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DIFFERENT OXIDANT SENSITIVITIES OF THE α_1 AND α_2 ISOFORMS OF Na^+/K^+-ATPase EXPRESSED IN BACULOVIRUS-INFECTED INSECT CELLS

Z. Xie*[1], M. Jack-Hays*, Y. Wang*, S.M. Periyasamy*,
G. Blanco†, W.-H. Huang*, and A. Askari*

*Department of Pharmacology, Medical College of Ohio, Toledo, OH 43699

†Department of Cell Biology and Physiology, Washington University
School of Medicine, St. Louis, MO 63110

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Summary: Inhibition of Na^+/K^+-ATPase by partially reduced oxygen metabolites is an early event in the course of cell injury caused by oxidative stress. We showed before that isoforms of the enzyme obtained from different sources have different oxidant sensitivities. To evaluate the role of tissue-specific impurities in this difference, cDNAs of α_1 and α_2 isoforms were expressed in Sf-9 insect cells, and the effects of H_2O_2 on the resulting isolated enzymes were studied. The expressed α_2 was significantly more sensitive than α_1 to H_2O_2. These findings, together with our previous data showing different oxidant sensitivities of α_1 and α_3 in a cardiac enzyme preparation, indicate that differential oxidant sensitivities of Na^+/K^+-ATPase isoforms of various tissues are dictated by the primary sequences of α_1, α_2, and α_3 subunits.

Na^+/K^+-ATPase is the plasma membrane enzyme that catalyzes the active transports of Na^+ and K^+ in most eukaryotic cells. Studies on the interactions of partially reduced oxygen metabolites (hydrogen peroxide, hydroxyl radical, and superoxide radical) with the enzyme are of interest because there is evidence to suggest that inhibition of Na^+/K^+-ATPase by these oxidants may be involved in post-ischemic injury to heart and other organs (1-4). Na^+/K^+-ATPase consists of α and β subunits, both of which are essential for function (5,6). The α-subunit which contains the catalytic site and the binding site for digitalis drugs (e.g., ouabain) has three well characterized isoforms with different ouabain sensitivities (6,7). Our recent studies suggested that α_2 and α_3 isoforms have higher oxidant sensitivities than α_1, raising the possibility that isoform compositions of the various tissues may contribute to their relative susceptibilities to oxidative stress (3,4).

[1]FAX Number of Corresponding Author: 419-381-2871.

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Different oxidant sensitivities of isoforms were first suspected in studies of oxidant effects on intact rat cardiac myocytes (1), and then demonstrated in comparative studies of purified kidney enzymes containing α_1, and a partially purified axolemmal enzyme containing a mixture of α_2 and α_3 (3). One could argue, however, that different sensitivities of isolated enzyme preparations to an inhibitor may be due to tissue-specific factors or impurities. In the case of α_1 versus α_3, this possibility became unlikely when we showed the different oxidant sensitivities of the two in a ferret heart preparation containing a mixture of α_1 and α_3 (3). The clear demonstration of the different sensitivities of α_1 and α_2, however, remained to be made. We have now expressed rat α_1 and α_2, each with rat β_1, in insect cells which have little or no endogenous Na^+/K^+-ATPase (8), and we have compared the sensitivities of the resulting active enzymes to H_2O_2.

Methods

Recombinant baculoviruses containing rodent α_1, α_2, and β_1 cDNAs were constructed and used to infect Sf-9 insect cells as described previously in detail (8,9). After 2-3 days of incubation at room temperature, cells were harvested when viability dropped to about 90% as assayed by trypan blue exclusion.

To make a membrane preparation for enzyme assay, Sf-9 cells were sedimented by centrifugation at 2,500 x g for 10 min, suspended ($3\text{-}4\text{x}10^7$ cells/5 ml) in 0.25 M sucrose, 30 mM histidine, 1 mM EDTA (pH 6.8), and sonicated on ice 3 times each time for 10 s (Branson Sonifier, constant cycle, output control setting 1). The suspension was centrifuged at 4,000 x g for 10 min, and the supernatant was removed and centrifuged at 150,000 x g for 1 h. The pellet (4-5 mg of protein) was suspended in 1 ml of a solution containing 1% CHAPS, 20% glycerol, 160 mM KCl and 20 mM MOPS (pH 7.2), using a hand homogenizer. After standing on ice for 40 min with intermittent homogenization, the suspension was centrifuged at 130,000 x g for 30 min, the soluble supernatant was mixed with phosphatidylcholine (30 mg/ml), and vortexed. The resulting suspension was placed on a 5 ml Sephadex G-25-300 column which had been equilibrated with 100 mM KCl, 20 mM MOPS (pH 7.2); allowed to stand for 1 h at 4°C, and centrifuged at 2,000 x g for 5 min. The collected material from several columns were combined and centrifuged at 150,000 x g for 90 min to obtain a sedimented membrane preparation.

To assay Na^+/K^+-ATPase and the time-course of its inactivation by H_2O_2, the above membrane preparation, suspended in 100 mM KCl, 20 mM MOPS (pH 7.2), was mixed with alamethicin (100 μg/mg of protein) to assure the opening of any sealed vesicles (10). An aliquot was added to a solution containing 100 mM NaCl, 25 mM KCl, 1 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.4), and a fixed concentration of H_2O_2. A control membrane sample was similarly treated but without H_2O_2. After various incubation times at 37°C, sufficient catalase (2,000 units/ml) was added to cause immediate decomposition of H_2O_2 and to stop the inactivation of Na^+/K^+-ATPase. ATPase assay was then started by the rapid and sequential addition of 1 mM EGTA, 5 mM sodium azide, and 2 mM Mg [γ-^{32}P]ATP. (EGTA and azide inhibit Ca^{2+}-ATPase and mitochondrial ATPase respectively.). ^{32}Pi release after an appropriate assay time was then measured (3). The assay of each control and H_2O_2-treated sample was done with and without 1 mM ouabain, and the difference was taken as Na^+/K^+-ATPase activity. In preliminary experiments with purified Na^+/K^+-ATPase, it was established that catalase as used here, has no effect on Na^+/K^+-ATPase activity.

Na^+/K^+-ATPases from rat brain stem, rat kidney, and canine kidney were prepared as described before (3). Western blot analyses using isoform-specific antibodies were also done as previously indicated (3,9). Protein was measured using Amido Black (11). ATP_2, alamethicin, and catalase (bovine liver) were obtained from Sigma. [γ-^{32}P]ATP was bought from DuPont-New England Nuclear.

Results

In our previous work (2,3), irreversible inactivation of Na^+/K^+-ATPase by H_2O_2 was studied by incubation of the enzyme with H_2O_2, removal of H_2O_2 by dilution or by centrifugation of the enzyme, and subsequent assay of activity. Because it was difficult to use such procedures for experiments on small quantities of the expressed enzymes, we developed the procedures described in Methods, involving the use of catalase for the termination of H_2O_2 action, to measure the time-course of H_2O_2-induced inactivation. Experiments of Fig. 1 show the use of this assay for comparing the inactivation rates, at a fixed H_2O_2 concentration, of three enzyme preparations from rat brain stem, rat kidney and canine kidney. The data confirm our previous conclusions (3) by showing that H_2O_2 sensitivity of the axolemmal enzyme containing a mixture of α_2 and α_3 isoforms is significantly higher than those of the two kidney enzymes that contain two different variants of the α_1 isoform.

In experiments of Figs. 2 and 3, the H_2O_2 sensitivities of the $\alpha_1\beta_1$ and $\alpha_2\beta_1$, each expressed separately in insect cells, were compared under identical

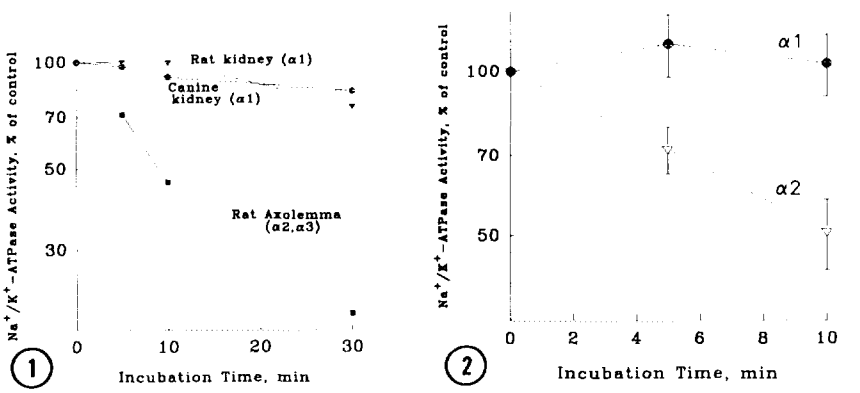

Fig. 1. H_2O_2-induced inactivations of the enzymes isolated from rat brain stem, rat kidney, and canine kidney. The enzymes were preincubated at 37°C with 10 mM H_2O_2 for the indicated times, catalase was added to remove H_2O_2, and ATPase activities were assayed as indicated in Methods.

Fig. 2. Time-courses of the H_2O_2-induced inactivations of $\alpha_1\beta_1$ and $\alpha_2\beta_1$ expressed in insect cells. Membranes isolated from the infected insect cells were exposed to 1 mM H_2O_2 for the indicated times and then assayed for activity. Values are mean ± SE from 3-5 separate experiments.

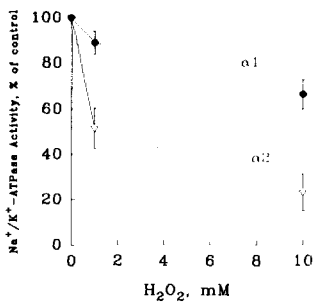

Fig. 3. Inactivations of $\alpha_1\beta_1$ and $\alpha_2\beta_1$ expressed in insect cells by different H_2O_2 concentrations. Membranes were exposed to H_2O_2 for 10 min and then assayed for activity. Values are mean ± SE from 5 separate experiments.

conditions. Time-dependent inactivations of the two enzymes at the same H_2O_2 concentration (Fig. 2), and inactivations caused by different H_2O_2 concentrations at a fixed time (Fig. 3) clearly show the greater sensitivity of α_2 than α_1 to H_2O_2.

In agreement with our previous observations (8,9), Western blots using isoform-specific antibodies showed the expressions of $\alpha_1\beta_1$ and $\alpha_2\beta_1$ in Sf-9 insect cells (data not presented). Ouabain-sensitive Na^+/K^+-ATPase activities (μmol Pi released/mg/h) of the various preparations used in experiments of Figs. 2 and 3 were in the range of 0.71 - 1.49 for $\alpha_1\beta_1$, and 1.37 - 2.09 for $\alpha_2\beta_1$. Ouabain-sensitive activity in membranes of uninfected cells was negligible.

Discussion

The data of Figs. 2 and 3 rule out the possibility that tissue-specific factors other than Na^+/K^+-ATPase subunits could be the cause of different oxidant sensitivities of α_1 and α_2 isoforms existing in different tissues. Our previous experiments on the ferret heart enzyme (3) also ruled out the possibility of tissue-specific impurities being responsible for the different H_2O_2 sensitivities of α_1 and α_3 isoforms. Taken together, these findings indicate that the determinants of the differential oxidant sensitivities of Na^+/K^+-ATPase isoforms are within the primary structures of α_1, α_2, and α_3 subunits. Considering that rat and canine α_1 isoforms which exhibit similar oxidant sensitivities (Ref. 3 and Fig. 1), have widely different ouabain sensitivities (12), it is also clear that structural features controlling oxidant and ouabain sensitivities are not the same.

Although amino acid differences occur throughout the lengths of the three α chains, there are two major areas of high sequence diversity that have been exploited for the production of isoform-specific antibodies (5,7,13,14).

One of these is on the intracellular N-terminal segment (residues 1-68 of α_1), and the other (residues 408-590 of α_1) on the large intracellular central loop between the fourth and fifth transmembrane segments. It is reasonable to suggest, therefore, that studies aimed at the characterization of the structural bases of the differential oxidant sensitivities of the isoforms should focus on the sequence differences within these two segments.

Acknowledgments

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