Aspirin but not Meloxicam Attenuates Early Atherosclerosis in Apolipoprotein E Knockout Mice

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ABSTRACT: **Background:** Atherosclerosis is a complex vascular inflammatory disease. In the last decade it was suggested that non-steroidal anti-inflammatory drugs (NSAIDs) and in particular inhibition of cyclooxygenase (COX)-2 are associated with an increase in cardiovascular morbidity and mortality. Aspirin is known to reduce the incidence and mortality from ischemic heart disease and is a mainstay in the prevention of vascular complications of atherosclerosis.

Objectives: To examine the effect of meloxicam, a selective COX-2 inhibitor, or low dose aspirin on the development of experimental atherosclerosis in apoE knockout (KO) compared to wild-type (WT) mice. We aimed to test the hypothesis that meloxicam, a potential vasculitis inducer, would exacerbate atherosclerotic lesions while aspirin, which is known to reduce the incidence of thrombosis occlusive events, would increase protection in this model.

Methods: We randomly divided 36 male apoE KO and 36 WT mice, 8 weeks old. Mice were treated for 10 weeks with 0.1 mg/ml aspirin, or 0.05 mg/ml meloxicam, dissolved in their drinking water. Control groups received regular drinking water. At sacrifice, the hearts were removed for histochemical staining and plaque size and composition were examined.

Results: Aspirin-treated animals displayed a decreased atherosclerotic lesion area compared to the untreated control mice, while meloxicam had a null effect on the extent of atherosclerosis in Apo E KO mice.

Conclusions: These results suggest that low dose aspirin reduces early atherosclerosis, while inhibition of COX-2 by meloxicam is not associated with an increase in atherosclerotic plaque size in this mouse model.

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KEY WORDS: aspirin, cyclooxygenase (COX)-2 inhibitor, atherosclerosis, apolipoprotein E, mouse model

Inflammation plays a central role in the development of atherosclerosis, from the early phases of lesion formation to plaque rupture, the main underlying cause of acute ischemic syndromes. Atherosclerosis remains the major cause of ischemic coronary artery disease and cerebrovascular disease. Moreover, current predictions estimate that by the year 2020 cardiovascular disease, notably atherosclerosis, will become the leading global cause of the total disease burden [1]. Although many generalized or systemic risk factors predispose to its development, atherosclerosis is causally associated with multiple cardiovascular risk factors, including dyslipidemia, hypertension, and cigarette smoking [2].

Metabolism of arachidonic acid is implicated in the pathophysiology of ischemic diseases and much attention has been focused on the pathway catalyzed by cyclooxygenase, which leads to the production of prostanoids. To date, three COX isoenzymes have been identified: COX-1, COX-2 and COX-3. The COX isoenzymes differ in terms of regulatory mechanisms of expression, tissue distribution and substrate specificity. COX-1 is a constitutively expressed isoform that maintains the integrity of the gastrointestinal mucosa and supports the beneficial homeostatic functions, whereas COX-2 is not expressed in the normal-appearing mucosa but is induced by inflammatory or neoplastic stimuli. COX-3 is a splice variant of COX-1, also known as COX-1b and is considered to play a key role in the biosynthesis of prostanoids known to be important mediators in pain and fever [3]. While the role of COX-1 in acute ischemic diseases is well established, the role of COX-2 is not entirely clear.

Selective inhibitors of COX-2 (coxibs) were originally designed to separate the anti-inflammatory actions of COX-2 inhibition from the physiological effects of COX-1-derived prostaglandins on the gastrointestinal tract. Thus, the COX-2 inhibitors are associated with reduced gastrointestinal mucosal damage, reduced bleeding, lesser ulcer formation, and clinically relevant complications [4]. Several prospective randomized trials have demonstrated that COX-2 selective inhibitors increase the risk for cardiovascular events, mainly myocardial infarction. One well-known example is the Adenomatous

COX = cyclooxygenase

Polyp Prevention on Vioxx (APPROVe) trial which showed a doubling of cardiovascular risk after 18 months of treatment with rofecoxib in patients without overt cardiovascular disorders [5]. A documented increased cardiovascular risk associated with rofecoxib use eventually led to the withdrawal of this drug from the market. Two trials – PreSAP (Prevention of Colorectal Sporadic Adenomatous Polyps) and APC (Adenoma Prevention Celecoxib) – confirmed the class effect of COX-2 inhibitors as these trials were terminated earlier due to an increase in cardiovascular risk [6,7].

The animal model most closely associated with human atherosclerosis is the apolipoprotein E knockout mouse model, which mimics atherosclerotic involvement in terms of plaque composition. The apoE-deficient (apoE$^{-/-}$) mouse is considered the best available model for human lipoprotein disorders and atherosclerosis. The lack of a functional *apoE* gene renders these mice unable to produce a key glycoprotein, apoE, which is synthesized in the liver, brain and in macrophages and is a constituent of chylomicrons and their remnants, very low density lipoproteins, intermediate density lipoproteins, and high density lipoprotein. ApoE plays a major physiological role in lipoprotein metabolism, mediating the high affinity binding of apoE-containing lipoproteins to the cell surface LDL-receptor and the chylomicron-remnant receptor, and is therefore essential for the transport, lipid metabolism, and hepatic metabolic clearance of circulating cholesterol [8]. The apoE KO mice are healthy when born, but have a markedly altered plasma lipid profile compared to normal mice and rapidly develop spontaneous atherosclerotic lesions even on a standard chow diet [9]. Absence of apoE in mice is associated with a fourfold increase in plasma total cholesterol levels and a twofold increase in plasma triglyceride levels, similar to humans with apoE deficiency. These data indicate that the absence of apoE significantly influences cholesterol metabolism in mice, which may be similar to apoE deficiency/abnormalities in humans [10].

Meloxicam is a member of the enolcarboxamide class of non-steroidal anti-inflammatory drugs and a relatively new COX inhibitor in the oxicam family that preferentially inhibits COX-2. Meloxicam is generally used in the treatment of rheumatoid arthritis, osteoarthritis and other joint pains. The efficacy of meloxicam was shown to be similar to that of other NSAIDs, whereas the incidence of adverse events, particularly gastrointestinal, was lower than with other NSAIDs. Meloxicam undergoes fast elimination, leading to a shorter half-life, compared to other NSAIDs. Although the drug is relatively well tolerated in humans, it was recently identified among the NSAID class as a culprit in dermatological reactions. Cutaneous and ocular adverse reactions have been reported in dogs following meloxicam administration as well as vasculitis in the deep dermis [11], suggesting that this drug may have adverse effects, inducing or exacerbating vascular injury and/or vasculitis in animal models.

Aspirin, a compound of salicylic acid, is one of the major preventive treatments against cardiovascular events in high risk adults and a mainstay in the prevention of vascular complications of atherosclerosis. It is rapidly absorbed in the stomach and upper small intestine, primarily by passive diffusion of non-dissociated acetylsalicylic acid [12]. The mechanism of action of aspirin occurs through permanent inactivation of COX-1 and COX-2, which catalyze the conversion of arachidonic acid to PGH_2, a substrate for several downstream isomerases that generate bioactive prostanoids, including thromboxane A_2 and prostacyclin. Higher levels of aspirin are required to inhibit COX-2 than to inhibit COX-1. These differences partly account for the need to use higher aspirin doses to achieve analgesic and anti-inflammatory effects, whereas anti-platelet effects can be obtained with daily doses as low as 30 mg [13]. Randomized trials indicate that low dose aspirin can prevent arterial thrombosis, including first vascular events among low risk healthy subjects and recurrent vascular events among patients with known acute or chronic occlusive vascular disease.

The current study examined the effects of meloxicam, as a potential vasculitis inducer, and aspirin in the development of atherosclerotic plaques in apoE KO as compared to wild-type mice. We tested the hypothesis that meloxicam would exacerbate atherosclerotic lesions while aspirin would increase protection. Here we show that meloxicam did not have any significant effects on the size of the plaques, whereas low dose aspirin was sufficient to prevent atherosclerosis in the apoE-deficient mice.

MATERIALS AND METHODS

ANIMALS AND EXPERIMENTAL DESIGN

Thirty-six male apoE knockout mice aged 8 weeks old on a C57BL/6J background were purchased from Jackson Laboratories (Bar Harbor, ME, USA); wild-type non-genetically modified age and gender-matched mice (n=36) were purchased from Harlan (Jerusalem, Israel). All animals underwent a common 2 week acclimatization period and were maintained on a 12 hour light-dark cycle at the local animal facility at Tel Aviv Sourasky Medical Center, Tel Aviv, under specific pathogen-free conditions.

At age 10 weeks the mice were randomly divided into 6 groups (n=12) and into subgroups of 6 animals per cage, and included control groups (untreated), aspirin or meloxicam-treated mice, either WT or apoE KO. The drugs were administered in the drinking water for 10 weeks and freshly replaced daily. Control mice received regular water.

apoE = apolipoprotein E
LDL = low density lipoprotein
KO = knockout
NSAID = non-steroidal anti-inflammatory drug

WT = wild-type

The oral bioavailability of regular aspirin is approximately 40–50% over a wide range of doses [12]. Aspirin has a half-life of 15–20 minutes in plasma and is rapidly cleared from the circulation. Meloxicam, on the other hand, has a bioavailability of 89% after oral administration, is strongly bound to plasma proteins, and its half-life is 20–24 hours [14]. The meloxicam dose was determined based on published pharmacokinetic studies [12,14]. The low dose of aspirin (0.1 mg/ml) chosen for the study was based on previously published literature [15].

The mice were treated with either 0.1 mg/ml aspirin (acetylsalicylic acid, Sigma, Israel) or 0.05 mg/ml meloxicam [4-hydroxy-2-methyl-N-(5-methyl-2-thiazolyl)-2H-1,2-benzothiazine-3-carboxamide-1,1 dioxide] (Sigma, Israel) and compared to untreated age-matched mice in the control groups. The mice were fed a standard chow, containing 4.5% fat by weight (0.02% cholesterol) for 10 weeks, ad libitum and were euthanized with sodium thiopental overdose (100 mg/kg) at age 20 weeks.

At sacrifice, the hearts were removed for staining and histochemistry. The upper section of the aorta was removed, immediately embedded in optimal cutting temperature compound, fresh-frozen and analyzed for extent of atherosclerosis. Plaque size and composition (lymphocyte infiltration, lipid core size, fibrous cap size) were assessed. All procedures were conducted in accordance with the institutional guidelines for animal research and the protocol was previously approved by the Institutional Ethics Committee for Use of Animals at Tel Aviv Sourasky Medical Center.

ASSESSMENT OF ATHEROSCLEROSIS AND PLAQUE STABILITY MARKERS

Quantification of atherosclerotic lesions was performed by calculating the lesion size in the aortic sinus as described elsewhere [16]. In brief, the heart and upper section of the aorta were removed from the animals, and the peripheral fat was cleaned away carefully. The upper section was embedded in OCT compound and frozen. A plaque cross-sectional area was measured in slices taken at the level of the aortic sinus. The aorta was pinned out and the lesion area was quantified as a percentage of total surface area. Sections were evaluated for atherosclerosis after staining with Oil-red O for estimation of lesion size. Images of the aortas were captured with a color video camera connected to a microscope (Olympus AX70, Olympus, Center Valley, PA, USA) and analyzed. An examiner blinded to the experimental groups performed the image analysis to prevent any bias in the interpretation of the results.

Plaque stability markers were examined using staining with Masson's trichrome to assess collagen content, delineate the fibrous area, and determine the ratio of fibrous cap to lipid core by quantitative morphometry. The method is useful for mild or early disease stage.

STATISTICAL ANALYSIS

Plaque sizing and features were compared between the groups using one-way ANOVA. Statistical significance was calculated using Student's *t*-test. $P < 0.05$ was considered significant.

RESULTS

We studied the effect of meloxicam and aspirin in apoE KO as compared to WT mice. Both drugs were administered in the drinking water and freshly replaced daily. The mice were individually numbered and weighed daily, and water and food consumption were carefully monitored each day. There was no difference in water and food consumption or in weight among the mice in the different groups.

Atherosclerotic lesions (atheromata) are asymmetric focal thickenings of the innermost layer of the artery, the intima. They consist of cells, connective tissue elements, lipids and debris. The atheroma is preceded by a fatty streak, an accumulation of lipid-laden cells beneath the endothelium. Most of these cells are macrophages and lymphocytes. In the center of an atheroma, extracellular lipid droplets form a core region, which is surrounded by a cap of smooth muscle cells and a collagen-rich matrix.

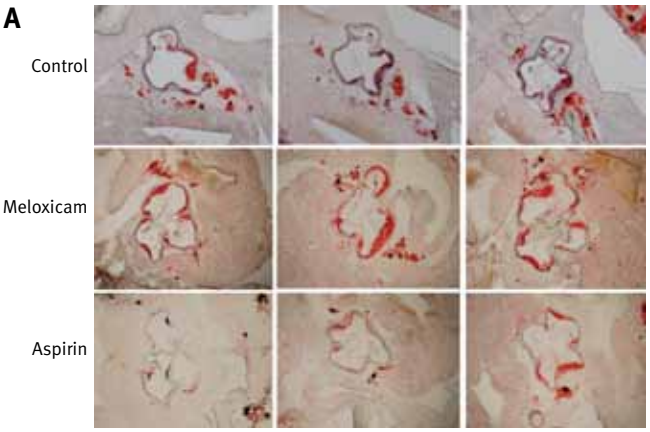
Treatment with aspirin was associated with a decreased atherosclerotic lesion area compared to controls at 20 weeks of age ($P = 0.033$). Thus, the average size of lesions in the aspirin-treated apoE KO mice was 7959.93 ± 1953.28 μm². Atherosclerotic aortic sinus lesion size was not significantly different in meloxicam-treated apoE KO mice compared to untreated control mice (11,804.36 ± 5106.41 vs. 11,130 ± 4038.16 μm² respectively, $P = 0.755$) [Figure 1].

Examination of representative Oil-red O-stained cross-sections of the aorta through the aortic sinus revealed that aspirin-treated but not meloxicam-treated mice displayed smaller atherosclerotic lesions at 20 weeks of age. Thus, plaques from aspirin-treated apoE KO mice had smaller lipid cores, indicating that the lesions were more stable [Figure 2].

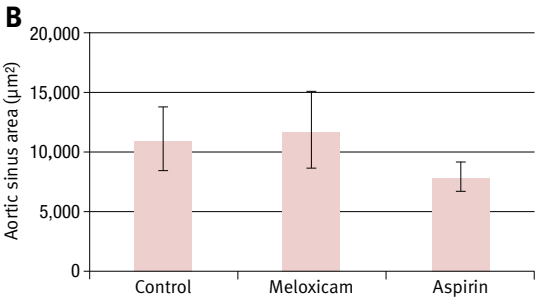
Assessment of plaque stability by evaluating fibrous cap and lipid core using Masson's trichrome staining and quantitative morphometry demonstrated that neither meloxicam nor aspirin had any effect on the percentage of fibrosis and collagen content in the apoE KO treated mice compared to the controls, and no statistically significant differences were found in the fibrous cap area [Figure 3]. Thus, the mean percentage of collagen was 33.21 ± 10.48% and 30.85 ± 16.73% in the meloxicam-treated and aspirin-treated groups, respectively, compared to 34.89 ± 13.42% in the apoE KO untreated group. Based on statistical analysis, the *P* value in the meloxican vs. control was 0.808 and 0.618 in the aspirin vs. control group, respectively and, therefore, not statistically significant. Based

OCT = optimal cutting temperature

Figure 1. Effect of meloxicam and aspirin on atherosclerotic plaque lesions in apoE KO mice. On killing, the aortic sinus from all mice was removed, embedded with OCT, and stained with Oil-red O for estimation of lesion size

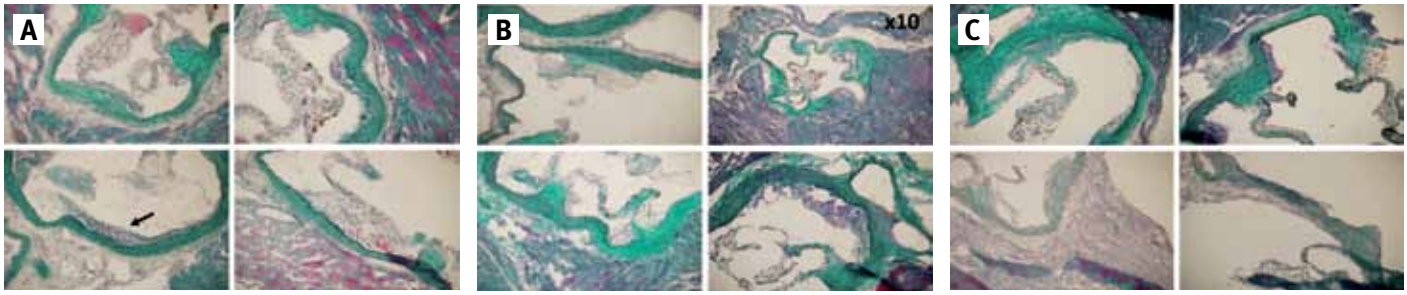


[A] Representative sections stained with Oil-red O



[B] Quantitative results of atherosclerotic lesions size of apoE knockout mice treated with either meloxicam (0.05 mg/ml) or aspirin (0.1 mg/ml) as compared to untreated apoE KO mice. Statistical significance was determined by Student's *t*-test of treated groups vs. control: *P* meloxicam 0.755 (NS), *P* aspirin 0.033

Figure 2. Masson's trichrome staining in apoE KO and control mice. Representative sections stained for Masson's thrichrome

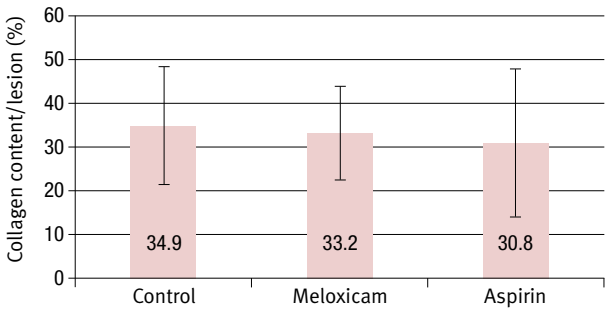


[A] Masson's trichrome staining in apoE KO control mice (group 1)

[B] Masson's trichrome staining in apoE KO meloxicam-treated mice (group 2)

[C] Masson's trichrome staining in apoE KO aspirin-treated mice (group 3)

Figure 3. Assessment of collagen content. Graphic presentation of fibrosis and collagen content in apoE KO mice treated with either meloxicam or aspirin as compared to apoE KO control mice. The percentage of collagen was determined by quantitative morphometry. Statistical significance was determined by Student's *t*-test of treated groups vs. control: *P* meloxicam 0.808 (NS), *P* aspirin 0.618 (NS)



on the histochemical analysis, no differences were visualized in the amount of cells infiltrating the caps between the three groups. Data on the WT animals are shown in Figure 4. WT mice, fed a standard chow diet, did not develop atherosclerotic plaques, as can be clearly seen in Figure 4.

DISCUSSION

The data presented here provide direct evidence that aspirin can attenuate early atherosclerosis in the apoE KO mouse model. The beneficial effects of aspirin in the secondary prevention of vascular disease are well established [17,18]. However, the role of aspirin and other NSAIDs in affecting and modulating the progression of vascular atherosclerotic lesions has been less studied. It is generally thought that the short-term beneficial effects of aspirin in secondary prevention relates to its platelet inhibitory effects while long-term effects might be associated with its anti-inflammatory action. In the current study we

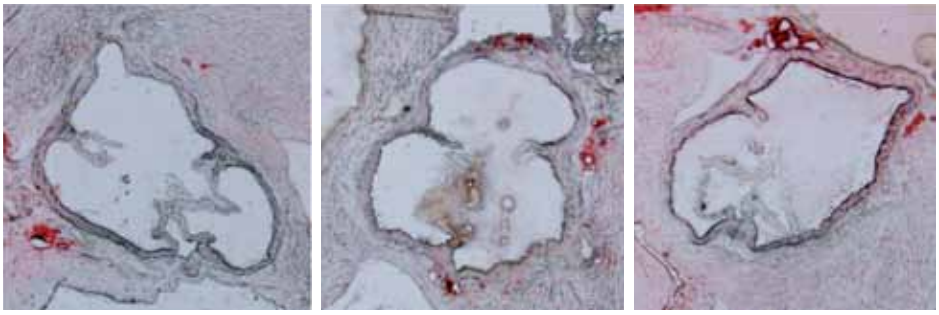


Figure 4. Oil-red O staining of wild-type mice. Representative sections of wild-type (WT) C57B/6J negative control mice stained with Oil-red O. The WT mice, fed on a standard chow diet, did not develop atherosclerotic plaques

examined the effects of low dose aspirin and meloxicam on the development of atherosclerotic lesions in apoE KO compared to age-matched untreated apoE KO and WT mice.

Our data show that aspirin reduced the size of the atherosclerotic plaque lesions in apoE KO mice, whereas meloxicam had no significant effect on the size of the lesion. The effect of low dose aspirin is supported by a study [19] conducted in LDL receptor-deficient mice (LDLR $^{-/-}$) showing that it retards the progression of established atherosclerotic lesions by a mechanism involving suppression of bioactive lipid formation and vascular inflammation, thereby reducing the progression of atherogenesis. Yamamoto et al. [20] studied the effect of long-term administration of differential doses of aspirin in apoE$^{-/-}$/LDRD$^{-/-}$ double KO mice and found that low dose aspirin improved endothelial dysfunction and reduced progression of atherosclerosis in these mice. In addition, a meta-analysis of six different trials [21] evaluating the benefits and risks of low dose aspirin in the secondary prevention of thromboembolic events showed that aspirin reduced all-cause mortality by 18%, the number of strokes by 20%, and other vascular events including myocardial infarction by 30%. Thus, the use of aspirin is generally considered to have a favorable benefit-to-risk profile.

Although various mechanisms have been proposed [13], inhibition of platelet COX-1 is sufficient to explain the antithrombotic effects of low dose aspirin. In a study by Shen et al. [22], the progression of atherosclerotic lesions was found to be associated with platelet activation and natural autoimmune mediated regulatory mechanisms in murine models of atherosclerosis, both LDLR$^{-/-}$ and apoE$^{-/-}$ KO mice. These mechanisms should be taken into account when examining the effects of aspirin treatment on the size of atherosclerotic plaques in these mice.

Daily administration of 30 mg aspirin results in the complete inhibition of platelet thromboxane A_2 production after 1 week through a cumulative process of fractional acetylation of unacetylated platelet COX-1 by successive daily doses of aspirin. Thus, regular regimens of 75–100 mg of aspirin per day clearly exceed the minimal effective dose required for a full pharmacodynamic effect. Previous studies have shown that the relative COX-1 selectivity of low dose aspirin accounts in part for the residual COX-2-dependent prostacyclin biosynthesis in vivo at daily doses of 20–80 mg aspirin [23].

Although low dose aspirin reduced the size of atherosclerotic lesions in our study and seemed to increase lesion stability, neither aspirin nor meloxicam affected the percentage of fibrosis and collagen content in the apoE KO treated mice and no statistically significant differences were found. One of the hallmarks of atherosclerosis is vascular dysfunction, observed mainly in large vessels and generally defined by a decrease in endothelium-dependent vasodilation that precedes the development of atherosclerosis and predisposes to structural vascular changes in humans. It should be noted that this concept is not fully supported by studies in the murine model of spontaneous atherosclerosis [8]. Aortic rings isolated from either young (6–18 weeks old) or adult (20–35 weeks old) apoE KO mice fed with a standard chow diet showed preserved endothelial function when compared to WT control mice. Furthermore, it was shown that endothelium-dependent relaxation remains undisturbed for up to 6 months [24]. On the other hand, in aged animals (72 weeks old), the aortic endothelial dysfunction seems to be strictly associated with plaque formation [25]. Therefore, endothelial function in the aortas of apoE KO mice is seemingly normal at the early stages of the pathology and we would not expect to see changes in plaque formation during early atherosclerosis in mice. The lack of effect of meloxicam and aspirin and the fact that no morphological changes were observed does not necessarily imply that the two drugs do not have an impact on plaque composition but further support the notion that endothelial dysfunction in mice is a focal (plaque-related) defect and typical of later stages of the disease. Whether aspirin has a more profound action on endothelial function, in particular, remains a subject of speculation and further investigation.

Studies have shown that COX-2 is involved in atherosclerotic lesions and promotes inflammation. Pharmacological inhibition of COX-2 or its down-regulation in mice genetically deficient in COX-2 suggests that this inflammatory

mediator is more likely to contribute to lesion initiation, and not progression, because in advanced lesions expression of COX-2 is reduced.

Taken together, our findings delineate the central role of aspirin in preventing atherosclerosis and add further support for its cardiovascular protecting ability, with no toxicity. The use of a COX-2 inhibitor, particularly meloxicam, was not found to be efficacious.

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Capsule

Growing back hearing?

Hair cells do not normally regenerate in the mammalian ear, and it has been thought that permanent damage to human hair cells in the cochlea inexorably resulted in hearing loss. However, Bramhall et al. have found that supporting cells in the cochlea taken from newborn mice can turn into hair cells. In a chemical model of damage, explant cultures were treated with gentamycin, and lineage tracing was done to track cell populations. New hair cells arose at a low level from a subpopulation of supporting cells that expressed the *Lgr*5 (leucine-rich repeat-containing G protein-coupled receptor 5) marker, a protein in the *Wnt* signaling pathway. Previous studies had shown that inhibition of the *Notch* signaling pathway can help restore hearing in mice with noise-induced deafness. Here, Bramhall and colleagues found that *Notch* inhibition by a gamma secretase inhibitor increased the fraction of supporting cells that transdifferentiated into hair cells and that the effects of *Notch* were dependent on β-catenin. It is not yet known whether this process can be triggered in older animals.

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