Mechanism of depression in cardiac sarcolemmal Na\textsuperscript{+}-K\textsuperscript{+}-ATPase by hypochlorous acid

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Kato, Kiminori, Qiming Shao, Vijayan Elimban, Anton Lukas, and Naranjan S. Dhalla. Mechanism of depression in cardiac sarcolemmal Na\textsuperscript{+}-K\textsuperscript{+}-ATPase by hypochlorous acid. Am. J. Physiol. 275 (Cell Physiol. 44): C826–C831, 1998.—Oxidative stress during pathological conditions such as ischemia-reperfusion is known to promote the formation of hypochlorous acid (HOCl) in the heart and to result in depression of cardiac sarcolemmal (SL) Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity. In this study, we examined the direct effects of HOCl on SL Na\textsuperscript{+}-K\textsuperscript{+}-ATPase from porcine heart. HOCl decreased SL Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity in a concentration- and time-dependent manner. Characterization of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity in the presence of different concentrations of MgATP revealed a decrease in the maximal velocity (V_max) value, without a change in affinity for MgATP on treatment of SL membranes with 0.1 mM HOCl. The V_max value of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase, when determined in the presence of different concentrations of Na\textsuperscript{+}, was also decreased, but affinity for Na\textsuperscript{+} was increased when treated with HOCl. Formation of acylphosphatase by SL Na\textsuperscript{+}-K\textsuperscript{+}-ATPase was not affected by HOCl. Scatchard plot analysis of \[^3H\]ouabain binding data indicated no significant change in the affinity or maximum binding capacity value for ouabain binding following treatment of SL membranes with HOCl. Western blot analysis of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase subunits in HOCl-treated SL membranes showed a decrease (34 ± 9% of control) in the \(\beta_1\)-subunit without any change in the \(\alpha_1\)- or \(\alpha_2\)-subunits. These data suggest that the HOCl-induced decrease in SL Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity may be due to a depression in the \(\beta_1\)-subunit of the enzyme.

oxidative stress; sarcolemmal sodium-potassium-adenosinetriphosphatase; sarcolemmal ouabain binding; pig heart; sodium-potassium-adenosinetriphosphatase subunits

METHODS

Preparation of cardiac SL membrane. Porcine hearts were obtained from a slaughterhouse or were freshly obtained in our animal holding facility. Hearts were cut into small pieces and frozen (−70°C). SL membrane was isolated according to the method of Pitts (23) as modified by Kaneko et al. (9). Marker enzyme activities (2, 9, 21) revealed a 16- to 18-fold purification of membranes with respect to Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity and minimal (2–4%) cross contamination with other subcellular organelles.

Either l-methionine (10 mM) or DTT (1 mM) was used as an antioxidant for HOCl (29). The SL membrane (0.4 mg/ml) was incubated separately with 0.1 mM HOCl in the absence and presence of antioxidant for 10 min. HOCl was prepared by vacuum distillation of sodium hypochlorite after adjusting the pH to 6.2 with dilute sulfuric acid.

Measurement of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity and \(K^+\)-dependent p-nitrophosphophatase activities. Estimation of Na\textsuperscript{+}K\textsuperscript{+}-ATPase activity was carried out as previously described (2). Briefly, SL membranes (10 µg) were preincubated at 37°C with 1.0 mM EGTA (Tris), pH 7.4, 5 mM NaN₃, 6 mM MgCl₂, 100 mM

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NaCl, 10 mM KCl, 2.5 mM phosphoenolpyruvate (PEP), and 10 U/ml pyruvate kinase. PEP and pyruvate were used as an ATP-regenerating system to maintain the ATP concentration in the incubation medium. The reaction was started by the addition of 0.025 ml of 80 mM Na₂ATP (pH 7.4) and stopped after 10 min with 0.5 ml ice-cold 12% TCA. Liberated phosphate was measured by the method of Taussky and Shorr (31). In experiments using different concentrations of MgATP, the amounts of Mg²⁺ and ATP required to achieve the final concentration of MgATP were calculated using the SPECS Fortran program developed by Fabiato (7). In parallel experiments, either Na⁺ plus K⁺ or Mg²⁺ was omitted from the reaction medium. Na⁺-K⁺-ATPase activity was calculated as the difference between activities with and without Na⁺ plus K⁺. Mg²⁺-ATPase activity was estimated as the difference between activities obtained with and without Mg²⁺, in the absence of Na⁺ plus K⁺, in the medium.

The K⁺-dependent p-nitrophenylphosphatase (K⁺-pNPPase) activity was determined at 37°C in 1.0 ml of reaction volume using a modified method of Pierce et al. (21, 22). The assay medium contained 30 mM imidazole-HCl, pH 7.8, 5 mM MgCl₂, 1 mM EGTA, 20 mM KCl, and 10 µg SL membrane. The reaction was started by adding 5 mM p-nitrophosphate and stopped after 10 min by addition of 2 ml 1 N NaOH. The amount of p-nitrophenol formed was measured at 410 nm.

Measurement of lipid peroxidation and sulfhydryl group content. Lipid peroxidation in the SL membrane was estimated from the malondialdehyde (MDA) concentration by using the thiobarbituric acid method (28). Total sulfhydryl (SH) groups in the SL were determined with DTNB (1).

Measurement of acylphosphate. A modified method of Elmoselhi et al. (6) was used for the formation of acylphosphate.

Fig. 1. Effect of hypochlorous acid (HOCl) on porcine heart sarcolemmal Na⁺-K⁺-ATPase (C) and Mg²⁺-ATPase (●) activities at different concentrations of HOCl (B). Each value is mean ± SE of 5 experiments. A: *P < 0.05 compared with value in 0 min incubation time. B: *P < 0.05 compared with value in 0 mM HOCl.

Fig. 2. Effect of 0.1 mM HOCl on porcine heart sarcolemmal Na⁺-K⁺-ATPase activities at different concentrations of MgATP. Each value is mean ± SE of 5 experiments. Inset: Lineweaver-Burk plot of a representative experiment. ○, Control; ●, 0.1 mM HOCl. *P < 0.05 compared with control.

Fig. 3. Effect of 0.1 mM HOCl on porcine heart sarcolemmal Na⁺-K⁺-ATPase activities at different concentrations of Na⁺. Each value is mean ± SE of 5 experiments. Inset: Lineweaver-Burk plot of a representative experiment. ○, Control; ●, 0.1 mM HOCl. *P < 0.05 compared with control.
Table 1. Kinetic characteristics of porcine cardiac sarcolemmal Na\(^+\)-K\(^+\)-ATPase treated with or without 0.1 mM HOCI

<table>
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<tr>
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<th>Control</th>
<th>HOCI</th>
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<tr>
<td>Kinetic parameters for MgATP</td>
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<tr>
<td>(V_{\text{max}}), (\mu\text{mol} \text{ P} \cdot \text{mg protein}^{-1} \cdot \text{h}^{-1})</td>
<td>20.00 ± 1.75</td>
<td>6.67 ± 1.75*</td>
</tr>
<tr>
<td>(K_m), mM</td>
<td>0.33 ± 0.04</td>
<td>0.41 ± 0.08</td>
</tr>
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| Kinetic parameters for Na\(^+\) \(\text{ATPase}\) |         |      |
| \(V_{\text{max}}\), \(\mu\text{mol} \text{ P} \cdot \text{mg protein}^{-1} \cdot \text{h}^{-1}\) | 28.57 ± 2.05 | 4.44 ± 0.02* |
| \(K_a\), mM | 14.29 ± 2.74 | 1.42 ± 0.07* |

Values are means ± SE of 5 experiments. Values for maximum velocity \((V_{\text{max}})\), Michaelis-Menten constant \((K_m)\), and apparent rate constant \((K_a)\) were calculated from data shown in Figs. 2 and 3 by employing Lineweaver-Burk plot analysis. *\(P < 0.05\) vs. control group.

RESULTS

Catalytic activity of Na\(^+\)-K\(^+\)-ATPase. Incubation of cardiac SL membranes with HOCI diminished the Na\(^+\)-K\(^+\)-ATPase activity with respect to concentration and time (Fig. 1). The data in Fig. 2 show the kinetic characteristics of Na\(^+\)-K\(^+\)-ATPase for MgATP, whereas Fig. 3 shows the characteristics for Na\(^+\). The results summarized in Table 1 indicate that \(V_{\text{max}}\), when determined in the presence of variable concentrations of MgATP, was decreased by HOCI, but \(K_m\) was not changed significantly between control and HOCI-treated preparations. In the kinetic study employing different concentrations of Na\(^+\), both \(V_{\text{max}}\) and \(K_a\) were significantly decreased by HOCI. The data in Table 2 show that the HOCI-induced depression in Na\(^+\)-K\(^+\)-ATPase activity was partially prevented by 10 mM L-methionine or 1 mM DTT. On the other hand, the decrease in K\(^+\)-pNPPase activity by HOCI was completely prevented by either 10 mM L-methionine or 1 mM DTT. L-Methionine or DTT alone did not exert any effect on the Na\(^+\)-K\(^+\)-ATPase or K\(^+\)-pNPPase activities (data not shown). HOCI increased the SL MDA formation; both L-methionine and DTT prevented this effect (Table 2). HOCI decreased the SL SH group content and L-methionine prevented this effect (Table 2); DTT was not used in this experiment because it interferes with the SH group content estimation.

Acylphosphates and ouabain binding. Figure 4 illustrates the results of treatment with 0.1 mM HOCI on acylphosphate formation. These bands were located at

Table 2. Effect of 0.1 mM HOCI on porcine heart sarcolemmal Na\(^+\)-K\(^+\)-ATPase, K\(^+\)-pNPPase, MDA content, and SH group content

<table>
<thead>
<tr>
<th></th>
<th>(\mu\text{mol} \text{ P} \cdot \text{mg protein}^{-1} \cdot \text{h}^{-1})</th>
<th>MDA content</th>
<th>SH group content</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Na(^+)-K(^+)-ATPase</td>
<td>K(^+)-pNPPase</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>18.86 ± 2.03</td>
<td>2.745 ± 0.055</td>
<td>51.64 ± 3.97</td>
</tr>
<tr>
<td>HOCI</td>
<td>2.16 ± 1.05*</td>
<td>1.633 ± 0.033*</td>
<td>67.33 ± 3.41*</td>
</tr>
<tr>
<td>HOCI-LM</td>
<td>13.61 ± 2.44</td>
<td>2.538 ± 0.122</td>
<td>48.27 ± 3.59</td>
</tr>
<tr>
<td>HOCI-DTT</td>
<td>15.56 ± 1.94</td>
<td>2.668 ± 0.113</td>
<td>52.22 ± 4.28</td>
</tr>
</tbody>
</table>

Values are means ± SE of 5 experiments. K\(^+\)-pNPPase, K\(^+\)-dependent p-nitrophosphophatase; MDA, malondialdehyde; SH, sulfhydryl. *\(P < 0.05\) compared with control group.
~110 kDa. Treatment of SL membranes with 0.1 mM HOCl in the absence and presence of 10 mM L-methionine or 1 mM DTT did not produce any significant effect on the formation of acylphosphate. Figure 5 shows the effect of HOCl on ouabain binding. As indicated, there are no changes in either Bmax or Kd values between control and 0.1 mM HOCl-treated preparation.

Western blot analysis of Na\(^+\)-K\(^+\)-ATPase subunits. The bands of both the \(\alpha_1\)- and \(\alpha_2\)-subunits of the SL Na\(^+\)-K\(^+\)-ATPase in our study are located at ~110 kDa (Fig. 6), which agrees with previous reports (see review in Ref. 30). On the other hand, the \(\beta\)-subunit band was located at ~55 kDa rather than at 35 kDa as reported by others (30). However, Pedemonte and Kaplan (20) reported the band of the Na\(^+\)-K\(^+\)-ATPase \(\beta\)-subunit to be at 55 kDa, which agrees with our results. Such a difference was explained by the fact that the electrophoretic mobility of the \(\beta\)-subunit was influenced largely by N-linked carbohydrate groups (30). Figure 7 shows

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**Fig. 4.** Effect of HOCl (0.1 mM), L-methionine (LM; 10 mM), and dithiothreitol (DTT; 1 mM) on acylphosphate intermediate of porcine heart sarcolemmal Na\(^+\)-K\(^+\)-ATPase. A representative autoradiograph is shown at top.

**Fig. 5.** Scatchard plot analysis of specific [\(^3\)H]ouabain binding to porcine heart sarcolemmal preparation. Values are taken from a typical experiment in which 3 control and 3 HOCl treatment heart preparations were employed. Inset: results of dissociation constant (Kd) and maximum binding capacity (Bmax). Each value is a mean of 3 experiments. ○, Control; ●, 0.1 mM HOCl.

**Fig. 6.** Representative Western blots depicting the effect of treatment with HOCl on expression of the \(\alpha_1\)-, \(\alpha_2\)-, and \(\beta\)-subunits of porcine heart sarcolemmal Na\(^+\)-K\(^+\)-ATPase. Lane 1, molecular mass markers (kDa); lane 2, control; lane 3, 0.1 mM HOCl; lane 4, HOCl + 10 mM LM; lane 5, HOCl + 1 mM DTT. A band of nonspecific binding was seen at ~75 kDa in all blots.

**Fig. 7.** Summary of Western blot analysis of subunit expression of porcine heart sarcolemmal Na\(^+\)-K\(^+\)-ATPase. Bars, data obtained from 4 Western blots for each subunit. I, control; II, 0.1 mM HOCl; III, HOCl + 10 mM LM; IV, HOCl + 1 mM DTT. *P < 0.05 compared with control.
that HOCl decreased the density of β-subunit of Na\(^+\)-K\(^+\)-ATPase and that L-methionine or DTT significantly prevented this effect. On the other hand, HOCl had no effect on both α\(_1\)- and α\(_2\)-subunits of the SL Na\(^+\)-K\(^+\)-ATPase.

**DISCUSSION**

This study demonstrates that treatment of SL membranes with HOCl causes a decrease in Na\(^+\)-K\(^+\)-ATPase activity and SH group content but an increase in MDA content. Other studies (13, 17) also report a depression in cardiac Na\(^+\)-K\(^+\)-ATPase by HOCl, which is a highly reactive oxidant (5, 25). Oxyradical generation under in vitro conditions also decreases Na\(^+\)-K\(^+\)-ATPase activity and SH group content of SL membranes but increases formation of MDA (12, 26). It is likely that the observed depression in the SL Na\(^+\)-K\(^+\)-ATPase activity by HOCl may be due to a decrease in the SH group content of the enzyme, since SH groups are important for the activity of Na\(^+\)-K\(^+\)-ATPase (12, 25). On the other hand, deleterious effects of different oxidants and oxyradicals may reflect increased formation of lipid peroxides (8, 9, 12, 17, 25, 26), and thus it is possible that the HOCl-induced depression may be due to the observed increase in the MDA content of the SL membranes. Treatment of SL membranes with L-methionine, which prevented the HOCl-induced changes in MDA and SH group content due to its antioxidant property, partially prevented (≈70% protection) the HOCl-induced depression in Na\(^+\)-K\(^+\)-ATPase activity. Furthermore, DTT, which prevents the oxidation of SH groups, completely attenuated the HOCl-induced increase in MDA content and partially prevented the HOCl-induced decrease in Na\(^+\)-K\(^+\)-ATPase activity (≈80% protection). Thus both elevated levels of MDA and depressed content of SH groups may be involved in the decreased Na\(^+\)-K\(^+\)-ATPase activity in HOCl-treated membranes. Inactivation of Na\(^+\)-K\(^+\)-ATPase by HOCl in cardiac membrane (13) and uncoupling of Na\(^+\) pump by HOCl in coronary artery (6) are also postulated mechanisms for the HOCl-induced changes in Na\(^+\)-K\(^+\)-ATPase.

The depression in SL Na\(^+\)-K\(^+\)-ATPase activity by HOCl was associated with a decrease in V\(_{\text{max}}\) of the enzymatic reaction when determined in the presence of different concentrations of MgATP or Na\(^+\). Because HOCl-treated membranes exhibited a decrease in the activity of K\(^+\)-pNPPase and this effect was prevented by L-methionine or DTT, it is possible that the observed depression in Na\(^+\)-K\(^+\)-ATPase activity reflects a depressant effect of HOCl on the catalytic sites of the enzyme. However, this may not be the case because the affinity for MgATP (1/K\(_m\)) was unaltered and the affinity for Na\(^+\) (1/K\(_n\)) was in fact increased on treatment of membranes with HOCl. Furthermore, neither the binding of ouabain nor the formation of acylphosphate was affected by HOCl. It should be mentioned that Na\(^+\), K\(^+\), ATP, and ouabain binding occur at the catalytic sites represented by α-subunits of Na\(^+\)-K\(^+\)-ATPase (20, 30). Because no change in the content of the α\(_1\)- and α\(_2\)-subunits of the enzyme was seen on HOCl treatment, the observed depression in Na\(^+\)-K\(^+\)-ATPase activity by HOCl may not be due to its effect on the catalytic sites of the enzyme.

Unlike the α\(_1\)- and α\(_2\)-subunits of SL Na\(^+\)-K\(^+\)-ATPase, the content of β-subunit was decreased by HOCl treatment. This observation suggests that the β-subunit of Na\(^+\)-K\(^+\)-ATPase may be more sensitive to HOCl than the α\(_1\)- and α\(_2\)-subunits of the enzyme. Differential effects of various oxidants on α\(_1\)-, α\(_2\)- and α\(_3\)-subunits of Na\(^+\)-K\(^+\)-ATPase have been observed by other investigators (8, 16, 32). Our lack of effect of HOCl on the α\(_1\)- and α\(_2\)-subunits of the porcine heart Na\(^+\)-K\(^+\)-ATPase may be due to species differences (3) or to the techniques used in this study. Nonetheless, it should be pointed out that the depressed activity of porcine kidney Na\(^+\)-K\(^+\)-ATPase in hypothyroidism is associated with a decrease in the abundance of β-subunit without any change in the α-subunit (18). Furthermore, inactivation of Na\(^+\)-K\(^+\)-ATPase by a high concentration of 2-mercaptoethanol at high temperature is also associated with deterioration of the β-subunit without any effect on the α-subunits (10). The observed effect of HOCl on the β-subunit of Na\(^+\)-K\(^+\)-ATPase, unlike the α-subunits, may be due to the presence of SH groups and disulfide bonds in the β-subunit (11). Earlier studies have shown that the β-subunit is required for the enzymatic activity of Na\(^+\)-K\(^+\)-ATPase because it cannot be separated from the α-subunits without an irreversible loss of enzyme function (18, 30). It should also be noted that SL Na\(^+\)-K\(^+\)-ATPase activity is decreased on incubation with antibody for the β-subunit (24). Thus it appears that the observed depression in SL Na\(^+\)-K\(^+\)-ATPase activity by HOCl may be mainly due to the malfunction of β-subunit of the enzyme.

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