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
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 α, 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 restriction site-directed mutagenesis kit (Stratagene). Bacteria were transformed with recombinant wild-type and mutant plasmids and plated for activity assay, Western blotting, or additional purification.

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, the human 5-LOX was overexpressed as nonfusion protein in E. coli. Five-milliliter liquid cultures of E. coli expressing the different 5-LOX mutants were grown, and lysis supernatants were prepared as described in the Methods section. Aliquots of the supernatants representing equal amounts of total lysis proteins were applied to SDS-PAGE, and the Western blot was developed using a polyclonal anti-human 5-LOX antibody. As reference, the purified human 5-LOX was used.

Activity Assays
The enzymatic activity was assayed by RP-HPLC quantification of the arachidonic acid oxygenation products (sum of 5-HpETE and LTC₄-hydrolysis products). For this purpose, the bacterial lysate supernatants or the purified enzyme preparations were incubated for 10 minutes in 0.5 mL Tris-HCl buffer, pH 7.4, containing 0.1 mmol/L arachidonic acid, 0.4 mmol/L CaCl₂, 40 μg/mL dipalmitoyl phosphatidylcholine, and 0.1 mmol/L ATP. The hydroperoxyl compounds were formed, were added with sodium borohydride. The mixture was acidified to pH 3, and 0.5 mL of ice-cold methanol was added. The protein precipitate 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. The resulting compounds were eluted with the solvents system n-hexane/2-propanol/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 lysate supernatants containing similar amounts of total protein were applied to SDS gel electrophoresis. Proteins were transferred to a nitrocellulose membrane by a semidyry procedure, and the blots were probed with a polyclonal rabbit antibody raised against the recombinant human 5-LOX. For generation of the antisera, E. coli–expressed 5-LOX protein was purified by SDS polyacrylamide gel electrophoresis. The electroeluted 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 carboxylic 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 \( \mu \text{mol/L} \), evaluating the data with Lineweaver-Burk plots. A reference mixture of the LTA\(_4\) hydrolysis products was prepared by incubating 5 \( \mu \text{g} \) 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 \( \text{mol/L} \), and the methyl esters of the resulting diol isomers were hydrolyzed to the free acids. After acidification to pH 3, the products formed by recombinant 5-LOX species. Five-milliliter cultures of \( \text{E. coli} \) expressing wt 5-LOX and its V646I double mutant were grown, and lysis supernatants were set (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 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, ie, 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%, \( \pm \) represents SD), the following results were obtained: Wt, 100 \( \pm 12.9\% \); I645V, 12.9 \( \pm 9.2\% \); V646I, 5.6 \( \pm 3.5\% \); and I645V+V646I, 14.7 \( \pm 9.8\% \). The differences between wt 5-LOX and the mutant enzyme species were significant at \( P<5\times10^{-7} \). 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 \( \mu \text{mol/L} \) and a \( V_{\text{max}} \) of 1.90 \( \mu \text{g} \text{5-LOX per min per g 5-LOX protein} \) 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, ( \mu \text{g 5-LOX product/\mu g enzyme} )</th>
<th>( K_m ), ( \mu \text{mol/L} )</th>
<th>( V_{\text{max}} ), ( \mu \text{g products/\mu g 5-LOX per min} )</th>
<th>Enantioselectivity, S/R ratio</th>
<th>Relative 5-LOX Activity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>1.47 ( \pm 0.05 )</td>
<td>55.6 ( \pm 18.1 )</td>
<td>1.90 ( \pm 0.33 )</td>
<td>88.12</td>
<td>100</td>
</tr>
<tr>
<td>I645V</td>
<td>0.25 ( \pm 0.01 )</td>
<td>17.0</td>
<td>0.36 ( \pm 0.08 )</td>
<td>82.18</td>
<td>11.0 ( \pm 1.1 )*</td>
</tr>
<tr>
<td>V646I</td>
<td>0.15 ( \pm 0.01 )</td>
<td>10.4</td>
<td>0.14 ( \pm 0.01 )</td>
<td>75.25</td>
<td>18.9 ( \pm 3.0 )*</td>
</tr>
<tr>
<td>I645V+V646I</td>
<td>0.18 ( \pm 0.01 )</td>
<td>11.2</td>
<td>0.22 ( \pm 0.02 )</td>
<td>88.12</td>
<td>11.8†</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; \( n=5 \); \( \pm \) indicates SD). \( K_m \) and \( V_{\text{max}} \) values (columns 4 and 5) were determined by measuring the reaction rates in the substrate concentration range between 10 and 80 \( \mu \text{mol/L} \), and the data were evaluated with Lineweaver-Burk plots (0.96 \( < R^2 < 0.98 \); \( \pm \) 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); \( \pm \) 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 A_4. To find out whether mutations of the I645 and V646 may also impact the LT synthase activity, the enzyme was incubated in our standard assay system, leaving arachidonic acid but using 50 μmol/L 5(S)-HpETE as substrate instead. It can be seen from Figure 3 that large amounts of LTA_4 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 LTA_4 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. During the last decade, research was focused predominantly on the 12/15-LOX family, because these LOXs are capable of oxidizing low-density lipoprotein to an atherogenic form. However, the precise role of these enzymes has not yet been completely understood. In some animal models, 12/15-LOXs seem to exhibit a proatherogenic activity, and in others they act antiatherogenic. Apart from these unresolved questions concerning 12/15-LOXs, 5-LOX metabolites such as LT have been discussed for more than 10 years as possible mediators of proinflammatory activity, and, thus, proinflammatory LTs have been suggested to promote the disease. Several months ago we reported that 5-LOX and LT receptors are abundantly expressed in human atherosclerotic plaques, and similar data were recently obtained in our laboratory for murine lesions. When LDL receptor-deficient mice that lacked just one 5-LOX allele (LDL-R^-/-/5-LOX^-/-) were fed a cholesterol-rich diet for 8 weeks, they developed significantly fewer lesions than their 5-LOX-sufficient counterparts. These data indicated that a modest decrease in 5-LOX 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% LTB_4 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 I645 and V646 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 region of the 5-LOX gene are less susceptible for treatment of the athero-resistant 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

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References
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