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Essential role of ATF-1 in induction of NOX1, a catalytic subunit of NADPH oxidase: involvement of mitochondrial respiratory chain

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NADPH oxidase is the major source of superoxide production in cardiovascular tissues. We and others reported that PG (prostaglandin) $F_{2\alpha}$, PDGF (platelet-derived growth factor) and angiotensin II cause hypertrophy of vascular smooth muscle cells by induction of NOX1 (NADPH oxidase 1), a catalytic subunit of NADPH oxidase. We found DPI (diphenylene iodonium), an inhibitor of flavoproteins, including NADPH oxidase itself, almost completely suppressed induction of NOX1 mRNA by $PGF_{2\alpha}$ or PDGF in a rat vascular smooth muscle cell line, A7r5. Exploration into the site of action of DPI using various inhibitors suggested the involvement of mitochondrial oxidative phosphorylation in $PGF_{2\alpha}$- or PDGF-induced increase in NOX1 mRNA. In a luciferase reporter assay, activation of the CRE (cAMP-response element)-dependent gene transcription by $PGF_{2\alpha}$ was attenuated by oligomycin, an inhibitor of mitochondrial F_oF_1-ATPase. Oligomycin and other mitochondrial inhibitors also suppressed $PGF_{2\alpha}$-induced phosphorylation of ATF (activating transcription factor)-1, a transcription factor of the CREB (CRE-binding protein)/ATF family. Silencing of the ATF-1 gene by RNA interference significantly reduced the induction of NOX1 by $PGF_{2\alpha}$ or PDGF, while overexpression of ATF-1 recovered NOX1 induction suppressed by oligomycin. Taken together, ATF-1 may play a pivotal role in the up-regulation of NOX1 in rat vascular smooth muscle cells.

Key words: activating transcription factor 1 (ATF-1), mitochondrion, NADPH oxidase, NOX1, prostaglandin $F_{2\alpha}$.

INTRODUCTION

ROS (reactive oxygen species), including superoxide (O_2^-) and hydrogen peroxide (H_2O_2), are recognized as important signalling molecules in cardiovascular tissues. It has been shown that NADPH oxidases are the major source of O_2^- in vascular cells and myocytes [1–4]. Homologues of the catalytic subunit of the phagocyte NADPH oxidase ($gp91^{phox}$; NOX2) have been found in VSMCs (vascular smooth muscle cells). Among them, NOX1 (NADPH oxidase 1) was implicated in the pathogenesis of arteriosclerosis and reperfusion injury, since it mediates the proliferation and hypertrophy of VSMCs [5–7]. Expression of NOX1 is increased by stimulation with PDGF (platelet-derived growth factor), angiotensin II, phorbol ester or FBS (foetal bovine serum) [5,6]. We reported previously that PG (prostaglandin) $F_{2\alpha}$, one of the primary prostanoids generated in the vascular tissue, causes hypertrophy of VSMCs by induction of NOX1 and subsequent increase in O_2^- generation [7]. It thus appears that O_2^- derived from NADPH oxidase serves as a signalling molecule that elicits vascular hypertrophy.

On the other hand, there is little information on the exact molecular mechanisms underlying the up-regulation of NOX1 gene expression in VSMCs. We have found that a flavoprotein inhibitor DPI (diphenylene iodonium) markedly suppresses induction of NOX1 mRNA by $PGF_{2\alpha}$, PDGF, FBS or PMA. As DPI is commonly used as an inhibitor of NADPH oxidase by forming adducts with reduced flavin [8–10], experiments were carried out to elucidate the site of action for DPI on the signalling pathway leading to the induction of NOX1 gene expression. In the present paper, we report the first evidence for the roles of mitochondria and ATF (activating transcription factor)-1, a member of the CREB (cAMP-response-element-binding protein)/ATF family, in the regulation of rat NOX1 gene expression.

EXPERIMENTAL

Materials

[α-^{32}P]UTP and [α-^{32}P]dCTP (3000 Ci/mmol) were obtained from ICN Biomedicals (Costa Mesa, CA, U.S.A.). PDGF-AB, PMA, DPI chloride, tiron, rotenone, antimycin A and oligomycin were purchased from Sigma (St. Louis, MO, U.S.A.). MnTBAP [Mn(III)tetrakis(4-benzoic acid)porphyrin chloride], EUK-8 and CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) were obtained from Calbiochem-Novabiochem (San Diego, CA, U.S.A.). $PGF_{2\alpha}$, G418 disulphate and protease inhibitor cocktails were purchased from Nacalai Tesque (Kyoto, Japan). Plasmids containing specific enhancer element–luciferase fusion DNA (Mercury Pathway Profiling Systems) were obtained from Clontech (Palo Alto, CA, U.S.A.).

Northern blot analysis

A7r5 cells obtained from the A.T.C.C. (Manassas, VA, U.S.A.) were maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % FBS. Before the addition of

Abbreviations used: AP1, activating protein 1; ATF, activating transcription factor; CRE, cAMP-response element; CREB, CRE-binding protein; DMEM, Dulbecco's modified Eagle's medium; DPI, diphenylene iodonium; ds, double-stranded; ERK, extracellular-signal-regulated kinase; FBS, foetal bovine serum; MnTBAP, Mn(III)tetrakis(4-benzoic acid)porphyrin chloride; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; NOS, nitric oxide synthase; NOX1, NADPH oxidase 1; PDGF, platelet-derived growth factor; PG, prostaglandin; PKCε, protein kinase Cε; SOD, superoxide dismutase; VSMC, vascular smooth muscle cell.

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reagents, cells were cultured in DMEM containing 0.5 % FBS for 48 h. Cells were then incubated with 100 nM $PGF_{2\alpha}$, 20 ng/ml PDGF-AB, 10 % FBS or 100 nM PMA in the presence or absence of various inhibitors for 24 h. RNA isolation and Northern blot analyses were performed essentially as described in [7]. Cell viability was determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay essentially as described previously [11].

Luciferase assay

A7r5 cells were seeded in six-well plates and were cultured for 24 h in DMEM supplemented with 10 % FBS. Luciferase plasmids (1 μg/well) and pSV-β-galactosidase control vector (0.5 μg/well; Promega, Madison, WI, U.S.A.) were co-transfected into the cells with FuGENE 6 Transfection Reagent (Roche, Indianapolis, IN, U.S.A.). After transfection, cells were cultured for 24 h in DMEM supplemented with 10 % FBS, and then in the absence of FBS for another 24 h. Cells were then incubated with 100 nM $PGF_{2\alpha}$ in the presence or absence of 2 μg/ml oligomycin for 24 h. Cell lysates were prepared, and luciferase activity normalized to β-galactosidase activity was determined according to the manufacturer's instructions.

Western blot analysis

A7r5 cells cultured in the absence of FBS for 48 h were pre-treated with mitochondrial inhibitors for 30 min, and then incubated with 100 nM $PGF_{2\alpha}$ for 5 min. Nuclear extracts were prepared as described previously [11,12]. Briefly, cells were suspended in buffer A (10 mM Hepes/KOH, pH 7.9, 1.5 mM $MgCl_2$ and 10 mM KCl) containing protease inhibitor cocktails, 0.5 mM dithiothreitol, 1 mM NaF, 20 mM β-glycerophosphate and 1 mM Na_3VO_4. After centrifugation, the pellets were resuspended in buffer C (20 mM Hepes/KOH, pH 7.9, 25 % glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$ and 0.2 mM EDTA) containing the inhibitors described above. The supernatants obtained after centrifugation were used as nuclear extracts. The extracts (10 μg) were separated by SDS/12.5 % PAGE, and transferred on to PVDF membranes (Millipore, Bedford, MA, U.S.A.). Membranes were incubated with anti-phospho-CREB antibody (reactive with phosphorylated forms of CREB and ATF-1; Cell Signaling Technology, Beverly, MA, U.S.A.) or monoclonal anti-ATF-1 antibody (25C10G; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), and then with anti-rabbit IgG or anti-mouse IgG conjugated with horseradish peroxidase respectively. Immunoreactive bands were detected using ECL® (enhanced chemiluminescence) Plus System (Amersham Biosciences, Piscataway, NJ, U.S.A.).

Synthesis of an anti-ATF-1 ds (double-stranded) RNA

A dsRNA expression vector, pcPURU6icassette, was constructed by insertion of puromycin *N*-acetyltransferase gene to pU6icassette vector [13,14], which contains a human U6 promoter and two *Bfu*AI (*Bsp*MI) sites. An anti-ATF-1 dsRNA was designed that targeted nucleotides 247–267 of the rat cDNA clone BU671705. Sense or antisense oligonucleotides containing the hairpin sequence, the terminator sequence and overhanging sequences were synthesized, amplified by PCR, digested by *Bfu*AI, and inserted into the *Bfu*AI site of the pcPURU6icassette.

Establishment of clones stably expressing an anti-ATF-1 dsRNA

The dsRNA expression vector (pcPURU6icassette containing an anti-ATF-1 dsRNA sequence) was transfected into A7r5 cells as described above. Stable transfectants were selected by single-cell cloning in the presence of puromycin (5 μg/ml). For mock transfection, the pcPURU6icassette vector was transfected and selected with puromycin.

Establishment of clones stably expressing ATF-1

The coding region of the rat ATF-1 cDNA was amplified by RT (reverse transcriptase)-PCR and inserted into pcDNA3. The plasmid was transfected into A7r5 cells as described above, and stable transfectants were selected by single-cell cloning in the presence of G418 (1 mg/ml).

Statistical analysis

Values were expressed as 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

DPI suppresses induction of NOX1 mRNA

As we reported previously, $PGF_{2\alpha}$ increases NOX1 mRNA levels in rat VSMCs, A7r5 [7]. In the course of the investigation on the signalling pathways that mediate $PGF_{2\alpha}$-induced NOX1 expression, we found that 100 nM DPI, an inhibitor of NADPH oxidase, almost completely suppressed induction of NOX1 mRNA by $PGF_{2\alpha}$. DPI also suppressed increased NOX1 mRNA induced by PDGF, 10 % FBS or PMA (Figure 1A). The MTT assay demonstrated that more than 85 % of the cells were viable when cells were incubated in the presence of 100 nM DPI for 24 h (results not shown). In these cells, induction of c-fos by 10 % FBS was clearly observed (Figure 1B). These findings suggest that the suppressive effect of DPI on NOX1 induction is not due to cell damage.

Scavengers of O_2^- have no effect on induction of NOX1 mRNA

To elucidate further the effect of DPI on NOX1 induction, we first examined whether scavengers of O_2^-, the reaction product of NADPH oxidase, could affect NOX1 gene expression. MnTBAP, a cell-permeant SOD (superoxide dismutase) mimetic and peroxynitrite scavenger, and tiron, a cell-permeant O_2^- scavenger, did not affect induction of NOX1 by $PGF_{2\alpha}$. Furthermore, EUK-8, a synthetic salen–manganese complex with high SOD, catalase and oxyradical scavenging activities, showed no effect on NOX1 induction by $PGF_{2\alpha}$ (Figure 2). These results suggest that NOX1 induction is not mediated by O_2^-, H_2O_2 or oxyradicals, and that the effect of DPI on NOX1 induction is not due to the inhibition of NADPH oxidase activity by DPI. DPI is also known as an inhibitor of NOS (nitric oxide synthase) [15]. N^G-monomethyl-L-arginine (L-NMMA), an inhibitor of NOS, however, did not suppress NOX1 induction by $PGF_{2\alpha}$ (results not shown). Thus involvement of NO in induction of NOX1 mRNA was ruled out.

Inhibitors of the mitochondrial respiratory chain suppress induction of NOX1 mRNA

DPI inhibits complex I in the mitochondrial respiratory chain in addition to NADPH oxidase [16]. Therefore involvement of the electron transport system in NOX1 induction was examined next. Rotenone and antimycin A, inhibitors of complexes I and III respectively, blocked induction of NOX1 by $PGF_{2\alpha}$ almost completely. Similarly, NOX1 induction by $PGF_{2\alpha}$ was suppressed by an inhibitor of F_oF_1-ATPase, oligomycin, and by an uncoupler of oxidative phosphorylation, CCCP (Figure 3A). All of these

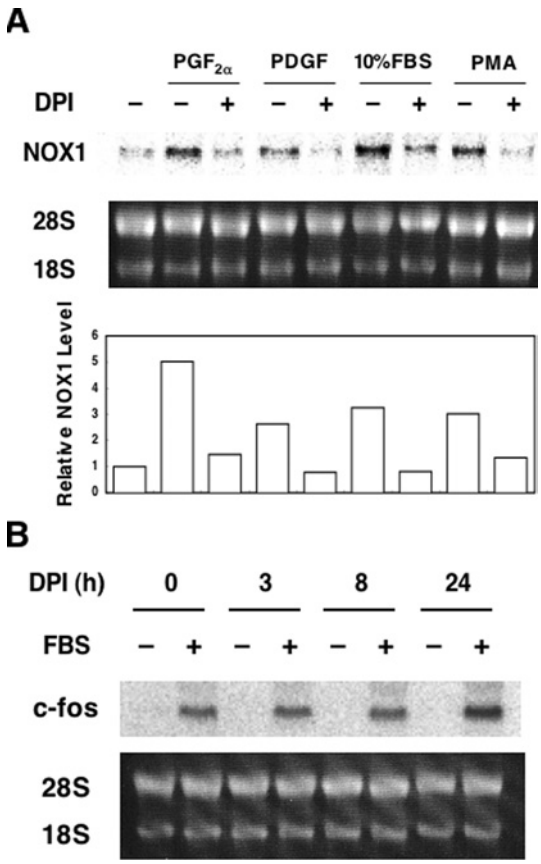

Figure 1 DPI suppressed induction of NOX1 mRNA, but not of c-fos mRNA

(**A**) Effects of DPI on induction of NOX1 mRNA. A7r5 cells, maintained in DMEM with 0.5 % FBS for 48 h, were incubated with 100 nM $PGF_{2\alpha}$, 20 ng/ml PDGF-AB, 10 % FBS or 100 nM PMA for 24 h in the presence or absence of 100 nM DPI. A representative autoradiograph of three experiments is demonstrated. Average expression levels of NOX1 normalized to 28 S RNA are shown below the blot. (**B**) Effects of DPI on induction of c-fos mRNA by FBS. Growth-arrested A7r5 cells were incubated with 100 nM DPI for the indicated times and then stimulated with 10 % FBS for 30 min. Northern blot analysis was performed as described in the Experimental section.

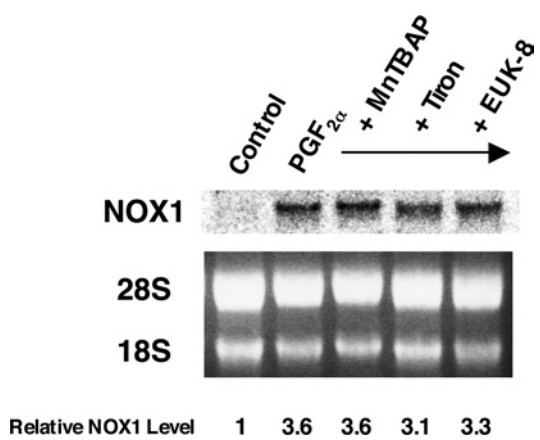

Figure 2 Scavengers of O_2^- had no effect on induction of NOX1 mRNA by $PGF_{2\alpha}$

A7r5 cells, maintained in DMEM with 0.5 % FBS for 48 h, were incubated with 100 μM MnTBAP, 10 mM tiron or 50 μM EUK-8 for 24 h in the presence of 100 nM $PGF_{2\alpha}$. A representative autoradiograph of three experiments is demonstrated. Relative expression levels of NOX1 normalized to 28 S RNA are shown below the blot.

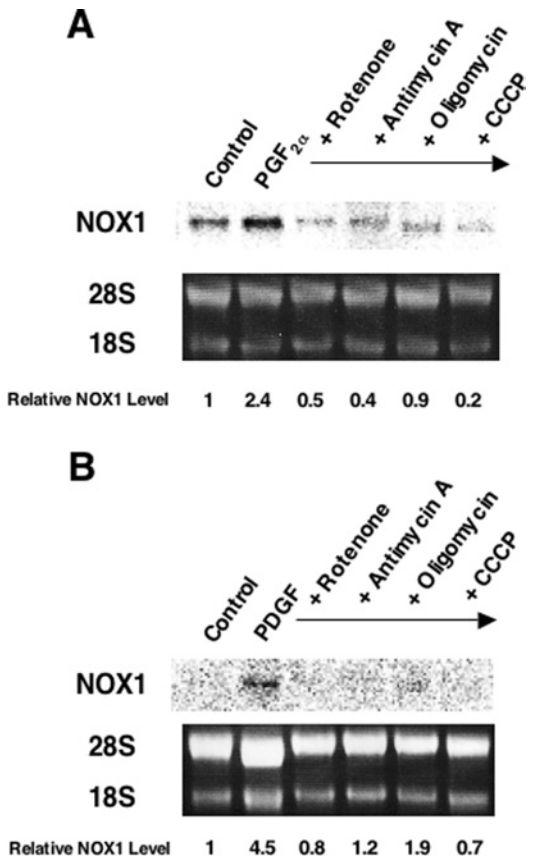

Figure 3 Inhibitors of the mitochondrial respiratory chain suppressed induction of NOX1 mRNA

A7r5 cells, maintained in DMEM with 0.5 % FBS for 48 h, were incubated with 500 nM rotenone, 100 ng/ml antimycin A, 2 μg/ml oligomycin or 10 μM CCCP for 24 h in the presence of 100 nM $PGF_{2\alpha}$ (**A**) or 20 ng/ml PDGF-AB (**B**). Representative autoradiographs of three experiments are demonstrated. Relative expression levels of NOX1 normalized to 28 S RNA are shown below the blots.

inhibitors also suppressed PDGF-induced expression of NOX1 (Figure 3B). In the presence of these mitochondrial inhibitors, induction of c-fos by 10 % FBS was preserved (see Supplementary Figure 1 at http://www.BiochemJ.org/bj/386/bj3860255add.htm). In a flow-cytometric analysis using a fluorescent dye, JC-1, we confirmed that the same concentration of CCCP markedly reduced mitochondrial membrane potential (results not shown). In the MTT assay, over 75 % of cells were viable in the presence of rotenone or CCCP. As for antimycin A and oligomycin, these inhibitors did not affect cell viability (results not shown). These results therefore suggest that inhibition of mitochondrial respiratory chain suppresses NOX1 induction by $PGF_{2\alpha}$ and PDGF.

CRE (cAMP-response element)-dependent transcriptional activation by $PGF_{2\alpha}$ is suppressed by oligomycin

To clarify the signal transduction pathway that mediates mitochondria to the nucleus, we screened transcription factors that are activated by $PGF_{2\alpha}$ and inhibited by mitochondrial inhibitors. A7r5 cells were transfected with plasmids containing specific enhancer elements fused to luciferase DNA, and stimulated with $PGF_{2\alpha}$ in the presence or absence of oligomycin. Among AP1 (activating protein 1)-binding site, CRE, glucocorticoid-response element, heat-shock element, NF-κB (nuclear factor κB)-binding site and serum-response element, only CRE-dependent transcription was enhanced by $PGF_{2\alpha}$ greater than 2-fold over the

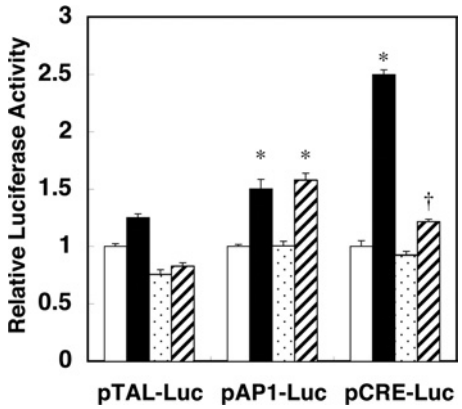

Figure 4 CRE-dependent transcriptional activation by $PGF_{2\alpha}$ was suppressed by oligomycin

Luciferase plasmids containing various enhancer elements were co-transfected with β-galactosidase control vector into A7r5 cells. The activity of luciferase was normalized by that of β-galactosidase. Results are means $\pm$ S.E.M. ($n = 3$). pTAL-Luc, the control vector lacking the enhancer element. pAP1-Luc, the luciferase plasmid containing the AP1-binding site. pCRE-Luc, the luciferase plasmid containing the CRE. Open bars, control; closed bars, $PGF_{2\alpha}$; dotted bar, oligomycin; hatched bar, $PGF_{2\alpha}$ + oligomycin. $^*P < 0.001$ compared with the control. $\dagger P <$ 0.001 compared with $PGF_{2\alpha}$.

control. As shown in Figure 4, AP1-dependent transcriptional activity was slightly enhanced by $PGF_{2\alpha}$, which appeared to be linked to the concomitant induction of c-fos by $PGF_{2\alpha}$ that binds to AP1 sites [17]. In contrast with the AP1-dependent activation, however, the CRE-dependent transcriptional activation by $PGF_{2\alpha}$ was significantly suppressed by oligomycin.

Inhibitors of the mitochondrial respiratory chain suppress phosphorylation of ATF-1 by $PGF_{2\alpha}$

CRE-dependent transcription is mediated by transcription factors of the CREB/ATF family. Of particular interest is the fact that the involvement of CREB and ATF-1 in angiotensin-II-induced hypertrophy and thrombin-induced growth of VSMCs has been reported [18,19]. We therefore examined whether $PGF_{2\alpha}$ induces phosphorylation of these proteins. As shown in Figure 5(A), a 35 kDa protein was detected in the nuclear extracts of $PGF_{2\alpha}$-stimulated cells by the anti-phospho-CREB antibody, which reacts with phosphorylated forms of CREB and ATF-1. While the phosphorylated form of CREB (43 kDa) was not detected in these cells, a time-dependent increase in phosphorylation of ATF-1 was observed up to 15 min. These findings suggest that $PGF_{2\alpha}$ elicits phosphorylation of ATF-1, but not that of CREB. PDGF also elicited phosphorylation of ATF-1 in a similar time course (results not shown). Effects of mitochondrial inhibitors on $PGF_{2\alpha}$-induced phosphorylation of ATF-1 were next examined. As shown in Figure 5(B), DPI and all other mitochondrial inhibitors suppressed phosphorylation of ATF-1 induced by $PGF_{2\alpha}$. DPI also suppressed PDGF-induced phosphorylation of ATF-1 (results not shown).

Gene silencing of ATF-1 attenuates induction of NOX1 mRNA

To verify further the involvement of ATF-1 in the induction of NOX1 gene expression, a dsRNA targeted at the rat ATF-1 mRNA sequence was introduced into A7r5 cells. Following single cell cloning of the transfectants, two clones (RNAi-5 and RNAi-16) stably expressing the dsRNA were isolated (Figure 6A, left-hand panel). In RNAi-5 and RNAi-16, the levels of ATF-1, but not those of CREB, were significantly reduced compared with mock-

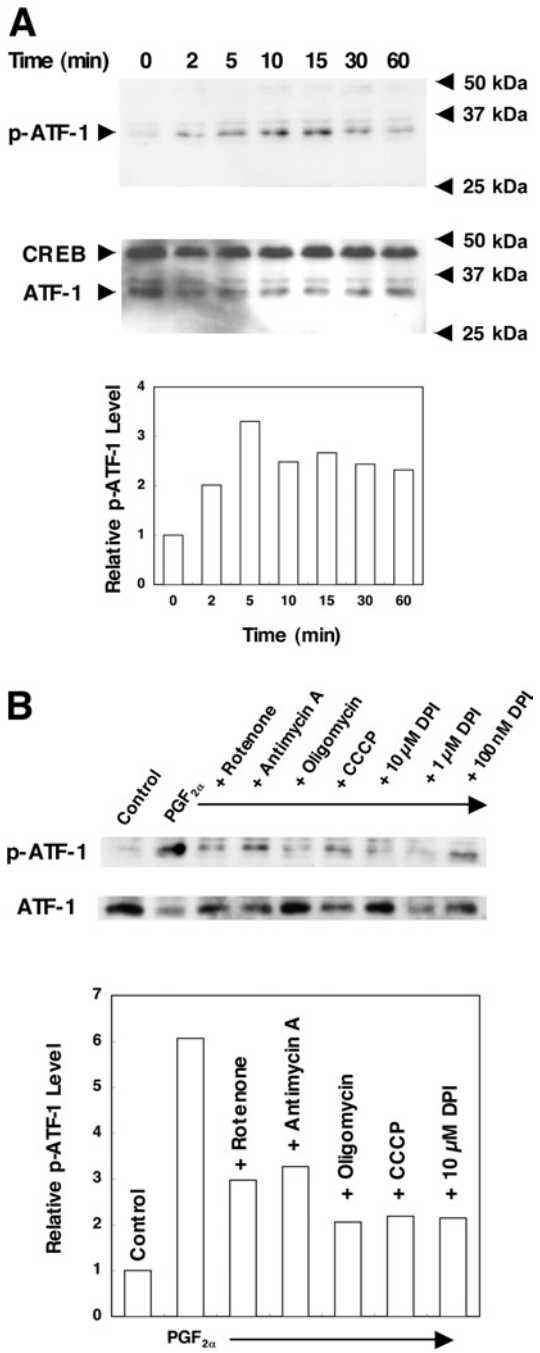

Figure 5 Inhibitors of the mitochondrial respiratory chain suppressed phosphorylation of ATF-1 by $PGF_{2\alpha}$

(**A**) Time course of ATF-1 phosphorylation by $PGF_{2\alpha}$. Serum-starved A7r5 cells were incubated with 100 nM $PGF_{2\alpha}$ for the indicated times. (**B**) Effects of various inhibitors of the mitochondrial respiratory chain. Serum-starved A7r5 cells were pre-incubated with 500 nM rotenone, 100 ng/ml antimycin A, 2 μg/ml oligomycin, 10 μM CCCP or the indicated concentrations of DPI for 30 min, and stimulated with 100 nM $PGF_{2\alpha}$ for 5 min. Western blot analysis was performed as described in the Experimental section. Representative results of three experiments are shown. Averaged relative intensities of the bands for phosphorylated ATF-1 are shown below the blots.

transfected cells (Figure 6A, right-hand panel). As demonstrated in Figure 6(B), induction of NOX1 mRNA by $PGF_{2\alpha}$ in these cells was abolished almost completely. In addition, induction of NOX1 mRNA by PDGF, 10 % FBS or PMA was also inhibited

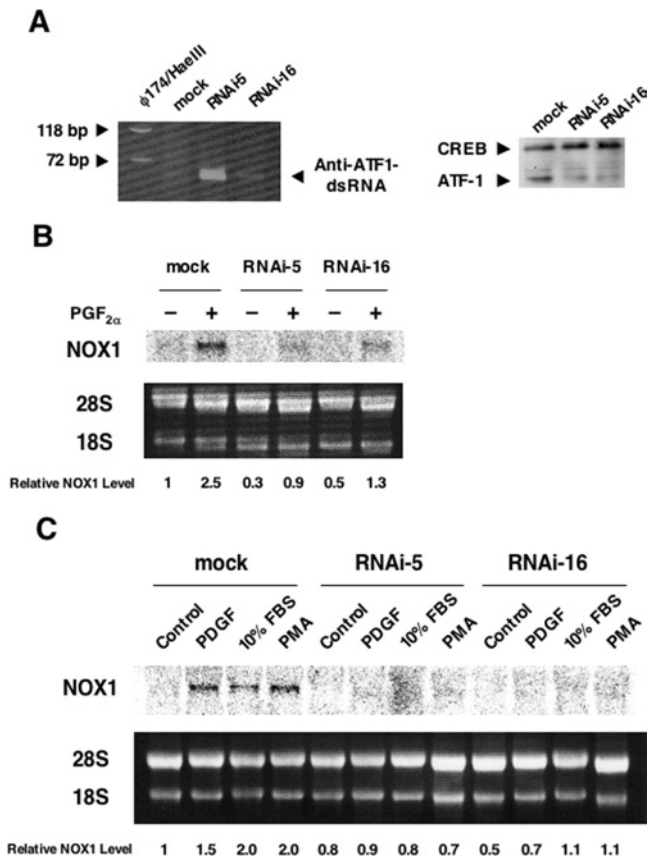

Figure 6 Gene silencing of ATF-1 attenuated induction of NOX1 mRNA

(**A**) Expression of the anti ATF-1 dsRNA (left-hand panel) and silencing of ATF-1 expression (right-hand panel) in the clones RNAi-5 and RNAi-16. Total RNA was reverse-transcribed with random nonomers, and the cDNA fragment (57 bp) was amplified by PCR. The product was separated by 15% PAGE. Western blot analysis was performed as described in the Experimental section. (**B**) Induction of NOX1 mRNA by $PGF_{2\alpha}$ was suppressed in RNAi-5 and RNAi-16. (**C**) Induction of NOX1 mRNA by PDGF, 10% FBS or PMA was suppressed in RNAi-5 and RNAi-16. Northern blot analysis was performed as described in the Experimental section. Relative expression levels of NOX1 normalized to 28 S RNA are shown below the blots.

in these clones (Figure 6C). In these clones, induction of c-fos by 10% FBS was clearly observed (see Supplementary Figure 2 at http://www.BiochemJ.org/bj/386/bj3860255add.htm).

Overexpression of ATF-1 restores $PGF_{2\alpha}$-induced NOX1 expression suppressed by oligomycin

To examine the effects of overexpression of ATF-1 on NOX1 induction, an expression plasmid containing the coding region of rat ATF-1 cDNA was introduced into A7r5 cells. Following single cell cloning of the transfectants, two clones stably expressing ATF-1, ATF1-3 and ATF1-7 were isolated (Figure 7A). As shown in Figure 7(B), induction of NOX1 by $PGF_{2\alpha}$ in these clones was observed in the presence of oligomycin. These findings, together with the results of gene silencing of ATF-1, clearly indicate that ATF-1 is an essential transcription factor that mediates expression of NOX1 gene in rat VSMCs.

DISCUSSION

The present findings suggest that ATF-1, a transcription factor of the CREB/ATF family, plays an essential role in the induction of NOX1 mRNA. The major lines of evidence provided in the present study are that: (i) DPI, which inhibits NADPH

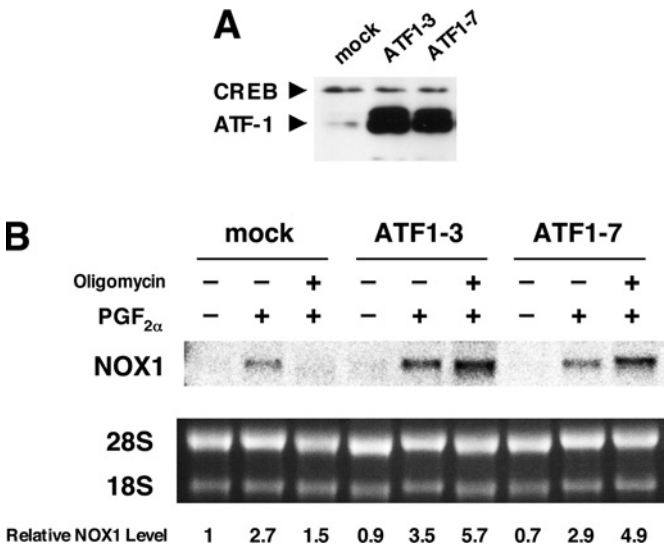

Figure 7 Overexpression of ATF-1 restored $PGF_{2\alpha}$-induced NOX1 expression suppressed by oligomycin

(**A**) Overexpression of ATF-1 in the clones ATF1-3 and ATF1-7. Western blot analysis was performed as described in the Experimental section. (**B**) Induction of NOX1 mRNA by $PGF_{2\alpha}$ was not affected by oligomycin in ATF1-3 and ATF1-7. Northern blot analysis was performed as described in the Experimental section. Relative expression levels of NOX1 normalized to 28 S RNA are shown below the blot.

oxidase in addition to complex I in the mitochondrial respiratory chain, almost completely abolished induction of NOX1 mRNA by $PGF_{2\alpha}$, PDGF, FBS or PMA; (ii) the increase in NOX1 mRNA induced by $PGF_{2\alpha}$ or PDGF was attenuated by inhibitors of the mitochondrial respiratory chain; (iii) CRE-dependent transcriptional activation by $PGF_{2\alpha}$ was suppressed by oligomycin, an inhibitor of mitochondrial F_oF_1-ATPase; (iv) phosphorylation of ATF-1 induced by $PGF_{2\alpha}$ was attenuated by inhibitors of the mitochondrial respiratory chain; (v) induction of NOX1 mRNA by $PGF_{2\alpha}$, PDGF, FBS or PMA was suppressed by RNA interference targeted at the ATF-1 mRNA sequence; and (vi) overexpression of ATF-1 restored NOX1 expression that was suppressed by oligomycin.

The flavoprotein inhibitor DPI is often used as an inhibitor of NADPH oxidase [8–10], because it reduces the activity of NADPH oxidase by forming adducts with reduced flavin [20]. The present findings suggest that DPI is not only an enzyme inhibitor of NADPH oxidase, but also a down-regulator of NOX1, a catalytic subunit of the enzyme. In the latter effect of DPI, implication of the mitochondrial respiratory chain was demonstrated.

In the same member of the CREB/ATF family, CREB was reported to be one of the transcription factors activated by mitochondrial dysfunction. Arnould et al. [21] reported that CREB is involved in the signalling pathway that communicates the state of the activity of mitochondria to the nucleus. In the mtDNA (mitochondrial DNA)-depleted cell line, CREB was constitutively activated by phosphorylation on Ser^{133}, and metabolic inhibitors of mitochondria led to phosphorylation of CREB in human embryonic kidney cells [21]. In contrast, metabolic inhibitors of mitochondria suppressed $PGF_{2\alpha}$-induced phosphorylation of ATF-1 in A7r5 cells. Such a discrepancy in the relationship between the phosphorylation status of CREB/ATF and mitochondrial activities may be attributed to the difference in the cell lineage used in these studies.

ATF-1, but not CREB, was phosphorylated in the cells stimulated by $PGF_{2\alpha}$, although high sequence similarity is found in

the sites phosphorylated by serine/threonine kinases. This suggests that strict mechanisms that distinguish phosphorylation sites of ATF-1 and CREB exist, and ATF-1 and CREB may have distinct functional roles in VSMCs. In fact, ATF-1 was reported to be involved in thrombin-induced growth of VSMCs, while CREB was implicated in angiotensin-II-induced hypertrophy of VSMCs [18,19]. We reported previously that $PGF_{2\alpha}$-induced hypertrophy of VSMCs was mediated by induction of NOX1 mRNA [7]. In the light of the present findings, involvement of ATF-1 in $PGF_{2\alpha}$-induced hypertrophy of VSMCs was clearly depicted. It is intriguing that the angiotensin AT_1 receptor, the thrombin receptor and the prostanoid FP (PGF) receptor are all $G_{q/11}$-coupled receptors. These receptors may be coupled to as yet unidentified activation units that discriminate between ATF-1 and CREB.

The molecular mechanisms by which $PGF_{2\alpha}$- or PDGF-induced signals are propagated to mitochondria and to the transcription factor ATF-1 are still unknown. $PGF_{2\alpha}$ exerts its biological actions through binding to its specific receptor, FP, on plasma membranes [22]. FP is coupled to phosphoinositide turnover and ensuing mobilization of cytosolic Ca^{2+} [23]. $PGF_{2\alpha}$ also induces translocation of PKCε (protein kinase Cε) to the myocyte membrane [17]. On the other hand, previous studies have demonstrated that PKCδ and PKCε are targeted to mitochondria upon activation, and PKCδ changes mitochondrial membrane potential [24–26]. It is also reported that PKCε co-localizes with ERK (extracellular-signal-regulated kinase) in cardiac mitochondria, and forms PKCε–ERK signalling modules that take part in PKCε-mediated cardioprotection [27]. PDGF, whose effects are mediated by the receptor tyrosine kinase, is also reported to activate ERK by a PKC-dependent manner [28]. In another line of study, we observed that $PGF_{2\alpha}$-induced expression of NOX1 mRNA was significantly suppressed by PD98059, an inhibitor of MEK1/2 (mitogen-activated protein kinase/ERK kinase 1/2) that blocks ERK1/2 pathway (C. Fan, M. Katsuyama, T. Nishinaka and C. Yabe-Nishimura, unpublished work). As localization of CREB was also reported in brain mitochondria [29], it can be postulated that these PKCs may mediate $PGF_{2\alpha}$- or PDGF-induced signals to mitochondria, and that ERK and/or ATF-1 closely associated with mitochondria may play pivotal roles in transducing the signals to the nucleus, leading to expression of the NOX1 gene. It is as yet unclear whether ATF-1 directly or indirectly activates transcription of NOX1. A $PGF_{2\alpha}$-responsive element(s) has not been found by the luciferase reporter assay using a construct containing approx. 2 kb of the 5′-flanking region of the mouse NOX1 gene (results not shown). Further studies on the promoter and enhancer of the NOX1 gene should lead to the better understanding of the regulation of NOX1 gene expression in VSMCs. The present study provides the first evidence for the roles of mitochondria and ATF-1, a member of CREB/ATF family, in the regulation of NOX1 gene expression.

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