Transactivation of the EGF receptor and a PI3 kinase–ATF-1 pathway is involved in the upregulation of NOX1, a catalytic subunit of NADPH oxidase

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Abstract **We previously reported that hypertrophy of vascular smooth muscle cells caused by prostaglandin (PG) $F_{2\alpha}$ is mediated by the induction of NOX1, a catalytic subunit of NADPH oxidase that generates superoxide. The signal transduction pathway(s) involved in this process, however, remained unresolved. $PGF_{2\alpha}$ enhanced the phosphorylation of the epidermal growth factor (EGF) receptor, and a selective inhibitor of EGF receptor kinase, tyrphostin AG1478, significantly suppressed $PGF_{2\alpha}$-induced NOX1 expression. AG1478 also blunted the $PGF_{2\alpha}$-induced phosphorylation of extracellular signal-regulated protein kinase (ERK)1/2 and Akt. Phosphoinositide 3 (PI3) kinase inhibitors not only reduced $PGF_{2\alpha}$-induced NOX1 expression, but also suppressed the phosphorylation of ATF-1, a transcription factor previously shown to play a key role in the induction of NOX1. Accordingly, the transactivation of the EGF receptor and the activation of ERK1/2, PI3 kinase, and ATF-1 constitute the signaling pathways involved in the upregulation of NOX1.**

Keywords: NOX1; NADPH oxidase; Prostaglandin $F_{2\alpha}$; Epidermal growth factor receptor; Phosphoinositide 3 kinase

1. Introduction

Reactive oxygen species (ROS), including superoxide (O_2^-) and hydrogen peroxide (H_2O_2), have been recognized to act as intrinsic signaling molecules in cardiovascular tissues. It has been shown that NADPH oxidases are the major source of O_2^- in vascular cells and cardiac myocytes [1–3]. Recently, several homologs of the catalytic subunit of the phagocyte NADPH oxidase (gp91phox; NOX2) were found in vascular smooth muscle cells (VSMC). Among them, NOX1 was implicated in the pathogenesis of atherosclerosis, hypertension, and restenosis after angioplasty, since it mediates the proliferation and hypertrophy of VSMC. Expression of the NOX1 gene is induced by angiotensin II, platelet-derived growth factor (PDGF), phorbol ester, and fetal bovine serum (FBS) [4,5]. We previously reported that prostaglandin (PG) $F_{2\alpha}$, one of the primary prostanoids generated in the vascular tissue, causes hypertrophy of VSMC by the induction of NOX1 and the subsequent increase in O_2^- generation [6]. Recently, we found that ATF-1, a transcription factor of the CREB/ATF family, is essential for the upregulation of NOX1 by $PGF_{2\alpha}$, PDGF, and other factors, including phorbol ester and FBS [7].

$PGF_{2\alpha}$ exerts its biological actions through binding to its specific G-protein-coupled receptor, FP [8]. FP is coupled to phospholipase C and elicits the mobilization of cytosolic Ca^{2+}. In VSMC isolated from rat aorta, the activation of extracellular signal-regulated kinase (ERK) 2, c-Jun-N-terminal kinase (JNK) 1, phosphoinositide 3 (PI3)-kinase, and p70 S6 kinase by $PGF_{2\alpha}$ has been reported [9]. It is as yet unknown, however, whether these protein kinases take part in the upregulation of NOX1 that leads to the generation of the signaling molecule, O_2^-. Here, we report that the upregulation of NOX1 by $PGF_{2\alpha}$ involves the transactivation of the EGF receptor, and ensuing activation of its downstream ERK1/2 and PI3 kinase–ATF-1 pathways.

2. Materials and methods

2.1. Materials

Tyrphostin AG1478 and Wortmannin were purchased from Sigma (St. Louis, MO). $PGF_{2\alpha}$ was acquired from Nacalai Tesque (Kyoto, Japan). Antibodies against EGF receptor or phosphorylated EGF receptor were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Biosource International (Nivelles, Belgium), respectively. Antibodies against ERK or phosphorylated ERK were purchased from New England Biolabs (Beverly, MA). Antibodies against Akt or phosphorylated Akt and horseradish peroxidase-linked anti-rabbit or anti-mouse antibodies were purchased from Cell Signaling Technology (Beverly, MA). PP2, GM6001, PD98059, SB203580, and SP600125 were purchased from Merck (Tokyo, Japan). LY294002 was purchased from Cayman Chemical (Ann Arbor, MI). [α-^{32}P]dCTP and [α-^{32}P]UTP were obtained from ICN Biomedicals (Costa Mesa, CA).

2.2. Cell treatment and Northern blot analysis

The rat VSMC line A7r5 obtained from American Type Culture Collection (Rockville, MD) was seeded in 10-cm dishes (1×10^6 cells/dish) and cultured for 24 h in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. The cells were then cultured for a further 48 h in DMEM containing 0.5% FBS. After pretreatment with or without various inhibitors for 1 h, the cells were subsequently incubated with 100 nM $PGF_{2\alpha}$ for 24 h. Northern blot analysis was performed as described previously [6]. Representative autoradiographs of the three experiments are shown in figures.

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Abbreviations: PG, prostaglandin; EGF, epidermal growth factor; ERK, extracellular signal-regulated protein kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MMP, matrix metalloproteinase; PI3, phosphoinositide 3

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2.3. Western blot analysis

A7r5 cells cultured in the absence of FBS for 48 h were pretreated with various inhibitors for 1 h, incubated with 100 nM $PGF_{2\alpha}$, washed twice with PBS, and then frozen with liquid nitrogen. The cells were lysed in a buffer containing 1% Triton, 0.5% sodium deoxycholate, 10 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 20 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM sodium fluoride, and 1 mM phenylmethylsulfonyl fluoride. The lysate was centrifuged and the supernatant was saved for analysis. The aliquots containing equal amounts of protein (10 μg) were subjected to SDS–polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). After hybridization with a primary antibody and horseradish-peroxidase-linked secondary antibody, immunoreactive bands were detected using an ECL-Plus System (Amersham Biosciences, Piscataway, NJ). For the detection of ATF-1, nuclear extracts were prepared and Western blot analyses were performed as described previously [7]. Representative results of the three experiments are shown in figures.

2.4. Statistical analysis

Values were expressed as means ± S.E.M. Statistical analysis was performed with Student's *t* test. For multiple treatment groups, one-way ANOVA followed by Bonferroni's *t* test was applied.

3. Results

3.1. $PGF_{2\alpha}$ transactivates EGF receptor

The mitogenic effects of angiotensin II are mediated through AT_1 receptor and subsequent transactivation of the EGF receptor [10]. Similar to the AT_1 receptor, the prostanoid FP receptor is among the Gq-protein-coupled receptors [8]. To examine whether $PGF_{2\alpha}$ transactivates the EGF receptor in A7r5 cells, phosphorylation of the EGF receptor was analyzed by Western blotting using a phospho-specific antibody. As shown in Fig. 1A, a time-dependent increase in the phosphorylation of the EGF receptor was demonstrated in the cells stimulated with $PGF_{2\alpha}$. Increased phosphorylation of the EGF receptor was clearly observed 0.5 min after stimulation with $PGF_{2\alpha}$, and the level reached a maximum at 2 min.

3.2. Inhibitors of EGF receptor kinase, Src, and matrix metalloproteinase (MMP) suppress the induction of NOX1 mRNA by $PGF_{2\alpha}$

To examine whether the transactivation of the EGF receptor is involved in NOX1 induction, A7r5 cells were stimulated with $PGF_{2\alpha}$ for 24 h in the presence or absence of tyrphostin AG1478, a selective inhibitor of EGF receptor kinase. As shown in Fig. 1B, tyrphostin AG1478 significantly suppressed the $PGF_{2\alpha}$-induced increase in NOX1 mRNA. Transactivation of the EGF receptor by angiotensin II is mediated by the activation of tyrosine kinases Src and Pyk2, and the ADAM family of MMPs, leading to the excision of heparin-binding EGF [10]. Therefore, effects of inhibitors of Src and MMPs were investigated. As shown in Fig. 1C, a Src inhibitor PP2 and a MMP inhibitor GM6001 suppressed the $PGF_{2\alpha}$-induced increase in NOX1 mRNA. These results suggest that the activation of Src and MMPs is involved in the transactivation of the

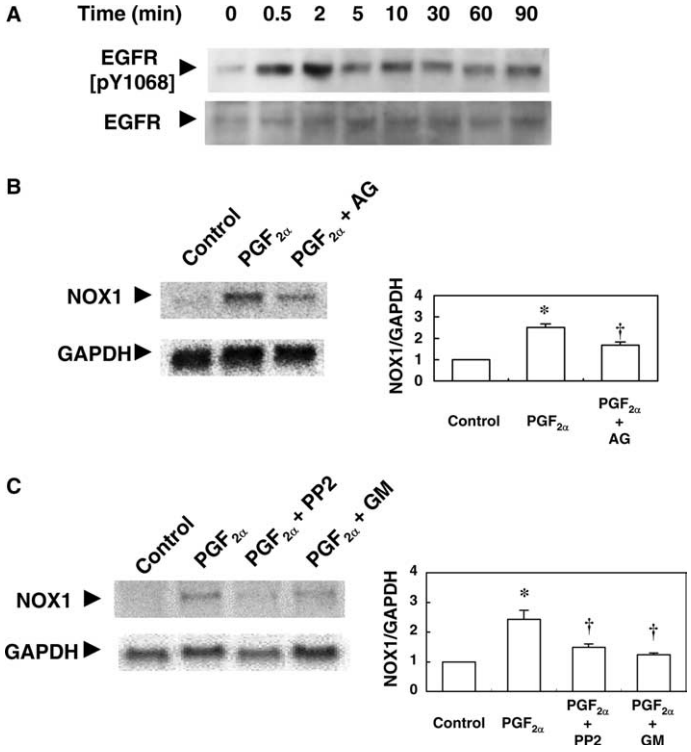

Fig. 1. Involvement of EGF receptor transactivation in $PGF_{2\alpha}$-induced NOX1 expression. (A) Time course of transactivation of the EGF receptor by $PGF_{2\alpha}$. Growth-arrested A7r5 cells were incubated with 100 nM $PGF_{2\alpha}$ for the indicated period and the cell lysates (10 μg) were subjected to Western blot analyses. The phosphorylation status of the EGF receptor was analyzed with anti-phospho-specific EGF receptor antibody. (B) Suppression of $PGF_{2\alpha}$-induced NOX1 expression by tyrphostin AG1478. Growth-arrested A7r5 cells were treated with 10 μM tyrphostin AG1478 for 1 h and subsequently incubated with 100 nM $PGF_{2\alpha}$ for 24 h. (C) Suppression of $PGF_{2\alpha}$-induced NOX1 expression by PP2 and GM6001. Growth-arrested A7r5 cells were treated with 50 μM PP2 or 20 μM GM6001 for 1 h and subsequently incubated with 100 nM $PGF_{2\alpha}$ for 24 h. Bar graphs represent means ± S.E.M. of the expression levels of NOX1 normalized to those of GAPDH obtained from three experiments. $^*P < 0.05$ vs. control; $^\dagger P < 0.05$ vs. $PGF_{2\alpha}$-treated cells.