

CARTO™ Images after Heart Transplantation

Masao Takemoto¹, Mari Nishizaka¹, Ryuji Matsukawa¹, Yoshikazu Kaji³,
Akiko Chishaki² and Kenji Sunagawa¹

Key words: heart transplantation, electroanatomical mapping, arrhythmia

(*Inter Med* 47: 813-814, 2008)

(DOI: 10.2169/internalmedicine.47.0880)

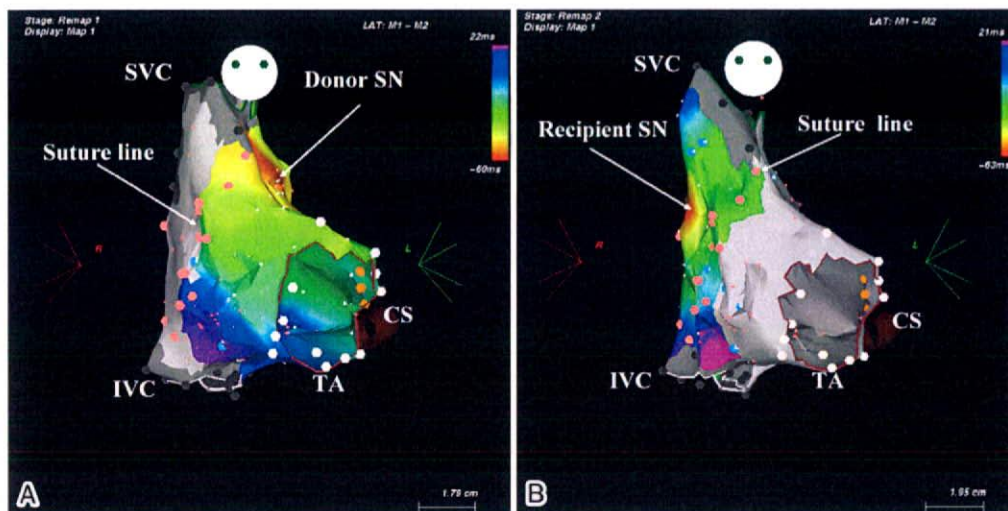


Figure 1. RAO view of CARTO™-activation maps showing the donor right atrium (A) and the recipient right atrium (B). The donor and recipient atriums were completely disconnected and dissociated. SN: sinus node, SVC: superior vena cava, IVC: inferior vena cava, CS: coronary sinus, TA: tricuspid valve annulus.

A 51-year-old man presented to the electrophysiological laboratory with a 3-week history of nonsustained narrow QRS tachycardia 6 weeks after an orthotopic heart transplantation for his dilated cardiomyopathy. Acute and chronic rejections had been already excluded by coronary angiography, echocardiography, and myocardial biopsy on a regular basis. Both the donor and the recipient right atrial (RA) activations were mapped during sinus rhythm using conventional and electroanatomical mapping system (CARTO™, Biosense Webster Ltd., Tokyo, Japan). Intracardiac electrograms and CARTO™-activation maps (Picture 1) revealed

that the donor and recipient atrias were completely dissociated. Electrophysiological studies demonstrated evidence of neither the accessory pathways nor the dual atrio-ventricular nodal pathways. Furthermore, programmed stimulations of the RAs or the ventricle could not induce any tachyarrhythmias even under isoproterenol administration. Thus, the patient continued medical treatment with beta-adrenergic receptor antagonist. He has remained well without any symptoms 2 years after the heart transplantation. This is the first reported case of CARTO™ images after a heart transplantation in Japan.

¹Department of Cardiovascular Medicine, Graduate School of Medical Sciences Kyushu University, Fukuoka, ²Department of Health Sciences, Faculty of Medical Sciences, Graduate School of Medical Sciences Kyushu University, Fukuoka and ³Internal Medicine, PS Clinic, Fukuoka
Received for publication January 4, 2008; Accepted for publication February 4, 2008

Correspondence to Dr. Masao Takemoto, matakemo@cardiol.med.kyushu-u.ac.jp

Acknowledgement

We thank Yoshihiro Miyawaki for his technical assistance with the electrophysiological study in the cardiac catheterization laboratory.

© 2008 The Japanese Society of Internal Medicine
<http://www.naika.or.jp/imindex.html>

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 antiatherogenic 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 inhibited by nicotinamide but not by TSA.^{11,12}

Sirtuins are highly preserved among numerous species and are associated with longevity, cell cycle regulation, apoptosis, DNA damage repair, and muscle differentiation.^{13,14} It is generally believed that longevity is mainly promoted by SIRT1,¹⁵ 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.¹⁶ Mammalian cells express 2 types of Ang II receptors, Ang II type 1 receptor (AT1R)¹⁷ and Ang II type 2 receptor (AT2R).¹⁸ 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.¹⁹ However, most of the traditional cardiovascular effects of Ang II such as vasoconstriction and water and sodium retention are mediated by AT1R.^{20,21} It was also reported that inhibition of Ang II function by AT1R antagonist prolonged the life span of hypertensive rats.²²

We, therefore, hypothesized that resveratrol may inhibit the renin-angiotensin system and examined whether resvera-

Original received October 10, 2007; final version accepted March 30, 2008.

From the Departments of Cardiovascular Medicine (R.M., T.I., T.H., K.I., I.I., K.S.) and Advanced Therapeutics for Cardiovascular Diseases (T.I.), Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan; and the Cardiovascular Research Institute (J.S.), University of Medicine and Dentistry of New Jersey, Newark.

Correspondence to Toshihiro Ichiki, MD, Department of Cardiovascular Medicine, Kyushu University Graduate School of Medical Sciences, 3-1-1 Maidashi, Higashi-ku, 812-8582 Fukuoka, Japan. E-mail ichiki@cardiol.med.kyushu-u.ac.jp

© 2008 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol is available at <http://atvb.ahajournals.org>

DOI: 10.1161/ATVBAHA.108.166991

rol 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 Signaling. [α - 32 P]dCTP and [γ - 32 P]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 radioactivity of the AT1R mRNA bands was counted with an imaging analyzer and was normalized with the radioactivity of rRNA. The expression level of AT1R was indicated as a ratio of AT1R mRNA to rRNA. To analyze mRNA stability of AT1R, Actinomycin D (5 μ g/mL) was added after 6 hours of stimulation with resveratrol (100 μ mol/L). VSMCs were harvested at 3, 6, 12, 24 hours after addition of actinomycin D. The expression level of AT1R mRNA was determined by Northern blot analysis.

To determine whether resveratrol-induced AT1R mRNA down-regulation requires de novo protein synthesis, VSMCs were pretreated with or without cycloheximide (CHX, 10 μ mol/L) for 30 minutes and incubated in the presence or absence of resveratrol (RV, 100 μ mol/L) for 12 hours. Then the AT1R mRNA level was determined by Northern blot analysis.

Western Blot Analysis

Western blot analysis of AT1R was performed as described previously.²⁴ The specific band was scanned with an imaging analyzer, and α -tubulin was used as a loading control. The expression level of AT1R was indicated as a ratio of AT1R to α -tubulin. Expression of SIRT 1 was examined by the same method.

Measurement of AT1R Gene Promoter Activity

Five deletion mutants of AT1a gene promoter were prepared by digestion with restriction endonucleases and ligated to luciferase gene as described previously.²⁵ AT1R promoter/luciferase DNA constructs and LacZ gene were introduced to VSMCs with the DEAE-dextran method. Then VSMCs were stimulated with resveratrol (100 μ mol/L) for 12 hours. Promoter activity of AT1R gene was measured by luciferase assay and normalized by β -galactosidase activity as described previously.²⁶ The AT1R promoter-luciferase construct with mutation in the GC-box-related sequence (wild-type: TGCAGAGCAGCGACGCCCCCTAGGC mutant: TGCAGAGCAGCGA CGTTTCCTAGGC) was a generous gift from Dr Sugawara (Tohoku University, Japan).

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 80% 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, 0.5mmol/L DTT), and then lysed for 10 minutes on ice in the buffer A containing 0.1% Nonident 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 mmol/L DTT), incubated for 15 minutes at 4°C, and centrifuged for 10 minutes at 10 000g. The supernatant was used as nuclear extracts.

A synthetic DNA probe (AT1R gene promoter: -40 bp to -6 bp; GGAACCTGCAGAGCA GCGACGCCCCCTAGGCTATA: containing Sp1 site) was labeled with 32 P by using [γ - 32 P] ATP and T4 polynucleotide kinase, and purified by Sephadex G-50 column. And A synthetic DNA probe with mutation in Sp1 site (GGAACCTGCAGAGCAGCGACGTTTCCTAGGCTATA) was also prepared and used as a competitor. Twenty μ g of nuclear extracts was incubated with 1 \times 10⁵ cpm of labeled DNA probe and 2 μ g of poly (dI-dC) in a buffer containing 10 mmol/L Tris-HCl, pH7.5, 1mmol/L EDTA, 4% glycerol, 100 mmol/L NaCl, 2.5mmol/L DTT, 100 mg/L bovine serum albumin for 15 minutes at room temperature. Then the samples were electrophoresed on 5% acrylamide/0.25 \times TBE gel (1 \times 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, UEDA). 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 \pm SEM. $P < 0.05$ was considered to be statistically significant.

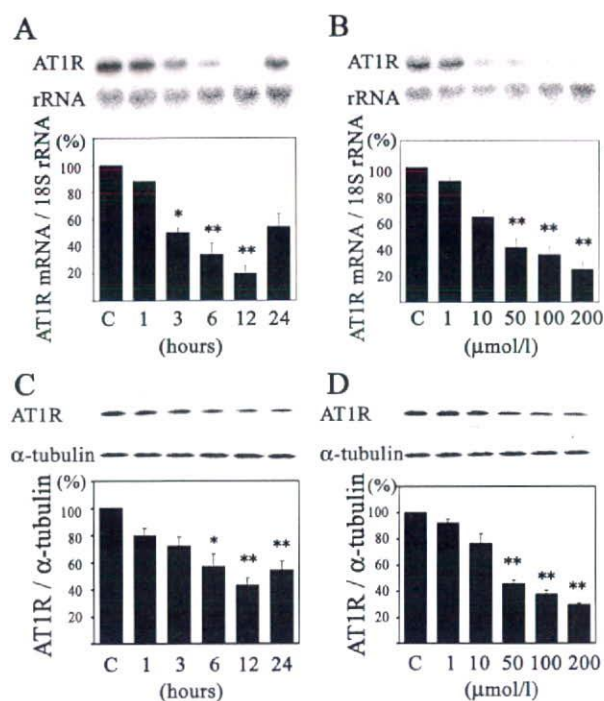


Figure 1. Suppression of AT1R mRNA and protein expression by resveratrol in VSMCs. A and C, VSMCs were incubated with resveratrol for varying periods indicated in the figure ($n=6$ to 7). B and D, VSMCs were incubated with resveratrol at varying concentrations for 12 hours ($n=6$ to 8). A and B, Expression of AT1R mRNA was determined by Northern blot analysis. C and D, Expression of AT1R protein was detected by Western blot analysis. Values are expressed as a percent of control (C) culture (100%). * $P<0.05$, ** $P<0.01$ vs Control.

Results

Resveratrol Suppresses AT1R Expression in VSMCs

VSMCs were incubated with resveratrol ($100\mu\text{mol/L}$) 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 ($100\mu\text{mol/L}$) 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 ($200\mu\text{mol/L}$) for 24 hours. Resveratrol did not decrease the viability of VSMCs (in percent of viable cells: Control $97.7\pm 2.0\%$, Resveratrol $98.3\pm 1.0\%$, 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

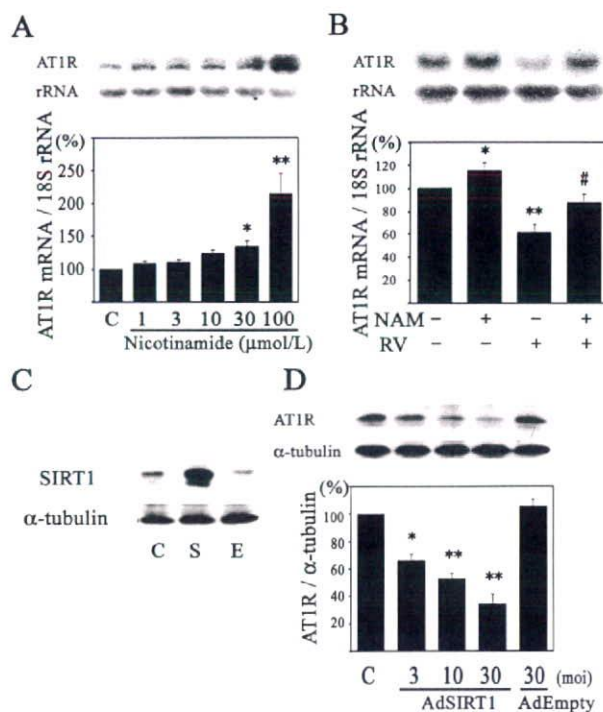


Figure 2. SIRT1 mediates downregulation of AT1R mRNA expression. A, VSMCs were incubated with nicotinamide at varying concentrations for 6 hours ($n=6$). B, VSMCs were incubated with resveratrol and/or nicotinamide for 12 hours ($n=8$). Expression level of AT1R mRNA was examined. C, VSMCs were infected with AdSIRT1 or AdEmpty and expression of SIRT1 was examined. D, VSMCs infected with AdSIRT1 or AdEmpty. Expression of AT1 protein was examined. Values are expressed as a percent of control (C) culture (100%). * $P<0.05$, ** $P<0.01$, vs C. # $P<0.05$ vs RV.

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 $100\mu\text{mol/L}$, Figure 2A). Resveratrol (RV: $50\mu\text{mol/L}$)-induced suppression of AT1R was significantly reversed by addition of nicotinamide (NAM, $30\mu\text{mol/L}$; 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 (100nmol/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).

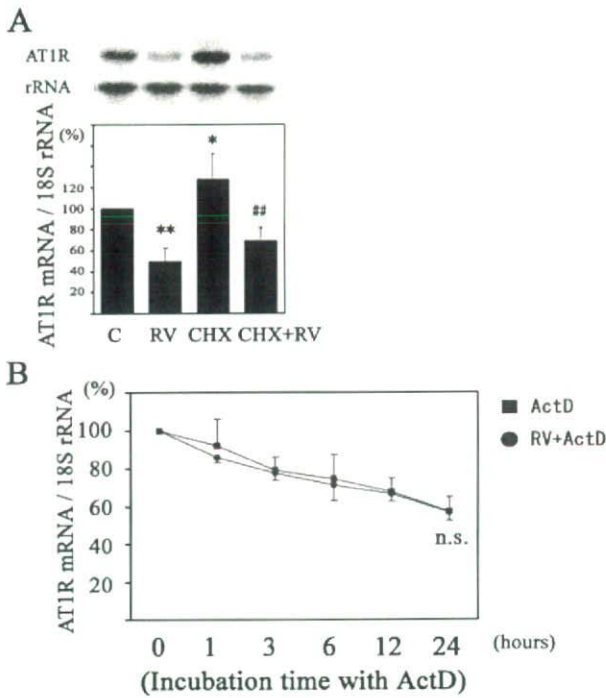


Figure 3. Effect of cycloheximide and actinomycin D on resveratrol-induced AT1R downregulation. **A**, Effect of cycloheximide (CHX) on resveratrol (RV)-induced AT1R mRNA downregulation was examined by Northern blot analysis. Values are expressed as a percent of control (C) culture (100%; n=8). **P*<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.

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 upregu-

lated 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

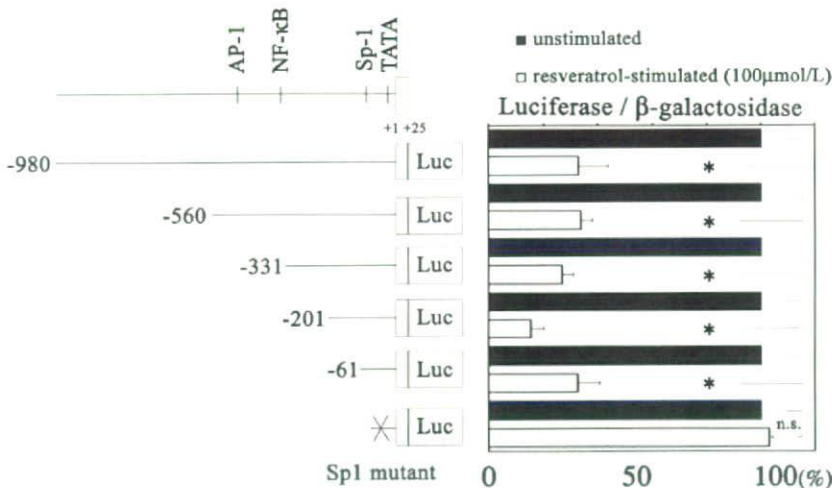


Figure 4. Resveratrol downregulates AT1R mRNA expression through transcriptional mechanisms. **Left**, The scheme of deletion mutants of AT1R promoter/luciferase fusion DNA construct and Sp1 mutant construct is shown. **Right**, The bar graphs indicate luciferase activity normalized by β-galactosidase 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.

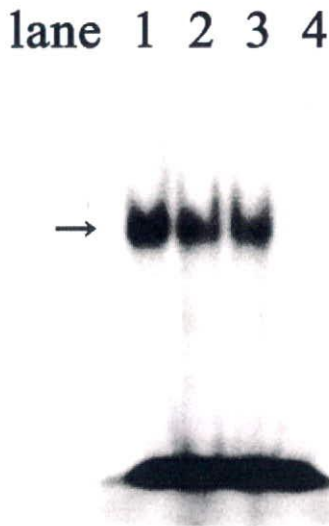


Figure 5. Gel mobility shift assay of nuclear protein of VSMCs. Binding activity of AT1R gene promoter (–40 bp to –6 bp), containing Sp1 site, was examined in the nuclear protein from unstimulated (lane 1) or resveratrol (100 μ mol/L)-stimulated (lane 2) VSMCs, by gel mobility shift assay. Fifty times molar excess of unlabeled mutant probe or wild-type probe were added to the reaction mixture (lane 3 and 4, respectively).

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.²⁹ 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-Goretta 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

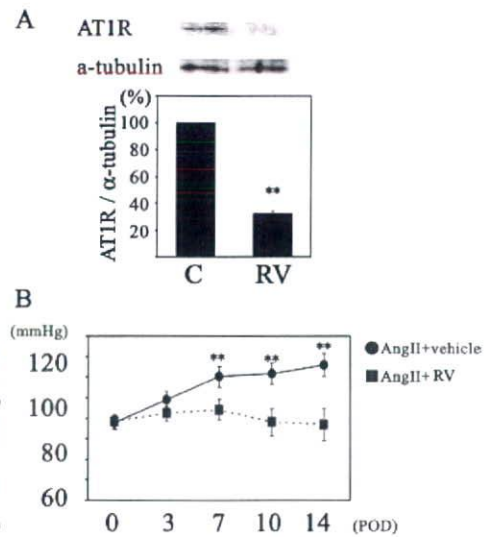


Figure 6. Effect of resveratrol on AT1R protein expression in the mouse aorta. A, AT1R expression in the membrane fractions of the aorta of mice treated with either resveratrol (RV) or water (C) was analyzed with Western blot analysis. The values are expressed as a percentage of water treated group (100%; n=4). ** P <0.01 vs water-treated group. B, Mice were treated with either resveratrol or vehicle (water). Ang II was administered in both groups and blood pressure was measured (n=4). ** P <0.01 vs vehicle-treated group. POD indicates postoperative days after implantation of osmotic minipump.

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.³¹ 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 VSMC. 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 Sp1.³⁵ 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,³⁶ and Ang II-induced hypertension was blunted by resveratrol. Because resveratrol inhibits AT1R signaling,²⁹ 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.^{6,7} It was also reported that inhibition of Ang II function by angiotensin converting enzyme inhibitor³⁷ and AT1R antagonist²² 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 life-span extension, inhibition of atherosclerosis and inflammation.

Sources of Funding

This study was supported in part by Grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (19590867) and Kimura Foundation Research Grant 2007 to T.I.

Disclosures

None.

References

- Soleas GJ, Diamandis EP, Goldberg DM. Wine as a biological fluid: history, production, and role in disease prevention. *J Clin Lab Anal.* 1997; 11:287–313.
- Nonomura S, Kanagawa H, Hashimoto A. Chemical constituents of polygonaceous plants. Studies on the components of Ko-jokon (*Polygonum Caspidatum* Sieb. et Zucc) *Yakugaku Zasshi.* 1963;83: 988–990.
- Baur JA, Sinclair DA. Therapeutic potential of resveratrol: the in vivo evidence. *Nat Rev Drug Discov.* 2006;5:493–506.
- Wang Q, Xu J, Rottinghaus GE, Lubahn D, Sun GY, Sun AY. Resveratrol protects against global cerebral ischemic injury in gerbils. *Brain Res.* 2002;958:439–447.
- Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CW, Fong HH, Farnsworth NR, Kinghorn AD, Mehta RG, Moon RC, Pezzuto JM. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science.* 1997;275:218–220.
- Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, Zipkin RE, Chung P, Kisilewski A, Zhang LL, Sscherer B, Sinclair DA. Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. *Nature.* 2003;425:191–196.
- Valenzano DR, Terzibasi E, Genade T, Cattaneo A, Domenici L, Cellerino A. Resveratrol prolongs lifespan and retards the onset of age-related markers in a short-lived vertebrate. *Curr Biol.* 2006;16: 296–300.
- Baur JA, Pearson KJ, Price NL, Jamieson HA, Lerin C, Kalra A, Prabhu VV, Allard JS, Lopez-Lluch G, Lewis K, Pistell PJ, Poosala S, Becker KG, Boss O, Gwinn D, Wang M, Ramaswamy S, Fishbein KW, Spencer RG, Lakatta EG, Le Couteur D, Shaw RJ, Navas P, Puigserver P, Ingram DK, de Cabo R, Sinclair DA. Resveratrol improves health and survival of mice on a high-calorie diet. *Nature.* 2006;444:337–342.
- Okamoto H, Fujioka Y, Takahashi A, Takahashi T, Taniguchi T, Ishikawa Y, Yokoyama M. Trichostatin A, an inhibitor of histone deacetylase, inhibits smooth muscle cell proliferation via induction of p21(WAF1). *J Atheroscler Thromb.* 2006;13:183–191.
- Denu JM. Linking chromatin function with metabolic networks: Sir2 family of NAD(+)-dependent deacetylases. *Trends Biochem Sci.* 2003; 28:41–48.
- Imai S, Christopher MA, Matt K, Leonard G. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. *Nature.* 1999;403:795–800.
- Bitterman KJ, Anderson RM, Cohen HY, Latorre-Esteves M, Sinclair DA. Inhibition of silencing and accelerated aging by nicotinamide, a putative negative regulator of yeast sir2 and human SIRT1. *J Biol Chem.* 2002;277:45099–45107.
- Dryden SC, Nahhas FA, Nowak JE, Goustin AS, Tainsky MA. Role for human SIRT2 NAD-dependent deacetylase activity in control of mitotic exit in the cell cycle. *Mol Cell Biol.* 2003;23:3173–3185.
- Blander G, Guarente L. The Sir2 family of protein deacetylases. *Annu Rev Biochem.* 2004;73:417–435.
- Cohen HY, Miller C, Bitterman KJ, Wall NR, Hekking B, Kessler B, Howitz KT, Gorospe M, de Cabo R, Sinclair DA. Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. *Science.* 2004;305:390–392.
- Goodfriend TL, Elliott ME, Catt KJ. Drug therapy: angiotensin receptors and their antagonists. *N Engl J Med.* 1996;334:1649–1654.
- Sasaki K, Yamano Y, Bardhan S, et al. Cloning and expression of a complementary DNA encoding a bovine adrenal angiotensin II type-1 receptor. *Nature.* 1991;351:230–233.
- Kambayashi Y, Bardhan S, Takahashi K, et al. Molecular cloning of a novel angiotensin II receptor isoform involved in phosphotyrosine phosphatase inhibition. *J Biol Chem.* 1993;268:24543–24546.
- Horiuchi M, Akishita M, Dzau VJ. Recent progress in angiotensin II type 2 receptor research in the cardiovascular system. *Hypertension.* 1999;33: 613–621.
- Touyz RM, Schiffrin EL. Signal transduction mechanisms mediating the physiological and pathophysiological actions of angiotensin II in vascular smooth muscle cells. *Pharmacol Rev.* 2000;52:639–672.
- Paradis P, Dali-Youcef N, Paradis FW, Thibault G, Nemer M. Overexpression of angiotensin II type 1 receptor in cardiomyocytes induces cardiac hypertrophy and remodeling. *Proc Natl Acad Sci U S A.* 2000; 97:931–936.
- Wolfgang L, Holger H, Bernward AS, Gabriele W. Long-term angiotensin II type 1 receptor blockade with Fosalartan doubles lifespan of hypertensive rats. *Hypertension.* 2000;35:908–913.
- Ichiki T, Usui M, Kato M, Funakoshi Y, Ito K, Egashira K, Takeshita A. Downregulation of angiotensin II type 1 receptor gene transcription by nitric oxide. *Hypertension.* 1998;31:342–348.
- Imayama I, Ichiki T, Inanaga K, Ohtsubo H, Fukuyama K, Ono H, Hashiguchi Y, Sunagawa K. Telmisartan downregulates angiotensin II type 1 receptor through activation of peroxisome proliferator-activated receptor. *Cardiovasc Res.* 2006;72:184–190.
- Guo DF, Uno S, Ishihata A, Nakamura N, Inagami T. Identification of a cis-acting glucocorticoid responsive element in the rat angiotensin II type 1A promoter. *Circ Res.* 1995;77:249–257.
- Tokunou T, Ichiki T, Takeda K, Funakoshi Y, Iino N, Shimokawa H, Egashira K, Takeshita A. Thrombin induces interleukin-6 expression through the cAMP response element in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol.* 2001;21:1759–1763.
- Fukuyama K, Ichiki T, Takeda K, Tokunou T, Iino N, Masuda S, Ishibashi M, Egashira K, Shimokawa H, Hirano K, Kanaide H, Takeshita A. Downregulation of vascular angiotensin II type 1 receptor by thyroid hormone. *Hypertension.* 2003;41:598–603.

28. Alcendor RR, Kirshenbaum LA, Imai S, Vatner SF, Sadoshima J. Silent information regulator 2alpha, a longevity factor and class III histone deacetylase, is an essential endogenous apoptosis inhibitor in cardiac myocytes. *Circ Res*. 2004;95:971-980.
29. Haider UG, Roos TU, Kontaridis MI, Neel BG, Sorescu D, Griendling KK, Vollmar AM, Dirsch VM. Resveratrol inhibits angiotensin II- and epidermal growth factor-mediated Akt activation: role of Gab1 and Shp2. *Mol Pharmacol*. 2005;68:41-48.
30. Actis-Goretti L, Ottaviani JJ, Fraga CG. Inhibition of angiotensin converting enzyme activity by flavanol-rich foods. *J Agric Food Chem*. 2006;54:229-234.
31. J G, Cq W, Hh F, Hy D, Xl X, Ym X, By W, Dj H. Effects of resveratrol on endothelial cells and their contributions to reendothelialization in intima-injured rats. *J Cardiovasc Pharmacol*. 2006;47:711-721.
32. Mnjoyan ZH, Fujise K. Profound negative regulatory effects by resveratrol on vascular smooth muscle cells: a role of p53-p21(WAF1/CIP1) pathway. *Biochem Biophys Res Commun*. 2003;311:546-552.
33. Das S, Fraga CG, Das DK. Cardioprotective effect of resveratrol via HO-1 expression involves p38 map kinase and PI-3-kinase signaling, but does not involve NFkappaB. *Free Radic Res*. 2006;40:1066-1075.
34. Hung JJ, Wang YT, Chang WC. Sp1 deacetylation induced by phorbol ester recruits p300 to activate 12(S)-lipoxygenase gene transcription. *Mol Cell Biol*. 2006;26:1770-1785.
35. Zhao S, Venkatasubbarao K, Li S, Freeman JW. Requirement of a specific Sp1 site for histone deacetylase-mediated repression of transforming growth factor beta type II receptor expression in human pancreatic cancer cells. *Cancer Research*. 2003;63:2624-2630.
36. Tomasz JG, Nyssa EH, Kathryn AB, Louise AM, Ayaz R, Sergey D, Jorg G, Cornelia W, and David GH. Role of the T cell in the genesis of angiotensin II-induced hypertension and vascular dysfunction. *J Exp Med*. 2007;204:2449-2460.
37. Linz W, Jessen T, Becker RHA, Schölkens BA, Wiemer G. Long-term ACE inhibition doubles lifespan of hypertensive rats. *Circulation*. 1997; 96:3164-3172.

Effect of anaesthesia-induced alterations in haemodynamics on *in vivo* kinetics of nitroxyl probes in electron spin resonance spectroscopy

TAKAKI TSUTSUMI¹, TOMOMI IDE¹, MAYUMI YAMATO², MAKOTO ANDOU¹,
TAKESHI SHIBA², HIDEO UTSUMI³, & KENJI SUNAGAWA¹

¹Department of Cardiovascular Medicine, Graduate School of Medical Sciences, ²Department REDOX Medicinal Science, and ³Department of Biofunctional Science, Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan

Accepted by Dr E. Niki

(Received 13 November 2007; in revised form 11 February 2008)

Abstract

Although the advent of *in vivo* electron spin resonance (ESR) spectroscopy has allowed analysis of the redox status of living animals, whether the haemodynamic condition affects the signal decay rate remains unknown. Three kinds of haemodynamic conditions were generated by changing the anaesthetic dosage in mice. Haemodynamics was analysed ($n = 6$ each) and *in vivo* ESR was performed to measure the signal decay rates of three nitroxyl spin probes (carbamoyl-, carboxy- and methoxycarbonyl-PROXYL) at the chest and head regions ($n = 6$ for each condition and probe). Haemodynamic analysis revealed negative inotropic and chronotropic effects on the cardiovascular system depending on the depth of anaesthesia. Although signal decay rates differed among three probes, they were not affected by heart rate alteration. In this study we report the haemodynamics-independent signal decay rate of nitroxyl probes.

Keywords: ESR, nitroxide, reactive oxygen species

Abbreviations: BP, blood pressure; 3-carbamoyl-PROXYL, 3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl; 3-carboxy-PROXYL, 3-carboxy-2,2,5,5-tetramethylpyrrolidine-1-oxyl; CmP, 3-carbamoyl-PROXYL; CxP, 3-carboxy-PROXYL; ESR, electron spin resonance; FS, fractional shortening; HR, heart rate; LV, left ventricle; LVEDD, left ventricular end-diastolic dimension; LVEDP, left ventricular end-diastolic pressure; LVESD, left ventricular end-systolic dimension; 3-methoxycarbonyl-PROXYL, 3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl; MCP, 3-methoxycarbonyl-PROXYL; ROS, reactive oxygen species.

Introduction

Free radicals and reactive oxygen species (ROS) are important mediators in the pathogenesis of several diseases. Experimental studies have shown that free radicals and ROS cause lipid peroxidation [1], protein oxidation [2] and DNA damage [3], resulting in cellular damage. The evaluation of ROS generation and/or redox status *in vivo* is important in understanding the pathogenic mechanisms of oxidative stress.

In the last few decades, the development of *in vivo* electron spin resonance (ESR) spectroscopy has made it possible to measure exogenously administered paramagnetic species in living animals [4,5]. Furthermore, nitroxyl radicals are used as spin probes in a variety of biological experiments in which ESR spectroscopy was used to detect ROS [6–9] and redox status [10]. The *in vivo* ESR signal decay rates of spin probes are enhanced by ROS such as hydroxy radical [3] and superoxide [11,12].

Correspondence: Tomomi Ide, MD PhD, Department of Cardiovascular Medicine, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582 Japan. Tel: +81-92-642-5359. Fax: +81-92-642-5374. Email: tomomi_i@cardiol.med.kyushu-u.ac.jp

The signal decay rate also depends on kinetic factors such as the distribution of the spin probe from blood to tissues and vice versa [13], urinary excretion via the kidney [14,15], faecal excretion via the liver and bile and transport into specific tissues/organs [15]. These factors are affected by anaesthesia-related physiological responses such as the haemodynamic changes and anaesthetics are often required in experiments using living animals. Anaesthetics are known to have significant negative inotropic effects on cardiovascular parameters [16–19]. They depress the cardiac function and decrease the peripheral vascular resistance, causing bradycardia, hypotension and decreased cardiac output. However, little is known about the effect of haemodynamic change on signal decay rates of spin probes *in vivo*.

In the present study, we evaluated whether the haemodynamic change affects the signal decay rates of spin probe *in vivo*. For this purpose, mice were anaesthetized by varying doses of pentobarbital sodium and divided into three groups of low-, intermediate- and high-heart rate (HR), which was used as an index of haemodynamic parameter.

Materials and methods

Animals and experimental protocol

Male CD-1 mice weighing 30–35 g were purchased from Kyudo Ltd. (Fukuoka, Japan). The mice were housed in a temperature- and humidity-controlled room. They were fed by a commercial diet and provided water *ad libitum*. Mice were randomly assigned to three groups: high-HR group, intermediate-HR group and low-HR group, and then anaesthetized by intraperitoneal injection of pentobarbital sodium at doses of 30, 40 and 50 mg/kg body weight, respectively ($n=6$ in each group for *in vivo* ESR) (Figure 1). After 15 min of anaesthesia, we measured rectal temperature quickly and performed ESR analysis. We also measured their rectal temperature during ESR analysis (at 3 min after the injection of spin probe). Although rectal temperature tended to be $\sim 1^\circ\text{C}$ lower during EPR measurement than before, there was no statistical significance among the groups (Table I). At 30 min after the injection of pentobarbital, blood samples were taken from LV for blood gas analysis. There was no difference in blood pH, pCO_2 or pO_2 among groups. All of the procedures were done within 40 min.

All procedures and animal care were approved by the Committee on Ethics of Animal Experiment, Kyushu University Graduate School of Medical and Pharmaceutical Sciences and carried out in accordance with the Guideline for Animal Experiment, Kyushu University and the Law (No.105) and Notification (No.6) of the Government. The investi-

gations conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Chemicals

3-Carbamoyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (3-carbamoyl-PROXYL) and 3-carboxy-2,2,5,5-tetramethylpyrrolidine-1-oxyl (3-carboxy-PROXYL) were purchased from Aldrich Chemical Co. (Milwaukee, WI). 3-Methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (3-methoxycarbonyl-PROXYL) was synthesized as described previously [20]. Each nitroxyl probe was dissolved in physiological saline to a final concentration of 100 mmol/L. The three isotonic probe solutions were analysed by X-band ESR and adjusted to contain the same concentration of nitroxyl radicals. All other reagents used were of the highest commercially available purity.

In vivo ESR spectroscopy

For the *in vivo* ESR measurements, 3 $\mu\text{L/g}$ body weight of an isotonic PROXYL solution (100 mmol/L) was administered intravenously. ESR spectra were recorded every minute at the chest or head region using a L-band ESR spectrometer (JEOL Co. Ltd., Akishima, Japan) with a loop-gap resonator (33 mm i.d. and 30 mm in length). The power of the 1.1 GHz microwave was 10 mW. The amplitude of the 100-kHz field modulation was 0.063 mT. Signal intensity was estimated from the height of the first positive peak in the spectrum. The signal decay rate was determined from the semi-logarithmic plot of signal intensity vs time after probe injection.

ECG acquisition

Needle electrodes were inserted subcutaneously into each of the four limbs. HR was calculated from electrocardiogram that was amplified and digitized online at 200 Hz by a 12-bit analogue-to-digital converter (AIO ADA 12-32/2(CB)F; Contec Co., CA). The digitized data and HR were stored on a hard disk for subsequent off-line analysis.

Echocardiography and haemodynamics measurements

Physiological evaluation with echocardiography and left heart catheterization was conducted as an independent experiment ($n=6$, each group). At 15 min after anaesthesia, a 7.5-MHz transducer connected to a dedicated ultrasonographic system (SSD-5500, Aloka Co., Tokyo) was applied to the left hemithorax. Two-dimensional targeted M-mode imaging was obtained from the short-axis view at a level showing the greatest left ventricular (LV)

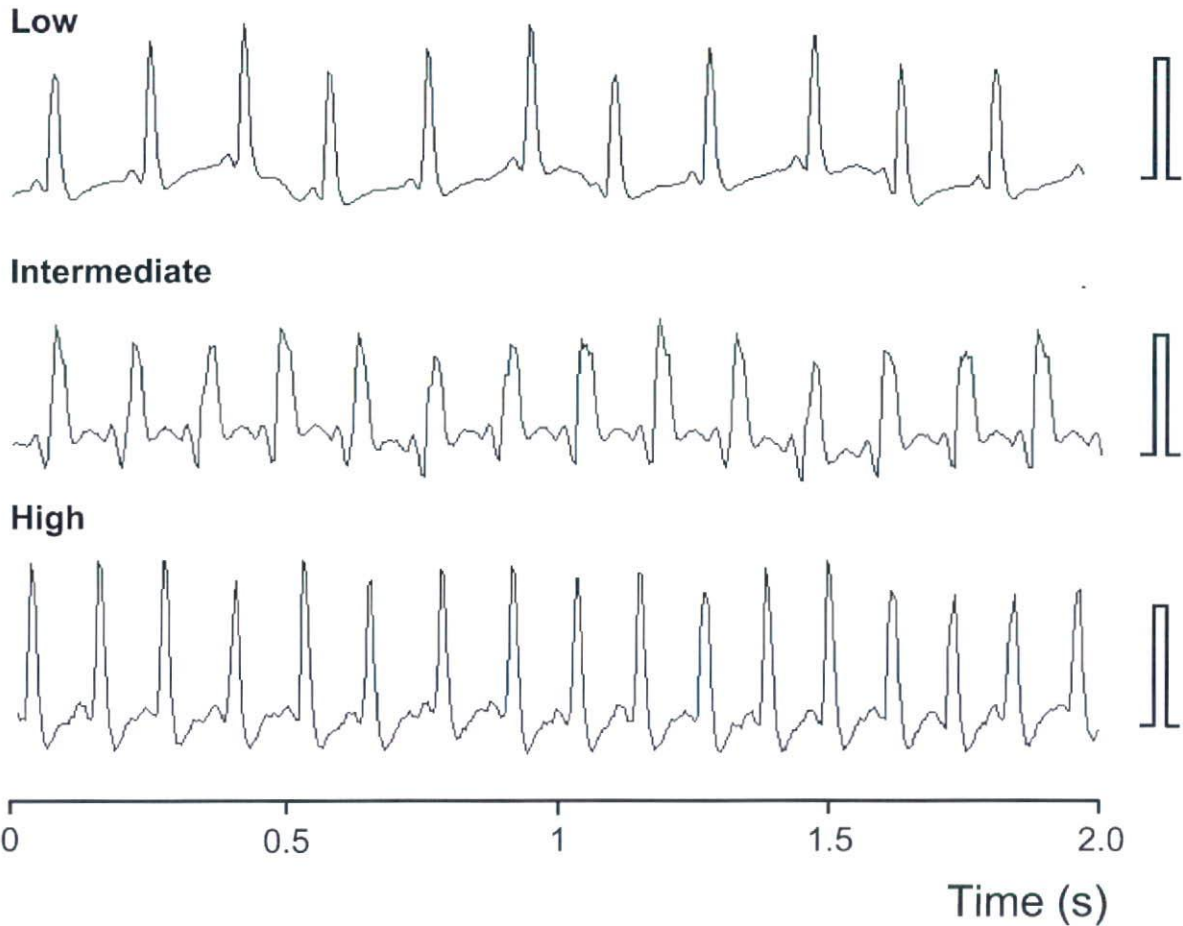


Figure 1. Typical electrocardiograms recorded from mice in the low-HR (HR: 330 bpm), intermediate-HR (HR: 420 bpm) and high-HR (HR: 510 bpm) groups. The height of the square on the right represents 1 mV.

dimension and end-diastolic (LVEDD) and end-systolic LV dimension (LVESD) were measured. Fractional shortening (FS) was calculated as follows.

$$\text{FS (\%)} = (\text{LVESD} - \text{LVEDD}) / \text{LVEDD} \times 100$$

After echocardiographic recording, a 1.4 F micro-manometer-tipped catheter (Millar Instruments, Houston, TX) was inserted into the right common carotid artery and advanced into the ascending aorta and LV for pressure measurement. All of the procedures ended within 40 min for each mouse.

Statistical analysis

Each value represents the mean \pm SEM. Inter-group differences were analysed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Values of *P* less than 0.05 were accepted as statistically significant.

Results

HR and cardiac function

Echocardiography and left heart catheterization were performed to evaluate haemodynamics under different HR conditions induced by anaesthesia. As summarized in Table II, a marked negative inotropic and chronotropic effect on the cardiovascular system was observed dependent on the depth of anaesthesia. The HR ranged from 287–359 bpm in low-, from

Table I. Physiological variables.

	Low-HR	Intermediate-HR	High-HR
<i>n</i>	6	6	6
pH	7.15 \pm 0.11	7.17 \pm 0.09	7.19 \pm 0.06
P _{CO₂} , mmHg	38.5 \pm 1.9	40.3 \pm 8.7	40.4 \pm 7.6
P _{O₂} , mmHg	113.9 \pm 15.8	119.6 \pm 17.0	113.1 \pm 28.3
Temperature, °C (before ESR)	36.1 \pm 1.7	35.5 \pm 1.6	36.6 \pm 1.0
Temperature, °C (after ESR)	35.0 \pm 1.9	34.5 \pm 1.4	35.6 \pm 1.0

Each value represents the mean \pm SEM.

Table II. Characteristics of animal models.

	Low-HR	Intermediate-HR	High-HR
<i>n</i>	6	6	6
Heart rate, bpm	317 ± 9	434 ± 6*	538 ± 8*†
Echocardiographic data			
LVEDD, mm	4.1 ± 0.1	3.7 ± 0.1	3.5 ± 0.1*
LVESD, mm	2.2 ± 0.1	1.9 ± 0.1	1.6 ± 0.1*
Fractional shortening, %	45 ± 1	49 ± 3	54 ± 2
Haemodynamic data			
Mean BP, mmHg	65.2 ± 4.0	83.1 ± 4.8	101.2 ± 2.6*†
Systolic BP, mmHg	83.1 ± 4.1	102.0 ± 5.4	122.0 ± 2.3*†
Diastolic BP, mmHg	48.9 ± 3.4	62.4 ± 5.1	79.1 ± 2.5*†
LVEDP, mmHg	3.5 ± 0.4	3.1 ± 0.7	3.2 ± 0.7
dP/dt _{max} , mmHg/s	5890 ± 670	8730 ± 650	13 500 ± 870*†
dP/dt _{min} , mmHg/s	-4510 ± 430	-6640 ± 310	-9 900 ± 1190*†

LV indicates left ventricular; EDD, end-diastolic diameter; ESD, end-systolic diameter; BP, blood pressure; EDP, end-diastolic pressure. Value are mean ± SEM. **P* < 0.05 vs low-HR. †*P* < 0.05 vs intermediate-HR.

426–451 bpm in intermediate- and from 510–545 bpm in high-HR group. LVESD and LVEDD were significantly larger and FS was significantly lower in low-HR mice compared with high-HR mice. Aortic pressure and the first derivative of LV pressure (dP/dt_{max}) were also depressed in low-HR group compared to high-HR group. There were no significant differences in left ventricular end-diastolic pressure (LVEDP) among three groups.

In vivo ESR spectroscopy

ESR spectra of three spin probes (Figure 2A) were recorded at the chest or head region in mice with different haemodynamic conditions. The *in vivo* spectrum (Figure 2B) consisted of sharp triplet lines, the intensity of which decreased gradually with time after probe injection (Figure 2C). Although the rate of signal decay recorded at the chest differed among

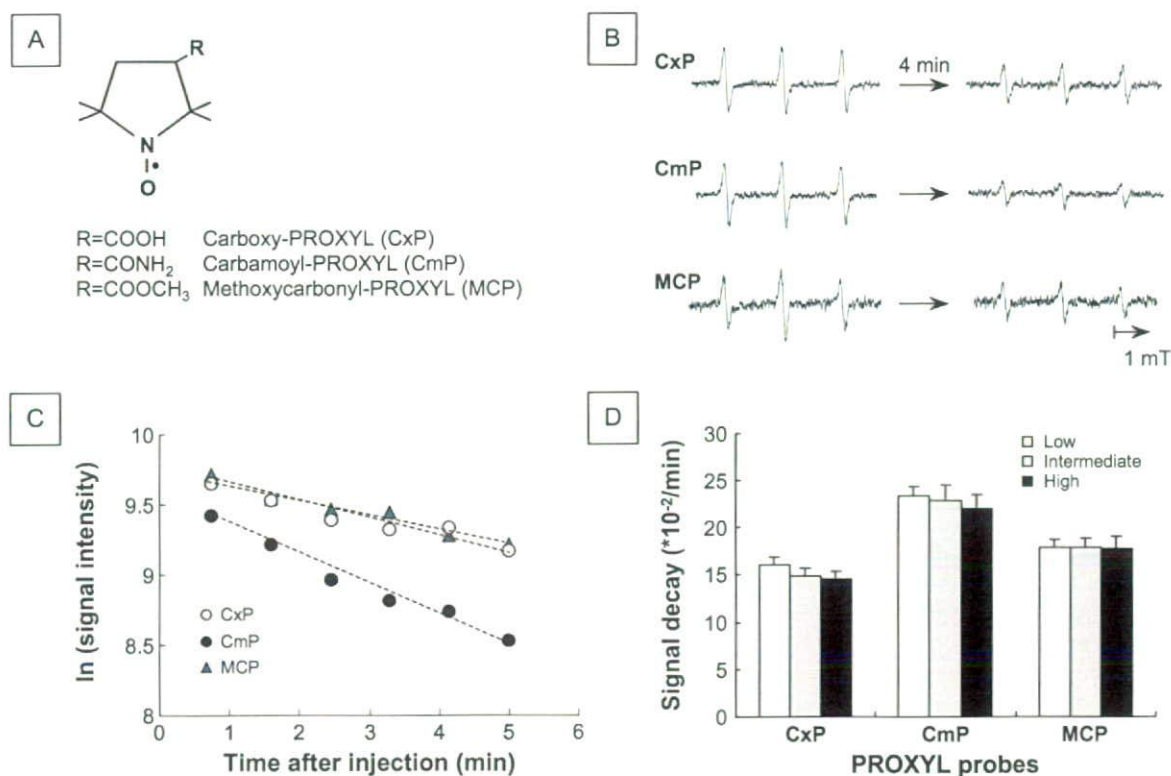


Figure 2. The chemical structure of nitroxide spin probes (A); typical ESR spectra of three spin probes (B); *in vivo* ESR signal decay curves of three spin probes from spectra recorded at chest region in the high-HR group (C); and signal decay rates of spin probes in low-HR, intermediate-HR and high-HR groups (D). Values are means ± SEM. The data of (C) and (D) were obtained from six animals.

Table III. Signal decay rate at the head region ($\times 10^{-2}/\text{min}$).

	Low-HR	Intermediate-HR	High-HR
Carboxy-PROXYL	5.2 \pm 0.9	6.2 \pm 0.3	5.7 \pm 1.7
Carbamoyl-PROXYL	9.5 \pm 1.0	9.3 \pm 1.6	10.3 \pm 2.2
Methoxycarbonyl-PROXYL	17.5 \pm 1.3	17.0 \pm 1.3	17.6 \pm 1.5

Values are mean \pm SEM. No statistical significant differences among low-HR, intermediate-HR and high-HR were observed in each probe.

probes, they were not affected by the HR alteration (Figure 2D). Similarly, the signal decay is the highest with Methoxycarbonyl-PROXYL compared with the other two probes at the head level; however, HR did not affect the decay rate at all (Table III).

Discussion

Although *in vivo* ESR spectroscopy is a powerful tool to determine the free radical generation and redox status in living animals, the effect of haemodynamic change associated with the depth of anaesthesia on the decay rate of ESR signals remains unclear. In this study, we demonstrated that the signal decay rate of particular nitroxyl probes was independent of the haemodynamic condition.

The kinetics of the intravenously administered spin probes are influenced by several factors [13–15]. However, in performing *in vivo* ESR spectroscopy, spin probe has to be administered intravenously and its transport to the target organs is dependent on the blood flow. Therefore, the haemodynamic condition is considered to have considerable effect on the decay kinetics of spin probes. In this study, for the purpose of generating a broad range of haemodynamic states, we chose pentobarbital sodium among many anaesthetics, because of its wide use in experiments using rodents and its profound cardiac depressive effect compared with other intravenous or inhalation anaesthetics [19,21]. Mice were divided into three HR groups by changing the pentobarbital dosage. The average HR and dp/dt_{max} in high-HR mice were ~ 550 bpm and 13500 mmHg/s, respectively, which were close to the values reported in conscious mice [22]. In contrast, in mice of the low-HR group, HR and dp/dt_{max} were profoundly depressed, supporting the success of producing various haemodynamic conditions by varying the anaesthetic dose. Among these three groups, there were no significant differences in signal decay rate when each spin probe was used to acquire ESR spectra at chest and head levels.

Several mechanisms may be proposed to explain the haemodynamics-independent signal decay rate. The first factor is the acquisition of ESR spectra 30–50 s after injection of the probe. In general, the circulation time of the probes ranges from a few

seconds to 1 min at the longest. Therefore, it probably takes a few seconds to a few minutes for the nitroxyl probes to reach an equilibrium state at the target organ. Takeshita et al. [23] have reported that the ESR signal obtained from mouse skin increased up to 2–3 min after injection of carbamoyl-PROXYL and then began to decrease in a first-order kinetics. However, such lag before decay was not observed in ESR spectra recorded at the chest and head levels in this study. Therefore, the time to reach equilibrium probably depends on the amount of blood supply and the vasculature structure in each organ. However, once equilibrium is achieved, the nitroxyls are reduced to reaction adduct such as secondary amine and their corresponding hydroxylamine without being affected by haemodynamics, consequently demonstrating loss of paramagnetism in a first order kinetics [24]. As we started ESR spectroscopy a few minutes after spin probe injection, the equilibrium condition has already been achieved in all groups. The second factor is the potential tissue transfer of the nitroxyl probes. Methoxycarbonyl-PROXYL is a lipophilic agent that passes through cell membrane [20,25,26]. Although carboxy-PROXYL are relatively hydrophilic [20,25,26], it was reported to be promptly taken up into tissues through the organic anion transporter [15]. Thus, a high potential of tissue transfer is also considered to be associated with the haemodynamics-independent signal decay.

There are a couple of unresolved questions in this study. First, there is a possibility that haemodynamic effects may induce biochemical alteration in longer period. Since we observed only a short-term change, further investigation is necessary for the long-time assessment. Secondly, we observe a slight tendency of the HR dependent decrease of the signal decay in carboxy-PROXYL and carbamoyl-PROXYL but not in MC-PROXYL. This might be due to the hydrophobicity of the spin probes. To clear this, we need to repeat a similar experiment in animals with more extreme conditions, which may be technically difficult in small animals.

On the other hand, there are some interesting observations in this study. After intravenous administration, the spin probes distribute from the blood to

the tissues [13], where they are reduced by enzymatic reaction [27–30]. There might be organ-specific differences in the enzymatic reduction of each probe. The order of clearance constant at the chest was as follows: carboxy- < methoxycarbonyl- < carbamoyl-PROXYL. In contrast, methoxycarbonyl-PROXYL showed the fastest signal decay rate at the head. Although the mechanisms of these differences were not elucidated in this study, the determinants of signal decay rate of each probe may depend not only on the target organs but also on the characteristics of the probe. Further studies are required to clarify these differences between spin probes.

In Conclusion, the haemodynamic condition does not affect the decay rate of ESR signal when using the *in vivo* ESR spectroscopy spin probe method. The variation in HR by anaesthetics may be compensated by homeostatic control *in vivo*, resulting in unaltered signal decay of spin probes.

Acknowledgements

This study was supported in part by grants from the Ministry of Education, Science and Culture, Health and Labor Sciences Research Grant for Comprehensive Research in Aging and Health Labor and Welfare of Japan, Japan Cardiovascular Research Foundation and Uehara Memorial Foundation. A part of this study was conducted in Kyushu University Station for Collaborative Research II.

References

- [1] Kato N, Yanaka K, Hyodo K, Homma K, Nagase S, Nose T. Stable nitroxide Tempol ameliorates brain injury by inhibiting lipid peroxidation in a rat model of transient focal cerebral ischemia. *Brain Res* 2003;979:188–193.
- [2] Hayashi T, Suda K, Imai H, Era S. Simple and sensitive high-performance liquid chromatographic method for the investigation of dynamic changes in the redox state of rat serum albumin. *J Chromatogr B Analyt Technol Biomed Life Sci* 2002;772:139–146.
- [3] Offer T, Samuni A. Nitroxides inhibit peroxy radical-mediated DNA scission and enzyme inactivation. *Free Radic Biol Med* 2002;32:872–881.
- [4] Berliner LJ, Wan XM. *In vivo* pharmacokinetics by electron magnetic resonance spectroscopy. *Magn Reson Med* 1989;9:430–434.
- [5] Ferrari M, Colacicchi S, Gualtieri G, Santini MT, Sotgiu A. Whole mouse nitroxide free radical pharmacokinetics by low frequency electron paramagnetic resonance. *Biochem Biophys Res Commun* 1990;166:168–173.
- [6] Yamato M, Egashira T, Utsumi H. Application of *in vivo* ESR spectroscopy to measurement of cerebrovascular ROS generation in stroke. *Free Radic Biol Med* 2003;35:1619–1631.
- [7] Yamada K, Nakamura T, Utsumi H. Enhanced intraarticular free radical reactions in adjuvant arthritis rats. *Free Radic Res* 2006;40:455–460.
- [8] Kasazaki K, Yasukawa K, Sano H, Utsumi H. Non-invasive analysis of reactive oxygen species generated in NH₄OH-induced gastric lesions of rats using a 300 MHz *in vivo* ESR technique. *Free Radic Res* 2003;37:757–766.
- [9] Phumala N, Ide T, Utsumi H. Noninvasive evaluation of *in vivo* free radical reactions catalyzed by iron using *in vivo* ESR spectroscopy. *Free Radic Biol Med* 1999;26:1209–1217.
- [10] Kuppusamy P, Li H, Ilangovan G, Cardounel AJ, Zweier JL, Yamada K, Krishna MC, Mitchell JB. Noninvasive imaging of tumor redox status and its modification by tissue glutathione levels. *Cancer Res* 2002;62:307–312.
- [11] Krishna MC, Grahame DA, Samuni A, Mitchell JB, Russo A. Oxoammonium cation intermediate in the nitroxide-catalyzed dismutation of superoxide. *Proc Natl Acad Sci USA* 1992;89:5537–5541.
- [12] Samuni A, Krishna CM, Mitchell JB, Collins CR, Russo A. Superoxide reaction with nitroxides. *Free Radic Res Commun* 1990;9:241–249.
- [13] Takechi K, Tamura H, Yamaoka K, Sakurai H. Pharmacokinetic analysis of free radicals by *in vivo* BCM (Blood Circulation Monitoring)-ESR method. *Free Radic Res* 1997;26:483–496.
- [14] Daryani A, Basu S, Becker W, Larsson A, Riserus U. Antioxidant intake, oxidative stress and inflammation among immigrant women from the Middle East living in Sweden: associations with cardiovascular risk factors. *Nutr Metab Cardiovasc Dis* 2007;17:748–756.
- [15] Ichikawa K, Sato Y, Kondo H, Utsumi H. An ESR contrast agent is transported to rat liver through organic anion transporter. *Free Radic Res* 2006;40:403–408.
- [16] Smith TL, Hutchins PM. Anesthetic effects on hemodynamics of spontaneously hypertensive and Wistar-Kyoto rats. *Am J Physiol* 1980;238:H539–H544.
- [17] Shimosato S, Etsen BE. Effect of anesthetic drugs on the heart: a critical review of myocardial contractility and its relationship to hemodynamics. *Clin Anesth* 1969;3:17–72.
- [18] Yang XP, Liu YH, Rhaleb NE, Kurihara N, Kim HE, Carretero OA. Echocardiographic assessment of cardiac function in conscious and anesthetized mice. *Am J Physiol* 1999;277:H1967–H1974.
- [19] Janssen BJ, De Celle T, Debets JJ, Brouns AE, Callahan MF, Smith TL. Effects of anesthetics on systemic hemodynamics in mice. *Am J Physiol Heart Circ Physiol* 2004;287:H1618–H1624.
- [20] Sano H, Matsumoto K, Utsumi H. Synthesis and imaging of blood-brain-barrier permeable nitroxyl-probes for free radical reactions in brain of living mice. *Biochem Mol Biol Int* 1997;42:641–647.
- [21] Oguchi T, Kashimoto S, Yamaguchi T, Nakamura T, Kumazawa T. Is pentobarbital appropriate for basal anesthesia in the working rat heart model? *J Pharmacol Toxicol Methods* 1993;29:37–43.
- [22] Kass DA, Hare JM, Georgakopoulos D. Murine cardiac function: a cautionary tail. *Circ Res* 1998;82:519–522.
- [23] Takeshita K, Takajo T, Hirata H, Ono M, Utsumi H. *In vivo* oxygen radical generation in the skin of the protoporphyria model mouse with visible light exposure: an L-band ESR study. *J Invest Dermatol* 2004;122:1463–1470.
- [24] Utsumi H, Muto E, Masuda S, Hamada A. *In vivo* ESR measurement of free radicals in whole mice. *Biochem Biophys Res Commun* 1990;172:1342–1348.
- [25] Sano H, Naruse M, Matsumoto K, Oi T, Utsumi H. A new nitroxyl-probe with high retention in the brain and its application for brain imaging. *Free Radic Biol Med* 2000;28:959–969.
- [26] Yamato M, Egashira T, Utsumi H. Application of *in vivo* ESR spectroscopy to measurement of cerebrovascular ROS generation in stroke. *Free Radic Biol Med* 2003;35:1619–1631.

- [27] Iannone A, Tomasi A, Vannini V, Swartz HM. Metabolism of nitroxide spin labels in subcellular fraction of rat liver. I. Reduction by microsomes. *Biochim Biophys Acta* 1990; 1034:285-289.
- [28] Iannone A, Tomasi A, Vannini V, Swartz HM. Metabolism of nitroxide spin labels in subcellular fractions of rat liver. II. Reduction in the cytosol. *Biochim Biophys Acta* 1990; 1034:290-293.
- [29] Quintanilha AT, Packer L. Surface localization of sites of reduction of nitroxide spin-labeled molecules in mitochondria. *Proc Natl Acad Sci USA* 1977;74:570-574.
- [30] Utsumi H, Shimakura A, Kashiwagi M, Hamada A. Localization of the active center of nitroxide radical reduction in rat liver microsomes: its relation to cytochrome P-450 and membrane fluidity. *J Biochem (Tokyo)* 1989;105:239-244.

Atorvastatin Reduces Oxidative Stress in the Rostral Ventrolateral Medulla of Stroke-Prone Spontaneously Hypertensive Rats

TAKUYA KISHI, YOSHITAKA HIROOKA,
HIROAKI SHIMOKAWA, AKIRA TAKESHITA, AND
KENJI SUNAGAWA

Department of Cardiovascular Medicine, Kyushu University Graduate School of
Medical Sciences, Fukuoka, Japan

Previously, we demonstrated that atorvastatin has sympatho-inhibitory effects with the upregulation of nitric oxide synthase in the brain in stroke-prone spontaneously hypertensive rats (SHRSP), and that reactive oxygen species in the rostral ventrolateral medulla (RVLM), where the vasomotor center is located, mediate the sympatho-excitatory effect. The aim of the present study was to determine if atorvastatin reduces oxidative stress in the RVLM of SHRSP along with the sympatho-inhibitory effect. SHRSP and Wistar-Kyoto (WKY) rats received standard feed with atorvastatin (50mg/kg per day) or standard feed for 30 days. Systolic blood pressure and heart rate were evaluated using the tail-cuff method. Urinary norepinephrine excretion was measured for 24 hours. After 30 days in SHRSP, blood pressure and urinary norepinephrine excretion were significantly lower in the atorvastatin group than in the control group. Thiobarbituric acid-reactive substance (TBARS) levels in the RVLM tissue obtained using the micropunch technique were used as measures of oxidative stress. Prior to the treatment, TBARS levels in the RVLM of SHRSP were significantly higher than those of WKY. After 30 days, TBARS levels in the RVLM of SHRSP were significantly lower in the atorvastatin group than in the control group. After 30 days in WKY, however, there were no differences in blood pressure, urinary norepinephrine excretion, and TBARS levels between the atorvastatin and control groups. These results suggest that atorvastatin reduces oxidative stress in the RVLM of SHRSP, which might contribute to the sympatho-inhibitory effects of atorvastatin in SHRSP.

Keywords statin, hypertension, brain, oxidative stress, sympathetic nervous system

Submitted July 16, 2005; accepted February 27, 2007.

Address correspondence to Yoshitaka Hirooka, M.D., Ph.D., Department of Cardiovascular Medicine, Kyushu University Graduate School of Medical Sciences, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan; E-mail: hyoshi@cardiol.med.kyushu-u.ac.jp

Introduction

Oxidative stress is implicated in the pathogenesis of hypertension (1,2). Among the target organs of hypertensive vascular diseases, the brain is the most affected by oxidative stress (3,4). Previously, we demonstrated that the generation of reactive oxygen species (ROS) is enhanced in the rostral ventrolateral medulla (RVLM) of the brain stem (i.e., where the vasomotor center is located) of stroke-prone spontaneously hypertensive rats (SHRSP), and that the oxidative stress in the RVLM induces a sympatho-excitatory effect (5). These findings suggest that oxidative stress in the RVLM is an important target in the treatment of hypertension.

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) are potent inhibitors of cholesterol biosynthesis. These agents slow the progression and foster the regression of atherosclerosis, resulting in an improvement of cardiovascular outcomes in humans with elevated and normal serum cholesterol levels (6–9). Moreover, statins might activate endothelial nitric oxide synthase (eNOS), improve endothelial function, increase vascular nitric oxide (NO) bioavailability, reduce oxidative stress, and improve endothelial progenitor cell function as a pleiotropic effect (10–19). In humans, four weeks of simvastatin treatment improves endothelial function independently of the decreases in LDL cholesterol, at least in part by reducing oxidative stress (20). The oral administration of atorvastatin has sympatho-inhibitory effects in SHRSP via an increase in NO production with upregulation of eNOS in the brain (21). The increase in NO production in the brain is thought to be mediated not only by eNOS upregulation in the brain by atorvastatin, but also by the antioxidant effect of atorvastatin, because NO is scavenged by ROS and statin has an antioxidant effect (10–19). Therefore, the aim of the present study was to determine if atorvastatin reduces oxidative stress in the RVLM of SHRSP along with the sympatho-inhibitory effect.

Materials and Methods

This study was reviewed and approved by the committee on ethics of Animal Experiments, Kyushu University Graduate School of Medical Sciences, and conducted according to the Guidelines for Animal Experiments of Kyushu University.

Measurements of Blood Pressure, Heart Rate, and Urinary Norepinephrine Excretion

Male Wister-Kyoto (WKY)/Izm and SHRSP/Izm (15 weeks old, SLC Japan, Hamamatsu, Japan) were put on standard feed or on standard feed supplemented with atorvastatin (gift from Pfizer Pharmaceuticals Inc.) and had free access to drinking water. The animals received atorvastatin at a dose of 50 mg/kg body weight per day, which was calculated according to the daily food intake (11,21), for 30 days. In rats, this dose produces plasma concentrations that are comparable to those achieved after the oral administration of similar doses of atorvastatin in humans (22). Systolic blood pressure (SBP) and heart rate (HR) were recorded using the tail-cuff method. Urine was collected for 24 hours using a metabolic cage, as described in our previous studies (5,21,23–26). Urinary norepinephrine was measured for 24 hours as a parameter of sympathetic nerve activity (5,21,23–26). To obtain RVLM tissues, the rats were deeply anesthetized with sodium pentobarbital (100 mg/kg IP) and transcardially perfused with phosphate buffered saline (5,21). The brain was removed quickly, and

1 mm-thick sections were obtained with a cryostat (5). The RVLM was defined according to a rat brain atlas (27), and the RVLM tissue was obtained using the micro-punch technique (5).

Measurement of TBARS Levels in the RVLM and Whole Brain

Thiobarbituric acid-reactive substances (TBARS) level was measured as a parameter of oxidative stress in the RVLM and whole brain (3,5). The RVLM and whole brain tissues were homogenized in 1.15% KCl (pH 7.4), 0.4% sodium dodecyl sulfate, and 7.5% acetic acid adjusted to pH 3.5 with NaOH. Thiobarbituric acid (0.3%) was added to the homogenate. The mixture was maintained at 5°C for 60 minutes, followed by heating to 100°C for 60 minutes. After cooling, the mixture was extracted with distilled water and *n*-butanolpyridine (15:1) and centrifuged at 1600g for 10 minutes. The absorbance of the organic phase was measured at 532 nm. The amount of TBARS was determined by absorbance with a molecular extinction coefficient of 156 000 and expressed as $\mu\text{mol/g}$ wet wt. All values were expressed as the mean \pm SEM, and unpaired Student's *t*-test was performed to compare the atorvastatin-treated and the control groups. *p* values of less than 0.05 were considered significant.

Results

Effects of Atorvastatin on Blood Pressure, Heart Rate, and Urinary Norepinephrine Excretion

Prior to treatment with atorvastatin, SBP, HR, and urinary norepinephrine excretion were significantly higher in SHRSP than in WKY (194 ± 4 mmHg vs. 139 ± 6 mmHg, 388 ± 11 beats/min vs. 348 ± 22 beats/min, 1.38 ± 0.06 μg vs. 0.91 ± 0.06 μg , $p < .05$; $n = 5$ for each). After 30 days, SBP and urinary norepinephrine excretion in SHRSP were significantly lower in the atorvastatin-treated group than in the control group (173 ± 9 mmHg vs. 199 ± 6 mmHg, 1.07 ± 0.07 μg vs. 1.41 ± 0.04 μg ; $p < .05$, $n = 5$ for each; see Figure 1). After 30 days in WKY, however, there was no significant difference in SBP and urinary norepinephrine excretion between the atorvastatin-treated group and the control group. HR was not significantly different between the atorvastatin-treated group and the control group in SHRSP and WKY (SHRSP: 384 ± 13 beats/min vs. 372 ± 18 beats/min; WKY: 352 ± 26 beats/min vs. 336 ± 24 beats/min).

Effects of Atorvastatin on TBARS Levels in RVLM and Whole Brain

Prior to treatment with atorvastatin, TBARS levels were significantly higher in the RVLM and whole brain of SHRSP than WKY (RVLM: 0.78 ± 0.06 vs. 0.32 ± 0.06 $\mu\text{mol/g}$ wet wt; whole brain: 0.88 ± 0.06 vs. 0.42 ± 0.08 $\mu\text{mol/g}$ wet wt, $p < .05$; $n = 5$ for each). After 30 days, TBARS levels of RVLM and whole brain in SHRSP were significantly lower in the atorvastatin-treated group than in the control group (RVLM: 0.55 ± 0.03 $\mu\text{mol/g}$ wet wt vs. 0.75 ± 0.04 $\mu\text{mol/g}$ wet wt; whole brain: 0.63 ± 0.04 $\mu\text{mol/g}$ wet wt vs. 0.84 ± 0.07 $\mu\text{mol/g}$ wet wt; $p < .05$, $n = 5$ for each; see Figure 2). After 30 days in WKY, however, there was no significant difference in TBARS levels of RVLM and whole brain between the atorvastatin-treated group and the control group.

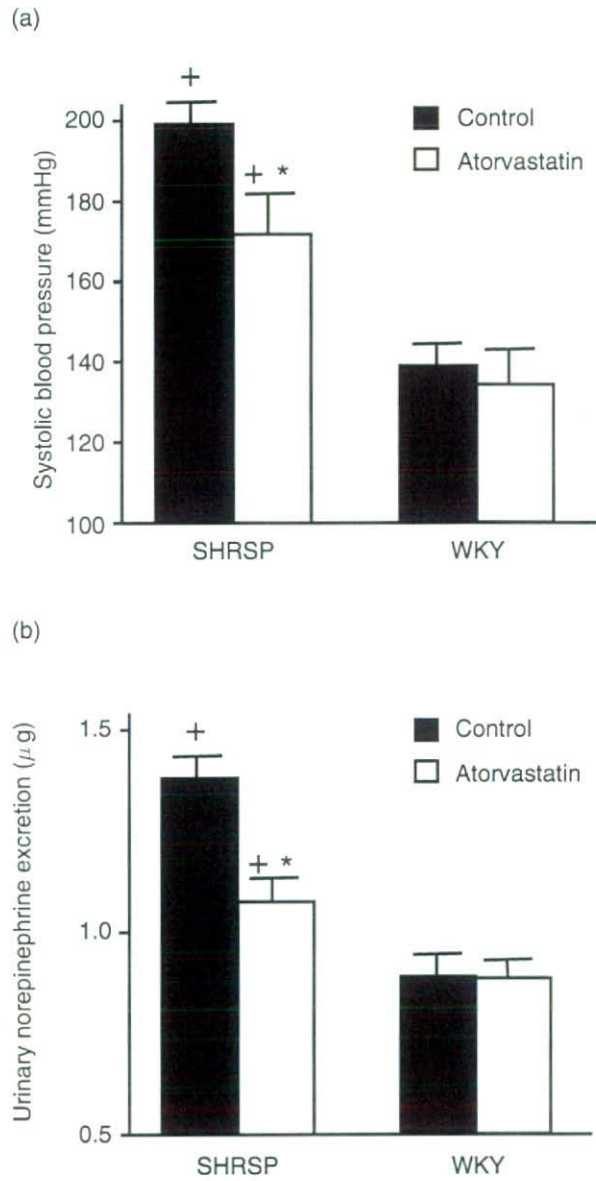


Figure 1. Effects of treatment with atorvastatin for 30 days on (A) systolic blood pressure, and (B) 24-hour urinary norepinephrine excretion in SHRSP and WKY. Male SHRSP and WKY (15 weeks old) were placed on a standard feed diet (control, filled square) or on a standard feed diet supplemented with atorvastatin (50 mg/kg of body weight per day, open square) for 30 days. Systolic blood pressure was assessed with rats in the conscious state using the tail-cuff method. Urinary norepinephrine excretion over 24 hours was measured by high performance liquid chromatography. Data are shown as mean \pm SEM ($n = 5$ per group). * $p < .05$ versus control group in the same strain; † $p < .05$ versus control group in WKY rats after 30 days.

Discussion

The major finding of the present study was that atorvastatin reduced oxidative stress, as measured by a reduction in TBARS levels, in the RVLM of SHRSP, which was significantly increased in SHRSP compared with WKY, and that the antioxidant effect of atorvastatin contributes to the sympatho-inhibitory effect of atorvastatin.