NADPH Oxidase Is Involved in Prostaglandin \( F_{2\alpha} \)-induced Hypertrophy of Vascular Smooth Muscle Cells

INDUCTION OF NOX1 BY PGF \( _{2\alpha} \)

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Prostaglandin (PG) \( F_{2\alpha} \), one of the primary prostanoids generated in vascular tissue, is known to cause hypertrophy in vascular smooth muscle cells. To clarify the molecular mechanisms underlying PGF \( _{2\alpha} \)-induced hypertrophy, the involvement of reactive oxygen species was examined in a rat vascular smooth muscle cell line, A7r5. PGF \( _{2\alpha} \) and (+)-fluprostenol, a selective agonist of the PGF receptor, significantly increased intracellular \( O_2 \) in A7r5. The PGF \( _{2\alpha} \)-induced \( O_2 \) increase was suppressed by diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase that has been reported to be the major source of \( O_2 \) in vascular cells. The augmented synthesis of the protein induced by PGF \( _{2\alpha} \) or (+)-fluprostenol was suppressed in the presence of DPI. In PGF \( _{2\alpha} \) or (+)-fluprostenol-treated cells, a dose-dependent increase in the expression of NOX1, a homolog of the catalytic subunit of the phagocyte NADPH oxidase gp91 \( ^{\text{phox}} \), was demonstrated by Northern blot analysis. Finally, depletion of NOX1 mRNA in the cells transfected with ribozymes targeted for three independent cleavage sites on the mRNA sequence significantly reduced the PGF \( _{2\alpha} \)-induced increase in protein synthesis. Taken together, these results suggest that hypertrophy of vascular smooth muscle cells caused by PGF \( _{2\alpha} \) is mediated by NOX1 induction and the resultant overproduction of \( O_2 \) by NADPH oxidase.

Prostanoids are metabolites of arachidonic acid that exert a variety of biological actions in tissue. Prostaglandin (PG) \( F_{2\alpha} \), a vasoactive factor that causes constriction and hypertrophy of vascular smooth muscle cells (VSMC) and cardiac myocytes (1–3). PGF \( _{2\alpha} \) exerts its biological actions through binding to its specific receptor, FP, on plasma membranes (4). FP is coupled to phospholipase C and elicits mobilization of cytosolic Ca\(^{2+}\). Although several protein kinases are reported to be involved in the PGF \( _{2\alpha} \)-induced hypertrophy of VSMC or myocytes, the signaling pathway(s) mediating this effect is still unknown (5–7).

Reactive oxygen species (ROS) including superoxide (\( O_2^- \)) and hydrogen peroxide (\( H_2O_2 \)) are recognized as important signaling molecules in cardiovascular tissues. It has been shown that NADPH oxidases are the major source of \( O_2 \) in vascular cells and myocytes (8–10), and their activities are increased by vasoactive factors such as angiotensin II and thrombin (11–13). The phagocyte NADPH oxidase is composed of two plasma membrane-spanning subunits, gp91 \( ^{\text{phox}} \) (NOX2) and p22 \( ^{\text{phox}} \); two cytosolic subunits, p47 \( ^{\text{phox}} \) and p67 \( ^{\text{phox}} \); and a small G protein, Rac. The catalytic subunit NOX2 contains binding sites for NADPH, FAD, and hemes. To date, six homologs of the NOX2, NOX1, NOX3, NOX4, NOX5, DUOX1, and DUOX2 have been reported (14–16). Among them NOX1, NOX4, and NOX5 have been detected in VSMC (16–18). NOX1 is also highly expressed in a colon carcinoma cell line Caco-2 (17, 19), and increased cell growth along with a transformed appearance were reported in NOX1-transfected NIH3T3 cells (17, 20). Platelet-derived growth factor (PDGF), angiotensin II, phorbol ester, and fetal bovine serum (FBS) were shown to induce the expression of NOX1 mRNA in VSMC (17, 18). Accordingly, NOX1/NOX1/NOX1-derived oxidase \( O_2^- \) may be implicated in the pathogenesis of vascular lesions mediating the proliferation and hypertrophy of VSMC.

Since PGF \( _{2\alpha} \) is known to elicit hypertrophy of VSMC we examined whether this effect of PGF \( _{2\alpha} \) is mediated by \( O_2^- \) generated by NOX1/NADPH oxidase.

EXPERIMENTAL PROCEDURES

Materials—Ribozyme expression vector pPUR-KE, which contains the human tRNA \( _{Val} \)-promoter between the pcDNA3.1 and pCMVneo vector, was a gift from Professor K. Taira of the Graduate School of Engineering, University of Tokyo. [\( ^{\text{35S}} \)]-EXPRESS Protein Labeling Mix was purchased from PerkinElmer Life Sciences. DPI chloride, polyethylene glycol-conjugated catalase, PDGF-AB, and puromycin were from Sigma. Mn\( \text{III} \)-tetakis(4-benzoic acid)porphyrin chloride (MnTBAP) was obtained from Calbiochem-Novabiochem. Measurement of Superoxide—A7r5 cells obtained from American Type Culture Collection were seeded in 6-well plates (2.5 × 10\(^5\) cells/well) and cultured for 24 h in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS. Cells were subsequently cultured in DMEM containing 0.5% FBS and then incubated with 1 \( \mu \)M PGF \( _{2\alpha} \) (Nacalai Tesque, Kyoto, Japan) or 1 \( \mu \)M (+)-fluprostenol (Cayman Chemicals) for another 24 h. After being harvested with trypsin cells were resuspended in Hank’s balanced salt solution and incubated for an additional 30 min at 37 °C with 5 \( \mu \)M hydroethidine (Polysciences) in the presence or absence of 100 nM DPI. The ethidium fluorescence (ex: 488 nm; em: 610 nm) resulting from the specific oxidation of hydroethidine by \( O_2^- \) (18) was measured with a flow cytometer (FCASCalibur, Becton Dickinson). The geometric mean of ethidium fluorescence intensity was used for analysis.

Measurement of Protein Synthesis—Cells were seeded in 24-well plates (5 × 10\(^4\) cells/well) and cultured for 24 h in DMEM supplemented with 10% FBS. After 48 h of culture in DMEM containing 0.5% FBS, cells were incubated for another 24 h with 1 \( \mu \)Ci/ml of [\( ^{\text{35S}} \)]-EXPRESS
Protein Labeling Mix and 100 nm PGF₂α or 100 nm (+)-fluoprostenol in the presence or absence of 100 nm DPI, 100 μM Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP), or 500 units/ml of polyethylene glycol-conjugated-catalase. Labeled cells were washed with phosphate-buffered saline, trypsinized, and precipitated with an equal volume of 10% trichloroacetic acid. After freezing and thawing, the mixture was passed through a glass fiber filter (GFC, Whatman). The filter was washed three times with 5% trichloroacetic acid, and the radioactivity on the filter was measured with a liquid scintillation counter.

Northern Blot Analysis—Total RNA was isolated from A7r5 cells by the acid guanidinium thiocyanate-phenol/chloroform method (21). Ten micrograms of RNA were separated by electrophoresis on a 1.5% agarose gel and transferred onto a nylon membrane (Hybond-N+, Amer sham Biosciences).

Rat CDNA fragments for NOX1, NOX4, and p22phox were amplified by RT-PCR. The fragments were cloned into a vector and linearized with an appropriate restriction enzyme. Using these DNAAs as templates, antisense RNA probes were synthesized with SP6 or T7 RNA polymerase and labeled with T4 polynucleotide kinase in the presence of [γ-32P]UTP. Hybridization was carried out at 68 °C for 15 h in 6× SSC, 2× Denhardt’s solution, 0.2% SDS, 0.05 mM sodium phosphate (pH 6.5), 250 μg/ml of heat-denatured salmon sperm DNA, 200 μg/ml of yeast tRNA, and the 32P-labeled probe. Membranes were washed twice at 70 °C in 0.1× SSC containing 0.1% SDS. Hybrids were detected with a Fuji BAS 2000 Bioimaging Analyzer (Fuji photo film, Tokyo, Japan). Blots were then rehybridized with a 32P-labeled DNA probe for glyceraldehyde-3-phosphate dehydrogenase. Hybridization was carried out at 68 °C for 15 h in 6× SSC containing 5× Denhardt’s solution and 0.5% SDS. Membranes were washed twice at 68 °C for 30 min in 2× SSC containing 1% SDS. The radioactivity on the membrane was quantitated with the bioimaging analyzer, and the levels of NOX1 mRNA were normalized based on the levels of glyceraldehyde-3-phosphate dehydrogenase mRNA.

Synthesis of Anti-NOX1 Ribozymes—Hammerhead ribozymes against rat NOX1 mRNA were designed using the MFOLD program in the following way. Rzm168 is targeted at the GUU triplet located at nucleotides 241–243, and Rzm243 is targeted at the GUA located at nucleotides 244–246. Rzm243 is targeted at the GUA triplet located at nucleotides 241–243, and Rzm603 is targeted at the GCU spanning nucleotides 601–603 of the rat NOX1 mRNA sequence (17). Schematic diagrams of these ribozymes are shown in Fig. 5. The construction of plasmids for ribozyme expression was performed essentially as described previously (22, 23).

Establishment of Clones Stably Expressing Anti-NOX1 Ribozymes—Ribozyme expression plasmids (pPUR-KE containing NOX1 ribozyme sequence) were transfected into A7r5 cells using GenePORTER2 transfection reagent (Gene Therapy Systems). Stable transfectants were selected by single cell cloning in the presence of puromycin (10 μg/ml). For mock transfection the pPUR-KE vector was transfected and selected with puromycin. The expression of ribozymes was verified as described previously (22). Briefly, total RNA (10 μg) was separated by electrophoresis on a 2.4% agarose gel and transferred onto a nylon membrane. Oligonucleotides complementary to the respective ribozyme sequences (see Fig. 5) were labeled with T4 polynucleotide kinase in the presence of [γ-32P]ATP. Hybridization was carried out at 60 °C for 4 h in 6× SSC, 5× Denhardt’s solution, 0.5% SDS, 0.01 mM sodium phosphate (pH 6.5), 100 μg/ml of heat-denatured salmon sperm DNA, and the 32P-labeled probe. Membranes were washed twice at 60 °C in 2× SSC containing 1% SDS for 15 min.

Statistical Analysis—Values are expressed as the mean ± S.E. Statistical analysis was performed with Student’s t test. For multiple treatment groups one-way analysis of variance followed by Bonferroni’s t test was applied.

RESULTS

Measurements of O₂⁻ in A7r5 Cells—To examine whether PGF₂α induces production of O₂⁻ in A7r5 cells, oxidation of intracellular hydroethidine by O₂⁻ was measured by flow cytometry. As shown in Fig. 1A, significant increases in mean fluorescence on oxidation of hydroethidine to fluorescent ethidium were observed in the cells stimulated with PGF₂α or (+)-fluoprostenol, a selective PGF receptor (FP) agonist. DPI, an inhibitor of NADPH oxidase, significantly suppressed the PGF₂α-induced increase in fluorescence (Fig. 1B).

Protein Synthesis in A7r5 Cells—Next, the effects of FP agonists and DPI on protein synthesis in A7r5 cells were investaged (Fig. 2). Growth-arrested cells were stimulated for 24 h with 100 nm PGF₂α or 100 nm (+)-fluoprostenol in the presence or absence of 100 nm DPI. Increased synthesis of protein as determined from the incorporation of [35S]methionine was observed in the cells stimulated with PGF₂α or (+)-fluoprostenol. On the other hand, the incorporation of [35S]methionine induced by these FP agonists was significantly suppressed in the presence of DPI. Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP), a cell-permeable superoxide dismutase mimetic, significantly suppressed PGF₂α-induced [35S]methionine incorporation at 100 μM, whereas polyethylene glycol-conjugated catalase did not affect the incorporation even at the concentration of 500 units/ml (data not shown). These results suggest that the PGF₂α-induced hypertrophy of VSMC is mediated by O₂⁻ generated by NADPH oxidase.

Expression of NOX1 mRNA in A7r5 Cells—To further clarify the mechanism underlying the FP agonist-induced increase in O₂⁻ generation, effects of FP agonists on the expression of NOX1, a member of the catalytic subunit superfamily of NADPH oxidase, was examined. Growth-arrested A7r5 cells were stimulated with 10% FBS, 20 μg/ml PDGF- AB, or 1 μM PGF₂α. The expression of NOX1 mRNA was determined by Northern blot analysis. As shown in Fig. 3, a small NOX1 mRNA signal was detected at ~2.6 kb in growth-arrested A7r5 cells. When cells were stimulated with 10% FBS or PDGF, the level of NOX1 mRNA was markedly increased as previously reported in VSMC isolated from rat aorta (17, 18). In line with these findings, PGF₂α significantly increased the level of NOX1 mRNA.

A dose-dependent induction of NOX1 mRNA was demonstrated in the cells stimulated with either PGF₂α, or (+)-fluoprostenol for 24 h (Fig. 4A). The level of NOX1 mRNA began to increase at 10⁻¹⁰ M and reached a plateau at 10⁻⁹ M of PGF₂α. When stimulated with (+)-fluoprostenol, the level of NOX1 mRNA.
PGF$_{2\alpha}$ Induces NOX1

Fig. 2. DPI suppresses the PGF$_{2\alpha}$-induced increase in protein synthesis. Growth-arrested A7r5 cells were incubated for 24 h with 1 µCi/ml $^{[35]S}$-EXPRESS Protein Labeling Mix and 100 nM PGF$_{2\alpha}$, or 100 nM (+)-fluprostenol in the presence or absence of 100 nM DPI. $^{[35]S}$-Methionine incorporation was determined from six independent samples. *p < 0.01 versus control; †p < 0.01 versus PGF$_{2\alpha}$; ††p < 0.01 versus (+)-fluprostenol-treated cells.

mRNA first detected at 10$^{-12}$ M increased dose dependently and reached a maximum at 10$^{-7}$ M. By contrast, no noteworthy alteration in the expression levels of NOX4 (another homolog of NOX2) and p22$^{	ext{phox}}$ was detected in the cells stimulated with PGF$_{2\alpha}$. As shown in Fig. 4B, an increase in NOX1 expression was clearly observed 3 h after stimulation with 10$^{-7}$ M PGF$_{2\alpha}$. The level of NOX1 mRNA reached a maximum at 12 h and remained high until 48 h after the stimulation.

Effects of Other Prostanoids on NOX1 Expression—We also examined the effects of other prostanoids on NOX1 mRNA expression. PGE$_{2\alpha}$, U-46619 (a thromboxane A$_{2}$ receptor agonist), and carbaprostacyclin (a PGL$_{2}$ receptor agonist) induced expression of NOX1 mRNA at concentrations higher than 10$^{-7}$ M. However, thromboxane A$_{2}$ receptor (TP) antagonists SQ 29,548 and I-SAP did not affect induction of NOX1 expression by TP agonists U-46619 and I-BOP (data not shown).

Effects of Ribozymes Targeted at NOX1 mRNA—To verify the involvement of NOX1 in PGF$_{2\alpha}$-induced hypertrophy in VSMC, a ribozyme that cleaves the targeted sequence of mRNA was designed to control the expression of NOX1 in A7r5 cells. hammerhead ribozymes targeting three independent sites of the NOX1 mRNA sequence are illustrated in Fig. 5. Ribozyme expression plasmids were constructed and transfected into A7r5 cells that gave cell clones stably expressing Rzm168, 243, and 603 (Fig. 6A). Almost complete suppression in the expression of NOX1 mRNA was demonstrated in these clones. When stimulated with PGF$_{2\alpha}$, the increase in NOX1 mRNA level was effectively suppressed in these clones compared with the mock-transfected cells (Fig. 6B). Furthermore, a PGF$_{2\alpha}$-induced increase in O$_{2}^{\bullet-}$ production as well as the basal level of cellular O$_{2}^{\bullet-}$ was significantly reduced in these clones compared with the mock-transfected cells (Fig. 6C).

As demonstrated in Fig. 7, PGF$_{2\alpha}$ elicited more than a 2-fold increase in $^{[35]S}$-methionine incorporation in mock-transfected cells. On the other hand, the extent of the PGF$_{2\alpha}$-induced increase in $^{[35]S}$-methionine incorporation was significantly reduced in the ribozyme-expressing cells. These results denoted the involvement of NOX1/NADPH oxidase in PGF$_{2\alpha}$-induced hypertrophy of VSMC.

**DISCUSSION**

The present findings indicate that PGF$_{2\alpha}$-induced hypertrophy of VSMC is mediated by O$_{2}^{\bullet-}$ generated by NADPH oxidase through induction of NOX1 expression. The following four major lines of evidence are provided in this study. 1) PGF$_{2\alpha}$ evoked generation of intracellular O$_{2}^{\bullet-}$ in A7r5 vascular smooth muscle cells. 2) The PGF$_{2\alpha}$-induced increase in protein synthesis was attenuated by DPI, an inhibitor of NADPH oxidase. 3) A dose-dependent induction of NOX1, a catalytic subunit of NADPH oxidase, was produced by FP agonists PGF$_{2\alpha}$ and (+)-fluprostenol. 4) The extent of the PGF$_{2\alpha}$-induced increase in protein synthesis was significantly suppressed when NOX1 mRNA was depleted by transfection of ribozymes specifically targeted at the NOX1 mRNA sequence.

Augmented production of O$_{2}^{\bullet-}$ caused by overexpression of NOX1 constituting NADPH oxidase appears to be important to the action of PGF$_{2\alpha}$ leading to hypertrophy of VSMC. PGF$_{2\alpha}$ is synthesized from arachidonic acid, which is converted to PGH$_{2}$ through oxygenation and reduction by prostaglandin H syn-
thase (cyclooxygenase). Subsequent conversion of PGH₂ (or via PGE₂) by PGF synthase generates PGF₂α, one of the primary prostanoids produced by vascular cells in response to various stimuli. Increased expression of COX-2, an inducible isoform of cyclooxygenase, is reported in VSMC after balloon angioplasty or pinching injury of the carotid artery (25) as well as in endothelial cells exposed to tumor necrosis factor-α (TNF-α) (26). It has also been demonstrated that PGF₂α is released from endothelial cells exposed to hypoxia (27). Thus PGF₂α is synthesized and released from vascular lesions under inflammatory conditions following various injuries including ischemia-reperfusion. Our findings indicate that the hypertrophic effects of PGF₂α on VSMC are mediated by O₂⁻ generated by cellular NOX1/NADPH oxidase activity.

Among the NOX superfamily, NOX1, NOX4, and NOX5 have been detected in VSMC (16–18). In the present study, no alteration in the expression levels of NOX4 and p22phox was detected in A7r5 cells stimulated with PGF₂α. As stimulation of

![Diagram of ribozymes targeted at rat NOX1 mRNA](image)

**Fig. 5.** Schematic diagram of ribozymes targeted at rat NOX1 mRNA. Sequences of ribozymes are depicted under the targeted sites of the mRNA sequence. A, ribozyme targeted at the GUU triplet located at nucleotides 166–168 of NOX1 mRNA (Rzm168). B, ribozyme targeted at the GUA located at nucleotides 241–243 (Rzm243). C, ribozyme targeted at the GUC located at nucleotides 601–603 (Rzm603).

![Expression of NOX1 mRNA and production of O₂⁻ are suppressed in ribozyme-expressing cells](image)

**Fig. 6.** Expression of NOX1 mRNA and production of O₂⁻ are suppressed in ribozyme-expressing cells. A, expression of ribozyme, Rzm168, 243, and 603 in the cell clones transfected with the respective expression plasmids. **P**-labeled oligonucleotides complementary to the ribozyme sequences were hybridized as described under “Experimental Procedures.” B, expression of NOX1 mRNA in the cell clones transfected with the respective ribozyme expression plasmids. Cells were untreated or treated with 100 nM PGF₂α for 24 h. C, ethidium fluorescence in the cells untreated (control, open bar) or treated with 10 nM PGF₂α (closed bar) for 24 h. Mean values were calculated from four samples. *p < 0.01 versus control mock-transfected cells.

![PGF₂α-induced increase in protein synthesis is suppressed in ribozyme-expressing cells](image)

**Fig. 7.** PGF₂α-induced increase in protein synthesis is suppressed in ribozyme-expressing cells. Growth-arrested cells were incubated for 24 h with 1 μCi/ml [³⁵S]-EXPRESS Protein Labeling Mix and 10 nM PGF₂α. [³⁵S]Methionine incorporation determined from six independent samples was expressed as a percentage of control (untreated cells). *p < 0.05; **p < 0.01 versus mock-transfected cells treated with PGF₂α.
PGF$_{2\alpha}$-induced production of O$_2$ demonstrated in A7r5 cells seems to be minimal.

The induction of NOX1 expression by PGF$_{2\alpha}$ seems to be mediated specifically by FP, not by other prostanooid receptors. There are several possible explanations for this. First, (+)-fluprostenol, which increased NOX1 mRNA in a dose-dependent fashion, is a highly selective FP agonist. It was reported that this compound does not react with other prostanooid receptors (28). Second, expression of FP was demonstrated in A7r5 cells (2), and we also verified the expression by RT-PCR (data not shown). Third, the concentration of PGF$_{2\alpha}$ that elicited NOX1 expression was $10^{-10}$ M, much lower than for other vasoactive prostanooids investigated such as PGE$_{2\omega}$, U-46619 (a thromboxane A$_2$ receptor agonist), and carbaprostacyclin (a PGI$_2$ receptor agonist). They induced NOX1 expression at concentrations greater than $10^{-7}$ M suggesting that these prostanooids exerted their action by cross-reacting with FP. Thromboxane (TX) A$_2$ is also known as a prostanooid causing proliferation and hypertrophy of VSMC (1, 29). Yet binding or functional responsiveness to TXA$_2$ receptor (TP) agonist I-BOP was not observed in A7r5 cells (30). When examined by RT-PCR the level of TP mRNA in our A7r5 cells was also found to be very low (data not shown). Furthermore, TP antagonists SQ 29,548 and I-SAP did not affect the induction of NOX1 expression by TP agonists U-46619 and I-BOP. Thus, as far as A7r5 cells are concerned, induction of NOX1 by prostanooids seems to be mediated specifically by FP. We cannot exclude the possibility, however, that the mitogenic effects of TXA$_2$ on VSMC are mediated by FP—K. Taira and Dr. Y. Kato of Kyoto Prefectural University of Medicine for valuable discussion and advice. We also thank H. Takahashi, M. Yamasaki, and B. Shin of Kyoto Prefectural University of Medicine for assistance.

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