Reversible Oxidative Modification
A Key Mechanism of Na\textsuperscript{+}-K\textsuperscript{+} Pump Regulation

Gemma A. Figtree,* Chia-Chi Liu,* Stephanie Bibert, Elisha J. Hamilton, Alvaro Garcia, Caroline N. White, Karin K.M. Chia, Flemming Cornelius, Kaethe Geering, Helge H. Rasmussen

Abstract—Angiotensin II (Ang II) inhibits the cardiac sarcolemmal Na\textsuperscript{+}-K\textsuperscript{+} pump via protein kinase (PK)C-dependent activation of NADPH oxidase. We examined whether this is mediated by oxidative modification of the pump subunits. We detected glutathionylation of \( \beta_1 \), but not \( \alpha_1 \), subunits in rabbit ventricular myocytes at baseline. \( \beta_1 \) Subunit glutathionylation was increased by peroxynitrite (ONOO\textsuperscript{−}), paraquat, or activation of NADPH oxidase by Ang II. Increased glutathionylation was associated with decreased \( \alpha_1/\beta_1 \) subunit coimmunoprecipitation. Glutathionylation was reversed after addition of superoxide dismutase. Glutaredoxin 1, which catalyzes deglutathionylation, coimmunoprecipitated with \( \beta_1 \) subunit and, when included in patch pipette solutions, abolished paraquat-induced inhibition of myocyte Na\textsuperscript{+}-K\textsuperscript{+} pump current \( (I_p) \). Cysteine (Cys46) of the \( \beta_1 \) subunit was the likely candidate for glutathionylation. We expressed Na\textsuperscript{+}-K\textsuperscript{+} pump \( \alpha_1 \) subunits with wild-type or Cys46-mutated \( \beta_1 \) subunits in Xenopus oocytes. ONOO\textsuperscript{−} induced glutathionylation of \( \beta_1 \) subunit and a decrease in Na\textsuperscript{+}-K\textsuperscript{+} pump turnover number. This was eliminated by mutation of Cys46. ONOO\textsuperscript{−} also induced glutathionylation of the Na\textsuperscript{+}-K\textsuperscript{+} ATPase \( \beta_1 \) subunit from pig kidney. This was associated with a \( \approx 2\text{-fold decrease in the rate-limiting } E_2 \rightarrow E_1 \text{ conformational change of the pump, as determined by RH421 fluorescence. We propose that kinase-dependent regulation of the Na}\textsuperscript{+}-K\textsuperscript{+} pump occurs via glutathionylation of its \( \beta_1 \) subunit at Cys46. These findings have implications for pathophysiological conditions characterized by neurohormonal dysregulation, myocardial oxidative stress and raised myocyte Na\textsuperscript{+} levels. (Circ Res. 2009;105:185-193.)

Key Words: glutathionylation ■ glutaredoxin ■ Na\textsuperscript{+}-K\textsuperscript{+} pump ■ angiotensin ■ NADPH oxidase

The ATP-dependent Na\textsuperscript{+}-K\textsuperscript{+} pump transports Na\textsuperscript{+} out of cells in exchange for extracellular K\textsuperscript{+}, against their respective electrochemical gradients. The Na\textsuperscript{+} and K\textsuperscript{+} gradients that it generates maintain the membrane potential essential for cellular electric excitability. However, the gradients also have an important broader role because they serve in coupled co- and countertransport processes for other ions and organic compounds. Regulation of the Na\textsuperscript{+}-K\textsuperscript{+} pump, usually attributed to phosphorylation, is poorly understood. We have found that the \( \varepsilon \) isoform of protein kinase C (\( \varepsilon \)PKC) mediates angiotensin II (Ang II)–induced inhibition of the pump in rabbit ventricular myocytes.\(^1\) However, inhibition could not be attributed to phosphorylation of the pump molecule or of the closely associated “FXYD protein” phospholemman that has been implicated in PKC-dependent pump regulation in cardiac myocytes.\(^2\) Instead, the dependence of Ang II–induced pump inhibition on NADPH oxidase and superoxide (\( O_2\textsuperscript{−} \))\(^3\) strongly suggests a role for oxidative signaling.

Oxidative signaling can occur via modifications of susceptible protein sulfhydryl groups (protein–SH). Their reactivities are too low to make them realistic targets for oxidative modification induced by \( O_2\textsuperscript{−} \),\(^3\) but \( O_2\textsuperscript{−} \) can combine with nitric oxide (NO) to form ONOO\textsuperscript{−} or be dismutated to H\textsubscript{2}O\textsubscript{2}, either of which can oxidize protein–SH. Of the possible oxidative modifications, formation of a mixed disulfide with the tripeptide glutathione (GSH) is a good candidate for mediating signaling: glutathionylation is facilitated by an abundance of GSH with a high negative redox potential,\(^4\) results in the addition of 305 Da negatively charged adduct with the potential for steric effects akin to those of phosphorylation,\(^5\) and is readily reversible. Glutathionylation has recently been shown to mediate regulation of important cell proteins including SERCA\(^a\) and actin.\(^7\)

Glutathionylation of cysteines with typical redox potentials is not thermodynamically favored by an intracellular environment that is reducing\(^8\) but can be facilitated by subcellular compartmentalization.\(^6\) Coimmunoprecipitation of the Na\textsuperscript{+}-K\textsuperscript{+} pump with NADPH oxidase subunits in cardiac myocytes\(^1\) suggests that the pump is in a microdomain that supports oxidative signaling. We examined whether

Original received October 31, 2008; resubmission received April 21, 2009; revised resubmission received May 21, 2009; accepted June 10, 2009.
From the North Shore Heart Research Group (G.A.F., C.-C.L., E.J.H., C.N.W., K.K.M.C., H.H.R.), Kolling Institute, University of Sydney, Australia; Department of Cardiology (G.A.F., A.G., K.K.M.C., H.H.R.), Royal North Shore Hospital, Sydney, Australia; Department of Pharmacology and Toxicology (S.B., K.G.), University of Lausanne, Switzerland; and Department of Physiology and Biophysics (F.C.), Aarhus University, Denmark.
*Both authors contributed equally to this work.
Correspondence to Helge H. Rasmussen, Department of Cardiology, Royal North Shore Hospital, University of Sydney, St Leonards NSW 2065, Australia. E-mail helger@med.usyd.edu.au
© 2009 American Heart Association, Inc.

Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/CIRCRESAHA.109.199547

185
any of the sulphydryl groups in its α/β heterodimer are “reactive,” ie, susceptible to S-glutathionylation and found that a specific cysteine in the β1 subunit was glutathionylated by oxidant stimuli, including Ang II–induced activation of NADPH oxidase. We propose reversible glutathionylation offers an explanation for Na\(^{+}\)-K\(^{+}\) pump regulation that does not involve phosphorylation of the pump molecule or associated FXYD proteins. However, because NADPH oxidase activation is phosphorylation-dependent, the scheme retains the firmly established role of protein kinases. Such oxidative regulation of the Na\(^{+}\)-K\(^{+}\) pump has important physiological and pathophysiological implications.

Materials and Methods

Myocytes
We measured Na\(^{+}\)-K\(^{+}\) pump current (\(I_{\text{p}}\)) as a ouabain-induced shift in holding current of voltage-clamped isolated rabbit cardiac myocytes. To detect S-glutathionylation of pump subunits, myocytes were loaded with biotinylated GSH. After lysis the biotin-tagged glutathionylated subfraction was precipitated using streptavidin-sepharose beads and immunoblotted for \(\alpha_{1}\) and \(\beta_{1}\) Na\(^{+}\)-K\(^{+}\) pump subunits. Separate experiments immunoblotted \(\beta_{1}\) subunit immunoprecipitate with an antibody against glutathionylated protein (anti-GSH antibody).

Oocytes
Xenopus oocytes were injected with Xenopus \(\alpha_{1}\) and wild-type \(\beta_{1}\) or \(\beta_{1}C46W\) mutant cRNAs or human \(\alpha_{1}\) and \(\beta_{1}\) or \(\beta_{2}\) or \(\beta_{3}\) cRNAs. They were loaded with biotinylated GSH and glutathionylated protein precipitated with streptavidin-sepharose beads and immunoblotted for \(\beta_{1}\) subunit. Measurements of Na\(^{+}\)-K\(^{+}\) pump current were performed by the 2-electrode voltage clamp technique.

Pig Kidney Na\(^{+}\)-K\(^{+}\) ATPase
The hydrolytic activity of pig kidney Na\(^{+}\)-K\(^{+}\) ATPase was measured under varying conditions of Na\(^{+}\), K\(^{+}\), and ATP, and enzyme conformational changes were detected with RH421 fluorescence. An expanded Materials and Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Results

Glutathionylation of the Na\(^{+}\)-K\(^{+}\) Pump
To determine whether the \(\alpha_{1}\) and \(\beta_{1}\) subunits of the Na\(^{+}\)-K\(^{+}\) pump have reactive cysteine residues susceptible to glutathionylation, myocytes were loaded with biotin-GSH. They were lysed and biotin-tagged glutathionylated proteins were precipitated using streptavidin beads. We performed immunoblotting on this glutathionylated subfraction for the most abundant isoforms of the \(\alpha\) and \(\beta\) subunits of the pump, \(\alpha_{1}\) and \(\beta_{1}\). Both \(\alpha_{1}\) and \(\beta_{1}\) were easily detectable in total cell lysate and there was a strong signal for \(\beta_{1}\) in the biotin-tagged glutathionylated subfraction (Figure 1A). However, there was no evidence of glutathionylated \(\alpha_{1}\) subunits. The biotin-tagged glutathionylated \(\beta_{1}\) subunit was not detected when the lysate was incubated with 1 mmol/L dithiothreitol (DTT) before precipitation by streptavidin. This sensitivity to 1 mmol/L DTT is supportive of a mixed disulfide bond between the \(\beta_{1}\) subunit and GSH.

We next examined the effect of a chemical oxidant on \(\beta_{1}\) subunit glutathionylation. Myocytes loaded with biotin-GSH were exposed to solutions containing 100 μmol/L ONOO\(^{-}\) for 10 minutes. ONOO\(^{-}\) increased \(\beta_{1}\) subunit glutathionylation detected by immunoblotting the glutathionylated protein subfraction (GSS-protein) precipitated by streptavidin as shown in Figure 1B. The \(\alpha_{1}\) subunit was not detected in the GSS-protein subfraction, even after ONOO\(^{-}\) exposure (data not shown). To obtain independent support for glutathionylation of \(\beta_{1}\) subunit, we immunoprecipitated \(\beta_{1}\) subunit, and immunoblotted the precipitate with antibody to GSS-protein. As seen with the biotin-GSH technique, a moderate degree of \(\beta_{1}\) glutathionylation was present at baseline, and exposure of the myocytes to ONOO\(^{-}\) increased glutathionylation as shown in Figure 1C. The reverse immunoprecipitation experiment was performed, as shown in Figure 1D. Cell lysate was precipitated with the GSH antibody and immunoblotted for either \(\alpha_{1}\) or \(\beta_{1}\) subunit. ONOO\(^{-}\) increased glutathionylation of the \(\beta_{1}\) subunit, whereas \(\alpha_{1}\) was not detected in the protein subfraction immunoprecipitated by the GSH antibody.

The effect of additional oxidant stresses on \(\beta_{1}\) subunit glutathionylation was investigated. Myocytes loaded with biotin-GSH were exposed to H\(_{2}\)O\(_{2}\) for 15 minutes. This increased \(\beta_{1}\) subunit glutathionylation as shown in Figure 2A. Exposure of myocytes to 100 μmol/L paraquat, which increases O\(_{2}\)\(^{-}\)-sensitive fluorescence also increased \(\beta_{1}\) subunit glutathionylation (Figure 2B). Because membrane-permeable, pegylated superoxide dismutase (SOD) abolishes the paraquat-induced increase in O\(_{2}\)\(^{-}\)-sensitive fluorescence, we examined the effect of SOD on glutathionylation of the \(\beta_{1}\) subunit. Incubation of myocytes with 200 IU/mL pegylated SOD starting 5 minutes before exposure to para-
Paraquat abolished the increase in β1 subunit glutathionylation (Figure 2B). Paraquat-induced glutathionylation may partially be attributable to a direct effect of paraquat as a “redox-cycler,” producing O$_{2}^\cdot$ anions. Alternatively, paraquat uncouples NO synthase (NOS), resulting in O$_{2}^\cdot$ synthesis. We examined whether there is a physical association of endothelial (e)NOS and the pump. Cell lysate was immunoprecipitated with monoclonal antibodies to the α1 and β1 subunit of the pump and then immunoblotted for eNOS. Figure 2C shows that eNOS coimmunoprecipitated with the α1 and β1 subunits.

To examine whether pathophysiological conditions known to be associated with increased oxidative stress might induce β1 Na$^+$$-$$K^+$ pump subunit glutathionylation, we examined myocardium from a sheep model of infarction. As shown in Figure 2D, β1 subunit from myocardium immediately adjacent to an infarct zone showed increased glutathionylation compared with that in normal myocardium.

Receptor-Coupled Oxidant Signaling and Glutathionylation of the β1 Subunit

Because Ang II inhibits the cardiac Na$^+$$-$$K^+$ pump via PKC-dependent activation of NADPH oxidase, and NADPH oxidase subunits are physically associated with the pump, we examined the effect of Ang II on glutathionylation of the β1 pump subunit. We exposed myocytes to control solution or solution containing 100 mmol/L Ang II for 10 minutes before lysis. Figure 3A shows that Ang II increased glutathionylation of the β1 subunit as detected by the biotin-GSH technique. Results shown in Figure 3B confirm this with the independent GSH antibody technique.

Ang II–induced pump inhibition is abolished by SOD. Figure 3C shows that Ang II–induced glutathionylation persisted in myocytes preincubated with 100 IU/mL catalase to eliminate H$_2$O$_2$. However, it was abolished by preexposing myocytes to 100 μmol/L ebselen to scavenge ONOO$^-$ or 1 mmol/L N$^\text{4}$-nitro-L-arginine methyl ester (L-NAME) to inhibit NOS. L-NAME also reduced baseline β1 subunit glutathionylation.

Reversibility of Na$^+$$-$$K^+$ Pump β1 Subunit Glutathionylation

Figure 4A shows the increase in β1 subunit glutathionylation with either 10 minutes or 15 minutes of exposure of myocytes to paraquat. We examined whether the increase in glutathionylation is reversible by adding pegylated SOD to a final concentration of 200 IU/mL after 10 minutes of exposure to paraquat. The myocytes were then lysed after an additional 5 minutes. Scavenging of O$_{2}^\cdot$ with SOD significantly reduced glutathionylation of β1 subunit compared to the glutathionylation in myocytes exposed to paraquat alone for either 10 or 15 minutes (Figure 4A).

Glutathionylation is catalyzed by glutaredoxin 1 (GRx1). We examined whether this oxidoreductase is physically associated with the β1 subunit of the Na$^+$$-$$K^+$ pump. Cell lysate was immunoprecipitated with antibody to the β1 subunit and immunoblotted with monoclonal antibody to GRx1. Figure 4B shows that GRx1 coimmunoprecipitates with the β1 pump subunit. We used the whole-cell patch-clamp technique to examine whether GRx1 has functional effects on Na$^+$$-$$K^+$ pump current, I$_{p}$. The patch pipette solution included 100 μmol/L paraquat or was paraquat-free. Paraquat induced a large decrease in I$_{p}$. We included 10 μmol/L recombinant GRx1 in the pipette solution, and, to facilitate the conversion of GRx1 from its oxidized to its reduced form, we added 100 mmol/L recombinant GRx1 to the pipette solution.
Oxidative Modification of the β1 Subunit and α/β Subunit Coimmunoprecipitation

Because a heterodimer of the catalytic α subunit and the regulatory β subunit is essential for Na<sup>+</sup>-K<sup>+</sup> pump function, oxidation might inhibit pump activity by altering subunit interaction. Myocytes were exposed to control solutions or solutions that included 200 or 500 μmol/L ONOO<sup>·</sup> for 10 minutes. ONOO<sup>·</sup> had no effect on detection of α<sub>1</sub> or β<sub>1</sub> subunits in cell lysate by standard Western blot techniques (Online Figure I). Myocyte lysate was immunoprecipitated with α<sub>1</sub> subunit antibody and immunoblotted for α<sub>1</sub> or β<sub>1</sub> subunit. The total α<sub>1</sub> subunit immunoprecipitated by α<sub>1</sub> antibody was similar in cells exposed to ONOO<sup>·</sup> or control solutions. However, ONOO<sup>·</sup> reduced the β<sub>1</sub> subunit coimmunoprecipitated with α<sub>1</sub> subunit as shown in Figure 5A. Reverse coimmunoprecipitation experiments were also performed. Myocyte lysate was immunoprecipitated using the β<sub>1</sub> antibody and immunoblotted for α<sub>1</sub> subunit. As shown in Figure 5B, ONOO<sup>·</sup> induced a reduction in α<sub>1</sub> subunit detected in the β<sub>1</sub> immunoprecipitate. A receptor-mediated oxidant signal induced by exposing myocytes to Ang II also markedly decreased α/β subunit coimmunoprecipitation, both when myocyte lysate was immunoprecipitated with α<sub>1</sub> and immunoblotted with β<sub>1</sub> (Figure 5C), and vice versa (Figure 5D).

Candidate β1 Subunit Cysteine

The β<sub>1</sub> subunit has 7 cysteine residues, but 6 are linked in 3 disulfide bridges in the ectodomain leaving one, Cys46<sup>10</sup> (numbering of amino acids corresponds to Xenopus β<sub>1</sub> subunits), free as the likely candidate for oxidant modification. To investigate whether Cys46 is reactive, and whether it mediates oxidative regulation of the Na<sup>+</sup>-K<sup>+</sup> pump, we expressed Xenopus Na<sup>+</sup>-K<sup>+</sup> pump α<sub>1</sub> subunits with wild-type or Cys46Trp mutant Xenopus β<sub>1</sub> subunits in Xenopus oocytes. We have previously shown that this C46W substitution has no significant effect on the structural and functional maturation of the Na<sup>+</sup>-K<sup>+</sup> pump.<sup>10</sup> Figure 6A shows that most β<sub>1</sub> subunits identified by Western blot analysis were core-glycosylated after 2 days of expression (lanes 7 to 10), reflecting the continuous synthesis from injected cRNA. However, a population of fully glycosylated subunits also appeared, (lanes 7 to 10). No β<sub>1</sub> subunit could be detected in noninjected oocytes (lanes 11 and 12), in agreement with the low expression of endogenous, oocyte subunits.<sup>13</sup> Oocytes were injected with biotin-GSH, and we examined for glutathionylation using streptavidin pull-down and immunoblotting. ONOO<sup>·</sup> induced glutathionylation of the core-glycosylated wild-type β<sub>1</sub> subunit (lane 2). In control experiments, injection of inactivated ONOO<sup>·</sup> did not induce glutathionylation (data not shown). In contrast to wild-type β<sub>1</sub> subunit, C46W mutant β<sub>1</sub> subunit was not glutathionylated (lane 4).

To examine whether glutathionylation of Cys46 had an effect on Na<sup>+</sup>-K<sup>+</sup> pump function, we measured Na<sup>+</sup>-K<sup>+</sup> pump current in Na<sup>+</sup>-loaded oocytes (I<sub>max</sub>) expressing wild-type or C46W β<sub>1</sub> subunits. Oocytes showed a 5 to 6 fold increase in I<sub>max</sub> compared with I<sub>max</sub> measured in oocytes not injected with cRNA (Figure 6B). Injection with ONOO<sup>·</sup> significantly decreased I<sub>max</sub> in oocytes expressing wild-type, but not mutant C46W β<sub>1</sub> subunits (Figure 6B). This decrease was observed without injection of GSH as ex-
A 99% cross-species homology of the transmembrane domain of the β1 subunit suggests the transmembrane domain of the β1 subunit is important for Na+/K+ pump function. We examined whether oxidant pump regulation also occurs for the human Na+/K+ pump. We expressed human wild-type α1 and β1 pump subunits in Xenopus oocytes and measured Na+/K+ pump current. Injection with ONOO− significantly decreased $I_{max}$ as shown in Figure 7. The limited homology between the transmembrane domains of the β1 subunit and the β2 and β3 subunits of the Na+/K+ pump (57% to 61%) includes the key difference that β2 and β3 subunits have no free cysteine residues. Figure 7 shows that ONOO− has no effect on $I_{max}$ in oocytes expressing β2 or β3 subunits, as was the case for the cysteine-free β1 Xenopus mutant.

**Oxidative Modification of the β1 Subunit and Na+/K+ ATPase Kinetics**

The effect of oxidative modification on Na+/K+ pump kinetics was examined in Na+/K+ ATPase-enriched membranes from pig kidney. β1 Subunit glutathionylation was detected at baseline using the GSH antibody technique without supplemental GSH. This was substantially increased by ONOO−, implying the presence of GSH in the preparation (Online Figure II, A). A GSH assay indicated that the concentration in the preparation was approximately 75 μmol/L. ONOO−-induced glutathionylation was associated with decreased $\alpha_1/\beta_1$ subunit coimmunoprecipitation (Online Figure II, B).

We investigated effects of ONOO−/GSH on Na+/K+ ATPase steady state kinetics. Experimental conditions, Hill equations and the fitting parameters are shown in the Online Data Supplement. Activation of hydrolytic activity of control enzyme was compared with activation of enzyme incubated with 0.5 mmol/L ONOO− for 5 minutes. The enzyme was then incubated with 0.5 mmol/L GSH for 15 minutes to ensure GSH was not rate-limiting.

The effect of ONOO−/GSH on activation of Na-K ATPase by Na+ is shown in Figure 8A. Exposure to ONOO−/GSH inhibited maximal enzyme activity ($V_{max}$), whereas the apparent Na+ affinity ($K_{Na}^*$) was unchanged (Online Table I). GSH itself did not affect either $V_{max}$ or $K_{Na}^*$ (data not shown). The activation of hydrolytic activity by K+ is shown in Figure 8B. Again, ONOO−/GSH significantly inhibited maximal enzyme activity by ≈25%, whereas the apparent K+ affinity ($K_{K}^*$) was unchanged (Online Table II). Activation by ATP is shown in Figure 8C. Exposure to ONOO−/GSH significantly decreased both $V_{max}$ and the apparent ATP affinity constant ($K_{ATP}^*$) (Online Table III), whereas GSH alone affected neither $V_{max}$ nor $K_{ATP}^*$ (data not shown). Exposure of the enzyme to 2 mmol/L DTT significantly reversed the inactivation induced by ONOO−/GSH from ≈30% to 10% (data not shown). This is very unlikely to be attributable to an effect on protein disulfide bonds, for example in the ectodomain of the β1 subunit, because much higher concentrations of DTT are required to disrupt these.

We measured the vanadate sensitivity to test whether the decrease in $K_{ATP}^*$ was secondary to a ONOO−/GSH-induced shift in the $E_1/E_2$ conformational equilibrium...
toward $E_1$. Figure 8D shows that ONOO$^-$/GSH right-shifted the vanadate inhibition curve. There was a significant increase in the inhibition constant, $K_{II}$, from $0.62 \pm 0.03$ to $4.22 \pm 0.01$ μmol/L (Online Table IV). This indicates stabilization of the $E_1$ conformation with the lower vanadate affinity.

We followed the ATP phosphorylation reaction by stopped-flow fluorescence of the membrane probe RH421 starting either from an $E_1$ or an $E_2$ conformation of the enzyme. Because the $E_2 \rightarrow E_1$ transition is the main rate-determining step of the overall reaction cycle, it rate limits the phosphorylation reaction that follows. Thus, the rate of the $E_2 \rightarrow E_1$ reaction can be measured as the rate of the phosphorylation reaction when the enzyme is initially stabilized in the $E_2$ conformation. Two independent sets of experiments with 5 stopped-flow measurements each were performed under the different conditions. Representative traces are shown in Online Figure IV. Exposure of the enzyme to ONOO$^-$/GSH significantly decreased the rate constant of the $E_1 \rightarrow E_2P$ reaction from $45.2 \pm 3.0$ to $26.9 \pm 2.8$.

Figure 5. Effect of ONOO$^-$ on α/β Na$^+$/K$^+$ pump subunit coimmunoprecipitation. Representative α1 and β1 immunoblots after immunoprecipitation with α1 antibody (A) and after immunoprecipitation with β1 antibody (B). Effect of Ang II on α/β Na$^+$/K$^+$ pump subunit coimmunoprecipitation. Representative immunoblots after immunoprecipitation with α1 antibody (C) and after immunoprecipitation with β1 antibody (D). The histograms show mean densitometry of blots from 3 experiments, each normalized against control (%). *P<0.05 vs control.

Figure 6. Candidate β1 subunit cysteine. Xenopus oocytes expressing Xenopus Na$^+$/K$^+$ ATPase α1 subunit and either wild-type β1 subunit or β1.C46W mutant were injected with biotin-GSH and ONOO$^-$ as indicated. A, Effect of ONOO$^-$ on β1 subunit glutathionylation. Microsomes were prepared and either directly loaded on gels (lanes 7 to 12) or first immunoprecipitated with streptavidin beads (lanes 1 to 6). Proteins were immunoblotted with β1 subunit antibody. cg indicates core-glycosylated; fg, fully glycosylated β1 subunit. One of 3 similar experiments is shown. Histograms showing the effect of ONOO$^-$ on maximal Na$^+$/K$^+$ pump currents (mean $I_{max}$ from 10 oocytes from 2 different batches) (B), [3H]ouabain binding (n=16 from 2 different batches) (C), and turnover number of Na$^+$/K$^+$ ATPase (calculated as the ratio between the maximal Na$^+$/K$^+$ pump current and the number of ouabain binding sites) (D). *P<0.05 vs control.
sec\(^-1\) and for the E\(_2\)→E\(_1\)→E\(_2\)P reaction from 31.1±2.8 to 16.8±2.5 sec\(^-1\).

In some experiments, the enzyme was exposed to ONOO\(^-\) without supplemental GSH. Consistent with the presence of GSH in the enzyme preparation and the \(\beta_1\) subunit glutathionylation induced by ONOO\(^-\) without supplemental GSH, supplemental GSH had no significant effect on Na\(^+\)-K\(^+\) pump kinetics (Online Tables I through IV).

Discussion

We have shown that oxidation induces glutathionylation of the \(\beta_1\) subunit of the Na\(^+\)-K\(^+\) pump in rabbit cardiac myocytes, Xenopus oocytes, and pig kidney. In all models, glutathionylation was associated with inhibition of Na\(^+\)-K\(^+\) pump activity, specifically a decrease in \(I_p\) in cardiac myocytes, a decrease in \(I_{\text{max}}\) and turnover number in Xenopus oocytes, and a decrease in the steady-state hydrolytic activity and rate constant for the E\(_2\)→E\(_1\) conformational change of the pump in pig kidney membrane. Mutational studies indicated that this oxidative regulation was mediated by glutathionylation of Cys46 of the \(\beta_1\) subunit of the pump.

Glutathionylation of the \(\beta_1\) subunit of the Na\(^+\)-K\(^+\) pump is reversible in intact cardiac myocytes (Figure 4A). The rate of spontaneous breakage of disulfide bonds in the process of deglutathionylation is very low and would not allow efficient signaling.\(^9\) However, the process is catalyzed by a family of intracellular oxidoreductases. Of these, GRx1 has exclusive selectivity for GSS-protein versus other protein-mixed disulfides.\(^8\) We show that GRx1 coimmunoprecipitates with the \(\beta_1\) subunit (Figure 4B) and, when added to patch pipette solutions perfusing cardiac myocytes, abolishes paraquat-induced pump inhibition. These results showing enzymatically mediated reversibility support a functional role for glutathionylation of the \(\beta_1\) subunit in regulation of Na\(^+\)-K\(^+\) pump activity.

The crystal structure of the Na\(^+\)-K\(^+\) pump indicates that the reactive Cys46 of the \(\beta_1\) subunit is located in the bulk lipid of the membrane\(^18\) and therefore is expected to be poorly accessible to the cytosolic, hydrophilic GSH. However, the crystal structure was determined with the pump fixed in a state analogous to the E\(_2\)2K\(^+\)+Pi configuration, and large-scale structural changes that include the \(\beta_1\) subunit may occur during the E\(_2\)→E\(_1\) conformational change.\(^18\) Such structural change may make Cys46 accessible to GSH. In support of this, Cys46 is susceptible to oxidation induced by the watersoluble Cu-phenanthroline.\(^19\) The accessibility of Cys46 to the aqueous cytosol, at least after it has been glutathionylated, is also supported by the role of GRx1 because GRx1 is restricted to the cytosol.\(^8\)
As expected from the lack of transmembrane cysteine residue in the β1 or β3 isoforms, heterodimer pumps, which include these subunits, were not regulated by an oxidant signal. They may serve in a “housekeeping” role, or they may be regulated by an alternative mechanism. These differences suggest that the expression of specific β subunit isoforms in particular cell types or subcellular domains contribute to differential regulation of the Na+/K+ pump.

Redox signaling may be mediated by direct modification of cysteine residues at the active sites of target proteins. Alternatively, oxidative modification may alter the tertiary structure of the protein and its interaction with other proteins. Regulation of the Na+/K+ pump by redox signaling may arise from a change in the interaction of the β3 subunit with the catalytic α1 subunit. This is supported by the decrease in coimmunoprecipitation of α1/β1 subunits associated with glutathionylation. This decreased coimmunoprecipitation likely reflects a change in the physical interaction of the subunits revealed with exposure to detergents, although not necessarily that glutathionylation completely disrupts the heterodimer in vivo. Nevertheless, a very distinct effect of glutathionylation on α1/β3 interaction is suggested by the complete absence of α1 subunit in the glutathionylated protein subfraction precipitated with streptavidin. If the subunits remained closely associated, the α1 subunit would be expected to be indirectly precipitated by streptavidin via its association with the glutathionylated β1 subunit.

The critical role of the β subunit for Na+/K+ ATPase function and ion-binding was recently demonstrated in a high-resolution structure of the shark Na+/K+ ATPase. It may be of particular importance that Cys46 is only separated from one of the hydrogen bonds linking αM7 with the β1 subunit by 1 amino acid. The 2 units of the α1/β3 heterodimer move relative to each other during the transition from the E2 to E1 conformational state described by the Post–Albers scheme, and charged residues on the β1 subunit may affect this movement and hence functional properties of the holoenzyme.

We used the pig kidney Na+/K+ ATPase preparation to examine effects of glutathionylation on the kinetic properties of the pump because purity, specific activity, and properties of this widely used preparation are well documented and widely accepted. Exposure of the preparation to ONOO− inhibited enzymatic activity, and RH421 fluorescence studies indicated that ONOO− decreased the rate constant for the E2→E1 conformational change. The shift in the conformational poise toward E2 may explain the ONOO− induced increase in apparent affinity to ATP. The E2→E1 reaction is a good candidate for physiologically significant regulation because, together with regulation by changes in the intracellular K+ and Na+ concentrations, it is the main rate-limiting step in the overall pump cycle.

Kinetic studies on isolated Na+/K+ ATPase do not necessarily directly predict functional properties of the enzyme in the complex environment of the intact cell. This reservation is particularly important for interpretation of the data from patch clamped myocytes in this study. We included ATP in patch pipette solutions perfusing the intracellular compartment, but the diffusion coefficient for ATP is many orders of magnitude lower in the subsarcolemmal space than in the compartment perfused by the pipette solutions. Because ATP is continuously consumed by membrane ATPases, large concentration gradients within myocytes and a low concentration at the membrane can occur. This has important effects on the E2→E1 conformational change we identified as susceptible to oxidative signaling because the E2→E1 conformational change becomes sensitive to K+ at low ATP levels. The overall forward reaction is therefore inhibited by K+. The degree of inhibition is tissue-dependent, particularly evident in cardiac Na+/K+ ATPase, and a high intracellular K+ near physiological levels may enhance effects of oxidative signaling on the Na+/K+ pump in cardiac myocytes.

It should be noted that there was baseline glutathionylation of the β1 subunit of the pump in both heart and kidney as also reported for some other proteins, allowing for the possibility that deglutathionylation from baseline may cause Na+/K+ pump stimulation. Inhibition could be induced by Ang II receptor–coupled activation of oxidative signaling emphasizing its physiological relevance. Oxidative regulation of pump function may also be of pathophysiological importance, in particular in heart failure. An increase in cardiac myocyte oxidant stress and raised levels of intracellular Na+ are believed to contribute to its clinical manifestations. Oxidative modification of the key export route for Na+, the Na+/K+ pump, suggests that oxidant stress and raised myocyte Na+ are interrelated. Intuitively, one might therefore expect that treatment with antioxidants would be beneficial. However, clinical trials do not support such treatment. Mudd and Kass proposed that the effects of reactive oxygen species are dependent on the site of their synthesis in the myocyte and that a targeted inhibition is likely to be more successful than broad-acting antioxidants. Our findings that Ang II mediates Na+/K+ pump inhibition via activation of colocalized NADPH oxidase are in good agreement with this proposal and may partially explain the well-established clinical efficacy of angiotensin-converting enzyme inhibitors.

Acknowledgments
We thank Prof Stephen Hunyor and colleagues for kindly providing sheep myocardial samples.

Sources of Funding
Supported by grants from the North Shore Heart Research Foundation and the National Health & Medical Research Council (Australia). G.A.F. was supported by a Royal Australasian College of Physicians/High Blood Pressure Research Foundation Fellowship and the Medical Foundation, University of Sydney. K.G. was supported by Swiss National Science Foundation grant 3100A0-107513; and F.C. was supported by The Danish Medical Research Council.

Disclosures
None.

References
2. Han F, Bosuuyt J, Despa S, Tucker AL, Bero DM. Phospholemman phosphorylation mediates the protein kinase C-dependent effects on...


Reversible Oxidative Modification: A Key Mechanism of Na+-K+ Pump Regulation
Gemma A. Figtree, Chia-Chi Liu, Stephanie Bibert, Elisha J. Hamilton, Alvaro Garcia, Caroline N. White, Karin K.M. Chia, Flemming Cornelius, Kaethi Geering and Helge H. Rasmussen

Circ Res. 2009;105:185-193; originally published online June 18, 2009;
doi: 10.1161/CIRCRESAHA.109.199547

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/105/2/185

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2009/06/18/CIRCRESAHA.109.199547.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
REVERSIBLE OXIDATIVE MODIFICATION: A KEY MECHANISM OF Na⁺-K⁺ PUMP REGULATION

ONLINE SUPPLEMENT

EXPANDED METHODS

Animals and Cells

*Myocytes:* Ventricular myocytes were isolated from a total of 18 male White New Zealand rabbits\(^1\). They were used on the day of isolation only, and were stored at room temperature in Krebs-Henseleit buffer solution until used. Details of anaesthesia, excision of the heart and cell isolation techniques have been described previously\(^1\).

*Sheep:* Myocardium was obtained from a sheep model of myocardial infarction (MI). The animals were anesthetized with 20 mg/kg of thiopental sodium as induction, intubated, and ventilated with 1.5 L/min of oxygen, 2 L/min of nitrous oxide, and isoflurane (1.5% to 1.8%). Maintenance fluid was provided through peripheral venous access with Hartmann’s solution (at a rate of 1 mL/kg of body weight per hour). The electrocardiograph was monitored with electrodes clipped to the extremities. MI with reperfusion was induced via a cardiac catheterisation procedure, and involved an occlusion of the LAD coronary artery immediately distal to the dominant diagonal vessel, using an over-the-wire balloon catheter. The artery was occluded for 90 minutes and the balloon subsequently deflated for reperfusion. The sheep was euthanased > 24 hours post induction of MI, and tissue immediately harvested for protein studies.
**Xenopus oocytes**: Stage V-VI oocytes were obtained from a total of 20 *Xenopus laevis*².

**Pig kidney enzyme preparation**: Na⁺,K⁺-ATPase from pig kidney outer medulla was prepared by the SDS extraction procedure of Jorgensen³. Pig kidneys were obtained from the slaughterhouse. The protein concentration was about 4.5 mg/ml measured using the Peterson’s modification⁴ of the Lowry method⁵ using bovine serum albumin as a standard. The specific activity of the preparation was ~30 µmol·mg⁻¹·min⁻¹ at 37 °C.

Experimental protocols were approved by the animal ethics committee of the institution at which experiments were performed.

**Immunodetection of S-glutathionylated proteins in rabbit ventricular myocytes**

To detect S-glutathionylation of specific pump-related proteins, isolated myocytes were loaded with biotinylated glutathione (GSH) (500 µmol/L; 1 hour). Biotinylated GSH ester was made by mixing 25 mmol/L sulfo-NHS-biotin with 25 mmol/L GSH ethyl ester in 50 mmol/L NaHCO₃ at pH 8.5 for 2 h followed by the addition of 125 mmol/L NH₄HCO₃ at pH 8.5 for 1 h. After incubation in biotinylated GSH ester, cells were washed 3 times with cold phosphate buffer and lysed in buffer (50 mmol/L Tris-HCl, pH 7.4, 1% Nonidet P-40, 150 mmol/L NaCl, 50 µmol/L diethylenetriaminepentaaeic acid, 2 mmol/L phenylmethylsulfonyl fluoride) containing 10 mmol/L N-ethylmaleimide to block further thiol reactions. The biotin-tag was used to precipitate S-glutathionylated proteins using modifications of previously described methods⁶. Approximately 0.5 mg of protein was mixed with streptavidin-sepharose beads for 1 h. The beads were washed five times with lysis buffer with 0.1% SDS, and the final precipitate was incubated for 15 min with 40 µl of elution
buffer (lysis buffer + 20 mmol/L DTT) to release S-glutathionylated proteins. After adding Laemmli buffer, GSS-protein pulled down by this technique was separated by gel electrophoresis, transferred to a membrane and probed with antibodies to the $\alpha_1$ and $\beta_1$ subunit of the Na$^+$-K$^+$ pump.

Separate experiments were performed using an antibody against glutathionylated protein (anti-GSH antibody). Myocytes were treated with ice-cold lysis buffer containing 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 8.0), 1% Triton X-100, 2 mM EDTA, and protease inhibitors (Complete EGTA-free, Roche Diagnostics). After 5 min at 4°C, the lysate was clarified by centrifugation at 16,000 g for 20 min. The supernatant (0.25–1 mg protein) was precleared and incubated with the appropriate antibody and then with protein A/G plus agarose beads. The proteins bound to the collected beads were subjected to SDS-PAGE and probed with appropriate antibodies. This protocol was also used to detect co-immunoprecipitation of pump-subunits with glutaredoxin 1 (GRx1).

The studies outlined above were performed on control myocytes, or myocytes exposed to either chemical oxidants, or the receptor-coupled oxidative stimulus, Ang II. The protocols for Ang II exposure were designed to parallel previous patch-clamp studies that were restricted in duration. The concentrations of Ang II were chosen to assure saturation binding within this time period.

**Immunodetection of S-glutathionylation of $\beta_1$ subunit in *Xenopus* oocytes**

Oocytes were injected with *Xenopus* $\alpha_1$ (10 ng) and wild type $\beta_1$ (1 ng)$^9$ or $\beta_1$C46W mutant (2 ng)$^9$ cRNAs. After two days of expression, oocytes were injected with biotinylated GSH ester and incubated for 45 min at 19°C. Since in *Xenopus* oocytes, the basal GSH level was estimated at 2.5 mmol/L$^{10}$ and could compete with
exogenous biotinylated GSH, 50 nl/oocyte of 25 mmol/L biotinylated GSH ester were injected to achieve a final concentration of 2.5 mmol/L, assuming an intracellular water space of 0.5 µl/oocyte. S-glutathionylation was then activated by injection of oocytes with 50 nl/oocyte of 1 mmol/L peroxynitrite at pH 7.4.

In view of the short half-life of peroxynitrite at pH 7.4, fresh peroxynitrite solutions were prepared from stock solutions after injection of each batch of 40 oocytes. Stock solutions were diluted in MBS (Modified Earth’s solution) buffer to a final concentration of 1 mM containing a volume of HCl determined in preliminary experiments to buffer the solution at pH 7.4. The final ionic strength of the MBS buffer is 183 mmol/l for control solution, and 193 mmol/l for ONOO- containing solution. The control oocyte population was either injected with decomposed peroxynitrite stored for 7 months, or was not injected with peroxynitrite. After 15 min incubation at 19 °C, oocyte microsomes were prepared as described with buffers containing 10 mmol/L N-ethylmaleimide. The protein content was determined by the method of Lowry. Microsomal proteins (10 µg) were directly subjected to SDS-PAGE or first pulled down with Streptavidin-Sepharose beads (200 µg), and then transferred overnight at 40 V to nitrocellulose membranes. Membranes were blocked with 10% nonfat dried milk in Tris-buffered saline containing 0.1% Tween-20 and incubated with Xenopus β1 primary antibody (1/1000)11 and peroxidase coupled secondary antibodies (1/10,000, Amersham Biosciences), and the complex was revealed with the ECL chemiluminescence kit (Amersham Biosciences) according to the manufacturer’s protocol.

Measurement of electrogenic Na⁺-K⁺ pump current (Iₚ) in rabbit cardiac myocytes
We measured electrogenic Na\(^+\)-K\(^+\) pump current (arising from the 3:2 Na\(^+\):K\(^+\) exchange ratio) in single myocytes using the whole-cell patch clamp technique. Solutions were designed to minimize non-pump membrane currents. We used wide-tipped patch pipettes (4-5 µm) filled with solutions containing (in mmol/L): HEPES 5; MgATP 2; ethylene glycol-bis(\(\beta\)-aminoethyl ether)-N,N',N'-tetraacetic acid (EGTA) 5; potassium glutamate 70, sodium glutamate 10 and tetramethylammonium chloride (TMA-Cl) 80, and L-arginine 0.010. They were titrated to a pH of 7.20 at 35 °C with KOH. Characteristics of patch pipettes filled with these solutions has been described\(^{12}\). We included recombinant GRx1 and/or GSH in pipette solutions as indicated. A holding potential of -40 mV was used to inactivate voltage-sensitive Na\(^+\) channels. While we established the whole-cell configuration myocytes were superfused with solution containing (in mmol/L): NaCl 140; KCl 5.6; CaCl\(_2\) 2.16; MgCl\(_2\) 1; glucose 10; NaH\(_2\)PO\(_4\) 0.44; N-2-hydroxyethyl piperazine-N'-2-ethane-sulphonic acid (HEPES) 10. It was titrated to a pH of 7.40 at 35 °C with NaOH. Two to three minutes after the whole cell configuration was established we switched to a superfusate that was designed to block membrane current arising from transmembrane K\(^+\) and Ca\(^{2+}\) gradients. It was nominally Ca\(^{2+}\)-free and contained and 0.2 mmol/L CdCl\(_2\) and 2 mmol/L BaCl\(_2\).

We used Axoclamp 2A and 2B voltage clamp amplifiers, supported by pClamp version 7 and Axotape version 2 (Axon Instruments, Ca, USD) to record currents. Na\(^+\)-K\(^+\) pump current (I\(_p\)) was identified as the difference between holding currents, sampled at 1 Hz before and after Na\(^+\)-K\(^+\) pump blockade with 100 µmol/L ouabain. Since pump currents are small, their identification is susceptible to contamination by currents from any source, and it is critical that only experiments with stable holding currents are included. The criteria for identification of stable currents and the
ouabain-induced changes in them from samples obtained with an electronic cursor have been reported\textsuperscript{12}. The effect of ouabain is not reversible within the time frame stable holding currents can be reliably measured\textsuperscript{12, 13}, and wash-out of the effect of ouabain was not attempted. $I_p$ was normalized for membrane capacitance.

\textbf{Maximal Na$^+$-K$^+$ pump current measurements in \textit{Xenopus} oocytes}

Oocytes were injected with \textit{Xenopus} $\alpha_1$ and wild type $\beta_1$\textsuperscript{8} cRNA or C46W $\beta_1$ mutant\textsuperscript{9} cRNA, or with human $\alpha_1$ (12 ng) and $\beta_1$, $\beta_2$ or $\beta_3$ (1 ng)$^{14}$ cRNAs. Measurements of Na$^+$-K$^+$ pump current were performed by the two-electrode voltage clamp technique, two days after oocyte injection. Oocytes were loaded with Na$^+$ by overnight incubation in a K$^+$-free medium\textsuperscript{15}. The membrane potential was set at -50 mV and the Na$^+$-K$^+$ pump current ($I_{\text{max}}$) of these Na$^+$-loaded myocytes was measured at room temperature as the outward current induced by the addition of 10 mmol/L K$^+$.

For the determination of effects of ONOO$^-$ on $I_{\text{max}}$ of Na$^+$-K$^+$ ATPase, oocytes were incubated for 2 h with 1 mmol/L biotinylated GSH ester at 19 °C and then injected with ONOO$^-$, 15 min before measurements.

\textbf{3H-ouabain binding on intact oocytes}

The total number of Na$^+$-K$^+$ ATPase expressed at the cell surface was determined by $^3$H-ouabain binding on non-injected oocytes or two days after incubation, on oocytes injected with \textit{Xenopus} $\alpha_1$ cRNA and wild type $\beta_1$ or C46W $\beta_1$ cRNA. Briefly, oocytes were loaded with Na$^+$ in a K$^+$-free solution containing 90 mmol/L NaCl, 1 mmol/L CaCl$_2$, 1 mmol/L MgCl$_2$, 10 mmol/L Hepes, pH 7.4 for 2 h at 19 °C. Oocytes were then incubated at 19°C for 2h in the K$^+$-free solution containing 1 mmol/L biotinylated
GSH ester. After incubation, oocytes were injected or not with ONOO\(^-\), and incubated at room temperature for 15 min. Oocytes were then incubated in a K\(^+\) free solution containing 0.3 $\mu$mol/L [21,22-\(^3\)H] ouabain (Amersham, specific activity 15 Ci/mmol) and 0.7 $\mu$mol/L cold ouabain for 30 min. Non-specific ouabain binding was determined in the presence of 300 $\mu$mol/L cold ouabain and amounted to about 5% of total binding. Oocytes were extensively washed with a buffer containing 90 mmol/L NaCl, 30 mmol/L imidazole, pH 7.4, individually transferred to scintillation tubes and solubilized with 100 $\mu$L of 5% SDS. Solubilized oocytes were counted after addition of 2 ml of Scintillator 299 (Packard).

**Measurement of GSH**

Ellman's assay\(^{16}\) was used to measure GSH in the pig kidney membrane Na\(^+\)-K\(^+\) ATPase preparation. A 100 $\mu$L of sample solution was mixed with 850 $\mu$L 0.1 mol/L NaH\(_2\)PO\(_4\), 5 mmol/L EDTA (pH 7.5) and 100 $\mu$L 6 mmol/L DTNB. Each sample solution was incubated for 5 minutes at room temperature. The absorbance at 412 nm was measured. GSH concentrations were calculated using a molar extinction of 13,600 Lcm\(^{-1}\)mol\(^{-1}\).

**Kinetics of pig kidney Na\(^+\)-K\(^+\) ATPase**

The hydrolytic activity of pig kidney Na\(^+\)-K\(^+\) ATPase was measured under varying conditions of Na\(^+\), K\(^+\), and ATP as described below\(^{17}\). The temperature was 23 °C and pH was 7.4.
Na\textsuperscript{+}-activation was measured at 20 mmol/L K\textsuperscript{+}, 3 mmol/L Mg\textsuperscript{2+}, 3 mmol/L ATP, 30 mmol/L histidine. The enzyme was incubated with 0.5 mmol/L ONOO\textsuperscript{−} for 5 min at 37 °C with or without subsequent incubation with 0.5 mmol/L GSH for 15 min. A Hill equation:

$$V = V_{max}/(1+10^{(\log K_{0.5}-[Na^+])n_H})$$

was fitted to the Na\textsuperscript{+}-induced activation of hydrolytic activity. $V_{max}$ is the maximum activity, $K_{0.5}$ is the half-maximum Na\textsuperscript{+}-concentration and $n_H$ is the Hill coefficient.

K\textsuperscript{+}-activation was measured at 100 mmol/L Na\textsuperscript{+}, 3 mmol/L Mg\textsuperscript{2+}, 3 mmol/L ATP, 30 mmol/L histidine. A Hill equation:

$$V = (V_{max} - V_0)/(1+10^{(\log K_{0.5}-[K^+])n_H}) + V_0$$

was fitted to the K\textsuperscript{+}-induced activation of hydrolytic activity. $K_{0.5}$ is the K\textsuperscript{+}-concentration half-way between baseline ($V_0$, Na\textsuperscript{+}-K\textsuperscript{+} ATPase activity) and maximum Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity ($V_{max}$), and $n_H$ is the Hill coefficient.

ATP-activation was measured at 130 mmol/L Na\textsuperscript{+}, 20 mmol/L K\textsuperscript{+}, 3 mmol/L Mg\textsuperscript{2+}, and 30 mmol/L histidine.

Vanadate is a transition-state analogue of inorganic phosphate and binds preferentially to the E\textsubscript{2} conformation of Na\textsuperscript{+}-K\textsuperscript{+} ATPase. The vanadate sensitivity of the enzyme can therefore be used as an index of the steady-state E\textsubscript{1}/E\textsubscript{2} distribution.\textsuperscript{18, 19} Vanadate inhibition of hydrolytic activity was measured at 100 mmol/L Na\textsuperscript{+}, 20 mmol/L K\textsuperscript{+}, 2 mmol/L Mg\textsuperscript{2+}, 100 µmol/L M ATP and 30 mmol/L histidine. A Hill equation:

$$V = (V_{max} - V_0)/(1+10^{(\log K_{0.5}-[VO_3])n_H}) + V_0$$
was fitted to the vanadate-induced inhibition of hydrolytic activity. $K_i$ is the vanadate concentration in µmol/L that gives an activity ($V$) half-way between baseline ($V_0$) and maximum ($V_{\text{max}}$) and $n_H$ is the Hill coefficient. $V_{\text{max}}$ is not the global maximum activity since the ATP concentration is suboptimal (100 µmol/L).

The phosphorylation reaction induced by ATP was studied by stopped-flow fluorescence measurements using the molecular probe RH421. The RH421 styryl dye partitions into membranes and is sensitive to formation of the E$_2$P phosphoform. The measurements were performed at 20°C using an SX.17MV spectrofluorimeter (Applied Photophysics, U.K.). The flow volume was ~150 µl. The excitation wavelength was set at 546 nm and fluorescence emission was measured using a 630 nm cut-off filter. The dead time was ~1.5 ms. For each fluorescence trace 1000 data points were collected, and 5 experimental traces were averaged before fitting single exponential equation with floating end-point to the data.

ATP phosphorylation initiated from either an E$_1$ or an E$_2$ enzyme conformation was detected by incubating the enzyme (~25 µg/ml) in histidine buffer (30 mmol/L, pH 7.4) plus 0.1 mmol/L EDTA in the presence or absence of Na$^+$ and ATP to stabilize the E$_1$ or E$_2$ conformations, respectively. Thus, to stabilize the enzyme in the E$_1$ conformation it was incubated in the histidine/EDTA medium with addition of Na$^+$ (130 mmol/L M) and ATP (30 µmol/L), and to stabilize the E$_2$ conformation Na$^+$ and ATP were omitted. In both cases the sample containing 200 nmol/L RH421 was loaded in syringe 1 of the stopped-flow equipment. Phosphorylation was initiated by mixing with the second syringe containing either 5 mmol/L Mg$^{2+}$ in histidine/EDTA buffer (E$_1$), or 130 mmol/L Na$^+$, 5 mmol/L Mg$^{2+}$ and 30 µmol/L ATP in histidine/EDTA buffer (E$_2$). In the first case the high-fluorescence E$_2$P conformation is formed directly from preformed E$_1$, whereas in the second case it is formed following an initial E$_2$ to
E₁ conformational change: E₂ → E₁ → E₂P. In the latter case the rate constant for phosphorylation will decrease if the phosphorylation reaction is rate-limited by the initial conformational change ²².

**Materials**

Peroxynitrite (ONOO⁻) and sulfo-NHS biotin were obtained from Merck. Streptavidin-sepharose was obtained from GE Healthcare Bio-Sciences. Angiotensin II, GSH ethyl ester, ouabain, L-NAME (L-Nitro-Arginine Methyl Ester) and paraquat were purchased from Sigma. Mouse monoclonal antibodies were obtained from the following vendors: α₁- and β₁-subunits of Na⁺-K⁺-ATPase from Upstate Biotechnology; eNOS from Alexis Biochemicals; Grx1 from Sata Cruz Biotechnology, and anti-GSH from Invitrogen. The goat anti-mouse IgG-HRP secondary antibody and chemiluminescence ECL kit were purchased from Pierce Protein A/G plus agarose was purchased from Santa Cruz Biotechnology. Recombinant GRx1 was purchased from United Bioresearch Products. TMA.Cl was purum grade and obtained from Fluke Chemicals. All other chemicals used in Tyrodes solutions were analytical grade and were obtained from BDH. RH421 was from Molecular Probes (OR, USA) and dissolved in DMSO at 2 mg/ml. ATP was from Boehringer Mannheim (Germany).

**Data analysis**

Each presented immunoblot is representative of at least three separate experiments. The band densities were quantified by densitometry (Fujifilm, LAS-3000). Data are expressed as the mean ± SE. Statistical comparisons were made with a Student’s t
test or 1 way ANOVA as appropriate. P<0.05 was considered to be statistically significant.
DATA SUPPLEMENT

Figures

Online Figure I. Effect of A. ONOO−; and B. Ang II on detection of α₁ and β₁ subunit by Western blot of cell lysate prior to immunoprecipitation.
Online Figure II. Glutathionylation of β₁ subunit and α₁/β₁ subunit co-immunoprecipitation in pig kidney membranes without supplemental GSH. Na⁺-K⁺ ATPase enriched membranes were exposed to ONOO⁻ for 15 min. A. ONOO⁻ increased glutathionylation as shown by the representative immunoblot, and the average densitometry from 3 experiments. B. ONOO⁻ decreased α₁/β₁ co-immunoprecipitation as shown by the representative immunoblot and the average densitometry from 3 experiments. * indicates significant difference versus control (p<0.05).
Online Figure III. Representative traces showing the effect of glutaredoxin 1 (GRx1) on paraquat induced Na⁺-K⁺ pump inhibition. The patch pipette solutions contained GRx1 (10 µmol/L), GSH (1 mmol) or paraquat (100 µmol/L) as indicated. Changes in superfusates are indicated. It is an important feature shown by the traces that holding currents before and after exposure to ouabain were stable to allow identification of the small Na⁺-K⁺ pump currents, according to pre-determined criteria. Na⁺-K⁺ pump current (Iₚ, pA) was identified from sampling raw data with an electronic cursor.
Online Figure IV. Stopped-flow fluorescence transients of pig kidney Na,K-ATPase labelled with 200 nM RH421 induced by ATP phosphorylation. In the left panel the enzyme in one syringe is stabilized in the E1-conformation by incubating in 50 mmol/L NaCl, 2 mmol/L ATP, 0.1 mmol/L EDTA and 30 mmol/L histidine, pH 7.4. In the right panel the enzyme is stabilized in the E2-conformation by omitting NaCl and ATP. Phosphorylation from the E1 conformation was initiated by mixing with the second syringe containing 130 mmol/L NaCl, 0.1 mM EDTA, 30 mmol/L histidine pH 7.4 plus 5 mmol/L MgCl₂. In case where the enzyme was phosphorylated from the E2-conformation the second syringe contained 130 mmol/L NaCl, 0.1 mmol/L EDTA, 30 mmol/L histidine pH 7.4 plus 2 mmol/L ATP and 5 mmol/L MgCl₂. The traces were fitted using a monoexponential time function. For the E1→E2P reaction (left panel) the rate constants for control and ONOO⁻/GSH treated enzyme were $42.3 \pm 3.8 \text{ s}^{-1}$ and $29.3 \pm 3.7 \text{ s}^{-1}$. For the E2→E1→E2P reaction (right panel) the rate constants were $31.5 \pm 3.9 \text{ s}^{-1}$ and $13.4 \pm 4.9 \text{ s}^{-1}$.
### Fitting Parameters for Na\(^+\)-K\(^+\) ATPase kinetics in pig kidney membrane.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ONOO'/GSH</th>
<th>ONOO(^-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(V_{\text{max}}) (µmol-mg(^{-1})-h(^{-1}))</td>
<td>322.1 ± 6.2</td>
<td>235.5 ± 5.2</td>
<td>228.5 ± 3.8</td>
</tr>
<tr>
<td>(K_{0.5}) (mmol-L(^{-1}))</td>
<td>14.8 ± 0.2</td>
<td>13.9 ± 0.4</td>
<td>13.9 ± 0.4</td>
</tr>
<tr>
<td>(n_H)</td>
<td>1.59 ± 0.08</td>
<td>1.47 ± 0.08</td>
<td>1.42 ± 0.05</td>
</tr>
</tbody>
</table>

**Online Table I.** Na\(^+\) activation of hydrolytic activity.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ONOO'/GSH</th>
<th>ONOO(^-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(V_0)</td>
<td>23.3 ± 2.3</td>
<td>16.5 ± 1.9</td>
<td>16.9 ± 1.8</td>
</tr>
<tr>
<td>(V_{\text{max}}) (µmol-mg(^{-1})-h(^{-1}))</td>
<td>315.1 ± 2.9</td>
<td>233.4 ± 2.6</td>
<td>230.9 ± 2.3</td>
</tr>
<tr>
<td>(K_{0.5}) (mmol-L(^{-1}))</td>
<td>0.59 ± 0.03</td>
<td>0.64 ± 0.04</td>
<td>0.59 ± 0.04</td>
</tr>
<tr>
<td>(n_H)</td>
<td>1.56 ± 0.05</td>
<td>1.46 ± 0.05</td>
<td>1.48 ± 0.05</td>
</tr>
</tbody>
</table>

**Online Table II.** K\(^+\) activation of hydrolytic activity.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ONOO'/GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>(V_{\text{max}}) (µmol-mg(^{-1})-h(^{-1}))</td>
<td>352 ± 10</td>
<td>227 ± 3</td>
</tr>
<tr>
<td>(K_{\text{ATP}}) (µmol-L(^{-1}))</td>
<td>629 ± 44</td>
<td>493 ± 19</td>
</tr>
</tbody>
</table>

**Online Table III.** ATP-activation of hydrolytic activity.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ONOO'/GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>(V_0)</td>
<td>1.1 ± 0.1</td>
<td>0.21 ± 0.08</td>
</tr>
<tr>
<td>(V_{\text{max}}) (µmol-mg(^{-1})-h(^{-1}))</td>
<td>22.8 ± 0.2</td>
<td>21.2 ± 0.1</td>
</tr>
<tr>
<td>(K_{0.5}) (µmol-L(^{-1}))</td>
<td>0.62 ± 0.03</td>
<td>4.22 ± 0.06</td>
</tr>
<tr>
<td>(n_H)</td>
<td>−1.59 ± 0.08</td>
<td>−1.18 ± 0.03</td>
</tr>
</tbody>
</table>

**Online Table IV.** Vanadate inhibition of hydrolytic activity.
References


