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# Evidence that 5-lipoxygenase and acetylated cyclooxygenase 2-derived eicosanoids regulate leukocyte–endothelial adherence in response to aspirin

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**1** Unlike other nonsteroidal anti-inflammatory drugs that inhibit formation of cyclooxygenase (COX)-dependent eicosanoids, acetylation of COX-2 by aspirin switches eicosanoid biosynthesis from prostaglandin $E_2$ ($PGE_2$) to 15-epi-lipoxin $A_4$ (15-epi-$LXA_4$ or aspirin-triggered lipoxin, ATL). ATL formation by activated leukocytes (PMN) requires the intervention of 5-lipoxygenase (5-LOX), an enzyme that is involved in leukotriene $B_4$ ($LTB_4$) formation.

**2** In the present study, we have examined the role of acetylated COX-2 and 5-LOX in modulating antiadhesive effects of aspirin on adhesion of PMN to endotoxin (LPS)-primed human umbilical endothelial cells (HUVEC).

**3** Treating PMN/HUVEC cocultures with aspirin resulted in a concentration-dependent inhibition of cell-to-cell adhesion induced by LPS. Treating HUVEC with selective COX-2 inhibitors, celecoxib and rofecoxib, caused an $\approx$70% reversion of antiadhesive effect of aspirin. In contrast, inhibition of neutrophil's 5-LOX pathway with 1 $\mu$M ZD2138, a selective 5-LOX inhibitor, 1 $\mu$M BAY-X-1005, a FLAP inhibitor, or 100 $\mu$M licofelone, a dual COX/5-LOX inhibitor, did not affect antiadhesive properties of aspirin.

**4** Exposure to celecoxib (100 $\mu$M) or rofecoxib (10 $\mu$M) completely suppressed ATL formation caused by aspirin without affecting $LTB_4$ levels. ZD2138, licofelone and BAY-X-1005 inhibited ATL formation as well as $LTB_4$ generation.

**5** Treatment with $LXA_4$ reduced PMN adhesion to HUVEC and counteracted the proadhesive effect of celecoxib. In contrast, exposure to Boc-1, an $LXA_4$ antagonist, counteracts the antiadhesive activities of aspirin. Exposure to U75302, an $LTB_4$ receptor antagonist, enhances the antiadesive effect of aspirin.

**6** Reversal of antiadhesive activities of aspirin by celecoxib was associated with increased expression of LFA-1 on PMN and E-selectin on HUVEC. Addition of $LXA_4$, ZD2138 and U75302 inhibited these changes.

**7** The present results support the notion that inhibition of ATL formation is mechanistically linked to the reversal of the antiadhesive activity of aspirin caused by selective COX-1 inhibitors and suggests that the $LTB_4$/ATL balance modulates pro- and antiadhesive activity of nonsteroidal anti-inflammatory drugs at the leukocyte–endothelial cell interface.
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**Keywords:** Leukotriene B4; lipoxin; aspirin-triggered lipoxin; neutrophils/endothelium adherence; aspirin; 5-lipoxigenase; cyclooxygenase; celcoxib; rofecoxib

**Abbreviations:** ATL, aspirin-triggered 15-epi-$LXA_4$; COX-1 and COX-2, cyclooxygenase-1 and –2; licofelone, 2,2-dimethyl-6-(4-chlorophenyl)-7-phenyl-2,3-dihydro-1H-pyrrolizine-5- yl]-[2'-14C]-acetic acid; 5-LOX, 5-lipooxygenase; $LTB_4$, leukotrienes B4; $LXA_4$, lipoxin $A_4$; PMN, polymorphonuclear neutrophil; U75302, (6-(6-(3-hydroxy-1E,5Z-undecadienyl)-2-pyridinyl)-1,5-hexane diol); ZD2138, [6-[3-fluoro-5-[4-methoxy-3,4,5,6-tetrahydro-2H-pyran-4-yl])phenoxy-methyl]-1-methyl-2-quinolone]

## Introduction

Adhesive interactions between neutrophils and endothelial cells play a major role in inflammatory diseases (Alpin *et al.*, 1998). Aspirin is a widely used antithrombotic, anti-inflammatory and analgesic drug (Patrono, 1994). However, unlike other nonsteroidal anti-inflammatory drugs (NSAIDs) that inhibit formation of eicosanoids from cyclooxygenase (COX)-1 and -2, aspirin switches COX-2-mediated eicosanoid biosynthesis from prostaglandin $E_2$ ($PGE_2$) to 15-epi-lipoxin $A_4$ (15-epi-$LXA_4$) or aspirin-triggered lipoxin (ATL) (Funk,

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2001; Lecomte *et al*., 1994; Clarià & Serhan, 1995; Planagumà *et al*., 2002; Serhan, 2002). Similar to endogenous $LXA_4$, ATL acts at target tissues as a braking signal limiting neutrophils' activation/recruitment (Serhan, 2002). In some tissues, aspirin also triggers the formation of the 5-lipoxygenase (5-LOX)-derived leukotriene $B_4$ ($LTB_4$), a potent PMN activator and chemoattractant (Planagumà *et al*., 2002), suggesting that exposure to aspirin supports the formation of both pro- and anti-inflammatory mediators.

Adhesive interactions involved in leukocyte recruitment in inflamed tissues represent a major target for anti-inflammatory drugs and aspirin (Carlos & Harlan, 1994; Alpin *et al*., 1998; Pillinger *et al*., 1998; Jordan *et al*., 1999). The expression of adhesion molecules on endothelial cells and leukocytes is tightly regulated by the availability of potent LTs, such as $LTB_4$, which in conjunction with other inflammatory mediators (cytokines and CXC chemokines) attract leukocytes at the site of inflammation (Yokomizo *et al*., 2000). $LXA_4$ modulates LTs bioactivity and synthesis, suggesting that by amplifying this endogenous anti-inflammatory circuit, ATL mediates, at least in part, some of the antiadhesive activities of aspirin (Scalia *et al*., 1997; Takano *et al*., 1997; Filep *et al*., 1999; Gronert *et al*., 2001; Serhan, 2002).

The treatment of pain and inflammation has changed dramatically in recent years after the introduction in the therapy of a new class of anti-inflammatory drugs that selectively inhibits the formation of COX-2-derived prostanoids: the coxibs (FitzGerald & Patrono, 2001). As these drugs do not inhibit COX-1 and are devoid of antiplatelet activity, it has been suggested that low doses of aspirin might be coadministered in combination with selective COX-2 inhibitors to patients with cardiovascular diseases that need an anti-inflammatory/analgesic therapy (Silverstein *et al*., 2000). However, since the acetylated form of COX-2 is required for ATL formation, its inhibition might, in theory, alter the balance between $LTB_4$ and ATL at target tissues, dampening the antiadhesive activity of aspirin (Serhan & Oliw, 2001).

As an increased adherence of leukocytes to endothelial cells is mechanistically involved in many inflammatory and degenerative process, we designed a study to assess the effect of aspirin and selective COX-2 inhibitors, celecoxib and rofecoxib, on leukocyte/endothelial cell adherence induced by bacterial endotoxin (LPS). Here we demonstrate that coxibs inhibit ATL synthesis without interfering with $LTB_4$ generation. Since ATL and $LTB_4$ exert divergent effects on leukocyte/endothelial cell adhesion, celecoxib and rofecoxib significantly impair the antiadhesive effects of aspirin. Interestingly, the simultaneous inhibition of $LTB_4$ and ATL formation obtained with the dual COX/5-LOX inhibitor licofelone did not interfere with the antiadhesive properties of aspirin, suggesting that $LTB_4$/ATL balance modulates antiadhesive activities of aspirin at the leukocyte–endothelial cell interface.

## Methods

### *Materials*

Aspirin, lipopolysaccharide (LPS; *Escherichia coli* 0111:B4 serotype) and Boc1 (*N*-*t*-butoxycarbonyl-methionine-leucine-phenylalanine) were from Sigma Chemical (St Louis, MO, U.S.A.). Licofelone, previously named ML-3000, 2,2-dimethyl-6-(4-chlorophenyl)-7-phenyl-2,3-dihydro-1H-pyrrolizine-5-yl]-acetic acid, was kindly provided by Merckle (Germany). Celecoxib was synthesized as previously described (Fiorucci *et al*., 2002). ZD2138 (Smith *et al*., 1995), rofecoxib (Nicoll-Griffith *et al*., 2000) and BAY-X-1005 (Muller-Peddinghaus *et al*., 1993) were synthesized by Dr Stefan Laufer (Department fur Pharmazie-Zentrum fur Pharmaforschung, University of Tubingen, Germany). Mouse anti-human monoclonal antibody anti-CD11a (LFA-1), anti-CD11b, and anti-E-selectin were from Immmunotech (Marseille, France). U-75302, a selective $LTB_4$ antagonist, was from Biomol (Plymouth Meeting, PA, U.S.A.) and Interleukin 1$\beta$ (IL-1$\beta$) from R&D Systems (Minneapolis, MN, U.S.A.).

### *HUVEC*

Primary cultures of human umbilical endothelial cells (HUVEC) were from Istituto Zooprofilattico of Brescia (Brescia, Italy). Culture media and fetal bovine serum (FBS) were from GIBCO (Milan, Italy). HUVEC were grown in endothelial basal medium supplemented with bovine brain extract ($12\,\mu g\,ml^{-1}$), human epithelial growth factor ($10\,ng\,ml^{-1}$), hydrocortisone ($1\,\mu g\,ml^{-1}$), penicillin ($100\,U/ml^{-1}$), streptomycin ($100\,\mu g\,ml^{-1}$), and gentamycin ($5\,\mu g\,ml^{-1}$), at 37°C in a humidified atmosphere containing 5 and 2% FBS. Cells were used between passages 2 and 5.

### *PMN isolations*

Fresh peripheral blood was isolated from healthy donors who had refrained from taking aspirin or other medications for at least 2 weeks. Neutrophils (PMN) were isolated using standard dextran sedimentation and gradient separation on Histopaque-1077 (Sigma Chemical Co., St Louis, MO, U.S.A.). This procedure yields a PMN population that is 95–98% viable (trypan blue exclusion) and 98% pure (acetic acid–crystal violet staining).

### *PMN–HUVEC cocultures*

HUVECs were seeded on gelatin-coated, 24-well plates for 48 h. Confluency was confirmed by microscopical inspection before each experiment. Confluent cells were then incubated for 24 h in the presence of $1\,ng\,ml^{-1}$ IL-1$\beta$ and $10\,ng\,ml^{-1}$ LPS (Clarià & Serhan, 1995; Filep *et al*., 1999; Gronert *et al*., 2001). For eicosanoid generation experiments, $2 \times 10^5$ HUVEC, treated as described above, was incubated with $500\,\mu M$ aspirin for 20 min and then for 30 min with $5\,\mu M$ A23187 and $20\,\mu M$ arachidonic acid (AA) and then coincubated with $2 \times 10^6$ PMN for further 30 min (Clarià & Serhan, 1995). To inhibit COX-2 activity, celecoxib, rofecoxib or licofelone was added directly to HUVEC and cells preincubated for 20 min before neutrophil addition. To inhibit 5-LOX activity, PMN were preincubated for 30 min with ZD2138, licofelone or BAY-X-1005 and then added to HUVEC cocultured for 30 min. Thus, each cell type was exposed only to a single drug. In experiments where Boc-1, $LXA_4$, $LTB_4$ and U75302 were used, these agents were added directly to PMN/HUVEC cocultures.

*HUVEC–PMN adhesion assay*

Freshly isolated PMN were washed twice with labelling medium (RPMI 1640 plus 1% FBS) and then incubated for 1 h (37°C; 5% $CO_2$) with $^{51}CrO_4$ (sodium salt; DuPont NEN, Boston, MA, U.S.A.; $3-5\,\mu Ci/5\times10^7$ cells). The labelled leukocytes were washed four times with labelling medium and then resuspended in fresh labelling medium. For static adhesion assays, 50 $\mu$l of labelled neutrophil suspension ($1\times10^7$ cells) was added to each well of endothelial cells (10 : 1 ratio of leukocytes to endothelial cells) and incubated for 30 min at 37°C on an orbital shaker at 90 rpm in the presence of aspirin. At the end of the incubation period, the medium from each well was aspirated and saved for radioactive counting. The monolayer was gently washed three times with cold PBS to remove loosely adherent or unattached neutrophils; collected washes were combined with medium and counted, yielding a measure of nonadherent leukocytes. After the final wash, monolayers were lysed for 1 h with 1 M NaOH; counting of the lysate (in counts $min^{-1}$(cpm)) yielded a measure of adherent leukocytes. Adhesion was quantitated as follows: %PMN adherence = lysate (cpm)/(supernatant (cpm) + wash (cpm) + lysate (cpm)), where cpm is counts per minute.

*CD11b, LFA-1 and E-selectin flow cytometry*

Surface expression of adhesion molecules was quantified by flow cytometry. To investigate the expression of CD11a (LFA-1) and CD62E (E-selectin) on PMN and HUVEC, activated HUVEC (see above) were treated with appropriate agents (see Figure 6) and then cocultured for 30 min with PMN. At the end of the incubation, both PMN and HUVEC were harvested by extensive wash and culture trypsinization. PMN were identified by staining with 20 $\mu g\,ml^{-1}$ of mouse anti-human Cd11b (MAC-1). HUVEC were identified either by staining with CD54 (ICAM-1) or by size (forward and side scatter). After staining with specific antibodies, cells were washed twice and incubated with FITC-conjugated sheep anti-mouse $F(ab')_{2s}$ Ab (1 : 400 dilution; Sigma Chemical Co., St Louis, MO, U.S.A.) for 45 min at 4°C. Stained cells were washed once and fixed in 1% ($v\,v^{-1}$) formaldehyde in PBS. Flow cytometry was performed on an Epics XL instrument (Coulter-Beckman, Milan, Italy). After gating out small-sized (i.e. noncellular) debris, 50,000 events were collected for each analysis. The levels of E-selectin and CD11b for each cell type for each experiment were normalized against the value of the isotype-matched control antibody (background).

*15-epi-$LXA_4$ (ATL) assay*

The 15-epi-$LXA_4$ concentrations were measured using a commercial assay (Neogen Corporation, Lansing, MI, U.S.A.) following the manufacturer's instructions. Samples were extracted according to a previously published method (Romano & Serhan, 1992). The antibody used in this assay specifically recognizes 15-epi-$LXA_4$, and has been characterized previously by others (Chiang *et al*., 1998). The identity of 15-epi-$LXA_4$ in gastric homogenates was also confirmed using a dual pump reversed-phase (RP)-HPLC analysis (Fiorucci *et al*., 2002). The column (Waters Symmetry $C_{18}$, 3 $\mu$m, $2.1\times150$ mm) was eluted with MeOH/$H_2O$/acetic acid (65 : 35 : 0.01; $v\,v^{-1}\,v^{-1}$) at a flow rate of 0.2 ml $min^{-1}$. Postrun analysis was performed with the Millennium 32 chromatography manager. $LXA_4$ and 15-epi-$LXA_4$ were identified by online UV spectral analysis and comparison of their retention times with those of authentic standards ($LXA_4$ was from Cascade Biochem Ltd, Reading, England and 15-epi-$LXA_4$-methyl ester was kindly provided by Dr C.N. Serhan). 15-epi-$LXA_4$-methyl ester was converted to free acid by saponification as described elsewhere (Romano & Serhan, 1995).

*$LTB_4$, $PGE_2$, $PGI_2$ assay*

These assays were carried out in duplicate on cell supernatants using a commercially available ELISA kit (Cayman Chemical Co., Milan, Italy) following the manufacturer's instructions.

*Statistical analysis*

All data are presented as the mean ± s.e.m. Comparisons of groups of data were performed using a one-way analysis of variance followed by the Student–Newman–Keuls *post hoc* test. An associated probability (*P*-value) of less than 5% was considered significant.

## Results

In a preliminary set of experiments, we have assessed the time course and the concentration dependency of PMN adhesion to HUVEC (data not shown). As adhesion of PMN was maximal when HUVEC were incubated for 24 h with 1 ng $ml^{-1}$ IL-1$\beta$ and 10 $\mu g\,ml^{-1}$ LPS, these concentrations and time-frame were used for all the following experiments.

As illustrated in Figure 1a, aspirin inhibited in a concentration-dependent manner the adhesion of PMN to activated HUVEC. At the dose of 100 $\mu$M (Figure 1b), aspirin caused an $\approx$80% reduction of these adhesive interactions ($n=6$; $P<0.001$ *versus* LPS alone). To dissect mediators involved in this effect, LPS-primed HUVEC were incubated with celecoxib and rofecoxib, two selective COX-2 inhibitors, or licofelone, a dual COX/5-LOX inhibitor, and adhesion assessed. As shown in Figure 1b–d, celecoxib and rofecoxib, but not licofelone, caused a concentration-dependent reversion of the antiadhesive activity of aspirin ($n=6$; $P<0.001$ *versus* aspirin alone).

As shown in Figure 2, adhesion of PMN to IL-1$\beta$- and LPS-primed HUVEC resulted in a significant stimulation of $PGE_2$, $PGI_2$ and $LTB_4$ generation in comparison with untreated cells ($n=6$ experiments, $P<0.01$). Addition of aspirin, 100 $\mu$M, switched prostanoid biosynthesis from $PGE_2$ to ATL, without affecting $LTB_4$ generation (Figure 2, $n=6$, $P<0.01$). Exposure to celecoxib, 100 $\mu$M, and rofecoxib, 10 $\mu$M, abrogated ATL generation induced by aspirin ($n=6$, $P<0.01$ *versus* aspirin alone) and caused a further inhibition of $PGE_2$ and $PGI_2$ synthesis ($n=6$, $P<0.01$ *versus* aspirin alone). This treatment, however, did not affect $LTB_4$ generation ($n=6$, $P>0.05$ *versus* aspirin alone). In contrast, preincubating HUVEC/PMN cocultures with licofelone resulted in a 70% reduction of $LTB_4$ and ATL formation ($n=6$, $P<0.01$ *versus* aspirin), but failed to potentiate the inhibitory effect of aspirin on $PGE_2$ and $PGI_2$. In control experiments where celecoxib and rofecoxib were added to PMN, no effect was observed on $LTB_4$ production (data not shown).

Since these data demonstrated that coxibs reverse the antiadhesive activity of aspirin and inhibit the formation of antiadhesive mediators, ATL, without interfering with the accumulation of pro-adhesive agents, $LTB_4$, we have examined whether modulation of ATL and $LTB_4$ is mechanistically involved in the effect of aspirin on neutrophils' adherence to activated HUVEC. As illustrated in Figure 3a, adding 10 ng ml$^{-1}$ $LXA_4$ directly to HUVEC/PMN cocultures significantly inhibited adhesion ($n=6-8$ experiments, $P<0.01$) and potently counteracted the proadhesive effects of celecoxib and rofecoxib ($n=6-8$, $P<0.001$). The effect of $LXA_4$ (Figure 3b) was concentration-dependent ($n=6-8$ experiments, $P<0.05$ *versus* LPS plus 100 $\mu$M celecoxib or 10 $\mu$M rofecoxib). The relevance of ATL inhibition in explaining the proadhesive effect of coxibs was further confirmed by treating the cells with Boc-1 (Perretti *et al*., 2001; Fiorucci *et al*., 2002), an $LXA_4$ receptor antagonist (Figure 3c and d). Indeed, not only addition of Boc-1 to HUVEC/PMN cocultures significantly inhibited the antiadhesive activity of $LXA_4$ ($n=6$, $P<0.01$ *versus* $LXA_4$ alone), confirming that this compound is an $LXA_4$ receptor antagonist (Figure 3c), but, as shown in Figure 3d, it also impaired the antiadhesive effect of aspirin ($n=8$, $P<0.01$ *versus* aspirin alone), demonstrating that ATL is involved in the antiadhesive activity of aspirin. Exposure to Boc-1, as shown in Figure 3d, did not increase adhesion caused by celecoxib and rofecoxib ($n=8$, $P>0.05$ *versus* celecoxib and rofecoxib alone). Thus, treating PMN/HUVEC cocultures with $LXA_4$ inhibits adhesion induced by LPS and counteracts the reversal of antiadhesive activity of aspirin caused by coxibs. In contrast, inhibition of endogenous $LXA_4$ activity with a selective $LXA_4$ receptor antagonist enhances the antiadhesive activities of aspirin.

To evaluate the role of 5-LOX-derived LTs on antiadhesive activity of aspirin, PMN were incubated with ZD2138, a potent and selective inhibitor of 5-LOX (Smith *et al*., 1995), BAY-X-1005, a FLAP inhibitor, before addition to activated HUVEC. As illustrated in Figure 4, ZD2138, BAY-X-1005 and U75302, an $LTB_4$ receptor antagonist, significantly reduced adhesion of PMN to activated HUVEC ($n=6-8$, $P<0.01$ *versus* LPS alone) and increased the antiadhesive activity of aspirin ($n=8-10$, $P<0.01$ *versus* aspirin alone). While inhibition of COX-2 activity with celecoxib dampened the antiadhesive effect of aspirin, inhibition of $LTB_4$ formation with ZD2138 and BAY-X-1005 restored the antiadhesive property of aspirin ($n=8-10$, $P<0.001$ *versus* celecoxib). Similar to licofelone, exposure of aspirin-treated cocultures to ZD2138 and BAY-X-1005 resulted in a significant inhibition of $LTB_4$ and ATL formation (Figure 5, $n=6-8$, $P<0.01$ *versus* cells incubated with LPS alone). As expected, adding U75302, a selective $LTB_4$ antagonist, to HUVEC/PMN

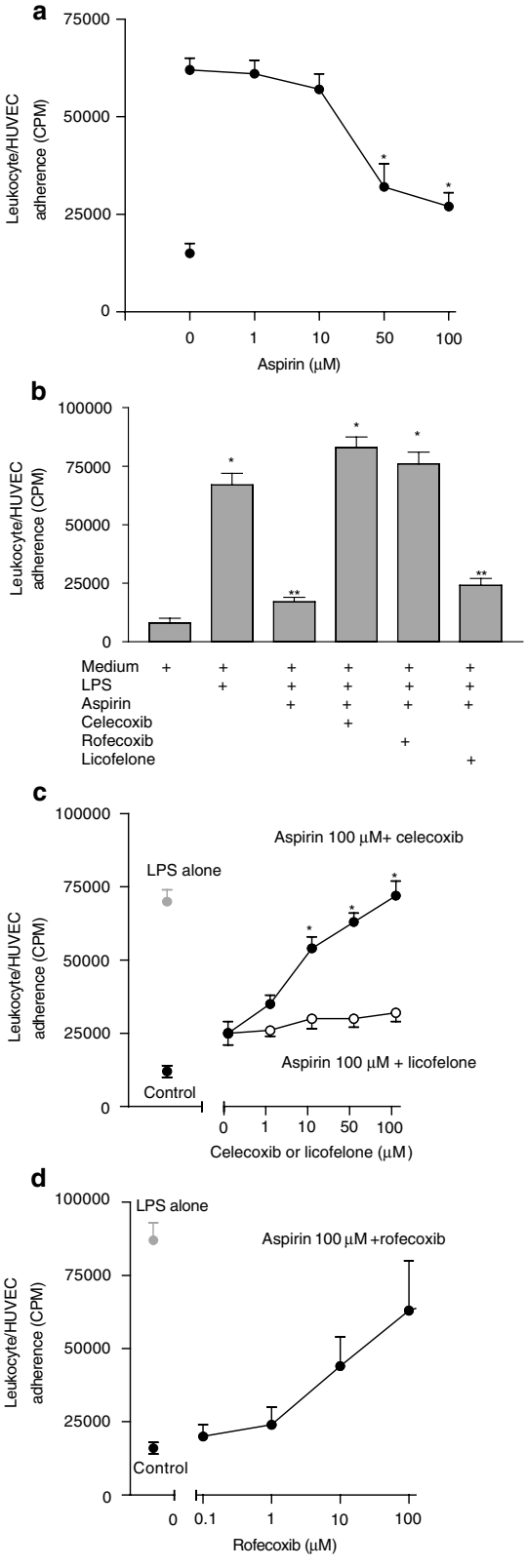



**Figure 1** Panel (a) Aspirin causes a concentration-dependent inhibition of cell to cell adhesion in PMN/HUVEC cocultures. Data are mean ± s.e. of six experiments. Asterisk denotes $P<0.001$ *versus* LPS alone. Panel (b) COX-2-derived eicosanoids are required for antiadhesive effects of aspirin. Exposure of PMN/HUVEC cocultures to 100 $\mu$M celecoxib or 10 $\mu$M rofecoxib but not to the dual COX-5-LOX inhibitor, licofelone, abrogates the antiadhesive activities of aspirin. Data are mean ± s.e. of six experiments. *$P<0.001$ *versus* control; **$P<0.0001$ *versus* LPS alone. Panel (c) Celecoxib, but not licofelone, causes a concentration-dependent reversal of antiadhesive properties of aspirin in PMN/HUVEC cocultures. Data are mean ± s.e. of six experiments. *$P<0.01$ *versus* aspirin alone. Panel (d) Rofecoxib causes a concentration-dependent reversal of antiadhesive properties of aspirin in PMN/HUVEC cocultures. Data are mean ± s.e.of six experiments. *$P<0.01$ *versus* aspirin alone.

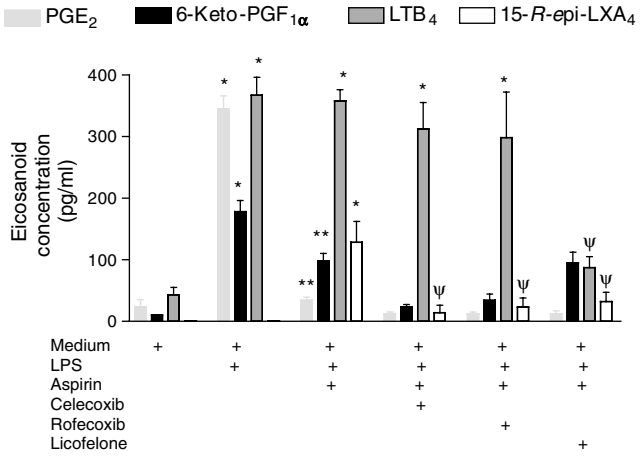
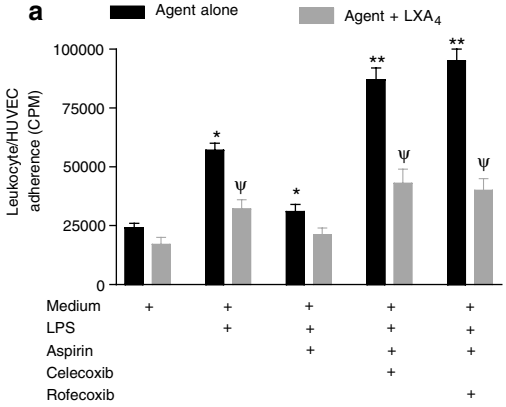


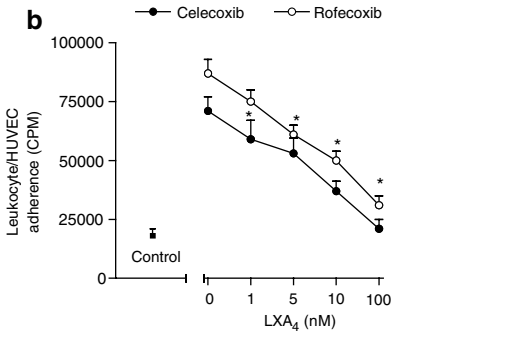

**Figure 2** Effect of COX and 5-LOX inhibitors on eicosanoid formation. Data are mean ± s.e. of six experiments. $P<0.001$ *versus* control; $^{*}P<0.01$ *versus* cells incubated with medium alone; $^{**}P<0.01$ *versus* cells incubated with LPS, $^{\psi}P<0.01$ *versus* cells incubated with LPS plus aspirin.

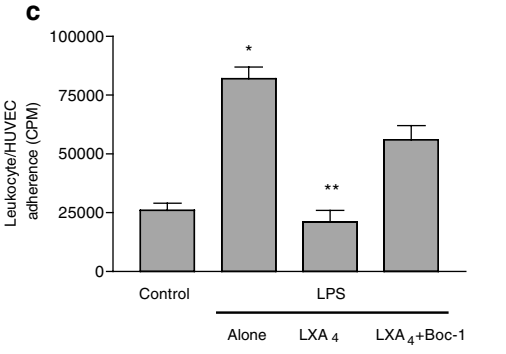
cocultures also reversed proadhesive effects of celecoxib but had no effect on eicosanoid generation (Figure 4). Similar to licofelone, the effects exerted by ZD2138, BAY-X-1005 and U75302 were concentration-dependent (Figure 5c–e).

Consistent with the observation that inhibition of the 5-LOX pathway reverses the proadhesive effect of celecoxib, the addition of $LTB_4$ to PMN–HUVEC cocultures reverted antiadhesive activity of aspirin as well as the activity of ZD2138 and BAY-X-1005 (Figure 5; $n=6$–8, $P<0.01$ *versus* aspirin alone). Also the effect exerted by U75302 was reverted by $LTB_4$, although a higher concentration, 10 $\mu$M, was required (data not shown).

To investigate whether reversal of antiadhesive effects of aspirin caused by celecoxib was mediated by changes in adhesion molecule expression, we assessed the expression of LFA-1 and E-selectin on PMN and HUVEC cocultures. Figure 6 shows that LFA-1 and E-selectin expression on PMN and HUVEC surface was markedly increased after exposure to LPS ($n=6$, $P<0.01$ *versus* control cells); this effect was not modified by aspirin. While celecoxib by itself had no effect on expression of these adhesion molecules (data not shown), it significantly enhanced both LFA-1 and E-selectin expression ($n=6$–8, $P<0.05$ *versus* aspirin alone) in PMN/HUVEC cocultures challenged with LPS plus aspirin. This effect was concentration-dependent (Figure 6d). Interestingly, while addition of 10 nM $LXA_4$ significantly reduced the upregulation of LFA-1 and E-selectin induced by LPS ($n=6$–8; $P<0.01$),



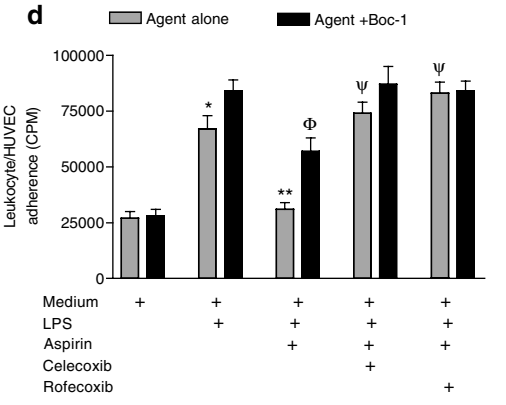




**Figure 3** Panel (a) $LXA_4$ reverses the antiadhesive activities of coxibs. LPS-treated PMN and HUVEC were treated with 10 ng ml$^{-1}$ LXA in the presence of 100 $\mu$M aspirin alone or aspirin plus celecoxib or rofecoxib and adhesion assessed. Data are mean ± s.e.of six to eight experiments. $^{*}P<0.01$ *versus* control; $^{**}P<0.001$ *versus* LPS plus aspirin; $^{\psi}P<0.05$ *versus* LPS plus aspirin and celecoxib or rofecoxib. Panel (b) LXA causes a concentration-dependent reversion of proadhesive effects of celecoxib and rofecoxib. Data are mean ± s.e. of six to eight experiments. $^{*}P<0.05$ *versus* cells incubated without $LXA_4$. Panel (c) Boc-1 antagonizes the antiadhesive activity of $LXA_4$. Data are mean ± s.e. of six experiments. $^{*}P<0.01$ *versus* control; $^{**}P<0.01$ *versus* LPS plus $LXA_4$. Panel (d) Boc-1, a selective $LXA_4$ receptor antagonist, reverses antiadhesive activities of aspirin. Data are mean ± s.e. of six experiments. $^{*}P<0.01$ *versus* control. $^{**}P<0.001$ *versus* LPS alone; $^{\Phi,\psi}P<0.01$ *versus* aspirin alone.

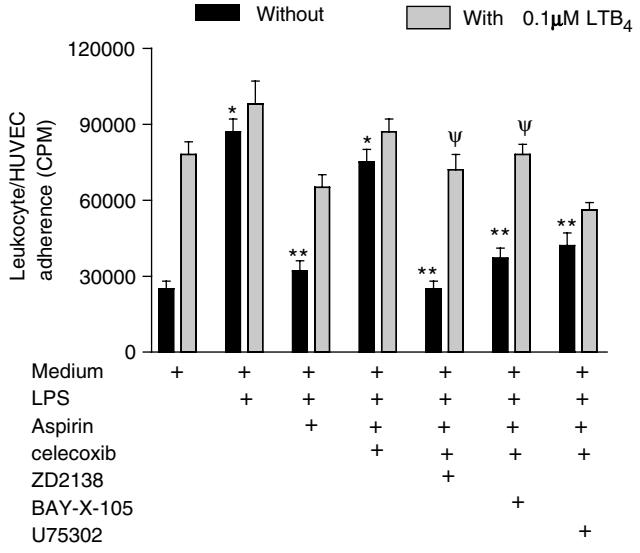



**Figure 4** LTB4 reverts antiadhesive activities of aspirin. Data are mean ± s.e. of six to eight experiments. $^{*}P<0.01$ *versus* cells incubated with medium alone; $^{**}P<0.01$ *versus* cells incubated with LPS; $^{\psi}P<0.01$ *versus* cells incubated with LPS plus celecoxib in combination with aspirin, ZD2138, BAY-X-1005 or U75302.

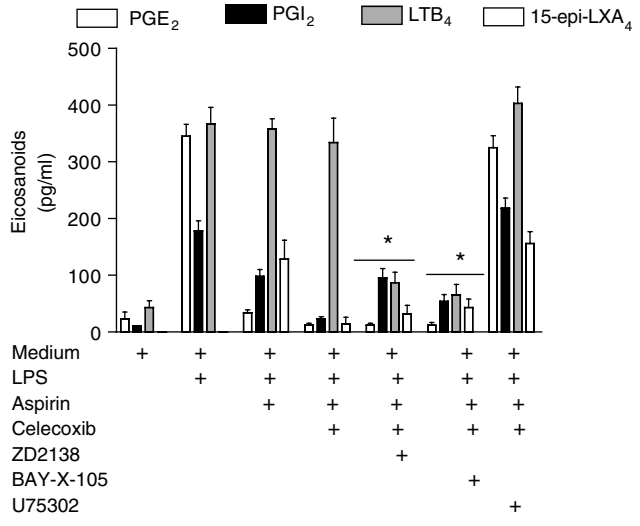



**Figure 5** Effect of aspirin and celecoxib in combination with ZD2138, BAY-X-1005 and U75302 on eicosanoid generation by LPS-primed PMN-HUVEC cocultures. Data are mean ± s.e. of six to eight experiments. $^{*}P<0.01$ *versus* LPS alone. Panel (c)–(e). The antiadhesive effect exerted by ZD2138, BAY-X-1005 and U75302 is concentration-dependent. $^{*}P<0.01$ *versus* cells incubated with LPS plus celecoxib.

the $LXA_4$ antagonist Boc-1 had the opposite effect ($P<0.05$ *versus* aspirin alone). Inhibition of 5-LOX with 1 $\mu$M ZD2138 also downregulated the expression of LFA-1 and E-selectin ($n=6$–8; $P<0.01$) induced by LPS. Finally, confirming the role of LFA-1 and E-selectin in PMN/HUVEC adhesion, exposure to licofelone to inhibit the formation of COX- and 5-LOX-derived eicosanoids downregulated the expression of E-selectin on HUVEC ($n=6$ experiments; $P<0.01$ *versus* aspirin plus LPS).

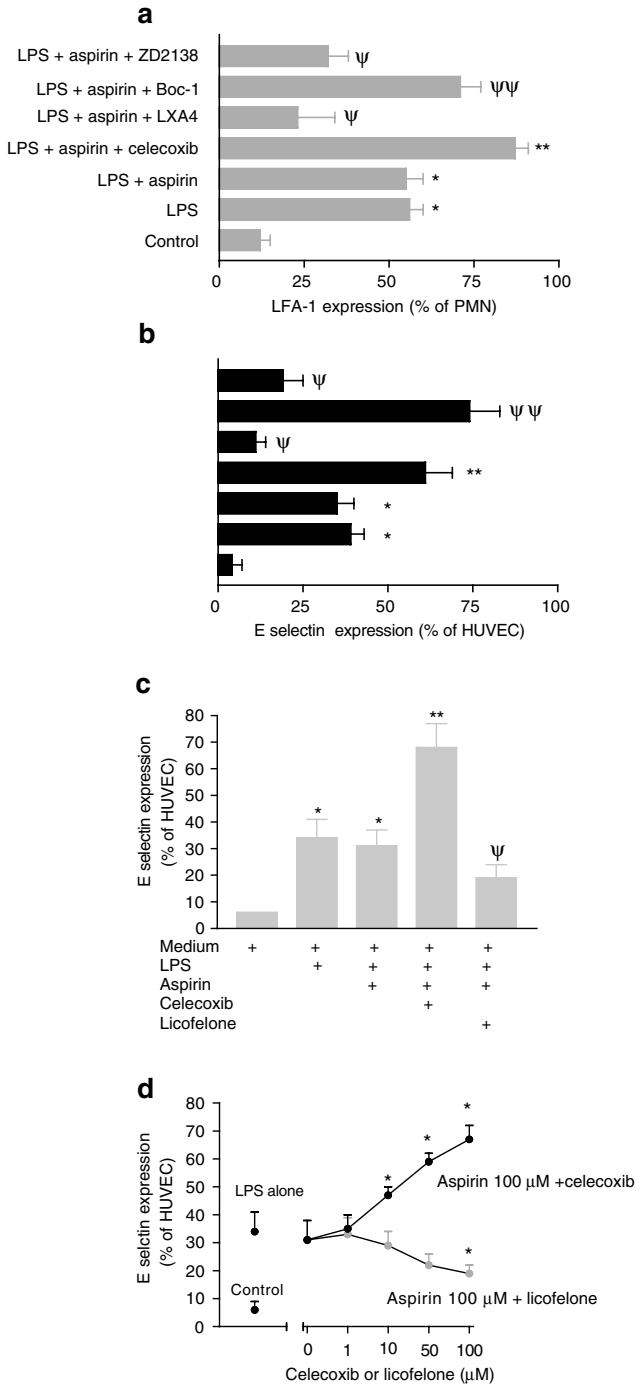



**Figure 6** $LXA_4$ and 5-LOX inhibition reverses upregulation of adhesion molecules caused by celecoxib on PMN (panel a) and HUVEC (panel b). Data are mean ± s.e. of six to eight experiments. $^{*}P<0.01$ *versus* control; $^{**}P<0.05$ *versus* LPS + aspirin; $^{\psi}P<0.01$ *versus* LPS plus aspirin; $^{\psi\psi}P<0.05$ celecoxib *versus* LPS plus aspirin. Panels (c) and (d). Induction of E-selectin expression on HUVEC caused by celecoxib is concentration dependent. Data are mean ± s.e. of six to eight experiments. $^{*}P<0.01$ *versus* untreated HUVEC; $^{**}P<0.01$ *versus* aspirin; $^{\psi}P<0.01$ *versus* aspirin plus celecoxib. Panel (d) Licofelone causes a concentration-dependent inhibition of E-selectin expression on HUVEC. Data are mean ± s.e. of six to eight experiments. $^{*}P<0.01$ *versus* HUVEC incubated with LPS plus aspirin.

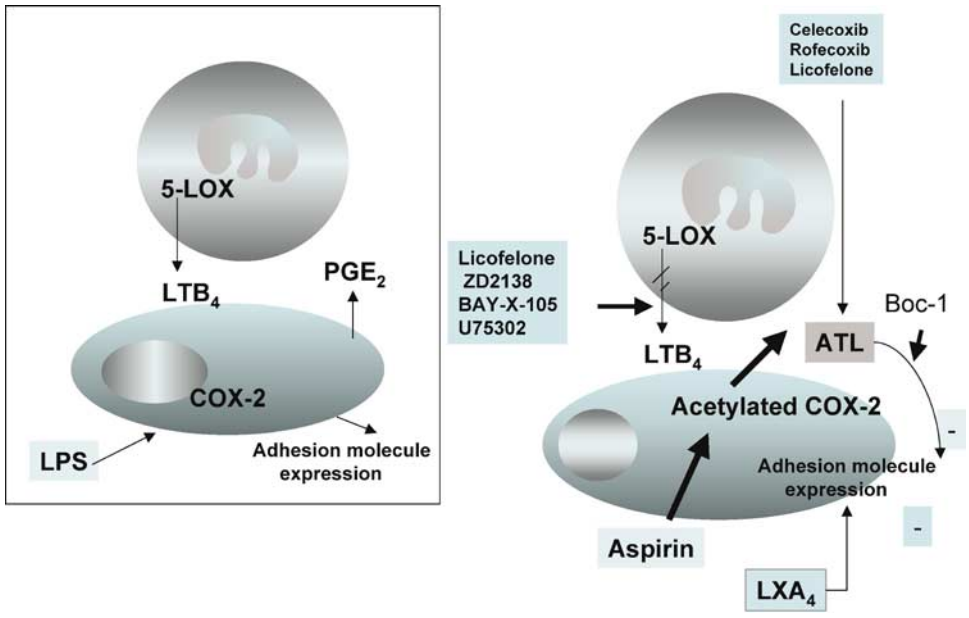



**Figure 7** Schematic drawing of the pathways activated by aspirin in LPS-primed PMN/HUVEC cocultures. (Inset) Exposure to LPS stimulates 5-LOX and COX-2 to generate proinflammatory ($PGE_2$) and proadhesive ($LTB_4$) eicosanoids. In the presence of aspirin, COX-2 is acetylated and eicosanoid metabolism is switched from $PGE_2$ to ATL and $LTB_4$. Inhibition of $LTB_4$ with celecoxib causes the reversal of antiadhesive activities of aspirin. Inhibition of 5-LOX with licofelone or ZD2138 enhances antiadhesive activity of aspirin. Addition of $LXA_4$ restores the antiadhesive activity of aspirin in the presence of celecoxib.

## Discussion

In the present study, we demonstrated that exposure of LPS-primed PMN/HUVEC cocultures to coxibs inhibits ATL formation and dampens the antiadhesive activity of aspirin.

Similar to $LXA_4$ , ATL is emerging as an endogenous braking signal for neutrophils and exerts a potent inhibitory effect on several inflammatory mechanisms, including cytokine and chemokine generation (Gronert *et al*., 2001), leukocyte responses to cytokines or to microbial stimulation (Gewirtz *et al*., 1998; Takano *et al*., 1998), neutrophil and eosinophil migration (Fiore & Serhan, 1995), as well as cell surface expression of P-selectin and LFA-1 (Filep *et al*., 1999). ATL is a tetraene-containing eicosanoid structurally related to $LXA_4$ generated in the presence of aspirin (see Serhan, 2002 for a review). $LXA_4$ is released by a transcellular biosynthetic route by endothelial cells/leukocytes after exposure to proinflammatory cytokines such as IL-1$\beta$ or TNF-$\alpha$ *or* bacterial products (Clarià & Serhan, 1995; Serhan, 2002). In this proinflammatory setting, aspirin switches COX-2 catalytic activity from prostaglandin intermediates to 15-*R*-HETE, which is released and transformed *via* transcellular routes to form 15-epi-$LXA_4$ by leukocytes in close proximity (Clarià & Serhan, 1995; Serhan, 2002). The interest in this pathway is that COX-2 is present in abundance at the PMN/endothelial cell interface (FitzGerald & Patrono, 2001) in inflammatory reactions, and is in place when individuals take aspirin for its therapeutic benefit. Thus, the finding that selective COX-2 inhibitors abrogate ATL formation induced by aspirin might be of pharmacological and clinical relevance. Indeed, by limiting the production of this potentially protective mediator, celecoxib and rofecoxib reverse the antiadhesive activities of aspirin on PMN/HUVEC cocultures, and might therefore interfere with the antiadhesive activity of aspirin *in vivo*.

The most obvious explanation for the effect of celecoxib and rofecoxib is that these drugs inhibit the acetylated form of COX-2. Indeed, while aspirin acetylates both COX-1 and COX-2 (Patrono, 1994), and COX-1 acetylation results in irreversible inhibition, acetylation of COX-2 leads to an enzyme that performs an incomplete reaction transforming AA into 15*R*-HETE (Clarià & Serhan, 1995). We have recently demonstrated (Fiorucci *et al*., 2002), and confirmed in the present study in a different setting, that celecoxib and rofecoxib abrogate ATL synthesis triggered by aspirin, suggesting that selective COX-2 inhibitors inhibit both the acetylated and the nonacetylated form of COX-2. The observation that nonsteroidal antiinflammatory drugs demonstrate either equal or slightly decreased potency for inhibition of the acetylated form of COX-2 compared with the native enzyme, further supports this concept (Mancini *et al*., 1997).

Consistent with the view that ATL inhibition is the key mechanism responsible for the proadhesive activity of celecoxib, we have demonstrated that exogenous $LXA_4$ can compensate for inhibition of acetylated COX-2 and restores the antiadhesive activity of aspirin (Scalia *et al*., 1997; Takano *et al*., 1997; 1998) in neutrophils/HUVEC cocultures exposed to a coxib. Further supporting the role of COX-2-derived ATL in the antiadhesive activity of aspirin, we demonstrated that antagonism of $LXA_4$ with Boc-1 enhances adhesion caused by celecoxib (Perretti *et al*., 2001).

An equally important finding of the present study was the demonstration that exposure of PMN/HUVEC cocultures to LPS triggered the formation of $LTB_4$, and that inhibition of 5-LOX activity with ZD2138 (Smith *et al*., 1995), a selective 5-LOX inhibitor, or licofelone (Laufer *et al*., 1994), a dual 5LOX-COX inhibitor, or BAY-X-1005, a FLAP inhibitor, greatly enhances the antiadhesive activity of aspirin. The

finding that $LTB_4$ enhances adhesion of neutrophils to activated HUVEC, and that U75302, an $LTB_4$ receptor antagonist, dampens this effect, supports the notion that, despite the ability of aspirin to switch eicosanoid biosynthesis from $PGE_2$ to ATL, a family of proinflammatory mediators that counteracts anti-inflammatory activities of aspirin is generated at the PMN/HUVEC interface during inflammation by the 5-LOX pathway. $LTB_4$, the main LTs generated by activated 5-LOX (Samuelsson *et al*., 1987; Yokomizo *et al*., 2000), is a potent proinflammatory agent and is involved in targeting PMN at the site of inflammation and is a well-known inducer of adhesion molecules on both endothelial cells and neutrophils (Samuelsson *et al*., 1987). As licofelone, BAY-X-1005 and ZD2138 failed to impair antiadhesive activities of aspirin in LPS-primed PMN/HUVEC cocultures, despite the fact that they block the conversion of 15R-HETE to ATL, it appears that 5-LOX-derived $LTB_4$ is operative in this experimental setting and counteracts the antiadhesive properties of locally generated ATL (Figure 6).

We have further confirmed that $LTB_4$/ATL balance is mechanistically linked to modulation of PMN adhesion to HUVEC, by showing that addition of $LXA_4$ or inhibition of 5-LOX with ZD2138 modulates the expression of adhesion molecules on PMN and HUVEC. $LTB_4$ is a potent chemoattractant for PMN and is known to induce LFA-1 expression on their surface (Samuelsson *et al*., 1987; Yokomizo *et al*., 2000). While our results demonstrated that aspirin at the concentrations used in this study did not modify the expression of LFA-1 on PMN and E-selectin on HUVEC (Pillinger *et al*., 1998), treating the cells with $LXA_4$ concentration-dependently abrogated upregulation of these adhesion molecule expression induced by LPS and celecoxib (Scalia *et al*., 1997). The observation that exposure to Boc-1 enhanced LFA-1 and E-selectin expression in the presence of aspirin is a further evidence that native LTs and ATL are released in response to LPS (Figure 7).

It has recently been shown that a third COX isoform (Chandrasekharan *et al*., 2002), named COX-3, is expressed in mammalian tissues. COX-3 is made from the COX-1 gene together with a smaller COX-1-derived protein (partial COX-1 or PCOX-1). This isoenzyme is selectively inhibited by acetaminophen but is also potently inhibited by aspirin. Although it is unknown whether COX-3 acetylation will result in the formation of new eicosanoids, it has previously been shown (Boutaud *et al*., 2002) that this isoenzyme is responsible for $PGI_2$ formation by human endothelial cells, raising the possibility that interactions of NSAIDs with aspirin might interfere with antiadhesive activity of aspirin by modulating COX-3 activity.

In conclusion, the results presented here support the notion that treating LPS-primed PMN/HUVEC cocultures with therapeutic concentrations of aspirin triggers a switch in the prostanoid metabolism from $PGE_2$ to ATL. The formation of ATL is mechanistically linked to the antiadhesive activity of aspirin as demonstrated by the fact that inhibition of ATL formation with the selective COX-2 inhibitor celecoxib alters the $LTB_4$/ATL balance, and greatly impairs the antiadhesive properties of aspirin. Inhibition of $LTB_4$ formation with dual COX/5-LOX inhibitors significantly enhances the antiadhesive properties of aspirin, highlighting the fact that aspirin triggers the release of both pro- and anti-inflammatory mediators.

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