Aging associated with mild dyslipidemia reveals that COX-2 preserves dilation despite endothelial dysfunction

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Gendron MÈ, Thorin-Trescases N, Villeneuve L, Thorin E. Aging associated with mild dyslipidemia reveals that COX-2 preserves dilation despite endothelial dysfunction. Am J Physiol Heart Circ Physiol 292: H451–H458, 2007. First published September 15, 2006; doi:10.1152/ajpheart.00551.2006.—The endothelial function declines with age, and dyslipidemia (DL) has been shown to hasten this process by favoring the generation of reactive oxygen species (ROS). Cyclooxygenase-2 (COX-2) can be induced by ROS, but its contribution to the regulation of the endothelial function is unknown. Since COX-2 inhibitors may be deleterious to the cardiovascular system, we hypothesized that DL leads to ROS-dependent endothelial damage and a protective upregulation of COX-2. Dilations to acetylcholine (ACh) of renal arteries isolated from 3-, 6-, and 12-mo-old wild-type (WT) and DL mice expressing the human ApoB-100 were recorded with or without COX inhibitors and the antioxidant N-acetyl-l-cystein (NAC). Nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF) were inhibited using Nω-nitro-l-arginine (l-NNA) and a depolarizing solution, respectively. In WT mice, the dilation to ACh declined at 12 mo but was insensitive to COX-1/2 inhibition alone or with NAC. DL led to an early endothelial dysfunction at 6 mo, normalized, however, by NAC. At 12 mo, vascular sensitivity to ACh was further reduced by DL. At this age, selective COX-2 inhibition reduced the dilation, whereas addition of NAC improved it. In 3- and 6-mo-old WT mice, l-NNA significantly reduced the dilation, whereas it limited the dilation only in 3-mo-old DL mice. EDHF-dependent dilation remains identical in both groups. These data suggest that COX-2 activity confers endothelium-dependent vasodilatory function in aged DL mice in the face of a prooxidative environment. Upregulation of this pathway compensates for the early loss of the contribution of NO in DL mice.

oxidative stress; endothelial function; cyclooxygenase and N-acetyl-l-cystein

RISK FACTORS FOR CARDIOVASCULAR diseases are associated with an early endothelial dysfunction. Previous studies on diabetes, hypertension, atherosclerosis, and hypercholesterolemia examined possible alterations in nitric oxide (NO) and PGI2 responses (3, 8, 14, 19, 28, 34). It was reported that oxidative stress was a key element contributing to endothelial cell damage and enhancement of PGI2 formation (17, 30). The recent clinical trials demonstrating that cyclooxygenase-2 (COX-2) inhibition was associated with cardiovascular events in an elderly population with combined risk factors for cardiovascular diseases (26, 32) strongly emphasize the cardiovascular protective role of prostanoids, most likely PGI2. COX-2 inhibition could have deleterious cardiovascular effects by reducing PGI2 production (27), favoring thromboxane A2 function (1), increasing blood pressure (35), decreasing angiogenesis, and destabilizing the plaque (12).

Whereas some preclinical data incriminate COX-2 activity as deleterious and proatherogenic (5–7), others (11, 12, 29) support the protective role of PGI2 in atherogenic conditions. To increase the level of complexity, oxidative stress favors the expression of an inflammatory phenotype that leads to the induction of COX-2 (11, 22, 29). Thus far, the protective role of PGI2 is opposed to the deleterious effects of thromboxane A2 by regulating platelet aggregation and thrombosis (27). The role of COX-2-derived PGI2 as endothelium-derived relaxing factor still needs to be clarified. In animal models, large arteries such as the aorta and renal arteries are vessels of choice to study the contribution of prostanooids in the regulation of vascular tone. The contractile and dilatory responses of these vessels are sensitive to indomethacin and are affected by pathological conditions (34). We hypothesized that dyslipidemia is associated with oxidative stress that induces an endothelial damage characterized by a compensatory and protective upregulation of COX-2. To validate this hypothesis, we investigated the evolution of the endothelial function of the renal artery isolated from wild-type (WT) and dyslipidemic (DL) mice through age.

MATERIALS AND METHODS

Three-, six-, and twelve-month-old male C57Bl/6 mice (WT, Charles River, St. Constant, QC, Canada) were used as control and compared with 3-, 6-, and 12-mo-old male DL mice expressing the human apolipoprotein B-100 gene (hApoB⁺/⁺) (20). Cholesterol and triglyceride concentrations were increased in the plasma of DL compared with WT mice (Table 1). The procedures and protocols were performed in accordance with the Guide for the Care and Use of Laboratory Animals of Canada and were approved by the Animal Care and Use Committee of the Montreal Heart Institute (University of Montreal, Montreal, QC, Canada).

Vascular reactivity studies. Experiments were conducted in isolated and pressurized (100 mmHg) mouse renal arteries (external diameter ~400 μm) as previously described (Living System, Burlington, VT) (16, 20). After a 50-min equilibration period, the resting myogenic tone (a reduction in diameter induced by the rise in intraluminal pressure) was measured. Renal arteries were then pre-constricted by phenylephrine (PE; 30 μmol/l, Sigma, St. Louis, MO), and concentration-response curves to ACh (0.001–30 μmol/l, Sigma) were constructed. The contribution of PGI2 to ACh-induced dilation was determined using indomethacin (10 μmol/l, Sigma), a nonselective inhibitor of COX; NS-398 (10 μmol/l, Cayman Chemical, Ann Arbor, MI), a selective COX-2 inhibitor; or valeryl salicylate (1

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mmol/L; Cayman Chemical), a selective COX-1 inhibitor. The contribution of NO to ACh-induced dilation was determined in the presence of Nω-nitro-arginine (L-NNA; 10 μmol/L; Sigma), a NO synthase (NOS) inhibitor. The contribution of endothelium-derived hyperpolarizing factor (EDHF) to ACh-induced dilation was determined in the presence of a depolarizing solution containing 40 mM KCl. The effects of endogenous free radicals were inhibited by acute pretreatment with a depolarizing solution containing 40 mM KCl. The presence of a depolarizing solution containing 40 mM KCl. The effects of endogenous free radicals were inhibited by acute pretreatment with sodium nitroprusside (0.001–300 μmol/L; Sigma) for 30 min before the dose-response curve was performed. Only one concentration-response curve was performed on each vessel. The endothelium-independent dilation was assessed using sodium nitroprusside (0.001–300 μmol/L; Sigma).

Aortic endothelial cell culture and Western blot analysis. Endothelial cells were cultured from the mouse aorta (MAEC) by explant by using a method slightly modified from a previous work (33). No difference in growth rate was detected. The endothelial origin of the cells (>95%) was confirmed by double immunostaining for von Willebrand factor and platelet endothelial cell adhesion molecule (CD31) (data not shown). MAEC were used after the first passage only. For Western blot analysis, proteins (35 μg/lane) were separated and transferred to nitrocellulose. Blots were incubated with the primary antibodies [rabbit polyclonal anti-COX-2, 1:1,000, Cayman Chemical; rabbit polyclonal anti-COX-1, 1:250, Cayman Chemical; rabbit polyclonal anti-endothelial NOS (eNOS), 1:1,200, BD Transduction Laboratories, San Jose, CA]. Equal protein loading was verified by using β-actin, rabbit polyclonal anti-β-actin, 1:1,000, Abcam, Cambridge, MA).

Immunofluorescence studies. Sections (14 μm) of the aorta were incubated with anti-COX-1 (dilution 1:100; Cayman Chemical), anti-COX-2 (dilution 1:300; Cayman Chemical), or anti-4-hydroxynonenal (HNE; dilution 1:400; Alpha Diagnostic International, San Antonio, TX) diluted in PBS containing 1% normal donkey serum and 0.1% (vol/vol) Triton X-100. The slides were washed and incubated with a 1:500 (vol/vol) dilution of the appropriate secondary antibody (Alexa 647-conjugated donkey anti-rabbit antibody; Invitrogen). For each secondary antibody, negative control experiments were performed in the absence of primary antibody. Fluorescence was visualized with the use of a scanning confocal microscope (model LSM 510, Zeiss, Oberkochen, Germany).

### Table 1. Body weight, total cholesterol, and level of triglycerides in 3-, 6-, and 12-mo-old WT and DL hApoB<sup>++/+</sup> mice

<table>
<thead>
<tr>
<th>Age, mo</th>
<th>Weight, g</th>
<th>Total Cholesterol, mg/dl</th>
<th>Triglycerides, mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>27±1</td>
<td>107±8</td>
<td>147±24</td>
</tr>
<tr>
<td>6</td>
<td>38±1*</td>
<td>103±6</td>
<td>92±7</td>
</tr>
<tr>
<td>12</td>
<td>46±2†</td>
<td>148±15</td>
<td>64±7</td>
</tr>
<tr>
<td>hApoB&lt;sup&gt;++/+&lt;/sup&gt; (DL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>32±1</td>
<td>159±14‡</td>
<td>204±38</td>
</tr>
<tr>
<td>6</td>
<td>42±1*</td>
<td>168±12‡</td>
<td>305±47‡</td>
</tr>
<tr>
<td>12</td>
<td>51±2†</td>
<td>227±18‡</td>
<td>288±76‡</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 10 mice per group. WT, wild type; DL, dyslipidemic. *P < 0.05 compared with 3-mo-old mice; †P < 0.05 compared with 3- and 6-mo-old mice; ‡P < 0.05 compared with age-matched WT mice.

Statistical analysis. In every case, n refers to the number of animals used in each protocol. Continuous variables are expressed as means ± SE. Half-maximum effective concentration (EC<sub>50</sub>) of ACh was measured from individual concentration-response curves only when a maximal response was obtained. The pD<sub>2</sub> value, the negative log of the EC<sub>50</sub>, was obtained. At the end of the protocol, the maximal diameter (∆<sub>d</sub>) was determined by changing the physiological saline solution (PSS) to a Ca<sup>2+</sup>-free PSS containing sodium nitroprusside (10 μmol/L; Sigma) and EGTA [ethylene glycol-bis(β-aminoethyll)ether]-N,N',N'-tetracetic acid (1 mmol/L; Sigma). For each protocol, basal diameter in no-flow condition was determined at the end of the 50-min equilibration period. Myogenic tone was measured at 100 mmHg and expressed as a percentage of the passive diameter (∆<sub>d</sub>). Myogenic tone was calculated according to the following formula: [(passive diameter − diameter at equilibrium/passive diameter) × 100]. ACh-induced dilation is expressed as a percentage of the maximal diameter. ANOVA studies followed by a Scheffe’s F-test were performed to compare the maximal effect (∆<sub>Dmax</sub>; maximal response observed for the highest dose tested) and pD<sub>2</sub> of dose-response curves. Unpaired t-tests were performed for Western blot analysis. Differences were considered to be statistically significant for a P value <0.05.

RESULTS

Baseline parameters. After 50 min of equilibration at an intraluminal pressure of 100 mmHg, internal diameters of isolated WT (n = 32) and DL (n = 32) renal arteries were 335 ± 25 and 354 ± 23 μm, 365 ± 17 and 412 ± 28 μm, and 321 ± 28 and 411 ± 30 μm at 3, 6, and 12 mo of age, respectively (P > 0.05). Significant (P < 0.05) myogenic tone had developed without differences between groups (Table 2). Similarly, the contractions induced by PE or KCl were similar in vessels with a comparable myogenic tone, PE-induced preconstriction, and 40 mM PSS-induced preconstriction measured in renal arteries isolated from 3-, 6-, and 12-mo-old WT and DL hApoB<sup>++/+</sup> mice.

### Table 2. Level of myogenic tone, PE-induced preconstriction, and 40 mM PSS-induced preconstriction measured in renal arteries isolated from 3-, 6-, and 12-mo-old WT and DL hApoB<sup>++/+</sup> mice

<table>
<thead>
<tr>
<th>Age, mo</th>
<th>Myogenic Tone, %</th>
<th>PE Preconstriction, %</th>
<th>KCl Preconstriction, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>17±5</td>
<td>66±6</td>
<td>66±3</td>
</tr>
<tr>
<td>6</td>
<td>33±4</td>
<td>66±5</td>
<td>47±5</td>
</tr>
<tr>
<td>12</td>
<td>36±9</td>
<td>52±5</td>
<td>64±4</td>
</tr>
<tr>
<td>hApoB&lt;sup&gt;++/+&lt;/sup&gt; (DL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>21±6</td>
<td>76±2</td>
<td>62±9</td>
</tr>
<tr>
<td>6</td>
<td>13±5</td>
<td>70±2</td>
<td>68±1</td>
</tr>
<tr>
<td>12</td>
<td>21±6</td>
<td>69±2</td>
<td>66±3</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6–8 mice per group. Myogenic tone expressed as percentage of the maximal dilation induced by a calcium-free physiological saline solution (PSS); PE, phenylephrine.
Evolution of the endothelium-dependent dilation to ACh in renal arteries of WT mice through aging. In PE-precontracted WT arteries, ACh induced a maximal endothelium-dependent dilation (E_max) of the renal artery isolated from 3-mo- old WT arteries, ACh induced a maximal endothelium-dependent dilation of the renal artery isolated from 3-mo-old WT arteries, which was similar at 6 mo (Fig. 1A and Tables 3 and 4). The age-dependent decrease (P < 0.05) in endothelial function was only apparent at 12 mo and was accompanied by a reduced (P < 0.05) vascular sensitivity (pD2) to ACh when compared with 3- and 6-mo-old WT mice (Tables 3 and 4).

The lack of contribution of COX-derived dilatory products during ACh-induced renal artery dilation is highlighted by the use of indomethacin. In vessels isolated from WT mice, indomethacin did not reduce the dilation at all ages (Tables 3 and 4).

To assess the potential deleterious impact of reactive oxygen species (ROS) during ACh-induced dilation, arteries were exposed to NAC in the presence of indomethacin. As revealed in Table 4, NAC did not modify the dilatory response to ACh.

To assess the contribution of NO and the EDHF to the dilation of WT renal arteries, we used l-NNA and a 40 mM KCl depolarizing solution, respectively. At 3 mo of age and further at 6 mo of age, l-NNA reduced (P < 0.05) the sensitivity and the maximal dilation to ACh (Fig. 3). The use of a depolarizing solution also decreased (P < 0.05) the sensitivity and the dilation to ACh in vessels isolated from 3- and 6-mo-old mice. At 12 mo of age, only the depolarizing solution reduced (P < 0.05) the maximal dilation induced by ACh (Tables 3 and 4 and Fig. 3).

Evolution of the endothelium-dependent dilation to ACh in renal arteries of DL mice: importance of ROS and COX-2 activity. ACh induced a maximal endothelium-dependent dilation of the renal artery isolated from 3-mo-old DL mice (Fig. 1B and Table 4), which was identical to the response obtained in vessels isolated from 3-mo-old WT mice (Table 4). In contrast with WT mice, however, the maximal dilation induced by ACh was prematurely reduced (P < 0.05) in vessels isolated from 6-mo-old DL mice (Fig. 1B and Table 4). At 12 mo of age, the maximal dilation was not further reduced—and similar in amplitude to that measured in WT mice—whereas a marked decrease (P < 0.05) in vascular sensitivity to ACh (Table 3) was observed.

Table 3. pD2 values of ACh-induced dilation of pressurized renal arteries of 3-, 6-, and 12-mo-old WT and DL hApoB+/+ mice preconstricted with 30 μM PE

<table>
<thead>
<tr>
<th>Age, mo</th>
<th>Control</th>
<th>Indo</th>
<th>Indo + NAC</th>
<th>l-NNA</th>
<th>KCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>6.7 ± 0.1</td>
<td>6.5 ± 0.2</td>
<td>6.0 ± 0.2c</td>
<td>5.8 ± 0.2c</td>
<td>6.1 ± 0.3c</td>
</tr>
<tr>
<td>6</td>
<td>7.0 ± 0.2</td>
<td>6.6 ± 0.2</td>
<td>6.4 ± 0.1</td>
<td>5.9 ± 0.2c</td>
<td>6.2 ± 0.3c</td>
</tr>
<tr>
<td>12</td>
<td>6.1 ± 0.2e</td>
<td>6.0 ± 0.2</td>
<td>5.9 ± 0.2c</td>
<td>5.9 ± 0.2c</td>
<td>5.5 ± 0.2c</td>
</tr>
<tr>
<td>DL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>6.4 ± 0.2</td>
<td>6.2 ± 0.2</td>
<td>5.7 ± 0.2</td>
<td>5.3 ± 0.1c</td>
<td>6.3 ± 0.2c</td>
</tr>
<tr>
<td>6</td>
<td>6.2 ± 0.1c</td>
<td>6.4 ± 0.4</td>
<td>6.5 ± 0.2</td>
<td>6.0 ± 0.3</td>
<td>5.6 ± 0.3</td>
</tr>
<tr>
<td>12</td>
<td>5.7 ± 0.2c</td>
<td>5.2 ± 0.1ab</td>
<td>5.8 ± 0.3</td>
<td>5.0 ± 0.3</td>
<td>6.1 ± 0.5</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6–8 mice per group. *P < 0.05 compared with WT mice; †P < 0.05 compared with 3-mo-old mice; ‡P < 0.05 compared with 3- and 6-mo-old mice; §P < 0.05 compared with 6-mo-old mice; ¶P < 0.05 compared with control.

Table 4. Maximal dilation values of ACh-induced dilation of pressurized renal arteries of 3-, 6-, and 12-mo-old WT and DL hApoB+/+ mice preconstricted with 30 μM PE

<table>
<thead>
<tr>
<th>Age, mo</th>
<th>Control</th>
<th>Indo</th>
<th>Indo + NAC</th>
<th>l-NNA</th>
<th>KCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>90 ± 5</td>
<td>88 ± 3</td>
<td>86 ± 4</td>
<td>68 ± 4c</td>
<td>35 ± 6c</td>
</tr>
<tr>
<td>6</td>
<td>81 ± 6</td>
<td>95 ± 8</td>
<td>87 ± 3</td>
<td>38 ± 11c</td>
<td>54 ± 4c</td>
</tr>
<tr>
<td>12</td>
<td>64 ± 10c</td>
<td>61 ± 10c</td>
<td>64 ± 9c</td>
<td>59 ± 11</td>
<td>16 ± 4c</td>
</tr>
<tr>
<td>DL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>93 ± 4</td>
<td>85 ± 6</td>
<td>79 ± 10</td>
<td>57 ± 12c</td>
<td>34 ± 9c</td>
</tr>
<tr>
<td>6</td>
<td>61 ± 10bc</td>
<td>66 ± 7a</td>
<td>95 ± 11c</td>
<td>77 ± 6c</td>
<td>36 ± 8c</td>
</tr>
<tr>
<td>12</td>
<td>64 ± 12c</td>
<td>29 ± 13ab</td>
<td>65 ± 13c</td>
<td>41 ± 13d</td>
<td>17 ± 9bc</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6–8 mice per group. *P < 0.05 compared with WT mice; †P < 0.05 compared with 3-mo-old mice; ‡P < 0.05 compared with 3- and 6-mo-old mice; §P < 0.05 compared with 6-mo-old mice; ¶P < 0.05 compared with control; ‡P < 0.05 compared with Indo.

Fig. 1. Acetylcholine-induced dilation of renal arteries isolated from 3-, 6-, and 12-mo-old wild-type mice (WT; A) and dyslipidemic (DL) mice expressing the human apolipoprotein B-100 gene (hApoB+/+; B); n = 6–8. *P < 0.05 compared with 3-mo-old mice.
The premature endothelial dysfunction observed in the renal artery of 6-mo-old mice was insensitive to indomethacin but reversed to normal (a dilation similar in amplitude to that measured at 3 mo of age) by acute exposure of the vessels to NAC (Table 4).

At the age of 12 mo, vascular sensitivity to ACh was reduced ($P < 0.05$), demonstrating that the endothelial dysfunction further worsens with age in DL mice (Table 3). At this age, however, indomethacin strongly limited ($P < 0.05$) the efficacy and potency of ACh (Tables 3 and 4), contrasting with the lack of effect of this inhibitor in WT mice. To determine the COX isoform involved in this response, selective COX-1 and COX-2 inhibitors were used. The deleterious effect of indomethacin on ACh-induced dilation of renal arteries isolated from 12-mo-old DL mice was closely mimicked by the COX-2 inhibitor NS-398, whereas the COX-1 inhibitor valeryl salicylate had no effect (Fig. 2). In the presence of NS-398, the COX-2 inhibitor NS-398, whereas the COX-1 inhibitor valeryl salicylate had no effect (Fig. 2). In the presence of NS-398, the $pD_2$ was $5.0 \pm 0.2$ ($n = 6$; $P < 0.05$ compared with the control condition without inhibitor, $pD_2 5.7 \pm 0.2$; $n = 6$) and similar to the $pD_2$ value obtained in the presence of indomethacin ($pD_2 5.2 \pm 0.1$; $n = 6$), whereas valeryl salicylate had no effect ($pD_2 5.7 \pm 0.2$; $n = 6$).

The protecting effect of NAC observed at 6 mo of age in DL mice was conserved at 12 mo of age in the presence of indomethacin (Tables 3 and 4) and NS-398 ($pD_2 5.6 \pm 0.2$, maximal response of $64 \pm 8$%; $n = 6$).

Finally, we assessed the contribution of NO and EDHF to the dilation induced by ACh of arteries isolated from DL mice. At 3 mo of age, l-NNA and the depolarizing solution reduced ($P < 0.05$) the dilation. At 6 and 12 mo, only the depolarizing solution decreased ($P < 0.05$) the dilation (Tables 3 and 4 and Fig. 3).

**Immunofluorescence and protein expression in cultured endothelial cells.** Immunofluorescence and quantification of protein expression by Western blot analysis in cultured endothelial cells demonstrate that COX-1 (Fig. 4A) and eNOS (Fig. 5) expression did not change between the ages of 3 and 12 mo in endothelial cells isolated from WT mice, whereas the expression of COX-2 increased ($P < 0.05$) significantly at 12 mo (Fig. 4B).

In endothelial cells isolated from DL mice, the expression of COX-1 decreased ($P < 0.05$) only at 12 mo of age (Fig. 4A). The expression of COX-2 was very low at 3 and 6 mo but significantly ($P < 0.05$) increased at 12 mo (Fig. 4B), whereas the level of expression of eNOS declined ($P < 0.05$) at 12 mo compared with the expression quantified at 6 mo (Fig. 5).

We observed a steady immunofluorescence for HNE, an indicator of lipid peroxidation, in aortic sections of WT mice, whereas in DL mice, it increased with age and was maximal at 12 mo (Fig. 6).

**DISCUSSION**

In the present study, we show that the endothelial dysfunction associated with aging is accelerated when associated with dyslipidemia. We report that COX-2 activity is mandatory to maintain some dilatory function in the pro-oxidative environment associated with dyslipidemia. The upregulation of this pathway compensates for the early loss of the contribution of NO in DL mice. The data obtained in isolated renal arteries from WT and DL mice confirm that aging is associated with a reduction of the endothelium-dependent dilation. Our results are therefore in line with the literature (for review, see Ref. 4). In the C57Bl/6 background (WT), however, neither the antioxidant NAC nor COX inhibition by indomethacin had an impact on the dilatory response induced by ACh at all ages. The absence of effect of indomethacin suggests that, during normal aging, dilatory prostanooids do not contribute to the dilation or that their inhibition is compensated by NO and/or EDHF. Our data reveal that the contribution of NO to the dilation induced by ACh increases from the age of 3 mo to 6 mo but is absent at 12 mo, another demonstration of the deleterious effects of aging. In contrast, the contribution of EDHF appears to be constant during normal aging. On the other hand, the absence of effect of the antioxidant NAC on the dilatory response induced by ACh at all ages in WT mice suggests that the rise in oxidative stress is not sufficient to acutely limit the dilatory effects of NO and EDHF.

It is known that risk factors for cardiovascular diseases hasten endothelial dysfunction (2, 10, 13, 18). As we previously reported, dyslipidemia has no deleterious effects on the endothelial function at 3 mo of age (20, 21). As in WT mice, neither indomethacin nor NAC influenced ACh-induced dilation of the renal artery. At 6 mo of age, however, arteries from DL mice showed impaired responses to ACh, 6 mo earlier than that in arteries isolated from WT mice. Whereas the inhibition of COX-1/2 had no effect, NAC normalized the dilation, suggesting that oxidative stress plays a role in the limitation of the dilation to ACh of arteries isolated from 6-mo-old DL mice. This is confirmed by the sustained increase in the expression of HNE in aortic sections of DL mice (Fig. 6), a marker of the accumulation of oxidative damage (15). This is in agreement with our recent observations that ROS production is increased in arteries isolated from aging DL mice in response to angiotensin II when compared with aged WT mice (20). The
deleterious effects of ROS on the dilation induced by ACh may originate from the inactivation of NO (9) or EDHF (20, 23–25). The data obtained in the presence of L-NNA reveal that NO plays a significant dilatory role at 3 mo but that its contribution is abrogated at 6 and 12 mo. The contribution of EDHF, however, appears to be conserved.

At the age of 12 mo, arteries isolated from DL mice became highly sensitive to indomethacin: inhibition of COX-1/2 reduced by more than 50% the dilation induced by ACh. Most importantly, the effects of indomethacin were reproduced by the selective COX-2 inhibitor NS-398 but not by the selective COX-1 inhibitor (valeryl salicylate). This strongly suggests that COX-2-derived prostanoids are important dilators in the renal artery isolated from aging DL mice. The cumulative negative impact of ROS is, however, still present since NAC improved dilation in the presence of indomethacin or NS-398, demonstrating that the origin of the oxidative stress induced by dyslipidemia is independent of COX activity.

This contrasts with the lack of effect of indomethacin as well as NAC in arteries isolated from 12-mo-old WT mice. It is, therefore, tempting to speculate that dyslipidemia leads to an early excess of free radicals by inactivating the buffering antioxidative capacity of the cells and/or increasing their production: this may, in turn, induce the expression of COX-2 that would act as a protective mechanism. To confirm this hypothesis, one would have to treat mice with a potent antioxidant from the age of 3 mo onward. Our data collected in cultured endothelial cells and aortic sections, however, reveal that the expression of COX-2 increases earlier in WT than in DL mice. Hence, oxidative stress (at least that associated with dyslipidemia) may not be directly responsible for the increased expression of COX-2. It is more likely that, by inactivating NO, ROS reveal the dilatory action of COX-2-derived prostanoids, possibly prostacyclin, although this was not determined in this study. In addition to potentially inactivating NO, we observed that the expression of eNOS decreases with age, a trend that is significant in DL mice. In healthy vessels isolated from WT mice, the lack of overt oxidative stress likely protects the “classical” dilatory pathways, i.e., NO and EDHF, although the latter appears rather insensitive to dyslipidemia.

Although we observed a rise in COX-2 expression, we were unable to detect inducible NOS in our cultured endothelial cells as well as in intact arteries by immunohistochemistry (data not
Fig. 4. Immunofluorescence from aortic sections and Western blot for COX-1 (A) and COX-2 (B) from cultured mouse aorta endothelial cells (MAEC) of 3-, 6-, and 12-mo-old WT and DL hApoB^{+/+} mice. β-actin was used to normalize for loading variations; n = 3 blots. *P < 0.05 compared with 3-mo-old mice; †P < 0.05 compared with 6-mo-old mice.
shown). An uncontrolled excessive NO production could lead to the formation of damaging peroxynitrites (9), which have been shown to favor nitration and inactivation of the prostacyclin synthase (31). The contrary has, however, also been reported, that is, an increased PGI2 production in conditions of excess NO and ROS production in human sepsis and after LPS treatment of bovine aortic muscle cells (17, 30). Hence, the origin of the excess in free radicals sensitive to NAC needs to be resolved.

In conclusion, our data highlight the importance of investigating the impact of dyslipidemia through age. To investigate such dynamic processes, one has to consider age and risk factors in the algorithm of the experimental design to be able to determine the biological changes that take place. Our data reinforce the current concept that uncontrolled oxidative stress takes place with risk factors and suggest that COX-2-derived prostanoids become essential endothelium-protecting autacoids in DL mice.

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