



CIRCADIAN MODULATION OF SODIUM-POTASSIUM ATPase AND SODIUM - PROTON EXCHANGER IN HUMAN ERYTHROCYTES: *IN VITRO* EFFECT OF MELATONIN

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Abstract

Melatonin (N-acetyl-5-methoxytryptamine) is a pineal secretory product which is involved in the regulation of circadian rhythm and participates in many physiological functions. It also acts as a potent antioxidant and a powerful free radical scavenger. The membrane-associated Na⁺/K⁺-ATPase and Na⁺/H⁺ exchanger in erythrocytes play an important role in maintaining cytosolic pH, ionic homeostasis, cell osmolarity and in the regulation of transmembrane ion movement. The present work was undertaken to determine the role of melatonin in modulating the activity of Na⁺/K⁺-ATPase and Na⁺/H⁺ exchanger in human erythrocytes. Our observation shows circadian modulation of Na⁺/K⁺-ATPase and Na⁺/H⁺ exchanger which may have important therapeutic implications. Exogenous melatonin modulated the activities of Na⁺/K⁺-ATPase and Na⁺/H⁺ exchanger in human red blood cells, this effect may in part be explained due to the antioxidative effect of melatonin and also due to modulation of membrane fluidity. Further work is needed to understand the mechanism of action.

Key words: Melatonin, erythrocytes, circadian, Na⁺/K⁺-ATPase, NHE.

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Abbreviations: **FXD2:** γ -subunit of Na⁺/K⁺-ATPase-associated protein; **NHE:** Sodium-Hydrogen (sodium-proton) Exchanger; **NHE1:** Sodium-proton exchanger (Isoform 1); **NHE3:** Sodium-proton exchanger (Isoform 3); **per, cry, bmal, clock:** (*period, cryptochrome, brain and muscle ARNT-like 1, clock*) clock gene mutants; **RBC:** Red Blood Cells; **ROS:** Reactive Oxygen Species.

INTRODUCTION

Melatonin (N-acetyl-5-methoxytryptamine) is involved in the regulation of circadian rhythm. The endogenous pineal hormone is a derivative of an essential amino acid L-tryptophan and is involved in several physiological functions such as cardioprotection, immuno-stimulation, thermoregulation, sleep-induction and gerontoprotection (17). It provides protection against neurodegeneration, and is active in tumour-suppression and oncostasis. Melatonin acts as a potent antioxidant and a powerful free radical scavenger (17). In addition, melatonin's amphiphilic nature enables it to transcend any morphophysiological barrier, a property which enhances its free radical-scavenging activity in cellular organelles. While pineal melatonin is as an efficient free-radical scavenger, pharmacological melatonin has also received acceptance as a novel drug against pro-oxidant imbalance in the past few years (20). This indole neurohormone has been reported to curb

oxidative disturbances through several mechanisms, some of which reportedly display circadian changes (10).

Sodium-potassium ATPase (sodium pump) or Na^+/K^+ -ATPase is a membrane-linked ATP-driven ionic pump located in the plasma membrane that allows the exchange of Na^+ and K^+ ions across the membrane and contributes to the resting membrane potential in excitable cells. Exacerbated cellular response to oxidative damage involves activation of Na^+/K^+ -ATPase that plays an important role in ionic homeostasis (13). This enzyme maintains electrochemical sodium and potassium gradient of tissues and cells. It also regulates intracellular calcium homeostasis (28). Na^+/K^+ -ATPases help in the active cotransport of ions such as H^+ , Ca^{2+} , K^+ and have been implicated in metabolic energy production as well as in the uptake, storage, and metabolism of catecholamines, serotonin, and glutamate. The ion gradient produced by Na^+/K^+ -ATPase influences cell volume and osmotic pressure and acts as a driving force for inward cotransport of amino acids and monosaccharides. It is widely believed that impairment in Na^+/K^+ -ATPase may play a major role at the cellular level in the pathophysiology of many complications of insulin-dependent and non-insulin-dependent diabetes mellitus (3).

Another ion-exchange system present in the plasma membrane of most mammalian cells is sodium-proton (Na^+/H^+) or sodium-hydrogen antiport. It is one of the most studied plasma membrane mechanisms involved in proton transport. Na^+/H^+ exchangers (NHE) are amiloride-sensitive antiport proteins which mediate electroneutral exchange of Na^+ and H^+ across plasma membrane for acid-base and electrolyte homeostasis. The isoform NHE1 is entrusted with pH regulation; NHE3 isoform plays a significant role in cellular transport of Na^+ in the gut which is activated during metabolic acidosis and hypovolemia. The sarcolemmal exchanger, NHE1 serves as an effective acid-extrusion system that couples H^+ efflux to Na^+ influx in an equal ratio (1:1) under the driving force of a Na^+ gradient formed by the Na^+ pump.

The red blood cells experience oxidative damage at various levels of cellular composition, including membrane-associated changes (33). The present study was undertaken to determine the circadian modulation of erythrocyte Na^+/K^+ -ATPase and Na^+/H^+ antiport and evaluate the

effect of exogenous melatonin in regulating their activities in accordance with circadian signals.

MATERIALS AND METHODS

Selection of subjects

The study was carried out on 31 normal healthy donors of both sexes between the ages of 20-32 who gave informed consent for the use of their blood samples for the study. The criteria for screening of volunteers included non-smoking individuals having no acute or chronic diseases (such as diabetes mellitus, asthma, or tuberculosis) or organ dysfunction and had not taken any medication (21). The protocol of study was in conformity with the guidelines of the University of Allahabad Institutional Ethical Committee.

Collection of blood samples

Blood samples were collected by venipuncture in heparinized vials (10 IU/mL) at two different timings viz., 10:00 hrs. (At the offset of melatonin secretion) and 22:00 hrs. (At the onset of melatonin secretion) of the day. The red blood cells (RBCs) were sedimented at 1000g for 10 min at 4°C and washed three times with cold phosphate-buffered saline pH 7.4 (PBS) containing 0.154 mM NaCl and 10 mM Na_2HPO_4 . The supernatant and buffy coat was carefully removed after each wash. The erythrocyte membrane from the leukocyte-free red cells was prepared following the method of Marchesi and Palade (11).

Determination of Na^+/K^+ -ATPase activity

Na^+/K^+ -ATPase activity (ouabain-sensitive) was assayed as described (22). The final assay mixture contained 0.4–0.9 mg/mL protein, 140 mmol/L NaCl, 20 mmol/L KCl, 3 mmol/L MgCl_2 , 30 mmol/L imidazole (pH 7.25), 5×10^{-4} mol/L ouabain and 6 mmol/L ATP. Incubation was carried out for 30 minutes at 37°C; the reaction was stopped by adding 3.5 ml of a solution containing 0.5 mol/L H_2SO_4 , 0.5% ammonium molybdate and 2% SDS. The amount of liberated inorganic phosphate was estimated by the method of Fiske and Subbarow (5).

Measurement of NHE activity

NHE activity in isolated erythrocytes was estimated in terms of amiloride-sensitive H^+ -efflux from acid loaded intact red blood cells as reported previously (22). The activity of the antiport is extrapolated by the difference in hydrogen efflux rates from acid loaded erythrocytes in the absence and presence of the inhibitor amiloride. Briefly, 0.2 mL of packed erythrocytes were suspended into a 3.8 mL solution containing 150 mmol/L NaCl, 1 mmol/L KCl, 1 mmol/L MgCl_2 , 10 mmol/L glucose and were incubated at 37°C for 5 minutes under magnetic stirring. The cell suspension was brought to pH ranging between 6.35–6.45 within 10 minutes using 0.2 mol/L HCl solution in 150 mmol/L NaCl. DIDS (4, 4-diisothiocyanatostilbene-2, 2-disulfonic acid) was added (0.2 mmol/L final concentration) and the pH of the medium was brought to 7.95–8.00 using 0.05 mol/L NaOH solution in 150 mmol/L NaCl. In a parallel experiment, amiloride (0.5 mmol/L final concentration) was added with DIDS. Thereafter, proton efflux in the first minute was registered. The rate of NHE, expressed in $\mu\text{mol/L}$ of cells/h, derives from the difference in rates of medium acidification in the absence (ΔpH_1) and presence (ΔpH_2) of amiloride, corrected by the buffer

capacity of the incubation medium (b-value), the cell volume in the suspension and the incubation time.

Determination of the in vitro effect of melatonin on Na⁺/K⁺-ATPase and Na⁺/H⁺ antiport activity

A stock solution (10mM) of melatonin was prepared in absolute ethanol; further dilutions (10^{-3} M - 10^{-10} M) were done with PBS. The concentration of ethanol was always <0.01% (v/v) in the final solution. The *in vitro* effect of melatonin was evaluated by adding different doses (10^{-4} M - 10^{-11} M final concentration) of melatonin to the assay medium and incubating for 30 minutes at 37°C, prior to Na⁺/K⁺-ATPase assay. For Na⁺/H⁺ antiport assay, the erythrocytes were incubated with melatonin at different doses in PBS containing 5mM glucose for 30 min at 37°C, prior to the assay. After exposure to the indoleamine, RBCs were washed twice with PBS to remove any amount of the compound and subjected to Na⁺/H⁺ antiport assay. Appropriate controls were run in parallel with solvent alone where the final concentration of ethanol did not exceed 0.01% (v/v).

RESULTS

We observe circadian oscillation in the activities of Na⁺/K⁺-ATPase and Na⁺/H⁺ antiport. The effect of melatonin on ion-transport mechanisms in erythrocytes follows rhythmic modulation with day/night cycle. The samples collected at two different timings of the day show different response to melatonin treatment. Na⁺/K⁺-ATPase of erythrocyte membrane shows higher activity in diurnal samples (Fig. 1). The concentration-dependant change is significant in samples collected at night. No significant change was observed in the activity of this enzyme even at abnormally high doses of melatonin (10^{-4} M - 10^{-5} M) for the morning samples. The enzyme activity attains maximum stimulation at a millimolar concentration proximal to the maximal nocturnal level of endogenous melatonin (10^{-6} M - 10^{-8} M). However, at very low concentration of melatonin (10^{-9} M - 10^{-11} M) the activity of the sodium-potassium ion-pump is inhibited which shows similarity to its oscillatory effect on the Na⁺/K⁺ ion pump at normal physiological concentration (9).

The Na⁺/H⁺ exchanger, on the other hand, exhibits rise in its baseline activity in the night samples in comparison to the morning samples (Fig. 2). The nocturnal upregulation is synchronous with the onset of pineal secretion at night. NHE shows stimulated activity at very high molar concentration of melatonin (10^{-4} M - 10^{-5} M) which may be attributed to melatonin's non-specific interactions with the antiport. Such alteration in the activities of membrane-linked

ion-transport systems at supraphysiological dosages of melatonin may have some specific regulatory mechanisms apart from photic stimuli. However, the antiport attains a significant sharp crest in activity in the morning samples in response to nanomolar concentration of exogenous melatonin; this pattern bears similarity to its upregulated activity in renal tissues after midnight when pineal melatonin content is at its peak (30).

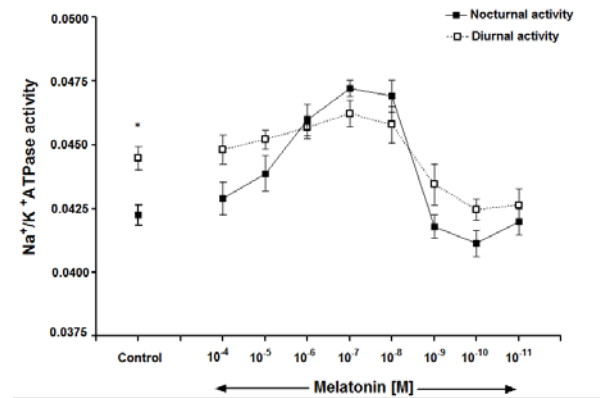


Figure 1. Circadian variation in the activity of erythrocyte Na⁺/K⁺-ATPase. The points (□) and (■) represent the effect of melatonin in diurnal and nocturnal blood samples respectively. Significant change was observed in diurnal and nocturnal activities of the sodium pump in control samples (* p < 0.05). Na⁺/K⁺-ATPase activity expressed in terms of μmol pi released/h/mg membrane protein at 37 °C. Values represent means ± S. D.

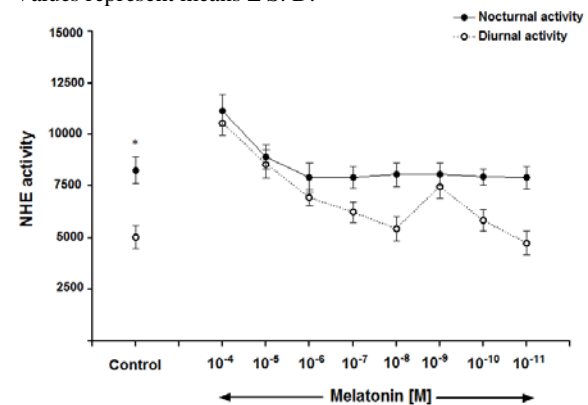


Figure 2. Circadian variation in the activity of erythrocyte Na⁺/H⁺ exchanger. The points (○) and (●) represent the effect of melatonin in diurnal and nocturnal blood samples respectively. Significant change was observed in diurnal and nocturnal activities of the sodium-proton exchanger in control samples (* p < 0.05). NHE activity expressed as proton efflux μmol/L erythrocyte/h at 37 °C. Values represent means ± S.D.

Erythrocyte membrane protein was estimated following the Folin-ciocalteu method (10).

Statistical analyses of the data were performed employing ANOVA followed by student's t-test using the software PRISM4 (GraphPad Software, San Diego, CA). Results showing $p < 0.05$ were assumed to be significant.

DISCUSSION

ATPases are lipid-dependent membrane-bound enzymes. Any change in the normal functioning of ATPases causes changes in electrophysiological homeostasis of plasma membrane. Circadian regulations of the Na^+/K^+ -ion-exchange system and its functions have been reviewed recently (9). The enhanced spontaneity of firing rates of action potential of SCN neurons during the daytime and altered activities of ion-transport systems at different timings of the day highlight the involvement of circadian regulation of sodium/potassium ion channels (30, 31). Na^+/K^+ -ATPase activity of SCN neurons exhibits rhythmicity following a photic stimulation, which is higher during the day (30). The nocturnal rise in pineal secretion of melatonin seems to impede the Na^+/K^+ -ATPase activity due to the rise in the level of some ATPase inhibitor in the blood (26). Regulation of the pump occurs at several levels consisting of gene expression and recruitment/internalization of the active pumps across the plasma membrane.

In the last few years evidence for a completely new mechanism of regulation of Na^+/K^+ -ATPase has emerged; the intrinsic properties of Na^+/K^+ -ATPase can be modulated by association with a small single span membrane protein belonging to the "FXFD" family (32). FXFD proteins encounter oxidative attack resulting in improper functioning of Na^+/K^+ -ATPase. Following any cardiovascular or cerebral trauma, rapid rise in free radicals is accompanied by consequent fall in the levels of ATP. As a result, ATP-dependent Na^+/K^+ -ATPase pump fail, allowing an inflow of sodium, chlorine and calcium into neurons, coupled with an outflow of potassium. Melatonin plays an essential role in enhancing energy supply for proper functioning of the cell (25). At millimolar concentrations ($10^{-4}\text{M} - 10^{-5}\text{M}$), the pro-oxidative nature of melatonin is predominant which may be having deleterious effects on membrane lipid-protein environment, resulting in minimal difference in diurnal Na^+/K^+ -ATPase; the change in Na^+/K^+ -ATPase at such high dosages is,

however, significant in nocturnal samples. The pronounced effect of the drug on erythrocyte sodium pump at relatively lower (micromolar) concentrations indicates its stimulatory effect on Na^+/K^+ -ATPase enzyme which can be discussed with regard to membrane fluidity alterations. However, the nadir obtained at nanomolar dosages is similar to the nocturnal inhibition of sodium pump in the presence of melatonin, as reviewed by Ko and Pang (9).

Modulation in NHE activity is brought about by changes in the phosphorylated state of the cytoplasmic carboxyl-terminal domain of the exchanger. A number of metabolic pathways eventually cross at NHE to meet their respective ends (7). Altered NHE activity has been linked to the pathogenesis of several diseases, including diabetes, essential hypertension, congenital secretory diarrhoea, tissue damage caused by ischemia / reperfusion and oncogenesis (1, 18). Mammalian NHE proteins show stimulated activity at night, when serum melatonin reaches its peak after midnight. Our result is in conformity with previous studies (14, 23). Since the expression of NHE isoforms is under the transcriptional control of clock genes (*per*, *cry*, *bmal*, *clock*) the argument is made that clock genes directly interact with the promoter to generate a rhythm in mRNA of NHE3 exchanger and also its activity (23). Higher incidences of cardiovascular events occurring after awakening from sleep has been reviewed recently which emphasized on the role of melatonin and proper timing of indole drug administration in lowering the risk of cardiovascular problems (4, 24). This reaffirms the relationship between the circadian neurohormone and Na^+/H^+ exchangers. The scotopic stimulation of erythrocyte NHE observed in the presence of millimolar concentrations of melatonin is relative to the antioxidative efficacy of this indole drug at supraphysiological dosages. The sudden sharp peak at nanomolar dose in diurnal samples depicts proximity to its maximum upregulation in the second phase of the night till early morning hours.

It is widely accepted that tissues under oxidative stress are characterised by altered fluidity of cell membranes (29). Na^+/K^+ -ATPases are membrane-linked proteins that are bound to get sensitized by alterations in lipid-protein environment of plasma membrane (27). Decrease in membrane fluidity causes decrease in Na^+/K^+ -ATPase activity (16). However, the reverse

happens to NHE activity which undergoes inhibition with increase in membrane fluidity (2). The dose-dependent effect of melatonin can be partly explained in the light of the antioxidative role of melatonin on the red blood cells. Melatonin, due to its antioxidative nature, may protect the cells from oxidant-induced membrane damage and reduce the loss of membrane fluidity (15). Experimental evidences have also confirmed the pro-oxidative behaviour of melatonin at very high concentration (12). Such evidences are supportive of the dose-dependent effect of melatonin at lower concentration.

Exogenous melatonin also modulates the membrane fluidity in the cells (6). Experimental evidences reveal that the indoleamine inflicts changes in Na^+/K^+ -ATPase and Na^+/H^+ exchanger activities which may be related to its interactions with membrane phospholipids. Melatonin, showing its amphiphilicity, crosses the cell membranes with ease and maintains the permeability of plasma membrane. The rise in Na^+/H^+ exchanger activity in presence of exogenous melatonin is in conformity with earlier observations (30). The dark phase secretion of melatonin may have enhanced the membrane phospholipid fluidity by inferring malondialdehyde production, thus improving ion-transport functions across the cell membranes.

Melatonin may play a key role in cytoskeletal rearrangements through its calmodulin antagonism. The indoleamine interaction with calmodulin may allow the hormone to modulate rhythmically many cellular functions. It is suggested that at a nanomolar (physiological) concentration cytoskeletal effects of melatonin are mediated by its antagonism to Ca^{2+} /calmodulin. At micromolar concentration non-specific binding of melatonin to tubulin occurs, thus overcoming the melatonin antagonism to Ca^{2+} /calmodulin (8). The non-specific binding of melatonin to the membrane-bound lipids may be helpful in understanding the changes in the transmembrane ion-transporters at supraphysiological dosages. Low affinity binding to calmodulin in the micromolar range of melatonin may promote ROS production (19). Functional importance of melatonin at such high doses needs further investigations.

Our investigation opens novel prospectives towards reducing oxidative stress-induced membrane-associated alterations. Na^+/K^+ -ATPase and Na^+/H^+ exchanger play significant role in physiological functions. Both the ion-transport

systems are modulated by melatonin as evidenced by their variation in activity during the day and night. In addition, exogenous melatonin has effects on these two systems which may have important therapeutic implications. Ample evidences are in favour of the antioxidative potential and membrane stabilising properties of melatonin (6, 20). The observed effects of melatonin on erythrocyte Na^+/K^+ -ATPase and Na^+/H^+ exchanger can in part be explained on the basis of its antioxidative effects and changes in plasma membrane fluidity; however the exact mechanism needs further study.

Other articles in this theme issue include references (34-49).

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