Research Paper

Possible involvement of membrane lipids peroxidation and oxidation of catalytically essential thiols of the cerebral transmembrane sodium pump as component mechanisms of iron-mediated oxidative stress-linked dysfunction of the pump's activity

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ABSTRACT

The precise molecular events defining the complex role of oxidative stress in the inactivation of the cerebral sodium pump in radical-induced neurodegenerative diseases is yet to be fully clarified and thus still open. Herein we investigated the modulation of the activity of the cerebral transmembrane electrogenic enzyme in Fe^{2+}-mediated in vitro oxidative stress model. The results show that Fe^{2+} inhibited the transmembrane enzyme in a concentration dependent manner and this effect was accompanied by a biphasic generation of aldehydic product of lipid peroxidation. While dithiothreitol prevented both Fe^{2+} inhibitory effect on the pump and lipid peroxidation, vitamin E prevented only lipid peroxidation but not inhibition of the pump. Besides, malondialdehyde (MDA) inhibited the pump by a mechanism not related to oxidation of its critical thiols. Apparently, the low activity of the pump in degenerative diseases mediated by Fe^{2+} may involve complex multi-component mechanisms which may partly involve an initial oxidation of the critical thiols of the enzyme directly mediated by Fe^{2+} and during severe progression of such diseases; aldehydic products of lipid peroxidation such as MDA may further exacerbate this inhibitory effect by a mechanism that is likely not related to the oxidation of the catalytically essential thiols of the ouabain-sensitive cerebral electrogenic pump.

Introduction

Reports have shown that the activity of Na^+/K^+-ATPase in many tissues and organs of mammalian systems can be profoundly inhibited under conditions of oxidative stress related diseases. Thus the pathophysiology, molecular and biochemical mechanisms underlying the reduced activity of the electrogenic enzyme in several free-radical linked degenerative and metabolic pathologies have been an area of intense study [1–5]. Although Na^+/K^+-ATPase is widely expressed in quite a number of tissues, the transmembrane protein is mainly expressed in brain and neuronal cells. Consequently, dysfunction of the pump has been characterized by neuronal hyperexcitability, depolarization and swelling [6]. Hence, the malfunctioning of the cerebral sodium pump has been associated with some specific neurological disorders such as diabetes, Alzheimer's disease, epilepsy and bipolar disorder among others. In these neurological pathologies, constant depolarization across the cell membrane induces an imbalance in the amount of neurotransmitters that are released within the cell [3,4,7–10].

More importantly, it has been observed that neurological disorders associated with low activity of the cerebral sodium pump have also been characterized by increased oxidative stress indices in the brain of such human or disease models. Thus there is an inverse relationship between extent of oxidative stress mediated damage and the activity of the Na^+/K^+-ATPase in these neurological dysfunctions [11]. However, earlier investigators have attempted to unravel the underlying mechanisms that participate in free radicals-induced dysfunction of the pump. In this regard, emerging data led to some speculative conclusions.

Firstly, it was suggested that reactive oxygen species (ROS) may evoke an inhibition on the enzyme indirectly. Herein, the ROS may attack lipids in the membrane that anchor the transmembrane

enzyme and consequently leading to an altered membrane microviscosity, which ultimately result in loss of the enzyme function [12,13]. On the other hand, ROS have been shown to directly attack and eventually modify amino acid residues of the enzyme thus leading to loss of activity of the transmembrane protein [14]. Yet some authors have speculated that aldehydic products which arise from the lipid peroxidation process may also interact with the transmembrane enzyme ultimately leading to its inactivation [15,16].

Despite these findings and observations however, the precise biochemical and molecular events that characterize free radical-induced dysfunction of the cerebral enzyme appear complex and far from being completely understood. Partly, the complexity arises from the multifactorial agents and factors that mediate oxidative stress. Therefore, in order to better understand the molecular events associated with oxidative stress-induced inactivation of the cerebral pump, the study of the participation of these agents and factors must be holistic, all inclusive and total. Evidently, unravelling these complex mechanism(s) is thus still open.

One of the most potent neurotoxic agent that exhibit prooxidant effect under in vitro and in vivo conditions is iron especially Fe^{2+}. In fact, reports have shown that impaired iron metabolism is an initial cause of some neurodegeneration mediated by ROS [17]. Moreso, several common genetic and sporadic neurodegenerative disorders have been associated with dysregulated iron homeostasis in the central nervous system (CNS) [17]. In general, iron accumulation causes neurodegeneration chiefly by inducing the formation of free radicals which eventually damage cellular macromolecules such as lipids, DNA and proteins. Furthermore, iron-induced oxidative stress is particularly dangerous because it can cause further iron release from iron-containing proteins such as ferritin, heme-proteins, and Fe–S clusters, forming a destructive intracellular positive-feedback loop that exacerbates the toxic effects of brain iron overload [17].

The harmful and deleterious effect of iron especially in the brain has been the prime interest of researchers. Primarily, this is because the mammalian brain is highly susceptible to free radical attack due to high oxygen tension and a high content of polyunsaturated fatty acids in cell membrane phospholipids [18]. Evidences suggest that iron invasion is an initial cause of neuronal cell death and axonal degeneration [19–21]. Moreso, a cardinal pathology associated with several common sporadic neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and Huntington's disease has been associated with iron accumulation. Findings have shown that under in vitro models relating to these pathologies, there is a strong coincidence between localized disturbed iron homeostasis and neuronal cell death in the brain [19,20,22–26]. Furthermore, several in vivo studies of iron regulatory proteins using transgenic rodents [27,28] have indeed suggested that iron may be a pathogenic factor for neurodegeneration. Indeed experimental evidences have suggested that iron chelators [20,29,30] and genetic manipulation resulting in low iron levels [29,31,32] are neuroprotective strategies against these iron-induced neurodegenerative diseases. Other central nervous system (CNS) disorders that have been proposed to be associated with disturbed iron homeostasis include the Freiderich's Ataxia [26], Restless Leg Syndrome [33], Ischaemic/Haemorrhagic Stroke [34] and Multiple Sclerosis [25].

From the foregoing, it is apparent that iron-mediated ROS production may be a suitable model in unravelling the precise mechanisms of ROS-mediated dysfunction of the ouabain-sensitive electrogenic sodium pump. Really, some authors have studied iron-induced oxidative stress models to study the inactivation of the cerebral pump's activity. In this regard, Rauchova and colleagues observed that iron-induced altered membrane fluidity thus

leading to the inhibition of the Na^+/K^+-ATPase activity at the initial process of lipid peroxidation. However, as lipid peroxidation progresses, the inhibition of the transmembrane enzyme strongly correlates only with the production of thiobarbituric acid reactive species (TBARS) and conjugated dienes (CD) [11]. However, their study is largely inconclusive and thus still open. Primarily, their study did not take into consideration the fact that the transmembrane enzyme is a sulphydryl protein and thus the possibility of a direct or indirect interaction between iron and the enzyme was not clarified and hence the need for the present study.

Materials and methods

Chemicals

Adenosine triphosphate (ATP), α-tocopherol, cysteine, reduced glutathione, dithiothreitol, thiobarbituric acid (TBA), were obtained from Sigma (St. Louis, MO). All other chemicals which are of analytical grade were obtained from standard commercial suppliers.

Animals

Male adult Wistar rats (200–250 g) from our own breeding colony were used. Animals were kept in separate animal cages, on a 12-h light: 12-h dark cycle, at a room temperature of 22–24 °C, and with free access to food and water. The animals were used according to standard guidelines on the Care and Use of Experimental Animal Resources.

Preparation of tissue homogenate

Rats were decapitated under mild ether anaesthesia and the cerebral tissue (whole brain) was rapidly removed, placed on ice and weighed. The brain was immediately homogenized in cold 10 mM Tris–HCl, pH 7.4 (1/10, w/v) with 10 up-and-down strokes at approximately 1200 rev/min in a Teflon–glass homogenizer. The homogenate was centrifuged for 10 min at 4000g to yield a pellet that was discarded and a low-speed supernatant (S1).

Incubation systems for TBARS and sodium pump assays

Aliquot of S1 was used for the assays of thiobarbituric acid reactive substances (TBARS) as well as Na^+/K^+-ATPase activity. For both assays the reaction mixture contained 3 mM MgCl, 125 mM NaCl, 20 mM KCl and 50 mM Tris–HCl, pH 7.4, $FeSO_4$ (final concentrations range of 1–100 μM), with and without α-tocopherol (final concentration, 1–100 μM) and with and without dithiothreitol (final concentration, 2 mM), with or without malondialdehyde (MDA) (1–100 μM) and 100–180 μg protein in a final volume of 500 μl. The reaction was initiated by addition of ATP to a final concentration of 3.0 mM. Controls were carried out under the same conditions with the addition of 0.1 mM ouabain. The reaction mixture was incubated at 37 °C for 30 min. At the end of the incubation time period, tubes were assayed for sodium pump activity and TBARS production.

Assay of sodium pump

The reaction system for the assay of the activity of cerebral Na^+/K^+-ATPase was essentially the same as described above under the section "incubation systems for TBARS and sodium pump". However, at the end of the incubation time period (30–60 min), the reaction was stopped by addition of 5% trichloroacetic acid. Released inorganic phosphorous (P_i) was measured by the method

of Fiske and Subbarow [35]. Na^+/K^+-ATPase activity was calculated by the difference between two assays (with and without ouabain). All the experiments were conducted at least three times and similar results were obtained. Protein was measured by the method of Lowry et al. [36], using bovine serum albumin as standard.

For all enzyme assays, incubation times and protein concentration were chosen to ensure the linearity of the reactions. All samples were run in duplicate. Controls with the addition of the enzyme preparation after mixing with trichloroacetic acid (TCA) were used to correct for non-enzymatic hydrolysis of substrates. Enzyme activity was expressed as nmol of phosphate (P_i) released min^{-1} mg protein^{-1}.

Assay of TBARS

The reaction system for the assay of production of the cerebral TBARS was essentially the same as described above under the section "incubation systems for TBARS and sodium pump". However, at the end of the incubation time period (30–60 min) production of TBARS was determined as described by the method of Ohkawa et al. [37] except that the buffer of colour reaction has a pH of 3.4. The colour reaction was developed by adding 200 µl 8.1% SDS to S1, followed by sequential addition of 500 µl acetic acid/HCl (pH 3.4) and 500 µl 0.8% of thiobarbituric acid (TBA). This mixture was incubated at 100 °C for 1 h. TBARS produced were measured at 532 nm and the absorbance was compared to that of a standard curve obtained using malondialdehyde (MDA).

Oxidation of thiols

The rate of dithiothreitol (DTT) oxidation was determined in the presence of 50 mM Tris–HCl, pH 7.4, and varying concentration of Fe^{2+} (10–100 µM). The rate of thiol oxidation was evaluated by measuring the disappearance of –SH groups. Free –SH groups were determined according to Ellman [38]. Incubation at 37 °C was initiated by the addition of DTT (final concentration, 2 mM). Aliquots of the reaction mixture (100 µl) were checked (at 1 h interval for 3 h) for the amount of –SH groups at 412 nm by the addition of the colour reagent 5'5'-dithio-bis (2-nitrobenzoic) acid (DTNB).

Statistical analysis

Results were analysed by appropriate analysis of variance (ANOVA) and this is indicated in text of results. Duncan's Multiple Range Test was applied. Differences between groups were considered to be significant when $p < 0.05$.

Results

Effect of Fe^{2+}, DTT and vitamin E on cerebral MDA production

In order to better explore the experimental results, separate and combined analysis of variance were conducted on the experimental data. As depicted in Fig. 1 (panel a), one-way ANOVA analysis indicate that Fe^{2+} elicited a biphasic effect on the production of aldehydic product of lipid peroxidation with lower
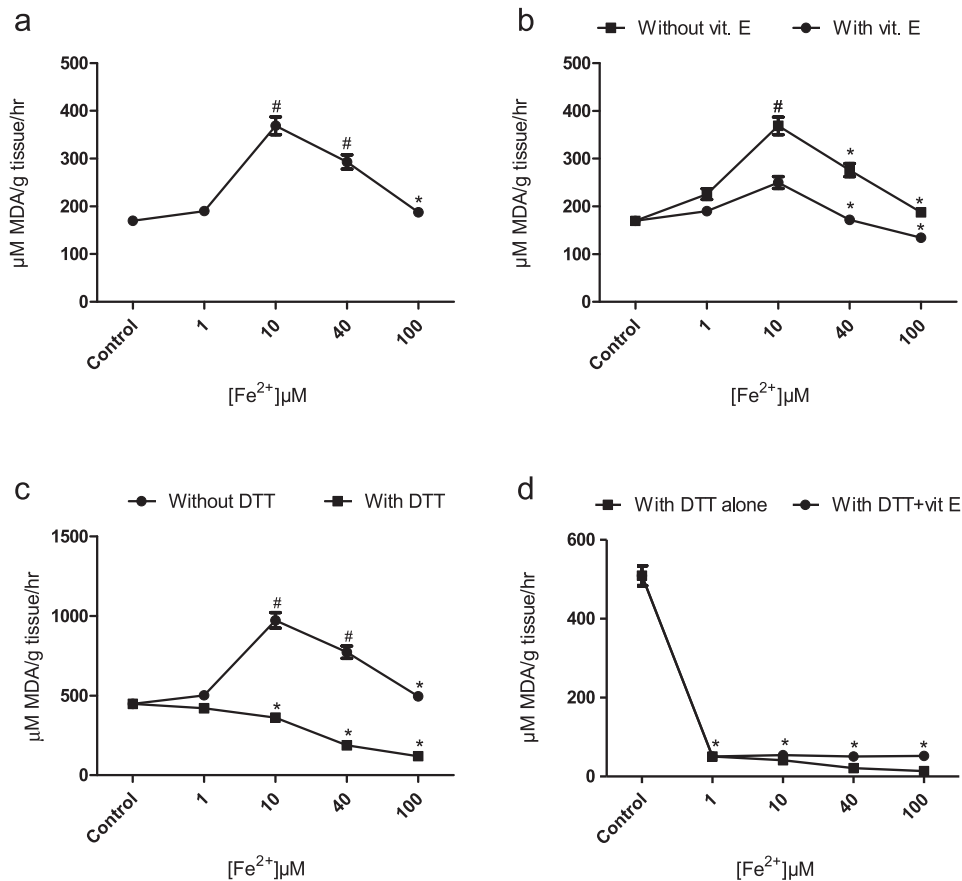

Fig. 1. Biphasic effect of iron (II) on MDA production from cerebral tissue homogenate (panel a). The influence of vitamin E (100 µM) (panel b), DTT (2 mM) (panel c) and combination of both 100 µM vitamin E and 2 mM DTT (panel d) on the biphasic effect iron (II) are shown as indicated. Data are presented as mean ± SEM of at least three independent experiments carried out in different days. Data were analysed using appropriate analysis of variance followed by Duncan test as indicated in the description of results. *Significantly lower from control; #significantly higher than control ($p < 0.05$).

concentrations evoking an increase in lipid peroxidation and higher concentrations of Fe^{2+} appear to be counteracting the production of these aldehydic products of lipid peroxidation. Conversely, in the presence of vitamin E, a potent antioxidant, two-way ANOVA [(with and without vitamin E) × (5 concentrations of Fe^{2+})], of the results obtained revealed that the production of aldehydic products of lipid peroxidation elicited by Fe^{2+} was markedly hindered (Fig. 1, panel b). However, since earlier data has shown that DTT could protect the oxidation of thiols especially on the cerebral sodium pump, attempt was made to investigate the possible inhibitory effect of this dithiol on lipid peroxidation. Similar to the result presented in Fig. 1 (panel b), two-way ANOVA [(with and without DTT) × (5 concentrations of Fe^{2+})] clearly revealed that DTT have marked inhibitory effect on the biphasic pattern of aldehydic productions elicited by Fe^{2+} (Fig. 1, panel c). The possible effect of the combination of both vitamin E and DTT on the lipid peroxidation process was also tested. In this regard, three-way ANOVA [(with and without DTT) × (with and without vitamin E) × (5 concentrations of Fe^{2+})] clearly revealed that the lipid peroxidation process was inhibited in the presence of vitamin E whether alone or in combination with DTT (Fig. 1, panel d). Suffice to mention that for all analysis, mean values are considered significant from each other when $p < 0.05$.

Effect of Fe^{2+}, vitamin E and DTT on cerebral Na^+/K^+-ATPase activity

In the reaction system for the assay of the cerebral sodium pump, the effect of increasing concentration of Fe^{2+} on the activity of the pump was tested. Herein it was observed that one-way ANOVA distinctly revealed that Fe^{2+} evoked a concentration-dependent decrease in the activity of the cerebral transmembrane sodium pump (Fig. 2, panel a). Based on this observed effect of

Fe^{2+} on the activity of the cerebral pump and considering Fig. 1a, it is rational to speculate that lipid peroxidation process may be linked to the observed inhibitory effect of Fe^{2+} on the activity of the enzyme. Hence the possible protective effect of vitamin E, a potent inhibitor of lipid peroxidation on the inhibitory effect of Fe^{2+} on the activity of the cerebral pump was tested. Two-way ANOVA analysis [(with and without vitamin E) × (5 concentrations of Fe^{2+})] of the data obtained revealed that vitamin E had no protective effect on Fe^{2+}-induced inhibition of the activity of the cerebral pump (Fig. 2, panel b). Furthermore, since DTT has already been documented to protect against oxidation of sulphydryl groups of the enzyme, the possible involvement of oxidation of the critical thiols of the enzyme as a component of its inhibition by Fe^{2+} was also investigated. Two-way ANOVA [(with and without DTT) × (5 concentrations of Fe^{2+})] showed that DTT exerted considerable protective effect on the activity of the enzyme (Fig. 2, panel c). In fact, three-way ANOVA (with and without vitamin E) × (with and without DTT) × (5 concentrations of Fe^{2+}) revealed that when both vitamin E and DTT were combined they exerted a marked protective effect on the activity of the enzyme (Fig. 2, panel d).

Effect of vitamin E on activity of Na^+/K^+-ATPase

The effect of increasing concentration of vitamin E on the activity of the ouabain sensitive cerebral electrogenic pump is presented in Fig. 3. One-way ANOVA apparently revealed that the transport function of the electrogenic pump was not affected by the potent antioxidant.
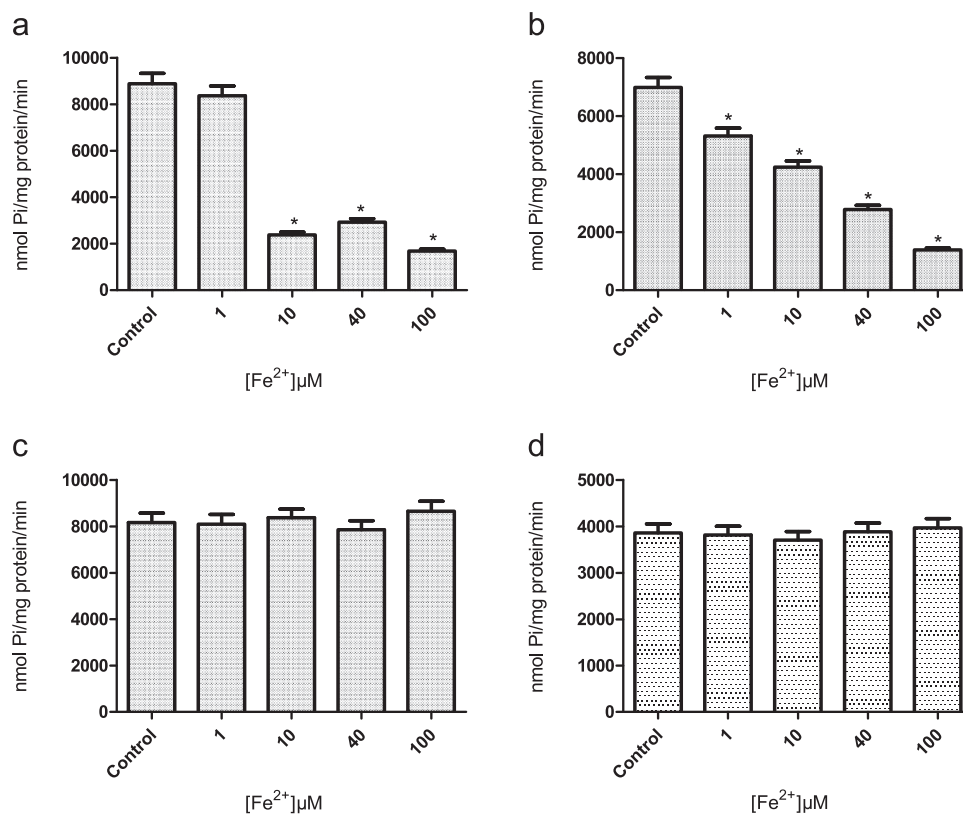

Fig. 2. Effect of iron (II) on the activity of the cerebral sodium pump (a). The different panels shows the effect of 100 μM vitamin E (panel b), 2 mM DTT (panel c) and combination of both vitamin E and DTT (panel d) on the activity of the cerebral sodium pump subjected to iron (II) assault. Data are presented as mean ± SEM of at least three independent experiments carried out in different days. Data were analysed using appropriate analysis of variance followed by Duncan test as indicated in the description of results. *Significantly lower from control; #significantly higher than control ($p < 0.05$).
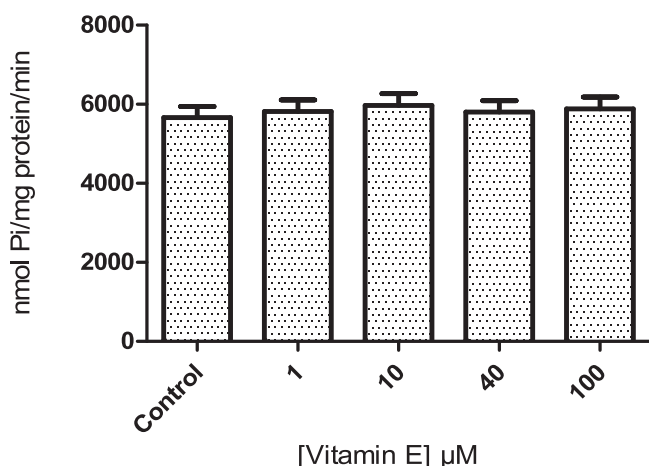
Fig. 3. Effect of vitamin E on Na^+/K^+-ATPase activity. Data are presented as mean ± SEM of three independent experiments carried out in different days. Data were analysed using one-way ANOVA followed by Duncan test ($p < 0.05$).

Effect of Fe^{2+} on oxidation of dithiothreitol

As shown in Fig. 4, the rate of DTT oxidation was increased by Fe^{2+}. Two-way ANOVA (control/Fe^{2+} × 4 sample times) or (control/4 concentrations of Fe^{2+} × 4 sample times) revealed a significant Fe^{2+} versus time interaction ($p < 0.001$). In fact, the oxidation of DTT was increased as a function of time in the presence of Fe^{2+}.

Effect of MDA on activity of Na^+/K^+-ATPase

Fig. 5 (panel a) shows the effect of increasing concentration of MDA on the activity of Na^+/K^+-ATPase. One-way ANOVA indicates that MDA inhibits the pump in a concentration dependent manner. Furthermore the inhibition of the cerebral enzyme by MDA was not markedly ($p < 0.05$) modified in the presence of DTT (Fig. 5, panel b).
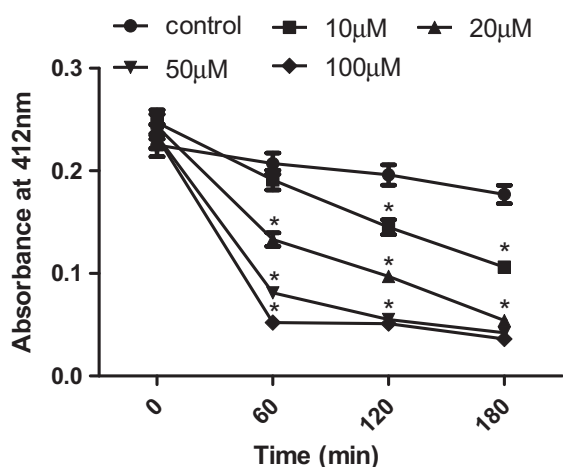

Fig. 4. Effects of iron on the rate of dithiothreitol oxidation. The rate of oxidation was evaluated at the indicated times and concentrations of iron. Data are the means of five to seven independent experiments carried out in different days. Data are expressed as mean ± SEM and post-hoc comparisons were done by Duncan's multiple range test. *Significant difference in relation to the control.

Discussion

Excellent reports and reviews have clearly shown that the transmembrane Na^+/K^+-ATPase is a target in oxidative stress-linked degenerative diseases (such as diabetes, Alzheimer diseases etc.) in which free radicals participate in their pathophysiology [39,40] and thus in human and animal models of these degenerative diseases, there is an inverse relationship between extent of oxidative stress and activity of this important transmembrane enzyme [41,42]. However, the precise molecular events that characterize the role of oxidative stress in the dysfunction of the enzyme's activity is yet to be fully clarified. In any case, a definitive answer to the molecular puzzle on the precise role of oxidative stress in the loss of transport function of the Na^+/K^+-ATPase in degenerative diseases may represent a key step in the development of the proper treatment of such diseases.

Several authors have made elegant attempts to unravel the precise mechanisms that are involved in the pathophysiological processes leading to the inactivation of the pump's activity under conditions of oxidative stress. Thus far, available data clearly indicate that oxidative stress mediated altered membrane dynamics strongly correlated with altered activity of the sodium pump [43,44]. In this regard also, the sequential molecular events that eventually led to pump's malfunction remain obscure and thus experimental data may be warranted to further clarify these molecular processes.

Based on earlier findings [19,20,22–28], we decided to use an in vitro model of Fe^{2+}-mediated oxidative stress to simulate possible in vivo oxidative stress situation in the brain. In fact, iron has been shown to react with superoxide anion ($O_2^{\bullet}$) and hydrogen peroxide (H_2O_2) to produce the hydroxyl radical ($^{\bullet}OH$) via the Fenton reaction [57]. These radicals can also lead to the formation of other reactive oxygen species (ROS) [56]. Ultimately, the overproduction of ROS can directly attack the polyunsaturated fatty acids of the cell membranes and induce lipid peroxidation. Hence, this model may be ideal to study the possible correlation between oxidative stress and the transport function of the transmembrane enzyme under study.

Firstly, it is worthwhile mentioning that at low concentrations, Fe^{2+} markedly increased the formation of aldehydic products of lipid peroxidation which is indicated by increase in the calculated malondialdehyde content of the brain tissue. However, at high concentration of Fe^{2+} this transition metal appears to inhibit the lipid peroxidation process (Fig. 1, panel a). This observation is a paradox and quite intriguing. In any case, earlier authors have suggested that when in excess, Fe^{2+} could compete with lipids as electron donors for $Fe^{3+}O_2^{-\bullet}$ (perferryl ion), thereby inhibiting hydrogen abstraction and thus lipid peroxidation [45–47]. Conversely, there is the likelihood that the quantification of lipid peroxidation products using thiobarbituric acid could also detect lipid hydroperoxides. Hence, at high iron concentrations, there is the strong possibility that excess iron could likely decompose lipid hydroperoxides and ultimately decrease the observed level of thiobarbituric acid reactive substances observed in this study. Notwithstanding, at low concentrations, Fe^{2+}-induced cerebral lipid peroxidation may be an excellent model to simulate in vivo situations involving oxidative stress assault on plasma membrane lipids in the brain and nervous cells. The implication of the above argument is that Fe^{2+} could disorganize membrane lipid bilayer arrangement of the brain cells and leading to altered membrane integrity and ultimately perturbed membrane fluidity. Expectedly, these series of events may lead to a parallel disorganization and eventual loss of function of the membrane bound protein.

Consistent with the above reasoning, Fig. 2 (panel a) clearly shows that Fe^{2+} inhibited the activity of the ouabain-sensitive electrogenic pump in a concentration dependent manner. In sharp
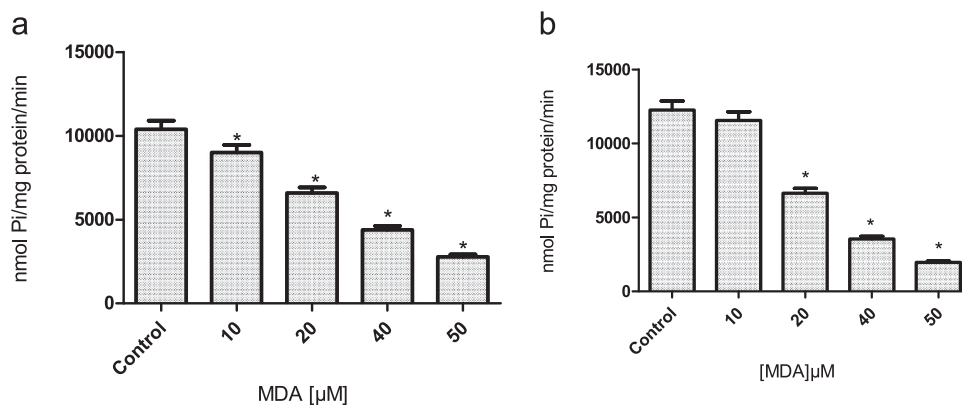
Fig. 5. Effect of varying concentrations of MDA on the activity of Na^+/K^+-ATPase in the absence (a) and presence of DTT (b). Data are presented as mean ± SEM of three independent experiments carried out in different days. Data were analysed using one-way ANOVA followed by Duncan test. *Significant difference from control at $p < 0.05$.

contrast with what was observed in Fig. 1 (panel a), high concentrations of the transition metal exerted a more profound inhibitory effect on the activity of the cerebral pump. Interpretation of this intriguing data may be complex. However, one could speculate that at low doses, Fe^{2+} may ultimately generate the highly reactive radical •OH via Fenton reactions. Next the reactive radical may assault cerebral membrane bound lipids, causing their peroxidation and ultimately altering membrane fluidity. In this case, the structural integrity of the transmembrane pump is adversely altered leading to a loss of its activity. On the other hand, there is a strong line of evidence from the present result to suggest that Fe^{2+} may bind directly to essential groups on the enzyme thus evoking a loss of its activity. This reason is plausible considering the fact that at high concentration there was inhibition of lipid peroxidation and yet the activity of the enzyme (Fig. 2, panel a).

Based on the above findings, we developed some conditions to further clarify the possible inter-relationship between of oxidative stress and the activity of the pump. In this regard, attempt was made to inhibit lipid peroxidation with an antioxidant agent that would possibly not affect the activity of the pump. Herein, vitamin E, a classical antioxidant was evaluated for its possible inhibitory effect on the activity of the cerebral sodium pump. The result (Fig. 3) clearly shows that this potent antioxidant when acting alone has no effect on the activity of the transmembrane sodium pump. With this fascinating result, this classical antioxidant was then employed to counteract the oxidative assault evoked by Fe^{2+} on brain lipids. As demonstrated in Fig. 1 (panel b), vitamin E effectively inhibited the lipid peroxidation process mediated by Fe^{2+} assaults at all concentrations tested. Conversely, while this potent antioxidant inhibited lipid peroxidation, it did not protect against inhibitory effect of Fe^{2+} on the activity of the enzyme (Fig. 2, panel b). This fact strongly suggests that in addition to inducing a peroxidation of cerebral lipids, the transition metal may also be interacting directly with this transmembrane protein.

Therefore, based on the latter speculation above, a reaction system was also developed to investigate the possibility of a direct interaction between Fe^{2+} and the transmembrane sodium pump that ultimately led to loss of its function. The reaction system was developed based on previous knowledge on the structure and function of the cerebral pump. Earlier authors and emerging data in our laboratory have consistently demonstrated that the cerebral sodium pump has sulphydryl groups that are critical to its activity and that these thiols can be inhibited by oxidizing agents such as selenium-containing compounds and inorganic mercury [39,40,48–53]. In these reports [48,51,52], the authors observed that these compounds oxidize critical thiol groups on the enzyme and that endogenous thiols could prevent or relieve the inhibition

imposed on the enzyme by these thiol oxidizing compounds.

Therefore in order to explore the possibility of Fe^{2+} oxidizing the catalytically important sulphhydryl groups of the cerebral sodium pump, an in vitro method was developed to investigate the ability of Fe^{2+} to oxidize dithiothreitol alone. Interestingly, as shown in Fig. 4, Fe^{2+} profoundly increased the oxidation of the dithiol in a time dependent fashion. This observation when translated may indicate that the toxic effect of this transition metal on the activity of the cerebral pump may be partly linked with its ability to oxidize essential thiols on the electrogenic pump that may be critical to its catalytic function.

In order to verify the possible participation of the oxidation of essential thiol on the electrogenic sodium pump by Fe^{2+} as a component of its inhibitory mechanism, the incubation condition developed allowed for the pre-incubation of dithiothreitol (DTT) with the cerebral enzyme before the assault of Fe^{2+}. The data obtained (Fig. 2, panel c) shows that when DTT was preincubated with the enzyme before the assault of Fe^{2+}, the dithiol completely prevented the inhibition of the enzyme's activity earlier evoked by Fe^{2+} assault as previously observed in Fig. 2 (panel a). Intriguing as this finding may seem, it is noteworthy that DTT also exerted a marked inhibitory effect on the lipid peroxidation process initiated by Fe^{2+} (Fig. 1, panel c). As a strong reductant, the mechanism by which DTT inhibited lipid peroxidation may be partly related to its ability to reduce the transition metal.

With the above findings describing the individual effect of vitamin E and the dithiol on both the activity of the cerebral sodium pump as well as the inhibition of lipid peroxidation processes, another incubation condition was carried to evaluate the combined effect of both vitamin E and the dithiol on the lipid peroxidation process and the activity of the pump. The results obtained clearly show that combination of vitamin E and DTT prevented lipid peroxidation evoked by Fe^{2+} (Fig. 1, panel d) and also relieved the inhibitory effect of Fe^{2+} on the activity of the electrogenic sodium pump (Fig. 2, panel d). From the foregoing, it is rational to summarize that there may be a strong basis to partly conclude that Fe^{2+} evoke a deleterious effect on the activity of the pump through direct and indirect mechanisms as earlier reported [11].

Moreso, it is a common knowledge that Fe^{2+} can ultimately generate the highly reactive •OH, and that this reactive oxygen species can attack proteins and ultimately damage several amino acid residues, including histidine, tryptophan, cysteine, proline, methionine, arginine and lysine. Hence, oxidative damage to several of these amino acid residues and/or to the peptide backbone of proteins has been reported to generate carbonyl products [48,54]. Thus the activity of the enzyme can also be compromised under direct assault of •OH. Suffice to mention that the

quantification of these carbonyl products was not a component of this study and may be a subject of future research.

However, Esterbauer et al. [55] have observed that the degradation products of lipid peroxidation process can have direct effect on the activity of enzyme protein molecule. Basically, lipid peroxidation products are mainly aldehydic products and can be generally quantified by reacting them with thiobarbituric acid to give a purple/pink colour. Among the aldehydic products, malondialdehyde (MDA) have been widely reported as the key aldehyde. Therefore, the study sought to also investigate the possible effect of malondialdehyde on the activity of the cerebral pump. As presented in Fig. 5 (panel a), MDA significantly inhibited the activity of the cerebral sodium pump in a concentration dependent manner. Further study also reveals that the inhibitory effect of MDA on the activity of the pump was not protected by the dithiol (Fig. 5, panel b).

In conclusion, within the limit of our present data and following the arguments thus far, it is apparent that the low activity of the pump in degenerative diseases mediated by Fe^{2+} may involve complex multi-component mechanisms which may partly involve an initial oxidation of the critical thiols of the enzyme directly mediated by Fe^{2+}. To the best of our knowledge, this fact has not been presented in the literature. In addition, however, the transition metal may also assault membrane lipids and thus evoke an altered membrane fluidity which ultimately leads to altered structural arrangement of the cerebral sodium pump with a concomitant loss or reduced activity of the enzyme. However, during severe oxidative-stress mediated progression of such degenerative diseases; aldehydic products of lipid peroxidation such as MDA may further exacerbate deleterious effect of Fe^{2+} by a mechanism that is likely not related to the oxidation of the essential thiols of the ouabain-sensitive cerebral electrogenic pump.

Acknowledgements

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