Available online at www.sciencedirect.com

ScienceDirect

Journal of Nutritional Biochemistry 26 (2015) 1338–1347

Journal of Nutritional Biochemistry

Nicotinic acid inhibits vascular inflammation via the SIRT1-dependent signaling pathway☆,☆☆

Yanxiang Li[a,d], Guangde Yang[b], Xiaofeng Yang[a], Yanhao He[a], Weirong Wang[c], Jiye Zhang[b], Tingting Li[a], Wei Zhang[a], Rong Lin[a,d,*]

[a]Department of Pharmacology, Health Science Center, Xi'an Jiaotong University, Xi'an, Shaanxi 710061, China
[b]School of Pharmacy, Health Science Center, Xi'an Jiaotong University, Xi'an, Shaanxi 710061, China
[c]Laboratory Animal Center, Health Science Center, Xi'an Jiaotong University, Xi'an, Shaanxi 710061, China
[d]Cardiovascular Research Center, Health Science Center, Xi'an Jiaotong University, Xi'an, Shaanxi 710061, China

Received 8 January 2015; received in revised form 17 June 2015; accepted 6 July 2015

Abstract

Nicotinic acid (NA) has recently been shown to inhibit inflammatory response in cardiovascular disease. Sirtuin1 (SIRT1), a NAD^+-dependent class III histone deacetylase, participates in the regulation of cellular inflammation. We hypothesized that dietary supplementation of NA could attenuate vascular inflammation via modulation of SIRT1 pathway. New Zealand White rabbits received chow or chow supplemented with 0.6% (wt/wt) NA for 2 weeks. Acute vascular inflammation was induced in the animals by placing a non-occlusive silastic collar around the left common carotid artery. At 24 h after collar implantation, the collar-induced production of C-reactive protein and monocyte chemotactic protein-1 was significantly suppressed in the NA-supplemented animals. Meanwhile, NA also decreased the expression of cluster of differentiation 40 (CD40) and CD40 ligand, but up-regulated SIRT1 expression, both in rabbits and in lipopolysaccharide-stimulated endothelial cells. Moreover, knockdown of SIRT1 reversed the inhibitory effect of NA on CD40 expression. Further study revealed that NA also decreased the expression of CD40 partly through mammalian target of rapamycin. These results indicate that NA protects against vascular inflammation via the SIRT1/CD40-dependent signaling pathway.

Keywords: Nicotinic acid; SIRT1; Inflammation; Cluster of differentiation 40; Endothelium

1. Introduction

Nicotinic acid (NA), an essential pellagra-preventing nutrient, has also been a widely used lipid-modifying agent to prevent atherosclerotic cardiovascular disease [1]. In addition to regulating the plasma lipid metabolism, recent evidences suggest that NA possesses anti-inflammatory [2] and antioxidant [3] properties independent of their lipid-lowering action. Some studies indicate that NA was able to decrease the production of pro-inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) in stimulated human monocytes/macrophage [4,5]. Moreover, similar effect of NA was also observed in a guinea pigs vascular inflammation model induced by high fat diet [6]. In our previous study, we found that fenofibrate can protect endothelial cells from C-reactive protein (CRP)-induced injury by decreasing the expression of cluster of differentiation 40 (CD40) [7], the key regulator of inflammatory response [8]. In a further study we also showed that lovastatin significantly diminished CD40 expression involved in cardiovascular diseases [9]. While extensive research is dedicated to the effect of an inflammatory reaction of lipid regulating drugs, little is known about the effect of NA on CD40 and the triggering pathway of inflammation.

Sirtuin 1 (SIRT1), a NAD^+-dependent deacetylase, plays a pivotal role in a wide variety of cellular processes such as senescence, differentiation and metabolism [10]. There is mounting evidence that SIRT1 has proven to be an important mediator in inflammation during cardiovascular diseases [11,12]. Up-regulation of SIRT1 attenuated the cigarette smoke extract-induced IL-6 and TNF-α in endothelial cells [13]. In addition, SIRT1 knockdown resulted in enhanced endothelial expression of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) in $ApoE^{-/-}$ mice [14]. In our previous study, we found that SIRT1 exerted anti-inflammatory property by regulating TNF-α-induced expression of CD40 in adipocytes [15]. Moreover, a recent report demonstrated that administration of NA up-regulated SIRT1 activity in livers of high-fat fed OLETF rats [16]. However, whether SIRT1 is involved in the regulation of NA on inflammation response by blockade of the CD40 pathway and the associated mechanisms remains unknown.

☆ Conflicts of interest: none.

☆☆ Funding: This study was supported by the National Natural Science Foundation of China (No. 81270347 and No. 81470549) and Shaanxi Science and Technology Innovation Project (No. 2013KTCL03-02).

* Corresponding author at: Department of Pharmacology, Health Science Center, Xi'an Jiaotong University, Xi'an, Shaanxi 710061, P.R. China. Tel.: +86 29 82657691; fax: +86 29 82657833.
E-mail address: linrong63@aliyun.com (R. Lin).

http://dx.doi.org/10.1016/j.jnutbio.2015.07.006
0955-2863/

Thus, in this study, we investigated the effect of NA on the expression of SIRT1 and CD40 both in New Zealand White (NZW) rabbits with a periarterial carotid collar and lipopolysaccharide (LPS)-stimulated endothelial cells. We then examined whether NA was able to inhibit acute vascular inflammation via the SIRT1/CD40-dependent signaling pathway so as to elucidate its anti-inflammatory mechanisms.

2. Materials and methods

2.1. Materials

NA, LPS and resveratrol (RSV) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Rabbit polyclonal anti-human SIRT1, CD40 and β-actin antibodies were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal anti-rabbit CD40 as well as mouse monoclonal anti-rabbit β-actin antibodies were purchased from Abcam (Cambridge, MA, UK). Rabbit polyclonal anti-rabbit SIRT1 antibody was obtained from Biosynthesis Biotechnology (Beijing, China). Anti-rabbit or anti-mouse immunoglobulin G horseradish peroxidase (Abcam, Cambridge, UK) were used as secondary antibodies. Lipofectamine 2000 transfection reagent was ordered from Invitrogen (Life Technologies, NY, USA). Protease inhibitor cocktail was purchased from Roche (Mannheim, Germany). Envision HRP system was provided by Dako Cytomation (Glostrup, Denmark). Rabbit interleukin-1β (IL-1β) and human IL-1β ELISA assay kits were products of RapidBio Lab (Calabasas, CA, USA) and R&D Systems (Minneapolis, MN, USA), respectively.

2.2. Animal care

Male NZW rabbits (2.0–2.5 kg) were provided by Medical Experimental Animal Center of the Xi'an Jiaotong University (Xi'an, China). The experimental protocol was in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and was approved by the Institutional Animal Care Committee of Xi'an Jiaotong University.

Four groups of NZW rabbits were maintained on a regular chow diet (Groups 1 and 2) or chow supplemented with 0.6% (wt/wt) NA (Groups 3 and 4). The animals were treated with the SIRT1 pharmacological activator RSV (80 mg/kg, p.o.) (Groups 2 and 4) or a vehicle daily (Groups 1 and 3) for 14 days before and for 24 h after inserting a non-occlusive silastic collar (total length 20 mm; internal diameter along bore, 4 mm; internal diameter at ends, 1 mm) around the left common carotid artery as described [2]. The contralateral right carotid artery was sham-operated. RSV has been reported as a potent activator of SIRT1 [17,18]. The animals were euthanized 24 h after the insertion of the collar. The collared section of the left common carotid artery and the corresponding section of the non-collared right common carotid artery, as well as liver, heart, spleen and kidney, were placed in ice-cold sterile saline and cleaned of fat and connective tissue. Three rings were cut from each collar-wrapped segment: one was formalin-fixed for 24 h, and the other two used for RNA and protein isolation as described [19].

2.3. Immunohistochemicals detection of CD40

Immunostaining was performed on deparaffinized sections of formalin-fixed, paraffin-embedded rabbit collared and non-collared carotid arteries. The fixed sections were de-waxed, rehydrated and incubated overnight at 4°C with anti-rabbit CD40 antibody (1: 100). Negative control was performed by omitting the primary antibody during the first incubation. Staining was visualized using Dako Cytomation Envision HRP system, followed by counter staining with haematoxylin. The sections were imaged using an upright light microscope (Olympus, Tokyo, Japan) at 20× magnification and analysed using ImageJ software (National Institutes of Health, Bethesda, Maryland). The threshold for positive staining was defined by a pathologist who was unaware of the identity of the samples. Positively stained areas were quantified by de-convolution. The results were expressed as the average positively stained area above the threshold for each section and the number of pixels representing endothelial CD40 positive staining was divided by the circumference of the lumen. The resulting values were presented as image units.

2.4. Cell culture

Human umbilical vein endothelial cells (HUVECs) were cultured in medium M199 supplemented with 15% fetal bovine serum, 3 ng/ml β-EC growth factor, 4 U/ml heparin, and 100 U/ml penicillin-streptomycin as described [20]. To determine whether the effect of NA on CD40 expression was mediated by SIRT1 in endothelial cells, the SIRT1 activator RSV and SIRT1 RNA interference were used in our study. In some experiments, HUVECs were pre-incubated for 1 h with the mammalian target of rapamycin (mTOR) pharmacological inhibitor rapamycin (100 nM, Houston, TX, USA), then incubated in the presence of 1 mM NA for a further 24 h without or with LPS (100 ng/ml).

2.5. Gene silencing

To silence SIRT1 expression, HUVECs were transfected at 37°C for 48 h with 20 μM synthesized specific siRNA targeting human small interfering RNA (siRNA) and scrambled siRNA control (Shanghai GenePharma Corporation, Shanghai, China). Briefly, the siRNA and lipofectamine 2000 were separately diluted in serum-free medium according to the manufacturer's instructions. Then the two solutions were softly mixed and incubated for a further 20 min to form siRNA-lipofectamine complexes. The HUVECs (2×10^5 cells/six-well plate) were transfected with the complexes for 48 h and then were used in the experiments as described above.

2.6. Western blotting analysis

The arteries and HUVECs were washed with ice-cold phosphate-buffered saline and lysed in RIPA lysis buffer containing protease inhibitor cocktail. Cellular proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were incubated overnight at 4°C using an anti-human SIRT1 (1:200), CD40 (1:100), β-actin (1:400) and anti-rabbit SIRT1 (1:100), CD40 (1:100), β-actin (1:200) antibody. As secondary antibody, anti-mouse IgG HRP (1:10000) or anti-rabbit IgG HRP (1:5000 or 1:10000) was used. Immunoreactive proteins were detected by chemiluminescent substrate and analyzed using Quantity One 1-D Analysis Software (BioRad, Hercules, CA, USA).

2.7. Real-time polymerase chain reaction (PCR)

Heart, liver, spleen, kidney and carotid artery segments of rabbits were incubated at 4°C for 24 h in RNAlater solution (Ambion, Austin, TX, USA) then stored at −80°C until use. Total RNA was isolated from the RNAlater-treated frozen tissues TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and extracted from HUVECs using RNAiso Plus reagent (TaKaRa Bio, Otsu, Japan). cDNAs were synthesized from 2 μg sample of total RNA using PrimeScript™ RT Master Mix kit (TaKaRa) following the manufacturer's instructions. Real-time PCR was performed with the SYBR Premix Ex Taq™ II (TaKaRa) by using an iQ5 real-time PCR detection system (BioRad, CA, USA). Relative changes in mRNA levels were analyzed by the ΔΔCT method, using GAPDH level as control. Primer pair sequences are shown in Table 1.

2.8. Enzyme-linked immunosorbent assay (ELISA)

Blood was collected at the commencement of the study and prior to euthanasia of the rabbits. The level of IL-1β in serum was detected using ELISA as described [21]. In addition, the concentration of IL-1β in cell culture supernatants was also measured by ELISA assay. All the assay kits were commercially available and the kit instructions were followed.

2.9. Statistical analysis

The results are expressed as mean±S.E.M. Statistical analysis was carried out using one-way analysis of variance and the Newman-Keul's test, as appropriate. $P<.05$ was considered statistically significant.

3. Results

3.1. Effects of NA on plasma lipid levels in rabbits

To examine the effect of NA on plasma lipid levels in rabbits, plasma lipid and lipoprotein levels of the rabbits were evaluated before NA treatment and when euthanized. There were no significant

Table 1
Primer sequences used in real-time PCR

Gene	Primer	Sequence (5′-3′)
Rabbit SIRT1	F	TGACTGTGAAGCTGTACGAGGAG
	R	GGAAGACCCAATAACAATGAGGAG
Rabbit CD40	F	GCAGGGAAAATCAGTACCAAGTG
	R	CGCCATTAGAGCAAGGAAGG
Rabbit CD40L	F	CCCGCAGTTCCTCCAAAA
	R	ACATTCACAAACACCGAAGCA
Rabbit MCP-1	F	GCAGAAGTGGGTCCAGGATG
	R	TAGTCGTGTGTTCTTGGGTTGTG
Rabbit CRP	F	CCTTTTCCTCCACTCCCACA
	R	TCTTCTCCATCTCCCTCCACA
Rabbit GAPDH	F	CCACTTTGTGAAGCTCATTTCCT
	R	TCGTCCTCCTCTGGTGCTCT
Human SIRT1	F	GGACTCCAAGGCCACGGATA
	R	GTTCGAGGATCTGTGCCAATCA
Human CD40	F	CACTGTACGAGTGAGGCCTGTGA
	R	TTGCACAACCAGGTCTTTGGTC
Human GAPDH	F	GCACCGTCAAGGCTGAGAAC
	R	TGGTGAAGACGCCAGTGGA

differences in plasma total cholesterol, high-density lipoprotein cholesterol, triglyceride, non-esterified fatty acid, and apolipoprotein A-I levels between the groups (data not shown). The results indicate that treatment with NA has no effect on plasma lipid levels in rabbits.

3.2. NA increases the expression of SIRT1 in rabbits

Our previous study has shown that SIRT1 is decreased in TNF-α-treated endothelial cells [22]. To investigate whether SIRT1 expression could be regulated by collar implantation in NZW rabbit carotid artery, the expression of SIRT1 was determined by western blotting analysis and quantitative real-time PCR. The results showed that SIRT1 protein and mRNA expressions were reduced in collared arteries, as compared with the non-collared arteries in the control animals ($P<.05$, Fig. 1A and B).

To confirm whether SIRT1 expression could be regulated by NA *in vivo*, the rabbits were treated with NA (chow supplemented with 0.6% NA) for 14 days. Relative to the control animals, dietary supplementation with NA increased SIRT1 protein and mRNA expression both in the non-collared and collared carotid arteries (Fig. 1A and B). In addition, we also observed the effects of NA on mRNA levels of SIRT1 in organ systems of the control animals. As shown in Fig. 1C and D, dietary supplementation with NA also increased liver, heart and spleen SIRT1 mRNA levels, respectively. However, NA supplementation showed little effect on SIRT1 expression in kidney (Fig. 1D).

3.3. NA protects against vascular inflammation and reduces serum IL-1β in rabbits

The implantation of a periarterial carotid collar or treatment with NA did not affect the intimal cross-sectional area or intimal/medial area ratio (data not shown). We further observed the effects of NA on the expressions of CD40, CD40 ligand (CD40L), CRP and monocyte chemotactic protein-1 (MCP-1) in the arteries of rabbits. Relative to the control animals, dietary supplementation with 0.6% NA decreased the endothelial expression of CD40 both in the non-collared and collared carotid arteries (Fig. 2A and B). Under suggestion of the results with the immunohistochemistry method, we further evaluated the effects of NA on protein and mRNA expressions of CD40 in the carotid arteries of rabbits. As shown in Fig. 2C and D, NA inhibited the collar-mediated increase in CD40 protein and mRNA expression. Moreover, dietary supplementation with NA also decreased the mRNA levels of CD40L, CRP and MCP-1 (Fig. 2E, F, G). In addition, we also observed a notable reduction of serum level of IL-1β in rabbits with NA supplementation ($P<.01$, Fig. 2H). These results indicate that NA has ability to reduce expressions of CD40 and CD40L, and to repress CRP and MCP-1 production in the arteries of rabbits, as well as serum IL-1β in the animals.

3.4. Relationship between effects of NA on vascular inflammation and SIRT1 in rabbits

Given the above results, it is important to determine whether SIRT1 is involved in the inhibitory effect of NA on collar-induced vascular inflammation. Therefore, an additional study was carried out in which SIRT1 was activated by RSV, a well-known SIRT1 activator. Four groups of NZW rabbits ($n=6$/group) received regular chow or chow supplemented with 0.6% (wt/wt) NA for 14 days preceding carotid collar implantation, meanwhile, SIRT1 activator was conducted daily. As shown in Fig. 3A and B, treatment of SIRT1 activator increased arterial SIRT1 protein and mRNA expression. Moreover, relative to the

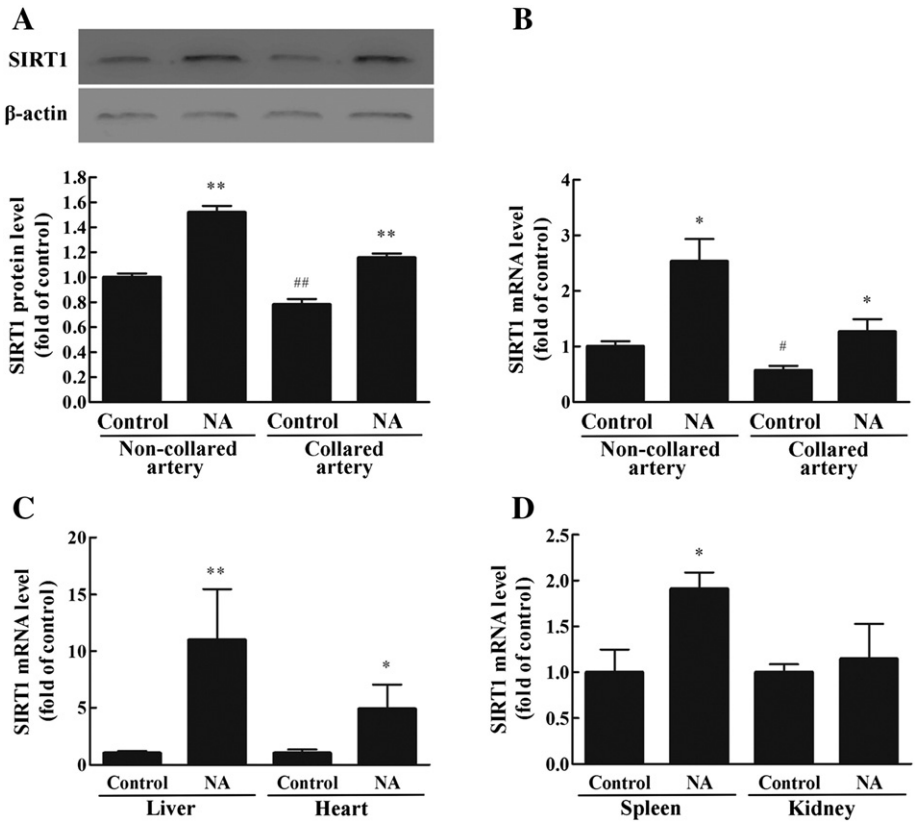

Fig. 1. NA up-regulates SIRT1 expression in NZW rabbits. NZW rabbits were maintained for 14 days on a regular chow diet (Control) or chow supplemented with 0.6% (wt/wt) NA before the implantation of a non-occlusive collar around the left carotid artery. The animals were euthanized 24 h after collar insertion. (A and B) The expression of SIRT1 protein and mRNA in the non-collared and collared carotid arteries was determined by western blotting and real-time PCR, respectively. Data are expressed as mean±S.E.M., $n=9$. (C and D) Liver, heart, spleen and kidney SIRT1 mRNA levels in animals that received regular chow or chow supplemented with 0.6% (wt/wt) NA. Data are expressed as mean±S.E.M., $n=6$. $^{*}P<.05$ and $^{**}P<.01$ vs. control; $^{\#}P<.05$ and $^{\#\#}P<.01$ vs. non-collared artery without NA treatment.

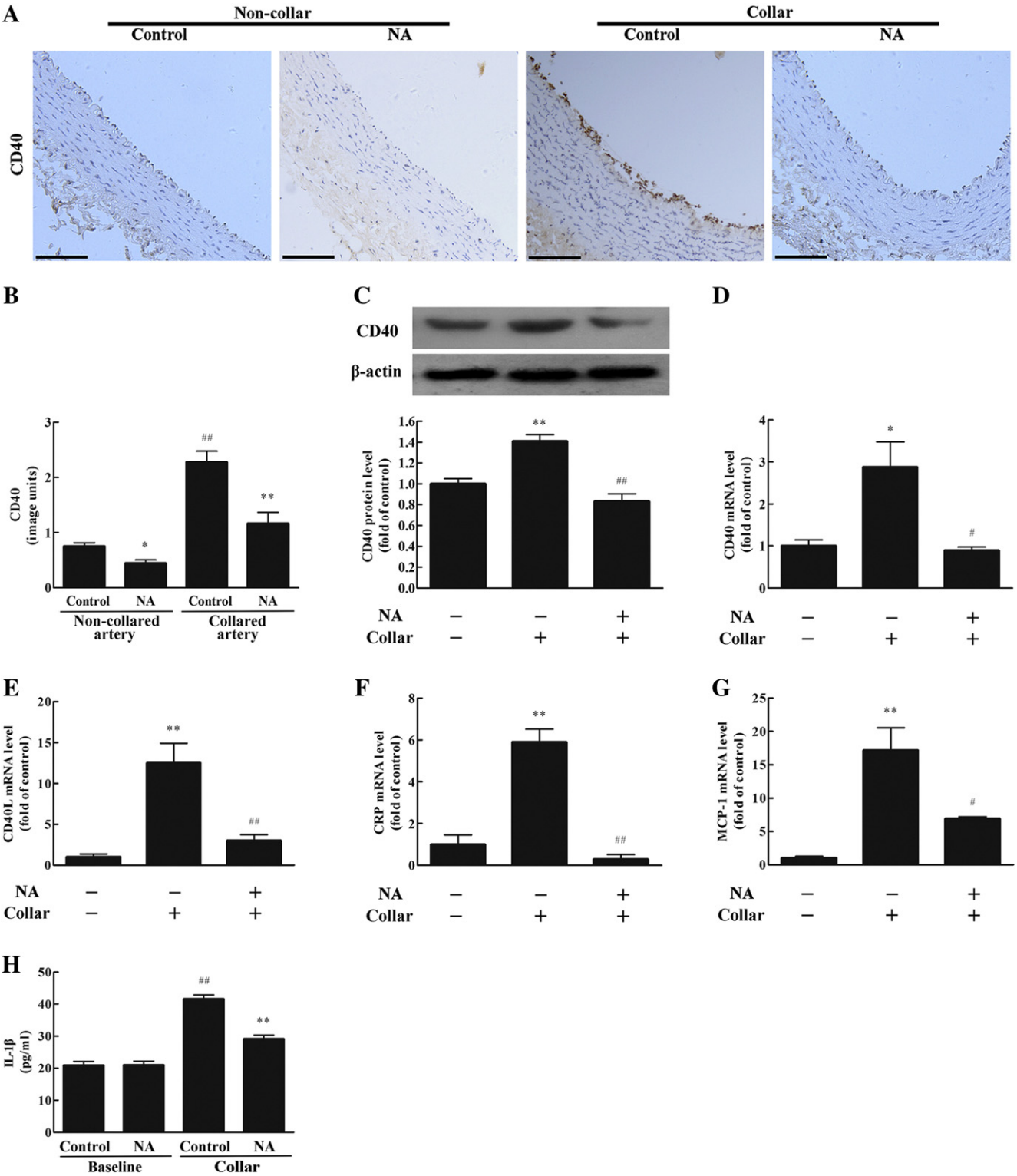

Fig. 2. NA inhibits acute vascular inflammation in NZW rabbit collared carotid arteries and IL-1β level in serum. Periarterial collars were inserted into NZW rabbits receiving regular chow (Control) or chow supplemented with 0.6% NA (wt/wt) for 14 days as described in the legend to Fig. 1. (A and B) Representative carotid artery sections immunostained for CD40 (bar= 50 μm). Quantification of CD40 staining in non-collared arteries and collared arteries. $^{*}P<.05$ and $^{**}P<.01$ vs. control; $^{\#\#}P<.01$ vs. non-collared artery without NA treatment. (C and D) The expression of CD40 protein and mRNA in the non-collared and collared carotid arteries was determined by western blotting and real-time PCR, respectively. $^{*}P<.05$ and $^{**}P<.01$ vs. non-collared artery; $^{\#}P<.05$ and $^{\#\#}P<.01$ vs. collared artery. (E, F and G) CD40L, CRP and MCP-1 mRNA levels in non-collared arteries and collared arteries are shown. $^{**}P<.01$ vs. non-collared artery; $^{\#}P<.05$ and $^{\#\#}P<.01$ vs. collared artery. (H) Blood was collected at the commencement of the study (Baseline) and at 24 h after collar insertion (Collar). Serum IL-1β was detected by ELISA analysis. $^{**}P<.01$ vs. control; $^{\#\#}P<.01$ vs. baseline. Data are expressed as mean±S.E.M., $n=6$.

NA supplementation alone, treatment of NA with RSV increased SIRT1 protein and mRNA levels by 1.35± 0.05 and 2.45±0.45 fold, respectively (Fig. 3A and B). In contrast, treatment of the animals with a combination of the SIRT1 activator and NA synergistically inhibited CD40, CRP and MCP-1 expression in comparison with treatment of NA alone (Fig. 3C, F, G). These data suggest that the modulatory effects of NA on vascular inflammation may be associated with up-regulation the expression of SIRT1.

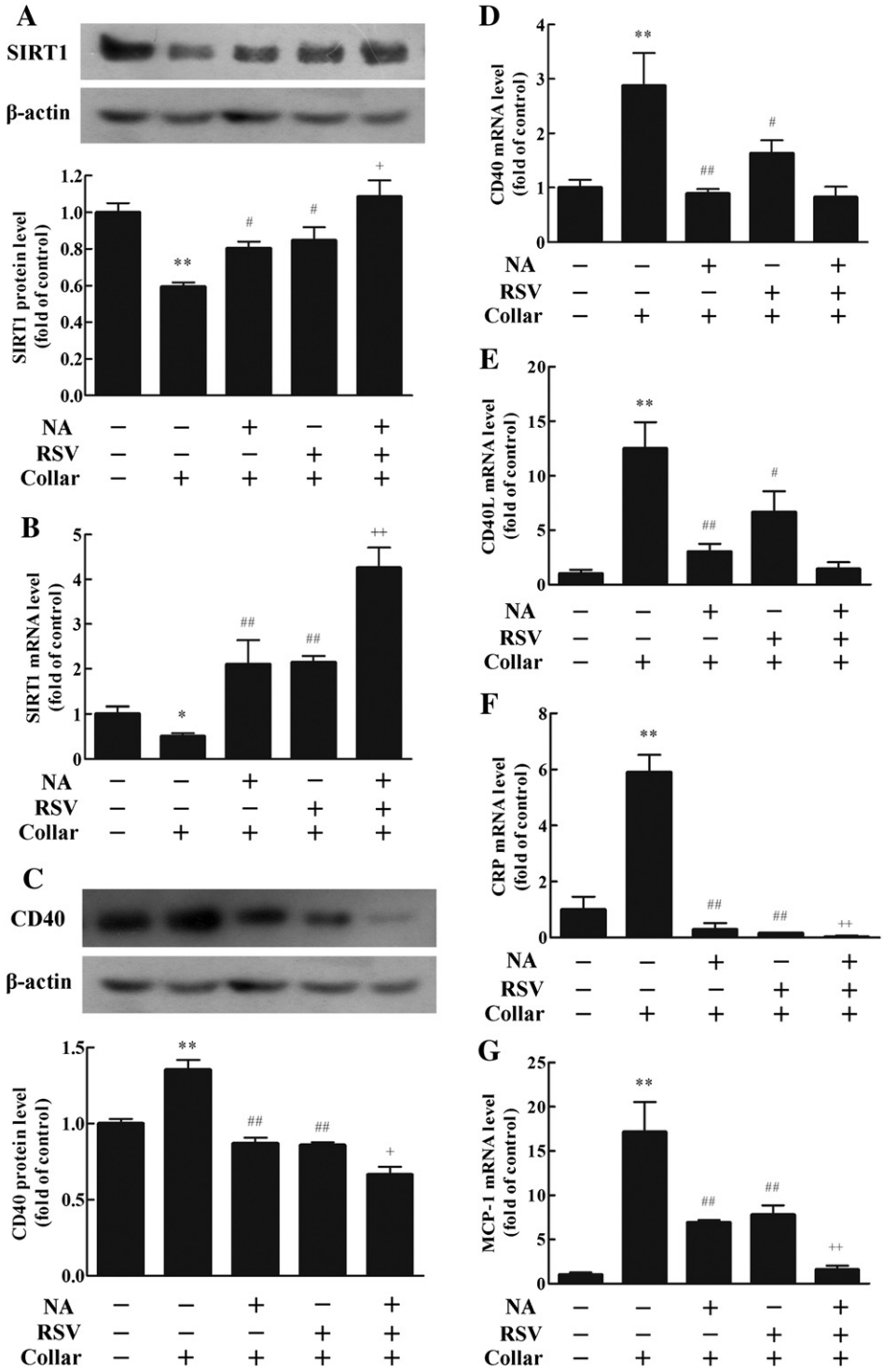

Fig. 3. Activation of SIRT1 synergistically protects against vascular inflammation in NZW rabbit collared carotid arteries. NZW rabbits were maintained on regular chow or chow supplemented with 0.6% (wt/wt) NA for 14 days preceding periarterial collars insertion. Vehicle or SIRT1 activator RSV was administrated daily. (A and B) The expression of SIRT1 protein and mRNA in the non-collared and collared carotid arteries was determined by western blotting and real-time PCR, respectively. (C and D) CD40 protein and mRNA levels in the collared carotid arteries are shown. (E, F and G) The expression of CD40L, CRP and MCP-1 was determined by real-time PCR. $^{*}P<.05$ and $^{**}P<.01$ vs. non-collared artery; $^{\#}P<.05$ and $^{\#\#}P<.01$ vs. collared artery without treatment; $^{+}P<.05$ and $^{++}P<.01$ vs. treatment of NA alone. Data are expressed as mean±S.E.M., $n=6$.

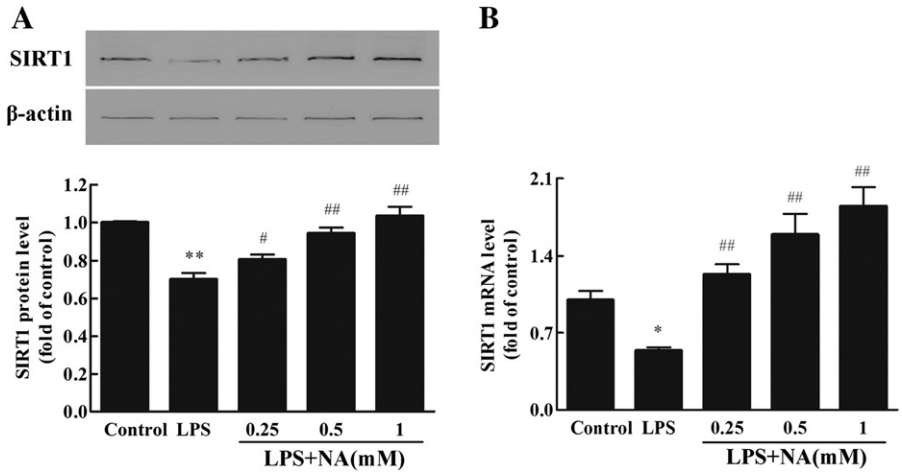

Fig. 4. NA up-regulates SIRT1 expression in LPS-stimulated HUVECs. (A and B) Endothelial cells were pretreated with NA at different concentrations (0.25, 0.5 and 1 mM) for 1 h, and then stimulated with LPS (100 ng/ml) for 24 h. The expression of SIRT1 protein and mRNA was determined by western blotting and real-time PCR, respectively. $^{*}P<.05$ and $^{**}P<.01$ vs. control; $^{\#}P<.05$ and $^{\#\#}P<.01$ vs. LPS group. Data are expressed as mean±S.E.M., $n=3$.

3.5. NA increases the expression of SIRT1 in LPS-stimulated HUVECs

In light of the above-mentioned results *in vivo*, effects of NA on SIRT1 expression in LPS-stimulated HUVECs were further determined. HUVECs were pretreated with NA at different concentrations (0.25, 0.5, and 1 mM) for 1 h and then treated with LPS (100 ng/ml) for 24 h. The protein and mRNA expression of SIRT1 was determined by western blotting and real-time PCR analysis, respectively. As shown in Fig. 4A and B, treatment of endothelial cells with LPS decreased the protein and mRNA expression of SIRT1, whereas NA up-regulated SIRT1 expression in LPS-stimulated endothelial cells in a concentration-dependent manner.

3.6. NA inhibits LPS-induced CD40 expression in HUVECs

To further determine the effect of NA on the expression of CD40 in HUVECs, the cells were pretreated with different concentrations of NA (0.25, 0.5, 1 mM) for 1 h and then stimulated with LPS (100 ng/ml) for 24 h. The protein and mRNA expression of CD40 was determined by western blotting and real-time PCR analysis, respectively. As shown in Fig. 5A and B, LPS increased CD40 expression at protein and mRNA levels in endothelial cells, as compared with the control group ($P<.05$ for protein and $P<.01$ for mRNA). However, pretreatment of the cells with NA significantly concentration-dependently inhibited the expression of CD40 in endothelial cells stimulated with LPS. This suggests that the anti-inflammatory effects that were observed in the NA-treated NZW rabbits were also observed *in vitro*.

It has been demonstrated that transfection of dendritic cells with recombinant adenovirus AdV-CD40L up-regulated IL-1β expression [23]. Therefore, we also observed the effect of NA on IL-1β secretion in LPS-stimulated HUVECs by ELISA. As shown in Fig. 5C, NA treatment significantly reduced the secretion of IL-1β in the cell culture supernatants, suggesting that the inhibitory effect of NA on the IL-1β production may be related with CD40.

3.7. NA inhibits LPS-induced CD40 expression via SIRT1-dependent pathway in HUVECs

Given our findings that NA could increase SIRT1 expression and inhibit CD40 expression *in vivo* and *in vitro*, we further evaluated whether SIRT1 is involved in the inhibitory effect of NA on LPS-induced CD40 expression. SIRT1 pharmacological activator RSV and SIRT1 RNA interference were used in our experiment. As expected, RSV increased the expression of SIRT1, but attenuated CD40 expression in LPS-stimulated endothelial cells (Fig. 6A and B). Furthermore, HUVECs were pretreated with RSV (20 μM) for 1 h, subsequently treated with NA (1 mM) for 1 h, and then stimulated with LPS (100 ng/ml) for 24 h. The results showed that SIRT1 activator increased SIRT1 protein expression by 1.37±0.10 fold ($P<.01$, Fig. 6C). Moreover, relative to treatment of the cells with NA alone, a combination of the SIRT1 activator and NA synergistically reversed LPS-induced changes of CD40 protein expression in endothelial cells ($P<.01$, Fig. 6D).

To determine whether the inhibition of NA on CD40 expression is mediated by SIRT1-dependent pathway, HUVECs were transfected with SIRT1 siRNA or scrambled siRNA (NC siRNA). Then endothelial cells transfected with SIRT1-specific siRNA were pretreated with NA (1 mM) prior to stimulation with LPS (100 ng/ml). As expected, endothelial cells transfected with SIRT1-specific siRNA showed decreased expression of SIRT1. Knockdown efficiency of SIRT1 was ~74% by western blotting analysis (Fig. 6E). In contrast to activated HUVECs with LPS and NA, pre-incubation of the SIRT1 siRNA-transfected endothelial cells with NA attenuated the increase effect of NA on SIRT1 expression ($P<.01$, Fig. 6F). Moreover, the inhibitory effect of NA on CD40 expression was abolished by knockdown of SIRT1 ($P<.01$, Fig. 6G). Taken together, our findings suggest that the inhibitory effect of NA on CD40 expression in endothelial cells is mediated via SIRT1-dependent pathway.

3.8. The mTOR pathway is involved in the protection effect of NA against LPS-induced CD40 expression in HUVECs

To examine whether mTOR pathway was involved in the inhibition of NA on LPS-induced CD40 expression, the endothelial cells were pretreated with mTOR inhibitor rapamycin (100 nM) for 1 h, followed by the addition of NA (1 mM) for 1 h, and then stimulated with LPS (100 ng/ml) for 24 h. The expression of CD40 protein and mRNA expression was determined by western blotting and real-time PCR analysis, respectively. As shown in Fig. 7A, rapamycin pretreatment augmented the inhibitory effect of NA on LPS-induced CD40 protein expression in endothelial cells ($P<.05$, Fig. 7A). Furthermore, the effect of mTOR was further confirmed by real-time PCR ($P<.01$, Fig. 7B). These preliminary results indicate that the inhibitory effect on CD40 that is observed in the NA-treated HUVECs may have been partly mediated by mTOR pathway.

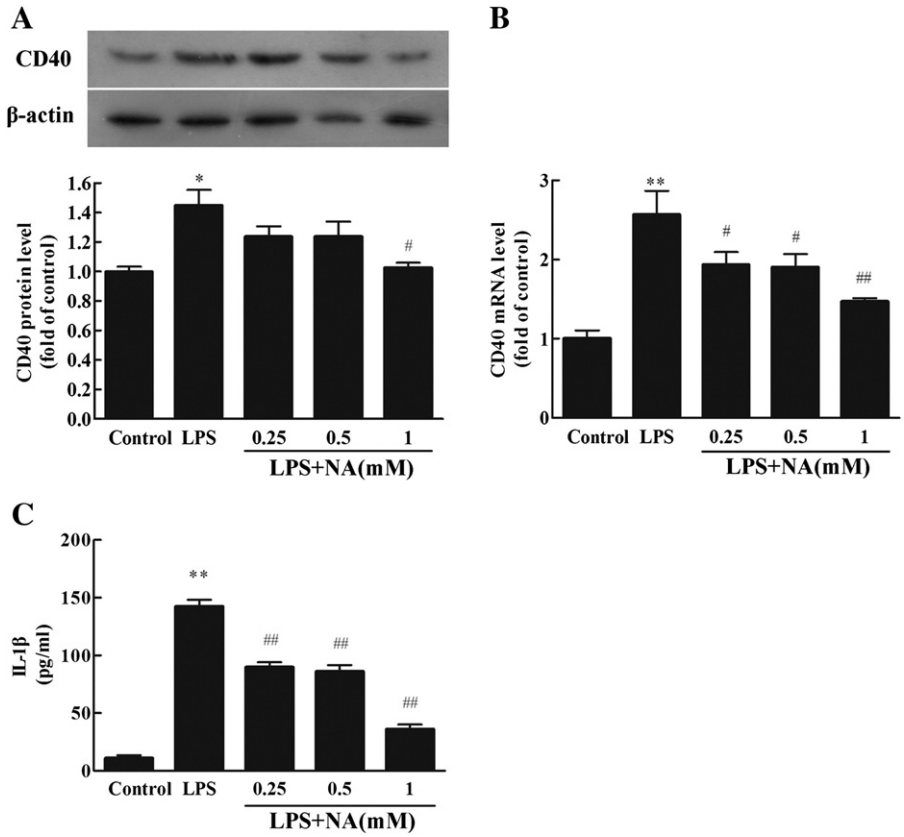

Fig. 5. NA inhibits LPS-induced CD40 expression and IL-1β level in HUVECs. Endothelial cells were pre-incubated with NA at different concentrations (0.25, 0.5 and 1 mM) for 1 h, then stimulated for 24 h with LPS (100 ng/ml). (A and B) The expression of CD40 protein and mRNA was determined by western blotting and real-time PCR, respectively. (C) The level of IL-1β in culture medium was measured by ELISA analysis. $^{*}P<.05$ and $^{**}P<.01$ vs. control; $^{\#}P<.05$ and $^{\#\#}P<.01$ vs. LPS group. Data are expressed as mean±S.E.M., $n=3$.

4. Discussion

Accumulating evidences suggest that the CD40/CD40L system plays a crucial role in the onset of the inflammatory reaction in cardiovascular disease [24,25]. Our previous studies have showed that fenofibrate [7] and lovastatin [9] suppress the inflammation by decreasing the CD40 expression in cardiovascular disease. In the present study, we found that NA partially antagonized the CD40 and CD40L expression in the arteries, which were triggered by placing a non-occlusive silastic collar around the carotid artery in the NZW rabbits. Meanwhile, NA inhibited the endothelial expression of CD40 in the vessel of the rabbits. In addition to *in vivo* experiment, our *in vitro* study has also revealed that NA evidently suppressed LPS-induced mRNA and protein expression of CD40. It has been demonstrated that CD40 deficiency inhibited the expression of IL-1β in the carotid arteries of mice after ligation injury [26]. Our study also showed that the inhibitory effect of NA in IL-1β production was accompanied by a decrease in the expression of CD40. Furthermore, it has been reported that a combination of NA and simvastatin decreased soluble CD40L in patients with coronary artery stenosis [27]. In combination, the present results indicate that NA exerts a potential role in the inhibition of the CD40 and CD40L expression.

In the past few years, the beneficial effects of NA have historically been regarded as a result of its lipid-modifying activity [28,29]. However, a growing number of studies suggested that NA has the anti-inflammatory potential in cardiovascular disease. A previous study showed that NA can reduce the generations of MCP-1 in TNF-α-stimulated human aortic endothelial cells [3]. Another report indicated that NA decreased Lp-PLA_2 level in the coronary artery disease patients [30]. Our results also demonstrated that treatment with NA repressed the increase in serum IL-1β of the rabbits. In addition, we found that the expression of MCP-1 and CRP in arteries was significantly increased in the rabbits with a periarterial carotid collar. When NA was given to the animals, the MCP-1 and CRP expression were significantly decreased. These results imply that NA may also have a potential to protect against carotid collar-induced vascular inflammation. In our previous study, we found that fenofibrate and lovastatin possess anti-inflammatory effects independent of their lipid-lowering action [7]. Importantly, our present study showed that there were no significant differences in plasma lipid levels of rabbits after NA treatment. Meanwhile, NA inhibited the expression of CD40 both in the animal study and LPS-stimulated endothelial cells. Wu et al. [31] also observed that NA protected against TNF-α-induced VCAM-1 and ICAM-1 expression in aortic rings from rabbits. In light of these findings, we speculate that NA exerts an inhibitory effect on vascular inflammation that is independent of changes in plasma lipids.

It has been well accepted that SIRT1 regulates the inflammatory response in cardiovascular disease [14,32,33]. Therefore, we wondered whether the potential mechanisms of NA were related to the expression of SIRT1. A recent report revealed that aspirin attenuated IL-6 secretion via up-regulating SIRT1 expression in vinorelbine-treated endothelial cells [34]. Our present study showed that the stimulation of collar placement leaded to the decrease of SIRT1 expression in the arteries of rabbits. After the administration of NA, the protein and mRNA expression of SIRT1 in arteries significantly increased. In addition, Yang et al. [16] reported that SIRT1 activity in livers of high-fat fed OLETF rats was up-regulated after administration of NA. Interestingly, our results also indicated that NA increased SIRT1

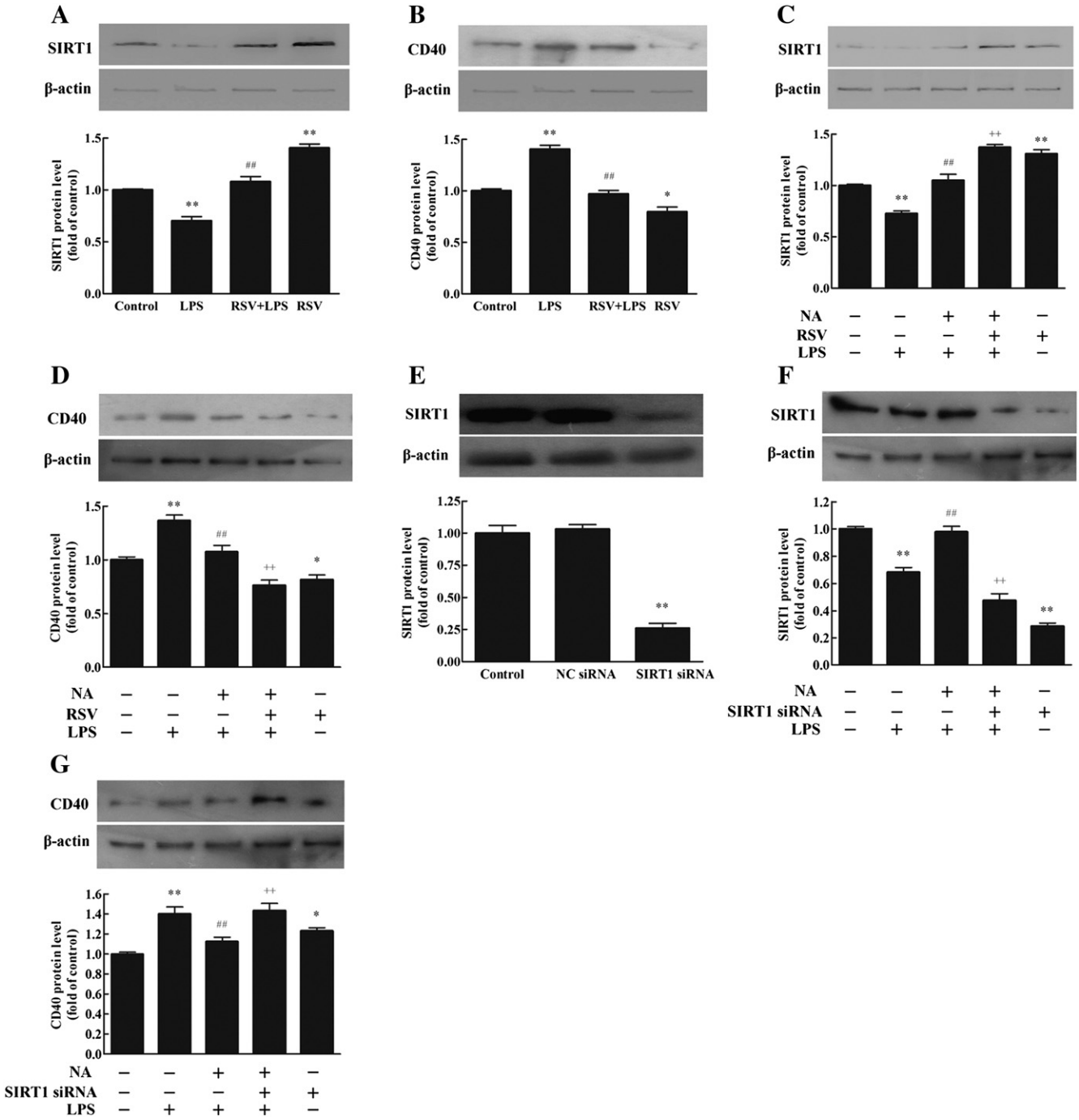

Fig. 6. NA inhibits LPS-induced CD40 expression through SIRT1 in HUVECs. (A and B) Endothelial cells were pretreated with SIRT1 activator RSV (20 μM), and subsequently stimulated with LPS (100 ng/ml) for 24 h. The expression of SIRT1 and CD40 was determined by western blotting. $^{*}P<.05$ and $^{**}P<.01$ vs. control; $^{\#\#}P<.01$ vs. LPS group. (C and D) Endothelial cells were pretreated with RSV (20 μM), and then incubated with NA (1 mM) for 1 h, followed by stimulation with LPS (100 ng/ml) for 24 h. $^{*}P<.05$ and $^{**}P<.01$ vs. control; $^{\#\#}P<.01$ vs. LPS group; $^{++}P<.01$ vs. NA+ LPS group. (E) Endothelial cells were transfected with SIRT1 siRNA or negative control siRNA (NC siRNA) for 48h, and the SIRT1 expression was determined by western blotting. $^{**}P<.01$ vs. NC siRNA. (F and G) Endothelial cells transfected with SIRT1-specific siRNA or NC siRNA were pretreated with NA (1 mM) for 1 h, and then stimulated with LPS (100 ng/ml) for 24 h. The expression of SIRT1 and CD40 was determined by western blotting. $^{*}P<.05$ and $^{**}P<.01$ vs. NC siRNA group; $^{\#\#}P<.01$ vs. NC siRNA+ LPS group; $^{++}P<.01$ vs. NC siRNA+ NA+ LPS group. Data are expressed as mean±S.E.M., $n=3$.

expression in livers, hearts, and spleens of rabbits. These accumulating data reveal that NA significantly increases SIRT1 levels, not only in the arteries but also in various other organs, e.g., liver, heart, and spleen. Moreover, our earlier studies suggested that fenofibrate was able to increase the SIRT1 expression in 3T3-L1 adipocytes [35]. Here, we also found that NA could up-regulate SIRT1 expression in a concentration-dependent manner in LPS-stimulated endothelial cells. The present *in vivo* and *in vitro* studies indicate that the ability of NA to inhibit vascular inflammation may be related to the increased expression of SIRT1.

On the basis of the results mentioned above, we further investigated whether the anti-inflammation effect of NA was mediated via SIRT1-dependent pathway. In our previous report, we found that SIRT1 exerted anti-inflammatory property by regulating

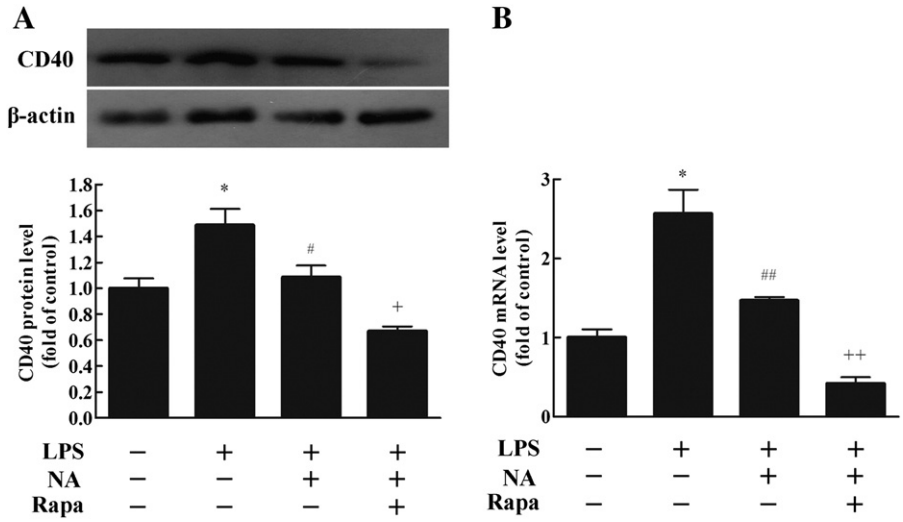

Fig. 7. NA inhibits LPS-induced CD40 expression partly through mTOR in HUVECs. (A and B) Endothelial cells were pretreated with mTOR inhibitor rapamycin (Rapa, 100 nM) for 1 h, and then incubated with NA (1 mM) for 1 h, followed by stimulation with LPS (100 ng/ml) for 24 h. The expression of CD40 protein and mRNA was determined by western blotting and real-time PCR, respectively. $^{*}P<.05$ vs. control; $^{\#}P<.05$ and $^{\#\#}P<.01$ vs. LPS group; $^{+}P<.05$ and $^{++}P<.01$ vs. NA+ LPS group. Data are expressed as mean±S.E.M., $n=3$.

TNF-α-induced expression of CD40 in adipocytes [15]. Our present study showed that SIRT1 activator obviously reduced the expression of CD40 and CD40L, and inhibited collar-induced changes of MCP-1 and CRP in the arteries of rabbits. In an *in vitro* study, we also found that SIRT1 activation obviously decreased LPS-induced CD40 expression. These results suggest that SIRT1 exerts anti-inflammatory property by regulating the expression of CD40. In addition, treatment of the animals with both the SIRT1 activator and NA synergistically suppressed CD40 and CD40L expression, which enriched the inhibitory effect of NA on MCP-1 and CRP expression in the arteries of rabbits. In light of the above-mentioned results *in vivo*, further study was determined in LPS-stimulated endothelial cells. Our results showed that the activation of SIRT1 augmented the inhibitory effect of NA on LPS-mediated CD40 expression in endothelia cells. This seems to be consistent with the fact observed in the collar-induced vascular inflammation of rabbits. However, there is still much to learn about the relationship between the inhibitory effects of NA on CD40 expression and SIRT1. Accordingly, SIRT1 siRNA was applied to endothelial cells to clarify whether SIRT1 was involved in the CD40 inhibitory effects of NA. We found that the up-regulation effect of NA on SIRT1 expression could be attenuated by SIRT1 siRNA. Furthermore, knockdown of SIRT1 abolished the inhibitory effect of NA on LPS-induced the expression of CD40 in endothelial cells, further suggesting that SIRT1 is associated with the inhibitory effects of NA on LPS-induced CD40 expression. These findings support the notion that NA protects against vascular inflammatory responses through SIRT1/CD40-dependent signaling pathway.

Recently, great interest exists in the critical role of mTOR in the inflammatory process in various cell types, including monocytes [36], macrophages [37] and adipocytes [38]. Wang et al. [39] reported that the mTOR inhibition decreased VCAM-1 expression in arteries in TNF-treated mice. Moreover, the inhibition of mTOR antagonized the expression of CD40 and ICAM-1 in TNF-α and IFN-γ stimulated-human islet endothelial cells [40]. In the present study, treatment of the cells with both the mTOR blocker and NA synergistically suppressed LPS-induced changes of CD40 expression. These preliminary findings show that the inhibitory effect of NA on CD40 expression in endothelial cells may be partly mediated by mTOR.

In conclusion, our data provides the evidence that NA can inhibit vascular inflammation in rabbits triggered by collar implantation. Moreover, NA exerts its anti-inflammatory effect via the SIRT1/CD40-dependent signaling pathway. Another preliminary finding is the capability of NA to inhibit inflammation response partly via mTOR. These findings provide new mechanisms to beneficial effects of NA in the prevention and treatment of cardiovascular disease.

References

[1] Meyers CD, Kamanna VS, Kashyap ML. Niacin therapy in atherosclerosis. Curr Opin Lipidol 2004;15:659–65.
[2] Wu BJ, Chen K, Barter PJ, Rye KA. Niacin inhibits vascular inflammation via the induction of heme oxygenase-1. Circulation 2012;125:150–8.
[3] Ganji SH, Qin S, Zhang L, Kamanna VS, Kashyap ML. Niacin inhibits vascular oxidative stress, redox-sensitive genes, and monocyte adhesion to human aortic endothelial cells. Atherosclerosis 2009;202:68–75.
[4] Digby JE, Martinez F, Jefferson A, Ruparelia N, Chai J, Wamil M, et al. Anti-inflammatory effects of nicotinic acid in human monocytes are mediated by GPR109A dependent mechanisms. Arterioscler Thromb Vasc Biol 2012;32:669–76.
[5] Lipszyc PS, Cremaschi GA, Zorrilla-Zubilete M, Bertolino ML, Capani F, Genaro AM, et al. Niacin modulates pro-inflammatory cytokine secretion. A potential mechanism involved in its anti-atherosclerotic effect. Open Cardiovasc Med J 2013;7:90–8.
[6] Si Y, Zhang Y, Zhao J, Guo S, Zhai L, Yao S, et al. Niacin inhibits vascular inflammation via downregulating nuclear transcription factor-kappa B signaling pathway. Mediators Inflamm 2014;2014:263786. http://dx.doi.org/10.1155/2014/263786.
[7] Lin R, Liu J, Gan W, Yang G. C-reactive protein-induced expression of CD40-CD40L and the effect of lovastatin and fenofibrate on it in human vascular endothelial cells. Biol Pharm Bull 2004;27:1537–43.
[8] Antoniades C, Bakogiannis C, Tousoulis D, Antonopoulos AS, Stefanadis C. The CD40/CD40 ligand system: linking inflammation with atherothrombosis. J Am Coll Cardiol 2009;54:669–77.
[9] Lin R, Liu J, Peng N, Yang G, Gan W, Wang W. Lovastatin reduces nuclear factor kappa B activation induced by C-reactive protein in human vascular endothelial cells. Biol Pharm Bull 2005;28:1630–4.
[10] Finkel T, Deng CX, Mostoslavsky R. Recent progress in the biology and physiology of sirtuins. Nature 2009;460:587–91.
[11] Hubbard BP, Gomes AP, Dai H, Li J, Case AW, Considine T, et al. Evidence for a common mechanism of SIRT1 regulation by allosteric activators. Science 2013;339:1216–9.
[12] Gorenne I, Kumar S, Gray K, Figg N, Yu H, Mercer J, et al. Vascular smooth muscle cell sirtuin 1 protects against DNA damage and inhibits atherosclerosis. Circulation 2013;127:386–96.
[13] Zhang QJ, Wang Z, Chen HZ, Zhou S, Zheng W, Liu G, et al. Endothelium-specific overexpression of class III deacetylase SIRT1 decreases atherosclerosis in apolipoprotein E-deficient mice. Cardiovasc Res 2008;80:191–9.
[14] Stein S, Schafer N, Breitenstein A, Besler C, Winnik S, Lohmann C, et al. SIRT1 reduces endothelial activation without affecting vascular function in ApoE$^{-/-}$ mice. Aging (Albany NY) 2010;2:353–60.
[15] Lin QQ, Yan CF, Lin R, Zhang JY, Wang WR, Yang LN, et al. SIRT1 regulates TNF-alpha-induced expression of CD40 in 3T3-L1 adipocytes via NF-kappa B pathway. Cytokine 2012;60:447–55.

[16] Yang SJ, Choi JM, Kim L, Park SE, Rhee EJ, Lee WY, et al. Nicotinamide improves glucose metabolism and affects the hepatic NAD-sirtuin pathway in a rodent model of obesity and type 2 diabetes. J Nutr Biochem 2014;25:66–72.
[17] Sinclair DA, Guarente L. Small-molecule allosteric activators of sirtuins. Annu Rev Pharmacol Toxicol 2014;54:363–80.
[18] Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, et al. Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. Nature 2003;425:191–6.
[19] Tabet F, Remaley AT, Segaliny AI, Millet J, Yan L, Nakhla S, et al. The 5A apolipoprotein A-I mimetic peptide displays antiinflammatory and antioxidant properties in vivo and in vitro. Arterioscler Thromb Vasc Biol 2010;30:246–52.
[20] Xiao H, Lu M, Lin TY, Chen Z, Chen G, Wang WC, et al. Sterol regulatory element binding protein 2 activation of NLRP3 inflammasome in endothelium mediates hemodynamic-induced atherosclerosis susceptibility. Circulation 2013;128:632–42.
[21] Fang Z, Lin R, Yuan B, Liu Y, Zhang H. Tanshinone IIA inhibits atherosclerotic plaque formation by down-regulating MMP-2 and MMP-9 expression in rabbits fed a high-fat diet. Life Sci 2007;81:1339–45.
[22] Yang L, Zhang J, Yan C, Zhou J, Lin R, Lin Q, et al. SIRT1 regulates CD40 expression induced by TNF-alpha via NF-kB pathway in endothelial cells. Cell Physiol Biochem 2012;30:1287–98.
[23] Liu Y, Zhang X, Zhang W, Chen Z, Chan T, Ali K, et al. Adenovirus-mediated CD40 ligand gene-engineered dendritic cells elicit enhanced $CD8^{+}$ cytotoxic T-cell activation and antitumor immunity. Cancer Gene Ther 2002;9:202–8.
[24] Rizvi M, Pathak D, Freedman JE, Chakrabarti S. CD40-CD40 ligand interactions in oxidative stress, inflammation and vascular disease. Trends Mol Med 2008;14:530–8.
[25] Hakkinen T, Karkola K, Yla-Herttuala S. Macrophages, smooth muscle cells, endothelial cells, and T-cells express CD40 and CD40L in fatty streaks and more advanced human atherosclerotic lesions. Colocalization with epitopes of oxidized low-density lipoprotein, scavenger receptor, and CD16 (Fc gammaRIII). Virchows Arch 2000;437:396–405.
[26] Song Z, Jin R, Yu S, Rivet JJ, Smyth SS, Nanda A, et al. CD40 is essential in the upregulation of TRAF proteins and NF-κB-dependent proinflammatory gene expression after arterial injury. PLoS One 2011;6:e23239. http://dx.doi.org/10.1371/journal.pone.0023239.
[27] Lee K, Ahn TH, Kang WC, Han SH, Choi IS, Shin EK. The effects of statin and niacin on plaque stability, plaque regression, inflammation and oxidative stress in patients with mild to moderate coronary artery stenosis. Korean Circ J 2011;41:641–8.
[28] Lukasova M, Hanson J, Tunaru S, Offermanns S. Nicotinic acid (niacin): new lipid-independent mechanisms of action and therapeutic potentials. Trends Pharmacol Sci 2011;32:700–7.
[29] Kamanna VS, Kashyap ML. Nicotinic acid (niacin) receptor agonists: will they be useful therapeutic agents? Am J Cardiol 2007;100:S53–61.
[30] Kuvin JT, Dave DM, Sliney KA, Mooney P, Patel AR, Kimmelstiel CD, et al. Effects of extended-release niacin on lipoprotein particle size, distribution, and inflammatory markers in patients with coronary artery disease. Am J Cardiol 2006;98:743–5.
[31] Wu BJ, Yan L, Charlton F, Witting P, Barter PJ, Rye KA. Evidence that niacin inhibits acute vascular inflammation and improves endothelial dysfunction independent of changes in plasma lipids. Arterioscler Thromb Vasc Biol 2010;30:968–75.
[32] Csiszar A, Labinskyy N, Podlutsky A, Kaminski PM, Wolin MS, Zhang C, et al. Vasoprotective effects of resveratrol and SIRT1: attenuation of cigarette smoke-induced oxidative stress and proinflammatory phenotypic alterations. Am J Physiol Heart Circ Physiol 2008;294:H2721–35.
[33] Csiszar A, Labinskyy N, Jimenez R, Pinto JT, Ballabh P, Losonczy G, et al. Anti-oxidative and anti-inflammatory vasoprotective effects of caloric restriction in aging: role of circulating factors and SIRT1. Mech Ageing Dev 2009;130:518–27.
[34] Tsai KL, Huang PH, Kao CL, Leu HB, Cheng YH, Liao YW, et al. Aspirin attenuates vinorelbine-induced endothelial inflammation via modulating SIRT1/AMPK axis. Biochem Pharmacol 2014;88:189–200.
[35] Wang W, Lin Q, Lin R, Zhang J, Ren F, Zhang J, et al. PPARalpha agonist fenofibrate attenuates TNF-alpha-induced CD40 expression in 3T3-L1 adipocytes via the SIRT1-dependent signaling pathway. Exp Cell Res 2013;319:1523–33.
[36] Park S, Regmi SC, Park SY, Lee EK, Chang JH, Ku SK, et al. Protective effect of 7-O-succinyl macrolactin A against intestinal inflammation is mediated through PI3-kinase/Akt/mTOR and NF-kappa B signaling pathways. Eur J Pharmacol 2014;735:184–92.
[37] van den Bosch MW, Palsson-Mcdermott E, Johnson DS, ONeill LA. LPS induces the degradation of programmed cell death protein 4 (PDCD4) to release Twist2, activating c-Maf transcription to promote interleukin-10 production. J Biol Chem 2014;289:22980–90.
[38] Tsai CW, Liu KL, Lin YR, Kuo WC. The mechanisms of carnosic acid attenuates tumor necrosis factor-alpha-mediated inflammation and insulin resistance in 3T3-L1 adipocytes. Mol Nutr Food Res 2014;58:654–64.
[39] Wang C, Qin L, Manes TD, Kirkiles-Smith NC, Tellides G, Pober JS. Rapamycin antagonizes TNF induction of VCAM-1 on endothelial cells by inhibiting mTORC2. J Exp Med 2014;211:395–404.
[40] Cantaluppi V, Biancone L, Romanazzi GM, Figliolini F, Beltramo S, Ninniri MS, et al. Antiangiogenic and immunomodulatory effects of rapamycin on islet endothelium: relevance for islet transplantation. Am J Transplant 2006;6:2601–11.