Physiol. Res. 48:73-78, 1999

The Role of Membrane Fluidity Changes and Thiobarbituric Acid-Reactive Substances Production in the Inhibition of Cerebral Cortex Na^+/K^+-ATPase Activity

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Received April 10, 1998
Accepted October 26, 1998

Summary

Lipid peroxidation of rat cerebral cortex membranes was induced by Fe^{2+}/ADP and ascorbate. The rate of Na^+/K^+-ATPase inhibition was correlated with the increase of thiobarbituric acid-reactive substances (TBARS) and conjugated dienes (CD) and with membrane fluidity changes. Our data showed that membrane fluidity changes (evaluated by fluorescence steady-state anisotropy measurements) can participate in Na^+/K^+-ATPase inhibition during the initial period of lipid peroxidation process, whereas during the following period the enzyme inhibition correlates only with TBARS and CD production.

Key words

Cerebral cortex membranes • Na^+/K^+-ATPase • Lipid peroxidation • Membrane fluidity

Introduction

A large integral plasma membrane-bound transport enzyme Na^+/K^+-ATPase controls directly or indirectly many essential cellular functions. Its primary function is the maintenance of intracellular K^+ concentration and active excretion of Na^+ from the cell. This enzyme is inhibited by oxygen reactive substances as was demonstrated in the brain (Brechtlová *et al.* 1996, Domanska-Janik and Bourre 1990, Jamme *et al.* 1995, Kaplán *et al.* 1997, Mishra *et al.* 1989), cerebrovascular endothelial cells (Lo and Betz 1986, Stanimirovic *et al.* 1995), kidneys (Kako *et al.* 1988, Rauchová *et al.* 1995, Thomas and Reed 1990), the heart (Kim and Akera 1987) or erythrocytes (Rohn *et al.* 1996, Stojadinovic *et al.* 1996).

Although the problem concerning lipid peroxidation and plasma membrane Na^+/K^+-ATPase activity is known for more than twenty years (Sun 1972, Svoboda *et al.* 1984), it is still open.

The mechanism of enzyme inactivation is not fully clarified because at least two independent mechanisms may be involved in Na^+/K^+-ATPase inhibition. The enzyme may be inactivated indirectly by changes in membrane microviscosity (Jamme *et al.* 1995, Stanimirovic *et al.* 1995) or directly by modification of protein polypeptide molecules of the enzyme complex by oxygen reactive substances

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(Boldyrev and Bulygina 1997) or by aldehydic products which arise from the lipid peroxidation process (Mark *et al.* 1997, Siems *et al.* 1996).

Plasma membrane Na^+/K^+-ATPase plays an important role in the functional activity of nervous cells and brain which are considered as highly vulnerable to free radical attack due to high oxygen tension and a high content of polyunsaturated fatty acids in cell membrane phospholipids (Halliwell 1992). We therefore used cerebral cortex membranes for obtaining more details of the molecular mechanisms participating in the inactivation of Na^+/K^+-ATPase by oxygen reactive substances.

In our present study we evaluated the time course of Na^+/K^+-ATPase inactivation after induction of lipid peroxide formation by Fe^{2+}/ADP and the ascorbate prooxidant system. We correlated these changes of enzyme activity with changes in membrane fluidity, with the production of thiobarbituric acid-reactive substances (TBARS) and with conjugated dienes (CD) formation. Our data showed that the effect of membrane fluidity changes can be considered during the first period of the enzyme inactivation, whereas the decrease of residual enzyme activity is associated only with the direct effect of oxygen reactive substances and peroxidation products on the enzyme molecule.

Methods

The experimental animals were Wistar rats of both sexes fed a standard diet. Rats were decapitated and the brains were rapidly dissected on a cooled block. The cerebral cortex was placed into ice-cold 250 mM sucrose, 10 mM Tris-HCl, 1 mM Na-EDTA, pH 7.4 and the 10 % homogenate was stored at −20 °C. Frozen-thawed cerebral cortex homogenates were centrifuged for 60 min at 100 000 x *g* in a Beckman L 90 ultracentrifuge. Pellets of total cerebral cortex membrane fraction were suspended in 250 mM sucrose, 10 mM Tris-HCl, 1 mM Na-EDTA, pH 7.4.

The samples of cerebral cortex membranes (final concentration: 0.5 mg protein/ml) were incubated in 154 mM NaCl in duplicate under aerobic conditions at 37 °C in a shaking water bath. Lipid peroxidation was induced by 50 μM $FeSO_4$ and 3 mM ADP (Fe^{2+}/ADP) and 0.4 mM ascorbate. Samples were taken at the appropriate time period for Na^+/K^+-ATPase, TBARS, CD and fluorescence steady-state anisotropy measurements.

For determination of TBARS formation, 1 ml of incubation suspension (0.5 mg protein) was mixed with 1 ml of a solution containing TCA-TBA-HCl reagent (Buege and Aust 1978). The mixture was allowed to proceed in the dark for approximately 20 h at room temperature as described by Erdahl *et al.* (1991). Then the samples were centrifuged to remove the precipitate and the absorbance of supernatant was determined at 535 nm. The results were expressed as nmol TBARS formed/mg protein using an extinction coefficient (E_{535}=1.56 x 10^{-5} mol^{-1} cm^{-1}).

CD formation was measured as an index of A_{233nm}/A_{215nm}. Aliquots of suspended or incubated membranes containing 0.010 and 0.020 mg protein per ml were added to 10 mM K-phosphate buffer, pH 7.4 with 1 % Lubrol similarly as was done by Kaplán *et al.* (1995).

Membrane order was evaluated by using two probes: nonpolar 1,6-diphenyl-1,3,5-hexatriene (DPH) and polar 1-[4(trimethylamino)phenyl]-1,3,5-hexatriene (TMA-DPH). Samples of membranes (about 0.080 mg protein per ml) were suspended in 0.1 M K-PO_4 buffer, pH 7.4. The final concentrations of DPH and TMA-DPH probes were 2.10^{-7} M. Fluorescence steady-state anisotropy values were determined using a Perkin-Elmer luminescence spectrometer LS 50 B. The excitation and emission wavelengths were 350 and 430 nm, respectively.

Na^+/K^+-ATPase activity was determined spectrophotometrically as the release of inorganic phosphate. The reaction mixture for total ATPase contained about 0.020 mg protein in 50 mM Tris-HCl, 100 mM NaCl, 20 mM KCl and 5 mM $MgCl_2$ (final volume: 0.75 ml). The Na^+/K^+-ATPase activity was calculated as the difference between total ATPase and the activity in the presence of 5 mM ouabain in the reaction mixture containing 0.020 mg protein, 50 mM Tris-HCl, 120 mM NaCl and 5 mM $MgCl_2$ (final volume: 0.75 ml). ATP was added after 5 min preincubation to start the Na^+/K^+-ATPase reaction. After 10 min incubation at 37 °C the reaction was terminated by the addition of 0.375 ml of 12 % ice-cold trichloroacetic acid (final concentration was 4 %). The samples were kept on ice, centrifuged at 2 000 x *g* at 4 °C for 15 min. The concentration of inorganic phosphate liberated from ATP was determined in the supernatant by the method of Taussky and Shorr (1953). Protein concentrations were determined by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as the standard.

All chemicals used were of the purest grades available. L-ascorbic acid, ADP, ATP, ouabain, Tris(hydroxymethyl)aminomethane (Tris), and thiobarbituric acid were obtained from Sigma Chemical

Co. (USA), fluorescent probes from Molecular Probes (USA). Other chemicals used were purchased from Lachema (Czech Republic).

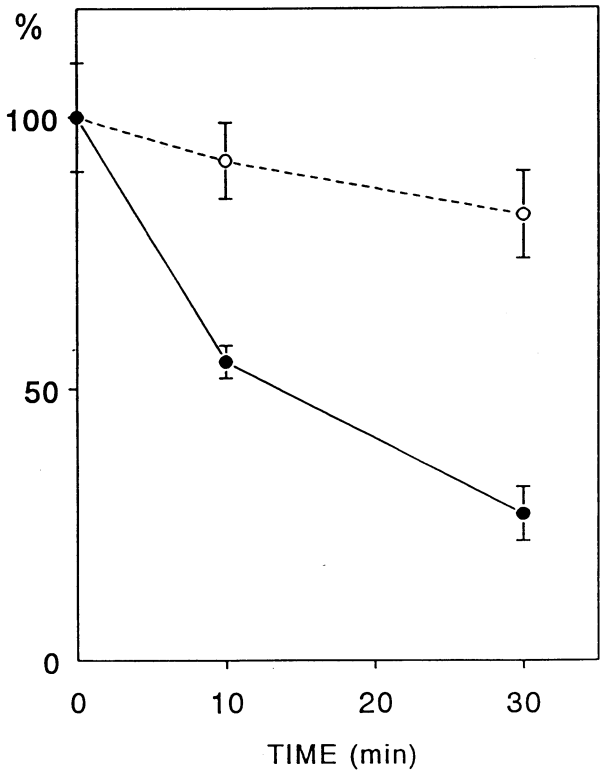

Fig. 1. *Inhibition of Na^+/K^+-ATPase activity in isolated cerebral cortex membranes in the absence and in the presence of Fe^{2+}/ADP and ascorbate (peroxidizing conditions). The enzyme activity is expressed as percentage of the control values. The 100 % corresponds to 600.47±62.24 nmol P/min/mg protein. Empty circles represent control values, full circles peroxidizing conditions. Each point represents the mean of four experiments ± S.E.M.*

Results

Figure 1 demonstrates the decrease of Na^+/K^+-ATPase activity in cerebral cortex membranes during incubation in the absence and presence of lipid peroxide inducers (Fe^{2+}/ADP and ascorbate). After 30 min of incubation in the presence of lipid peroxide inducers the enzyme activity was decreased by 73 %. In the absence of lipid peroxide inducers the activity during 30 min of incubation only decreased by 18 %. Figure 1 also shows that in the first 10 min of incubation the rate of enzyme inactivation was faster than during the following period of incubation.

Table 1. Steady-state anisotropy values using DPH and TMA-DPH probes in isolated cortex membranes in the absence and in the presence of Fe^{2+}/ADP and ascorbate (peroxidizing conditions)

Steady-state anisotropy (min)	Without Fe^{2+}/ADP + ascorbate	With Fe^{2+}/ADP + ascorbate
DPH probe		
0	0.193 ± 0.002	–
10	0.196 ± 0.004	0.244 ± 0.004
30	0.194 ± 0.002	0.246 ± 0.004
60	–	0.238 ± 0.001
TMA-DPH probe		
0	0.247 ± 0.005	–
10	0.245 ± 0.005	0.267 ± 0.006
30	0.248 ± 0.002	0.265 ± 0.007
60	–	0.267 ± 0.008

Data present means ± S.E.M. values of four separate experiments.

Figure 2A depicts the time course of the increase of TBARS production induced by Fe^{2+}/ADP and ascorbate. The rate of TBARS formation shows a progressive enhancement during 2 h incubation. The small amount of TBARS (0.28 nmol/mg protein, i.e. 1 %) was also found in non-incubated membranes and in membranes incubated for 10 min in the absence of lipid peroxide inducers.

Figure 2B illustrates the increase of CD formation in the presence of Fe^{2+}/ADP and ascorbate.

In membrane fluidity studies, we used two probes, nonpolar probe DPH and polar probe TMA-DPH to evaluate the membrane fluidity of surface and lipid core of cerebral cortex membranes. Table 1 presents the fluorescence steady-state anisotropy values for DPH and TMA-DPH probes. After a 10 min period of peroxidation, anisotropy values of both probes were significantly increased in comparison with non-incubated samples or samples incubated in the absence of lipid peroxide inducers. During the 10 or 30 min period of incubation no further changes in fluorescence steady-state anisotropy were found. The prolonged peroxidation time (3-6 hours) did not increase the values of steady-state anisotropy (data not shown).

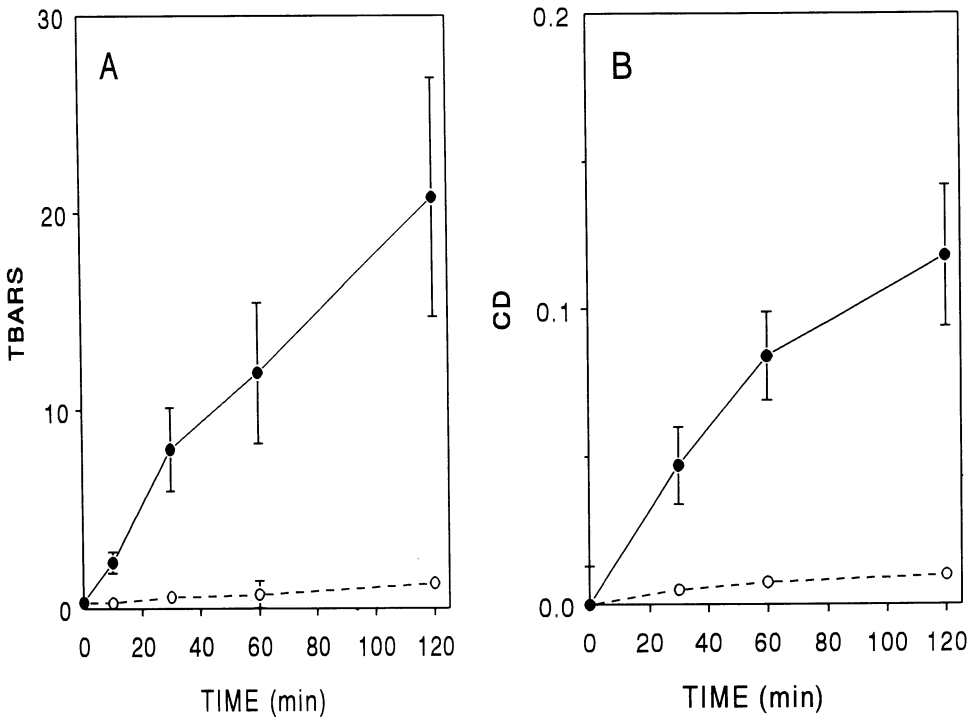

Fig. 2. *Time-course studies of TBARS formation (A) and CD formation (B) in isolated cerebral cortex membranes in the absence and in the presence of Fe^{2+}/ADP and ascorbate (peroxidizing conditions. A. The values were corrected by subtraction of the value of endogenous TBARS. Results are expressed as nmol TBARS/mg protein. Empty circles represent control values, full circles peroxidizing conditions. Each point represents the mean of four experiments ± S.E.M. B. The values were corrected by subtraction of the value of endogenous CD. Empty circles represent control values, full circles peroxidizing conditions. Each point represent the mean of four experiments ± S.E.M.*

Discussion

Lipid peroxidation is one of the main events induced by oxidative stress. Biological membranes altered by lipid peroxidation are postulated to be one of the underlying causes of a variety of physiological or pathological states (aging, ischemia, injury neurodegenerative diseases). Good evidence exists that the major damaging role is played by oxidants, e.g. in reoxidation injury or posttraumatic degeneration of the brain (Braughler and Hall 1989, Hall and Braughler 1989).

One of the consequences of lipid peroxidation degenerative processes can also result in enzyme activity changes. To find the optimum conditions for protection of membrane-bound enzymes that are affected by oxygen reactive substances, more data are required for elucidating the molecular mechanisms of enzyme inactivation. Two mechanisms which may inhibit the enzyme activity must be considered. The first one is the indirect effect of oxygen reactive substances on enzyme activity through modification of membrane fluidity, because the activity of most membrane-bound enzymes is regulated by the physicochemical state of their lipid environment. Experimental data have shown that parallel changes of membrane fluidity and activity of enzymes are associated with membrane lipids (Amler *et al.* 1990, Yu *et al.* 1992) or changes in receptor binding (Ghosh *et al.* 1993). The second mechanism through which membrane-bound enzymes may be inhibited by oxidative stress is the direct action of oxygen reactive substances (Boldyrev and Bulygina 1997) and degradation products of lipid peroxidation process on the enzyme protein molecule (Esterbauer *et al.* 1991). In order to obtain some additional data that could help to elucidate this problem, we correlated the time course of inhibition of the Na^+/K^+-ATPase activity during the peroxidation process induced by Fe^{2+}/ADP and ascorbate with changes of fluorescence steady-state anisotropy and production of TBARS and CD.

Our data have revealed two different rates of Na^+/K^+-ATPase inactivation: a rapid one during the first

period after induction of the lipid peroxidation process which is followed by slower rate of inhibition of residual enzyme activity. During the rapid phase, the enzyme activity may depend on both mechanisms, i.e. on an indirect effect of membrane fluidity changes (changes of lipoprotein interactions) and on the direct effect of oxygen reactive substances and TBARS on enzyme molecule. Because membrane fluidity changes reached a maximal plateau after 10 min peroxidation, subsequent inactivation of residual enzyme activity could be due to the direct effect of oxygen reaction substances and malondialdehyde products on the enzyme molecule. We also found that the time course of membrane fluidity changes and TBARS and CD production induced by Fe^{2+}/ADP and ascorbate is quite different. This may indicate differences in the capacity of particular processes. The membrane fluidity changes can be modified within very narrow limits and thus might be more rapid and detectable in the shorter incubation period. These findings further support our previous conclusions that two different mechanisms, namely an indirect effect of membrane fluidity changes and a direct effect of oxygen reactive substances and TBARS, can participate in the inhibition of Na^+/K^+-ATPase activity.

It is surprising that the production of TBARS and CD is going on after 10 min, while fluorescence steady-state anisotropy values reach their maximum and do not increase further after a longer time period. This effect could be explained by DPH (or TMA-DPH) quenching in regions where the production of free radicals takes place. In such a case, the contribution of DPH (or TMA-DPH) molecules to the total fluorescence intensity from this region is relatively lower with respect to DPH (or TMA-DPH) population from more fluid parts of the membrane, having consequences for the fluorescence steady-state anisotropy values.

Acknowledgments

This work was partially supported by research grants 303/95/0615 and 303/98/0473 from the Grant Agency of the Czech Republic.

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