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Aspirin reduces endothelial cell senescence

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Abstract

We report here the effect of aspirin on the onset of replicative senescence. Endothelial cells that were cultured until cumulative population doublings 40 showed clear signs of aging. Incubation with aspirin inhibited senescence-associated β-galactosidase activity and increased telomerase activity. Along with the delayed onset of senescence, aspirin decreased reactive oxygen species and increased nitric oxide (NO) and cGMP levels. Furthermore, aspirin reduced the elaboration of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NO synthase, and up-regulated the activity of dimethylarginine dimethylaminohydrolase, the enzyme that degrades ADMA. These effects were specific in that other nonsteroidal anti-inflammatory drugs, such as ibuprofen or acetaminophen, did not prevent the onset of endothelial senescence. The NO synthase inhibitor L-NAME, but not its inactive D-enantiomer, led to complete inhibition of aspirin-delayed senescence. These findings demonstrate that aspirin delays the onset of endothelial senescence by preventing a decrease in NO formation/generation. This might provide a therapeutic strategy aimed at blocking aging-induced NO inhibition.

Keywords: Aspirin; Aging; Oxidative stress; Nitric oxide; Asymmetric dimethylarginine; Dimethylarginine dimethylaminohydrolase; L-NAME; Telomerase; Reactive oxygen species; Senescence

Endothelial cells have a finite life span when cultured in vitro and enter an irreversible growth arrest known as senescence [1]. In senescence state, cells are viable and metabolically active, but are unable to divide. Furthermore, the capacity of endothelial cells to generate nitric oxide (NO) decreases [2,3]. This phenomenon may result from decreased expression and activity of NO synthase [2,4] or accumulation of the cardiovascular risk factor and NO synthase inhibitor asymmetrical dimethylarginine (ADMA) [5,6] as well as increased production of reactive oxygen species (ROS) [3,7]. These findings suggest that in vivo, cellular senescence is an important factor contributing to the increased vascular risk associated with aging [8].

Telomeres, repetitive DNA sequences at the ends of eukaryotic chromosomes, shorten as a linear function of increasing cellular division, and a critically short telomere length triggers the onset of senescence. Telomerase, a ribonucleoprotein, can synthesize new telomeric repeats and restore telomere length. In the absence of telomerase or when this enzyme is expressed at very low levels, DNA synthesis during cell division results in the progressive shortening of telomeric DNA. Interestingly, studies indicate that the shortening of telomeres and the reduction of telomerase activity are not solely linked to the number of cell divisions, but can be altered by several different factors [3,9–11]. Recently, we have demonstrated that ADMA, an endogenous inhibitor of

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NO synthase, increases the rate of telomere shortening and accelerated endothelial senescence [3]. We proposed that an increase in ROS formation and a decrease in NO formation may be responsible for the premature senescence. In agreement with this, suppression of oxidative stress or application of NO-donors delayed the onset of replicative senescence in cell culture [3,9–11].

Aspirin, the most widely prescribed nonsteroidal anti-inflammatory drug, is used in the treatment and prevention of common cardiovascular disorders [12]. Accumulating evidence suggests that aspirin, in addition to antithrombotic and anti-inflammatory effects, may have additional biological properties on the vasculature that contribute to increased NO formation and protect the endothelium from deleterious effects of oxidative stress [13–17].

Accordingly, the present study was performed to examine whether aspirin delays the onset of replicative senescence by counteracting the adverse effect of decreased NO formation and increased oxidative stress on aging of endothelial cells in culture.

Materials and methods

Materials. Aspirin was obtained from Bayer AG (Leverkusen, Germany). Ibuprofen, acetaminophen, L-NAME, and D-NAME were delivered by Sigma (Steinheim, Germany). Human umbilical vein endothelial cells (HUVECs) and the cell culture medium were obtained from Clonetics/Cambrex (Kerviers, Belgium).

Cell culture. Human umbilical vein endothelial cells (HUVECs) were cultured in endothelial basal medium supplemented with hydrocortisone (0.5 mg/ml), gentamicin (30 μg/ml), amphotericin B (15 μg/ml), hEGF (10 μg/ml), hFGF-B (1 μg/ml), VEGF (2 μg/ml), ascorbic acid (75 mg/ml), R^3-IGF-1 (5 μg/ml), heparin (1 mg/ml), and 2% FCS at a density of 8000 cells/cm^2 per 75 cm^2 flasks or 6-well plates. After reaching confluence (between 8 and 9 days), endothelial cells were trypsinized and counted in a Neubauer cell chamber. Viability was determined by means of staining with Trypan blue (0.5%) in physiological saline. Viability after trypsinization was usually >95%. Population doubling (PD) was calculated at each passage until growth arrest by 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 number of cells seeded). Cumulative population doubling (CPD) was calculated as the sum of all the changes in PD. HUVECs were characterized at passage 12 by specific staining for CD31 (PECAM-1) using the fluorescence-activated flow cytometry assay (FACS), as previously described [3].

Study protocol. Starting at the fourth passage, HUVECs were treated with aspirin (100 μM), ibuprofen (100 μM), acetaminophen (100 μM), aspirin (100 μM) plus L-NAME (100 μM), aspirin (100 μM) plus D-NAME (100 μM), or vehicle every 48 h until twelfth passage. Every 2 passages, the cells and the supernatants were harvested and saved for the measurements described below. The total cellular protein was measured using BCA protein assay kit (Pierce, Bonn, Germany).

Detection of senescence. HUVECs were fixed and stained for senescence-associated β-galactosidase (SA β-gal) activity according to the procedure described by Dimri et al. [18]. The percentage of SA β-gal positive cells was determined by counting the amount of blue cells within a sample of 1000 cells.

Telomerase activity. 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) based on telomeric repeat amplification protocol (TRAP) assay. For telomerase activity 2 μg protein was used by polymerase chain reaction.

Measurement of nitrate, nitrite, and cGMP. The determination of nitrate and nitrite was carried out in accordance with the method described by Tsikas et al. [19]. 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.

The levels of cGMP were assayed using cyclic GMP immunoassay (R&D Systems, Wiesbaden, Germany) according to manufacturer's instructions.

Determination of ADMA. For the determination of ADMA, we adopted the HPLC-mass spectrometry method for plasma and urine published previously by our group [20]. The calibration covers the range of 0.15–6 μM. The calibration function was linear and the limit of detection was found to be 0.05 μM for ADMA. The intra-day precision was 4.65%, while the inter-day precision was 3.3%.

DDAH activity. Dimethylarginine dimethylaminohydrolase (DDAH) activity was estimated by directly measuring the amount of ADMA metabolized by the enzyme described by Lin et al. [21]. The ADMA level in each group was measured as described above.

Detection of oxidative stress. The intracellular thiol concentration was measured by 5-chloromethylfluorescein diacetate (CMFDA) staining in flow cytometry [22]. Briefly, cell samples were stained with CMFDA (Molecular Probes, Eugene, OR, USA) at a final concentration of 12.5 μM in phosphate-buffered saline for 15 min at room temperature. After washing, the cells were fixed in 1% paraformaldehyde and analyzed within 2 h by flow cytometry at $\lambda_{EX} = 490$ nm/$\lambda_{EM} = 520$ nm (Epics XL-MCL; Coulter, Krefeld, Germany).

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

The levels of intracellular thiols and ROS were assessed by measuring mean fluorescence intensities (mfi) of stained probes versus negative controls.

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

Results

β-Galactosidase and telomerase activities

In order to establish a link between senescence and aspirin exposure, human endothelial cells were cultured until cumulative population doubling (CPD) 40 and incubated in the presence of aspirin, which was replaced every 48 h starting from CPD 20. During serial passages of endothelial cells, the activity of senescence-associated β-galactosidase (SA β-gal) increased 7-fold at CPD 40 compared with CPD 20. Incubation with aspirin inhibited SA β-gal activity significantly. In contrast to aspirin, β-galactosidase activity was significantly elevated by ibuprofen and acetaminophen. Addition of the NOS inhibitor L-NAME abolished the aspirin-reduced SA

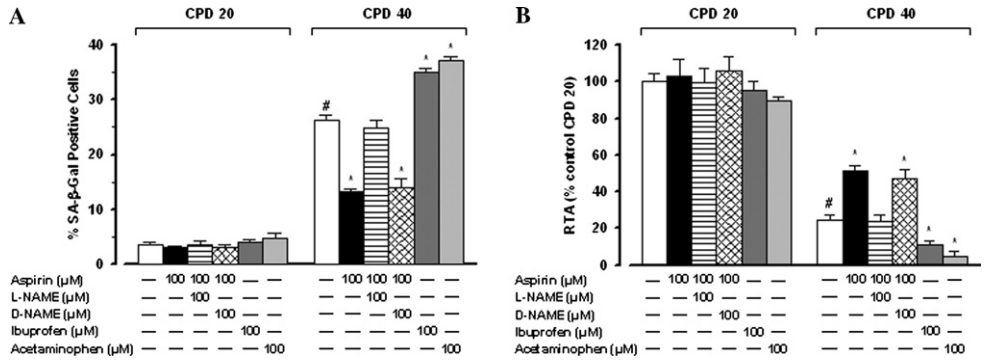

Fig. 1. Effect of aspirin, ibuprofen, and acetaminophen on senescence (A) and telomerase activity (B) of endothelial cells (EC). EC were incubated with aspirin, ibuprofen, acetaminophen, L-NAME, and D-NAME starting from cumulative population doubling (CPD) 20 and replaced every 48 h until CPD 40. (A) Endothelial senescence was detected by senescence-associated β-galactosidase (SA-β-gal) positive cells. (B) Relative telomerase activity (RTA) was detected by ELISA. Each point represents the mean ± SEM of results from three experiments. $^{*}P < 0.05$ versus corresponding CPD of control cells; $^{\#}P < 0.01$ versus CPD 20 control.

β-gal activity, whereas the enantiomer D-NAME was without effect (Fig. 1A).

In order to investigate the effect of aspirin on telomerase activity during aging of endothelial cells, cell lysates were prepared from cultures between CPD 20 and CPD 37. The activity of telomerase decreased and reached the value of 24.9 ± 2.8 % at CPD 37. Aspirin increased telomerase activity significantly compared to control. In contrast, ibuprofen and acetaminophen significantly reduced telomerase activity. Co-incubation with L-NAME significantly blocked the effect of aspirin on telomerase activity (Fig. 1B).

ROS and thiol content

In order to provide insight into the cellular mechanism of aspirin-induced delay of endothelial senescence, the content of ROS and thiols in endothelial cells was determined. The intracellular level of ROS was increased 4-fold at CPD 40 compared with CPD 20. As shown in Fig. 2A, incubation with aspirin prevented the increase in ROS formation. In contrast to aspirin, ibuprofen and acetaminophen produced a further increase in ROS formation. The NOS blocker L-NAME inhibited the effect of aspirin on endogenous oxidative stress.

Total intracellular thiol content was measured by detection of CMFDA. In endothelial cells treated with aspirin or ibuprofen, the formation of endogenous thiols was similar to control, whereas incubation with acetaminophen was followed by a loss of intracellular thiols (Fig. 2B).

NO, cGMP, and ADMA levels

The above results suggested that oxidative stress in endothelial cells is down-regulated by aspirin during aging. In order to investigate this possibility in more detail, NO was measured from cultures between CPD 20 and 40. In control cells, NO synthesis was reduced by 46% at CPD 40 compared to CPD 20. Aspirin enhanced NO formation, whereas ibuprofen and acetaminophen significantly decreased NO formation (Fig. 3A). In addi-

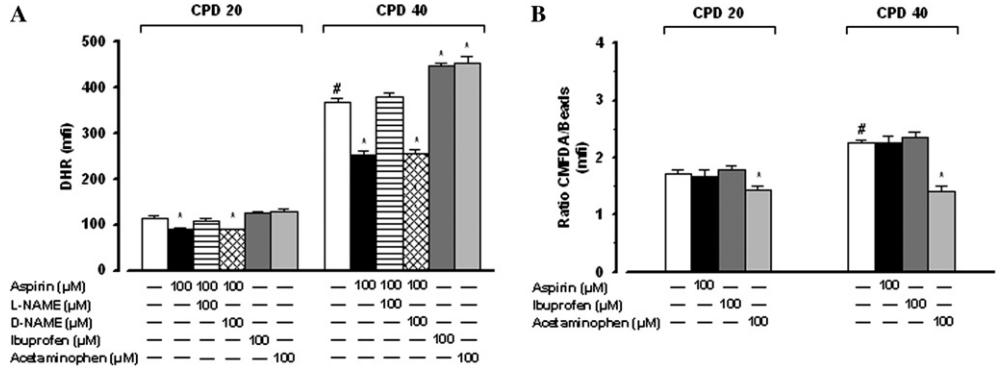

Fig. 2. Effect of aspirin, ibuprofen, and acetaminophen on ROS (A) and thiol (B) content. EC were incubated with aspirin, ibuprofen, acetaminophen, L-NAME, and D-NAME starting from CPD 20 and replaced every 48 h until CPD 40. (A) Endogenous ROS formation was measured with DHR using a FACS analysis. (B) Total intracellular thiol content was determined by CMFDA staining in flow cytometry. The levels of intracellular thiols and ROS were assessed by mean fluorescence intensities (mfi) of stained probes versus negative controls. $^{*}P < 0.05$ versus corresponding CPD of control cells; $^{\#}P < 0.05$ versus CPD 20 control.

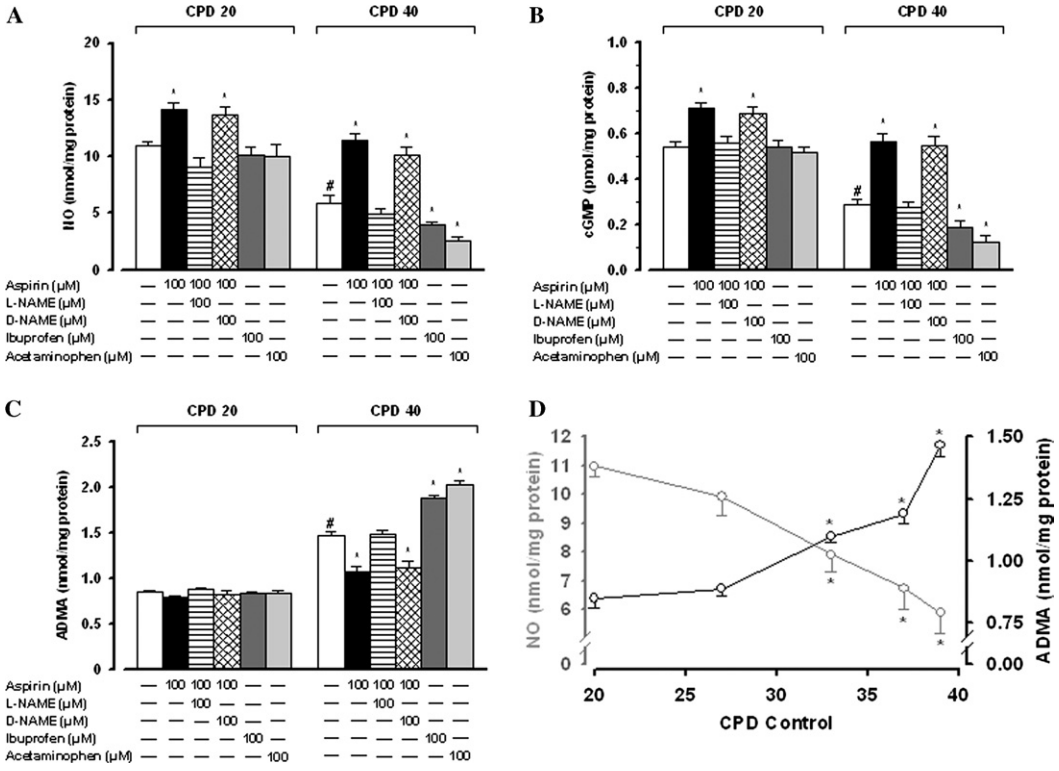

Fig. 3. Effect of aspirin, ibuprofen, acetaminophen on NO (A), cGMP (B), and ADMA (C) formation. EC were incubated with aspirin, ibuprofen, acetaminophen, L-NAME, and D-NAME starting from CPD 20 and replaced every 48 h until CPD 40. $^{*}P < 0.05$ versus corresponding CPD of control cells; $^{\#}P < 0.01$ versus CPD 20 control. (D) The decrease in NO synthesis during aging of EC was accompanied by a corresponding increase in ADMA accumulation. EC were continuously passaged until CPD 39. $^{*}P < 0.05$ versus CPD 20 control.

tion to increased NO synthesis, aspirin led to a specific elevation of cGMP levels that was not observed with ibuprofen and acetaminophen (Fig. 3B). Addition of the NOS inhibitor L-NAME abolished the aspirin-induced increase in NO synthesis and cGMP levels, whereas D-NAME had no effect (Figs. 3A and B).

The elevation in NO synthesis in the aspirin group was associated with a reduction of ADMA accumulation. As demonstrated in Fig. 3C, aspirin diminished ADMA concentrations, whereas ibuprofen and acetaminophen caused accumulation of ADMA. Co-incubation with L-NAME abrogated the effect of aspirin on ADMA levels.

In addition, Fig. 3D shows that during aging, ADMA was increased by 72% at CPD 39 compared with CPD 20, whereas NO synthesis was reduced by 46% in untreated endothelial cells.

DDAH activity

Because ADMA levels were decreased by supplemental aspirin, we hypothesized that this reduction is due to increased degradation of ADMA by DDAH. In order to test the effect of aspirin on DDAH in aging endothelial cells, DDAH activity was determined during constant passaging of endothelial cells by assessing the rate of degradation of exogenous ADMA added to the cell lysates. DDAH activity decreased in parallel to increased

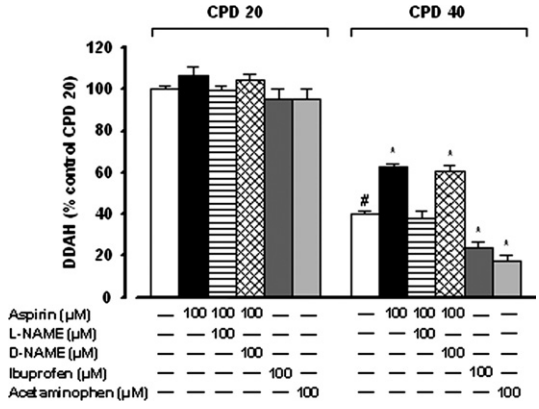

Fig. 4. Effect of aspirin, ibuprofen, and acetaminophen on DDAH activity. EC were incubated with aspirin, ibuprofen, acetaminophen, L-NAME, and D-NAME starting from CPD 20 and replaced every 48 h until CPD 40. In all experiments, DDAH activity in cell lysates is expressed as the percentage of amount of ADMA metabolized by control at CPD 20, which is defined as 100% for every experiment. $^{*}P < 0.05$ versus corresponding CPD of control cells; $^{\#}P < 0.01$ versus CPD 20 control.

ADMA formation and reached the value of 40% at CPD 40 compared with CPD 20. Incubation with aspirin enhanced DDAH activity, whereas ibuprofen and acetaminophen diminished DDAH activity. L-NAME impaired DDAH activity and prevented the increase in DDAH activity caused by aspirin (Fig. 4).

Discussion

The results of the present study demonstrate for the first time that aspirin, at therapeutically relevant concentrations, prevents aging of endothelial cells. This effect seems to be due to increased nitric oxide (NO) synthesis and decreased oxidative stress. Consistent with these findings, the formation of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NO synthase, was reduced and the activity of dimethylarginine dimethylaminohydrolase (DDAH), the enzyme that degrades ADMA, was enhanced. Other nonsteroidal anti-inflammatory drugs, such as ibuprofen or acetaminophen, did not prevent the onset of endothelial senescence. The data provide a novel mechanism for the aspirin-induced delay in endothelial senescence in vitro.

The free radical theory of aging proposes that degenerative senescence is largely the result of the cumulative effect of oxidative end products [23]. Emerging data have indicated a possible linkage between increased oxidative stress and reduction of telomerase activity [11,24]. We and others have previously demonstrated that the adverse effect of these stimuli can be reversed in vitro by antioxidants, which reduce oxidative stress, preserve the activity of telomerase, and delay the onset of replicative senescence [3,11]. Aspirin has been shown to possess antioxidant properties [13–17], which may explain the aspirin-induced endothelial protection against oxidative injury during aging observed in our study. Several mechanisms have been proposed to underlie the potential role of aspirin as an antioxidant. For example, aspirin protects endothelial cells from the deleterious effects of iron-dependent oxygen radical formation [25], through induction of ferritin synthesis, a protein with cytoprotective and antioxidant properties. Furthermore, chronic treatment with aspirin markedly reduced vascular production of superoxide anion through lowering NAD(P)H oxidase activity in both normotensive and hypertensive rats as well as in cultured aortic smooth muscle cells [14]. Moreover, aspirin increased heme oxygenase-1 (HO-1) protein levels and enzymatic activity in cultured endothelial cells, via NO-dependent and COX-independent pathways [26]. For this novel aspirin action, recent work [27] offers a possible mechanistic explanation by introducing another HO-1 inducer, 15-epi-lipoxin A4, a lipid metabolite and anti-inflammatory mediator of aspirin in vivo. Using a stable aspirin-triggered lipoxin A4 analog, the authors found that HO-1 induction occurs both at the mRNA and protein levels and appears to be mediated by activation of the G-protein-coupled lipoxin A4 receptor. Taken together, all the above information supports the fact that aspirin possesses antioxidative properties which could reduce reactive oxygen species (ROS) generation [17].

Results from the present study are consistent with these findings. We have also shown that aspirin prevents the increase of intracellular ROS formation during aging and consequently up-regulates telomerase activity and delays the onset of cellular senescence.

Recently, we have reported that inhibition of NO synthesis by exogenous ADMA was accompanied by an increase in ROS formation and a corresponding acceleration of EC senescence [3]. Moreover, application of NO-donors delayed the onset of replicative senescence [9]. These results underline the important role of NO in the regulation of endothelial senescence. Accumulating evidence suggests that aspirin regulates NOS activity and thereby induces NO release from vascular endothelium. These effects on the NO signaling system seem to be specific for aspirin and are independent of COX inhibition or blockade of superoxide-mediated NO degradation [15,16,28]. It is conceivable and supported by recent observations that aspirin stimulates NO formation through its unique ability to trigger the synthesis of 15-epi-lipoxin A_4 [28]. Aspirin is known to acetylate COX-2 within the endothelium, thus triggering 15-epi-lipoxin A_4 which, in turn, elicits NO synthesis from both eNOS and iNOS.

In the experiments presented here, aspirin increases NO bioavailability and reduces endothelial senescence. Aspirin-induced NO synthesis is associated with a corresponding stimulation of endothelial cGMP levels as a sensitive marker of intracellular NO formation. This finding is supported by the observation that co-incubation with the inhibitor of NOS, L-NAME, but not its D-enantiomer completely inhibited the effect of aspirin on the NO/cGMP system confirming the involvement of NO in endothelial senescence.

The effects on endothelial senescence and the NO/cGMP system are specific for aspirin and not elicited by other nonsteroidal anti-inflammatory drugs such as ibuprofen and acetaminophen, suggesting that the observed effect occurs independently of COX-inhibition.

In the vascular system, bioavailability of NO can be impaired by various mechanisms, including decreased NO production by eNOS, and/or enhanced NO breakdown due to increased oxidative stress. Reduced activity or sensitivity of eNOS is often associated with accumulation of its endogenous inhibitor, asymmetric dimethylarginine (ADMA) [29]. ADMA is actively metabolized by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) [30]. The activity of DDAH seems to be particularly susceptible to inhibition by oxidative stress. A wide range of pathological stimuli as well as aging induce endothelial oxidative stress and consequently reduce DDAH activity in vitro and in vivo [3,22,31–33]. These observations suggest a role for oxidative stress as a mechanism to control and limit enzymatic activity of DDAH. The adverse effects of these stimuli can be reversed by antioxidants, which preserve DDAH activity. In our investigation, increased ROS formation was accompanied by a corresponding decrease in DDAH

activity, which, in turn, led to ADMA accumulation during endothelial aging. These effects were completely abolished by aspirin, which suggests that aspirin-sensitive targets such as NO synthase, indeed, exert a crucial regulatory function in the DDAH–ADMA system.

In contrast, ibuprofen and acetaminophen, which were previously shown to leave NO synthase and antioxidant activity unaltered did not prevent dysregulation of the DDAH–ADMA system.

This investigation is consistent with the hypothesis that aspirin-dependent prevention of endothelial senescence is probably mediated by increased NO bioavailability and decreased oxidative stress.

In summary, we have shown, for the first time, that aspirin delays endothelial cell senescence and we propose the reduction of ADMA levels as a novel mechanism for "anti-aging" drugs. This approach might provide a future therapeutic strategy aimed at blocking age-dependent NO inhibition and progression of cardiovascular diseases.

Acknowledgments

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