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$_2$O$_2$ 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$_2$O$_2$-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$_2$O$_2$ and superoxide anion (O$_2^-$), 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$_2$O$_2$ and O$_2^-$ 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
signalling systems [8,9,11]. Redox regulation signalling has also been demonstrated by treating cells with exogenous H$_2$O$_2$ 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 (PTPase) activity due to the increase of intracellular ROS generated by PDGF stimulation in Rat-1 cells [14]. In the redox regulation of PDGF$\alpha$ 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 PDGF$\alpha$ 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$_2$O$_2$ or that produced after PDGF stimulation. Intracellular H$_2$O$_2$ 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 sulphydryl 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$_2$O$_2$ induces PDGF$\alpha$ tyrosine phosphorylation, and intracellular H$_2$O$_2$ 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$_2$O$_2$ 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$_2^-$, which spontaneously or enzymatically dismutates to H$_2$O$_2$. 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 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$_2$O$_2$ 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 PDGF$\alpha$ signalling pathway that are involved in PDGF-induced intracellular H$_2$O$_2$ generation and that can contribute to the redox regulation of PDGF$\alpha$ 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$_2$O$_2$ production, c-Src kinase activation and PDGF$\alpha$ tyrosine phosphorylation have also been investigated, given that c-Src binding to PDGF$\alpha$ 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 PDGF$\alpha$ tyrosine phosphorylation has not been found [3,31–35].

1. Materials and methods

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$_2$O$_2$ for 20 min. In some experiments, the cells were pretreated for 30 min with different inhibitors: 100 $\mu$M Vanadate, or 5, 10, 20, $\mu$M Bisindoleyl maleimide 1 (GF), or 2-(4-morpholinyl)-8-phenylchromone (LY294002), or Diphenilene Iodonium Chloride (DPI), or 250 $\mu$M, 1 mM Amminoetil benzenil sulphonil fluoride (AEBSF), or 300 $\mu$M N-Arginine-methyl estere (NAME), or 100 $\mu$M Rotenone, or 300 $\mu$M Oxypurinol, or 5 $\mu$M Nordihydroguaiaretic acid (NDGA), or 3, 6, 10 $\mu$M 4-amino-5-(4-chlorophenyl)-7-(r-butyl)pyrazole(3,4-d)pyrimidine (PP2), or 6 $\mu$M 4-amino-7-phenylpyrazole(3,4-d) pyrimidine (PP3), or 20, 40 $\mu$M 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. Western blot and immunoprecipitation analysis

At the end of treatments, dishes were washed with ice cold PBS at pH 7.4. To determine immunoochemical detection of PDGF$\alpha$ or cytoplasm proteins or c-Src tyrosine phosphorylation, the cells were collected in 300 $\mu$l 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 $\times$ g for 10 min. Immunoochemical detection of p47-phox and p67-phox was...
performed in the membrane fraction [22]. In this case, after various treatments, cells were scraped in PBS, containing protease inhibitor cocktail and sonicated on ice with two 10-s 50-W bursts. The sonicated was centrifuged at 2000 × g for 10 min at 4 °C and the supernatant was centrifuged at 100,000 × g for 60 min at 4 °C. The precipitate was collected and the plasma membranes were resuspended in PBS with protease inhibitors. Equal protein amounts from whole-cell extract or membrane fraction, determined by the method of BCA [12], were subjected to SDS/PAGE on 8%, 10%, or 12% (w/v) gel to detect PDGFr tyrosine phosphorylation, pTyr416 c-Src and p47 phox or p67 phox respectively and electrotransferred to a PVDF membrane (Millipore). Proteins were visualized by incubating the membranes with specific antibodies: mouse monoclonal anti-phosphotyrosine PY20 (anti-pTyr), rabbit polyclonal anti-PDGFr-β (anti-PDGFr), rabbit anti-c-Src, rabbit polyclonal anti-p47 phox or anti-p67 phox (Santa Cruz), rabbit anti-pTyr416 c-Src (BD). The results obtained by the Western blots of cell lysates were confirmed by Western blots of immunoprecipitated tyrosine phosphorylated proteins or immunoprecipitated c-Src performed as previously reported [12]. Subsequently, membranes were stripped and incubated with anti-PDGFr or anti-c-Src antibodies to normalize and to perform densitometric analysis. Antigen–antibody complexes were detected with a chemiluminescence reagent kit (Amersham Pharmacia Biotec). The antibody complexes were visualized by incubating the membrane with specific antibodies: mouse monoclonal anti-phosphotyrosine PY20 (anti-pTyr), rabbit polyclonal anti-PDGFr-β (anti-PDGFr), rabbit anti-c-Src, rabbit polyclonal anti-p47 phox or anti-p67 phox (Santa Cruz), rabbit anti-pTyr416 c-Src (BD). The results obtained by the Western blots of cell lysates were confirmed by Western blots of immunoprecipitated tyrosine phosphorylated proteins or immunoprecipitated c-Src performed as previously reported [12]. Chemidoc-Quantity-One software (Biorad Laboratories) was used to perform quantitative analyses.

2.3. Assay of intracellular \( \text{H}_2\text{O}_2 \) and superoxide production

The intracellular production of \( \text{H}_2\text{O}_2 \) was assayed as previously described [13] by fluorescence spectrofotometric analysis at 510 nm, with 5 μg/ml 2′,7′-Dichlorofluorescin diacetate (H2DCF-DA) added in culture medium 3 min before the end of the various treatments. The data have been normalized on total protein content.

\( \text{O}_2^- \) production and NADPH oxidase activity were determined by lucigenin-enhanced chemiluminescence [20,36] with light modifications. Briefly, after various treatments, the cells were tripinized, collected by centrifugation and suspended in buffer containing 340 mM sucrose, 10 mM Tris, 1 mM EDATA, 1 mM PMSF pH 7.1, and sonicated on ice with two 10-s 50-W bursts. The sonicated was clarified by centrifugation at 15,000 × g for 15 min and the supernatant was used for the determinations. In the assay mixture, 200 μM lucigenin and 500 μM NADPH were added and chemiluminescence was recorded using Berthold LB 9505 luminoimeter, repeated measurements were made over 15 min. In some samples, 10 mM Tiron (4, 5-dihydroxy-1, 3-benzenedisulfonic acid), cell-permeable \( \text{O}_2^- \) scavenger, was also added to measure background chemiluminescence signal. For the determination of a single value, it was performed the average of five readings over 5 min after NADPH addition to the assay mixture. The data were normalized on total protein content. Statistical analyses were evaluated using Student’s t-test.

3. Results and discussion

3.1. Role of NADPH oxidase, PI3K and PKC on tyrosine phosphorylation of PDGFr and cytoplasm proteins

Previous data, obtained in PDGF-stimulated NIH3T3 cells after catalase treatment, demonstrated a significant decrease in PDGFr tyrosine phosphorylation (about 40–50%) and a simultaneous decrease in the pattern of cytoplasm protein tyrosine phosphorylation [12]. Therefore, we investigated if NADPH oxidase activity and some factors related to the activation of this enzyme were involved in both PDGFr tyrosine phosphorylation and PDGF-induced \( \text{H}_2\text{O}_2 \) production. For this purpose, we compared, in PDGF-stimulated cells, the decrease in PDGFr tyrosine phosphorylation caused by catalase treatment with that obtained in fibroblasts treated with DPI or AEBSF, specific inhibitors of flavine oxidase and NADPH oxidase respectively [19–21]. AEBSF, in particular, is an inhibitor of NADPH oxidase and is more specific than DPI since it interferes directly with the interaction of p47phox and/or p67phox with cytochrome b558 [37]. The tyrosine phosphorylation level of PDGFr was evaluated using an anti-phosphotyrosine immunoblot (Fig. 1A). Fig. 1B shows that after stripping and immunoblotting with anti-PDGFr, the tyrosine phosphorylated band of approximately 180 kDa is effectively that of the PDGFr. These bands were used for normalization and quantitative determinations. The results shown in Fig. 1A demonstrate that the inhibition of PDGFr phosphorylation (approximately 50%) in NIH3T3 fibroblasts after both DPI and AEBSF treatment in comparison to the control is similar to that obtained in cells after catalase incubation. Similarly, the decrease in tyrosine phosphorylation of cytoplasm proteins was also evident in catalase-, DPI- or AEBSF-treated cells after PDGF stimulation (Fig. 1A). No significant decrease in PDGFr phosphorylation was obtained after NIH3T3 treatment with specific inhibitors of other enzymatic systems that produce ROS, such as NAME, oxypurinol, rotenone, NDGA which inhibit nitric oxidase synthase, xanthine oxidase, NADH dehydrogenase and lipoxygenase activity respectively (data not shown) [20]. These data confirm the specific involvement of NADPH oxidase activity and the physiological role of endogenous \( \text{H}_2\text{O}_2 \) on the activation and signalling of PDGFr in NIH3T3 fibroblasts [12,13]. These results are also in agreement with those obtained in vascular smooth muscle cells, A431 cells and RAT2 fibroblasts [10,11,19]. Effectively, intracellularly generated \( \text{H}_2\text{O}_2 \) can activate PDGFr through the autocrine mechanism and thus mimic the ligand action in NIH3T3 fibroblasts, as well as that observed in other cells [8,10,12]. On the contrary,
various PDGFr mutants expressed in HepG2 cells, which are not able to induce H₂O₂ production after PDGF stimulation, were fully autophosphorylated [11]. These results indicate that PDGFr redox regulation may be cell specific. Subsequently, in order to clarify the mechanisms of NADPH oxidase activation after PDGF stimulation and the redox regulation of PDGFr, we investigated the involvement of PI3K and PKC activity in PDGF-induced tyrosine phosphorylation and H₂O₂/O₂⁻ production. Indeed, both these enzymes can contribute to the translocation and assembly of various components of NADPH oxidase on the membrane in phagocyte cells stimulated by cytokines or other factors [12,16,21,22]. In non-phagocyte cells, the PI3K product can effectively activate Rac1, which is necessary for PDGF-induced H₂O₂ production [11]. PI3K is also one of the proteins recruited from PDGFr on specific binding sites and it is necessary for its signalling [1–3,5]. PKC activation is also related to the PDGF signalling pathway in NIH3T3 [1,2], but it is unclear whether PKC activity participates in the activation of non-phagocyte oxidase [21].

PDGFr tyrosine phosphorylation has been determined in PDGF-stimulated cells using different concentrations of LY, a specific inhibitor of PI3K [11,38], or GF, an inhibitor of all PKC isoforms [16,39]. The data reported in Fig. 1A were obtained by using concentrations of inhibitors that bring about the highest decrease in PDGFr phosphorylation compared to the control without any apparent cell damage.

Both inhibitors significantly decrease receptor phosphorylation by about 40%–50% compared to the control (Fig. 1A), and no increase in the inhibition effect on PDGFr phosphorylation was obtained in NIH3T3 cells simultaneously pretreated with GF and LY. Moreover, previous findings exclude direct effects of two inhibitors, at used concentrations, on PDGFr tyrosine kinase and on other tyrosine kinase such as c-Src kinase, which can affect PDGFr phosphorylation [38,39], confirming the specificity of their effect.

These data suggest that PKC and PI3K activity affects PDGFr phosphorylation through the same pathway considering the results obtained in NIH3T3 fibroblasts simultaneously treated with both inhibitors at concentrations, which cause the highest effect of inhibition. Therefore, we performed other experiments to correlate PKC and PI3K effect to PDGF-induced NADPH oxidase activation and H₂O₂ production.

### 3.2 Role of NADPH oxidase, PI3K and PKC on PDGF-induced H₂O₂ and O₂⁻ production

In order to study the relationship between these enzymes and PDGF-induced H₂O₂ production, we measured the H₂O₂ level in PDGF-stimulated or non-stimulated cells, which were treated or non-treated with the inhibitors, used in this study. As previously demonstrated, H₂O₂ production, measured by a fluorescence-based assay with H2DCF-DA,
in PDGF-stimulated NIH3T3 cells increased significantly after 5 min of stimulation and reached the maximal increase in fluorescence intensity after 10 min of stimulation compared to non-stimulated cells [13]. Fig. 2A shows that H₂O₂ production, measured after 10 min of PDGF stimulation, decreased notably in cells treated with DPI, AEBSF, GF or LY, confirming the involvement of NADPH oxidase, PI3K and PKC activity in PDGF-induced H₂O₂ production. These data indicate also that PI3K or PKC activity inhibition can effectively decrease PDGFr auto-phosphorylation through the inhibition of intracellular H₂O₂ production.

We determined also NADPH oxidase activity measuring directly O₂⁻ production in a cell-free preparation using lucigenin and chemiluminescence measurements [20,36]. Fig. 2B shows an O₂⁻ increase, of about 100%, after 10 min of PDGF stimulation, compared to non-stimulated cells. All inhibitors significantly decrease O₂⁻ production in PDGF-stimulated cells (Fig. 2B) confirming data observed about H₂O₂ production. No change in H₂O₂ or O₂⁻ production was obtained in the presence of NAME, rotenone or oxypurinol (data not shown).

In order to confirm NADPH oxidase activation by PDGF and PKC activity, the p47-phox and p67-phox translocation in membrane fraction was determined in NIH3T3 cells stimulated by PDGF and phorbol ester acetate (PMA, PKC activator). We performed also some experiments in PDGF-stimulated cells after GF treatment. Fig. 3A and B show that the content of these proteins in the membrane fraction increases in PDGF- or PMA-stimulated cells compared to non-stimulated cells; on the contrary, no significant translocation occurred in GF-treated cells. These data agree with those reported in macrophages, in which treatment with vanadate plus PMA led to the activation of PKC, NADPH oxidase and cellular protein tyrosine phosphorylation [16,22]. Indeed, we observed PDGFr tyrosine phosphorylation in NIH3T3 fibroblasts, stimulated with 250 nM PMA for 30 min, in the presence of 100 µM vanadate, and this effect was inhibited by treatment with DPI or catalase (data not shown). These data exclude a direct effect of PKC activation on PDGFr tyrosine phosphorylation. The treatment with vanadate, a specific inhibitor of tyrosine phosphatases, is necessary to detect tyrosine phosphorylation due to low levels of H₂O₂ [12,16]. NADPH oxidase activation has been associated with increased tyrosine phosphorylation enhanced by treatment with vanadate [17]. In conclusion, PKC and PI3K activation contribute to the increase of oxidative intracellular state and PDGFr tyrosine phosphorylation. Recently, PI3K activation has been also related to PDGF-induced phosphatase inactivation by oxidation [40] confirming the role of PI3K in PDGF-induced H₂O₂ production and tyrosine phosphorylation activation by phosphatase inhibition.
3.3. Role of c-Src tyrosine kinase on redox regulation of PDGFr tyrosine phosphorylation

In order to clarify the early events related to the H₂O₂ production induced by PDGF and the role of endogenous H₂O₂ on the redox regulation of PDGFr tyrosine phosphorylation, we investigated whether the H₂O₂ effect was directed only on PDGFr tyrosine kinase or was also related to other tyrosine kinases involved in PDGFr phosphorylation, such as c-Src family kinases. It has, in fact, been demonstrated in HepG2 cells, that, after PDGF induced receptor dimerization, c-Src binding to the 579 and 581 juxtamembrane tyrosines is the prerequisite for tyrosine phosphorylation in position 857 of the activation loop [3,5]. This event is necessary for subsequent phosphorylation of other tyrosines in multiple SH2-domain binding sites, including tyr-751 (PI3K, binding site) [3,5,25], and it is required for the full activation of the exogenous substrates involved in the PDGF signalling cascade. Other data report that some redox-sensitive pathways are mediated by Src family kinase [31–35]. For this purpose, we studied PDGFr and cytoplasm protein tyrosine phosphorylation in NIH3T3 cells stimulated with PDGF or exposed to exogenous H₂O₂ after treatment with AG1296 and PP2 inhibitors of tyrosine kinase activity. AG1296 has high specificity for PDGFr tyrosine kinase and it does not affect c-Src tyrosine kinase [41], whereas PP2 is a specific inhibitor for c-Src tyrosine kinase in many cell types [42], and in NIH3T3 fibroblasts inhibits also PDGFr [26,43]. Initially, we determined the concentrations of AG1296 and PP2 which cause the maximum decrease of PDGFr tyrosine phosphorylation in NIH3T3 cells after PDGF stimulation. Fig. 4A shows the effect of both inhibitors at the concentrations which almost totally decreased PDGFr-induced tyrosine phosphorylation, whereas no significant change was observed in cells treated with PP3, a PP2 structurally inactive analogue. Subsequently, we studied the effect of these inhibitor concentrations in cells exposed to 0.5 or 1 mM H₂O₂ for 20 min following treatment with vanadate. We used these H₂O₂ concentrations because PDGF stimulation induces the intracellular production of about 0.1–1 mM H₂O₂ [10] and vanadate treatment was performed to better detect tyrosine phosphorylation as reported in our previous study performed in NIH3T3 fibroblasts [12]. Fig. 4C shows that 0.5 mM H₂O₂ increases PDGFr tyrosine phosphorylation in the presence of vanadate as previously reported [12], and differently from that observed in Fig. 4A, the highest and significant decrease in PDGFr tyrosine phosphorylation was obtained in PP2-treated cells with respect to that measured in AG1296-treated cells. We obtained similar results in cells treated for 20 min with 1 mM H₂O₂ (data not shown). These data indicate an important role of c-Src kinase activation in H₂O₂-induced PDGFr phosphorylation. In fact, the concentrations used for both inhibitors block almost completely PDGFr tyrosine kinase activation due to PDGF stimulation (Fig. 4A), whereas this does not occur in H₂O₂-treated cells after AG1296 treatment (Fig. 4C). These findings confirm that PDGFr tyrosine phosphorylation induced by H₂O₂ can effectively involve other kinases and demonstrate that c-Src kinase can be one of these. Indeed, Kazlauskas reports that the inactive kinase form of the PDGFr became tyrosine phosphorylated following sodium pervanadate treatment, indicating that a kinase other than the receptor itself may be responsible for this phosphorylation [3]. Moreover, receptor tyrosine kinases appear activated when the activated form of c-Src was expressed [44]. Many data are in disagreement about the role of c-Src kinase on PDGFr tyrosine phosphorylation and signalling. Some results demonstrate that c-Src activation is necessary for the phosphorylation of certain tyrosine residues required for full activation of PDGFr and PDGFr-associated proteins [3,5,25] and others show c-Src involvement at different levels of PDGFr signal pathways such as Ras/MAPKinase cascade activation, proliferation, cell cycle advance and disease progression [26–30]. On the contrary, Klinghoffer et al. demonstrate in mice embryonic cells that Src family kinase does not affect PDGF-induced proliferation and chemotaxis as well as PDGFr tyrosine phosphorylation and signalling [45]. Other researchers have also shown that PDGFr mutants which do not activate Src family kinase, are fully capable of mediating myc expression and DNA synthesis [46]. Probably these different results are due to different approaches and cell types used i.e. to different structural and functional characteristics of PDGFr and cell protein component. These differences can also explain the different PP2 effect on PDGFr tyrosine kinase of various cell types [43]. We investigated also GF and LY effect on H₂O₂-induced PDGFr phosphorylation, and no significant change was observed (data not shown), confirming that there is no redox activation of these kinases contributing to PDGFr phosphorylation differently than that observed by c-Src kinase. On the other hand, PDGF-induced activation of these kinases is essential to increasing ROS production. This confirms the cross-talk between mechanisms which regulate growth factor receptor activity and ROS production as also proposed by other researchers [3,8,9,15], in fact the activation of these receptors determines and regulates intracellular levels of ROS and these can contribute to regulate receptor activity.

Subsequently, we evaluated H₂O₂ effect and the role of PKC, PI3K and NADPH oxidase activity on H₂O₂- and PDGF-induced c-Src kinase activation. For this purpose, we studied the c-Src-activated form that is Tyr416 phosphorylated by a specific antibody [24,47] in H₂O₂ and PDGF-stimulated NIH3T3 fibroblasts.

Fig. 5A shows a significant increase in Tyr416 phosphorylation of c-Src in 0.5 mM H₂O₂-treated cells as compared to non-stimulated cells treated with vanadate alone, similar results were observed in 1 mM treated cells (data not shown). Moreover, no change in H₂O₂-induced c-Src phosphorylation was observed in cells pre-treated with GF, LY or AEBSF, indicating that PKC, PI3K and NADPH oxidase activity are not related to c-Src activation due to
H$_2$O$_2$, as well as to H$_2$O$_2$-induced PDGFr phosphorylation. Whereas, similarly to that observed in H$_2$O$_2$-induced PDGFr phosphorylation, both PP2 and AG1296 decrease H$_2$O$_2$-induced c-Src activation and the highest inhibition observed in PP2-treated cells was significantly different than that obtained in the presence of AG1296. These data demonstrate that H$_2$O$_2$ can activate c-Src through its kinase and in part by PDGFr tyrosine kinase. Finally, Fig. 5C shows that H$_2$O$_2$ generated after PDGF stimulation also notably affects PDGF-induced c-Src activation, considering the effect of catalase. Moreover, PKC, PI3K and NADPH oxidase activity are also involved on PDGF- induced c-Src phosphorylation. Therefore, H$_2$O$_2$ intracellular increase, due to PDGF stimulation, is necessary for maximum activation of both PDGFr and c-Src kinase. These results, in their whole, suggest that a mutual redox regulation of c-Src and PDGFr tyrosine kinase activity is possible. Indeed, previous data show that H$_2$O$_2$-induced c-Src activation can play an important physiological role in the activation of factors downstream of the signalling pathways, such as c-Jun NH2-terminal kinases, which are related to inflammatory cytokines and stress oxidative signalling in both VSMC and...
fibroblasts [6, 7, 34, 44, 47]. However, there are no data on c-Src redox regulation due to growth factor receptor-induced H₂O₂ production. With regard to the involvement of tyrosine phosphatase activity on H₂O₂-induced c-Src kinase activation, we suggest that PTPase inhibition does not directly activate c-Src tyrosine kinase but it is necessary to enhance and detect c-Src tyrosine phosphorylation induced by H₂O₂. In fact, we previously demonstrated that NIH3T3 fibroblasts pre-treated or not with 10 μM GF or 20 μM LY or 250 μM AEBSF or 20 μM AG1296 or 6 μM PP2 or 6 μM PP3 for 30 min, in the presence or not of 100 μM vanadate, were stimulated or not with 0.5 mM H₂O₂ for 20 min (A). Starved NIH3T3 fibroblasts pre-treated or not with 6000 U/ml catalase for 24 h or 10 μM DPI or 250 μM AEBSF or 10 μM GF or 20 μM LY for 30 min were stimulated with 20 ng/ml PDGF for 5 min (C). Western blotting with anti-p-Tyr⁴¹⁶ c-Src antibodies of cell lysates was performed. The same filters were re-probed with anti-c-Src antibodies for the normalization of cell lysates (B and D). The arrows indicate Tyr⁴¹⁶ phosphorylated form of c-Src or total c-Src present in the cell lysates. Bottom B and D: the normalized values, obtained by densitometric analysis, are reported as mean percentage of phosphorylation ± S.E. relative to the value obtained in cells stimulated with H₂O₂ without inhibitors, or PDGF alone (control, 100%) of four independent experiments. *P < 0.05 compared to the control, §P < 0.05 compared to AG1296-treated cells.

Taken together, these results demonstrate for the first time that the redox regulation of PDGF⁰ tyrosine autophosphorylation and its signalling are related to NADPH oxidase activity through PKC and PI₃K activation which is an upstream occurrence of PDGF-induced H₂O₂ production. Moreover, we demonstrate the involvement of PKC activity in NADPH oxidase activation induced by PDGF, as it occurs in phagocyte cells stimulated by various factors. It has also been demonstrated that the full c-Src kinase activation, which is a downstream event of PDGF-induced H₂O₂ generation, can contribute to PDGF redox regulation. Therefore, we suggest a redox circuit as follows: PDGF

![Fig. 5. Effect of specific enzyme inhibitors and catalase on c-Src activation after H₂O₂ or PDGF stimulation. Starved NIH3T3 fibroblasts pre-treated or not with 10 μM GF or 20 μM LY or 250 μM AEBSF or 20 μM AG1296 or 6 μM PP2 or 6 μM PP3 for 30 min, in the presence or not of 100 μM vanadate, were stimulated or not with 0.5 mM H₂O₂ for 20 min (A). Starved NIH3T3 fibroblasts pre-treated or not with 6000 U/ml catalase for 24 h or 10 μM DPI or 250 μM AEBSF or 10 μM GF or 20 μM LY for 30 min were stimulated with 20 ng/ml PDGF for 5 min (C). Western blotting with anti-p-Tyr⁴¹⁶ c-Src antibodies of cell lysates was performed. The same filters were re-probed with anti-c-Src antibodies for the normalization of cell lysates (B and D). The arrows indicate Tyr⁴¹⁶ phosphorylated form of c-Src or total c-Src present in the cell lysates. Bottom B and D: the normalized values, obtained by densitometric analysis, are reported as mean percentage of phosphorylation ± S.E. relative to the value obtained in cells stimulated with H₂O₂ without inhibitors, or PDGF alone (control, 100%) of four independent experiments. *P < 0.05 compared to the control, §P < 0.05 compared to AG1296-treated cells.](image)

![Fig. 6. Factors involved in the proposed redox circuit for PDGF⁰ activation. The dashed arrow indicates a possible role of c-Src kinase on PDGF⁰ autophosphorylation induced by PDGF.](image)
through its receptor activates c-Src kinase, PI3K and PKC, these two last enzyme activity are involved in NADPH oxidase activation and H₂O₂ increase, which in turn contributes to the full activation of both PDGF and c-Src tyrosine phosphorylation (Fig. 6). A role of c-Src kinase itself on PDGF activation in the presence of intracellular increased oxidative state is demonstrated, and a possible relationship between PDGF and c-Src tyrosine kinase redox regulation is indicated. However, the precise sequence of these events and the various factors related to mechanisms through which ROS activate these kinases remain to be clarified, as well as the role of PDGF induced c-Src kinase activation on PDGF tyrosine autophosphorylation.

PDGF redox regulation involves various factors, which participating to sequential activation of PDGF signalling can modulate different biological processes related to this receptor. Finally, knowledge of the redox mechanisms, which regulate the early passages of growth factor receptor signalling, can be useful in identifying specific targets, which, if altered, can be responsible for disease onset related to intracellular oxidative stress and/or to the development of hyperproliferative disorders such as cancer and atherosclerosis.

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References


