Extracellular Superoxide Dismutase Polymorphism in Mice

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Objective—In this study, we describe a previously unrecognized murine extracellular superoxide dismutase (ecSOD) allele and examine its distribution among various strains and its effect on the ecSOD phenotype.

Methods and Results—Polymerase chain reaction analysis of genomic and cDNA from apolipoprotein E/LDLR−/− mice indicates the presence of 2 distinct transcripts for this enzyme independent of the extent of atherosclerosis or age. Sequencing and genotyping analyses reveal the presence of 2 alleles for ecSOD. One is a short variant with a 10-base pair deletion in the 3′UTR, accompanied by a single nucleotide substitution (position 61) found in the 129P3/J strain of mice. By contrast, all other strains examined carry the long form. Both free and heparin-releasable ecSOD activities in the 129P3/J strain are more than 3-fold higher than those in the C57Bl/6 mice. Corresponding differences in plasma enzyme mass are observed by immunoblotting. A clear allele dose effect can be observed in F2 hybrids of these 2 strains; free and total ecSOD activities in mice homozygous for the short allele are twice those of mice homozygous for the long allele, with the heterozygote values in between.

Conclusions—These data clearly demonstrate the allele-specific effects on the ecSOD phenotype independent of other strain-specific factors and underline the need for backcrossing of genetically modified mice. (Arterioscler Thromb Vasc Biol. 2003;23:1820-1825.)

Key Words: extracellular superoxide dismutase ■ apolipoprotein E−/−/LDLR−/− mice ■ atherosclerosis ■ antioxidant response ■ oxidation

Oxidative damage to cellular lipid, protein, or DNA is a prominent feature of the pathologies of atherosclerosis, various neurodegenerative diseases, and tissue inflammation.1–3 A major component of the cellular response to various neurodegenerative diseases, and tissue inflammation is a family of enzymes referred to as superoxide dismutases (SOD). The extracellular form of this enzyme (ecSOD) may be particularly important in the maintenance of the extracellular redox homeostasis and provide protection, along with other enzymes, against the production of oxidized lipids and lipoproteins, major initiators of the atherogenic process in the arterial wall. Extracellular SOD activity in the arterial wall may also be essential for the maintenance of the antiatherogenic and vasodilating effects of nitric oxide. Extracellular SOD is a 135-kDa homotetrameric glycoprotein and in blood vessels is mainly secreted by smooth muscle cells.4 However, the bulk of the ecSOD in atherosclerotic lesions may be derived from macrophage foam cells.5 The enzyme is anchored to heparan sulfate proteoglycans of the extracellular matrix through a positively charged, 6 amino acid carboxy-terminal heparin-binding domain.6 Free, unbound ecSOD is in an equilibrium with the matrix-bound compartments in vivo; the dissociation of accessible ecSOD from the matrix by heparin injection leads to a large increase in plasma SOD activity.8 Because of the involvement of oxidative processes in atherosclerosis and inflammation,1,3 ecSOD is a key enzyme that has the potential to reduce oxidative stress. Although the exact role of ecSOD in atherosclerosis remains to be defined, ecSOD colocalizes with oxidized LDL epitopes in atherosclerotic lesions.9 After adenoviral-mediated transfer, eSOD is atheroprotective in LDLR−/− mice10 and inhibits LDL oxidation by cultured aortic endothelial cells.11 In human studies, circulating free and vascular ecSOD activities are low in patients with coronary artery disease compared with healthy subjects,12,13 and cardiovascular risk factors such as body mass index and smoking correlate inversely with plasma ecSOD activity.14

We previously reported on the atheroprotective effect of the hyperbaric oxygen (HBO) treatment of cholesterol-fed rabbits.15 We became interested in the potential role of ecSOD in the HBO-mediated protection of the arterial wall of apolipoprotein (apo) E−/−/LDLR−/− mice. Specifically, we were interested in the potential role of HBO in the appearance of the truncated form of the ecSOD transcript, reportedly derived from a macrophage foam cell posttranscriptional modification that progressively increases with age and severity of the lesions.5 Instead, we report here that the appearance of the putative, posttranscriptionally derived truncated mRNA of ecSOD in apoE−/−/LDLR−/− mice is in fact the result of a heterozygous population of apoE−/−/LDLR−/− mice, expressing 2 different alleles of ecSOD, one derived from the 129P3/J (129) strain, the source of the embryonic
stem cells, and the other derived from the C57Bl/6 (C57) parental strain.

The 129 sequence is distinguished from the C57 sequence by a 10-base pair (bp) deletion in the 3′ UTR and a point mutation (from A to G at bp 61). The point mutation leads to an asn to asp change at amino acid 21 in the immature protein. These changes correspond exactly to those reported by Fukai et al but are attributable to the presence of 2 alleles for the enzyme and not posttranscriptional modification, as suggested. The existence of these alleles was confirmed by genomic DNA sequencing and genotyping analysis developed in our laboratory.

The 129 strain is homozygous for the short ecSOD, whereas the C57, BALB/c, C3H, and outbred SW mice were shown to bear the long allele. The 2 murine alleles of ecSOD are associated with different plasma and heparin-releasable ecSOD activities. The products of both alleles seem to have similar specific activities. Our studies also demonstrate that caution must be exercised when using genetically modified mice, especially when insufficiently backcrossed with the parental strain.

Methods

Animals

C57, 129, BALB/c, B6.129 apoE−/−/LDLR−/−, and wild-type F2 hybrid B6.129 mice were obtained from Jackson Laboratories (Bar Harbor, Maine). Swiss Webster and C3H mice were purchased from Harlan Sprague-Dawley. Mice were maintained on normal chow and water until the ages indicated. Experimental protocols were approved by the UNT HSC Institutional Animal Care and Use Committee.

Animal Procedures

For determination of free ecSOD in plasma, blood was collected into heparinized syringes by cardiac puncture. Mice were allowed to recover for 2 weeks before additional blood collection. To measure total, heparin-accessible ecSOD, mice were injected with 100 U heparin via the tail vein to liberate matrix-bound ecSOD. Blood was collected by cardiac puncture 10 minutes after the heparin injection. Mice were then killed by cervical dislocation.

Reverse Transcriptase–Polymerase Chain Reaction and Sequencing

DNA and total RNA were isolated from liver or cultured peritoneal macrophages, as indicated, using TRI reagent (Molecular Research Center Inc). Total RNA was used to obtain the corresponding cDNA by using the Retroscript Kit (Ambion, Inc) and oligo-dT primers. One quarter of this reaction mixture was used for the subsequent polymerase chain reaction (PCR) reaction using primers spanning the 10-bp deletion in the ecSOD gene. In initial PCR reactions, the 10-bp deletion in the ecSOD gene. In initial PCR reactions, the primers amplified a product 133- or 123-bp long, depending on the presence or absence of 10-bp deletion. PCR amplicons from liver genomic DNA using primers without linkers were concentrated with a PCR concentration kit (Qiagen). Concentrated PCR reactions were electrophoresed and gel purified using a gel purification kit (Qiagen) and sequenced (ACGT Inc).

DNA Isolation and Genotyping

MacVector (v6.5, Oxford Molecular, Ltd) was used to identify unique restriction enzyme cut sites in the amplified regions of DNA. Genomic DNA was isolated from 100 µL of blood by vortexing with 0.5 mL Tris-EDTA pH 7.5 and centrifuging at 13 000g for 4 minutes at 4°C repeatedly until hem was removed from the pellet, followed by digestion of the pellet with 10 mg/ml proteinase K in 1X Perkin Elmer PCR buffer with 1% Triton and 0.5% Tween 20 at 56°C for 1 hour. Proteinase K was then heat-inactivated by incubation at 95°C for 10 minutes. PCR of genomic or cDNA was carried out to genotype for a single nucleotide substitution at position 61, as follows: forward: 5′-GGGAGACATTCCACAGGTGAC-3′; back- ward: 5′-TGTCTCGTACGTGAACGTGGAC-3′, and the 10-bp deletion in the 3′ UTR, as follows: forward: 5′-TGGGAGACGAGAACAGAAAGGAG-3′; reverse: 5′-CGCCTGGAGACATCTATGG-3′ of the ecSOD gene. After the PCR, 15 µL of the reaction was digested with 1 µL MboI (SNP at position 61) or HaeIII (10-bp deletion) for 3 hours at 37°C. The reaction products were separated on a 12% polyacrylamide gel in TBE buffer and visualized by ethidium bromide staining.

Isolation of ecSOD

Mouse plasma (150 µL) was applied to a 1-cm ConA-Sepharose (Sigma) column equilibrated in a 50-mmol/L HEPES buffer, pH 7.0, containing 0.25 mol/L NaCl, as described by Marklund.16 The enzyme was eluted with the equilibration buffer containing 0.5 mol/L α-methyl mannoside, pH 6.0, and assayed, as described below. Alternatively, mouse plasma was fractionated in PBS containing 1 mmol/L EDTA, pH 7.2, for 1 hour. FPLC using 2 10×300-nm Sepharose 6 columns (Amersham Pharmacia Biotech) set in tandem, were assayed to identify those containing SOD activity. The carbohydrate moiety of ecSOD causes the 135-kDa protein to migrate as 150 kDa.16 The fractions containing SOD activity around a 150-kDa marker were therefore pooled and assayed in subsequent experiments. This enabled us to exclude the cytosolic Cu/Zn SOD (30 kDa) or mitochondrial MnSOD (85 kDa),17 occasional contaminants in plasma. The 2 methods gave similar qualitative results.

Immunoblotting ecSOD

Aliquots of whole mouse plasma (0.5 µL), boiled in Laemmli buffer,18 were separated by SDS-PAGE using 12% gel and transferred to polyvinylidene fluoride membranes (Bio-Rad) at 100 V for 1 hour. After blocking for 1 hour at room temperature (RT) with TBS containing Tween-20 (TBST, 20 mmol/L Tris, pH 7.6, containing 137 mmol/L NaCl and 0.4% Tween) in the presence of 5% nonfat dry milk, the membranes were incubated with the primary antibody in TBST containing 1% nonfat dry milk at RT for 2 hours. Secondary antibodies (goat anti-rabbit IgG) conjugated to horseradish peroxide (Jackson Immunoresearch) in TBST containing 1% nonfat dry milk were added for 2 hours at RT (1:2500 dilution). After this incubation, the blots were washed five times for 3 minutes each in TBST containing 0.2% nonfat dry milk. Bound secondary antibodies were detected using an ECL detection system (Amersham Pharmacia Biotech).

A mouse-specific 21-amino acid peptide was synthesized corresponding to amino acids 4 to 23 in the mature protein (NH2- DLADRLDPVEKIDRLDLVEKC-COOH). A carboxy-terminal cysteine was added to the sequence to aid in conjugation to keyhole limpet hemocyanin before immunization. Whole rabbit antiserum was used in a 1:10 000 dilution.

Measurement of ecSOD

Plasma ecSOD activity was determined using a system based on the oxidation of NAD(P)H. Briefly, 0.1 mL of sample was combined with 0.8 mL of 100-mmol/L triethanolamine/diethanolamine-HCl buffer, pH 7.4, 25 µL of 100-mmol/L EDTA/50-mmol/L MnCl2 solution, and 40 µL of 7.5-mmol/L NAD(P)H. The reaction was started with the addition of 0.1 mL of 100-mmol/L mercaptoethanol. Superoxide is generated by molecular oxygen in the presence of EDTA, MnCl2, and mercaptoethanol. Superoxide oxidizes NAD(P)H at a predictable rate and thereby lowers its absorbance at 340 nm. The decrease in absorbance is inhibited in the presence of SOD. Samples were tested for NAD(P)H oxidase activity before addition of mercaptoethanol. Extracellular SOD activity was estimated from a standard curve constructed by measuring the activity of increasing
Results

PCR Amplicons of the 3′ UTR of the ecSOD Gene in apoE/LDLR−/− Mice Show Heterogeneity in Size

We monitored the appearance of the ecSOD transcript variant reported by Fukai et al in the apoE−/−/LDLR−/− mice as a function of age and treatment with HBO. As shown in Figure 1, RT-PCR amplicons of the 3′ UTR of the ecSOD gene from peritoneal macrophage mRNA of some of the apoE−/−/LDLR−/− mice were found to migrate faster than others. The difference in migration corresponds to approximately a 10-bp difference, consistent with previous findings. The presence of the shorter amplicon, however, was random; it did not correspond to the extent of disease, age, or presence or absence of treatment with hyperbaric oxygen.

Presence of Short ecSOD mRNA Is Related to Genomic Differences

Because the presence of the shorter ecSOD transcript found in our mice was unrelated to the extent of atherosclerosis, we examined the PCR product using liver genomic DNA as a template and compared it with the product of RT-PCR using peritoneal macrophage mRNA of the B6.129 apoE−/−/LDLR−/− mice. The amplicons derived from the genomic DNA were always identical to the amplicons derived from cDNA of the same animal but not identical from animal to animal. An example is shown in Figure 2. In fact, of the 30 apoE−/−/LDLR−/− mice (19 female and 11 male) examined, 15 were homozygous for the long transcript, 5 were homozygous for the short transcript, and 10 appeared to be heterozygous. Clearly, these data indicate that the presence of differ-

Statistical Analysis

Results from experiments were reported as mean±SEM. Statistical significance was determined using statistical analysis software (Interactive Statistical Programs) and Student’s t test. P≤0.05 was considered statistically significant.

Genotyping Assay for the Short and Long ecSOD Allele

Because B6.129 apoE−/−/LDLR−/− mice are a mixture of the C57 and 129 strains, we set out to genotype the wild-type C57 and 129 strains as well as other commonly used strains in generating knockout mice. The assay requires 2 separate PCR reactions amplifying the regions of the ecSOD gene that encompass a 127-bp region containing bp 61 as well as the 3′ UTR region that can contain the 10-bp deletion. As shown in Figure 3, the resulting amplicons may or may not contain the following restriction enzyme cleavage sites; the 10-bp sequence deleted in the short allele contains a HaeIII site, whereas the A61G change creates a MboI site. Several inbred mouse strains were analyzed, as shown in the Table, including C57 (n=20), BALB/c (n=4), and C3H (n=4), as well as the outbred Swiss Webster (n=20). All of the strains were found to be homozygous for the long ecSOD allele. In contrast, the analysis of genomic DNA from the 129 mice (n=9) found them to be homozygous for the short ecSOD allele, also containing the A61G nucleotide substitution (see the Table). Fifty percent of the control B6.129 mice, used as wild-type controls for the apoE/LDLR−/− mice, were heterozygous, whereas 25% were homozygous for the long or short allele, respectively (n=20), as would be expected for a F2 hybrid of C57 and 129 mice.

Clearly, these data confirm the existence of 2 ecSOD alleles in mouse strains examined. Only the 129 strain carries the short (deletion/single nucleotide substitution) variant of the ecSOD gene, whereas all others tested carry the full-length allele. Of particular significance is the fact that 129-derived stem cells are frequently used for the creation of genetically modified mice, the apparent source of the modified ecSOD allele in some of the animals.
Plasma ecSOD Activity and Mass in C57 and 129 Mice

The 2 ecSOD alleles differ in a single amino acid substitution (Asn to Asp). The 10-bp deletion is in the noncoding region (3' UTR) but may have an effect on transcript stability. It is therefore not unreasonable to suggest that mice bearing the different allele may exhibit different ecSOD activities. To examine this, plasma ecSOD activities were measured in C57 and 129 mice, which are homozygous for the long and short alleles, respectively. As described in the Methods section, we measured both free (circulating) and total (free plus heparin-accessible, matrix-bound) ecSOD activity. The difference between these values defines the matrix-bound, heparin-accessible ecSOD activity in the vasculature.

As shown in Figure 4A, plasma levels of free ecSOD in 129 mice are more than 3-fold higher than those of C57 mice, whereas total activity is nearly 4-fold higher in 129 mice. The calculated heparin-accessible matrix-bound ecSOD activity is 4.6-fold higher in 129 mice. As shown in Figure 4B, these profound differences in enzyme activities are supported by corresponding changes in enzyme mass, as determined by immunoblotting.

Effect of ecSOD Alleles on ecSOD Activity in B6.129 Mice

Based on these data, there is a clear indication that the ecSOD allele has a profound effect on the ecSOD phenotype. However, the results may be confounded by a host of other likely differences in the gene expression pattern between the 2 strains of mice and not directly attributable to the ecSOD gene product. The availability of the F2 hybrids of these 2 strains (B6.129) provides an excellent opportunity to evaluate ecSOD allele-specific effects. These results are shown in Figure 5. The profound differences in the ecSOD phenotype are immediately apparent. It is clear that the homozygous expression of the short allele is associated with the highest heparin-accessible matrix-bound as well as free ecSOD activity, whereas the homozygous expression of the long allele is associated with the lowest total and free ecSOD activity. Mice heterozygous for ecSOD exhibit, as expected, activities that are in between the values from the homozygous animals. These results confirm that the activities associated with the expression of a specific allele of ecSOD are inherently attributable to the allele product and not the environment the enzyme is operating in.

Discussion

Oxidative stress and the superoxide radical are thought to play an important role in the development of several diseases, including atherosclerosis, ischemia/reperfusion injury, inflammation, and cancer. Therefore, ecSOD may be an important component of the antioxidant defense mechanism. It has been shown to play a role in the development of atherosclerosis in vivo and in vitro, and its activity in the context of atherosclerosis or any other oxidation-driven disease should be of particular interest. In this study, we have shown for the first time that a polymorphism exists at the mouse ecSOD locus that gives rise to the short and long ecSOD alleles and leads to significant differences in ecSOD activities in the vasculature.

The short allele, present in the 129 strain and some genetically modified mice derived from it, is characterized by a single substitution at position +61, changing adenine to guanine. This leads to a change from Asn to Asp at amino...
Our observations contrast those reported on vascular ecSOD expression and atherosclerosis. The presence of a smaller ecSOD transcript in apoE−/− or LDLR−/− mice in those studies was attributed to a posttranscriptional modification that occurred as a result of disease progression. Data reported here clearly demonstrate that the reported presence of 2 different ecSOD transcripts in some of the knockout mice used in that study is attributable to genetic heterozygosity through inadequate backcrossing of the mice.

The significant differences observed in allele-specific ecSOD activities seem to be attributable to differences in the amount of enzyme secreted. These differences in enzyme mass in the free and heparin-accessible matrix-bound compartment may well be related to potential differences in the intracellular processing of the enzyme. The noted mutations support this hypothesis. First, the A61G substitution, which leads to the Asn21Asp change in the signal sequence, has the potential to affect cleavage and secretion of the ecSOD preprotein. The first amino acid in the mature protein corresponds to amino acid 25 of the immature protein. Computer-assisted prediction of the cleavage site in the signal sequence predicts the cut site to occur between amino acid 18 and 19 or 20 and 21. It is not known, however, which processing mechanism is responsible for the removal of the remaining 4 amino acids (amino acids 21 through 24) absent in the mature protein, giving rise to the possibility that the point mutation could have an effect on ecSOD processing before or after it is secreted.

In addition to changes in the way ecSOD is processed and secreted, the 10-bp deletion in the 3′ UTR may alter the stability of ecSOD mRNA and therefore rates of ecSOD synthesis. Although the details regarding the regulation of mRNA stability are poorly understood, it is accepted that regulatory elements that determine mRNA half-life and control posttranscriptional regulation reside in the 3′ UTR. Because ecSOD has been shown to be regulated posttranscriptionally, based on a disparity between mRNA and activity levels in various tissues, it is possible that the 10-bp deletion in the 3′ UTR may alter the stability of ecSOD mRNA and therefore rates of ecSOD synthesis.

### Figure 4

**A**. Extracellular SOD was isolated from plasma by Con A-Sepharose affinity chromatography and assayed for activity, as described in the Methods section. Equal aliquots of total plasma proteins were separated by SDS-PAGE, and ecSOD mass was detected by Western blot analysis, as described. Results represent an average ± SEM of 5 animals per group. *Statistically different at P < 0.05.

### Figure 5

**A**. Plasma activity of ecSOD in C57 and 129 mice. Free (open) and total (free plus heparin-releasable, matrix-bound) ecSOD (filled) was measured in 6-month-old C57 and 129 mice. Total values represent ecSOD activity after heparin injection (100 U). A, Extracellular SOD was isolated from plasma by Con A-Sepharose affinity chromatography and assayed for activity, as described in the Methods section. B, Equal aliquots of total plasma proteins were separated by SDS-PAGE, and ecSOD mass was detected by Western blot analysis, as described. Results represent an average ± SEM of 5 animals per group. *Statistically different at P < 0.05.
deletion may somehow affect these events, but additional studies are needed.

The observed differences in ecSOD activities and mass make it particularly important to sufficiently backcross genetically modified mice to be used to study diseases or processes that are affected by superoxide generation and concentration. It is reasonable to assume that their response would be affected, at least partially, by their capacity to scavenge superoxide. In general, extensive backcrossing is essential to maximize gene homozygosity of genetically modified mice. Additional characterization of the ecSODs derived from the 2 alleles is necessary to elucidate differences in enzyme synthesis, processing, and secretion.

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