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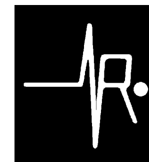

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Original Contribution

Protein phosphatase 1α is tyrosine-phosphorylated and inactivated by peroxynitrite in erythrocytes through the *src* family kinase *fgr*

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Abstract

Protein serine/threonine phosphorylation is a significant component of the intracellular signal that together with tyrosine phosphorylation regulates several processes, including cell-cycle progression, muscle contraction, transcription, and neuronal signaling. Cross-talk between phosphoserine/threonine- and phosphotyrosine-mediated pathways is not yet well understood. In this study we found that peroxynitrite, a physiological oxidant formed by the fast radical–radical reaction between the nitric oxide and the superoxide anion, induced tyrosine phosphorylation of the serine/threonine protein phosphatase 1α (PP1α) in human erythrocytes through activation of *src* family kinases. We have previously shown in mouse red cells that upregulation of the *src* kinase *fgr* phosphorylates PP1α, acting as an upstream negative regulator of PP1α, and downregulates K-Cl cotransport. Here we found that PP1α is a selective substrate of peroxynitrite-activated *fgr* and that tyrosine phosphorylation of PP1α corresponds to an inhibition of its enzymatic activity. Despite *fgr* activation and PP1α downregulation, peroxynitrite stimulated in a dose-dependent fashion the function of the K-Cl cotransporter. In an attempt to understand the mechanism of K-Cl cotransport activation, we found that the effect of peroxynitrite is completely reversed by dithriothreitol, suggesting that peroxynitrite acts as an oxidizing agent by an SH-dependent and PP1α-independent mechanism. These findings highlight a novel function of peroxynitrite in regulating the intracellular signal transduction pathways involving serine/threonine phosphorylation and the functional role of proteins that are targets of these phosphatases.
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Keywords: Peroxynitrite; Serine/threonine protein phosphatase; *Src* family tyrosine kinase; K-Cl cotransport; Tyrosine phosphorylation; Oxidative stress; Red blood cell

Introduction

Cell signaling responses to extra/intracellular stress result in functional changes in proteins mediated by phosphorylation and dephosphorylation. Reversible protein serine/threonine phosphorylation and tyrosine phosphorylation are significant components of the intracellular signal transduction machinery. Although regulation of the large family of protein kinases has been a major focus of research for several decades, the regulation of protein phosphatases has only recently been studied in detail. The protein phosphatase 1 family (PP1) is one of four serine/threonine phosphatases involved in signaling pathways in different cell types. PP1, PP2A, and PP2B (calcineurin) are structurally related to each other, whereas PP2C appears to have a distinct evolutionary background. Since the PP1 phosphatase family is phylogenetically well conserved, PP1 isoforms (α, γ, and δ) may be present in virtually all mammalian cells [1]. PP1 is a major

Abbreviations: fgr, *f*eline *G*ardner-*R*asheed; *hck*, *h*ematopoietic *c*ell *k*inase; *lyn*, *l*ck/*y*es-related *n*ovel tyrosine kinase; PP1, protein phosphatase 1 family; $ONOO^-$, peroxynitrite; SIN-1, 3-morpholinosydnonimine; •NO, nitric oxide; KCC, K-Cl cotransporter; NEM, *N*-ethylmaleimide; DTT, dithiothreitol; p-NPP, *para*-nitrophenyl phosphate; pY, phosphotyrosine; DMSO, dimethyl sulfoxide; MOPS, 3(*N*-morpholino)propanesulfonic acid; DTPA, diethylenetriaminepentaacetic acid; PMSF, phenylmethylsulfonyl fluoride.

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eukaryotic serine/threonine protein phosphatase that regulates different cellular processes such as cell-cycle progression, protein synthesis, muscle contraction, carbohydrate metabolism, transcription, and neuronal signaling [1]. The protein phosphatase holoenzyme is a trimeric complex formed by a regulatory subunit, a variable subunit, and a catalytic subunit. The combination of the catalytic subunit with the other subunits characterizes the localization and specificity of the enzyme [2,3].

PP1 and PP2A are phosphorylated in vitro by *c-src*, *v-src,* and *v-abl* on a residue located toward the C-terminus, probably Tyr 306 in PP1α, and phosphorylation by *src* induces enzyme inactivation [1,4,5]. Regulation of PP1 and PP2A by tyrosine phosphorylation would represent a means for *src*, or other tyrosine kinases, to regulate the phosphoserine/phosphothreonine level of proteins that are targets of these two phosphatases.

The K-Cl cotransport system is a fast gradient-driven membrane pathway and is abnormally activated by cell swelling, cell acidification, reduced red cell magnesium content, membrane oxidative damage, and cell age [6–18]. Other factors modulating the K-Cl cotransport are urea and *N*-ethylmaleimide (NEM) [11,19]. Studies with inhibitors of the families of phosphatases and kinases suggest that the K-Cl cotransport is regulated by a cycle of phosphorylation and dephosphorylation that is responsible for the resting/activation states of the transporter (KCC). Studies with specific inhibitors of serine/threonine protein phosphatases have proposed a role of membrane-associated PP1 and PP2A in regulating the KCC activity in red cells [9,10,16,20–22]. In a mouse model lacking *src* kinases *fgr* and *hck*, red cells are denser with a lower red cell K^+ content and an activated K-Cl cotransport system compared with control mice, suggesting functional cross-talk between *src* kinases and K-Cl cotransport [23,24]. The functional cross-talk between the *src* family tyrosine kinases and serine/threonine phosphatase PP1α in regulating K-Cl cotransport has been studied in a mouse model for high and low magnesium, a factor known to modulate the transport system [22,25,26]. Using this mouse model, we have shown that *fgr* phosphorylates PP1α acting as an upstream negative regulator of PP1α and that upregulation of *src* kinases downregulates KCC activity [23,24]. A role for *src* kinases in modulating KCC activity has also been reported in sickle red cells exposed to cyclic oxygenation–deoxygenation, which is known to activate the K-Cl system abnormally [27–29].

Oxidative stress represents an important factor in the modulation of KCC activity. The abnormal activation of K-Cl cotransport through oxidative damage has been demonstrated in vivo in hereditary hemoglobin disorders such as sickle cell disease and β thalassemia, which are characterized by membrane oxidative damage, and in vitro by a variety of oxidizing agents, including nitric oxide (•NO)-derived oxidants [14,30–33].

One of the cellular effects of the redox signals may be the inactivation of tyrosine phosphatases (PTPs), through the oxidation of critical sulfhydryl groups, as well as activation of specific kinases. Recent evidence suggests that the activities of serine/threonine phosphatases are differentially modulated by oxidative and nitrosative stress [33].

Peroxynitrite ($ONOO^-$), a pathophysiological oxidant formed by a fast radical–radical reaction between •NO and the superoxide anion, is a strong oxidant that is presumed to be formed in vivo under intense oxidative stress. One of the major protein modifications induced by $ONOO^-$ is oxidation/nitrosation/nitration of cysteines, tyrosines, and tryptophans [30,34–38]. The oxidation of cysteines and tyrosines by •NO-related oxidants has been shown to modify significantly the enzymatic function of several target proteins [38–41].

In red cells, we have shown that $ONOO^-$ upregulates band 3 tyrosine phosphorylation with consequent activation of glycolysis [42]. The $ONOO^-$-dependent upregulation of band 3 phosphotyrosine signaling in erythrocytes involves the inhibition of PTPs as well as the activation of *src* kinases through cysteine-dependent and independent processes [42–44]. In different cell types, $ONOO^-$-induced oxidation of thiols and metal-containing proteins, in particular of protein cysteinyl residues, regulates a multitude of redox-sensitive pathways and triggers the activation of specific signaling events [45,46].

In this study, we investigated whether, through the activation of *src* tyrosine kinases, $ONOO^-$ affects tyrosine phosphorylation and the activity of PP1α and PP2A, toward modulation of the KCC function. Our results indicate that $ONOO^-$ induces: (i) tyrosine phosphorylation and inactivation of PP1α via activation of *fgr*; (ii) an increase in KCC activity, via an SH-dependent and phosphatase-independent mechanism.

Materials and methods

Drugs and chemicals

NaCl, KCl, sulfamic acid (SFa), tris(hydroxymethyl) aminomethane (Tris), 3(*N*-morpholino)propanesulfonic acid (MOPS), ouabain, bumetanide, and nystatin were purchased from Sigma Chemical Co (St. Louis, MO). $MgCl_2$, $Mg(NO_3)_2$, dimethyl sulfoxide (DMSO), and *n*-butyl phthalate were purchased from Fisher Scientific Co. Choline chloride was purchased from Calbiochem-Boehring (San Diego, CA). Bovine serum albumin fraction V was purchased from Boehringer Mannheim (Mannheim, Germany). All solutions were prepared using double-distilled water. [γ-^{32}P]ATP (>3000 Ci/mmol) was obtained from DuPont NEN (Boston, MA). Antibodies were obtained from the following sources: monoclonal anti-phosphotyrosine antibodies (clone 4G10) from Upstate Biotechnology (Lake Placid, NY); anti-*lyn*, anti-*hck*, anti-*fgr*, anti-*syk*, agarose-conjugate anti-PP1α,

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