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CELLULAR & MOLECULAR BIOLOGY LETTERS
http://www.cmbl.org.pl

Received: 17 July 2010
Final form accepted: 03 February 2011
Published online: 16 February 2011

Volume 16 (2011) pp 226-235
DOI: 10.2478/s11658-011-0002-3

Research article

THE MITOCHONDRIA MEDIATE THE INDUCTION OF NOX1 GENE EXPRESSION BY ALDOSTERONE IN AN ATF-1-DEPENDENT MANNER

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Abstract: High aldosterone (Ald) levels can induce hypertrophy of vascular smooth muscle cells (VSMCs), which carries high risks of heart failure. A previous study showed that Ald induces hypertrophy of VSMCs by up-regulating NOX1, a catalytic subunit of NADPH oxidase that produces superoxides. However, the precise mechanism remains unknown. Diphenylene iodonium (DPI) is known as an inhibitor of complex I in the mitochondrial respiratory chain, and it was also found to almost completely suppress the induction of NOX1 mRNA and the phosphorylation of activating transcription factor (ATF-1) by PGF2α or PDGF in a rat VSMC cell line. In this study, we found that the Ald-induced phosphorylation of ATF-1 and NOX1 expression was significantly suppressed by DPI. Silencing of ATF-1 gene expression attenuated the induction of NOX1 mRNA expression, and over-expression of ATF-1

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Abbreviations used: ALD – aldosterone; ATF – activating transcription factor-1; CRE – cAMP-response element; CREB – CRE-binding protein; DMEM – Dulbecco's modified Eagle's medium; DPI – diphenylene iodonium; FBS – fetal bovine serum; MnTBAP – Mn(III)tetrakis(4-benzoic acid)porphyrin chloride; MR – mineralocorticoid receptor; MTT,3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; NOX1 – NADPH oxidase 1; ROS – reactive oxygen species; VSMC – vascular smooth muscle cell

restored Ald-induced NOX1 expression. On the basis of this data, we show that the mitochondria mediate aldosterone-induced NOX1 gene expression in an ATF-1-dependent manner.

Key words: Aldosterone, Mitochondria, ATF-1, NOX1, VSMC

INTRODUCTION

Recently, more and more studies have demonstrated that Ald has direct effects on the cardiovascular system independent of renal salt and water regulation [1-3]. Ald induces cardiac fibrosis independently of other renin-angiotensin system components. Accumulation and hypertrophy of VSMCs are characteristic for atherosclerotic, restenotic and hypertensive vascular diseases [4]. Many factors are involved in the mediation of VSMC growth. Among them, ROS play a vital role. A variety of cellular enzymes are potential sources of ROS, including microsomal cytochrome P-450, xanthine oxidase, and those involved in arachidonic acid metabolism and mitochondrial electron transportation. However, NADPH oxidase is the major source of O_2^- in vascular cells and myocytes [5]. NOX1, a catalytic subunit of NADPH oxidase found in VSMCs, was involved in the pathogenesis of arteriosclerosis and reperfusion injury, as it mediated the proliferation and hypertrophy of VSMCs [6, 7]. We reported that aldosterone alone and aldosterone plus salt induced hypertrophy of VSMCs by up-regulating NOX1, and the mitochondria and ATF-1 had been found to be involved the induction of NOX1 by PGF2α in a previous study [8]. Despite these discoveries, the molecular signaling pathway that elicits the expression of NOX1 is still unclear. To explore whether aldosterone and PGF2α share the same mechanism in inducing NOX1, we conducted *in vitro* experiments and found that the mitochondria mediated aldosterone-induced NOX1 gene expression in an ATF-1-dependent manner.

MATERIALS AND METHODS

The A7r5 rat aortic smooth muscle cell line was obtained from the American Type Culture Collection (Rockville, MD). The control solutions contained the appropriate amount of the vehicle, ethanol, for the aldosterone (Geel, Belgium). DPI chloride, tiron, rotenone, antimycin A and oligomycin were purchased from Sigma (St. Louis, MO, USA). Mn(III) tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP), EUK-8 and carbonyl cyanide m-chlorophenylhydrazone (CCCP) were obtained from Calbiochem-Novabiochem (San Diego, CA, USA). G418 disulphate and protease inhibitor cocktails were purchased from Nacalai Tesque (Kyoto, Japan).

Real-time RT-PCR

The A7r5 cells were seeded in 10-cm dishes (1 x 10^6 cells/dish) and cultured for 24 h in DMEM supplemented with 10% FBS followed by a further 48-h

incubation without FBS. The cells were incubated with or without some inhibitors (100 nM DPI, 500 nM rotenone, 100 ng/ml antimycin A, 2 ng/ml oligomycin, 10 μM CCCP) for 1 h, followed by incubation with 1 nM Ald for 24 h. The total RNA was isolated from the cells using an acid guanidinium thiocyanate/ phenol/chloroform mixture, and was amplified via real-time PCR [9] using an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) with the SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen). All of the samples were investigated in triplicate. The primers for NOX1 amplification were as follows: Forward, 5'-CAC GCT GAG AAA GCC ATT GGA TCAC-3'; Reverse, 5'-GGA TGG AAT AGG CTG GAG AGA ACA-3'.

Western blot analysis

A7r5 cells were cultured in the absence of FBS for 48 h, then pre-treated with mitochondrial inhibitors for 30 min, and incubated with 1 nM Ald for 15 min. Nuclear extracts were prepared as described previously [10]. The extracts (10 μg) were separated by SDS/12.5% PAGE, and transferred onto PVDF membranes (Millipore, Bedford, MA, USA). The membranes were incubated with anti-phospho-CREB antibody (reacting with phosphorylated forms of CREB and ATF-1; Cell Signaling Technology, Beverly, MA, USA) or monoclonal anti-ATF-1 antibody (reacting with ATF-1; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and then respectively with anti-rabbit IgG or anti-mouse IgG conjugated with horseradish peroxidase. Immunoreactive bands were detected using the ECL (enhanced chemiluminescence) Plus System (Amersham Biosciences, Piscataway, NJ, USA).

Synthesis of anti-ATF-1 dsRNA

A dsRNA expression vector, pcPURU6i cassette, was constructed by inserting puromycin N-acetyltransferase gene into a pU6i cassette vector [11, 12], which contains a human U6 promoter and two BfuAI (BspMI) 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 via PCR, digested via BfuAI, and inserted into the BfuAI site of the pcPURU6i cassette.

Establishment of clones that stably express anti-ATF-1 dsRNA

The dsRNA expression vector (pcPURU6i cassette containing an anti-ATF-1 dsRNA sequence) was transfected into A7r5 cells [8]. Stable transfectants were selected by single-cell cloning in the presence of puromycin (5 μg/ml). The pcPURU6i cassette vector was used for mock transfection.

Establishment of clones stably expressing ATF-1

The coding region of the rat ATF-1 cDNA was amplified via RT-PCR and inserted into pcDNA3.1. The plasmid was transfected into A7r5 cells [8], and stable transfectants were selected by single-cell cloning in the presence of G418 (1 mg/ml).

MTT assay

The cell viability was determined using the MTT assay. Following incubation with Ald (1 nM) for 24 h, MTT (at a final concentration of 0.45 mg/ml) was added to the culture medium of A7R5 seeded in 96-well plates. The cells were incubated for 4 h, and the medium was removed. The formazan crystals formed after the reduction of MTT by mitochondrial dehydrogenases in the living cells were solubilized in demethyl sulfoxide and examined spectrophotometrically at 450 nm.

Statistical analysis

The values were expressed as means ± SEM. 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 the induction of NOX1 mRNA

Ald was found to significantly induce NOX1 expression. There was no significant difference between the results for 1, 3, 6, 12 and 24 h (data not show). 100 nM DPI almost completely suppressed the induction of NOX1 mRNA by Ald in a time-dependent manner (Fig. 1A). The MTT assay demonstrated that more than 85% of the cells were viable when the cells were incubated in the presence of 100 nM DPI for 24 h (data not shown). We also observed induction of c-fos by 10% FBS in these cells (Fig. 1B). These findings suggest that the suppressive effect of DPI on NOX1 induction is not due to cell damage.

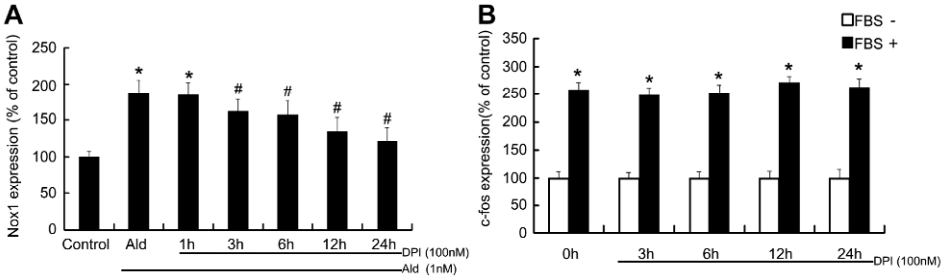

Fig. 1. 100 nM DPI almost completely suppressed the induction of NOX1 mRNA by Ald in a time-dependent manner (A). The induction of c-fos by 10% FBS was observed in these cells. DPI suppresses induction of NOX1 mRNA (B). A representative autoradiograph of three experiments is shown. *$P < 0.05$ compared with the control; #$P < 0.05$ compared with aldosterone.

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

To further elucidate the effect of DPI on NOX1 induction, we first examined whether scavengers of O_2^- could affect NOX1 gene expression. MnTBAP, a cell-permeant superoxide dismutase (SOD) mimetic and peroxynitrite scavenger, and tiron, a cell-permeant O_2^- scavenger, did not affect the induction

of NOX1 by Ald. Furthermore, EUK-8, a synthetic salen–manganese complex with high SOD, catalase and oxyradical scavenging activities, showed no effect on NOX1 induction by Ald (Fig. 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.

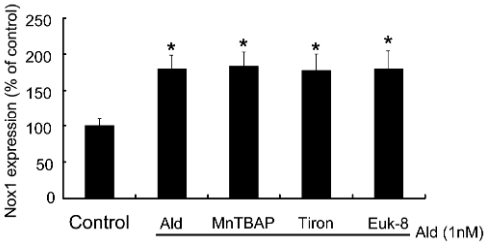

Fig. 2. Scavengers of O_2^- have no effect on the induction of NOX1 mRNA. The cells were incubated with 100 μM MnTBAP, 10 mM tiron or 50 μM EUK-8 for 24 h in the presence of 1 nM Ald. A representative autoradiograph of three experiments is demonstrated. *$P < 0.05$ compared with the control.

Inhibitors of the mitochondrial respiratory chain suppress the induction of NOX1 mRNA

DPI inhibits complex I in the mitochondrial respiratory chain as well as NADPH oxidase [13]. The involvement of the electron transport system in NOX1 induction was examined next. Rotenone and antimycin A, inhibitors of complexes I and III respectively, blocked the induction of NOX1 by Ald almost completely. Similarly, NOX1 induction by Ald was suppressed by an inhibitor of FoF1-ATPase, oligomycin, and by an uncoupler of oxidative phosphorylation, CCCP (Fig. 3). In the MTT assay, over 75% of the cells were viable in the presence of rotenone or CCCP. As for antimycin A and oligomycin, these inhibitors did not affect cell viability (data not shown). These results suggest that inhibition of the mitochondrial respiratory chain suppresses NOX1 induction by Ald.

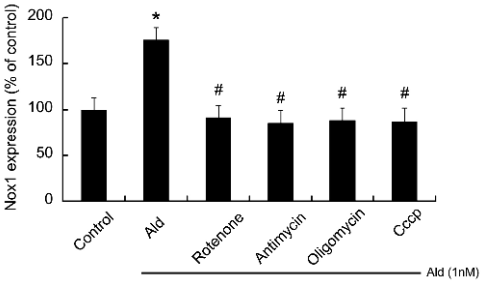

Fig. 3. Inhibitors of the mitochondrial respiratory chain suppress the induction of NOX1 mRNA. The cells were incubated with or without 100 nM DPI, 500 nM Retenone, 100 ng/ml antimycin A, 2 ng/ml oligomycin and 10 μM CCCP for 1 h, followed by 1 nM Ald for 24 h. A representative autoradiograph of three experiments is demonstrated. *$P < 0.05$ compared with the control, #$P < 0.05$ compared with aldosterone.

Inhibitors of the mitochondrial respiratory chain suppress phosphorylation of ATF-1 induced by Ald

CRE-dependent transcription is mediated by transcription factors of the CREB/ATF family. We reported previously that inhibitors of the mitochondrial respiratory chain suppressed phosphorylation of ATF-1by $PGF_{2\alpha}$ [8]. Therefore, we examined whether Ald induces phosphorylation of these proteins. As shown in Fig. 4A, a 35-kDa protein was detected in the nuclear extracts of Ald-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 the phosphorylation of ATF-1 was observed from 10 to 90 min. These findings suggest that Ald elicits phosphorylation of ATF-1, but not that of CREB. The effects of mitochondrial inhibitors on Ald-induced phosphorylation of ATF-1 were then examined. As shown in Fig. 4B, DPI and all other mitochondrial inhibitors suppressed the phosphorylation of ATF-1 induced by Ald.

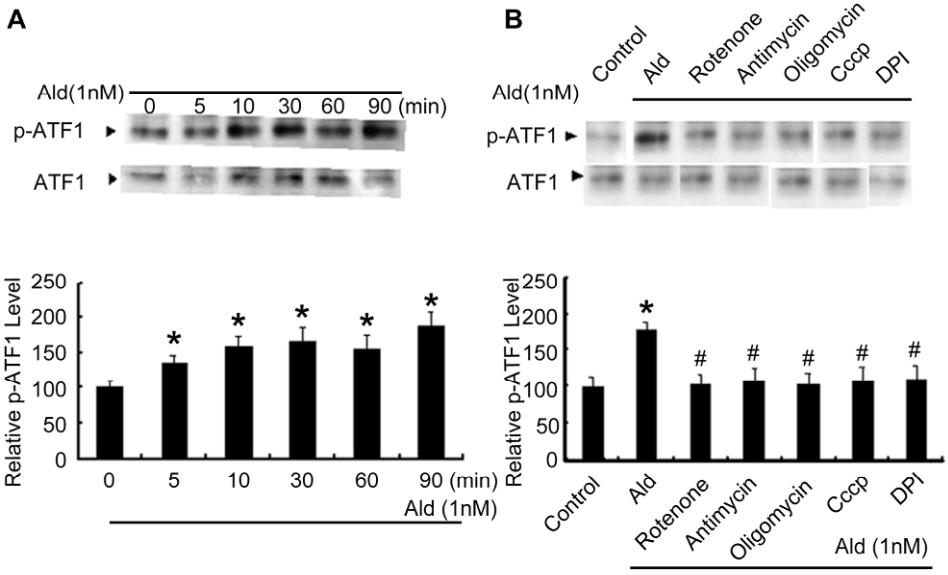

Fig. 4. The inhibitors of the mitochondrial respiratory chain suppress the phosphorylation of ATF-1 by Ald. A time-dependent increase in the phosphorylation of ATF-1 was observed from 10 to 90 min induced by Ald (A). DPI and all other mitochondrial inhibitors suppressed the phosphorylation of ATF-1 induced by Ald (B). A representative autoradiograph of three experiments is shown. pATF-1 presented as normalized to total ATF-1. *$P < 0.05$ compared with the control, #$P < 0.05$ compared with aldosterone.

Gene silencing of ATF-1 attenuates the induction of NOX1 mRNA

To further verify the involvement of ATF-1 in the induction of NOX1 gene expression, a dsRNA targeting the rat ATF-1mRNA sequence was introduced into A7r5 cells [8]. Following single cell cloning of the transfectants, two clones stably expressing the dsRNA (RNAi-5 and RNAi-16) were isolated (Fig. 5A,

left-hand panel) In RNAi-5 and RNAi-16, the levels of ATF-1, but not those of CREB, were significantly reduced compared with those for mock-transfected cells (Fig. 5A, right-hand panel). As shown in Fig. 5B, in the absence of aldosterone, the expression of NOX1 had no significant difference compared with mock-transfected cells, but the induction of NOX1 mRNA by Ald in these cells was reduced greatly. These results indicated that ATF-1 regulates the expression of NOX1 induced by Ald.

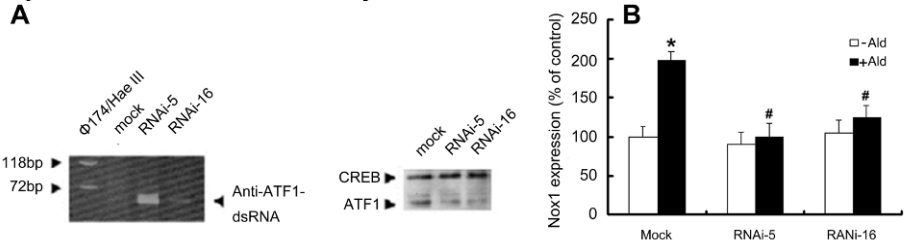

Fig. 5. Gene silencing of ATF-1 attenuates the induction of NOX1 mRNA. A – Left-hand panel: two clones stably expressing the dsRNA (RNAi-5 and RNAi-16) were isolated. Right-hand panel: in RNAi-5 and RNAi-16, the levels of ATF-1, but not those of CREB, were significantly reduced compared with the mock-transfected cells. B – Induction of NOX1 mRNA by Ald in RNAi-5 and RNAi-16 was reduced greatly. *$P < 0.05$ compared with control, #$P < 0.05$ compared with Ald.

Overexpression of ATF-1 restores Ald-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 [8], two clones stably expressing ATF-1, ATF1-3 and ATF1-7 were isolated (Fig. 6A). As shown in (Fig. 6B), induction of NOX1 by Ald in these clones was observed in the presence of oligomycin. These findings, together with the results of the gene silencing of ATF-1, clearly indicate that ATF-1 is an essential transcription factor that mediates the expression of the NOX1 gene in rat VSMCs.

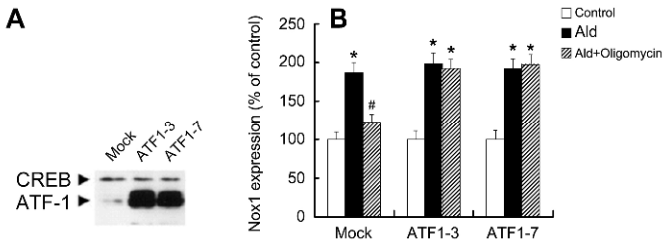

Fig. 6. Overexpression of ATF-1 restores Ald-induced NOX1 expression suppressed by oligomycin. Two clones stably expressing ATF-1, ATF1-3 and ATF1-7 were isolated (A). Overexpression of ATF-1 restores the Ald-induced NOX1 expression suppressed by oligomycin (B). *$P < 0.05$ compared with the control, #$P < 0.05$ compared with Ald.

DISCUSSION

NADPH oxidase is the main enzymatic source of superoxides in VSMCs and myocytes, and overproduction of O_2^- plays an essential role in the hypertrophy of these cells [4, 5]. NOX1 is a catalytic subunit of NADPH oxidase, and it has been detected in the VSMCs. The expression of NOX1 mRNA can be induced by platelet-derived growth factor (PDGF), angiotensin II, $PGF_{2\alpha}$, phorbol ester, and fetal bovine serum (FBS) [6-8].

This study showed that aldosterone could also induce NOX1 gene expression, and this induction could be attenuated by inhibitors of the mitochondrial respiratory chain. These results indicate that the mitochondrial respiratory chain is involved in the regulation of NOX1 gene expression.

ATF-1 is a responsive factor to cAMP, a second intracellular messenger that has profound effects on the transcriptional activity of many cellular and viral promoters [14]. It is reported to be implicated in thrombin and $PGF_{2\alpha}$-induced VSMC hypertrophy [15]. Our studies showed that Ald could induce ATF-1 phosphorylation, and inhibitors of the mitochondrial respiratory chain attenuated this induction. The induction of NOX1 mRNA by Ald was restrained by RNA interference targeting the ATF-1 mRNA sequence, and overexpression of ATF-1 restored NOX1 expression that had been suppressed by oligomycin. These findings suggest that ATF-1 is an important factor in regulating NOX1 expression induced by Ald, and that there must be a relationship between mitochondrial function, ATF-1 phosphorylation and nuclear reactions. It was reported previously that the inducible expression of NOX1 is governed by the activating transcription factor-1-myocyte enhancer factor 2B (MEF2B) cascade downstream of phosphoinositide 3 (PI3) kinase [16]. It is tempting to speculate that mitochondrial dysfunction can suppress phosphorylation of ATF-1 through an unknown mechanism, and then attenuate NOX1 expression resulting in a decrease in the activation of NADPH oxidase. Seung Bum Lee *et al.* [17] demonstrated that serum withdrawal promoted the production of ROSs in human 293T cells by stimulating both the mitochondria and NOX1. That means that ROS take the role of connection between mitochondrial function and nuclear reaction. By contrast, the scavengers of superoxides did not influence the induction of NOX1 mRNA in A7r5 cells. Such a discrepancy in the relationship between the mitochondria and the nucleus may be attributed to the difference in the cell lineage used in these studies.

The molecular mechanisms of Ald-induced NOX1 mRNA expression have yet to be established. Aldosterone produces cardiac hypertrophy and fibrosis via the mediation of mineralocorticoind receptors (MRs) [18]. The MRs are expressed not only in the renal cortical collecting duct but also in many other tissues, including heart and vasculature [19]. Ald binds to MR, which then serves as a transcription factor [20-21]. Further investigations are needed to explore the detailed mechanisms mediating NOX1 gene expression induced by Ald.

Our study revealed that the mitochondria mediate aldosterone-induced NOX1 gene expression in an ATF-1-dependent manner in VSMCs. However, there remains an urgent need to discover the connection of Ald and the mitochondria, and the pathway of mitochondrial function affecting ATF-1 phosphorylation. This also serves as a point of departure for further studies on vascular protection.

Acknowledgements. This study was supported by a grant from the Japanese Ministry of Health, Labor and Welfare 2005.

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