Mitochondrial Integrity and Function in Atherogenesis

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Background—Coronary atherosclerotic disease remains the leading cause of death in the Western world. Although the exact sequence of events in this process is controversial, reactive oxygen and nitrogen species (RS) likely play an important role in vascular cell dysfunction and atherogenesis. Oxidative damage to the mitochondrial genome with resultant mitochondrial dysfunction is an important consequence of increased intracellular RS.

Methods and Results—We examined the contribution of mitochondrial oxidant generation and DNA damage to the progression of atherosclerotic lesions in human arterial specimens and atherosclerosis-prone mice. Mitochondrial DNA damage not only correlated with the extent of atherosclerosis in human specimens and aortas from apolipoprotein E\textsuperscript{−/−} mice but also preceded atherogenesis in young apolipoprotein E\textsuperscript{−/−} mice. Apolipoprotein E\textsuperscript{−/−} mice deficient in manganese superoxide dismutase, a mitochondrial antioxidant enzyme, exhibited early increases in mitochondrial DNA damage and a phenotype of accelerated atherogenesis at arterial branch points.

Conclusions—Mitochondrial DNA damage may result from RS production in vascular tissues and may in turn be an early event in the initiation of atherosclerotic lesions. (Circulation. 2002;106:544-549.)

Key Words: atherosclerosis ■ muscle, smooth ■ antioxidants

Reactive species (RS) define a collective grouping of reactive oxygen and nitrogen species that can alter the biological functions of essential molecules such as lipids, proteins, and DNA. Numerous studies have linked excess RS generation with vascular lesion formation and functional defects.\textsuperscript{1–3} This association has been reported for various RS models and species.\textsuperscript{4–6} A role for RS in atherogenesis is supported by epidemiological evidence of links between common risk factors for coronary artery disease and increased levels of RS.\textsuperscript{7–9} Among the extensively studied intracellular systems capable of generating RS in vascular cells are the NADH/NADPH oxidase, xanthine oxidase, lipoygenase, and cyclooxygenase systems.\textsuperscript{6,10–12}

Mitochondria are biologically important sources and targets for RS.\textsuperscript{13,14} However, their role as mediators of oxidative disease processes such as atherogenesis has not been examined. We recently reported that exposure of vascular cells to RS in vitro results in preferential mitochondrial DNA (mtDNA) damage and dysfunction and that mtDNA damage is a very sensitive marker for RS-mediated cellular effects.\textsuperscript{15} In addition to the potential role of mtDNA damage as a marker of ambient oxidative stress, oxidative damage to the mitochondrion can lead to decreased oxidative energetic capacity (via impaired oxidative phosphorylation) and increased generation of intracellular RS.\textsuperscript{15–17} Thus, we hypothesized that mitochondrial dysfunction accentuates atherosclerosis by modulating the phenotype of vascular cells and that measurements of mtDNA damage reflect RS-mediated atherosclerosis risk.

Using human aortic specimens and a murine model of early atherosclerosis (the apolipoprotein E null, apoE\textsuperscript{−/−}), we examined the correlation between mtDNA damage and atherogenesis and sought to determine whether mtDNA damage is a cause or an effect in this process. ApoE\textsuperscript{−/−} mice lack apoE, a high-affinity ligand for lipoprotein receptors, which are important for LDL uptake from the bloodstream\textsuperscript{18}; as a result, these mice have significantly elevated levels of serum LDL cholesterol and triglycerides and develop atherosclerotic lesions similar to humans.\textsuperscript{19,20} In such mice fed a 4\% fat diet, monocytes attach to endothelial cells by 8 to 10 weeks of age, foam cells develop after 10 weeks of age, and fibrous plaques appear after 20 weeks of age.\textsuperscript{19,20} We hypothesized that mtDNA damage would be greater in atherosclerosis-prone apoE\textsuperscript{−/−} mice than in age-matched controls. Because the timing of lesion formation is predictable in these mice, we analyzed mtDNA damage in those with early and advanced lesions, as well as in apoE\textsuperscript{−/−} mice (and age-matched controls) before any detectable atherosclerotic lesions formed, to...
determine whether mtDNA damage and dysfunction preceded lesion formation or simply resulted from the highly oxidative milieu produced by advanced lesions. We extended these studies by modulating mitochondrial superoxide dismutase (SOD2) levels in the apoE−/− background by crossing apoE−/− mice with mice heterozygous at the SOD2 locus (apoE−/−, SOD2+/−) to determine the effects of mitochondrial oxidative stress and DNA damage on atherosclerotic lesion formation.

Methods

Mice

C57BL/6j control and apoE−/− (C57BL/6j background) mice were purchased (Jackson Laboratories, Bar Harbor, Maine) when 5 weeks old and fed chow diets (4% fat: Harlan Teklad diet 7001) until 10 or 34 weeks old. We bred pairs of apoE−/− or C57BL/6j mice to obtain 3-week-old apoE−/− or control pups, respectively. SOD2+/− mice, which have been described previously,24 were backcrossed at least 8 times into the C57BL/6j background. All animal protocols were approved by the University of Texas Medical Branch Institutional Animal Care and Use Committee.

Hybrid apoE−/−, SOD2+/− animals were generated by crossing heterozygous SOD2+/− knockout and homozygous null apoE−/− mice (F1). F1 double heterozygotes were backcrossed with apoE−/− mice to generate appropriate F2 breeders (apoE−/−, SOD2+/−). Genotypes were determined through polymerase chain reaction (PCR) analysis of tail clips. SOD2+/− mice were used as breeders because the SOD2+/− animal dies within 2 weeks of birth.21

Histochemical Analysis

Tissues were collected after intraperitoneal injection of ketamine (60 mg/kg)/xylazine (10 mg/kg). Aortas were fixed in 4% paraformaldehyde, embedded in paraffin, cut into 5-μm sections, dewaxed, rehydrated, and stained with hematoxylin and eosin, and atherosclerotic lesion size was assessed. Alternatively, whole aortas were dissected free from the root to the bifurcation, rinsed in PBS buffer, incised longitudinally, and fixed in a flat position with 10% formaldehyde. Aortas were then washed with PBS, stained with oil red O (saturated 60% isopropanol solution), rehydrated, and stained with hematoxylin and eosin, and atherosclerotic lesions were determined by the biotin-streptavidin horseradish peroxidase method and developed with diaminobenzidine.

Results

We examined hematoxylin and eosin–stained sections of 10- and 34-week-old aortas from apoE−/− and control mice to determine the extent and distribution of atherosclerotic lesions. Our findings in the apoE−/− mice were consistent with previous observations.19,20 We found no lesions in vascular sections from 10-week-old control or apoE−/− mice. We did find atherosclerotic lesions in 34-week-old apoE−/− mice but not in age-matched controls.

Aortic tissues from apoE−/− mice had significantly increased mtDNA damage before (10 weeks) and after (34 weeks) histologically detectable lesions developed (Figure 1A). PCR of 10-week-old apoE−/− aortic DNA demonstrated a 60.6% decrease in mtDNA amplification (reflecting an increase in DNA lesions) compared with age-matched, healthy controls (0.582±0.123 mtDNA lesions/10 kb versus 0.0±0.198 lesions/10 kb, P=0.018). The 34-week-old apoE−/− mice had 3-fold greater mtDNA damage than age-matched controls (1.325±0.257 lesions/10 kb versus 0.453±0.162 lesions/10 kb; P=0.007). In contrast to the effects of the apoE−/− background on mtDNA damage, no significant differences were observed between control and apoE−/− groups for damage at the β-globin gene locus, used as a marker for nuclear DNA damage (10-week-old group, P=0.187; 34-week-old group, P=0.098), which indicates the increased sensitivity of the mitochondrial genome to damage. Aortic mtDNA damage increased 2.3-fold with age in apoE−/− mice (Figure 1B, 10-week-old versus 34-week-old mice; P=0.013). Control mice had increases in mtDNA damage that were not statistically significant (P=0.088).

We examined aortas from 3-week-old apoE−/− and control mice to determine whether mtDNA damage was present before the earliest stages of atherogenesis (before inflammatory cell adherence to the vessel wall). ApoE−/− mice had significantly higher levels of mtDNA damage than control animals (Figure 1C; P=0.001), which confirms that mtDNA damage occurs early in aortas predestined to develop atherosclerotic lesions. Thus, mtDNA damage precedes atherogenesis in apoE−/− mice. The lesion incidence in 3-week-old apoE−/− mice was higher than in 10-week-old mice and exposed to phosphor screens for 12 to 14 hours. A standard with one half (7.5 ng) of control genomic template was included in each PCR series to ensure quantitative conditions. DNA lesion frequencies were calculated as described previously.24 Statistical analysis used the independent Student t test.

SOD2 Activity

SOD2 activity assay is based on the ability of SOD to inhibit the reduction of cytochrome C by xanthine/xanthine oxidase.25 SOD2 activity was determined in the presence of 3 mmol/L potassium cyanide, which inhibits SOD1 and SOD3 activities.

3-Nitrotyrosine Immunohistochemistry

Aortic roots were sectioned, deparaffinized, rehydrated, and washed in PBS–Tween 20 buffer, pretreated with 0.3% H2O2 to inhibit endogenous peroxidase activity, and washed. Sections were incubated with PBS–Tween 20 containing 10% serum, excess serum was blotted, and sections were incubated with anti-3-nitrotyrosine antibody (diluted 1:400 in PBS/0.5% serum). The nitrotyrosine primary antibody is revealed by the biotin-streptavidin horseradish peroxidase method and developed with diaminobenzidine.
comparable to that in 34-week-old mice. This could be because of a decreased mtDNA/nuclear DNA ratio in the aortas of younger mice caused by age, disease, or hormonal effects on DNA repair enzymes.

We used QPCR (with human primers) to assess mtDNA damage in human aortic tissues. mtDNA damage was significantly greater in atherosclerotic than in nonatherosclerotic specimens (Figure 2). The marker for nuclear DNA damage, the β-globin gene cluster, did not sustain significant damage in atherosclerotic aortic specimens compared with controls (P=0.15). Accordingly, our findings in human aortas were consistent with the theory that mtDNA damage is an integral feature in atherogenesis. Because increased mtDNA damage was consistent in both human and mouse atherosclerosis, we hypothesized that mitochondrial dysfunction is an important event in atherogenesis, possibly by modulation of the phenotype of vascular cells involved in atherosclerotic lesion formation.

To determine whether mtDNA damage is more likely a cause than an effect of atherosclerotic lesion formation, we examined whether genetic perturbation of mitochondrial antioxidant capacity accentuated mtDNA damage in mouse aortas and whether this exacerbated lesion formation. SOD2 was chosen for these studies because of its importance in maintaining mitochondrial redox state; heterozygous (+/−) SOD2 mice on the C57BL/J6 background have demonstrated decreased mitochondrial function and SOD2 activity compared with wild-type mice. We were also interested in examining SOD2-mediated effects on mitochondrial function in atherogenesis, given the regulation of SOD2 by shear stress forces and the putative role of shear stress in atherogenesis.

ApoE−/−, SOD2−/− mice and apoE−/− controls were fed a 4% fat diet and killed when 17 weeks old. Functional SOD2 activity in apoE−/−, SOD2−/− aortas was 44% less than in apoE−/− mice (P<0.001; data not shown). Histologically, apoE−/−, SOD2−/− mice developed earlier lesions at physiologically relevant sites, including the ascending aorta at the origin of the cerebral vessels. However, the most impressive finding was that oil red O-positive lesions were present throughout the descending aorta at every branch point of the spinal arteries in the apoE−/−, SOD2−/− mice (Figure 3A). Analogous lesions were rare in both 17- and 34-week-old apoE−/− mice. ApoE+/−, SOD2−/− mice did not develop atherosclerotic lesions, regardless of age or diet, which indicates that the phenotype of the apoE−/−, SOD2−/− mice represents an interaction between a proatherogenic lipid milieu and increased mitochondrial RS generation. Overall, the apoE−/−, SOD2−/− mice had 2.5-fold as many atherosclerotic lesions (P=0.02) as their apoE−/− littermates (Figure 2).
discuss mitochondrial dysfunction plays a role in atherogenesis, (2) mtDNA damage at an early stage accurately reflects RS-mediated atherosclerotic risk, and (3) SOD2 prevents atherogenesis in areas of low shear stress by protecting the mitochondrial genome from oxidative damage.

The concept that mitochondrial damage and dysfunction are important in chronic, age-related diseases is not new.\textsuperscript{14,28–30} The paradigm derived from studies over the past decade is that mitochondrial damage accumulates in tissues over time, which causes cellular oxidative phosphorylation potentials to decline while RS production increases, thus accelerating cellular dysfunction.\textsuperscript{14} Of particular relevance are reports of pathogenic mtDNA mutations associated with risk factors for heart disease,\textsuperscript{22,29,30} as well as increased mtDNA mutations in human cardiac tissue.\textsuperscript{28} Our results differ in that the mtDNA damage we measured is not likely to be caused by genetically transmitted mutations or deletions in the mitochondrial genome but rather by an accumulation of discrete oxidative lesions.\textsuperscript{15} Because oxidative stress is present in individuals with many of the traditional risk factors for atherosclerosis (cigarette smoking, diabetes mellitus, hypertension, hypercholesterolemia, hyperhomocysteinemia, and increased age), we hypothesize that mtDNA damage is a common mechanism by which these factors contribute to atherogenesis.

SOD2 mutant (SOD2\textsuperscript{−/−}) mice exhibit inhibition of the respiratory chain enzymes NADH-dehydrogenase (complex I) and succinate dehydrogenase (complex II), as well as inactivation of redox-sensitive enzymes, such as aconitase, accompanied by accumulation of oxidative mitochondrial DNA damage.\textsuperscript{31} Because both complexes I and II contain several iron-sulfur clusters, they are prone to oxidative damage.\textsuperscript{31}

SOD2\textsuperscript{−/−} mice also exhibit an increase in superoxide anion levels concomitant with the 50% decrease in manganese (Mn) SOD activity in mitochondria (compared with SOD2\textsuperscript{+/+} mice).\textsuperscript{26} When quantified in vitro, vascular smooth muscle cells isolated from SOD2\textsuperscript{−/−} mice have 3-fold greater constitutive superoxide levels than SOD2\textsuperscript{+/+} mice (S.K. Moon, PhD, and M.S.R., unpublished data, 2002). The discrepancy in SOD2 activity and superoxide levels suggests that decreased oxidative phosphorylation caused by impairment of complexes I and III might have a chain propagative effect on superoxide production. Consistent with this hypothesis, mice with defects in oxidative phosphorylation exhibit increased levels of RS.\textsuperscript{13} Additionally, the low MnSOD activity in SOD2\textsuperscript{−/−} mice is associated with a 30% decrease in mitochondrial glutathione levels and increased mtDNA modification (protein carbonyl moieties and 8-hydroxydeoxyguanosine).\textsuperscript{28} When one considers the notion that mtDNA damage and mitochondrial RS generation may be important mediators of demands on cardiac tissue. Oxidative mtDNA damage in the aorta, although harmful enough to cause accelerated atherogenic phenotype, does not result in apoptosis as measured by DNA laddering.

**Discussion**

Our findings support the hypotheses that (1) mitochondrial dysfunction plays a role in atherogenesis, (2) mtDNA damage at an early stage accurately reflects RS-mediated atherosclerotic risk, and (3) SOD2 prevents atherogenesis in areas of low shear stress by protecting the mitochondrial genome from oxidative damage.

3B). Although the small size of atherosclerotic lesions precluded measurement of mtDNA damage at branch points, QPCR revealed increased aortic mtDNA damage in the apoE\textsuperscript{−/−}, SOD2\textsuperscript{−/−} mice (\(P=0.006\); Figure 3C). Besides demonstrating that mitochondrial dysfunction resulting from oxidative mtDNA damage is important in atherogenesis, these findings support the theory that under normal circumstances, decreased SOD2 expression in response to low shear stress promotes atherogenesis at arterial branch points.

Compared with controls, 34-week apoE\textsuperscript{−/−} mice had higher levels of 3-nitrotyrosine staining in aortic sections, consistent with higher superoxide and RS levels, the effectors of oxidative mitochondrial DNA damage (Figure 4). To determine whether cumulative mitochondrial DNA damage results in apoptosis, we performed DNA laddering experiments on aortic samples from wild-type, apoE\textsuperscript{−/−}, and apoE\textsuperscript{−/−}, SOD2\textsuperscript{−/−} mice and found no DNA laddering (data not shown). However, substantial DNA laddering with accumulation of fragments <600 bp was observed in left ventricles of apoE\textsuperscript{−/−}, SOD2\textsuperscript{−/−} mice, which reflects higher energetic
vascular cell function and atherogenesis, it is useful to recall that atherogenesis occurs preferentially at sites of disturbed flow and decreased shear stress. In vitro and in vivo, alterations in shear stress result in altered gene expression. Interestingly, SOD2, the predominant mitochondrial antioxidant enzyme, is among the genes regulated by shear stress forces. Because SOD2 expression is increased with high shear stress and decreased with low shear stress, it is likely decreased at branch points in vivo, and the susceptibility of mitochondria to RS-mediated damage would be greatest at these sites, consistent with our observations.

The principal results of these studies—that mtDNA damage precedes atherosclerotic lesion formation and that increased mitochondrial RS generation (due to deficiency in SOD2) increases mtDNA damage and accelerates atherosclerotic lesion formation—are consistent with the notion that oxidative damage to mtDNA leads to further mitochondrial dysfunction and oxidant generation. This “vicious circle” model is supported by studies in mice that lack the adenine nucleotide translocator ANT1 and thus have uncoupling of oxidative phosphorylation due to impaired exchange of ADP and ATP across the mitochondrial inner membrane. In the absence of ANT1, mice have increased levels of oxidative species and mtDNA damage. However, recent studies of mice that express a proofreading-defective mitochondrial polymerase in the heart failed to demonstrate increased RS generation. These results indicate that although the vicious circle hypothesis is plausible and attractive, it may not fully explain the association between mtDNA damage and mitochondrial dysfunction. Additional studies are warranted to determine how signaling and cellular energetics affect the response to mtDNA damage in vascular disease.

In summary, atherogenesis is a complex process of which inflammatory responses are an important component. Our hypothesis, that mtDNA damage is an important initiating event in atherogenesis, is complementary to “inflammatory response” theories. Increased RS production from exposure of the vascular endothelium to an inflammatory milieu results in endothelial dysfunction, inflammatory cell attachment, and initiation of atherosclerotic lesion formation. The mtDNA damage reported here contributes to compromised metabolic processes (in particular, oxidative phosphorylation) that likely participate in both endothelial cell dysfunction and vascular smooth muscle cell phenotypic alterations that are key components of atherogenesis. Thus, our findings, although not conclusive, support current theories that the accumulation of RS-mediated mitochondrial damage in vascular tissues, which leads to compromised vascular cell energetic capacities and dysfunction, is an important early event in atherogenesis.

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References


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