Apurinic/apyrimidinic endonuclease 1 inhibits protein kinase C-mediated p66shc phosphorylation and vasoconstriction

Sang Ki Lee†, Jae In Chung†, Myoung Soo Park†, Hee Kyung Joo†, Eun Ji Lee†, Eun Jung Cho†, Jin Bong Park†, Sungwoo Ryoo‡, Kaikobad Irani§, and Byeong Hwa Jeon†*

†Infection Signaling Network Research Center, Research Institute of Medical Sciences, Department of Physiology, School of Medicine, Chungnam National University, 6 Munhwa-dong, Jung-gu, Daejeon 301-131, Korea; ‡Department of Biological Sciences, Kangwon National University, Chuncheon 200-701, Korea; §Cardiovascular Institute, Department of Medicine, Vascular Medicine Institute, and Department of Pharmacology and Chemical Biology, University of Pittsburgh Medical Center, Pittsburgh, PA 15213, USA

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Aims Phosphorylation of the adaptor protein p66shc is essential for p66shc-mediated oxidative stress. We investigated the role of the reducing protein/DNA repair enzyme apurinic/apyrimidinic endonuclease 1 (APE1) in modulating protein kinase CβII (PKCβII)-mediated p66shc phosphorylation in cultured endothelial cells and PKC-mediated vasoconstriction of arteries.

Methods and results Oxidized low-density lipoprotein (oxLDL) induced p66shc phosphorylation at serine 36 residue and PKCβII phosphorylation in mouse endothelial cells. Adenoviral overexpression of APE1 resulted in reduction of oxLDL-induced p66shc and PKCβII phosphorylation. Phorbol 12-myristate 13-acetate (PMA), which stimulates PKCs, induced p66shc phosphorylation and this was inhibited by a selective PKCβII inhibitor. Adenoviral overexpression of PKCβII also increased p66shc phosphorylation. Overexpression of APE1 suppressed PMA-induced p66shc phosphorylation. Moreover, PMA-induced p66shc phosphorylation was augmented in cells in which APE1 was knocked down. PMA increased cytoplasmic APE1 expression, compared with the basal condition, suggesting the role of cytoplasmic APE1 against p66shc phosphorylation. Finally, vasoconstriction induced by phorbol-12,13, dibutyrate, another PKC agonist, was partially inhibited by transduction of Tat-APE1 into arteries.

Conclusion APE1 suppresses oxLDL-induced p66shc activation in endothelial cells by inhibiting PKCβII-mediated serine phosphorylation of p66shc, and mitigates vasoconstriction induced by activation of PKC.

Keywords p66shc • Apurinic/apyrimidinic endonuclease1 • Oxidized LDL • Protein kinase C • Endothelial cells

1. Introduction Oxidative stress negatively impacts vascular homeostasis and controls a number of signalling pathways relevant to vascular disease. P66shc belongs to the ShcA family of adaptor proteins and mediates oxidative stress in many cell types and tissues. Mice lacking the adaptor protein p66shc have increased resistance to oxidative stress and a 30% increase in life span. In the vasculature, p66shc plays an important role in endothelial dysfunction associated with pathophysiological conditions such as diabetes and hyperlipidaemia. ShcA proteins are phosphorylated at tyrosine residues in response to stimulation by a variety of growth factors and cytokines. However, p66Shc is functionally different from the p46 and p52 isoforms in that it also undergoes phosphorylation mainly at Ser36 after exposure to oxidative stress such as ultraviolet or H₂O₂. Phosphorylation at Ser36 is required for conferring increased susceptibility to oxidative stress and is critical for the cell death response elicited by oxidative damage. Therefore, prevention of this phosphorylation may have a therapeutic impact on diseases that are associated with oxidative damage. Activation of endothelial cells by oxidized low-density lipoprotein (oxLDL) is an early key event in hyperlipidaemia-induced endothelial dysfunction and atherosclerosis. OxLDL signals, in part, through p66shc. Apoptosis...
induced by oxLDL is associated with up-regulation of p66shc expression in intestinal cells. Moreover, p66shc phosphorylation is induced by protein kinase Cβ (PKCβ) which is activated by oxidative conditions in atherosclerosis.  

Among PKC isoenzymes, PKCβII was focused on the pathogenesis in cardiovascular disorder. Previously, reports showed that oxLDL-induced PKCβII membrane translocation and other PKC isoforms including PKCβ revealed no change in membrane translocation by oxLDL.  Also, other studies suggested that oxidative stress and hyperglycemia are able to trigger the activation of PKCβII. Diacylglycerol activates one or more PKC isoenzymes, PKCβII is preferentially activated in vascular smooth muscle and endothelial cells.  

Apurinic/apyrimidinic endonuclease1/redox factor-1 (APE1) is a multi-functional protein involved in base excision DNA repair and in transcriptional regulation of gene expression, and has a pleiotropic role in controlling cellular response to oxidative stress. APE1 reduces intracellular reactive oxygen species (ROS) production. Overexpression of APE1 suppresses tumour necrosis factor-alpha-induced monocyte adhesion to endothelial cells and inhibits hypoxia-induced endothelial cell apoptosis. Moreover, APE1 inhibits balloon injury-induced neointimal formation in rats, suggesting that it has an anti-inflammatory function in the vascular endothelium. However, whether APE1 affects p66shc, and in what context is unknown. Hypothesizing that APE1 suppresses cellular oxidative stress in part by inhibiting p66shc, we investigated the role and mechanism of APE1 in p66shc phosphorylation in endothelial cells.

2. Methods

2.1 Cell culture and reagent
Mouse MS-1 endothelial cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in Dulbecco's modified Eagle medium with 10% foetal bovine serum, 10 U/mL penicillin, and 10 µg/mL streptomycin. Antibodies to PKCβII (SC-210, APE1 (SC-17774), β-actin (SC-1616), GAPDH (SC-25778), and β-galactosidase (Adw1,856-b-galactosidase) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to phospho-ser36-p66shc (CIN56807, Calbiochem, La Jolla, CA, USA), phospho-PKCβII (AB5785, Abcam, Cambridge, MA, USA), SHC (C610082, BD Biosciences, Franklin Lakes, NJ, USA), and p84/N5 (Gene Tex, San Antonio, TX, USA) were used. Native LDL was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-human APE1 antibodies were purchased from Calbiochem, Go6976 was purchased from Tocris Bioscience (Bristol, UK). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

2.2 Adenoviral vector construction
Adenoviruses encoding β-galactosidase (Adβgal) and full-length APE1(A-dAPE1) were generated by homologous recombination in human embryonic kidney 293 cells, and have been described previously. Adβgal was used as an adenoviral control. Overexpression of human PKCβII in endothelial cells was accomplished with a replication-incompetent adenovirus created by the Viralpower adenovirus expression system (Invitrogen, Carlsbad, CA, USA). Briefly, human PKCβII was isolated from PKCβII cDNA in pBlsucreptR (C#M51010–7294004, Open Biosystems, Huntsville, AL, USA), which was matched with Genbank accession number BC036472 using the following primers with the restriction enzyme linker: sense primer: 5′- CGC GGA TCC ATG GCT GAC CGG GCT GCG-3′ (containing a BamH1 restriction site), anti-sense primer: 5′- CCG TGA TGG TAC GCT GAC TCG CCG-3′ (containing a XhoI restriction site). After BamHI and XhoI digestion, full-length hPKCβII constructs were cloned into the pENTR-CMV-FLAG vector, which has attL sites for site-specific recombination with a Gateway destination vector and an entry vector (Invitrogen). The site-specific recombination between hPKCβII cDNA in the pENTR-CMV-FLAG and the adenovirus vector pAdPL-DEST was achieved with LR clonase II. Cells were grown until an 80% cytopathic effect was seen, and then harvested to prepare the stock recombination adenovirus. The adenovirus was amplified in 293A cells and purified using the CsCl2 gradient technique, as described previously. Cells were infected with 200 multiplicity of infection (MOI; particle forming units per cell) of adenovirus for 18 h. The virus was removed, and the cells were incubated for another 24 h.

2.3 Oxidized LDL
To produce oxLDL, 1 mL of human native LDL (density 1.019–1.063 g/mL, 5 mg/mL) was diluted to 1.5 mL in EDTA-free PBS and incubated with 20 µM CuSO4 for 18 h at 37°C. At the end of the incubation, 0.1 mM EDTA was added to prevent further oxidation, and the oxLDL was concentrated to 1.4 mg/mL. The extent of LDL oxidation was assessed in a TBARS assay.

2.4 Small interfering RNA for APE1
Mouse small interfering RNA (siRNA) and scrambled siRNA were purchased from Bioneer Co. (Deajeon, South Korea). The cells were transfected with 20 nM chemically synthesized siRNA targeting mouse APE1 (5′-GUC UGG UAA GAC UGG AGU ACC-3′), and scrambled siRNA was used as a control. siRNA and lipofectamine 2000 were separately combined, and incubated for an additional 20 min followed by adding it to the cells for 48 h.

2.5 Recombinant Tat-APE1 protein expression and purification
Full-length human APE1 cDNA was cloned into the pTAT bacterial expression vector (pTAT-2.1, kindly provided by Steven Dowdy), which contains a six-histidine tag, for easy purification. pTAT-APE1 plasmids were then transformed into the BL21(DE3) strain of Escherichia coli. Following 4 h of IPTG induction, the cells were sonicated in buffer Z (8 M urea, 100 mM NaCl, and 20 mM HEPES), and recombinant proteins were purified on an Ni-NTA agarose column (Qiagen, Valencia, CA, USA). After washing, Tat-APE1 was eluted using 250 mM imidazole-containing buffer Z followed by desalting on a PD-10 column (Amersham Pharmacia Biotech, Piscataway, NJ, USA) in PBS. The eluate was frozen in 10% glycerol at −80°C.

2.6 Western blot analysis
MS-1 cells were harvested in 100 µL of lysis buffer containing 20 mM Tris-Cl (pH 7.5), 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1 mM Na3VO4, 1 mM β-glycerophosphate, 4 mM Na pyrophosphate, 5 mM NaF, 1% Triton X-100, and a protease inhibitor cocktail. The lysate was centrifuged at 12 000 rpm for 20 min, and the supernatant was collected. Protein (20–40 µg) was separated on 10% SDS–PAGE and was electrotransferred onto nitrocellulose membranes. After blocking with 5% skimmed milk for 2 h at room temperature, blots were incubated overnight at 4°C with specific primary antibody (1:1000) and subsequently with horseradish peroxidase-conjugated secondary antibody. Blots were developed for visualization using an enhanced chemiluminescence detection kit (Pierce Biotechnology, Rockford, IL, USA). Cells were serum starved for 18 h in some experiments to reduce basal phosphorylation of specific proteins. Nuclear and cytoplasmic extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit.

APE1 inhibits PKC-mediated p66shc phosphorylation
Protein concentration was measured with a Coomassie Protein assay kit (Pierce) using bovine serum albumin as the standard.

### 2.7 Immunofluorescent staining

For transfection and immunofluorescent staining, $5 \times 10^4$ cells of MS-1 were grown on glass coverslips, and then transiently transfected with 1 µg of pEGFP-APE1 by using lipofectamine 2000, as recommended by the manufacturer (Invitrogen). pEGFP-APE1 was generated by insertion of full length of human APE1 cDNA into pEGFP-N1 (Clonetech, Mountain View, CA, USA). Twenty-four hours after transfection, the cells were exposed to 100 nM PMA for 30 min and then fixed with ice-cold acetone. Coverslips were mounted on microscope slides, and fluorescence signals were visualized with a Zeiss fluorescent microscope.

### 2.8 Measurement of vasoreactivity

Male Sprague-Dawley rats 5–6 weeks old (150–200 g) were anaesthetized with a mixture of ketamine (80 mg/kg) and xylazine (12 mg/kg) intraperitoneally. Aortas were isolated, cleaned, and cut into rings (2–3 mm in width). Each ring was connected to an isometric force transducer (MultiMyograph 610M, Danish Myo Technology, Aarhus, Denmark), suspended in an organ chamber filled with 7.5 mL of Krebs buffer solution (NaCl, 99 mM; KCl, 4.7 mM; CaCl$_2$, 1.9 mM; MgSO$_4$, 1.2 mM; K$_2$HPO$_4$, 1.03 mM; NaHCO$_3$, 25 mM; Glucose 11.1 mM; pH 7.4) and aerated with 95% O$_2$/5% CO$_2$. Isometric tension was recorded continuously, as previously described. After a 1 h equilibration period, the rings were pre-contracted with PDBu (100 nM) and then isometric tension was observed with Tat-APE1 or Tat-GFP (100 nM). Relaxation was expressed as a percentage of the pre-contracted tension obtained with PDBu (100 nM). All in vivo procedures were in accordance with US National Institutes of Health guidelines and were approved by the institutional animal care and use committee of Chungnam National University (South Korea).

### 2.9 Statistical analysis

Values are expressed as means ± SEM. The statistical evaluation was conducted with a one-way analysis of variance followed by a Tukey’s post hoc test, and $P < 0.05$ was considered statistically significant.

### 3. Results

#### 3.1 Oxidized LDL-induced p66shc and PKCβII phosphorylation

To investigate whether oxLDL activated p66shc and PKCβII phosphorylation, we treated endothelial cells with oxLDL (80 µg/mL) for various times. As shown in Figure 1, oxLDL induced p66shc phosphorylation (Ser 36) and PKCβII phosphorylation (T641). p66shc and PKCβII phosphorylation were induced by oxLDL within 15 min, which remained for 60 min. However, total Shc and PKCβII were not changed by oxLDL in endothelial cells.

#### 3.2 APE1 suppressed oxLDL-induced p66shc phosphorylation and PKCβII phosphorylation

We investigated the effect of APE1 on oxLDL-induced p66shc phosphorylation. In Adβgal-infected endothelial cells, oxLDL (80 µg/mL) also increased p66shc phosphorylation within 30–60 min; however, APE1 overexpression using adenoviral APE1 gene transfer significantly reduced oxLDL-induced p66shc phosphorylation in endothelial cells (Figure 2A and B). Next, we investigated the effect of APE1 overexpression on oxLDL-induced PKCβII phosphorylation. oxLDL (80 µg/mL) increased PKCβII phosphorylation within 15 min, which returned to basal levels within 24 h. However, APE1 overexpression

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**Figure 1** Oxidized LDL (oxLDL) increases p66shc and PKCβII phosphorylation in the MS-1 endothelial cell line. Cells were harvested after treatment with oxidized LDL (80 µg/mL) for the indicated times. Expression of p66shc phosphorylation and PKCβII phosphorylation were measured by western blotting. (A) Typical representative data. (B and C). Summarized data. Each bar represents the ratio of endogenous p66shc or PKCβII. Each bar shows the mean ± SEM ($n=4$); *$P < 0.05$ vs. control.
significantly reduced oxLDL-induced PKCβII phosphorylation (Figure 2C and D).

3.3 PMA or oxLDL-induced p66shc phosphorylation: involvement of PKCβII
To investigate whether p66shc phosphorylation was activated by PKCβII, we examined the effect of a PKC antagonist on PMA or oxLDL-induced p66shc phosphorylation. The exposure of PMA (100 nM) or oxLDL (80 μg/mL) for 30 min markedly increased p66shc phosphorylation, which was inhibited significantly by 10 nM PKCβi, a specific inhibitor of PKCβII (PKCβi, aniline-monoindolylmaleimide inhibitor) (Figure 3A and B). To confirm whether p66shc phosphorylation was related to PKCβII activation, we examined the effect of PKCβII overexpression using an adenoviral flag-tagged PKCβII gene transfer on p66shc phosphorylation. Adenoviral flag-tagged PKCβII gene transfer in a range of 100–200 MOI successfully induced PKCβII expression, as assessed by western blot for FLAG. AdPKCβII-infected endothelial cells showed an increase in p66shc phosphorylation compared with Adβgal-infected endothelial cells, suggesting PKCβII-induced p66shc phosphorylation (Figure 3C). Therefore, our data suggested that PMA-induced p66shc phosphorylation can be mediated by PKCβII activation in endothelial cells.

3.4 APE1 suppressed PMA-induced p66shc phosphorylation
As we established that PMA induced p66shc phosphorylation, we next examined the effect of APE1 overexpression on p66shc phosphorylation in endothelial cells. PMA (100 nM, 30 min) markedly induced p66shc phosphorylation (S36), which was significantly inhibited by Go6976 (1 μM), an inhibitor of the PKCa/β isoenzyme. However, APE1 overexpression using AdAPE1 significantly suppressed PMA-induced p66shc phosphorylation, as shown in Figure 4A. A densitometric analysis is plotted in Figure 4B. To investigate whether basal APE1 influences PMA-induced p66shc phosphorylation, we evaluated the effect of APE1 siRNA on PMA-induced p66shc phosphorylation. Following the treatment of endothelial cells with 20 nM APE1-specific siRNA for 48 h, endogenous APE1 expression was efficiently reduced compared with scrambled siRNA-transfected cells. PMA (1–100 nM) increased p66shc phosphorylation in a dose-dependent manner in scrambled siRNA-transfected cells. Even basal p66shc phosphorylation was not increased by the treatment with APE1 siRNA, but PMA-induced p66shc phosphorylation was increased in endothelial cells transfected with APE1 siRNA (Figure 4C). These data suggested that gene APE1 silencing increased PMA-induced p66shc phosphorylation in endothelial cells.

3.5 PKC activations induced cytoplasmic translocation of APE1
Under basal conditions, APE1 is localized mostly in the nucleus, whereas p66shc is mainly localized in the cytoplasm and/or mitochondria. We next focused on how APE1 inhibited PKC-induced p66shc phosphorylation. In the pEGFP-APE1-transfected MS-1, we investigated the effect of PMA on APE1 translocation. Twenty-four hours after transfection, the cells were exposed to 100 nM of PMA for 30 min and then visualized with fluorescent microscopy. As shown in Figure 5A, in the basal state, APE1 was mainly localized to the nucleus of MS-1 cells (a.c). However, cytoplasmic APE1 expression

![Figure 2](https://academic.oup.com/cardiovascres/article-abstract/91/3/502/308015)}
Figure 3 PKCβII activates p66shc phosphorylation in the MS-1 endothelial cell line. (A) Effect of PKCβi on PMA (100 nM)-induced p66shc phosphorylation. PKCβi (10 nM), a specific PKCβII inhibitor (PKCβi, aniline-monoindolylmaleimide inhibitor). (B) Effect of PKCβi on oxLDL (80 μg/mL)-induced p66shc phosphorylation. PKCβi was pretreated for 10 min before exposure to PMA or oxLDL. (C) Effect of PKCβII overexpression on p66shc phosphorylation. PKCβII overexpression with AdPKCβII was confirmed by western blot for FLAG-PKCβII. Total adenoviral gene transfection was balanced with 200 MOI Adβgal as a control virus. Each bar shows the mean ± SEM (n = 3); *P < 0.05 vs. control.

Figure 4 APE1 controls PMA-induced p66shc phosphorylation in the MS-1 endothelial cell line. (A) APE1 overexpression significantly suppressed PMA-induced p66shc phosphorylation. Go6976 (1 μM) was pretreated for 10 min before exposure to PMA. (B) Denstometric analysis of p66shc phosphorylation. Each bar shows the mean ± SEM (n = 4); *P < 0.05 vs. PMA control; #P < 0.05 vs. Adβgal. (C) Gene silencing of APE1 with siRNA increased PMA-induced p66shc phosphorylation. (D) Denstometric analysis of p66shc phosphorylation. Cells were transfected with scrambled siRNA (sc siRNA) or APE1 siRNA (20 nM) using lipofectamine 2000 for 48 h. Each bar shows the mean ± SEM (n = 4); *P < 0.05 vs. control siRNA.
was detected in the cells that were exposed to PMA for 30 min (bd). Under a non-stimulated condition, APE1 was detected mainly in the nuclear fraction, as assessed by western blotting. However, cells treated with PMA for 15–60 min showed increased cytoplasmic APE1 expression, suggesting APE1 cytoplasmic translocation (Figure 5B and C).

### 3.6 Cell-permeable APE1 (Tat-APE1) inhibited PKC-induced vasoconstriction

Tat-APE1 is a cell-permeable APE1 that inhibits monocyte adhesion in endothelial cells. Finally, we investigated whether Tat-APE1 affected PKC agonist-induced vasoconstriction in rat aorta. To activate PKC in the rat aortas, we used PDBu to activate PKC in rat aorta. PDBu (100 nM)-induced tonic contraction in aortic rings, as shown in Figure 6A. However, Tat-APE1 (10–100 nM) exposure inhibited PDBu-induced contraction compared with Tat-GFP used as a control (Figure 6B).

### 4. Discussion

It is widely accepted that the p66shc adaptor protein controls oxidative stress and life span in mammals and genetic deletion of the p66shc adaptor protein prevents endothelial dysfunction and oxidative stress. As p66shc is activated by oxidative stress, the endogenous biomolecules for preventing p66shc phosphorylation may have a therapeutic impact on oxidative stress diseases. APE1 is a multi-functional protein involved in base excision DNA repair and transcriptional regulation of gene expression. The multifunctional nature of APE1 is being uncovered and has been extensively studied in the cellular response against oxidative stress.

In the present study, we demonstrated that APE1 inhibited oxLDL or PKC-induced p66shc phosphorylation in endothelial cells. The inhibitory action of APE1 on oxLDL-induced p66shc phosphorylation was mediated by inhibiting PKC\(_{\beta II}\) phosphorylation. Furthermore, cell-permeable APE1 inhibited PKC-induced vasoconstriction, suggesting that APE1 plays a crucial inhibitory role in p66shc phosphorylation.

Oxidative stress, such as hydrogen peroxide, is able to phosphorylate p66shc via PKC\(_{\beta II}\) activation in MEF cell lines. Hispidin, a specific blocker of a PKC\(_{\beta II}\) isoform, inhibits p66shc phosphorylation. However, it is unknown which type of PKC\(_{\beta}\) isoforms affect p66shc phosphorylation in endothelial cells. In the present study, our data showed that transfection of PKC\(_{\beta I}\) increased p66shc phosphorylation, but PKC\(_{\beta I}\) did not increase it (Supplementary material online, Figure S1). Also, we verified that inhibiting PKC\(_{\beta II}\) reduced PKC agonist-induced p66shc phosphorylation. The PKC\(_{\beta}\) inhibitor, an anilino-monoindolylmaleimide compound, acts as a potent, cell-permeable, reversible, and ATP-competitive inhibitor of PKC\(_{\beta}\) isoforms (IC\(_{50}\) = 5 nM and 21 nM for human PKC\(_{\beta I}\) and \(\beta I\), respectively) and displays greater selectivity over PKC\(_{\alpha}, \rho, \text{ and } \varepsilon\) (IC\(_{50}\) = 331 nM, >1 \(\mu M\), and 2.8 \(\mu M\), respectively). We used 10 nM of a PKC\(_{\beta}\) inhibitor to specifically inhibit PKC\(_{\beta II}\). Treatment with 10 nM of a PKC\(_{\beta}\) inhibitor suppressed PKC agonist-induced p66shc phosphorylation, suggesting the involvement of PKC\(_{\beta II}\). We also confirmed that adenoviral overexpression of human-specific PKC\(_{\beta II}\) cDNA increased p66shc phosphorylation (Figure 3). Therefore, our data strongly suggested that PKC\(_{\beta II}\) is upstream of p66shc phosphorylation in endothelial cells.
OxLDL is an important cardiovascular disease biomarker. Hypercholesterolaemia increases ROS production, resulting in lipid and protein oxidation and peroxidation. p66shc(–/–) mice are more resistant to high-fat diet-induced atherogenesis and reduced expression of oxidation markers. Among the PKCs, PKC is a classic PKC, which is activated by calcium, diacylglycerol, and phorbol esters. Activation of PKC isoforms causes vascular dysfunction. Recent data have shown that a reduction of PKC attenuates neointimal formation in response to acute arterial injury in mice. In the present study, we confirmed that oxLDL exposure increased PKC phosphorylation and p66shc phosphorylation in endothelial cells. Moreover, oxLDL-induced PKC phosphorylation was suppressed by APE1 overexpression. These data suggest that APE1 plays an inhibitory role against oxLDL-induced p66shc phosphorylation by inhibition of PKC phosphorylation in endothelial cells. In rat aorta, we also demonstrated that cellular transduction of APE1 using Tat-APE1 inhibited PKC-induced vasoconstriction. This finding indicates that the cellular transduction of APE1 may be useful to reduce PKC-induced vascular dysfunction (Figure 6). Endothelial dysfunction is mediated by multiple mechanisms in the endothelium. Increase in p66 phosphorylation due to PKC activation could lead to endothelial dysfunction through several mechanisms including increase in endothelial production of ROS, and decrease in endothelial nitric oxide. Our in vitro data in endothelial cells, and ex vivo data in vascular rings, taken together, with the known role of endothelial p66shc in mediating endothelial dysfunction, suggests that APE1 mitigates endothelial dysfunction induced by PKC.

Some evidence suggests that APE1 expression and subcellular localization are finely tuned. The subcellular distribution of APE1 may be regulated by both nuclear import and export. APE1 is a ubiquitous protein, but its expression pattern differs based on the cell type. APE1 subcellular localization is mainly nuclear, but cytoplasmic staining has also been reported, the latter being associated with mitochondria and/or the endoplasmic reticulum. Subcellular localization of APE1 is crucial for its activity. In the present study, we investigated how APE1 is involved in the regulation of p66shc activation. Basal cytoplasmic APE1 was ~20% of nuclear APE1 in endothelial cells. However, exposure to PKC agonists, such as PMA, increased cytoplasmic APE1, suggesting the cytoplasmic translocation of APE1 in endothelial cells. As acute exposure of PMA for 1 h has limited ability to induce de novo APE1 protein expression, increased cytoplasmic APE1 by PMA would be due to cytoplasmic translocation of APE1 in endothelial cells. Recently, Yuk et al. showed that forced cytoplasmic APE1 overexpression profoundly attenuates up-regulation of HMGB1-mediated ROS, cytokine secretion, and cyclooxygenase-2 expression in primary monocytes and macrophage-like THP-1 cell lines. Therefore, it is reasonable that cytoplasmic APE1 has been proposed as a potential therapeutic modality for inflammatory diseases.

In the present study, there was some limitation. PKC inhibitors, such as PKCi and Go6976, did not completely suppress PMA-induced p66shc phosphorylation. Go6976 further suppressed PMA-induced p66shc phosphorylation in APE1-overexpressed endothelial cells, indicating that APE1 inhibition of PMA-induced p66shc phosphorylation may also involve a PKC-independent pathway in addition to PKC pathway. However, the role of APE1 on PKC-independent pathway needs further investigation.

Because APE1 is affected by p66shc phosphorylation in response to oxLDL or PKC, the present finding will reasonably improve the understanding of the crosstalk between APE1 and p66shc and potentially produce therapeutic modes against oxidative vascular diseases.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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