ROS

RESEARCH ARTICLE

Erk1/2 and Elk1 Are Involved in PKCα-Induced Nox4 Expression in Human Endothelial Cells

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ABSTRACT | In a previous study, we have shown that activation of protein kinase C-alpha (PKCα) leads to an upregulation of Nox4 expression in human endothelial cells. The present study was conducted to analyze the signaling pathway linking PKCα activation to Nox4 upregulation. Treatment of human EA.hy 926 endothelial cells with phorbol 12-myristate 13-acetate (PMA) increased Nox4 expression, which was prevented by the PKC inhibitor Gö 6983 and by siRNA-mediated knock-down of PKCα. PKC activation is known to stimulate the Ras/Raf/MEK/Erk/Elk1 pathway. Indeed, PMA treatment of EA.hy 926 cells induced Erk1/2 phosphorylation and Elk1 upregulation. The PMA-induced Nox4 expression in EA.hy 926 cells could be prevented by the MEK1 inhibitor PD98059 and by siRNA-mediated knock-down of Erk1/2 or Elk1, indicating a dependency of Nox4 expression on Erk1/2 and Elk1. In promoter activity analyses, PMA treatment of EA.hy 926 cells increased the activity of the Nox4 promoter fragments of 2160 bp and 672 bp in length, but not that of 495 bp. Consistently, a potential Elk1 binding site was found between –644 bp and –629 bp in the Nox4 promotor by in silico analysis. In conclusion, Erk1/2 and Elk1 are involved in the signaling pathway linking PKCα activation to Nox4 expression.

KEYWORDS | Elk1; Erk1/2; Gene expression; NADPH oxidase; Nox4; Protein kinase C

ABBREVIATIONS | DMSO, dimethyl sulfoxide; Nox, NADPH oxidase; PKCα, protein kinase C-alpha; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species

CONTENTS

1. INTRODUCTION

Oxidative stress, which arises when the intrinsic antioxidant defense is overwhelmed by excess production of reactive oxygen species (ROS) [1], is linked to cardiovascular risk factors as well as the formation of atherosclerosis [2, 3]. While in the vascular wall many enzymes, including enzymes of the respiratory chain, cytochrome P450 monooxygenases, and uncoupled endothelial nitric oxide synthase (eNOS) [4], produce ROS, evidence suggests that in coronary arteries of diseased humans the major ROS producers are nicotinamide adenine dinucleotide phosphate oxidases (NADPH oxidase, Nox) and xanthine oxidase, contributing to 60% and 25% of the ROS burden, respectively [5].

NADPH oxidases facilitate the transport of electrons over biological membranes wherefore they produce ROS [6]. NADPH oxidases are enzyme complexes consisting of various cytosolic, regulatory subunits as well as of two subunits (Nox protein and $p22^{phox}$) anchored to the cell membrane [7, 8]. There are seven members in the Nox family: Nox1, Nox2, Nox3, Nox4, and Nox5, as well as dual oxidase (Duox) 1 and Duox2 [6]. Nox4 is the most prominent isoform expressed in the vasculature [8]. Apart from its dominant expression in the vascular wall, Nox4 additionally exhibits two special properties: (1) it mostly produces hydrogen peroxide whereas the other isoforms generate superoxide [9]; and (2) Nox4 is independent of the regulatory, cytosolic subunits, and only in need of the subunit $p22^{phox}$. Consequently, Nox4-mediated ROS production is dependent on its gene expression level [6].

Various factors have been linked to Nox4 transcription regulation [10]. An enhancement of Nox4 expression has been shown in human aortic smooth muscle cells after tumor necrosis factor-α (TNF-α) or interferon-γ (IFN-γ) treatment via NF-κB activation [11] or the JAK/STAT pathway [12], respectively. In contrast, peroxisome proliferator-activated receptor-gamma ligands down-regulate Nox4 expression in endothelial cells [13]. An enzyme regularly associated with Nox activation in vasculature is the protein kinase C (PKC) [5, 14]. In addition to the regulatory role on Nox activity, PKC also regulates Nox expression. Our previous study has demonstrated that activation of PKCα leads to an upregulation of Nox4 in human endothelial cells [15]. The signaling cascade connecting PKC to Nox4 transcription, however, has not been identified. The present study was conducted to close that gap.

2. MATERIALS AND METHODS

2.1. Materials

Phorbol 12-myristate 13-acetate (PMA), 3-[1-[3-(dimethylamino)propyl]-5-methoxy-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione (Gö 6983), and 2′-Amino-3′-methoxyflavone (PD98059) were obtained from Calbiochem (Merck Biosciences, Darmstadt, Germany).

2.2. Cell Culture

EA.hy 926 endothelial cells were provided by Dr. Cora-Jean S. Edgell (University of North Carolina at Chapel Hill, NC, USA) [16] and cultured as previously described [15].

2.3. Real-Time RT-PCR

RNA was isolated with the PeqGOLD TriFast™ total RNA isolation kit (PEQLAB Biotechnologie GmbH/VWR, Erlangen, Germany) according to the manufacturer's protocol. RNA concentration and purity were analyzed using NanoDrop™ 1000 (Thermo Fisher Scientific, Waltham, MA, USA).

cDNA was generated with the High-Capacity cDNA reverse transcription kit (Applied Biosystems,

Foster City, CA, USA) following the manufacturer's instructions. For qPCR, an iCycler real-time PCR detection system (Bio-Rad, Hercules, CA, USA) with SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma-Aldrich, St. Louis, MO, USA) was used. For quantification of the relative mRNA levels of the target genes, their threshold C_T values were normalized to the housekeeping gene RNA polymerase 2A (POLR2A) [17]. Primer sequences (Eurofins MWG Synthesis GmbH, Ebersberg, Germany) were as following: POLR2A_forward: GCT ATA AGG TGG AAC GGC ACA T; POLR2A_reverse: ACC CGA TGC CCC ATC AT; Nox4_forward: TTT TCT CAG GCG TGC ATG TG; Nox4_reverse: CAT TCA GTT CAA CAA AGT CTT CAC TGT.

2.4. Knock-Down of PKCα, Erk1/2, and Elk1

Cells were seeded the day before transfection which was implemented once they reached 50–60% confluency. Transfection was performed with SaintRed (Synvolux Therapeutics, Groningen, Netherlands) according to the manufacturer's protocol. The siRNAs for PKCα (SASI_Hs01 00018817), Erk1/2 (SASI_Hs01 00190617), and Elk1 (SASI_Hs02 00326324) were purchased from Sigma-Aldrich.

2.5. Western Blot Analyses

Western blot analyses were carried out as previously described [18]. Total protein samples (30 µg each) from EA.hy 926 cells were used. Sample separation was performed on a bis-tris gel and transferred onto a nitrocellulose membrane. Unspecific antibody binding was blocked with 5% milk powder in TBS-T (10 mM Tris-HCl, pH 7.4, 150 mM NaCl with 0.1% Tween 20) for 1 h at room temperature. Primary antibodies were diluted in the blocking solution and left on the blots over night at 4°C followed by washing with TBS-T. The horseradish peroxidase-conjugated secondary antibody was also diluted in the described milk solution and used on blots for 1 h at room temperature. Washing was conducted with TBS-T and then with TBS alone. For immunodetection, the enhanced horseradish peroxidase/luminol chemiluminescence reagent (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA) was utilized as detailed by the manufacturer. Densitometry was achieved with Adobe Photoshop CS5™ (Adobe Systems Software Ireland Ltd, Dublin, Ireland). Primary antibodies against GAPDH (#2251-1, Epitopics, Leipzig, Germany), PKCα (#sc-8393, Santa Cruz Biotech, Dallas, TX, USA) and Elk1 (#04-226, Millipore, Burlington, MA, USA) were used. The secondary antibodies, a goat anti-rabbit (#A9169) and a rabbit anti-mouse (#A9044) antibody, were from Sigma-Aldrich.

2.6. Cloning of the 5′-Flanking Regions from the Human Nox4 Gene

Genomic DNA isolated from human EA.hy 926 cells was used for amplification of the 5′-flanking DNA of the human Nox4 gene. PCR was performed by using three different forward primers (ccg gat ccT TGG AAG CAA ACC CAG TTT C; ccg gat ccG GGC AAG GGG ATA AAG AAA C; ccg gat ccT TGG GGG AAA ACA ATC AGT C) and a reverse primer (ggc cat GGC AGC GGT TAC AGT TGT G) (extra nucleotides to generate Bam HI and Nco I restriction sites are displayed in lowercase letters). The sequences were based on Ensembl Gene ID ENSG00000086991. The resulting PCR fragments (of 2160 bp, 672 bp, and 495 bp, respectively) were restricted with Bam HI and Nco I and cloned into pGL3-Basic (containing a promoterless luciferase reporter gene; Promega, Madison, MI, USA) restricted with Bgl II and Nco I to generate pGL3-huNox4-2160, pGL3-huNox4-672, and pGL3-huNox4-495, respectively.

2.7. Luciferase Reporter Gene Assay

The day prior to transfection, EA.hy 926 cells were seeded with a density of 5×10^4 cells per well on 24-well plates to reach 60–80% confluency on the next day. Human Nox4 promoter fragments (2160 bp, 672 bp, and 495 bp in length, respectively) cloned into the pGL3-basic vector (pGl3_basic) were used. Transfection was performed with Nanofection (Q050-005; PAA laboratories, Pasching, Austria) according to the manufacturer's protocol. After siRNA transfection, cells were treated with PMA according to the experiment protocol. Then, cells were washed with phosphate-buffered saline (PBS) and lysed with Passive Lysis Buffer (PLB, Promega). Lysates were centrifuged at 4°C and 20,000 *g* for 15 min and the supernatant was transferred to a fresh tube. Protein concentration was determined with a BCA assay kit (Sigma-Aldrich). Luciferase activity was analyzed on

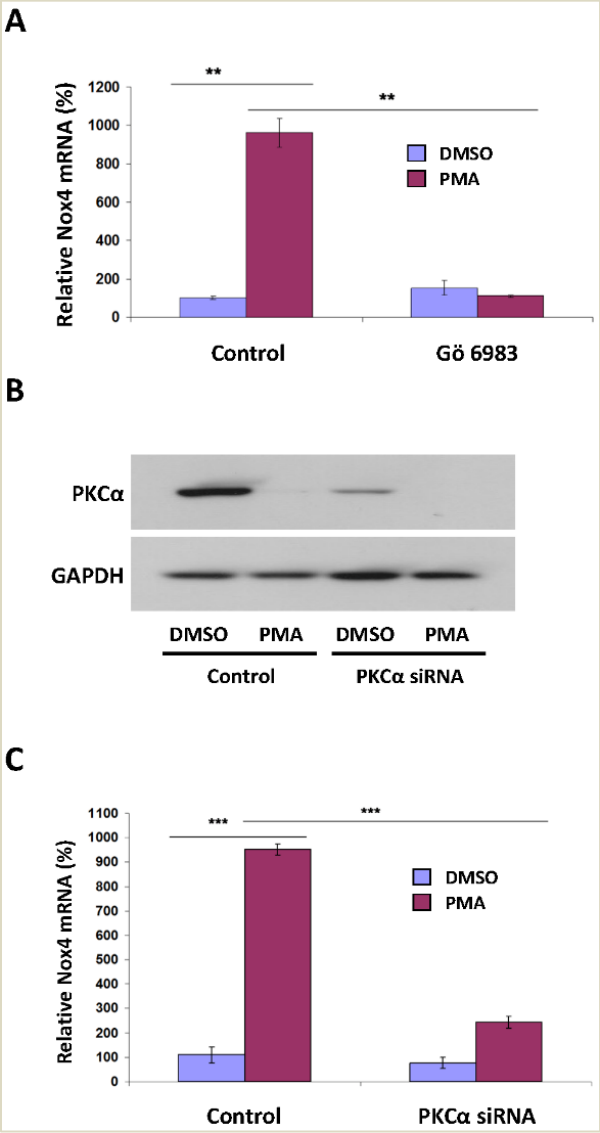

FIGURE 1. Regulation of Nox4 expression by PKCα. Human EA.hy 926 endothelial cells were treated with 10 nM PMA (dissolved in dimethyl sulfoxide, DMSO) for 48 h. Relative mRNA expression of Nox4 was analyzed by quantitative real-time RT-PCR (qPCR). Effects of PKC inhibition on Nox4 mRNA expression were studied by pretreatment with the pan PKC inhibitor Gö 6983 (1 μM) for 30 min before PMA was added (A). PKCα was knocked down by treatment with PKCα siRNA (50 nM) for 48 h. PKCα protein expression after siRNA-mediated knock-down was analyzed by western blot analysis. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The blots shown are representative for three independent experiments (B). 48 h after siRNA-mediated knock-down of PKCα, cells were treated with 10 nM PMA for 48 h. Relative mRNA expression of Nox4 was analyzed by qPCR (C). Columns represent mean ± SEM (n = 6). **, $p < 0.01$; ***, $p < 0.001$.

a Centro LB960 luminometer (Berthold, Wildbad, Germany).

2.8. Statistical Analysis

Data are expressed as the mean ± SEM. For comparison of two groups, Student's t test was used. To compare mean values between three or more groups, analysis of variance followed by Fisher's protected least significant difference test was used. Values of $p < 0.05$ were considered significantly different. The software used was GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA).

3. RESULTS

3.1. PKCα Activation Increases Nox4 Expression in Endothelial Cells

As reported in our previous study [15], treatment of human EA.hy 926 endothelial cells with PMA led to an upregulation of Nox4 mRNA expression (**Figure 1A**). The PMA-induced Nox4 upregulation could be completely prevented by Gö 6983, a pan PKC inhibitor (**Figure 1A**). In the cells treated with PMA for 48 h, PKCα was downregulated (**Figure 1B**). This is an indirect evidence of PKCα activation by PMA. PKC activation is typically associated with a translocation of the PKC proteins from the cytosol to the cell membrane, followed by a downregulation after prolonged PMA incubation (> 24 h). This is a well-known phenomenon, which has also been documented in our previous publications [15, 19]. To test the role of PKCα, this PKC isoform was knocked down with siRNA (**Figure 1B**). Knock-down of PKCα by siRNA significantly reduced the effect of PMA on Nox4 expression (**Figure 1C**), indicating a crucial role of the PKCα isoform for Nox4 transcription.

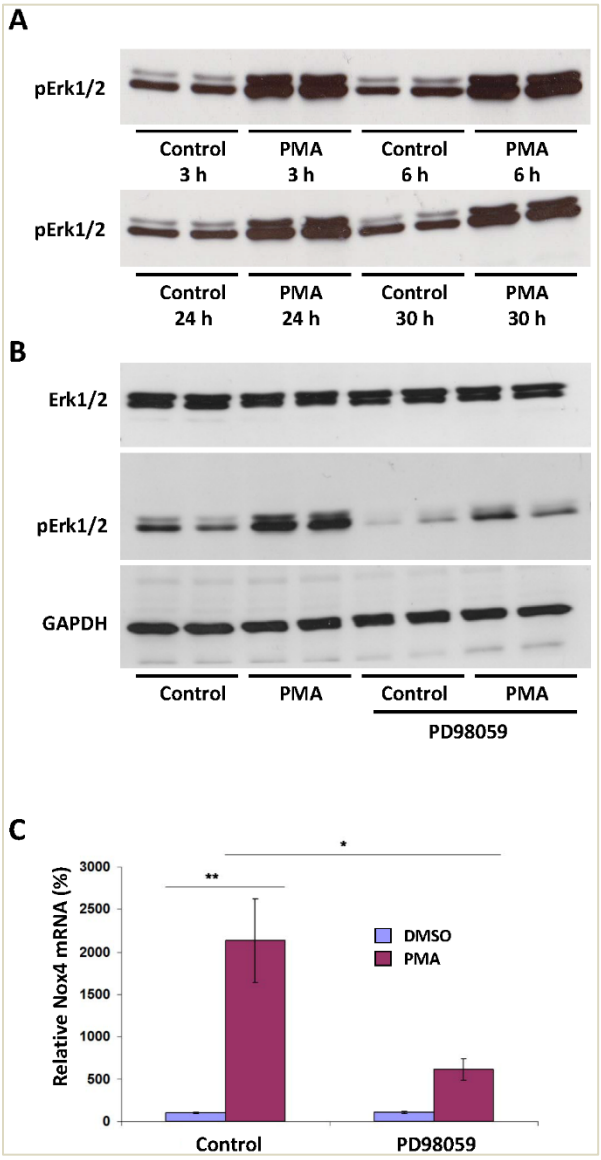

FIGURE 2. Role of Erk1/2 in PMA-induced Nox4 expression. Human EA.hy 926 endothelial cells were treated with 10 nM PMA and Erk1/2 phosphorylation was analyzed by western blot analysis (A). Effects of MEK1 inhibition, an upstream kinase of Erk1/2, on PMA-induced Erk1/2 phosphorylation were studied by treatment with the MEK1 inhibitor PD98059 (10 µM) 30 min prior to PMA application (10 nM for 48 h). Erk1/2 protein expression and protein phosphorylation were analyzed by western blot analysis (B). GAPDH was used as an internal control. Blots shown are representatives for three independent experiments. Relative mRNA expression of Nox4 was analyzed by qPCR in cells treated with PMA and PD98059 (C). Columns represent mean ± SEM (n = 6). *, $p < 0.05$; **, $p < 0.01$.

3.2. Erk1/2 Is Involved in PKCα-Induced Nox4 Expression

Treatment of human EA.hy 926 cells with PMA led to phosphorylation of Erk1/2 (**Figure 2A**). To verify whether Erk1/2 is involved in the effect of PMA on Nox4 expression, cells were treated with PD98059. PD98059 is an inhibitor of the mitogen-activated protein kinase kinase 1 (MEK1), which is an upstream kinase of the extracellular signal-regulated kinases 1/2 (Erk1/2). As shown in **Figure 2B** and **2C**, PD98059 prevented PMA-induced Erk1/2 activation and Nox4 upregulation. To further strength these results, Erk1/2 was downregulated by siRNA. Protein levels of Erk1/2 were significantly lowered between 2 days and 5 days after siRNA incubation (**Figure 3A**). Since siRNA-mediated Erk1/2 knock-down was a slow process, PMA induction was performed 72 h after siRNA exposure. As shown in **Figure 3B**, Erk1/2 knock-down significantly diminished PMA-induced Nox4 expression in human EA.hy 926 cells.

3.3. Elk1 Is Involved in PKCα-Induced Nox4 Expression

Treatment of human EA.hy 926 cells with PMA led to a time-dependent increase in Elk1 expression at the protein level (**Figure 4A**). Elk1 is a downstream target of Erk1/2. To examine the involvement of Elk1 in the PKCα-mediated Nox4 expression, Elk1 was knocked down with siRNA in human EA.hy 926 cells. A significant reduction of PMA-induced Nox4 mRNA expression was observed after siRNA-mediated Elk1 knock-down (**Figure 4B**).

3.4. PKCα Activation Increases Nox4 Promoter Activity in Endothelial Cells

To identify the transcription factor binding sites responsible for PMA-induced Nox4 transcription, Nox4 promotor activity was analyzed. For this purpose, plasmids containing Nox4 promotor fragments

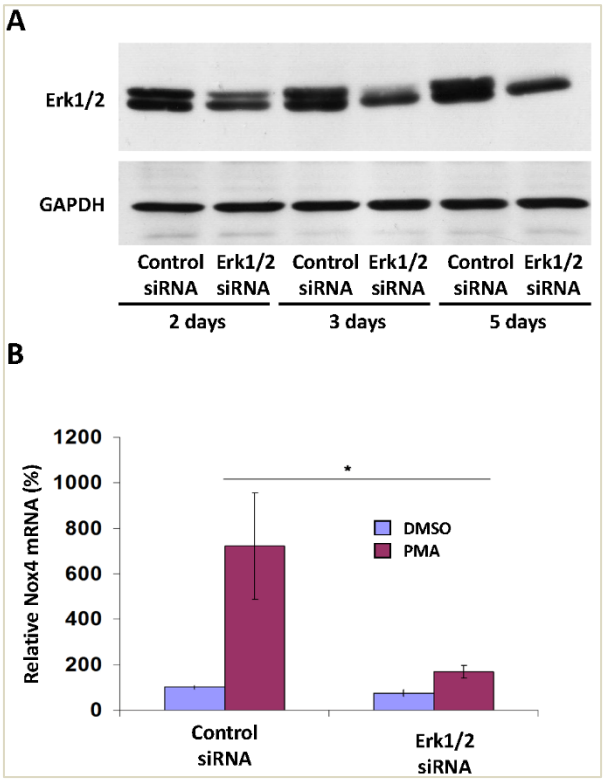

FIGURE 3. Prevention of PMA-induced Nox4 expression by Erk1/2 knock-down. Human EA.hy 926 endothelial cells were treated with scrambled (control) or Erk1/2 siRNA (50 nM). Erk1/2 protein expression was analyzed by western blot analysis. GAPDH was used as internal control. The displayed blots are representative for three independent experiments (A). 72 h after siRNA-mediated knock-down of Erk1/2, cells were treated with 10 nM PMA for 48 h. Relative mRNA expression was analyzed by qPCR (B). Columns represent mean ± SEM (n = 6). *, $p < 0.05$.

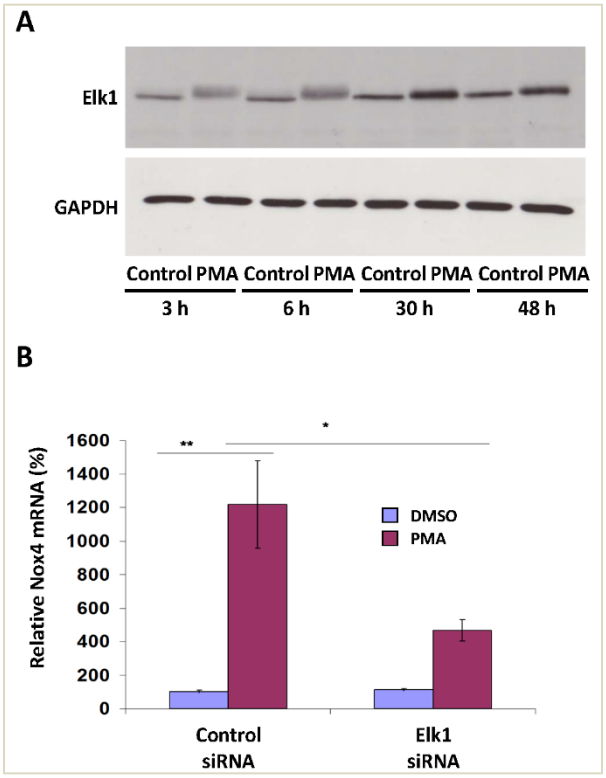

Figure 4. Role of Elk1 in PMA-induced Nox4 expression. Human EA.hy 926 endothelial cells were treated with 10 nM PMA for 3, 6, 30, or 48 h. Elk1 protein expression was analyzed by western blot analysis. GAPDH was used as an internal control. The blots shown are representative for 3 independent experiments (A). To knock-down Elk1, human EA.hy 926 cells were treated with 50 nM Elk1 siRNA for 48 h prior to PMA application (10 nM, 48 h). Relative mRNA expression was analyzed by qPCR (B). Columns represent mean ± SEM (n = 6). *, $p < 0.05$; **, $p < 0.01$.

in different lengths (2160 bp, 672 bp, and 495 bp) were transfected into human EA.hy 926 cells. PMA increased the activity of the 672 bp-long Nox4 promotor fragment, but not the 495 bp-long fragment (**Figure 5**). These data suggest that the elements responsible for PMA-induced Nox4 upregulation is likely to be localized between –672 bp and –495 bp of the human Nox4 promoter.

4. DISCUSSION

The present study demonstrates that (1) the Erk1/2-Elk1 signaling pathway is involved in PKCα-induced Nox4 upregulation, and (2) Elk1 is likely to function as a direct transcription factor for Nox4 transcription. We have previously shown that activation of PKCα led to an upregulation of Nox4 in endothelial cells [15]. However, the signaling pathway underlying this

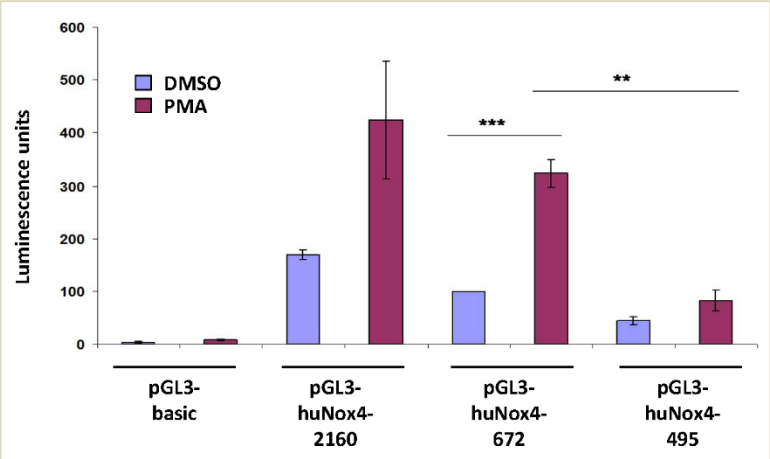

FIGURE 5. PMA-induced Nox4 promoter activation. Human EA.hy 926 were transfected with pGL3-basic plasmids containing Nox4 promotor fragments in different lengths (2160 bp, 672 bp, and 495 bp, respectively). 24 h after plasmid transfection, cells were treated with 10 nM PMA for 48 h. Promotor activity was measured by the luciferase assay. Columns represent mean ± SEM (n = 9). **, $p < 0.01$; ***, $p < 0.001$.

phenomenon was unclear. In the past, various PKC isoforms have been identified as important enzymes for the transduction of cellular stress signals, including cytotoxic chemicals, osmotic pressure, and physical forces [20]. So far three subclasses of PKC isoforms have been defined, namely, the so called conventional, novel, and atypical PKCs. The conventional PKCs (cPKC) rely on Ca^{2+}, diacylglycerol (DAG), and phosphatidylserine for activation, while novel PKCs (nPKC) are independent of Ca^{2+} and atypical PKCs (aPKC) need neither Ca^{2+} nor DAG [21]. Another means to activate cPKCs and nPKCs is phorbol ester analogs, such as PMA [22, 23]. A well-established downstream target of PKCs is the Ras/Raf/MEK/Erk cascade [24], a key pathway in regulating proliferation and differentiation. Activation of Erk leads to its translocation to the nucleus where it exhibits its kinase function with one of the targets being Elk1 [25].

In the present study, the effect of PKCα on Nox4 expression was demonstrated by using the PKC inhibitor Gö 6983 as well as the siRNA-mediated PKCα knock-down (**Figure 1**). In addition, PMA treatment of EA.hy 926 endothelial cells led to activation of Erk1/2, as evidenced by Erk1/2 phosphorylation (**Figure 2**). As mentioned above, Erk1/2 is activated by PKCα via the Ras/Raf/MEK signaling pathway [26]. Consistently, Erk1/2 phosphorylation was prevented by the MEK1 inhibitor PD98059 (**Figure 2**), as also shown in previous studies [23]. Importantly, inhibition of Erk1/2 by PD98059 largely prevented the PMA-induced Nox4 upregulation (**Figure 2A**), indicating the involvement of MEK1 and Erk1/2. This conclusion is further supported by siRNA experiments. Knock-down of Erk1/2 with siRNA prevented PMA-induced Nox4 upregulation in endothelial cells (**Figure 3**).

Elk1 is a well-known downstream target of Erk [25, 27]. Moreover, PMA treatment also increased the protein levels of Elk1 in EA.hy 926 endothelial cells (**Figure 4**). Importantly, siRNA-mediated knock-down of Elk1 led to a significant decrease in PMA-induced Nox4 mRNA expression (**Figure 4**), demonstrating that Elk1 is involved in PMA-mediated Nox4 transcription.

Previously, we have shown that c-Jun plays a crucial role in controlling Nox4 transcription [8]. c-Jun is a member of the Jun family, a class of transcription factors which together with members of the Fos family form the activating protein-1 (AP-1) transcription factor complexes [28]. Therefore, a possible mechanism for Nox4 transcription after Elk1 activation could be the Elk1-dependent expression of c-Jun which in turn leads to Nox4 expression. An-

other possibility could be a direct transcription induction of Nox4 by Elk1. To test this hypothesis, we created 3 Nox4 promotor fragments in different lengths and cloned these into a plasmid before the luciferase gene. PMA treatment increased the luciferase activity of the 672 bp promoter fragment, but not the 495 bp promoter fragment (**Figure 5**), indicating that the response elements are within the 177 bp-long sequence in the Nox4 promotor. In silico analysis (rVISTA 2.0, TRAP) revealed a binding site for Elk1 in this 177 bp-long promotor sequence with the binding motifs (TCGGGCTTCCACTCAG) being located between -644 bp and -629 bp in the Nox4 promotor sequence. Thus, it is likely that the stimulation of Nox4 transcription by PMA is mediated by a direct binding of Elk1 to the Nox4 promoter.

In conclusion, our data demonstrate that Erk1/2 and Elk1 are involved in PKCα-induced Nox4 expression in endothelial cells. The signaling cascade may involve Ras/Raf/MEK1 resulting in Erk1/2 phosphorylation. Elk1, in turn, may activate Nox4 transcription by binding to the Nox4 promoter region between –644 bp and –629 bp.

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