Mitochondrial Respiration Is Reduced in Atherosclerosis, Promoting Necrotic Core Formation and Reducing Relative Fibrous Cap Thickness

Emma P.K. Yu,* Johannes Reinhold,* Haixiang Yu, Lakshi Starks, Anna K. Uryga, Kirsty Foote, Alison Finigan, Nichola Figg, Yuh-Fen Pung, Angela Logan, Michael P. Murphy, Martin Bennett

Objective—Mitochondrial DNA (mtDNA) damage is present in murine and human atherosclerotic plaques. However, whether endogenous levels of mtDNA damage are sufficient to cause mitochondrial dysfunction and whether decreasing mtDNA damage and improving mitochondrial respiration affects plaque burden or composition are unclear. We examined mitochondrial respiration in human atherosclerotic plaques and whether augmenting mitochondrial respiration affects atherogenesis.

Approach and Results—Human atherosclerotic plaques showed marked mitochondrial dysfunction, manifested as reduced mtDNA copy number and oxygen consumption rate in fibrous cap and core regions. Vascular smooth muscle cells derived from plaques showed impaired mitochondrial respiration, reduced complex I expression, and increased mitophagy, which was induced by oxidized low-density lipoprotein. Apolipoprotein E–deficient (ApoE<sup>−/−</sup>) mice showed decreased mtDNA integrity and mitochondrial respiration, associated with increased mitochondrial reactive oxygen species. To determine whether alleviating mtDNA damage and increasing mitochondrial respiration affects atherogenesis, we studied ApoE<sup>−/−</sup> mice overexpressing the mitochondrial helicase Twinkle (Tw+/ApoE<sup>−/−</sup>). Tw+/ApoE<sup>−/−</sup> mice showed increased mtDNA integrity, copy number, respiratory complex abundance, and respiration. Tw+/ApoE<sup>−/−</sup> mice had decreased necrotic core and increased fibrous cap areas, and Tw+/ApoE<sup>−/−</sup> bone marrow transplantation also reduced core areas. Twinkle increased vascular smooth muscle cell mtDNA integrity and respiration. Twinkle also promoted vascular smooth muscle cell proliferation and protected both vascular smooth muscle cells and macrophages from oxidative stress–induced apoptosis.

Conclusions—Endogenous mtDNA damage in mouse and human atherosclerosis is associated with significantly reduced mitochondrial respiration. Reducing mtDNA damage and increasing mitochondrial respiration decrease necrotic core and increase fibrous cap areas independently of changes in reactive oxygen species and may be a promising therapeutic strategy in atherosclerosis.

Visual Overview—An online visual overview is available for this article. (Arterioscler Thromb Vasc Biol. 2017;37:2322-2332. DOI: 10.1161/ATVBAHA.117.310042.)

Key Words: atherosclerosis • mitochondria • reactive oxygen species • respiration • vascular smooth muscle

Mitochondria are cellular powerhouses, fuelling metabolic processes through their generation of ATP. Mitochondria are thus critical for cellular function, yet they hold a degree of independence, with their own genome and time-scale of replication. In addition, mitochondria have pivotal roles in the regulation of cell death, metabolism, and generation of reactive oxygen species (ROS). Damage to mitochondria can, therefore, impair cellular function, potentially promoting aging and disease.

Mitochondrial DNA (mtDNA) exists as a 16,569 bp circular molecule associated with the mitochondrial inner membrane. mtDNA contains 37 genes, encoding subunits of respiratory complexes I, III, and IV and the ATP synthase complex V, together with ribosomal and transfer RNAs. The respiratory complexes are central to oxidative phosphorylation, where electron transfer to oxygen is coupled with ATP production. However, ROS are formed as a byproduct of the respiratory chain making mitochondria a major source of cellular ROS. mtDNA is especially vulnerable to damage, partly because mtDNA lies close to the

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Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org DOI: 10.1161/ATVBAHA.117.310042
site of ROS production, and also because mtDNA lacks protective histones. mtDNA defects, such as mutations and deletions, can also be introduced through replication error.\(^4\)

mtDNA is replicated by the mtDNA replisome, which comprises the Twinkle helicase, the mtDNA polymerase, and mitochondrial single-stranded DNA-binding protein. Twinkle unwinds short stretches of double-stranded DNA in the 5'-3' direction and is an important regulator of mtDNA copy number.\(^5\) Mice expressing mutated Twinkle show multiple mtDNA deletions and develop chronic late-onset mitochondrial disease\(^6\); in contrast, transgenic mice expressing increased Twinkle levels (Tw+) show increased mtDNA copy number, potentially increasing mitochondrial function.\(^5\)

mtDNA damage can result in mitochondrial dysfunction, leading to the proatherogenic processes of inflammation and apoptosis. Indeed, mtDNA damage is present in the aortas, hearts, and circulating leukocytes of patients with atherosclerosis\(^7\)–\(^9\) and is an early event in atherogenesis in apolipoprotein E–deficient (ApoE\(^{-/-}\)) mice.\(^8\) Furthermore, large-scale induction of mtDNA damage can directly promote atherosclerosis and plaque vulnerability in ApoE\(^{-/-}\) mice independent of ROS.\(^9\) However, it is not clear what causes mtDNA damage in atherosclerosis and importantly whether the endogenous levels of mtDNA damage seen in mouse or human atherosclerosis are sufficient to cause mitochondrial dysfunction. It is also unclear whether alleviating mtDNA damage and improving mitochondrial respiration affect plaque burden or composition and whether any effects are independent of changes in ROS or metabolism.

We find that endogenous levels of mtDNA damage in mouse and human atherosclerosis are sufficient to reduce mitochondrial copy number and respiration. In contrast, increasing mtDNA integrity and copy number through overexpression of Twinkle increases mitochondrial respiration independent of changes in ROS, promotes vascular smooth muscle cell (VSMC) proliferation, and reduces VSMC and macrophage apoptosis. This results in decreased necrotic core and increased fibrous cap areas.

**Materials and Methods**

Materials and Methods are available in the online-only Data Supplement.

**Results**

**Mitochondrial Dysfunction Is Present in Human Atherosclerosis**

To determine whether mtDNA damage in human plaques results in functional consequences, such as reduced mtDNA copy number or mitochondrial respiration, human plaques were obtained from carotid endarterectomies and normal (undiseased) aortas from patients undergoing aortic valve surgery. Patient age and sex were similar in both groups (n=9 per group; mean age, 71.8±8.3 and 70.6±11.1 years for plaques and aortas, respectively, and 66.7% male for both groups). Human plaques had significantly reduced mtDNA copy number compared with normal aorta (Figure 1A); this was not because of reduced cell number because copy number was controlled against nuclear DNA. Mitochondrial respiration was determined using a Seahorse extracellular flux analyzer to assess mitochondrial oxygen consumption rate (OCR) and the respiratory reserve capacity, which assesses the potential to increase respiration after uncoupling electron transport. Plaques were microdissected into regions containing the media, the shoulder region, the fibrous cap, and the necrotic core (Figure I in the online-only Data Supplement) and normalized for wet weight. Cell nuclei were subsequently counted in each tissue segment to correct for differences in cellularity. OCR was similar in the media and plaque regions at baseline, but the cap (P=0.019) and core (P=0.024) regions showed much lower increases after carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) uncoupling (Figure 1B). Similarly, respiratory reserve capacity was markedly reduced in cap versus media with a similar trend in core versus media (Figure 1C).

**Plaque VSMCs Show Markedly Reduced Mitochondrial Respiration**

Plaques comprise several different cell types, and thus whole plaque assays do not identify which cells have reduced mitochondrial respiration. We therefore cultured VSMCs from plaques and normal vessels and analyzed their mitochondrial respiration by Seahorse. VSMCs derived from these endarterectomies are from the intima and part of the underlying media, and aortic VSMCs were from the media. OCR was similar in plaque versus normal aortic VSMCs at baseline but significantly reduced after uncoupling (Figure 1D and 1E). Western blot of the mitochondrial protein complexes within the electron transport chain (complexes I–V) showed significant heterogeneity of expression both within and between cell lines; however, complexes I and II expression were significantly reduced in cultured plaque versus normal aortic VSMCs normalized to citrate synthase (Figure 1F).

**Mitophagy Is Increased by Oxidized Low-Density Lipoprotein and in Plaque VSMCs**

Mitochondrial mass is determined by the balance between mitonidriogenesis and mitochondrial autophagy (mitophagy), both of which are highly regulated processes.\(^10\) Autophagy has been shown previously to protect against atherosclerosis\(^11\) while oxidized low-density lipoprotein (ox-LDL) can induce mitophagy in VSMCs.\(^12\) Mitophagy was determined by infecting human VSMCs with a lentivirus encoding a mitochondrially targeted keima protein, which fluoresces with peak emission at 620 nm and a pH-dependent excitation maximum of 440 nm at pH 7 to 8, the normal pH

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**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>ApoE(^{-/-})</td>
<td>apolipoprotein E–deficient mice</td>
</tr>
<tr>
<td>FCCP</td>
<td>carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone</td>
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<tr>
<td>mtDNA</td>
<td>mitochondrial DNA</td>
</tr>
<tr>
<td>OCR</td>
<td>oxygen consumption rate</td>
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<tr>
<td>ox-LDL</td>
<td>oxidized low-density lipoprotein</td>
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<tr>
<td>PINK1</td>
<td>PTEN-induced putative kinase 1</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>VSMC</td>
<td>vascular smooth muscle cell</td>
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Figure 1. Human atherosclerosis shows reduced mitochondrial copy number and respiration. A, Mitochondrial copy number in normal human aorta and plaque (n=9). B and C, Seahorse profile for oxygen consumption rate (OCR) in human plaque segments from the media, shoulder, cap, and core with treatment with oligomycin (oligo), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), and antimycin/rotenone (A/R; B) and respiratory reserve capacity (RRC; C; n=5–6). Representative Seahorse profile of human plaque vascular smooth muscle cells (VSMCs; blue) and normal aortic VSMCs (red; D) and OCR after FCCP (E) from n=5 to 7 cultures. F, Western blot and quantification of protein expression of mitochondrial protein complexes within the electron transport chain (complexes I–V) in normal human aortic or plaque VSMCs (n=3–5) relative to citrate synthase (CS). Data are mean±SEM. mtDNA indicates mitochondrial DNA.
of the mitochondrial matrix (coded green), or 586 nm at pH 4 to 5, the pH of lysosomes targeting mitochondria for mitophagy (coded red).

Because keima is resistant to lysosomal degradation, a ratio of red/green keima fluorescence can estimate mitophagy. Ox-LDL but not native LDL induced time-dependent mitophagy, which was also seen after FCCP (Figure 2A and 2B). Human plaque VSMCs also showed increased mitophagy compared with normal VSMCs, which was not further increased by ox-LDL or FCCP (Figure 2C).

mtDNA synthesis is regulated by several genes, including Twinkle, mtDNA polymerase-γ, and mitochondrial transcription factor A (TFAM). Expressions of Twinkle, polymerase-γ, and TFAM were similar in aortic and plaque VSMCs (Figure 2D and 2E). In contrast, mitophagy is regulated, in part, by the expression and localization of the serine-threonine kinase phosphatase and tensin homolog (PTEN)–induced putative kinase 1 (PINK1) and its interaction with the ubiquitin ligase parkin. PINK1 is normally expressed at low levels

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**Figure 2.** Mitophagy is increased by oxidized low-density lipoprotein (ox-LDL) and in plaque vascular smooth muscle cells (VSMCs). A, Confocal microscopic images of control normal human aortic or plaque VSMCs expressing mitochondrially targeted keima, either untreated or after treatment with 100 mg/mL ox-LDL or 5 mmol/L carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP). Insets show high-power views of outlined areas. B and C, Red/green keima ratios for individual normal aortic VSMCs either untreated (C) or treated with 100 mg/mL ox-LDL for 4 to 24 h, 100 mg/mL native LDL for 24 h, or 5 mmol/L FCCP (B), or individual normal aortic VSMCs (C) or human plaque VSMCs either untreated or treated with ox-LDL or FCCP for 24 h (C). n=20 images from 3 biological replicates. Western blots (D) and their quantification (E) for proteins involved in mitochondrial DNA synthesis and mitophagy in human aortic or plaque VSMCs, normalized to actin (n=3). PINK1 indicates PTEN-induced putative kinase 1; PolG, polymerase-γ; and TFAM, mitochondrial transcription factor A.
but is stabilized and becomes detectable during mitophagy. PINK1 expression was significantly increased in plaque versus normal VSMCs, consistent with increased mitophagy (Figure 2D and 2E). Together, these findings suggest that plaque VSMCs undergo increased mitophagy, most likely induced by ox-LDL, which is not compensated by increased mitochondrial biogenesis.

**mtDNA Damage and Dysfunction Are Present in Mouse Atherosclerosis**

mtDNA damage is found in ApoE−/− mouse aortas8,9; however, whether the mtDNA damage is sufficient to affect mitochondrial function and the cause of endogenous mtDNA damage are unclear. mtDNA damage was assessed by quantitative polymerase chain reaction for mtDNA adducts which halt polymerase progression; damage can therefore be quantified by comparing the amplification of a large mtDNA product to a short target to control for copy number.15 mtDNA damage was increased in ApoE−/− mice fed fat for 14 weeks compared with 6-week-old control mice (Figure 3A), associated with reduced abundance of the mitochondrial respiratory complexes that have mtDNA-encoded subunits (complexes I, III, IV, and V) but not citrate synthase (Figure 3B). Respirometry on permeabilized mouse aortas also showed that fat-fed ApoE−/− mice had reduced complex I- and complex IV-supported aortic respiration (Figure 3C and 3D).

mtDNA lies close to the site of ROS production, such that ROS may induce the mtDNA damage associated with atherosclerosis; however, assessment of mitochondrial ROS during atherogenesis has not been reported. Mitochondrial H2O2 was assessed in vivo in ApoE−/− mice using the mitochondria-targeted probe MitoB. MitoB forms MitoP on reaction with mitochondrial H2O2 (and also with peroxynitrite), changes that can be assessed by quantifying the MitoP/MitoB ratio by mass spectroscopy.16 MitoP/MitoB was increased in ApoE−/− mice fed fat for 14 weeks compared with normal ApoE−/− mice (Figure III in the online-only Data Supplement), mtDNA copy number was not increased in Tw+/ApoE−/− aortas or VSMCs, but mtDNA damage was significantly decreased in both (Figure III in the online-only Data Supplement). Conversely, Tw+/ApoE−/− macrophages showed increased mtDNA copy number but no change in mtDNA damage compared with ApoE−/− controls (Figure III in the online-only Data Supplement).

To determine the functional consequences of increased mtDNA copy number or reduced mtDNA damage, we assessed expression of mitochondrial respiratory complexes and respiratory function in Tw+/ApoE−/− and ApoE−/− control mice. Tw+/ApoE−/− aortas showed increased complex I abundance, and Tw+/ApoE−/− macrophages showed increased abundance of both complexes I and III, whereas cultured VSMCs showed no significant difference in complex expression between groups (Figure IV in the online-only Data Supplement). In contrast, Tw+/ApoE−/− aortas showed increased complex I-supported respiration (Figure 4A) but no differences in complex II- or IV-supported respiration (Figure V in the online-only Data Supplement), and both Tw+/ApoE−/− VSMCs (Figure 4B and 4C) and macrophages (Figure 4D and 4E) had increased OCR after uncoupling with FCCP compared with ApoE−/− controls.

Although, mitochondrial ROS were increased in aortas as atherosclerosis develops (Figure 3E), mtDNA damage can promote atherosclerosis independent of changes in ROS.9 We, therefore, assayed ROS in both tissues and cells of ApoE−/− and Tw+/ApoE−/− mice. There were no significant differences in the MitoP/MitoB ratio in a range of tissues between control ApoE−/− and Tw+/ApoE−/− mice after 14 weeks of high-fat diet (Figure 4F). Similarly, ROS levels in control ApoE−/− or Tw+/ApoE−/− VSMCs or macrophages determined using the ROS-sensitive dye dichlorodihydrofluorescein diacetate were similar at baseline and after stimulation with lipopolysaccharide or the free radical-generating agent tert-butyldihydroperoxide (Figure 4G and 4H), indicating that Tw overexpression does not affect ROS levels in vitro or in vivo.

**Tw+/ApoE−/− Mice Show Increased mtDNA Integrity, Copy Number, and Mitochondrial Respiration With No Change in ROS**

Our data indicate that atherosclerosis is associated with reduced mitochondrial respiration in human and mouse atherosclerosis. We, therefore, used Twinkle transgenic mice (Tw+) to determine whether alleviating mtDNA damage and increasing mitochondrial respiration could affect atherogenesis or plaque composition. Tw+ mice express mouse Twinkle helicase intron 4 cDNA under the control of the human β actin promoter, which results in widespread transgene expression, including in muscle-containing tissues.7 Tw+ mice show increased mtDNA copy number that varies between organs5 and reduced mtDNA damage after oxidative stress.16 We crossed C57Bl/6 Tw+ mice with ApoE−/− mice and examined Twinkle expression, copy number, mtDNA damage, and mitochondrial respiration in aortas and cultured cells. Tw+/ApoE−/− mice had increased Twinkle mRNA in aortas, aortic VSMCs, and bone marrow–derived macrophages compared with ApoE−/− controls although the extent of Twinkle expression varied between tissues (Figure II in the online-only Data Supplement). mtDNA copy number was not increased in Tw+/ApoE−/− aortas or VSMCs, but mtDNA damage was significantly decreased in both (Figure III in the online-only Data Supplement). Conversely, Tw+/ApoE−/− macrophages showed increased mtDNA copy number but no change in mtDNA damage compared with ApoE−/− controls (Figure III in the online-only Data Supplement). We, therefore, used Twinkle transgenic mice to determine whether alleviating mtDNA damage and mtDNA copy number or reduced mtDNA damage, we assessed expression of mitochondrial respiratory complexes and respiratory function in Tw+/ApoE−/− and ApoE−/− control mice. Tw+/ApoE−/− aortas showed increased complex I abundance, and Tw+/ApoE−/− macrophages showed increased abundance of both complexes I and III, whereas cultured VSMCs showed no significant difference in complex expression between groups (Figure IV in the online-only Data Supplement). In contrast, Tw+/ApoE−/− aortas showed increased complex I-supported respiration (Figure 4A) but no differences in complex II- or IV-supported respiration (Figure V in the online-only Data Supplement), and both Tw+/ApoE−/− VSMCs (Figure 4B and 4C) and macrophages (Figure 4D and 4E) had increased OCR after uncoupling with FCCP compared with ApoE−/− controls.

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**Tw+/ApoE−/− Mice Show Increased Fibrous Cap and Decreased Necrotic Core Areas**

To determine whether improved mitochondrial respiration could affect atherosclerosis, male and female Tw+/ApoE−/− and ApoE−/− littermate controls were fed fat from 6 to 20 weeks of age. There were no differences in body weight, blood pressure, blood counts, hemoglobin, serum lipids, or glucose between groups (Figure VI in the online-only Data Supplement). Detailed metabolic phenotyping using comprehensive laboratory animal monitoring system cages also showed no differences in food/water consumption, activity (as assessed by beam breaks), and total oxygen consumption (Figure VII in the online-only Data Supplement). Atherosclerosis was examined in aortic roots, brachiocephalic arteries, and the descending aorta. There was no difference
in plaque burden between Tw+/ApoE−/− and ApoE−/− mice in any vascular bed (Table; Figures 5 and 6A). However, Twinkle overexpression affected plaque composition, with Tw+/ApoE−/− plaques showing increased fibrous cap and decreased necrotic core areas (Table; Figures 5 and 6B). Tw−/ApoE−/− plaques also showed a borderline increase in smooth
muscle actin–positive area, significantly increased proliferating cells, and significantly decreased cells undergoing apoptosis (Table; Figure 5).

To distinguish between the effects of Twinkle overexpression on vessel wall or bone marrow–derived cells, 6-week-old ApoE−/− mice were irradiated and transplanted with Tw+/−.
ApoE− or control ApoE− bone marrow, fat fed from 6 to 20 weeks, and atherosclerosis examined. There was no difference in body weight, blood counts, hemoglobin or serum lipids, or glucose between mice receiving Tw+/ApoE− or control ApoE− bone marrow (Figure VI in the online-only Data Supplement). Twinkle expression and complex I-, II-, or IV-supported respiration were similar in the aortas from mice transplanted with control or Tw+ marrow (Figure VIII in the online-only Data Supplement). Although there was no difference in the extent of atherosclerosis (Figure 6C), Tw+ marrow transplantation decreased the necrotic core area while fibrous cap areas were similar between groups (Figure 6D).

Effects of Twinkle Overexpression on VSMCs and Macrophages

The presence of increased fibrous cap and decreased necrotic core areas in Tw+/ApoE− mice, together with increased numbers of proliferating cells and reduced numbers of apoptotic cells, suggests that Twinkle overexpression may have direct effects on cells in the atherosclerotic plaque. Indeed, Tw+/ApoE− VSMCs showed increased cell counts after 4 days in culture (Figure 6E), and because there was no difference in baseline apoptosis (Figure 6F), this indicates increased proliferation compared with control ApoE− VSMCs. Tw+ VSMCs and macrophages also showed reduced apoptosis after tert-butyl hydroperoxide–induced oxidative stress (Figure 6F and 6G) while Tw+/ApoE− macrophages showed no difference in the secretion of a range of cytokines (Figure IX in the online-only Data Supplement).

Discussion

Mitochondria regulate multiple processes that are key to both cellular and organismal health, including ATP and ROS generation, cell death, inflammation, and systemic metabolism. We and others have shown that human and mouse atherosclerotic plaques show mtDNA damage and that extensive, supra-physiological levels of mtDNA damage promote atherosclerosis through effects on apoptosis, inflammation, and systemic metabolism. However, it is unclear whether the endogenous levels of mtDNA damage observed in mouse and human atherosclerosis are sufficient to reduce mitochondrial respiration, whether improving mitochondrial respiration affects atherosclerosis, and what the mechanisms involved are.

Our study demonstrates several important findings. We show for the first time that the mtDNA damage seen in human and mouse atherosclerosis is sufficient to reduce mitochondrial respiration, is most marked in the cap and core regions, and is associated with reduced mtDNA copy number in human plaques. Cultured human plaque VSMCs show reduced mitochondrial respiration and reduced expression of complex I. Ox-LDL induces mtDNA damage and mitophagy in VSMCs, suggesting a possible cause for the mtDNA damage and dysfunction in atherosclerosis, and human plaque VSMCs show increased mitophagy. The Twinkle helicase increases mtDNA copy number and respiratory complex expression, resulting in improved mitochondrial respiration in the whole aorta, VSMCs, and macrophages. Twinkle increases VSMC proliferation and protects VSMCs and macrophages against oxidant

Table. Analysis of Atherosclerotic Plaques From Control ApoE−/− and Tw+/ApoE−/− Mice

<table>
<thead>
<tr>
<th></th>
<th>Control ApoE−/− (n=14)</th>
<th>Tw+/ApoE−/− (n=17)</th>
<th>P Value</th>
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<tbody>
<tr>
<td>Aortic root area (%)</td>
<td>25.04±2.8</td>
<td>25.13±1.6</td>
<td>0.98</td>
</tr>
<tr>
<td>Brachiocephalic plaque area (%)</td>
<td>18.71±6.2</td>
<td>15.80±4.7</td>
<td>0.70</td>
</tr>
<tr>
<td>Descending aortic plaque area (%)</td>
<td>2.15±0.5</td>
<td>1.08±0.3</td>
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<tr>
<td>Aortic root plaques, %</td>
<td></td>
<td></td>
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<tr>
<td>Necrotic core area</td>
<td>48.56±4.6</td>
<td>38.9±1.7</td>
<td>0.031</td>
</tr>
<tr>
<td>Fibrous cap area</td>
<td>35.53±3.0</td>
<td>49.83±1.5</td>
<td>&lt;0.0001</td>
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<tr>
<td>SMA-positive area</td>
<td>27.58±3.4</td>
<td>38.73±3.7</td>
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<tr>
<td>MAC-positive area</td>
<td>27.14±2.9</td>
<td>32.02±2.3</td>
<td>0.20</td>
</tr>
<tr>
<td>TUNEL-positive cells</td>
<td>7.48±1.3</td>
<td>3.63±0.4</td>
<td>0.018</td>
</tr>
<tr>
<td>ki67-positive cells</td>
<td>2.50±0.3</td>
<td>3.93±0.4</td>
<td>0.02</td>
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</table>

All areas were quantified by planimetry and expressed as % of the cross-sectional vessel area (plaque areas), or % plaque area expressing SMA or MAC3, or % cells showing TUNEL or ki67 expression. Data are means±SEM. ApoE− indicates apolipoprotein E–deficient; MAC, Mac3 macrophage marker; SMA, smooth muscle actin; TUNEL, terminal UTP nick-end labeling apoptosis marker; and Tw, Twinkle helicase.

ApoE−/− or control ApoE−/− bone marrow, fat fed from 6 to 20 weeks, and atherosclerosis examined. There was no difference in body weight, blood counts, hemoglobin or serum lipids, or glucose between mice receiving Tw+/ApoE− or control ApoE− bone marrow (Figure VI in the online-only Data Supplement). Twinkle expression and complex I-, II-, or IV-supported respiration were similar in the aortas from mice transplanted with control or Tw+ marrow (Figure VIII in the online-only Data Supplement). Although there was no difference in the extent of atherosclerosis (Figure 6C), Tw+ marrow transplantation decreased the necrotic core area while fibrous cap areas were similar between groups (Figure 6D).

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stress–induced apoptosis. Importantly, Twinkle overexpression during atherogenesis increases the size of the fibrous cap and reduces necrotic core areas while also increasing cell proliferation and reducing apoptosis in vivo. Reduced necrotic core areas were also seen after transplantation with Tw+ marrow, suggesting that effects on both bone marrow–derived cells and VSMCs are important. Our study suggests that preventing the mtDNA damage and dysfunction seen in atherosclerosis may be important in modulating plaque composition.

Mitochondria can sustain extensive damage without affecting overall function, in part because of compensatory mitochondrial biogenesis. However, we show that the mtDNA damage seen in mouse and human atherosclerosis is sufficient to reduce complex expression, mitochondrial respiration, and reserve capacity, particularly in the cap and core regions in human plaques. OCR and respiratory reserve capacity were corrected for both tissue weight and cell number, and reduced complex I- and IV-supported respirations were also seen in

Figure 6. Twinkle mouse plaques show reduced necrotic core and increased fibrous cap areas. Aortic root plaque % (A), necrotic core, and fibrous cap % areas (B) in control apolipoprotein E–deficient (ApoE−/−) and Tw+/ApoE−/− mice after fat feeding from 6 to 20 wk (n=11–18). Aortic root plaque % (C), necrotic core, and fibrous cap % areas (D) in ApoE−/− mice transplanted with control ApoE−/− or Tw+/ApoE−/− bone marrow at 6 wk and fat feeding from 6 to 20 w (n=8–10). E, Cell number at day 0 and day 4 in vascular smooth muscle cells (VSMCs) derived from control ApoE−/− and Tw+/ApoE−/− mice (n=3). Apoptosis at baseline and after 50 μmol/L tert-butyl hydroperoxide (t-BHP) for 16 h in VSMCs (F) and macrophages (G) from control ApoE−/− and Tw+/ApoE−/− mice (n=4–5). BMT indicates bone marrow transplant; and Tw, Twinkle helicase.
Mitochondrial Dysfunction and Plaque Composition

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Mitochondria are a major source of cellular ROS, but the exact relationship between mtDNA damage, dysfunction, and ROS is unclear. Improving mtDNA integrity might reduce ROS levels, and reduced ROS has been observed previously in Twinkle-overexpressing cells. In contrast, although Twinkle overexpression improved mitochondrial respiration, we found no changes in ROS in either arteries in vivo or VSMCs or macrophages in vitro. Our study shows that while aortic mitochondrial ROS do increase during murine atherogenesis, augmenting mtDNA integrity and mitochondrial respiration can affect plaque composition independently of changes in ROS.

We find that selectively improving mtDNA copy number, integrity, and respiration increases fibrous cap and reduces necrotic core areas and increases cell proliferation and reduces apoptosis in Tw+/ApoE−/− mice. There were no systemic metabolic changes in Tw+/ApoE−/− mice, suggesting that the changes in plaque composition are because of changes in vessel wall cells and macrophages. VSMCs provide the protective fibrous cap in established plaques, and their apoptosis and senescence promote cap thinning. In contrast, Tw+/ApoE−/− VSMCs show increased cell proliferation, and Tw+/ApoE−/− macrophages, and plaques show reduced apoptosis. The independent effect of Twinkle on myeloid cells is also demonstrated by the reduction in core size seen after transplantation with Tw+ marrow.

Our data support a model where ROS/ox-LDL induce mtDNA damage, which is sufficient to decrease copy number, reduce complex I expression, promote mitophagy without compensatory mitochondrial biogenesis, and reduce mitochondrial respiration. Mitochondrial dysfunction promotes VSMC and macrophage apoptosis and reduces VSMC proliferation leading to increased necrotic core and decreased fibrous cap areas. However, recovery of mtDNA integrity, for example, by Twinkle overexpression, improves mitochondrial respiration, promotes VSMC proliferation, and protects against VSMC and macrophage apoptosis. Plaque composition is thus altered, with decreased necrotic core and increased fibrous cap areas.

Our study has some limitations. First, although the reduced mtDNA copy number and mitochondrial respiration in human plaques and plaque-derived VSMCs may be because of ox-LDL–mediated mtDNA damage and mitophagy, we were not able to directly examine the cause of reduced mitochondrial respiration in human plaques or examine mitophagy in mouse plaques. Second, we examined respiration in normal aortic versus diseased carotid VSMCs, and cell origin might affect our results. However, our previous studies have shown that DNA damage is similar in normal (undiseased) arteries from different vascular beds. Third, we studied mouse bone marrow–derived macrophages rather than resident macrophages exposed to the high-fat diet. However, plaque macrophages are mainly derived from bone marrow precursors, although as bone marrow–derived macrophages are cultured ex vivo for 7 days, the extent the cells retain the effects of high-fat feeding is uncertain. Last, the Tw+ mice show improved mtDNA integrity, copy number, and respiration over the timeframe under study (up to 20 weeks) and in specific tissues. However, Twinkle overexpression has different effects on different tissues, and we cannot exclude beneficial effects of Twinkle overexpression on other cells in the plaque, such as endothelial cells. In addition, high Twinkle expression can cause respiratory complex deficiency, particularly in tissues with high mitochondrial content. Twinkle itself is, therefore, unlikely to be a therapeutic target although protecting and augmenting mitochondrial function by pharmacological means may be more successful.

In summary, we show that the endogenous levels of mtDNA damage in mouse and human atherosclerosis are associated with significant reductions in mitochondrial copy number and respiration. Twinkle overexpression improves mtDNA integrity and copy number, resulting in improved mitochondrial respiration and increased fibrous cap and decreased necrotic core areas. Maintaining and restoring mtDNA integrity and mitochondrial function is, therefore, a promising therapeutic strategy.

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Disclosures
None.

References

Highlights
- Mitochondrial respiration is markedly reduced in human atherosclerosis and in vascular smooth muscle cells derived from plaques.
- Mitophagy is increased in plaque vascular smooth muscle cells and after oxidized high-density lipoprotein.
- Augmentation of mitochondrial respiration by overexpression of the Twinkle helicase reduces multiple features of plaque vulnerability.
- Twinkle promotes vascular smooth muscle cell proliferation and reduces apoptosis of vascular smooth muscle cells and macrophages.
- Augmentation of mitochondrial respiration may be a therapeutic target in atherosclerosis.
Mitochondrial Respiration Is Reduced in Atherosclerosis, Promoting Necrotic Core Formation and Reducing Relative Fibrous Cap Thickness
Emma P.K. Yu, Johannes Reinhold, Haixiang Yu, Lakshi Starks, Anna K. Uryga, Kirsty Foote, Alison Finigan, Nichola Figg, Yuh-Fen Pung, Angela Logan, Michael P. Murphy and Martin Bennett

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Supplemental Figure I. Illustration of sample areas for Seahorse experiments of carotid endarterectomies.
Hematoxylin and eosin stain of human carotid endarterectomy sample. Top right: lumen. Sample areas used for Seahorse experiments and scale bar are depicted for illustration.
Supplemental Figure II. Twinkle mice show increased levels of twinkle expression in aortas, VSMCs and macrophages

qPCR for fold expression of Twinkle mRNA in (A) aortas after 14 weeks HFD, (B) cultured VSMCs or (C) cultured bone marrow-derived macrophages from Control ApoE−/− mice or Tw+/ApoE−/− mice. Data are expressed as fold expression in Tw+/ApoE−/− vs. ApoE−/− mice. n=5-7.
Supplemental Figure III: Twinkle mice show tissue-specific increases in mtDNA copy number or reductions in mtDNA adducts

(A-F) MtDNA copy number and mtDNA adducts in aorta (A-B), VSMCs (C-D) or bone marrow-derived macrophages (E-F) of control ApoE\(^{-/-}\) and Tw\(^{+/}\)/ApoE\(^{-/-}\) mice. (n=4-17).
Supplemental Figure IV. Twinkle increases expression of mitochondrial respiratory complexes in aorta and macrophages

(A-C) Western blot (left) and quantification of western blots (right) for mitochondrial respiratory complex I-V or citrate synthase (CS) expression from aorta (A), VSMCs (B), and bone marrow-derived macrophages (C) derived from control ApoE⁻/⁻ or Tw⁺⁺/ApoE⁻/⁻ mice  n=4-6. Tub = tubulin.
Supplemental Figure V. Twinkle mice show no difference in aortic complex II or IV respiration. Ex-vivo respirometry for complex II (A) and complex IV (B)-supported respiration in aortas from control ApoE⁻/⁻ or Tw⁺/ApoE⁻/⁻ mice after 14 weeks HFD (n=4-8).
Supplemental Figure VI. Twinkle mice show normal blood counts, weights and blood pressure

Body weight, blood pressure, white cell count (WCC), Hemoglobin concentration (Hb) and platelet count (Plts), serum cholesterol (Chol) or glucose in control ApoE−/− and Tw+/ApoE−/− mice (A-D) or BMT of ApoE−/− mice with control ApoE−/− or Tw+/ApoE−/− marrow (E-G) and undergoing 14w HFD. n=7-14 (A-D), and 7-11 (E-G).
Supplemental Figure VII. Twinkle mice show normal activity and total oxygen consumption

Comprehensive laboratory animal monitoring system data of control ApoE\textsuperscript{−/−} and Tw\textsuperscript{+/−}/ApoE\textsuperscript{−/−} mice after 13 weeks of HFD (n=3). Food and water intake (A). Activity measured as the total number of beam breaks per 24 hours (B). Oxygen consumption (VO\textsubscript{2}) profile (C) or mean VO\textsubscript{2} normalised to body mass (D).
Supplemental Figure VIII. Tw+ bone marrow transplantation has no effect on aortic twinkle expression or respiration

(A-C) ApoE−/− mice were transplanted with control ApoE−/− mice or Tw+/ApoE−/− bone marrow and fat fed from 6-20w (n=3-4) and aortas examined for (A) twinkle expression, Complex I (B), II (C) and IV-supported respiration (D). Data for Tw expression are expressed as fold expression versus ApoE−/− mice.
Supplemental Figure IX. Tw+/ApoE−− macrophages show no difference in cytokine release
Cytokine release after treatment with 1µg/ml LPS from Tw+/ApoE−− (Tw+) and ApoE−− bone marrow derived macrophages (n=7). IL indicates interleukin, TNFα, tumour necrosis factor-α and MCP1, monocyte chemoattractant protein-1.
Materials and Methods

Mice
This research has been regulated under the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB). 6-week old male and female C57Bl6/J ApoE−/− mice (Stock number 002052, Jackson Laboratory, Bar Harbor, Maine) were either sacrificed or fed high fat for 14 weeks (21% fat, Special Diet Services # 829100). ApoE−/− mice that over-expressed Twinkle (Tw+/ApoE−/−) were generated by crossing C57Bl6Tw+ (from Dr Anu Suomalainen, Helsinki) with C57Bl6 ApoE−/− mice at least 3 times. Genotyping of ApoE−/− mice was performed by PCR using company protocols (Jackson Laboratory). Tw+ mice genotyping was performed by PCR using the following primers at 5µmol/L: FWD: TGCAGTTCATGATGGGTCAC, REV: TGCTGTCTGCAGTTCTTGT. The cycling parameters were 1 cycle of 94°C, 1 min and 30 cycles of 94°C, 30s; 56°C, 30s; 72°C, 1min. Expected product sizes were Tw+ allele 200 bp and wild type allele 350 bp.

Both male and female littermate experimental (Tw+/ApoE−/−) and control (ApoE−/−) mice were fat-fed from 6-20 weeks. (21% fat, Special Diet Services # 829100). Blood pressure was measured at 16 weeks of age, using Visitech BP-2000 Blood Pressure Analysis System (Visitech Systems Inc, USA) after prior familiarisation. At 2 weeks prior to sacrifice, fasting peripheral blood samples were collected. Full blood count was assayed using the ABC vet blood counter (ABX vet 16p, ABX Diagnostics Inc, USA). Lipid profiles and glucose were assayed on serum, using commercial enzymatic assays and high-performance liquid chromatography (Department of Clinical Biochemistry, Addenbrooke’s Hospital). After 13 weeks of high fat feeding, experimental mice were transferred to individual monitoring cages, kept at 22°C, under an alternating 12h:12h light-dark cycle. Following 24 hours of acclimatisation, mice were assessed for 48 hours. Water and food consumption, ambulatory activity, VCO2 and VO2 were measured in the comprehensive laboratory animal monitoring system (CLAMS, Columbus Instruments, OH, USA) fitted with an indirect calorimetry system (miniMOX; University of Cambridge, Cambridge, UK). Body weight was assessed before and after the procedure, and VO2 corrected for lean body weight.

For bone marrow transplantation experiments, 6-week old ApoE−/− mice received 2 doses of 5.5Gy total body irradiation, 4 hours apart. For repopulation, bone marrow cells were harvested from either Tw+/ApoE−/− or ApoE−/− mice. Tibias and fibulas were excised from donors, and after removal of the trabecular heads, the bone marrow was flushed out with Dulbecco’s Modified Eagle Medium (DMEM). The cells were washed with phosphate-buffered saline (PBS) and 10x106 cells were injected via the tail vein, 6 hours after the 1st dose of irradiation. Recipient mice received 0.025% Baytril antibiotic (Bayer AG, Germany) in their drinking water for 4 weeks, and peripheral counts were performed at 11 and 18 weeks of age to check for reconstitution. At sacrifice the mice underwent terminal anaesthesia with pentobarbital, and blood and tissues were removed.

In vivo mitochondrial hydrogen peroxide assessment
MitoB, a mitochondria-targeted ratiometric probe, was used to assess in vivo mitochondrial H2O2. MitoB has a triphenylphosphonium cation, which drives its accumulation within mitochondria, where it reacts with H2O2 to form MitoP. Quantifying the mitoP/mitoB ratio enables measurement of mitochondrial H2O2. On the day of sacrifice, mice were administered 25 nmol of mitoB via intravenous injection. After 4 hours of equilibration, tissues were harvested for analysis of mitoP/mitoB by liquid chromatography-tandem mass spectrometry.

Histological analysis
Ascending aortas and brachiocephalic arteries were fixed in 10% formalin, embedded in paraffin and sectioned at 5 µm intervals. The specimens were then stained with Masson’s Trichrome. Images were captured and analysed in Image-Pro Insight (Media Cybernetics) to
assess atherosclerotic lesion size and composition. Total plaque area was expressed as a percentage of vessel lumen area, and fibrous cap and necrotic core areas expressed as a percentage of plaque area. Descending aortas were dissected and incised longitudinally. The samples were washed with de-ionised water and 60% isopropranolol, before staining with 60% Oil Red O stain. Images were captured and the stained area was expressed as a percentage of total vessel area.

For immunohistochemical analysis of aortic root plaques, sections were blocked in bovine serum albumin (BSA) and incubated with primary antibodies specific for anti-smooth muscle actin (SMA, 1A4, Dako,1:500) and Ki67 (VP-K452-Ki67, Vector Laboratories,1:100). Sections were stained with biotinylated secondary antibodies detected with Avidin/Biotinylated enzyme Complex (ABC) reagents (Vector Laboratories), visualised with Vector Blue or diaminobenzidine (DAB, Vector Laboratories). Terminal deoxynucleotidyl transferase deoxyuridine triphosphate (dUTP) Nick End Labelling (TUNEL) assay was used for the detection of apoptosis. Slides were incubated with terminal deoxynucleotidyl transferase (TdT) and digoxigenin-dUTP (Roche). Detection was performed with an alkaline phosphatase-conjugated antibody to digoxigenin (Roche), visualised with 5-bromo-4-chloro-3-indoly-phosphate/p-nitroblue tetrazolium (Vector). 1% eosin was used as the counterstain. Digital images were acquired, and stained areas were expressed as a percentage of total plaque area (for SMA), or the number of positive cells was expressed as a percentage of the total number of plaque cells (Ki67 and TUNEL staining).

Human atherosclerosis
Following Ethical Committee approval (REC97/084), human atherosclerotic plaques were obtained following carotid endarterectomy, and normal aortas from patients undergoing aortic valve surgery or root replacement. DNA was isolated for mtDNA copy number from whole plaques or normal aorta. Plaques were also microdissected into regions containing the fibrous cap, shoulder region, necrotic core, and underlying media for analysis by Seahorse (see below). Human VSMCs were cultured from normal aortas and plaques as previously described and used below passage 3 for Seahorse, or expanded for lentivirus infection, qPCR or Western blotting.

Seahorse assay
Mitochondrial respiration was determined using a Seahorse XF24e extracellular flux analyzer (Seahorse Bioscience) as per the manufacturer’s instructions. Five tissue pieces of approximately 1 mg were dissected from carotid endarterectomies into regions representing underlying media, shoulder region, cap and core and placed into 24-well Seahorse islet capture plates filled with 250 µl pre-warmed Dulbecco’s modified Eagle’s medium (DMEM) sparing four temperature control wells. Islet capture screens were applied, medium replaced with 250 µl fresh DMEM and remaining air bubbles removed. Final assay concentrations were: oligomycin 10 µg/ml, FCCP 1 µmol/L, rotenone 10 µmol/L and antimycin 10 µmol/L as described previously. Four measurements were obtained at baseline and following injection of the above compounds. Following analysis, all tissue pieces were retrieved, excess fluid removed, weighed and fixed in neutral-buffered formalin before staining with hematoxylin and eosin (H+E) for confirmation of segment identity and assessment of cell number.

For cells, VSMCs or macrophages were plated into XF96 microplates and allowed to adhere for 24 hours. Basal oxygen consumption rate (OCR) was determined, and then sequential additions of oligomycin (1 µg/ml), FCCP (12 µmol/L) and antimycin/rotenone (10/1 µmol/L) performed. OCR was normalized to either wet weight and cell nuclei (plaque tissue) or protein content (cells) determined by the Bradford method.

Quantitative PCR for mtDNA damage and copy number
To assess for global mtDNA damage, amplification of a 10 kb segment of mitochondrial DNA spanning 60% of the mitochondrial genome was performed. To control for mtDNA copy number, this was compared to the amplification of a short target.

Long primers: used at 0.5 µM producing a 10150 bp product: FWD: GCCAGCCTGACCCATAGCCATAAT, REV: GAGAGATTATATGGGTGTAATGCG. Short primers: used at 0.5 µM producing a 127 bp product: FWD: GCCAGCCTGACCCATAGCCATAAT, REV: GCCGGCTGCGATTCTACGTTA.

QPCR was performed in triplicate, on 20 ng of sample DNA, in 20 µl reactions on a Rotor-Gene™ 6000 QPCR thermocycler (Qiagen). Rotor-gene SYBR Green Mix (Qiagen) was used for the short reaction. PFU Ultra II Hotstart PCR Master Mix (#600850 Agilent) was used for the long reaction, with 1 in 400 dilution of Eva Green (#31000 Biotium). Cycling parameters for the short reaction were 95°C for 5 minutes followed by 45 cycles of 95°C for 5s, 60°C for 10s. Conditions for the long amplification were 94°C for 2 minutes followed by 45 cycles of 92°C for 30s, 71°C for 30s, and 68°C for 5 minutes. Ct values were identified using the Rotorgene software, and the relative amplification of the 10kb product calculated by the comparative Ct method. DNA lesion frequencies were calculated using the Poisson transformation.

For assessment of mouse mitochondrial DNA copy number, the amplification of a short segment of mtDNA was compared to the amplification of a short segment of nuclear (β globin) DNA. The short mtDNA primers were used as above; Primers used at 0.5 µmol/L were as follows: MtDNA primers: producing a 127 bp product, FWD: GCCAGCCTGACCCATAGCCATAAT REV: GCCGGCTGCGATTCTACGTTA.

Nuclear primers: producing a 155 bp product
FWD: TTGAGACTGTGATTGGCAATGCCT, REV: CCAGAAATGCTGGGCGCTCACT.

Human mitochondrial DNA copy number was assessed using primers (900nmol/L) that have previously been described. Relative mtDNA expression was calculated for two short segments and the average of these measurements was used.

Mitochondrial primers:
FWD: CACCCCAAGAACCAGGTTTG
REV: TGGCCATGGGTATGTGTTAA

FWD: CGTCATTATGGCTCAA
REV: GATGGAGACATACAGAAATAG

Nuclear primers:
FWD: TGCTGTCTCCATGTTGTATCT
REV: TCTCTGCTCCCCACCTCAGTT

QPCR was performed in triplicate, on 10 (human) -20 (mouse) ng of sample DNA, on a Rotor-Gene™ 6000 QPCR thermocycler (Corbett Research). Rotor-gene SYBR Green Mix (Qiagen) was used for the reaction, with the following cycling parameters: Mouse: 95°C for 5 minutes followed by 45 cycles of 95°C for 5s, 60°C for 10s. Human: 50°C for 2 minutes and 95°C for 10 minutes followed by 40 cycles of 95°C for 15s and 60°C for 60s. Relative expression was calculated by the comparative Ct method.

RNA isolation and Quantitative PCR
As per manufacturer’s instructions, RNA was isolated from aortas and cells using TRIzol Reagent (Thermo Fisher Scientific). RNA was treated with RQ1 RNase-Free DNase treatment and cDNAs synthesized using Oligo(dT) primers and reverse transcription system (Promega). QPCR analysis for was performed using the following primers at 0.5 µmol/L:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Twinkle</td>
<td>GCCACGTGACTCTGGTCATTTC</td>
<td>CCATCAAAGCGATTCTTGAGACA</td>
</tr>
<tr>
<td>β actin</td>
<td>GGCACCACACCTCTCAACATG</td>
<td>GTGGTTGGGAGCTGAGGCGC</td>
</tr>
</tbody>
</table>

QPCR was performed in triplicate, in 20 µl reactions. Reaction reagents and conditions were as described for copy number qPCR.

**Western blotting**

Western blotting was performed on 20 µg of aortic, plaque or cellular protein loaded on to a 4-12% polyacrylamide gel and separated by electrophoresis. Proteins were transferred to PVDF membranes, blocked for 1 h in 5% non-fat milk and probed. Primary antibodies included total OXPHOS rodent antibody (1:500)(ab110413), citrate synthase (1:500-1:1000)(ab96600), manganese superoxide dismutase (1:5000)(ab13533), DNA polymerase gamma (1:1000)(ab128899), actin (1:10000)(ab8227)(all from abcam), tubulin (1:500)(2148s), PINK1, (1:1000)(6946), TFAM (1:1000)(7495s)(all from Cell Signaling Technologies), and PEO1 (Twinkle)(1:1000)(ARP36483_P050)(from Aviva Systems Biology).

**Aortic respiration**

To assess mitochondrial respiration, descending aortas were dissected and maintained in preservation solution (BIOPS; 10mM Ca-EGTA buffer, 0.1 µM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM MES, 0.5 mM dithiothreitol, 6.56 mM MgCl₂, 5.77 mM ATP, 15 mM phosphocreatine, pH 7.1) at 4°C. Samples were permeabilized in 50 µg/ml saponin solution and washed in respiration buffer (0.5 mM EGTA, 3 mM MgCl₂·6H₂O, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH₂PO₄, 20 mM HEPES, 110 mM mannitol, 0.3 mM dithiothreitol, 1 mg/ml fatty acid free BSA, pH 7.1). Respiration was assessed at 37°C with a Clarke-type oxygen electrode (Strathkelvin Instruments Ltd, UK).

Complex I-supported respiration rates were acquired using 10 mM glutamate and 5 mM malate. 5mM ADP was then added to stimulate State 3 respiration. After the addition of 1 µM rotenone, complex II-supported respiration was assessed with 10 mM succinate. Following complex III inhibition by 5 µM antimycin, 0.5 mM N,N,N′,N′-Tetramethyl-p-phenylenediamine (TMPD) and 2 mM ascorbate were used to induce complex IV respiration. The intactness of the outer mitochondrial membrane was assessed by adding 10 µM cytochrome C. Tissues were removed from the electrode chambers and dried, with oxygen flux expressed as nanomoles O₂ per minute per dry weight.

**Cell isolation and culture**

For the differentiation of bone marrow-derived macrophages (BMDMs) bone marrow cells were isolated and cultured for 7 days in RPMI 1640 (Invitrogen) supplemented with 20% Fetal Bovine Serum (FBS) and 15% L929-conditioned medium. Isolation purity was determined by flow cytometric staining for CD11b and F4/80. Vascular smooth muscle cells (VSMCs) were cultured from explanted aortas. The aortas were dissected free of surrounding fat and connective tissue, divided into small pieces, and cultured with Dulbecco’s Modified Eagle Medium (DMEM) (Sigma UK) supplemented with 20% FBS.

**Cellular ROS assay**
To assess ROS cells were incubated in 10 µM 5-(and 6) 2’7’-dichlorodihydrofluorescein diacetate (DCFDA) (Invitrogen #C6827) for 30 minutes at 37°C. Cells were washed with PBS and relative fluorescence index was measured (Excitation 485 nm, Emission 528 nm).

**Cellular proliferation and apoptosis assays**

VSMC proliferation was measured using the CyQuant cell assay (Invitrogen), a nucleic acid-based fluorescent assay, according to the manufacturer’s instructions. Fluorescence was measured on a Synergy HT Plate Reader (Biotek Instruments) with standard curves generated from known numbers of cells. For macrophage and VSMC apoptosis assessment, cells were seeded into 24-well plates and incubated with 50 µmol/L tert-butyl hydroperoxide (t-BHP) for 16 hours. Cells were then stained with propidium iodide and Annexin V Alexa Fluor 488 (Thermo Fisher Scientific) as per manufacturer’s instructions, and analyzed on an Accuri C6 flow cytometer (Becton Dickinson).

**Cytokine assay**

Bone marrow derived macrophages were incubated with 1 µg/ml lipopolysaccharide (LPS) *E. coli* strain 0111:B4 (Sigma # L2630) for 16 hours. The culture supernatant was collected and analysed for cytokines using Invitrogen mouse bead assays (Invitrogen) on a Becton Dickinson Accuri C6 flow cytometer (Becton Dickinson Inc, USA). Known concentrations of standards were used to generate standard curves, and cytokine concentrations calculated using FlowCytomix Pro software (eBioscience, USA).

**Mitophagy assay**

The plasmid containing mitochondrially-targeted Keima (Keima-pLESIP) was a gift from Dr. Toren Finkel, NHLBI, USA. Plasmids were propagated in One Shot OmniMAX™2-T1R chemically-competent *E. coli*. HEK-293T cells were co-transfected with packaging plasmids pMD2.G, psPAX and Keima-pLESIP using FuGENE 6 (Roche) according to standard techniques. The transfection medium was replaced with fresh culture medium after 24 hours, pseudo-lentivirus particles were harvested after 48 hours, concentrated using Amicon Ultra-15 centrifugal filter devices and lentivirus titers determined using qPCR lentivirus titration (titer) kit (abm, LV900) according to manufacturer’s instructions. VSMCs were transduced at an MOI of three in the presence of 8 µg/ml Polybrene (Sigma) and after an overnight incubation the medium was replaced with fresh culture medium. Infected cells were puromycin-selected and plated into MatTek gamma irradiated, poly-d-lysine coated, 35mm glass bottom wells before treatment, co-staining with Hoechst 33342 and imaging using an SP5 confocal microscope (Leica).

Assessment of mitophagy using mitochondrial keima has previously been described. In short, fluorescence of keima was imaged in two separate channels after consecutive excitation at 458nm (coded green) and 561nm (coded red) using a 580nm to 690nm emission range. Hoechst was imaged in a third channel after excitation by UV light. At least 20 z-stacks per group were acquired in at least three independent experiments from three different primary cell cultures per group using a 63x/1.40NA oil immersion objective. ImageJ plug-in, originally designed for assessing mitochondrial morphology (Richard Butler, Gurdon Inst, Cambridge), was used to quantify fluorescence and the mitophagy index (ratio red/green) as a marker of mitophagy was calculated.

**Statistical analysis**

Data were tested for a normal distribution by examination of the histogram and normal probability plot, and by using the Shapiro-Wilk test. Student’s t test was used for pair-wise comparison for data following a normal distribution, and Mann Whitney U test for non-parametric data. ANOVA with Bonferroni’s correction was used to compare means of multiple groups. Values are presented as means ± SEM. p<0.05 was considered significant.


