PKC δ mediates up-regulation of NOX1, a catalytic subunit of NADPH oxidase, via transactivation of the EGF receptor: possible involvement of PKC δ in vascular hypertrophy

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NADPH oxidase is the major source of superoxide production in cardiovascular tissues. We reported previously that PG (prostaglandin) F₂ α caused hypertrophy of vascular smooth muscle cells by induction of NOX1, a catalytic subunit of NADPH oxidase. PGF₂ α -induced NOX1 expression was mediated by transactivation of the EGF (epidermal growth factor) receptor and subsequent activation of ERK (extracellular-signal-regulated kinase) 1/2, PI3K (phosphoinositide 3-kinase) and ATF-1 (activating transcription factor-1), a member of the CREB (cAMP-response-element-binding protein)/ATF family. As the receptor for PGF₂ α is known to activate PKC (protein kinase C), involvement of PKC in up-regulation of NOX1 expression was investigated in A7r5 cells. GF109203x, a non-selective inhibitor of PKC, dose-dependently suppressed the induction of NOX1 mRNA by PGF₂ α . Whereas an inhibitor of the conventional PKC, Gö 6976, and a PKC ϵ translocation-inhibitor peptide had no effect, an inhibitor of PKC δ , rottlerin, significantly attenuated

the PGF₂ α -induced increase in NOX1 mRNA. Gene silencing of PKC δ by RNA interference significantly suppressed the PGF₂ α -induced increase in NOX1 mRNA, as well as phosphorylation of the EGF receptor, ERK1/2 and ATF-1. Silencing of the PKC δ gene also attenuated the PDGF (platelet-derived growth factor)-induced increase in NOX1 mRNA and transactivation of the EGF receptor. Moreover, the augmented synthesis of the protein induced by PGF₂ α or PDGF was abolished by gene silencing of PKC δ . These results suggest that PKC δ -mediated transactivation of the EGF receptor is elicited not only by PGF₂ α , but also by PDGF, and that the subsequent activation of ERK1/2 and ATF-1 leads to up-regulation of NOX1 gene expression and ensuing hypertrophy in the vascular cell lineage.

Key words: activating transcription factor-1 (ATF-1), NADPH oxidase, NOX1, platelet-derived growth factor (PDGF), prostaglandin F₂ α (PGF₂ α), protein kinase C δ (PKC δ).

INTRODUCTION

Superoxide (O₂⁻) and hydrogen peroxide (H₂O₂), the so-called reactive oxygen species, have been documented as intrinsic signalling molecules in cardiovascular tissues. The major source of O₂⁻ in vascular cells and cardiac myocytes is NADPH oxidase [1–3]. Among several homologues of the catalytic subunit of the phagocyte NADPH oxidase (gp91^{phox}; NOX2) recently identified, NOX1 has been implicated in the proliferation and hypertrophy of VSMC (vascular smooth muscle cells). Expression of NOX1 is induced by various vasoactive factors, including angiotensin II, PDGF (platelet-derived growth factor), phorbol ester and FBS (fetal bovine serum) [4,5]. Previously, we have shown that PG (prostaglandin) F₂ α , one of the primary prostanoids that is generated in the vascular tissue, also induces NOX1 and causes hypertrophy of VSMC through the increased generation of O₂⁻ [6]. A transcription factor of the CREB (cAMP-response-element-binding protein)/ATF (activating transcription factor) family, ATF-1, was determined to be essential for the up-regulation of NOX1 not only by PGF₂ α , but by PDGF, phorbol ester and FBS [7]. In addition, PGF₂ α -induced NOX1 expression was mediated by the transactivation of the EGF (epidermal growth factor) receptor and subsequent activation of ERK (extracellular-signal-related kinase) 1/2, PI3K (phosphoinositide 3-kinase), and ATF-1 [8].

The specific receptor for PGF₂ α , FP, is a G-protein-coupled receptor [9] that activates phospholipase C and elicits mobilization of cytosolic Ca²⁺. Since phorbol ester induces NOX1 expression in VSMC [5,7], we sought to investigate whether PKC (protein kinase C) is involved in PGF₂ α -induced NOX1 expression. In the present paper we report that PKC δ mediates the up-regulation of NOX1 not only by PGF₂ α , but also by PDGF. The involvement of transactivation of the EGF receptor was indicated in this process.

EXPERIMENTAL

Materials

GF109203x and rottlerin were purchased from Sigma (St. Louis, MO, U.S.A.). PGF₂ α was from Nacalai Tesque (Kyoto, Japan). Antibodies against the EGF receptor or phosphorylated EGF receptor were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) and Biosource International (Nivelles, Belgium) respectively. Antibodies against ERK or phosphorylated ERK were purchased from New England Biolabs (Beverly, MA, U.S.A.). Horseradish-peroxidase-conjugated anti-rabbit or anti-mouse antibodies were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.). Gö 6976 and a PKC ϵ

Abbreviations used: ATF-1, activating transcription factor-1; CREB, cAMP-response-element-binding protein; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; dsRNA, double-stranded RNA; EGF, epidermal growth factor; ERK, extracellular-signal-regulated protein kinase; FBS, fetal bovine serum; MMP, matrix metalloproteinase; PDGF, platelet-derived growth factor; PG, prostaglandin; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; cPKC, conventional PKC; nPKC, novel PKC; RNAi, RNA interference; RT-PCR, reverse transcriptase-PCR; VSMC, vascular smooth muscle cells.

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translocation-inhibitor peptide were purchased from Merck (Tokyo, Japan). The homodimer PDGF-BB was obtained from PeproTech (London, UK). [α - 32 P]dCTP and [α - 32 P]UTP were from ICN Biomedicals (Costa Mesa, CA, U.S.A.). [35 S]-EXPRESS Protein Labeling Mix was purchased from Perkin-Elmer Life Sciences (Boston, MA, U.S.A.). Hydroethidine was obtained from Polyscience (Warrington, PA, U.S.A.).

Cell culture and Northern blot analysis

A7r5 cells, obtained from American Type Culture Collection (U.S.A.), were seeded in 10 cm dishes (1×10^6 cells/dish) and cultured for 24 h in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % (v/v) FBS. Cells were incubated for a further 48 h in DMEM containing 0.5 % (v/v) FBS and were pre-treated with or without various inhibitors for 1 h and subsequently exposed to 100 nM PGF2 α for 24 h. Northern blot analysis was performed as described previously [6,7].

Western blot analysis

Cell lysates were prepared for the detection of the EGF receptor and ERK1/2 [8]. Nuclear extracts were prepared and Western blot analysis was performed for the detection of ATF-1 [7].

Synthesis of anti-PKC δ dsRNAs (double-stranded RNAs)

Anti-PKC δ dsRNAs were designed against nucleotides 381–410 [PKC δ -RNAi (RNA interference)-1] and 888–917 (PKC δ -RNAi-2) of the rat PKC δ mRNA sequence [10]. Sense or antisense oligonucleotides containing the hairpin sequence, the terminator sequence and overhanging sequences were synthesized, amplified by PCR and inserted into the pPUR-KE expression vector, which contains a tRNA^{Val} promoter. Establishment of A7r5 clones stably expressing an anti-PKC δ dsRNA was performed, essentially as described previously [6]. Measurements of O $_2^-$ production and protein synthesis were performed using hydroethidine and 35 S-labelled methionine as described previously [6].

Statistical analysis

Values were expressed as the means $\pm$ 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.

RESULTS

GF109203x suppresses PGF2 α -induced NOX1 expression

PGF2 α exerts its biological actions through binding to its specific receptor FP [9]. FP is coupled to phospholipase C and elicits mobilization of cytosolic Ca $^{2+}$ and activation of PKC in VSMC or ventricular myocytes [11,12]. Since phorbol ester induces the expression of NOX1 in VSMC [5,7], we first examined whether PKC is involved in the induction of NOX1 by PGF2 α . Pre-treatment of A7r5 cells with GF109203x, an isoform-non-selective inhibitor of PKC, dose-dependently reduced the PGF2 α -induced increase in NOX1 mRNA (Figure 1).

Rottlerin, but not Gö 6976 or a PKC ϵ translocation-inhibitor peptide, suppresses PGF2 α -induced NOX1 expression

We next investigated which PKC isoform is involved in NOX1 induction. As phorbol ester was shown previously to induce NOX1 expression in VSMC [5,7], an isoform of either a conventional PKC (cPKC) or a novel PKC (nPKC) which requires DAG (diacylglycerol) for its activation, is a likely candidate. In fact, no effect from a peptide inhibitor or a dominant-negative form

Figure 1 GF109203x suppresses PGF2 α -induced NOX1 expression

Growth-arrested A7r5 cells were treated with the indicated concentrations of PKC inhibitor for 1 h, and subsequently incubated with 100 nM PGF2 α for 24 h. Northern blot analysis was performed as described in the Experimental section. Representative results of three experiments are shown. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Bars represent the mean $\pm$ S.E.M. **P* < 0.05 compared with control; †*P* < 0.05 compared with PGF2 α treated cells.

of PKC ζ , an atypical PKC that does not require DAG for its activation, was observed on PGF2 α -induced NOX1 expression (results not shown). Since an inhibitor of cPKC, Gö 6976, did not affect NOX1 induction even at 1 μ M (Figure 2A), the PKC isoform involved in PGF2 α -induced NOX1 expression appeared to be a member of the nPKCs. Among nPKCs, transcripts for PKC δ and PKC ϵ , but not for PKC η , were detected in A7r5 cells by RT (reverse transcriptase)-PCR (results not shown). Therefore, the effects of a selective inhibitor of PKC δ , rottlerin [13], and a peptide that inhibits translocation of PKC ϵ were examined. As shown in Figure 2(B), rottlerin significantly suppressed the PGF2 α -induced increase in NOX1 mRNA at concentrations higher than 5 μ M, whereas the PKC ϵ translocation-inhibitor peptide had no effect (Figure 2C). These results suggest that PKC δ is the isoform involved in NOX1 induction by PGF2 α .

Gene silencing of PKC δ

To confirm the role of PKC δ in the up-regulation of NOX1 by PGF2 α , dsRNAs targeted at the rat PKC δ mRNA sequence were introduced into A7r5 cells. Following single-cell cloning of the transfectants, two clones, PKC δ -RNAi-1 and PKC δ -RNAi-2, which stably expressed the dsRNA targeted at the two independent sites of the mRNA sequence, were isolated (Figure 3A). In these clones, protein levels of PKC δ , but not of PKC ϵ , were reduced compared with the mock-transfected cells (Figure 3B).

Gene silencing of PKC δ attenuates transactivation of the EGF receptor by PGF2 α and downstream signalling

Involvement of PKC δ in transactivation of the EGF receptor has been documented in [14,15]. Previously, we demonstrated that PGF2 α -induced NOX1 expression is mediated by transactivation of the EGF receptor and subsequent activation of ERK1/2, PI3K and ATF-1 [7,8]. Based on these findings, we first examined whether gene silencing of PKC δ affects phosphorylation of these proteins. As shown in Figure 4(A), phosphorylation of the EGF

Figure 2 Rottlerin but not Gö 6976 or a PKC ϵ translocation-inhibitor peptide suppresses PGF_{2 α} -induced NOX1 expression

Growth-arrested A7r5 cells were treated for 1 h with the indicated concentrations of an inhibitor of cPKC, Gö 6976 (**A**), a selective inhibitor of PKC δ , rottlerin (**B**), or a PKC ϵ translocation-inhibitor peptide (**C**), and were subsequently incubated with 100 nM PGF_{2 α} for 24 h. Representative results of three experiments are shown. Bars represent the mean $\pm$ S.E.M. * P < 0.05 compared with control cells; † P < 0.05 compared with PGF_{2 α} treated cells.

Figure 3 Gene silencing of PKC δ by RNAi

(**A**) Expression of anti-PKC δ dsRNA precursors in the clones RNAi-1 and RNAi-2. Total RNA was reverse-transcribed with random nonomers, and the cDNA fragment (125 bp) was amplified by PCR. The product was separated on a 15% polyacrylamide gel. Φ 174HaeIII, molecular size marker. (**B**) Silencing of PKC δ expression in the clones RNAi-1 and RNAi-2. Western blot analysis was performed as described in the Experimental section.

receptor was significantly attenuated in PKC δ knocked-down clones. Similarly, the PGF_{2 α} -induced phosphorylation of ERK1/2 and ATF-1 was markedly suppressed in these clones (Figures 4B and 4C).

Gene silencing of PKC δ attenuates PGF_{2 α} -induced NOX1 expression, O₂⁻ production and protein synthesis

We next examined the effects of gene silencing of PKC δ on the level of NOX1 mRNA. As shown in Figure 5(A), induction of NOX1 mRNA by PGF_{2 α} was almost completely abolished in PKC δ knocked-down clones. In addition, PGF_{2 α} -induced O₂⁻ production as well as protein synthesis were abolished in these clones (Figures 5B and 5C). These results suggest that PKC δ is essential for the PGF_{2 α} -induced up-regulation of NOX1 and resultant hypertrophy of VSMC.

Gene silencing of PKC δ attenuates transactivation of the EGF receptor and activation of ERK1/2 by PDGF

In the previous investigation, we demonstrated that silencing of the ATF-1 gene significantly reduced the induction of NOX1 by PDGF [7,8]. We therefore examined whether gene silencing of PKC δ affects up-regulation of NOX1 by PDGF. As shown in

Figure 4 Gene silencing of PKC δ attenuates PGF $_{2\alpha}$ -induced transactivation of the EGF receptor and activation of downstream signalling pathways

PGF $_{2\alpha}$ -induced transactivation of the EGF receptor (EGFR) (**A**), activation of ERK1/2 (**B**) and phosphorylation of ATF-1 (**C**) were suppressed in clones RNAi-1 and RNAi-2. The prefix P denotes phosphorylation. Representative results of three experiments are shown. Bars represent the mean $\pm$ S.E.M. * $P < 0.05$ compared with control cells (mock –); † $P < 0.05$ compared with PGF $_{2\alpha}$ treated cells (mock +).

Figure 6(A), PDGF elicited phosphorylation of the EGF receptor in mock-transfected cells. In PKC δ knocked-down clones, phosphorylation of the EGF receptor was significantly attenuated, indicating that PKC δ is also involved in the transactivation of the EGF receptor by PDGF. In accord with these findings, phosphorylation of ERK1/2 induced by PDGF was significantly suppressed in these clones (Figure 6B).

Gene silencing of PKC δ attenuates PDGF-induced NOX1 expression and protein synthesis

Induction of NOX1 mRNA by PDGF was almost completely abolished in PKC δ knocked-down clones (Figure 7A). Furthermore, PDGF-induced protein synthesis was not observed in these clones (Figure 7B), suggesting that PKC δ is essential for the up-regulation of NOX1 and the ensuing vascular hypertrophy elicited not only by PGF $_{2\alpha}$, but also by PDGF.

DISCUSSION

The major lines of evidence provided in the present study are that: (i) a selective inhibitor of PKC δ , rottlerin, attenuated the induction of NOX1 by PGF $_{2\alpha}$, whereas an inhibitor of cPKC, Gö 6976, and a PKC ϵ translocation-inhibitor peptide had no effect; (ii) RNAi targeted at the PKC δ mRNA suppressed PGF $_{2\alpha}$ - or PDGF-induced phosphorylation of the EGF receptor; (iii) RNAi targeted at PKC δ suppressed the up-regulation of NOX1 induced

by PGF $_{2\alpha}$ as well as PDGF; (iv) RNAi targeted at PKC δ abolished the increased protein synthesis induced by PGF $_{2\alpha}$ or PDGF. Based on these findings and those of our earlier studies [7,8], it is reasonable to conclude that PKC δ -mediated transactivation of the EGF receptor and subsequent activation of ERK1/2, PI3K and ATF-1 constitute the signalling pathways that lead to up-regulation of NOX1, not only by PGF $_{2\alpha}$, but also by PDGF (summarized in Figure 8). To our knowledge, the present study provides the first evidence for the possible involvement of PKC δ in hypertrophy of VSMC.

The specific receptor for PGF $_{2\alpha}$, FP, is coupled to a G-protein and activates phospholipase C to elicit the mobilization of cytosolic Ca $^{2+}$ [9]. Some of the effects of PGF $_{2\alpha}$ are, however, mediated through the transactivation of the EGF receptor. In fact, the PGF $_{2\alpha}$ -induced increase in NOX1 mRNA involved the transactivation of the EGF receptor and subsequent activation of ERK1/2, PI3K and ATF-1 [8]. However, the identity of the molecule that transduces the signals from FP to the EGF receptor remains unknown. The present study provides the first evidence for the functional role of PKC δ in transducing signals from FP to the EGF receptor to activate the ERK1/2 and ATF-1 signalling pathways.

Involvement of PKC δ in the transactivation of the EGF receptor was reported in ATP-stimulated VSMC [14] and in angiotensin-II-stimulated hepatic C9 cells [15]. PKC δ is known to activate MMPs (matrix metalloproteinases) that cleave heparin-binding EGF [16,17]. The Src/Pyk2 (proline-rich tyrosine kinase 2) complex,

Figure 5 Gene silencing of PKC δ attenuates PGF_{2α}-induced NOX1 expression, production of O₂⁻ and protein synthesis

(A) Induction of NOX1 mRNA by PGF_{2α} was suppressed in clones RNAi-1 and RNAi-2. Northern blot analysis was performed as described in the Experimental section. Representative results of three experiments are shown. Bars represent the mean $\pm$ S.E.M. * P < 0.05 compared with control (mock -); † P < 0.05 compared with PGF_{2α} treated cells (mock +). GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (B) PGF_{2α}-induced O₂⁻ production was suppressed in RNAi-1 and RNAi-2. Ethidium fluorescence in the cells untreated (control; open bar) or treated with 100 nM PGF_{2α} (closed bar) for 24 h is shown. Mean values were calculated from four samples. * P < 0.05 compared with control. (C) PGF_{2α}-induced protein synthesis was suppressed in RNAi-1 and RNAi-2. Growth-arrested cells were incubated for 24 h with 1 μ Ci/ml [³⁵S]-EXPRESS Protein Labeling Mix and 100 nM PGF_{2α}. [³⁵S]-Methionine incorporation, determined from four independent samples, was expressed as a percentage of control (untreated cells). * P < 0.05 compared with control cells.

implicated in the transactivation of the EGF receptor, is also activated by a PKC δ -dependent mechanism [15]. These findings suggest that activation of both MMPs and Src/Pyk2 by PKC δ takes part in transactivation of the EGF receptor. In our previous study,

Figure 6 Gene silencing of PKC δ attenuated PDGF-induced transactivation of the EGF receptor and activation of ERK1/2

The PDGF-induced transactivation of the EGF receptor (EGFR) (A) and activation of ERK1/2 (B) were suppressed in clones RNAi-1 and RNAi-2. Representative results of three experiments are shown. Bars represent the mean $\pm$ S.E.M. * P < 0.05 compared with control cells (mock -); † P < 0.05 compared with PDGF treated cells (mock +).

inhibitors of Src and MMPs significantly suppressed the PGF_{2α}-induced increase in NOX1 mRNA [8]. Accordingly, PKC δ appears to activate both Src/Pyk2 and MMPs to elicit activation of the EGF receptor and downstream signalling pathways, which leads to the up-regulation of NOX1 gene expression.

This is the first report indicating the involvement of PKC δ in the PDGF-induced transactivation of the EGF receptor. In murine B82L fibroblasts and NIH-3T3 cells, transactivation of the EGF receptor by PDGF was documented [18,19]. Migration of fibroblasts and activation of p21-activated kinase, induced by PDGF, were dependent on the EGF receptor expressed in these cells. While the biological significance of the cross-talk between the PDGF- β receptor and the EGF receptor has been suggested, the precise mechanism of transactivation is still poorly understood. There is a report postulating heterodimer formation between the PDGF- β receptor and the EGF receptor in unstimulated VSMC [20]. PDGF- β receptor dimers form in response to binding of PDGF to its receptor, and breakdown of the heterodimers by treatment with a Src inhibitor abolishes PDGF-induced

Figure 7 Gene silencing of PKC δ attenuates PDGF-induced NOX1 expression and protein synthesis

(A) Induction of NOX1 mRNA by PDGF was suppressed in clones RNAi-1 and RNAi-2. Northern blot analysis was performed as described in the Experimental section. Representative results of three experiments are shown. Bars represent the mean $\pm$ S.E.M. * $P < 0.05$ compared with control (mock -); † $P < 0.05$ compared with PDGF treated cells (mock +). GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (B) PDGF-induced protein synthesis was suppressed in RNAi-1 and RNAi-2. Growth-arrested cells were incubated for 24 h with 1 μ Ci/ml [35 S]-EXPRESS Protein Labeling Mix and 20 ng/ml PDGF-BB. [35 S]Methionine incorporation, determined from four independent samples, was expressed as a percentage of control (untreated cells). * $P < 0.05$ compared with control cells.

transactivation of the EGF receptor [20]. The present study in PKC δ knocked-down cells clearly demonstrates that PKC δ is an essential component for transactivation of the EGF receptor, not only by a G-protein-coupled receptor, FP, but also by the PDGF- β receptor, a receptor tyrosine kinase. It may be that PKC δ is involved in stabilization of the PDGF- β -EGF-receptor heterodimer through activation of Src, which phosphorylates one of the receptors, or a protein that links the receptors [20].

However, elucidating the different roles of PKC δ in VSMC seems to be somewhat elusive. Recent proteomic and metabolomic analysis of VSMC obtained from PKC δ -deficient mice demonstrated that PKC δ is crucial in regulating glucose and lipid metabolism, controlling the cellular redox state and maintaining VSMC differentiation [21]. In contrast, another study indicated that PKC δ mediates mechanical-stress-induced de-differentiation and migration of VSMC, a key event in vascular remodelling [22]. Angiotensin-II-induced activation of protein kinase D is also regulated by PKC δ in VSMC [23]. We have demonstrated in the present study that increased protein synthesis induced by

Figure 8 Diagram illustrating the proposed signalling pathways that lead to up-regulation of NOX1 in VSMC

FP, PGF; PDGF- β R, PDGF- β receptor; EGFR, EGF receptor; MEK, MAPK (mitogen-activated protein kinase)/ERK kinase.

PGF2 α or PDGF was abolished in cells in which expression of the PKC δ gene is knocked-down by RNAi. In this context, the role of PKC δ in the up-regulation of NOX1 supports the notion that PKC δ is involved in vascular remodelling by mediating the proliferation and hypertrophy of VSMC.

In the previous study, we reported that inhibitors of the mitochondrial respiratory chain suppressed the phosphorylation of ATF-1 and NOX1 gene expression induced by PGF2 α , PDGF, FBS or phorbol ester [7]. Previous studies have demonstrated that PKC δ is targeted to mitochondria upon activation by phorbol ester, H₂O₂, or a δ -opioid agonist [24–26]. The translocation of PKC δ to mitochondria elicits changes in the mitochondrial membrane potential and initiates the apoptotic pathway or confers cardioprotection to the ischaemic myocardium [24–26]. However, a close link between mitochondria and EGF receptor signalling has been reported. A-kinase anchor protein 121, a protein anchored to the outer membrane of the mitochondria, traps and inhibits protein tyrosine phosphatase D1, a Src-associated phosphatase that activates EGF receptor signalling [27]. This implies that tethering or redistributing the signalling molecule to mitochondria modulates the Src-dependent EGF receptor transduction pathway. If mitochondria are involved in transducing signals from PKC δ to the EGF receptor, the organelle may play an important role in regulating superoxide generation by NOX1/NADPH oxidase. Further studies may cast light on the role of mitochondria in the regulation of gene expression mediated by the PKC δ -EGF receptor signalling pathway.

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