ORIGINAL RESEARCH PAPER

Biochemistry.

MEMBRANE CHOLESTEROL IS A BETTER PREDICTOR OF NA+/K+-ATPASE ACTIVITY THAN SERUM CHOLESTEROL IN HYPOTHYROID PATIENTS.

KEY WORDS: Hypothyroidism, Na+/K+-ATPase activity, membrane cholesterol, serum cholesterol.

Dr. Biplab Biswas	MBBS, Junior resident, 3rd year MD (Biochemistry). Dept. of Biochemsitry, Calcutta National Medical College, Kolkata.
Dr. Satwika Sinha	MD (Biochemistry), Assistant Professor, Dept. of Biochemsitry, Calcutta National Medical College, Kolkata.
Dr. Kaushik Kar	MD (Biochemistry), Associate Professor, Dept. of Biochemsitry, Calcutta National Medical College, Kolkata.
Dr. Anindya Dasgupta*	MD (Biochemistry), Professor & Head, Dept. of Biochemsitry, Calcutta National Medical College, Kolkata. *Corresponding Author

ABSTRACT

Background: Dyslipidemia is a major complication of hypothyroidism. Thyroid deficiency alters the membrane function also. The present study was aimed to assess the predictive role of membrane cholesterol on RBC membrane sodium pump activity in comparison to serum cholesterol level.

Methods: In a hospital based cross sectional observational study, serum TSH, fT4, total cholesterol, LDLc and HDLc were measured in 50 hypothyroid cases and 50 control subjects using spectrophotometric techniques. RBC mmbrane cholesterol and Na+/K+-ATPase were assayed by chloroform extraction method and kinetic assay method respectively.

Results: Na+/K+-ATPase activity and membrane cholesterol levels were significantly reduced in the hypothyroid group with significantly increased total cholesterol and LDLc levels (P<.001). Multiple linear regression analysis suggested a direct dependence of Na+/K+-ATPase activity on membrane cholesterol level only in the hypothyroid group.

Conclusion: Membrane cholesterol levels are directly linked to the optimal functioning of the Na+/K+-ATPase pump in biomembranes. Excessive reduction in membrane cholesterol levels may hamper its activity to subnormal level.

INTRODUCTION:

Thyroid hormone is essential for cell differentiations during development and helps to maintain caloriegenic and metabolic homeostasis at adult life. Hypothyroidism is a one of the commonest endocrine disorders worldwide(1). It occurs due to deficient hormone secretion or from their impaired action to its cognate receptor. Hypothyroidism presents in a spectrum of disease from asymptomatic subclinical (elevated TSH and normal serum fT4 level) to symptomatic or overt hypothyroidism (elevated TSH and decreased fT4 level).

Derangement of serum lipid profile and different proteins like thyroperoxidase have long been known in hypothyroid patient(2, 3). Moreover alteration of membrane cholesterol in hypothyroid patient has been shown in different studies(4).

Na+/K+-ATPase (the sodium pump) is an ATP-dependent transmembrane enzyme protein composed of two major classes of subunits (α and β), which help in the overall regulation of its activity under different metabolic conditions. Membrane cholesterol in plasma membrane helps to maintain its fluidity and also helps in functioning of different transporter. Na^+K^+ATPase pump reside in membrane caveola which is rich in cholesterol, one of the major transporter(5). So an optimum level of cholesterol in biomembrane is needed for proper functioning of Na^+K^+ATPase pump.

Keeping these factors in mind the hypothesis of the present study is formulated that there changes in membrane cholesterol level is linked to alterations in Na+K+ATPase activity along with changes in lipid profile in hypothyroid patients.

MATERIAL AND METHODS:

Study design:

It was a hospital based non interventional, case-control observational study.

Selection of cases and control subjects:

Cases were selected from the diagnosed hypothyroid patients attending the Department of thyroid clinic in this tertiary care medical college of Kolkata by the method of convenience. After attaining proper clinical history, final diagnosis was made by measuring the serum TSH and fT4 values in the Dept. of Biochemistry. Patients suffering from any other metabolic, endocrinological or chronic diseases, any malignant or premalignant condition, any chronic inflammatory diseases, any history of addiction to alcohol and use of tobacco in any form or any other drug were excluded from the study. Pregnant population were excluded to avoid the pregnancy associated endocrinological changes.

Control subjects were selected from the same geographical area with similar socioeconomic and nutritional status in age and sex matched way during the same period. Subjects falling between 25to 50 years were considered for both case and control selection. Following these inclusion and exclusion criteria 50 subjects were finally selected in each case and control group each within a study period of July 2015 to June 2016.

Ethical consideration: Informed consent were taken from patients and control. The study was initiated after getting written consent from the properly constituted institutional ethical committee.

Measurement of study parameters:

A) Measurement of Na+K+ATPase activity in RBC membrane: This was done in two steps*(6):*

Step I: Preparation of membrane suspension from RBC hemolysate:

The packed red cells were extracted by centrifugation at 4°C, 450 g for 15min and was resuspended and diluted in 25 volumes of 0.011 mol/L Tris-HCl buffer at pH 7.4. The haemolysed cells was centrifuged for 30 min at 12,000 rpm at 4^0 C and the membrane pellet was resuspended in 30 ml of 0.011 mol/L Tris-HCl buffer. This centrifugation step was repeated three times. The membrane suspension obtained was stored at -80^0 C until the assay will be performed.

Step II: Measurement of Na+K+ATPase Activity:

A reaction mixture consisting of 1.15 ml of Tris HCl buffer (pH 7.4), 0.15 ml of ionic solution (30 mmol/L $MgSO_4$, 1 mol/L NaCl, 200 mmol/KCl), 0.025 ml of NADH (10mg/ml), 0.015 ml of pyruvate kinase (3 units), 0.025 ml of phosphoenolpyruvate (0.1 mol/L), 0.005 ml of LDH and 0.045 ml of deionized water was prepared.

The reaction mixture was put in a UV spectrophotometer and the baseline was established by measuring the extinction at 340 nm for 1-2 min. At the end of this time, 50µl of suitably prepared sample was added and the reaction was initiated by adding 100µl of sodium ATP solution (0.1 mol/L). Once a good linear rate was established, 100µl of ouabain (10 mmol/L) was added and the rate was measured after this addition for 3 minutes and ΔA/minute was calculated.

The Na+K+ATPase activity was calculated from the differences between two ΔA/minute .Once the difference between the above two rates were measured, the enzyme activity was calculated as follows:

***Step III: Calculation of enzyme activity*:**
ΔA/minute for Na+K+ATPase = ΔA/minute before addition of ouabain - ΔA/minute after addition of ouabain.

- ΔA/minute for Na+K+ATPase obtained was then multiplied with 50 to convert it into nmol of ADP.

The final value was expressed in units or milli units (1µmol/min=1 Unit) per mg of tissue protein obtained by using the Lowry's method.

B) Measurement of serum TSH and Ft4 levels:
Serum *TSH and Ft4 levels* were measured using the technique of non-competitive sandwich and competitive ELISA method respectively. The ELISA kits were obtained from Accubind, USA.

C) Measurement of lipid profiles: Serum cholesterol, LDLc and HDLc were measured by standard spectrophotometric assays. Serum cholesterol was assayed using the CHOD-PAP method whereas the LDL and HDL cholesterol were assayed using standard homogenous spectrophotometric techniques. All assays were performed in the XL 600 autoanalyzer from ERBA using the reagent kits obtained from ERBA, USA.

D) Measurement of membrane cholesterol: Membrane cholesterol was assayed from RBC membrane using chloroform extraction technique as described by Macchia et al (1991)(7).

D) *Quality control of study procedure:* Coefficient of variation (CV) was monitored for the precision of each assay and was found to be limited within 5 percent.

RESULTS:
Following the inclusion and exclusion criteria 50 subjects were finally selected in both the case and control group. All data obtained were found to follow a normal distribution pattern approximately. Independent t test in table 1 shows significant altered levels of both serum and membrane cholesterol in the case group, but in opposite directions. Serum cholesterol level was significantly higher in the case group with markedly lowered membrane cholesterol content. Na+K+ATpase activity was also found to be decreased significantly in the case group.

Table 1: Group statistics showing differences of mean values of study parameters between the case and control groups.

	Cases (n=50) (Mean/SD)	Controls (n=50) (Mean/SD)	t value	P value
TC	216/46	179/35	3.9	<0.001*
TG	169/43	122/20	4.7	<0.001*
HDL	41/8.3	52/11	-4.9	<0.001*
LDL	129/35	101/28	3.6	<0.001*
Na+K+ATpase activity	3.5/.90	6.9/1.8	-11.4	<0.001*
Membrane cholesterol	1.73/.34	2.76/.46	-11	<0.001*

*P value considered to be significant at a level of P < 0.05 for 95% confidence interval.

To obtain the dependency of the Na+K+ATpase activity on serum and membrane cholesterol we performed multiple linear regression analysis. Results show that the enzyme activity was significantly dependent only on the membrane cholesterol inversely.

Table 2: Multiple linear regression analysis showing the dependence of sodium pump activity on serum and membrane cholesterol levels in hypothyroid patients.

Coefficients[a]						
Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	2.337	.870		2.688	.010
	Membrane cholesterol	1.073	.349	.403	3.073	.004*
	Serum total cholesterol	-.003	.003	-.158	-1.204	.234
a. Dependent Variable: Na+K+ATpase activity						

*P value considered to be significant at a level of P < 0.05 for 95% confidence interval.

DISCUSSION:
The Na+K+ATPase pump activity in RBC is correlated with the cell membrane integrity of other tissues like liver, kidney, myocardial and neuronal tissue (8-10). As membrane cholesterol is an important modulator of the membrane fluidity and hence regulates several integral membrane protein activity including that of the sodium pump (11, 12). Cholesterol is a member of membrane lipids that exerts a significant influ ence on the structural integrity of the sodium pump. The bind ing of lipids such as phosphatidylserine and cholesterol stabiliz es particular sites of the sodium pump. Purified detergent-solu ble recombinant αβ or αβ/FXYD sodium pump complexes have been found show specific functional effects in response to the binding of phospholipids and cholesterol at different sites of the enzyme, with distinctive structural selectivity. These findings have underscored the crucial role of phospholipids and choles terol, and the interactions thereof, in maintaining the stability and molecular activity of the sodium pump(13). Changes in the cholesterol content of biological membranes are known to alter the properties of the lipid lamella and hence affect the activity of membrane-bound enzymes. High concentrations of sodium pumps have been reported in the membrane caveolae, which are rich in cholesterol (containing up to 25% to 30% of total cell cholesterol), reflecting the impor tance of a cholesterol-rich environment for their optimum func tion(14).

Keeping in track with the above explanations, membrane cholesterol content was significantly decreased in the hypothyroid patients in spite of having a raised serum cholesterol level. This finding is further explained by the fact that deficiency of thyroid hormones affects both the synthesis of cholesterol at intracellular level and its uptake by the cells from blood due to impaired LDL receptor activity(15).

Results of multiple linear regression showed a significant positive dependence of sodium pump activity on the membrane cholesterol content (Table.2, β=0.403, p value = 0.004) without any such effect on the serum cholesterol level signifying that membrane cholesterol is a better indicator of the effect of thyroid hormones on cholesterol homoeostasis compared to the serum cholesterol. Furthermore, the study indicates a major cue regarding the contribution of membrane cholesterol on regulating the sodium pump activity in hypothyroid patients that may elucidate the deranged ionic balance in signal transduction observed in hypothyroid patients.

ACKNOWLEDGEMENT: Nil.

REFERENCES:
1. Unnikrishnan AG, Kalra S, Sahay RK, Bantwal G, John M, Tewari N. Prevalence of

hypothyroidism in adults: An epidemiological study in eight cities of India. Indian J Endocrinol Metab. 2013 Jul;17(4):647-52.
2. Duntas LH. Thyroid disease and lipids. Thyroid. 2002 Apr;12(4):287-93.
3. Ris-Stalpers C, Bikker H. Genetics and phenomics of hypothyroidism and goiter due to TPO mutations. Mol Cell Endocrinol. 2010 Jun 30;322(1-2):38-43.
4. Roy S, Dasgupta A. The Effects of Altered Membrane Cholesterol Levels on Sodium Pump Activity in Subclinical Hypothyroidism. Endocrinol Metab (Seoul). 2017 Mar;32(1):129-39.
5. Cornelius F, Habeck M, Kanai R, Toyoshima C, Karlish SJ. General and specific lipid-protein interactions in Na,K-ATPase. Biochim Biophys Acta. 2015 Sep;1848(9):1729-43.
6. Noori S, Zafar H, Mahboob T. Biochemical effectiveness of cocoa powder on electrolytes homeostasis, liver and cardiac specific enzymes and renal function. Pak J Nutr 2009;8:882-6.
7. Macchia T, Mancinelli R, Barbini DA, Taggi F, Avico U, Cantafora A. Determination of membrane cholesterol in normal and pathological red blood cells. Clin Chim Acta. 1991 May 31;199(1):59-67.
8. Kumar AR, Kurup PA. Membrane Na+ K+ ATPase inhibition related dyslipidemia and insulin resistance in neuropsychiatric disorders. Indian J Physiol Pharmacol. 2001 Jul;45(3):296-304.
9. Ravi Kumar A, Kurup PA. Digoxin and membrane sodium potassium ATPase inhibition in cardiovascular disease. Indian Heart J. 2000 May-Jun;52(3):315-8.
10. Prasad R, Mond R, Jain S, Kaur G, Chugh KS. Modulation of ouabain sensitive sodium potassium pump of erythrocytes from patients with chronic renal failure: role of acute hemodialysis. Biochem Mol Biol Int. 1996 Dec;40(6):1087-94.
11. Turner, NigelElse, Paul L. Sodium pump molecular activity and membrane lipid composition in two disparate ectotherms, and comparison with endotherms. Journal of Comparative Physiology B 2005. Issue 4–5, pp 296–302.
12. Roy S, Dasgupta A, Banerjee U, Chowdhury P, Mukhopadhyay A, Saha G, et al. Role of membrane cholesterol and lipid peroxidation in regulating the Na(+)/K(+)-ATPase activity in schizophrenia. Indian J Psychiatry. 2016 Jul-Sep;58(3):317-25.
13. Cornelius F. Modulation of Na,K-ATPase and Na-ATPase activity by phospholipids and cholesterol. I. Steady-state kinetics. Biochemistry. 2001 Jul 31;40(30):8842-51.
14. Yosef E, Katz A, Peleg Y, Mehlman T, Karlish SJ. Do Src Kinase and Caveolin Interact Directly with Na,K-ATPase? J Biol Chem. 2016 May 27;291(22):11736-50.
15. Rizos CV, Elisaf MS, Liberopoulos EN. Effects of thyroid dysfunction on lipid profile. Open Cardiovasc Med J. 2011;5:76-84.