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Aspirin resistance with genetic dyslipidemia: contribution of vascular thromboxane generation

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Frisbee JC, Goodwill AG, Stapleton PA, Frisbee SJ, d’Audiffret AC. Aspirin resistance with genetic dyslipidemia: contribution of vascular thromboxane generation. Physiol Genomics 42: 331–341, 2010. First published June 8, 2010; doi:10.1152/physiolgenomics.00090.2010.—One clinical intervention against the negative outcomes associated with atherothrombotic vascular disease (AVD) is low-dose, chronic aspirin therapy. However, epidemiological studies suggest that recurrence of adverse vascular events with aspirin therapy is growing and associated with therapy duration. The contributors to this outcome are unclear and include poor patient compliance and aspirin-resistant platelet thromboxane A2 (TxA2) production. Based on previous results in hypercholesterolemic mice, we hypothesized that elevated aspirin-insensitive arachidonic acid (AA)-induced TxA2 production by the vascular endothelium contributes to aspirin resistance in AVD independent of platelet behavior. AA-induced dilation was blunted in aortic rings and in arterioles from apolipoprotein E (ApoE) and low-density lipoprotein receptor (LDLR) gene deletion mice (vs. C57/Bl6/J), partially due to elevated TxA2 production. Acute inhibition of cyclooxygenases or TxA2 synthase attenuated the increased TxA2 production in ApoE and LDLR and improved AA-induced dilation, responses that were mirrored by chronic treatment with low-dose aspirin of 16 wk duration. However, this effect was not temporally stable, and, with longer-duration therapy, the beneficial impact of aspirin on outcomes diminished. A similar, though less robust, pattern to the impact of chronic aspirin therapy on vascular outcomes was identified with chronic antioxidant treatment (TEMPOL). These results suggest that in dyslipidemic mice, the beneficial impact of chronic aspirin therapy on improving vascular outcomes decay with time and that a contributing element to subsequent negative vascular events may be the development of aspirin-resistant TxA2 production by the vasculature itself.

Peripheral vascular disease; arachidonic acid metabolism; regulation of vascular tone; atherothrombotic vascular disease

Chronic aspirin therapy is one of the most commonly prescribed prophylactic regimens for the clinical prevention of atherothrombosis and has also been shown to be effective in the acute treatment for, or primary/secondary prevention of, myocardial infarction and stroke (4, 22, 35). The mechanistic basis for this therapy resides in the ability of chronic aspirin therapy to abrogate the production of thromboxane A2 (TxA2) within the circulating platelets of the patient, thus attenuating the impact of TxA2 on platelet aggregation and the potential for the initiation of thrombo-embolism formation. However, while previous study has demonstrated that chronic aspirin therapy can be highly effective in preventing the recurrence of atherothrombotic events, myocardial infarction, and stroke, an increasing number of studies have suggested that a population of individuals afflicted with atherothrombotic vascular disease (AVD) exist for whom aspirin therapy demonstrates a declining efficacy in relation to the duration of the treatment regimen (14, 17). It has been estimated that a many as 40% of individuals afflicted with AVD receiving chronic aspirin treatment may manifest this growing resistance to the intended ameliorative effects (22). Aspirin resistance, which can be conceptualized as an idiopathic diminished antiatherothrombotic response in the face of continuous, therapeutically significant, aspirin levels has been demonstrated to be clinically relevant in that ~40% of the aspirin-resistant population experiences a major secondary cardiovascular event within 2 yr following an initial event compared with ~4% in the population of individuals that are sensitive to aspirin therapy (22). While a portion of aspirin resistance in this population may reside within patient compliance and dosing issues (8), investigation into aspirin resistance may suffer from a lack of a true analytical test and definition for classifying an individual as “aspirin resistant” (2).

The majority of the complications from AVD arise from rupturing or fissuring of atherosclerotic plaques, followed by generation of either occlusive or subocclusive thrombi, with the ensuing coagulation cascade and negative cardiovascular outcomes associated with the excessive amounts of TxA2 released from platelets, monocytes, and macrophages (20, 25). However, our recent studies using mouse models of AVD, including familial hypercholesterolemia and familial combined hyperlipidemia, have clearly suggested that one of the contributing elements to a negative vascular outcome under these conditions is an elevation in the vascular (i.e., platelet-independent) production of TxA2 (16). Specifically, in vessels studied under in vitro conditions following removal of all blood components, from the low-density lipoprotein receptor (LDLR) and from the apolipoprotein E (ApoE) gene deletion mice, both models demonstrated that vascular production of TxA2 was significantly elevated compared with levels determined in their control, normolipidemic strain, the C57/Bl6/J mouse (16).
Building on our previous results suggesting that AVD in ApoE and LDLR gene deletion mice is associated with a shift in arachidonic acid metabolism toward vascular TxA2 production, in the present study we sought to determine if the phenomenon of aspirin resistance within this setting is associated with an evolving resistance of arachidonic acid-induced TxA2 production by the vasculature to inhibition via aspirin. These experiments evaluated the hypothesis that with genetic dyslipidemia, the increase in arachidonic acid-induced TxA2 production by the vasculature becomes increasingly resistant to chronic pharmacological intervention by aspirin therapy. This may ultimately result in the creation of an environment in the setting of elevated AVD risk wherein the likelihood for adverse vascular outcomes increases with time, despite chronic ingestion of low-dose aspirin.

**MATERIALS AND METHODS**

**Animals.** The present study used three strains of mice: the C57B6/J (C57) as the control strain and theapolipoprotein E gene deletion (B6.129P2-Apoe<sup>+/−</sup>/J; ApoE) and low-density lipoprotein receptor gene deletion (B6.12957-<i>Ldlr</i><sup>−/−</sup>/J) mice on the C57B6/J background. All mice were purchased from Jackson Laboratories (Bar Harbor, ME) at 6–7 wk of age. The ApoE mouse manifests familial combined hyperlipidemia, in which both plasma cholesterol and triglyceride levels are elevated (29). In contrast, the LDLR mouse is a model of familial hypercholesterolemia, manifesting a profound increase in serum LDL levels while ingesting a normal diet (18). Both familial combined hyperlipidemia and familial hypercholesterolemia are substantial risk factors for the development of AVD. Male mice of each strain were fed standard chow and drinking water ad libitum and were housed in an animal care facility at the West Virginia University (WVU) Health Sciences Center; all procedures had received prior approval by the Institutional Animal Care and Use Committee.

At the time of final usage, mice were anesthetized with injections of pentobarbital sodium (50 mg/kg ip) and received tracheal intubation to facilitate maintenance of a patent airway. In all mice, a carotid artery was cannulated for determination of arterial pressure. Blood aliquots were drawn from the jugular vein cannula for determination of glucose (Freestyle), a lipid profile (Wako), plasma nitrotyrosine (Cayman), and an inflammatory marker and insulin profile (Millipore).

**Preparation of isolated skeletal muscle resistance arterioles.** In anesthetized mice, the intramuscular continuation of the gracilis artery was removed and cannulated, as described previously (34). These first-order arterioles were extended to their approximate in situ length and were equilibrated at 80% of mean arterial pressure to approximate first-order arterioles were extended to their approximate in situ length and were equilibrated at 80% of mean arterial pressure to approximate in situ pressure. The organ baths contained physiological salt solution at 37°C and aerated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. Rings were preconditioned by treatment with 10<sup>−7</sup> M phenylephrine for 5 min, at which time 10<sup>−4</sup> M methacholine or 10<sup>−4</sup> M sodium nitroprusside was added to the bath to assess endothelium-dependent and endothelium-independent dilator integrity. Any ring that failed to demonstrate both a brisk constrictor response to phenylephrine and vable dilator endothelial function was discarded. For the assessment of dilator reactivity, rings were pretreated with 10<sup>−6</sup> M phenylephrine and exposed to increasing concentrations of arachidonic acid (10<sup>−10</sup>–10<sup>−4</sup> M). To assure that reactivity comparisons between aortic rings was representative of the response of an equivalent tissue mass, all rings were weighed subsequent to analyses.

In both arterioles and aortic rings, reactivity patterns in response to increasing concentrations of arachidonic acid were assessed under control conditions, following inhibition of cyclooxygenases (COX) with indomethacin (10<sup>−5</sup> M), COX-1 with aspirin (10<sup>−5</sup> M), inhibition of TxA2 synthase with dazoxiben (10<sup>−5</sup> M), or treatment with the antioxidant TEMPOL (10<sup>−4</sup> M), as required for the individual protocol.

**Preparation of aortic rings.** In each mouse, the thoracic aorta was removed and cannulated, as described previously (34). These experiments evaluated the hypothesis that with genetic dyslipidemia, the increase in arachidonic acid-induced TxA2 production by the vasculature becomes increasingly resistant to chronic pharmacological intervention by aspirin therapy. This may ultimately result in the creation of an environment in the setting of elevated AVD risk wherein the likelihood for adverse vascular outcomes increases with time, despite chronic ingestion of low-dose aspirin.

**Determination of vascular metabolites of arachidonic acid.** Vascular production of 6-keto-prostaglandin F<sub>1α</sub> (6-keto-PGF<sub>1α</sub>, the stable breakdown product of prostacyclin (PGI<sub>2</sub>); Ref. 24) and 11-dehydro-thromboxane B<sub>2</sub> (11-dehydro-TXB<sub>2</sub>, the stable plasma breakdown product of TXA<sub>2</sub>) in response to challenge with arachidonic acid within the three mouse strains was assessed using pooled conduit arteries (femoral, saphenous, iliac, carotid) from each mouse. Produced TXA<sub>2</sub> is rapidly degraded via non-enzymatic processes to form thromboxane B<sub>2</sub> (TXB<sub>2</sub>) and, although levels are extremely low and labile under non-enzymatic conditions, TXB<sub>2</sub> can be metabolized by 11-hydroxy-thrombox-ane dehydrogenase to form 11-dehydro-TXB<sub>2</sub>, or by β-oxidation to form 2,3-dinor-TXB<sub>2</sub> and 11-dehydro-TXB<sub>2</sub> being more stable (10, 19) and thus a more accurate estimator of TXA<sub>2</sub> production. Vessels were incubated in microcentrifuge tubes in 500 µl of physiological salt solution for 30 min under control conditions, after which time arachidonic acid (10<sup>−6</sup> M) was added to the tube for an additional 30 min. After the second 30 min period, the PSS was transferred to a new tube, frozen in liquid N<sub>2</sub> and stored at −80°C. Metabolite release by the vessels was determined using commercially available EIA kits for 6-keto-PGF<sub>1α</sub> and 11-dehydro-TXB<sub>2</sub> (Cayman). In specific experiments, vessels were also incubated with indomethacin (10<sup>−5</sup> M) dazoxiben (10<sup>−5</sup> M), aspirin (10<sup>−5</sup> M), or TEMPOL (10<sup>−4</sup> M), as required for the individual protocol.

**Experimental protocols.** In the initial experiments, mice were aged to 22–23 wk, at which time they were used for study with the procedures outlined above. In the second series of experiments, mice were treated with aspirin (100 µg/day; Refs. 5, 27, 33) starting at 6–7 wk of age. Treatments were maintained for 16, 22, and 28 wk, at which time the procedures outlined above were repeated. In all animals, tissues were harvested for biochemical analyses as described above.

**Data and statistical analyses.** Mechanical responses following challenge with arachidonic acid were fit with the three-parameter logistic equation:

\[ y = \min + \left[ \frac{\max - \min}{1 + 10^{(\log ED_{50} - x)}} \right] \]

where \( y \) represents the isometric tension, “max” and “min” represent the upper and lower bounds, respectively, of the change in tone with agonist concentration, \( x \) is the logarithm of the agonist concentration, and \( \log ED_{50} \) represents the logarithm of the agonist concentration (x) where the response (y) is halfway between the bounds. The use of the three-parameter logistic equation is appropriate for the analysis of sigmoidal concentration-response relationships, as it simultaneously provides estimates of the curve maximum (upper bound), minimum (lower bound) and the dose at which the dependent variable reaches 50% of maximum (ED<sub>50</sub>).

All data are presented as means ± SE. Significant differences between groups were determined using analysis of variance. In all cases, Student-Newman-Keuls post hoc test was used when appropriate and \( P < 0.05 \) was taken to reflect statistical significance.

**RESULTS**

Table 1 presents data summarizing the baseline characteristics of the three mouse strains in the present study at 22–23, 28–29, and 34–35 wk of age. While, within an age cohort,
mice were of approximately the same mass, both ApoE and LDLR exhibited increased mean arterial pressure and impaired glycemic control, evidenced by elevated plasma insulin despite similar blood glucose. Expectedly, both ApoE and LDLR exhibited a striking dyslipidemia, with ApoE demonstrating a severe elevation in plasma cholesterol levels in LDLR only. Both ApoE and LDLR exhibited significant elevations in plasma marker of oxidant stress, nitrotyrosine, versus levels in C57. Finally, active tone of isolated arterioles and the magnitude of aortic ring preconstriction with phenylephrine did not differ significantly between the three mouse strains at any age.

Responses of isolated resistance arterioles from C57, ApoE, and LDLR mice in response to increasing concentrations of arachidonic acid are summarized in Fig. 1. Dilator responses of arterioles from both ApoE and LDLR were significantly reduced compared with responses in C57, although pretreatment of arterioles with the COX inhibitor indomethacin nearly abolished mechanical responses to challenge with arachidonic acid (Fig. 1A). Treatment with the TxA2 synthase inhibitor dazoxiben (Fig. 1B) or aspirin (Fig. 1C) improved dilator responses of arterioles from ApoE and LDLR to arachidonic acid, although neither of these agents had a significant effect on responses in C57. Pretreatment of arterioles with the antioxidant TEMPOL significantly improved dilator responses to arachidonic acid in arterioles from ApoE, although this effect failed to reach statistical significance in arterioles from LDLR (Fig. 1D).

Figure 2 presents the relaxation responses of preconstricted aortic rings from the three mouse strains in response to increasing concentrations of arachidonic acid. Similar to that pre-

| Table 1. Characteristics of mouse groups in the present study at the different age ranges |
|---------------------------------|-------|-------|-------|-------|-------|-------|-------|-------|
|                                | C57   | ApoE  | LDLR  | C57   | ApoE  | LDLR  | C57   | ApoE  | LDLR  |
| Mass, g                        | 31 ± 3| 30 ± 2| 29 ± 3| 35 ± 3| 36 ± 4| 35 ± 4| 41 ± 4| 40 ± 3| 40 ± 4|
| MAP, mmHg                      | 94 ± 5| 101 ± 5| 106 ± 5*| 102 ± 5| 109 ± 4| 110 ± 5*| 104 ± 6| 110 ± 4*| 118 ± 5*|
| Insulinplasma, ng/ml           | 1.1 ± 0.2| 1.8 ± 0.4| 3.5 ± 0.5*| 1.1 ± 0.2| 2.0 ± 0.4| 3.4 ± 0.6*| 1.3 ± 0.3| 3.5 ± 0.9*| 3.5 ± 1.0*|
| Glucomannan, mg/dl             | 92 ± 7| 106 ± 9| 103 ± 7| 98 ± 9| 107 ± 10| 116 ± 11| 104 ± 7| 120 ± 11| 116 ± 14|
| Cholesterolplasma, mg/dl       | 88 ± 7| 388 ± 24*| 402 ± 16*| 94 ± 10| 296 ± 29*| 386 ± 31*| 104 ± 9| 312 ± 24*| 394 ± 28*†|
| Triglyceridesplasma, mg/dl     | 114 ± 14| 296 ± 32*| 158 ± 10*| 128 ± 19| 244 ± 24*| 140 ± 18†| 130 ± 19| 320 ± 28*| 146 ± 22†|
| Nitrotyrosineplasma, ng/ml     | 14 ± 3| 44 ± 11*| 29 ± 8*| 15 ± 5| 48 ± 12*| 44 ± 10*| 18 ± 5| 55 ± 11*| 44 ± 13*|
| Active diameter, μm            | 68 ± 4| 72 ± 6| 64 ± 6| 72 ± 3| 74 ± 4| 69 ± 4| 73 ± 5| 71 ± 4| 70 ± 5|
| Passive diameter, μm           | 109 ± 6| 112 ± 5| 107 ± 6| 118 ± 5| 111 ± 4| 114 ± 6| 116 ± 5| 115 ± 5| 118 ± 5|
| Active tone, μm                | 37 ± 3| 35 ± 5| 40 ± 4| 38 ± 4| 33 ± 5| 39 ± 4| 37 ± 5| 38 ± 4| 39 ± 4|
| Initial aortic tension, g      | 0.81 ± 0.14| 0.78 ± 0.18| 0.84 ± 0.16| 0.79 ± 0.14| 0.76 ± 0.20| 0.82 ± 0.18| 0.79 ± 0.15| 0.81 ± 0.18| 0.76 ± 0.17|

*P < 0.05 vs. C57/Bl6/J (C57); †P < 0.05 vs. apolipoprotein E (ApoE); LDLR, low-density lipoprotein receptor; MAP, mean arterial pressure.
presented in Fig. 1, dilator responses of rings from ApoE and LDLR mice were attenuated compared with that determined for C57. Furthermore, while indomethacin abolished reactivity to arachidonic acid in all three strains (Fig. 2A), treatment with either dazoxiben (Fig. 2B) or aspirin (Fig. 2C) increased dilator responses of vascular rings in both ApoE and LDLR. In contrast to the data presented for resistance arterioles, the effectiveness of TEMPOL in restoring vascular dilation to arachidonic acid in ApoE and LDLR was more robust in the aortic rings (Fig. 2D).

Vascular TxA2 and PGI2 production levels within the three mouse strains are presented in Fig. 3. These data indicate that, following challenge with arachidonic acid, arteries from ApoE and LDLR exhibit an increased production of TxA2, estimated from its stable breakdown product 11-dehydro TxB2, compared with that in C57 (Fig. 3A). However, pretreatment of vessels with dazoxiben, aspirin, or TEMPOL significantly reduces TxA2 production, with dazoxiben and aspirin being most effective. In contrast, vascular PGI2 production, estimated from its stable breakdown product 6-keto-PGF1α, was relatively consistent across the three mouse strains, was largely abolished following pretreatment of vessels with indomethacin, and was generally unaffected by pretreatment of the vessels with either dazoxiben or TEMPOL (Fig. 3B).

Data describing the baseline characteristics of the three mouse strains in the present study following chronic treatment with aspirin are summarized in Table 2. While minor fluctuations between the strains were apparent, chronic aspirin therapy did not result in a significant alteration in any of the measured variables presented in Table 2 between the three mouse strains.

Figure 4 presents data describing the impact of chronic aspirin therapy on resistance arteriolar responses to challenge with arachidonic acid. After 16 wk of treatment (22–23 wk of age), dilator responses of arterioles from ApoE and LDLR mice to arachidonic acid were increased compared with responses in untreated mice (Fig. 4A). Following 22 wk of treatment (28–29 wk of age), the beneficial effects of chronic aspirin therapy were reduced from that determined at 16 wk of treatment, although a significant improvement to arteriolar responses to arachidonic acid were still evident in ApoE and LDLR mice (Fig. 4B). In contrast, at 28 wk of treatment (34–35 wk of age), the impact of chronic aspirin therapy on arachidonic acid-induced dilation in arterioles from ApoE and LDLR mice was further muted and was no longer statistically significant (Fig. 4C). The percentage change in the upper bound of the concentration-response relations (from the untreated control values) in each of the three strains after each of the three durations of aspirin therapy are summarized in Fig. 4D.

The impact of acute treatment of arterioles with dazoxiben or TEMPOL on the arachidonic acid-induced dilation in aspirin treated ApoE and LDLR mice is summarized in Fig. 5. After 16 wk of aspirin therapy, pretreatment of vessels with neither dazoxiben nor TEMPOL impacted reactivity to arachidonic acid in ApoE or LDLR mice (Fig. 5A). Figure 5B demonstrates that after 22 wk of aspirin therapy, treatment of arterioles from ApoE with dazoxiben resulted in a significant improvement to dilator responses to arachidonic acid, although in arterioles from LDLR mice, only treatment with TEMPOL resulted in a significant increase in dilator reactivity (Fig. 5C). However, by the time the animals had reached 34–35 wk of age (28 wk of aspirin treatment), treatment with either dazoxiben or TEMPOL resulted in an improved arteriolar dilation to arachidonic acid in ApoE and LDLR mice. The percentage change in the upper bound of the concentration-response relations (from...
from aspirin-treated ApoE and LDLR to arachidonic acid was increased compared with responses in untreated mice (Fig. 6A). At both 28–29 and 34–35 wk of age (following 22 and 28 wk of treatment, respectively), the beneficial effects of chronic aspirin therapy on arachidonic acid-induced aortic relaxation were attenuated such that chronic aspirin therapy was not as effective in improving aortic relaxation compared with untreated controls (Fig. 6, B and C, respectively). The percentage changes in the lower bound of the concentration-response relations (from the untreated control values) in each of the three strains after each of the three durations of aspirin therapy are summarized in Fig. 6D.

Figure 7 presents data describing the effect of acute treatment of aortic rings from aspirin-treated ApoE and LDLR with dazoxiben or TEMPOL on arachidonic acid-induced relaxation. Comparable to results determined in resistance arterioles presented above, treatment of aortic rings with either dazoxiben or TEMPOL exhibited a progressive effect on increasing aortic relaxation to arachidonic acid. Following 16 wk of aspirin therapy (Fig. 7A), treatment of rings from ApoE and LDLR with either dazoxiben or TEMPOL did not improve arachidonic acid-induced relaxation. However, following 22 wk of therapy, treatment with dazoxiben or TEMPOL significantly improved relaxation responses to arachidonic acid in ApoE, although responses in aortic rings from LDLR were not altered (Fig. 7B). Data presented in Fig. 7C demonstrate that, after 28 wk of aspirin treatment, pretreatment of aortic rings from both ApoE and LDLR with either dazoxiben or TEMPOL results in a significant improvement to the vascular relaxation responses induced by arachidonic acid. The percentage changes in the lower bound of the concentration-response relations (from the respective aspirin-treated values) in aortic rings from ApoE and LDLR mice after each of the three durations of aspirin therapy are summarized in Fig. 7D.

Vascular production of TxA2 in C57, ApoE, and LDLR after the three durations of aspirin therapy is summarized in Fig. 8. After 16 wk of aspirin therapy (Fig. 8A), the elevated levels of TxA2 production, estimated through 11-dehydro-TxB2, normally demonstrated in arteries of ApoE and LDLR are significantly reduced compared with levels determined in untreated mice. Additional treatment of vessels with dazoxiben further reduced TxA2 production from that determined as a result of chronic aspirin therapy alone, although treatment with TEMPOL was

the respective aspirin-treated values) in the ApoE and LDLR mice strains after each of the three durations of aspirin therapy are summarized in Fig. 5D.

Figure 6 presents data summarizing the effects of chronic aspirin therapy on the dilator responses of aortic rings from C57, ApoE, and LDLR mice following application of increasing concentrations of arachidonic acid. At 22–23 wk of age (following 16 wk of treatment), relaxation of the aortic rings

Table 2. Characteristics of mouse groups in the present study in response to chronic aspirin treatment of 16, 22, and 28 wk duration starting at 6–7 wk of age

<table>
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<th>+16 Weeks/22–23 Wk Old</th>
<th></th>
<th>+22 Weeks/28–29 Wk Old</th>
<th></th>
<th>+28 Weeks/34–35 Wk Old</th>
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<tr>
<td></td>
<td>C57</td>
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<td>LDLR</td>
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<td>ApoE</td>
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<td>31 ± 3</td>
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<td>34 ± 3</td>
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<td>MAP, mmHg</td>
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<td>102 ± 5</td>
<td>96 ± 5</td>
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<td>Insulin (pmol/L)</td>
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<td>1.2 ± 0.3</td>
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<td>Glucose (mg/dL)</td>
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<td>Cholesterol (mg/dL)</td>
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<td>364 ± 27*</td>
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<td>Triglycerides (mg/dL)</td>
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<td>Nitrotyrosine (pmol/L)</td>
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<td>Initial aortic tension, g</td>
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*P < 0.05 vs. C57; †P < 0.05 vs. ApoE.
without significant effect. These responses were altered following 22 wk (Fig. 8B) and 28 wk (Fig. 8C) of aspirin therapy in ApoE and LDLR mice, as the effectiveness of the chronic aspirin treatment to reduce TxA2 production was progressively attenuated to the point where these effects were no longer statistically significant at 28 wk of treatment. Furthermore, the impact of dazoxiben on reducing vascular TxA2 production in both ApoE and LDLR mice was progressively increased with time. A directionally similar pattern to that for dazoxiben was also identified following treatment with TEMPOL, although this failed to reach statistical significance. Taken together, these data clearly suggest that the ability of chronic aspirin therapy to blunt the vascular production of TxA2 in ApoE and LDLR mice is increasingly attenuated in proportion to the duration of the aspirin treatment.

DISCUSSION

Recent epidemiological data suggest that the incidence and prevalence of risk factors that predispose individuals to the development of atherothrombotic vascular disease are increasing in severity and impacting many diverse demographic groups (9, 13, 23). One of the most critical of these risk factors is hyperlipidemia, in terms of both cholesterol and/or triglyceride profiles, and a common clinically employed pharmacological treatment for afflicted individuals is the chronic ingestion of a low-dose aspirin regimen as a means for attenuating platelet-induced TxA2 generation and the progression of thrombo-embolic events (20, 25). However, as recent studies have suggested that a subset of individuals receiving chronic aspirin therapy suffer from subsequent negative cardiovascular events more rapidly than predicted (22) and that this may be associated with a developing resistance to the pharmacological impacts of the aspirin treatment (2, 8), it is imperative to determine how chronic aspirin therapy impacts vascular function with increasing treatment duration in a model of AVD.

Previous reports from Pfister (28) have suggested that, in the setting of diet-induced hypercholesterolemia in rabbits, signaling through the vascular thromboxane receptors is a critical element of the alterations to the patterns of vascular reactivity associated with this pathological condition. Similarly, our previous studies suggest that combined hyperlipidemia in ApoE gene deletion mice and hypercholesterolemia in LDLR gene deletion mice is associated with impairments to the normal patterns of vasodilator reactivity owing, in part, to an increased vascular production of, and potentially increased sensitivity to, TxA2 (16). The initial observations of the current study support this previous work, as dilator responses of skeletal muscle resistance arterioles (Fig. 1) and relaxation of preconstricted aortic rings (Fig. 2) following challenge with arachidonic acid were attenuated in both ApoE and LDLR mice. Acute pharmacological intervention suggested that these impairments to dilator reactivity in both vascular segments were dependent on an increased production of TxA2 from the vascular endothelium via cyclooxygenase and thromboxane synthase, with a partial contribution via increased oxidant stress (which can shift arachidonic acid metabolism toward an increased production of TxA2; Refs. 36, 37). An interesting element of the data presented in Fig. 2 is the transient increase in vascular tone with the lower concentrations of arachidonic acid that was abolished by blockade of TxA2 with dazoxiben, further suggesting an increased constrictor effect of TxA2 production. These data are also supported by the biochemical determination of arachidonic acid metabolites, which suggests...
that vascular TxA2 production, estimated by its stable breakdown product 11-dehydro-TxB2, is elevated in ApoE and LDLR mice, but that it is largely correctable by pretreatment of vessels with aspirin or with the antioxidant TEMPOL (Fig. 3). The primary interpretation from these data is that both models of genetic dyslipidemia in LDLR and ApoE mice are associated with an impaired vascular dilation/relaxation in response to challenge with arachidonic acid and that this is associated with an increased vascular production of TxA2. However, at this age (22–23 wk), acute treatment with aspirin can reduce vascular TxA2 production and improve mechanical responses toward levels determined in vessels from control C57 mice.

Pursuant to these results, ApoE and LDLR mice were chronically treated with a low-dose aspirin therapy from 6–7 wk of age. After 16 wk of aspirin treatment, when mice were age-matched with those discussed above, the chronic aspirin regimen was highly effective at improving mechanical responses of both arterioles (Fig. 4) and aortic rings (Fig. 6) in response to arachidonic acid. However, this beneficial impact of chronic aspirin therapy in terms of improving vascular reactivity was not temporally stable as, with increasing duration of the treatment regimen, the ability of the intervention to improve vascular mechanical responses was progressively attenuated at therapy durations greater than 16 wk such that the calculated bounds of the arachidonic acid concentration-response relationships were largely unaffected between the time control results and 28 wk of treatment in both strains (Figs. 4D and 6D).

The data presented in Figs. 5 and 7 provide further support for the concept of a developing aspirin resistance of the proximal microcirculation and conduit vasculature, respectively, in dyslipidemic mice. In both strains, application of the TxA2 synthase inhibitor (dazoxiben) or the antioxidant (TEMPOL) had no impact on vascular dilation/relaxation responses to arachidonic acid compared with those determined following 16 wk of aspirin therapy (Figs. 5A and 7A), suggesting that chronic aspirin therapy was successful in blunting vascular TxA2 production in either strain at this age. However, with increasing therapy duration, incubation of vessels with dazoxiben or TEMPOL elicited an improvement to arachidonic acid-induced mechanical responses such that, after 28 wk of treatment, application of either pharmacological agent improved dilation/relaxation (Figs. 5 and 7, B and C). As summarized in Figs. 5D and 7D, the ability of either dazoxiben or TEMPOL to improve vascular reactivity to arachidonic acid exhibited a progressive increase in the calculated bound of the concentration-response relationships across the three treatment durations. These observations provide further support for the declining ability of chronic aspirin therapy to blunt vascular (i.e., platelet-independent) TxA2 production under the settings of familial hypercholesterolemia and combined hyperlipidemia in mice.

As a final support for the mechanical data, the ability of chronic aspirin therapy to blunt vascular TxA2 production clearly suggests that the ability of the treatment regimen to reduce the elevated generation of the constrictor prostanooid is progressively attenuated with increasing duration under the setting of genetic dyslipidemia (Fig. 8). While chronic aspirin treatment reduced vascular TxA2 production in arteries from ApoE and LDLR mice at 16 wk of age, this effect was progressively attenuated such that by 28 wk of treatment, this reduction failed to attain statistical significance. However, given that acute treatment of arteries with dazoxiben nearly abolished TxA2 production in all mouse strains and conditions,
these data clearly suggest that a developing resistance to the pharmacological impact of low-dose aspirin therapy had evolved, rather than a resistance of the pathways of TxA2 production to pharmacological intervention, per se.

One area that warrants particular attention is defining the role of vascular oxidant stress as a contributing element to the developing aspirin resistance. While the present results suggest that alterations to the mechanical responses of the
vasculature from ApoE and LDLR mice are highly dependent on TxA2 production/action, these results also suggest that a considerable portion of this role for TxA2 in contributing to poor vascular outcomes may be associated with an increased vascular oxidant stress. This possibility was also demonstrated in our initial studies of vascular reactivity in the dyslipidemic mice (7), thus allowing a potential avenue for not only maintenance of prostanoid production, but also “aspirin resistance” via COX-2-dependent TxA2 production. Related to this, it is also possible that the kinetic rates of, and recovery in, PGI2 production exceed that of TxA2 production (2, 8), leaving a better maintained or recovered PGI2 production in the aspirin-treated state, conditions that could be exploited by the chronic aspirin therapy at low dosages (11, 12). Furthermore, our previous studies suggest that the development of lipoxygenase-

disease risk, aspirin therapy, and TxA2 synthesis. In that study, the authors determined that the persistent arachidonic acid-induced TxA2 production and platelet aggregation during the acute phase following myocardial infarction was effectively attenuated by combined treatment with aspirin and an HMG-CoA reductase inhibitor, atorvastatin. Given that this timeframe is too rapid for any lipid-lowering effects of the statin, the authors concluded that their results likely reflected one of its major pleiotropic effects. Pignatelli et al. (30) examined the mechanism of one of the major pleiotropic effects of atorvastatin treatment, that of antioxidant behavior, in patients suffering from hypercholesterolemia, demonstrating a profound reduction in soluble gp91phox expression and multiple markers of oxidative stress. Furthermore, the recent work from Bulckaen et al. (5) suggests that chronic low-dose aspirin therapy minimized endothelial dysfunction in the vasculature associated with aging of normal rats through a chronic reduction in systemic markers of oxidative stress. However, it should be noted that chronic aspirin treatment was not associated with a reduction in systemic oxidant stress in the dyslipidemic mice of the present study (Table 2).

Aspirin inhibits COX-1 primarily by constitutive enzyme binding to the serine 529 residue (21). However, one of the more intriguing aspects from the present study was that pharmacologic inhibition of COX-1 with aspirin appeared to have a disparate impact on metabolites distal to that enzymatic locus. As shown in Figs. 1C and 2C, treatment of the vascular preparations with aspirin either had no impact on dilator responses to arachidonic acid or, in the cases of ApoE and LDLR mice, increased mechanical responses. This observation contrasts with the results in panels A of those same figures, where treatment of vessel with indomethacin (a nonspecific COX inhibitor) abolished nearly all of responses to challenge with arachidonic acid, leading to the obvious question of why acute inhibition of cyclooxygenase abolishes arachidonic acid-induced reactivity in once case (indomethacin) and fails to achieve comparable results in another (aspirin). In general, these data suggest that acute aspirin treatment at $10^{-5}$ M may result in an incomplete inhibition of COX compared with that for indomethacin. However, as this acute dose of aspirin did severely attenuate TxA2 production (Fig. 3) in vessels from the mouse strains, this also suggests a preferential inhibition of TxA2 as opposed to PGI2 production. Furthermore, this disparity may also have been present in the results from the chronic aspirin treatment, as vessels still retained reactivity to arachidonic acid despite the aspirin therapy. Given the current state of knowledge, several speculative explanations for these outcomes should be considered. First, aspirin, while an effective inhibitor of COX-1, does not effectively antagonize COX-2 (21, 26, 32), the expression of which has been demonstrated to be significantly elevated in ApoE (3, 6) and LDLR mice (7), thus allowing a potential avenue for not only maintenance of prostanoid production, but also “aspirin resistance” via COX-2-dependent TxA2 production. Related to this, it is also possible that the kinetic rates of, and recovery in, PGI2 production exceed that of TxA2 production (2, 8), leaving a better maintained or recovered PGI2 production in the aspirin-treated state, conditions that could be exploited by the chronic aspirin therapy at low dosages (11, 12). Furthermore, our previous studies suggest that the development of lipoxygenase-

![Fig. 8. Vascular production of TxA2 (estimated from 11-dehydro-TxB2) in arteries from C57, ApoE, and LDLR mice. Data (means ± SE) are presented following 16 wk (A), 22 wk (B), and 28 wk (C) of aspirin therapy in the 3 strains of mice. Within each panel, data are presented for vascular TxA2 production under control conditions, after chronic aspirin treatment, and after pretreatment of vessels from aspirin-treated animals with either dazoxiben or TEMPOL. *P < 0.05 vs. Control; †P < 0.05 vs. Control within that strain; ††P < 0.05 vs. aspirin treatment alone within that strain.](http://physiolgenomics.physiology.org/doi/10.220.33.4)
dependent metabolites of arachidonic acid evolve in the ApoE and LDLR mice as a compensatory mechanism in response to a decay in the efficacy of normal mechanisms present in the C57 mouse, which could be largely unaffected by treatment with indomethacin (34). A similar observation is the important emergence of lipooxygenase-based metabolites of arachidonic acid, which has also recently been found to be present in other models of hypercholesterolemia, including the high cholesterol-fed rabbit (1). Additionally, the presence of additional comorbidities, especially impaired glycemic control, which was determined in both ApoE and LDLR mice with increasing age, has been previously demonstrated as a significant contributor to poor treatment effectiveness of chronic aspirin therapy in the clinical setting (2, 8). Finally, several recent meta-analytic studies have suggested that the prevalence of aspirin resistance in clinical populations is associated with genetic polymorphisms within the cascades of arachidonic acid metabolism (15), although this has not been evaluated in animal models. Each of these potential contributors may represent a key mechanistic element comprising the phenomenon of aspirin resistance and represent justified avenues for future investigation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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