

Vascular wall–produced prostaglandin E2 exacerbates arterial thrombosis and atherothrombosis through platelet EP3 receptors

Sabrina Gross, Peggy Tilly, Didier Hentsch, Jean-Luc Vonesch, and Jean-Etienne Fabre

Institut de Génétique et de Biologie Moléculaire et Cellulaire, Institut National de la Santé et de la Recherche Médicale U596, Centre National de la Recherche Scientifique UMR7104, Université Louis Pasteur, 67400 Illkirch, France

Prostanoids, bioactive lipids derived from arachidonic acid (AA), are important for vascular homeostasis. Among them, prostaglandin E2 (PGE2) enhances aggregation of platelets submaximally stimulated in vitro. This results from activation of EP3, one of the four PGE2 receptors, which decreases the threshold at which agonists activate platelets to aggregate. Although PGE2 altered venous thrombosis induced by administration of AA, its role in pathophysiological conditions has remained speculative. We report that arterial walls subjected to inflammatory stimuli produce PGE2. In several models, we show that PGE2 produced by the arterial wall facilitates arterial thrombosis. Next, we detected PGE2 in mouse atherosclerotic plaques. We demonstrate that this plaque–produced PGE2 is not altered and is still able to activate EP3. In addition, we present evidence that PGE2 can leave the plaque and activate EP3 on blood platelets. Consistent with these findings, we observed that atherothrombosis induced in vivo by mechanical rupture of the plaque was drastically decreased when platelets lacked EP3. In conclusion, PGE2 facilitates the initiation of arterial thrombosis and, hence, contributes to atherothrombosis. Inhibition of the platelet EP3 receptor should improve prevention of atherothrombosis.

Atherosclerosis is an inflammatory disease that thickens the intimal part of the arterial wall by focal plaques, which are confined by a fibrous cap and covered with the endothelial layer (1, 2). Atherothrombosis is the occurrence of thrombosis on the surface of these plaques. This major complication is induced by either a mere denudation of the endothelium, also called endothelial erosion, or a frank rupture of the fibrous cap. The endothelial erosion exposes subendothelial collagen, releases von Willebrand factor, and promotes platelet aggregation. In addition to these mechanisms, the rupture releases tissue factor contained in the lipid core (3) and activates the coagulation pathway, which increases the thrombogenic stimulus. However, a superficial erosion may trigger massive thrombosis (4, 5), whereas a rupture may induce only limited thrombosis (6). Therefore, the resulting thrombotic response must be influenced by other factors, such as

platelet aggregability, which might play a pivotal role in regulating the response to stimuli.

The chronic inflammatory condition prevailing in the plaque explains the presence of numerous macrophages and the activation of their phospholipase A2, which releases AA from membrane phospholipid pools. Cyclooxygenases (COXs), particularly COX-2, are expressed in plaques (7, 8) and convert arachidonic acid (AA) into the cyclic endoperoxide prostaglandin H2 (PGH2). The structure of PGH2 is rearranged by specific enzymes to yield several prostanoids, such as thromboxane A2 (TXA2), prostacyclin (PGI2), or PGE2, depending on the tissue considered. In the plaque, PGH2 is thought to be preferentially converted into PGE2. This is consistent with in vitro preferential production of PGE2 by sustained stimulation of macrophages (9, 10) and with identification in the plaque of microsomal prostaglandin E synthase-1 (mPGES-1), which converts PGH2 into PGE2 (8, 11). Indeed, dissected mouse *ApoE*^{-/-} aorta surviving in culture media were shown to produce PGE2 (12).

CORRESPONDENCE

Jean-Etienne Fabre:
jefabre@igbmc.u-strasbg.fr

Abbreviations used: AA, arachidonic acid; COX, cyclooxygenase; mPGES-1, microsomal prostaglandin E synthase-1; NO, nitric oxide; PGE2 and PGH2, prostaglandin E2 and H2, respectively; PGI2, prostacyclin; TP, thromboxane prostanoid; TXA2, thromboxane A2.

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In vitro, PGE₂ by itself does not induce aggregation of platelets but modulates the response to their agonists. High levels of PGE₂ ($>10^{-5}$ M) inhibit platelet aggregation through nonspecific activation of IP, the receptor for PGI₂, whereas low levels ($<10^{-6}$ M) increase aggregation of submaximally stimulated platelets (13, 14), an effect named potentiation. The specific actions of PGE₂ are mediated through binding to four different G protein-coupled heptahelical receptors: EP1, EP2, EP3, and EP4 (15). EP2, EP3, and EP4 mRNAs have been identified in mouse platelets, where EP3 expression largely predominates (16). Studies of mice selectively lacking each of the four known EP receptors have indicated that the potentiating effect of PGE₂ is mediated solely by EP3 (14). Activation of EP3 on platelets inhibits adenylate cyclase, decreases the cAMP intracellular level (14, 16), and decreases the platelet threshold of activation (14), which explains the potentiating effect. In brief, low concentrations of PGE₂ in vitro activate its EP3 receptor on platelets and increase their sensitivity to agonists,

leading to complete aggregation even when they are submaximally stimulated.

In vivo significance of the PGE₂-induced potentiation of platelet aggregation observed in vitro requires that inflammation produces PGE₂ in the low range to specifically activate the EP3 receptor, not the IP receptor. We and others (14, 16) have shown that mice lacking EP3 developed less severe thrombosis after exogenous AA was delivered in the venous bed. These data indicate that although its precursor was supplied in abundance, PGE₂ was not produced at inhibiting concentrations but instead increased the aggregation induced by TXA₂ (14), a potent agonist of platelets.

We therefore hypothesized that PGE₂ enhances atherothrombosis. To test our hypothesis, we initially examined whether PGE₂ can modify the hemostatic balance in arterial flow, because it is a highly inhibitory environment for platelet aggregation. We found that PGE₂ is produced by the arterial wall in response to inflammation and, using EP3-deficient mice, that PGE₂ facilitates local arterial thrombosis. We have

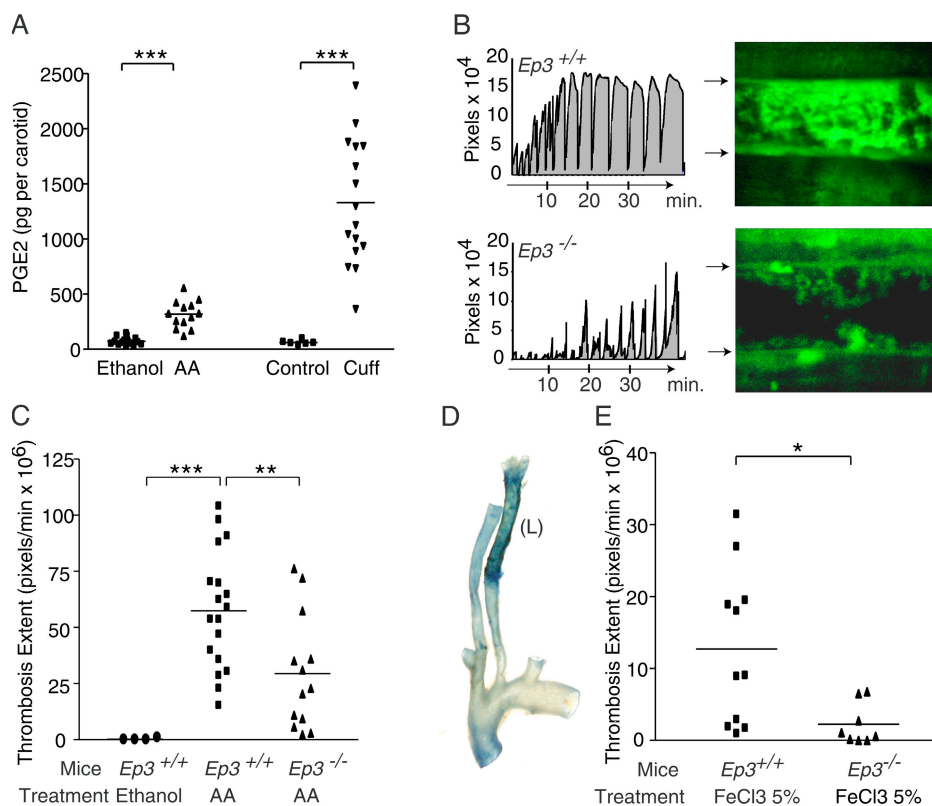


Figure 1. Vascular wall-produced PGE₂ facilitates arterial thrombosis through its EP3 receptor. (A) PGE₂ content of mouse carotid arteries after periadventitial delivery of ethanol versus 50 mg/ml AA or after placement of a polyethylene collar for 4 wk (cuff) versus nonexposed carotids (control). ***, $P < 0.0001$. (B, left) Representative curves of thrombosis induced in the carotid of one *Ep3*^{+/+} versus one *Ep3*^{-/-} mouse by periadventitial application of 100 mg/ml AA. Each point on the curve represents the number of green pixels detected on each of the 12 video-recorded images per second and reflects the number of accumulating platelets. Each peak represents the formation, growth, and

detachment of a thrombus carried away by bloodstream. Thrombosis extent was measured as the gray area under the curve. (B, right) Images of AA-induced thrombus (green light) observed at 15 min in one *Ep3*^{+/+} (top) versus one *Ep3*^{-/-} (bottom) mouse. Arrows indicate the carotid walls. (C) Extent of vehicle (ethanol)- or AA-induced thrombosis in *Ep3*^{+/+} versus *Ep3*^{-/-} mice. ***, $P < 0.001$; **, $P < 0.01$. (D) Endothelium denudation as indicated by Evans blue staining after exposure of the left (L) mouse carotids to ferric chloride. (E) Extent of ferric chloride (FeCl₃, 5%)-induced thrombosis in *Ep3*^{+/+} versus *Ep3*^{-/-} mice. *, $P = 0.011$. Horizontal lines indicate the mean value for each group.

Table I. Effect of the placement of a collar on prostaglandin contents of the carotid wall

Group	Control (pg/carotid)	Collar (pg/carotid)	Bilateral <i>t</i> test	Ratio
PGE2	60.6 ± 8.4 (<i>n</i> = 6)	1,330 ± 141.6 (<i>n</i> = 16)	<i>P</i> < 0.0001	21.93
6keto PGF1 ^a	48.1 ± 15.5 (<i>n</i> = 8)	334.7 ± 54.3 (<i>n</i> = 8)	<i>P</i> = 0.001	6.98
TXB2 ^b	22.9 ± 6.3 (<i>n</i> = 8)	127 ± 22.1 (<i>n</i> = 8)	<i>P</i> = 0.002	5.55

^aPGI2 metabolite.^bTXA2 metabolite.

detected PGE2 in mouse plaques, and we demonstrate that the plaque-produced PGE2 enhances atherothrombosis induced by mechanical rupture of the plaque.

RESULTS

The healthy arterial wall produces PGE2 in response to inflammation

We tested the ability of the arterial wall to produce PGE2 from locally delivered AA. We observed that the PGE2 content of a healthy carotid wall (72 ± 9 pg/carotid; *n* = 15) increased significantly after we topically applied 50 mg/ml AA onto the adventitia (317 ± 34 pg/carotid; *n* = 13; *P* < 0.0001; Fig. 1 A). Thus, the arterial wall can convert AA into PGE2. To test the production of PGE2 by arterial tissue in more pathophysiological conditions, we placed a collar loosely maintained around the carotid for 4 wk. This procedure, known to locally induce a chronic inflammatory lesion (17), predominantly enhanced the tissue content of PGE2 by 22-fold, whereas the PGI2 and TXB2 increased by only 7- and 6-fold, respectively (Table I). These data show that inflammation stimulates the arterial wall to produce PGE2.

PGE2 modulates arterial thrombosis induced by topical delivery of AA

Endothelium produces potent inhibitors of platelet aggregation, such as PGI2 or nitric oxide (NO), to prevent any inappropriate local thrombosis. To test whether PGE2 produced by the arterial wall can oppose PGI2 and NO to alter the local arterial hemostatic balance, we adapted a previously described model of inflammatory venous thrombosis (14). Topical superfusion of 100 mg/ml AA onto the mouse carotid induced intraarterial thrombosis. The inability of eicosatrienoic acid, which differs from AA by only a double bond, to induce thrombosis suggests that thrombus formation in this model

depends on formation of AA metabolites (unpublished data). Indeed, this thrombosis was triggered by TXA2, because the visual semiquantitative thrombotic score (18) dropped from 2.62 ± 0.32 (*n* = 8) in WT mice to 0.01 ± 0.01 (*n* = 8; *P* < 0.0001) in mice lacking thromboxane prostanoid (TP), the receptor for TXA2. Hence, periadventitial delivery of AA leads to its conversion into both TXA2 and PGE2 by the arterial wall. To examine whether the TXA2-induced thrombosis is modulated by PGE2 in this model, we quantified the extent of thrombosis in mice deficient for EP3. The targeted disruption of the EP3 locus did not alter the production of prostaglandins by the carotid wall (Table II). After the mice were injected with fluorescently labeled platelets, the entire clotting process was measured by counting the number of green fluorescent pixels on images acquired through a fluorescence microscope (Fig. 1 B). The extent of arterial thrombosis measured in the absence of EP3 (29.5 ± 7 × 10⁶ pixels/min; *n* = 13) was significantly reduced when compared with WT mice (57.4 ± 6.2 × 10⁶ pixels/min; *n* = 18; *P* = 0.006; Fig. 1 C). Thus, PGE2 produced by local periadventitial delivery of AA facilitated arterial thrombosis induced by TXA2 (Video S1, available at <http://www.jem.org/cgi/content/full/jem.20061617/DC1>), suggesting that it successfully opposed PGI2 and NO to shift the hemostatic balance toward a prothrombotic state.

PGE2 modulates arterial thrombosis induced by topical delivery of ferric chloride

Because its conversion by cyclooxygenases leads to PGE2 biosynthesis, the local delivery of AA to the arterial wall might have enforced PGE2 production and exaggerated its role in thrombosis formation. To test whether endothelial injury induced by inflammation produces enough PGE2 to alter local hemostasis, we topically applied ferric chloride (5%) onto the

Table II. Effect of Ep3 gene disruption on prostaglandin content of the carotid wall stimulated or not by ferric chloride

Genotype	Control (pg/carotid)			FeCl ₃ (pg/carotid)		
	<i>Ep3</i> ^{+/+}	<i>Ep3</i> ^{-/-}	<i>t</i> test	<i>Ep3</i> ^{+/+}	<i>Ep3</i> ^{-/-}	<i>t</i> test
PGE2	68.45 ± 9.21 (<i>n</i> = 5)	69.72 ± 6.09 (<i>n</i> = 5)	<i>P</i> = 0.91, NS	150 ± 25.81 (<i>n</i> = 5)	122 ± 16.62 (<i>n</i> = 5)	<i>P</i> = 0.34, NS
6keto PGF1 ^a	14.09 ± 3.12 (<i>n</i> = 7)	15.09 ± 3.86 (<i>n</i> = 6)	<i>P</i> = 0.84, NS	23.95 ± 6.04 (<i>n</i> = 7)	29.73 ± 5.69 (<i>n</i> = 6)	<i>P</i> = 0.50, NS
TXB2 ^b	4.91 ± 0.79 (<i>n</i> = 6)	5.71 ± 0.57 (<i>n</i> = 6)	<i>P</i> = 0.43, NS	50.75 ± 13.89 (<i>n</i> = 6)	45.12 ± 11.39 (<i>n</i> = 6)	<i>P</i> = 0.76, NS

^aPGI2 metabolite.^bTXA2 metabolite.

carotid. After its delivery, ferric chloride crosses the arterial wall and triggers thrombosis by inducing endothelial cell death (Fig. 1 D), subsequent release of ADP, and exposure of underlying collagen (19). In addition, the lesion induces local inflammation and increased the arterial wall production of AA metabolites, as shown in Table II. In this setting, fluorescent thrombosis appeared ~ 8 min after ferric chloride application. It reached $12.7 \pm 3.3 \times 10^6$ pixels/min ($n = 11$) in WT mice but was significantly reduced to 2.2 ± 10^6 pixels/min ($n = 8$; $P = 0.011$; Fig. 1 E) in mice lacking EP3. These results show that PGE2 produced in response to aggression of the arterial wall is sufficient to facilitate platelet activation induced by ADP, TXA2, and/or collagen in arterial flow.

The mechanism of PGE2-induced facilitation of thrombosis

PGE2 facilitates thrombosis by decreasing the activation threshold of platelets, making them more sensitive to their agonists (14). In vivo, PGE2 is produced by the arterial wall, but perhaps also by activated platelets themselves (20). In the latter case, PGE2 might facilitate the effect of ADP and TXA2 secreted by activated platelets to recruit more platelets. This would suggest that PGE2 plays a role in thrombosis amplification rather than in thrombosis driven by inflammation of the vascular wall. To address this possibility, we examined whether in vitro aggregation of isolated platelets is EP3 dependent. Platelets stimulated with low concentrations of collagen elicited partial aggregation that was not modified by the presence or the absence of EP3 ($47.6 \pm 2.1\%$ vs. $47.9 \pm 4.2\%$ [$n = 4$] at $1.5 \mu\text{g/ml}$ collagen [$P = 0.95$] and $58.8 \pm 4.2\%$ vs. $57.2 \pm 3\%$ [$n = 9$] at $2 \mu\text{g/ml}$ collagen [$P = 0.75$], respectively; Fig. 2). The absence of potentiation shows that activated platelets do not produce enough PGE2 to amplify aggregation in vitro.

To further ascertain that platelet production of PGE2 does not alter aggregation in vivo, we looked for a model of throm-

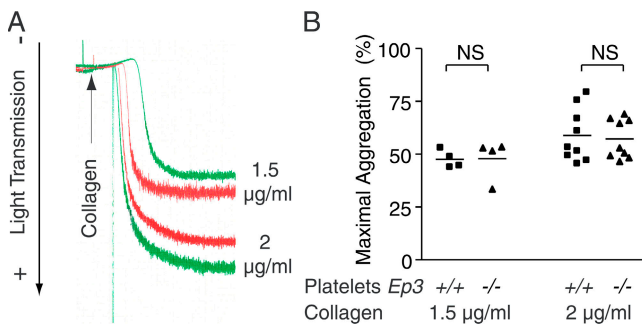


Figure 2. The in vitro facilitating effect of PGE2 is not induced by activated platelets. (A) Representative traces of aggregation of WT (green traces) versus EP3-deficient (red traces) platelets activated by 1.5 or 2 $\mu\text{g/ml}$ of collagen. These doses were chosen to induce partial and submaximal aggregation, respectively, to ensure that any potentiation could be evidenced. The increase in light transmission indicates an increase in the aggregation of platelets. (B) Quantitative analysis showing that the maximal aggregation of platelets was not altered by absence of the EP3 receptor to PGE2. Horizontal lines indicate the mean value for each group.

basis in which mural PGE2 is not produced when thrombosis starts. We tested a model of endothelial injury induced by oxidative stress resulting from local excitation of Rose bengal by a laser beam. Under standard conditions (21), we observed that a few clots were already visible at 4 s and that 80% of the arterial diameter was visually obstructed at 45 s (Fig. 3 A). PGE2 levels in these injured carotids were found in the range of control values and were not significantly different between 4 s (55 ± 6 pg/carotid; $n = 6$) and 45 s (73 ± 11 pg/carotid; $n = 6$; $P > 0.05$; Fig. 3 B). Thus, we used this model deprived of PGE2 at the initiation of thrombosis to examine whether aggregating platelets could produce enough PGE2 to alter amplification of thrombosis. Consistent with our in vitro data, carotids of *Ep3*^{-/-} mice infused with EP3-deficient platelets were totally occluded after 30 min of laser exposure, as were WT mice ($n = 5$ in each group). We concluded that platelets did not produce enough PGE2 in vivo to facilitate amplification. Collectively, our data show that thrombosis is facilitated primarily by arterial wall-produced PGE2.

Mouse atherosclerotic plaques contain PGE2

To further substantiate its role in pathophysiological conditions, we examined whether PGE2 modulates thrombosis on

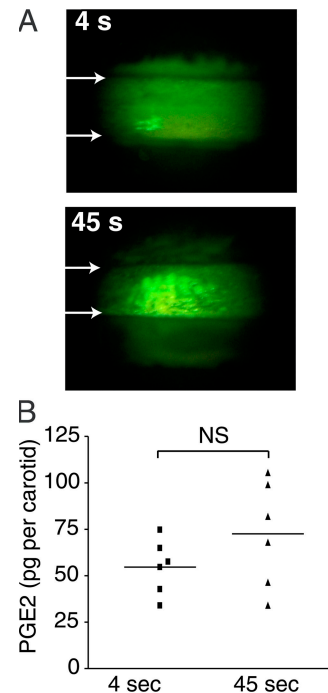


Figure 3. In vivo Rose bengal-induced oxidative stress of endothelium does not stimulate local production of PGE2. (A) Representative images of thrombosis (green light) induced by oxidative stress due to laser excitation of Rose bengal. Only a few clots are formed at 4 s, whereas massive thrombosis is already seen at 45 s. Arrows indicate carotid walls. (B) Amount of PGE2 detected in carotid tissue after 4 or 45 s of oxidative stress, showing that PGE2 was not increased at the beginning of thrombosis (compare with Fig. 1 A), nor when thrombosis was massive. Horizontal lines indicate the mean value for each group.

atherosclerotic plaques. To confirm previous data that suggested the ability of plaque to produce PGE2 (8, 12), we quantified it directly in mouse plaques. *ApoE*^{+/+} aorta contained 661 ± 98 pg PGE2 (*n* = 12), whereas its amount in *ApoE*^{-/-} aorta was found to be about fourfold higher, at 2,483 ± 485 pg (*n* = 14; *P* < 0.05). After we fed *ApoE*^{-/-} mice a high fat diet, which is known to increase the size of atherosclerotic lesions, the difference was even more impressive (5,018 ± 705 pg/aorta; *n* = 23; *P* < 0.001; Fig. 4 A). Thus, atherosclerotic plaques produce PGE2.

Plaque-produced PGE2 is functional and able to activate EP3 on platelets in vitro

Because numerous macrophages are recruited in the plaque (22, 23) and produce reactive oxygen species (24), we examined whether PGE2 produced by the plaque was functionally altered. Homogenates of whole atherosclerotic plaques added to platelet suspension in vitro trigger their aggregation, as a consequence of their high content of collagen (25). We observed that low concentrations of homogenized plaques added to EP3-deficient platelets induced aggregation that reached only 14.6 ± 3% of WT platelet aggregations (*n* = 6; Fig. 4 B). We concluded that mouse atherosclerotic plaques contain a molecule able to activate EP3. To rule out that a molecule other than PGE2 could activate EP3 (26), we treated mice with high doses of aspirin (500 mg/kg for 8 d) to inhibit cyclooxygenases. As expected, the treatment depressed the PGE2 levels in plaques (401.1 ± 163.8 pg/aorta; *n* = 4) down to the range of *ApoE*^{+/+} aorta values. Aggregation elicited by EP3-deficient platelets in response to homogenized suspensions of these aspirin-treated plaques reached 101 ± 15.1% of the aggregation elicited by WT platelets (Fig. 4 C). Thus, the EP3 receptor was not activated when PGE2 production is inhibited, indicating that the potentiating effect of homogenized plaques was indeed caused by PGE2. Hence, PGE2 produced by atherosclerotic plaques can activate EP3.

PGE2 contained in atherosclerotic plaques activates EP3 on blood platelets

We wondered whether PGE2 can exit from the plaque to act efficiently on blood platelets. To address the question, we enforced the plaque production of PGE2 by exposing its endoluminal side to low concentrations of AA (2 mg/ml; Fig. 5 A). We observed thrombi forming spontaneously when blood flow returned after the plaque has been incubated with AA (Fig. 5 B, middle). Importantly, thrombosis was detected only at the contact of the plaque, indicating that the plaque produced a platelet agonist that triggered local aggregation. Conversely, healthy the arterial wall was left unchanged by AA incubation, showing that the chosen dose of AA was too low to induce thrombosis at its contact (Fig. 5 B, top). We examined whether the plaque-produced PGE2 could modulate the local thrombosis elicited by the plaque, using *Ep3*^{-/-} mice crossed with *ApoE*^{-/-} mice. In these >55-wk-old double-mutant mice (*ApoE*^{-/-} × *Ep3*^{-/-}), the lack of EP3 did

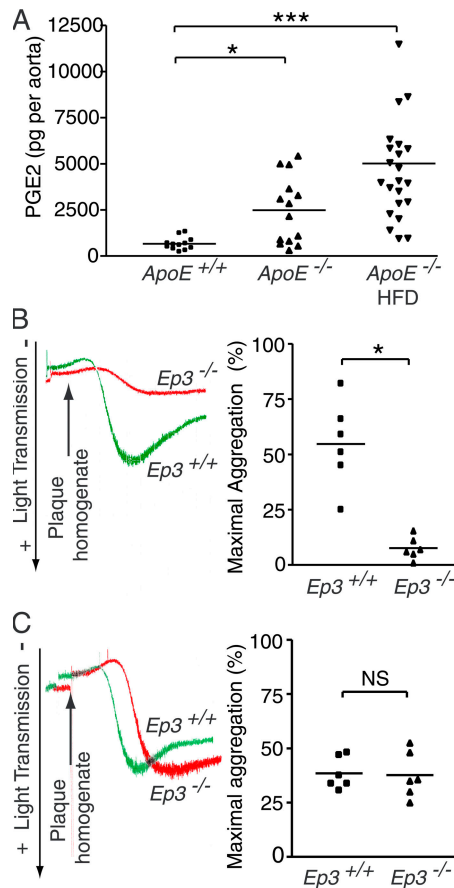


Figure 4. Mouse atherosclerotic plaques produce functional PGE2. (A) PGE2 content of aortic arteries from *ApoE*^{+/+} versus *ApoE*^{-/-} mice, showing that PGE2 tissue content is increased by the mere presence of atherosclerotic plaques, especially when the development of plaques was accelerated by a high fat diet for 3 wk. *, *P* < 0.05; ***, *P* < 0.001. HFD, high fat diet. (B) Representative traces and quantitative analysis showing *Ep3*^{+/+} versus *Ep3*^{-/-} platelet aggregation in response to 0.5 mg/ml of mouse homogenized plaques. The lack of EP3 on platelets strikingly reduced their response to the plaque material, indicating that a molecule produced inside the plaques activates EP3. *, *P* = 0.002. (C) Representative traces and quantitative analysis of platelet aggregation induced by mouse homogenized plaques extracted from *ApoE*^{-/-} mice treated by high doses of aspirin, documenting the lack of difference between *Ep3*^{+/+} versus *Ep3*^{-/-} platelet responses when PGE2 production is depressed. Horizontal lines indicate the mean value in each group.

not alter the extent of atherosclerosis, because their plaques covered 49.3 ± 4.1% (*n* = 10) of the total aortic surface versus 49.8 ± 5.1% (*n* = 9; *P* = 0.94) in *ApoE*^{-/-} × *Ep3*^{+/+} controls. The extent of thrombosis induced by intraluminal AA delivery reached 5.3 ± 1.5 × 10⁶ pixels/min (*n* = 10) in the presence of WT platelets but was drastically reduced to 0.9 ± 0.2 × 10⁶ pixels/min (*n* = 10; *P* < 0.05) when injected fluorescent platelets lacked EP3 (Fig. 5 C and Video S2, available at <http://www.jem.org/cgi/content/full/jem.20061617/DC1>). This experiment shows that AA-induced atherothrombosis in these mice was modulated by PGE2,

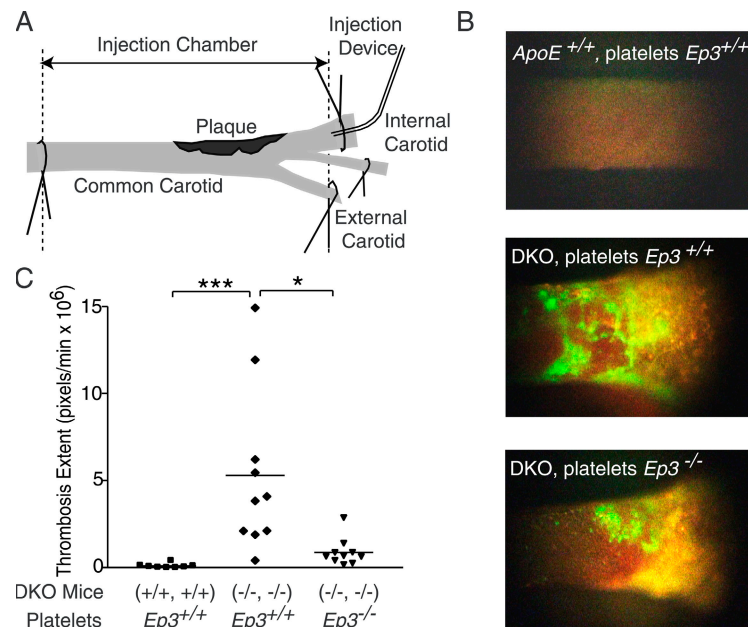


Figure 5. PGE2 found in mouse atherosclerotic plaques can act on blood platelets. (A) Model depicting the in vivo set-up used for endoluminal delivery of AA at low concentrations to the plaque. (B) Images from videos recorded under fluorescence, showing a segment of carotid with a plaque in yellow (autofluorescence) and thrombi in green. (top) An example of the absence of response to 2 $\mu\text{g/ml}$ AA in healthy carotid. Conversely, thrombosis developed on plaques after the same amount of AA was delivered in carotids of double-mutant (*ApoE*^{-/-} \times *Ep3*^{-/-}) mice

injected with WT (middle) or *Ep3*^{-/-} (bottom) platelets. (C) Quantitative analysis of experiments depicted in A. The chosen dose of AA never induced thrombosis in the absence of plaque (control, platelets *Ep3*^{+/+}), whereas thrombosis observed on plaques in double mutants injected with WT platelets (*ApoE*^{-/-} \times *Ep3*^{-/-}, platelets *Ep3*^{+/+}) was significantly decreased when platelets lacked EP3 (*ApoE*^{-/-} \times *Ep3*^{-/-}, platelets *Ep3*^{-/-}). ***, $P < 0.001$; *, $P < 0.05$. Horizontal lines indicate the mean value for each group. DKO, double knockout.

which indicates that the plaque-produced PGE2 was able to act on blood platelets through EP3.

Plaque-produced PGE2 promotes atherothrombosis induced by rupture of the plaque

In the last step, we tested the role of PGE2 in pathophysiological conditions. Rupture of the fibrous cap allows blood platelets to be activated by their agonists present inside the lesion, such as collagen (25), lysophosphatidic acid (27), or tissue factor (28) that generates thrombin. We therefore examined whether a mere rupture of the plaque, the most frequent cause of myocardial infarction in human pathology, could induce atherothrombosis. After a tiny curved needle was introduced through a collateral of internal carotid and scratched the plaque (Fig. 6, A–C), we observed small, non-occluding, but nevertheless detectable thrombosis on the surface of the ruptured plaque. Thrombi associated platelets (seen in fluorescence), red blood cells (seen on cross section; Fig. 6 C), and fibrin (Fig. 6 B, bottom). Although the ruptured areas measured by scanning electronic microscopy were similar in both groups of mice ($34,200 \pm 7,691 \mu\text{m}^2$ [$n = 13$] vs. $35,600 \pm 7,454 \mu\text{m}^2$ [$n = 10$], respectively; $P = 0.89$), we observed an impressive reduction in the development of thrombosis according to the presence ($0.16 \pm 0.05 \times 10^6$ pixels/min; $n = 13$) or absence ($0.007 \pm 0.005 \times 10^6$ pixels/min; $n = 10$; $P = 0.008$) of EP3 on platelets injected in

ApoE^{-/-} mice (Fig. 6 D). Thus, our data support the hypothesis that PGE2 produced in the plaques aggravates atherothrombosis.

DISCUSSION

It was not possible to predict what the in vivo action of PGE2 would be, if any, from its in vitro effects, which are both inhibition and potentiation of platelet aggregation. Studies identified EP3 as the receptor mediating the potentiating effect and showed that in vivo PGE2 might exert its potentiating effect on venous thrombosis (14, 16). However, these studies did not establish whether PGE2 is produced in pathophysiological conditions nor whether PGE2 modifies the hemostatic balance in arterial flow, where thrombosis is tightly controlled by endothelial production of potent inhibitors. Our present experiments show that PGE2 is produced by the arterial wall in response to inflammation and shifts the local hemostatic balance toward a prothrombotic state. These data allow us to conclude that PGE2 has an in vivo prothrombotic role. Moreover, our studies of the atherosclerotic plaques demonstrate that thrombosis induced by a rupture of the fibrous cap is clearly enhanced by the plaque-produced PGE2. This suggests that PGE2 modulates the thrombogenic potential of the plaque.

However, the impact of low PGE2 levels on thrombosis was examined in a recent study using mice deficient for one

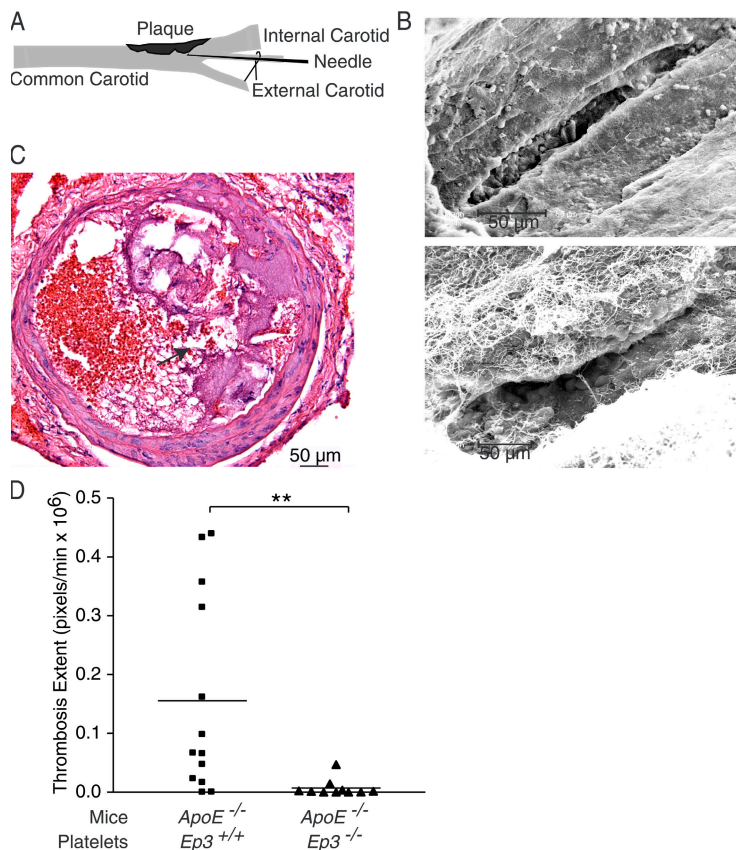


Figure 6. Atherothrombosis triggered by rupture of the plaque is exacerbated by PGE2. (A) Model depicting the set-up used to rupture mouse plaques in vivo with a needle. (B) Scanning electron microscopy photographs showing the rupture induced by the passage of the needle (top) and a case where fibrin network was still observable near the rupture after the wash (bottom). Bars, 50 μ m. (C) Thrombus on the needle-

induced lesion of the plaque. The arrow indicates the fracture. Bar, 50 μ m. (D) Quantitative analysis of thrombosis measured at the surface of ruptured plaques in *ApoE*^{-/-} mice, showing that absence of EP3 on platelets almost suppressed atherothrombosis that developed in the presence of WT platelets. **, $P = 0.0081$. Horizontal lines indicate the mean value for each group.

of the enzymes catalyzing the last step in PGE2 biosynthesis, mPGES-1 (21). To our surprise, arterial thrombosis was not decreased in *mPGES-1*^{-/-} mice, suggesting that PGE2 did not potentiate in vivo platelet aggregation, at least when thrombosis was induced by endothelial oxidative stress resulting from local excitation of Rose bengal. Using the same model of endothelial injury, we could not observe any difference in the extent of fluorescent thrombosis detected in mice injected with WT versus EP3-deficient platelets. However, we were also unable to detect substantial levels of PGE2 in the arterial walls of WT mice subjected to this oxidative stress. The Rose bengal–laser model induces thrombosis before inflammatory mechanisms could produce the PGE2 level required for its potentiating effect, and it is likely that inflammation takes place secondarily when PGE2 can no longer influence an already maximal thrombosis. Thus, the inability of this model to produce PGE2 when thrombosis develops explains the lack of difference between WT and *mPGES-1*^{-/-} mice. On another hand, this limitation highlights the fact that some models of thrombosis might not have a clear pathophysiological significance. This prompted us to

examine the effect of PGE2 in a clearly more pathophysiological condition such as atherothrombosis.

A role for PGE2 in atherothrombosis has been previously suggested, but through an indirect mechanism. From association studies showing colocalization of macrophages, mPGES-1, and metalloproteases, it was deduced that PGE2 might stimulate metalloproteases to thin the fibrous cap. The subsequent increased vulnerability of the plaque might favor atherothrombosis. This concept links PGE2 to atherothrombosis through a suggested action on plaque stability (8). Our study used direct tissue detection and confirmed that atherosclerotic plaques produce PGE2. Above all, our work links PGE2 to atherothrombosis through its direct action on platelets and thrombosis, showing that it increases the platelet sensitivity to their agonists in vivo.

To establish this direct link, we set up a model of atherothrombosis in which we simply scratched the plaque without interrupting the blood flow (Fig. 6 B). This mechanical disruption induces local thrombosis at the contact of the plaque (Fig. 6 C). Although our model looks histologically close to ruptures observed in autopsy studies, creating a defect in the

fibrous cap and exposing the thrombogenic core to circulating blood, its main limitation is linked to the mechanism of rupture. The current paradigm links inflammation to the plaque vulnerability through the action of metalloproteases. This mechanism implies that the most inflammatory plaques are the most vulnerable. As a consequence, rupture happens mainly on high-grade inflamed plaques. In contrast, we scratched plaques without knowledge of their inflammatory status, and some of them were very likely low-grade inflamed. This potential limitation might explain the variable response to the scratch in the control group (Fig. 6 D). However, it conversely strengthens our conclusion that PGE2 enhances atherothrombosis, because the more inflamed a plaque, the more PGE2 it might produce.

We established this direct link in mice, not in humans. However, a high homology (in the range of 80–90%) was found between mouse and human gene sequences coding for the PGE2 receptors (15). In addition, mouse and human distribution patterns of EP receptors on platelets are very similar, because RT-PCR showed obvious EP3 and EP4 bands in both species, whereas the EP2 band was really faint (16, 29). This is consistent with *in vitro* platelet responses to PGE2, which are very similar in mice (14) and in humans (13, 30, 31). Collectively, these data show a high similarity between mouse and human platelet behavior in response to PGE2, suggesting that PGE2 also facilitates arterial thrombosis in humans.

PGE2 appears in our study as a molecule playing a role in vascular wall homeostasis through its action in limited healing thrombosis. Indeed, the fact that PGE2 facilitated thrombosis in the arterial flow in response to inflammation indicates that its *in vivo* effect is sufficient to oppose PGI2. Therefore, even when a small vascular lesion releases or exposes low amounts of agonists, the concentrations of which are below the platelet activation threshold, the presence of PGE2 produced by local inflammation may sensitize platelets and foster healing thrombosis. Conversely, PGI2 produced upon inflammation by neighboring functional endothelial cells limits the extent of thrombosis (32) to restrict it to the lesion. Hence, PGE2 catalyzes healing thrombosis on small lesions, whereas PGI2 restricts its amplification to keep it focalized (Fig. 7).

On the contrary, PGE2 produced by the plaques must be regarded as a harmful molecule, because it aggravates atherothrombosis. In this study, PGE2 present in subendothelial connective tissue sensitizes platelets that adhered to a mere erosion and facilitates local thrombosis. Providing that the surrounding endothelium on the plaque is dysfunctional (33, 34), the nascent thrombosis is incompletely controlled by the deficient local production of NO and/or PGI2. Thus, PGE2 might trigger a massive and occluding thrombosis on a mere endothelial erosion.

To prevent this dreadful sequence, the effect of PGE2 should be inhibited. PGE2 has numerous functions in various physiological systems, implying that inhibition of its production might induce unexpected side effects. Inhibiting the EP3 receptor instead might be safer, especially considering that

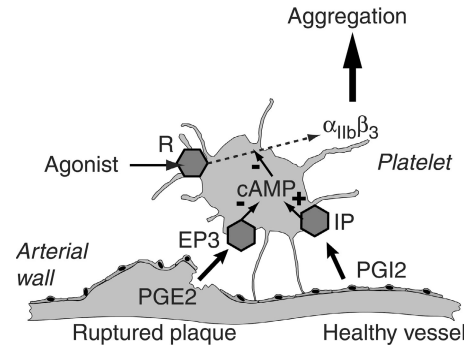


Figure 7. Proposed model for *in vivo* action of PGE2/EP3 upon thrombosis. Platelet agonists activate their specific receptor (R), triggering the signaling cascade and exposing the integrin $\alpha_{IIb}\beta_3$, which is crucial for platelet aggregation. Inflammation of the vessel or rupture of the plaque releases PGE2 locally. Activation of its EP3 receptor on platelets decreases the internal level of cAMP, whereas activation of IP receptor by endothelium-produced PGI2 increases it. This balance between PGE2 and PGI2 eventually determines the intracellular level of cAMP. Because the cAMP level inhibits some steps of the signaling cascade activated by R, the balance ultimately regulates the platelet capability to aggregate in response to its agonists. Hence, platelet rupture or vessel wall inflammation enhances platelet aggregability via the release of PGE2.

the EP3 receptor has multiple isoforms (35). Therefore, a drug targeting the platelet EP3 isoform, which inhibits adenylate cyclase, might be much more specific than a drug inhibiting PGE2 production. Such a drug should improve the current limited efficiency in preventing myocardial infarction (36, 37). Indeed, aspirin at low doses, the treatment of reference, inhibits COX-1 in platelets but does not alter the COX-2 that produces PGE2 in plaques. In addition, specific inhibition of COX-2 that could be beneficial in decreasing the PGE2 level has in fact been detrimental because of concomitant inhibition of PGI2 (38). Specific inhibition of the platelet EP3 receptor will shift the hemostatic balance toward an antithrombotic state by inhibiting the facilitating effect of PGE2 and allowing PGI2 to predominate. Finally, keeping in mind that PGE2 is produced by the arterial wall in response to inflammation, such a treatment would theoretically decrease only thrombosis triggered by inflammation.

In conclusion, we have shown that the potentiating effect of PGE2 has a clear *in vivo* significance. Through its platelet EP3 receptor, PGE2 is a full actor in intravascular hemostasis in inflammatory conditions, appearing as another link between inflammation and thrombosis. As a major consequence, we demonstrated that PGE2 exacerbates atherothrombosis. This unexpected role for PGE2 opens new possibilities for preventing myocardial infarction or stroke.

MATERIALS AND METHODS

Mice. Homozygous *ApoE*^{-/-} mice were obtained from the Jackson Laboratory. *Ep3*^{-/-} mice (provided by B.H. Koller, University of North Carolina at Chapel Hill, Chapel Hill, NC) and *Tp*^{-/-} mice (provided by T.M. Coffman, Duke University, Durham, NC) were previously described (39, 40). *Ep3*^{-/-} and *ApoE*^{-/-} mice have been intercrossed to obtain double-mutant mice. All of these strains were maintained on a C57BL/6 genetic

background. Animal procedures were approved by the local committee for animal ethics (Comité Régional d'éthique en Matière d'Expérimentation Animale de Strasbourg, Strasbourg, France). The investigator was unaware of the genotype of mice throughout the experiments.

Prostaglandin detection. Prostaglandin levels were determined from mashed tissues, using specific enzyme immunoassays (GE Healthcare). In the first experiment, the left common carotid of mice was dissected free, and a strip of parafilm was slipped below the carotid for topical application of 100% EtOH (vehicle) or 40 μ L AA (50 mg/ml in EtOH; Sigma-Aldrich) for 10 min. The carotid was harvested and snap frozen 30 min later. In a second series of experiments, a nonconstrictive polyethylene collar (0.38-mm inner diameter; Biotrol) was placed loosely around the left common carotid, as previously described (17), for 4 wk. In a third group, aortas of at least 35-wk-old *ApoE*^{-/-} mice were harvested from the aortic valves to the renal bifurcation and snap frozen. In the final group, *ApoE*^{-/-} mice were fed a high fat diet (D12336; Research Diets, Inc.) for 4 wk.

Visual scoring of arterial thrombosis. Carotids were exposed to 100 mg/ml AA for 10 min, and thrombosis was scored 2 h later under magnification using a visual scale from 0 to 4 (18).

Quantification of thrombosis induced in healthy carotids. Whole blood collected on 0.38% sodium citrate was centrifuged at 100 *g* to allow separation of platelets. Washed platelets were incubated with 300 ng/ml calcein AM (Invitrogen) in Tyrode's buffer for 15 min in the dark. Washed labeled platelets were injected in the jugular vein of receiver mice. The treated carotid was placed under a fluorescent microscope (MacroFluo; Leica) for video recording of thrombosis at 480 nm. Images extracted from the 40-min recorded video were computer processed for counting the number of green pixels. Thrombosis extent was expressed as the total number of green pixels divided by the duration of the experiment in minutes (pixels/min).

Models of thrombogenesis. The carotid was exposed to 100mg/ml AA or eicosatrienoic acid for 25 min, and thrombosis was recorded. Thrombosis induced by 5% ferric chloride (1 μ L; Sigma-Aldrich) was recorded immediately after the topical application. To demonstrate ferric chloride-induced denudation, three mice received 200 μ L of 2% Evans blue and 30 μ L heparin (100 U/ml). The photochemical injury followed the procedure previously described (21), except that thrombosis was quantified by fluorimetry, as described in the previous section. Rose bengal (Fischer Scientific) was diluted in PBS and injected in the jugular vein in a volume of 0.12 ml at a concentration of 50 mg/kg. The 1.5-mW, 540-nm laser (model 05-LGR-193; Melles Griot) was applied from a distance of 5 cm for 4 s, 45 s, or 30 min. The carotid was then harvested and snap frozen for PGE2 detection.

Platelet aggregation. Aggregation tests were performed in an optical aggregometer (model 570-VS; Chrono-log Corporation), using 250 μ L of platelet-rich plasma adjusted to 300,000 platelets/ μ L. Collagen was obtained from Chrono-log Corporation. Homogenates were obtained by mashing plaques in saline at a concentration of 80 mg/ml. Aspirin-treated *ApoE*^{-/-} mice received 500 mg/kg aspirin (Sanofi-Synthelabo) i.p. daily for 8 d. Each homogenized plaque was used for both PGE2 detection and aggregation tests.

AA-induced atherothrombosis. Mice >55 wk old received fluorescent platelets, and one carotid was exposed. An injection chamber was made by isolating an arterial segment bearing at least one plaque from blood flow with temporary ligatures (Fig. 5 A). Once washed, the chamber was filled with DMSO for 10 min before the blood flow returned. A 10-min video ensured that DMSO did not induce thrombosis. After ligatures were placed again, the vascular chamber was filled with a 2- μ L solution of 2 mg/ml AA (in DMSO) for 10 min. Once blood reflowed, thrombosis was recorded through a double bandpass optical filter (525 and 605 nm) for 30 min.

Rupture-induced atherothrombosis. After its tip was curved, a clean 30-gauge needle was introduced in 60-wk-old *ApoE*^{-/-} mice through a small collateral of internal carotid (Fig. 6 A). Under magnification, the tip of the needle was positioned next to the distal edge of the plaque, applied to the plaque, and pulled backward under visual control. The collateral artery was ligatured, and the mouse was placed under the fluoromicroscope for 20 min for video recording. The carotid was washed, harvested, fixed in 2.5% glutaraldehyde overnight, fixed in cacodylate buffer for a few minutes, post-fixed for 60 min in 4% osmium tetroxide, dehydrated in increasing ethanol concentrations, critical point dried with hexamethyldisilazane, mounted, sputter coated with palladium, and examined with a scanning electron microscope (XL20; Philips). Rupture areas were measured using Scion Image software. In two additional cases, the carotid was snap frozen, and 7- μ m cross sections were stained by hematoxylin and eosin.

En face atherosclerosis analysis. Anesthetized mice were infused with 10% neutral buffered formalin. Aorta were dissected, placed overnight in formalin at 4°C, opened longitudinally, and stained with 0.5% Sudan black. Measurements of the plaque area were performed using Scion Image software.

Statistics. Statistical tests were bilateral and nonpaired student *t* tests. When variances were not comparable, we used the bilateral nonpaired Welch-corrected *t* test. To compare more than two groups with non-Gaussian distribution, we used the Kruskal-Wallis test, followed by Dunn's multiple comparison test. Means are expressed \pm SEM. All tests were performed using GraphPad Prism (version 3.0 for Windows; GraphPad Software). *P* < 0.05 denotes statistical significance.

Online supplemental material. Video S1 shows the effect of EP3 on the thrombotic response induced by periaortic delivery of AA onto the mouse carotid artery. Video S2 shows atherothrombosis developing at the surface of a mouse carotid plaque in response to intraluminal delivery of AA, when EP3 is expressed or not. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20061617/DC1>.

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