 0.05$). Correction of the diabetes with insulin restored this ratio to control values. The ratio of saturated to unsaturated fatty acids was also lower in CHD versus control synaptosomes; however, this difference did not reach statistical significance.

4. Discussion

These experiments are the first to demonstrate that two hyperosmolal states, hypernatremia and hyperglycemia, result in increased cerebral membrane fluidity. This change correlated with an adaptive increase in the uptake of taurine, an osmoprotective molecule. The response to an in vivo perturbation is demonstrable in vitro using synaptosomes, sealed terminal nerve fragments. The normalization of taurine transport and lipid fluidity changes by administration of insulin to the hyperglycemic STZ-diabetic animals suggests that hyperosmolality directly influences membrane structure and function in a reversible manner and that the two membrane properties are interrelated.

The reversibility of the synaptosomal membrane fluidity in the STZ-diabetic rats with insulin is consistent with previous reports that insulin lowers the fluidity of hepatocyte cell membranes [18]. This effect of insulin may be related to membrane lipid composition changes. While we were unable to detect changes in fatty acid and total phospholipid composition, the ratio of saturated to unsaturated fatty acids was decreased in hyperosmolal states. These differences were in the absence of changes of lipid peroxidation. These compositional changes could account for the altered membrane fluidity, which may modulate the V_{max} of synaptosomal taurine transport.

During hyperosmolal disturbances, cells maintain their size by altering the cytosolic content of electrolytes and non-perturbing organic osmolytes. Shifts in osmotic equilibrium have been shown to alter the movement and transcellular distribution of these solutes through resident membrane channels and/or transporters [5,6,10,11,19]. In addition, the rate of gene transcription for the synthesis of the specific osmolyte

Table 2

	Control	STZ	STZ + insulin	CHD
Cholesterol (μmol/mg protein)	0.304 ± 0.057	0.331 ± 0.053	0.324 ± 0.073	0.292 ± 0.041
Phospholipid (μmol/mg protein)	0.378 ± 0.049	0.439 ± 0.066	0.397 ± 0.077	0.396 ± 0.053
Cholesterol/phospholipid (mol/mol)	0.804 ± 0.128	0.754 ± 0.118	0.816 ± 0.171	0.737 ± 0.101
Saturated/unsaturated ratio	0.399 ± 0.091	0.270 ± 0.031 *	0.411 ± 0.132	0.358 ± 0.032

* $P < 0.05$, compared to control, STZ, streptozocin, CHD, chronic hypernatremic dehydration.

transport proteins is augmented in response to increased osmolality. This coordinated adaptive response serves to expand the cytoplasmic pool of osmoprotective solutes, reducing the plasma to cell osmolal gradient, and limiting cell shrinkage. The activity of solute carriers is influenced by the physical state of the membrane, and alterations of fluidity may represent a more rapid regulatory response which is capable of modulating cytosolic osmolyte content in response to fluctuations of ambient osmolality.

The relationship between membrane fluidity and cellular functions is well described, and most of these effects are thought to be due to the influence of membrane lipid physical state on the activity of cell membrane proteins. Various cellular functions have been shown to be influenced by membrane fluidity including enzyme activity [20,21], transmembrane solute and ion transport [13,14], as well as passive permeability characteristics [22]. This interaction has been extensively studied for Na^+/K^+-ATPase. Arrhenius plots indicate that enzyme activity is dependent upon the physical state of the membrane, evidenced by a distinct thermotropic transition point. Alterations in membrane fluidity due to age [16,23], hormones [14,20], cell membrane cholesterol content [21] and chemical modifications of cell membranes [24], all modulate Na^+/K^+-ATPase activity. However, the relationship between membrane fluidity and transport activity is not always direct. While phosphate transport changes parallel membrane fluidity alterations [25], glucose transport increases as membrane fluidity decreases [26].

Membrane fluidity is determined by factors which describe cell membrane composition, including the protein to lipid ratio, phospholipid, fatty acid and cholesterol content, and chain length and degree of saturation of phospholipid fatty acyl side chains [27]. In addition, chemical modification of membrane lipids also alters fluidity. STZ-induced diabetes increased phospholipid n-methylation in erythrocytes, which exhibited decreased Na^+/K^+-ATPase activity [28]. In our experiments, increased synaptosomal taurine uptake and membrane fluidity were associated with subtle changes in membrane lipid composition. This suggests that hyperosmolality directly alters membrane fluidity as a result of compositional changes.

An alternative mechanism by which hyperosmolality alters fluidity may be by promoting lipid peroxidation of cell membranes. A recent study from our laboratory showed that hyperglycemia caused increased lipid peroxidation production in cultured rat mesangial cells; addition of taurine to high glucose media prevented glucose-induced lipid peroxidation [29]. However, in the present study, we were unable to demonstrate increased synaptosomal conjugated diene content under hyperosmolal conditions. This is in contrast to other studies which documented increased lipid peroxidation and conjugated diene content in cerebral microvessels isolated from diabetic rats. These studies failed to demonstrate changes in lipid composition or synaptosomal fluidity as hyperosmolality did not cause a change of thermotropic transition temperature [30]. Hypoxia-induced lipid peroxidation in cultured pulmonary arterial endothelial cells was associated with increased membrane fluidity and 5-hydroxytryptamine transport [31].

In conclusion, our studies suggest that the adaptive increase in cerebral taurine transport during hyperosmolal states may occur as a result of altered synaptosomal membrane lipid composition and fluidity. This may be an immediate and direct effect of hyperosmolality which enables enhanced transmembrane flux of osmoprotective molecules. This action is supplemented by a second mechanism in chronic hyperosmolal states, namely the long term induction of increased synthesis of new osmolyte carriers, resulting in increased V_{max} of taurine transport. These changes together result in the net accumulation of non-perturbing organic solutes in the cytosol and help limit changes in cell volume. An additional benefit of these two adaptive responses would be to increase the availability of taurine, among other osmolytes.

Acknowledgements

Part of this work was presented at the 25th Annual Meeting of the American Society of Nephrology, November, 1992. This work was supported in part by Grants from the American Heart Association, New York State Affiliate (M.S.M.) #91–020G, the National Institutes of Health (M.S.M), #HL 35739, and the Juvenile Diabetes Fund International (H.T.) #191845. We would like to acknowledge the excellent technical assistance of Stephen Futterweit.

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