SIRT1, a Longevity Gene, Downregulates Angiotensin II Type 1 Receptor Expression in Vascular Smooth Muscle Cells

Ryohei Miyazaki, Toshihiro Ichiki, Toru Hashimoto, Keita Inanaga, Ikuyo Imayama, Junichi Sadoshima, Kenji Sunagawa

Objective—Resveratrol (3,5,4′-trihydroxystilbene), a polyphenol found in red wine, is known to activate sirtuin1 (SIRT1), a longevity gene. Previous studies have demonstrated that resveratrol extends the life span of diverse species through activation of SIRT1. It was also reported that inhibition of angiotensin II function by angiotensin II type I receptor (AT1R) antagonist prolonged rat life span. We, therefore, hypothesized that resveratrol may inhibit the renin-angiotensin system and examined whether resveratrol affects AT1R expression in vascular smooth muscle cells (VSMCs).

Methods and Results—Northern and Western blot analysis revealed that resveratrol significantly decreased the expression of AT1R at mRNA and protein levels in a dose- and time-dependent manner. Overexpression of SIRT1 reduced AT1R expression whereas nicotinamide, an inhibitor of SIRT1, increased AT1R expression and reversed the resveratrol-induced AT1R downregulation. AT1R gene promoter activity was decreased by resveratrol, but resveratrol did not affect the AT1R mRNA stability. Deletion analysis showed that the most proximal region of AT1R gene promoter containing Sp1 site is responsible for downregulation. Administration of resveratrol suppressed AT1R expression in the mouse aorta and blunted angiotensin II–induced hypertension.

Conclusion—Resveratrol suppressed AT1R expression through SIRT1 activation both in vivo and in vitro. The inhibition of the renin-angiotensin system may contribute, at least in part, to the resveratrol-induced longevity and antitherapeutic effect of resveratrol. (Arterioscler Thromb Vasc Biol. 2008;28:1263-1269)

Key Words: resveratrol ■ SIRT1 ■ angiotensin II receptor ■ vascular smooth muscle cell

Resveratrol (3,5,4′-trihydroxystilbene) is one of the polyphenols contained in red wine,1 pomegranates, and Polygonum Caspidatum used in traditional Chinese and Japanese medicine.2 Resveratrol has various beneficial effects on cardiovascular diseases,3 cerebral ischemic injuries,4 and cancer.5 Previous studies have demonstrated that resveratrol extends the life span of diverse species through activation of silent information regulator 2 (SIR2)6,7 even on a high-calorie diet.8 Mammals have 7 SIR2 homologs (sirtuins, SIRT1 to 7), and sirtuins belong to class III histone/protein deacetylases (HDACs). Class I and II HDACs consume a water molecule for direct hydrolysis of the acetyl group, and they are inhibitable by trichostatin A (TSA).9 On the other hand, sirtuins require nicotinamide adenine dinucleotide (NAD⁺) as a cosubstrate for the deacetylation reaction: Sirtuins catalyze a reaction in which the cleavage of NAD⁺ and protein deacetylation are coupled to the formation of O-acetyl-ADP-ribose, nicotinamide and deacetylated lysine residue.10 Sirtuins are highly preserved among numerous species and are associated with longevity, cell cycle regulation, apoptosis, DNA damage repair, and muscle differentiation.11,12 It is generally believed that longevity is mainly promoted by SIRT1,13,14 and resveratrol affects various aspects of cell function through SIRT1 in mammalian cells.

Angiotensin II (Ang II) plays important roles in the pathogenesis of atherosclerosis and hypertension.15 Mammalian cells express 2 types of Ang II receptors, Ang II type 1 receptor (AT1R)16 and Ang II type 2 receptor (AT2R).17 AT1R and AT2R belong to the 7-transmembrane, G protein–coupled receptor family. These receptors exert opposite effects in terms of cell growth and blood pressure regulation.18 However, most of the traditional cardiovascular effects of Ang II such as vasoconstriction and water and sodium retention are mediated by AT1R.19,20 It was also reported that inhibition of Ang II function by AT1R antagonist prolonged the life span of hypertensive rats.21,22

We, therefore, hypothesized that resveratrol may inhibit the renin-angiotensin system and examined whether resvera-
trol affects AT1R expression in vascular smooth muscle cells (VSMCs).

Materials and Methods

Materials
Dulbecco’s modified Eagle medium (DMEM) was purchased from Gibco BRL. Fetal bovine serum (FBS) was from JRH Biosciences Inc. Resveratrol, bovine serum albumin (BSA), actinomycin D, phorbol-12-myristate 13-acetate (PMA), TSA, and nicotinamide were purchased from Sigma Chemical Co. Ang II was purchased from PEPTIDE Inc. Rabbit polyclonal antibodies against AT1R and α-tubulin were from Santa Cruz Biotechnology. An anti-SIRT1, anti–extracellular signal regulated protein kinase (ERK), and anti-phospho ERK (pERK) antibodies were purchased from Cell Signal-ing. [α-32P]dCTP and [γ-32P]ATP were purchased from Perkin-Elmer Life Sciences. Other chemical reagents were purchased from Wako Pure Chemicals unless mentioned specifically.

Cell Culture
VSMCs were isolated from the thoracic aorta of Sprague-Dawley rats and maintained in DMEM supplemented with 10% FBS at 37°C in a humidified atmosphere of 95% air-5% CO2. VSMCs were grown to confluence, cultured in DMEM with 0.1% BSA for additional 2 days, and used in the experiment. Cells between passages 5 and 12 were used.

Northern Blot Analysis
Total RNA was prepared by an acid guanidinium thiocyanate-phenol-chloroform extraction method, and Northern blot analysis of AT1R and 18s ribosomal (r) RNA was performed as described previously.23 The specific band was scanned with an imaging analyzer, and was normalized with the expression level of AT1R. ACT1R and 18s ribosomal (r) RNA was performed as described previously.23 The radioactivity of the AT1R mRNA bands was measured by luciferase assay and normalized by [α-32P]dCTP and [γ-32P]ATP.

Measurement of AT1R Gene Promoter Activity

Measurement of Cell Viability
Confluent VSMCs were serum deprived for 48 hours and then treated with resveratrol. After 24 hours incubation, attached cells were harvested with trypsin-EDTA. Cells in the medium were collected by centrifugation. These cells were stained with 0.4% trypan blue. The number of total and dead cells was counted with an hemocytometer.27

Adenovirus Vector Expressing SIRT1
A recombinant adenovirus vector expressing a wild-type SIRT1 (AdSIRT1) was described previously.28 VSMCs were grown to confluence, washed twice with phosphate-buffered saline (PBS), and incubated with AdSIRT1 or empty viral vector (AdEmpty) under gentle agitation for 2 hours at room temperature. Then the cells were washed 3 times with PBS, cultured in DMEM with 0.1% BSA for 2 days, and used for the experiments. Multiplicity of infection (moi) indicates the number of virus per cell added to culture dish.

Preparation of Nuclear Extracts and Gel Mobility Shift Assay
Cells were scraped off, washed in ice-cold PBS followed by ice-cold hypotonic buffer (buffer A: 10mmol/L HEPES, pH7.9, 1.5mmol/L MgCl2, 10mmol/L/ KCl, 0.5mmol/L PMSF, 0.5mol/L DTT), and then lysed for 10 minutes on ice in the buffer A containing 0.1% Nonidet P-40. The lysates were centrifuged for 10 minutes at 10 000g. The pelleted nuclei were suspended in lysis buffer (20 mmol/L HEPES, pH 7.9, 420 mmol/L NaCl, 1.5 mmol/L MgCl2, 0.2 mmol/L EDTA, 25% glycerol, 0.5 mmol/L PMSF, 0.5 mol/L DTT), incubated for 15 minutes at 4°C, and centrifuged for 15 minutes at 10 000g. The supernatant was used as nuclear extracts.

A synthetic DNA probe (AT1R gene promoter: −40 bp to −6 bp; GGACCTGCGAGAGCA GGAGGCCCCTAGGCTATA: containing Sp1 site) was labeled with 32P by using [γ-32P] ATP and T4 polynucleotide kinase, and purified by Sephadex G-50 column. A synthetic DNA probe with mutation in Sp1 site (GGACCTGCGAGCAGCGACGTTTCCTAGGCTATA) was also prepared and used as a competitor. Twenty μg of nuclear extracts was incubated with 1×105 cpm of labeled DNA probe and 2 μg of poly (dl-dC) in a buffer containing 10mmol/L Tris-HCl, pH7.5, 1mmol/L EDTA, 4% glycerol, 100mmol/L NaCl, 2.5mmol/L DTT, 100 mg bovine serum albumin for 15 minutes at room temperature. Then the samples were electrophoresed on 5% acrylamide/0.25×TBE gels (1×TBE 90mmol/L of tris borate, 2mmol/L of EDTA). After electrophoresis, gels were dried and exposed to x-ray film at −70°C.

Animal Experiment
All procedures were approved by the institutional animal use and care committee, and were conducted in conformity with institutional guidelines. Nine-week-old C57/B6 mice were purchased from Kyudo Co Ltd (Japan). Resveratrol was suspended in water at 0.1 mg/mL and administered ad libitum. The estimated dose of orally ingested resveratrol was 10 mg/kg/d. In Ang II group, 490 ng/min/kg of Ang II was administered subcutaneously via osmotic minipump (Alzet). Blood pressure and heart rate were measured using tail-cuff method (UR-5000, UDIA). After 1 week, mice were euthanized under pentobarbital anesthesia. Aortas were quickly removed, minced into small pieces, and homogenized on ice in a buffer (0.25 mol/L sucrose, 5 mmol/L Tris-HCl at pH7.5, 1 mmol/L MgCl2). The homogenates were centrifuged at 2000 rpm for 15 minutes at 4°C and the supernatants were centrifuged at 100 000g for 30 minutes at 4°C. The pellets were used as a membrane fraction and subjected to Western blot analysis of AT1R and α-tubulin.

Statistical Analysis
Statistical analysis was performed with performed 1- or 2-way ANOVA and Fisher test, if appropriate. Data are shown as mean±SEM. P<0.05 was considered to be statistically significant.
Results

Resveratrol Suppresses AT1R Expression in VSMCs

VSMCs were incubated with resveratrol (100 μmol/L) for varying time periods, and expression level of AT1R mRNA was determined by Northern blot analysis. The expression level of AT1R mRNA was significantly reduced by resveratrol at 6 hours compared with the control level, and the reduction was reached a maximum at 12 hours of stimulation (Figure 1A). Incubation with varying concentrations of resveratrol resulted in downregulation of AT1R mRNA in a dose-dependent manner (Figure 1B). Western blot analysis revealed that resveratrol (100 μmol/L) reduced AT1R protein level in VSMCs. The reduction was reached a maximum at 12 to 24 hours (Figure 1C), and resveratrol downregulated AT1R protein in a dose-dependent manner (Figure 1D). To exclude a possible toxic effect of resveratrol on VSMC, we next assessed cell viability by trypan blue staining. VSMCs were incubated with resveratrol (200 μmol/L) for 24 hours. Resveratrol did not decrease the viability of VSMCs (in percent of viable cells: Control 97.7 ± 2.0%, Resveratrol 98.3 ± 1.0%, not significant n = 4). In addition, resveratrol did not affect SIRT1 expression level (supplemental Figure I, available online at http://atvb.ahajournals.org).

SIRT1 Mediates AT1R mRNA Downregulation

Resveratrol is known to activate SIRT1. We examined whether SIRT1 is involved in the resveratrol-induced AT1R downregulation. The expression level of AT1R mRNA was significantly increased by incubation with nicotinamide, a noncompetitive inhibitor of SIRT1 compared with the control level in a dose-dependent manner (1 to 100 μmol/L, Figure 2A). Resveratrol (RV: 50 μmol/L)-induced suppression of AT1R was significantly reversed by addition of nicotinamide (NAM, 30 μmol/L; Figure 2B). However, resveratrol downregulated AT1R mRNA in the presence of TSA, class I or II HDAC inhibitor (supplemental Figure II). To achieve high expression levels of SIRT1 in VSMCs, we used a recombinant adenovirus vector expressing wild-type of SIRT1 (S, 10 moi) and AdEmpty (E, 10 moi; Figure 2C). Overexpression of SIRT1 (3 to 30 moi) significantly suppressed AT1R expression compared with the cells infected with AdEmpty (30 moi; Figure 2D). To clarify whether AT1R downregulation is functional, we examined ERK phosphorylation. AngII (100 nmol/L)-induced ERK phosphorylation was inhibited in SIRT1 overexpressing cells, but not in empty vector-infected cells, suggesting that downregulation of AT1R attenuated the response of VSMCs to AngII (supplemental Figure IIIA). However, PMA-induced ERK phosphorylation was not affected in the same condition, suggesting that the pathway to ERK activation is almost intact in SIRT1 overexpressing cells (supplemental Figure IIIB).
De Novo Protein Synthesis Is Not Required for Resveratrol-Induced Downregulation of AT1R Expression

To examine whether resveratrol-induced downregulation of AT1R mRNA requires de novo protein synthesis, we examined the effect of cycloheximide (10 μmol/L). Although incubation with cycloheximide alone for 12 hours upregulated the AT1R mRNA expression, resveratrol significantly suppressed the AT1R mRNA level in the presence of cycloheximide (Figure 3A). These data suggest that resveratrol-induced AT1R downregulation does not require de novo protein synthesis. We next examined whether resveratrol affects AT1R mRNA stability. In control, AT1R mRNA levels were reduced by 50% after 24 hours, and resveratrol did not affect the degradation rate of AT1R mRNA (Figure 3B).

Resveratrol Inhibits AT1R Expression at the Transcriptional Level

To locate the DNA element responsible for resveratrol-induced AT1R suppression, we examined the transcription activity of the deletion mutants of AT1a gene promoter/luciferase fusion DNA (Figure 4A). The suppression was observed in all mutants, suggesting that the response element exists in the DNA segment between −61 bp and +25 bp, which contains Sp1 site. The luciferase construct with mutation in Sp1 site failed to respond to resveratrol, indicating the important role of Sp1 site in resveratrol-induced downregulation.

Reduction of Sp1 Binding by Resveratrol

We examined the DNA binding protein bound to the Sp1 site using gel mobility shift assay (Figure 5). When nuclear extracts from resveratrol-stimulated VSMC were used, DNA binding protein (arrow) was decreased compared with those from unstimulated VSMC (lanes 1 to 2). Addition of 50 times molar excess of unlabeled probe (lane 4) but not the Sp1 mutant probe (lane 3) eliminated this band, confirming the specificity of the binding.

Resveratrol Suppresses AT1R Expression In Vivo

Finally, we examined whether resveratrol affects AT1R expression in vivo. Nine-week-old mice were allotted to resveratrol group or water group in a random manner. Blood pressure, heart rate, and body weight were not significantly different between resveratrol- and water-treated mice after 1 week (supplemental Table I). After 1 week, membrane protein of aorta was extracted and Western blot analysis was performed. Western blot revealed that resveratrol reduced expression level of AT1R protein in the aorta (Figure 6A). And

![Figure 3](http://atvb.ahajournals.org/)

**Figure 3.** Effect of cycloheximide and actinomycin D on resveratrol-induced AT1R downregulation. A, Effect of cycloheximide (CHX) on resveratrol (RV)-induced AT1R mRNA downregulation was examined by Northern blot analysis. Values are expressed as a percent of control (C) culture (100%; n=8). *P<0.05, **P<0.01 vs control, ###P<0.01 vs CHX. B, Effect of actinomycin D (ActD) on resveratrol-induced AT1R mRNA downregulation was examined by Northern blot analysis. The expression level of AT1R mRNA in VSMCs before addition of actinomycin D in each group was set as 100% (n=5). n.s: not significant.

![Figure 4](http://atvb.ahajournals.org/)

**Figure 4.** Resveratrol downregulates AT1R mRNA expression through transcriptional mechanisms. Left, The scheme of deletion mutants of AT1R promoter/luciferase fusion DNA construct and Sp1 mutant construct is shown. Right, The bar graphs indicate luciferase activity normalized by β-galactosidase activity derived from the corresponding deletion or Sp1 mutant. The luciferase activity of resveratrol-stimulated VSMCs (white bars) relative to unstimulated VSMCs (black bars) in each group is indicated. Values (mean±SEM) are expressed as a percent (n=6). *P<0.01 vs unstimulated.
then, we examined the effect of resveratrol on AngII-induced hypertension. We found that Ang II–induced hypertension was markedly blunted by resveratrol administration (Figure 6B). Heart rate and body weight were not significantly different between Ang II+vehicle and Ang II+resveratrol-treated mice after 2 weeks (supplemental Table II).

Discussion

In the present study, we demonstrated that resveratrol reduced the expression of AT1R in VSMCs and in mice aorta. Resveratrol reduced AT1R promoter activity without an effect on mRNA stability. Overexpression of SIRT1 suppressed AT1R expression, and nicotinamide increased AT1R expression. To our knowledge, this is the first report showing the downregulation of vascular AT1R by resveratrol. Furthermore, we demonstrated that SIRT1 overexpression suppressed Ang II–induced ERK phosphorylation, suggesting that downregulation of AT1R attenuated AT1R signaling.

We showed that nicotinamide, a SIRT1 natural inhibitor, upregulated the expression level of AT1R mRNA, and overexpression of SIRT1 significantly suppressed AT1R protein expression. Furthermore, resveratrol-induced suppression of AT1R was reversed by nicotinamide. These data suggested that resveratrol suppressed AT1R expression through SIRT1 activation at least in part.

Haider UG et al reported that resveratrol interfered with Ang II signaling pathways in VSMCs.26 Resveratrol inhibited Ang II–induced tyrosine-phosphorylation of Gab1 and its association with the p85 subunit of phosphatidylinositol-3-kinase. Therefore, resveratrol inhibits AT1R signaling as well as gene expression. Actis-Goretti L reported that red wine inhibits ACE activity more effectively than white wine. However resveratrol was ineffective in the inhibition of ACE activity in rat tissues.30 We supposed that resveratrol may suppress renin-angiotensin system not via ACE downregulation but via AT1R downregulation and inhibition of AT1R signaling.

In a rat model of injured aorta, administration of resveratrol accelerated reendothelialization and inhibited neointimal formation.29 Because Ang II and AT1R play an important role in the neointimal formation after vascular injury, downregulation of AT1R expression by resveratrol may be involved in the suppression of neointimal formation. In addition, resveratrol has been reported to inhibit serum-induced VSMC growth.32 Therefore, direct inhibition of VSMC growth by resveratrol may also play a role.

It was previously reported that resveratrol increased the endothelial nitric oxide synthase (eNOS) mRNA stability in human EA.hy 926 endothelial cells.33 We investigated whether resveratrol affects the AT1R mRNA stability in VSMCs. Resveratrol reduced AT1R gene promoter activity, but resveratrol did not affect AT1R mRNA stability. These data suggest that resveratrol suppressed AT1R gene expression at the transcriptional level rather than posttranscriptional level. The deletion analysis of the AT1R gene promoter revealed that suppression of AT1R expression by resveratrol is dependent on the most proximal promoter region (from −61 bp to +25 bp), which contains Sp1 binding site (GC box). The luciferase construct with mutation in GC box failed to respond to resveratrol, indicating an important role of Sp1 site in resveratrol-induced AT1R downregulation. Gel shift assay showed that DNA binding protein bound to the Sp1 site was decreased in resveratrol-stimulated VSMCs. A recent report showed that Sp1 is constitutively acetylated at Lys703,34 and deacetylation of Sp1 induced activation of
12(s)-lipoxygenase gene expression. Conversely, another report showed that HDAC1 mediates repression of transforming growth factor (TGF) β type II receptor gene transcription by deacetylation of Sp1. Therefore, the effects of deacetylation of Sp1 on gene expression may be context-dependent. Our data suggest that resveratrol inhibits Sp1 binding to AT1R gene promoter and AT1R gene transcription, supporting the results of the latter report. However, it is not clear how SIRT1 inhibits Sp1 binding at this moment, and further study is needed.

As shown in Figure 6B, chronic low-dose AngII infusion via osmotic minipump developed hypertension in mice as described previously, and Ang II–induced hypertension was blunted by resveratrol. Because resveratrol inhibits AT1R signaling, we supposed that this was attributable to inhibition of AT1R signaling and AT1R downregulation as shown Figure 6A by resveratrol. However, it is difficult to dissect these 2 effects in terms of the blood pressure regulation.

Previous studies have demonstrated that resveratrol extends the life span of diverse species. It was also reported that inhibition of Ang II function by angiotensin converting enzyme inhibitor and AT1R antagonist prolonged the lifespan of hypertensive rats. Considering that oral administration of resveratrol suppressed the AT1R protein expression in the aorta of mice, the inhibition of the renin-angiotensin system via AT1R suppression may contribute, at least in part, to the longevity by resveratrol. The downregulation of AT1R may also account for antiatherogenic effect of resveratrol.

In conclusion, we demonstrated that resveratrol downregulated AT1R expression through SIRT1. The suppression of AT1R expression may contribute to resveratrol-induced lifespan extension, inhibition of atherosclerosis and inflammation.

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Disclosures

None.

References


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Supplementary Figure 1

SIRT1

α-tubulin

SIRT1 / α-tubulin (%)

C  1  3  6  12  24 (hours)

n.s.
Legends for supplementary figure.

Supplementary Figure 1. Resveratrol did not affect SIRT1 expression level. VSMCs were incubated with resveratrol (100µmol/L) for varying periods indicated in the figure (N=3). Expression of SIRT1 protein and α–tubulin was detected by Western blot analysis. The bar graph indicates the ratio of AT1R to α-tubulin. Values are expressed as a percent of control (C) culture (100%). n.s: not significant.

Supplementary Figure 2. Resveratrol suppressed AT1R mRNA expression in the presence of trichostatin A. VSMCs were pretreated with or without trichostatin A (TSA, 1µmol/L) for 24 hours and then incubated in presence or absence of resveratrol (RV, 100µmol/L) for 12 hours. Expression of AT1R mRNA was examined by Northern blot analysis. Values are expressed as a percent of control (C) culture (100%) (N=8). **P<0.01 vs control.

Supplementary Figure 3. AngII-induced ERK phosphorylation after overexpression of SIRT1. VSMCs were infected with AdSIRT1 (30moi) or AdEmpty (30moi). A, Forty eight hours after infection, the cells were incubated with or without AngII (100nM) for 5 minutes. B, The cells were incubated with or without PMA (100nM) for 5 minutes. Phosphorylation of ERK and EPK protein expression were detected by Western blot analysis. Values are expressed as a percent of control culture (100%). *P<0.01 vs Control, **P<0.01 vs Ad Empty+AngII.
Supplementary Table 1. Body weight, heart rate and blood pressure of control and resveratrol-treated mice.

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BW, body weight; HR, heart rate; SBP, systolic blood pressure; Values are mean±SEM
Supplementary figure 3

(A) pERK

EPK (%)

phospho ERK / EPK

AII 100nmol/L

- - + +
AdEmpty AdSIRT1

(B)

pERK

EPK

phospho ERK / EPK

PMA 100nmol/L

- + - +
AdEmpty AdSIRT1
Supplementary table 2. Body weight, heart rate and blood pressure of AngII and resveratrol-treated mice.

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BW, body weight; HR, heart rate; SBP, systolic blood pressure; Values are mean±SEM