MEDICAL SCIENCE MONITOR

LAB/IN VITRO RESEARCH

e-ISSN 1643-3750
© Med Sci Monit, 2019; 25: 4137-4148
DOI: 10.12659/MSM.913240

Received: 2018.09.17
Accepted: 2019.02.12
Published: 2019.06.03

Screening Analysis of Sirtuins Family Expression on Anti-Inflammation of Resveratrol in Endothelial Cells

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Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Source of support: This work was supported by grants from the National Natural Science Foundation of China (No. 81670258, 81373785), Natural Science Foundation of Fujian (2017J01247) and the Projection of Excellent Yang Doctor Training Plan in Fujian Provincial Health System (No. 2013-ZQN-ZD-2)

Background: Resveratrol has been shown to possess beneficial activities including antioxidant, anti-inflammatory, and cardioprotective effects through activating a nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylase family member sirtuin-1 (SIRT1) protein. The current study was undertaken to investigate the role of sirtuin family members (SIRT1–SIRT7) on the anti-inflammation activities of resveratrol in endothelial cells.

Material/Methods: Primary human umbilical vein endothelial cells (HUVECs) were pretreated with resveratrol before tumor necrosis factor (TNF)-α (10–20 μg/L) stimulation. Cell viability was measured using the Cell Counting Kit-8 method. Total RNA was extracted after different treatments and the NimbleGen Human 12×135K Gene Expression Array was applied to screen and analyze SIRTs expression. Quantitative real-time polymerase chain reaction and western blot were applied to verify the results of the gene expression microarrays. Reactive oxygen species (ROS) production was examined using flow cytometry analysis.

Results: Microarray analysis showed that the expressions of SIRT1, SIRT2, SIRT3, SIRT5, SIRT6, and SIRT7 showed the tendency to increase while SIRT4 showed the tendency to decrease. SIRT1, SIRT2, SIRT5, and SIRT7 gene expression could be upregulated by pretreatment with resveratrol compared with TNF-α alone while there were no obvious differences of SIRT3, SIRT4, and SIRT6 expressions observed in TNF-α alone treated cells and resveratrol-TNF-α co-treated cells. Interestingly, SIRT1, SIRT2, SIRT3, SIRT4, and SIRT5 siRNA could reverse the effect of resveratrol on ROS production; SIRT1 and SIRT5 siRNA could significantly increase CD40 expression inhibited by resveratrol in TNF-α treated cells.

Conclusions: Our results suggest that resveratrol inhibiting oxidative stress production is associated with SIRT1, SIRT2, SIRT3, SIRT4, and SIRT5 pathways; attenuating CD40 expression was only associated with SIRT1 and SIRT5 pathways in TNF-α-induced endothelial cells injury.

MeSH Keywords: Endothelial Cells • Inflammation • Sirtuins

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Background

Sirtuins belong to class III histone deacetylases that are dependent on nicotinamide adenine dinucleotide (NAD) for their activity which was evolutionarily conserved from bacteria to humans [1]. Seven different homologues of yeast Sir2, which are a set of genes collectively called sirtuins (SIRT1–SIRT7), exist in mammals [2]. Sirtuins appear to have a prominent role in vascular biology, and in preclinical models, they promote a variety of physiological effects, which would be expected to oppose atherogenesis. Preclinical studies suggest roles for SIRTs in protecting endothelial cells from the deleterious effects associated with lipid deposition, oxidative stress, and inflammation. SIRT1 is known to play a crucial role in cell survival, metabolism, oxidative stress, and inflammation [3]. SIRT3 plays important roles in regulating mitochondrial and maintaining homeostasis and cellular metabolic functions [4]. Recently, Xu et al. reported that SIRT6 can suppress vascular inflammation and attenuate endothelial dysfunction that reduces the formation of atherosclerotic lesions [5].

Resveratrol (3', 5', 4', trihydroxy-trans-stilbene) is a natural polyphenol compound found in more than 70 plant species and their derivatives such as red wine or grape juice. It has been reported that resveratrol has a wide range of health-promoting abilities including extended lifespan, anti-aging, anti-cancer, and other cardiovascular protective effects which are associated with antioxidation, anti-inflammation and anti-apoptosis [6]. The protection effects of resveratrol have been proven to be involve in multiple signal pathways [7,8]. Many studies have proposed that the beneficial effects of resveratrol are mainly mediated through activation of the SIRT1 pathway *in vitro* and *in vivo* [9,10]. Our previous study also proved that resveratrol could regulate immune inflammatory response through the SIRT1/NF-κB/CD40 pathway [11]. Yu et al. found that resveratrol protected cardiomyocytes from oxidative-stress induced apoptosis by activating SIRT1, SIRT3, SIRT4, and SIRT7 [12]. Schirmer et al. showed that resveratrol did not change the mRNA levels of SIRT1 but decreased the expression levels of the SIRT3 and SIRT4 in wild-type adult zebrafish liver [13]. Interestingly, as yet, no data has systematically analyzed the role of sirtuins family, in particular the role SIRT2–SIRT7, in endothelial cells where resveratrol inhibits immune inflammatory response.

Inflammation plays important roles in the pathogenesis of atherosclerotic cardiovascular disease. Seven closely-related SIRT family members, SIRT1–SIRT7, have been identified in mammals. The present study aimed to investigate whether the effect of resveratrol on inhibiting inflammatory activities were mediated by other sirtuins pathways, through providing screening detection of resveratrol on SIRT1–SIRT7 using human whole genome microarrays in HUVECs. Hence, this study constitutes a step forward in the understanding of the potential of resveratrol on the gene expression profiles of the sirtuins family. In addition, we sought to correlate the relationship between sirtuins gene expression and endothelial inflammation.

Material and Methods

Reagents

Tumor necrosis factor (TNF)-α (300-01A) was purchased from PeproTech (Rocky Hill, NJ, USA). Resveratrol (SML0963) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Endothelial cell medium (ECM), fetal bovine serum (FBS), endothelial cell growth supplement (ECGS), and penicillin/streptomycin solution (P/S) (1001) were purchased from Sciencell (CA, USA). Fluorescein (FITC)-conjugated affiniPure Goat Anti-Rabbit IgG (H+L) (111-095-144) was purchased from Jackson ImmunoResearch Company (USA). Rabbit Anti-Factor VIII related antigen (BA0046) was purchased from Boster (China). SIRT1 (ab32441), SIRT2 (ab51023), SIRT3 (ab86671), SIRT4 (ab105039), SIRT5 (ab105040), SIRT6 (ab62739), SIRT7 (ab105038) antibodies, and BCA protein assay kit (ab102536) were provided by Abcam (Abcam, USA) [11].

Cell culture

Our study was approved by the Ethics Committee of the Fujian Provincial Hospital (No. K2014-021-01). To obtain qualified HUVECs samples, human umbilical cords were collected from a total of 20 healthy pregnant women continuously during our experiment, they were strictly examined without hepatitis B/C, human immunodeficiency (HIV) infection, syphilis and meconium-stained amniotic fluid. Every sample was obtained after receiving a written informed consent document for each patient. All aspects of the study complied with the declaration of Helsinki. Primary HUVECs cultures were separated from human umbilical cords within 6 hours of delivery according to the methodology of a collagenase treatment provided by Marin et al. [14]. HUVECs were cultured in ECM supplemented with 5% FBS, 50 ug/mL ECGS and 1% P/S at 37°C in a humidified atmosphere of 5% CO_2/95% air. HUVECs were maintained in the medium replaced every 3 days. All experiments were performed using HUVECs from passages 3–5. Primary HUVECs culture was in Figure 1.

Treatment and experimental design

HUVECs were placed in 6-well plates (1×10^6 cells/well) containing medium. The cultivated cells were pretreated with 20 μmol/L resveratrol 4 hours before 10 μg/L TNF-α stimulation for 24 hours. The mRNA and protein levels of sirtuins were measured by real-time quantitative polymerase chain reaction (RT-qPCR) and western blot.

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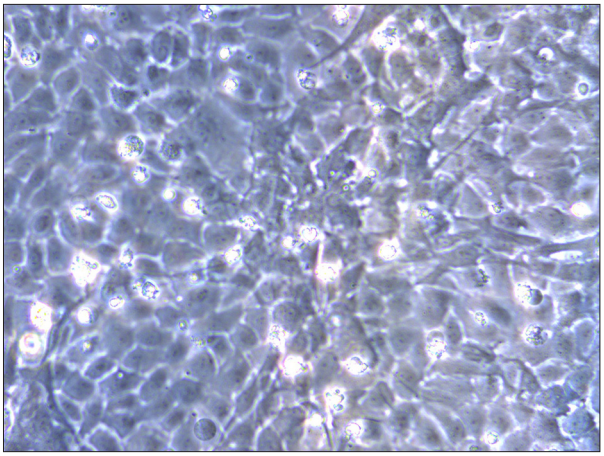

Figure 1. Primary HUVECs culture (200×). HUVECs – human umbilical vein endothelial cells.

RNA interference1

HUVECs were transfected with synthesized small interfering RNA (siRNA) targeting human SIRT1–SIRT7 or a negative control siRNA (Nc siRNA) as previously describe. The siRNA and Lipofectamine2000 transfection agents were separately diluted with serum-free medium, according to the manufacturer's instructions and then were mixed together. HUVECs were transfected with siRNA-Lipofectamine 2000 complexes, and the silencing effect of the siRNA was evaluated by RT-qPCR as well as western blot after 48 hours transfection. After successful transfection by PCR methods, HUVECs were treated with resveratrol and TNF-α according to the experimental design mentioned aforementioned. SiRNA sequence were as shown in Table 1 [11].

Cytotoxicity assay

HUVECs were dispensed in 96-well microtiter at a density of 1×10^4 cells/well for 48 hours. A Cell Counting Kit-8 (CCK-8) assay was used to determine the anti-toxicity effect of each drug. After treatment with resveratrol or TNF-α over a range of concentrations (resveratrol: 0 (control), 10, 20, 40, and 80 μmol/L, TNF-α: 0 (control), 1, 10, and 100 μg/L), 10 μl of CKK-8 reagent was added to 100 μL of the media in each well, followed by incubation for 4 hours at 37°C, absorbance of each well was determined at 450 nm (reference wavelength 630 nm) by a multiplate reader. Cell viability was calculated using the formula (absorbance of treated cells)/(absorbance of control cells)×100.

RNA labeling, hybridization, microarray processing, scanning, data collection, and normalization

Invitrogen SuperScript ds-cDNA synthesis kits were used to synthesized double-strand cDNA (ds-cDNA) from 5 μg of total RNA, then the synthesis ds-cDNA was cleaned and labeled in accordance to the NimbleGen Gene Expression Analysis protocol and quantified by NanoDrop ND-1000. For Cy3 labeling of cDNA, the NimbleGen One-Color DNA labeling kit was used according to the manufacturer's guideline. Then, 100 pmol of deoxynucleoside triphosphates and 100 U of the Klenow fragment (New England Biolabs, USA) were added and the mix incubated at 37°C for 2 hours [15]. Microarrays were hybridized at 42°C during 16 to 20 hours with 4 μg of Cy3 labeled ds-cDNA in NimbleGen hybridization buffer/hybridization component A in a hybridization chamber (Hybridization System – NimbleGen Systems, Inc., Madison, WI, USA). Following hybridization, washing was performed using the NimbleGen Wash Buffer kit (NimbleGen Systems, Inc., Madison, WI, USA). After being washed in an ozone-free environment, the slides were scanned using the Axon GenePix 4000B microarray scanner (Molecular Devices Corporation) piloted by GenePix Pro 6.0 software (Axon). Scanned images (TIFF format) were then imported into NimbleScan software (version 2.5) for grid alignment and expression data analysis. Expression data were normalized through quantile normalization and the Robust Multichip Average (RMA) algorithm included in the NimbleScan software. The Probe level (*_norm_RMA.pair) files and Gene level (*_RMA.calls) files were generated after normalization. All gene level files were imported into Agilent GeneSpring GX software (version 12.1) for further analysis [16]. First, high quality expression values (for example, where at least 3 out of 9 samples have values within cut-off (cut-off=100) will be selected for analysis. Then differentially expressed genes were identified through fold change filtering between 2 samples or *t*-test filtering between 2 groups. The genes that were consistently altered in both arrays with differences in mean expression ratios that

Table 1. SiRNA sequence.

SiRNA name	Sequence
Negative siRNA	Sense: 5'-UUCUCCGAACGUGUCACGUTT-3' Anti-sense: 5'-ACGUGACACGUUCGGAGAATT-3'
SIRT1 siRNA	Sense: 5'-CCAAGCAGCUAAGAGUAAUTT-3' Anti-sense: 5'-AUUACUCUUAGCUGCUUGGTT-3'
SIRT2 siRNA	Sense: 5'-CGGCCUCUAUGACAACCUATT-3' Anti-sense: 5'-UAGGUUGUCAUAGAGGCCGTT-3'
SIRT3 siRNA	Sense: 5'-CCAGCAUGAAAUACAUUUATT-3' Anti-sense: 5'-UAAAUGUAUUUCAUGCUGGTT-3'
SIRT4 siRNA	Sense: 5'-CAUCCAGCAUGGUGAUUUUTT-3' Anti-sense: 5'-AAAAUCACCAUGCUGGAUGTT-3'
SIRT5 siRNA	Sense: 5'-CUCGCCCACUGUGAUUUAUTT-3' Anti-sense: 5'-AUAAAUCACAGUGGGCGAGTT-3'
SIRT6 siRNA	Sense: 5'-ACGCAGUACGUCCGAGACATT-3' Anti-sense: 5'-UGUCUCGGACGUACUGCGUTT-3'
SIRT7 siRNA	Sense: 5'-GGAAGUGUGAUGACGUCAUTT-3' Anti-sense: 5'-AUGACGUCAUCACACUUCCTT-3'

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Table 2. PCR primers sequences for SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, and SIRT7.

Gene	Forward sequence	Reverse sequence
SIRT1	5'-GATTAGTAGGCGGCTTGATGGT-3'	5'-TCTTCTAAACTTGGACTCTGGCAT-3'
SIRT2	5'-CAAGCCAACCATCTGTCACTACT-3'	5'-CTCCACCAAGTCCTCCTGTTC-3'
SIRT3	5'-TGTTGACTCTCCATACTCAGCCTC-3'	5'-TCCACCTCCTATGTCACAATCCAG-3'
SIRT4	5'-CGTTGTGGAGAGTTGCTGCCT-3'	5'-TTGAATGGGAACTGGAATCTGTC-3'
SIRT5	5'-CCTTGGAATGCCTGCTGTC-3'	5'-ATGATAGTGTCTTGTTGCCTGTT-3'
SIRT6	5'-GCAGTCTTCCAGTGTGGTGTTC-3'	5'-CGTCTTACACTTGGCACATTCT-3'
SIRT7	5'-CAGAACTGTGACGGGCTCCA-3'	5'-AGGATGGTGTCTGCTCTGCTG-3'
CD40	5'-ACACTGCCACCAGCACAAATAC-3'	5'-GATAAAGACCAGCACCAAGAGGAT-3'
GAPDH	5'-GAGAAACCTGCCAAGTATGATGAC-3'	5'-AGAGTGGGAGTTGCTGTTGAAG-3'

were greater than 2-fold on average were selected as differentially expressed genes. Hierarchical clustering was performed using R scripts. GO analysis and pathway analysis were performed using the standard enrichment computation methods.

Validation by RT-qPCR

After harvesting HUVECs, total RNA was extracted by using the QIAGEN RNeasy® Mini Kit and was verified purified when the ratio between the absorbance values at 260 and 280 nm between 1.8–2.0. RT-qPCR was performed using GAPDH as the internal control. Thermal cycling conditions were as: 95°C for 2 minutes for the activation of Taq DNA polymerase followed by 40 cycles of amplification at 95°C for 1 minute, 1 minute at 55°C, and 1 minute at 72°C. The PCR primer sequences for SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, and SIRT7 are listed in Table 2.

Western blotting

Cells were processed for protein extraction, and western blotting was performed by standard procedures as described earlier using sirtuins and CD40 antibodies against proteins under detection. Primary antibodies were used at 1: 1000 dilutions overnight, whereas HRP-conjugated secondary antibodies were used at 1: 2000 dilutions for 1 hour. Then β-actin was used as loading control. Antigen-antibody complexes were visualized with the enhanced chemiluminescence (ECL) plus western blotting detection system and the signal detected using a LAS-3000 image analyzer.

Reactive oxygen species (ROS) determination

HUVECs were plated (5×10^5 cells/well) in wells and the media was replaced with serum-free media along with different medium for intervention. After incubation, a solution of 2',7'-dihydrochloroflurorescein acetate (DCFH-DA) (10 μm/L) was added to the cells and incubated for 20 minutes in the dark. After staining, the cells were collected for determination. The fluorescence of DCFH-DA labelled cells was examined using flow cytometry analysis.

Statistical analysis

Each experiment was performed 3 times independently. All data are expressed as the means ± standard error of the mean (SEM). The mean values groups were subjected to one-way ANOVA. A value of $P < 0.05$ was considered as significant. SPSS software was used for all statistical analysis.

Results

Cytotoxicity test

To examine the cytotoxicity of resveratrol and TNF-α, HUVECs were treated with TNF-α in a range of 0–100 μg/L or resveratrol in a range of 0–80 μmol/L for 24 hours respectively. The data indicated that TNF-α of 10 μg/L statistically decreased the cell viability. The results also demonstrated that resveratrol in the concentration range of 0–40 μmol/L are largely non-toxic to HUVECs. To further investigate whether resveratrol could affect the cell viability reduced by TNF-α, HUVECs were treated with different concentrations of TNF-α combined with 20 μmol/L resveratrol. The results demonstrated that resveratrol at 20 μmol/L reversed the side effects of TNF-α significantly (Figure 2). So, we applied 20 μmol/L resveratrol and 10 μg/L TNF-α for our following experiment.

Effects of resveratrol on sirtuins family gene expression by microarray analysis

Perturbation of sirtuins gene expression in TNF-α and resveratrol treated HUVECs were determined by a Whole Human Genome Oligo Microarray platform. The heat map (Figure 3) showed the altered sirtuins genes expression from SIRT1–SIRT7 in HUVECs

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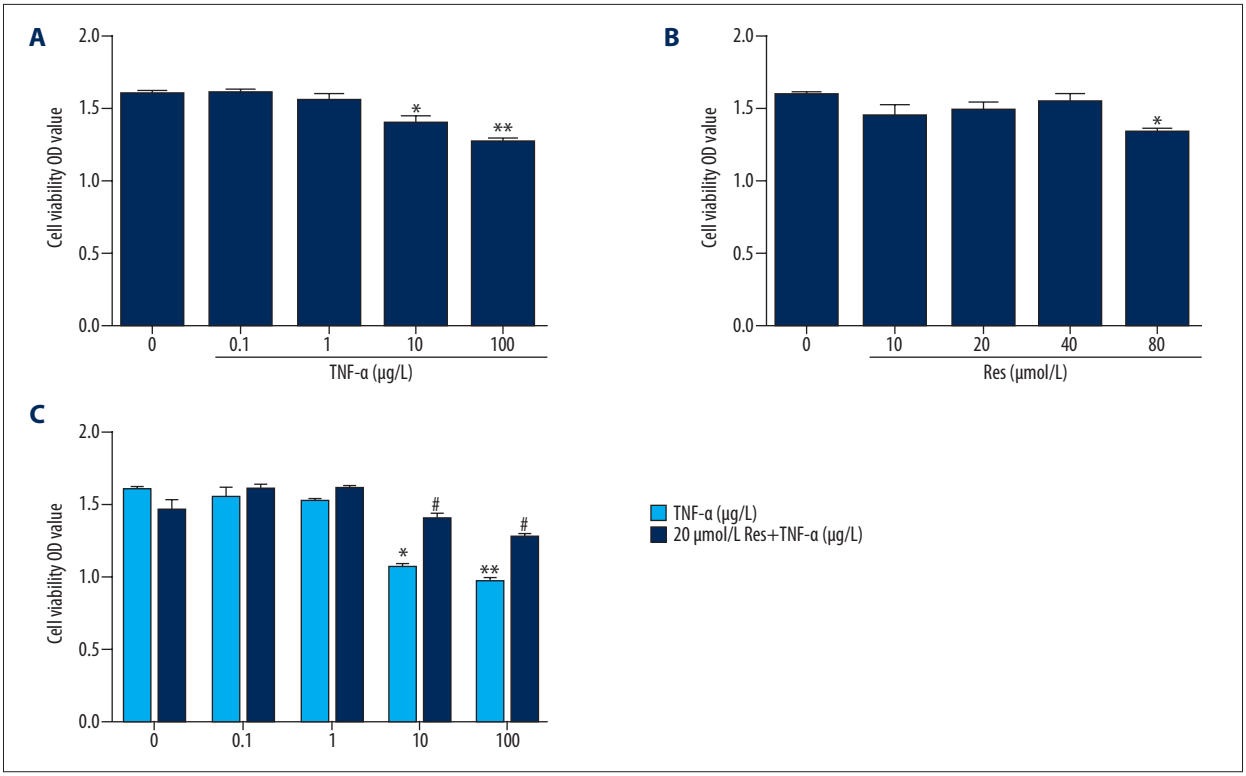

Figure 2. Cell viability assay. (**A**) Effects of different concentrations of TNF-α on HUVECs viability. (**B**) Effects of different concentrations of resveratrol on HUVECs viability. (**C**) Effects of different concentrations of TNF-α combined with 20 umol/L resveratrol on HUVECs viability. Compared with negative control, * $P<0.05$, ** $P<0.01$. Compared with 10 ug/L TNF-α stimulated HUVECs, # $P<0.05$. TNF – tumor necrosis factor; HUVECs – human umbilical vein endothelial cells.

treated with TNF-α alone or together with resveratrol. As we known, SIRT1–SIRT7 were involved in the regulating histone acetylation. The data showed SIRT2 gene expression was more than 2-fold increased by resveratrol compared with the TNF-α alone. SIRT1, SIRT2, SIRT3, SIRT5, SIRT6, and SIRT7 all showed the tendency to increase with more than 1-fold change while SIRT4 showed the tendency to decrease with more than 1-fold change. As shown in Figure 3, the data suggested that there was a tendency that SIRT4 was downregulated while other sirtuins were upregulated after resveratrol pretreatment but without statistical significance.

Effects of resveratrol on sirtuins SIRT1–SIRT7 mRNA expression

Quantitative real-time PCR analysis was performed to verification results of the microarray analysis. As shown in Figure 4, SIRT1–SIRT7 mRNA expression in 10 μmol/L TNF-α stimulated HUVECs all significantly decreased. The mRNA expression of SIRT1, SIRT2, SIRT5, and SIRT7 were statistically increased in resveratrol pre-treated cells compared with the treated TNF-α cells alone. However, SIRT3, SIRT4, and SIRT6 genes expression was shown no statistical significance in HUVECs.

Effects of resveratrol on sirtuins protein expression

We observed that the protein expressions of SIRT1–SIRT7 in HUVECs were all decreased by 10μg/L TNF-α stimulation. As shown in Figure 5, resveratrol at 20 μmol/L could reverse the repression of SIRT1, SIRT2, SIRT5, and SIRT7 induced by TNF-α. However, there is no statistical significance observed on SIRT3, SIRT4, and SIRT6 expression.

Effects of resveratrol on ROS through activating SIRT1, SIRT2, SIRT3, SIRT4, and SIRT5

Our previous study showed that resveratrol significantly decreased the reactive oxygen species (ROS) production through activating the SIRT1 pathway. In this study, we attempt to explore the relationships between the inhibitory effects of resveratrol on the anti-oxidative function and other sirtuins pathway. HUVECs were pre-transfected with synthesized small-interfering RNA (siRNA) targeting human SIRT1–SIRT7, then the ROS production in cells was detected by fluorescence-activated cell sorting. As shown in Figure 6, the inhibitory effect of resveratrol on TNF-α induced ROS production was reversed by siRNA targeting to SIRT1, SIRT2, SIRT3, SIRT4, and SIRT5. However, transfection with siRNA specific to SIRT6 and SIRT7 showed

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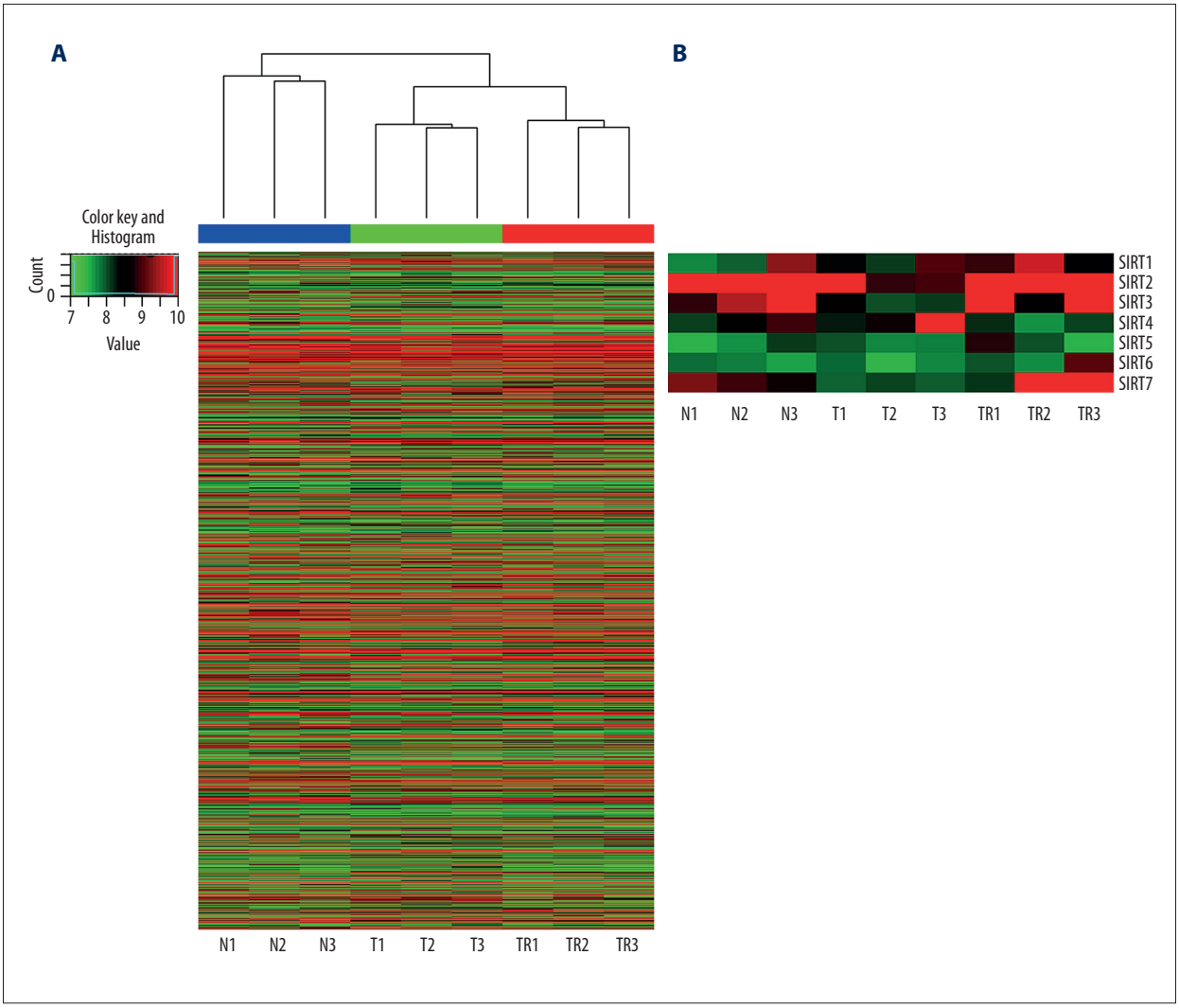

Figure 3. Microarray analysis of sirtuins family gene expression changes in HUVECs treated with 10 μg/L TNF-α alone or together with 20 μmol/L resveratrol. (**A**) Heat map of differently expressed genes. (**B**) Heat map of SIRT1–SIRT7 genes expression. HUVECs – human umbilical vein endothelial cells; TNF – tumor necrosis factor.

no effect, suggesting that resveratrol may attenuate oxidative stress production in TNF-α induced HUVECs through activating SIRT1, SIRT2, SIRT3, SIRT4,and SIRT5 pathways.

Relationship of resveratrol on CD40 expression and sirtuins pathways

The CD40-CD40L system is a pathway which is associated with both pro-thrombotic and pro-inflammatory effects. Our previous study showed that resveratrol treatment attenuates the increased expression of CD40 triggered by TNF-α stimulation through activating the SIRT1 pathway. To determine whether relationship of resveratrol on anti-inflammatory response and other sirtuins pathway, HUVECs were transfected with siRNAs specific to SIRT1-7 for 48 hours. After treatment with 20 μm/L resveratrol, HUVECs were stimulated with 10 μg/L TNF-α. As it shows in Figure 7, the representative histograms and quantitative data showed that SIRT1 and SIRT5 siRNA could reverse the effect of resveratrol on CD40 mRNA in TNF-α stimulated HUVECs. SIRT2, SIRT3, SIRT4, SIRT6, and SIRT7 siRNA could not reverse the effect of resveratrol on CD40 gene expression.

Discussion

As we known, oxidative stress has a major role in endothelial dysfunction molecular mechanisms. Resveratrol possesses multiple protective properties in the vasculature, including anti-oxidative and anti-inflammatory effects and improvement of endothelial function [17]. Seven members (SIRT1–SIRT7) of the sirtuin family have been identified in mammals. The sirtuin family are localized to different subcellular compartments such as the

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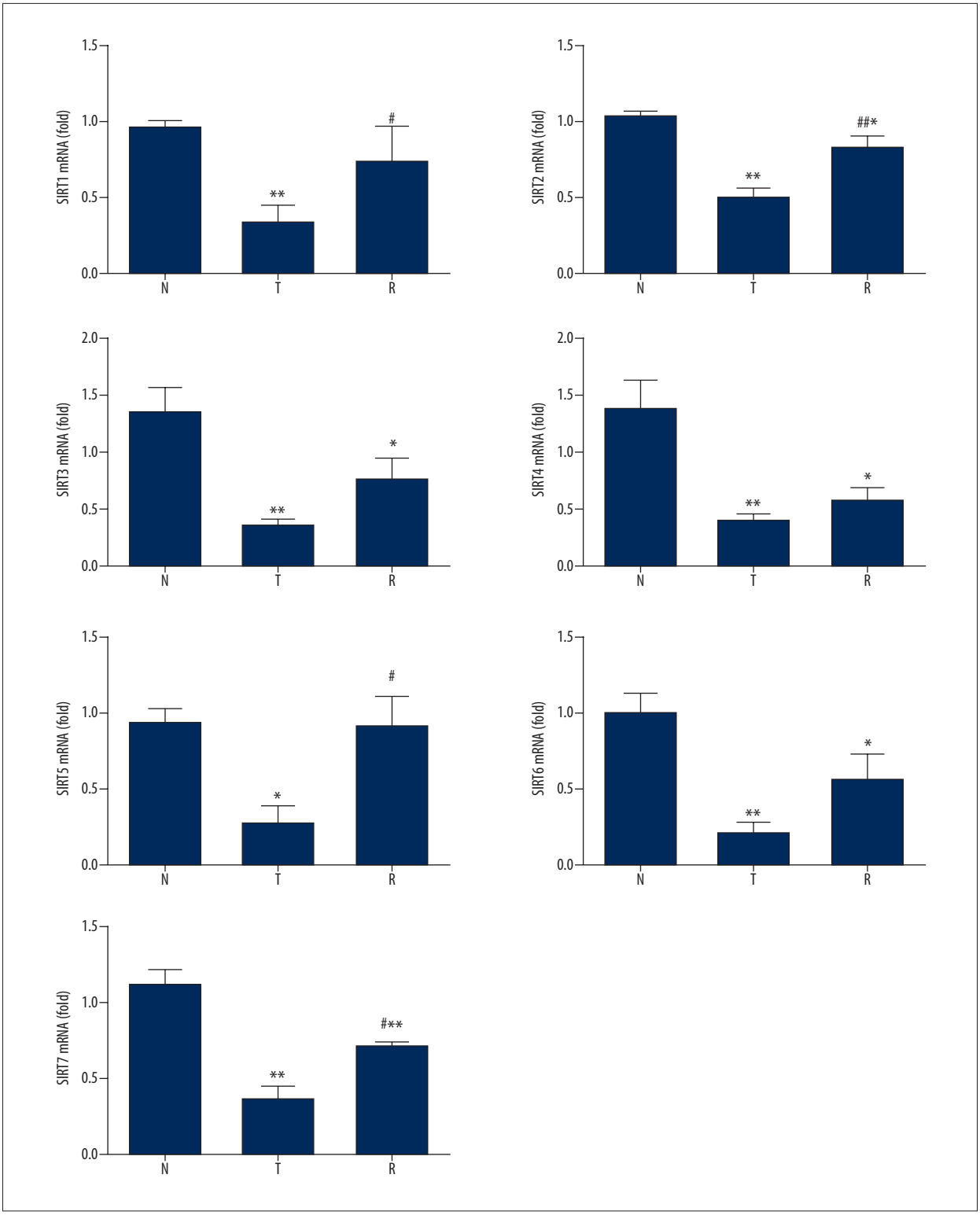

Figure 4. Validation of SIRT1-SIRT7 genes expression by real-time PCR. Relative mRNA expression was calculated with the $2^{-\Delta\Delta T}$ equation. Data were expressed as mean ±SEM. * *P*<0.05 versus control, # *P*<0.05 versus cells treated with TNF-a alone. The experiment was repeated 3 times respectively. PCR – polymerase chain reaction, SEM – standard error of the mean; TNF – tumor necrosis factor.

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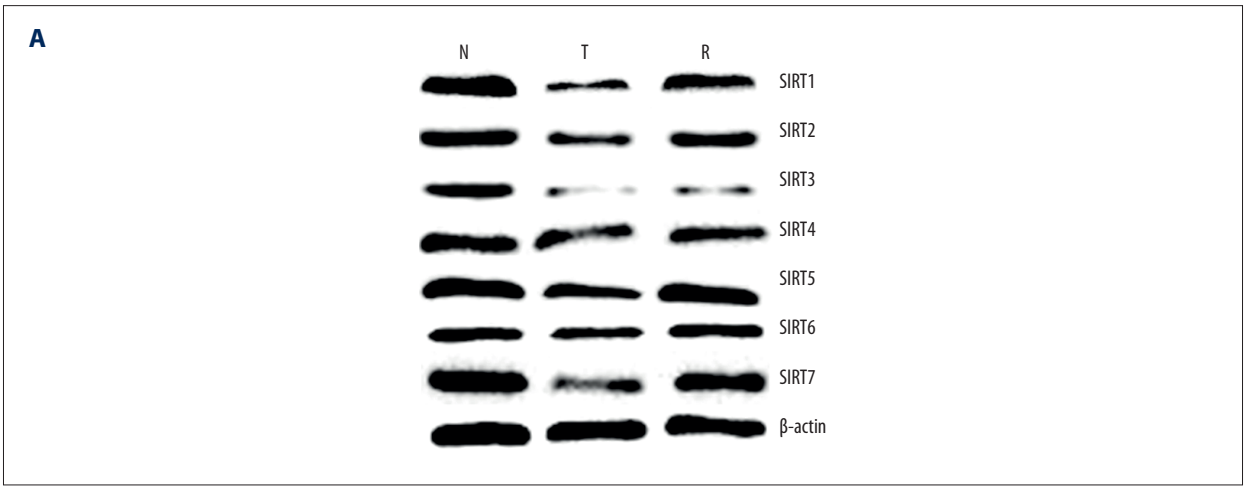
nucleus (SIRT1, SIRT2, SIRT6, and SIRT7), cytoplasm (SIRT1 and SIRT2), and the mitochondria (SIRT3, SIRT4, and SIRT5) [18]. This is the first study to systemically analyze whether SIRT1–SIRT7 were involved in resveratrol on anti-inflammatory and anti-oxidative functions in HUVECs induced by TNF-α.

In this study, we treated HUVECs with TNF-α and resveratrol to systemically analyze the expression of other sirtuins. First of all, from the whole human genome microarray analysis, our results revealed the distinction in sirtuins family gene expression profiles on TNF-α stimulated HUVECs with or without resveratrol pretreatment. SIRT1, SIRT2, SIRT3, SIRT5, SIRT6, and SIRT7 all showed the tendency of increase with more than 1-fold change while SIRT4 showed the tendency of decrease with more than 1-fold change. Accompanying with our further verified RT-PCR and western blot analysis, the results showed that the mRNA and protein expression of SIRT1, SIRT2, SIRT5, and SIRT7 were all increased by resveratrol compared with the TNF-α alone, while the expression of SIRT3, SIRT4, and SIRT6 show no the statistical change.

SIRT1 regulates many cellular processes through modification of histones and many nonhistone protein which are important for apoptosis, inflammation, stress resistance. Many previous studies showed that the effect of resveratrol through sirtuins mostly focus on SIRT1 pathway [19]. Our preliminary study also proved that resveratrol may exert anti-inflammatory and anti-oxidative effect by SIRT1-NF-κB signal pathway in HUVECs [11]. SIRT1 has also been proven to be the molecular target of resveratrol, which can be induced by a restricted calorie diet [20]. Surprisingly, we found that SIRT5 kept pace with SIRT1. The SIRT5 gene and protein expression significantly increased by resveratrol and SIRT5 siRNA significantly reversed the decrease of ROS production and CD40 gene expression by resveratrol in HUVECs. SIRT5 is discovered to be located in mitochondria and predominantly expressed in lymphoblasts and heart muscle [21]. Our results that showed resveratrol significantly decreased the ROS production and attenuated the increased expression of CD40 in HUVECs consistent with the Gertz and Steegborn study [22]. Li et al. also identified the role of SIRT5 deacetylase activity in regulating the expression of mitochondrial steroidogenic P450 by using resveratrol [23]. Resveratrol inhibited inflammation through SIRT1 pathway and then downregulated the expression of MMP-9, iNOS, IL-1β, and IL-6 in a dose-dependent manner [24]. Resveratrol as an activator of SIRT1 was found to be sufficient to activate AMPK and the improved cardiac function [25]. However, the physiological function of SIRT5 deacylation remains to be under investigated.

SIRT2 has been proved to be expressed in a wide range of tissues and organs especially higher in the brain than all other organs [26]. There is growing evidence showed that resveratrol could opposite the neural abnormal conditions through SIRT2 [27]. Previous studies showed that SIRT2 suppressed inflammatory responses probably through p65 deacetylation in mice and mediates microtubule reorganization through tubulin deacetylation [28,29]. Our results also showed that resveratrol reduced the ROS levels in TNF-α induced HUVECs, and SIRT2 siRNA significantly reversed the effect. These data suggest that resveratrol inhibiting ROS production is also associated with SIRT2 pathway. This results coordinate with previous results that show SIRT2 may mediate mitochondrial biogenesis by deacetylating PGC-1α, upregulating antioxidant enzyme expression by deacetylating FOXO3a [30]. However, our result demonstrated that SIRT2 siRNA had no inhibitory effect on resveratrol in reducing CD40 expression, suggesting that resveratrol on regulating CD40 did not mediated by SIRT2 pathway in TNF-α induced HUVECs. Interestingly, no data has studied the role of SIRT2 under conditions where resveratrol suppresses CD040-CD40L axis in endothelial cells.

Recently, SIRT3 has been shown to have important roles in maintaining homeostasis and cellular metabolic functions, particularly under conditions of stress [4]. SIRT3 provides protection



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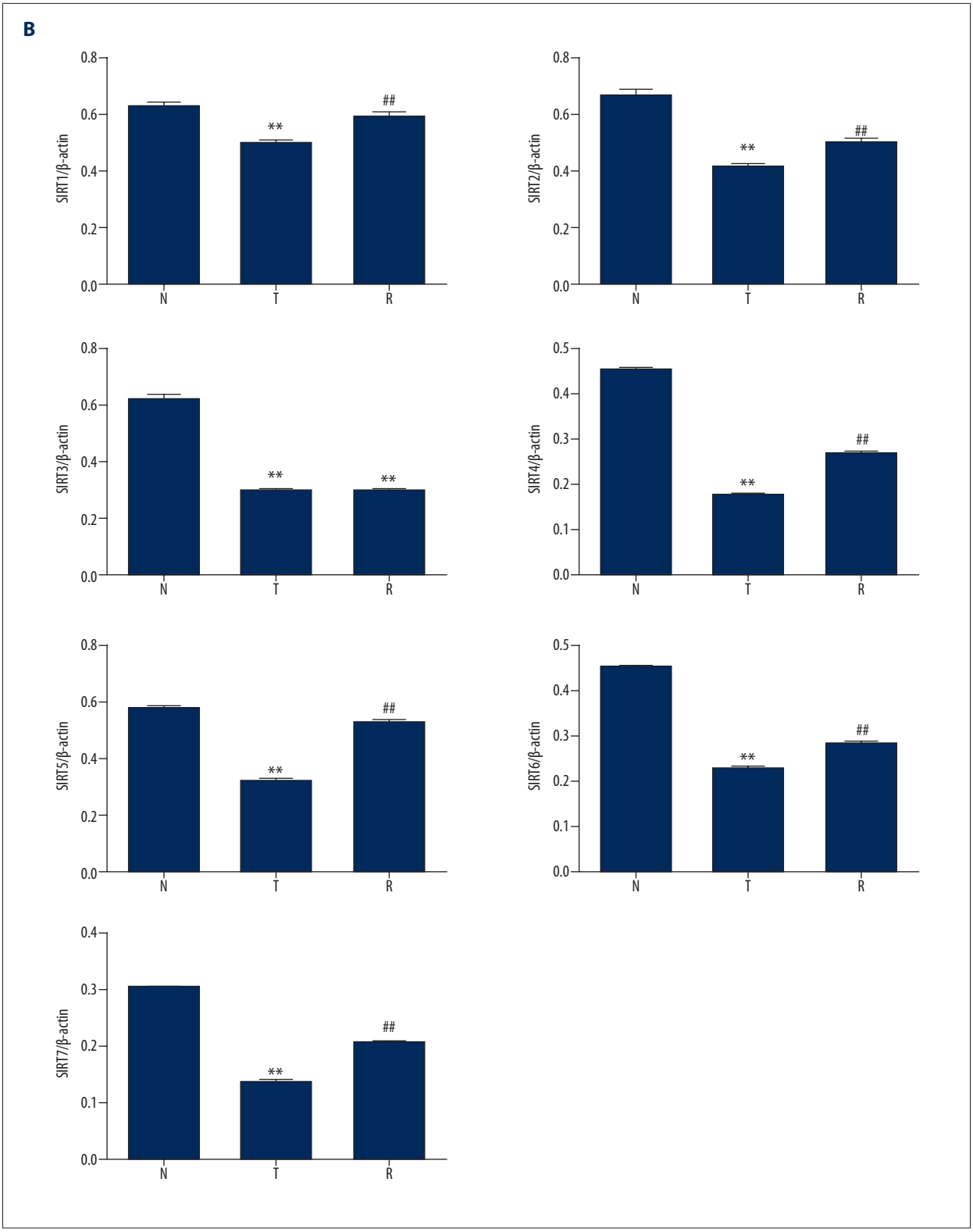

Figure 5. Effects of resveratrol on sirtuins protein expression. (**A**) Bands of cell extracts for target proteins as well as β-actin determination. (**B**) The representative histograms and quantitative data were shown. ** *P*<0.01 versus control. ## *P*<0.01 versus TNF-α, (n=3). TNF – tumor necrosis factor.

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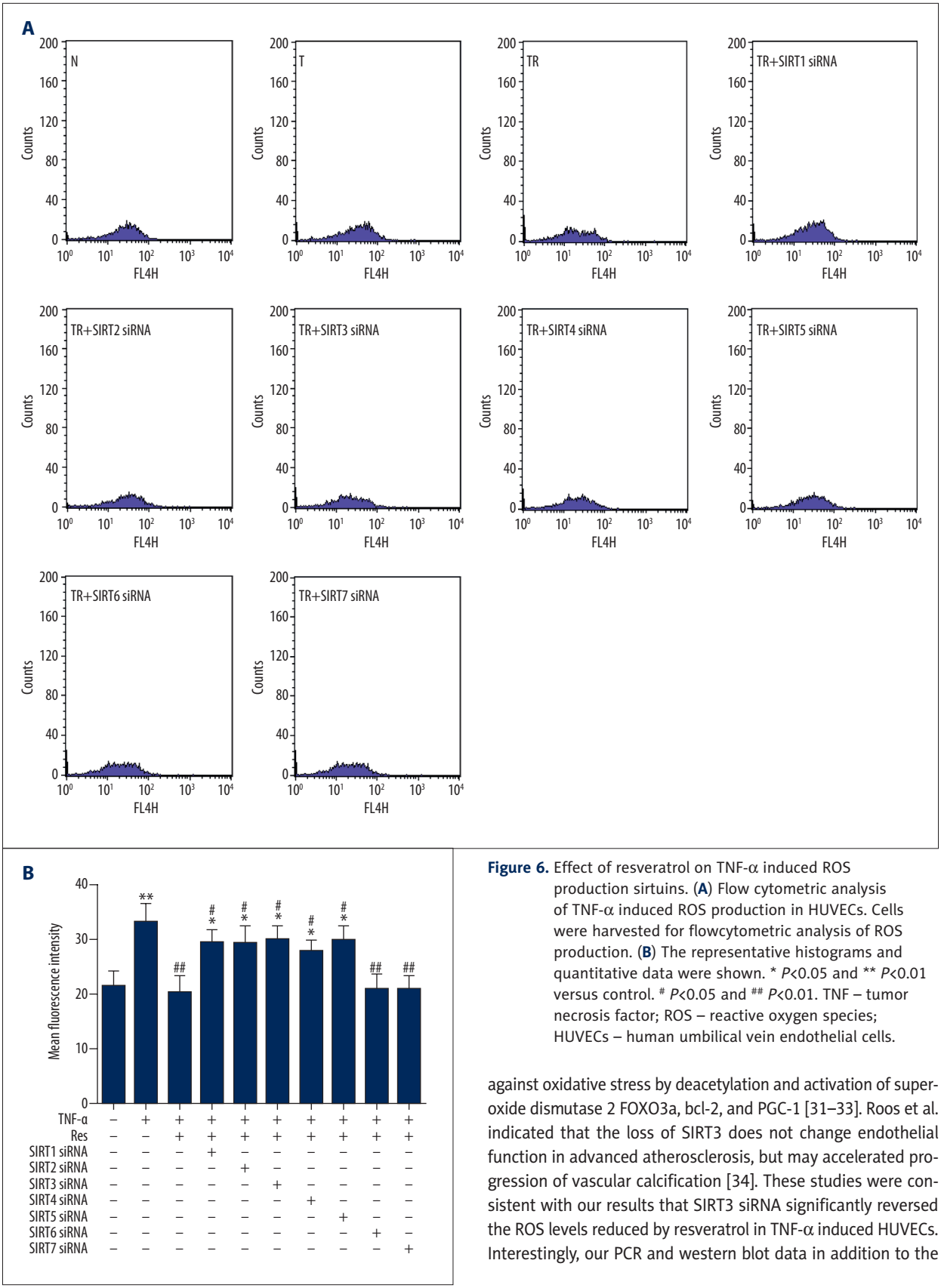

Figure 6. Effect of resveratrol on TNF-α induced ROS production sirtuins. (**A**) Flow cytometric analysis of TNF-α induced ROS production in HUVECs. Cells were harvested for flowcytometric analysis of ROS production. (**B**) The representative histograms and quantitative data were shown. * $P<0.05$ and ** $P<0.01$ versus control. # $P<0.05$ and ## $P<0.01$. TNF – tumor necrosis factor; ROS – reactive oxygen species; HUVECs – human umbilical vein endothelial cells.

against oxidative stress by deacetylation and activation of superoxide dismutase 2 FOXO3a, bcl-2, and PGC-1 [31–33]. Roos et al. indicated that the loss of SIRT3 does not change endothelial function in advanced atherosclerosis, but may accelerated progression of vascular calcification [34]. These studies were consistent with our results that SIRT3 siRNA significantly reversed the ROS levels reduced by resveratrol in TNF-α induced HUVECs. Interestingly, our PCR and western blot data in addition to the

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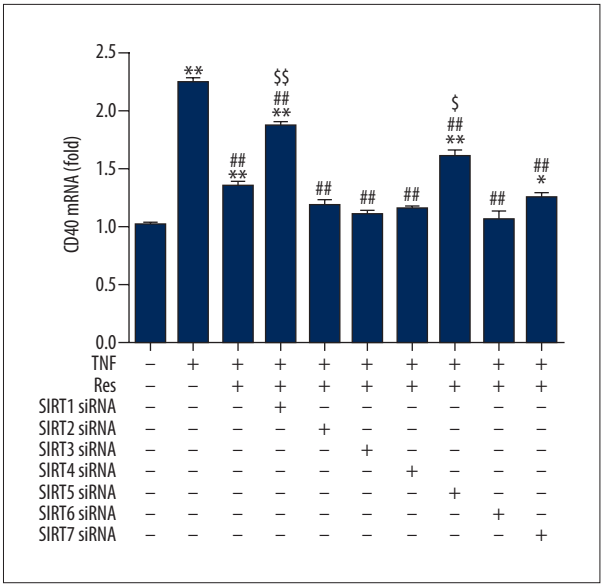

Figure 7. Effects of resveratrol on CD40 expression in siRNA SIRT1-SIRT7 pretreatment. HUVECs relative mRNA expression was calculated with the $2^{-\Delta\Delta T}$ equation. Data were expressed as mean ±SEM. * $P<0.05$ and ** $P<0.01$ versus control. # $P<0.05$ and ## $P<0.01$ versus TNF-α. $ $P<0.05$ versus TNF-α+resveratrol (n=3). HUVECs – human umbilical vein endothelial cells; TNF – tumor necrosis factor; SEM – standard error of the mean.

microarray analysis showed that resveratrol didn't statistically change the transcriptional and translational expression of SIRT3 which might be owing to the location of SIRT3 and might be also related to the physiological action of STRI3, which not only might change endothelial function, but might accelerated progression of vascular calcification [18,34]. Some studies have found that SIRT4 was involved in lipid storage, and increased the mitochondrial and fatty acid metabolism in hepatocytes, liver, and muscles [35]. Our results also showed that SIRT4 could significantly affect the oxidative stress production by using siRNA for SIRT4 and our PCR and western blot data with microarray analysis showed that resveratrol didn't statistically change the transcriptional and translational expression of SIRT4, which was similar with results for SIRT3. So, we concluded that resveratrol didn't change the gene or protein expression of SIRT3 and SIRT4 but might prompt the enzymatic activity of SIRT3 and SIRT4, the active deacetylation of downstream molecule might be involved in the reduction of the production of ROS in mitochondrial complex I. Further study is needed in this area.

There is strong evidence that links SIRT1 and SIRT6 to aging processes and stress responses [36]. SIRT6 has also been implicated as a potential regulator of longevity FoxO3 [37]. However, our results showed that resveratrol didn't change the gene and protein expression of SIRT6, which was inconsistent with previous studies [36,37], speculating that it might have contributed to mainly nuclear distribution of SIRT6. SIRT7 was also been reported to reside in the nucleolus [18]. But recent research seldom focuses on the function of SIRT7. Its protein substrate is still unknown. A study of SIRT7 in knockout mice found that cardiac hypertrophy accompanying reduced lifespan and cardiac dysfunction was linked to p53 hyperacetylation [38]. Our data showed that resveratrol could significantly increase the transcriptional and translational levels of SIRT7, however, the function of SIRT7 still needs to be further studied. Recently, many new insights into the mechanism, including hormonal controls and specific molecule pathways such as miRNAs are pointing to regulation of SIRT6 and SIRT7 activity. For example, SIRT6 is reported to be the direct target of miR-34a in HKCs [39]. SIRT7 has also been reported to bind to the promoter of miR-34a and deacetylases the H3K18ac in human gastric cancer tissues [40].

Conclusions

Our study was the first systematic analysis of the effect of resveratrol on sirtuins family. Our data showed that resveratrol could regulate the transcriptional and translational levels of SIRT1, SIRT2, SIRT5, and SIRT7. We used siRNA for different sirtuins, and found that siRNA targeting to SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, but not SIRT6 and SIRT7 siRNA, could reverse the effect of resveratrol on TNF-α induced ROS production in HUVECs. Further study suggested that only SIRT1 and SIRT5 siRNA could reverse the effect of resveratrol on CD40 expression. So, our results revealed that resveratrol might inhibit oxidative stress production by activating SIRT1, SIRT2, SIRT3, SIRT4, and SIRT5 pathway and attenuate CD40 expression by activating SIRT1 and SIRT5 pathways. Based on the aforementioned data, we concluded that the effects of resveratrol suppressing inflammation and immune response was closely-related with SIRT1 and SIRT5 pathways in endothelial cells.

Acknowledgements

We thank the head nurse Changfang Lin and the staff in the Department of Obstetrics in Fujian Provincial Hospital for assistance in obtaining the umbilical cord samples. We also would like to thank Changsheng Xu for technical guidance.

Conflict of interest

None.

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