Rosmarinic Acid Attenuates the Lipopolysaccharide-Provoked Inflammatory Response of Vascular Smooth Muscle Cell via Inhibition of MAPK/NF-κB Cascade

Ching-Pei Chen 1, You-Cian Lin 2,3, Yu-Hui Peng 4, Han-Min Chen 5, Jiun-Tsai Lin 6 and Shao-Hsuan Kao 4,7,*

1 Division of Cardiology, Department of Internal Medicine, Changhua Christian Hospital, Changhua 50006, Taiwan; 782809@cch.org.tw
2 Cardiovascular Division, Surgical Department, China Medical University Hospital, Taichung 404332, Taiwan; lh0203@gmail.com
3 School of Medicine, China Medical University, Taichung 404332, Taiwan
4 Institute of Medicine, College of Medicine, Chung Shan Medical University, Taichung 402306, Taiwan; me5124ya@gmail.com
5 Institute of Applied Science and Engineering, Catholic Fu Jen University, New Taipei 242048, Taiwan; 056489@mail.fju.edu.tw
6 Energenesis Biomedical Co., Ltd., Taipei 114694, Taiwan; jt@energenesis-biomedical.com
7 Department of Medical Research, Chung Shan Medical University Hospital, Taichung 402306, Taiwan
* Correspondence: kaosh@csmu.edu.tw; Tel.: +886-4-2473-0022 (ext. 11681)

Abstract: Rosmarinic acid (RA) is a phenolic compound that has several bioactivities, such as anti-inflammatory and antioxidant activities. Here, we further investigate the anti-inflammatory effect of RA on rat A7r5 aortic smooth muscle cells with exposure to lipopolysaccharide (LPS). Our findings showed that low-dose RA (10–25 µM) did not influence the cell viability and morphology of A7r5 cells and significantly inhibited LPS-induced mRNA expression of the pro-inflammatory mediators TNFα, IL-8, and inducible NO synthase (iNOS). Consistently, RA reduced the production of TNFα, IL-8, and NO by A7r5 cells with exposure to LPS. Signaling cascade analysis showed that LPS induced activation of Erk, JNK, p38 mitogen-activated protein kinase (MAPK), and NF-κB, and RA treatments attenuated the activation of the three MAPKs and NF-κB. Moreover, cotreatment with RA and Erk, JNK, p38 MAPK, or NF-κB inhibitors further downregulated the mRNA expression of TNFα, IL-8, and iNOS, and decreased the production of TNFα, IL-8, and NO by A7r5 cells. Taken together, these findings indicate that RA may ameliorate the LPS-provoked inflammatory response of vascular smooth muscle cells by inhibition of MAPK/NF-κB signaling.

Keywords: rosmarinic acid; vascular smooth muscle cell; proinflammatory response; mitogen-activated protein kinase; NF-κB

1. Introduction

Chronic and insistent inflammation plays a pivotal role in the initiation and development of vascular disorders, such as periphery artery disease, atherosclerosis, and hypertension [1–3]. Increasing evidence indicates that vascular smooth muscle cell (VSMC) activation plays an important role in the development and progression of vascular disorders [4]. Activated VSMCs exhibit higher proliferative activity, induce a spectrum of proinflammatory genes, and produce various proinflammatory mediators, which alter vascular structure and function and lead to vascular injury. Therefore, inhibition of vascular inflammation and VSMC activation has been regarded as a promising strategy for inflamed vascular disorders.

Previous studies have demonstrated that Toll-like receptor (TLR)-mediated signaling cascades play a central role in atherosclerosis and cardiac hypertrophy, and is involved in regulating the apoptosis, differentiation, and proliferation of VSMCs [5]. With the
inflammatory stimulus of bacterial lipopolysaccharide (LPS), a potent TLR activator, VSMCs are activated and subsequently express proinflammatory mediators, such as inducible nitric oxide synthase (iNOS), tumor necrosis factor (TNF)-α, and IL-8, thereby contributing to VSMC injury and cardiovascular diseases [6,7]. Activation of TLR by LPS also induces mitogen-activated protein kinases (MAPKs) and NF-κB signaling in VSMCs [8]. MAPKs, including extracellular signal-regulated kinases (Erk), c-Jun N-terminal kinase (JNK), and p38 MAPK, are highly involved in the LPS-triggered transcriptional activity of nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) [9]. NF-κB is a key transcription factor that governs the expression of various proinflammatory genes in response to LPS [9,10]. Therefore, inhibition of MAPK and NF-κB cascades plays an important role in attenuating LPS-triggered pro-inflammatory responses.

Rosmarinic acid (RA) is one of the major phenolic compounds in rosemary (Rosmarinus officinalis L.) which has been widely used in folk medicine for its various pharmacological effects, including anti-inflammatory and antioxidant activities [11,12]. In addition, Konishi et al. report that RA can be absorbed in the gastrointestinal tract by passive diffusion across the intestinal epithelium and reach the maximal plasma concentration 30 min after oral administration [13]. RA treatment has been also reported to alleviate neuropathic pain via anti-apoptotic and anti-inflammatory activities in a chronic constriction injury rat model [14]. However, whether RA attenuates the proinflammatory response of VSMCs in response to LPS and the underlying mechanism are still incompletely known. Therefore, the present study aimed to explore the in vitro effects of RA on the LPS-induced proinflammatory response of VSMCs and the mechanistic pathways. The cytotoxicity of RA to murine aortic SMC A7r5 cells and the inhibitory effect of RA on LPS-induced expression of proinflammatory mediators were assessed and analyzed. The involvement of MAPKs and NF-κB signaling in the inhibitory effect of RA on A7r5 cells with exposure to LPS was also evaluated.

2. Results

2.1. Low-Dose RA Does Not Significantly Influence the Cell Morphology and Cell Viability of A7r5 Cells

We first explored the effects of RA on the cell viability and cell morphology of the rat aortic smooth muscle cell A7r5. As shown in Figure 1, high-dose RA treatments at 100–400 µM significantly altered the cell morphology of A7r5 cells and reduced the cell viability of A7r5 cells to 53.1 ± 2.4% of the control (p < 0.005). On the contrary, low-dose RA (10–50 µM) did not influence the cell morphology of A7r5 cells and the cell viability of A7r5 cells compared with the control (C). These findings indicate that high-dose RA exhibits cytotoxicity to A7r5 cells, but low-dose RA has no significant effects on A7r5 cells. Therefore, low-dose RA was used to investigate whether RA alleviates the pro-inflammatory response of A7r5 cells in response to LPS stimuli.

2.2. RA Downregulated the mRNA Expression of TNFα, IL-8, and iNOS and Reduced the Production of TNFα, IL-8, and NO by A7r5 Cells

Proinflammatory mediators such as TNFα, IL-8, and iNOS play an important role in atherosclerosis-related inflammation and are highly associated with the development of atherosclerosis [15,16]. Therefore, the effect of RA on these proinflammatory mediators was assessed. As shown in Figure 2A–C, LPS stimulus significantly upregulated the mRNA expression of TNFα, IL-8, and iNOS up to 7.7 ± 0.9-fold, 45.1 ± 2.3-fold, and 6.2 ± 0.9-fold as compared to the control, respectively. These upregulations of mRNAs were dose-dependently downregulated by RA treatments 3.8 ± 0.6-fold, 9.8 ± 1.8-fold, and 2.3 ± 0.6-fold as compared to LPS alone, respectively (RA at 50 µM, p < 0.01). In addition to mRNA expression, production of TNFα and IL-8 was also determined using a quantitative ELISA assay. As shown in Figure 2D,E, TNFα and IL-8 production was significantly increased by LPS stimuli, and the increased TNFα and IL-8 production was lowered by RA treatments in a dose-dependent manner (p < 0.01 as compared to LPS alone). Meanwhile, NO production by A7r5 cells with exposure to LPS was also increased, and the elevated NO production was significantly reduced by RA treatments (p < 0.005...
to as compared to LPS alone). Collectively, these findings reveal that RA treatments significantly reduce LPS-induced TNFα, IL-8, and iNOS in both mRNA expression and protein secretion/catalytic product.

![Image](image_url)

**Figure 1.** Effects of rosmarinic acid (RA) on the cell viability and morphology of A7r5 cells. Rat A7r5 aortic smooth muscle cell was treated with serial concentrations (0–400 μM) of RA for 24 h and then the resulting cell viability and cell morphology were assessed using SRB assay and light microscopy at 200×, respectively. Data are presented as mean ± SD. Three independent experiments were performed for statistical analysis: ** and ***, $p < 0.01$ and $p < 0.005$ as compared to the control.

2.3. RA Inhibited the Activation of Erk, JNK, and p38 MAPK and NF-κB Signaling in A7r5 Cells in the Presence of LPS

MAPKs and NF-κB signaling play a central role in LPS-provoked inflammatory response [8–10]. Next, the involvement of MAPK and NF-κB signaling in RA-attenuated proinflammatory response was investigated. As shown in Figure 3A, LPS stimuli significantly induced the phosphorylation of Erk1/2, JNK, and p38 MAPK in A7r5 cells, and the LPS-induced phosphorylation of Erk1/2, JNK, and p38 MAPK was inhibited by RA treatments ($p < 0.01$ as compared with LPS alone). LPS stimuli also triggered NF-κB signaling, including increased IkBα phosphorylation and nuclear translocation of NF-κB/p65 (Figure 3B). Notably, the increased IkBα phosphorylation and nuclear translocation of NF-κB were significantly reduced by RA treatments ($p < 0.05$ as compared to LPS alone). Taken together, these results indicate that RA treatments significantly inhibit LPS-induced Erk1/2, JNK, and p38 MAPK activation and NF-κB signaling.
Figure 2. RA downregulated the mRNA expression and production of pro-inflammatory mediators by A7r5 cells in the presence of LPS. Cells were pretreated with RA at 10, 25, or 50 μM for 1 h, then treated with LPS for 6 h or 24 h. The 6 h LPS-treated cells were subjected to (A–C) mRNA analysis using qPCR, and the 24 h LPS-treated cell culture medium was collected for (D–F) pro-inflammatory mediator production analysis using ELISA or Greiss reaction. The change in mRNA expression was presented as the ratio of treatment/control. *, **, and *** p < 0.05, < 0.01 as compared to the control; # p < 0.05 as compared with RA alone.

Figure 3. Effect of RA treatments on LPS-induced MAPK activation and NF-κB signaling. Cells were pretreated with RA at 25 or 50 μM for 1 h, then treated with LPS for 24 h. The treated cells were subjected to immunodetection of (A) MAPKs and their phosphorylations or (B) cytosol/nucleus fractionation and subsequent immunodetection of IκBα phosphorylation and nuclear NF-κB. GAPDH and Histone H1 were used as an internal control for the cytosol and nucleus fraction, respectively. Quantitative data were acquired by densitometric analysis from three independent experiments. a, p < 0.01 as compared to the control; b and c, p < 0.05 and p < 0.01 as compared with LPS alone.
2.4. Involvement of MAPKs and NF-κB Signaling in the RA-Inhibited Proinflammatory Response of A7r5 Cells in the Presence of LPS

Based on the results that RA inhibited MAPK and NF-κB signaling in A7r5 cells in response to LPS stimuli, the involvement of MAPK and NF-κB signaling in the RA-inhibited proinflammatory response was further explored. In order to clearly observe the combined inhibitory effects of RA with MAPK inhibitors, RA at 25 µM with a moderate inhibitory activity was used for the test. As shown in Figure 4A–C, LPS upregulated mRNA expression of TNFα, IL-8, and iNOS; RA treatments reduced the upregulated mRNA expressions; and cotreatment with RA and Erk inhibitor PD98059 (PD) further downregulated the mRNA expression of these proinflammatory mediators (p < 0.05 as compared with RA alone). Similarly, cotreatment with RA and JNK inhibitor SP600125 (SP) or RA and p38 MAPK inhibitor SB203580 (SB) also further downregulated the mRNA expression of these proinflammatory mediators (p < 0.05 as compared with RA alone). In parallel with MAPK inhibitors, cotreatment with RA and IκB kinase (IKK) inhibitor BAY 11-7082 (BAY) further downregulated the mRNA expression of TNFα, IL-8, and iNOS in A7r5 cells with exposure to LPS as compared to those treated with RA alone (p < 0.05).

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Involvement of MAPK activation and NF-κB signaling in RA-downregulated mRNA expression and production of pro-inflammatory mediators by A7r5 cells with LPS stimuli. Cells were pretreated with RA at 25 µM or RA combined with PD98059 (PD), SP600125, or SB203580 (SB) for 2 h, then treated with LPS for 6 h or 24 h. The 6 h LPS-treated cells were subjected to (A–C) mRNA analysis using qPCR, and the 24 h LPS-treated cell culture medium was collected for (D–F) pro-inflammatory mediator production analysis using ELISA or Greiss reaction. The change in mRNA expression was presented as the ratio of treatment/control. a, p < 0.01 as compared to the control; b, p < 0.01 as compared with LPS alone; c, p < 0.05 as compared with the LPS and RA pretreatment.

Moreover, the effects of cotreatment with RA and the MAPK inhibitors on the production of proinflammatory mediators by A7r5 cells were then investigated. As shown in Figure 4D–F, LPS stimuli increased the production of TNFα, IL-8, and nitric oxide (NO) by A7r5 cells, and RA treatments reduced the increased production of these proinflammatory mediators. Consistent with the effects on mRNA expression, cotreatment with RA and PD,
SP, SB, or BAY significantly lowered the production of these proinflammatory mediators as compared to treatment with RA alone ($p < 0.05$). Collectively, these observations suggest that Erk, JNK, p38 MAPK, and NF-κB signaling may be involved in the RA-attenuated proinflammatory response of A7r5 cells to LPS stimuli.

3. Discussion

In this study, our findings show that low-dose RA has non-cytotoxicity to VSMC A7r5 cells and inhibits the mRNA expression and production of proinflammatory mediators by A7r5 cells in response to LPS stimuli. Mechanistically, RA reduces the LPS-induced Erk, JNK, and NF-κB signaling cascade, which may contribute to the downregulation of the proinflammatory mediators. These findings indicate that low-dose RA may have potent anti-inflammatory activity in VSMCs in response to LPS stimulus and that the Erk/JNK/p38 MAPK and NF-κB axis may play an important role in the anti-inflammatory activity. Similarly, previous studies reported that RA is compatible with hemodialysis fluid commonly used in hemodialysis and that RA supplementation can inhibit the mRNA expression and production of inflammatory cytokines by human vein epithelial cells in response to LPS stimuli [17].

Consistent with in vitro findings, several studies using animal models have revealed that RA and its derivatives have potential in vivo anti-inflammatory activities, including suppression of NLRP3 activation in streptozotocin-induced diabetic rats [11], improvement of dextran sodium sulfate-induced ulcerative colitis in mice [18], and alleviation of doxorubicin-induced cardiotoxicity in rats [19]. These in vivo studies indicated that RA had low toxicity in a murine model and suggested that RA could be a promising phenolic acid for treatment of inflammatory diseases. However, further in vivo animal models may provide more evidence to demonstrate the preclinical benefits of RA on vascular inflammation.

Proinflammatory mediators are highly associated with the pathogenesis of inflamed vascular disorders, including atherosclerosis and sepsis. TNFα is a multipotential cytokine and thought to be a major mediator of the host response that triggers septic shock [20]. In addition, TNFα also promotes the development of atherosclerotic lesions and plaque by modulating the proliferation and apoptosis of VSMCs [21]. IL-8, also known as CXCL8, is a chemokine that attracts neutrophils, monocytes, and T cells. Moreover, IL-8 is also regarded as an effective predictor of cardiovascular disorders after percutaneous coronary intervention [22]. Our results show that RA diminishes the mRNA expression and production of TNFα and IL-8 by A7r5, suggesting that RA may alleviate LPS-triggered vascular injury and inflammation.

The release of endotoxins such as LPS by bacteria and the concomitant vascular inflammatory response are the major characteristics of inflamed vascular diseases such as sepsis. The inflammatory mediators not only cause hemodynamic instability but also impair respiratory functions [23]. Among inflammatory mediators, NO produced by iNOS is highly associated with vascular cell death and tissue damage [24]. Although three isoforms of NOS have been identified and their NO productions are involved in pathophysiological processes, it is proposed that an excess of iNOS-produced NO primarily causes the vascular disorders that occur in shock [25]. Our findings reveal that RA significantly reduces the expression of iNOS and the production of NO by VSMCs, indicating that RA may alleviate vascular damage in inflamed vascular diseases.

Activation of MAPKs by LPS is one of the critical signal cascades in modulating the expression and production of inflammatory cytokines and mediators and in managing NF-κB activation [26]. Inflammation-associated MAPKs mainly consist of ERK1/2, JNK, and p38 MAPK, and they can be activated by many extracellular stimuli, such as LPS, cytokines, growth factors, and radiation [27,28]. The activated MAPKs subsequently regulate expression and production of pro-inflammatory cytokines and enzymes, including iNOS, COX-2, TNFα, and IL-8 [29]. Here, our findings indicate that RA significantly inhibits the phosphorylation of Erk1/2, JNK, and p38 MAPK, and reduces phosphorylation of IκBα and nuclear translocation of NF-κB in LPS-stimulated A7r5 cells. Moreover, RA combined
with the inhibitors of Erk, JNK, or p38 MAPK further reduces the mRNA expression of TNFα, IL-8, and iNOS, as well as the production of TNFα, IL-8, and NO by A7r5 cells. Accordingly, we suggest that RA attenuates LPS-induced pro-inflammatory cytokines and mediators via inhibition of MAPKs and NF-κB signaling pathways.

4. Materials and Methods

4.1. Chemicals and Reagents

Bovine serum albumin (BSA), Griess reagent, lipopolysaccharide (LPS), sulforhodamine B (SRB), rosmarinic acid (RA), protease and phosphatase inhibitor cocktail, HEPES, KCl, MgCl2, EDTA, dithiothreitol (DTT), Igepal CA-630, PMSE, 1 mM Na3VO4, NaCl, NaF NaNO2, Na2HPO4, Tris-HCl, Triton X-100, and Tween-20 were purchase from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco’s-modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY, USA).

4.2. Cell Culture and Treatments

Murine aortic SMC A7r5 cells were obtained from the Bioresource Collection Research Center (Hsinchu, Taiwan) and cultured with Dulbecco’s modified minimal essential medium (DMEM) containing 10% FBS, 100 units/mL penicillin, and 100 units/mL streptomycin. Cells were maintained in a 37 °C humidified incubator with 5% CO2. For RA treatments, A7r5 cells were incubated with 1% FBS-DMEM overnight, pretreated with DMSO or RA at indicated concentrations for 1 h, and then treated with 1 µg/mL LPS in 1% FBS-DMEM for 1 h (Western blotting), 6 h (quantitative real-time PCR analysis), or 24 h (cell viability assay, morphology, and nitric oxide production). Thereafter, cells were collected and washed with phosphate-buffered saline (PBS, pH 7.4) for the subsequent analysis.

4.3. Cell Viability Assay

Cell viability was determined using an SRB assay as previously described [30]. Briefly, 2 × 104 cells were seeded in a 24-well plate, cultured with a complete medium for 24 h, and treated with RA at concentrations of 50, 100, 200, and 400 µM for 24 h. After the treatments, the cells were fixed with 10% trichloroacetic acid, stained with SRB for 30 min, and washed with 1% acetic acid. The protein-bound dye was dissolved in a 10 mM Tris base solution, and the absorbance at 510 nm of the solution was measured using a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Data were presented as percentage of control (DMSO treatment).

4.4. RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Total RNA was extracted and purified using the RNeasy kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer’s instructions. The purified RNA was used as a template to generate first-strand cDNA synthesis using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas. Life Sciences, St. Leon-Rot, Germany). The primer sequences used for qPCR were: TNFα forward 5’-TCCCAACAAGGAGGAAGT-3’, reverse 5’-TGTTATGAACTGGCAAATC-3’; IL-8 forward 5’-ACATTATTTTACGATGCTGGTCTTTCA-3’, reverse 5’-GGCTACCACCTTTTAAACTGCACAAT-3’; and iNOS forward 5’-CCACGCCCTTTGCTGTACTCAAC-3’, reverse 5’-AAGGGCTTGTCACTCGAG-3’. qPCR was conducted using the ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA). For mRNA quantitation, FastStart Universal SYBR Green Master (Roche Applied Science, Mannheim, Germany) was used for Taqman PCR. The threshold cycle numbers were calculated using the ∆∆CT relative value method and normalized to GAPDH. qPCR experiments were performed in triplicate for statistical analysis.
4.5. Quantitation of IL-8 and TNFα Using ELISA

For determination of IL-8 and TNFα production, cells were seeded onto a 6-well plate at an initial density of $5 \times 10^4$ cells/mL and incubated with RA and/or LPS for 24 h. The resulting culture supernatants were collected and the concentrations of IL-8 and TNFα were determined using Rat TNFα Quantikine® ELISA Kits (RTA00, R&D Systems, Abingdon, UK) and the rat IL-8 ELISA Kit (ABIN2535650, antibodies GmbH, Aachen, Germany) according to the manufacturer’s instructions.

4.6. Nitric Oxide Production Assay

Nitric oxide (NO) concentrations in culture supernatants were determined by means of the Griess reaction. Briefly, cells were pretreated with 10, 25, or 50 µM for 1 h, followed by incubation with 1 µg/mL LPS for 24 h. Nitrite in the supernatants was mixed with the same volume of Griess reagent (1% sulfanilamide and 0.1% N-[1-naphthyl]-ethylenediamine dihydrochloride in 5% phosphoric acid) and determined by measuring absorbance at 540 nm. Quantitation of NO was carried out by sodium nitrite (NaNO₂) standards.

4.7. Subcellular Fractionation

Cells were harvested and lysed using a lysis buffer (10 mM HEPES, pH 7.6; containing 15 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, 0.05% v/v Igepal CA-630 and 1 mM PMSE, 1 mM Na₃VO₄, 50 mM NaF). The cell lysates were centrifuged at 2500 × g for 10 min at 4 °C to remove the insoluble fraction, and the supernatant containing the cytosol fraction was further centrifuged at 20,000 × g for 15 min at 4 °C. The resulting pellets containing nuclei were washed with PBS, resuspended in nuclear buffer (25 mM HEPES, pH 7.6, 0.1% v/v Igepal CA-630, 1 M KCl, 0.1 mM EDTA, 1 mM PMSE, 1 mM Na₃VO₄, 2 mM NaF), and then centrifuged at 10,000 × g for 15 min at 4 °C. The resulting supernatants were collected as the nuclear fraction.

4.8. Protein Extraction and Western Blot Analysis

Cells were lysed in RIPA buffer containing a protease and phosphatase inhibitor cocktail (Sigma-Aldrich) at 4 °C for 30 min, centrifuged at 20,000 × g at 4 °C for 15 min to remove cell debris, and then the supernatant was used as crude protein extract. A protein assay was conducted using the Bradford method according to the manufacturer’s instruction (Bio-Rad Laboratories, Hercules, CA, USA). The crude proteins were separated using SDS-polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene difluoride membrane (Immobilon, Merck, Billerica, MA, USA). The membrane was incubated with 3% (w/v) BSA in PBS for 1 h and then incubated with primary antibodies (1000-fold dilution) for 2 h. Thereafter, the membrane was washed with PBS containing 0.5% Tween-20 (PBST) and incubated with secondary antibodies (2000-fold dilution) for 2 h. The bound antibodies were detected using an ECL chemiluminescence reagent (SuperSignal West Dura HRP Detection Kit; Pierce Biotechnology, Rockford, IL, USA), and the resulting chemiluminescence signals were recorded and semi-quantitated with an image analysis system (Fujifilm, Tokyo, Japan). Signals from DMSO treatment were used as control.

4.9. Statistical Analysis

Quantitative data were presented as means ± standards deviations (SD) from three independent experiments. The Student’s t-test was used to compare the differences between two groups. p < 0.05 was considered statistically significant.

5. Conclusions

VSMCs play an important role in the development of inflamed vascular diseases. In this study, our findings indicate that although high-dose RA is cytotoxic for A7r5 cells, low-dose RA is not only noncytotoxic to A7r5 cells but also reduces the mRNA expression and the production of proinflammatory mediators by A7r5 cells in response to LPS stimuli. These inhibitory effects of RA on inflamed A7r5 cells may result from suppression of
MAPK/NF-kB signaling. It is suggested that RA has potential benefits in alleviating vascular inflammation.

Author Contributions: Conceptualization, C.-P.C., Y.-C.L. and S.-H.K.; methodology, C.-P.C., Y.-C.L. and Y.-H.P.; validation, C.-P.C., Y.-C.L. and S.-H.K.; formal analysis, C.-P.C., Y.-C.L. and Y.-H.P.; investigation, H.-M.C. and J.-T.L.; resources, H.-M.C. and J.-T.L.; data curation, Y.-C.L. and S.-H.K.; writing—original draft preparation, S.-H.K.; writing—review and editing, S.-H.K.; supervision, S.-H.K.; funding acquisition, C.-P.C. and S.-H.K. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Changhua Christian Hospital, Changhua, Taiwan (grant no. CSMU CCH-108-03) and the Ministry of Science and Technology, Taiwan (grant no. MOST 108-2320-B-040-026-MY3).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article.

Conflicts of Interest: The authors declare no conflict of interest.

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