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Endogenous Nitric Oxide Synthesis Inhibitor Asymmetric Dimethyl L-Arginine Accelerates Endothelial Cell Senescence

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Objectives—Asymmetrical dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide synthase (NOS), and its accumulation has been associated with cardiovascular disease. We aimed to investigate the role of ADMA in endothelial cell senescence.

Methods and Results—Endothelial cells were cultured until the tenth passage. ADMA was replaced every 48 hours starting at the fourth passage. ADMA significantly accelerated senescence associated with β-galactosidase activity. Additionally, the shortening of telomere length was significantly accelerated and the telomerase activity was significantly reduced. This effect was associated with an increase of oxidative stress: allantoin, a marker of oxygen free radical generation, and intracellular reactive oxygen species (ROS) increased significantly after ADMA treatment compared with control, whereas cellular thiol status and NOx synthesis decreased. Furthermore, ADMA-increased oxidative stress was accompanied by a decrease in the activity of dimethylarginine dimethylaminohydrolase (DDAH), the enzyme that degrades ADMA, which could be prevented by the antioxidant pyrrolidine dithiocarbamate. Exogenous ADMA also stimulated secretion of MCP-1 and interleukin-8. Coincubation with the methyltransferase inhibitor S-adenosylhomocysteine abolished the effects of ADMA.

Conclusions—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 endothelial senescence. (Arterioscler Thromb Vasc Biol. 2004; 24:1816-1822.)

Key Words: asymmetrical dimethylarginine ■ senescence ■ oxidative stress ■ telomerase activity ■ DDAH

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 These links underscore the need for biological indicators of aging in evaluating the cause of these age-related disorders. A novel risk factor for cardiovascular disease is asymmetrical dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase (NOS). Elevations in plasma ADMA may contribute to the vascular pathophysiology observed in atherosclerosis, hypertension, hypercholesterolemia, and renal failure. Increased oxidative stress seems to play an important role in the pathogenesis of these clinical conditions. Moreover, ADMA seems to be an independent predictor of cardiovascular mortality.4 The synthesis of ADMA requires the enzyme protein arginine methyltransferase type I, which methylates arginine residues and uses S-adenosylmethionine as methyl group donor by human endothelial cells.5 ADMA is derived from the catabolism of proteins containing methylated arginine residues, and free ADMA is released during proteolytic breakdown. Recent findings suggest a significant positive correlation between age and plasma ADMA levels.6,7 We have previously demonstrated that ADMA concentration-dependently increased oxidative stress measured as superoxide production by cultured human endothelial cells.7 ADMA also activated the oxidant-responsive transcription factor NF-κB, resulting in enhanced monocyte chemoattractant protein-1 (MCP-1) and endothelial adhesiveness for monocytes.7 MCP-1 and interleukin-8 (IL-8) are major chemokines for leukocyte trafficking and were found to be highly expressed in human atherosclerotic lesions.8

Recently 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 stress,9 and it is reduced by NO donor treatment.2 Therefore, in the present study we investigate if ADMA, a novel cardiovascu-
lar risk factor, influences cell senescence in cell culture and increases oxidative stress.

Materials and Methods

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were cultured in endothelial basal medium supplemented with hydrocortisone (0.5 mg/mL), gentamicin (30 μg/mL), amphotericin B (15 μg/mL), human endothelial growth factor (10 μg/mL), human fetal growth factor-B (1 μg/mL), VEGF (2 μg/mL), ascorbic acid (75 mg/mL), R-IGF-1 (5 μg/mL), heparin (1 mg/mL), and 2% fetal calf serum until tenth passage. ADMA, S-adenosylhomocysteine (SAH), and pyrrodinenedihiocarbamate (PDTC) were replaced every 48 hours starting at the fourth passage. After reaching confluence (between 8 to 9 days), endothelial cells were trypsinized and seeded at a density of 2500 cells/cm² per 75-cm² flasks or 6-well plates. Population doubling (PD) was calculated at each passage until growth arrest by the following formula: PD = (log10 Y − log10 X)/log2 (Y indicates number of cells counted of the end the passage; X number of cells seeded). Cumulative population doubling (CPD) was calculated as the sum of all the changes in PD. The endothelial cells were counted in a Neubauer cell chamber (Assistent) and the vitality was determined by means of staining with trypan blue (0.5%; Sigma) in physiological saline. Viability after trypsinization was usually >95%. HUVEC were characterized at passage 10 by specific physiological saline. Viability after trypsinization was usually >95%. HUVEC were characterized at passage 10 by specific staining for CD31 (PECAM-1) and the vitality was determined by means of staining with trypan blue (0.5%; Sigma) in physiological saline. Viability after trypsinization was usually >95%. HUVEC were characterized at passage 10 by specific staining for CD31 (PECAM-1) and the vitality was determined by counting, of 1000 cells, the number of blue cells.

Staining for Senescence-Associated β-Galactosidase Activity

HUVEC were fixed and stained for senescence-associated β-galactosidase (SA-β-gal) activity according to the procedure of Dimri et al.11 The percentage of SA-β-gal-positive cells was determined by counting, of 1000 cells, the number of blue cells.

Telomerase Assay and Telomere Length

Quantitative determination of telomerase activity was performed according to the manufacturer’s protocol of the TeloTAGGG telomerase polymerase chain reaction ELISA Kit (Roche Diagnostic GmbH) based on telomeric repeat amplification protocol assay. For telomerase activity, 1.5 μg protein was used by polymerase chain reaction. Telomere length was analyzed by the terminal restriction fragment length assay using a TeloTAGGG Telomere Length Assay Kit (Roche). Genomic DNA was isolated from HUVEC using a Qiagen DNA isolation kit.

Measurement of Nitrate and Nitrite

The determination of nitrate and nitrite in cell culture supernatants was performed in accordance to the method described by Tsikas et al.12 In our laboratory, the intra-day precision test yields a relative standard deviation of 3.8% for nitrite and 1.3% for nitrate, respectively. The inter-day precision test yields a relative standard deviation of 4.4% for nitrite and 4.2% for nitrate.

Dimethylarginine Dimethylaminohydrolase Activity Assay

Dimethylarginine dimethylaminohydrolase (DDAH) activity in endothelial cells was estimated by directly measuring the amount of ADMA metabolized by the enzyme described by Lin et al.13 The ADMA level in each group was measured by high-performance liquid chromatography mass spectrometry as described by our group previously.14

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.15 In brief, after addition of the internal standard [15N]-allantoin, the mixture was evaporated in vacuum. The residue was derivatized with N-tert-butylmethyldisil- N-methyltrifluoroacetamide and analyzed by gas chromatography mass spectrometry. The intra-day precision test yields a relative standard deviation of 2.1% and the inter-day precision of 3.8%, respectively, with an accuracy of 2.8%.

The intracellular thiol concentration was measured by 5-chloromethylfluoresceindiacetate (CMFDA) staining in flow cytometry.16 Briefly, cell samples were stained with CMFDA (Molecular Probes, Eugene, Ore) at a final concentration of 12.5 μmol/L in phosphate-buffered saline for 15 minutes at room temperature. After washing, the cells were fixed in 1% paraformaldehyde and analyzed within 2 hours by flow cytometry at λEX=490 nm/λEM=520 nm (Epics XL-MCL; Coulter).

Dihydorhoradin 123 (DHR) was used as a marker for intracellular reactive oxygen species (ROS). The cells were incubated for 20 minutes at 37°C in presence of 10 μmol/L DHR123 with gentle agitation. The reaction was stopped by cooling on ice for 1 minute and subsequent addition of 500 μL phosphate-buffered saline followed by 2 washing steps. After a final fixation with 1% paraformaldehyde, the cells were analyzed by flow cytometry (Epics XL-MCL; Coulter).

HUVEC were defined by forward/side scattering and live-gated for analysis. The levels of intracellular thiols and ROS were indicated by mean fluorescence intensities of stained probes versus negative controls. Fluorogenic beads (Flow check; Coulter) added to each sample were used for internal calibration of the quantitative measurement.

Measurement of MCP-1 and IL-8

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

Materials

ADMA, SAH, and PDTC were delivered by Sigma (Steinheim, Germany). HUVEC and the cell culture medium were obtained from Cell System/Clonetics (Solingen, Germany).

Statistical Methods

All data are given as mean±SEM from at least 3 independent experiments. Statistical significance was tested with repeated measures ANOVA using LSD post hoc test or ANOVA for multiple comparisons (SPSS Software 11.0). Differences were considered significant with P<0.05.

Results

Exogenously Applied ADMA Accelerates Endothelial Senescence by Inhibiting Telomerase Activity

To investigate the link between EC 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 μmol/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, separated, and telomere restriction fragment length was measured.

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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 enzymatic activity of an RNA protein complex called telomerase, we measured the relative activity of telomerase in HUVEC. 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).

**Effect of SAH on ADMA-Accelerated Senescence, NOx Synthesis, and Allantoin Production**

To test whether SAH could attenuate the effect of ADMA (50 μmol/L) on cellular senescence, HUVEC were incubated in the presence of SAH (100 μmol/L) starting from CPD 20. Interestingly, a 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 significantly delayed and the telomerase activity was significantly increased (Figure 2B and 2C). The increased senescence in the presence of ADMA was completely reversed in the presence of SAH.

To assess the onset of endothelial senescence, the levels of NOx and allantoin, a marker of oxygen free radical generation, in conditioned media were determined. Figure 2D illustrates that exposure of endothelial cells to ADMA resulted in a significant reduction of NOx synthesis. After SAH treatment, NOx levels were increased significantly compared with control cells (P < 0.05). The inhibitory effects of ADMA on endothelial NOx release were abolished by SAH.

Concomitantly with the inhibition of NOx synthesis, incubation with ADMA increased allantoin levels. As shown in Figure 2E, incubation of endothelial cells with ADMA led to a significant increase in allantoin levels compared with control (P < 0.05). Incubation with SAH decreased the allantoin formation. The stimulatory effects of ADMA on allantoin production were reversed by coincubating the cells with SAH.

**ADMA-Inhibited NO Synthesis Was Associated With an Increase in Endogenous Oxidative Stress and a Decrease in DDAH Enzyme Activity During Endothelial Cell Aging**

To attribute whether intracellular oxidative stress is responsible for ADMA-accelerated endothelial senescence, the content of ROS and thiols in endothelial cells were determined.

The 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 μmol/L) advanced the increase in ROS formation (P < 0.05). The antioxidant PDTC (10 μmol/L) prevented the effect of ADMA on endogenous oxidative stress.

Simultaneously, the total intracellular thiol content was measured by detection of CMFDA. The formation of endogenous thiols was increased starting from CPD 29 and decreased starting from CPD 35 in control cells (Figure 3B). Likewise, the treatment with ADMA decreased the thiol content at CPD 34. ADMA resulted in significantly decreased intracellular thiol levels at CPD 34 (P < 0.05). Taken together, these data suggest an imbalance between oxidant and antioxidant forces in favor of oxidation by ADMA-treated cells starting at CPD 34.

To examine whether the ADMA-induced oxidative stress is caused by 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 cell lysates. Concomitantly with the significant increase in ROS formation, DDAH...
activity significantly decreased at CPD 29 in control cells (Figure 3C). 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 ADMA-inhibited NO synthesis were blocked by PDTC (Figure 3D). Moreover, PDTC abolished the effect of ADMA on allantoin production (Figure 3E) and endothelial senescence (Figure 3F).

**Exogenous ADMA Increases MCP-1 and IL-8 Secretion During Endothelial Cell Aging**

In Figure 4, the change in MCP-1 (Figure 4A) and IL-8 (Figure 4B) secretion in endothelial cells supernatants are shown as a function of passage number. Treatment of cultured endothelial cells with ADMA (50 μmol/L) induced a significant secretion of MCP-1 and IL-8 compared with control ($P<0.05$). When cultured endothelial cells were incubated with SAH (100 μmol/L), the secretion of MCP-1 and IL-8 were significantly reduced compared with untreated cells ($P<0.05$). The stimulatory effects of ADMA on MCP-1 and IL-8 secretion were significantly diminished by SAH.

**Flow Cytometry**

To confirm the phenotype of cultured endothelial cells at the eleventh passage, surface expression of adhesion molecules was measured by flow cytometry using fluorescein isothiocyanate-labeled antibodies against CD31 (PECAM-1). FACS analysis of the passaged HUVEC cultures did not reveal any contamination with nonendothelial cells. ADMA and ADMA with SAH-treated or PDTC-treated cells demonstrated that surface expression of CD31 was similar to the untreated cells (data not shown).

**Discussion**

Endothelial dysfunction is emerging as a key component in the pathophysiology of diverse cardiovascular abnormalities associated with atherosclerosis and aging. Recent studies have reported that atherosclerosis alters the rate of telomere attrition as a function of age. Plasma ADMA levels are positively correlated with risk factors for atherosclerosis 

The rate of endothelial senescence is increased after the repeated addition of ADMA. An important marker for cellu-
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 protein arginine methyltransferase type I. A major source of methyl groups used for various methylation reactions is S-adenosylmethionine, and SAH inhibits the methyltransferase-catalyzed reactions.5

In this article, we could show that SAH attenuates significantly the endothelial senescence by increasing the activity of telomerase compared with control cells. The increased production rate of endothelial senescence in the presence of ADMA was completely reversed in the presence of SAH.

The issue to be addressed is why simultaneous administration of exogenous SAH and ADMA completely inhibited the ability of exogenous ADMA to produce its biological effects. It could be assumed that intracellular ADMA levels remained unchanged despite the SAH-induced inhibition of endogenous ADMA formation of ADMA5 because of the effect of exogenous ADMA. However, it is difficult to explain how lowering of endogenous ADMA formation could fully counteract exogenous ADMA administered at a concentration as high as 50 \( \mu \text{mol/L} \). A possible explanation would be the activation of DDAH, the enzyme that selectively degraded ADMA21 and whose Michaelis-Menten constant (\( K_m \)) for ADMA is \( \approx 180 \mu \text{mol/L} \), so that increased DDAH activity could deal even with supraphysiological ADMA concentrations. This enzyme seems to be exquisitely sensitive to oxidative13,22 and nitrosative stress,23 and we observed a decrease of the formation of allantoin and consequently of the oxidative13,22 and nitrosative stress, 23 and we observed a decrease of the formation of allantoin and consequently of the generation of superoxide anions. As recently shown by the Cooke group, even modest changes in ADMA levels induced by DDAH activity can have significant effects on NO synthesis.24 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 coincubation 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 regulated by ROS formation.25 Similarly, in the present study, we found that ADMA-increased ROS accelerated the rate of cellular senescence, which was reduced by incubation with the antioxidant PDTC. So, decreased ADMA synthesis induced by SAH and increased DDAH activity induced by PDTC delayed the onset of cellular senescence.

In the present report, we have also investigated the effects of ADMA on secretion of MCP-1 and IL-8 in endothelial cell supernatants. MCP-1 and IL-8 are major chemokines for leukocyte trafficking and were found to be highly expressed in human atherosclerosis lesions.8 Upregulation of these proteins by ADMA in vitro indicates that these effects are due to increased synthesis and secretion of these proteins by ADMA. So, simultaneously administering exogenous ADMA and SAH completely inhibited the effects of ADMA on the secretion of MCP-1 and IL-8.

Uncoupling of NOS catalytic activity is observed in the presence of NO\(^{-}\)-monomethyl-L-arginine (L-NMMA),20 an endogenous inhibitor of NOS. In this situation, optimal electron flow within the 2 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.7 In the experiments presented here, the inhibition of NO synthase by ADMA decreases the elaboration of NOx synthesis and cellular thiol status and increases endothelial oxidative stress measured by intracellular ROS and allantoin production. Allantoin, a marker of oxygen free radical generation, results from further oxidation of uric acid. Allantoin may occur in human tissue exclusively as a result of nonenzymatic reaction with highly reactive oxygen species, because the enzyme involved, uricase, is not expressed in humans. Therefore, elevated levels of allantoin are considered as a marker of increased oxidative stress.15

Which may be the underlying mechanism of ADMA to accelerate cellular senescence? We would speculate that ADMA increases oxidative stress, eg, by uncoupling NOS activity.

Uncoupling of NOS catalytic activity is observed in the presence of G-rich sequence at the end of chromosomes. Interestingly, the telomere length in ADMA-treated cells is significantly shortened compared with control cells. The telomeres are synthesized by enzymatic activity associated with an RNA protein complex called telomerase. A significant reduction in telomerase activity is observed in ADMA-treated cells compared with control cells.

Figure 4. Effect of ADMA and SAH on the secretion of MCP-1 (A) and IL-8 (B) in HUVEC. EC were incubated with ADMA (50 \( \mu \text{mol/L} \)) and SAH (100 \( \mu \text{mol/L} \)) starting from CPD-20 and replaced every 48 hours.
proteins is induced via enhanced formation of intracellular oxidative stress, which activates the oxidant-responsive transcription factor NF-κB, leading to increased expression and secretion of MCP-1 and IL-8. 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.

Study Limitations
Do our ADMA concentrations correspond to physiological and pathophysiologial plasma concentrations? Several investigators indicated that circulating concentrations of ADMA in plasma from healthy humans were in the range 0.3 to 1.0 μmol/L. ADMA levels were increased in plasma of human with hypercholesterolemia or atherosclerosis. ADMA plasma levels were elevated to 2.2±0.2 μmol/L in young, clinically asymptomatic hypercholesterolemic individuals. In elderly patients with peripheral arterial disease and generalized atherosclerosis, ADMA levels were in the range 2.5 to 3.5 μmol/L, corresponding to the severity of the vascular disease. We have previously published that ADMA plasma concentrations were 3.8±0.3 μmol/L in healthy elderly people and 3.8±0.3 μmol/L in patients with peripheral arterial occlusive disease and hyperhomocyst(e)inemia. Because in this study we used ADMA-HCl, the exact concentrations of ADMA were 3.7, 37, and 74 μmol/L. ADMA levels in lysed endothelial cells were 8- to 12-fold higher than those in conditioned media, amounting to 5 to 40 μmol/L.

Clinical Implications
It has been shown that in atherosclerosis plasma, ADMA levels are positively correlated with risk factors for atherosclerosis. Miyazaki et al reported a positively correlation for ADMA and endothelial function in prospective studies as surrogate parameters for the increased risk of future cardiovascular events.

In summary, these data suggest that at concentrations corresponding to plasma levels at pathophysiological conditions, ADMA accelerates aging in endothelial cells and provokes increased oxidative stress. Coincubation with SAH abolished these effects. We speculate that modest changes of intracellular ADMA levels are associated with significant effects on slowing endothelial cell senescence. This would provide a mechanism through which elevated plasma ADMA levels observed in the elderly and in the presence of atherosclerotic risk factors might be a hallmark of a hazard of endothelial senescence and atherogenesis. Furthermore, appearance of the senescent phenotype concomitantly with increased oxidative stress within foci of the endothelium could further impair NO and potentiate endothelial dysfunction, creating a kind of vicious circle. Recently, it could be shown that senescence was associated with decreases in basal and shear stress-induced eNOS expression, NOx release, and with augmented endothelial adhesiveness for monocytes, all of which could be prevented by stable transfection of human telomerase.


