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ALTERATION IN RABBIT BRAIN ENDOPLASMIC RETICULUM Ca^{2+} TRANSPORT BY FREE OXYGEN RADICALS IN VITRO

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SUMMARY. The involvement of free oxygen radicals in ischemia-reperfusion injury is generally accepted. We describe here the loss of efficiency of reticular membranes to sequester Ca^{2+} due to free oxygen radical damage *in vitro*. Based on experimental results we suggest that the primary effect of free oxygen radicals is alteration of the lipid component of the membrane manifested in the increase of passive Ca^{2+} leak. Prolonged treatment of microsomes with free oxygen radical generating systems also led to the decrease of Ca^{2+}-ATPase activity which is caused as we suppose by modulation of the lipid-Ca^{2+} pump interactions. A protective effect of butylated hydroxytoluene on the depression in the Ca^{2+} uptake and Ca^{2+}-ATPase activities supports our suggestions.

Calcium is an important second messenger, which triggers and regulates many cellular processes in all cell types including neurons (1). Excitation of cells is caused by the entry of extracellular calcium via electrically and chemically operated Ca^{2+} channels localized on the plasma membrane and by the release of calcium from the endoplasmic reticulum (ER). ER is believed to play an important role in the intracellular Ca^{2+} handling in the brain as well (2). This intracellular organelle appears to include the intracellular Ca^{2+} pool responsive to the $InsP_3$-mediated agonists and/or Ca^{2+} pool sensitive to caffeine (3). In addition, endoplasmic reticulum modulates its own filling by the entry of extracellular Ca^{2+} via a putative unidentified mediator (4). Ca^{2+} overload is thought to be an important factor in irreversible ischemic cell damage (5) and impairment of intracellular Ca^{2+} transport might be one of the leading causes of disorder in the Ca^{2+} homeostasis. Lack of oxygen during ischemia leads to decreased ATP level and therefore to the failure of ATP-driven ion transport systems (5). On the other hand, ischemia and mainly reperfusion are believed to create conditions favourable for the generation of free oxygen radicals (6). The latter have been implicated as the possible mediators of cellular injury through nonspecific modification and disruption of proteins, phospholipids and nucleic acids (7,8).

So far, the effect of free oxygen radicals on Ca^{2+} dependent ATPase and Ca^{2+} transport activities of sarcoplasmic reticulum from different muscle types has been investigated (9,10); but, there is no report describing the effect of free oxygen radicals on Ca^{2+} transport and Ca^{2+}-ATPase activities in brain endoplasmic reticulum. This study addresses examination of the possible changes in the brain endoplasmic reticular Ca^{2+} transport activity due to the presence of free oxygen radicals generated *in vitro*.

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METHODS

The brains of adult rabbits were removed after decapitation and the cerebrum was separated from cerebellum and mesencephalon. The endoplasmic reticular fraction was isolated from the cerebrum according to Edelman et al. (11). The final preparation was suspended in 5 mM phosphate buffer pH=7.4 containing 0.32 M sucrose. The purity of the final microsomal preparation was examined by measuring the sensitivity of Ca^{2+} uptake to oxalate as well as to 5 μM of vanadate. Unlike plasmalemmal enzyme, the ER Ca^{2+}-stimulated ATPase activity is inhibited by higher concentrations of vanadate ($K_{0.5}$=10-100 μM) (12,13). The Ca^{2+} uptake values in the presence of 5 μM vanadate reached 90.4 per cent of control levels. There was no Ca^{2+} uptake in the absence of oxalate.

Isolated microsomes (7 mg/ml) were preincubated with free oxygen radical generating systems for appropriate times at 0°C or at 37°C. Hydroxyl radicals were generated by the Fenton reaction by adding 0.185 μmol of H_2O_2/mg of microsomal protein with subsequent addition of 0.185 μmol $FeSO_4$-EDTA /mg of protein. Alkoxy radicals and peroxy radicals were generated by adding 0.185 μmol $FeSO_4$-EDTA/mg of protein and 0.185 μmol $FeCl_3$-EDTA/mg of protein, respectively. Ions of Fe^{2+} and Fe^{3+} react with lipoperoxides, naturally occurring in biomembranes as a product of spontaneous oxidation of polyunsaturated fatty acids, to produce alkoxy and peroxy radicals which initiate further lipid peroxidation (14). When the effect of H_2O_2 was investigated, 0.185 μmol of H_2O_2/mg of protein was added to the isolated microsomes. In the experiments where the effect of butylated hydroxytoluene (BHT) was assayed, samples were prepared by preincubation of microsomes with 0.185 μmol/mg protein of BHT for 30 min. prior to the incubation with oxidants.

ATP dependent Ca^{2+} uptake was measured by rapid filtration technique. Microsomes (100 μg of protein) were preincubated at 37°C for 5 min. in 1.9 ml of medium containing 100 mM HEPES pH=7.0, 100 mM KCl, 5 mM $MgCl_2$, 10 mM K_2-oxalate, 5 mM NaN_3 and 1 mM EDTA. $CaCl_2$ was added to yield 10 μM of ionized Ca^{2+}. Ca^{2+} uptake was initiated by adding 100 μl of 100 mM ATP pH=7.0. After 10, 20, 30, 45 and 60 min. of incubation at 37°C, aliquots of 200 μl were immediately filtered through Millipore filters (HA, 0.45 μm), then washed with 5 ml of stop solution (20 mM imidazole-HCl pH=7.0, 100 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA) and dried. Radioactivity was measured by liquid scintillation spectrometer TriCarb 300 C. Ca^{2+} uptake values were calculated from the intercept of cpm dependence on incubation time.

Ca^{2+} dependent ATPase activity was determined by coupled enzyme assay as described by Michelangeli et al.(15) with slight modification. Microsomes (13.5 μg of protein) were incubated at 37°C in medium containing 5 mM HEPES pH=7.0, 5mM $MgCl_2$, 1 mM EGTA, 5 mM NaN_3, 0.44 mM phosphoenolpyruvate, 0.4 mM NADH, 81 μg Triton X-100, pyruvate kinase (10 units) and lactate dehydrogenase (22 units) in a total volume of 0.5 ml. ADP production was monitored by measurement of NADH oxidation at 340 nm (Pharmacia LKB Ultrospec III). The Ca^{2+} dependent activity was calculated by subtracting the activity in the absence of Ca^{2+} (basal rate) from that obtained after addition of the appropriate concentration of Ca^{2+}.

Free Ca^{2+} concentration was calculated by Cabuf software, generously provided by Dr. G. Droogmans (K. U. Leuven, Belgium).

Protein concentration was determined by the Lowry et al. (16) method.

Results are presented as mean ± S.E.M. and Student's t-test was used to determine differences between the means. Values statistically different from the control are indicated by asterisks ($P<0.05$) or by double asterisks ($P<0.005$).

RESULTS

The effect of free oxygen radicals, generated *in vitro*, on ATP dependent Ca^{2+} uptake was investigated at 0°C (Fig. 1) and at 37°C (Table 1). As shown, the preincubation of microsomes

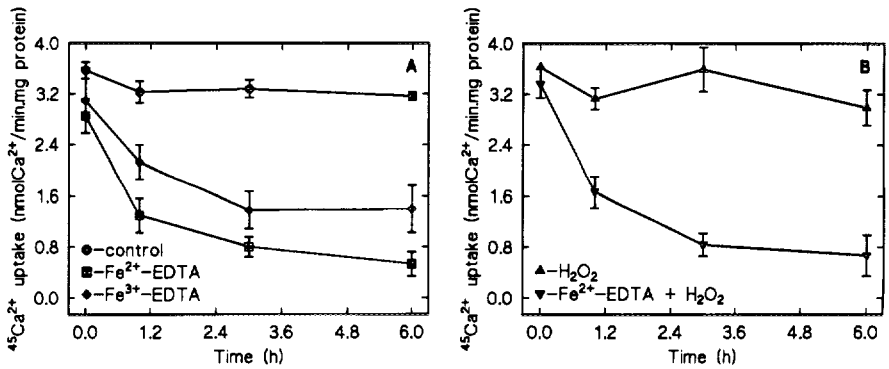

Fig. 1.Effects of free oxygen radicals on the microsomal Ca^{2+} transport

Results are shown as mean ± S.E.M. of 3-6 experiments. Microsomes were preincubated for indicated times at 0°C with appropriate compounds, and then the activities were assayed. Concentrations of compounds and uptake measurement were as indicated in Methods.

either with Fe^{2+}-EDTA complex or with H_2O_2 plus Fe^{2+}-EDTA complex led at both temperatures to the rapid decrease of Ca^{2+} uptake activity. The decline of Ca^{2+} uptake rate was not so rapid when microsomes were preincubated with Fe^{3+}-EDTA complex and reached a minimum constant level, after about 3 hours. Control samples and samples preincubated with H_2O_2 under the same conditions showed no significant loss of Ca^{2+} uptake in the given time period.

Ion dependent ATPase activity is an inherent hydrolytic property of P-type ionic pump protein including sarco/endoplasmic reticular Ca^{2+} pump (17). In the next series of experiments Ca^{2+} dependent ATPase activities were measured by preincubation of microsomes with free oxygen radical generating compounds at 0°C. As can be seen in Fig. 2, Fe^{2+}-EDTA and H_2O_2 plus Fe^{2+}-EDTA complex also caused decrease of Ca^{2+}-ATPase activity; however, this process was

Table 1 Effects of free oxygen radicals on the microsomal Ca^{2+} transport

	ATP-dependent Ca^{2+} uptake (nmol Ca^{2+}/min.mg protein)	
Sample	- BHT	+ BHT
Control	3.55 ± 0.37	3.16 ± 0.44
Fe^{2+}-EDTA	0.28 ± 0.06**	2.95 ± 0.54
Fe^{2+}-EDTA + H_2O_2	0.33 ± 0.06**	2.97 ± 0.36
Fe^{3+}-EDTA	0.95 ± 0.16**	3.01 ± 0.50
H_2O_2	3.20 ± 0.31	2.61 ± 0.31

Results are shown as mean ± S.E.M. of 3-4 experiments. Microsomes were preincubated for 30 min at 37°C with appropriate compounds, and then the activities were assayed. Samples with BHT were preincubated with antioxidant for 30 min at 0°C before treatment with oxidants. Concentrations of compounds and uptake measurement were as indicated in Methods.

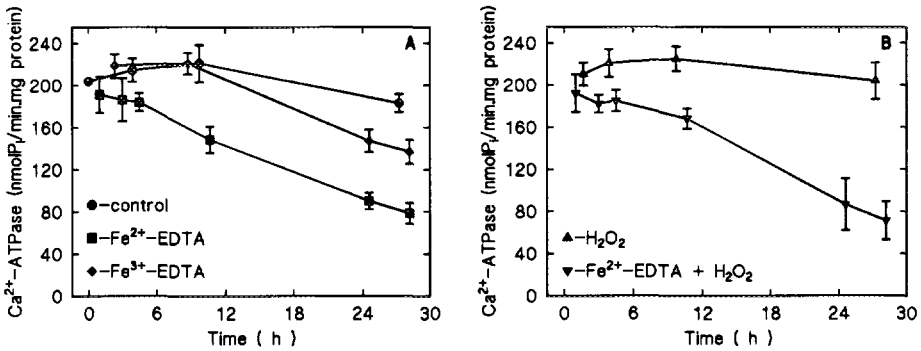

Fig. 2.Effects of free oxygen radicals on the microsomal Ca^{2+} ATPase activity

Results are shown as mean ± S.E.M. of 3 experiments. Microsomes were preincubated for indicated times at 0°C with appropriate compounds, and then the activities were assayed. Concentrations of compounds and activity measurement were as indicated in Methods.

not so rapid as in the case of Ca^{2+} uptake. More significant changes in Ca^{2+}-ATPase activities were observed only after about 10 hours of preincubation with alkoxy and hydroxyl radical generating systems. At the same time, only a slight decrease of Ca^{2+}-ATPase activity was shown with Fe^{3+}-EDTA complex. The control samples as well as samples preincubated with hydrogen peroxide showed no significant change in the Ca^{2+}-ATPase activity. Table 2 shows the changes caused by oxygen free radicals on the kinetic parameters of Ca^{2+} dependent ATPase activity after 24 hours of incubation. Fe^{2+}-EDTA and H_2O_2 plus Fe^{2+}-EDTA complex led to the drop of V_{max} values without significant changes in the affinity for Ca^{2+}. Only slight decreases of V_{max} value have been observed when microsomes were preincubated with Fe^{3+}-EDTA complex.

Table 2 Effects of free oxygen radicals on the kinetical parameters of microsomal Ca^{2+} ATPase activity

Sample	$K_{0.5}$ (μM)	V_{max} (nmol P_i/min.mg protein)
Control	0.48 ± 0.06	183.5 ± 7.8
Control + BHT	0.49 ± 0.04	177.5 ± 11.7
Fe^{2+}-EDTA	0.44 ± 0.04	88.6 ± 4.4**
Fe^{2+}-EDTA + BHT	0.47 ± 0.01	183.4 ± 38.1
Fe^{2+}-EDTA + H_2O_2	0.41 ± 0.03	88.6 ± 5.2**
Fe^{2+}-EDTA + H_2O_2 + BHT	0.51 ± 0.06	154.2 ± 1.7
Fe^{3+}-EDTA	0.46 ± 0.07	136.9 ± 11.9*
Fe^{3+}-EDTA + BHT	0.46 ± 0.01	203.3 ± 30.3
H_2O_2	0.45 ± 0.07	173.3 ± 4.8
H_2O_2 + BHT	0.40 ± 0.08	181.3 ± 0.2

Results are shown as mean ± S.E.M. of 3-4 experiments performed in triplicate. Microsomes were preincubated for 24 hours at 0°C with appropriate compounds, and then the activities were assayed. Samples with BHT were preincubated with antioxidant for 30 min at 0°C before treatment with oxidants. Concentrations of compounds and activity measurement were as indicated in Methods.

The effect of membrane-soluble antioxidant BHT (7) was also investigated. Table 1 shows the results of experiments using the antioxidant activity of BHT on microsomal Ca^{2+} uptake. Preincubation of membranes with BHT led to the recovery in Ca^{2+} uptake levels in all investigated samples (Table 1). In addition, BHT demonstrated a protective effect on the depression in V_{max} for Ca^{2+}-ATPase activity (Table 2).

DISCUSSION

Production of radicals during brain ischemia and reperfusion was experimentally documented, but the nature of those radicals is unknown (18). The aim of this study was to determine the effect of free oxygen radicals generated in vitro on the rabbit brain Ca^{2+} transport across endoplasmic reticulum membranes. Experiments were partly thought as modelling for ischemia-reperfusion injury. Several mechanisms of reticular Ca^{2+} transport alteration can be considered:
- free oxygen radicals and/or iron may initiate lipid peroxidation and fatty acid chain disruption (7,14). This disruption can lead to "leakiness" of the membrane for Ca^{2+} ions and to modulation of membrane proteins including the Ca^{2+} pump
- free radicals can cause oxidation of sulfhydryl groups in Ca^{2+} pump protein (19) and other modifications of Ca^{2+}-ATPase polypeptide chain as was described for a number of proteins (20).

As seen in Figs. 1 and 2, Fe^{2+} and Fe^{3+} complexes, as well as the hydroxyl radical generating system led to the decrease of both Ca^{2+} uptake and Ca^{2+}-ATPase activities; however, the decrease of Ca^{2+}-ATPase activity was not so rapid as was observed for Ca^{2+} uptake rate. It appears, that our results, like the results of Dinis et al. (10) and Okabe et al. (21), indicate that free oxygen radicals preferentially impair the membrane integrity. The likely cause is the increase of the passive Ca^{2+} leak and therefore the decrease of net Ca^{2+} uptake. In fact, free oxygen radicals are known to initiate chain reactions with membrane polyunsaturated fatty acids (PUFA) which produce lipoperoxides and consequently the break in the fatty acid chain (7,14). Damage to PUFA tends to alter membrane fluidity (10), and to change membrane permeability allowing passage of substances that do not normally cross it (Ca^{2+} ions) as documented by Okabe et al. (21) regarding cardiac sarcoplasmic reticulum membrane. In our study we have also shown the protective effect of the membrane-soluble antioxidant BHT on Ca^{2+} uptake as well as on Ca^{2+}-ATPase activity (Table 1, 2).

The decrease of Ca^{2+}-ATPase activity occurred only after prolonged preincubation with free oxygen radical generating systems. It is generally believed that hydrolysis of ATP comprises only partial reaction of complex Ca^{2+} transport cycle. We suppose that the significant decrease of ATPase activity occurring only after prolonged incubation is due to continued long-term fragmentation of fatty acid chains, which leads to the more profound changes in membrane composition and therefore to subsequent apparent modulation of Ca^{2+}-ATPase activity (22). Similarly the results obtained by Moore et al. (23) indicate that for optimal function the Ca^{2+}-ATPase requires a "fluid" membrane containing unsaturated phospholipid acyl chains. Hydrogen peroxide, thought to be a sulfhydryl group oxidizing agent (24), seems to be controversial regarding its effect on cellular Ca^{2+} transport. It inhibited the heart sarcolemmal Ca^{2+}-ATPase

(25) and demonstrated an inhibitory effect on sarcoplasmic Ca^{2+}-ATPase in a concentration-dependent manner (26). In another series of experiments at lower concentration it did not, however, affect the Ca^{2+}-ATPase (27). Similarly in our study, while it is considered to be an SH-group oxidant, it did not significantly affect either Ca^{2+} uptake or Ca^{2+}-ATPase activities of brain ER.

In summary, the involvement of oxygen free radicals in ischemia-reperfusion injury is generally accepted, but is not yet fully understood. Loss of ability of the brain endoplasmic reticulum to sequester Ca^{2+} due to free radical mediated damage, described in this *in vitro* study, may also contribute to understanding the *in vivo* processes leading to the intracellular Ca^{2+} overload associated with ischemia-reperfusion injury in the brain. We suggest that the primary effect of free oxygen radicals is alteration of the lipid portion of the membrane which appears to be expressed in the increased passive Ca^{2+} leak and which, after prolonged incubation, also causes changes in lipid-protein interactions and the consequent inhibition of Ca^{2+}-ATPase activity.

An increasing body of evidence has been presented demonstrating that Ca^{2+} influx can be triggered in many cells by depletion of the intracellular Ca^{2+} store (3). Recently it has been proved that a small messenger released after depletion of Ca^{2+} store, stimulates Ca^{2+} influx (4). It is an interesting question as to whether damage of reticular Ca^{2+} transport by free radicals and subsequent disability to store Ca^{2+} can affect the control of plasma membrane Ca^{2+} permeability in the brain.

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