Amino Acid Differences in the Deduced 5-Lipoxygenase Sequence of CAST Atherosclerosis-Resistance Mice Confer Impaired Activity When Introduced Into the Human Ortholog

Hartmut Kuhn, Monika Anton, Christa Gerth, Andreas Habenicht

Objectives—The mouse strain CON6, which was generated by breeding athero-resistant CAST mice into an athero-susceptible B6 background, exhibits almost complete resistance to atherosclerosis. An athero-resistance gene cluster has been localized at the central region of chromosome 6, and among the candidate genes of this locus, the 5-lipoxygenase has attracted particular attention because of its involvement in the biosynthesis of proinflammatory leukotrienes. Comparison of 5-lipoxygenase genomic sequences of B6 and CON6 mice indicated 2 conserved amino acid exchanges in the CON6 animals, but the functional impact of these mutations has not been defined.

Methods and Results—We analyzed the functionality of these amino acid exchanges relative to essential catalytic properties (specific activity, substrate affinity, and reaction specificity) and found that these mutations confer an impaired lipoxygenase and leukotriene A4-synthase activity when introduced into the human enzyme. In contrast, substrate affinity, enantiomer selectivity, and positional specificity remained unchanged.

Conclusions—These data are consistent with the possibility that naturally occurring conservative mutations in the coding region of the murine 5-lipoxygenase gene can significantly affect enzyme activity and that this loss of function may be involved in CAST/CON6 athero-resistance. (Arterioscler Thromb Vasc Biol. 2003;23:1072-1076.)

Key Words: eicosanoids ■ leukotrienes ■ inflammation ■ mutation ■ atherogenesis

The 5-lipoxygenase (5-LOX) is the key enzyme in the biosynthesis of leukotrienes (LTs), which constitute powerful proinflammatory mediators.1,2 Expression of the enzyme has been reported in different types of leukocytes,2 including in foamy macrophages of atherosclerotic lesions3 and in the aorta of apolipoprotein E–deficient and LDL receptor–deficient mice.4 Moreover, the number of transcripts of both 5-LOX and LT-receptors seems to expand during lesion progression,3 suggesting a causal relation between 5-LOX expression and atherogenesis. The genes for human and murine 5-LOX were mapped to syntenic regions of chromosomes 10 and 6, respectively.5 To identify genes with potential impact on atherogenesis, athero-resistant CAST mice were crossed with athero-susceptible B6 animals. This crossbreeding yielded the congenic mouse strain CON6, which exhibits a similarly pronounced athero-resistance as the parent CAST strain.6 Importantly, CON6 mice retained the athero-resistance gene locus of the CAST animals,6 and genotyping of the 2 strains identified the 5-LOX gene immediately underneath the linkage peak.4 Moreover, bone marrow transplantation suggested the importance of leukocytes in the athero-resistance of CON6 mice.4 Thus, the leukocyte 5-LOX emerged as an attractive candidate gene that could contribute to athero-resistance of CAST and CON6 mice. To address this hypothesis, 5-LOX expression was compared between athero-resistant (CON6) and athero-susceptible (B6) mouse strains, and it was found that CON6 mice exhibited 5-fold lower expression levels of both 5-LOX mRNA and protein than the corresponding B6 control animals.4 Moreover, comparison of the genomic 5-LOX sequences indicated 2 nucleotide exchanges in the 5-LOX coding region, which resulted at the protein level in 2 adjacent point mutations: isoleucine 645 of B6 mice was exchanged to valine (I645V) and valine 6461 was mutated to isoleucine (V646I). 4 These mutations are rather conservative in nature, and, thus, no major functional consequences were initially expected. However, amino acid alignments of all mammalian 5-LOX species indicated an absolute conservation of these residues, suggesting their functional importance.

To explore whether these amino acid exchanges may be associated with alterations of 5-LOX functionality, we overexpressed the human enzyme as recombinant nonfusion protein in Escherichia coli, mutated the corresponding amino acids, and characterized the resulting LOX species with respect to major enzymatic properties, such as specific activity (LOX and LTA4-synthase activity), positional speci-
ficity, and substrate affinity. We found that each point mutation (I645V and V646I) as well as the double mutant (I645V+V646I) exhibited reduced catalytic activities but retained their positional specificity and substrate affinity. These data suggest that athero-resistant CON6 mice express a catalytically less active 5-LOX, and they also raise the important possibility that similar loss of function mutations may occur in other species. Thus, our findings may encourage experimental approaches to search for functional 5-LOX open reading frame mutations in human populations with a modified risk of cardiovascular disorders.

Methods

Chemicals
The chemicals used were from the following sources: (5Z,8Z,11Z,14Z)-eicosa-5,8,11,14-tetraenoic acid (arachidonic acid), (5S,6E,8Z,11Z,14Z)-5-hydro(pero)xyeicosa-6,8,11,14-tetraenoic acid [5S-H(p)ETE], CaCl₂, EDTA, ATP, and sodium borohydride from Serva; ampicillin from Gibco; dipalmitoyl phosphatidylcholine, isopropyl-β-D-thiogalactopyranoside (IPTG), and ATP-sepharose from Sigma-Aldrich; and HPLC standards of hydroxy fatty acids and LT A₄ methyl ester from Cayman Chemical (distributed by Alexis GmbH). HPLC solvents were obtained from Merck. Restriction enzymes were purchased from New England Biolabs. Phage T₄ ligase, PWO-polymerase, and sequencing kits were bought at Boehringer Mannheim, and the BioIabs. Phage T4 ligase, PWO-polymerase, and sequencing kits were purchased from Invitrogen. Oligonucleotide synthesis was carried out by Tib-Molbiol.

Bacterial Expression, Site-Directed Mutagenesis, and Enzyme Purification
The human 5-LOX was overexpressed as nonfusion protein in E. coli, and for this purpose its cDNA was subcloned into the bacterial expression plasmid PKK 233-2. First, we introduced an NcoI restriction site at the starting ATG and a HindIII site just behind the expression plasmid PKK 233-2. Then bacteria were transformed with the recombinant plasmid. Then bacteria were cultured at 37°C, bacteria were spun down, washed with PBS, and kept on ice for 10 minutes. Then the cells were lysed by sonication with a Labsonic U tip-sonifier, cell debris was removed by centrifugation, and the lysis supernatant was kept on ice for 10 minutes. Then the cells were lysed by sonication with a Labsonic U tip-sonifier, cell debris was spun down, and aliquots of the clear supernatant were injected to HPLC for quantification of the LOX products. HPLC was carried out on a Shimadzu system connected to a Hewlett Packard diode array detector 1040. Reverse-phase HPLC was performed on a Nucleosil C-18 column (Macherey-Nagel, KS-system, 250×4 mm, 5-μm particle size) coupled with an appropriate guard column (30×4 mm, 5-μm particle size). A solvent system of methanol/water/acetic acid (80/20/0.1, by volume) was used at a flow rate of 1 mL/min. The chromatographic scale was calibrated (5-point calibration) by injecting known amounts of 15S-HETE (conjugated dienes) and LTB₄ (conjugated trienes). 5(R/S)-HETE enantiomers were separated on a Chiralcel OB column (250×4 mm, 5-μm particle size), and the hydroxy fatty acid methyl esters were eluted with the solvents system n-hexane/2-propanol/acetic acid (100/40/1, by volume) at a flow rate of 1 mL/min.

Immunoblotting
For immunoblotting, the bacteria were lysed as described for the activity assay. Aliquots of the lysis supernatants containing similar amounts of total protein were applied to SDS gel electrophoresis. Proteins were transferred to a nitrocellulose membrane by a semidy procedure, and the blots were probed with a polyclonal rabbit antibody raised against the recombinant human 5-LOX. For generation of the antiserum, E. coli-expressed 5-LOX protein was purified by SDS polyacrylamide gel electrophoresis. The eluted antigen was then directly injected into lymph nodes of a rabbit with one boost. The immunoreactive bands (see Figure 1) were quantified densitometrically, and a linear calibration curve (0.08 to 0.3 μg) was established using an electrophoretically homogenous human 5-LOX preparation.

Miscellaneous Methods
Protein concentration was determined with the Roti-Quant detection system (Roth) that is based on the Bradford method. For chiral phase HPLC, the carboxylate group of the free hydroxy fatty acids was methylated with diazomethane in diethylether, and the resulting
methyl esters were repurified by RP-HPLC. $K_M$ values were determined by varying substrate concentrations in the range of 10 to 80 μmol/L, evaluating the data with Lineweaver-Burk plots. A reference mixture of the LTA$_4$-hydrolysis products was prepared by incubating 5 μg of LTA$_4$ methyl ester for 2 hours at pH 2 (epoxide ring opening). Then KOH was added to a final concentration of 0.5 mol/L, and the methyl esters of the resulting diol isomers were hydrolyzed to the free acids. After acidification to pH 3, the hydrolysis mixture was directly injected for RP-HPLC and the hydrolysis products were prepared.

**Results**

When we expressed the wild-type (wt) human 5-LOX and the 3 mutants I645V, V646I, and I645V+V646I in E. coli, we observed by immunoblotting variable expression levels of the recombinant enzyme species (9 independent small-scale preparations). When all expression experiments are taken together, the wt enzyme showed always the highest expression level (approximately 100%) whereas expression of the mutants varied between 55% and 90% in different fermentation samples. An immunoblot of a representative experiment is shown in Figure 1. Because of the expression variability, the results of the activity assays had to be normalized to wt 5-LOX protein, as determined by densitometry of 5-LOX immunoblotting. To obtain reliable activity data, 3 clones were randomly selected for wt 5-LOX and for each mutant, and activity assays were carried out. In Figure 2, representative chromatograms obtained for wt human 5-LOX and for the I645V+V646I double mutant are compared. It can be seen that wt LOX produced significantly higher amounts of arachidonic acid oxygenation products, i.e., 5-HETE and the LTA$_4$ hydrolysis products, than the double mutant. In the Table, the normalized 5-LOX activities of lysis supernatants are summarized. These data indicated that the specific activities of the 5-LOX mutants are strongly reduced compared with wt 5-LOX.

These results were clearly surprising, because the mutations carried out were rather conservative in nature. To exclude methodological artifacts, an additional set of experiments determining the normalized arachidonic acid oxygenase was performed. For this purpose, bacteria were retransformed with sequenced recombinant plasmids, and well-separated clones of wt enzyme and of each mutant were randomly selected. In this series of experiments (4 independent measurements using single clones of the wt 5-LOX and of each mutant; mean of the wt activity was set 100%, ± represents SD), the following results were obtained: Wt, 100±12.9%; I645V, 12.9±9.2%; V646I, 5.6±3.5%; and I645V+V646I, 14.7±9.8%. The differences between wt 5-LOX and the mutant enzyme species were significant at $P<5\times10^{-6}$. By contrast, normalized activities were not significantly different when the various mutants were compared with each other. These data revealed that each single amino acid exchange as well as the double mutation exhibited an impaired 5-LOX activity compared with the wt enzyme.

To explore the possibility of whether the impaired activities of the mutant enzymes are the result of a reduced substrate affinity, basic kinetic parameters were determined. For the wt enzyme, a $K_M$ of 55.6 μmol/L and a $V_{max}$ of 1.90 μmol oxygenation products/μg 5-LOX protein was calculated (Table). This $K_M$ is somewhat higher than the corresponding

### Enzymatic Properties of 5-LOX Mutants

<table>
<thead>
<tr>
<th>Enzyme Species</th>
<th>Absolute 5-LOX Activity, μg product/μg 5-LOX per min</th>
<th>Relative 5-LOX Activity, %</th>
<th>$K_M$, μmol/L</th>
<th>Relative 5-LOX Activity, %</th>
<th>$V_{max}$, μg product/μg 5-LOX per min</th>
<th>Enantioselctivity, S/R ratio</th>
<th>Purified Enzyme</th>
<th>Relative 5-LOX Activity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>1.47±0.05</td>
<td>100</td>
<td>55.6±18.1</td>
<td>1.90±0.33</td>
<td>88.12</td>
<td>50</td>
<td>100</td>
<td>10.0±1.1*</td>
</tr>
<tr>
<td>I645V</td>
<td>0.25±0.01</td>
<td>17.0</td>
<td>47.9±21.4</td>
<td>0.36±0.08</td>
<td>82.18</td>
<td>11.0±1.1*</td>
<td>18.9±3.0*</td>
<td></td>
</tr>
<tr>
<td>V646I</td>
<td>0.15±0.01</td>
<td>10.4</td>
<td>24.8±5.3</td>
<td>0.14±0.01</td>
<td>75.25</td>
<td>11.8±1.1*</td>
<td>18.9±3.0*</td>
<td></td>
</tr>
<tr>
<td>I645V+V646I</td>
<td>0.18±0.01</td>
<td>11.2</td>
<td>49.9±7.3</td>
<td>0.22±0.02</td>
<td>88.12</td>
<td>11.8±1.1*</td>
<td>18.9±3.0*</td>
<td></td>
</tr>
</tbody>
</table>

Five-milliliter cultures were grown for each LOX species, and activity assays with the bacterial lysis supernatants were carried out as described in Methods (columns 2 and 3: $n=5$, ± indicates SD). $K_M$ and $V_{max}$ values (columns 4 and 5) were determined by measuring the reaction rates in the substrate concentration range between 10 and 80 μmol/L, and the data were evaluated with Lineweaver-Burk plots (0.96<-$R^2$<0.98; ± indicates SD). 5-HETE enantiomers were resolved as methyl esters by chiral phase HPLC (column 6). For enzyme purification, two independent experiments were performed (column 7); ±± indicates the error range; ±one purification experiment only.
value determined for the purified native enzyme, and this may be attributable to the presence of fatty acid–binding proteins in the E. coli lysate supernatant. $K_M$ values for the mutant enzyme species were found to be in the same range. Moreover, the $V_{max}$ values for the 5-LOX mutants were strongly reduced (Table), indicating their impaired catalytic activities even under $V_{max}$ conditions.

In many cases, LOX mutants with low specific activity show a reduced reaction specificity, eg, arachidonic acid is oxygenated to a complex mixture of stereo-random oxygenation products. To examine whether the 5-LOX mutants prepared in this study retained stereochemical control, the enantiomer composition of the major oxygenation product (5-HETE) was analyzed. We found that the S/R ratio determined by chiral phase HPLC was similar for the wt enzyme and the mutants (Table). These data indicated that the high degree of stereo-specificity of the wt enzyme was retained in the mutant 5-LOX species, supporting the conclusion that these mutations did not alter the substrate alignment at the active site.

To exclude that non-LOX proteins in the bacterial lysate may have impacted the activity assay, bacterially expressed enzymes were purified by affinity chromatography on ATP-sepharose. Comparable amounts of the purified enzyme preparations were used for activity assays, and the activity data were normalized for an equal LOX content. Here again, we observed that the 5-LOX mutants exhibited a strongly impaired enzymatic activity (Table).

The 5-LOX is a multifunctional enzyme that exhibits an arachidonic acid 5-oxygenase activity but also is capable of converting 5(S)-HpETE, its primary oxygenation product, to LT A4. To find out whether mutations of the I645 and V646 may impact the LT synthase activity, the enzyme was incubated in our standard assay system, leaving out arachidonic acid but using 50 $\mu$mol/L 5(S)-HpETE as substrate instead. It can be seen from Figure 3 that large amounts of LTA4 hydrolysis products were detected when the wild-type enzyme was used. In contrast, with the mutant enzyme species, much smaller amounts were analyzed. These data suggest that I645V and V646I exchange does not only impair the oxygenase but also LTA4 synthase activity of the enzyme.

**Discussion**

Because LOXs are lipid-peroxidizing enzymes, they have been implicated in the pathogenesis of atherosclerosis in the frame of the LDL oxidation hypothesis. Among the genes located in this region, the 5-LOX was shown to have a high expression level of 5-LOX mRNA and protein, respectively. CON6 bone marrow cells indicated a 3- to 5-fold lower expression might have major antiatherogenic effects in vivo. CON6 mice that had been generated by crossing the atheroresistant CAST mice onto a B6 background carry an antiatherogenic gene cluster in the central region of chromosome 6 that confers almost complete athero-resistance. Among the genes located in this region, the 5-LOX was particularly attractive because it is involved in the biosynthesis of proinflammatory mediators. Reverse-transcriptase–polymerase reaction, Northern blots, and immunoblotting of CON6 bone marrow cells indicated a 3- to 5-fold lower expression level of 5-LOX mRNA and protein, respectively. Interestingly, bone marrow cells prepared from CON6 mice only produced less than 5% LT$_B$ compared with the corresponding B6 cells, suggesting the importance of posttranslational mechanisms. Comparison of the coding sequences of the 5-LOX of CON6 and B6 mice revealed 2 amino acid exchanges (I645V and V646I). These mutations are rather conservative, and thus, no functional consequences were immediately apparent. Moreover, modeling of the 5-LOX structure based on the X-ray coordinates of rabbit 15-LOX suggested that I645V and V646I are located in a surface helix with no obvious connection to the active site, casting addi-
tional doubt on the functionality of these mutations. On the other hand, I645 and V646 and the entire primary structure region surrounding these residues are absolutely conserved among all mammalian 5-LOXs. Such a high degree of amino acid conservation suggests a functional importance of this structural element. Here we report that even conservative amino acid exchanges, such as I645V and V646I, may lead to mutant enzyme species that exhibited strongly impaired catalytic activities, whereas other important enzyme properties (substrate affinity and positional specificity) remained unaltered. Considering the low expression levels of the 5-LOX in CON6 mice and the impaired specific activities of the mutant enzyme expressed in these animals (data reported here), a strongly reduced 5-LOX pathway must be postulated for the athero-resistant CON6 mice. This conclusion is consistent with low-level formation of LTB4 by CON6 bone marrow cells.4

It has been reported before that a subpopulation of human asthma patients carrying mutations in the central promoter region of the 5-LOX gene are less susceptible for treatment with drugs impacting LT metabolism.22 Thus, this regulatory polymorphism seems to impact the pathogenesis of an allergic disease. It would be of particular interest to find out whether a structural polymorphism (open reading frame mutations) also exists for the 5-LOX gene and whether such polymorphisms may correlate with the risk for cardiovascular disorders. Identification of such functional mutants and the corresponding correlation studies may help to examine whether 5-LOX is related to the risk of developing inflammatory cardiovascular disorders in humans.

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