Disruption of the 5-lipoxygenase pathway attenuates atherogenesis consequent to COX-2 deletion in mice

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Suppression of cyclooxygenase 2 (COX-2)–derived prostacyclin (PGI_2) is sufficient to explain most elements of the cardiovascular hazard from nonsteroidal antinflammatory drugs (NSAIDs). However, randomized trials are consistent with the emergence of cardiovascular risk during chronic dosing with NSAIDs. Although deletion of the PGI_2 receptor fosters atherogenesis, the importance of COX-2 during development has constrained the use of conventional knockout (KO) mice to address this question. We developed mice in which COX-2 was deleted postnatally, bypassing cardiorenal defects exhibited by conventional KOs. When crossed into ApoE-deficient hyperlipidemic mice, COX-2 deletion accelerated atherogenesis in both genders, with lesions exhibiting leukocyte infiltration and phenotypic modulation of vascular smooth muscle cells, as reflected by loss of α-smooth muscle cell actin and up-regulation of vascular cell adhesion molecule-1. Stimulated peritoneal macrophages revealed suppression of COX-2–derived prostanoids and augmented 5-lipoxygenase product formation, consistent with COX-2 substrate rediversion. Although deletion of the 5-lipoxygenase activating protein (FLAP) did not influence atherogenesis, it attenuated the proatherogeneic impact of COX-2 deletion in hyperlipidemic mice. Chronic administration of NSAIDs may increasingly confer a cardiovascular hazard on patients at low initial risk. Promotion of atherogenesis by postnatal COX-2 deletion affords a mechanistic explanation for this observation. Coincident inhibition of FLAP may offer an approach to attenuating such a risk from NSAIDs.

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Seven placebo-controlled trials of nonsteroidal antinflammatory drugs (NSAIDs) designed to inhibit specifically prostaglandin G/H synthase-2, commonly known as cyclooxygenase-2 (COX-2), reveal that these drugs confer a cardiovascular hazard (1). Observational studies consistent with a cardiovascular risk have been reported for several traditional NSAIDs, which also exhibit selectivity for inhibition of COX-2, such as diclofenac and meloxicam (2–5). The clinically manifest elements of this hazard (a predisposition to thrombotic events, an elevation of blood pressure, cardiac failure, and arrhythmogenesis) have been recapitulated in rodent models in which the COX-2–dependent formation of prostacyclin (PGI_2) or its action is disrupted (6–8). This mechanism would be expected to be influenced by the underlying cardiovascular risk of an individual patient (9). Overview analysis of placebo-controlled trials of celecoxib showed that the likelihood of myocardial infarction was indeed influenced by cardiovascular risk before treatment as well as by the degree of drug exposure (10).

Three of the seven placebo-controlled trials of NSAIDs were performed to assess their value in chemoprevention of colonic adenomata. The patients in these trials were selected to be at low cardiovascular risk ab initio; despite this, evidence for a cardiovascular hazard in the NSAID-treated group emerged when treatment had continued for more than a year. This result raises the prospect that continued suppression of COX-2–dependent PGI_2 formation might result in an alteration in cardiovascular risk status in patients initially deemed to be at low risk (9). The gradual, delayed dissipation of elevated risk after drug discontinuation (11) would also be consistent with induction of structural changes in the vasculature due to NSAIDs (12). In this regard, deletion of the I prostanoid (IP) receptor for PGI_2 promotes initiation and early development of atherogenesis in hyperlipidemic mice (13, 14), but deletion of COX-2 or its pharmacological inhibition has been variously reported to promote (15) or restrain (16) atherogenesis in such models or to leave it unaltered (17, 18). Residual effects of COX-2 disruption during development severely constrain the use of conventional knockout (KO) mice (19, 20). We developed mice in which global deletion of COX-2 could be attained postnatally and crossed them into hyperlipidemic mice to mimic the administration of a selective inhibitor on atherogenesis.

Although suppression of COX-2–dependent PGI_2 is sufficient to explain the cardiovascular hazard from NSAIDs (1), it is possible that this effect is amplified by rediversion of the arachidonic substrate to other products. Here, we disrupted the 5-lipoxygenase pathway by deleting the 5-lipoxygenase activating protein (FLAP) in hyperlipidemic mice with and without concomitant deletion of COX-2. Postnatal deletion of COX-2 accelerates atherogenesis, and this effect is attenuated by deletion of FLAP. These results afford a mechanism by which chronic therapy with NSAIDs might elevate cardiovascular risk in humans and raises the possibility of a therapeutic strategy to attenuate this phenomenon.

Results

Characterization of Ind.COX-2 KO Mice. The COX-2 gene was intact in both wild-type (WT) and Ind.COX-2 KO mice (Fig. 1*A Left*). Efficient COX-2 deletion was shown in the KOs after TM induction in mouse tail DNA (Fig. 1*A Right*). COX-2 protein expression was reduced by ~95% in lipopolysaccharide (LPS)-stimulated peritoneal macrophages and kidney medulla of Ind.COX-2 KOs compared with WT mice (Fig. 1 *B* and *C*). Consistent with protein reduction, LPS-evoked macrophage prostanoid production was greatly reduced in Ind.COX-2 KO mice (Fig. 1*D*). Aspirin was added to inhibit COX-1 and then washed away before LPS stimulation. Macrophage COX-2–dependent prostanoids were markedly reduced in Ind.COX-2 KO mice (Fig. 1*D*). The induced COX-2 deletion is stable as shown by sustained suppression of COX-2 mRNA after 15 wk in both stimulated peritoneal macrophages and kidneys (Fig. 1*E*). Mice deficient in COX-2 throughout gestation exhibit multiple cardiorenal defects (19, 20). In contrast, 15 wk after deactivation of COX-2, Ind.COX-2 KO mice have a normal body weight, gross morphology of the kidneys, and renal function (Fig. S1 *A–C*). Baseline systemic blood pressure was unaltered (Fig. S1*E*). Furthermore,

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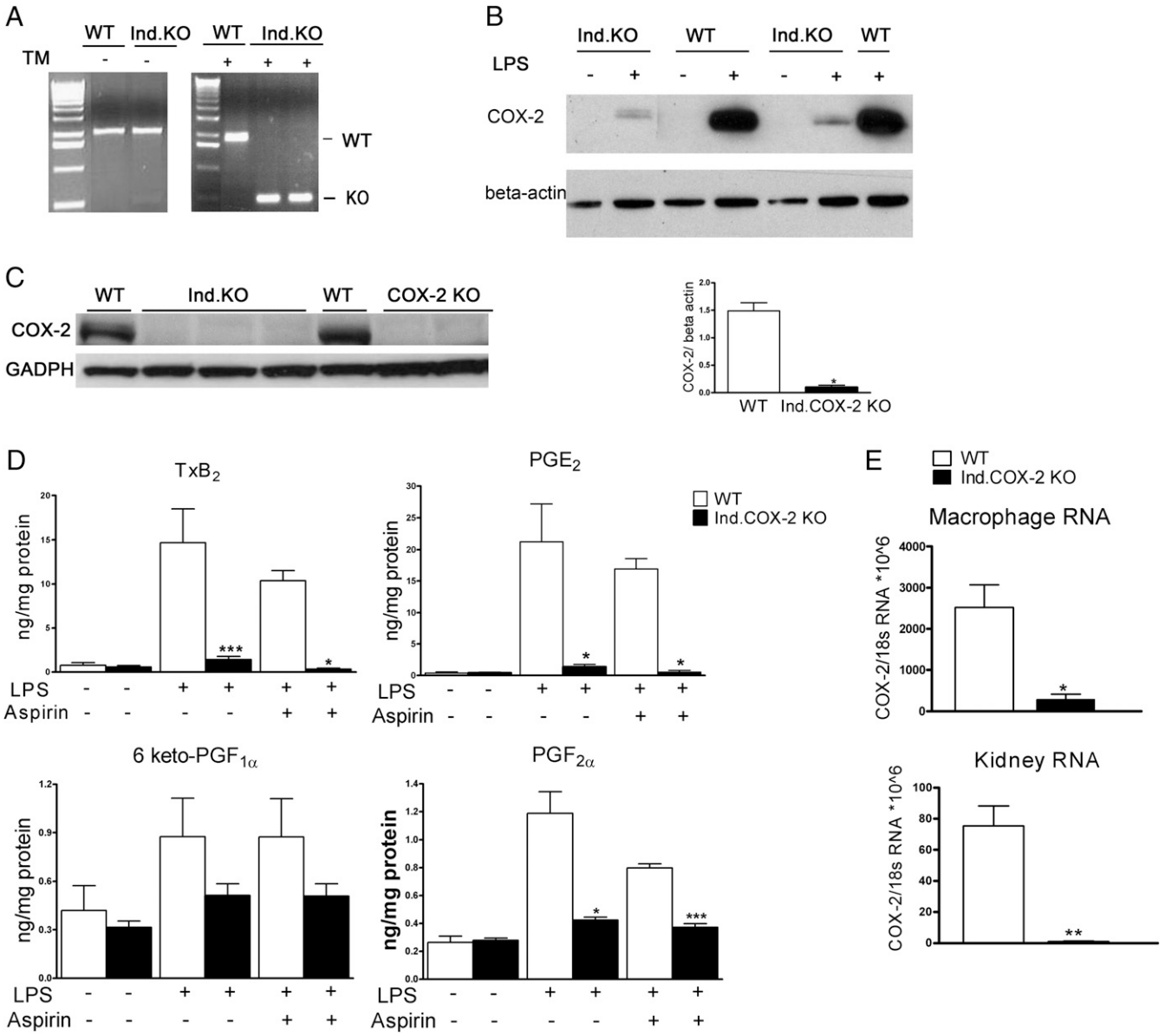

Fig. 1. Characterization of inducible COX-2 deletion in mice. (*A*) The COX-2 gene was normally expressed under baseline and TM successfully induced the postnatal deletion of the gene. Ind.KO indicates Ind.COX-2 KO mice. (*B*) COX-2 protein expression was reduced by ~95% in LPS stimulated peritoneal macrophages. Two-tailed *t* test, $***P < 0.001$. Error bars indicate SEM of the mean. (*C*) Kidney medulla COX-2 protein expression was diminished in Ind.COX-2 KO mice. (*D*) The most abundant prostanoids (TxB_2, PGE_2) are reduced significantly by COX-2 deletion in peritoneal macrophage cultures; aspirin (500 μM) was added and washed away before stimulation to inhibit COX-1–derived products. Error bars indicate SEM. Two-tailed *t* test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $n = 4–7$. (*E*) Macrophage and kidney COX-2 mRNA expression 15 wk after COX-2 deletion. Two-tailed *t* test, $*P < 0.05$, $**P < 0.01$; WT and Ind.COX-2 KO, $n = 6$. Error bars indicate SEM.

15 wk after COX-2 deletion in these mice, there was no evidence of increased cardiac fibrosis (Fig. S1*D*).

Inducible COX-2 Deletion Accelerates Atherogenesis. Atherosclerotic lesion burden (Fig. 2) was increased significantly in Ind.COX-2 KOs in both genders after 4 mo on a high-fat diet (HFD). However, weight gain (Fig. S2*A*) and plasma total cholesterol and triglyceride levels (Fig. S2 *B* and *C*) did not differ between genotypes. Consistent with the *en face* data, cross-sectional analysis of aortic root samples revealed a significant increase in the total lesion area in the aortic root from female Ind.COX-2 KO mice fed HFD for 4 mo (Fig. 3*C*). Lesional analysis of the aortic root revealed a disproportionate accumulation of CD11c- and CD11b-positive myeloid cells in the Ind.COX-2 KO mice (Fig. 3 *A a* and *b* and *B*). Although medial vascular smooth muscle cells (VSMCs) were evident in the lesions of both groups, there was an increase in the KOs of neointimal "stromal" cells, characteristic of activated VSMC, based on morphology, loss of α-smooth muscle cell actin (SMA) and up-regulation of vascular cell adhesion molecule (VCAM)-1 (Fig. 3 *A c–f* and *B*). Ind.COX-2 KO lesions showed an increase in necrotic regions (Fig. 3 *A a–h*) and cells staining positive for COX-2 (Fig. 3*Ag*) were depleted in KO lesions (Fig. 3*Ah*). At this advanced stage of lesional development, these COX-2–positive cells were heterogeneous with expression of CD11b as well as staining positive for VCAM-1, previously reported as marker of activated VSMCs (21).

Restraint of the Impact of COX-2 Disruption on Atherogenesis by the Concomitant Deletion of FLAP. Disruption of the FLAP alone did not influence plaque burden (Figs. 2 and 3 *A* and *C*). However, the acceleration of atherogenesis due to COX-2 deletion was

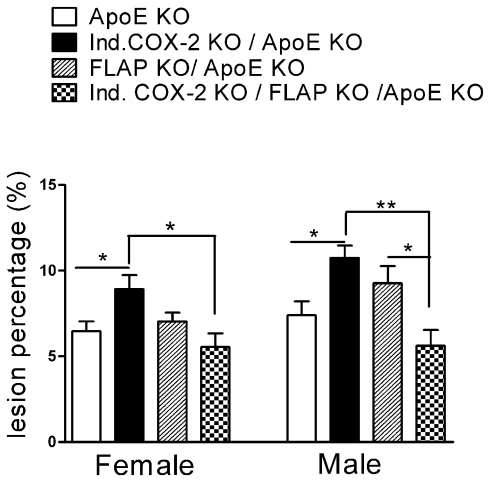

Fig. 2. Effects of COX-2 deletion and/ or FLAP deletion on atherogenesis. Aortic atherosclerotic lesion formation, represented by the ratio of lesion area *en face* to total aortic area, in mice after 4 mo on a HFD. One-way ANOVA reveals a significant effect of genotype on lesion progression in both genders (*$P < 0.05$, $n = 10–20$). Tukey's multiple comparison tests shows $P < 0.05$ for ApoE KO vs. Ind.COX-2 KO/ApoE KO in both genders. Lesion areas in male Ind. COX-2 KO/FLAP KO/ApoE KO mice were significantly reduced compared with those in FLAP KO/ ApoE KO mice (*$P < 0.05$); FLAP deletion significantly attenuated the pro-atherogenic impact of COX-2 deletion in both females (*$P < 0.05$) and males (**$P < 0.01$). No difference was detected between FLAP KO/ ApoE KO and ApoE KO mice in both genders. Error bars indicate SEM.

attenuated by concomitant FLAP deletion; indeed, atherogenesis was not significantly accelerated in the triple KO mice (Figs. 2 and 3*C*). Lesions appeared more matrix rich, with less necrosis as macrophage accumulation (Fig. 3 *Aj* and *B*) and VSMC phenotypic modulation in the neointima were restrained by FLAP deletion in these mice (Fig. 3 *A l–n* and *B*).

Rediversion of Arachidonic Acid Metabolism in KO Mice. Both evoked thromboxane A_2 (TxA_2) and prostaglandin E_2 (PGE_2) were markedly depressed in cultured peritoneal macrophages from Ind.COX-2 KOs on a hyperlipidemic background, as were the less abundant products, PGI_2 and PGD_2 (Fig. 4*A*). Coincident with suppression of prostanoid formation, macrophage 5-lipoxygenase product formation was increased (Fig. 4*B*). Systemic production of PGI_2 was significantly decreased in Ind.COX-2 KO/ApoE KO mice, as measured by urinary PGI-M production, consistent with its dominant origin from this enzyme in humans and mice (Fig. 4*C*). Biosynthesis of other prostanoids, reflected by urinary PGD-M, Tx-M, and PGE-M, where COX-1 is a major contributor, revealed a significant contribution from COX-2 deletion only in the sex with the most abundant of these metabolites (Fig. 4*C*). Systemic production of PGE_2, PGD_2, PGI_2, and Tx, as reflected by major urinary metabolites significantly increased in FLAP KO mice (Fig. S3*A*). Only PGE_2, the most abundant prostanoid formed by peritoneal macrophages, was significantly increased in FLAP KOs compared with WTs following LPS stimulation (Fig. S3*B*). In contrast to the data under control conditions, systemic prostanoid biosynthesis was not detectably altered in FLAP KO/ApoE KOs compared with ApoE KOs after 4 mo on a HFD (Fig. S3*C*). Single nucleotide polymorphism (SNP) analysis shows all mouse lines for the atherosclerosis study achieved 97–98% purity on the C57BL/6 background (Fig. S4).

Discussion

Seven placebo-controlled trials of NSAIDs purposefully designed to inhibit specifically COX-2 have revealed a cardiovascular hazard attributable to three structurally distinct drugs: rofecoxib, valdecoxib and celecoxib (1). Although disruption of COX-2/PGI_2 synthesis/response pathway is the primary mechanism by which this hazard occurs (6–8), its manifestation at the individual level is conditioned by factors that influence drug exposure, concomitant therapies, and underlying pretreatment cardiovascular risk (9).

Evidence for emergence of a cardiovascular hazard in low-risk patients where NSAID treatment had continued for more than 1 y (22–25) raises the possibility that cardiovascular risk might increase in otherwise healthy individuals treated chronically with NSAIDs selective for inhibition of COX-2. Consistent with this hypothesis, deletion of the IP fosters initiation and early development of atherogenesis (13, 14). However, attempts to address the issue more directly, using either COX-2 KOs or NSAIDs in hyperlipidemic mice have yielded conflicting results (15–18). In some cases, this may have reflected limitations of conventional COX-2 KO mice, which breed poorly and exhibit cardiorenal anomalies reflective of the absence of COX-2 during development (19, 20). The severity of this phenotype is also influenced by genetic background that differed between these studies. In other cases, pharmacological probes were used to elucidate the role of COX-2. However, the biochemical selectivity of the regimens for inhibition of COX-2 was rarely characterized. As deletion of either COX-1 or the receptor for its dominant product in platelets, TxA_2 retards atherogenesis, nonspecific COX inhibition by such regimens could have confounded interpretation of these results.

Here, we deleted COX-2 in a tamoxifen (TM)-dependent manner postnatally, attaining ~95% suppression of enzyme expression and marked inhibition of prostanoid formation. These mice do not exhibit the myocardial fibrosis, hypertension and glomerulosclerosis typical of classical COX-2 KOs. When crossed into hyperlipidemic ApoE KO mice, COX-2 deletion accelerates atherogenesis in both genders, affording a mechanism for increased cardiovascular risk and the delayed emergence of vascular occlusive events.

Previous studies have shown that COX-1 and COX-2 have different preferences in coupling with distinct downstream prostaglandin synthases in vivo. COX-1 is highly expressed in mature human platelets and is the major source of TxA_2 biosynthesis (26), whereas PGI_2, a dominant product of endothelium (27), is largely derived from COX-2 under physiological conditions in both humans (28) and mice (29). Here, we show that deletion of COX-2 significantly suppresses urinary PGI-M in both genders. Urinary excretion of the other metabolites, dominantly (Tx-M, PGD-M) or substantially (PGE-M) derived from COX-1, reveals a weaker signal; they are only suppressed significantly in the sex in which they are most abundant. Although suppression of COX-2–dependent PGI_2 formation is the primary mechanism by which NSAIDs result in cardiovascular hazard, reflecting the importance of this prostanoid as a restraint on endogenous mediators of platelet activation, vasoconstriction, and vascular proliferation, it is only one among many mediators that interact to maintain cardiovascular homeostasis (9). All may affect the events consequent to suppression of PGI_2. Similarly, secondary effects [such as loss of PGI_2-dependent up-regulation of thrombomodulin and consequently, activated protein C (30) or restraint of oxidant stress due to up-regulation of heme oxygenase-1 (13)] may serve to amplify the primary effects of this eicosanoid mediated via the IP. Similarly, rediversion of the arachidonic acid substrate of COX-2 to other metabolic enzymes may be of importance. Here, we found that products of the 5-lipoxygenase increased, coincident with depression of prostanoid synthesis, in peritoneal macrophages obtained from hyperlipidemic mice lacking COX-2. COX inhibitors have been reported to increase 5-lipoxygenase products in bronchoalveolar lavage fluid (31), colon cancer tissue (32), osteoarthritic synovial membranes (33) in humans, and in peritoneal macrophages in mice (34).

Products of 5-lipoxygenase exhibit properties relevant to atherogenesis. 5-HETE evokes chemotaxis in leukocytes (35) and is taken up by coronary artery endothelial cells, where it restrains PGI_2 production (36). In neutrophils, dendritic cells, and

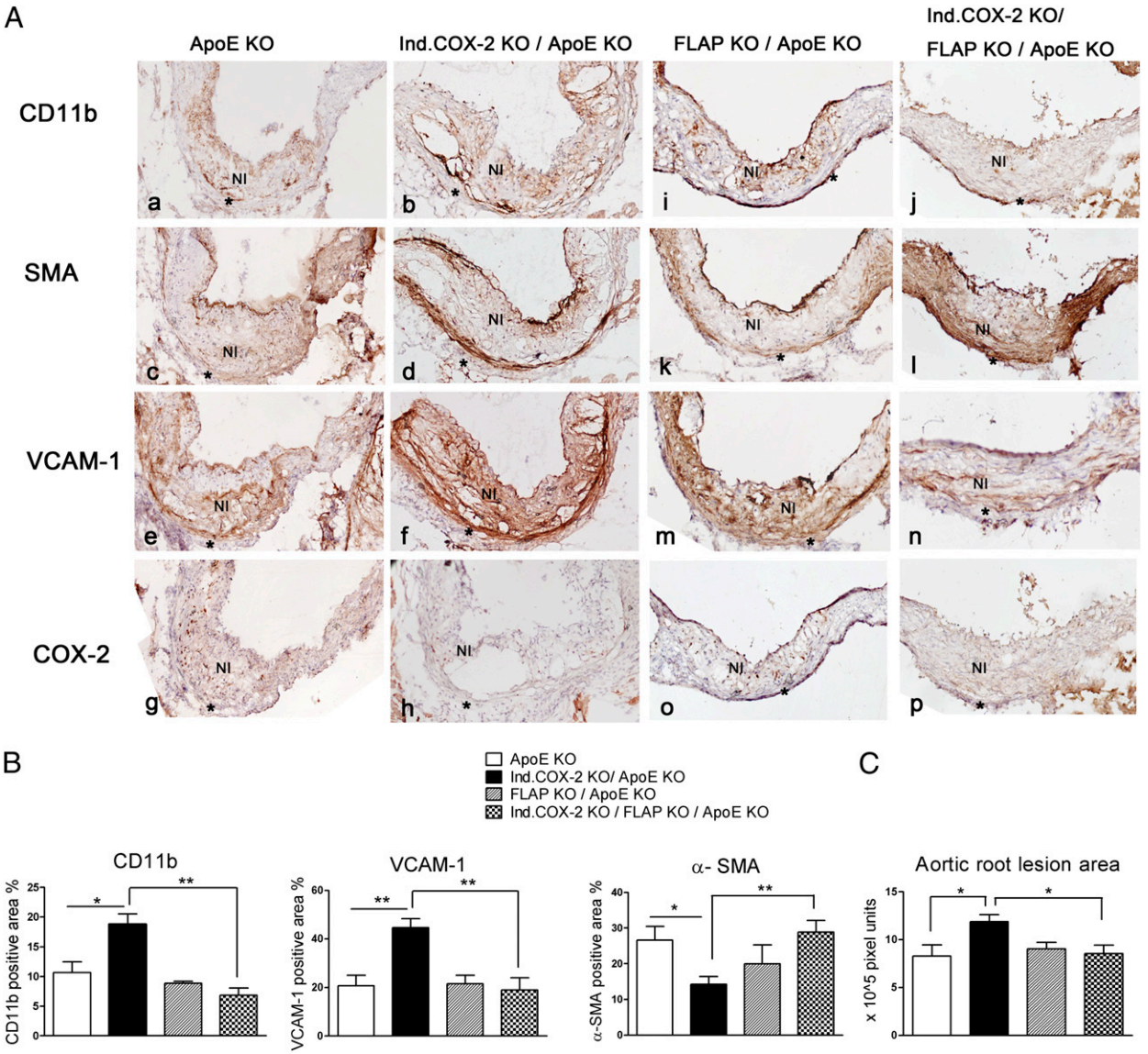

Fig. 3. Morphometric consequences of COX-2 and FLAP deletion on lesion development. (*A*) Lesion morphology analysis in aortic roots from mice on a HFD for 4 mo: representative CD11b (*a*), SMA (*c*), VCAM-1 (*e*), and COX-2 (*g*) staining in ApoE KO; representative CD11b (*b*), SMA (*d*), VCAM-1 (*f*), and COX-2 (*h*) staining in Ind.COX2 KO/ApoE KO; representative CD11b (*i*), SMA (*k*), VCAM-1 (*m*), and COX-2 (*o*) staining in FLAP KO/ ApoE KO; representative CD11b (*j*), SMA (*l*), VCAM-1 (*n*), and COX-2 (*p*) staining in Ind.COX-2 KO/FLAP KO/ApoE KO aortic root sections. NI: neointima; *: media. (*B*) Quantification of immunohistochemistry staining of CD11b, SMA, and VCAM-1. Two-tailed *t* test, $*P < 0.05$, $**P < 0.01$, $n = 3–5$. Error bars indicate SEM. (*C*) Quantification of aortic root lesion burden. Two-tailed *t* tests. $*P < 0.05$, $n = 3–5$. Error bars indicate SEM.

monocyte/macrophages, 5-HETE can be converted to 5-oxo-ETE (37, 38), which is ∼10-fold more potent than 5-HETE in stimulating monocyte migration. Elements of the LTB_4 synthesis/response pathway have been detected in human plaque (39). Pharmacological inhibition or genetic deletion of BLT1, a receptor of LTB_4, restrains atherogenesis (40, 41). Cysteine leukotrienes, LTC_4 and LTD_4, induce concentration-dependent contractions in atherosclerotic coronary arteries (42). LTE_4, the stable metabolite of LTC_4, mediates pulmonary inflammation and platelet activation through the P2Y12 receptor (43). However, the importance of 5-lipoxygenase in atherogenesis has been debated. Both 5-lipoxygenase and FLAP are strong candidate genes for atherosclerosis susceptibility in humans (44), and an initial report suggested that 5-lipoxygenase deletion retards atherogenesis in mice (45). Further analyses with 5-lipoxygenase-deficient mice on a variety of atherosclerotic backgrounds failed to support that notion (34, 46). A FLAP inhibitor, MK886, restrains the accelerated atherogenesis observed in ApoE KO mice expressing a dominant negative form of the TGFβ receptor type II on $CD4^+$ and $CD8^+$ T lymphocytes (47). In another study, MK-886 retards atherosclerosis development in ApoE/LDLR double knockout mice (48). However, in both of these models, atherogenesis is accelerated compared with ApoE deficiency alone with coincident up-regulation of the 5-lipoxygenase pathway.

We used mice deficient in FLAP to address the hypothesis that 5-lipoxygenase products were functionally relevant to the atherogenesis consequent to COX-2 deletion. We observed no significant effect of FLAP deletion on atherosclerosis lesion size in the ApoE KOs in this study. This result is consistent with the failure of 5-lipoxygenase inhibition or deletion to restrain atherogenesis in ApoE-deficient mice (34, 46, 49). Here, we show that both macrophage PGE_2 and systemic biosynthesis of PGE_2, PGI_2, and PGD_2 are increased under basal conditions in FLAP

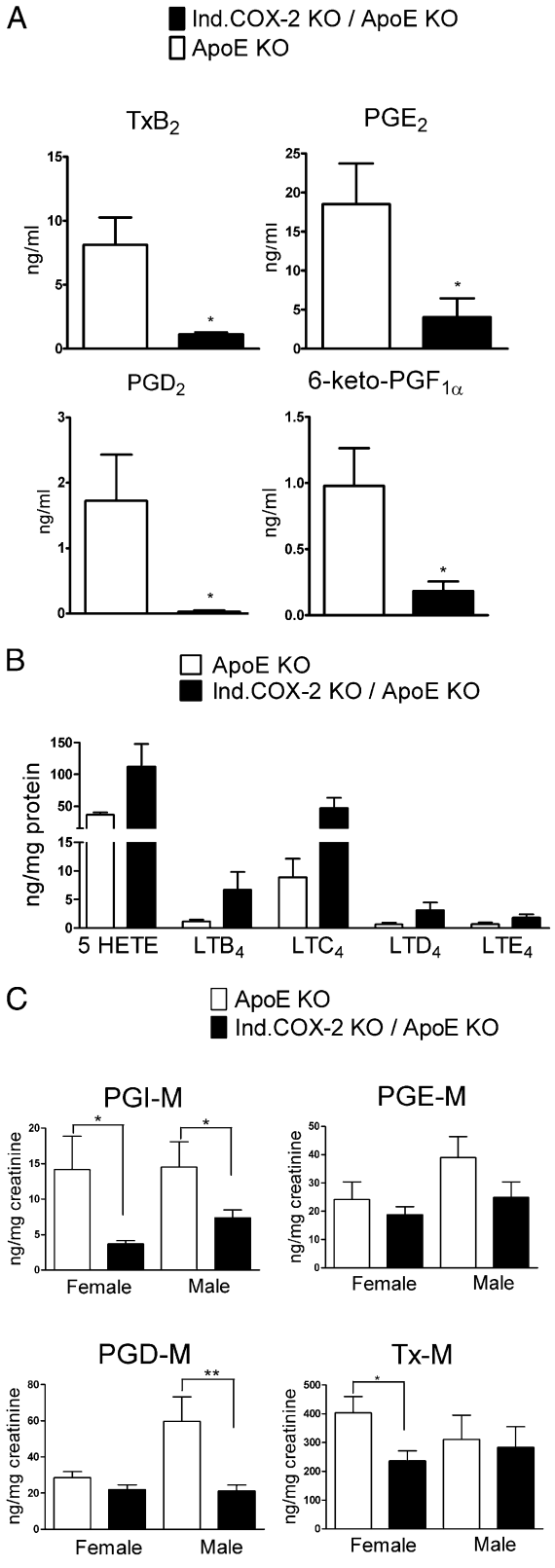

Fig. 4. Impact of COX-2 deletion on production of prostanoids and 5-lipoxygenase products. (*A*) Production of TxA_2 and PGE_2 in LPS-treated (5 μg/mL) peritoneal macrophages were markedly depressed in cultured peritoneal macrophages from Ind.COX-2 KOs/ApoE KOs, as were the less-abundant products, PGI_2 and PGD_2. One-tailed *t* test, $*P < 0.05$, $n = 4$–6. Error bars indicate SEM. (*B*) Peritoneal macrophages were incubated with A23187 (1 μM) for 3 h, washed, and then incubated with arachidonic acid for 30 min. Evidence consistent with substrate rediversion was the significant increase in 5-lipoxygenase pathway products as reflected by two-way ANOVA, $F = 8.11$, $P < 0.01$, $n = 7$–8. Subsequent pairwise analysis revealed significant ($P < 0.05$) differences in the most abundant 5-lipoxygenase pathway products, 5-HETE and LTC_4. Comparative *P* values obtained for LTB, LTD_4, and LTE_4 are 0.055, 0.056, and 0.058, respectively. Error bars indicate SEM. (*C*) Urinary PGI-M was significantly decreased in Ind.COX-2 KO/ApoE KO mice (one-tailed *t* test, $*P < 0.05$, $n = 9$–15). Urinary PGD-M (males) Ind.COX-2 KO (one-tailed *t* test, $**P < 0.01$) and Tx-M (females) were only reduced significantly in the genders in which their abundance was greatest in Ind.COX-2 KO/ApoE KO mice (one-tailed *t* test, $*P < 0.05$). Error bars indicate SEM.

KOs. It is possible that increased production of prostanoids (especially PGE_2) may have offset the protective effects of reducing 5-lipoxygenase products in FLAP KOs during the early stages of atherogenesis. However, evidence for enhanced prostanoid production was not observed after 4 mo on a HFD in FLAP KOs on a hyperlipidemic ApoE-deficient background.

Despite the failure of FLAP deletion to influence directly atherogenesis in ApoE KOs, it attenuated the disease acceleration in Ind.COX-2 KOs at this time. Macrophages from Ind.COX-2 KOs revealed evidence consistent with substrate rediversion to the 5-lipoxygenase pathway with increased formation of their most abundant products, 5-HETE and LTC_4. Thus, these mice may more closely correspond to those models described above in which 5-lipoxygenase induction accompanies further acceleration of atherosclerosis than observed with ApoE deficiency alone and in which the FLAP inhibitor MK 886 was effective. Pharmacological inhibition of FLAP has not been assessed in ApoE-deficient mice. However, the comparison might be complicated by additional inhibition of both COX-1 (50) and mPGES-1(51) by MK 866, possible differences in the degree and/or chronicity of suppression of FLAP activity or in the extent to which they result in substrate rediversion.

The results of the present study, taken together with those in IP KOs (13), afford a mechanism whereby prolonged treatment with NSAIDs selective for inhibition of COX-2 (either those designed to be so, such as celecoxib or older drugs with these properties, such as diclofenac) may result in increased cardiovascular risk in patients initially at low demographic risk of cardiovascular events. Such a possibility is consistent with the outcome of clinical trials but has not been addressed directly. Given the implications for the public health, an assessment of the propensity of NSAIDs to accelerate plaque burden formation in humans would seem timely. Should they do so, FLAP inhibitors, currently under investigation in asthma (52), may afford a novel approach to risk reduction.

Materials and Methods

Generation of Ind.COX-2 KO Mice. Mice with the COX-2 gene flanked by two LoxP sites, $COX\text{-}2^{f/f}$ mice, were previously generated (8) onto a 129Sv/B6 background (~75% B6, ~25% 129Sv). $COX\text{-}2^{f/f}$ mice were crossed with B6 Cre-ER mice (53) to obtain Cre-ER +, $COX\text{-}2^{f/+}$ mice, which were then intercrossed with $COX\text{-}2^{f/+}$ to obtain Cre-ER +, $COX\text{-}2^{f/f}$ (the so called Ind.COX-2 KO) mice and $COX\text{-}2^{f/f}$ mice as their littermate WT controls.

TM Induction of COX-2 Deletion. TM 4.5 mg/40 g body weight/day was injected i.p. for 5 consecutive days (53). TM administration was performed at age 7–9 wk and, unless specified, characterization experiments were performed 4 wk after the last day of TM injection. For simplification, these mice are referred as "TM-injected mice."

Peritoneal Macrophage Culture. Peritoneal macrophages from TM-injected Ind.COX-2 KO mice were collected 4 d after i.p. injection of 0.5 mL of 10% (vol/vol) thioglycollate. Nonadherent cells were removed after 2-h incubation. For Fig. 1*D*, aspirin (500 μM, Sigma) was added before LPS stimulation to inhibit

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COX-1–derived products. All wells were then thoroughly washed three times with culture medium to remove any trace amounts of aspirin. Adherent cells were treated with LPS (Sigma) 5 μg/mL for 6 h for RNA and 18 h for collection of protein and supernatants for prostanoid measurements. Cells were treated with A23187 (1 μM, Sigma) for 3 h for stimulation of the 5-lipoxygenase pathway, washed, and then incubated with arachidonic acid (30 μM, Cayman Chemical) for 10 min. Supernatants were collected for measurement of leukotrienes.

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