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Redox regulation of platelet-derived-growth-factor-receptor: Role of NADPH-oxidase and c-Src tyrosine kinase

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Abstract

This study identifies some early events contributing to the redox regulation of platelet-derived growth factor receptor (PDGFr) activation and its signalling in NIH3T3 fibroblasts. We demonstrate for the first time that the redox regulation of PDGFr tyrosine autophosphorylation and its signalling are related to NADPH oxidase activity through protein kinase C (PKC) and phosphoinositide-3-kinase (PI3K) activation and H_2O_2 production. This event is also essential for complete PDGF-induced activation of c-Src kinase by Tyr^{416} phosphorylation, and the involvement of c-Src kinase on H_2O_2-induced PDGFr tyrosine phosphorylation is demonstrated, suggesting a role of this kinase on the redox regulation of PDGFr activation. Finally, it has been determined that not only PI3K activity, but also PKC activity, are related to NADPH oxidase activation due to PDGF stimulation in NIH3T3 cells, as it occurs in non-phagocyte cells. Therefore, we suggest a redox circuit whereby, upon PDGF stimulation, PKC, PI3K and NADPH oxidase activity contribute to complete c-Src kinase activation, thus promoting maximal phosphorylation and activation of PDGFr tyrosine phosphorylation.

Keywords: Platelet-derived growth factor receptor; Redox regulation; c-Src kinase; NADPH oxidase; Tyrosine phosphorylation

1. Introduction

The platelet-derived growth factor (PDGF) plays an important role in cell proliferation, differentiation and chemotaxis and is involved in proliferative disorders, such as cancer and atherosclerosis [1].

The PDGF receptor (PDGFr) is a tyrosine kinase receptor expressed in many cell types including fibroblasts [2]. The binding of PDGF to its receptor on the cell surface induces receptor dimerization and intrinsic tyrosine kinase activation [3,4]. This leads to receptor autophosphorylation and activation, along with the recruitment and tyrosine phosphorylation of numerous other cellular proteins containing Src homology (SH2) domains [2,3]. These events initiate the PDGF signalling pathways. Complete receptor autophosphorylation is due to a temporally determined specific sequence of various tyrosine phosphorylation types, and the regulation mechanisms of the tyrosine phosphorylation receptor can be different and related to the diverse receptor functions [3,5–7]. The mechanisms that regulate PDGFr activation and the subsequent events are still largely unknown. Recent studies have revealed that various cellular signalling pathways can be regulated at different levels by variations in the intracellular redox state [8,9]. Reactive oxygen species (ROS), such as H_2O_2 and superoxide anion ($O_2^{\cdot-}$), have been shown to be generated in a wide variety of cells stimulated by various ligands, such as cytokine, growth factors and agonists of G protein-linked receptors [10,11]. H_2O_2 and $O_2^{\cdot-}$ may act as second messengers in signal transduction. These activate signalling molecules, such as protein tyrosine kinases (PTKs), serine/threonine kinases and phospholipases, resulting in the stimulation of downstream

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signalling systems [8,9,11]. Redox regulation signalling has also been demonstrated by treating cells with exogenous H_2O_2 or with systems generating an increase in the intracellular oxidative state, which enhance tyrosine phosphorylation of the growth factor receptor and cytoplasm proteins, whereas reductants or the peroxide-scavenging enzyme, catalase, inhibit these events [8,9,12,13]. Recently, it has been demonstrated that PDGF-dependent tyrosine phosphorylation is affected by the inhibition of phosphotyrosine phosphatase (PTP) activity due to the increase of intracellular ROS generated by PDGF stimulation in Rat-1cells [14]. In the redox regulation of PDGFr activation and autophosphorylation, low molecular weight phosphotyrosine phosphatase (LMW-PTP) seems also to be involved [15]. In fact, in NIH3T3 fibroblasts, LMW-PTP activity is related to a negative regulation of PDGFr phosphorylation and all downstream signals and its activity is redox-regulated. Decreases in LMW-PTP activity occur by reversible oxidation of two vicinal cysteines present in the catalytic domain and this oxidation is induced by both extracellular H_2O_2 or that produced after PDGF stimulation. Intracellular H_2O_2 increases inhibit phosphotyrosine phosphatases (PTPase), enhancing tyrosine phosphorylation, and conditions of oxidative stress induce tyrosine phosphorylation of cell proteins strongly potentiated by combination with vanadate, a known inhibitor of PTPase [12,15,16]. It has also been suggested that direct oxidation of specific sulfhydryl groups of tyrosine kinases similar to that observed for tyrosine phosphatases can occur [9,12,17]. Indeed, previous data demonstrated that, in NIH3T3 fibroblasts, extracellular H_2O_2 induces PDGFr tyrosine phosphorylation, and intracellular H_2O_2 produced by the receptor itself can contribute to its maximum phosphorylation by reversible effects with the involvement of SH-groups [12]. Moreover, GSH, the principal thiol responsible for the intracellular redox state, is also involved in the early events of PDGF signalling [18]. However, the specific steps and/or factors that are involved in the redox regulation of receptor tyrosine phosphorylation are not yet known. PDGF induces H_2O_2 generation in some cells, by the activation of a multi-component NADPH oxidase system [10,11,19,20], which has been studied extensively in phagocyte cells [8,21,22]. This enzyme system produces $O_2^{\cdot-}$, which spontaneously or enzymatically dismutates to H_2O_2. The phagocyte NADPH oxidase complex is constituted from two citosolic components, p47phox and p67phox, and flavo-heme membrane proteins, such as gp91phox and p22phox. NADPH oxidase activation requires the association of these proteins on the membrane also including the small GTP-binding protein RAC (either RAC1 or RAC2). The NADPH oxidase system seems to be functionally similar in non-phagocyte cells. In fibroblasts, the overproduction of RAC1 has been related to H_2O_2 production [21,23] and gp91phox homologues, named Nox, have been described in mammals [8,11,21]. However, in non-phagocyte cells, NADPH oxidase activation mechanisms are poorly characterized.

The aim of this study is to identify factors related to early events in the PDGFr signalling pathway that are involved in PDGF-induced intracellular H_2O_2 generation and that can contribute to the redox regulation of PDGFr autophosphorylation and its signalling. In particular, in PDGF-stimulated NIH3T3 fibroblasts, we studied the role of PI3K and PKC enzyme activity in NADPH oxidase activation. The relationships between PDGF-induced H_2O_2 production, c-Src kinase activation and PDGFr tyrosine phosphorylation have also been investigated, given that c-Src binding to PDGFr seems to constitute one of the early processes necessary for the receptor signalling activation in different cell types including fibroblasts [3,24–30]. Moreover, previous studies have shown that c-Src kinase is involved in signal cascades stimulated by ROS, but a relationship to the redox regulation of PDGFr tyrosine phosphorylation has not been found [3,31–35].

2. Materials and methods

2.1. Cell culture and treatments

NIH3T3 murine fibroblasts were obtained from ATCC and cultured in Dulbecco's modified Eagle Medium as previously reported [12]. 24 h starved cells were stimulated in fresh medium with 20 ng/ml PDGF for 5 or 10 min or exposed to 0.5 or 1 mM H_2O_2 for 20 min. In some experiments, the cells were pretreated for 30 min with different inhibitors: 100 μM Vanadate, or 5, 10, 20, μM Bisindoleyl maleimide I (GF), or 2-(4-morpholinyl)-8-phenylchromone (LY294002), or Diphenilene Iodonium Chloride (DPI), or 250 μM, 1 mM Amminoetil benzenil sulfonil fluoride (AEBSF), or 300 μM *N*-Arginine-methyl-estere (NAME), or 100 μM Rotenone, or 300 μM Oxypurinol, or 5 μM Nordihydroguaiaretic acid (NDGA), or 3, 6, 10 μM 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazole(3,4-d)pyrimidine (PP2), or 6 μM 4-amino-7-phenylpyrazole(3,4-d) pyrimidine (PP3), or 20, 40 μM tyrphostin (AG1296). Some experiments were also performed with 6000 U/ml catalase added to the starvation medium for 24 h [12].

Cell viability during the course of some experiments was evaluated using trypan blue exclusion, in all cases viability was =90%.

2.2. Western blot and immunoprecipitation analysis

At the end of treatments, dishes were washed with ice cold PBS at pH 7.4. To determine immunochemical detection of PDGFr or cytoplasm proteins or c-Src tyrosine phosphorylation, the cells were collected in 300 μl of lysis buffer (50 mM Tris/HCl pH 7.5, 1% Triton X100, 150 mM NaCl, 100 mM NaF, 2 mM EGTA, phosphatase and protease inhibitor cocktail, Sigma). The cell lysates, after 15 min on ice, were centrifuged at 11,600 × *g* for 10 min. Immunochemical detection of p47-phox and p67-phox was