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Original Contribution

Involvement of Na/K-ATPase in hydrogen peroxide-induced activation of the Src/ERK pathway in LLC-PK1 cells

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ABSTRACT

We have shown that Na/K-ATPase interacts with Src. Here, we test the role of this interaction in H_2O_2-induced activation of Src and ERK. We found that exposure of LLC-PK1 cells to H_2O_2 generated by the addition of glucose oxidase into the culture medium activated Src and ERK1/2. It also caused a modest reduction in the number of surface Na/K-ATPases and in ouabain-sensitive Rb^+ uptake. These effects of H_2O_2 seem similar to those induced by ouabain, a specific ligand of Na/K-ATPase, in LLC-PK1 cells. In accordance, we found that the effects of H_2O_2 on Src and ERK1/2 were inhibited in α1 Na/K-ATPase-knockdown PY-17 cells. Whereas expression of wild-type α1 or the A420P mutant α1 defective in Src regulation rescued the pumping activity in PY-17 cells, only α1, and not the A420P mutant, was able to restore the H_2O_2-induced activation of protein kinases. Consistent with this, disrupting the formation of the Na/K-ATPase/Src complex with pNaKtide attenuated the effects of H_2O_2 on the kinases. Moreover, a direct effect of H_2O_2 on Na/K-ATPase-mediated regulation of Src was demonstrated. Finally, H_2O_2 reduced the expression of E-cadherin through the Na/K-ATPase/Src-mediated signaling pathway. Taken together, the data suggest that the Na/K-ATPase/Src complex may serve as one of the receptor mechanisms for H_2O_2 to regulate Src/ERK protein kinases and consequently the phenotype of renal epithelial cells.

The Na/K-ATPase is a transmembrane enzyme responsible for pumping Na^+ and K^+ ions across the plasma membrane by hydrolyzing ATP [1]. Recent studies have revealed many non-pumping functions of the Na/K-ATPase, especially the α1 isoform [2]. For example, the α1 Na/K-ATPase is involved in the formation of membrane structures such as caveolae and tight junctions [3,4]. Moreover, the α1 Na/K-ATPase tethers and regulates multiple protein and lipid kinases as well as membrane receptors (e.g., Src, epidermal growth factor receptor (EGFR), and PI3K) [2,5]. Specifically, we have reported that the α1 subunit of Na/K-ATPase interacts with Src via two pairs of domain interactions [6]. Functionally, these interactions allow a cell to regulate Src and Src effectors through at least two potential pathways. First, changes in the expression of either α1 or Src would alter the interaction equilibrium between these two proteins, resulting in changes in Src activity because these interactions keep Src in an inactive state [7]. In accordance, a decrease in the α1 expression correlates with a large increase in Src activity in cultured cells as well as in vivo [7–9]. Moreover, we have recently demonstrated that the expression of α1 Na/K-ATPase in the plasma membrane is significantly reduced in highly proliferative cells in which an increase in active Src is detected [10]. Second, the Na/K-ATPase/Src complex functions as a receptor for cardiotonic steroids and other pump ligands [11]. Based on early studies, the α1 Na/K-ATPase adopts two major distinct conformations, namely E1 and E2 [12]. The equilibrium between E1 and E2 could be regulated by many factors including pump substrates and cardiotonic steroids such as ouabain [12]. Whereas E1 Na/K-ATPase keeps Src in an inactive state, conversion of E1 to E2 by ouabain activates the Na/K-ATPase-associated Src [11]. It is important to note that normal epithelial cells express millions of α1 Na/K-ATPases (roughly five times the amount of Src) [13]. Therefore, the Na/K-ATPase/Src interaction could represent a critical mechanism of Src regulation [13,14], working in concert with the other well-established pathways, including tyrosine phosphorylation of Src [15,6].

Src is the prototypic member of a family of nonreceptor tyrosine kinases that associate with the plasma membrane.

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It contributes to the regulation of several important cellular activities such as cell proliferation, survival, motility, and migration through activation of multiple protein and lipid kinase cascades via phosphorylation-mediated pathways [16]. Src contains a positive-regulatory phosphorylation site at Tyr418 (Y418) and a negative phosphorylation site at Tyr529 (Y529). Phosphorylation of Y418 induces a full activation of Src. Many intracellular and extracellular stimuli affect Src activities [15]. One class of these regulators is reactive oxygen species (ROS) [17].

"Reactive oxygen species" refers to a group of oxygen derivatives, including superoxide, H_2O_2, and hydroxyl radical. Although excessive production of ROS could induce DNA damage, oxidation of protein and lipids, and cell death, recent studies have demonstrated ROS to be important second messengers [18]. For example, we have shown that increases in ROS are required for ouabain-induced growth stimulation of cardiac myocytes [19,20] and marinobufugenin-induced collagen production of cardiac fibroblasts [21]. ROS have also been shown to regulate renal proximal tubule sodium handling and contribute to renal fibrosis, diabetic nephropathy, and hypertension [22,23]. Furthermore, as second messengers, ROS exerts many regulatory effects on key signaling proteins [17]. For example, increases in cellular ROS could activate protein kinase cascades, including the transactivation of EGFR and consequently the activation of extracellular signal-regulated kinase 1/2 (ERK1/2). Interestingly, inhibition of Src by chemical Src inhibitors (such as PP2) or the expression of dominant-negative mutant of Src or Src RNAi attenuates ROS-induced activation of these kinases [17,24]. These findings point to Src as a key upstream target of ROS-initiated signal transduction [25,26]. Although direct oxidation/reduction of Src by ROS appears to be involved in regulation of Src activity, there is also evidence that ROS could regulate Src by affecting the interaction between Src and its regulatory proteins [17]. To this end, it is known that ROS affects the Na/K-ATPase directly [20,27–30]. The fact that the Na/K-ATPase is a major regulator of Src in epithelial cells led us to propose that the Na/K-ATPase could be involved in ROS-mediated regulation of Src and consequently other protein kinases such as ERK1/2. To test this hypothesis, we have measured the effects of H_2O_2 on cellular Na/K-ATPase, Src, and ERK1/2 in control LLC-PK1 cells, Na/K-ATPase-knockdown cells, and both wild-type and Src regulation-defective α1 mutant-rescued cells. These studies suggest that the Na/K-ATPase/Src complex plays an important role in ROS-induced activation of Src and ERK1/2.

Material and methods

Materials

The antibodies used in this work and their resources were as follows: the polyclonal anti-ERK1/2 antibody, the monoclonal anti-phospho-ERK1/2 antibody, the monoclonal anti-c-Src antibody (B12), the monoclonal anti-insulin receptor β subunit antibody, and the anti-rabbit- and the anti-mouse-conjugated horseradish peroxidase second antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); the polyclonal anti-phospho-Src pY418 and the polyclonal anti-phospho-Src pY529 antibodies were obtained from Invitrogen (Carlsbad, CA. USA); the monoclonal anti-α1 antibody α6 f was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa and used for Western blot; the recombinant human Src was from Upstate (Lake Placid, NY, USA). Chemiluminescence ECL kit was purchased from PerkinElmer (Rockford, IL, USA). [^{3}H]Ouabain was purchased from DuPont NEN (Boston, MA, USA). Radioactive $^{86}Rb^+$ was from PerkinElmer Life Science Products (Boston, MA, USA). Other chemicals of the highest purity were all obtained from Sigma–Aldrich (St. Louis, MO, USA).

Cell culture and treatment

The pig renal proximal tubule cell line, LLC-PK1, was obtained from the American Type Culture Collection and cultured as described before [31]. After reaching 80–90% confluence, cells were serum-starved overnight before they were used for experiments. PY-17 cells were generated from LLC-PK1 cells that were stably transfected with α1-specific small interfering RNA (siRNA) [7]. AAC-19 cells were derived from PY-17 cells rescued by wild-type rat α1 carrying silent mutations of siRNA-targeting sequence [7]. LL-A416P-4 cells and LL-A420P-20 cells were derived from PY-17 cells rescued by A416P and A420P rat α1 mutants, respectively [14].

Assay of cell surface expression of Na/K-ATPase by [^{3}H]ouabain binding and pumping activity by ouabain-sensitive $^{86}Rb^+$ uptake

Cell surface Na/K-ATPase content was determined using the [^{3}H]ouabain binding assay as we previously reported [8]. Briefly, cells were cultured in 12-well plates, serum-starved, and treated with glucose oxidase. Afterward, the cells were incubated in K^+-free Krebs solution (NaCl 137 mM, $CaCl_2$ 2.8 mM, NaH_2PO_4 0.6 mM, $MgSO_4$ 1.2 mM, dextrose 10 mM, Tris 15 mM, pH 7.4) for 15 min and then exposed to 200 nM [^{3}H]ouabain for 30 min at 37 °C in the presence of 20 μM monensin. At the end of incubation, the cells were washed three times with ice-cold K^+-free Krebs solution, solubilized in 0.1 M NaOH–0.2% (w/v) SDS, and counted in a scintillation counter for [^{3}H]ouabain. Nonspecific binding was measured in the presence of 1 mM unlabeled ouabain and subtracted from total binding. [^{3}H]Ouabain binding data were calculated as binding sites per milligram of protein. To assess the pumping activity, cells were cultured in 12-well plates and $^{86}Rb^+$ uptake was performed as previously described [8]. All counts were normalized to protein amount.

Western blot and immunoprecipitation

Immunoblotting and Src immunoprecipitation were performed as described previously [32,33]. Activation of Src and ERK1/2 was determined by using anti-phospho-Src and anti-phospho-ERK1/2 antibodies as we previously described [8]. The same blots were stripped and probed with antibodies that recognize the total amount of Src and ERK1/2 to account for equal loading. Detection was performed using the chemiluminescence ECL kit. Films were scanned and quantified using ImageJ software.

Cell surface biotinylation and streptavidin precipitation

Biotinylation assay was performed according to Liang et al. [8]. Cells were cultured on 60-mm petri dishes until they reached 90% confluence. The cells were then rinsed twice with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) and incubated with 2 ml of NHS-SS-biotin (1.5 mg/ml) freshly dissolved into biotinylation buffer (10 mM triethanolamine, 150 mM NaCl, pH 9.0) for 25 min at 4 °C on a gently shaking rocker. After being rinsed twice with PBS containing 100 mM glycine, the cells were incubated in the same buffer for 20 min at 4 °C to quench the unreacted biotin and solubilized in 400 μl of lysis buffer (1% (v/v) Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.5) for 60 min on a rocker with gentle motion. Cell lysates were collected by centrifugation at 16,000*g* for 10 min. The cell lysates (250 μg) were incubated with 150 μl of streptavidin–agarose beads in lysis buffer in a total volume of 800 μl at 4 °C with

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