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Role of resveratrol in regulation of membrane transporters and integrity of human erythrocytes

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ABSTRACT

An altered ion homeostasis due to impaired membrane transporters is known to be involved in the pathophysiology of many chronic diseases. Resveratrol, a phytoalexin, has been reported to elicit pleiotropic health-promoting effects, however, the mechanism(s) which underlie these effects remain speculative. The present study investigate the modulatory role of resveratrol on erythrocyte membrane Ca^{2+}ATPase (PMCA pump), Na^+/K^+-ATPase (NKA pump), and Na^+/H^+ exchanger (NHE) in control and experimental-oxidative stress conditions. Results suggest that resveratrol is a potent modulator of membrane transporters evidenced by stimulation of PMCA and NKA pumps and down-regulation of NHE. The observed effects on membrane transporters correlated with susceptibility of erythrocyte membrane to oxidative damage. The findings provide an insight into the role of membrane transporters and their involvement in the health beneficial effects of resveratrol.

1. Introduction

Ion homeostasis is crucial for successful cell physiology. Membrane bound enzymes and exchangers play a vital role in maintaining ion homeostasis essential for generating ion gradient, regulating cell volume, uptake of nutrients, cell growth, action potential of smooth muscle, nerve conduction, and for many other physiological processes [1–3].

The plasma membrane Ca^{2+}-ATPase (PMCA pump) is a highly regulated transporter involved in maintaining Ca^{2+} homeostasis vital for cellular functions [4]. The Na^+/K^+-ATPase (NKA pump) is a transmembrane protein, which regulates intra- and extra-cellular Na^+ and K^+ concentrations linked to hydrolysis of ATP [1]. The Na^+/H^+ exchanger (NHE) is an amiloride sensitive, electro neutral ion exchange system in plasma membrane, involved in alkalinization and control of intracellular acidosis by removal of hydrogen and influx of sodium [3].

A close relationship has been established between the impairment in activities of various membrane transporters causing loss of ion homeostasis and development of pathologies [1,2,4]. Exhaustive studies have provided evidence that oxidative stress is among the prime reasons which disturb membrane characteristics thereby alteration in transporters [5,6]. Altered expression of membrane bound transporters has been reported in many chronic diseases including cancer, diabetes, neuro, and heart maladies, and aging [3,4,7,8].

During the last decade, resveratrol a natural phytoalexin present in skin of grapes and in red wine has received much attention due to its pleiotropic biological activities. Exhaustive studies on different model systems and human clinical trials have suggested that resveratrol elicits strong anti-cancer, anti-diabetic, and cardio protective properties [9,10]. Resveratrol has also been reported to counteract age-associated maladies and enhance life span in many organisms [11,12]. Multiple mechanism of action have been proposed behind diverse health-promoting effects of resveratrol including, anti-oxidant, activation of sirtuins, calorie restriction (CR) mimetic, and recycling of ascorbic acid [9–12], however, the molecular targets of resveratrol that mediate its diverse biological effects remain speculative.

Vast amount of studies on resveratrol and different targets of cells/tissues are available but reports on membrane transporters modulatory effects of resveratrol are few and in case of human cells, very limited. The present study was conducted to evaluate the dose as well as time dependent effect of resveratrol on human erythrocyte PMCA and NKA pumps, and NHE activities, in control and experimental-oxidative stress condition. Effect of resveratrol on erythrocyte membrane integrity was also studied.

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2. Materials and methods

2.1. Selection of subjects

The study was carried out on 33 normal healthy subjects of both sexes between the ages of 21 and 45 years. The criteria of selection were as published earlier [12,13]. All volunteers were aware of the study protocol and gave their informed consent for the use of their blood samples for the study. The study protocol was in conformity with the guidelines of the Allahabad University Ethical Committee.

2.2. Isolation of packed red blood cells and preparation of erythrocyte ghosts

Venous blood under fasting condition was obtained in the morning by venipuncture in sterile polystyrene tubes containing heparin. Packed red blood cells (PRBCs) obtained after the removal of plasma, buffy coat, and the upper 15% of red blood cells (RBCs), were washed twice with cold phosphate buffered saline (PBS) (0.9% NaCl and 10 mM Na_2HPO_4; pH 7.4). Erythrocyte 'ghosts' from leucocyte-free RBCs were prepared by an osmotic shock procedure as described in an earlier [14] and protein content was determined with the method of Lowry et al. [15].

2.3. Determination of PMCA pump activity

Erythrocyte PMCA pump activity was determined as earlier [8]. Briefly, RBC ghosts were incubated for 30 min at 37 °C in medium containing 3 mM $MgCl_2$, 80 mM NaCl, 15 mM KCl, 0.1 mM EGTA; 50 mM Tris–HCl (pH 7.4) and 0.5 mM ouabain in the presence and absence of 0.2 mM $CaCl_2$. The reaction was started by the addition of 6 mM ATP. Calmodulin (40 units/ml) was present in all assays. The reaction was stopped by adding 0.5 M H_2SO_4, 0.5% ammonium molybdate and, 2% SDS. After 10 min, 0.04 ml of a solution containing 1.2% sodium metabisulfite, sodium sulfite and 0.2% of 1-amino-2-naphthol-4-sulfonic acid was added to each tube. After 30 min incubation followed by centrifugation at $800\times g$ for 5 min at 37 °C, the absorbance was determined at 650 nm. The activity of PMCA pump is expressed as μmol inorganic phosphate (Pi)/mg protein/h at 37 °C.

2.4. Measurement of NKA pump activity

The NKA pump activity was evaluated according to the methods of Suhail and Rizvi [16]. The final assay mixture contained 0.4–0.9 mg protein/ml, 140 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 30 mM imidazole (pH 7.25), with or without 0.5 mM ouabain and 6 mM ATP. Samples were incubated for 30 min at 37 °C and the reaction was stopped by the addition of 3.5 ml of a solution containing 0.5 M H_2SO_4, 0.5% ammonium molybdate and 2% SDS. The amount of Pi liberated was estimated according to the method of Fiske and Subbarow [17] and NKA pump is expressed as μmol Pi released/mg protein/h at 37 °C.

2.5. Estimation of NHE activity

The NHE activity in isolated erythrocytes was estimated as reported by Matteucci et al. [18]. Briefly, PRBCs were suspended in 150 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$ and 10 mM glucose, at 37 °C for 5 min under magnetic stirring. The cell suspension was brought to pH 6.35–6.45 within 10 min using 0.2 M HCl solution in 150 mM NaCl. Then, 4,4-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) was added and the pH of the medium was brought to 7.95–8.00 using 0.05 M NaOH solution in 150 mM NaCl. In a parallel experiment, amiloride was added with DIDS. Thereafter, proton efflux in the first minute was recorded. The rate of NHE is expressed as proton efflux μmol/l RBC per h at 37 °C, derived from differences in the rates of medium acidification in the absence and presence of amiloride.

2.6. Determination of osmotic stability of erythrocytes

The erythrocyte osmotic fragility was determined as described previously [19]. Briefly, PRBCs was added to tubes containing increasing concentrations (0%, 0.1%, 0.2%, 0.3%, 0.5%, 0.7%, 0.8%, and 0.9%) of phosphate-buffered sodium chloride (NaCl) solution at pH 7.4 in a final volume of 10 ml and incubated for 30 min at 37 °C. After centrifugation at $1500\times g$ for 10 min, absorbance of the supernatant was measured at 540 nm. Hemolysis in each tube was expressed as a percentage, using hemolysis in distilled water (0% NaCl) as 100%. The experiments were run in replicates of three or more to obtain statistically reliable data.

2.7. Experiments with resveratrol and induction of oxidative stress

Effect of resveratrol on membrane transporters was studied by incubating erythrocyte ghosts (0.8–1.5 mg of protein) with the resveratrol, at different doses in PBS (pH 7.4) for 1 h at 37 °C, prior to assay. Oxidative stress was induced *in vitro* by incubating washed erythrocytes/erythrocyte ghosts with 10 μM tertbutyl hydroperoxide (t-BHP) for 60 min at 37 °C. The concentration and duration of t-BHP used to induce oxidative stress in erythrocytes and ghosts was the same as described in previously published reports [12,13]. The effect of resveratrol was evaluated by co-incubating erythrocytes/erythrocyte ghosts with t-BHP and resveratrol for 60 min at 37 °C with mild shaking. After incubation period, for measuring membrane transporter activities, erythrocyte ghosts and to measure osmotic fragility intact erythrocytes, were washed twice with PBS at room temperature and subjected to assay.

2.8. Statistical analyses

Statistical analyses were performed using the software PRISM 5 for Windows (Graphpad Software Inc., San Diego, Calif., USA). Statistical differences were analyzed with Student's *t* test, and the differences were considered to be significant when $p < 0.05$. Data are expressed the as mean ± SD of 10–12 independent experiments.

3. Results

In vitro treatment of resveratrol with erythrocyte membrane caused stimulation of PMCA pump. The effect of resveratrol was concentration dependent; about 69% ($p < 0.001$) activation was observed at 10 μM whereas at 1 μM stimulation was 41% ($p < 0.05$) of the basal activity. Effect of resveratrol was insignificant at 0.1 μM (Fig. 1A). In the time dependent study, resveratrol (10 μM) showed maximum effect until 60 min of incubation, increasing the time beyond this period resulted in a lesser activation in enzyme activity. The Ca^{2+}-ATPase activity returned to basal level at 2 h of incubation with resveratrol (Fig. 1B). Inducing oxidative stress by t-BHP, caused a 32% ($p < 0.01$) inhibition of PMCA pump which was significantly ($p < 0.05$) protected by resveratrol at 1 μM. Resveratrol at 10 M further activated PMCA pump but the protection by resveratrol at 0.1 μM was not significant (Fig. 1C).

Incubation of resveratrol with erythrocyte membrane caused over expression of NKA pump which was about 66% ($p < 0.005$) at 10 μM and 37% ($p < 0.05$) at 1 μM than basal. The effect of

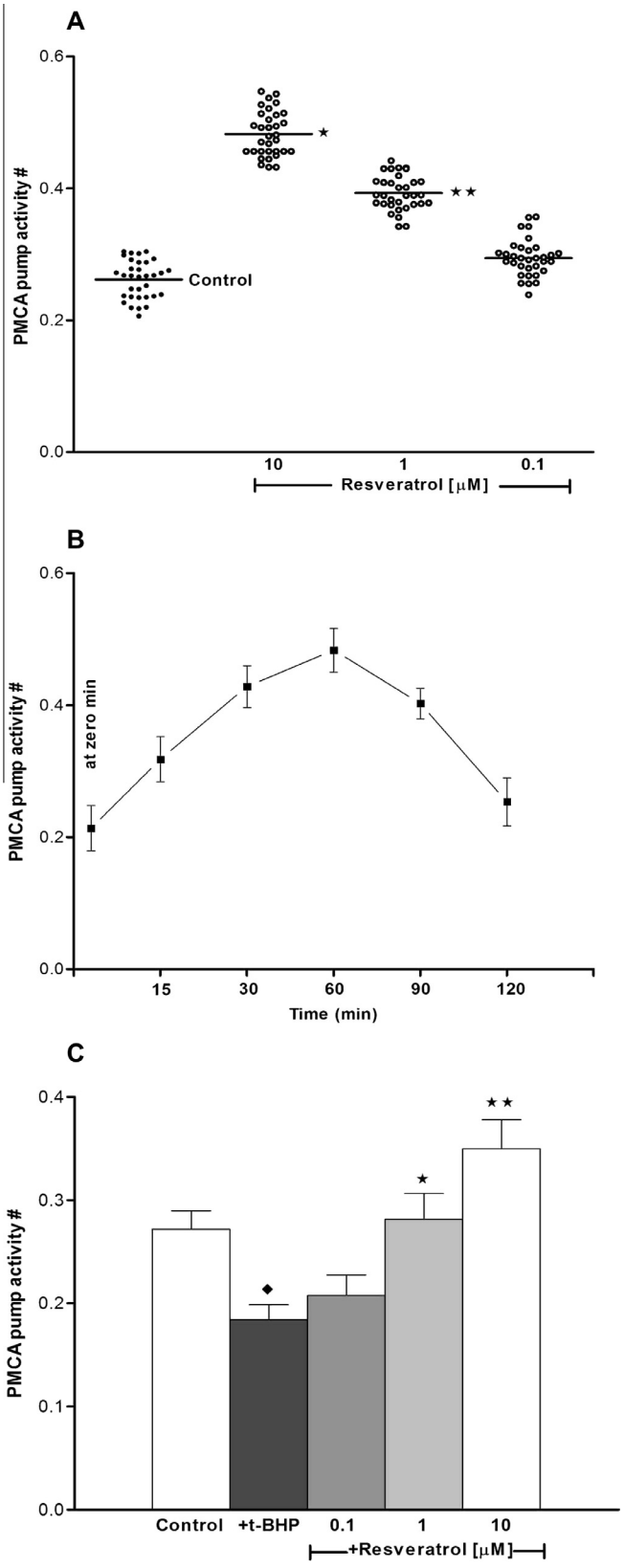

Fig. 1. (A) Concentration-dependent effect of resveratrol on erythrocyte PMCA pump activity. $n = 33$; $^{*}p < 0.001$ and $^{**}p < 0.05$ when compared with basal control. (B) Time-dependent effect of resveratrol (10 μM) on erythrocyte PMCA pump activity. (C) Dose-dependent effect of resveratrol on t-BHP induced oxidatively stressed erythrocyte PMCA pump activity. $n = 33$; $^{\blacklozenge}p < 0.01$, in comparison to control, $^{*}p < 0.05$, and $^{**}p < 0.001$ when compared with t-BHP treated. # PMCA pump activity is expressed as μmol of Pi released/h/mg membrane protein at 37 °C. Values are means ± SD of 10–12 independent experiments.

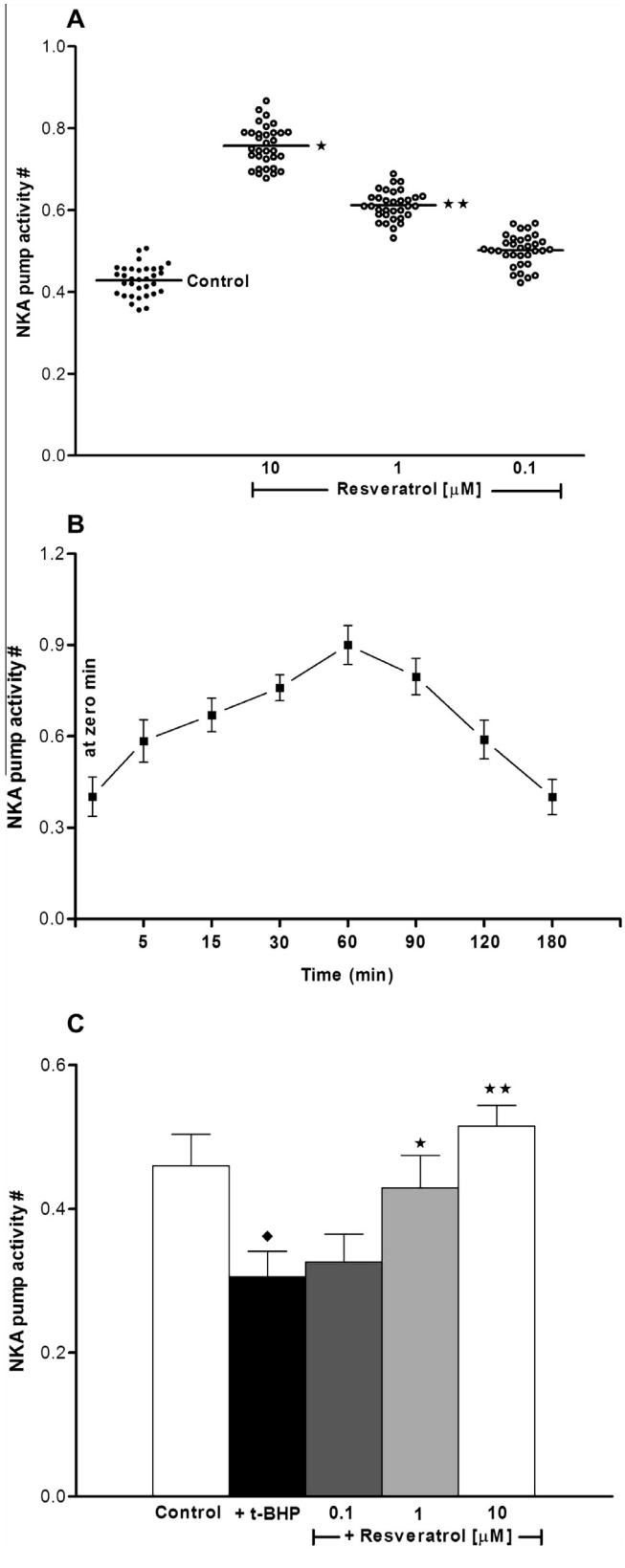

Fig. 2. (A) Concentration-dependent effect of resveratrol on erythrocyte NKA pump activity. $n = 33$; $^{*}p < 0.001$ and $^{**}p < 0.05$ when compared with basal control. (B) Time-dependent effect of resveratrol (10 μM) on erythrocyte NKA pump activity. (C) Dose-dependent effect of resveratrol on t-BHP induced oxidatively stressed erythrocyte NKA pump activity. $n = 33$; $^{\blacklozenge}p < 0.05$, in comparison of control, $^{*}p < 0.05$ and $^{**}p < 0.005$, in comparison to t-BHP treated. # NKA pump activity is expressed in terms of μmol pi released/h/mg membrane protein at 37 °C. Values are means ± SD of 10–12 independent experiments.

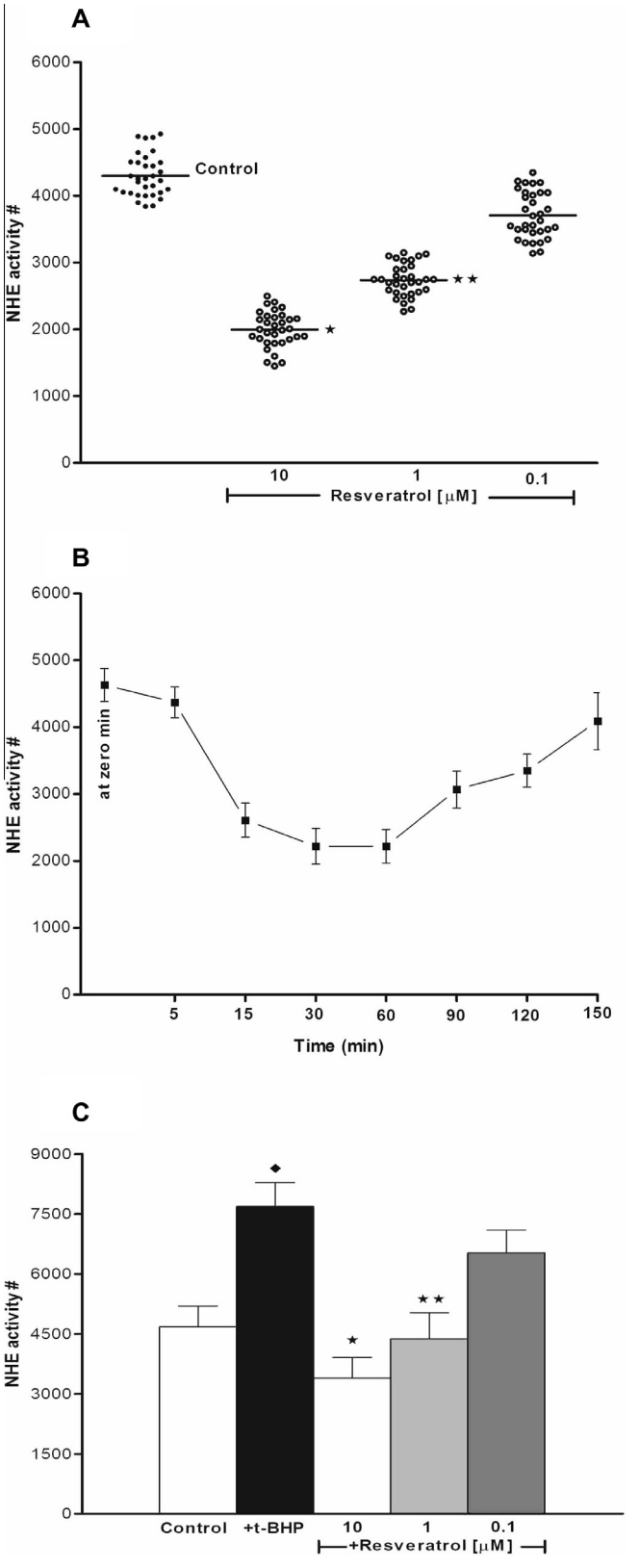

Fig. 3. (A) Concentration dependent effect of resveratrol on NHE activity. $n = 33$; $^{*}p < 0.001$ and $^{**}p < 0.05$ when compared with basal control. (B) Time-dependent effect of resveratrol (10 μM) on erythrocyte NHE activity. (C) Dose-dependent effect of resveratrol on t-BHP induced oxidatively stressed erythrocyte NHE activity. $n = 33$; $^{\blacklozenge}p < 0.005$, in comparison to control, $^{*}p < 0.001$ and $^{**}p < 0.01$, in comparison to t-BHP treated. # The NHE activity is expressed as proton efflux μmol/L RBC/h at 37 °C. Values are means ± SD of 10–12 independent experiments.

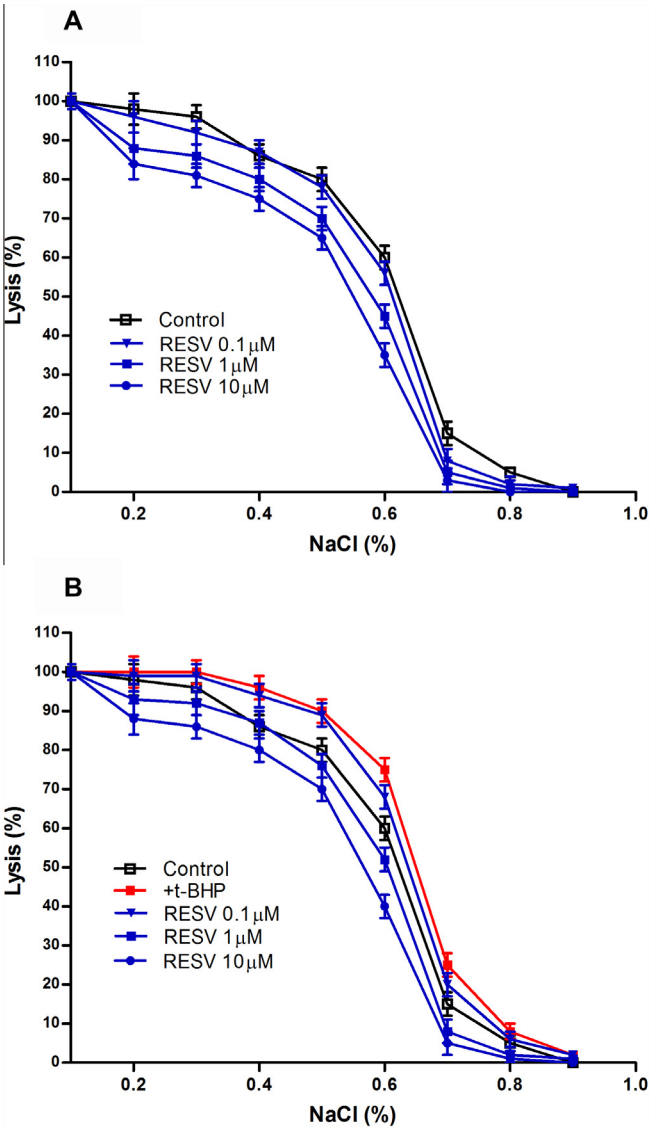

Fig. 4. Concentration dependent effect of resveratrol on osmotic fragility pattern of (A) control human erythrocytes. (B) t-BHP induced oxidative stressed human erythrocytes. Values are means ± SD of 10–12 independent experiments.

resveratrol on NKA pump was insignificant at 0.1 μM (Fig. 2A). The effect of resveratrol on NKA pump was fast; a significant ($p < 0.01$) activation of enzyme activity observed within 15 min of incubation. Optimum activation was noted at 60 min but resveratrol treatment was effective until 3 h of incubation (Fig. 2B). Subjecting erythrocyte ghosts to oxidative stress by t-BHP caused a 30% ($p < 0.05$) inhibition in the activity of NKA pump. Resveratrol at 1 μM concentration was able to neutralize almost inhibitory effect of t-BHP. No significant protection against t-BHP induced inhibition of NKA pump was observed at 0.1 μM resveratrol (Fig. 2C).

Treatment of erythrocytes ghosts with resveratrol down regulated the NHE activity in dose dependent manner. Resveratrol at 10 μM caused 54% ($p < 0.001$) and at 1 μM, 36% ($p < 0.05$) down regulation of the basal NHE activity. Similar to effect on ATPases, resveratrol at 0.1 μM concentration did not show significant modulation of NHE (Fig. 3A). Rapid response of resveratrol on NHE expression was observed; on 15 min of incubation significant ($p < 0.05$) effect was elicited by resveratrol which was increased until 60 min and after then reduction was observed, however it

was effective until 150 min of incubation (Fig. 3B). Induction of oxidative stress *in vitro* by t-BHP resulted in 62% ($p < 0.005$) activation of NHE, which was significantly reversed by co-incubation of 10 μM and 1 μM resveratrol. Resveratrol at 1 μM neutralized almost effect of t-BHP. The effect at 0.1 μM was insignificant (Fig. 3C).

Resveratrol protected the erythrocyte membrane integrity in dose dependent manner; higher protection at higher concentration (Fig. 4A). Erythrocytes showed increased susceptibility to osmotic fragility when incubated with t-BHP. Co-treated erythrocytes with resveratrol at different concentrations provided significant protection against t-BHP induced enhanced osmotic fragility and improved the osmotic stability of the erythrocytes (Fig. 4B).

4. Discussion

PMCA pump acts as a fine tuner of cytosolic Ca^{2+}, the important role of this enzyme is also reflected by its isoform-specific ubiquitous expression among different cell types [2]. Up-regulation of erythrocyte PMCA pump by resveratrol assumes significance since altered Ca^{2+} regulation due to diminished PMCA pump activity appears to play a major role in the development of cardiomyopathy as characterized by reduced contractibility, relaxation, and diastolic complications [20]. Reduced activity of PMCA pump may represent a common underlying abnormality linking the metabolic, cardiovascular, ocular, and neural manifestations [4,20,21]. Reduced activity of PMCA pump has also been reported during aging in humans [8].

Oxidative stress at cellular level has been documented in many pathological conditions [22]. Inhibited activity of PMCA pump during oxidative stress causes intracellular calcium overload which results in the loss of membrane integrity and glycoproteins that may lead to alterations in signal transduction pathways and ultimately development of cellular abnormalities [23,24]. Thus, the protection of oxidative stress induced PMCA pump inhibition by resveratrol may also be beneficial in impaired Ca^{2+} homeostasis mediated pathologies. Our results corroborate previous findings which documented that resveratrol attenuated ischemia/reperfusion in cardiomyocytes and maladies of acute pancreatitis by up-regulating PMCA pump, enhancing antioxidant potential and alleviating calcium overload in rats [25,26].

The ion gradient produced by NKA pump influences cell volume and osmotic pressure, acts as a driving force for inward co-transport of amino acids and monosaccharides [1]. Reduced NKA pump activity has been demonstrated in several degenerative diseases including Alzheimer, diabetes, cerebral ischemia, and vascular complications [16,26,27]. A decreased erythrocyte NKA pump activity has been implicated in the pathogenesis of neuropathy, neural electrophysiological abnormalities, and atherosclerosis in diabetes [27,28]. Besides, changes in NKA pump activity may also affect excitable tissues, skeletal muscle cells, and the pacemaker fibers of the heart [26,29]. Thus, up-regulation of NKA pump by resveratrol demonstrates the possible protective role of this stilbene against such degenerative diseases.

Oxidative stress mediated reduction in the activity of NKA pump has been repetitively reported during impairment of internal milieu of cells and implications in pathologies [8,30,31]. The inhibition of pump may be due to direct attack of free radicals resulting in Ca^{2+} overload in oxidative stress condition [31]. Resveratrol's ameliorative effect on NKA pump activity signifies the role of resveratrol in maintaining cellular redox state.

Inhibition of PMCA and NKA pumps by free radicals has been demonstrated in a variety of systems [24,31]. Diminished activities of both the pumps in pathological events and in oxidative stress has been hypothesized to be due to alteration in membrane fluidity [5,6,32]. Since PMCA and NKA pump are lipid dependent transmembrane enzymes, the cross-linking between the lipid peroxidation products and membrane proteins may be a possible explanation for the deactivation of membrane bound pumps in oxidatively stressed cells. Besides this, oxidation of thiol groups present in the structures of both the pumps may also provide a target, which could influence reduction in activity of these transporters [30–32]. In our previous studies, we have reported that resveratrol, in a dose dependent manner, prevented oxidative stress mediated oxidation of proteins, thiols, and lipids [12–14]; the antioxidative effect of resveratrol could also explain its observed effect on PMCA and NKA pumps.

^{13}C and NMR studies have reported that resveratrol interacts with the surface polar head groups of lipid bilayer [33]. We hypothesize that interaction of resveratrol at surface of bilayers may act to provide reduced access of membrane surface by free radicals, thus protecting the oxidative stress induced inhibition of ATPases. Stimulation of PMCA and NKA pumps by resveratrol may also be explained on the basis of the inhibitory effect of resveratrol on protein kinase C (PKC) [34], a negative regulator of the pump activity [35]. PKC modulates the activity of PMCA pump by phosphorylating target residues in its C-terminal tail, which is the main regulatory site of PMCA pump located towards cytosol, and binds with calmodulin (CaM). The phosphorylation by PKC decreased the stimulation by CaM, thereby down regulating the PMCA pump [36]. Inhibition of PKC by resveratrol may prevent phosphorylation, thereby stimulating the PMCA pump.

Regulated intracellular pH is necessary for several critical biological functions such as cell proliferation, invasion and metastasis, drug resistance and apoptosis [3,37]. Since activation of NHE is an early event in the response of cells to mitogenic growth factors, it appears likely that activation of NHE and resulting cellular alkalinization is a key mechanism in oncogenic transformation and is necessary for the development and maintenance of the transformed phenotype [38]. Inhibition of intracellular pH regulation may thus provide a target for effective cancer chemotherapy. In this context, down-regulation of NHE by resveratrol assumes significance and may provide a lead in prevention of carcinogenesis. A number of studies have frequently reported anti-cancer and tumor preventive effect of resveratrol in different model systems [9,10].

It has been suggested that down-regulation of NHE in erythrocyte membrane by resveratrol may be mediated *via* caspase-dependent repression of the gene promoter activity, without inducing cell death [39]. Resveratrol may trigger early activation of caspase 3 and late activation of caspase 6, which are not interdependent. Whereas, caspase 3 activation appeared to be a direct effect of resveratrol, and caspase 6 activation was mediated via intracellular hydrogen peroxide production and iron [39].

The NHE is a major mechanism by which the heart adapts to intracellular acidosis during ischemia and recovers from acidosis after reperfusion. Studies have proved the efficacy of NHE inhibitors as effective agents for limiting cardiac ischemic–reperfusion damage [3,40]. It has been suggested that selective NHE1inhibitors have clinical utility since they are able to markedly reduce infarct size when given before or during ischemia [41]. Evidence also implicates NHE1 in other cardiac disease states; the exchanger may be particularly critical to post infraction-remodeling responses resulting in development of hypertrophy and heart failure [40]. Our observation of an inhibitory effect of resveratrol on NHE also emphasizes the cardio-protective role of this stilbene.

An activated erythrocyte NHE has been reported in oxidative stress mediated degenerative diseases including heart diseases, oncogenic transformation, diabetes, and aging [3,7,8]. Activation of NHE was also observed during induced oxidative stress in erythrocytes after treatment with t-BHP. Over-expression of NHE activity may be correlated with reduced PMCA pump followed by

intracellular Ca^{2+} accumulation [40]. Inhibitory effect of resveratrol on oxidative stress mediated stimulation of NHE activity correlates with strong antioxidant potential of resveratrol.

In time dependent studies with resveratrol, all the transporters showed similar time periods required for maximum activity albeit slightly altered patterns, (Figs. 1B, 2B, 3B). Significant modulation of all the three membrane transporters by resveratrol within 15 min of treatment provides evidence that resveratrol interacts fast with membrane proteins. PMCA pump activity returned to basal level at 2 h of incubation but for NKA pump and NHE, it took more than 2 h for the same.

The effect of resveratrol on the activities of the NHE, PMCA, and NKA pumps in control as well as in oxidative stressed membranes correlate with the effect of resveratrol on stability of erythrocytes and its susceptibility to oxidative stress measured in terms of osmotic fragility (Fig. 4A and B). The red blood cell osmotic fragility profile provides an index of membrane integrity [19]. Enhanced osmotic fragility in t-BHP induced oxidatively stressed erythrocytes provides evidence for vulnerability of erythrocyte membrane to free radicals. Resveratrol treated erythrocytes were able to counteract the effect of free radicals and to restore the integrity of the membrane thus restoring normal transporter activities.

5. Conclusion

Results of the study prompt us to conclude that resveratrol is an effective modulator of PMCA, NKA pumps, NHE, and strong protector of membrane integrity. Since impaired ion homeostasis due to altered membrane transporters are involved in development and progression of many diseases and metabolic conditions, resveratrol may provide some protection against such pathologies.

Conflict of interest

Nil.

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