Interleukin-13 Upregulates Vasodilatory 15-Lipoxygenase Eicosanoids in Rabbit Aorta

Xin Tang, Nancy Spitzbarth, Hartmut Kuhn, Pavlos Chaitidis, William B. Campbell

Objective—Vasorelaxation of rabbit aorta is mediated by factors released from the vascular endothelium. In the aortic endothelium, arachidonic acid (AA) is metabolized via the 15-lipoxygenase pathway to the vasodilatory compounds 11,12,15-trihydroxyeicosatrienoic acid (THETA) and 15-hydroxy-11,12-epoxyeicosatrienoic acid (HEETA). Interleukin-13 (IL-13) increases 15-lipoxygenase expression and activity in several types of cells. We tested the hypothesis that IL-13 upregulates the 15-lipoxygenase pathway in rabbit aorta by inducing 15-lipoxygenase expression, thus increasing vascular relaxation mediated by THETA and HEETA.

Methods and Results—Aorta rings and cultured endothelial cells were treated with IL-13, and 15-lipoxygenase expression was analyzed by reverse transcription–polymerase chain reaction and immunoblotting. 15-Lipoxygenase expression was increased by IL-13 in a concentration- and time-dependent manner. Aortic rings were incubated with [14C]AA, and the metabolites were extracted and resolved by high-performance liquid chromatography. IL-13 treatment increased the production of 15-hydroxyeicosatetraenoic acid, HEETA, and THETA. Indomethacin-resistant vasorelaxation to AA was significantly greater in IL-13–treated vessels than in controls. The relaxation responses to sodium nitroprusside were not altered by IL-13 treatment.

Conclusions—These data indicate that in the vascular endothelium, IL-13 induces the expression of 15-lipoxygenase and increases the production of the vasodilatory eicosanoids HEETA and THETA. (Arterioscler Thromb Vasc Biol. 2003; 23:1768-1774.)

Key Words: endothelium • arachidonic acid • trihydroxyeicosatrienoic acid • hydroxyeicosatetraenoic acid • endothelium-derived hyperpolarizing factor

On stimulation with acetylcholine, ATP, or bradykinin, vascular endothelial cells synthesize and release a number of vasoactive compounds that are involved in the regulation of vascular tone.1 Arachidonic acid is metabolized in vascular endothelium via cyclooxygenase, lipoxygenase, and cytochrome P450 pathways to a broad spectrum of eicosanoids and induces endothelium-dependent vessel relaxation in a concentration-dependent manner.2–4 In rabbit aorta, arachidonic acid–induced relaxation is blocked by lipoxygenase inhibitors but not cyclooxygenase inhibitors. 15-Lipoxygenase (15-LO) metabolizes arachidonic acid to 15-hydroperoxyeicosatetraenoic acid, which is reduced to 15-hydroxyeicosatetraenoic acid (15-HETE) or further converted to the vasodilatory compounds 15-hydroxy-11,12-epoxyeicosatrienoic acid (HEETA) and 11,12,15-trihydroxyeicosatrienoic acid (THETA). HEETA and THETA relax precontracted rabbit aorta,5 and thus, modulation of the 15-LO pathway might represent a mechanism for regulation of vascular activity.

Mammalian 15-LO metabolizes free or esterified polyunsaturated acid.6 The reticulocyte-type 15-lipoxygenase (15-LO-I) was first cloned from the rabbit7 and later detected in various cells and tissues of many species (for reviews, see Kuhn et al8 and Kuhn and Thiele9). It synthesizes bioactive compounds involved in red blood cell maturation, inflammation, atherogenesis, cancer, and oxidative stress.10–12 Activity of this enzyme is tightly regulated at transcriptional and posttranslational levels,13 and the Th2 cytokines such as interleukin-4 (IL-4)14 and IL-1315 might act as modulators of the 15-LO pathway. IL-4 and IL-13 are genetically and structurally related proteins. They share a common receptor subunit, IL-4Rα, and have overlapping biologic functions.16,17 IL-4 and IL-13 upregulate 15-LO activity in human monocytes,14 human epithelial cells,18 the lung carcinoma cell line A549,19 and human endothelial cells,20 but the physiologic relevance of this stimulatory effect is still unclear. In this study, we used the rabbit aorta as the model to test the hypothesis that IL-13 increases the production of vasodilatory eicosanoids by stimulating the 15-LO pathway. Our data indicate that expression of 15-LO is upregulated by IL-13 treatment in a time- and concentration-dependent manner and that the synthesis of the major metabolites of 15-LO pathway, 15-HETE, THETA and HEETA, were augmented. Moreover, we found that IL-13–treated vessels relaxed more to arachi-
donic acid stimulation than did control vessels. These results suggest that modulation of the 15-LO pathway in aortic endothelium might play an important role in regulation of vascular tone.

Methods

Aortic Tissue and Endothelial Cell Incubation

Aortas were dissected from 4- to 6-week-old New Zealand White rabbits. Vessels were cut into rings and incubated with vehicle or human recombinant IL-13 (0.05 to 1 nmol/L; R&D Systems Inc) at 37°C in Krebs’ bicarbonate solution (NaCl 119 mmol/L, KCl 4.8 mmol/L, NaHCO3 24 mmol/L, KH2PO4 1.2 mmol/L, MgSO4 1.2 mmol/L, glucose 11 mmol/L, EDTA 0.02 mmol/L, and CaCl2 3.2 mmol/L).21 Endothelial cells were cultured from rabbit thoracic aortas in 75-cm2 plastic flasks at 37°C in an atmosphere of 5% CO2 in air with minimum essential medium containing 10% rabbit serum, 10% fetal bovine serum, 1% L-glutamine, 1% antibiotic/antimycotic, and 1% ampicillin. Vehicle or 0.25 nmol/L IL-13 was added directly to the medium. Cells were harvested after being treated for 12 to 48 hours.

Reverse Transcription–Polymerase Chain Reaction

15-LO-I and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNAs were quantified in IL-13–treated and untreated aortas by reverse transcription–polymerase chain reaction (RT-PCR). Total RNA was isolated with use of a commercially available kit (Quick Prep micro RNA kit). RNA (3 μg) was reverse-transcribed at 37°C for 90 minutes in 45 μL of 80 mmol/L Tris-HCl buffer, pH 8.2, containing 8 mmol/L MgCl2, 50 mmol/L KCl, 1 mmol/L dithiothreitol, 100 μg/mL bovine serum albumin, 30 U RNase inhibitor, 0.166 mmol/L of each dNTP, 150 pmol oligo-dT primer, and 15 U avian myeloblastosis virus reverse transcriptase. To stop the RT reaction, the samples were heated to 95°C for 10 minutes. RT products (2 μL) were used for amplification of 15-LO. The following set of intron-spanning PCR primers was used: 5'-GTC CCC CAC CTG CGA TAC ACC-3' and 5'-TCG TCT ACA GCC TCG TCG GTT-3'. The PCR sample (total volume, 22 μL) contained 66 ng cDNA, 2 U Taq polymerase, 130 μmol/L dNTP, 1 mmol/L MgSO4, and 5 pmol PCR primers. For amplification, the sample was heated first for 2 minutes at 94°C, and then 30 PCR cycles consisting of a denaturation phase (45 seconds at 95°C), an annealing period (30 seconds at 60°C), and a synthesis phase (3 minutes at 68°C) were performed. The PCR was stopped by a 10-minute, isothermic postrun at 70°C, and then the reaction products were analyzed by electrophoresis on a 2% agarose gel. The gels were stained with ethidium bromide and evaluated densitometrically. The PCR products were sequenced to confirm their identity. (Additional Methods are available online on http://www.atvb.ahajournals.org.)

Immunohistochemistry

Rabbit aortic segments (internal diameter, 4 to 5 mm) were fixed in 4% paraformaldehyde in physiologic salt solution containing (all in mmol/L) 119 NaCl, 4.7 KCl, 1.8 KH2PO4, 1.17 MgSO4, 25 NaHCO3, 1.6 CaCl2, 0.026 EDTA, 10 HEPES, and 5.5 glucose for 1 hour; filled with physiologic salt solution containing 2% agar; and embedded in an agarose-containing cartridge on dry ice. On a cryostat, the arteries were cut into 6-μm sections and placed on glass slides etched with 8% nitric acid.22,23 The sections were refixed in 4% paraformaldehyde for 30 minutes and permeabilized by incubation with 0.2% Triton X-100 for 15 minutes. Sections were incubated with either anti-actin (Sigma), anti-platelet and endothelial cell adhesion molecule (PECAM; kindly provided by Dr Peter Newman, Milwaukee, Wis), or anti-rabbit 15-LO antibody (kindly provided by Dr Thomas Poole, University of Colorado) overnight at 4°C. The slides were then rinsed and incubated with the appropriately labeled secondary antibody (1:200 diluted) for 1 hour at 25°C. The slides were then rinsed and incubated with 1% 4,6-diamidino-2-phenylindole (Sigma) for 5 minutes. After a final rinse, the slides were edge mounted with mounting medium (Immuno Fluor, ICN) and protected by a glass coverslip. Nomarski and fluorescence images were captured (400×, Nikon Eclipse E600 microscope; Spot Advanced software).

Metabolism of [14C]Arachidonic Acid

Control and IL-13–treated aortic rings were placed into 5 mL HEPES buffer (in mmol/L: 10 HEPES, 150 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, and 6 glucose; pH 7.4). Vessels were incubated at 37°C with 10−3 mol/L indomethacin for 10 minutes, and then [U-14C]arachidonic acid (0.5 μCi) was added to a final concentration of 10−7 mol/L. After 5 minutes, calcium ionophore A23187 (10−6 mol/L) was added. After another 10 minutes, all reactions were stopped by adding ethanol (15% final concentration), and the mixtures were stored at −40°C until analyzed. The buffer was acidified to pH 3.5 with glacial acetic acid and extracted on octadecylsilane (Bond Elute) columns.24 The extracted lipid metabolites were analyzed by reverse-phase, high-pressure liquid chromatography (HPLC) with solvent system I and a C-18 (5 μm, 4.6x250 mm; Nucleosil) column. A 40-minute linear gradient from 50% solvent B (acetonitrile with 0.1% glacial acetic acid) in solvent A (deionized water) to 100% solvent B was used. The flow rate was 1 mL/min. The column effluent was collected in 0.2-mL fractions, and the radioactivity was determined. The fractions corresponding to the THETAs (fractions 27 to 35; 5 to 7.5 minutes) were collected, acidified, extracted with cyclohexane/ethyl acetate (50:50, vol/vol), and rerechromatographed. In solvent system II, solvent A was water containing 0.1% glacial acetic acid, and solvent B was acetonitrile. The program used a 5-minute isocratic phase with 35% B in A, followed by a 35-minute linear gradient to 85% B with a flow rate of 1 mL/min. The column eluate was collected in 0.2-mL aliquots, and radioactivity was determined as described earlier. The fractions containing the THETAs (fractions 87 to 93, 17.5 to 18.5 minutes) were collected, acidified, extracted with cyclohexane/ethyl acetate (50:50, vol/vol), dried under a stream of N2, derivatized, and analyzed by gas chromatography/mass spectrometry (GC/MS).5 (Additional Methods are available online at http://www.atvb.ahajournals.org.)

Immunoblotting of 15-LO

Control and IL-13–treated rabbit aortas were homogenized in tissue lysis buffer consisting of 10 mmol/L HEPES, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L sodium bisulfite, and protease inhibitors (Roche Molecular Biochemicals). Endothelial cells were incubated on ice in the flasks for 10 minutes in lysis buffer (50 mmol/L HEPES, 150 mmol/L NaCl, 1.5 mmol/L MgCl2, 1 mmol/L EGTA, 10% glycerol, 1% Triton X-100, and protease inhibitors). Protein (30 μg) was loaded in each lane and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 10% resolving gel and a 4% stacking gel. Protein was then transferred to nitrocellulose membranes. Nonspecific binding was blocked with TBS buffer containing 20 mmol/L Tris base, 150 mmol/L NaCl, 0.1% Na3VO4, and 3% bovine serum albumin overnight at 4°C. Guinea pig antibody against the rabbit 15-LO was used.23 Membranes were exposed to primary antibody (dilution, 1:2000) in blocking buffer for 1 hour at room temperature and rinsed with TBS buffer containing 0.1% Tween-20. Membranes were then incubated with 1:5000 horseradish peroxidase–conjugated goat anti-guinea pig IgG for 1 hour at room temperature and washed with TBS buffer. Immunoreactive bands were identified with a chemiluminescence detection kit (Renaissance) and film (Kodak BioMax ML).

Vascular Activity

Aortic rings from the IL-13–treated and control groups were suspended in a 6-mL tissue bath with Krebs’ bicarbonate buffer at 37°C and bubbled with 95% O2 and 5% CO2. Isometric tension was measured with force-displacement transducers, and the vessels were gradually adjusted to a basal tension of 1.75 g. They were pretreated with 10−3 mol/L indomethacin for 10 minutes and then contracted with 10−7 to 10−6 mol/L phenylephrine to 50% of the maximal KCl contraction. When contraction was stabilized, cumulative aliquots of arachidonic acid (10−6 to 3×10−4 mol/L), acetylcholine (10−6 to 10−2 mol/L), or sodium nitroprusside (10−8 to 10−2 mol/L) were added to the bath, and
changes in isomeric tension were measured. Vasorelaxation was expressed as a percentage of maximum precontraction. Statistical comparison of the data obtained from treated and control groups was performed with a 1-way ANOVA, with $P<0.05$ considered statistically significant.

Results

Upregulation of 15-LO Expression by IL-13

15-LO expression in rabbit aortic tissue was determined by Western immunoblotting. In untreated aortas, 15-LO is expressed at low levels. 15-LO was increased in a concentration-dependent manner (0.05 to 0.75 nmol/L) by IL-13 (Figure 1A). When aortic rings were incubated for different time intervals with vehicle (negative control), the low level of 15-LO expression was reduced after 6, 12, and 24 hours of incubation. In contrast, in the presence of IL-13 (0.25 nmol/L), 15-LO was augmented after 12 hours but decreased after 24 hours (Figure 1B). A PCR band migrating at the expected size (305 bp) was observed when the RNA of untreated aortas was amplified with 15-LO–specific primers (Figure 1C). After treatment with IL-13, this band was 1 order of magnitude more intense. Because intron-spanning primers were selected, amplification of genomic DNA could be excluded. When PCR for 15-LO was performed for 28, 30, and 32 cycles, we always observed a more intense signal with IL-13–treated aorta. Sequencing of the PCR fragments indicated their identity. To find out whether 15-LO was induced in the endothelium of the arterial wall, we studied the induction of 15-LO expression by IL-13 in cultured rabbit aortic endothelial cells in flasks at 80% confluence. Immunoblotting showed that 15-LO expression was higher in cells treated with IL-13 for 36 and 48 hours compared with control cells. Maximal induction occurred after 36 hours of incubation (See online Figure I available at http://atvb.ahajournals.org).

Immunohistochemical Location of Aortic 15-LO

Immunofluorescence was performed on rabbit aortas treated with vehicle or IL-13 for 12 hours. Vascular endothelium and smooth muscle were well preserved after incubation, as indicated by PECAM-1 and α-actin immunostaining, respectively (data not shown). No attached or resident blood or inflammatory cells were detected on the vascular wall. 15-LO immunofluorescence was strongly associated with vascular endothelial cells (Figure 2). After IL-13 treatment, 15-LO staining increased markedly in endothelial cells as well as in some smooth muscle cells located close to the endothelium. Thus, it is possible that 15-LO expression in both cell types is upregulated by IL-13. No staining was observed when the primary anti–15-LO antibody was omitted.

Effects of IL-13 Treatment on Arachidonic Acid Metabolism by Rabbit Aortas

Rabbit aortas converted arachidonic acid to metabolites that comigrated with the THETAs, HEETAs, and 15-HETE (Figure 3). Quantification of the metabolites indicated increased formation after 12 and 24 hours of incubation with IL-13. Compared with controls, the production of 15-HETE, THETA, and HEETA was increased by 96±17.5%, 40±10.9%, and 47±9.0%, respectively, after 12 hours (Figure 3A and 3B) and by 118±19.2%, 90±25.0%, and 68±13.1%, respectively, after 24 hours (Figure 3C and 3D) of IL-13 treatment ($n=5$). 12-HETE increased by 69±23.1% after a 12-hour incubation and by 81±49.6% after a 24-hour incubation, whereas production of prostaglandins was not significantly altered. Rabbit aortas were denuded of their endothelium, treated with IL-13 for 12 hours, and incubated with [14C]arachidonic acid (online Figure IIA and IIB). There...
was very little metabolism of [14C]arachidonic acid by the endothelium-denuded vessel. No THETA or HEETA and very little 15-HETE synthesis was detected. There was no increase with IL-13 treatment. Thus, IL-13 primarily increases 15-LO activity and production of 15-LO–derived metabolites in vascular endothelial cells.

The column fractions (Figure 3D) containing the THETAs (fractions 27 to 35) from IL-13–treated vessels were collected and further purified by reverse-phase HPLC with solvent system II. A single, major, radioactive peak was observed that eluted in fractions 87 to 93 (Figure 4A). Analysis of the THETA fraction by positive-ion chemical- ionization GC/MS indicated the presence of 2 products that eluted at 13.73 and 13.85 minutes. Both metabolites had similar mass spectra with major ions (mass-to-charge ratio [m/z]) of 585 [M+1], 569 [M-15, loss of CH3], 405 [M-179, loss of (CH3)3 SiOH and (CH3)2 SiO], 283 (M-301, ((CH3)3 SiO)-CH-(CH2-CH=CH2)-CH2-COOCH3), and 173 [M-411, ((CH3)3 SiO)-(CH2)2 CH3]. The 2 metabolites differed in the intensity of the 173 and 283 m/z ions, indicating the favored cleavage between the 14,15 and 11,12 vicinal diols, respectively.5 The derivatized metabolite that eluted at 13.73 minutes had a mass spectrum consistent with 11,12,15-THETA (Figure 4B). The metabolite that eluted at 13.85 minutes had a mass spectrum consistent with 11,14,15-THETA (Figure 4C). This pattern of regioisomers was identical to that of control vessels. These findings indicate that arachidonic acid is metabolized to a mixture of 11,12,15- and 11,14,15-THETA in both control and IL-13–treated vessels.
Arachidonic Acid–Induced Vasorelaxation Is Upregulated by IL-13 Treatment

Because HEETA and THETA might be involved in the regulation of vascular tone, enhanced production of these compounds might result in an increase in vascular relaxation. Arachidonic acid caused a concentration-related relaxation in both IL-13–treated and control vessels precontracted by phenylephrine (Figure 5). IL-13 treatment increased vasorelaxation to 30, 100, and 300 µmol/L of arachidonic acid by 19%, 46%, and 43%, respectively. Acetylcholine- and sodium nitroprusside–induced vasorelaxations were not significantly different between IL-13–treated and control rings (online Figure III). These data indicate that arachidonic acid–induced vasorelaxations that are mediated by 15-LO–derived eicosanoids are enhanced by IL-13.

Discussion

In rabbit aorta, arachidonic acid induces endothelium-dependent relaxation mediated by factors other than nitric oxide and prostaglandins. Several vasodilatory lipoxygenase metabolites of arachidonic acid, THETA and HEETA, have been identified in the past. These compounds are formed via the 15-LO pathway and are involved in the regulation of vascular tone. Our results indicate that inflammatory cytokines such as IL-13 increase 15-LO expression in vascular tissue, increase the production of THETA and HEETA, and increase vessel relaxation. 15-LO expression is also upregulated by IL-4, IL-13, or both in a variety of nonvascular cells, such as human peripheral monocytes, macrophages, human bronchial epithelial cells, and human epithelial lung carcinoma cells (A549). In A549 cells, inverse regulation of pro(15-LO) and antioxidative enzymes (phospholipids, hydroperoxide, and glutathione peroxidases) leads to upregulation of intracellular lipid peroxidation. In our studies, the maximal increase in 15-LO protein and enzymatic activity was detected after 12 hours of treatment of aortic tissue but after 36 hours in cultured endothelial cells. This different time profile might be due to the different experimental environments of freshly isolated tissue and cultured cells.

We tried many different approaches to establish incubation conditions that preserved vascular integrity and normal function after IL-13 incubation at 37°C. We selected serum-free Krebs’ buffer as the incubation buffer. Endothelium-dependent vasorelaxation to arachidonic acid and acetylcholine was well preserved after 12 hours of incubation under these conditions. In all vessels treated with IL-13, we observed an increased vasorelaxation by arachidonic acid compared with controls. It might be possible that vasodilatation is further upregulated after longer incubation periods, but it was not possible to keep isolated aortic tissue functional for >12 hours. In fact, both phenylephrine-induced contraction and arachidonic acid–induced vasorelaxation were almost completely abolished after 24 hours of incubation at 37°C in Krebs’ buffer.

Many studies have shown IL-13 upregulates 15-LO expression through activation of Jak2 and Tyk2 kinase and...
Stat transcription factors. The phosphorylation of Stat 6 is important for 15-LO induction by IL-13 in human airway epithelial cells, monocytes, and macrophages.\textsuperscript{29–31} Xu et al.\textsuperscript{32} showed that Stat 1, 3, and 5 are also involved in this process. Although a variety of studies have been published on the effects of IL-4 and IL-13 in nonvascular cells, there is only limited information for vascular tissue. In cultured human endothelial cells, treatment with IL-4 causes a time-dependent induction of 15-LO mRNA expression.\textsuperscript{20} Surprisingly, no 15-LO protein was expressed, and arachidonic acid metabolism of IL-4–treated cells was unaltered. It was concluded that transcription of the 15-LO gene was switched on by IL-4, but its translation was prevented by inhibitory proteins.\textsuperscript{33,34} These inhibitory proteins were detected in the cytosol of IL-4–treated cells. In this study, we show for the first time an upregulation of 15-LO protein expression by IL-13 in the vascular wall of any mammalian species. This increase was associated predominately with endothelial cells and to a lesser extent with smooth muscle cells close to the endothelium. Removal of the endothelium inhibited HEETA, THETA, and 15-HETE synthesis.

15-LO exhibits a proatherogenic effect because of its ability to oxidize nonatherogenic LDL to an atherogenic form.\textsuperscript{12} There is convincing evidence for this hypothesis in mice.\textsuperscript{35–37} On the other hand, a study in transgenic hypercholesterolemic rabbits, which overexpressed 15-LO in monocyte/macrophages, suggested an antiatherogenic activity for this lipid-peroxidizing enzyme.\textsuperscript{38} Because vasorelaxation is an antiatherogenic effect, the results presented here might in part contribute to the antiatherogenic activity of the enzyme in this particular rabbit atherosclerosis model.

In summary, our data suggest that IL-13 treatment induces 15-LO expression in rabbit aortas and cultured aortic endothelial cells, which enhances production of arachidonic acid metabolites from the 15-LO pathway. This treatment increased vasorelaxation stimulated by arachidonic acid. These data further support the important role of HEETA and/or THETA in regulation of vascular tone.

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