Lipoxin (LX)A₄ and Aspirin-triggered 15-epi-LXA₄ Inhibit Tumor Necrosis Factor 1α-initiated Neutrophil Responses and Trafficking: Regulators of a Cytokine–Chemokine Axis

By Mohamed Hachicha,* Marc Pouliot,* Nicos A. Petasis,† and Charles N. Serhan*

Summary

The impact of lipoxin A₄ (LXA₄) and aspirin-triggered lipoxins (ATLs) was investigated in tumor necrosis factor (TNF)-α–initiated neutrophil (polymorphonuclear leukocyte) responses in vitro and in vivo using metabolically stable LX analogues. At concentrations as low as 1–10 nM, the LXA₄ and ATL analogues each inhibited TNF-α–stimulated superoxide anion generation and IL-1β release by human polymorphonuclear leukocytes. These LXA₄–ATL actions were time and concentration dependent and proved selective for TNF-α, as these responses were not altered with either GM-CSF– or zymosan-stimulated cells. TNF-α–induced IL-1β gene expression was also regulated by both anti-LXA₄ receptor antibodies and LXA₄–ATL analogues. In murine air pouches, 15R/S-methyl-LXA₄ dramatically inhibited TNF-α–stimulated leukocyte trafficking, as well as the appearance of both macrophage inflammatory peptide 2 and IL-1β, while concomitantly stimulating IL-4 in pouch exudates. Together, these results indicate that both LXA₄ and ATL regulate TNF-α–directed neutrophil actions in vitro and in vivo and stimulate IL-4 in exudates, playing a pivotal role in immune responses.

Key words: eicosanoids • leukocytes • lipid mediators • antiinflammatory receptors • wound healing

Lipid and protein mediators of inflammation, such as cytokines and chemokines, have a profound impact on the formation and actions of each other (1). In particular, the cytokines TNF-α and IL-1β play major roles in inflammation, septic shock, and tissue injury. PMN perform a range of well acknowledged, specialized functions, including chemotaxis, generation of reactive oxygen species (ROS), and biosynthesis of potent lipid mediators (2). In this regard, TNF-α stimulates PMN to transcribe and release cytokines such as IL-1β, enhances leukotriene biosynthesis, and upregulates adhesion molecules (3). As PMN represent ~70% of the peripheral blood leukocytes and are in many instances the initial cell type recruited to interstitial sites, they are now considered a significant source of “proinflammatory” cytokines, including TNF-α and IL-1β. These as well as other PMN–derived cytokines and chemokines can, in turn, affect the course of inflammatory and immune responses (4). In certain clinical settings, including respiratory distress syndrome, myocardial reperfusion injury, gout, and rheumatoid arthritis (RA), PMN contribute to ongoing damage of host tissues (2, 5, 6). Thus, it is of interest to understand the complex relationships between lipid mediators and TNF-α–evoked PMN responses in order to gain insight for new approaches in controlling these events.

The contribution of leukotriene (LT)B₄ in inflammation is well established in view of its potent ability to attract PMN. Another series of bioactive lipid mediators, termed lipoxins (LX) and aspirin-triggered lipoxins (ATLs), inhibits, within the nanomolar range, FMLP– and LTB₄–stimulated PMN adhesion and transmigration (1, 7–9) and hence represent proposed counterregulatory signals operative in the resolution of inflammatory sites (10). In human tissues, three main pathways are known for LX generation. An in-

Abbreviations used in this paper: ATLs, aspirin-triggered lipoxins; ATL analogue, 15R/S-methyl-LXA₄; methyl ester; LT, leukotriene; LX, lipoxin; LXA₄, 5,6R,15S-trihydroxy-7,9,13-trans-11-cis-eicosatetraenoic acid; LXA₄ analogue, 16-phenoxy-lipoxin A₄; methyl ester; 15-epi-LXA₄, 5,6R,15R-trihydroxy-7,9,13-trans-11-cis-eicosatetraenoic acid; MIP, macrophage inflammatory peptide; RA, rheumatoid arthritis; ROS, reactive oxygen species.

* M. Hachicha and M. Pouliot contributed equally to this study.

† A abbreviations used in this paper: ATL, aspirin-triggered lipoxins; ATL analogue, 15R/S-methyl-LXA₄; methyl ester; LT, leukotriene; LX, lipoxin; LXA₄, 5,6R,15S-trihydroxy-7,9,13-trans-11-cis-eicosatetraenoic acid; LXA₄ analogue, 16-phenoxy-lipoxin A₄; methyl ester; 15-epi-LXA₄, 5,6R,15R-trihydroxy-7,9,13-trans-11-cis-eicosatetraenoic acid; MIP, macrophage inflammatory peptide; RA, rheumatoid arthritis; ROS, reactive oxygen species.

From the *C enter for Experimental T herapeutics and R eperfusion I njury, D epartment of A nesth esiology, P erioperative and P ain M edicine, B righam and W omen ’ s H ospital, H arvard M edical School, BOSTON, M ASSACHUSETTS 02115; and the †D epartment of C hemistry, U niversity of SOUTHERN C alifornia, L OS A NGEL ES, C alifornia 90089

M. Hachicha and M. Pouliot contributed equally to this study.

http://www.jem.org

Volume 189, Number 12, June 21, 1999 1923–1929

J. Exp. Med. © The Rockefeller University Press • 0022-1007/99/06/1923/07 $2.00
traluminal source of LX is exemplified by PMN–platelet interactions that utilize sequential transcellular biosynthetic routes with the PMN 5 lipoygenase (LO) product LAT and platelet 12-LO. The mucosal and/or interstitial source of these eicosanoids involves cell–cell interactions with leukocyte 5-LO and 15-LO present in, for example, eosinophils, gastrointestinal or tracheal epithelium controlled by IL-4 and IL-13 (for review see reference 1). The third and most recently elucidated pathway also represents a novel mechanism of action for aspirin that triggers the endogenous biosynthesis of 15R epimers of native LX, termed ATL, generated via transcellular biosynthesis (8).

LX are generated during cell–cell interactions via transcellular biosynthesis (1) and are produced in vivo during angioplasty and in immune complex glomerulonephritis and sensitive asthmatics and is generated by leukocytes from patients with asthma and RA (12, 13). Like most autacoids and lipid mediators, LX are rapidly biosynthesized, act within a local microenvironment, and are rapidly enzymatically inactivated. To advance our understanding of LX and ATL roles in vivo, metabolically stable LX analogues were designed that resist rapid inactivation and mimic the in vitro actions of naturally occurring LX and ATL (14). Here, we report that these compounds are potent inhibitors of TNF-α-driven PMN–associated inflammatory events in vitro as well as in vivo. Moreover, LX A4-epimers of native LX, termed ATL, are generated via transcellular biosynthesis (8).

Materials and Methods

Human and mouse rTNF-α and human rGM-CSF were obtained from Boehringer Mannheim. Dulbecco’s PBS (Mg2+ and Ca2+ free), RPMI 1640, and FCS were purchased from BioWhitaker, Inc. Ficoll-Hyphaque was from Organon Teknika Corp., and HBSS was purchased from Gibco BRL. BSA, dextran, antibiotics, L-glutamine, cytochrome C, superoxide dismutase, and zymosan were obtained from Sigma Chemical Co. The assessment of human IL-1β in supernatants was performed by using an immunometric assay with acetylcholine esterase (Cayman Chemical). Mouse IL-1β was assessed using an ELISA from Endogen. ELISAs for IL-4 and IL-10 were from Amersham Corp.; MIP-2 and IL-13 ELISAs were from R & D Systems, Inc. LX A4 and ATL metabolically stable analogues were prepared and characterized, including nuclear magnetic resonance spectroscopy, as in reference 14. Concentrations of each LX analogue were determined using an extinction coefficient of 50,000/M/cm just before each experiment and stored in cold (4°C) Hank’s medium (supplemented with 1.6 mM Ca2+, 0.1% FCS, 2 mM L-glutamine, 1% penicillin, and 2% streptomycin, pH 7.4). Cell preparations were >98% PMN, as determined by Giemsa-stained Wright’s cells, and cell viability was >98% for freshly isolated PMN and >92% for PMN incubated for 20 h, as determined by trypan blue exclusion using light microscopy. To examine superoxide production, PMN (109/ml) were placed at 37°C (3 min) and then exposed to vehicle (0.1% ethanol) or synthetic LX A4, 15R/S-methyl LX A4, or 16-phenoxy-LX A4 for 5 min at 37°C. Before adding TNF-α (50 ng/ml), PMN were incubated with cytochrome C (0.7 mg/ml) for 10 min at 37°C. Superoxide dismutase-dependent reduction of cytochrome C was terminated by rapidly placing tubes in an ice water bath. The extent of cytochrome C reduction in each supernatant was determined at 550 nm in reference and compared with control values obtained when superoxide dismutase was added before a stimulus or vehicle control. Cytochrome C reduction was quantitated using the extinction coefficient of 21.1/mmol/liter.

Materials and Methods

Human and mouse rTNF-α and human rGM-CSF were obtained from Boehringer Mannheim. Dulbecco’s PBS (Mg2+ and Ca2+ free), RPMI 1640, and FCS were purchased from BioWhitaker, Inc. Ficoll-Hyphaque was from Organon Teknika Corp., and HBSS was purchased from Gibco BRL. BSA, dextran, antibiotics, L-glutamine, cytochrome C, superoxide dismutase, and zymosan were obtained from Sigma Chemical Co. The assessment of human IL-1β in supernatants was performed by using an immunometric assay with acetylcholine esterase (Cayman Chemical). Mouse IL-1β was assessed using an ELISA from Endogen. ELISAs for IL-4 and IL-10 were from Amersham Corp.; MIP-2 and IL-13 ELISAs were from R & D Systems, Inc. LX A4 and ATL metabolically stable analogues were prepared and characterized, including nuclear magnetic resonance spectroscopy, as in reference 14. Concentrations of each LX analogue were determined using an extinction coefficient of 50,000/M/cm just before each experiment and stored in cold (4°C) Hank’s medium (supplemented with 1.6 mM Ca2+, 0.1% FCS, 2 mM L-glutamine, 1% penicillin, and 2% streptomycin, pH 7.4). Cell preparations were >98% PMN, as determined by Giemsa-stained Wright’s cells, and cell viability was >98% for freshly isolated PMN and >92% for PMN incubated for 20 h, as determined by trypan blue exclusion using light microscopy. To examine superoxide production, PMN (109/ml) were placed at 37°C (3 min) and then exposed to vehicle (0.1% ethanol) or synthetic LX A4, 15R/S-methyl LX A4, or 16-phenoxy-LX A4 for 5 min at 37°C. Before adding TNF-α (50 ng/ml), PMN were incubated with cytochrome C (0.7 mg/ml) for 10 min at 37°C. Superoxide dismutase-dependent reduction of cytochrome C was terminated by rapidly placing tubes in an ice water bath. The extent of cytochrome C reduction in each supernatant was determined at 550 nm in reference and compared with control values obtained when superoxide dismutase was added before a stimulus or vehicle control. Cytochrome C reduction was quantitated using the extinction coefficient of 21.1/mmol/liter.

R N A Isolation and Northern analysis. Total RNA extraction and Northern blot analyses were performed as in reference 7. pSM 320 vector containing cDNA for IL-1β was purchased from American Type Culture Collection.

Figure 1. LX A4 and ATL stable analogues inhibit TNF-α–stimulated superoxide generation by human neutrophils. Human PMN were incubated with vehicle alone or indicated concentrations of LX A4, 15R/S-methyl LX A4, or 16-phenoxy-LX A4 for 5 min and then with TNF-α (50 ng/ml) for an additional 10 min. Values are the mean ± SEM for LX A4 (n = 3), 15R/S-methyl-LX A4 (n = 4), or 16-phenoxy-LX A4 (n = 3). LX A4 and analogues at all concentrations tested, led to a statistically significant inhibition of TNF-α–induced IL-1β appearance (P < 0.01). Inset: TNF-α concentration–dependent superoxide production. Human PMN were incubated with indicated concentrations of TNF-α. Values are the mean ± SEM (n = 3). TNF-α alone (50 ng/ml) gave 0.76 ± 0.12 nmol cytochrome C–reduced cells per 106 cells compared with FMLP (10 nM), another physiologically relevant stimulus, that gave 6.02 ± 0.05 nmol cytochrome C–reduced cells per 106 cells.
Murine Air Pouches. 6-8-wk-old male BALB/c mice were obtained from Taconic Farms, Inc. Air pouches were raised on the dorsum by subcutaneous injection of 3 ml of sterile air on days 0 and 3. All experiments were conducted on day 6 (16). Individual air pouches (one per mouse) were injected with vehicle alone (0.1% ethanol), TNF-α, 15R/S-methyl-LXA₄, or TNF-α plus 15R/S-methyl-LXA₄, and each was suspended in 1 ml endotoxin-free PBS immediately before injection into pouch cavities. At given intervals, the mice were killed, and individual air pouches were lavaged three times with sterile PBS (1 ml). The exudates were centrifuged at 2,000 rpm (5 min), and the supernatants were removed. Cell pellets were suspended in PBS (200 μl) for enumeration and assessed for viability. 50 μl of each cell suspension was mixed with 150 μl 30% BSA and then centrifuged onto microscope slides at 500 rpm for 5 min using a cyto spin centrifuge, air dried, and stained with Giemsa-Wright.

Results and Discussion

Inhibition of TNF-α–stimulated Superoxide Generation. TNF-α, although a modest agonist of O₂⁻ generation by human PMN, is a physiologically relevant stimulus for the generation of ROS by nonadherent human PMN (17) that can play critical roles in local tissue injury during both inflammation and reperfusion (17–19). In Fig. 1, we evaluated the impact of LXA₄ and ATL-related bioactive stable analogues on TNF-α–stimulated superoxide anion production. TNF-α gave a concentration-dependent increase in superoxide anion dependence (Fig. 1, inset) with nonadherent PMN; therefore, TNF-α (50 ng/ml) was used to examine the analogues. Native LXA₄ and the analogues (15R/S-methyl-LXA₄ and 16 phenoxy-LXA₄) inhibited TNF-α–stimulated superoxide anion generation in a concentration-dependent fashion. Their rank order of potency at 10 nM was 15R/S-methyl-LXA₄ (81.3 ± 14.1% inhibition) > 16-phenoxy-LXA₄ (93.7 ± 3.2%) > LXA₄ (34.3 ± 2.3%). 15R/S-methyl-LXA₄ covers both LXA₄ and ATL in structure, and 16-phenoxy-LXA₄ is an LXA₄ analogue (Fig. 1). Each analogue competes at the LXA₄R (7). LXA₄, 15R/S-methyl-LXA₄, and 16 phenoxy-LXA₄, at concentrations up to 1 μM added to cells alone, did not stimulate generation of ROS (data not shown). 15R/S-methyl-LXA₄ and 16-phenoxy-LXA₄ were approximately three times more potent than native LXA₄ and proved to be powerful inhibitors of TNF-α–stimulated superoxide generation by PMN. However, neither LXA₄ nor its analogues inhibit PMA (100 nM)- or fMLP (100 nM)-stimulated O₂⁻ production (n = 3; data not shown). Inhibition of ROS by LXA₄ and its analogues is of interest in a context of ischemia/reperfusion, where ROS are held to be primary mediators of tissue injury (15).

Suppression of TNF-α–stimulated IL-1β Release. PMN express and release interleukin-1β, which is a potent pro-inflammatory cytokine (20). Therefore, we next investigated the actions of native LXA₄ and its analogues on TNF-
1926 LXA₄ and 15-epi-LXA₄ Inhibit TNF-α-initiated Neutrophil Responses

Dramatically increase their staining (Fig. 2 A, inset), suggesting that the ATL did not reduce PMN viability during the time courses of these experiments.

PMN were exposed to increasing concentrations of 15R/S-methyl-LXA₄, 16-phenoxy-LXA₄, or native LXA₄ in the presence of TNF-α (10 ng/ml) or vehicle alone. At a concentration of 100 nM, 15R/S-methyl-LXA₄ inhibited ~60% of IL-1β release, and 16-phenoxy-LXA₄ at equimolar levels gave ~40% inhibition (values comparable to those obtained with native LXA₄; data not shown). Time course and concentration dependence were carried out with 15R/S-methyl-LXA₄ (Fig. 2 B). At 10 nM, 15R/S-methyl-LXA₄ gave clear, statistically significant inhibition, which was evident within 6 h and more prominent after 24 h (Fig. 2 B). Inhibition of IL-1β by these LX analogues was at least in part, the result of a downregulation in gene expression, because the IL-1β messenger RNA levels in cells treated with TNF-α (10 ng/ml) plus 15R/S-methyl-LXA₄ (100 nM) were decreased by ~60% when compared with cells treated with TNF-α alone (Fig. 3). Therefore, as IL-1β and TNF-α are two cytokines that are considered important in inflammation, the inhibition of IL-1β observed (Figs. 1 and 2) suggested that 15R/S-methyl-LXA₄ might exert a potent in vivo anticytokine action (vide infra).

Involvement of LXA₄R. To investigate whether LXA₄R was involved in the regulation of TNF-α–stimulated IL-1β release, the rabbit polyclonal antibodies against a portion of the third extracellular domain (ASWGGTPEERLK) of LXA₄R prepared earlier (21) were used. PMN were incubated with ~50 μg/ml of either preimmune protein A–purified IgG or IgG directed against LXA₄R for 1 h at 4°C before exposure to TNF-α (10 ng/ml) and 15R/S-methyl-LXA₄ (100 nM). Anti-LXA₄R antibodies prevented IL-1β release by TNF-α, suggesting that the third extracellular loop plays a crucial role in LXA₄R activation (Fig. 4). 15R/S-methyl-LXA₄ inhibited ~50% of IL-1β

15R/S-methyl-LXA₄ downregulates TNF-α–triggered IL-1β gene expression. PMN were incubated with either 0.1% ethanol (vehicle) or 15R/S-methyl-LXA₄ at 10, 100, and 1,000 nM, in the presence or absence of TNF-α (10 ng/ml), for 6 h at 37°C. Northern blot analyses were performed in order to detect IL-1β mRNA. The results presented are from one experiment, which is representative of two others performed with different donors.

α–induced IL-1β release. Incubation of PMN with physiologically relevant concentrations of TNF-α, GM-CSF, or phagocytic particles (zymosan) resulted in a concentration-dependent increase in the levels of IL-1β present in supernatants. Approximate EC₅₀ for each agonist were: TNF-α, 10 ng/ml; GM-CSF, 10 U/ml; and zymosan, 100 μg/ml. Native LXA₄ specifically inhibited TNF-α–induced IL-1β release (Fig. 2 A), whereas similar amounts of IL-1β were released in the presence or absence of LXA₄ when PMN were exposed to either GM-CSF or zymosan. The viability of PMN exposed to ATL or TNF-α was examined using trypan blue exclusion. PMN exposed to these agents did not

Figure 3. 15R/S-methyl-LXA₄ downregulates TNF-α–triggered IL-1β gene expression. PMN were incubated with either 0.1% ethanol (vehicle) or 15R/S-methyl-LXA₄ at 10, 100, and 1,000 nM, in the presence or absence of TNF-α (10 ng/ml), for 6 h at 37°C. Northern blot analyses were performed in order to detect IL-1β mRNA. The results presented are from one experiment, which is representative of two others performed with different donors.

Figure 4. Involvement of LXA₄R. PMN were incubated with either IgG purified from preimmune serum (50 μg/ml) or anti-LXA₄R (50 μg/ml) for 1 h at 4°C and then exposed to agonists for 12 h at 37°C and 5% CO₂. Values are expressed as mean ± SD from an experiment performed in triplicate, which is representative of three distinct experiments, each performed with different donors (*P < 0.01).
release. When added together, anti-LX₄R antibodies and 15R/S-methyl-LX₄A₄ in the presence of TNF-α did not inhibit IL-1β appearance, and neither anti-LX₄R antibodies nor 15R/S-LX₄A₄ alone stimulated significant amounts of IL-1β to appear in supernatants. The results of these experiments are twofold: first, they indicated that the inhibitory action of 15R/S-methyl-LX₄A₄ is transduced via LX₄R and, second, that the anti-LX₄R antibodies alone activate LX₄R and lead to inhibition of IL-1β release.

Inhibition of TNF-α-directed Leukocyte Trafficking In Vivo. As TNF-α evokes leukocyte infiltration in a chemokine-dependent fashion in the murine six-day air pouch (16, 22), we evaluated the impact of 15R/S-methyl-LX₄A₄ in this model to determine whether LX₄ or ATL also intersects the cytokine-chemokine axis in vivo. 15R/S-methyl-LX₄A₄ is the most subtle modification to native LX₄A and ATL structure, with addition of a methyl at carbon 15. Murine TNF-α (10 ng/ml) caused a transient infiltration of leukocytes to the air pouch in a time-dependent fashion, with maximal accumulation at 4 h. 15R/S-methyl-LX₄A₄ at 25 nmol inhibits the TNF-α-stimulated recruitment of leukocytes to the air pouch by 62% (Fig. 5). Inhibition was evident at 1 h and maximal between 2 and 4 h. At these intervals, a 60% reduction in leukocyte infiltration was noted that remained significantly reduced at 8 h (Fig. 5, inset). Injection of pouches either with vehicle or the analogue alone did not cause a significant leukocyte infiltration. Also, inflammatory exudates were collected 4 h after injection with vehicle alone, TNF-α, 15R/S-methyl-LX₄A₄ alone, or TNF-α plus 15R/S-methyl-LX₄A₄, and cell types were enumerated. In the six-day pouches given TNF-α, PMN constituted the major cell type present within the exudates at 4 h and ranged from 80 to 85% of total cell number. Administration of both 15R/S-methyl-LX₄A₄ and TNF-α into the six-day air pouch cavity inhibited migration of PMN and eosinophils/basophils as well as mononuclear cells (Table I).

Table I. TNF-α–induced Leukocyte Infiltration in Murine Air Pouches: Inhibitory Action of 15R/S-methyl-LX₄A₄

| Injection                  | N neutrophils | Eosinophils/basophils | M monocytes/macrophages |
|---------------------------|---------------|-----------------------|-------------------------|
| TNF-α                     | 2.40 ± 0.10*  | 0.30 ± 0.01*          | 0.20 ± 0.01*            |
| 15R/S-methyl-LX₄A₄        | 0.98 ± 0.10†  | 0.13 ± 0.01†          | 0.10 ± 0.01†            |
| + TNF-α                   | (59.1%)       | (56.0%)               | (50%)                   |
| 15R/S-methyl-LX₄A₄        | 0.25 ± 0.01   | 0.03 ± 0.01           | 0.14 ± 0.01*            |
| Vehicle                   | 0.30 ± 0.01   | 0.07 ± 0.01           | 0.06 ± 0.01             |

Air pouches were raised as described in Materials and Methods. Each mouse was injected with 1 ml PBS containing vehicle (0.1% ethanol), TNF-α (10 ng), 15R/S-methyl-LX₄A₄ (25 nmol), or TNF-α plus 15R/S-methyl-LX₄A₄. Leukocyte infiltration was determined 4 h after injection. Results present the mean ± SEM of three different mice. Percent inhibition is indicated in parentheses. Statistically different from *vehicle-injected mice (P < 0.01) and ‡TNF-α-injected mice (P < 0.01).
methyl-LXA₄ alone in the air pouch did not stimulate MIP-2 or IL-1β release. In sharp contrast, 15R/S-methyl-LXA₄ stimulated the appearance of IL-4 within the exudates. This stimulation of IL-4 was observed both in the absence as well as the presence of TNF-α. Neither IL-10 nor IL-13 was detected within the pouch exudates. These results demonstrate that administration of 15R/S-methyl-LXA₄ modified the cytokine-chemokine axis in TNF-α-initiated acute inflammation, and, interestingly, this reorientation of the cytokine-chemokine axis paralleled the reduction in leukocyte infiltration.

Several different strategies have been explored in an attempt to attenuate nondesirable action of TNF-α in inflammatory diseases and ischemia reperfusion injury, including treatment of patients suffering from RA with rTNF-α linked to human Ig as a fusion protein (26). Different steroidal and nonsteroidal drugs (27) to alleviate the pain and the severity of inflammatory responses are extensively used. However, certain clinical settings, such as reperfusion injury, are still not well controlled, and new therapeutic agents are needed. Our results indicate that LXA₄ and ATL, as evidenced by the actions of their metabolically stable analogues (16-phenoxy-LXA₄ and 15R/S-methyl-LXA₄), are potent cytokine-regulating lipid mediators that can also impact the course of inflammation initiated by TNF-α and IL-1β. These two cytokines are considered to be key components in orchestrating the rapid inflammatory-like events in ischemia reperfusion (within minutes to hours) and are major cytokines in RA and many other chronic diseases. Interestingly, in an exudate and skin wound model, 15R/S-methyl-LXA₄ not only inhibited the TNF-α-elicited appearance of IL-1β and MIP-2 but also concomitantly stimulated IL-4 (Figs. 5 and 6). This represents the first observation that lipoxins induce upregulation of a potential "antiinflammatory" cytokine such as IL-4. Hence, it is of particular interest that IL-4 inhibits PMN influx in acute antibody-mediated inflammation (28) and inhibits H₂O₂ production by IFN-γ-treated human monocytes (29). IL-4 is also an active antitumor agent and, most recently, was shown to be a potent inhibitor of angiogenesis (25). It is thus likely that the increase in IL-4 levels stimulated by metabolically stable LX analogues may in part mediate some of the in vivo impact of LXA₄ and aspirin-triggered 15-epi-LXA₄, a finding that provides a new understanding of the relationship between antiinflammatory cytokines and lipid mediators.

In conclusion, LXA₄ and ATL appear to be involved in controlling both acute as well as chronic inflammatory responses. The results presented here support the notion that aspirin may exert its beneficial action in part via the biosynthesis of endogenous ATL that can in turn act directly on PMN and/or the appearance of IL-4. Thus, LXA₄ and 15-epi-LXA₄ can protect host tissues via multilevel regulation of proinflammatory signals.

Figure 6. 15R/S-methyl-LXA₄ redirects the TNF-α-induced cytokine-chemokine profile in vivo. Experiments were conducted as described in the Fig. 5 legend. Quantitation for IL-1β, IL-4, IL-10, IL-13, and MIP-2 was performed using ELISA with air pouch cell-free exudates. The results are expressed as mean ± SEM from three different mice for each time point. Changes in IL-1β, MIP-2, and IL-4 were significant at all tested time intervals (P < 0.01). At 1 h, air pouches injected with TNF-α alone generated 384 ± 12 pg/pouch of MIP-2 and 14.9 ± 2.3 pg/pouch of IL-1β. 15R/S-methyl-LXA₄ alone induced 42.7 ± 0.7 pg/pouch of IL-4.

These studies were supported in part by National Institutes of Health grants, nos. GM-38765 and PO1-DK50305 (to C. N. Serhan), and a grant from Schering AG (to C. N. Serhan and N. A. Petasis). M. Pouliot is the recipient of a Centennial fellowship from the Medical Research Council of Canada.

Address correspondence to Charles N. Serhan, Center for Experimental Therapeutics and Reperfusion Injury, Thornd Building for Medical Research, 7th Fl., Brigham and Women's Hospital, 75 Francis St., Boston, MA 02115. Phone: 617-732-8822; Fax: 617-278-6957; E-mail: cnserhan@zeus.bwh.harvard.edu

M. Hachicha's present address is Pharmacopeia, Inc., 3000 Eastpark Blvd., Cranbury, NJ 08512.

Received for publication 11 February 1999 and in revised form 28 April 1999.
References

1. Serhan, C.N., J.Z. Haggström, and C.C. Leslie. 1996. Lipid mediator networks in cell signaling: update and impact of cytokines. FASEB J. 10:1147–1158.

2. Weiss, S.J. 1989. Tissue destruction by neutrophils. N. Engl. J. Med. 320:365–376.

3. Marucha, P.T., R.A. Zeff, and D.L. Kreutzer. 1991. Cytokine-induced IL-1β gene expression in the human polymorphonuclear leukocyte: transcriptional and post-transcriptional regulation by tumor necrosis factor and IL-1. J. Immunol. 147:2603–2608.

4. Lloyd, A.R., and J.J. Oppenheim. 1992. Poly’s lament: the neglected role of the polymorphonuclear neutrophil in the afferent limb of the immune response. Immuno. Today. 13:169–172.

5. Hachicha, M., P.H. Naccache, and S.R. McColl. 1995. Inflammatory microcrystals differentially regulate the secretion of macrophage inflammatory protein 1 and interleukin 8 by human neutrophils: a possible mechanism of neutrophil recruitment to sites of inflammation in synovitis. J. Exp. Med. 182:2019–2025.

6. Hansen, P.R. 1995. Role of neutrophils in myocardial ischemia and reperfusion. Circulation. 91:1872–1885.

7. Takano, T., S. Fiore, J.F. Maddox, H.R. Brady, N.A. Petasis, and C.N. Serhan. 1997. Aspirin-triggered 15-epi-lipoxin A4 and LX A4 stable analogs are potent inhibitors of acute inflammation: evidence for antiinflammatory receptors. J. Exp. Med. 185:1693–1704.

8. Claria, J., and C.N. Serhan. 1995. Aspirin triggers previously undescribed bioactive eicosanoids by human endothelial cell-leukocyte interactions. Proc. Natl. Acad. Sci. U.S.A. 92:9475–9479.

9. Lee, T.H., C.E. Horton, U. Kyan-Aung, D.H. Spur. 1989. Lipoxin A4 and lipoxin B4 inhibit chemotactic responses of human neutrophils stimulated by leukotriene B4 and N-formyl-l-methionyl-l-leucyl-l-phenylalanine. Clin. Sci. 77:195–203.

10. Serhan, C.N. 1994. Lipoxin biosynthesis and its impact in inflammatory and vascular events. Biophys. A 12a:1212:1–25.

11. Papayianni, A., C.N. Serhan, M.L. Phillips, H.G. Rennke, and H.R. Brady. 1995. Transcellular biosynthesis of lipoxin A4 during adhesion of platelets and neutrophils in experimental immune complex glomerulonephritis. Kidney Int. 47:1295–1302.

12. Chavis, C., I. Vachier, P. Chanez, J. Bousquet, and P. Godard. 1996. 5(S),15(S)-dihydroxyeicosatetraenoic acid and lipoxin generation in human polymorphonuclear cells: dual specificity of 5-lipoxygenase towards endogenous and exogenous precursors. J. Exp. Med. 183:1633–1643.

13. Thomas, E., J.L. Leroux, F. Blotman, and C. Chavis. 1995. Conversion of endogenous arachidonic acid to 5,15-diHETE and lipoxins by polymorphonuclear cells from patients with rheumatoid arthritis. Inflamm. Res. 44:121–124.

14. Serhan, C.N., J.F. Maddox, N.A. Petasis, J. Akritopoulou-Zanze, A. Papayianni, H.R. Brady, S.P. Colgan, and J.L. Madara. 1995. Design of lipoxin A4 stable analogs that block transmigration and adhesion of human neutrophils. Biochemistry. 34:14609–14615.

15. Gronert, K., S.P. Colgan, and C.N. Serhan. 1998. Characterization of human neutrophil and endothelial cell ligand-occupied extracellular acidification rate by microphysiometry: impact of reoxygenation. J. Pham. Exp. T. Har. 285:252–261.

16. Tessler, P.A., P.H. Naccache, I. Clark-Lewis, R.P. Glade, K.S. N eote, and S.R. Mccoll. 1997. Chemokine networks in vivo: involvement of C-C-X-C and C-C chemokines in neutrophil extravasation in vivo in response to TNF-α. J. Immunol. 159:3595–3602.

17. Tsujimoto, M., S. Yokota, J. Viček, and G. Weissmann. 1986. Tumor necrosis factor provokes superoxide anion generation from neutrophils. Biod. Biophys. Res. Commun. 137:1094–1100.

18. Shibuya, H., N. O’kohchi, S. Tsukamoto, and S. Satomi. 1997. Tumor necrosis factor-induced, superoxide-mediated neutrophil accumulation in cold ischemic/reperfused rat liver. Hepatology. 26:113–120.

19. Jäckhe, H., A. Farhood, and C.W. Smith. 1990. N-oxides contribute to ischemia/reperfusion injury in rat liver in vivo. FASEB J. 4:3355–3359.

20. Dinarello, C.A. 1996. Biologic basis for interleukin-1 in disease. Blood. 87:2095–2147.

21. Fiore, S., and C.N. Serhan. 1995. Lipoxin A4 receptor activation is distinct from that of the formyl peptide receptor in myeloid cells: inhibition of CD11/18 expression by lipoxin A4 receptor interaction. Biochemistry. 34:16678–16686.

22. Sin, Y.M., A.D. Sedgwick, E.P. Chea, and D.A. Willoughby. 1986. Mast cells in newly formed lining tissue during acute inflammation: a six day air pouch model in the mouse. Ann. R. Hum. 45:873–877.

23. Maddox, J.F., M. Hachicha, T. Takano, N.A. Petasis, V.V. Fokin, and C.N. Serhan. 1997. Lipoxin A4 stable analogs are potent mimetics that stimulate human monocytes and THP-1 cells via a G-protein linked lipoxin A4 receptor. J. Biol. Chem. 272:6972–6978.

24. Isomaki, P., and J. Punnonen. 1997. Pro-and anti-inflammatory cytokines in rheumatoid arthritis. Ann. Med. 29:499–507.

25. Volpert, O.V., T. Fong, A.E. Koch, J.D. Peterson, C. Wardenbaugh, R.I. Tepper, and N.P. Bouch. 1998. Inhibition of angiogenesis by interleukin 4. J. Exp. Med. 188:1039–1046.

26. Orel, L.W., S.W. Baumgartner, M.H. Schiff, E.A. Tindal, R.M. Fleischmann, A.L. Weaver, R.E. Ettlinger, S. Cohen, W.J. Koopman, K. Mohler, et al. 1997. Treatment of rheumatoid arthritis with a recombinant human tumor necrosis factor receptor (p75)-Fc fusion protein. N. Engl. J. Med. 337:141–147.

27. Marriott, J.B., M. Westby, and A.G. Dalgleish. 1997. Therapeutic potential of TNF--α inhibitors old and new. DDT. 2:273–282.

28. Sáeien, S., Z. Dá, S.N. Coelho, B.T. Konieczny, K.J.M. Assmann, F.K. Badoua, and F.G. Lakks. 1998. IL-4 is an endogenous inhibitor of neutrophil influx and subsequent pathology in acute antibody-mediated inflammation. J. Immunol. 160:979–984.

29. Leh, M., W.Y. Weiser, S. Engelhorn, S. Gillis, and H.G. Remold. 1989. IL-4 inhibits H2O2 production and antielaidin-mobility of human cultured monocytes mediated by IFN--γ. J. Immunol. 143:3020–3024.