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Lipoxin A_4 Analogs Attenuate Induction of Intestinal Epithelial Proinflammatory Gene Expression and Reduce the Severity of Dextran Sodium Sulfate-Induced Colitis[1]

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The anti-inflammatory eicosanoid lipoxin A_4 (LXA_4), aspirin-triggered 15-epi-LXA_4, and their stable analogs down-regulate IL-8 secretion and subsequent recruitment of neutrophils by intestinal epithelia. In an effort to elucidate the mechanism by which these lipid mediators modulate cellular proinflammatory programs, we surveyed global epithelial gene expression using cDNA microarrays. LXA_4 analog alone did not significantly affect expression of any of the >7000 genes analyzed. However, LXA_4 analog pretreatment attenuated induction of ~50% of the 125 genes up-regulated in response to the gastroenteritis-causing pathogen *Salmonella typhimurium*. A major subset of genes whose induction was reduced by LXA_4 analog pretreatment is regulated by NF-κB, suggesting that LXA_4 analog was influencing the activity of this transcription factor. Nanomolar concentrations of LXA_4 analog reduced NF-κB-mediated transcriptional activation in a LXA_4 receptor-dependent manner and inhibited induced degradation of IκBα. LXA_4 analog did not affect earlier stimulus-induced signaling events that lead to IκBα degradation, such as *S. typhimurium*-induced epithelial Ca^{2+} mobilization or TNF-α-induced phosphorylation of IκBα. To establish the in vivo relevance of these findings, we examined whether LXA_4 analogs could affect intestinal inflammation in vivo using the mouse model of DSS-induced inflammatory colitis. Oral administration of LXA_4 analog (15-epi-16-para-fluoro-phenoxy-LXA_4, 10 μg/day) significantly reduced the weight loss, hematochezia, and mortality that characterize DSS colitis. Thus, LXA_4 analog-mediated down-regulation of proinflammatory gene expression via inhibition of the NF-κB pathway can be therapeutic for diseases characterized by mucosal inflammation. *The Journal of Immunology,* **2002, 168: 5260–5267.**

As an interface with the outside world, the intestinal epithelium dynamically alters its gene expression in response to the changing environments in both the intestinal lumen and the subepithelial domain. For example, in response to either lumenal enteric pathogens or subepithelial proinflammatory cytokines, the intestinal epithelium activates the expression of a panel of genes that promote an acute inflammatory response (1). As uncontrolled inflammation can result in tissue damage, the intestinal epithelium is also influenced by exogenous and endogenous anti-inflammatory mediators that attenuate proinflammatory responses. One example of this type of mediator and the focus of this study is that of the anti-inflammatory eicosanoid lipoxin (lipoxin A_4 (LXA_4)[3]).

Lipoxins such as LXA_4 are derived from arachidonate as a result of its exposure to the unique combinations of lipoxygenases that occur during specific heterotypic cell-cell interactions such as those occurring in inflammation (e.g., epithelial-neutrophil interactions). LXA_4-induced responses down-regulate events associated with inflammation in a variety of in vitro and in vivo models (2). Acetylation of cyclooxygenase by aspirin results in the biosynthesis of the 15-epimer of LXA_4 (3). Such 15-epi-LXA_4 as well synthetic analogs of LXA_4 resist enzymatic degradation and thus have longer-lasting anti-inflammatory bioactivity than the native eicosanoid (4). LXA_4 and its synthetic stable analogs attenuate the IL-8 expression that is induced in model epithelia in response to the gastroenteritis-causing pathogen *Salmonella typhimurium* and the proinflammatory cytokine TNF-α (5, 6). Lipoxin analogs also attenuate chemokine secretion by human colon, resulting in reduced neutrophil adherence and tissue damage (7).

The mechanism by which LXA_4 analogs down-regulate IL-8 expression is largely unknown, although we have shown that IL-8 mRNA levels are reduced (5), implying action at the level of transcription. While this bioaction is at least somewhat specific, in that the mRNA levels of actin are not affected by LXA_4 analogs, technology to broadly evaluate gene expression has not, until recently, been available. Microarray technology now permits simultaneous parallel measurement of the expression of thousands of genes, making it possible to evaluate the effect of a given mediator on global gene expression. We sought to use this technology to characterize LXA_4 bioaction and perhaps better predict its in vivo behavior. In this study, we use this approach to test the hypothesis that LXA_4 exerts its effects primarily on the transcriptional activation of genes involved in the proinflammatory epithelial response.

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[3] Abbreviations used in this paper: LXA_4, lipoxin A_4, CAT, chloramphenicol acetyl transferase; DSS, dextran sodium sulfate; IBD, inflammatory bowel disease; HSP, heat shock protein; NSAID, nonsteroidal anti-inflammatory drug.

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We observed that LXA_4 analogs did not directly affect gene expression but broadly induced proinflammatory gene expression, particularly that regulated by NF-κB. This result is not surprising when one considers that LXA_4 analogs are known to antagonize the effects of a number of proinflammatory agonists that signal through this transcription factor (as discussed above). Having identified a role for this factor, we next explored which elements of the signaling pathway were affected. Last, we tested the in vivo relevance of these findings in a mouse model of colitis.

Materials and Methods

Materials

15-(R/S)-methyl-LXA_4 was synthesized by Dr. N. Petasis (University of Southern California, Los Angeles, CA) as previously described (4). 15-epi-16-parafluoro-phenoxy-LXA_4 was supplied by Berlex Biosciences (Richmond, CA). Dextran sodium sulfate (DSS; m.w. ~40,000) was obtained from ICN Pharmaceuticals (Costa Mesa, CA). MG-132 was obtained from Calbiochem (La Jolla, CA).

Cell culture

Polarized model intestinal epithelia were prepared via culturing T84 cells on permeable supports as previously described (8). Model epithelia were used 6–14 days after plating after verification (for T84) that they had achieved a transepithelial electrical resistance of at least 1000 Ωcm^2. *S. typhimurium* was cultured and used to colonize model epithelia as previously described (5).

Microarray analysis

Total RNA was isolated with TRIzol (GIBCO, Gaithersburg, MD) following instructions by the manufacturer. mRNA was isolated, hybridization was performed by Incyte Genomics (Palo Alto, CA), and mRNA were analyzed as we have recently described (9). Each array condition was performed on RNA pooled from six individual 5-cm^2 model epithelia so as to minimize the effect of experimental variability that might occasionally arise in individual samples. Changes in gene expression of 2-fold or more are highly likely to be significant (9).

Transient transfections and CAT assays

HeLa cells (40–60% confluent) were transiently transfected using Superfect Reagent (Qiagen, Valencia, CA) with 2 μg of the reporter plasmid pIL-8-CAT (10) and variable quantities of the pCMV-myc-LXA_4R expression plasmid (encoding LXA_4 receptor) according to the manufacturer's instructions. All cotransfection reactions were balanced for total amount of expression plasmid DNA with pCMV-myc vector. Approximately 16–24 h after transfection, cells were washed with HBSS and incubated with 0–100 nM 15-(R/S)-methyl-LXA_4 for 1 h followed by TNF-α for 8 h. Cell lysates were prepared and assayed for chloramphenicol acetyl transferase (CAT) using the CAT ELISA kit from Roche (Basel, Switzerland).

IκB assays

Levels of IκBα and phospho-IκBα were assayed from whole cell lysates of model intestinal epithelia via immunoblotting using an IκBα Ab (Santa Cruz Biotechnology, Santa Cruz, CA) as previously described (11).

Ca^{2+} mobilization

Intracellular Ca^{2+} was measured in fura 2-loaded polarized model epithelia via spectrofluorometry as previously described (11). Briefly, polarized model epithelia were prepared on customized supports permitting their insertion into a standard fluorometry cuvette. Model epithelia were incubated with 5 mM fura 2-AM (Molecular Probes, Eugene, OR) added for 60 min, and unincorporated probe was removed with a 10-min washing. Fluorescence was read with emission at 505 nm while the excitation wavelength is changed from 340 to 380 nm. Values of intracellular Ca^{2+} were calculated via the Grynciewitz equation $(R - R_{min})/(R_{max} - R) \times K_d$. R_{max} and R_{min} are measured by adding digitonin (10 μM) and then EGTA (20 mM), respectively.

DSS colitis

Six- to 8-wk-old BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME). One week following arrival, mice were given drinking water containing 4% DSS and 0.05% ethanol (vehicle) or 10 μg/ml 15-epi-16-parafluoro-LXA_4 (following internal review board ap-

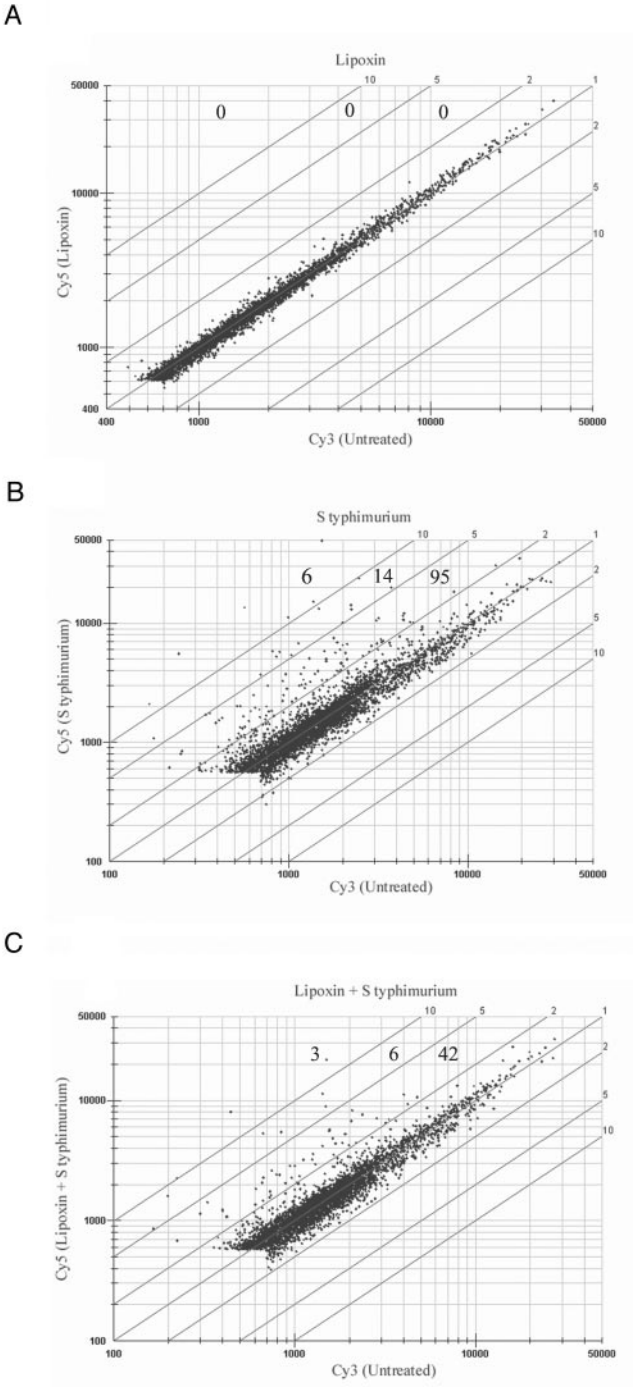

FIGURE 1. LXA_4 analog attenuates changes in epithelial gene expression induced by *S. typhimurium*: scatter plot of expression levels from control and LXA_4 analog-treated cells. Paired hextuplicate sets of T84 model epithelia were treated as follows for 4 h, at which time mRNA was isolated and analyzed via cDNA microarray hybridization. *A*, Epithelia were treated with 100 nM 15-(R/S)-methyl-LXA_4 and compared with untreated (except vehicle) control. *B*, Epithelia were treated with vehicle for 1 h followed by apical colonization with 10^9 CFU *S. typhimurium* and compared with untreated control. *C*, Epithelia were treated with 100 nM 15-(R/S)-methyl-LXA_4 vehicle for 1 h followed by apical colonization with 10^9 CFU *S. typhimurium* and compared with untreated control. Expression of each gene plotted as treated vs untreated so the diagonal represents no change from the control state. The diagonals represent indicated fold changes (above for positive) from control. The total number of genes between each set of diagonals is indicated.

Table I. *LXA_4 analog attenuates activation of proinflammatory gene expression*[a]

Gene Name	Accession No.	Control Fold Induction	LX Fold Induction	LX/Control Induction Ratio
Small inducible cytokine subfamily A (Cys-Cys), member 20	**D86955**	**31.8**	**14.2**	**0.45**
TNF-α-induced protein 3	**M59465**	**23.4**	**17.9**	**0.75**
Baculoviral IAP repeat-containing 3	**AI581499**	**22.5**	**9.9**	**0.44**
IL-1α	**M28983**	**12.3**	**7.8**	**0.63**
Superoxide dismutase 2, mitochondrial	**Y00472**	**11**	**6.4**	**0.58**
Nuclear factor of κ light gene enhancer in B-cells inhibitor	**M69043**	**10.9**	**7.8**	**0.72**
Apoptosis inhibitor 2 (baculoviral IAP repeat-contining 3)	**U37546**	**9.6**	**3.9**	**0.41**
Leukemia inhibitory factor (cholinergic differentiation factor)	**X13967**	**8.8**	**5.9**	**0.67**
Matrix metalloproteinase 7 (matrilysin, uterine)	L22524	7.1	3.1	0.44
Heat shock 70-kDa protein 1A	*M59828*	*6.3*	*0.59*	*0.09*
ESTs (chromosome 8 open reading frame 4)	AA128305	6.3	7.8	1.24
Syndecan binding protein (syntenin)	AF000652	6	2.3	0.38
ESTs (hypothetical protein FLJ23231)	AI225235	6	5.1	0.85
Apoptosis inhibitor 1 (baculoviral IAP repeat-contining 2)	**U37547**	**5.9**	**3.3**	**0.56**
UDP-Gal:βGlcNac β 1,4-galactosyltransferase, polypeptide 1	X13223	5.8	2.1	0.36
Heat shock 70-kDa protein 8 (HSC71)	*AL044172*	*5.3*	*0.67*	*0.13*
Laminin	X84900	5.2	1.8	0.35
c-*fos* oncogene	V01512	5.2	2.9	0.56
Protease inhibitor 3, skin-derived (SKALP)	**D13156**	**5**	**2**	**0.4**
Platelet factor 4 variant 1	M26167	4.9	3.7	0.76
GTP-binding protein overexpressed in skeletal muscle	U10550	4.7	2.5	0.53
Phosphoprotein regulated by mitogenic pathways	AJ000480	4.2	3.6	0.86
Heat shock 70-kDa protein (HASP70B′)	*X51757*	*3.9*	*0.77*	*0.2*
Heat shock 105-kDa	*AB003334*	*3.8*	*0.59*	*0.15*
DnaJ (Hsp40) homolog, subfamily B, member 4	*U40992*	*3.8*	*0.71*	*0.19*
Transmembrane protease, serine 2	U75329	3.8	3	0.79
Prostate differentiation factor	AA216685	3.8	3.4	0.89
NK cell transcript 4	AI539055	3.7	2.5	0.68
Proplatelet basic protein	M54995	3.7	2.9	0.78
Tissue inhibitor of metalloproteinase 3	AI245471	3.7	3	0.81
IFN (α, β, ω) receptor 2	L41942	3.7	3.1	0.84
Jagged1 (Alagille syndrome)	U61276	3.6	1.7	0.47
IFN-γR1	J03143	3.6	2.8	0.78
Ephrin-A1	M57730	3.5	2.9	0.83
Cytokeratin 20	X73501	3.4	1.5	0.44
Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	L25610	3.4	2.1	0.62
E74-like factor 3 (ets domain transcription factor)	U66894	3.4	3.2	0.94
ESTs (possessing ankyrin repeats)	AI744478	3.3	3	0.91
ESTs (dual adapter of phosphotyrosine and 3-phosphoinositides)	AA149868	3.2	2.2	0.69
Nuclear receptor subfamily 4, group A, member 1	L13740	3.2	2.3	0.72
Down syndrome candidate region 1	U85267	3.2	3.6	1.13
Ring finger protein	Y07828	3.1	1.6	0.52
Human Bcl-2 binding component 3 (bbc3)	U82987	3.1	2.6	0.84
Cytochrome P450, subfamily I (aromat. Comp.-inducible), polypeptide 1	K03191	3.1	3.2	1.03
Diphtheria toxin receptor	AC004634	3	2.4	0.8
IFN regulatory factor 1	X14454	3	2.9	0.97
SKI-like	U70730	2.9	1.6	0.55
Early growth response 1	M80583	2.9	1.9	0.66
Low density lipoprotein receptor (familial hypercholesterolemia)	L00352	2.9	2.1	0.72
Small inducible cytokine subfamily B (Cys-X-Cys), member 10	X02530	2.8	1.5	0.54
Stress-induced-phosphoprotein 1 (Hsp70/Hsp90-organizing protein)	M86752	2.7	0.59	0.22
Laminin, β 3 (nicein (125 kDa), kalinin (140 kDa), BM600 (125 kDa))	*U17760*	*2.7*	*1.7*	*0.63*
Ubiquitin C	AI565117	2.7	2.2	0.81
KIAA0127 gene product	D50917	2.7	2.2	0.81
Nef-associated factor 1	AJ011895	2.7	2.5	0.93
Heat shock 60-kDa protein 1 (chaperonin)	*M34664*	*2.6*	*0.71*	*0.27*
KIAA0291 protein (cytoplasmic linker 2) {GenBank AB006629}	AB006629	2.6	1.3	0.5
Myeloid cell leukemia sequence 1 (BCL2-related)	AF118124	2.6	1.5	0.58
Chromodomain helicase DNA binding protein 4	X86691	2.6	1.6	0.62
Growth arrest and DNA-damage-inducible, α	AI634658	2.6	2.2	0.85
CDP-diacylglycerol synthase (phosphatidate cytidylytransferase) 1	AI636223	2.5	1.7	0.68
TNFR superfamily, member 10b	AF016266	2.5	2	0.8
Heat shock 90-kDa protein 1, β	*M16660*	*2.4*	*0.83*	*0.35*
KIAA0585 protein (phosphotidylserine receptor)	AB011157	2.4	1	0.42

(*Table continues*)

[a] Model epithelia were treated with vehicle (0.1% ethanol or 100 nM 15-(R/S)-methyl-LXA_4) for 1 h, and then were colonized for 4 h with *S. typhimurium* at which time mRNA was isolated and analyzed via cDNA microarray hybridization. GenBank name and corresponding accession number are shown. Control fold induction and LXA_4 analog induction are the fold up-regulation for each gene observed in response to *S. typhimurium* relative to a common control (untreated T84 cell mRNA) in the presence (LX induction) and absence (control induction) of LXA_4 analog. LX/control induction ratio is the ratio of control induction to that observed in the presence of LXA_4 analog. Data are in descending order of control induction beginning with the most up-regulated genes. NF-κB-regulated genes are shown in bold. Genes for heat shock proteins are italicized.

Table I. *Continued*

Gene Name	Accession No.	Control Fold Induction	LX Fold Induction	LX/Control Induction Ratio
ESTs (GenBank N93892)	N93892	2.4	1.1	0.46
Cytochrome P450, subfamily IIIA, polypeptide 7	D00408	2.4	1.4	0.58
ESTs (type I transmembrane protein Fn14)	AI827127	2.4	1.7	0.71
Potassium channel, subfamily K, member 1 (TWIK-1)	U90065	2.4	1.9	0.79
Territin, heavy polypeptide 1	AA102267	2.4	2.7	1.13
Plasminogen activator, urokinase receptor	AC0076953	2.4	2.9	1.21
Tubulin, β5	X00734	2.3	1.5	0.65
Glucosamine-6-phosphate deaminase	L40636	2.3	1.9	0.83
Ephrin-B1	U09304	2.3	2.1	0.91
Small inducible cytokine D (Cys-X3-Cys), (fractalkine, neurotactin)	AC004382	2.3	2.2	0.96
EH domain containing 1	AF001434	2.3	2.4	1.04
Pim-1 oncogene	M54915	2.3	2.7	1.17
Related to t-complex 1/acetyl-Coenzyme A acetyltransferase 2	X52882	2.2	0.83	0.38
Early growth response 3	X63741	2.2	0.91	0.41
Splicing factor proline/glutamine rich	X70944	2.2	1.2	0.55
Cdc42 effector protein 2	AF001436	2.2	1.2	0.55
Ubiquitin B	U49869	2.2	1.7	0.77
TNF (TNF superfamily, member 2)	M10988	2.2	1.8	0.82
TGFα	X70340	2.2	1.8	0.82
Ladinin	U42408	2.2	2.1	0.95
Epiregulin	D30783	2.2	4.2	1.91
Tubulin, β polypeptide	X79535	2.1	1.2	0.57
ESTs (sirtuin 1)	AI378978	2.1	1.2	0.57
CDC28 protein kinase 2	X54942	2.1	1.2	0.57
Putative translation initiation factor (SUI 1)	AI832315	2.1	1.3	0.62
Protein phosphatase 1, regulatory subuint 10	Y13247	2.1	1.4	0.67
Early growth response 2 (Krox-20 (Drosophila) homolog)	J04076	2.1	1.4	0.67
Heat shock 70-kDa protein 5 (glucose-regulated protein, 78 kDa)	*AI878886*	*2.1*	*1.7*	*0.81*
Dual specificity phosphatase 5	U15932	2.1	1.7	0.81
ESTs (transcriptional cofactor with PDZ binding motif (TAZ))	AL050107	2.1	1.9	0.9
Epithelial membrane protein 1	U77085	2.1	2	0.95
Actin, α2, smooth muscle, aorta	AL048044	2.1	2.5	1.19
Lipocalin 2 (oncogene 24p3)	X99133	2.1	3	1.43
FK506-binding protein 4 (59kD)	M88279	2	0.63	0.31
Decay accelerating factor for complement (CD55)	AF052110	2	1	0.5
ESTs (solute carrier family 7)	N35555	2	1.1	0.55
Chromodomain helicase DNA binding protein 2	AF006514	2	1.1	0.55
Nucleoside phosphorylase	AA311617	2	1.3	0.65
EphA2	AA612998	2	1.3	0.65
ESTs (hypothetical protein MGC11034)	AA775792	2	1.4	0.7
B-cell translocation gene 1, anti-proliferative	AI560266	2	1.4	0.7
IL-2Rγ (severe combined immunodeficiency)	D11086	2	1.5	0.75
Carcinoembryonic Ag gene family member 6	X52378	2	1.5	0.75
Carbonic anhydrase II	J03037	2	1.5	0.75
Mitogen-activated protein kinase kinase kinase 8	D14497	2	1.6	0.8
GTP cyclohydrolase 1 (dopa-responsive dystonia)	S44053	2	1.7	0.85
6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	AA463459	2	1.7	0.85
Forkhead (*Drosophila*)-like 8	U59831	2	2.2	1.1
Incyte EST (Incyte PD:58522)		1.9	2.1	1.11
Ornithine decarboxylase 1	M81740	1.8	2.8	1.56

proved protocol). Water consumption was measured per cage and corrected for leakage/evaporation by comparison to identical water bottles placed in empty cages. Water was changed every 5 days, a time at which HPLC analysis indicated that degradation of the LXA_4 analog had not occurred. Body mass was measured daily by a technician blinded to the drug protocol, who also checked mice daily for gross rectal bleeding and occult blood in stools via Hemoccult Sensa (Beckman Coulter, Fullerton, CA). Bleeding score was 0 (hemoccult negative), 1 (mild positive), 2 (strong positive), and 4 (gross blood) following the well-established procedure developed by Cooper et al. (12). We did not consistently observe diarrhea in these mice and therefore did not include this parameter in our data presentation. Mice that were judged (by the blinded technician) to be moribund were sacrificed (and classified as nonsurvivors), although only one mouse in this study fell into this category (others were found dead upon daily check). Seven days following administration of DSS, mice were switched to DSS-free water containing vehicle or LXA_4 analog. Statistical significance was determined for body mass and survival data by Student's *t* test with $p < 0.05$ termed significant.

HPLC

HPLC analyses were conducted on LXA_4 analog-containing drinking water via a LUNA 5 μm C18(2) column (250 $\times$ 4.60 mm) using a ProStar HPLC (Varian, Palo Alto, CA) equipped with a diode array detector.

Results

LXA_4 analog attenuates changes in gene expression induced by S. typhimurium

To explore the mechanism of the anti-inflammatory bioactivity of LXA_4 and its stable analogs, the effect of this eicosanoid on global gene expression in control and inflamed (*S. typhimurium*-infected) model intestinal epithelia was examined by high-density cDNA microarray. First, we asked whether LXA_4 analogs alone globally influenced gene regulation, potentially up-regulating anti-

inflammatory effector molecules. Model epithelia (6×5 cm^2 epithelia per condition) were treated with vehicle (0.05% ethanol) or 100 nM of 15-(R/S)-methyl-LXA_4 (a concentration known to attenuate agonist-induced IL-8 mRNA expression (5)) for 4 h. mRNA was isolated from each set of samples and pooled, and microarray analysis was performed by hybridization to 7075 independent cDNA targets according to the protocol of Incyte Genomics as previously described (9). The expression levels of each gene from untreated (labeled with Cy3 before hybridization) epithelia vs that of LXA_4 analog-treated epithelia (Cy5 labeled) are plotted in Fig. 1. Accordingly, genes whose expression are unchanged lie on the central diagonal while, for example, genes whose expression is induced 2- to 5-fold are plotted between the upper diagonals labeled 2 and 5 (Fig. 1). Interestingly, LXA_4 analog by itself did not induce any significant changes (2-fold or more (9)) in any of the genes included in the array used. These data suggest that LXA_4 does not directly affect gene expression, and hence more likely modulates the signaling pathways by which proinflammatory agonists regulate gene expression.

Next we examined the effect of LXA_4 analog on the changes in epithelial gene expression that are induced by colonization with *S. typhimurium* (Table I). In previous experiments in which model epithelia were colonized with *S. typhimurium* for various lengths of time (A. Young, A. Gewirtz, and A. S. Neish, manuscript in preparation), up to 300 genes were significantly differentially and reliably regulated over a 2- to 6-h time course after attachment of this pathogen to the apical epithelial surface. The maximal number of differentially regulated genes was observed 4 h postcolonization, so this time point was selected to study potential effects of lipoxins on proinflammatory gene regulation. Thus, model epithelia were treated with vehicle (0.05% ethanol) or 100 nM of 15-(R/S)-methyl-LXA_4 for 90 min, and then apically colonized with *S. typhimurium* for 4 h, followed by mRNA isolation and analysis as described above. All of these experiments were performed in parallel with the same pool of common control RNA derived from vehicle-treated cells. Consistent with previous data, *S. typhimurium* colonization of T84 model epithelia resulted in significant up-regulation of 115 genes (1.57% of the specific mRNAs assayed). Of these 115 up-regulated genes, 57 (49%) exhibited reduced (by 25% or more) induction of transcript abundance in the presence of the LXA_4 analog. LXA_4 analog treatment led to an increased induction (by 25% or more) of only three genes while the remaining up-regulated genes were not significantly affected by LXA_4 analog. Relatively few genes (nine) exhibited decreased expression in response to *S. typhimurium*, and this diminution was not affected by LXA_4 analog (data not shown). While conclusions regarding effects on individual specific genes require verification via other means, this technique is effective at showing the overall effect of LXA_4 analog on such induced proinflammatory gene expression as is represented in Fig. 1. Many of the genes whose expression exhibited the greatest fold up-regulation in response to *S. typhimurium* were genes that are known to be regulated by the transcription factor NF-κB (see boldface in Table I). The induction of these NF-κB-mediated genes was attenuated by LXA_4 analog (average reduction, 44%), suggesting that this eicosanoid regulates activation of this transcription factor.

LXA_4 analog attenuates activation of NF-κB in model epithelia

In light of both the above microarray data and our previous finding that LXA_4 analogs down-regulate IL-8 secretion (5, 6) (also a NF-κB regulated gene), we next investigated whether LXA_4 analogs directly attenuated epithelial cell activation of NF-κB. As polarized model epithelia do not permit direct quantitation of NF-κB promoter activity (because they are not transfectable), we used HeLa epithelial cells, which were transiently cotransfected with plasmids encoding an NF-κB-responsive reporter gene (derived from the IL-8 promoter) in the presence and absence of a plasmid encoding the LXA_4 receptor. While such nonpolarized cells respond very differently to bacteria than polarized ones, both cell types respond to classic cytokine agonists such as TNF-α in a very similar manner. Thus, the effects of LXA_4 analog on NF-κB-mediated transcriptional activity was determined by comparing TNF-α-induced CAT reporter activity in LXA_4 analog-pretreated and untreated cells. LXA_4 analog attenuated NF-κB-mediated gene expression by ~50% (Fig. 2), consistent with the notion that attenuation of proinflammatory gene expression by LXA_4 analog is mediated via signaling through this transcription factor. Such LXA_4 analog attenuation of NF-κB activity was not seen in the absence of cotransfected LXA_4 receptor, indicating that this receptor is specifically required for the observed anti-inflammatory bioactivity.

We next investigated the mechanism by which LXA_4 analogs attenuate NF-κB activation. NF-κB activation requires the degradation of its physically associated negative regulator IκB. Thus, we measured whether the degradation of IκBα that we have previously shown occurs in *S. typhimurium*-colonized or TNF-α-treated model epithelia (8) was attenuated by LXA_4 analogs. Indeed, as shown in Fig. 3, LXA_4 analog treatment reduced such IκB degradation induced in model epithelia by either stimulus, although the affect was more clearly visible in TNF-α-treated epithelia, likely due to the uniform kinetics of the response to this soluble agonist. This attenuation of IκBα degradation was observed in the presence of as little as 1 nM LXA_4 analog, consistent with the concentration dependence of the attenuation of IL-8 secretion previously observed in the presence of LXA_4 analogs (5). IκBα degradation is regulated by induced phosphorylation of serines 32 and 36. While such phospho-IκBα is normally rapidly ubiquitinated and degraded by the proteasome, it can be stabilized via inhibiting its proteolysis with pharmacologic inhibitors. Thus, using such cell-permeant inhibitors of the proteasome, we analyzed generation of phospho-IκBα induced by TNF-α in the presence of a range of concentrations of 15-(R/S) methyl-LXA_4. In contrast to its inhibition of IκBα degradation, we did not observe a reduction in IκBα phosphorylation (phospho-IκBα can be distinguished from IκBα by its higher m.w.) in the presence of any of the tested concentrations of LXA_4 analogs. LXA_4 analogs had similar effects on IκBα phosphorylation and degradation induced by *S. typhimurium* or when assayed using a phospho-specific IκBα Ab (data not shown). Finally, we examined Ca^{2+} mobilization, one of

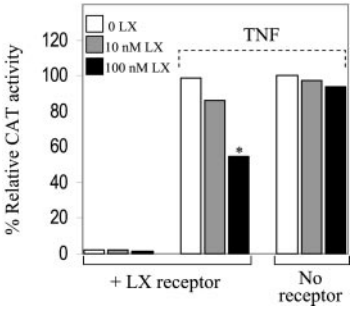

FIGURE 2. LXA_4 analog down-regulates epithelial activation of NF-κB. HeLa cells were transiently transfected with plasmids encoding NF-κB-CAT and empty vector or LXA_4R as described in *Materials and Methods*, treated with 100 nM 15-(R/S)-methyl-LXA_4 for 1 h, and then stimulated with TNF-α for 8 h. Cells were then lysed and CAT activity was measured via ELISA. Data are means of two separate experiments, each of which showed a similar pattern of results. *, Statistically significant difference ($p < 0.05$).

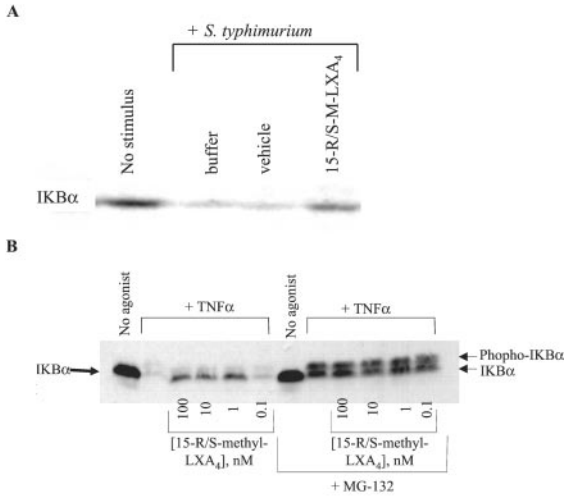

FIGURE 3. LXA_4 analog reduces degradation, but not phosphorylation, of IκBα. Model epithelia were treated with vehicle (0.1% ethanol or 15-(R/S)-methyl-LXA_4) for 1 h and stimulated, and whole cell lysates were assayed for IκBα levels via immunoblotting. *A*, Epithelia were colonized by 10^9 CFU *S. typhimurium* for 1 h in the presence or absence of 100 nM 15-(R/S)-methyl-LXA_4. *B*, Epithelia were treated with vehicle or indicated concentration of 15-(R/S)-methyl-LXA_4 in the presence or absence of the proteasome inihibitor MG-132 (50 μM), and then stimulated with TNF-α (10 ng/ml) for 45 min. Whole cell lysates were analyzed via SDS-PAGE immunoblotting using anti-IκBα. Note that phospho-IκBα is visible only upon proteasomal inhibition with MG-132. Data are individual experiments and are representative of three separate experiments.

the early signaling events that leads to IκBα phosphorylation in *S. typhimurium*-colonized epithelia (11). *S. typhimurium*-induced Ca^{2+} mobilization was not affected by a LXA_4 analog (Fig. 4). Together, these results suggest that LXA_4 analogs act subsequent to initial Ca^{2+} signal and resulting IκBα phosphorylation in the reduction of the IκBα degradation that attenuates proinflammatory gene activation.

LXA_4 analog is therapeutic for DSS colitis

Chronic inflammatory diseases of the intestine such as Crohn's disease and ulcerative colitis (i.e., inflammatory bowel disease (IBD)) are associated with, and possibly mediated by, increased levels of proinflammatory cytokines, many of which are NF-κB regulated, in the intestinal mucosa (13). Activated NF-κB is detectable in biopsies of IBD patients (14), and therapeutic agents that are effective in IBD are known to act, at least in part, through inhibition of NF-κB activation. Because LXA_4 analogs attenuated NF-κB-mediated gene expression in vitro, we next asked whether LXA_4 analogs might be therapeutic for intestinal inflammation in vivo. Due to the lack of established murine models of infectious gastroenteritis (mice get systemic illness rather than intestinal inflammation from *S. typhimurium*), we used a well-established chemically induced murine colitis model. Specifically, we examined whether an orally administered LXA_4 analog affected the colitis induced by DSS by measuring the clinical parameters that are the defined disease indicators in this widely used colitis model (Fig. 5). Colitis was induced in groups of five 8-wk-old mice via the addition of 4% DSS to their drinking water for 7 days. Simultaneous to the DSS administration, mice were also administered, via their drinking water, vehicle (0.05% ethanol) or 10 μg/ml 15-epi-16-para-fuoro-phenoxy-LXA_4, an analog of LXA_4 that has been shown to have local and systemic in vivo anti-inflammatory bioactivity (15). HPLC analysis of the LXA_4 analog recovered from the drinking water solutions demonstrated the structural integrity of the tetraene chromophore with only ester hydrolysis byproducts seen. Daily measurements of weight, occult blood, gross bleeding, and average (per group) water consumption were made. Because each mouse drank ~1 ml per day, the approximate daily dose of ingested compound was 10 μg per mouse.

DSS/vehicle-treated mice began to lose weight ~3–5 days (variance in different experiments) after the treatment began and continued to lose weight until ~3 days after the DSS treatment was stopped. Mice that did not die during this period then stopped intestinal bleeding (as assessed by gross observation and occult blood assay) and recovered their original weights over the next several days. The DSS/LXA_4 analog-treated mice lost weight with similar kinetics but on average lost significantly less weight than the DSS/vehicle-treated controls (Fig. 5). LXA_4 analog-treated mice also exhibited a trend toward less intestinal bleeding than their matched controls, although the difference did not quite reach statistical significance ($p = 0.062$ by Wilcox signed-rank test). These differences did not result from differing levels of DSS or water consumption, as water consumption was indistinguishable between the two groups. Nor were these differences likely the result of the LXA_4 analog reducing epithelial exposure to DSS via affecting epithelial chloride secretion, as LXA_4 analogs have been shown not to affect this secretory pathway (6). Perhaps most importantly, DSS/vehicle-treated mice had a mortality rate of 33% ($n = 15$) while, in contrast, we did not observe any mortality among the DSS/LXA_4 analog-treated mice ($n = 15$). About 3 days

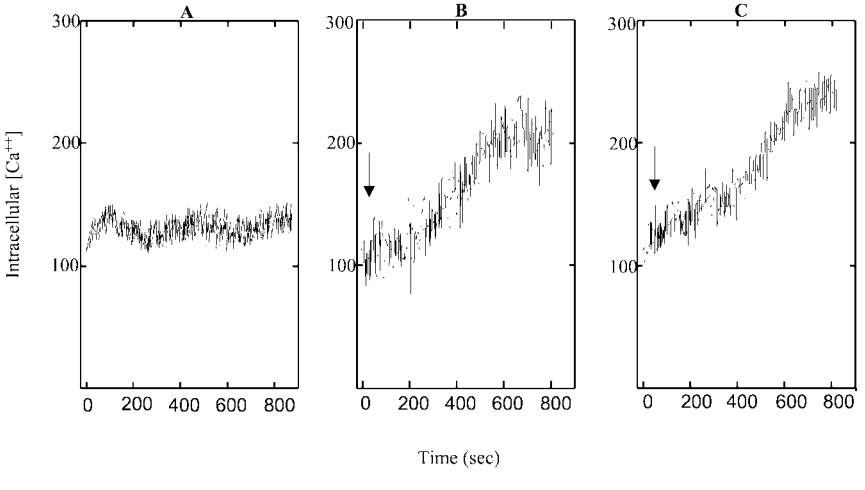

FIGURE 4. LXA_4 analog did not affect Ca^{2+} mobilization in response to *S. typhimurium*. Model epithelia were loaded with the Ca^{2+} indicator fura 2 (5 μM for 1 h) and intracellular Ca^{2+} measured by spectrofluorometry. *A*, Monitor of baseline Ca^{2+} in untreated epithelia. *B* and *C*, Fura 2-loaded epithelia were treated for 1 h with vehicle (*B*) (0.1% ethanol) or 100 nM 15-(R/S)-methyl-LXA_4 (*C*) and then apically treated with 10^9 CFU *S. typhimurium* during fluorometric measurement (addition of bacteria is indicated by arrow). Data are individual experiments and are representative of three separate experiments.

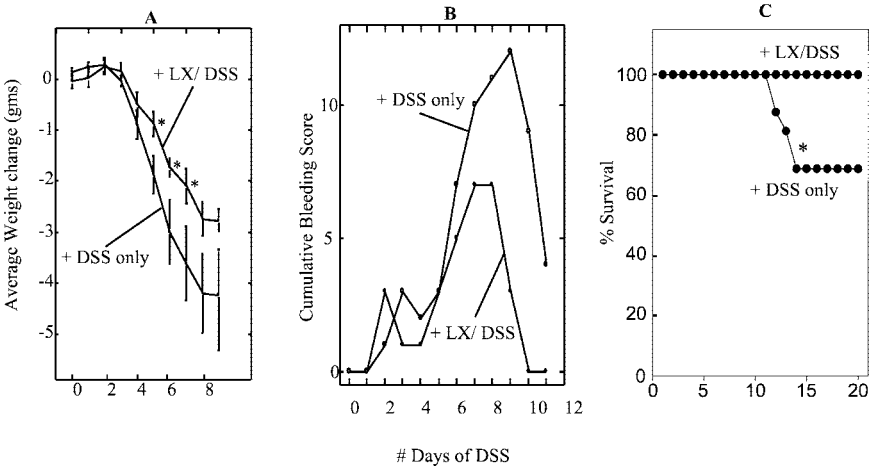

FIGURE 5. Oral administration of LXA$_4$ analog attenuates DSS-induced colitis. BALB/c mice were given 4% DSS in their drinking water along with vehicle or 10 μg/ml 15-epi-16-parafluoro-phenoxy-LXA$_4$. Body mass and bleeding (gross and occult, when stools could be obtained) were checked daily. *A* and *B*, Results (±SEM for body mass) of an experiment using six mice for each condition. The pattern is similar to two additional experiments using five mice per condition. *C*, Pooled results from the three replicated experiments. ∗, Statistically significant differences ($p < 0.05$).

after DSS treatment was suspended, both DSS/vehicle- and DSS/LXA$_4$ analog-treated mice stopped intestinal bleeding and began to recover their body weight. Excluding the DSS/vehicle-treated mice that died, the recovery rates did not differ significantly between mice receiving LXA$_4$ analogs vs their matched controls (data not shown). In the absence of DSS, mice treated with vehicle or LXA$_4$ analog exhibited indistinguishable slow weight gains of ~0.5 gm per week and did not have any detectable occult (or gross) bleeding. Together, these results indicate LXA$_4$ analogs can reduce the development of DSS colitis disease activity and its consequences on global physiological parameters of wellness, such as body weight.

Discussion

Chronic inflammatory diseases of the intestine (e.g., Crohn's disease and ulcerative colitis, collectively referred to as IBD) are a serious public health problem, particularly in the developed world. While chronic inflammatory events play a definitive role in these diseases, clinically significant flares are often associated with augmentation of the innate immune response, specifically neutrophil flux across the epithelial surface. Although the underlying causes of this response are generally unknown, proinflammatory cytokines, including those of epithelial origin, are thought to be essential mediators of this process (16). As shown in this work, stable analogs of the endogenous anti-inflammatory eicosanoid LXA$_4$ can attenuate epithelial proinflammatory gene expression. LXA$_4$ analog attenuation of proinflammatory gene expression was mediated through a central proinflammatory signaling pathway and lessened the disease activity in a well-established mouse model of colitis, thus suggesting that these compounds could be therapeutic for the active phase of IBD.

Acute flares of IBD resemble infectious colitis (e.g., salmonellosis) both histologically (characterized by massive neutrophil influx and transepithelial migration) and clinically (i.e., diarrhea, cramping). The clinical manifestations of salmonellosis are thought to be largely attributable to the mucosal innate (neutrophil-mediated) immune response to this organism, leading to the suggestion that IBD flares may result from an aberrant mucosal innate immune response to normally nonpathogenic gut flora (perhaps the end result of signals originating from cells of specific immunity) (1). Thus, it is encouraging that LXA$_4$ analogs broadly attenuated the changes in epithelial gene expression induced by *S. typhimurium*. Interestingly, LXA$_4$ analogs did not uniformly attenuate all such changes in gene expression but rather diminished some nearly completely while other induced changes in gene expression were unaffected. Categorization of many of these genes as well as their possible roles in inflammation are not yet well defined. One specific class of genes uniformly down-regulated by LXA$_4$ analogs were the heat shock proteins (HSP); the up-regulation of the *HSP* genes induced by *S. typhimurium* was completely reversed in the presence of LXA$_4$ analogs. Such regulation of *HSP* genes may play a role in LXA$_4$ analog bioactivity or may simply reflect the general reduced stress level of LXA$_4$ analog-treated epithelial cells. Consistent with the latter possibility, another family of stress-induced genes, the NF-κB-dependent genes, was also clearly down-regulated. As these genes are known to encode mediators of mucosal inflammation, this LXA$_4$ analog bioactivity likely plays a role in the observed therapeutic effects of this eicosanoid on DSS colitis in vivo.

Several nonsteroidal anti-inflammatory drugs (NSAID) including aspirin have also been shown to attenuate activation of the proinflammatory transcription factor NF-κB (17). However, LXA$_4$ analog attenuation of NF-κB differs from that of such agents in several important ways. LXA$_4$ analogs act via a specific receptor, whereas NSAID primarily directly inhibit proinflammatory enzymes. While both LXA$_4$ analogs and NSAID reduce IκBα degradation, LXA$_4$ analogs did so at nanomolar concentrations, while NSAID require much higher concentrations for this activity. In vitro studies indicate that the mechanism by which LXA$_4$ analog attenuated IκBα degradation also differs significantly from that of NSAID, in that LXA$_4$ analogs did not reduce the phosphorylation of IκBα, while NSAID, like other pharmacological attenuators of NF-κB, reduce IκBα degradation by preventing this phosphorylation event (18). However, because LXA$_4$ analogs are structurally similar to 15-epi-LXA$_4$, which is biosynthesized by cyclooxygenase that has been acetylated by aspirin (3), some of aspirin's attenuation of proinflammatory gene expression may yet result via this route. Mechanistically, LXA$_4$ analog attenuation of NF-κB more closely resembles that of non-proinflammatory bacteria that also block IκBα degradation but not phosphorylation (19). Such phosphorylation-independent regulation of IκBα may thus be common to agonists that activate endogenous anti-inflammatory pathways and may hold more promise to have fewer unwanted effects than global inhibitors of IκBα kinase.

LXA$_4$ analogs have in vivo anti-inflammatory activity when applied both locally (topically in the mouse ear) and systemically via tail vein (15, 20). Oral administration is somewhat equivalent to topical application in the gut, as it provides direct delivery of LXA$_4$ analog to the intestinal epithelium. However, this LXA$_4$ analog is rapidly absorbed following oral gavage in rodents with ~17% oral availability (B. Subramanyam, W. Guilford, J. Bauman, and J. Parkinson, unpublished observations) and thus may act

systemically. While LXA_4 analog is short-lived in plasma ($t_{1/2}$ <30 min for i.v.; B. Subramanyam, W. Guilford, J. Bauman, and J. Parkinson, unpublished observations) placement in the drinking water provides semicontinuous systemic delivery. A particularly likely target are neutrophils, which are known to be targets of LXA_4 and major immune mediators of this colitis model. LXA_4 analogs attenuate neutrophil chemotaxis (21), oxidative burst (22), and the release of granule proteases (23). Delivery of LXA_4 analogs via tail vein showed a similar trend as oral administration on DSS colitis but did not differ statistically significantly from control (DSS-treated) mice (data not shown). This could have resulted from a less-targeted delivery of compound to the inflammatory site or may have occurred because delivery in the drinking water maintained a continued presence of the LXA_4 analog while the injected compound is rapidly cleared from the circulation (24).

While it is important to sort out the precise cellular mechanism of LXA_4 analog in vivo bioactivity, regardless, oral administration seems to be an effective way for this compound to down-regulate intestinal inflammation. In contrast, NSAID, including cyclooxygenase-2-specific inhibitors, tend to damage the intestinal mucosa, resulting in causation or exacerbation of intestinal inflammation, while mAb-based drugs (e.g., imflixamab), although therapeutic, must be given i.v. Thus, this novel strategy of activating endogenous anti-inflammatory pathways with stable analogs of LXA_4 could be developed into an effective means of treating human colitis.

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