Aspirin triggered lipid mediators: novel inhibitors of leucocyte trafficking

Charles N Serhan, Clary B Clish

Despite nearly 100 years of wide usage, complete knowledge of the therapeutic impact of aspirin is still evolving[1–3] and new beneficial effects are being uncovered in cardiovascular disease and cancer.[4 5] The irreversible acetylation of cyclo-oxygenase (COX)-1 and its more recently discovered isoform COX-2[1 6] with inhibition of prostaglandins is well appreciated and explains some, but not all, of the pharmacological actions of aspirin.

Another series of bioactive lipid mediators on which aspirin has an impact are lipoxins (LX), and their C15 epimers (15R), denoted aspirin triggered lipoxins (ATL, fig 1), which are biosynthesised by separate routes involving transcellular circuits.[7] Native LX in the nanomolar range inhibit the adhesion and transmigration of polymorphonuclear leucocytes (PMNs)[8–10] and hence serve as counter-regulatory signals operative in the resolution of inflammatory sites.[11 12] Not only do LX serve counter-regulatory roles, but specific enantiomerically modified LX (ATL) may also be actual effectors of well established anti-inflammatory therapeutic actions of aspirin. The impact of 5S,6R,15S-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid (LXA_4) and ATL was investigated in tumour necrosis factor (TNFα) initiated PMN responses in vitro and in vivo using the metabolically more stable LX analogues 15R/S-methyl-LXA_4 and 15-epi-16-(parafluoro)-phenoxy-LXA_4. These compounds represent subtle modifications to the native LXA_4 and ATL structure that prevent rapid metabolic inactivation of the lipoxin and 15-epi-LX structures.[13 14] These compounds are also potent novel inhibitors of TNFα driven PMN associated inflammatory events in vitro as well as in vivo, as shown in a murine model with end points relevant to pulmonary inflammation.

Center for Experimental Therapeutics and Reperfusion Injury, Department of Anesthesiology, Perioperative and Pain Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115, USA
C N Serhan
C B Clish

Correspondence to:
Dr C N Serhan
cnserhan@zeus.bwh.harvard.edu

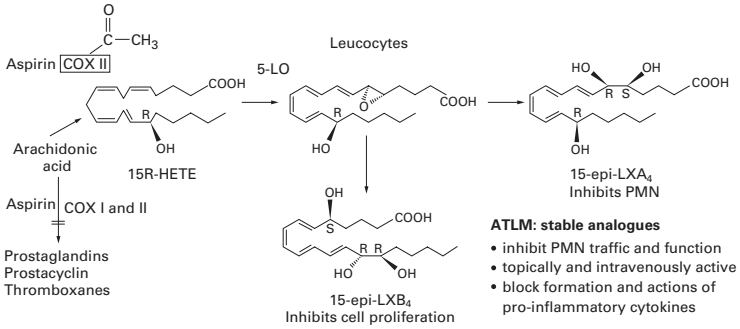

Figure 1 Generation of aspirin triggered lipid mediators (ATLM): anti-inflammation signals. Arachidonic acid can be transformed via COX-1 or its inducible isoform COX-2 to produce prostaglandins and thromboxane. Acetylation by aspirin blocks this conversion.[1 6] The lipoxins (LX) are biosynthesised through transcellular interactions and production of 15-epi-LX is triggered when COX-2 is acetylated by aspirin. LX and aspirin triggered lipoxins (ATL) have potent inhibitory actions since their longer acting stable analogues are potent in vivo[8 13 14] and appear to be involved in healing/resolution and anti-inflammation.

Methods

Six day air pouches were raised on the dorsum of 6–8 week old male BALB/c mice by subcutaneous injection of 3 ml sterile air and experiments were conducted on day 6.[13–15] Individual air pouches were injected with either vehicle alone (0.1% ethanol), TNFα, ATL stable analogue, or TNFα + ATL, with each suspended in 1 ml of endotoxin free phosphate buffered saline (PBS; pH 7.45) immediately before injection into pouch cavities. Mice were sacrificed and individual air pouches were lavaged, and the cell exudates were measured and analysed for cytokines/chemokines.[13]

Results

PMNs express and release several cytokines among which interleukin (IL)-1β is a potent proinflammatory agent.[16] We have recently investigated the actions of native LXA_4 and its stable analogues on TNFα induced IL-1β release.[13] PMNs from healthy donors were exposed to increasing concentrations of 15R/S-methyl-LXA_4 or native LXA_4 in the presence of TNFα (25 000–50 000 activity units/ml) or vehicle alone. At a concentration of 100 nM, 15R/S-methyl-LXA_4 inhibited approximately 60% of IL-1β release, a value which is comparable to that obtained with native LXA_4. Inhibition of IL-1β by LXA_4 and its stable analogue was, at least in part, the result of a down-regulation in gene expression, since the IL-1β messenger RNA levels in cells treated with TNFα (10 ng/ml) plus 15R/S-methyl-LXA_4 (100 nM) were decreased by approximately 60% compared with cells treated with TNFα alone.[13] Since IL-1β and TNFα are two cytokines considered to be important in inflammation, the inhibition of IL-1β suggested that ATL might exert an in vivo anti-cytokine action.

TNFα evokes leucocyte infiltration in a chemokine-dependent fashion in the murine six day air pouch.[15] We therefore evaluated the impact of ATL stable analogues 15R/S-methyl-LXA_4 and 15-epi-16-(para-fluoro)-phenoxy-LXA_4 in this model to determine whether they also intersect the cytokine/chemokine axis in vivo and inhibit leucocyte infiltration.[13 14] Murine TNFα gives a transient infiltration of leucocytes to the pouch cavity in a time-dependent fashion with maximal accumulation at four hours.[13] 15R/S-methyl-LXA_4 at a concentration of 25 nmol inhibited the TNFα stimulated recruitment of leucocytes by approximately 62%. Inhibition was evident at one hour and was maximal at 2–4 hours. At these intervals a reduction in leucocyte infiltration of more than 60% was noted that remained significantly reduced at eight hours. Inflamma-

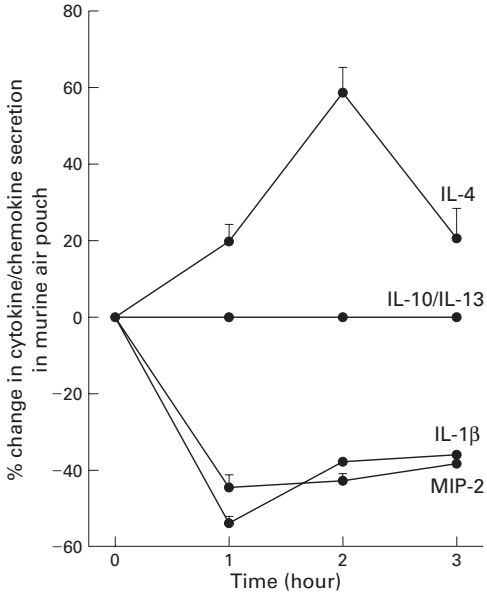

Figure 2 15R/S-methyl-LXA$_4$ redirects the TNFα induced cytokine/chemokine profile in vivo. Sterile PBS (~1 ml) containing either 0.1% ethanol, TNFα, 15R/S-methyl-LXA$_4$, or TNFα + 15R/S-methyl-LXA$_4$ was injected into the pouches and individual lavage exudates were collected at the indicated time periods. Measurements were performed with air pouch cell-free lavage exudates as described by Hachicha et al.[13] The results are expressed as the % change compared with the quantities of cytokines found in TNFα injected air pouches in the absence of LXA$_4$ (mean (SE) from three different mice for each time interval). Changes in IL-1β, MIP-2, and IL-4 secretion were statistically significant at all tested time intervals ($p<0.01$). At one hour the air pouches injected with TNFα alone generated 384 (12) pg MIP-2/pouch and 14.9 (2.3) pg IL-1β/pouch. 15R/S-methyl-LXA$_4$ alone generated 42.7 (0.7) pg IL-4/pouch.

tory exudates were collected four hours after injections and trafficking of cell types into the cavity was measured. In the six day pouches given TNFα, PMNs constituted the major cell type present within the exudates at four hours with a range of 80–85%. Administration of both 15R/S-methyl-LXA$_4$ and TNFα into the six day air pouch cavity inhibited migration of PMNs, eosinophils, and basophils as well as mononuclear cells.[13] The fluorinated ATL analogue was compared with aspirin in this model and, when injected into the pouch with TNFα, more than 75% inhibition was achieved with 10 μg. This analogue also showed increased stability in whole blood (mouse and human) and intravenous administration was as effective as administration into the air pouch (topical). This stable ATL analogue was also about 100 times more potent than aspirin in blocking PMN influx to the pouch.[14]

Since macrophage inflammatory peptide (MIP)-2 is the major chemokine involved in recruiting PMNs to the pouch cavity following injection of TNFα,[15] we determined the action of 15R/S-methyl-LXA$_4$ in this TNFα induced chemokine-cytokine axis. MIP-2 and IL-1β are important proinflammatory cytokines, and IL-4, IL-10, and IL-13 possess immunomodulatory properties. Exudates from selected time intervals were collected and cell-free supernatants assessed for the presence of these murine cytokines. 15R/S-methyl-LXA$_4$ (25 nmol) inhibited TNFα stimulated MIP-2 and IL-1β release by 48% and 30%, respectively (fig 2). 15R/S-methyl-LXA$_4$ alone in the air pouch did not stimulate MIP-2 or IL-1β release. In sharp contrast, 15R/S-methyl-LXA$_4$ stimulated the appearance of IL-4 within the exudates. This stimulation of IL-4 was observed both with and without TNFα. Neither IL-10 nor IL-13 was detected within the pouch exudates. These results show that administration of 15R/S-methyl-LXA$_4$ dramatically modified the cytokine/chemokine axis in TNFα initiated acute inflammation and, interestingly, this re-orientation of the cytokine/chemokine axis paralleled the reduction in leucocyte infiltration.

Discussion

LXA$_4$ and ATL (generated by separate biosynthetic mechanisms in vivo; fig 1 and Serhan *et al*[10–12]), as evidenced by the actions of the metabolically stable analogue 15R/S-methyl-LXA$_4$, are both potent cytokine regulating lipid mediators that can also have an impact on the course of inflammation initiated by TNFα. In this exudate and skin wound model,[13] 15R/S-methyl-LXA$_4$ not only inhibited TNFα elicited PMNs, monocytes, basophils, and eosinophils as well as the appearance of IL-1β and MIP-2, but also stimulated IL-4 appearance within the pouch. These results provide the first evidence to indicate that lipoxins and ATL induce upregulation of potential "anti-inflammatory" cytokines such as IL-4.[17] It is likely that both the inhibition of IL-1β and MIP-2 within exudates and IL-4 levels in the surrounding tissues by metabolically stable LX analogues may represent, in part, some of the in vivo impact of LXA$_4$ and aspirin triggered 15-epi-LXA$_4$. These findings provide a new understanding of the relationship between "anti-inflammatory" cytokines and lipid mediators and also open new avenues to investigate protective lipid and protein mediators in host defence.

In summary, these results suggest that LXA$_4$ and aspirin triggered LXA$_4$ appear to be involved in controlling not only acute inflammatory responses, but also mechanisms that can influence chronic inflammatory responses. The recent results reviewed here also support the notion that aspirin may exert its beneficial action in part via the biosynthesis of endogenous ATLMs such as 15-epi-LXA$_4$ that can, in turn, act directly on PMNs as well as affect the appearance of chemokines and cytokines. Thus, LX-ATL can protect host tissues via multi-level regulation of proinflammatory signals and may be relevant new lipid mediators of interest in airway diseases.

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