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VANADYL (VO^{2+}) INDUCED LIPOPEROXIDATION IN THE BRAIN MICROSOMAL FRACTION IS NOT RELATED TO VO^{2+} INHIBITION OF Na,K-ATPASE

PETR SVOBODA, JAN TEISINGER* and FRANTIŠEK VYSKOČIL

Institute of Physiology, Czechoslovak Academy of Sciences, Vídeňská 1083, Praha 4; and *Institute of Hygiene and Epidemiology, Šrobárova 48, Praha 2, Czechoslovakia

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Abstract—Vanadyl (VO^{2+}) is a potent inductor of the lipid peroxidation in brain microsomes. This effect, however, is obtained at concentrations by two orders of magnitude higher (10^{-4}–10^{-3} M) than those which effectively inhibit the brain microsomal Na,K-ATPase. At 10^{-6} M VO^{2+} which inhibits 50% of the Na,K-ATPase activity there is no measurable malonyldialdehyde production. Vanadate (VO_3^-) which is an equally potent inhibitor of Na,K-ATPase as VO^{2+} has almost no capacity to induce the lipoperoxidation. The addition of 10^{-4} M ascorbate to the brain microsomes stimulates the lipoperoxidation to the maximum level regardless of the presence or absence of exogenous vanadium ions. Ascorbate-induced inhibition of brain Na,K-ATPase which is known to be associated with lipoperoxidation is strictly additive with the vanadyl (VO^{2+}) inhibition of this enzyme. Even at submaximal concentrations there is no indication for any potentiation between these two inhibitory systems. The disparity between the mechanisms of ascorbate and vanadyl-induced inhibition of Na,K-ATPase is also documented by the effect of EDTA which inhibits the former type only. It is concluded, that the vanadium-induced inhibition of brain microsomal Na,K-ATPase is not related to induction of lipoperoxidative capacity of the brain.

The brain microsomal Na,K-ATPase was shown to be inhibited by vanadate (V^{5+}) as well as by vanadyl (V^{4+}) ions* (see preceding paper, ref. 1). Besides vanadium, there appears to be another candidate as a physiologically significant inhibitor of the brain Na,K-ATPase—namely ascorbate [2–4]. This is present as a soluble factor in cytosol (supernatant 100,000 *g*) fraction [4, 5].

It was suggested that some transition heavy metal ion, presumably iron, may be responsible for the blocking effect of ascorbic acid on Na,K-ATPase activity [5]. The mechanism of this inhibition appears to be identical to the radical damage of unsaturated membrane phospholipids (lipoperoxidation) induced by these agents [6–8]. Catecholamines and chelators like EDTA block the manifestation of the lipoperoxidative capacity of brain subcellular fraction and simultaneously protect the Na,K-ATPase activity against the ascorbate-induced inhibition [7–9].

Recent data of Inouye [10] showed that +4 oxidation state of vanadium together with Fe^{2+} were the two most effective cations potentiating sulfite-induced lipoperoxidation of liver microsomes. The possibility thus arose that the surprisingly high potency of V^{4+} to inhibit the brain Na,K-ATPase may also be related to ascorbate-induced lipoperoxidative degradation of Na,K-ATPase and/or fairly high lipoperoxidative capacity of the brain subcellular fractions. Hence, in the present report, the V^{4+} ions were tested in the presence or absence of ascorbic acid as pro-oxidants (inductors) of lipoperoxidative degradation of the brain microsomal phospholipids. The results obtained were correlated with Na,K-ATPase inhibition measured under identical conditions.

* Abbreviations: V^{4+}, vanadyl (VO^{2+}) ions; V^{5+}, vanadate (VO_3^-) ions; MDA, malonyldialdehyde.

MATERIALS AND METHODS

Preparation of microsomal fractions from the rat cerebral cortex. Brain subcellular fractions were prepared according to De Robertis *et al.* [11] from the cerebral cortex of white Wistar rats (160–180 g) as described in detail in the previous reports [8, 9]. The microsomal membrane particles were sedimented from the 12,000 *g* supernatant by centrifugation for 60 min at 100,000 *g*. The resulting sediment was suspended in 0.32 M sucrose, 10 mM Tris–HCl, pH 7.4 (microsomes). The samples (0.8–1.2 mg membrane protein per ml) were stored at −25° until use.

Measurement of lipid peroxidation. Lipid peroxidation was measured as malonyldialdehyde production using the thiobarbituric acid colour reaction [12] as was described before [8, 9]. Lipoperoxidative capacity of the brain subcellular fractions was expressed as malonyldialdehyde (MDA) production per hour per mg of membrane protein.

Measurement of enzyme activities. The activity of sodium plus potassium activated, magnesium-dependent adenosinetriphosphatase (Na,K-ATPase) of the brain microsomal membranes was measured as inorganic phosphate production as described before [8, 9].

Brain microsomal membranes were preincubated in 100 mM NaCl, 20 mM KCl, 100 mM Tris–HCl pH 7.4, 5 mM $MgCl_2$ (Na+K+Mg-ATPase reaction media) or in 120 mM NaCl, 100 mM Tris–HCl pH

7.4, 5 mM $MgCl_2$ plus 2×10^{-4} M ouabain (Mg-ATPase reaction media) for 5 min at 37°. The reaction was started by addition of ATP (Boehringer) to the final concentration of 2.5 mM and continued for 15 min at 37°. After precipitation of the protein by 1 N $HClO_4$, the inorganic phosphate was determined according to Taussky and Shorr [13]. Vanadyl ($VOSO_4$, Merck) and vanadate ($NaVO_3$, Merck) were applied to the ATPase reaction media from the freshly prepared 100 mM stock solutions in distilled water or in 0.1 N NaOH. Ascorbate was also freshly prepared before the enzyme assay. Neither ascorbate nor the vanadium compounds interferred with the inorganic phosphate determination [13].

The Na,K-ATPase activity was calculated as the difference between the Na+K+Mg-ATPase and the basal, ouabain-independent Mg-ATPase.

RESULTS

Vanadium and ascorbate-induced lipid peroxidation of brain microsomes. The influence of increasing vanadyl (V^{4+}) concentrations on the lipoperoxidative degradation (malonlydialdehyde formation) of brain microsomal membranes is indicated in Fig. 1. In the absence of ascorbic acid, the malonyldialdehyde production measured as function of increasing concentrations of V^{4+} was minimal up to the 10^{-5} M (see Fig. 1A). Significant peroxidation of membrane lipids was detected only at concentrations as high as 10^{-4}–10^{-3} M. The effect of V^{5+} was much lower when compared with V^{4+}; malonyldialdehyde production measured with 10^{-3} M vanadate (8 nmoles MDA/hr/mg protein) was 5–6 times lower than the maximum peroxidation (44–48 nmoles MDA/hr/mg protein) detected with 10^{-3} M V^{4+} (data not shown).

Quite a different situation was found in the presence of ascorbic acid (Fig. 1A). The addition of ascorbate stimulated lipoperoxidation to maximum levels (obtained at 10^{-4} M V^{4+} alone) already at the lowest vanadium concentrations studied, namely at 10^{-9} M. Under these conditions, i.e. in the presence of ascorbic acid there was no significant difference between +5 and +4 redox forms of vanadium (data not shown).

These results show that vanadium alone is not able to induce significant lipoperoxidative damage in the range of "physiological concentrations", i.e. within concentrations endogenously present in the brain tissue [14, 15]. On the other hand, ascorbate is an extremely potent inductor of lipoperoxidative damage and its action can not be further potentiated by exogenous vanadium. The effect of ascorbate in the absence of any exogenous heavy metal (Fig. 1A, point zero) may either be explained that very low, trace amounts of endogenous transition metals (iron, vanadium) are sufficient to catalyse the ascorbate effect or that ascorbate radicals themselves may induce membrane peroxidation [16].

To test the former possibility, the microsomal membranes were preincubated with 1 mM EDTA for 10 min at 0° with the aim to diminish the endogenous metal content. In the first set of experiments, the microsomes were washed once with sucrose–Tris buffer to remove the excess of EDTA. As is shown in Fig. 1(B), the presence of residual EDTA in the microsomal sediment inhibited the ascorbate-induced lipoperoxidation measured at low vanadium concentrations (10^{-9}–10^{-6} M). The minimum malonyldialdehyde production without ascorbate was not affected. Very high V^{4+} concentrations (10^{-4}–10^{-3}M) concentrated the EDTA effect and lipoperoxidation was activated again. The

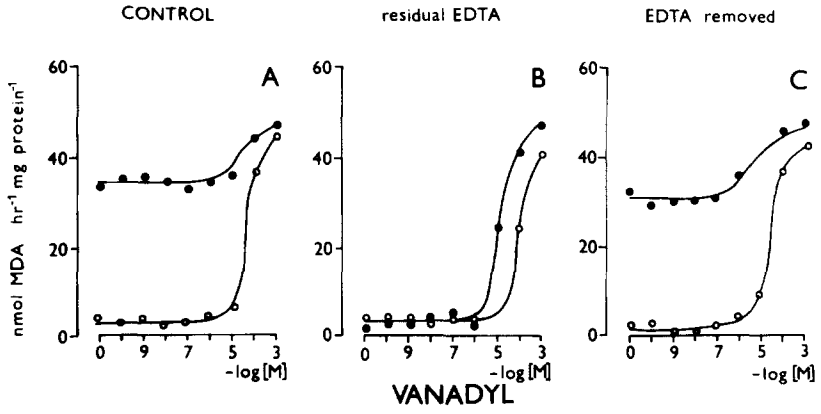

Fig. 1. Influence of increasing concentrations of vanadyl (V^{4+}) with or without ascorbate on the lipoperoxidative capacity of brain microsomal membranes. Microsomes were prepared as described in Methods and diluted 10 times in 0.2 M phosphate buffer, pH 5.9. Malonyldialdehyde production was measured by thiobarbituric acid colour reaction as described in Methods. (A) (Control)—microsomal membranes nontreated with EDTA. (B) (Residual EDTA)—microsomes (0.5–1 mg per ml) were incubated with 1 mM EDTA for 5 min at 0°. The incubation was terminated by centrifugation at 100,000 *g* for 30 min. The microsomal pellet was used for estimating of lipoperoxidation. (C) (EDTA removed)—microsomes with residual EDTA prepared according to protocol B were washed three times by centrifugation at 100,000 *g* for 30 min to remove the residual EDTA. ●——● $VOSO_4$ plus 10^{-4} ascorbate; ○——○ $VOSO_4$ without ascorbate. The data shown (nmol malonyldialdehyde produced per hour per mg membrane protein) represent the average from 3 experiments carried out in triplicates.

maximum level measured at 10^{-3} M V^{4+} was identical to that observed in the nontreated controls (Fig. 1A).

After carefully washing out EDTA by 3 times repeated centrifugation (Fig. 1C = EDTA removed), the EDTA-treated microsomes were equally sensitive as target membrane structures of radical damage as nontreated control membranes (compare Figs. 1C and A). This finding applies to the basal peroxidation without ascorbate as well as for maximum peroxidation manifested in the presence of ascorbate and vanadium ions. It means that the endogenous heavy metals are either tightly bound in the membranes and in this form not available to EDTA chelation or—as already mentioned—ascorbate radicals alone may induce the radical damage without any metal [16]. The contamination of commercial ascorbate by heavy metals may also be important in this respect.

The influence of ascorbate and vanadyl on the brain Na,K-*ATPase activity*. It was shown previously [6, 8] that the ascorbate-induced inhibition of Na,K-ATPase activity is directly proportional to the induction of lipoperoxidative capacity of brain microsomes. Maximum malonyldialdehyde production is measured at minimum enzyme activity (10^{-5}–10^{-4} M ascorbate). The increase of ascorbate concentrations to the millimolar range diminishes lipoperoxidation [17, 18] and simultaneously protects the Na,K-ATPase activity [6, 8]. The Na,K-ATPase is preferentially sensitive to lipoperoxidative damage, the basal Mg-ATPase is affected to a minor degree (data not shown, ref. [18]).

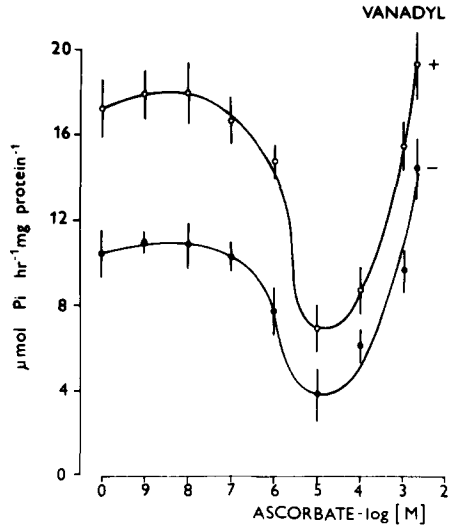

Fig. 2. Effect of ascorbic acid on the brain Na,K-ATPase measured in the presence or absence of 10^{-6} V^{4+}. Upper curve ○——○, the influence of increasing ascorbate concentrations on the brain microsomal Na,K-ATPase (control without vanadyl). Lower curve ●——●, the dose response curve for ascorbate-induced inhibition of Na,K-ATPase was measured in the presence of 10^{-6} M V^{4+}. Ascorbate plus vanadyl were added to the preincubation media and the assay of enzyme activity (after start of the reaction with ATP) was performed as described in Materials and Methods. The data shown are the means from triplicate determination of specific enzyme activities ± S.D. of 4 independent preparations of brain microsomal membranes.

The upper curve demonstrated in Fig. 2 corresponds well to the previously published data [4, 6, 8]. It shows the biphasic effect of ascorbic acid on Na,K-ATPase activity, i.e. inhibition at low (10^{-5} M) and protection at high (10^{-3} M) concentrations. The dual role of ascorbate as both a pro- and an anti-oxidant may explain this result [20].

The lower curve in Fig. 2 indicates that the addition of 10^{-6} M V^{4+} shifted the dose–response curve to much lower enzyme activities within the whole range of ascorbate concentrations studied. There was no potentiation of ascorbate inhibition by exogenous vanadium. Both types of inhibition are therefore strictly additive and there is no evidence for any potentiation or interaction between these two inhibitory systems. Rather, the relative efficiency of V^{4+} to inhibit enzyme was somewhat decreased at high ascorbate concentrations (above 10^{-3}M). The ability of ascorbate to decrease the blocking effect of V^{4+} is probably related to the chelating properties of this agent [20] which are manifested at millimolar concentrations.

The addition of EDTA to microsomes incubated in the presence of increasing ascorbate concentrations together with 10^{-6} M V^{4+} completely eliminated the ascorbate effect at 10^{-5}–10^{-4} M, while the vanadyl inhibition remained unchanged. The level of enzyme activities measured in the presence of EDTA+ascorbate+vanadyl (11 µmoles P_i/hr/mg) is very close to the activity measured in the presence of vanadyl alone (compare with Fig. 2 point zero, + vanadyl). Thus, with the help of EDTA which is known to diminish the lipoperoxidation, the ascorbate-induced inhibition of Na,K-ATPase activity could further be distinguished from the vanadyl inhibition.

DISCUSSION

Schaeffer *et al.* [4] have shown that catecholamines together with metal chelators such as EDTA increase Na,K-ATPase activity in microsomal membrane fractions of the rat brain by antagonizing the effect of the endogenous inhibitor present in the cytosol. One soluble inhibitor was identical as ascorbic acid [5] and its lipid-peroxidation inducing effect was shown to be responsible for the inhibition of the enzyme [7]. Similar observations were published by Matsuda [22] and Svoboda and Mosinger [8]. It was also shown that the stimulation of the brain Na,K-ATPase by catecholamines has no relationship to the specific β- or α-adrenergic receptor [9, 23].

Besides the ascorbate-induced lipoperoxidative damage there appears to be another factor which may act as a physiologically important Na,K-ATPase inhibitor, namely endogenous vanadium. Its effect is also blocked by catecholamines [24, 25]. Vanadate (V^{5+}) was found to inhibit Na,K-ATPase at concentrations as low as 10^{-8}–10^{-7} M [24–26] and the paper of Adam-Vizi *et al.* [15] showed that the rat neural tissue normally contains vanadium in concentrations sufficient to produce significant enzyme inhibition. The vanadate inhibition is reversed by

high concentrations of noradrenaline [25–27] but is unaffected by EDTA [14, 25]. Vanadyl, the +4 oxidation state of vanadium, at least in the brain, seems to inhibit the Na,K-ATPase activity with a similar affinity as V^{5+} and its effect is also blocked by noradrenaline and transferrin [1].

The Fe^{2+} and VO^{2+} cations were shown [10] to be the two most effective cations potentiating the sulfite-induced lipoperoxidation of liver microsomes. The possibility of mutually potentiating effect of V^{4+} and ascorbate in Na,K-ATPase inhibition was therefore tested in this work together with the capacity of vanadium compounds to induce the lipoperoxidative damage of brain microsomes.

High concentrations of vanadyl (10^{-4}–10^{-3} M) can stimulate the malonyldialdehyde formation to the same degree as 10^{-4} M ascorbate. Vanadyl (V^{4+}) ions may therefore be regarded as a strong pro-oxidant (inductor of lipoperoxidative damage) while the vanadate V^{5+} is almost without any effect. However, at low concentrations, the ability of vanadyl to stimulate lipoperoxidation is almost zero and the minimum V^{4+} concentration (10^{-5} M) which is needed for measurable malonyldialdehyde production is much higher than V^{4+} concentrations (10^{-7}–10^{-6} M) which effectively inhibit the Na,K-ATPase activity.

Thus, according to comparison between the dose–response curves (compare Figs. 1 in this and in the accompanying paper) there appears to be a clear dissociation between the vanadyl-induced inhibition of Na,K-ATPase activity and lipoperoxidation.

With the exception of very high concentration (10^{-4}–10^{-3} M), ascorbate-induced lipoperoxidation of brain microsomes is not dependent on exogenous vanadium. Surprisingly, the EDTA-washed microsomes exhibit the same ascorbate-induced lipoperoxidation as nontreated membranes. One explanation of this finding would be based on the idea of Goto and Tanaka [16] that the ascorbate radical alone, i.e. without endogenous, membrane-bound heavy metals, would induce lipoperoxidative damage. On the other hand, some tightly bound cations need not necessarily be removed by EDTA treatment or that the ultra-trace amount of exo- or endogenous metals may still serve as reaction centres for the initiation of radical chain reactions.

Post *et al.* [14] convincingly showed that EDTA forms a strong complex with V^{4+} and in this way eliminates the V^{4+}-induced inhibition of Na, K-ATPase. On the contrary, EDTA in our experiments, was not able to eliminate the V^{4+} inhibition. The explanation of this finding may probably be related to other data of Post *et al.* [14] showing that an excess of Mg^{2+} cations eliminated this EDTA effect. In our experiments, 10^{-4} M EDTA was added together with 5×10^{-3} M Mg^{2+}. This magnesium effect also makes it possible to differentiate between the V^{4+}-induced and ascorbate plus heavy metal (Fe^{2+})-induced lipoperoxidation. The latter type of lipoperoxidation and inhibition of Na,K-ATPase activity is inhibited by EDTA even in the presence of excess of Mg^{2+} (Fig. 3).

The concentration dependence of ascorbate-induced inhibition of Na,K-ATPase activity in the presence or absence of 10^{-6} M vanadyl (Fig. 2) showed that both inhibitory systems were independent of each other. There did not appear any potentiation between these two agents and even at submaximum concentrations of ascorbate (10^{-6} M) and vanadyl (10^{-7} M) the inhibitory effects were additive only (data not shown).

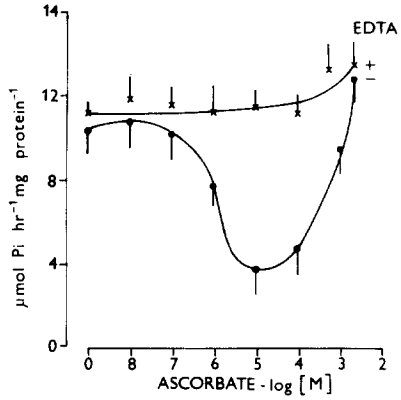

Fig. 3. The influence of EDTA on the brain microsomal Na,K-ATPase measured in the presence of increasing concentrations of ascorbate plus 10^{-6} M vanadyl (V^{4+}). The inhibitors ascorbate (10^{-8}–10^{-2} M) plus vanadyl (10^{-6} M) were mixed with 10^{-4} M EDTA in ATPase reaction media (see Methods) and preincubated for 5 min in the presence of microsomal membranes. After starting the reaction by ATP, Na,K-ATPase activities were determined as described in Materials and Methods. Upper curve ×——×, with 10^{-4} M EDTA; Lower curve ●——●, without EDTA. The data shown are the means from duplicate determination of specific enzyme activities ± S.D. of 3 independent preparations of brain microsomal membranes.

The results presented in this work and in the accompanying paper [1] differ from the conclusions of Adam-Vizi and Seregi [23], who also measured the influence of ascorbic acid and noradrenaline on both the redox state of vanadium species and Na,K-ATPase activity. Using ESR spectroscopy these authors [30] showed that in the presence of noradrenaline and ascorbate, the V^{5+} is quantitatively reduced to V^{4+}. Concomitantly with this reduction, Na,K-ATPase inhibition was eliminated. The blocking potency of ascorbate plus noradrenaline was explained exclusively in terms of the reduction of inhibiting V^{5+} to non-inhibiting V^{4+} [30]. We have shown however [1] that V^{4+} is also a strong inhibitor of brain Na,K-ATPase activity. This discrepancy may simply be explained by the fact that the millimolar concentration of ascorbate used by Adam-Vizi and Seregi [23] completely eliminates the lipoperoxidative damage of Na,K-ATPase (see Fig. 2 in this work) and that the 10^{-4} M noradrenaline blocks vanadyl (V^{4+})-induced inhibition (compare with Fig. 6 in ref. 1). Furthermore, vanadyl inhibition of Na,K-ATPase is partially eliminated at high ascorbate concentrations (see lower curve) in Fig. 2 in the present report).

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