Platelet-derived Growth Factor-induced H₂O₂ Production Requires the Activation of Phosphatidylinositol 3-Kinase*

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Autophosphorylation of the platelet-derived growth factor (PDGF) receptor triggers intracellular signaling cascades as a result of recruitment of Src homology 2 domain-containing enzymes, including phosphatidylinositol 3-kinase (PI3K), the GTPase-activating protein of Ras (GAP), the protein-tyrosine phosphatase SHP-2, and phospholipase C-γ1 (PLC-γ1), to specific phosphotyrosine residues. The roles of these various effectors in PDGF-induced generation of H₂O₂ have now been investigated in HepG2 cells expressing various PDGF receptor mutants. These mutants included a kinase-deficient receptor and receptors in which various combinations of the tyrosine residues required for the binding of PI3K (Tyr²⁰⁴ and Tyr²⁷⁰), GAP (Tyr²⁷⁰), SHP-2 (Tyr¹⁰⁶⁹), or PLC-γ1 (Tyr¹⁰⁵¹) were mutated to Phe. PDGF failed to increase H₂O₂ production in cells expressing either the kinase-deficient mutant or a receptor in which the two Tyr residues required for the binding of PI3K were replaced by Phe. In contrast, PDGF-induced H₂O₂ production in cells expressing a receptor in which the binding sites for GAP, SHP-2, and PLC-γ1 were all mutated was slightly greater than that in cells expressing the wild-type receptor. Only the PI3K binding site was alone sufficient for PDGF-induced H₂O₂ production. The effect of PDGF on H₂O₂ generation was blocked by the PI3K inhibitors LY294002 and wortmannin or by overexpression of a dominant negative mutant of Rac1. These results suggest that a product of PI3K is required for PDGF-induced production of H₂O₂ in nonphagocytic cells, and that Rac1 mediates signaling between the PI3K product and the putative NADPH oxidase.

Many cell types produce hydrogen peroxide (H₂O₂) in response to a variety of extracellular stimuli that include cytokines, such as transforming growth factor-β1 (1, 2), interleukin-1 (3–5), interferon-γ (4), and tumor necrosis factor-α (3, 6); peptide growth factors, such as platelet-derived growth factor (PDGF)¹ (7), epidermal growth factor (8), basic fibroblast growth factor (6), insulin (9), and granulocyte-macrophage colony-stimulating factor (5); and agonists of receptors with seven transmembrane domains, such as angiotensin II (10), thrombin (11), thyrotropin (4, 12), parathyroid hormone (4), lysophosphatidic acid (13), sphingosine 1-phosphate (14), mechanical shear strain (15), phorbol esters (16), and phosphatidic acid (17) also induce H₂O₂ production in various cell types. Receptor-mediated generation of H₂O₂ has been linked to the activation of transcription factors such as nuclear factor-κB (18) and AP1 (6, 19), mitogen-activated protein kinases (7, 13), phospholipase A₂ (20), protein kinase C (21), and phospholipase D (22, 23); it has also been linked to an increase in the cytosolic Ca²⁺ concentration (24), to the triggering of apoptosis (25), to the inhibition of protein-tyrosine phosphatases (26), and to modulation of ion transport (27). Because H₂O₂ is a small, diffusible, and ubiquitous molecule that can be synthesized, as well as destroyed, rapidly in response to external stimuli, it fulfills the important criteria for an intracellular messenger.

The mechanism of receptor-mediated generation of H₂O₂ has been studied extensively in phagocytic cells, in which O₂•⁻ is produced via the one-electron reduction of O₂ by a multicomponent NADPH oxidase system (28). The O₂•⁻ is then spontaneously or enzymatically dismutated to H₂O₂. The NADPH oxidase complex includes two cytosolic components (p47phox and p67phox) and two transmembrane flavocytochrome b components (gp91phox and p22phox). In addition, the small GTP-binding protein Rac (either Rac1 or Rac2) is required for activation of NADPH oxidase. In contrast, the mechanism of H₂O₂ generation in nonphagocytic cells remains unclear. Evidence suggests that the system responsible for H₂O₂ (O₂•⁻) production in nonphagocytic cells is structurally and genetically distinct from, but functionally similar to, the NADPH oxidase system of phagocytes (29, 30). Consistent with this notion, overproduction of Rac1 in fibroblasts was associated with increased production of H₂O₂ (31, 32) and a homolog of gp91-phox was found in several nonphagocytic cells (33).

The binding of growth factors to their receptors results in receptor autophosphorylation on specific tyrosine residues. These phosphotyrosine residues initiate cellular signaling by acting as high affinity binding sites for the Src homology 2 domains of various effector proteins. In the PDGF β receptor

¹ The abbreviations used are: PDGF, platelet-derived growth factor; PDGF/B, PDGF β receptor; PI3K, phosphatidylinositol 3-kinase; GAP, GTPase-activating protein of Ras; SHP-2, Src homology 2 domain-containing protein-tyrosine phosphatase-2; PLC-γ1, phospholipase C-γ1; MEM, modified Eagle’s medium; DCF, 2',7'- dichlorofluorescein; GEF, guanine nucleotide exchange factor; PI(3,4,5)P₃, phosphatidylinositol 3,4,5-trisphosphate; PI(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; p110CAAX, membrane-targeted p110 subunit of PI3K.
(PDGFβR), seven autophosphorylation sites have been identified as specific binding sites for Src family tyrosine kinases (Tyr759 and Tyr751), phosphatidylinositol 3-kinase (PI3K) (Tyr740 and Tyr751), the GTPase-activating protein of Ras (GAP) (Tyr771), Src homology 2 domain-containing protein-tyrosine phosphatase-2 (SHP-2) (Tyr1009), and phospholipase C-y1 (PLC-y1) (Tyr1021). A series of PDGFβR mutants was previously constructed that includes a kinase-deficient [kinase(-)] mutant and receptors in which the binding sites for PI3K, GAP, SHP-2, and PLC-y1 were changed individually or in various combinations to phenylalanine (34).

To characterize the mechanism of H2O2 production in nonphagocytic cells, we have now measured PDGF-dependent H2O2 generation in human hepatoma HepG2 cells expressing these various PDGFβR mutants. Our data indicate that PDGFβR-dependent H2O2 production requires both the intrinsic kinase activity of the receptor as well as the activation of PI3K. Experiments with a dominant negative mutant of Rac1 (N17Rac1) also suggest that Rac1 participates in the PDGFβR-induced production of H2O2 in HepG2 cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco’s modified Eagle’s medium, modified Eagle’s medium (MEM) without phenol red, Opti-MEM, fetal bovine serum, antibiotic-antimycotic mixture, and LipofectAMINE were obtained from Life Technologies, Inc.; expression plasmids encoding dominant negative Akt (Akt K179M) and constitutively activated Akt (Myr-Akt), PDGF-BB, and rabbit antibodies to Rac1 were from Upstate Biotechnology; LY294002 was from Bio-Mol, and 2′,7′-dichlorofluorescin diacetate. This compound is converted by intracellular esterases to 2′,7′-dichlorofluorescin, which is then oxidized by H2O2 to the highly fluorescent 2′,7′-dichlorodihydrofluorescein diacetate. This compound is converted by intracellular esterases to 2′,7′-dichlorofluorescein, which is then oxidized by H2O2 to the highly fluorescent 2′,7′-dichlorofluorescein diacetate (DCF). Culture dishes were transfected with a Zeiss Axiolab 15 inverted microscope that was equipped with a x20 Neofluor objective and Zeiss LSM 410 confocal attachment, and DCF fluorescence was measured with an excitation wavelength of 485 nm and emission at 515–540 nm. To avoid photodegradation of 2′,7′-dichlorofluorescin, the fluorescence image was collected by a single rapid scan (4-line average, total scan time of 4.33 s), with identical parameters (such as contrast and brightness) for all samples. After collection of the fluorescence image, the cells were imaged by digital interference contrast. Five groups of 10–20 cells each were randomly selected from each sample, and the mean relative fluorescence intensity for each group of cells was then measured by Karl Zeiss vision system (KS4000, version 3.0) and averaged. All experiments were repeated at least five times.

**Expression of N17Rac1 in HepG2 Cells**—HepG2 cells were plated at a density of 2 × 10^4/well in culture dishes and allowed to recover for 24 h. Adenovirus infection was performed at a multiplicity of infection of 200. After adenovirus infection for 72 h, the cells were then washed with MEM without phenol red, exposed for 30 min to PDGF-BB (100 ng/ml), and subjected to assay of H2O2 production. Western blot analysis of Myc-tagged form of N17Rac was performed using an antibody to rac1 (35).

**RESULTS**

Vallius and Kazlauskas (34) previously constructed the series of PDGFβR mutants shown in Fig. 1. The kinase(-) mutant was generated by mutating Lys635 to Arg; the F740/751 mutant by replacing the residues that constitute the PI3K binding site (Tyr740 and Tyr751) with Phe; the F771 mutant by replacing the GAP binding site (Tyr771) with Phe; the F1021 mutant by replacing the PLC-y1 binding site (Tyr1021) with Phe; the Y740/751/771 mutant by replacing Tyr740, Tyr751, and Tyr771 with Phe; and the F5 mutant by replacing the five tyrosine residues Tyr740, Tyr751, Tyr771, Tyr1009 (the SHP-2 binding site), and Tyr1021 with Phe (Fig. 1). In addition, the ability of F5 to signal via PI3K was selectively restored by changing Phe740 and Phe751 in this mutant back to Tyr, thereby generating Y740/751 (Fig. 1). Similarly, the ability of F5 to signal via PLC-y1 was restored by changing Phe1021 in this mutant back to Tyr, generating Y1021 (Fig. 1). The wild-type and mutant PDGFβR proteins were expressed, with the use of a retroviral expression vector, in HepG2 cells, which do not express detectable amounts of endogenous PDGFβR receptors. The level of PDGFβR expression was approximately 5 × 10^5 receptors/cell for both wild-type and mutant receptors (34).

The production of H2O2 by HepG2 cells was measured with a fluorescence-based assay with 2′,7′-dichlorofluorescin diacetate and laser-scanning confocal microscopy. Stimulation of HepG2 cells expressing wild-type PDGFβR with PDGF resulted in a time-dependent increase in the intensity of DCF fluorescence, with the maximal, 3.5-fold increase apparent 10–30 min after stimulation; fluorescence had returned to the baseline value after 60 min (Fig. 2A). In contrast, cells expressing the kinase(-) or F5 mutant receptors failed to produce H2O2 in response to PDGF (Fig. 2, B and C). These results suggest that PDGF-induced H2O2 production requires the kinase activity of PDGFβR as well as at least one of the five tyrosine phosphorylation sites mutated in F5.

We next measured PDGF-induced H2O2 production in cells expressing other PDGFβR mutants (Fig. 3). Whereas PDGF had no effect on H2O2 production in cells expressing F740/751 or F740/751/771 mutants, it increased H2O2 production in cells expressing the F771 or F1021 mutants to an extent similar to that observed in cells expressing the wild-type receptor. Cells expressing Y740/751 (equivalent to F5 in which the PI3K binding site has been restored) showed a response to PDGF that
was slightly greater than that of cells expressing the wild-type receptor. However, cells expressing Y1021 (equivalent to F5 in which the PLC-\(\gamma_1\) binding site has been restored) did not generate \(\text{H}_2\text{O}_2\) in response to PDGF. These results thus indicate that activation of PI3K, but not of GAP, SHP-2, or PLC-\(\gamma_1\), is necessary for \(\text{H}_2\text{O}_2\) production in response to PDGF.

The essential role of PI3K was further supported by the observation that prior incubation with 10 \(\mu\text{M}\) LY294002, a specific inhibitor of PI3K, completely blocked the PDGF-induced increase in \(\text{H}_2\text{O}_2\) production in HepG2 cells expressing wild-type or Y740/751 PDGF receptors (Fig. 4). Similar inhibition was also observed with wortmannin, another inhibitor of PI3K (data not shown). Prior incubation with the inhibitors had no effect on the basal level of \(\text{H}_2\text{O}_2\) production. PDGF also induced a transient increase in \(\text{H}_2\text{O}_2\) production, with the maximal, 6-fold increase apparent 5–10 min after stimulation, in NIH 3T3 cells, and this effect was also abolished by 10 \(\mu\text{M}\) LY294002 (data not shown).

We further examined whether activation of PI3K is sufficient to produce \(\text{H}_2\text{O}_2\) by transiently expressing in HepG2 cells the catalytic p110 subunit of PI3K with c-Myc epitope and farnesylation signal (CAAX) sequences at the NH\(_2\) and COOH termini, respectively. It was previously shown that even modest expression of the membrane-targeted p110 (p110-CAAX) is sufficient to trigger activation of downstream events (36, 37). Expression of p110-CAAX in HepG2 cells expressing F5 mutant receptor resulted in a 3-fold increase in \(\text{H}_2\text{O}_2\) production, and the effect was blocked by prior incubation of cells with LY294002 or wortmannin (Fig. 5).

To investigate the role of Rac1 in PI3K-dependent \(\text{H}_2\text{O}_2\) production, we infected HepG2 cells expressing the Y740/751 receptor either with recombinant adenovirus encoding N17Rac1, a dominant negative mutant of Rac1 (Ad.N17rac), or control adenovirus (35). Expression of N17Rac1 in the infected HepG2 cells was confirmed by immunoblot analysis (Fig. 6A). Whereas HepG2 cells expressing the Y740/751 receptor infected control adenovirus showed an increase in \(\text{H}_2\text{O}_2\) production in response to PDGF, those infected with recombinant adenovirus encoding N17Rac1 (Ad.N17rac) did not (Fig. 6B). Similar results were observed after infection of cells expressing wild-type PDGF\(\beta\)R with the recombinant adenovirus encoding

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**Fig. 2.** Effects of PDGF on \(\text{H}_2\text{O}_2\) production in HepG2 cells expressing wild-type PDGF\(\beta\)R or the kinase(−) or F5 mutants. A, time course of \(\text{H}_2\text{O}_2\) generation induced by PDGF-BB (100 ng/ml) in cells expressing the wild-type PDGF\(\beta\)R. The generation of \(\text{H}_2\text{O}_2\) was assayed on the basis of DCF fluorescence as described under “Experimental Procedures.” Data are expressed relative to the value for non-stimulated cells. B and C, DCF fluorescence of HepG2 cells expressing wild-type PDGF\(\beta\)R or the kinase(−) or F5 mutants after incubation for 30 min in the absence or presence of PDGF-BB (100 ng/ml). Representative microscopic fields are shown in (B) and relative fluorescence intensity relative to nonstimulated cells is shown in C. Data in A and C are mean ± S.E. of five independent experiments.

**Fig. 3.** Effects of mutation of specific effector binding sites in PDGF\(\beta\)R on PDGF-induced \(\text{H}_2\text{O}_2\) production. HepG2 cells transfected with the empty vector (pLXSN) or with vectors encoding either wild-type PDGF\(\beta\)R or the indicated receptor mutants were incubated for 30 min in the absence or presence of PDGF-BB (100 ng/ml). The production of \(\text{H}_2\text{O}_2\) was then measured on the basis of DCF fluorescence. Data are mean ± S.E. of values from five independent experiments.
Role of PI3-kinase in PDGF-induced H₂O₂ Production

FIG. 4. Effect of the PI3K inhibitor LY294002 on PDGF-induced H₂O₂ production. HepG2 cells expressing wild-type (left panels) or Y740/751 PDGF receptors were incubated for 45 min in the absence or presence of 10 μM LY294002 and then for 30 min with or without PDGF-BB (100 ng/ml), after which DCF fluorescence was measured with a confocal laser-scanning microscope.

FIG. 5. Effect of the expression of the membrane-targeted p110 subunit of PI3K (p110-CAAX) on H₂O₂ production. HepG2 cells expressing the F5 mutant PDGF receptor were plated at a density of 3 × 10⁴/well in 6-well plates and allowed to recover for 24 h. The cells were then incubated for 6 h with 1.3 μg of empty (pCMV) or MYC-p110-CAAX-encoding (pCMVp110-CAAX) plasmid and 6.7 μl of LipofectAMINE in 1 ml of opti-MEM medium (Life Technologies, Inc.), after which 1 ml of Dulbecco’s modified Eagle’s medium supplemented with 20% fetal bovine serum was added to each well and the cells were incubated for an additional 24 h. The cells were then incubated in the absence or presence of 10 μM LY294002 or 10 nM wortmannin for 45 min. The production of H₂O₂ was assayed on the basis of DCF fluorescence as described under “Experimental Procedures.” Data are expressed as the relative fluorescence of the values for pCMV-transfected cells in the absence of the PI3K inhibitors and are mean ± S.E. of three independent experiments.

N17Rac1 (data not shown). In contrast, expression of dominant negative mutants of other Rho-family members (N19RhoA or N17Cdc42) did not affect the PDGF-induced increase in H₂O₂ production in HepG2 cells expressing wild-type PDGFβR (data not shown).

DISCUSSION

The binding of peptide growth factors to their specific receptors results in receptor autophosphorylation at several tyrosine residues, thereby triggering the recruitment of Src homology 2 domain-containing effector enzymes that include Src, PI3K, GAP, SHP-2, and PLC-γ1 (34, 38). The interaction of growth factor receptors with their receptors also induces a transient increase in the intracellular concentration of H₂O₂ (5–9). In the present study, the role of the receptor-associated effectors in H₂O₂ production was investigated with HepG2 cells expressing various PDGFβR mutants. The PDGFβR was well suited for this study because the effector enzymes bind only when the receptor is phosphorylated at specific tyrosine residues (34). Mutation of these tyrosine residues individually prevents association of a specific effector without affecting the binding of other enzymes to the receptor mutant. In the case of other growth factor receptors, either the effector enzymes bind less specifically at multiple tyrosine residues or their binding sites are not well defined.

Our results suggest that, among the effector enzymes PI3K, GAP, SHP-2, and PLC-γ1, only the binding of PI3K to PDGFβR is necessary for PDGF-induced H₂O₂ production. The absence of the binding sites for GAP, SHP-2, and PLC-γ1 in the Y740/751 receptor mutant was actually associated with a slight, but reproducible, increase in the extent of PDGF-induced H₂O₂ production relative to that apparent with the wild-type receptor. This observation is consistent with the results of a previous study showing that PLC-γ1 and GAP negatively regulate the PDGF-induced activation of PI3K (38). Given that phosphorylation of Tyr⁷⁴⁰ and Tyr⁷⁵¹ is required to generate the binding site for PI3K, it follows that the intrinsic kinase activity of PDGFβR is also required for PDGF-induced generation of H₂O₂. We previously also showed that an epidermal growth factor receptor mutant lacking kinase activity did not induce an increase in H₂O₂ production in response to epidermal growth factor (8). In contrast, the observation that a membrane-bound NADPH oxidase prepared from human fat cells produced H₂O₂ in the absence of ATP in response to insulin suggested that this growth factor is able to induce H₂O₂ production independently of the kinase activity of its receptor (39). Activation of PI3K appears to be sufficient to produce H₂O₂ as indicated by the observation that expression of p110-CAAX...
induced H₂O₂ production. One of the major events following PI3K activation is the phosphorylation and activation of Akt, a serine/threonine kinase (40). Therefore, we determined whether Akt is involved in PDGF-induced H₂O₂ production. Transient expression of either a dominant negative Akt mutant in HepG2 cells expressing Y740/751 receptor or a constitutively active Akt in HepG2 cells expressing F5 receptor had no effect on H₂O₂ production (data not shown). These results suggest that Akt is not involved in the PDGF-induced H₂O₂ production.

It was previously shown that the blockage of the growth factor-induced H₂O₂ increase by catalase in vascular smooth muscle and A431 cells resulted in a marked decrease in tyrosine phosphorylation of various proteins including the growth factor receptors (7, 8). At the present time, we do not know why PDGF-Rs (F740/751, F740/751/771, and F5) that fail to induce H₂O₂ production were fully autophosphorylated in HepG2 cells (34, 38). It is possible that in HepG2 cells, H₂O₂ production makes a greater contribution to receptor tyrosine phosphorylation as compared with vascular smooth muscle and A431 cells.

An essential role for Rac in activation of the NADPH oxidase complex has been well established in phagocytes (28). Rac also appears important in this regard in nonphagocytic cells, as indicated by the observations that transient expression of an active form of Rac1 (V12Rac1) in NIH 3T3 cells itself resulted in an increase in H₂O₂ production and that expression of N17Rac1 inhibited the increase in H₂O₂ concentration induced by PDGF, epidermal growth factor, tumor necrosis factor-α, or interleukin-1 (31). Our observation that N17Rac1 blocked the Y740/751 receptor-induced generation of H₂O₂ indicates that Rac1 acts downstream of PI3K in the signaling pathway that leads to activation of NADPH oxidase. Moreover, signaling by this pathway appears independent of activation of GAP, SHP-2, and PLC-γ1.

Additional evidence suggests that Rac functions downstream of PI3K (41–44). Thus, the exchange of Rac-bound GDP for GTP catalyzed by guanine nucleotide exchange factors (GEFs) is stimulated by phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3), a product of the action of PI3K. A family of GEFs has been identified that mediate the activation of Rac-related proteins has been identified. All members of this family, including Vav, Sos, and Pix, contain a pleckstrin homology domain that binds inositol-containing phospholipids such as PI(4,5)P2 and PI(3,4,5)P3 (44–47). PI(4,5)P2, when bound to the pleckstrin homology domain of Vav, inhibited activation of Vav GEF activity by the protein-tyrosine kinase Lck, whereas PI(3,4,5)P3 enhanced phosphorylation and activation of Vav by Lck (42). Thus, it appears that PI3K might serve to convert a Vav inhibitor into an activator, resulting in a rapid transformation of inactive, GDP-bound Rac to its active, GTP-bound form. Sos possesses two distinct domains that allow it to function as a GEF for both Ras and Rac; the Rac GEF activity requires the binding of activated Ras to the Ras GEF domain of Sos as well as the binding of PI(3,4,5)P3 to the pleckstrin homology domain (43). Although not yet demonstrated, the GEF activity of Pix is also likely dependent on a product of PI3K.

The mechanism by which GTP-bound Rac increases the production of superoxide (and thus H₂O₂) in nonphagocytic cells is not clear. In phagocytes, activated Rac1 associates directly with pI67-phox to stimulate NADPH oxidase (48, 49). Although the NADPH oxidase system in nonphagocytic cells is not well characterized, a similar interaction might occur.

In summary, we have shown that the activation of PI3K is required for PDGF-induced H₂O₂ production. The essential role of PI3K is likely to provide PI(3,4,5)P3 for the activation of Rac, a component of the H₂O₂-generating system in both phagocytes and nonphagocytic cells.
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