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Protein Kinase C-δ Is Involved in Induction of *NOX1* Gene Expression by Aldosterone in Rat Vascular Smooth Muscle Cells

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Abstract—In this study, we focused on the relationship between aldosterone and *NOX1* expression in vascular smooth muscle cells (VSMCs). For the first time, with the use of specific inhibitors of protein kinase C (PKC), we report that PKCδ mediates upregulation of *NOX1* induced by 10 nM aldosterone in cultured VSMCs. Participation of PKC in the mediation of *NOX1* regulation was further confirmed by the effect of diacylglycerol, a PKC agonist, on the *NOX1* RNA in A7r5 cells with Northern blot analysis. To establish cause and effect, we next silenced the PKCδ gene partly by RNA interference and found knockdown of PKCδ gene attenuated aldosterone-induced *NOX1* expression, generation of superoxide, as well as protein synthesis in VSMCs. Taken together, these data indicated PKCδ might mediate aldosterone-dependent *NOX1* upregulation in VSMCs. In addition, we showed that the cascade from aldosterone to PKCδ activation had the participation of the mineralocorticoid receptor.

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Key words: aldosterone, NOX1, PKCδ

Recent studies have shown the potential participation of reactive oxygen species (ROS) in the pathophysiology of aldosterone-induced cardiovascular injury [1-3]. Vascular NADPH-oxidase activity and ROS generation were markedly increased in aldosterone/salt-treated hypertensive rats [2, 3].

It is recognized that aldosterone has rapid nongenomic effects in many kinds of tissues including vascular smooth muscle cells (VSMCs) [4, 5] and cardiac myocytes [6]. In VSMCs, aldosterone induces rapid cellular responses by interfering with intracellular Ca^{2+} and cAMP levels, Na^+/H^+ exchanger activity, and phosphorylation of signaling molecules such as protein kinase C (PKC), epidermal growth factor receptor, and mitogen-activated protein kinase (MAPK), c-Jun NH_2-terminal kinase (JNK), and extracellular signal-regulated kinases (ERKs) 1/2 [7-10]. We have previously shown that aldosterone increased the production of superoxide via upregulating *NOX1* in VSMCs in a dose and time dependent manner. We found the level of *NOX1* mRNA increased in a dose- and time-dependent way in the cells stimulated with various concentrations of aldosterone (0.1-100 nM). The level of *NOX1* mRNA in the treated cells and control cells differed significantly above 1 nM aldosterone [11]. Generally, the physiological concentration of aldosterone is 0.2-0.5 nM. To study the effect of aldosterone under pathology situation, we used 10 nM aldosterone in VSMCs for our further studies. Therefore, we investigated which signal transduction pathways might be involved in the aldosterone-induced *NOX1* mRNA upregulation.

Abbreviations: DAG, diacylglycerol; DHE, dihydroethidium; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAPK, mitogen-activated protein kinase; MR, mineralocorticoid receptor; PKC, protein kinase C; ROS, reactive oxygen species; SSC, standard saline citrate; VSMCs, vascular smooth muscle cells.

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ERK1/2 inhibitor PD98059 (10 μM), MAPK inhibitor SB203580 (10 μM), or JNK inhibitor SP600125 (5 μM) failed to affect the increased level of *NOX1* mRNA expression induced by aldosterone, negating the role of ERK1/2, MAPK, or JNK pathways [11].

The aims of the present study were to examine whether the effects of aldosterone on superoxide production in VSMCs were mediated by PKC and, if so, to determine the PKC isoforms involved by the use of isoform-selective inhibitors and the knockdown technique.

MATERIALS AND METHODS

Reagents. Aldosterone was purchased from Across Organics (Belgium). Dihydroethidium (DHE) was obtained from Molecular Probes (USA). GF109203x, rottlerin, and other reagents were obtained from Sigma (USA). Culture medium, fetal bovine serum (FBS), and antibiotics were obtained from Invitrogen (USA).

Cell culture. The A7r5 (CRL-1444), a rat aortic smooth muscle cell line [12], was obtained from the American Type Culture Collection (USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS in 5% CO_2 and humidified air at 37°C, passaged by trypsinization, and used between 5-20 passages.

Real-time PCR. A7r5 cells were seeded in 10-cm dishes ($1 \cdot 10^6$ cells/dish) and cultured for 24 h in DMEM supplemented with 10% FBS followed by a further 48 h without FBS. The cells were incubated with or without the specific inhibitors for 1 h, followed by 10 nM aldosterone for another 24 h. Total RNA isolated from the cells using acid guanidinium thiocyanate–phenol–chloroform was amplified by real-time PCR as described previously [13]. The specific primers for NOX1 were: forward, 5′-CACGCTGAGAAAGCCATTGGATCAC-3′; reverse, 5′-GGATGGAATAGGCTGGAGAGAACA-3′.

Synthesis of anti-PKCδ double-stranded RNAs (dsRNAs). Anti-PKCδ dsRNAs were designed against nucleotides 381-410 (PKCδ-RNAi-1) and 888-917 (PKCδ-RNAi-2) of the rat PKCδ mRNA sequence [14]. 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-PKCδ dsRNA was performed, essentially as described previously [15]. Ribozyme expression plasmids (pPUR-KE containing NOX1 ribozyme sequence) were transfected into A7r5 cells using GenePORTER2 transfection reagent (Gene Therapy Systems, USA). 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 [15].

Northern blot analysis. Total RNA was isolated from A7r5 cells stimulated by 10 nM aldosterone or 160 nM diacylglycerol (DAG) (a PKC agonist). In some experiments, cells were pre-exposed to 10 μM eplerenone (a mineralocorticoid receptor inhibitor). Ten micrograms of RNA were separated by electrophoresis on a 1.5% agarose gel and transferred onto a Nylon membrane (Hybond-N; Amersham Biosciences, USA).

Rat cDNA fragments for *NOX1* were amplified by RT-PCR. The fragments were cloned into a vector and linearized with an appropriate restriction enzyme. Using the DNA as template, antisense RNA probes were synthesized with SP6 or T7 RNA polymerase in the presence of [^{32}P]UTP. Hybridization was carried out at 70°C for 15 h in 5× SSC (standard saline citrate), 50% formamide, 5× Denhardt's solution, 0.2% SDS, 0.05 M sodium phosphate (pH 6.5), 100 μg/ml of heat-denatured salmon sperm DNA, 100 μg/ml of yeast tRNA, and the ^{32}P-labeled probe. Membranes were washed twice at 70°C in 0.1× SSC containing 0.1% SDS for 20 min. Hybrids were detected with a Fujix BAS 2000 Bioimaging Analyzer (Fuji Photo Film, Japan). Blots were then rehybridized with a ^{32}P-labeled DNA probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). 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 quantified with the bioimaging analyzer, and the levels of NOX1 mRNA were normalized based on GAPDH mRNA.

Measurement of protein synthesis. ^{3}H-Labeled phenylalanine incorporation was considered as a marker of VSMCs hypertrophy. Protein synthesis was measured as described previously [15].

Measurement of superoxide production. Intracellular superoxide production was revealed with DHE. A7r5 cells or PKCδ knockdown cells seeded in 10-cm dishes ($1 \cdot 10^6$ cells/dish) were cultured in serum-free DMEM for 48 h and stimulated with 10 nM aldosterone for a further 12 h, and in some experiments cells were pre-exposed for 1 h to 10 μM eplerenone. The cells were washed with phosphate buffered saline (PBS), and then DHE (10 μM) in Hanks' balanced salt solution was added to each dish. Cells were incubated in a light-protected humidified chamber at 37°C for 30 min, and images were obtained using a confocal laser-scanning microscope Leica TCS SPII AOBS (Leica, Germany).

Western blot analysis. A7r5 cells were cultured in the absence of FBS for 48 h and then incubated with 10 nM aldosterone for 24 h. Cell lysates and nuclear extracts were prepared and detected by Western blot as described previously [16].

Statistical analysis. Data were expressed as means ± SEM and were analyzed using the unpaired Student's *t*-

test for comparisons between two groups and by one-way ANOVA followed by the Tukey–Kramer test for multiple comparisons. A probability value below 0.05 was considered statistically significant.

RESULTS

PKCδ is involved in aldosterone-induced *NOX1* expression. Pre-treatment of A7r5 cells with 10 μM GF109203x, a non-selective PKC inhibitor, significantly reduced the aldosterone-induced *NOX1* mRNA (Fig. 1a).

Next we investigated which PKC isoform is involved in *NOX1* induction. As shown in Fig. 1 (b-d), rottlerin, a selective inhibitor of PKCδ, but not Go6976 (the conventional PKC-specific inhibitor) or a PKCε inhibitor (a peptide with myristoylated N-terminus allowing cell membrane permeability), suppressed aldosterone-induced *NOX1* expression. So PKCδ can mediate the increased expression of *NOX1* simulated by aldosterone in VSMCs.

To further confirm the participation of PKC in the mediation of *NOX1* regulation, the effect of a PKC agonist DAG was examined. Growth-arrested A7r5 cells were stimulated with 10 nM aldosterone and 160 nM DAG for 24 h. The expression of *NOX1* mRNA was determined by Northern blot analysis. As shown in Fig. 2, a 2.6-kb band was detected. With the stimulation of aldosterone or DAG, the amount of *NOX1* mRNA was increased remarkably. The other important problem is whether or not the mineralocorticoid receptor (MR) is involved in the cascade from aldosterone to PKCδ activation. The selective MR blocker eplerenone was used in this study. Figure 2 shows that eplerenone was able to block the induced upregulation of *NOX1* mRNA by aldosterone.

Gene silencing of PKCδ attenuates aldosterone-induced *NOX1* expression and decreases superoxide production. To confirm the role of PKCδ in the upregulation of *NOX1* by aldosterone, dsRNAs targeted at rat PKCδ mRNA sequence were introduced into A7r5 cells. Following single cell cloning of the transfectants, PKCδ knockdown cells, which stably expressed the dsRNA, were isolated. In this cell clone, protein levels of PKCδ, but not of PKCε, were reduced by at least 50% compared with the mock-transfected cells (Fig. 3).

As compared with the control group, PKCδ knockdown inhibited the effect of aldosterone in *NOX1* activation. Aldosterone treatment in the knockdown cell clones demonstrated similar amount of *NOX1* as control (Fig. 4a). In our study, application of PKCδ RNAi appeared to block *NOX1* upregulation under the stimulation of aldosterone. Aldosterone apparently increased *NOX1* mRNA levels in the mock twofold, whereas in the knocked-down cell with and without 10 nM aldosterone treatment, the amount of *NOX1* mRNA was similar to that of the mock-transfected cell. Therefore, it looks like PKCδ is involved in the *NOX1* gene expression stimulated by aldosterone.

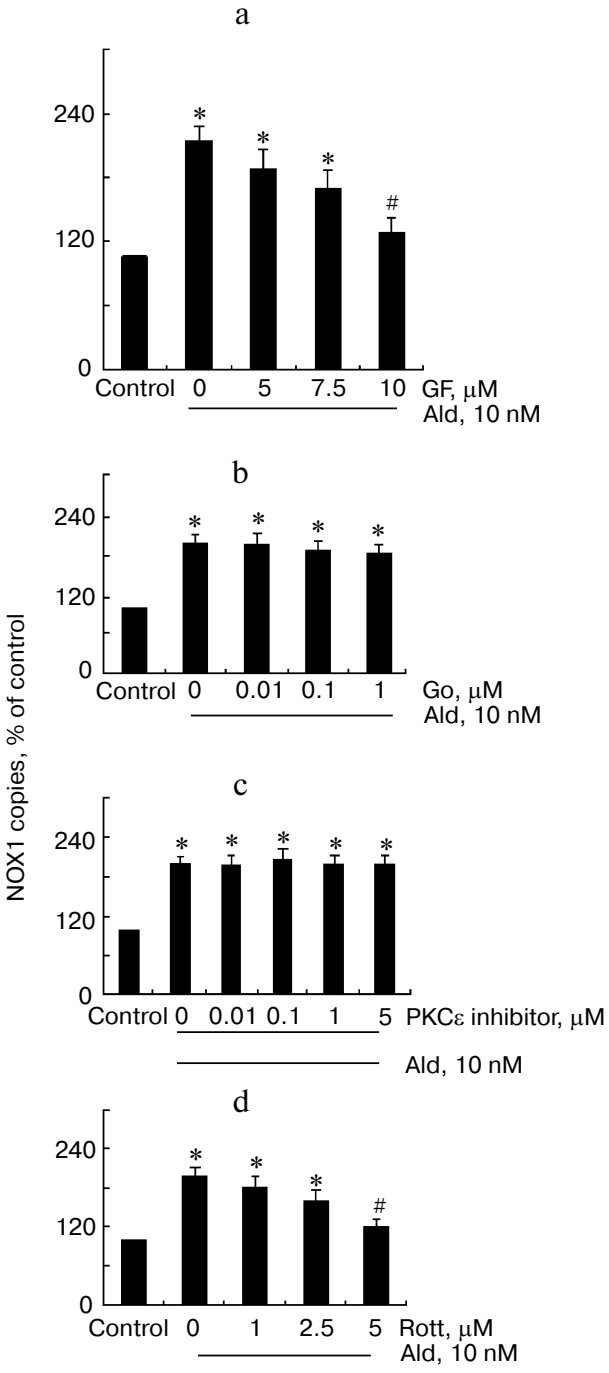

Fig. 1. Effects of different PKC inhibitors on aldosterone-induced *NOX1* mRNA expression in VSMC A7r5 cells. In the absence of FBS for 48 h, growth-arrested A7r5 cells were treated with the indicated concentrations of PKC inhibitor for 1 h and subsequently incubated with 10 nM aldosterone (Ald) for 24 h. Real-time PCR analysis was performed as described in "Materials and Methods". a) Non-selective PKC inhibitor GF109203x; b) conventional PKC specific inhibitor Go6976; c) PKCε translocation-inhibitor peptide; d) selective PKCδ inhibitor rottlerin (Rott). Mean data from 3-6 experiments are shown. Bars represent the mean ± SEM. * $P < 0.05$ compared with control; # $P < 0.05$ compared with aldosterone-treated cells.

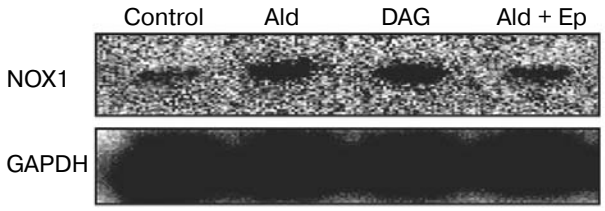

Fig. 2. Induction of *NOX1* expression by aldosterone and DAG. Growth-arrested A7r5 cells were stimulated with 10 nM aldosterone and 160 nM DAG for 24 h. The expression of *NOX1* mRNA was determined by Northern blot analysis.

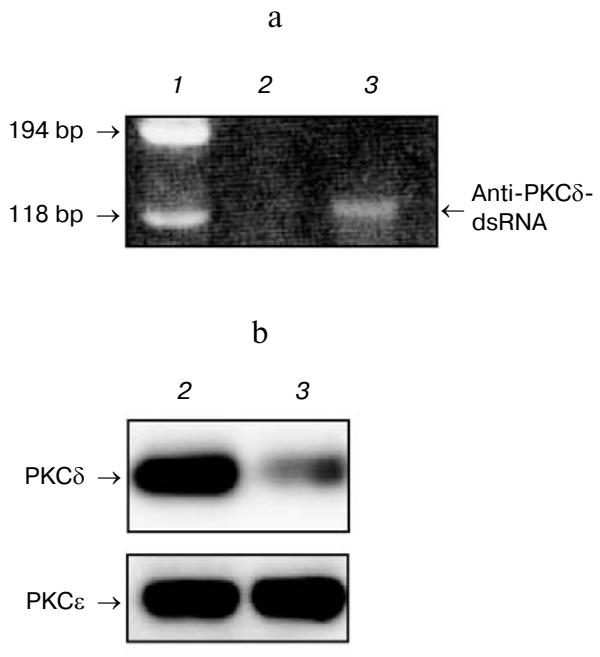

Fig. 3. Gene silencing of PKCδ by RNAi and decrease in PKCδ synthesis in PKC-knockdown cells. a) Expression of anti-PKCδ dsRNA precursors in the clone. Total RNA was reverse-transcribed with random nonomers, and the cDNA fragment (125 bp) was amplified by PCR. The product was separated on a 15% polyacrylamide gel. Lanes: *1*) molecular size markers (ϕ174HaeIII); *2*) mock-transfected cells (control); *3*) clone of PKCδ knockdown cells, which stably expressed the dsRNA. b) PKC expression was determined by Western blotting as described in "Materials and Methods". Lanes *2* and *3* are the same as in Fig. 3a.

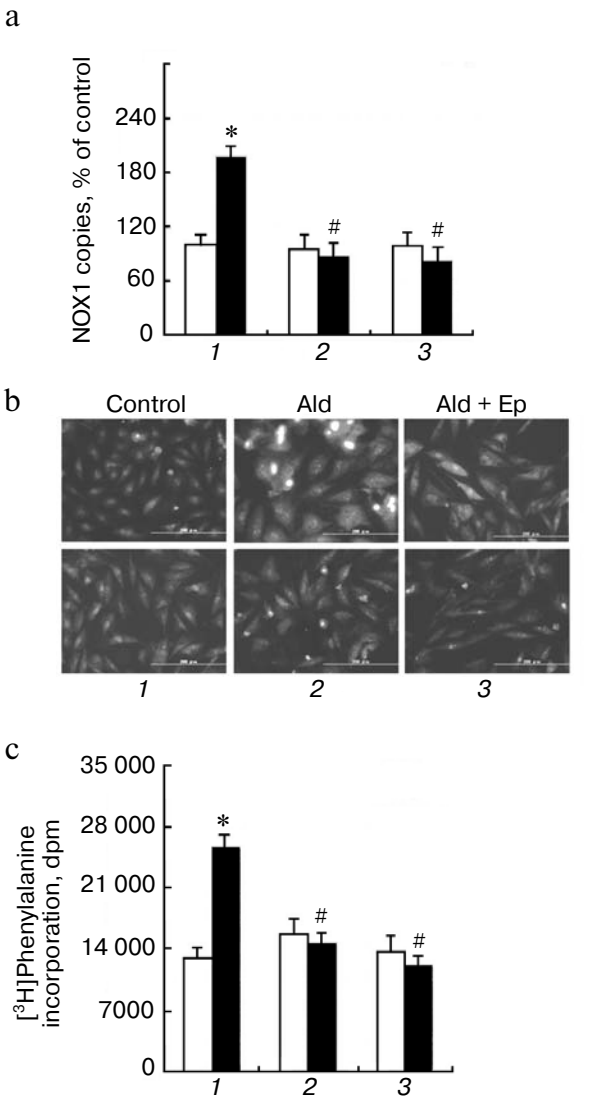

Fig. 4. a) Induction of *NOX1* mRNA by aldosterone. Real-time PCR was performed as described in "Materials and Methods". Here and on Fig. 4c: *1*) mock-transfected cells; *2*, *3*) two different clones of PKCδ-knockdown cells corresponding to two various coding regions in expression vector (RNAi-1 and RNAi-2; see "Materials and Methods"). White columns, control; black columns, 10 nM aldosterone. Bars represent the mean of 3-6 experiments ± SEM. * $P < 0.05$ compared with mock-transfected cells without 10 nM aldosterone; # $P < 0.05$ compared with aldosterone-treated mock cells. b) Superoxide production detected *in situ* with DHE. A7r5 cells or PKCδ knockdown cells were cultured and treated with 10 nM aldosterone (Ald) and in some experiments with 10 μM eplerenone (Ep) as described in "Materials and Methods" and stained with DHE. Control, normal A7r5 cells; Ald, mock-transfected cells with 10 nM aldosterone; Ald + Ep, mock-transfected cells with 10 nM Ald and 10 μM Ep. *1*) Mock-transfected cells; *2*, *3*) PKCδ knockdown cells treated with 10 nM Ald. Bar, 200 μm. c) Aldosterone effects on ^{3}H-labeled phenylalanine incorporation. A7r5 cells or PKCδ knockdown cells seeded in 24-well plate ($1 \cdot 10^5$ cells/dish) were cultured in serum-free DMEM for 48 h and stimulated with 10 nM aldosterone for further 24 h.

Because NOX1 is the major generation source of superoxide in VSMCs, intracellular superoxide was revealed by DHE staining. Figure 4b shows a low level of DHE fluorescence in the control cells. DHE fluorescence was increased after exposure of cells to aldosterone for 12 h, whereas silencing of PKCδ attenuated aldosterone-induced augmentation of DHE staining. Eplerenone (MR antagonist) pretreatment inhibited aldosterone-induced superoxide production. Furthermore, as demonstrated in Fig. 4b, aldosterone caused the hypertrophy of

VSMCs compared with the other groups. In line with the hypertrophy of VSMCs, ^{3}H-labeled phenylalanine incorporation increased in response to aldosterone (Fig. 4c), whereas in the PKCδ knockdown cells aldosterone did not induce ^{3}H-labeled phenylalanine incorporation.

DISCUSSION

We demonstrated here that aldosterone caused superoxide production through the upregulation of NOX1, a catalytic subunit of superoxide-generating NADPH oxidase. NOX1 was upregulated in VSMCs in the presence of 10 nM aldosterone and normalized by the MR antagonist eplerenone.

Effects of aldosterone have long been considered to be mediated through the genomic pathway. However, recent evidence has been provided for rapid effects of the hormone that might involve non-genomic mechanisms. Cathy et al. [17] suggested an interaction exists between the two kinds of signaling pathways. Blocking the early non-genomic pathway results in the inhibition of the late genomic response to aldosterone. In the present study, we also found that the genomic and non-genomic aldosterone actions appear to be interdependent. Inhibition of the PKCδ activation leads to the suppression of aldosterone-induced increase in *NOX1* gene expression. Aldosterone is supposed to activate PKCδ in non-genomic fashion, followed by genomic action in increasing NOX1 expression. As a result, we chose 24 h treatment with aldosterone. These data demonstrated aldosterone–MR mediated NOX1 upregulation depends, at least in part, upon PKCδ activation.

Aldosterone has been shown to activate PKC in leukocytes [18], gut [19], and renal cells [20]. Christ et al. [18] found that aldosterone (100 nM) stimulated translocation of PKCα in VSMCs. In the heart, rapid and non-genomic effects of aldosterone were mediated by PKCε [21]. In our study, PKCδ was identified to play roles in the aldosterone-inducible NOX1 upregulation in VSMCs. It is apparent that PKC isozymes can selectively couple to MAPK signaling pathways, although the particular isozymes and pathways might be cell and tissue specific. Western blotting and RT-PCR [22] have confirmed PKCα and PKCδ as the predominant isozymes presented in rat VSMCs. However in the cultured A7r5 cells, we found PKCε and PKCδ is abundant (data not shown). This study indicated that PKCδ but not PKCε plays an important role in the aldosterone dependent non-genomic effects on VSMCs. The present study provides the first evidence for the role of PKCδ in transducing signals of aldosterone-induced NOX1 upregulation in rat VSMCs.

In our studies, we found that the cultured A7r5 cells in serum-free medium with 10 nM aldosterone became larger and "stronger" than the cells without aldosterone. It was observed that ^{3}H-labeled phenylalanine incorporation increased under the stimulation of aldosterone. We speculate that aldosterone activates the generation of superoxide and initiates the transduction of intracellular signals related to protein synthesis in VSMCs.

Study of the change of NOX1 expression on the protein level is hampered by the lack of rat NOX1 antibody. Real-time RT-PCR as a sensitive quantitative assay and Northern blot analysis were therefore used in our study. Next, we plan to confirm the role of PKCδ in the action of aldosterone on *NOX1* gene expression in human primary VSMCs culture as a human antibody is available.

Whether MR takes part in the non-genomic effects on PKCδ generally occurring within a few seconds to minutes has not been measured in our study. Research into short-term effects of aldosterone on different cells has focused on identifying a specific receptor, distinct from the MR, although there is evidence that the non-genomic action can be mediated by the classical MR [23]. In MR knockout mice, it was reported that non-genomic actions of aldosterone still occurred in the absence of classical MRs [24]. Some reports have shown that non-genomic actions of aldosterone are not blocked by the classical MR antagonist. In contrast, others have demonstrated that MR antagonism inhibited signaling cascades activated by aldosterone [25-27]. Next, we will make certain whether the non-genomic effect of aldosterone on the activation of PKCδ needs MR or some other receptor. In addition, aldosterone-induced NADPH oxidase activation can be partly due to membranous translocation of cytosolic components [28, 29] or upregulation of catalytic subunit and other components [3, 30-32]. At present, we find that aldosterone increases the expression of *NOX1* in VSMCs mediated by PKCδ. Further *in vitro* studies will be needed to determine the effects of aldosterone on the expression of NADPH oxidase membrane components in VSMCs.

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