EXPERIMENTAL STUDY

Amlodipine Inhibits Vascular Cell Senescence and Protects Against Atherogenesis Through the Mechanism Independent of Calcium Channel Blockade

Hiromi Kayamori,[1] MD, Ippei Shimizu,[1,2] MD, Yohko Yoshida,[1,2] MD, Yuka Hayashi,[1] MD, Masayoshi Suda,[1] MD, Ryutaro Ikegami,[1] MD, Goro Katsuumi,[1] MD, Takayuki Wakasugi,[1] MD and Tohru Minamino,[1] MD

Summary

Vascular cells have a finite lifespan and eventually enter irreversible growth arrest called cellular senescence. We have previously suggested that vascular cell senescence contributes to the pathogenesis of human atherosclerosis. Amlodipine is a mixture of two enantiomers, one of which (S- enantiomer) has L-type channel blocking activity, while the other (R+ enantiomer) shows ~1000-fold weaker channel blocking activity than S-enantiomer and has other unknown effects. It has been reported that amlodipine inhibits the progression of atherosclerosis in humans, but the molecular mechanism of this beneficial effect remains unknown. Apolipoprotein E-deficient mice on a high-fat diet were treated with amlodipine, its R+ enantiomer or vehicle for eight weeks. Compared with vehicle treatment, both amlodipine and the R+ enantiomer significantly reduced the number of senescent vascular cells and inhibited plaque formation to a similar extent. Expression of the pro-inflammatory molecule interleukin-1β was markedly upregulated in vehicle-treated mice, but was inhibited to a similar extent by treatment with amlodipine or the R+ enantiomer. Likewise, activation of p53 (a critical inducer of senescence) was markedly suppressed by treatment with amlodipine or the R+ enantiomer. These results suggest that amlodipine inhibits vascular cell senescence and protects against atherogenesis at least partly by a mechanism that is independent of calcium channel blockade.

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Key words: Cellular senescence, p53, Calcium channel blockers

Cellular senescence was first detected as the finite replicative lifespan of human somatic cells in culture. Senescent cells enter irreversible growth arrest, exhibit a flattened and enlarged morphology, and express a different set of genes that include negative regulators of the cell cycle such as p53 and p21, as well as pro-inflammatory molecules.[1)] Because the growth potential of cultured cells correlates well with the mean maximum lifespan of the species from which the cells are derived, these phenotypic changes associated with senescence have been suggested to be involved in human aging.[1,2)] Primary cultured cells from patients with premature aging syndromes, such as Werner syndrome and Bloom syndrome, are known to have a shorter lifespan than cells from age-matched healthy persons,[3,4)] supporting the notion that cellular senescence is associated with aging and age-associated disease.

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The histology of human atherosclerotic lesions has been studied extensively, demonstrating the presence of both endothelial cells and vascular smooth muscle cells (VSMC) with the morphological features of cellular senescence.[5,6)] Vascular cells that are positive for senescence-associated β-galactosidase (SA β-gal), a biomarker of senescence, have been demonstrated in atherosclerotic plaques obtained from the coronary arteries of patients with ischemic heart disease.[7,8)] Interestingly, such SA β-gal-positive cells are not observed in the internal mammary arteries of the same patients where atherosclerotic changes are minimal.[7)] SA β-gal-positive VSMC are also detected in advanced plaque.[9)] SA β-gal-positive cells from human atheroma show increased expression of p53

From the [1]Department of Cardiovascular Biology and Medicine, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan and [2]Division of Molecular Aging and Cell Biology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan.

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Address for correspondence: Tohru Minamino, MD, Department of Cardiovascular Biology and Medicine, Niigata University Graduate School of Medical and Dental Sciences, 1-757 Asahimachidori, Chuo-ku, Niigata 951-8510, Japan. E-mail: tminamino@med.niigata-u.ac.jp or t_minamino@yahoo.co.jp

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and p21, which are markers of cellular senescence.[9] These cells also exhibit various functional abnormalities including decreased expression of eNOS and increased expression of pro-inflammatory molecules.[9] Inhibition of cellular aging signals has been shown to decrease the size of atherosclerotic plaque as well as the number of senescent vascular cells in a murine model of atherosclerosis.[10,11] Moreover, it has been shown that elimination of senescent cells could reversibly reduce atheroma formation.[12] Such findings suggest that cellular senescence occurs *in vivo* and contributes to the pathogenesis of human atherosclerosis.[13-15]

Calcium channel blockers (CCBs) were developed as vasodilators, and their use in the treatment of cardiovascular disease remains largely based on that mechanism of action. Although a number of drugs used to treat cardiovascular disease, including statins and angiotensin-converting enzyme inhibitors, have multiple well-described effects that are universally accepted as contributing to their benefit,[16,17] little attention has been paid to the potentially similar effects of CCBs. Evidence suggests that amlodipine has distinct pharmacologic actions in addition to L-type calcium channel blockade.[18] Amlodipine is a mixture of two enantiomers, among which the S-enantiomer has L-type Ca channel blocking activity. In contrast, the R+ enantiomer has a much weaker effect on the L-type Ca channel and little is known about its other actions.

In the present study, we found a novel mechanism by which amlodipine inhibits the development and progression of atherosclerosis. Compared with vehicle treatment, amlodipine and its R+ enantiomer both significantly reduced the number of senescent vascular cells and inhibited plaque formation to a similar extent, suggesting that a mechanism independent of calcium channel blockade was involved in the anti-atherogenic activity of amlodipine.

Methods

Mouse study: The animal experiments were approved by our institutional review board. *Apolipoprotein E*-deficient mice (C57BL/6 background) were obtained from the Jackson Laboratory (Bar Harbor, ME). Animals were housed under a 12-hour light/dark cycle, fed a high-fat diet from six weeks of age and simultaneously treated with vehicle, amlodipine (5 mg/kg), or its R+ enantiomer (5 mg/kg) for eight weeks. Mean blood pressure was measured using a noninvasive tail cuff system. Blood samples were obtained from the mice at the time of euthanizing. The aortas were removed after systemic perfusion with phosphate-buffered saline (PBS) for histological examination, western blotting, and RNA analysis. Amlodipine and the R+ enantiomer were kind gifts from Dainippon Sumitomo Pharma. We only used amlodipine and its R+ enantiomer for our experiments, because the S- enantiomer was not available due to the technical inability to purify this compound.

Histology: To examine cellular senescence in the aorta, samples were subjected to SA β-gal staining, immediately embedded in Optimal Cutting Temperature compound (Sakura Finetechinical, Tokyo, Japan), and snap-frozen in liquid nitrogen to prepare cryostat sections. The sections were then stained with an antibody for α-smooth muscle actin (Sigma), after which the number of cells showing double-positivity for SA β-gal activity and α-smooth muscle actin (senescent VSMC) was counted in 4-6 sections obtained from each aorta. To evaluate the extent of atherosclerosis, we measured the aortic intimal area using sections stained with hematoxylin-eosin (H&E).

Western blot analysis: Samples were prepared in lysis buffer (10 mM Tris-HCL, pH 8, 140 mM NaCl, 5 mM EDTA, 0.025% NaN_3, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 mM PMSF, 5 μg/mL leupetin, 2 μg/mL aprotinin, 50 mM NaF, and 1 mM Na_2VO_3). The lysates (30 μg) were resolved by SDS polyacrylamide gel electrophoresis. Proteins were transferred to a polyvinylidine difluoride membrane (Millipore, Bedford, MA) and incubated with the primary antibody followed by a horseradish peroxidase-conjugated anti-rabbit immunoglobulin G antibody or anti-mouse immunoglobulin G antibody (Jackson, West Grove, PA). The primary antibodies used for western blotting were anti-p21 antibody, anti-p53 antibody (Santa Cruz, Santa Cruz, CA), and anti-actin antibody (Sigma).

RNA analysis: Total RNA (5 μg) was extracted using RNA Bee (Tel Test, Friendswood, TX), according to the manufacturer's instructions. The levels of interleukin-1β were examined with a multi-probe ribonuclease protection assay system (BD Bioscience), according to the manufacturer's instructions.

Luciferase assay: Primary cultured human aortic VSMC were purchased from Cambrex (Walkersville, MD), and were grown according to the manufacturer's instructions. The reporter gene plasmid (1 μg) was transfected into VSMC in the presence or absence of hydrogen peroxide (500 μM) at 24 hours before the luciferase assay. The control vector encoding *Renilla* luciferase (0.1 μg) was co-transfected as an internal control. VSMC were treated with vehicle, amlodipine (10^{-7} M), or the R+ enantiomer (10^{-7} M) for 15 hours before the luciferase assay. In some experiments, VSMC were simultaneously treated with an NO synthase inhibitor (N-nitro-L-arginine methyl ester, 1 mM). Then the luciferase assay was carried out using a dual luciferase reporter assay system (Promega, Madison, WI), according to the manufacturer's instructions. pPG13-Luc was a gift from Dr. B Vogelstein (Johns Hopkins University, Baltimore, MD).

Statistical analysis: Data are shown as the mean±SEM. Differences between groups were examined by Student's *t*-test or analysis of variance, followed by Bonferroni's correction for comparison of means. For all analyses, $P < 0.05$ was considered statistically significant.

Results

Effect of amlodipine and its R+ enantiomer on atherogenesis: To investigate whether actions distinct from the L-type calcium channel blockade were involved in the anti-atherogenic activity of amlodipine, we fed a high-fat diet to apolipoprotein E (apoE)-deficient mice and treated these animals with amlodipine or its R+ enantiomer for eight weeks. A low dose was used to avoid the influence of reduced blood pressure on atherogenesis. We measured mean blood pressure after four and eight weeks of treat-

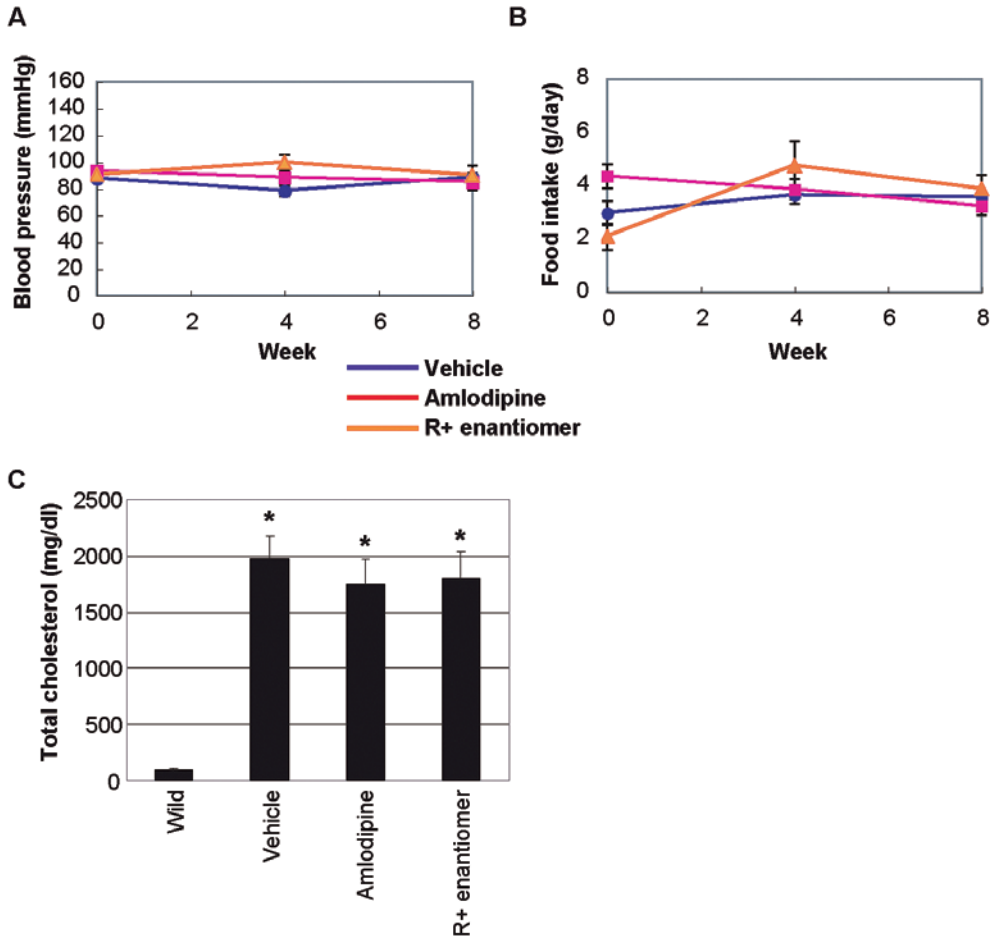

Figure 1. Effects of amlodipine and R+ enantiomer on physiological parameters. ApoE-deficient mice on a high-fat diet were treated with vehicle, amlodipine, or R+ enantiomer for 8 weeks. Blood pressure (**A**), food intake (**B**), and total cholesterol levels (**C**) were examined. *$P < 0.05$ versus wild-type (WT) mice ($n = 5$)

ment and confirmed that neither amlodipine nor its R+ enantiomer affected blood pressure at the doses used (Figure 1A). We also examined food intake during treatment and found that it was unaffected (Figure 1B). Serum total cholesterol levels were significantly higher in apoE-deficient mice receiving a high-fat diet than in wild-type (WT) mice on a normal diet, but treatment with either amlodipine or the R+ enantiomer did not cause a significant change of total cholesterol (Figure 1C).

We next examined aortic intimal formation after eight weeks. ApoE-deficient mice on a high-fat diet with vehicle treatment exhibited extensive intimal formation (Figure 2). In contrast, formation of aortic intima was significantly reduced by treatment with either amlodipine or the R+ enantiomer (Figure 2). The extent of reduction in the intimal area was similar in the latter two groups, suggesting that an effect independent of calcium channel blockade may be crucial for the anti-atherogenic activity of amlodipine.

Effect of amlodipine and the R+ enantiomer on vascular cell senescence: Vascular cells have a finite lifespan and eventually undergo senescence. The state of cellular senescence is accompanied by various changes of morphology, gene expression, and function.[1)] A number of studies have shown that many of the changes occurring in senescent vascular cells are consistent with changes seen in age-related vascular disease, such as decreased production of nitric oxide (NO), suggesting that cellular senescence has a role in vascular aging, including atherosclerosis.[13,19,20)] Therefore, we examined the effects of amlodipine and the R+ enantiomer on vascular cell senescence. About 30% of vascular cells in the intima and media were SA-β gal-positive in apoE-deficient mice on a high-fat diet treated with the vehicle (Figure 3A), whereas few positive cells were detected in the aortas of WT mice on a normal diet (data not shown). The number of SA-β gal-positive vascular cells was significantly decreased in apoE-deficient mice treated with either amlodipine or the R+ enantiomer when compared with the vehicle-treated group (Figure 3A). The inhibitory effect of the R+ enantiomer on cellular senescence was similar to that of amlodipine, suggesting that this enantiomer has anti-senescence activity independent of calcium channel blockade.

This notion is further supported by data on the expression of negative regulators of the cell cycle such as p53 and p21. ApoE-deficient mice treated with the vehicle

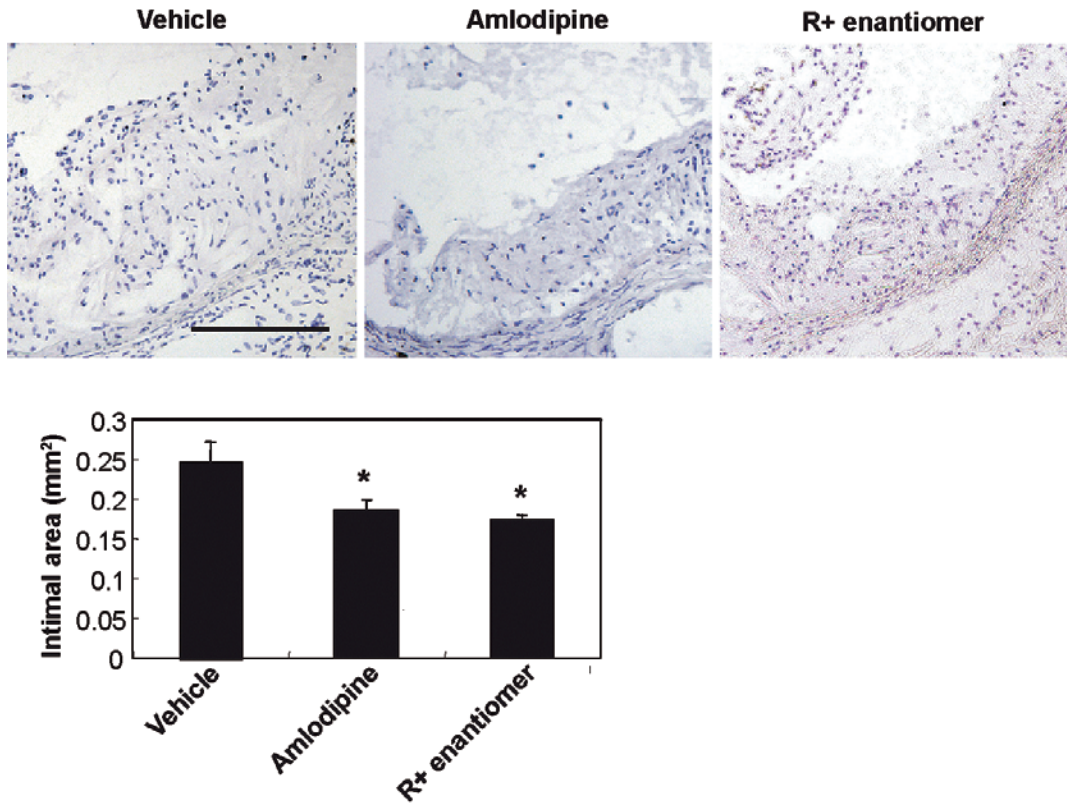

Figure 2. Effects of amlodipine and R+ enantiomer on atherogenesis. The mice prepared in Figure 1 were sacrificed at eight weeks after treatment, and the aortas were removed after systemic perfusion with phosphate-buffered saline (PBS) for histological examination. Scale bar: 500 μm. The graph indicates aortic intimal area. $*P < 0.05$ versus vehicle-treated mice ($n = 5$).

showed an increase in the aortic expression of these regulators compared with WT mice (Figure 3B and data not shown), while this increase was significantly and equally suppressed by treatment with amlodipine or the R+ enantiomer (Figure 3B). Since cellular senescence is associated with upregulation of pro-inflammatory molecules,[21] we examined the aortic expression of interleukin (IL)-1β, a pro-inflammatory cytokine that contributes to atherogenesis.[22] Consistent with previous reports, expression of IL-1β was markedly upregulated in apoE-deficient mice treated with the vehicle (Figure 3C), while such upregulation was significantly inhibited by treatment with either amlodipine or the R+ enantiomer (Figure 3C).

Potential anti-senescence mechanism of amlodipine: There is evidence that amlodipine has non-calcium related actions, such as stimulation of NO production and protection against oxidative stress[23,24]. It has been reported that chronic oxidative stress induces cellular senescence via the p53-dependent pathway and contributes to the development of atherosclerosis.[13,25] To investigate the possible mechanism underlying the anti-senescence activity of amlodipine, we treated VSMC with hydrogen peroxide to induce senescence and examined the effect of an NO synthase inhibitor (N-nitro-L-arginine methyl ester, L-NAME) on inhibition of cellular senescence by amlodipine. As a result, p53 transcriptional activity measured by the luciferase assay was markedly increased after treatment with hydrogen peroxide (Figure 4), while treatment with amlodipine or its R+ enantiomer significantly suppressed this increase to a similar extent (Figure 4). When NO production was inhibited, hydrogen peroxide-induced p53 activation was enhanced and the beneficial effect of these agents was abolished (Figure 4). Since NO is also known to reduce oxidative stress and inhibit cellular senescence,[26] these results suggested that the anti-senescence activity of amlodipine is attributable to its ability to stimulate NO production as well as to protect against oxidative stress.

Discussion

A number of clinical trials have shown that treatment with amlodipine significantly inhibits the progression of atherogenesis and reduces cardiovascular events. In the CAMLOT study,[27] patients with coronary artery disease and normal blood pressure were randomized to receive treatment with amlodipine, enarapril, or a placebo. Administration of amlodipine led to a decrease of adverse cardiovascular events, while a similar (but smaller and nonsignificant) effect was observed with enalapril. In subjects receiving amlodipine, intravascular ultrasound showed slowing of the progression of atherosclerosis. In the ASCOT-BPLA study,[28] patients with hypertension who had at least three other cardiovascular risk factors were assigned to either an amlodipine-based regimen or an atenolol-based regimen, and the amlodipine-based regimen

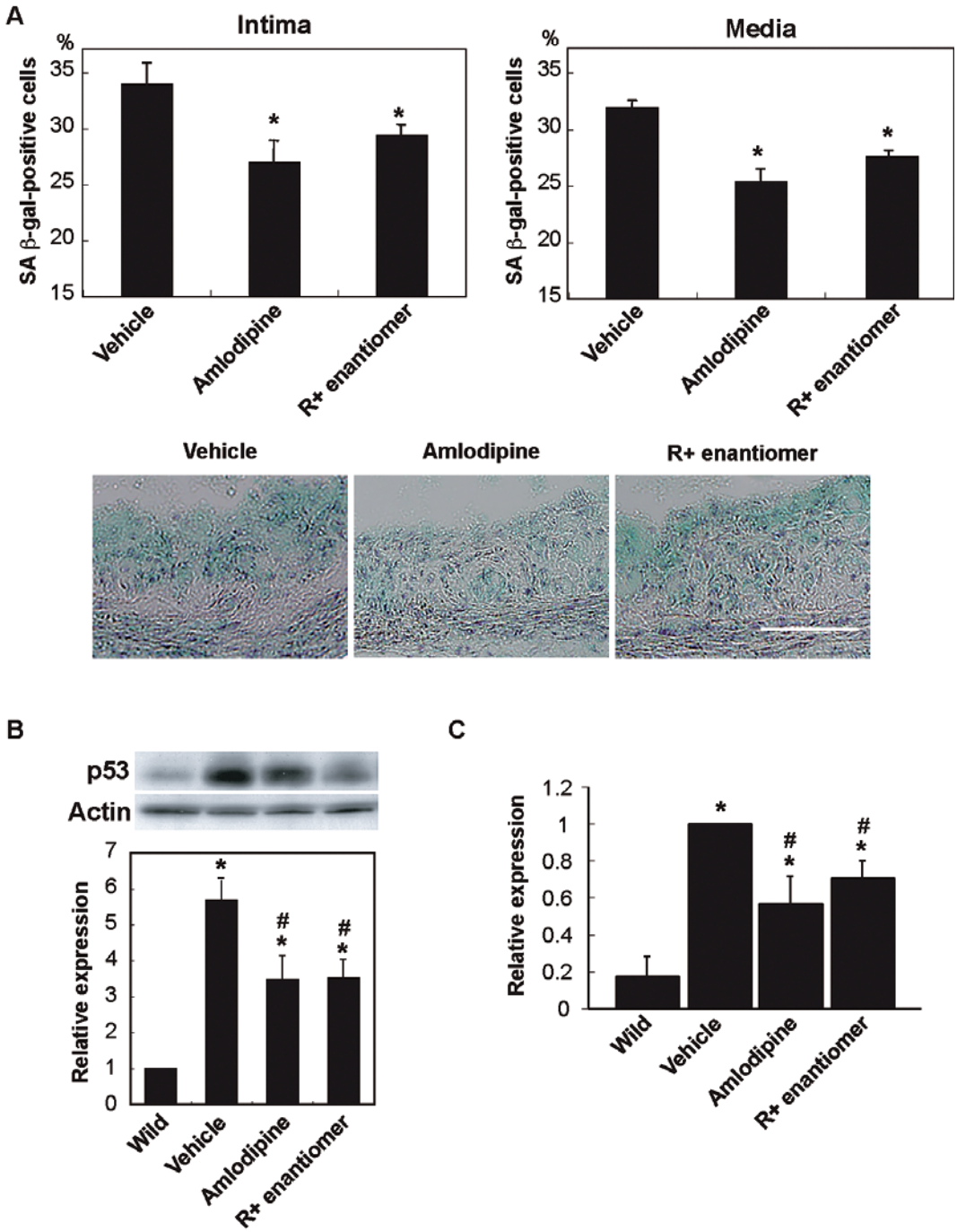

Figure 3. Effects of amlodipine and R+ enantiomer on senescence. **A:** The mice prepared in Figure 1 were sacrificed at eight weeks after treatment, and the aortas were removed after systemic perfusion with phosphate-buffered saline (PBS) for SA β-gal staining. The number of SA β-gal-positive cells was estimated. Scale bar: 100 μm. *$P < 0.05$ versus vehicle-treated mice ($n = 5$). **B:** The protein samples were extracted from the aortas prepared in Figure 3A and analyzed for p53 expression by western blotting. *$P < 0.05$ versus WT mice; #$P < 0.05$ versus vehicle-treated mice ($n = 5$). **C:** The RNA samples were extracted from the aortas prepared in Figure 3A and analyzed for IL-1β expression by a ribonuclease protection assay. *$P < 0.05$ versus WT mice; #$P < 0.05$ versus vehicle-treated mice ($n = 5$).

prevented major cardiovascular events more effectively. These effects of amlodipine might not be entirely explained by better control of blood pressure. It has also been reported that treatment of cardiovascular disease with amlodipine is as effective as treatment with other types of antihypertensive agents, such as angiotensin-converting enzyme inhibitors and angiotensin II receptor antagonists, which are well known to have additional non-blood

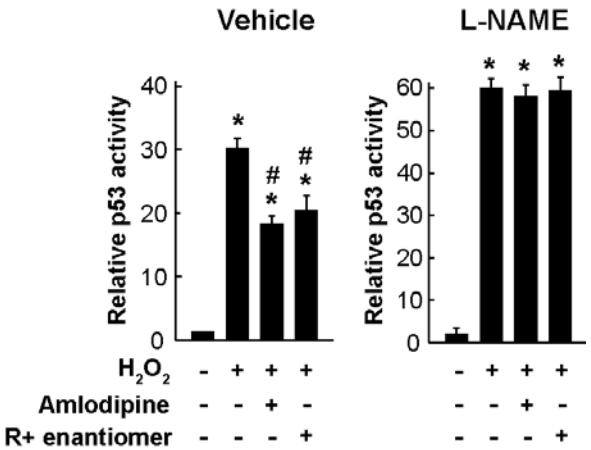

Figure 4. Effects of amlodipine and R+ enantiomer on p53 activity. The reporter gene plasmid (1 μg) was transfected into VSMC in the presence or absence of hydrogen peroxide (500 μg) at 24 hours before the luciferase assay. The control vector encoding *Renilla* luciferase (0.1 μg) was co-transfected as an internal control. VSMC were treated with vehicle, amlodipine (10^{-7} M), or R+ enantiomer (10^{-7} M) at 15 hours before the luciferase assay. VSMC were simultaneously treated with vehicle or an NO synthase inhibitor (L-NAME, 1 mM). Then the luciferase assay was carried out. *$P < 0.05$ versus the vehicle-treated group; #$P < 0.05$ versus the H_2O_2-treated group ($n = 6$).

pressure-related effects. The results of these clinical trials indicate that a mechanism independent of calcium channel blockade may be involved in the anti-atherogenic activity of amlodipine.

Our results suggest that other actions of amlodipine, besides a decrease of blood pressure, contribute to inhibiting the development of atherosclerosis, because the R+ enantiomer and amlodipine both markedly suppressed atherogenesis without any significant change of blood pressure or total cholesterol. Moreover, the R+ enantiomer inhibited vascular cell senescence through its ability to increase NO production and prevent oxidative damage, and this anti-senescence activity may represent another effect of amlodipine. The effects of the R+ enantiomer on NO production could be mediated by the angiotensin II type 2 receptor (AT2)-dependent pathway because it has been reported that the ability of the R+ enantiomer to release NO is inhibited by the AT2 blocker,[18] but in order to clarify underlying molecular mechanisms, further studies would be required. It is also possible that amlodipine exerts the anti-senescence property by modulating glucose metabolism, which was not addressed in the present study. Better understanding these effects will contribute to elucidating disease mechanisms and the rationale for amlodipine therapy. Such knowledge will help us to clarify the disease states for which amlodipine is most useful.

Disclosures

Conflicts of interest: All authors except for T.M. declare no conflicts of interest. T.M. discloses lecture fees from Amgen Astellas BioPharma K.K., Sanofi K.K., Nippon Boehringer Ingelheim, Co., Ltd., Mitsubishi Tanabe Pharma Corporation, MSD, Bayer Yakuhin Ltd., Daiichi Sankyo Co., Ltd., Takeda Pharmaceutical Co., Ltd. and research funds form Boehringer Ingelheim, Co., Ltd., Mitsubishi Tanabe Pharma Corporation, Astellas Pharma Inc., Daiichi Sankyo Co., Ltd., Pfizer Japan Inc., Bayer Yakuhin Ltd., Takeda Pharmaceutical Co., Ltd., Bristol-Myers Squibb, Novartis Pharma K.K., and AstraZeneca K. K. All the funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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