Low-dose aspirin prevents age-related endothelial dysfunction in a mouse model of physiological aging

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Bulckaen H, Prévost G, Boulanger E, Robitaille G, Roquet V, Gaxatte C, Garçon G, Corman B, Gosset P, Shirali P, Creusy C, Puisieux F. Low-dose aspirin prevents age-related endothelial dysfunction in a mouse model of physiological aging. Am J Physiol Heart Circ Physiol 294: H1562–H1570, 2008. First published January 25, 2008; doi:10.1152/ajpheart.00241.2007.—The age-related impairment of endothelium-dependent vasodilatation contributes to increased cardiovascular risk in the elderly. For primary and secondary prevention, aspirin can reduce the incidence of cardiovascular events in this patient population. The present work evaluated the effect of low-dose aspirin on age-related endothelial dysfunction in C57B/6J aging mice and investigated its protective antioxidative effect. Age-related endothelial dysfunction was assessed by the response to acetylcholine of phenylephrine-induced precontracted aortic segments isolated from 12-, 36-, 60-, and 84-wk-old mice. The effect of low-dose aspirin was examined in mice presenting a decrease in endothelium-dependent relaxation (EDR). The effects of age and aspirin treatment on structural changes were determined in mouse aortic sections. The effect of aspirin on the oxidative stress markers malondialdehyde and 8-hydroxy-2′-deoxyguanosine (8-OHdG) was also quantified. Compared with that of 12-wk-old mice, the EDR was significantly reduced in 60- and 84-wk-old mice (P < 0.05); 68-wk-old mice treated with aspirin displayed a higher EDR compared with control mice of the same age (83.9 ± 4 vs. 66.3 ± 5%; P < 0.05). Aspirin treatment decreased 8-OHdG levels (P < 0.05), but no significant effect on intima/media thickness ratio was observed. The protective effect of aspirin was not observed when treatment was initiated in older mice (96 wk of age). It was found that low-dose aspirin is able to prevent age-related endothelial dysfunction in aging mice. However, the absence of this effect in the older age groups demonstrates that treatment should be initiated early on. The underlying mechanism may involve the protective effect of aspirin against oxidative stress.

AGE-ASSOCIATED CHANGES SUCH as endothelial dysfunction are involved in the significantly increased risk of cardiovascular complications and microthrombus formation in the elderly patient population (44). The presence of endothelial dysfunction in the coronary or peripheral circulation has been shown to constitute a risk factor for cardiovascular events independent of the development of atherosclerosis or other vascular risk factors (22, 43–45). There is emerging evidence that age-associated endothelial dysfunction is related to the local formation of reactive oxygen and nitrogen species within and in the vicinity of the vascular wall (13, 25, 36, 43, 49). Therapeutic approaches capable of preventing or reversing age-related endothelial dysfunction may thus help to reduce cardiovascular risk in the elderly.

Age-related endothelial-dependent relaxation (EDR) decreases in the large vessels of different animal species including humans (36). EDR in response to acetylcholine (ACH) decreases with age in the rat aorta: the maximal relaxation effect of ACH is 100% in 4- to 6-wk-old, 50% in 3- to 6-mo-old, and 25% in 12- to 25-mo-old rats (29). The age-related decrease in EDR varies from one vessel to another and from one species to another (23).

Aspirin, a white crystalline compound of salicylic acid, is one of the most preventive treatments against cardiovascular events in high-risk adults (7, 38). The benefits of low-dose aspirin are well established in secondary prevention (1, 39, 42). In primary prevention, a recent meta-analysis of more than 50,000 women and 40,000 men taking part in six randomized trials has indicated that low-dose aspirin therapy is associated with a significant reduction in cardiovascular events in both men and women (3).

Aspirin preserves the integrity of the vascular wall through its free radical scavenging properties and its capacity to protect endothelial cells from the deleterious effects of hydrogen peroxide (16, 21, 40). The effects of aspirin in preventing cardiovascular events are attributed to its platelet-inhibitory function, which results from the irreversible inhibition of the activity of platelet cyclooxygenase and thromboxane B2, the major products of cyclooxygenase (COX-1) activity (39). Low-dose aspirin suppresses the age-related increase in oxidative stress via the modulation of NF-κB (27). The acetyl group provides aspirin with the capacity to increase endothelial nitric oxide (NO) synthesis and bioavailability (14, 15, 24, 46, 53). Aspirin reduces monocyte chemoattractant protein-1 and soluble ICAM-1 levels in low-density lipoprotein (LDL) receptor-deficient mice (8, 9). This reduction in adherence molecule expression has a functional effect, since aspirin inhibits monocyte adhesion to LDL-stimulated endothelial cells (15). Evidence from explorative clinical trials suggests that treatment with low-dose aspirin increases EDR. In humans, low-dose

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Aspirin administered over a 2-mo period has been found to improve endothelial function (34). In rats with vascular endothelial injury induced by an injection of native LDL, low-dose aspirin is able to reverse the EDR dysfunction (11).

The aim of the present study was to determine the long-term ability of low-dose aspirin to prevent endothelial dysfunction in a mouse model of physiological aging. We decided to perform our study on C57B/6J mice, which do not develop atherosclerotic risk factors such as hypertension, dyslipidemia, or obesity during aging. Age-related endothelial dysfunction was assessed by the response to ACh of phenylephrine (Phe)-induced precontracted aortic segments isolated from 12-, 36-, 60-, and 84-wk-old mice. The protective effect of 8 (initiated in 60-wk-old mice), and 16 (initiated in 60- and 96-wk-old mice) wk of aspirin treatment was studied. The effect of low-dose aspirin was then studied in animals 68, 76, and 112 wk of age, respectively. The effects of age and the preventive effects in this regard of low-dose aspirin on structural changes were determined in aortic samples. The effects of low-dose aspirin treatment on the oxidative markers malondialdehyde (MDA) and 8-hydroxy-2’-deoxyguanosine (8-OHdG) were also quantified.

MATERIALS AND METHODS

Animals

C57B/6J male mice were obtained from Janvier Laboratories (Le Genest-St-Isle, France). The same six mice per group were analyzed for the study of aortic reactivity and for the markers of oxidative stress. Five different mice per group were used for immunohistological investigations. All the experimental procedures including those regarding animal care were approved by our University Ethical Committee. The mice were housed in cages with five animals per cage at the animal care facilities under controlled conditions at a temperature of 21–23°C, a humidity level of 50–60%, and a 12:12-h light-dark cycle. The mice were fed a standard laboratory diet and provided with water ad libitum. They underwent a period of 2-wk acclimatization before any experiments were carried out.

Aspirin Treatment

The animals were randomized into two groups: the No Asp group, with no aspirin in their drinking water, and the Asp group, with aspirin in their drinking water (30 μg/ml; UPSA, Paris, France). The drinking water, with or without the addition of aspirin, was replaced every other day. The stability of salicylate activity over time was tested by fluorescent polarization immune assay 48 h after the addition of aspirin to the drinking water (AxSYM, Abbott, Abbott Park, IL). The results showed that salicylate activity remained stable, with a 13.3% maximal variation after 48 h (n = 4). Prior to the study, we established that C57B/6J mice drank 3–4 ml water/day. Considering that an adult C57B/6J male mouse weighs on average 30 g and drinks the night prior to the experiment.

Aortic Reactivity

To evaluate the effect of age on endothelial function, the endothelial response to ACh of Phe-precontracted aortic segments was studied in 12-, 36-, 60-, and 84-wk-old mice, whereas the effect of treatment with low-dose aspirin over an 8- or 16-wk period on the decrease in EDR was assessed in 60- and 96-wk-old mice.

After deep anesthesia induced by a 0.3-ml intraperitoneal injection of pentobarbital 6% (Ceva Santé Animal, Libourne, France), the thoracic aorta between the arch and the diaphragm was removed while the heart was still beating, and placed in oxygenated Krebs buffer containing (in mmol/l) 118 NaCl, 4.6 KCl, 27.2 NaHCO3, 1.75 CaCl2, 1.2 MgSO4, 0.026 EDTA, 1.2 KH2PO4, and 11.1 d-glucose; pH 7.40. The aorta was cleaned of its surrounding adventitial fatty and connective tissues. Aortic rings (3 mm) were suspended in individual organ chambers filled with oxygenated Krebs buffer to maintain the pH at 7.4 (Radnoti Glass Technology, Monrovia, CA). The isometric tension was measured with isometric transducers (Radnoti), digitized with a Workbench PC (Strawberry Tree, Sunnyvale, CA). The rings were equilibrated for 60 min at a 1.5-g resting level, previously determined as being the optimal level for their length-tension relationship. Tissue viability was assessed by 75-mmol KCl-induced contraction. Following a 30-min washout period, the cumulative concentration-response to Phe (Sigma-Aldrich, Saint Quentin Fullavier, France) was determined for each aortic ring. When the maximal Phe-induced concentration reached a plateau, the cumulative concentration-response to sodium nitroprusside (SNP, 10−4 to 10−5 mol/l; Sigma-Aldrich) was assessed to evaluate EDR status. To study the endothelium-independent relaxation (EiDR) status following a 30-min washout period, the cumulative concentration-response to sodium nitroprusside (SNP, 10−4 to 10−5 mol/l; Sigma-Aldrich) was evaluated after a single dose of Phe (3.10−7 mol/l). Contraction responses were expressed as a percentage of KCl-induced maximal contraction. The relaxation response was expressed as a percentage of Phe-induced precontraction, and Emax expressed the maximal effect obtained following the cumulative concentrations of the contracting or relaxant agent.

Histological Findings

To correlate the effects of age and the protective action of aspirin in this regard on aortic structure and vascular responsiveness, a histological analysis was performed.

Once the blood had been collected, the hearts were perfused with phosphate-buffered saline (PBS). Then the aortic arch was removed, and a section was fixed in formaldehyde 4% for 4 h before being embedded in paraffin. The morphometric study of the aorta was performed using hematoxylin-eosin-safranin staining (Leica Autstostainer-XL, Rueil-Malmaison, France). The mean intima/media aortic thickness was calculated as the mean of the maximal and minimal thickness in cross section. For the histological study, 4-μm aortic sections were used (Leica RM 2035, Wetzlar, Germany) and examined under a light microscope (Leica, DMR-B, Wetzlar, Germany) fitted with a 3-channel-coupled devices color video camera (Power HAD, Sony, Sherwood, Dallas, TX). Aortic areas were measured with an image-analysis system (Q-Win, Leica, Rueil-Malmaison, France). The elastin laminae were visualized by orcein staining and collagen III deposition by the Volgens-Gomori method. Immunohistological investigations were performed to qualitatively identify muscle actin (clone 1A4) that cross-reacts with mouse smooth muscle α-actin (M0851, DAKO, Copenhagen, Denmark). The antibody was diluted to 1:100 and staining was performed with a Nexes automatic stainer (Ventana, Tucson, AZ). Endogenous peroxidase was neutralized with 1% H2O2 for 30 min. The slides were then washed in PBS and incubated with primary antibody at 4°C for 20 min. After incubation, the slides were again washed in PBS and detection was carried out with an avidin-biotin-alkaline phosphatase method, a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA).

To analyze vascular (intima/media), collagen and elastin fiber changes, two aortic sections per animal were analyzed by two blinded pathologists for each staining. A qualitative analysis was performed using the image-analysis system Q-Win.
Study of Oxidative Stress Markers

Two major markers of oxidative stress were examined, namely MDA and 8-OHdG. They were investigated before low-dose aspirin treatment (60-wk-old mice) and 8 wk later in the aortas of No Asp and Asp animals (68-wk-old mice). After the animals had been killed, the blood was collected by intracardiac puncture. Then the thoracic aorta was carefully dissected and the samples were quickly frozen in liquid nitrogen and stored at −80°C until further study of these biological markers of oxidative stress.

Determination of MDA concentrations. MDA is extensively used as a marker of lipid peroxidation in processes associated with oxidative stress and vascular injury. It is an end product of the metabolically uncoupled peroxidation of polynsaturated fatty acids, stimulated by free radicals such as hydroxyl. Lipid peroxidation was evaluated using a high-performance liquid chromatography (HPLC) MDA assay (19). Two hundred microliters of aortic homogenates were mixed with 2 ml of 0.1 M HCl, extracted with 2×3 ml of ethyl acetate, stirred for 5 min, and centrifuged at 3,000 g for 10 min. After evaporation, the extracts were suspended in 200 μl of methanol. The HPLC system consisted of a Jasco PU-980 pump equipped with a Nucleosil column (C18, 150×4.6 mm, 5 μm particle size), a Rheodyne 7725 automated injector, a UV Jasco 975 detector, and a Shimadzu CR3A integrator (Vasse Industries, Lille, France). The mobile phase consisted of a mixture of 50 mM KH2PO4 and methanol 60:40 (vol/vol) adjusted to pH 6.8 with 1 M KOH. Tetraethoxypropane (Sigma-Aldrich), which hydrolyzes to yield one molecule of MDA, was used as standard, with thiobarbituric acid (TBA) as reagent. A 50-ml volume of standard solution or methanol extract was injected into the HPLC system and the MDA-TBA adducts were detected at λ = 532 nm (18).

Determination of 8-OHdG concentrations. 8-OHdG formation is frequently used to estimate oxygen radical-induced damage. They were investigated before low-dose aspirin treatment (60-wk-old mice) and 8 wk later in the aortas of No Asp and Asp animals (68-wk-old mice). They were investigated before low-dose aspirin treatment (60-wk-old mice) and 8 wk later in the aortas of No Asp and Asp animals (68-wk-old mice). 8-OHdG concentrations were determined in the aortic homogenates using the method originally described by Toyokuni et al. (48) and modified by García et al. (21). Briefly, DNA was extracted from the tissues using a DNaseasy Tissue Kit (Qiagen, Courtaboeuf, France), incubated at 100°C for 2 min, treated with 1 μl of 250 mM potassium acetate buffer (pH 5.4), 1 μl of 10 mM zinc sulfate, and 2 μl of P1 nuclease (6.25 U/μl; Sigma-Aldrich), left at 37°C overnight, and then treated with 2 μl of 0.5 M Tris·HCl (pH 8.3) and 2 μl of alkaline phosphatase (0.31 U/μl; Sigma-Aldrich), and left at 37°C for 2 h. 8-OHdG adducts were determined by use of an 8-OHdG ELISA kit (Japan Institute for the Control of Aging, Hruoka, Japan).

Determination of total protein content. Total protein content was determined with a BCA protein reagent (Sigma-Aldrich).

Study of Gene Expression of Cytochrome cyp 2e1

To determine the effective intake of low-dose aspirin, the expression in the liver of the cyp 2e1 gene was evaluated in the Asp mouse group. A previous study of the interactions between aspirin or sodium salicylate and the oxidative metabolism of several organic chemicals has demonstrated that both of the above-mentioned compounds, i.e., aspirin and sodium salicylate, induce the expression of cytochrome P450 2E1 (cyp 2e1) in rodent liver (10).

Real-time RT-PCR. Total RNA was extracted from liver tissues using an RNeasy mini kit (RNeasy Total RNA System, Qiagen, Courtaboeuf, France), according to the manufacturer’s instructions. Conditions for RT were as described previously (20). Briefly, for cDNA synthesis, 1 μg of total RNA was suspended in a final volume of 19 μl reaction buffer [50 mM Tris·HCl, 40 mM KCl, 5 mM MgCl2, 10 mM diithothreitol, 0.5% Tween 20 (vol/vol), 1 mM dNTP, 20 U RNase inhibitor, and 100 pmol oligo(dT)15 (Roche Diagnostics, Meylan, France)]. After the mixture had reached a temperature of 42°C, 50 U of Expand Reverse Transcriptase (Roche Diagnostics) were added to each tube and incubated for 60 min at 42°C. The reaction was stopped by denaturing the enzyme at 95°C for 5 min. Analysis for cyp 2e1 and glyceraldehyde-3-phosphate dehydrogenase (gapdh) cDNA was performed by using Power SYBR Green PCR Master Mix Applied Biosystems 7500 (Applied France SA, Courtaboeuf, France). Specific primer pairs and PCR thermocycling conditions were as previously described by Loephen et al. (32). The primer sequences synthesized (Prologr France SAS, Paris, France) were as follows: cyp 2e1 (sense: 5′-TCC GTA AGT ATC TTC CGT GA-3′, antisense: 5′-GTA ATC GAA GCC TGT GGT GA-3′); gapdh (sense: 5′-ACC ACA GTC CAT GCC ATC AC-3′, antisense: 5′-50-TCC ACC ACC CTG TTC CTG TA-3′). Gapdh was used as a housekeeping gene to account for any variance in the quality of mRNA and the amount of cDNA input. Following the method of Zhang et al. (54), standard curves were generated by plotting the threshold cycle (Ct) values vs. the log of the amount of RNA input equivalent for each transcript, and denaturation profiles were constructed to determine primer pair specificity. The difference in target gene cyp 2e1 mRNA expression between Asp-mice and Non-Asp-treated mice, corrected by the gapdh level, was determined by using the equation 2−ΔΔCt, where ΔCt = Ct(cyp2e1)−Ct(gapdh), and ΔΔCt = ΔCt(cyp2e1)−ΔCt(gapdh).

Data Analysis

For the investigation of alterations in the EDR, the Emax values were expressed as means ± SE. Statistical analysis was performed by one-way ANOVA. The software used was SPSS for Windows (SPSS, v11, 2002; Paris, France).

For the oxidative stress study, the results were expressed as mean values ± SD and range (minimum-maximum value). Statistical analyses were performed by the Mann-Whitney U-test. The software used was SPSS for Windows (SPSS, v10.05, 2000; Paris, France). Statistically significant differences were reported in terms of a P value of <0.05.

RESULTS

Effects of Age on Aortic EDR and Associated Histological Changes

Endothelial dysfunction. Although body weight increased with age, there were no differences in the glycemic and lipid profiles of 12-, 60-, and 84-wk-old mice (Table 1). A vascular responsiveness analysis demonstrated that the aortic contraction response to Phe as well as to EiD induced by SNP remained stable in the different age groups but that the Emax ACh-induced relaxation gradually decreased with age (Table 2). The Emax ACh-induced relaxation differed significantly between the 12- and 60-wk-old mice and between the 12- and 84-wk-old mice (P < 0.05) (Fig. 1A). No significant decrease in EDR was found between the 12- and 36-wk-old mice.

Histological changes. In parallel to the age-related EDR alterations, aortic structural changes were observed in the older mice (84-wk-old animals). A comparison was then made of the histological changes observed between the 12- and 84-wk-old mice.

Table 1. Weight, glycemic, and lipid profiles in mice of different ages

<table>
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<tr>
<th>Mouse Age, wk</th>
<th>12</th>
<th>60</th>
<th>84</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>Weight, g</td>
<td>22.2 ± 1.5</td>
<td>29 ± 2.9*</td>
<td>30.3 ± 3.7*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Glycemia, g/l</td>
<td>1.6 ± 0.3</td>
<td>1.5 ± 0.3</td>
<td>1.4 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>Cholesterol, g/l</td>
<td>1.2 ± 0.1</td>
<td>1.1 ± 0.2</td>
<td>1.2 ± 0.1</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides, g/l</td>
<td>0.8 ± 0.2</td>
<td>0.7 ± 0.2</td>
<td>0.6 ± 0.2</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are as means ± SE. *P < 0.001.
The mean intima/media thickness was significantly increased in the 84-wk-old mice (82 ± 100/140 n.m) compared with that in the 12-wk-old mice (52 ± 6 μm; P < 0.05) (Fig. 1, a and b). The orcein-stained elastin fibers (Fig. 1B, c and d) were disorganized and disrupted. The collagen III content formed a network perpendicular to the elastin fibers (Fig. 1B, e and f). The aortic medial vascular smooth muscle cell samples from the older mice were fewer in number and were scattered within the media compared with those from the younger mice (Fig. 1B, g and h). No atherosclerotic development was observed irrespective of the animal’s age.

Effect of Short-Term (8 Wk) Low-Dose Aspirin Treatment (Initiated in 60-Wk-Old Mice) on Endothelial Dysfunction, Age-Associated Histological Changes, and Modifications Due to Oxidative Stress

Since endothelial dysfunction had been established in middle-aged mice (60-wk-old), we decided to investigate the effect of low-dose aspirin treatment on EDR alteration in this age group. After 8 wk of aspirin treatment, no significant difference in body weight was found between the 68-wk-old (60 ± 8 wk) Asp and the No Asp mice receiving regular drinking water (29.09 ± 1.08 vs. 30.04 ± 0.87 g, respectively; P = 0.498).

To determine the effective intake of low-dose aspirin, cyp 2e1 gene expression was evaluated in the livers of 68-wk-old Asp and No Asp mice by real-time quantitative mRNA-PCR analysis. As shown in Fig. 2, a statistically significant ~10-fold increase in cyp 2e1 transcripts was observed in 68-wk-old mice exposed to low-dose aspirin treatment compared with controls, thereby demonstrating the effective intake and therapeutic effect of this compound (P < 0.001).

Endothelial dysfunction. The responsiveness to cumulative Phe concentrations as assessed by E_{max} showed no difference

Table 2. Decrease in endothelium-dependent (Ach-induced) relaxation with age in different groups of mice

<table>
<thead>
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<th>Mouse Age, wk</th>
<th>12</th>
<th>36</th>
<th>60</th>
<th>84</th>
</tr>
</thead>
<tbody>
<tr>
<td>E_{max} Phe contraction, %</td>
<td>184±18</td>
<td>167±11</td>
<td>180±16</td>
<td>191±10</td>
</tr>
<tr>
<td>E_{max} SNP relaxation, %</td>
<td>121±5</td>
<td>131±15</td>
<td>112±2</td>
<td>127±9</td>
</tr>
<tr>
<td>E_{max} ACh relaxation, %</td>
<td>75±5</td>
<td>68±7</td>
<td>59±4*</td>
<td>48±3*</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SE of experiments performed in 6 mice for each age group. E_{max}, maximal effect; Phe, phenylephrine; SNP, sodium nitroprusside; ACh, acetylcholine. *P < 0.05 compared to 12-wk-old animals.

Fig. 1. Effect of age on aortic endothelial dysfunction and associated histological changes. A: age-related endothelial-dependent relaxation (EDR) alteration. The maximal ACh-induced EDR was lower in 60- and 84-wk-old mice compared with 12-wk-old mice (*P < 0.05). No significant EDR impairment was found between 12- and 36-wk-old mice. Aortic structural changes as a result of age. Aortic structural changes were analyzed in 12- and 84-wk-old mice. Histological studies using hematoxylin-eosin-safranin (HES; a and b), orcein staining (c and d), and the Volgens-Gomori method (e and f) were performed for elastin fiber and collagen III analysis, respectively. α-Smooth muscle actin was evidenced using an anti-α-smooth muscle actin antibody (g and h). Arrows indicate structural changes.

C: aortic thickening with age. Intima/media aortic mean (maximal/minimal) thickening was significantly increased in 84-wk-old mice compared with 12-wk-old mice (*P < 0.05). The results are expressed as means ± SE of experiments performed on 5 mice for each age group.
LOW-DOSE ASPIRIN PREVENTS AGE-RELATED ENDOTHELIAL DYSFUNCTION

Fig. 2. Eight weeks of low-dose aspirin treatment initiated in 60-wk-old mice and the resulting increase in cyp 2e1 transcripts. To determine the effective intake of low-dose aspirin, the expression of the cyp 2e1 gene in the liver was evaluated. Real-time quantitative mRNA-PCR analysis of cytochrome P450 2e1 (cyp 2e1) mRNA levels in the livers of 68-wk-old no-aspirin (No Asp) mice was carried out and in those of 68-wk-old aspirin-receiving (Asp) mice. These values have been shown in terms of the mean ± SD of 6 vs. 5 mice for the No Asp and the Asp groups, respectively (**P < 0.001).

between the No Asp and the Asp groups. SNP-induced EiDR did not differ between the two groups [No Asp: 130 ± 6% vs. Asp: 114 ± 5%; not significant (NS)]. However, the decrease in ACh-induced EDR was significantly higher in the Asp group than in the No Asp group, with an ACh E max of 83.9 ± 4 vs. 66.3 ± 5% (P < 0.05) (Fig. 3A), suggesting that low-dose aspirin administered over an 8-wk period is able to prevent age-related endothelial dysfunction when treatment is initiated in 60-wk-old mice. The E max, ACh-induced relaxation did not differ significantly between the group of 12-wk-old mice that did not receive aspirin and those in the 68-wk-old Asp group (75 ± 5 vs. 83.9 ± 4%; P = NS) (Fig. 3B).

Histological changes. We also investigated whether the age-related decline in endothelial function and its improvement in response to low-dose aspirin treatment were associated with morphological vascular changes. The aortic histological study revealed some differences between the No Asp group and the Asp group in this regard (Fig. 3C, a and b). The elastin fibers were disorganized and disrupted in a similar manner in both groups (Fig. 3C, c and d). The staining intensity regarding the collagen III content was reduced in the Asp group and did not form a network perpendicular to the elastin fibers (Fig. 3C, e and f). No difference was found in the α-actin smooth muscle staining (Fig. 3C, g and h). In addition, the mean intima/media thickness did not differ between the two groups (P = NS) (Fig. 3D).

Oxidative stress changes. After 8 wk of low-dose aspirin treatment, 8-OHdG concentrations in the aorta were significantly lower in the Asp group compared with the No Asp group (12.5 ± 3.5 ng/μg DNA vs. 14.8 ± 4.4 ng/μg DNA, respectively; P < 0.05) (Fig. 3E). The aortic MDA concentrations did not decrease in the Asp group (1.01 ± 0.4 μM/mg protein vs. 0.99 ± 0.6 μM/mg protein; P = NS).

Effect on Endothelial Dysfunction of Long-Term (16 Wk) Low-Dose Aspirin Treatment Initiated in 60- and 96-Wk-Old Mice

After doubling the length of low-dose aspirin treatment initiated in 60-wk-old mice, i.e., for 16 wk instead of 8 wk, the protective effect of aspirin on EDR remained present and was relatively stable (E max = 79.9 ± 5.5 vs. 83.9 ± 4%, respectively; P = NS). However, when aspirin treatment was initiated in the 96-wk-old group, even for the longer period of 16 wk, the positive effect of aspirin treatment was not significant. In the 112-wk-old mice (96-wk-old mice + 16 wk of aspirin treatment), the E max Phe-induced contraction amounted to 184 ± 21% in the Asp group vs. 206 ± 7% in the No Asp group (P = NS), and the SNP-induced E max relaxation amounted to 111 ± 21 vs. 111.3 ± 3%, respectively (P = NS). The EDR status for ACh-induced relaxation also remained unchanged when 96-wk-old mice were treated for 16 wk; P = NS (Fig. 4). This result suggests that the protective effect of low-dose aspirin on endothelial dysfunction is greater when treatment is initiated sufficiently early on in the animal’s life.

DISCUSSION

The present study demonstrates that low-dose aspirin intake improves endothelial function when initiated in 60-wk-old-mice and that this effect is associated with a decrease in oxidative stress.

Several reports suggest that there may be an age-associated endothelial dysfunction in humans and in rats (29–31). The present results demonstrate an age-related impairment in endothelial function in C57B/6J mice with physiological aging. The decrease in EDR was found to gradually decline with age, and the E max relaxation was significantly different in the 60-wk-old compared with the 12-wk-old mice. The age-related histological modifications observed in the aorta corresponded to those observed in previous reports, namely an aortic intima/media thickening with semi-quantitative and qualitative alterations in the elastin and collagen fibers (31).

In humans, primary prevention trials have shown that the beneficial effects of low-dose aspirin treatment are maximal in middle-aged adults, i.e., persons aged 50 years or over. In our study, the E max, EDR differed significantly between the 60-wk-old compared with the 12-wk-old mice. Bearing this in mind, we decided to evaluate the effect of low-dose aspirin treatment in middle-aged mice (i.e., 60-wk-old mice at initiation of low-dose aspirin treatment) over a 2-mo period based on the approach of Cyrus et al. (8, 9). The increase in the hepatic induction of cyp 2e1 gene expression in 68-wk-old Asp mice (after 8 wk of aspirin treatment) compared with 68-wk-old No Asp mice showed that daily low-dose aspirin treatment had a therapeutic effect: after intake in the drinking water, the compound became enzymatically converted into reactive metabolites in the liver. Following the oral intake of low-dose aspirin, we observed the complete prevention of vascular wall changes and a significant decrease in age-induced oxidative stress and associated damage to the aorta, as evaluated through the formation of oxidative 8-OHdG adducts.

It is important to underline that the present results demonstrate the positive effect of low-dose aspirin on endothelial function in an animal model of physiological aging in the present study in C57B/6J mice that do not develop atherosclerosis, obesity, or hypertension with age. The functional changes observed following the administration of low-dose aspirin were associated with a decrease of collagen distribution in the media. A decrease in aortic collagen content following high-dose aspirin treatment has been previously described in an
Fig. 3. Effect of 8 wk of low-dose aspirin treatment on EDR alteration, associated histological changes, and oxidative stress modifications. Eight weeks of low-dose aspirin treatment initiated in 60-wk-old mice prevented age-related endothelial dysfunction (A). The maximal ACh-induced EDR reduction in middle-aged mice was prevented by low-dose aspirin treatment (3–4 mg·kg⁻¹·day⁻¹) (*P < 0.05). The maximal effect (Emax) ACh-induced relaxation did not differ significantly between the (young) 12-wk-old mice group that did not receive aspirin and the middle-aged Asp 68-wk-old group.

C: aortic structural changes with aging. Aortic structural changes with aging were analyzed in 68-wk-old mice after 8 wk of Asp (initiated at 60 wk of age) or No Asp treatment. A histological study was performed in this respect. Aortic morphology was studied by HES (a and b) and that of the elastin fibers by orcein staining (c and d), whereas collagen III content was assessed by the Volgens-Gomori method (e and f). α-Smooth muscle actin was evidenced using an anti-α-smooth muscle actin antibody (g and h). Arrows indicate structural change prevention by aspirin. D: aortic thickening after 8 wk of low-dose aspirin treatment. No significant difference was observed in the aortic arch mean (maximal/minimal) thickening between the 2 groups (No Asp and Asp). The results are expressed as means ± SE of experiments performed on 5 mice in each age group. E: 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels after 8 wk of low-dose aspirin treatment. 8-OHdG concentrations were found to be significantly higher in the Asp group (*P < 0.05).
Atherosclerotic rodent model (28). The decrease in collagen content may contribute to the enhanced bioavailability of the free radical gas, NO. NO is a bioactive mediator formed in the vasculature that plays an important role in regulating vascular tone in vivo. NO removal may occur through its reaction with free radicals formed by enzymes that are upregulated as part of the vascular degeneration process. The induction of free radical pathways that can consume NO include nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, lipoxygenases, heme peroxidases, and COXs (52). An involvement of platelet COX in regulating NO bioactivity has been indicated by numerous clinical studies such as that of Williams et al. (52). Aspirin is routinely prescribed to patients with vascular disease as an inhibitor of COX-1. It has been suggested that aspirin may improve NO bioactivity by inhibiting platelet COX-1-dependent NO consumption.

Indeed, as regards advanced glycation end products (AGEs), during aging cross-linking collagen leads to NO scavenging (2, 6). AGEs are involved in endothelial dysfunction, in particular in cases of diabetes mellitus (50). The engagement to their specific AGE receptor, RAGE, is followed by a series of cell reactions including NADPH-oxidase activation, reactive oxygen intermediate formation, and gene transcription such as VCAM-1 and VEGF that could induce vascular aging (4, 5, 51).

Clinical analysis has demonstrated that the age-associated reduction of brachial flow-mediated dilatation is related to the increase in endothelial nitrotyrosine which could serve as oxidative stress indicator (13). A reduction in endothelium-dependent dilatation with aging is inversely related to plasma markers of oxidative stress and is reversed by the administration of high doses of vitamin C, a potent antioxidant (17). 8 Iso-PGF\textsubscript{2\alpha} is an indicator of oxidative stress, and its levels in the urine decrease in elderly people treated with low-dose aspirin. Animal studies have demonstrated that O\textsubscript{2} generation increases with age in the rat aorta and contributes to the age-related decrease in vasodilatation (25). The incubation of tail artery segments with MDA has been found to reduce ACh-induced relaxation in both younger and older rats (43). In the present work, we have postulated that low-dose aspirin treatment could prevent such age-related oxidative damage to the vascular wall. The effects of low-dose aspirin treatment on MDA and 8-OHdG were quantified. The choice of both these biological markers of oxidative stress was mainly connected with the ability of reactive oxygen species formed within eukaryotic cells to oxidize macromolecules (lipids, nucleic acids, and proteins) that can play an important role in protecting endothelial cells from oxidative damage (11, 23, 46). The alteration in membrane phospholipids through lipid peroxidation is considered to be one of the key events in oxidative damage (12). The determination of MDA in a variety of pathological conditions has also been extensively performed to assess the extent of tissue damage caused by lipid peroxidation. Among the oxidized bases, 8-OHdG has been frequently considered as a marker for oxidative DNA damage (21, 35, 37). The hepatic gene expression of cytochrome P450 (CYP) 2E1 has also been studied, and it has been reported that the induction of CYP 2E1 by aspirin in rodents results in a significant increase in mRNA levels (10). We demonstrated a significant decrease in 8-OHdG concentrations in the aortas of the Asp group compared with those of the No Asp group, which further supported the present hypothesis. 8-OHdG is a specific marker of oxidative stress and is the product of the specific attack of a hydroxyl radical on the DNA (41). Levels of 8-OHdG rapidly increase after the induction of oxidative stress especially during glutathione (GSH) depletion. Aspirin treatment significantly decreased lipid peroxidation and significantly restored reduced GSH content (47). Both a rapid increase in 8-OHdG levels following the induction of oxidative stress and the specific activity of aspirin on GSH could explain the decrease in 8-OHdG levels that was demonstrated, without any difference in MDA aortic levels being observed between the two groups. The link between the functional change in ACh responsiveness and a reduction in oxidative stress has been reported by Wu et al. (53). A previous study by Rahmani et al. (42) was performed to investigate the involvement of the endothelium in aspirin-mediated effects by recording the active tension of denuded and non-denuded aortic rings from spontaneously hypertensive rats. The results showed that, in the presence of aspirin, denuded and non-denuded aortic rings from spontaneously hypertensive rats generated significantly higher active tension than in the absence of aspirin. The reactivity of aortic rings from Wistar-Kyoto control animals was not found to be significantly altered in the presence of aspirin, suggesting that the latter can also modulate aortic contractility through mechanisms other than its effects on the metabolites of arachidoncic acid (42). We investigated the hypothesis that the longer the low-dose aspirin treatment, the better was the EDR. In middle-aged mice (60 wk old at the initiation of low-dose aspirin treatment), after 16 wk of this treatment, the positive effect on the EDR remained present and stable, compared with the shorter treatment of 8 wk. We then tested the hypothesis that aspirin treatment could improve EDR status in older mice. In older animals (96-wk-old), the low-dose aspirin treatment over a 16-wk period did not significantly improve EDR status. This result suggests that this treatment must be initiated sufficiently early on in the mouse’s life for it to improve endothelial function.

In conclusion, our study shows that low-dose aspirin given daily over a 16-wk period improves endothelial function in middle-aged animals and that this positive effect may be mediated, at least in part, by a protective effect against oxiada-
tive stress. Therefore, in the primary prevention of cardiovascular disease, the efficiency of low-dose aspirin treatment may be linked to the prevention of vascular wall changes through limitation of the damage caused by oxidative stress. Further investigations on long-term low-dose aspirin treatment are therefore advocated in humans.

REFERENCES


