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Protective mechanisms of resveratrol derivatives against TNF-α-induced inflammatory responses in rat mesangial cells

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ABSTRACT

Introduction: Resveratrol has been reported to alleviate inflammatory responses and oxidative stress in mesangial cells and in several types of renal injury in animal models. Previously, the active resveratrol derivatives from the roots of *Vitis thunbergii* Sieb. & Zucc. (Vitaceae) were shown to have significant anti-platelet and anti-oxidative activities. However, the anti-inflammatory mechanisms of these resveratrol derivatives in rat mesangial cells (RMCs) have not been clarified fully.

Methods: The protective mechanisms of resveratrol derivatives involved in tumor necrosis factor-α (TNF-α)-induced inflammatory responses were assessed by Western blot analysis, real-time PCR, and RT–PCR. The involvement of various signaling molecules in these responses was investigated using selective pharmacological inhibitors.

Results: Nontoxic concentrations of the resveratrol derivatives significantly attenuated cytosolic phospholipase A_2 ($cPLA_2$) and cyclooxygenase 2 (COX-2) expression in RMCs challenged by TNF-α. These resveratrol derivatives inhibited TNF-α-activated ERK1/2 and JNK1/2 without affecting p38 phosphorylation. Next, we demonstrated that TNF-α induced NF-κB activation, translocation, and promoter activity, which was inhibited by pretreatment with resveratrol derivatives in RMCs.

Conclusion: The protective mechanisms of resveratrol derivatives against TNF-α-stimulated inflammatory responses via $cPLA_2$/COX-2/PGE_2 inhibition was caused by the attenuation of the JNK1/2, ERK1/2, and NF-κB signaling pathways in RMCs.

1. Introduction

The tumor necrosis factor-α (TNF-α) is a major proinflammatory cytokine in kidney pathogenesis that damages the glomerular permeability barrier with the development of albuminuria [1,2]. Blocking TNF-α activity with a recombinant TNF-α-binding protein reduced neutrophil infiltration and ameliorated ischemic injury in kidneys [3]. As an extrinsic source or positive feedback loop, binding of TNF-α to its membrane receptors can reactivate signaling pathways and further augment TNF-α production and the expression of inflammatory mediators in renal resident cells [1]. Some studies have shown that TNF-α is implicated in inflammation by upregulating inflammatory genes such

Abbreviations: COX, cyclooxygenase; $cPLA_2$, cytosolic phospholipase A_2; PGE_2, prostaglandin E_2; RMCs, rat mesangial cells; TNF-α, Tumor necrosis factor α

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as cyclooxygenase 2 (COX-2) and cytosolic phospholipase A_2 ($cPLA_2$) [4]. In addition, prostaglandin E_2 (PGE_2), which is one of the most abundant metabolites of arachidonic acid (AA), is generated through an enzymatic cascade controlled by COX enzymes and is a principal mediator of inflammation in diseases [4]. Therefore, $cPLA_2$ and COX-2 may play a crucial role in the development of a variety of renal inflammatory diseases. However, the molecular mechanisms underlying TNF-α stimulation of $cPLA_2$ and COX-2 in renal mesangial cells remain largely unknown.

Mitogen-activated protein kinases (MAPKs) are a type of serine/threonine protein kinases. They are phosphorylated by many stimuli, such as cytokines and growth factors [5]. It was found that inflammation elicited by various external stimuli activates NF-κB, which regulates the expression of many inflammatory-related genes, such as *cPLA$_2$* and *COX-2*. NF-κB activity is regulated by several pathways, including the activation of various MAPKs [4]. Thus, we explored the roles of NF-κB and MAPKs in $cPLA_2$ and COX-2 induction in rat mesangial cells (RMCs) challenged with TNF-α.

Resveratrol has been reported to ameliorate the increase in vasoconstrictors and intracellular calcium in mesangial cells and in several types of renal injury in animal models, including diabetic nephropathy and hyperuricemia [6]. Recently, many reports have described resveratrol derivatives that are more effective than resveratrol itself and inhibit tumor cell proliferation and inflammation [7,8]. Previously, active resveratrol derivatives, such as ampelopsin C (AC) and ampelopsin F (AF) from the roots of *Vitis thunbergii* Sieb. & Zucc. (Vitaceae), were shown to have significant anti-platelet and anti-oxidative activities [9,10]. Moreover, both AC and AF are stilbene oligomers; AC is a trimer form of resveratrol, whereas AF is a dimer form of resveratrol [8]. Polydatin (PD) is the glycoside form of resveratrol [11]. Accumulating evidence indicates that resveratrol derivatives may attenuate inflammatory signaling pathways [7,12,13]. PD has been shown to possess strong anti-oxidative bioactivity [14] and to alleviate experimental-diabetes-induced extracellular matrix accumulation through anti-inflammatory mechanisms in rat glomerular mesangial cells [15]. The nephroprotective effects of PD were attributed to its anti-inflammatory cascade, including the inhibition of the expression of the NF-κB (p65), COX-2, and inducible nitric oxide synthase (iNOS) proteins and the production of TNF-α, PGE_2, and IL-1β [16]. Qi et al. reported that ampelopsin has anti-inflammatory effects via the inhibition of the Phosphoinositide 3-kinase, Protein kinase B, and NF-κB signaling cascades in RAW264.7 murine macrophage-like cells [17]. Ampelopsin can inhibit LPS-induced inflammatory responses via the NF-κB and JAK2/STAT3 signaling pathways in microglial cells [18]. However, the anti-inflammatory mechanisms of ampelopsin in renal diseases *in vivo* or *in vitro* have not been reported.

Therefore, we investigated whether resveratrol derivatives inhibit TNF-α-induced $cPLA_2$ and COX-2 expression and elucidated the underlying mechanisms. Currently, our laboratory uses RMCs as an *in vitro* cell culture model to examine the effects of AC, AF, and PD on TNF-α-induced inflammatory responses. Here, we established for the first time in RMCs that resveratrol derivatives reduced $cPLA_2$/COX-2 expression and PGE_2 production via the JNK1/2, ERK1/2, and NF-κB signaling pathways in response to TNF-α.

2. Materials and methods

2.1. Plant materials

The roots of *V. thunbergii* were purchased in Taipei and identified by Mr. Jun-Chih Ou, a taxonomist previously with the National Research Institute of Chinese Medicine. The specimen (NRICM-05-018) is preserved at the National Research Institute of Chinese Medicine, Republic of China. AC and AF were isolated from the EtOH extracts of *V. thunbergii*, as described previously [10], and the purities of AC and AF were determined to be greater than 97% by high-performance liquid chromatography analysis. Their structures were determined by spectroscopic analyses and by comparison with those available in the literature [10]. PD was from Sigma-Aldrich (St. Louis, MO, USA). AC, AF, or PD was dissolved in dimethylsulfoxide (DMSO) and then further diluted with the assay medium (2, 5, 10 μg/ml). Solvent controls with DMSO were used in each assay.

2.2. Materials

The anti-phospho-c-Jun, anti-phospho-p65, anti-phospho-ERK1/2, anti-phospho-p38, anti-phospho-JNK1/2, anti-COX-2, and anti-$cPLA_2$ antibodies were from Cell Signaling Technology (Danvers, MA, USA). The anti-p65 and anti-lamin B antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). AACOCF3, Bay 11-7082, SP600125, SB202190, and U0126 were from Enzo Life Sciences (Farmingdale, NY, USA). Human recombinant TNF-α was from R&D Systems (Minneapolis, MN, USA). The anti-GAPDH antibody, enzymes, and other chemicals were from Sigma-Aldrich.

2.3. Cell culture

The rat mesangial cell line was bought from the American Type Culture Collection (ATCC; Rockville, MD, USA) and used according to the instructions provided. In experiments, cells were cultured with resveratrol derivatives or inhibitors of kinases for 1 h prior to TNF-α treatment in a 5% CO_2-humidified incubator at 37 °C.

2.4. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

RMCs (2.5×10^5 cells per well) were incubated at 37 °C with varying concentrations of AC, AF, and PD, followed by the addition of the MTT solution (0.25 mg/ml) for 30 min. After incubation, the DMSO solution was added to dissolve the formazan crystals, and the supernatants were surveyed at A540 nm on a microplate reader (SpectraMax 250, Molecular Device, CA, USA). The results were determined by comparing the optical density of the drug-treated group with that of the vehicle-treated group (1% DMSO).

2.5. Isolation of cell fractions

After treatment, cells were harvested, sonicated, and centrifuged and the cytoplasmic and nuclear proteins separated, as per the workflow steps described previously [19]. The translocation of NF-κB (p65) from the cytoplasm into the nucleus was analyzed by Western blotting.

2.6. Immunofluorescence staining

Growth-arrested RMCs were stimulated with TNF-α at different time points. In addition, cells were treated with inhibitors of kinases for 1 h prior to TNF-α treatment. After treatment, cells were washed with phosphate buffered saline, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with blocking buffer (Visual Protein Biotech Corporation, Taipei, Taiwan), and stained with an anti-NF-κB (p65) antibody, as per the workflow steps described previously [19]. The distribution of NF-κB (p65) movement and the nucleus were observed using a fluorescence microscope (DFC310 FX; Leica, Wetzlar, Germany).

2.7. Western blotting

After the treatment of cells with drugs and TNF-α, cell lysates were collected and the protein concentrations were adjusted, as described previously [19]. Briefly, samples were separated on sodium dodecyl sulphate–polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA), which were

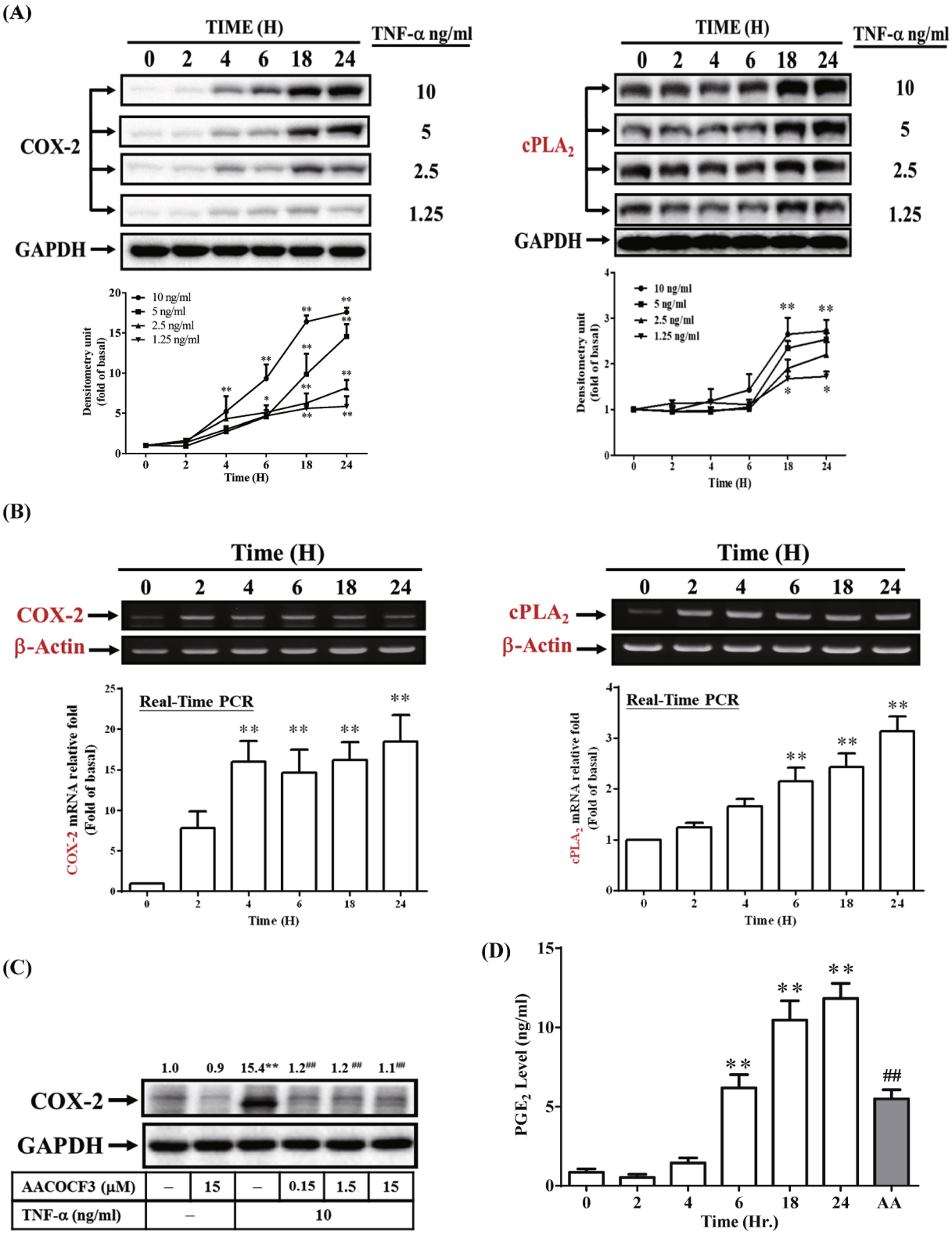

Fig. 1. Time and concentration dependence of TNF-α-stimulated COX-2 and $cPLA_2$ expression in RMCs. Cells were incubated with different concentrations of TNF-α (10, 5, 2.5, or 1.25 ng/ml) for various times. The expression of the COX-2 and $cPLA_2$ proteins was determined by Western blotting (A). The mRNA levels of COX-2 and $cPLA_2$ were determined by RT–PCR and real-time PCR (B). (C) Cells were pretreated with AACOCF3 for 1 h and then incubated with TNF-α (10 ng/ml) for 24 h. The expression of the COX-2 protein was determined by Western blotting. (D) Cells were treated with TNF-α (10 ng/ml) for the indicated times or pretreated with AACOCF3 for 1 h, followed by incubation with TNF-α (10 ng/ml) for 24 h. The media were collected and analyzed for PGE_2 release. Data are expressed as the mean ± S.E.M. of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ compared with cells exposed to the vehicle. $^{\#\#}P < 0.01$ compared with cells exposed to TNF-α alone.

then blocked and incubated with primary and secondary antibodies. Between this two-step process, membranes were washed with Tris buffered saline with Tween-20 solution. Immunofluorescent signals were developed using the LumiFlash Ultima chemiluminescent substrate HRP system (Visual Protein Biotech Corporation). The levels of specific proteins were quantified using Image LabTM 5.0 Software (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and normalized to GAPDH levels (which was used as a control).

2.8. RT–PCR and real-time PCR

Total RNA was extracted with TRIzol reagent (Invitrogen, CA, USA) and used as a template for cDNA synthesis. RNA concentration and purity were determined spectrophotometrically. The expression of inflammatory genes was measured using RT–PCR and real-time PCR system kits (Bio-Rad Laboratories). The PCR conditions were as follows: one cycle of initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 1 min, primer annealing at 58 °C for 1 min, and extension at 72 °C for 2 min; and one cycle of final extension at 72 °C for 12 min. The expression of β-actin was used as an internal control for the assay of a constitutively expressed gene. The primers used for RT–PCR were as follows: 5′–GAACCCTAAGGCCAACCGTG–3′ (forward) and 5′–TGGCATAGAGGTCTTTACGG–3′ (reverse) for β-actin; 5′–TGTTCAACA GAGTTTTGG–3′ (forward) and 5′–AACAGAGCAACGAGATGG–3′ (reverse) for $cPLA_2$; and 5′–CTGTATCCCGCCGCCCTGCTGGTG–3′ (forward) and 5′–ACTTGCGTTGATGGTGGCTGTCT–3′ (reverse) for COX-2. Real-time PCR was performed using the CFX Connect Real-time PCR Detection System (Bio-Rad Laboratories). Standard thermal conditions

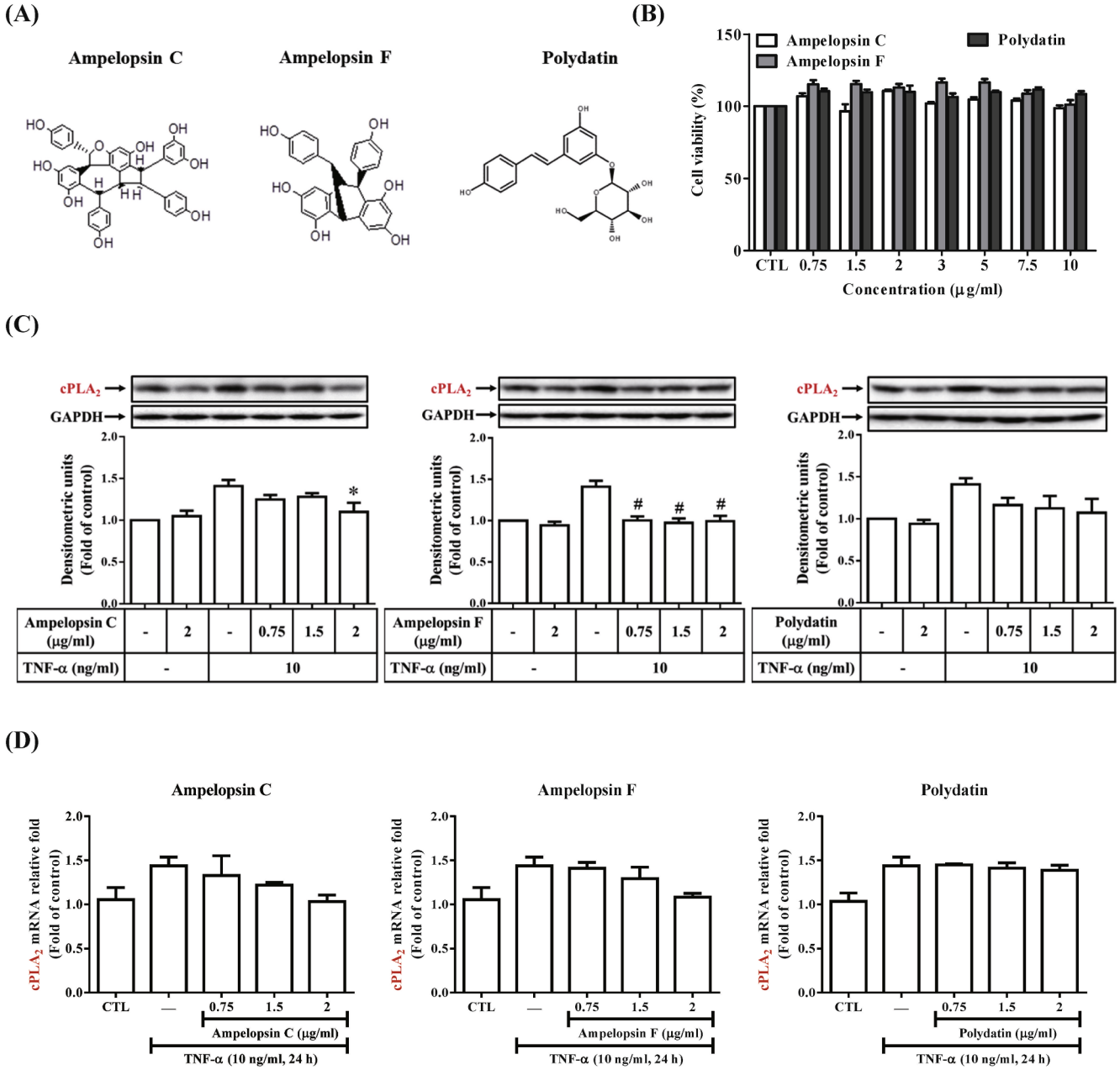

Fig. 2. Effects of AC, AF, and PD on TNF-α-induced $cPLA_2$ expression in RMCs. (A) Chemical structure of AC, AF, and PD. (B) Cells were treated with various concentrations of AC, AF, or PD for 24 h. Cell viability was assayed using the MTT assay. (C) Cells were pretreated with AC, AF, or PD for 1 h, and then exposed to TNF-α (10 ng/ml) for 24 h. The expression of the $cPLA_2$ protein was determined by Western blotting. (D) Cells were pretreated with AC, AF, or PD for 1 h, and then incubated with TNF-α for 6 h. The mRNA levels of $cPLA_2$ were analyzed by real-time PCR. Data are expressed as the mean ± S.E.M. of three independent experiments. $^{*}P < 0.05$; $^{\#}P < 0.01$ compared with cells exposed to TNF-α alone.

(3 min at 95 °C, 40 cycles of 10 s at 95 °C and 30 s at 58 °C) were used. Relative gene expression was determined by the $2^{-\Delta\Delta Ct}$ method, where Ct is the threshold cycle. Gene expression was normalized relative to unstimulated cells and fold variation was normalized to β-actin (an endogenous control). The primers used for real-time PCR were as follows: 5′–CGTGAAAAGATGACCCAGATCA–3′ (forward) and 5′–CTCCG GAGTCCATCACAATG–3′ (reverse) for β-actin; 5′–ACATTCAGGCAGCA GAGGA–3′ (forward) and 5′–CCACCACAGGCACATCAC–3′ (reverse) for $cPLA_2$; and 5′–CAAGAATCAAATTACCGCTGAAG–3′ (forward) and 5′–CGAAGGAAGGGAATGTTGTT–3′ (reverse) for COX-2.

2.9. Measurement of NF-κB-luciferase activity

Cells were transiently transfected with an NF-κB-luciferase reporter plasmid using the Gene Pulser Xcell Electroporation System (Bio-Rad Laboratories). Cells (1.0×10^6 cells/ml) were washed, suspended in Opti-MEM medium, and placed into a 2 mm electroporation cuvette (Bio-Rad Laboratories). A reporter gene (10 μg) was added into the cuvette containing the cells, which was subjected to a high-voltage electrical pulse at 260 V, 950 μF, ∞Ω, exponential decay pulse. After electroporation, cell suspensions were immediately transferred to 10% FBS growth medium (without antibiotics) and incubated for 24 h. The luciferase assay was performed after the electroporation. The medium was changed to fresh 10% FBS growth medium (without antibiotics) before RMCs were treated with or without resveratrol derivatives (10 μg/ml) for 1 h prior to TNF-α treatment. NF-κB-luc activity was analyzed using a luciferase assay system (Promega, Madison, WI, USA) and read using a SpectraMax i3x microplate reader. The luciferase activity was normalized to the β-galactosidase activity.

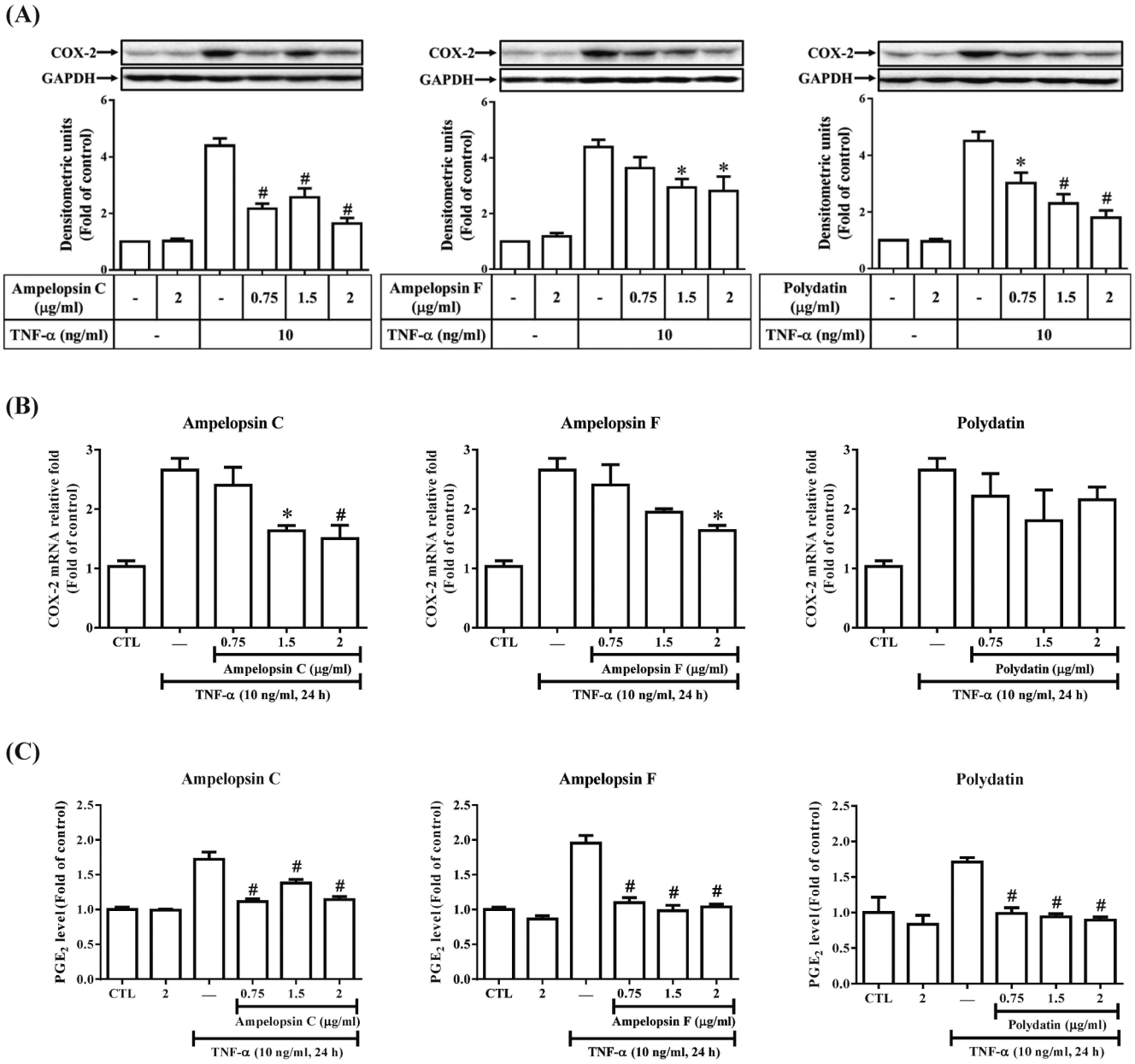

Fig. 3. Effects of AC, AF, and PD on TNF-α-induced COX-2 expression and PGE_2 production in RMCs. (A) Cells were pretreated with AC, AF, or PD for 1 h, and then exposed to TNF-α (10 ng/ml) for 24 h. The expression of the COX-2 protein was determined by Western blotting. (B) Cells were pretreated with AC, AF, or PD for 1 h, and then incubated with TNF-α for 6 h. The mRNA levels of COX-2 were analyzed by real-time PCR. (C) Cells were pretreated with AC, AF, or PD for 1 h, and then exposed to TNF-α (10 ng/ml) for 24 h. The media were collected and analyzed for PGE_2 release. Data are expressed as the mean ± S.E.M. of three independent experiments. $^{\#}P < 0.01$ compared with cells exposed to TNF-α alone.

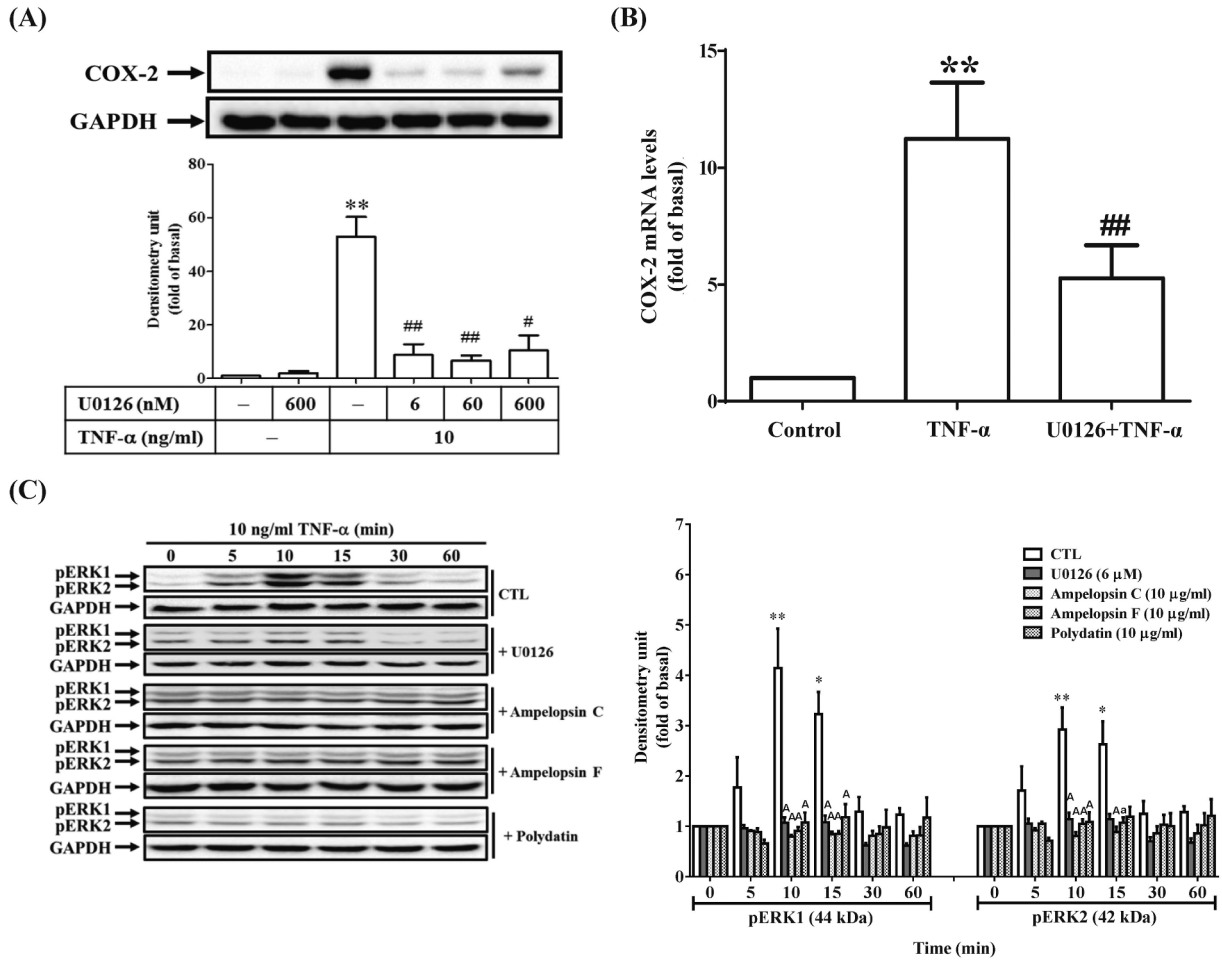

Fig. 4. AC, AF, and PD inhibit TNF-α-induced COX-2 expression via the inhibition of ERK1/2 activation in RMCs. (A) RMCs were pretreated with U0126 (6, 60, or 600 nM) for 1 h, and then incubated with TNF-α for 24 h. The expression of the COX-2 protein was determined by Western blotting. (B) Cells were pretreated with U0126 for 1 h, and then incubated with TNF-α for 6 h. The mRNA levels of COX-2 were analyzed by real-time PCR. (C) Cells were pretreated with or without U0126 (600 nM), AC (10 μg/ml), AF (10 μg/ml), or PD (10 μg/ml) for 1 h, and then treated with TNF-α for the indicated time. The expression of phospho-ERK1/2 was determined by Western blotting. Data are expressed as the mean ± S.E.M. of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ compared with the cells exposed to the vehicle. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ compared with cells exposed to TNF-α alone for 24 h. $^{a}P < 0.05$; $^{A}P < 0.01$ compared with the cells exposed to TNF-α alone for 10 or 15 min.

2.10. Determination of PGE_2 release

Confluent RMCs were treated with or without resveratrol derivatives for 1 h, followed by incubation with TNF-α for 24 h. The supernatants were then collected for PGE_2 production analysis. The expression of PGE_2 was measured using a PGE_2 ELISA kit (Enzo Life Sciences) according to the manufacturer's instructions.

2.11. Statistical analysis

Results were statistically analyzed using GraphPad Prism 6 Software (GraphPad Software, Inc., CA, USA). Quantitative data were analyzed by two-way ANOVA followed by Bonferroni's honestly significant difference between individual groups. Data are presented as the mean ± S.E.M. of at least three independent experiments. $P < 0.05$ was considered statistically significant.

3. Results

3.1. TNF-α induced proinflammatory cytokine expression in RMCs

The literature shows that TNF-α is implicated in the pathogenesis of kidney injury and glomerular permeability barrier damage by upregulating proinflammatory cytokines [1,2]. To determine the effects of TNF-α, RMCs were challenged with different concentrations of this molecule. As shown in Fig. 1A, TNF-α induced COX-2 and $cPLA_2$ expression in time- and concentration-dependent manners. In addition, cells were treated with TNF-α (10 ng/ml) for the indicated time intervals, and the mRNA expression of COX-2 and $cPLA_2$ was determined by RT–PCR and real-time PCR. As shown in Fig. 1B, TNF-α markedly enhanced COX-2 and $cPLA_2$ mRNA expression in a time-dependent manner in RMCs. PGE_2, which is one of the most abundant metabolites of AA, is generated through an enzymatic cascade controlled by COX enzymes and is a principal mediator of inflammation in diseases [4]. Thus, we further assessed whether TNF-α induces PGE_2 generation mediated by $cPLA_2$ and COX-2 upregulation. As shown in Fig. 1C, pretreatment with an inhibitor of $cPLA_2$ (AACOCF3) [20] significantly attenuated COX-2 expression in RMCs in response to TNF-α. In addition, TNF-α markedly induced PGE_2 release in a time-dependent manner (Fig. 1D). However, pretreatment with AACOCF3 also significantly attenuated TNF-α-induced PGE_2 production (Fig. 1D). These results suggest that TNF-α enhances PGE_2 generation via $cPLA_2$/COX-2 upregulation in RMCs.

3.2. Inhibitory effects of resveratrol derivatives on TNF-α-induced $cPLA_2$/COX-2 expression and PGE_2 release in RMCs

Fig. 2A shows the chemical structures of resveratrol derivatives (AC, AF, and PD). Here, we examined the effects of AC, AF, and PD on the viability of RMCs using an MTT assay. As shown in Fig. 2B, AC, AF, and PD (0.75–10 μg/ml) had no effects on the viability of RMCs. Therefore, we used AC, AF, and PD at a concentration < 10 μg/ml in this study and evaluated their potential anti-inflammatory and pharmacological properties in TNF-α-stimulated RMCs. Next, we investigated the effects of resveratrol derivatives on TNF-α-induced $cPLA_2$ expression. As shown in Fig. 2C, pretreatment with AC or AF at 2 μg/ml significantly attenuated TNF-α-induced $cPLA_2$ protein expression, but did not affect $cPLA_2$ mRNA levels significantly (Fig. 2D). In addition, pretreatment with PD did not affect TNF-α-induced $cPLA_2$ expression. We further evaluated the effects of AC, AF, and PD on TNF-α-induced COX-2 expression and PGE_2 release in RMCs. Cells were pretreated with AC, AF, or PD for 1 h, and then incubated with TNF-α for 24 h. As shown in Fig. 3A, pretreatment with AC, AF, or PD significantly attenuated TNF-α-induced COX-2 expression in a concentration-dependent manner. In addition, both AC and AF at 2 μg/ml significantly inhibited COX-2 mRNA levels (Fig. 3B). Finally, we demonstrated that TNF-α-induced PGE_2 release was reduced by pretreatment with AC, AF, or PD in RMCs (Fig. 3C), although increased concentrations (up to 2 μg/ml) did not yield an increase in the inhibitory effect. Taken together, these results showed that resveratrol derivatives (AC, AF, and PD) have an inhibitory effect on TNF-α-induced $cPLA_2$/COX-2 expression and PGE_2 production in RMCs.

3.3. Resveratrol derivatives inhibited TNF-α-induced COX-2 expression via the downregulation of ERK1/2 activation in RMCs

To investigate whether MAPKs are involved in TNF-α-induced COX-2 expression, cells were pretreated with an inhibitor of MEK1/2 (U0126) [21] for 1 h, and then incubated with TNF-α for 24 h. As shown in Fig. 4A, pretreatment with U0126 attenuated TNF-α-induced COX-2 expression in a concentration-dependent manner. In addition, TNF-α-induced COX-2 mRNA levels were also reduced by pretreatment with U0126 (Fig. 4B). We further showed that TNF-α markedly induced ERK1/2 activation in RMCs in a time-dependent manner (Fig. 4C). Finally, we investigated whether resveratrol derivatives (AC, AF, and PD) inhibited TNF-α-induced ERK1/2 activation. As shown in Fig. 4C, pretreatment with AC, AF, or PD significantly attenuated TNF-α-stimulated ERK1/2 phosphorylation. Taken together, these results indicate that, in RMCs, these resveratrol derivatives inhibited TNF-α-induced COX-2 expression via the downregulation of ERK1/2 activation.

3.4. Resveratrol derivatives inhibited TNF-α-induced COX-2 expression via a p38 MAPK-independent pathway

We determined whether p38 MAPK phosphorylation was required for TNF-α-induced COX-2 expression in RMCs. However, in this study, we found that pretreatment with SB202190 (a p38 MAPK inhibitor) [22] did not inhibit TNF-α-induced COX-2 mRNA accumulation (Fig. 5A). We further showed that TNF-α markedly induced p38 MAPK activation in RMCs in a time-dependent manner, which was reduced by pretreatment with SB202190 (Fig. 5B). Finally, we investigated

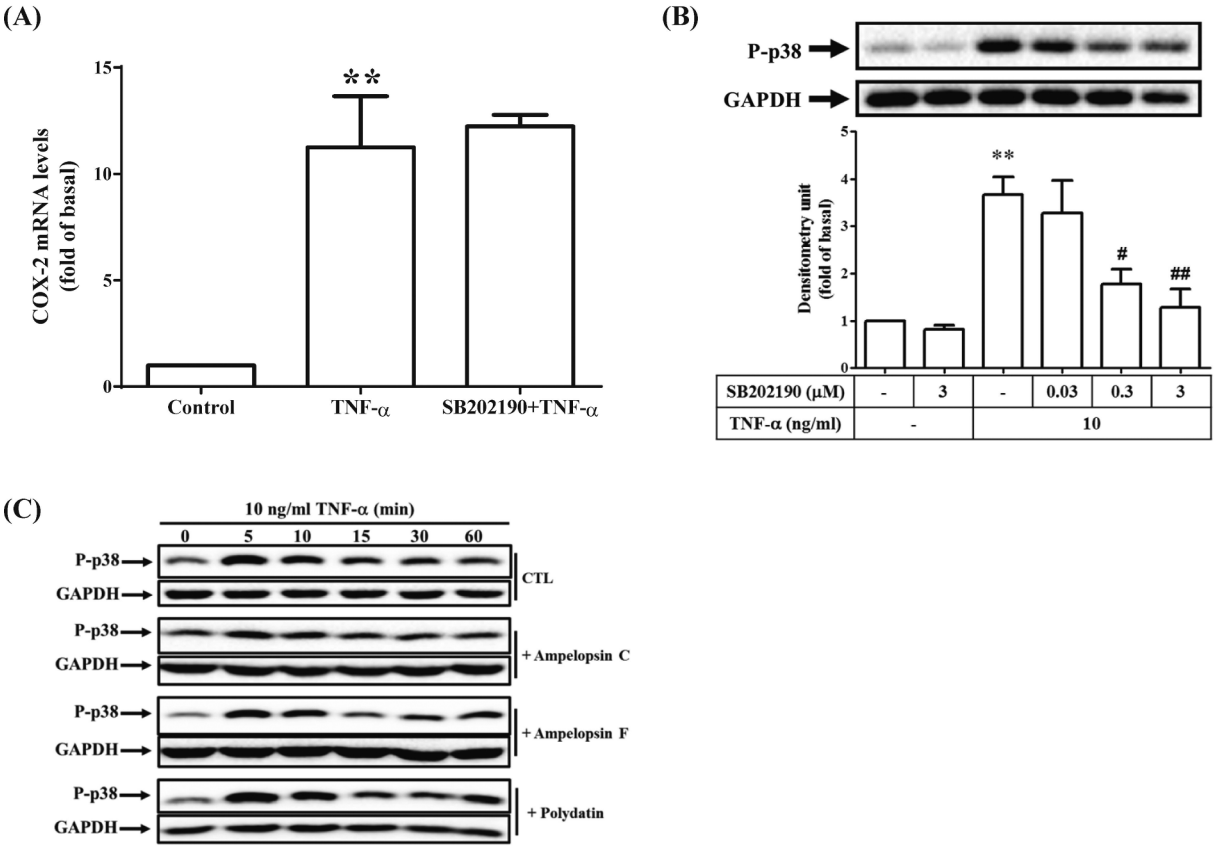

Fig. 5. AC, AF, and PD have no effects on TNF-α-induced p38 activation. (A) Cells were pretreated with SB202190 for 1 h, and then incubated with TNF-α for 6 h. The mRNA levels of COX-2 were analyzed by real-time PCR. (B) RMCs were pretreated with SB202190 (0.03, 0.3, or 3 μM) for 1 h, and then incubated with TNF-α for 5 min. The expression of phospho-p38 MAPK was determined by Western blotting. (C) Cells were pretreated with or without AC (10 μg/ml), AF (10 μg/ml), or PD (10 μg/ml) for 1 h, and then treated with TNF-α for the indicated time. The expression of phospho-p38 MAPK was determined by Western blotting. Data are expressed as the mean ± S.E.M. of three independent experiments. $^{**}P < 0.01$ compared with cells exposed to the vehicle. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ compared with cells exposed to TNF-α alone for 5 min.

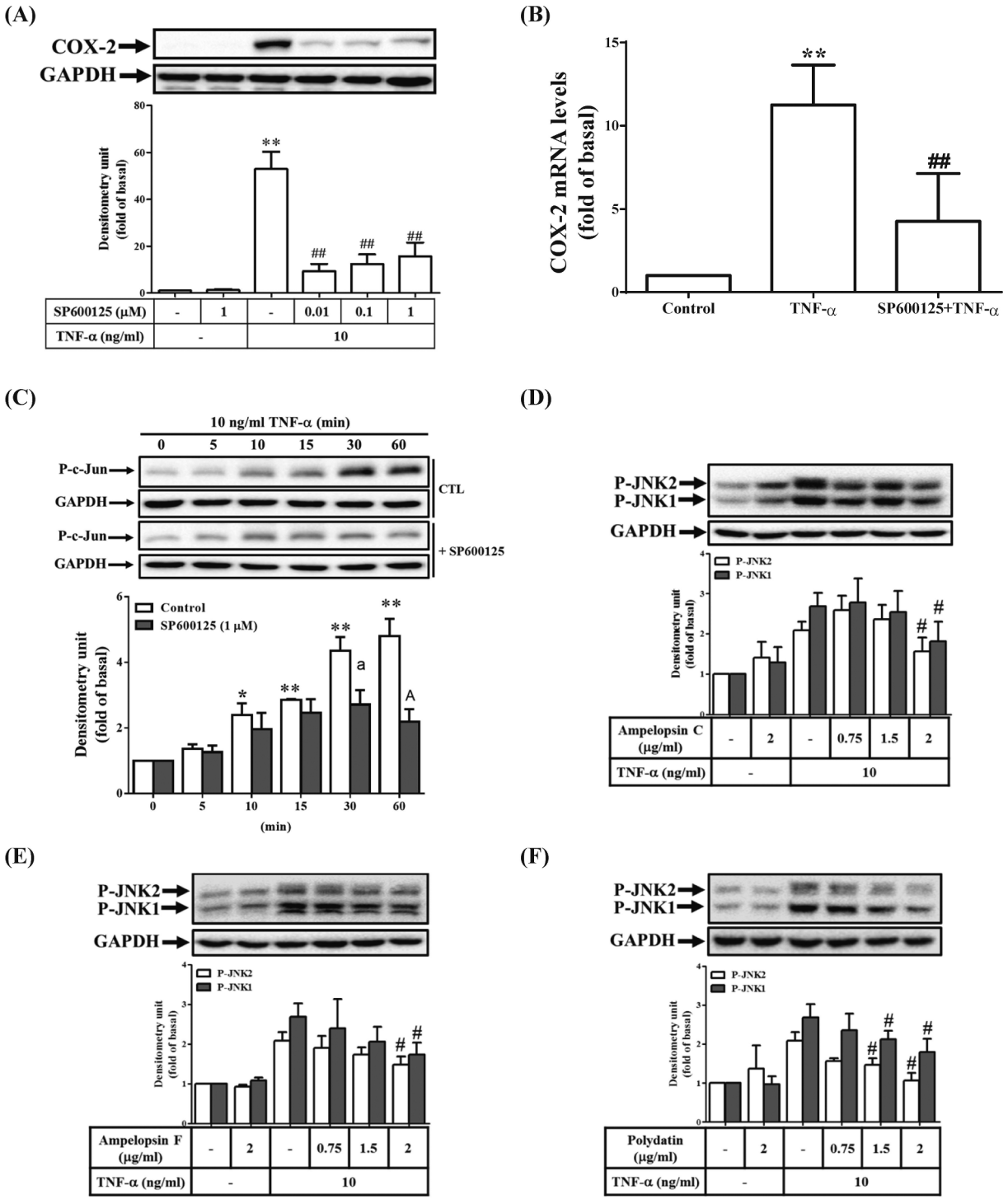

Fig. 6. AC, AF, and PD inhibit TNF-α-induced COX-2 expression via the reduction of JNK1/2 activation in RMCs. (A) RMCs were pretreated with SP600125 (0.01, 0.1, or 1 μM) for 1 h, and then incubated with TNF-α for 24 h. The expression of the COX-2 protein was determined by Western blotting. (B) Cells were pretreated with SP600125 for 1 h, and then incubated with TNF-α for 6 h. The mRNA levels of COX-2 were analyzed by real-time PCR. (C) Cells were pretreated with or without SP600125 for 1 h, and then incubated with TNF-α for the indicated time. The expression of phospho-c-Jun was determined by Western blotting. (D–F) Cells were pretreated with AC, AF, or PD for 1 h, and then incubated with TNF-α for 15 min. The expression of phospho-JNK1/2 was determined by Western blotting. Data are expressed as the mean ± S.E.M. of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ compared with cells exposed to the vehicle. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ compared with cells exposed to TNF-α alone. $^{a}P < 0.05$; $^{A}P < 0.01$ compared with cells exposed to TNF-α alone for 30 or 60 min.

whether resveratrol derivatives (AC, AF, and PD) inhibited TNF-α-induced p38 MAPK activation. As shown in Fig. 5C, pretreatment with AC, AF, or PD did not attenuate TNF-α-stimulated p38 MAPK phosphorylation. Taken together, these results indicate that, in RMCs, AC, AF, and PD inhibited TNF-α-induced COX-2 expression via a p38 MAPK-independent pathway.

3.5. Resveratrol derivatives inhibited TNF-α-induced COX-2 expression via the reduction of JNK1/2 activation in RMCs

JNK1/2 has been shown to regulate COX-2 expression in various cell types [23–25]. Here, we investigated whether JNK1/2 is involved in TNF-α-induced COX-2 expression. Cells were pretreated with an inhibitor of JNK1/2 (SP600125) [26] for 1 h, and then incubated with TNF-α for 24 h. As shown in Fig. 6A, pretreatment with SP600125 attenuated TNF-α-induced COX-2 expression. In addition, TNF-α-induced COX-2 mRNA levels were also reduced by pretreatment with SP600125 (Fig. 6B). We further showed that TNF-α markedly induced c-Jun activation in RMCs in a time-dependent manner, which was inhibited by pretreatment with SP600125 (Fig. 6C). Finally, we investigated whether resveratrol derivatives (AC, AF, and PD) inhibited TNF-α-induced JNK1/2 activation. As shown in Fig. 6D–F, pretreatment with AC, AF, or PD significantly attenuated TNF-α-stimulated JNK1/2 phosphorylation. Taken together, these results indicate that, in RMCs, resveratrol derivatives inhibited TNF-α-induced COX-2 expression via the down-regulation of JNK1/2 activation.

3.6. Resveratrol derivatives inhibited TNF-α-induced COX-2 expression via the reduction of NF-κB (p65) activation in RMCs

NF-κB has been shown to be involved in COX-2 induction in various cell types [27–29]. Here, we investigated whether NF-κB is involved in TNF-α-induced COX-2 expression. Cells were pretreated with an inhibitor of NF-κB (Bay 11–7082) [30] for 1 h, and then incubated with TNF-α for 24 h. As shown in Fig. 7A, pretreatment with Bay11-7082 attenuated TNF-α-induced COX-2 expression. In addition, TNF-α-induced COX-2 mRNA levels were also reduced by pretreatment with Bay11-7082 (Fig. 7B). We further showed that TNF-α markedly induced NF-κB (p65) activation in RMCs in a time-dependent manner, which was inhibited by pretreatment with Bay11-7082 (Fig. 7C). Finally, we investigated whether resveratrol derivatives (AC, AF, and PD) inhibited TNF-α-induced NF-κB (p65) activation. As shown in Fig. 7C, pretreatment with AC, AF, or PD significantly attenuated TNF-α-stimulated NF-κB (p65) phosphorylation. NF-κB activity is regulated by multiple mechanisms, including phosphorylation by various MAPKs [4]. Interestingly, in our study, we found that pretreatment of RMCs with U0126, SB202190, or SP600125 did not attenuate the TNF-α-induced phosphorylation of NF-κB (p65) (Fig. 7D). Thus, these results indicate that, in RMCs, these resveratrol derivatives reduced TNF-α-induced COX-2 expression via the inhibition of MAPK-independent NF-κB activation.

3.7. Resveratrol derivatives inhibited TNF-α-induced COX-2 expression via the reduction of NF-κB (p65) translocation

Here, we determined whether TNF-α induces NF-κB (p65) translocation in RMCs. As shown in Fig. 8A and B, TNF-α markedly induced NF-κB (p65) translocation from the cytosol to the nucleus in these cells in a time-dependent manner. We further investigated whether the resveratrol derivatives (AC, AF, and PD) inhibited TNF-α-induced NF-κB (p65) translocation. As shown in Fig. 8C, pretreatment with AC, AF, or PD partially attenuated TNF-α-stimulated NF-κB (p65) translocation. Moreover, we found that pretreatment of RMCs with U0126, SB202190, or SP600125, but not Bay11-7082, did not attenuate the TNF-α-induced

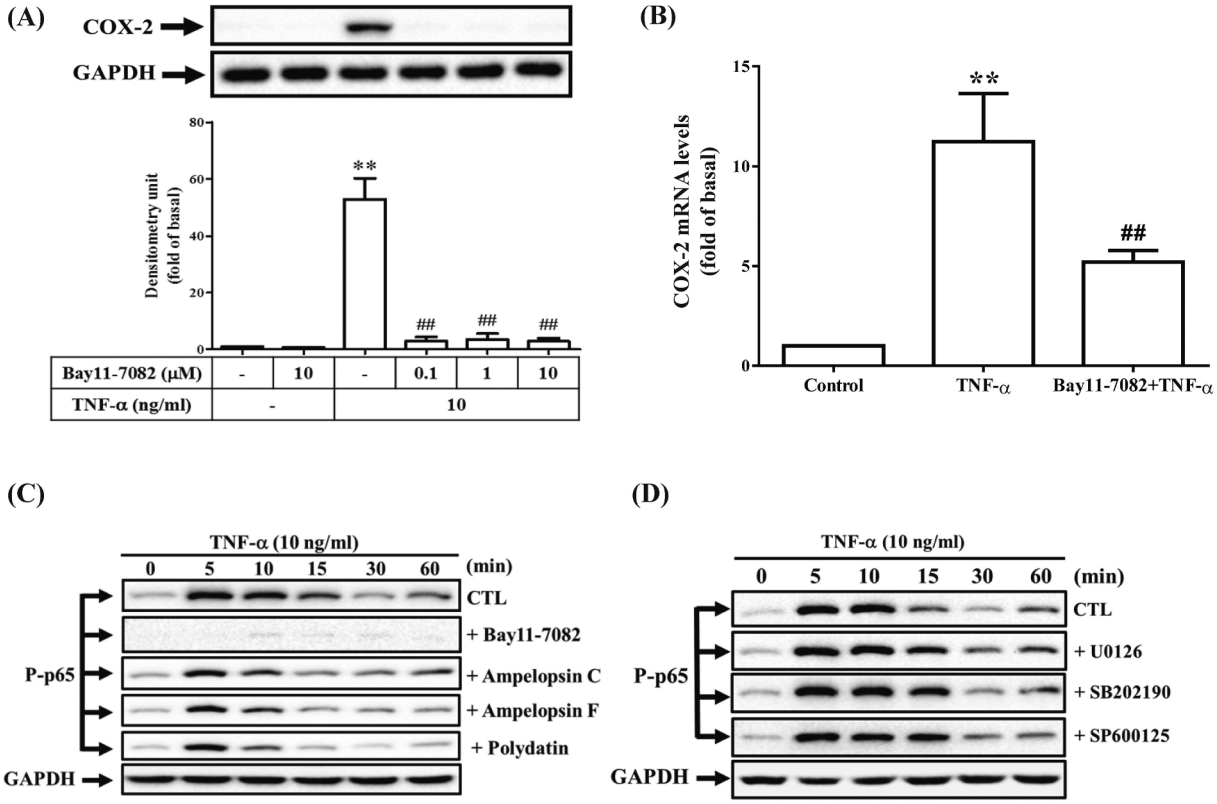

Fig. 7. AC, AF, and PD inhibit TNF-α-induced NF-κB (p65) phosphorylation. (A) RMCs were pretreated with Bay11-7082 (0.1, 1, or 10 μM) for 1 h, and then incubated with TNF-α for 24 h. The expression of the COX-2 protein was determined by Western blotting. (B) Cells were pretreated with Bay11-7082 for 1 h, and then incubated with TNF-α for 6 h. The mRNA levels of COX-2 were analyzed by real-time PCR. (C, D) Cells were pretreated with or without Bay11-7082 (10 μM), AC (10 μg/ml), AF (10 μg/ml), PD (10 μg/ml), U0126 (600 nM), SB202190 (3 μM), or SP600125 (1 μM) for 1 h, and then incubated with TNF-α for the indicated time. The expression of phospho-p65 was determined by Western blotting. Data are expressed as the mean ± S.E.M. of three independent experiments. $^{**}P < 0.01$ compared with cells exposed to the vehicle. $^{\#\#}P < 0.01$ compared with cells exposed to TNF-α alone.

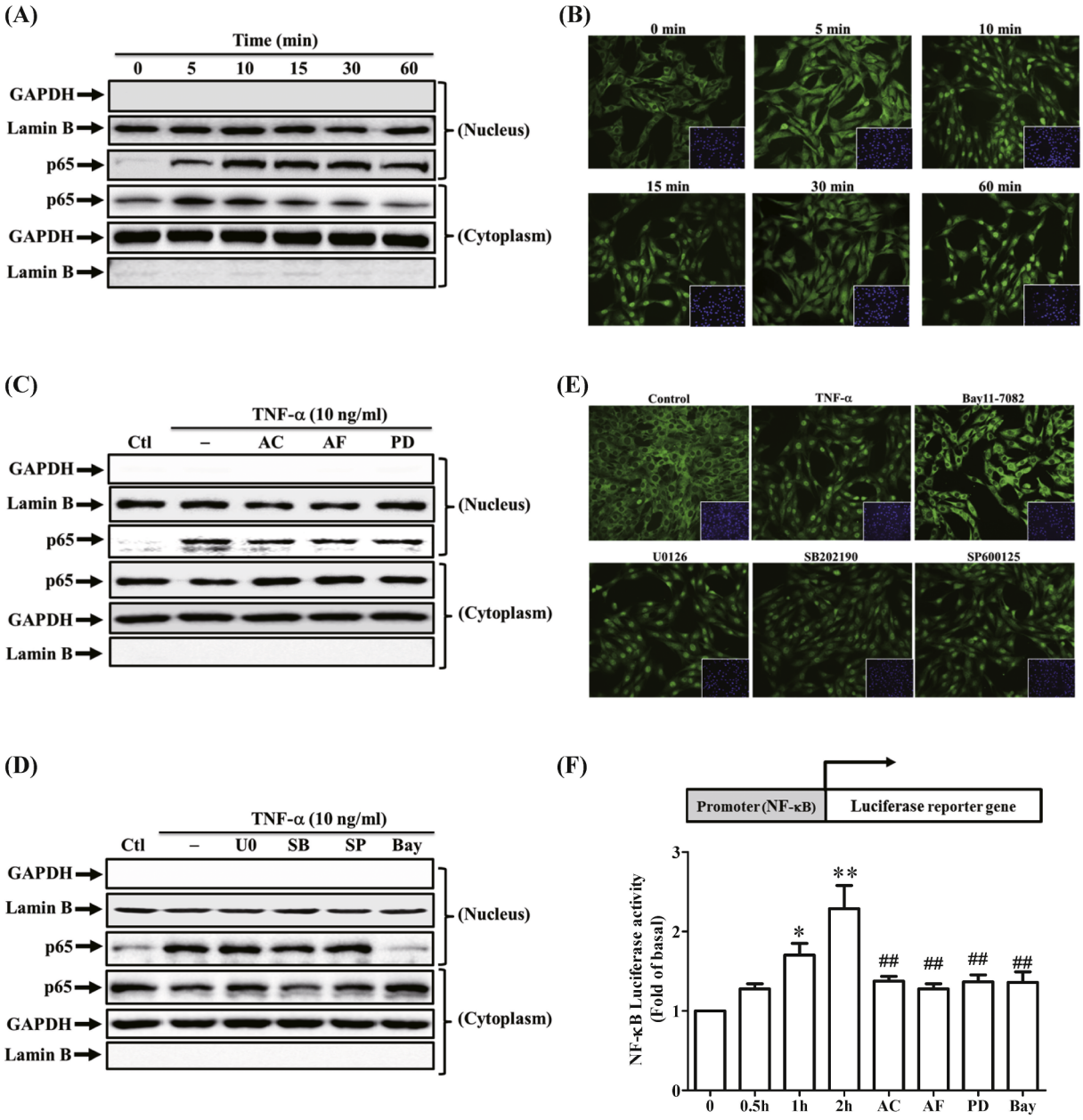

Fig. 8. AC, AF, and PD inhibit TNF-α-induced NF-κB (p65) translocation in RMCs. Cells were treated with TNF-α for the indicated time. The nuclear and cytosolic fractions were prepared and subjected to Western blotting using an anti-p65 antibody. Lamin B and GAPDH were used as marker proteins of the nuclear and cytosolic fractions, respectively (A). The translocation of NF-κB (p65) was observed using fluorescence microscopy (B). (C, D) Cells were pretreated without or with AC, AF, PD, U0126, SB202190, SP600125, or Bay11-7082 for 1 h, and then stimulated with 10 ng/ml TNF-α for 10 min. The nuclear and cytosolic fractions were prepared and subjected to Western blotting using an anti-p65 antibody. Lamin B and GAPDH were used as marker proteins of the nuclear and cytosolic fractions, respectively. (E) Cells were pretreated with or without U0126, SB202190, SP600125, or Bay11-7082 for 1 h, and then stimulated with 10 ng/ml TNF-α for 10 min. The translocation of NF-κB (p65) was observed using fluorescence microscopy. (F) Cells were treated with TNF-α for the indicated time or pretreated with AC, AF, PD, or Bay 11-7082 for 1 h, and then incubated with TNF-α for 2 h. NF-κB promoter activity was measured. Data are expressed as the mean ± S.E.M. of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ compared with cells exposed to the vehicle. $^{\#\#}P < 0.01$ compared with cells exposed to TNF-α alone.

translocation of NF-κB (p65) (Fig. 8D and E). Finally, we showed that TNF-α markedly induced NF-κB promoter activity in a time-dependent manner, which was reduced by pretreatment with the resveratrol derivatives (AC, AF, and PD) (Fig. 8F). These results indicate that resveratrol derivatives attenuated TNF-α-induced COX-2 expression via the inhibition of MAPK-independent NF-κB translocation.

4. Discussion

$cPLA_2$ and COX-2, which are regulated by various stimuli, are important in renal inflammation and may lead to several renal diseases. The inhibition of COX-2 can limit renal damage and inflammation [31]. Resveratrol has been reported to suppress inflammatory responses in mesangial cells and in several types of renal injury in animal models [32,33]. Moreover, resveratrol derivatives were shown to inhibit inflammatory pathways (COX activity), in part more effectively than resveratrol itself [7,8,12,13]. Previously, the active resveratrol derivatives from the roots of *V. thunbergii* Sieb. & Zucc. (Vitaceae) were shown to have significant anti-platelet and anti-oxidative activities. However, the anti-inflammatory mechanisms of these resveratrol derivatives in RMCs have not been clearly explained. In the present study, we found that resveratrol derivatives (AC, AF, and PD) can reduce $cPLA_2$/COX-2/

PGE_2 expression via the inhibition of JNK1/2, ERK1/2 and attenuate the NF-κB activation induced by TNF-α in RMCs. TNF-α induces JNK1/2- and ERK1/2-independent NF-κB activation and translocation in RMCs. Activated NF-κB is recruited to the promoter regions of COX-2, leading to an increase in COX-2 mRNA levels and protein expression. These responses may further lead to kidney inflammatory diseases. The results of the present study suggest that resveratrol derivatives from the roots of *V. thunbergii* Sieb. & Zucc. (Vitaceae) exerted a protective effect on renal inflammation by inhibiting $cPLA_2$/COX-2/PGE_2 expression.

TNF-α, which was initially reported to induce tumor cell apoptosis and cachexia, is now considered a central mediator of a broad range of biological activities, from cell proliferation, cell death, and differentiation to the induction of inflammation and immune modulation [34]. TNF-α exerts its biological responses via interaction with two cell surface receptors: TNFR1 and TNFR2 [35]. These receptors trigger shared and distinct signaling pathways upon TNF-α binding, which in turn results in cellular outputs that may promote tissue injury, but may also induce protective, beneficial responses [36]. Multiple studies have investigated the role of TNFRs in the development of early and late renal failure (diabetic nephropathy, nephroangiosclerosis, acute kidney transplant rejection, renal cell carcinoma, glomerulonephritis, sepsis, and obstructive renal injury) [37]. Renal expression and circulating levels of bioactive TNF-α are increased during clinical and experimental lupus nephritis and correlate with disease activity [38]. Low levels of the TNF-α mRNA can be detected in lupus-prone mice prior to renal injury [39]. Renal mesangial cells are responsible for glomerular PAF generation and, ultimately, are the victims of its excessive production [40]. The mesangial pathology is widely acknowledged to reflect glomerular damage, which culminates in glomerulosclerosis and proteinuria. Therefore, the modulation of mesangial cell responses would offer a pathophysiology-based therapeutic approach to prevent glomerular injury.

Plants contain numerous polyphenols, which have been shown to reduce inflammation and increase resistance to disease. Although resveratrol, a natural polyphenolic molecule with several biological activities, is a well-recognized anti-inflammation, anti-oxidant, anti-aging, and cancer chemopreventive agent [41,42], some resveratrol derivatives have been shown to inhibit inflammatory pathways (COX activity), in part more effectively than resveratrol itself [7,8]. Active resveratrol derivatives from the roots of *V. thunbergii* Sieb. & Zucc. (Vitaceae) are well-known folk medicines for the treatment of hepatitis and diarrhea in Taiwan [8]. Previously, active resveratrol derivatives, such as AC and AF, from the roots of *V. thunbergii* Sieb. & Zucc. (Vitaceae) have been shown to have significant anti-platelet and anti-oxidative activities [9,10]. Ampelopsin, a plant flavonoid, has potent anti-inflammatory properties *in vitro* and *in vivo*. It has been reported that ampelopsin inhibited NO production in LPS-stimulated RAW264.7 macrophages and reduced carrageenan-induced acute inflammation *in vivo* [43], which is consistent with another report that ampelopsin played an important role not only in reducing the production of pro-inflammatory mediators, such as IL-1β, IL-6, and TNF-α, but also in inhibiting the activity of iNOS. The nephroprotective effects of PD were attributed to its anti-inflammatory cascade, including the inhibition of the expression of NF-κB (p65), COX-2, and iNOS proteins and the production of TNF-α, PGE_2 and IL-1β [16]. PD has been reported to alleviate high-glucose-induced extracellular matrix accumulation through anti-inflammatory mechanisms in rat glomerular mesangial cells *in vitro* [15]. Here, we found that pretreatment with resveratrol derivatives inhibited $cPLA_2$ and COX-2 expression and PGE_2 release in RMCs. Moreover, the inhibitory effects of TNF-α-induced $cPLA_2$ and COX-2 expression detected in the AC and AF groups were stronger than those observed in the PD group. This may be because both AC and AF are stilbene oligomers (AC is a trimer form of resveratrol, whereas AF is a dimer form of resveratrol [8]). By contrast, PD is a glycoside form of resveratrol [11], which is the most abundant form of resveratrol in nature [44]. Therefore, in our study, both AC and AF were shown to inhibit $cPLA_2$/COX-2/PGE_2 activity more effectively than resveratrol itself (PD).

In addition, we also found that the cells treated with TNF-α prior to the administration of these inhibitors and resveratrol derivatives exhibited higher expression levels of $cPLA_2$ and COX-2 than did the cells that had been exposed to TNF-α alone (Supplementary data 1). This may be because the cells stimulated by TNF-α release many factors, including inflammatory cytokines, which might worsen the inflammatory responses. Therefore, when these inhibitors and resveratrol derivatives were added after the cells had already been exposed to TNF-α, they may not have reduced or inhibited inflammatory responses. Taken together, these results suggest that resveratrol derivatives might be useful as anti-inflammatory modulators of kidney inflammation.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.cyto.2018.10.008.

The compromised MAPK signaling pathways, which include JNK1/2, p38 MAPK, and ERK1/2, contribute to the pathology of diverse human diseases [45,46]. The JNK1/2 and p38 MAPK signaling pathways are activated by various types of cellular stresses, such as oxidative, genotoxic, and osmotic, as well as by proinflammatory cytokines, such as TNF-α and IL-1β [46]. Renal ERK1/2 and JNK1/2 activation was also increased in the glycerol model of myoglobinuric acute renal injury [47]. It seems that MAPK signaling pathways represent a potential target for therapeutic intervention. Here, we found that TNF-α-induced COX-2 expression was downregulated by an inhibitor of JNK1/2 or ERK1/2, but not of p38 MAPK, in RMCs. In addition, pretreatment with the resveratrol derivatives reduced TNF-α-induced ERK1/2, JNK1/2, and c-Jun activation in RMCs. Thus, we consider that the resveratrol derivatives inhibit TNF-α-induced inflammatory responses via the inhibition of ERK1/2 and JNK1/2 activation in RMCs.

NF-κB regulates the expression of cytokines, growth factors, and effector enzymes in response to the ligation of many receptors involved in immunity, including TNFR, CD40, and the TIR family [48]. NF-κB also regulates the expression of genes outside of the immune system and, hence, can influence multiple aspects of normal and disease physiology [49]. Moreover, we established that NF-κB played a key role in mediating TNF-α-induced inflammatory responses in RMCs. In addition, NF-κB activity is regulated by multiple mechanisms, including phosphorylation by various MAPKs [4]. Interestingly, we found that TNF-α induced NF-κB activation via a JNK1/2- and ERK1/2-independent pathway in RMCs. In the future, we will investigate the detailed signaling pathways involved in TNF-α-stimulated NF-κB activation in these cells. Moreover, we also showed that the resveratrol derivatives inhibited TNF-α-induced NF-κB (p65) activation and translocation. Thus, we also consider that the resveratrol derivatives inhibit TNF-α-induced inflammatory responses via the inhibition of NF-κB activation in RMCs.

In summary, as shown in Fig. 9, our results demonstrate that TNF-α induces JNK1/2- and ERK1/2-independent NF-κB activation and translocation in RMCs. Activated NF-κB is recruited to the promoter regions of COX-2, leading to the upregulation of the COX-2 mRNA and protein. Resveratrol derivatives (AC, AF, and PD) can reduce $cPLA_2$/COX-2/PGE_2 expression via the inhibition of the JNK1/2, ERK1/2, and NF-κB activation induced by TNF-α in RMCs.

5. Notes

The authors declare that there is no conflict of interest.

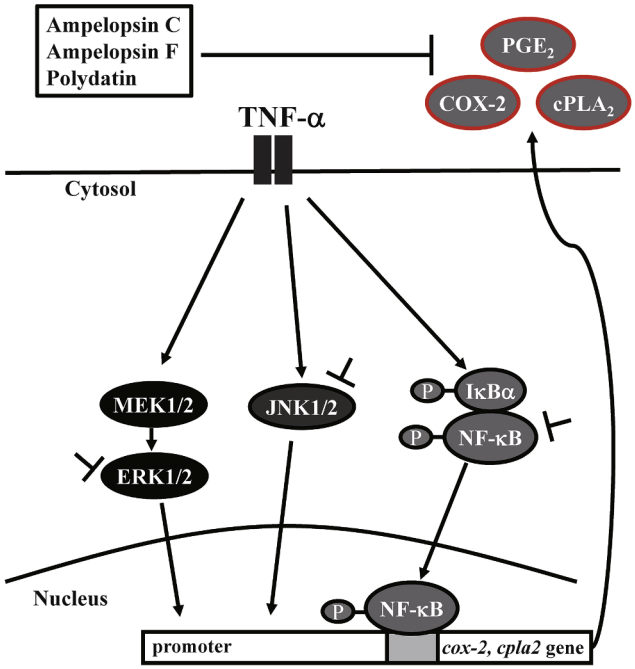

Fig. 9. Schematic diagram of the proposed signaling pathway involved in TNF-α-induced $cPLA_2$/COX-2 expression in RMCs. TNF-α induces JNK1/2- and ERK1/2-independent NF-κB activation and translocation in RMCs. The activated NF-κB is recruited to the promoter regions of COX-2, leading to the up-regulation of the COX-2 mRNA and protein. Resveratrol derivatives (AC, AF, and PD) downregulate $cPLA_2$/COX-2/PGE_2 expression via the inhibition of TNF-α-induced JNK1/2, ERK1/2, and NF-κB activation in RMCs.

Acknowledgments

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