Cigarette Smoke Exposure and Hypercholesterolemia Increase Mitochondrial Damage in Cardiovascular Tissues

Cynthia A. Knight-Lozano, BS; Christal G. Young, BS; David L. Burow, MS; Zhao Yong Hu, MD; Dale Uyeminami, BS; Kent E. Pinkerton, PhD; Harry Ischiropoulos, PhD; Scott W. Ballinger, PhD

Background—A shared feature among cardiovascular disease risk factors is increased oxidative stress. Because mitochondria are susceptible to damage mediated by oxidative stress, we hypothesized that risk factors (secondhand smoke and hypercholesterolemia) are associated with increased mitochondrial damage in cardiovascular tissues.

Methods and Results—Atherosclerotic lesion formation, mitochondrial DNA damage, protein nitration, and specific activities of mitochondrial proteins in cardiovascular tissues from age-matched C57 and apoE \(^{-/-} \) mice exposed to filtered air or secondhand smoke were quantified. Both secondhand smoke and hypercholesterolemia were associated with significantly increased mitochondrial DNA damage and protein nitration. Tobacco smoke exposure also resulted in significantly decreased specific activities of mitochondrial enzymes. The combination of secondhand smoke and hypercholesterolemia resulted in increased atherosclerotic lesion formation and even greater levels of mitochondrial damage.

Conclusions—These data are consistent with the hypothesis that cardiovascular disease risk factors cause mitochondrial damage and dysfunction. *(Circulation. 2002;105:849-854.)*

Key Words: atherosclerosis ■ smoking ■ hypercholesterolemia ■ mitochondria
Methods

Mice

C57 and apoE^{−/−} male mice were purchased from Jackson Laboratories (Bar Harbor, Maine). The apoE^{−/−} mouse lacks apolipoprotein E, a high-affinity ligand for lipoprotein receptors, and consequently has elevated levels of serum LDL cholesterol and triglycerides, developing atherosclerotic plaques in a fashion similar to humans. The C57BL/6 mouse shares the same genetic background and thus is the proper normocholesterolemic control. All mice were fed diets that contain 4.5% fat by weight (PicoLab Rodent Chow 20) and water ad libitum. Animal care was given in accordance with institutional guidelines.

SHS Exposure

Exposures were conducted at the Institute of Toxicology and Environmental Health inhalation facilities (University of California, Davis) in accordance with institutional guidelines. Animals (6 weeks old) were acclimated in filtered air chambers for 1 week. Commencing at 7 weeks of age, mice (N = 20/genotype per exposure group) were exposed to either 42 days of filtered air; 21 days of filtered air followed by 21 days of SHS; or 42 days of SHS, 6 hours per day, 5 days per week (Monday through Friday), for a total of 252 hours of exposure. A side-stream dose of 30 mg/m³ total suspended particulate (TSP) (equivalent to SHS from 2 cigarettes every 15 minutes) and 0.05% Triton X-100 and protease inhibitors) and centrifuged at 13 000 g for 10 minutes. Immunoprecipitations (0.5 mg) of the supernatant were performed with rabbit polyclonal SOD2 antibody (Research Diagnostics). Proteins A and G bound to Sepharose (Pierce Chemical Co) were used to pellet the specific immune complex by centrifugation at 3000g for 5 minutes. The immunoprecipitates were separated on 12% SDS-PAGE gels, transferred, and immunoblotted with 3-nitrotyrosine antibody.

Quantitative Polymerase Chain Reaction for Evaluating mtDNA Damage

Quantitative polymerase chain reaction (QPCR) was performed as previously described using primer pairs M13597 FOR (bps 13597 to 13620) and M13361 REV (bps 13361 to 13337). Copy number differences (mtDNA) were normalized using QPCR of an 80-bp region of the mitochondrial genome that yields products directly related to gene copy numbers (primers M13281F [bps 13261 to 13303] and M13361REV).

Statistical Analysis

Results are expressed as mean±SEM. Two-way ANOVA (genotype and SHS exposure time) was used in all instances except for analysis of mtDNA damage (3-way ANOVA was used; genotype, SHS time, and dose) to test for the global hypothesis that all samples were drawn from a single population. If this test yielded a significant value (P<0.05), a Student-Newman-Keuls test was used for group comparisons.

Results

SHS Exposure Increases Atherosclerotic Lesion Formation in apoE^{−/−} Mice

Hematoxylin-eosin staining of aortic sinuses revealed an increase in mean lesion size in SHS-exposed apoE^{−/−} mice compared with unexposed apoE^{−/−} mice (Figure 1, 76% and 156% at 21 and 42 days of SHS, respectively). By contrast, no obvious lesions were observed within the aortic sinus region from the C57 mouse exposure groups. Quantitative assessment of whole aortas (from the aortic root to the iliac artery) by oil red O staining revealed increases in the mean percentage of positive staining area in SHS-exposed apoE^{−/−} compared with unexposed apoE^{−/−} mice (4.5-fold, Figure 2, P<0.05). However, aortas from C57 mice exposed to SHS had 2.1- and 3.7-fold increases (21- and 42-day SHS exposures, respectively) in the mean percentages of positive staining compared with unexposed counterparts (Figure 2).
As expected, apoE\(^{-/-}\) mouse aortas had significantly higher levels of oil red O staining compared with C57 mice (\(P < 0.05\)). The effects of SHS were most pronounced in the apoE\(^{-/-}\) mice, consistent with significant interaction between hypercholesterolemia and SHS exposure (\(P = 0.036\)).

Predictably, the apoE\(^{-/-}\) mice had significantly higher plasma cholesterol levels compared with the C57 mice overall (587.04 ± 21.97 mg/dL versus 106.05 ± 1.51 mg/dL, apoE\(^{-/-}\) versus C57, all groups combined, \(P < 0.05\)). SHS exposure did not significantly change total cholesterol levels among the apoE\(^{-/-}\) or C57 mice when compared with unexposed genotype-matched controls (apoE\(^{-/-}\), 570.46 ± 38.55 mg/dL [unexposed] versus 584.49 ± 44.57 mg/dL and 606.17 ± 32.52 mg/dL after 21 and 42 days of SHS exposure, respectively; C57, 104.37 ± 3.19 mg/dL [unexposed] versus 107.53 ± 6.18 mg/dL and 106.25 ± 6.54 mg/dL after 21 and 42 days of SHS exposure, respectively), consistent with previous reports. There was no significant interaction between genotype and SHS exposure (\(P = 0.326\)).

**SHS Exposure Significantly Altered Mitochondrial Protein–Specific Activities and Increased Protein Nitration**

SHS was associated with a significant decrease in both SOD2- and ANT-specific activities in hearts from apoE\(^{-/-}\) and C57 mice (\(P < 0.05\)). Figure 3A shows that SOD2-specific activity was reduced after 21 and 42 days of 30 mg/m\(^3\) TSP exposure in apoE\(^{-/-}\) and C57 mice, respectively. Similarly, Figure 3B shows that ANT-specific activity was reduced in apoE\(^{-/-}\) and C57 mice after 42 days of SHS exposure (\(P < 0.05\)). There was significant interaction between SHS exposure and hypercholesterolemia in decreasing the specific activity of both enzymes (SOD2, \(P < 0.001\); ANT, \(P < 0.001\)). These data are consistent with the notion that increased oxidative stress reduces mitochondrial SOD and ANT activities.

![Figure 1. Hematoxylin-eosin staining of the aortic sinus from apoE\(^{-/-}\) mice exposed to either 42 days of filtered air (unexposed), 21 days of filtered air followed by 21 days of 30 mg/m\(^3\) TSP (21 days SHS exposure), or 42 days of 30 mg/m\(^3\) TSP (42 days SHS exposure). White arrows indicate obvious lesions. Numbers below represent mean±SEM for lesion area for each group of apoE\(^{-/-}\) mice.](image1)

![Figure 2. Percent oil red O staining area in unexposed, 21-day SHS-exposed, and 42-day SHS-exposed mice. Filled and open bars represent C57 and apoE\(^{-/-}\) mice, respectively. *Significant difference exists between exposed and unexposed apoE\(^{-/-}\) mice.](image2)

![Figure 3. Specific enzyme activities in unexposed, 21-day SHS-exposed, and 42-day SHS-exposed mice. A, SOD2-specific activity. B, ANT-specific activity. All activities are expressed relative to the unexposed C57 group. *Significant differences exist between genotype-matched exposed and unexposed groups. **Significant differences exist between apoE\(^{-/-}\) and C57 counterparts.](image3)
Because previous reports have indicated that SOD2 can be inactivated by tyrosine residue nitration, we assessed 3-nitrotyrosine adduct levels in proteins from SHS-exposed mice. Figure 4A shows that SHS exposure and hypercholesterolemia increased 3-nitrotyrosine levels, and their combination resulted in greater 3-nitrotyrosine levels (interaction between hypercholesterolemia and SHS exposure, \( P<0.001 \)). Finally, unexposed apoE \(-/-\) mice had significantly greater levels of aortic mtDNA damage compared with their C57 counterparts (Figure 5), suggesting that in addition to SHS exposure, hypercholesterolemia contributed to aortic mtDNA damage (\( P=0.013 \)). To examine the effects of a lower SHS dose, mtDNA damage was assessed in aortas from apoE \(-/-\) and C57 mice exposed to the same regimen, but at 1 mg/m\(^3\) TSP. The lower-dose SHS exposure was associated with significantly increased aortic mtDNA damage in both apoE \(-/-\) and C57 mice after 42 days of exposure (Figure 5), and hypercholesterolemia increased the effects of SHS exposure (42-day apoE \(-/-\) versus 42-day C57, \( P<0.001 \)), suggesting that relatively low levels of passive smoke exposure are capable of causing significant mtDNA damage and that hypercholesterolemia accentuates these effects (there was significant interaction between hypercholesterolemia and SHS exposure, \( P=0.002 \)). As expected, mtDNA damage was the greatest at 30 mg/m\(^3\) TSP in both genotypes of mice when compared with 1 mg/m\(^3\) TSP (\( P<0.001 \)).

**Discussion**

There is a growing consensus that increased oxidative stress mediates CVD. However, the biologically seminal events...
Mitochondrial damage could affect cardiovascular cell function through a variety of mechanisms. In addition to contributing to increased formation of reactive oxygen species and RNS (and thus modulating available NO nitric oxide levels), which are capable of oxidizing LDL (a key step in atherogenesis) and mitochondrial proteins, altered antioxidant function and radical production may modify redox signaling pathways mediated through the mitochondrion, potentially influencing cellular regulatory pathways. Chronic mitochondrial damage could also affect cellular energy production; however, because some tissues can have relatively low energetic thresholds (ie, require a minority of properly functioning mitochondria to meet energetic demands), we predict that the initial impact of mitochondrial damage contributes to cell dysfunction via altered signaling and NO availability. Chronic oxidant exposure would ultimately result in compromised energy production and cell death as well.

Increased levels of nitration products have been observed in human atherosclerotic tissues, and it has been hypothesized that the relative balance of NO and O$_2^-$ (superoxide) within the vascular environment are important factors in influencing the reactivity of NO toward the detrimental effects of RNS formation. We have shown that nitration of SOD2 is increased in apoE$^{-/-}$ mice and that SHS exposure increases these levels in apoE$^{-/-}$ and C57 mice. These data support the concepts that RNS exist intramitochondrially and that they are increased with CVD risk factors. It has been shown that certain mitochondrial proteins, including SOD2, are susceptible to reactive nitrogen-mediated damage and inactivation. The observed increase in SOD2 nitration and decrease in SOD2-specific activity associated with both SHS and hypercholesterolemia in this study are consistent with these observations and the notion that CVD risk factors increase mitochondrial damage. Moreover, the observation that SOD2-specific activities were not significantly different between the 42-day SHS-exposed C57 and apoE$^{-/-}$ mice (although they were significantly different from unexposed controls), despite the fact that the SHS-exposed apoE$^{-/-}$ mice had significantly higher levels of 3-nitrotyrosine compared with their C57 counterparts overall, is consistent with reports of differential susceptibility of SOD2 tyrosine residues to nitration. For example, whereas only 3 (Tyr34, Tyr45, and Tyr193) of the 9 total tyrosine residues in the SOD2 subunit seem to be susceptible to nitration, Tyr34 located near the manganese atom in the active site is the most susceptible to nitration and is believed to be the primary residue associated with inactivation of SOD2. The 2 remaining residues are less vulnerable to nitration and seem unrelated to enzyme inactivation. Consequently, the first SOD2 tyrosine residue to be nitrated by RNS is generally the one associated with enzyme inactivation. Whereas nitration of additional tyrosine residues can occur, it will not significantly contribute to decreased specific activity.

Although the mice in this study were relatively young (15 weeks of age), and the overall ANT-specific activities between the unexposed apoE$^{-/-}$ and C57 mice were not different, SHS exposure did result in decreased ANT specific activity in both C57 and apoE$^{-/-}$ mice, consistent with the concept that increased oxidative stress can alter ANT function. Because the SHS-exposed apoE$^{-/-}$ mice had lower ANT-specific activities, it is possible that chronic hypercholesterolemia imparts higher basal levels of oxidative stress in apoE$^{-/-}$ mice (supported by the increased levels of protein nitration observed in unexposed apoE$^{-/-}$ compared with C57 mice), making the ANT in these mice more sensitive to SHS exposure. Because oxidative stress purportedly increases with age, we suspect that ANT activity in older animals would be more susceptible to the effects of SHS exposure or hypercholesterolemia.

Because intermediate to advanced atherosclerotic lesions are typically not observed even in apoE$^{-/-}$ mice until 15 weeks of age or older when fed the chow diet (4% fat, as in this study), the lack of significant atherosclerotic lesion development in the normocholesterolemic C57 mice exposed to SHS is not surprising. Regardless, oil red O staining did increase with SHS exposure in normocholesterolemic mice 2.1- to 3.7-fold (after 21 and 42 days of exposure, respectively). It is possible that exposure to SHS over longer periods of time will be required to observe statistically significant lesion development in the C57 mice in the absence of preexistent hyperlipidemia (work in progress).

As expected, the highest dose of SHS (30 mg/m$^3$ TSP) had the greatest impact on mtDNA damage. However, aortic mtDNA damage was significantly increased in both groups of mice, even at lower levels of SHS exposure (1 mg/m$^3$ TSP), supporting the concept that mitochondria in cardiovascular tissues are susceptible targets for SHS-mediated damage. We have previously shown that increased mitochondrial damage in endothelial and smooth muscle cells is also associated with reduced mitochondrial function in vitro. Because the mitochondrion is integral for multiple cellular processes, including energy production, apoptosis, and cell signaling, significant mitochondrial damage in vivo may compromise multiple aspects of cell function.

It has become increasingly clear that inflammatory responses involving a variety of vascular factors are an important component of CVD development. Our hypothesis that mitochondrial damage is an important event in CVD is complementary to inflammatory response theories. Several studies have shown that oxidative stress is associated with increased mitochondrial DNA damage, lipid peroxidation, inhibition of electron transport, and inactivation of specific mitochondrial enzymes. The mitochondrial damage associated with SHS exposure and hypercholesterolemia...
reported here may compromise important metabolic processes that influence both endothelial and vascular smooth muscle cell function, key components of atherogenesis. Consequently, our findings are consistent with present theories that oxidative stress mediates CVD by causing mitochondrial damage and dysfunction in tissues, which ultimately lead to compromised energetic capacities and cell dysfunction, important early events in CVD.

Acknowledgments

This work was supported by National Institutes of Health grants ES09318-1 and ES01172-01, the Clayton Foundation for Research, and the John Sealy Foundation (Dr Ballinger) and the California Tobacco-Related Disease Research Program (Dr Pinkerton).

References

Cigarette Smoke Exposure and Hypercholesterolemia Increase Mitochondrial Damage in Cardiovascular Tissues

Cynthia A. Knight-Lozano, Christal G. Young, David L. Burow, Zhao Yong Hu, Dale Uyeminami, Kent E. Pinkerton, Harry Ischiropoulos and Scott W. Ballinger

_Circulation_. 2002;105:849-854; originally published online January 14, 2002;
doi: 10.1161/hc0702.103977

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/105/7/849

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/