Angiotensin II Type 2 Receptor Deletion Enhances Vascular Senescence by Methyl Methanesulfonate Sensitive 2 Inhibition

Li-Juan Min, Masaki Mogi, Jun Iwanami, Jian-Mei Li, Akiko Sakata, Teppei Fujita, Kana Tsukuda, Masaru Iwai, Masatsugu Horiuchi

Abstract—Vascular senescence is closely associated with age-related vascular disorders and is enhanced by angiotensin (Ang) II type 1 receptor stimulation. However, the role of Ang II type 2 receptor activation in vascular senescence is still an enigma. Ang II stimulation significantly increased senescence-associated β-galactosidase activity and the level of 8-hydroxy-2'-deoxyguanosine, with enhancement of oxidative stress and expression of Ki-ras2A, p53, and p21 in vascular smooth muscle cells (VSMCs) from wild-type (Agtr2+/-) mice, whereas these effects of Ang II were enhanced in VSMCs from Ang II type 2 receptor null (Agtr2-/-) mice. Administration of an Ang II type 1 receptor blocker, valsartan, attenuated these parameters, with less effect in Agtr2+/- VSMCs. Ang II stimulation increased methyl methanesulfonate sensitive 2 (MMS2) expression in Agtr2+/- VSMCs but not in Agtr2-/-VSMCs. MMS2 small-interfering RNA treatment enhanced Ang II–induced senescence-associated β-galactosidase activity and 8-hydroxy-2'-deoxyguanosine level with no significant changes in oxidative stress markers and the expression of Ki-ras2A, p53, and p21. Moreover, exposure of Agtr2+/- VSMCs to hydrogen peroxide and ultraviolet irradiation induced marked increases in senescence-associated β-galactosidase activity and 8-hydroxy-2'-deoxyguanosine level, which were further enhanced in Agtr2-/- and MMS2 small-interfering RNA–treated Agtr2+/- VSMCs. Agtr2-/- mice exposed to x-ray irradiation showed increases in senescence-associated β-galactosidase activity and 8-hydroxy-2'-deoxyguanosine level in the aorta, which were further exaggerated in the aorta of Agtr2-/- mice with a lower MMS2 level. These findings suggest that Ang II type 2 receptor signaling attenuates DNA damage and consequent vascular senescence at least in part through MMS2 transactivation and propose the beneficial effects of Ang II type 2 receptor stimulation with Ang II type 1 receptor blockers in age-related vascular disorders. (Hypertension. 2008;51:1339-1344.)

Key Words: angiotensin II type 2 receptor ■ vascular cell ■ senescence ■ methyl methanesulfonate sensitive 2 ■ DNA damage

Vascular senescence plays an important role in age-related vascular disorders.1 Angiotensin (Ang) II type 1 (AT1) receptor stimulation has recently been suggested to provoke vascular cell senescence.2 Treatment with AT1 receptor blockers (ARBs) showed protective effects on age-associated vascular diseases.3 Moreover, some articles and our study indicated that oxidative stress, Ki-ras2A (a member of the oncoprotein Ras family) and its downstream target (cell cycle transcriptional activator), p53/cyclin-dependent kinase inhibitor, and p21 pathway are involved in the senescence-promoting effect of the AT1 receptor.3,4,5 In contrast, the roles of activation of the Ang II type 2 (AT2) receptor in vascular senescence are totally unknown. AT2 receptor expression is reported to be upregulated in cardiac senescence,6 and AT2 receptor stimulation is believed to oppose AT1 receptor-mediated signaling and functions.7 Moreover, AT2 receptor stimulation has a unique mechanism in addition to a negative interaction with AT1 receptor signaling.8–10 Therefore, it is possible that AT2 receptor activation could prevent vascular senescence via both inhibition of AT1 receptor-mediated signaling and its own signaling mechanism. Recently, many genetic studies supported the important role of DNA repair in the protection of damaged DNA and the prevention of cellular senescence.11 Methyl methanesulfonate sensitive 2 (MMS2), a family of ubiquitin-conjugating enzyme variants, could act as a key factor for DNA repair.12 Moreover, we demonstrated previously that AT2 receptor stimulation induces MMS2 expression, resulting in DNA repair and neural differentiation.9,13 These findings led to a hypothesis that AT2 receptor signaling could prevent vascular senescence through ≥2 mechanisms: an MMS2-mediated protection system for DNA damage and counteracting the AT1 receptor–mediated signaling pathway.
Valsartan administration. Senescence-associated 
formed by 10 Gy of total-body x-ray irradiation before and after 

decreased x-ray irradiation–induced SA-
(8-OHdG), a DNA damage marker, was performed 
lucigenin chemiluminescence. Quantification of 8-hydroxy-2 
dinucleotide phosphate oxidase in VSMCs was measured by a 

Deletion of AT2 Receptor Signaling Enhanced 

Deletion of AT2 Receptor Signaling Enhanced 
Vascular Senescence and DNA Damage 

Deletion of AT2 Receptor Signaling Enhanced Vascular Senescence and DNA Damage 

Results 

Deletion of AT2 Receptor Signaling Enhanced Vascular Senescence and DNA Damage 

Methods 

This study was performed in accordance with the National Institutes of Health guidelines for the use of male wild-type (Agtr2+) mice (C57BL/6J; Clea Japan, Inc, Osaka, Japan) and AT2 receptor null (Agtr2−) mice (based on the C57BL/6J strain bred in our laboratory). Vascular smooth muscle cells (VSMCs) were isolated from the thoracic aorta of Agtr2+ mice and Agtr2− mice by the explant method. Ang II or other reagents were administrated to VSMCs as described previously. VSMCs were subjected to repeated 250 mJ/cm2 UV irradiation and 2 hours of 600 μmol/L of hydrogen peroxide (H2O2) exposure. The irradiated mouse model was performed by 10 Gy of total-body x-ray irradiation before and after valsartan administration. Senescence-associated β-galactosidase (SA-β-gal) activity was measured using a Senescence Detection kit. Immunoblot analysis and RNA interference of MMS2 were performed as described previously. Activity of nicotinamide-adenine dinucleotide phosphate oxidase in VSMCs was measured by a luminescence assay, and superoxide generation was determined by lucigenin chemiluminescence. Quantification of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a DNA damage marker, was performed 

Inhibition of AT2 Receptor–Mediated MMS2 
Associated With Enhancement of Vascular Senescence and DNA Damage 

Inhibition of AT2 Receptor–Mediated MMS2 
Associated With Enhancement of Vascular Senescence and DNA Damage 

Figure 1. SA-β-gal activity by Ang II stimulation in VSMCs (A) and in the aorta of irradiated mice (B). n=4 for each. Ang II is 10−7 mol/L. Val indicates valsartan (10−5 mol/L) and 3 mg/kg per day. PD123319 is 10−5 mol/L. *P<0.05 vs control in VSMCs or in aorta of Agtr2+ mice. †P<0.01 vs Ang II 10 days or irradiation (+) in Agtr2+ or Agtr2− mice. ‡P<0.01 vs 3, 5, and 10 days of stimulation of Ang II or irradiation (+) in Agtr2− mice.
an Ang II–mediated increase in MMS2 expression was not observed in Agtr2− VSMCs (Figure S3A), and x-ray irradiation–induced MMS2 expression in the aorta was attenuated in Agtr2− mice (Figure S3B). We chose H2O2 or UV irradiation as DNA damage and senescence-inducing factors to further explore the role of MMS2 and observed that the MMS2 level was markedly increased in Agtr2+ VSMCs after exposure to H2O2 (600 μmol/L) or UV irradiation (250 mJ/cm2), whereas this effect of H2O2 or UV irradiation was attenuated in Agtr2− VSMCs (Figure S3C).

We used the RNA interference method to investigate the effect of MMS2 on SA-β-gal activity and the 8-OHdG level. Under treatment with MMS2 small-interfering RNA (siRNA), the stimulatory effects of Ang II on SA-β-gal activity and 8-OHdG level in Agtr2+ VSMCs after 5 days of Ang II treatment were significantly increased compared with those under control-siRNA treatment (Figures 3A and S3D). SA-β-gal activity and the 8-OHdG level were also significantly increased in control-siRNA–treated Agtr2+ VSMCs after exposure to H2O2 or UV irradiation, and they were further enhanced in control-siRNA–treated Agtr2+ and MMS2-siRNA–treated Agtr2+ VSMCs. Moreover, SA-β-gal activity and 8-OHdG level induced by H2O2 or UV irradiation were higher in MMS2-siRNA–treated Agtr2+ VSMCs than in control-siRNA–treated Agtr2− and MMS2-siRNA–treated Agtr2− VSMCs (Figures 3B and S3E).

**Effect of MMS2 on Oxidative Stress and the Ki-ras2A-p53-p21 Pathway**

Finally, we examined the association of MMS2 with oxidative stress and the Ki-ras2A-p53-p21 cascade and observed that MMS2-siRNA treatment did not significantly influence the Ang II–induced superoxide production; nicotinamide adenine dinucleotide phosphate oxidase activity; and Ki-ras2A-p53-p21 expression in VSMCs (Figure 4A through 4C and Figure S4).

**Discussion**

Vascular senescence mediated by AT1 receptor stimulation has been highlighted, and ARBs have been shown to prevent vascular disorders associated with aging. It has been reported that AT1 receptor blockade and stimulation of unbound AT2 receptors by Ang II could contribute to vaso-
protective effects of ARBs, such as inhibition of vascular remodeling and atherosclerosis.15,16 However, the roles of AT1 receptor stimulation in vascular senescence are not well investigated, leading us to examine the possibility that AT2 receptor signaling could prevent vascular senescence and its possible mechanisms. We demonstrated that vascular senescence evaluated by SA-β-gal activity was more exaggerated in Agtr2−/− mice than in Agtr2+/+ mice and that treatment with an ARB, valsartan, prevented vascular senescence, whereas this protective effect of valsartan was weaker in Agtr2−/− mice in vitro and in vivo. We observed that AT2 receptor expression was upregulated in senescent vascular cells of Agtr2−/− mice. These results indicate that AT2 receptor signaling could play an important role in attenuating vascular senescence.

Oxidative stress and the oncoprotein Ras, which are essential for various signaling pathways, have been shown recently to trigger cellular senescence by regulating the level of the cyclin-dependent kinase inhibitor.5,17–19 We have demonstrated that AT1 receptor–mediated oxidative stress enhances the Ki-ras2A-p53-cyclin-dependent kinase inhibitor p21 pathway, resulting in VSMC senescence.4 In the present study, we addressed the issue that AT2 receptor signaling prevents vascular cell senescence possibly because of its inhibitory effect on the AT1 receptor–mediated signaling pathway. We demonstrated that oxidative stress; Ki-ras2A, p53, and p21 expression induced by Ang II stimulation; and x-ray irradiation were further enhanced in VSMCs and the aorta in Agtr2−/− mice compared with those in Agtr2+/+ mice. Therefore, we assumed a possible antagonistic effect of AT2 receptor signaling on AT1 receptor–mediated oxidative stress and the Ki-ras2A-p53-p21 signaling pathway could prevent vascular cell senescence. Although the detailed signaling mechanism of the antagonism of the AT1 receptor by the AT2 receptor is still poorly understood, AT2 receptor stimulation is known to activate a variety of phosphatases and to enhance NO production in VSMCs.20 Protein phosphatases could inactivate Ras-mediated signaling, such as mitogen-activated protein kinase, in response to mechanical stress.21 Therefore, the direct downstream targets of Ki-ras2A, such as extracellular signal regulated kinase, p38 mitogen-activated protein kinase, or phosphatidylinositol 3-kinase, may also play potential roles in AT2 receptor–mediated inhibition of vascular senescence.

Figure 3. Effects of MMS2-siRNA on Ang II– (A) and H2O2- or UV irradiation–induced (B) senescence in VSMCs. n=4 for each.

Figure 4. Effect of MMS2-siRNA on Ang II–induced superoxide anion production (A) in VSMCs. Effect of MMS2-siRNA on Ang II–induced Ki-ras2A (B) and p53 and p21 expression (C). n=4 for each.

*P<0.05 vs control in control-siRNA–treated Agtr2+ VSMCs. **P<0.01 vs H2O2 or UV irradiation (+) in control-siRNA–treated Agtr2+ VSMCs. #P<0.01 vs H2O2 or UV irradiation (+) in MMS2-siRNA–treated Agtr2+ VSMCs.
Ki-ras2A-p53-p21 pathway. Our findings support the idea that AT2 receptor–mediated antisenescence signaling is closely related to an MMS2-dependent protection system for DNA damage, such as DNA repair, in addition to antagonizing AT1 receptor signaling, and that the MMS2 cascade is an unique mechanism of AT2 receptor signaling. The signaling cascade from AT2 receptor stimulation to MMS2 activation and the detailed mechanism of MMS2 in mediating DNA repair and consequent inhibition of vascular senescence need to be further investigated.

**Perspectives**

Collectively, our findings suggest that AT2 receptor signaling exerts inhibitory effects on vascular senescence through an MMS2-dependent protection system for DNA damage, such as DNA repair, as well as counteracting AT1 receptor-mediated oxidative stress and the Ki-ras2A-p53-p21 pathway. We also demonstrated that treatment with an ARB, valsartan, attenuated vascular senescence and DNA damage and that the effects of valsartan were less in Agtr2−/− mice, suggesting that both AT2 receptor signaling and AT1 receptor blockade are necessary for preventing vascular senescence. We, therefore, propose important beneficial pharmacotherapeutic effects of ARBs, by stimulating the AT2 receptor, in the application of ARBs to age-related vascular disorders.

**Sources of Funding**

This work was supported by grants from the Ministry of Education, Science, Sports, and Culture of Japan (to M.H., M.M., and L-J.M.) and the Suzukien Memorial Foundation and Takeda Science Foundation (to M.M.).

**Disclosures**

None.

**References**


Angiotensin II Type 2 Receptor Deletion Enhances Vascular Senescence by Methyl Methanesulfonate Sensitive 2 Inhibition
Li-Juan Min, Masaki Mogi, Jun Iwanami, Jian-Mei Li, Akiko Sakata, Teppei Fujita, Kana Tsukuda, Masaru Iwai and Masatsugu Horiuchi

Hypertension. 2008;51:1339-1344; originally published online March 24, 2008; doi: 10.1161/HYPERTENSIONAHA.107.105692

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/51/5/1339

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2008/03/18/HYPERTENSIONAHA.107.105692.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org/subscriptions/
Angiotensin II Type 2 Receptor Deletion Enhances Vascular Senescence by Methyl Methanesulfonate Sensitive 2 Inhibition

Li-Juan Min, Masaki Mogi, Jun Iwanami, Jian-Mei Li, Akiko Sakata, Teppei Fujita, Kana Tsukuda, Masaru Iwai, and Masatsugu Horiuchi

Department of Molecular Cardiovascular Biology and Pharmacology,
Ehime University, Graduate School of Medicine

Short Title: Role of AT_2 receptor in vascular senescence

Correspondence to: Masatsugu Horiuchi, MD, PhD, FAHA
Department of Molecular Cardiovascular Biology and Pharmacology,
Ehime University, Graduate School of Medicine,
Tohon, Ehime 791-0295, Japan.
Tel: 81-89-960-5248, Fax: 81-89-960-5251
E-mail: horiuchi@m.ehime-u.ac.jp
Expanded Methods

Cell culture

Vascular smooth muscle cells (VSMC) were isolated from the thoracic aorta of adult male wild-type (Agtr2+) mice (based on C57BL/6J strain; Clea Japan Inc., Osaka, Japan) and AT2 receptor null (Agtr2-) mice (based on C57BL/6J strain bred in our laboratory) by the explant method. The cells were cultured on 100-mm dishes in Dulbecco’s Modified Eagle Medium (DMEM) (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37°C in a humidified atmosphere of 5% CO2 and 95% air. Cells at passage 3 to 8 were used for the experiment. Subconfluent cells were cultured in DMEM containing 0.1% FBS for 48 hours to induce a quiescent state before the experiments. Ang II was administrated to VSMC as previously described. Fifty-60% confluent and quiescent VSMC cultured on 6-well plates were stimulated with Ang II (10^{-7} mol/L) for 3 days, 5 days and 10 days, respectively by changing the medium every 24 hours with new medium containing freshly prepared Ang II. Valsartan (10^{-5} mol/L) was added after 5 days of Ang II stimulation group for an additional 5 days also by changing the medium every 24 hours with new medium containing freshly prepared Ang II plus valsartan.

Ultraviolet (UV) irradiation and hydrogen peroxide (H2O2) treatment of VSMC

Cultures of VSMC (50-60% confluent) were subjected to repeated UV stress. VSMC were washed once with phosphate-buffered saline (PBS) and exposed to a subcytotoxic dose of UV radiation (250 mJ/cm²) in a thin layer of PBS using a UV cross-linker (CL-1000, Funakoshi Corp., Tokyo, Japan), followed by washing and replacement of growth medium. Irradiation
was performed once a day for 5 days. For hydrogen peroxide (H₂O₂) treatment, 50-60% confluent VSMC were submitted to 2 hours’ exposure to a subcytotoxic dose of H₂O₂ (600 μmol/L) diluted in growth medium, followed by washing and replacement of growth medium. After exposure to repeated UV irradiation for 5 days or H₂O₂ for 2 hours, the cells were cultured under normal condition for 3 days and used for different experiments. Control cultures were performed in the same medium without UV or H₂O₂ treatment.

**Irradiated mouse model**

This study was performed in accordance with the National Institutes of Health guidelines for the use of experimental animals. All animal studies were reviewed and approved by the Animal Studies Committee of Ehime University. Mice were maintained in a pathogen-free environment and exposed to a lethal dose, 10 Gy total-body X-ray irradiation at room temperature, using an irradiator (MBR-1520R-3, Hitachi, Tokyo, Japan). Valsartan was administered at a dose of 3 mg/kg/day i.p. via an osmotic minipump (Alzet model 1002, Durect Corporation) implanted 7 days before irradiation. The dose of valsartan we chose was a nonhypotensive dose and did not significantly influence blood pressure of mice. At 3 days after irradiation, the thoracic aorta was isolated.

**Senescence-associated β-galactosidase (SA-β-gal) staining**

Senescence-associated β-galactosidase (SA-β-gal) activity in VSMC cultured on 6-well plates was measured as previously described. The cells were counterstained with 4’6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA) for 10 minutes to
count the numbers of total cells at ×20 magnification. Fresh thoracic aorta taken from mice were immediately trimmed of excess periadventitial tissue and opened vertically, and directly stained with SA-β-gal. Analysis of SA-β-gal activity in the aorta was performed using a computer-imaging software system (Densitograph, ATTO Corp., Tokyo, Japan).

**Immunoblot analysis**

Total proteins were prepared from VSMC cultured in 100-mm dishes and thoracic aorta under different experimental conditions. The proteins were subjected to SDS-PAGE and immunoblotted with anti-MMS2 antibody (Zymed Laboratories, Inc., South San Francisco, CA), anti-Ki-ras2A antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-p21, or p53 antibody (Cell Signaling Technology Inc., Beverly, MA), or anti-α smooth muscle actin antibody (Sigma-Aldrich, Inc., St. Louis, MO). The bands of proteins were visualized with an ECL system (Amersham Biosciences). Densitometric analysis was performed using NIH image software.5

**RNA interference of MMS2**

For small interfering RNA (siRNA) assay, VSMC were transiently transfected with control siRNA or MMS2-specific siRNA, a cocktail of three siRNAs designed by B-Bridge (Sunnyvale, CA) as previously described.2

**Determination of NADPH oxidase activity in VSMC**

Activity of NADPH oxidase in VSMC in total protein cell homogenates was measured by a
luminescence assay as previously described, using 500 μmol/L lucigenin as the electron acceptor and 100 μmol/L NADPH as the substrate.²

Detection of superoxide anion in VSMC

Superoxide generation of VSMC cultured on 6-well plates was evaluated using fluorogenic dihydroethidium (DHE, 5 μmol/L) as previously described.² Intensity of fluorescence was analyzed and quantified using computer-imaging software (Densitograph, ATTO Corp., Tokyo, Japan).

Quantification of 8-hydroxy-2'-deoxyguanosine (8-OHdG)

Immunoreactivity of 8-OHdG in DNA of VSMC and thoracic aorta of mice was detected by an enzyme-linked immunosorbent assay (ELISA).⁶ Briefly, genomic DNA was extracted using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). The 8-OHdG level in 20 μg of DNA samples was measured by an 8-OHdG Check ELISA Kit (Japan Institute for the Control of Aging). The ratio of ng 8-OHdG/mg DNA was calculated.

Ang II receptor binding assay

The changes in Ang II receptor densities in response to Ang II, UV radiation or H₂O₂ were determined by radioligand binding assay as described previously.⁷

Materials

Reagents not listed above were as follows: An AT₁ receptor blocker, valsartan, was donated by
Novartis Pharma AG (Basel, Switzerland). All other reagents not mentioned above were purchased from Sigma-Aldrich.

**Statistical analysis**

All values are expressed as mean ± SEM in the text and figures. Data were evaluated by ANOVA followed by post-hoc analysis for multiple comparisons. Differences with $P<0.05$ were considered significant.
References


Figure S1 A
Representative photographs of senescence-associated β–galactosidase (SA-β-gal)-stained VSMC after 10 days of Ang II stimulation from 5 randomly chosen different fields at ×20 magnification. Blue-stained cells represent SA-β-gal positive cells, and 4′6-diamidino-2-phenylindole (DAPI)-stained cells show total cells. Ang II, angiotensin II (10^{-7} mol/L); Val, valsartan (10^{-5} mol/L), PD123319 (10^{-5} mol/L).
Figure S1 B and C
8-OHdG level by Ang II stimulation in VSMC (B) and in the aorta of irradiated mice (C). n=4 for each. Ang II, angiotensin II (10^-7 mol/L); Val, valsartan (10^-5 mol/L) and (3 mg/kg/day). *P<0.05 vs. control in VSMC or in aorta of Agtr2^+ mice. #P<0.01 vs. Ang II 10 days or irradiation (+) in Agtr2^+ or Agtr2^- mice. †P<0.01 vs. 3 days, 5 days, and 10 days stimulation of Ang II or irradiation (+) in Agtr2^+ mice.
Figure S1 D
Ang II receptors binding densities by Ang II stimulation in VSMC performed by radioligand binding assay. n=4. *P<0.05 vs. Ang II 0 days. †P<0.01 vs. Ang II 3 days, 5 days, and 10 days, respectively.
Figure S2
Effect of Ang II stimulation on NADPH oxidase activity in VSMC. n=4. *P<0.05 vs. control in Agtr2+ VSMC. #P<0.01 vs. Ang II 10 days in Agtr2+ and Agtr2- VSMC, respectively. †P<0.01 vs. 3 days, 5 days, and 10 days stimulation of Ang II in Agtr2+ VSMC, respectively.
Figure S3 A
Effect of Ang II stimulation on MMS2 expression in VSMC performed by Western blot analysis. n=4. *P<0.05 vs. control in Agtr2+ VSMC. †P<0.01 vs. Ang II at 10 days in Agtr2+ VSMC.
Figure S3 B and C
(B) MMS2 expression in the aorta of mice exposed to X-ray irradiation determined by Western blot analysis. (C) Effect of H₂O₂ or UV irradiation on MMS2 expression in VSMC. n=4 for each. *P<0.05 vs. control in aorta or in VSMC in Agtr2⁺ mice. †P<0.01 vs. irradiation (+) in aorta or H₂O₂ or UV irradiation (+) in VSMC in Agtr2⁺ mice. #P<0.01 vs. irradiation (+) in aorta in Agtr2⁺ mice.
Figure S3 D and E
Effects of MMS2-siRNA on Ang II- (D) and H₂O₂- or UV irradiation-induced (E) 8-OHdG level in VSMC. n=4 for each. *P<0.05 vs. control in Control-siRNA-treated-Agrt2⁺ VSMC. †P<0.01 vs. H₂O₂ or UV irradiation (+) in Agrt2⁺ VSMC and Ang II, H₂O₂ or UV irradiation (+) in Control-siRNA-treated-Agrt2⁺ VSMC. #P<0.01 vs. H₂O₂ or UV irradiation (+) in MMS2-siRNA-treated-Agrt2⁻ VSMC.
**Figure S4**
Effect of MMS2-siRNA on Ang II-induced NADPH oxidase activity in VSMC. n=4. *P<0.01 vs. control in Control-siRNA-treated-VSMC. NS, no significance.