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Asymmetric dimethylarginine (ADMA) accelerates cell senescence

Stefanie M Bode-Böger, Fortunato Scalera and Jens Martens-Lobenhoffer

Abstract: Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide synthase and its accumulation has been associated with cardiovascular disease. We aimed to investigate the role of ADMA in endothelial cell senescence. Endothelial cells were cultured until the tenth passage. ADMA was replaced every 48 hours starting at the fourth passage. ADMA significantly accelerated senescence-associated β -galactosidase activity. Additionally, the shortening of telomere length was significantly speeded up and telomerase activity was significantly reduced. This effect was associated with an increase of oxidative stress: both allantoin, a marker of oxygen free radical generation, and intracellular reactive oxygen species increased significantly after ADMA treatment compared with control, whereas nitric oxide synthesis decreased. Furthermore, ADMA-increased oxidative stress was accompanied by a decrease in the activity of dimethylarginine dimethylaminohydrolase, the enzyme that degrades ADMA, which could be prevented by the antioxidant pyrrolidine dithiocarbamate. Exogenous ADMA also stimulated secretion of monocyte chemotactic protein-1 and interleukin-8. Co-incubation with the methyltransferase inhibitor *S*-adenosylhomocysteine abolished the effects of ADMA. These data suggest that ADMA accelerates senescence, probably via increased oxygen radical formation by inhibiting nitric oxide elaboration. This study provides evidence that modest changes of intracellular ADMA levels are associated with significant effects on slowing down endothelial senescence.

Key words: asymmetric dimethylarginine; DDAH; oxidative stress; senescence; telomerase activity

Introduction

The incidence of atherosclerosis and cardiovascular disease increases dramatically with age. The links between aging and atherosclerosis are not well established. Common pathways at the cellular level have been proposed for aging and atherosclerosis.^{1,2} There is accumulating evidence that telomeres and telomerase play a role in cellular senescence. Telomeres, the physical ends of the chromosomes, are involved in the control of chromosome stability, genetic integrity and cell viability.³ Primary mammalian cells have a finite life span in tissue culture. Two sources of signals have been identified to cause the induction of senescence in cultured cells. Intrinsic signals can be induced by a critical telomere shortening, which is a consequence

of telomere length reduction during each cell division ('mitotic clock').⁴ Cell culturing can induce stress signals that result in the expression of different cell cycle inhibitors, leading to a senescent phenotype. Furthermore, a disturbance of the telomere-associated proteins can cause induction of senescence. Telomere shortening forces human primary cells, including endothelial cells, to stop dividing when a critical minimum telomere length is reached.⁵ The enzyme telomerase, a ribonucleoprotein, counteracts the shortening of telomeres. Recently, it was reported that there is a higher rate of age-dependent telomere attrition in both the intima and the media of the distal compared with the proximal human abdominal aorta, accompanied by a negative correlation between telomere length and atherosclerotic grade.⁶ These links underscore the need for biological indicators of aging in evaluating the etiology of these age-related disorders. A novel risk factor for cardiovascular disease is asymmetric dimethylarginine (ADMA),⁷ an endogenous inhibitor of nitric oxide synthase (NOS). Elevations in plasma ADMA may contribute to the vascular pathophysiology observed in atherosclerosis, hypertension, hypercholesteremia and renal failure. Increased oxidative

Institute of Clinical Pharmacology, University Hospital Otto-von-Guericke University, Magdeburg, Germany

Address for correspondence: Professor Stefanie M Bode-Böger, Institute of Clinical Pharmacology, University Hospital, Otto-von-Guericke University, Leipziger Straße 44, D-39120 Magdeburg, Germany. Tel: +49 391 6713060; Fax: +49 391 6713062; E-mail: stefanie.bode-boeger@medizin.uni-magdeburg.de

stress seems to play an important role in the pathogenesis of these clinical conditions. Moreover, ADMA appears to be an independent predictor of cardiovascular mortality.⁸ Recent findings suggest a significant positive correlation between age and plasma ADMA levels.^{8,9} We have previously demonstrated that ADMA concentration dependently increased oxidative stress measured as superoxide production by cultured human endothelial cells.¹⁰

It has been reported that cellular senescence, the limited ability of primary human cells to divide when cultured *in vitro*, is accelerated by increased oxidative stress¹¹ and is reduced by nitric oxide (NO) donor treatment.²

In the present study we therefore investigated if ADMA, a novel cardiovascular risk factor, influences cell senescence in cell culture and increases oxidative stress.

Materials and methods

Cell culture

Human umbilical vein endothelial cells (HUVECs, Clonetics, Germany) were cultured in endothelial basal medium until the tenth passage. ADMA, *S*-adenosylhomocysteine (SAH) and pyrrolidine dithiocarbamate (PDTIC, Sigma, Germany) were replaced every 48 hours starting at the fourth passage. After reaching confluence (between 8 and 9 days) endothelial cells were trypsinized and seeded at a density of 2500 cells/cm² using 75 cm² flasks or six-well plates. Population doubling (PD) was calculated at each passage until growth arrest by using the following formula: $PD = (\log_{10} Y - \log_{10} X) / \log_{10} 2$ (*Y* indicates the number of cells counted at the end of the passage; *X* is the number of cells seeded). Cumulative population doubling (CPD) was calculated as the sum of all the changes in PD. The endothelial cells were counted and the vitality was determined. Viability after trypsinization was usually >95%. HUVECs were characterized at passage 10 by specific staining for CD31 (PECAM-1) using the fluorescence-activated flow cytometry assay (FACS). After detachment with trypsin telomerase activity, telomere length, FACS analyses or dimethylarginine dimethylaminohydrolase (DDAH) activity was performed.

Staining for senescence-associated β -galactosidase activity

HUVECs were fixed and stained for senescence-associated β -galactosidase (SA- β -gal) activity according to the procedure developed by Dimri et al.¹² The percentage of SA- β -gal positive cells was determined by counting the number of blue cells in 1000 cells.

Telomerase assay and telomere length

Quantitative determination of telomerase activity was performed according to the manufacturer's protocol of

the TeloTAGGG Telomerase PCR ELISA^{PLUS} Kit (Roche Diagnostic GmbH, Mannheim, Germany), which is based on the telomeric repeat amplification protocol assay. Telomerase activity was measured by the polymerase chain reaction using 1.5 μ g protein. Telomere length was analysed by the terminal restriction fragment length assay using a Telo TAGGG Telomere Length Assay Kit (Roche).

Measurement of nitrate and nitrite

The determination of nitrate and nitrite in cell culture supernatants was carried out in accordance with the method described by Tsikas et al.¹³

DDAH activity assay

DDAH activity in endothelial cells was estimated directly by measuring the amount of ADMA metabolized by the enzyme as described by Lin et al.¹⁴ The ADMA level in each group was measured by high-performance liquid chromatography-mass spectrometry as previously described by our group.¹⁵

Detection of oxidative stress

For the determination of allantoin (a marker of oxygen free radical generation) in cell culture supernatants we adopted the method for human plasma described by Doehner et al.¹⁶

Dihydrorhodamine 123 (DHR) was used as a marker for intracellular reactive oxygen species (ROS). The cells were incubated with gentle agitation for 20 minutes at 37°C in the presence of 10 μ M DHR123. The reaction was stopped by cooling on dry-ice for 1 minute and the subsequent addition of 500 μ l phosphate buffered solution followed by two washing steps. After final fixation with 1% paraformaldehyde the cells were analyzed by flow cytometry (Epics XL-MCL, Coulter). The level of ROS was indicated by mean fluorescence intensities (mfi) of stained probes versus negative controls.

Measurement of monocyte chemotactic protein-1 and interleukin-8

For the quantitative determination of monocyte chemotactic protein-1 (MCP-1) and interleukin (IL)-8 the cell supernatants were collected, centrifuged and stored at -20°C until assayed. The MCP-1 and IL-8 protein concentrations were assayed using the Quantikine human MCP-1 and IL-8 immunoassay (R&D Systems, Wiesbaden, Germany).

Statistical methods

All data were expressed as the mean \pm standard error of the mean (SEM) from at least three independent experiments. Statistical significance was tested with repeated analysis of variance (ANOVA) using a least significant difference post-hoc test or ANOVA for multiple comparisons (SPSS Software 11.0). Differences were considered significant at $p < 0.05$.

Results

ADMA accelerates endothelial senescence by inhibiting telomerase activity

To investigate the link between endothelial cell senescence and ADMA, human endothelial cells were cultured until CPD 40 and incubated in the presence of different concentrations of ADMA, which was replaced every 48 hours starting from CPD 20. All concentrations of ADMA (5, 50 and 100 $\mu\text{mol/l}$) significantly increased SA- β -gal activity compared with untreated cells in a dose-dependent manner (Figure 1A).

To examine the effect of ADMA on shortening of telomere length, genomic DNA was isolated and separated, and telomere restriction fragment length was measured. The telomere length was shortened with increasing passage in control cells (Figure 1B). The repeated addition of different concentrations of ADMA increased significantly the telomere shortening compared with control cells in a dose-dependent manner.

Because the synthesis of telomeres is associated with the enzymatic activity of an RNA-protein complex called telomerase, we measured the relative activity of telomerase in HUVECs. Telomerase activity decreased as a function of passage in control cells and was significantly further reduced in a dose-dependent manner in the ADMA treated cells (Figure 1C).

SAH-inhibited ADMA-accelerated senescence

To test whether SAH could attenuate the effect of ADMA (50 $\mu\text{mol/l}$) on cellular senescence, HUVECs were incubated in the presence of SAH (100 $\mu\text{mol/l}$) starting from CPD 20. It is interesting that a more significant decrease in SA- β -gal activity was observed in endothelial cells treated with SAH compared with the control cells (Figure 2A), with a maximal effect at the tenth passage (CPD 40). Additionally, the shortening of telomere length was substantially delayed and the telomerase activity was significantly increased (Figures 2B and 2C). The increased senescence in the presence of ADMA was completely reversed in the presence of SAH. Exposure of endothelial cells to ADMA resulted in a significant reduction of NO synthesis (CPD 40: ADMA, 2.59 ± 0.4 nmol/mg protein versus control, 4.18 ± 0.3 nmol/mg protein; $p < 0.05$ versus control). After SAH treatment, NO levels were increased significantly compared with control cells (CPD 40: SAH, 5.44 ± 0.3 nmol/mg protein; $p < 0.05$ versus control). The inhibitory effects of ADMA on endothelial NO release were abolished by SAH (CPD 40: SAH + ADMA, 4.03 ± 0.7 nmol/mg protein; $p < 0.05$ versus ADMA). Incubation of endothelial cells with ADMA led to a significant increase in allantoin levels compared with control (CPD 40: ADMA, 41.2 ± 1.8 nmol/mg protein versus control, 30.3 ± 1.6 nmol/mg protein; $p < 0.05$ versus

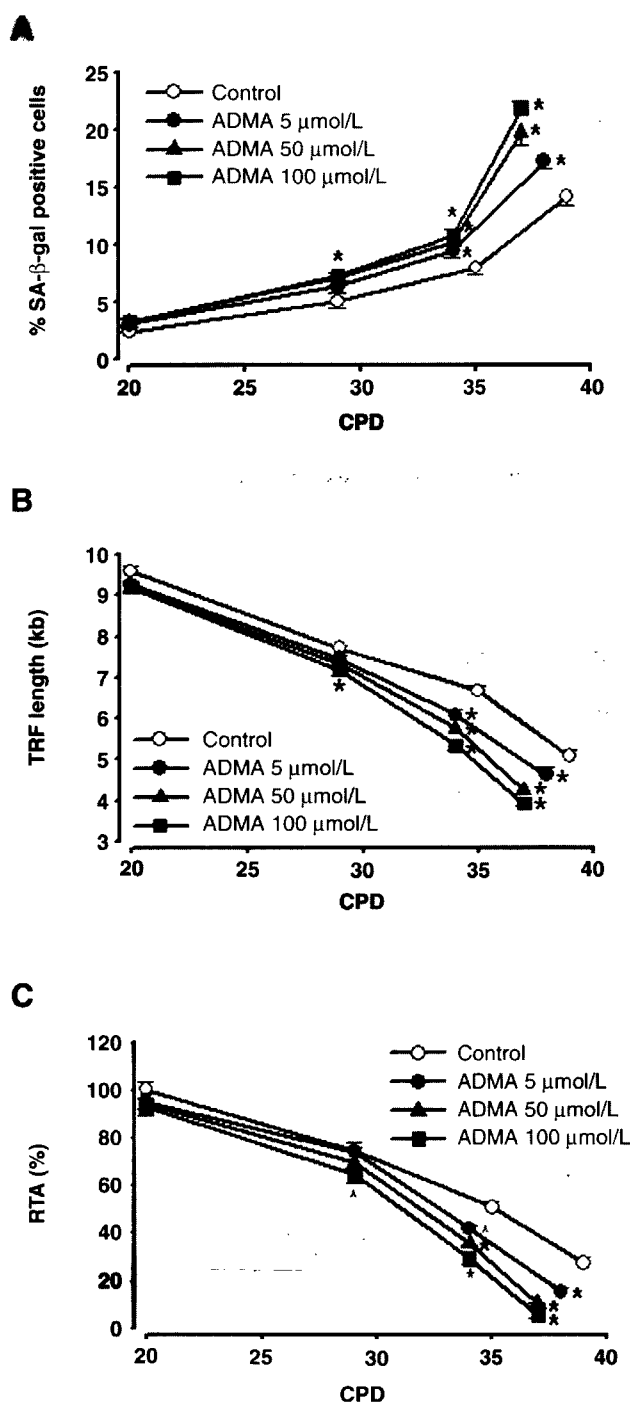


Figure 1 Effect of different concentrations of ADMA on senescence, telomere length and telomerase activity of endothelial cells, which were incubated with ADMA (5, 50 and 100 $\mu\text{mol/L}$) starting from cumulative population doubling (CPD) 20 and replaced every 48 hours (A through C): (A) endothelial senescence was detected by senescence associated β -galactosidase (SA- β -gal) positive cells; (B) terminal restriction fragment (TRF) lengths are plotted as a function of passage number; (C) relative telomerase activity (RTA). Each point represents the mean \pm SEM of results from three experiments. * $p < 0.05$ vs corresponding CPD of control cells. (Figure 1A, B and C reproduced by permission of the publisher Lippincott Williams & Wilkins from: Scalera F, Borlak J, Beckmann B et al. Endogenous nitric oxide synthesis inhibitor asymmetric dimethyl L-arginine accelerates endothelial cell senescence. *Arterioscler Thromb Vasc Biol* 2004; **24**: 1816–22.¹⁷)

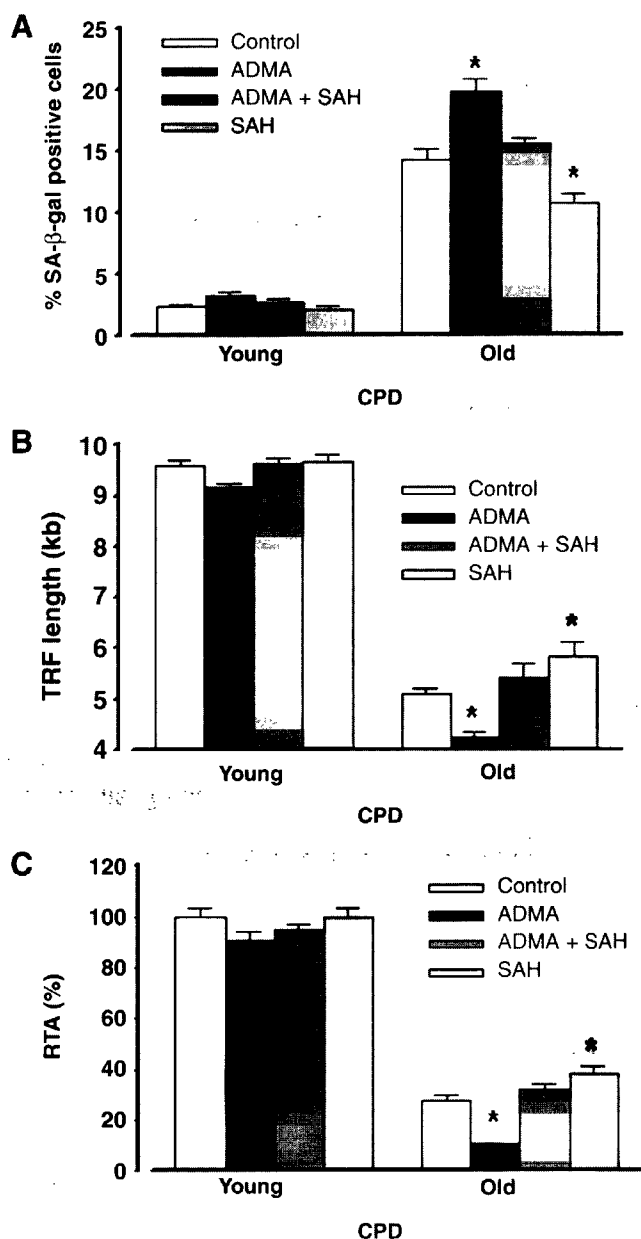


Figure 2 Effect of SAH on ADMA-accelerated senescence. Endothelial cells were incubated with ADMA (50 $\mu\text{mol/L}$) and SAH (100 $\mu\text{mol/L}$) starting from the fourth passage (young) and replaced every 48 hours until the tenth passage (old): (A) endothelial senescence was detected by senescence-associated β -galactosidase (SA- β -gal) positive cells; (B) terminal restriction fragment (TRF) lengths are plotted as a function of passage number; (C) relative telomerase activity (RTA). Each point represents the mean \pm SEM of results from three experiments. * $p < 0.05$ vs corresponding cumulative population doubling (CPD) of control cells.

control). Incubation with SAH significantly decreased allantoin formation. The stimulatory effects of ADMA on allantoin production were reversed by co-incubating the cells with SAH.

ADMA-induced senescence was associated with an increase in oxidative stress and a decrease in DDAH enzyme activity

Endogenous ROS formation was measured by detection of DHR during endothelial cell aging. The intracellular level of ROS was increased in control cells, starting at CPD 29 (Figure 3A). ADMA (5 $\mu\text{mol/L}$) augmented ROS formation ($p < 0.05$). The antioxidant PDTC (10 $\mu\text{mol/L}$) prevented an increase endogenous oxidative stress by ADMA.

To examine whether ADMA-induced oxidative stress is due to reduced degradation of ADMA by DDAH, DDAH activity was determined during continuous passaging of endothelial cells by assessing the rate of degradation of exogenous ADMA added to the

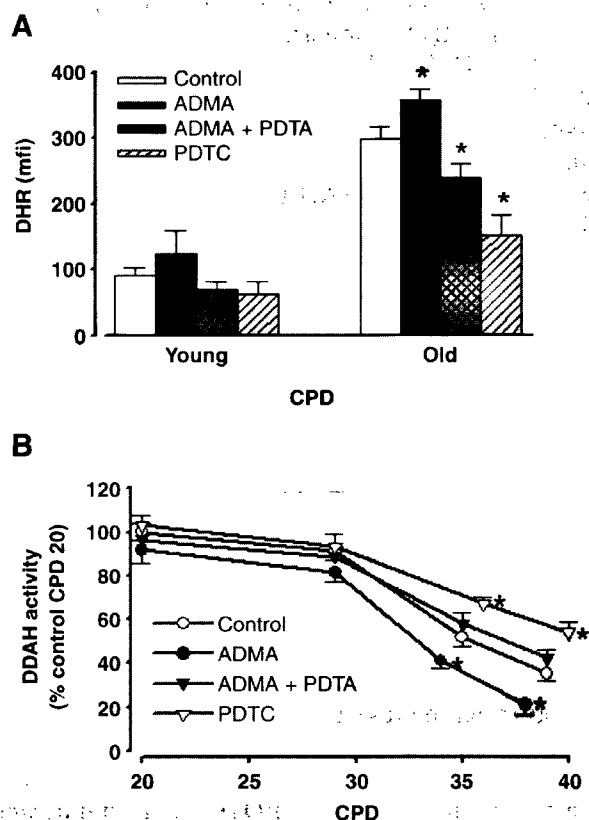


Figure 3 ADMA-inhibited NO synthesis was associated with an increase in endogenous oxidative stress and a decrease in endothelial DDAH enzyme activity. Endothelial cells were incubated with ADMA (5 $\mu\text{mol/L}$) and pyrrolidine dithiocarbamate (PDTC) (10 $\mu\text{mol/L}$) starting from the fourth passage (young) and added every 48 hours until the tenth passage (old): (A) endogenous reactive oxygen species formation was measured using the marker dihydrorhodamine (DHR) and the fluorescence-activated flow cytometry assay; (B) effect of ADMA and PDTC on endothelial DDAH activity. Each point represents the mean \pm SEM of results from three experiments. * $p < 0.05$ vs corresponding cumulative population doubling (CPD) of control cells. (Figure 3B reproduced by permission of the publisher Lippincott Williams & Wilkins from: Scalera F, Borlak J, Beckmann B et al. Endogenous nitric oxide synthesis inhibitor asymmetric dimethyl L-arginine accelerates endothelial cell senescence. *Arterioscler Thromb Vasc Biol* 2004; **24**: 1816–22.¹⁷)

cell lysates. Concomitantly with the significant increase in ROS formation, DDAH activity significantly decreased at CPD 29 in control cells (Figure 3B). The treatment of endothelial cells with ADMA reduced DDAH enzyme activity in cell lysates compared with untreated cells ($p < 0.05$). PDTC enhanced DDAH activity and prevented the impairment of DDAH activity by ADMA.

Furthermore, the effect of ADMA to inhibit NO synthesis was reversed by PDTC (data not shown). Moreover, PDTC abolished the effect of ADMA on allantoin production and endothelial senescence (data not shown).

MCP-1 and IL-8 secretion increased during ADMA-induced endothelial cell aging

The treatment of cultured endothelial cells with ADMA (50 $\mu\text{mol/l}$) induced a significant secretion of MCP-1 and IL-8 compared with control (MCP-1: CPD 40 ADMA, 3073 ± 67 pg/ml versus control, 2683 ± 51 pg/ml; IL-8: ADMA, 2461 ± 60 pg/ml versus control, 1968 ± 30 pg/ml; $p < 0.05$ versus control, respectively). When cultured endothelial cells were incubated with SAH (100 $\mu\text{mol/l}$), secretion of MCP-1 and IL-8 was significantly reduced compared with untreated cells (MCP-1: CPD 40 SAH, 2407 ± 67 pg/ml; IL-8: SAH, 1753 ± 58 pg/ml; $p < 0.05$ versus control, respectively). The stimulatory effects of ADMA on MCP-1 and IL-8 secretion were significantly diminished by SAH (MCP-1: SAH + ADMA, 2663 ± 52 pg/ml; IL-8: SAH + ADMA, 1849 ± 36 pg/ml; $p < 0.05$ versus ADMA, respectively).

Discussion

ADMA accelerates endothelial aging, as demonstrated by a more rapid reduction in telomere length and a decline in telomerase activity. The effect of ADMA on accelerating endothelial senescence seems to be due to increased oxidative stress because it can be reversed by the antioxidant PDTC. SAH significantly attenuates endothelial senescence, indicating that modest changes of intracellular ADMA levels are associated with substantial effects on endothelial cell senescence.

These data provide the first evidence for a novel mechanism of endothelial cell senescence mediated by ADMA.

What is the underlying mechanism by which ADMA accelerates cellular senescence? We speculate that ADMA increases oxidative stress, for example, by uncoupling NOS activity. Uncoupling of NOS catalytic activity is observed in the presence of N^G -monomethyl-L-arginine (L-NMMA),¹⁸ an endogenous inhibitor of NOS. In this situation optimal electron flow within the two catalytic domains of NOS is

impaired and molecular oxygen becomes the sole electron acceptor. This means that NOS generates superoxide radicals. We have shown that ADMA concentration dependently increases superoxide production by cultured human endothelial cells.¹⁰ In the experiments presented here, the inhibition of NOS by ADMA decreases the elaboration of NO and increases endothelial oxidative stress as measured by intracellular ROS and allantoin production.

Telomere length is an important marker for cellular aging. Our data suggest that replicative senescence can be modulated by various intracellular processes. Endothelial cells are capable of synthesizing ADMA by the protein arginine methyltransferase type one. A major source of methyl groups used for various methylating reactions is *S*-adenosylmethionine. This methylation reaction is inhibited by SAH.¹⁹ We found that SAH attenuates endothelial senescence by increasing the activity of telomerase compared with control cells. The increased rate of endothelial senescence in the presence of ADMA was completely reversed in the presence of SAH.

The issue to be addressed is why the administration of exogenous SAH was seen to inhibit the effect of exogenous ADMA to produce its biological effects. However, it is hard to understand how an SAH-induced reduction of endogenous ADMA formation could fully counteract high concentrations of exogenous ADMA (50 μM). A possible explanation would be the activation of DDAH, the enzyme that degrades ADMA.²⁰ DDAH has a K_m (Michaelis–Menten constant) for ADMA of about 180 μM , so increased DDAH activity could overcome supraphysiological ADMA concentrations. This enzyme seems to be exquisitely sensitive to oxidative^{14,21} and nitrosative stress.²² We observed a decrease in the formation of allantoin, reflecting the reduced generation of superoxide anions. As recently shown by Cooke's group, even modest changes in ADMA levels induced by DDAH activity can have significant effects on NO synthesis.²³ We were able to show in this study that incubation with SAH significantly increases the synthesis of NO.

This finding may indicate that intracellular levels of ADMA are more important than extracellular or systemic levels and/or that there may be compartmentalization of intracellular ADMA, with much higher levels in the vicinity of NOS.

Moreover, we observed in this investigation that ADMA-increased intracellular ROS formation was accompanied by a corresponding decrease in the activity of DDAH. This finding is supported by the observation that co-incubation with PDTC, a thiol antioxidant that protects sulfhydryl groups, reduced ROS formation and consequently the impairment of DDAH activity during ADMA-accelerated senescence. Recently, Haendler et al reported that the onset of replicative senescence of endothelial cells is regu-

lated by ROS formation.²⁴ Similarly, in the present study, we found that ADMA increased oxidative stress and accelerated the rate of cellular senescence. This effect of ADMA was reversed by the antioxidant PDTTC. Thus, a reduction in ADMA synthesis induced by SAH and increased DDAH activity induced by PDTTC both delayed the onset of cellular senescence.

We also investigated the effects of ADMA on the secretion of MCP-1 and IL-8 in endothelial cell supernatants. MCP-1 and IL-8 are major chemokines for leukocyte trafficking and have been found to be highly expressed in lesions of human atherosclerosis.²⁵ Upregulation of these proteins is induced by intracellular oxidative stress via the oxidant-responsive transcription factor NF- κ B.^{10,26} Indeed, we found that reduced NO production by ADMA was associated with increased oxidative stress and increased protein secretion of MCP-1 and IL-8. These effects were completely reversed by SAH.

Clinical relevance

Plasma ADMA levels are positively correlated with risk factors for atherosclerosis. Miyazaki et al reported a positive correlation for ADMA with age in individuals with no symptoms of coronary or peripheral artery disease.⁷ Celermajer et al reported that aging is associated with progressive endothelial dysfunction in normal humans.²⁷ Furthermore, endothelial senescence is associated with decreases in eNOS expression and activity, as well as with augmented endothelial adhesiveness for monocytes. These effects can be prevented by stably transfecting endothelial cells with human telomerase.²⁸ We speculate that high plasma levels of ADMA may accelerate the loss of telomerase activity, increase cellular senescence and induce endothelial dysfunction. Our data provide a new mechanism to explain the association of plasma ADMA or endothelial dysfunction with an increased risk of cardiovascular events.

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