Orbital fibroblasts orchestrate tissue remodeling in Graves disease, at least in part, because they exhibit exaggerated responses to proinflammatory cytokines. A hallmark of late stage orbital disease is vision-threatening fibrosis, the molecular basis of which remains uncertain. We report here that the Th2 cytokines, interleukin (IL)-4 and IL-13, can induce in these cells the expression of 15-lipoxygenase-1 (15-LOX-1) and in so doing up-regulate the production of 15-hydroxyeicosatetraenoic acid. IL-4 increases 15-LOX-1 protein levels through pretranslational actions. The increased steady-state 15-LOX-1 mRNA is independent of ongoing protein synthesis and involves very modestly increased gene promoter activity. Importantly, IL-4 substantially enhances 15-LOX-1 transcript stability, activity that localizes to a 293-bp sequence of the 3'-untranslated region. IL-4 activates Jak2 in orbital fibroblasts. Interrupting signaling through that pathway, either with the specific chemical inhibitor, AG490, or by transiently transfecting the cells with a Jak2 dominant negative mutant kinase, attenuates this induction by attenuating IL-4-dependent mRNA stabilization. 15-LOX-1 protein and its mRNA were undetectable in IL-4-treated dermal fibroblasts, despite comparable levels of cell surface IL-4 receptor and phosphorylated Jak2 and STAT6. Our findings suggest that orbital connective tissues may represent a site of localized 15-hydroxyeicosatetraenoic acid generation resulting from cell type-specific 15-LOX-1 mRNA stabilization by IL-4. These results may have relevance to the pathogenesis of orbital Graves disease, an inflammatory autoimmune condition that gives way to extensive fibrosis associated with a Th2 response.

Graves disease is associated with dramatic remodeling of the orbital connective tissue and muscles, a process known as thyroid-associated ophthalmopathy (TAO)\(^1\). It is an autoimmune disease that in some as yet unidentified way relates to pathology occurring in thyroid tissue. Important features of TAO include a dramatic inflammatory response and disordered accumulation of the nonsulfated glycosaminoglycan, hyaluronan. Late in the disease, inflammation abates and is followed by fibrosis (2). Orbital fibroblasts become activated in TAO, probably through their interactions with T lymphocytes and mast cells trafficked to the orbit (3). These immunocompetent cells elaborate multiple cytokines in Graves disease. Abundant cytokines belonging to both Th1 and Th2 classes have been demonstrated in affected orbital tissues (4). Th1 responses appear to predominate early, whereas Th2 cytokines drive later stages of tissue reactivity (5). Th2 cytokines and the T cells from which they derive are associated with fibrosis, a hallmark of the seemingly irreversible morbidity resulting from severe TAO (1). Thus, cytokines, such as IL-4, IL-5, and IL-13, may play important roles in end stage disease. With regard to TAO, nothing is currently known about the molecular targets for Th2 cytokines in the orbit that are responsible for attenuation of inflammation and provoking end stage tissue remodeling, including fibrosis. Identification of the proximate mechanisms involved in these changes could yield therapeutic strategies directed at preserving sight. Moreover, they may also have important implications for the tissue remodeling occurring in other autoimmune diseases, such as rheumatoid arthritis.

Lipoxygenases (LOX) are a family of lipid-peroxidizing enzymes that oxygenate polyunsaturated fatty acids to specific hydroperoxy derivatives (6); arachidonic acid is converted to distinct hydroperoxyeicosatetraenoic acids (HPETE). There are two human 15-LOX (EC 1.13.11.33) genes, 15-LOX-1 and 15-LOX-2 (7-10), each possessing a distinct profile of tissue expression and catalytic activities. 15-LOX-1 generates 12-HPETE as well as 15-HPETE from arachidonic acid, whereas 15-LOX-2 forms purely 15-HPETE (11). 15-LOX-1 is expressed in reticulocytes, eosinophils, macrophages, and monocytes (8). In reticulocytes from anemic animals, this enzyme mediates mitochondrial disruption during differentiation (12). The distribution of 15-LOX-2 appears restricted to certain epithelial cell types, including those of the hair root, skin, prostate, lung, and cornea (9). There is evidence that 15-LOX-2 functions as a negative regulator of cell cycle progression in normal prostate epithelium (13), and levels of the enzyme may be diminished substantially in prostate carcinoma (14).

15-LOX-1 can oxidize biomembranes, and because these activities profoundly disrupt cellular function, its expression must be tightly regulated. In developing reticulocytes, 15-LOX-1 mRNA exhibits translational silence until the final stages, despite its high relative abundance (15). IL-4 and IL-13 have been shown to up-regulate 15-LOX-1 expression in several mammalian cell types, including those of the fibroblast lineage derived from the synovial membrane and lung (16, 17). It may be in part through their up-regulation of 15-LOX-1 that the anti-inflammatory activities of Th2 cytokines exert their influence on tissue activa-
tion and remodeling (18). Many of these actions in connective tissues are thought to be mediated by resident fibroblasts.

Orbital fibroblasts exhibit attributes that set them apart from those derived from other connective tissue depots (19). They possess a distinctive morphology (20) and profiles of surface receptors (21) and gangliosides (22) and express several proteins differentially (23) when activated by cytokines. Moreover, they comprise a heterogeneous population of cells (24), subsets of which differentiate into distinct phenotypes (25). We hypothesize that the vulnerability of orbital connective tissue to Graves disease is a direct result of the unique attributes of its resident fibroblasts (19).

When orbital fibroblasts are activated by proinflammatory cytokines, such as IL-1β, leukoregulin, or CD154, they generate extraordinarily high levels of PGE2 (26, 27). This results from the coordinate induction of prostaglandin endoperoxide H synthase, the inflammatory cyclooxygenase, and microsomal PGE2 synthase (27). In the setting of inflammation, PGE2 conditions tissues to the actions of other factors and can alter the balance between Th1 and Th2, favoring the formation of the latter cytokines (28, 29). Thus, cells that generate particularly high levels of PGE2 exert an important influence over qualitative aspects of tissue remodeling and potentially promote profibrotic events. Other eicosanoid synthetic pathways have yet to be characterized in orbital fibroblasts. The actions of their products, like those of PGE2, can represent critical determinants of the immune response. Synthetic products of lipoxygenases have been insinuated in the pathogenesis of inflammatory and allergic responses and autoimmune diseases (6, 30). Their potential roles in Graves disease and TAO have not been examined previously.

We now report that IL-4 and IL-13 can up-regulate 15-LOX-1 expression in orbital fibroblasts from patients with severe TAO. This induction is the consequence of increased steady-state 15-LOX-1 mRNA levels and appears to be mediated through the Jak2/STAT6 signal transduction pathway. Unlike previous examples of IL-4-induced 15-LOX-1 expression that involve large increases in gene transcription, those reported here result from modest increases in gene promoter activity and substantially enhanced mRNA stability. Our results suggest that Th2 cytokines can activate an important component of the immunomodulatory machinery in orbital fibroblasts in an anatomic site- and disease-selective manner. They suggest a heretofore unrecognized action of IL-4 in orbital connective tissue that could underlie the Th2-driven manifestations of late stage TAO.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dexamethasone (1,4-pregnadien-9-fluoro-16α-methyl-11β,17α,21-triol-3,20-dione), 5,6-dichlorobenzimidazole, arachidonic acid, and cycloheximide were from Sigma. IL-1β, IL-4, IL-5, IL-12, IL-13, interferon γ, and tumor necrosis factor-α were purchased from BIOSOURCE (Camarillo, CA). AG940 was supplied by Calbiochem (La Jolla, CA). The plasmid containing a dominant negative (DN) mutant expression vector for Jak2 was generously provided by Dr. David E. Levy (New York University). 15-LOX-1 cDNA was from Oxford Biomedical Research (Oxford, MI). mAbs directed against human 15-LOX-1 and 15-LOX-2 were purchased from Cayman (Ann Arbor, MI). Pan-Ab and phosphorylated protein-specific Abs against Jak2, STAT6, and Tyk2 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) or Cell Signaling Technology (Danvers, MA). Anti-IL-4 receptor Abs were from BD Biosciences.

**Cell Culture**—Orbital fibroblast strains were initiated from tissue explants obtained as surgical waste during decompression surgery for severe TAO. Other strains were generated from normal appearing orbital tissues from patients undergoing surgery for eye diseases not involving the orbit. The Institutional Review Boards of the Harbor-UCLA Medical Center and Research and Education Institute and the UCLA Center for Health Sciences have approved these activities. Dermal fibroblasts were generated from punch biopsies of normal appearing skin or were purchased from the American Type Tissue Collection (Manassas, VA). Tissue fragments were generated by mechanical disruption of explants, and fibroblasts were then allowed to adhere to plastic culture plates. They were covered with Eagle’s medium to which 10% fetal bovine serum, glutamine (435 μg/ml), and penicillin/streptomycin were added, as described previously (31). Monolayers were disrupted by gentle treatment with trypsin/EDTA. Medium was changed every 3–4 days, and cultures were maintained in a 5% CO2 humidified incubator at 37 °C. Strains were utilized between the second and 12th passage. All experimental manipulations were conducted after a state of confluence had been reached. We have already characterized these cultures extensively (32). We found them to be essentially devoid of contamination with endothelial, epithelial, and smooth muscle cells.

**RNA Isolation and Northern Blot Hybridization and mRNA Stability Assays**—Fibroblasts were cultivated in 100-mm diameter plates to a confluent state and were then treated with the test agents specified in the figure legends. Cellular RNA was extracted from rinsed monolayers by the method of Chomczynski and Sacchi (33) with an RNA-isolating system purchased from Biotecex (Houston, TX). The nucleic acid was subjected to electrophoresis through denaturing 1% agarose, formaldehyde gels. Purity and integrity of the RNA were established by determining the 260/280 spectrophotometric ratios and by staining electrophoresed samples with ethidium bromide and inspecting them under UV light.

The RNA was transferred to Zeta-probe membrane (Bio-Rad), and immobilized samples were hybridized with [32P]dCTP-labeled 15-LOX-1 cDNA probes generated by the random primer method. Hybridization was conducted in ExpressHyb solution (Clontech, Palo Alto, CA) at 68 °C for 1 h. Membranes were washed under high stringency conditions, and then the RNA/DNA hybrids were visualized by autoradiography on X-Omat film (Eastman Kodak Co.) following exposure using intensifier screens at ~70 °C. Bands resulting from radioactive hybrids were scanned by densitometry. Membranes were then stripped according to the manufacturer’s instructions. They were rehybridized with a GAPDH cDNA probe, and band densities were normalized to this signal. In mRNA stabilization studies, cultures were pretreated with IL-4 (10 ng/ml) for 12 h. They were then rinsed extensively and treated without or with IL-4 for the times indicated along the abscissa in Fig. 7, A and B, in the presence of 5,6-dichlorobenzimidazole (20 μg/ml). Cellular RNA was isolated using a Qiagen (Valencia, CA) RNeasy Kit. Total RNA was treated with RNase-free DNase (Qiagen) at room temperature for 15 min. RNA (2 μg) was reverse-transcribed with an Omniscript reverse transcription kit (Qiagen) using oligod(T) primers (Invitrogen). cDNA was quantified with the SYBR green PCR kit (Qiagen) using real time PCR (Applied Biosystems, Foster City, CA). The primers used were forward 5′-CCCCCTGAAATATACCCT-CGG-3′ and reverse 5′-TCCCAAGCCCGCTCAGCATC-3′. In other mRNA stability studies, RNA was subjected to Northern blot analysis. In both studies, data were normalized to their respective GAPDH concentrations.

**HPLC Analysis**—Frozen fibroblast and monocyte pellets were thawed on ice in 100 μl of buffer (50 mM Tris with 100 mM NaCl, pH 7.5). The cells were then homogenized by sonication for 5 s using the microprobe of a Vibersonic 100 (Virtis Inc., Gardiner, NY) ultrasonicator at setting 6. Incubations using 50 μl of the sonicated cells were begun by the addition of 100 μM [1-14C]arachidonic acid (final concentration)
15-LOX-1 in Fibroblasts

(PerkinElmer Life Sciences) in 0.5 μl of ethanol (1% final volume). Samples were incubated for 45 min at 37 °C with continual agitation and terminated by the addition of 2.5 volumes of cold methanol. Following the addition of 1.25 volumes of dichloromethane and thorough mixing, samples were centrifuged to remove protein precipitates, and products were recovered in the mixed phase of methanol/water/dichloromethane. Samples were evaporated under a stream of nitrogen to remove most of the dichloromethane and methanol, water was added, and the products were extracted using a 1-ml C18 Waters Oasis cartridge. These materials were washed with water and eluted with 1 ml of MeOH. Extracts were analyzed by reversed-phase HPLC using a Waters Symmetry 5 μm C18 column (25 × 0.46 cm) with a solvent of methanol/water/glacial acetic acid 80:20:0.01 (by volume) at a flow rate of 1 ml/min (retention time of 15-HETE ~16.5 min). Unlabeled HETEs (100 ng each of 5-, 8-, 9-, 11-, 12-, and 15-HETEs) were added to each sample prior to HPLC analysis; this permitted an exact determination of the retention times of each HETE product within individual chromatographic runs. UV spectra and the profiles at 205, 220, 235, and 270 nm were recorded using a Hewlett-Packard 1040A diode array detector, and radioactivity was monitored on-line using a Radiomatic Instruments Flo-One detector.

Western Blot Analysis of Fibroblast Proteins—Cellular proteins were solubilized from rinsed fibroblast monolayers following the treatments indicated in the figure legends. The ice-cold harvest buffer contained 0.5% Nonidet P-40, 50 mM Tris-HCl (pH 8.0), and 10 μM phenylmethylsulfonyl fluoride. Lysates were taken up in Laemml buffer and subjected to SDS-PAGE; the separated proteins were transferred to polyvinylinide difluoride membrane (Bio-Rad). Primary Abs directed against human 15-LOX-1 gene promoter studies involved cloning a 338-bp fragment spanning nt 361 to 20 nt relative to the transcriptional start site was generated with the forward primer 5′-TTAAAGGTACCGTGGTA-CACACGGC-3′ and reverse sequence 5′-TTAAACCAAGCTTCTCGGTGGAGAAGAAGGTTGG-3′. Another, longer, 1025-bp fragment 263 was generated with the forward primer 5′-TTGGAGAAGAAGGTTGG-3′ and reverse primer 5′-TCCCTTCTGGTGGAGAAGAAGGTTGG-3′. The amplified fragment was sequenced and subcloned from pCR2.1 TOPO (Invitrogen) into a promoterless pGL2-luciferase reporter vector (Promega). A 293-bp fragment of the 3′-UTR spanning nt +2361 to +2653 was generated with the forward primer 5′-TGTTGTTTGAACAGACG-3′ and reverse primer 5′-CGTCCTAGTCTTCCCTTCTCAC-3′. The fragment was cloned into the XbaI site downstream from the luciferase reporter gene in the pG3 promoter vector (Promega). Transcription of this construct is driven by a SV40 promoter.

To transiently transfect fibroblasts, cultures were allowed to proliferate to 80–90% confluence in medium containing 10% fetal bovine serum. Constructs were transfected using the Lipofectamine PLUS system (Invitrogen). 0.75 μg of pGL2 promoter DNA and 0.1 μg of pRL-TK vector DNA (Promega), serving as a transfection efficiency control, were mixed with PLUS reagent for 15 min before being combined with Lipofectamine for another 15 min. The DNA-lipid mixture was added to culture medium for 3 h at 37 °C. Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum replaced the transfection mixture overnight. Transfected cultures were then serum-starved, and some received either IL-4 (10 ng/ml) for 2 h or nothing (control) as indicated. Cellular material was harvested in buffer provided by the manufacturer (Promega) and stored at −80 °C until assayed. Luciferase activity was monitored with the Dual-Luciferase Reporter Assay System (Promega) in an FB12 tube luminometer (Zylux). Values were normalized to internal controls, and each experiment was performed at least three times.

To interrupt the expression of potentially relevant signaling pathway components, the DN mutant kinase construct for Jak2 was ligated into pcDNA3.1 (Invitrogen). This was transiently transfected into cells as described above. Control cultures received a constant amount (2 μg) of empty vector DNA. Diminished levels of the kinases were documented by Western blotting an aliquot of the lysate with relevant antibodies.

Flow Cytometric Analysis of Cell Surface IL-4 Receptor Display—Fibroblasts were harvested by subjecting monolayers to gentle mechanical disruption, placing the cells in polypropylene tubes, and incubating for 20 min on ice with fluorochrome-conjugated mAbs (1 μg/10^6 cells). Cells were washed twice with staining buffer (phosphate-buffered saline and 4% bovine serum) and analyzed immediately. Analysis was performed on a FACS Calibur flow cytometer (BD Biosciences). Mean fluorescent intensity was calculated as a ratio of mean fluorescence sample/isotype fluorescence.

Statistics—Data are generally expressed as the mean ± range of duplicates or mean ± S.D. of three or more determinations. Significance was determined using Student’s t test.

RESULTS

IL-4 and IL-13 Induce the Expression of 15-LOX-1 in Orbital Fibroblasts—When orbital fibroblasts, in this case derived from a patient with TAO, were treated with IL-4 (10 ng/ml), 15-LOX-1 protein was induced in a time-dependent manner (Fig. 1A). The protein was undetectable in untreated fibroblasts, and the effect of IL-4 was first detected following 16 h of treatment. Maximal up-regulation occurred at 72 h, the duration of the study. In a total of three separate experiments, 15-LOX-1 protein was enhanced 68.4 ± 0.5-fold after 72 h (mean ± S.D., p < 0.05 versus control). IL-13 (10 ng/ml) also induced 15-LOX-1 protein expression (70 ± 2.5-fold at 72 h, p < 0.05 versus control) (Fig. 1B). In contrast, 5- and 12-LOX remained undetectable following treatment of the TAO orbital fibroblasts with either IL-4 or IL-13 (data not shown). The effects of IL-4 were concentration-dependent (Fig. 1C). Low level expression was observed at a cytokine concentration of 0.1 ng/ml (the lowest tested), and a nearly maximal effect was observed at 0.5 ng/ml. Higher concentrations of IL-4 (up to 10 ng/ml) failed to induce 15-LOX-1 further. In contrast to 15-LOX-1, 15-LOX-2 protein was constitutively expressed, albeit at low levels in TAO orbital fibroblasts (Fig. 1D). The levels of this protein were unaffected by IL-4 treatment up to 72 h, the duration of the study.

15-LOX-1 protein could be induced in multiple orbital fibroblast strains, each from a different donor with TAO. All four strains examined were found to exhibit substantial induction after 72 h of treatment with IL-4 (10 ng/ml) (9.85 ± 1.81-fold above controls, p < 0.005) (Fig. 2, A and C). In contrast, either extremely low or undetectable levels of expression were observed in three orbital fibroblast strains, each from a different donor without Graves disease following IL-4 treatment (p <
0.005 versus TAO orbital fibroblasts) (Fig. 2, B and C). No 15-LOX-1 protein induction could be detected in two strains of dermal fibroblasts harvested from normal appearing skin (Fig. 2B). Thus, it would appear that the induction of 15-LOX-1 by IL-4 is anatomic site-restricted to orbital fibroblasts derived from patients with TAO.

Glucocorticoid steroids exert broad anti-inflammatory effects and profoundly attenuate prostaglandin generation in IL-1β-treated orbital fibroblasts (26). Dexamethasone (10 nM), a specific, synthetic glucocorticoid, blocked the induction of 15-LOX-1 by IL-4 (IL-4, 3.13 ± 0.32-fold (mean ± range) greater than IL-4 plus dexamethasone) (Fig. 3A). A similar blockade was noted with regard to the induction by IL-13. Moreover, this action may be at least partially mediated by decreasing levels of 15-LOX-1 mRNA (see Fig. 5B). Often, Th1 cytokines oppose the actions of Th2 molecules. We thus determined the impact of interferon γ, a Th1 cytokine, on IL-4 and IL-13 effects and found that it could block the induction of 15-LOX-1 by both Th2 cytokines (IL-4, 8.13 ± 0.4-fold greater than IL-4 plus interferon γ (Fig. 3B)). A number of other cytokines were tested for their ability to induce 15-LOX-1. IL-5, IL-1β, IL-12, and tumor necrosis factor-α (10 ng/ml in every case) failed to induce the protein (data not shown). Thus, the expression of cytokine-dependent 15-LOX-1 in orbital fibroblasts appears to represent a specific Th2 immune response that is modulated by Th1 cytokines. The critical balance between Th1/Th2 may well represent a key determinant of enzyme expression.

Analysis of Arachidonic Acid Metabolism in Fibroblasts and Monocytes by Reversed-phase HPLC—We next compared 15-LOX-1 protein levels expressed in cytokine-treated human monocytes (a firmly established target for IL-4 action (8)) with those achieved in orbital fibroblasts. As the Western blot in Fig. 4A indicates, 15-LOX-1 protein levels in IL-4-treated monocytes were 3.8-fold greater on a “per cell” basis than those in fibroblasts after 72 h. We then determined whether the 15-LOX-1 protein induced by IL-4 was active in both cell types. Confluent orbital fibroblast cultures from a patient with severe TAO and monocytes from a healthy donor were treated with IL-4 (10 ng/ml) for 72 h. Cells were disrupted mechanically, and homogenates were incubated with [14C]arachidonic acid (see “Experimental Procedures”). Samples were extracted, and aliquots were analyzed by reversed-phase HPLC. Shown is analysis of control fibroblasts (Fig. 4B, top left), control monocytes (top right), and the corresponding IL-4-treated cells (bottom). Prior to injection on HPLC, a mixture of unlabeled HETE standards (5-, 8-, 9-, 11-, 12-, and 15-HETE) was added to each sample, allowing the profile of radiolabeled metabolites monitored by the radioactive detector to be compared with the retention times of these unlabeled HETEs monitored by the UV detector. The retention time of 15-HETE is indicated on each chromatogram, and the lower chromatograms also show the retention times of all six HETE standards (eluting in the order 15-, 11-, 12-, 8-, 9-, and 5-HETE). From these data, we can conclude that catalytically active 15-LOX-1 is expressed by orbital fibroblasts following treatment with IL-4.

15-LOX-1 Induction by IL-4 in Orbital Fibroblasts Is a Consequence of Increased Steady-state mRNA Levels—We next determined whether increased 15-LOX-1 expression provoked by IL-4 was a consequence of enhanced steady-state levels of the encoding mRNA. As the Northern blot hybridization shown in Fig. 5A indicates, hybridizable 15-LOX-1 mRNA is undetectable in control orbital fibroblast cultures from a patient with TAO. IL-4 substantially up-regulates transcript levels, migrating as a doublet of ~2.7- and 4-kb bands. The increase is not apparent at 6 h, but by 16 h, 15-LOX-1 mRNA has become abundant. Its levels continue to increase at 24 and 48 h, the duration of the study. To determine whether the up-regulation in this transcript constitutes a primary gene induction or is dependent on the ongoing synthesis of an
intermediate protein, cycloheximide (10 μg/ml) was added to cultures, either alone or in combination with IL-4. As Fig. 5B indicates, the inhibitor fails to alter the magnitude of the 15-LOX-1 induction by IL-4, suggesting strongly that it is not dependent on ongoing protein synthesis. With regard to the potential impact of interferon on pretranslational events, the addition of that cytokine to cultures receiving IL-4 abolished the induction of 15-LOX-1 mRNA (Fig. 5C).

**IL-4 Modestly Up-regulates 15-LOX-1 Gene Promoter Activity but Substantially Enhances mRNA Stability in Orbital Fibroblasts**—We next determined whether increases by IL-4 in steady state levels of 15-LOX-1 mRNA could be attributed to enhanced gene promoter activity. A 338-bp fragment of the 15-LOX-1 gene promoter, extending from −361 to −23 nt, was cloned, fused to a luciferase reporter gene, and transiently transfected into orbital fibroblasts. IL-4 (10 ng/ml) could enhance the activity of this reporter gene in a time-dependent manner (0 h, 2.81 ± 0.03 AU versus 12 h, 5.24 ± 0.01 AU (1.86 ± 0.01-fold), p < 0.001 versus control) (Fig. 6A). The effect then decayed back to base-line levels at 24 h. When interferon γ (100 units/ml) was added to the culture medium in combination with IL-4, no modulation of gene promoter activity was observed (data not shown). Because a consensus STAT6 binding site can be identified in the promoter at −952 nt, we cloned a longer 1025-bp fragment spanning nt −1045 to −20 and fused it to the
reporter gene. As Fig. 6B demonstrates, this longer construct exhibited only modest basal activity (≈2-fold) that failed to respond to IL-4, in contrast to the shorter fragment (control, 1.7 ± 0.09 AU versus IL-4, 2.91 ± 0.07 (1.7-fold, p < 0.001)). Thus, it would appear that enhancement of 15-LOX-1 gene transcription is not the predominant mechanism through which IL-4 exerts its impact on the expression of this enzyme.

Because the increase in gene promoter activity elicited by IL-4 was modest and transient, we investigated whether this cytokine also might enhance 15-LOX-1 mRNA stability. The addition of IL-4 enhanced survival of this transcript for at least 8 h, the duration of the study (Fig. 7A). In contrast, 15-LOX-1 transcript levels had declined in cultures not treated with IL-4 (0.11 ± 0.02 AU versus time 0, IL-4 versus control at 8 h, 12.2 ± 0.04-fold). When interferon γ was added to the medium in combination with IL-4, the impact of the latter on mRNA survival was attenuated (Fig. 7B). This result indicates that interferon γ modulates IL-4-dependent 15-LOX-1 expression at the post-transcriptional level by blocking IL-4-dependent transcript stability.

To further investigate the mechanism for the impact of IL-4 on mRNA stability, we cloned the entire 3′-UTR of 15-LOX-1, spanning nt +2030 to +2653, and fused it to the pGL3 reporter gene. This fragment failed to alter the activity of pGL3 transfected into TAO fibroblasts. Thus, we subsequently cloned a 293-bp fragment of the 15-LOX-1 3′-UTR spanning nt +2361 to +2653 and fused it to the reporter plasmid. When transfected into TAO orbital fibroblasts, the shorter 3′-UTR fragment substantially diminished the activity of the reporter (empty reporter, 9.28 ± 0.28 AU versus 3′-UTR, 4.5 ± 0.6 AU, p < 0.001, n = 3) (Fig. 7C). Treatment of the cultures with IL-4 (10 ng/ml) for 6 h partially restored the reporter gene activity (3′-UTR, 4.5 ± 0.6 versus 3′-UTR plus IL-4, 7.38 ± 0.51, p < 0.005), strongly implicating the distal 3′-UTR in mediating the instability of the mRNA and its enhancement following IL-4 treatment.
The Jak/STAT Pathway Is Activated by IL-4 in Orbital Fibroblasts and Mediates the Induction of 15-LOX-1 by That Cytokine—We next determined what signaling pathways might be involved in the cytokine-dependent up-regulation of 15-LOX-1 in TAO orbital fibroblasts. IL-4 can utilize the Janus kinase/signal transducer and activator of transcription (Jak/STAT) pathways in a number of different cell types. Among the multiple molecular targets of IL-4 and IL-13 is the induction of 15-LOX-1 (34–36). As the Western blot analysis in Fig. 8A demonstrates, treatment of orbital fibroblasts with IL-4 (10 ng/ml) results in the phosphorylation of Jak2 in a time-dependent manner. Abundance of phosphorylated Jak2 is increased at 10 min. The protein remains phosphorylated for at least 2 h and begins to disappear at 6 h. Levels of Jak2 protein are unaffected by IL-4 treatment (Fig. 8A). STAT6, an important down-stream target of Jak2, is also transiently phosphorylated in orbital fibroblasts following the addition of IL-4 (Fig. 8B). It is phosphorylated at 10 and 30 min, appears diminished at 2 h, and is undetectable thereafter. The abundance of phosphorylated Jak2 (Fig. 8A) and STAT6 (Fig. 8B) was equivalent in TAO orbit, normal orbit, and dermal fibroblasts. Tyk2 was somewhat difficult to detect in these fibroblasts (data not shown). Moreover, the levels of cell surface IL-4 receptor display, as assessed by changes in mean fluorescent intensity, were similar in the three cell types (Fig. 8C). The specific Jak2 inhibitor, AG490 (75 μM), blocks the induction of 15-LOX-1 by IL-4, as the Western analysis in Fig. 9A demonstrates. Using a molecular approach to disrupt Jak2-dependent signaling, TAO orbital fibroblasts were transiently transfected with a DN mutant Jak2 kinase or an empty vector control plasmid. The DN mutant kinase could block the induction by IL-4 of 15-LOX-1 (Fig. 9B). Thus, two approaches for interrupting the Jak2 pathway, chemical inhibition and introduction of a DN mutant kinase, yielded congruent results. In aggregate, these findings strongly suggest that the Jak2 pathway is critically involved in the induction of 15-LOX-1 by IL-4.

DISCUSSION

Cytokine regulation of 15-LOX-1 has been examined in several different cell types previously. IL-4 and IL-13 can up-regulate expression in A549 lung epithelial carcinoma cells (37), alveolar macrophages (38), human monocytes (8), the human colorectal carcinoma cell line, Caco-2 (39), and human bronchial epithelial cells (40). Enzyme up-regulation by Th2 cytokines occurs in a tissue- and cell type-specific manner and is currently thought to involve transcriptional events. The 5′-flanking region of the gene contains multiple copies of a sequence that, as an aggregate, functions as a “transcriptional silencer” in nonerythroid cell lines (41). This negative regulation maps to a 900-bp sequence containing nine binding sites for an...
as yet unidentified nuclear repressor factor. 15-LOX-1 gene promoter demethylation may be critical to transcriptional up-regulation by IL-4 (42). A key signaling event necessary for IL-4-dependent 15-LOX-1 expression is the phosphorylation of STAT6 (43). In A549 cells, nuclear histones ordinarily mask the STAT6 binding site on the gene. But IL-4 over several hours promotes the acetylation of both STAT6 and these histones, allowing the phosphorylated transcription factor to bind to the gene (43). In human umbilical vein endothelial cells, IL-4 also up-regulates 15-LOX-1 through the induction of gene transcription (44). A number of transcription factors are activated by the cytokine in these cells, leading to gene activity but no detectable 15-LOX-1 protein (44). IL-4 increases the DNA binding of multiple factors within 30 min. The authors of that study concluded that additional regulatory elements must provoke the translation of 15-LOX-1 protein. In human monocytes, rottlerin, a specific inhibitor of protein kinase C, could abolish the induction of 15-LOX-1 mRNA and protein by IL-13, whereas inhibition of conventional protein kinase C was ineffective (45). This induction by IL-13 in human monocytes is dependent on the tyrosine phosphorylation of Jak2 and Tyk2 (36). Transfecting cells with antisense oligodeoxyribonucleotides against Jak2 could attenuate the cytokine-dependent induction of the enzyme. Thus, it would appear that multiple signaling pathways are utilized by Th2 cytokines in 15-LOX-1 up-regulation and that some of these critical signaling events might vary in different cell types. In the current studies, a reporter construct containing a 1025-bp promoter fragment, including the palindromic 5’-TTCN_2-4-GGA-3’ STAT6 binding motif at nt −952 of the human 15-LOX-1 promoter (34). In another study, induction by IL-13 in human monocytes was dependent on the tyrosine phosphorylation of Jak2 and Tyk2 (36). 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differentiation of erythroid precursor cells involves proteins binding to a differentiation control (cis-acting) element (DICE) comprising cytidine-rich repetitive sequences located in the 3'UTR (48–52). Heterogeneous nuclear ribonucleoprotein K and E1 binding to DICE results in the imposition of translational control (53, 54). Two mRNAs can be detected in various rabbit tissues and IL-4-treated human monocytes (55). These include a 2.6-kb transcript (15-LOX mRNA1), the expression of which is confined to rabbit red blood cells and a 3.6-kb mRNA (15-LOX mRNA2). Both transcripts can be found expressed in human TAO orbital fibroblasts (Fig. 5). The larger mRNA contains a 1019-nt extension in the 3'UTR compared with the more abundant 2.6-kb transcript (55). It apparently arises as a consequence of alternate poly(A) site selection and contains a novel 23-nt GC-rich motif, DICE2, that interacts with as yet unidentified proteins (56). A 93-kDa lung cytosolic protein binding DICE2 can suppress heterogeneous nuclear ribonucleoprotein-imposed translational inhibition (56). In the human transcript, AU-, CU-, and pyrimidine-rich elements and a hairpin-like structure are found in the sequence spanning nt 2004–2665. Translational regulation of 15-LOX-1 mRNA derives from factors that appear independent of poly(A) tail length. Whether translational regulation is important in cells other than reticulocytes remains to be determined.

The role of 15-LOX-1 in human autoimmune disease is uncertain, but the enzyme and its products are suspected of dampening the initial inflammatory events and influencing qualitative aspects of tissue remodeling and tissue repair. Synovial fibroblasts from patients with rheumatoid arthritis have been shown to express 15-LOX-1 mRNA, although no mechanism for its induction by cytokines was identified in that earlier study (16). Here we report that IL-4 increases promoter activity modestly but substantially enhances 15-LOX-1 mRNA stability (Fig. 7A). mRNA destabilizing activity was localized to a 293-bp fragment of the 3'UTR, and IL-4 treatment partially reversed this instability (Fig. 7C). This IL-4 effect has not been reported previously and thus represents a new insight into the mechanism by which the cytokine influences 15-LOX-1 expression. In contrast to cultures from the orbit, dermal fibroblasts fail to exhibit this induction of either 15-LOX-1 mRNA or protein (Fig. 2). Moreover, the control orbital fibroblast strains from "normal" tissue were dramatically less responsive than those from patients with TAO. The influence on 15-LOX-1 mRNA stability exerted by IL-4 may represent a unique attribute of orbital fibroblasts in Graves disease and thus could influence the characteristic pattern of tissue remodeling observed in TAO. We found that the Jak2/STAT6 pathway is activated by IL-4 in orbital fibroblasts. Inhibiting Jak2 activity with a specific chemical inhibitor (Fig. 9A) or attenuating Jak2 protein expression by transiently transfecting a DN mutant kinase lowers 15-LOX-1 induction by IL-4 (Fig. 9B). This pathway thus may represent a target for interrupting Th2-driven processes late in the clinical course of TAO. With regard to its potential influence on immune responses in the orbit, 15-LOX-1 has been associated previously with decreased inflammation in a rat kidney model of glomerulonephritis (57). High levels of 15-LOX-1 are sometimes associated with anti-inflammatory changes in other eicosanoid pathways. For instance, the synthesis of leukotriene B4 in human mononuclear cells can be attenuated by
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15-LOX-1 in Fibroblasts

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