Mitochondrial Oxidative Stress Mediates Macrophage Pro-inflammatory Metabolic Switch in Atherosclerotic Vascular Disease in Aging

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ABSTRACT

Aging elevates cardiovascular disease risk, including atherosclerosis. Macrophages play crucial role vascular aging by promoting inflammation and atherosclerosis progression. Age-related increase in NOX4 NADPH oxidase expression correlates with mitochondrial dysfunction, inflammation, and atherosclerosis severity. We hypothesized that NOX4-dependent mitochondrial oxidative stress induces macrophage metabolic dysfunction and an inflammatory phenotype in aging-associated atherosclerotic disease.

Aortic and brachiocephalic artery lesion areas were comparable in 5-month-old (young) $Nox4^{-/-}/Apoe^{-/-}$ and $Apoe^{-/-}$ mice, increased significantly in 16-month-old (aged) mice, but were significantly lower in $Nox4^{-/-}/Apoe^{-/-}$ versus $Apoe^{-/-}$ mice. In aged $Nox4^{-/-}/Apoe^{-/-}$ mice, atherosclerotic lesions had reduced CD11b+ area, lower expression of CCL2, IL1β, and IL6, and fewer classically activated pro-inflammatory macrophages (CD38+CD80+). Notably, there was also an increased proportion of alternatively activated pro-resolving macrophages (CD163+CD206+). Spectral flow cytometry and t-SNE analysis revealed a significantly lower proportion of activated inflammatory macrophages and macrophage-like cells in atherosclerotic lesions of aged $Nox4^{-/-}/Apoe^{-/-}$ compared to $Apoe^{-/-}$ mice. Macrophages from aged $Apoe^{-/-}$ mice had altered metabolic function. In contrast, macrophages from $Nox4^{-/-}/Apoe^{-/-}$ mice were less glycolytic, more aerobic, and had preserved basal and maximal respiration and mitochondrial ATP production. $Nox4^{-/-}/Apoe^{-/-}$ macrophages had lower mitochondrial ROS and reduced IL1β secretion, compared with $Apoe^{-/-}$ mice. In aged $Apoe^{-/-}$ mice, inhibition of NOX4 using GKT137831 significantly reduced macrophage ROS and improved mitochondrial function. This resulted in a decreased CD68+CD80+ and increased CD163+CD206+ lesion macrophages and attenuated atherosclerosis.

Our results imply that NOX4-dependent mitochondrial oxidative stress in aging contributes to macrophage mitochondrial dysfunction, glycolytic metabolic switch, and pro-inflammatory phenotype, advancing atherosclerosis. Inhibition of NOX4 could alleviate vascular inflammation and atherosclerosis by improving mitochondrial function in macrophages.
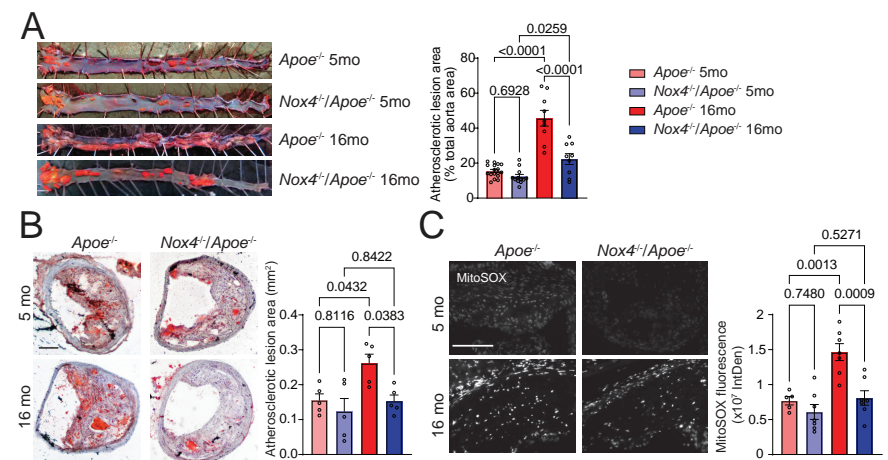
RESULTS

Figure 1. **Aging-associated atherosclerosis burden is attenuated in *Nox4*-deficient *Apoe⁻/⁻* mice.** (A) Representative images of oil red O-stained aortas and quantification of atherosclerotic lesion area in young (5-month-old) and aged (16-month old) $Apoe^{-/-}$ and $Nox4^{-/-}/Apoe^{-/-}$ mice fed Western diet for 3 months (mean±SEM, n=8). (B) Representative images of oil red O-stained brachiocephalic artery sections and quantification of atherosclerotic lesion area (mean±SEM, n=5). (C) Representative fluorescence microscopy images and fluorescence quantification in brachiocephalic artery sections stained with MitoSOX. Data are mean±SEM, n=7.
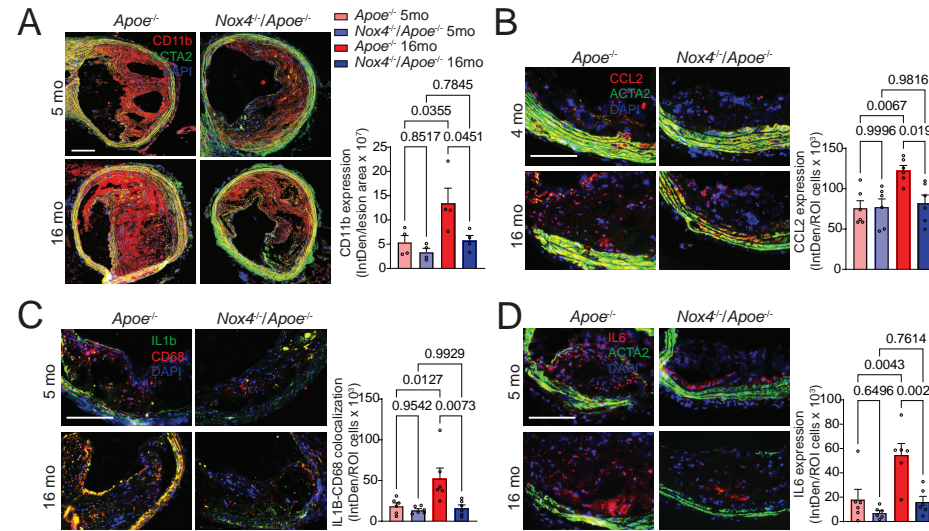

Figure 2. **Increased NOX4 expression in aging is associated with vascular inflammation.** (A) Representative fluorescence microscopy images and quantification of immunoreactive CD11b expression (red) in brachiocephalic artery sections stained for ACTA2 (green) and DAPI (blue). Data are fluorescence integrated density of CD11b expression per lesion area (mean±SEM, n=4). (B-D) Representative fluorescence microscopy images and quantification of immunoreactive CCL2 (B), IL1b (C), and IL6 (D) expression in brachiocephalic artery sections stained for ACTA2 (B&D) or CD68 (C) and DAPI (B-D). Data are fluorescence integrated density of expression/colocalization per lesion cell number (mean±SEM, n=6).
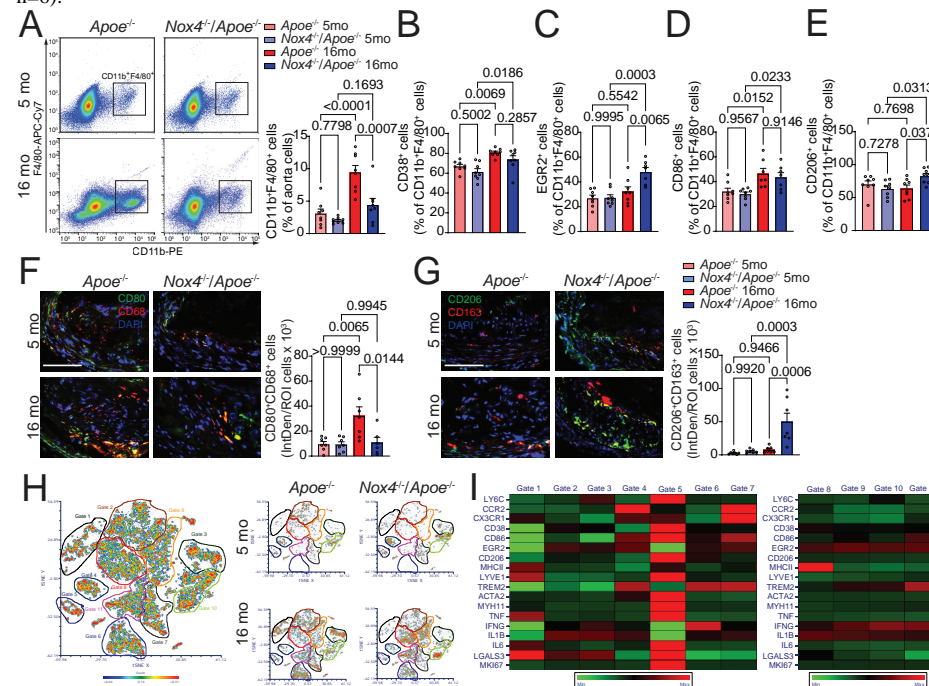

Figure 3. **_Nox4_ deficiency induces pro-resolving phenotype in atherosclerotic lesion macrophages in aged _Apoe⁻/⁻_ mice.** (A) Flow cytometry analysis and quantification of atherosclerotic lesion single cell suspension from young and aged $Apoe^{-/-}$ and $Nox4^{-/-}/Apoe^{-/-}$ mice for CD11b+F4/80+ macrophages. Data are macrophage fraction of aorta cells (mean±SEM, n=6). (B-E) Flow cytometry analysis and quantification of CD38+ (B) and EGR2+ (C) or CD86+ (D) and CD206+ (E) cell fraction of CD11b+F4/80+ aorta macrophages (mean±SEM, n=6). (F-G) Representative fluorescence microscopy images and quantification of CD80+CD68+ (F) and CD206+CD163+ (G) macrophages in brachiocephalic artery sections from young and aged $Apoe^{-/-}$ and $Nox4^{-/-}/Apoe^{-/-}$ mice. Data are fluorescence colocalization integrated density per lesion cell number (mean±SEM, n=6). (H) t-SNE clustering of spectral flow cytometry data from aortic atherosclerotic lesions. (I) Heat-map representation of macrophage markers relative expression for each distinct cluster.
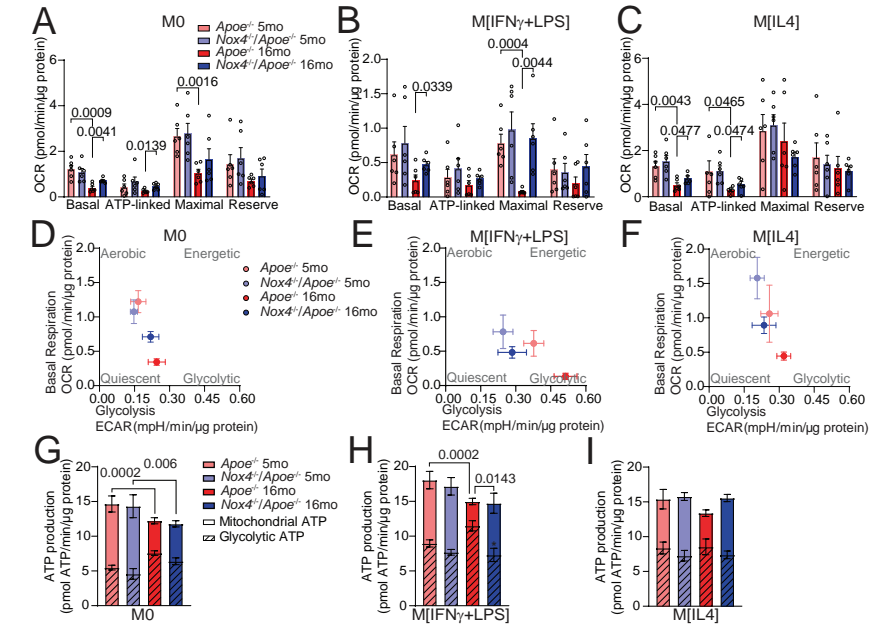

Figure 4. **Mitochondrial function and metabolic profiling of macrophages from young and aged _Apoe⁻/⁻_ and _Nox4⁻/⁻Apoe-/-_ mice.** (A-C) Oxygen consumption rate (OCR) measurements and mitochondria bioenergetic parameters were determined in control M0 (A), M[IFNγ+LPS] (B), and M[IL4] (C) cultured macrophages (mean±SEM, n=6). (D-E) Metabolic profiling showing basal respiration and glycolysis relations in control M0 (D), M[IFNγ+LPS] (E), and M[IL4] (F) cultured macrophages (mean±SEM, n=6). (G-I) Mitochondrial and glycolytic contribution to ATP production in control M0 (G), M[IFNγ+LPS] (H), and M[IL4] (I) cultured macrophages (mean±SEM, n=6).
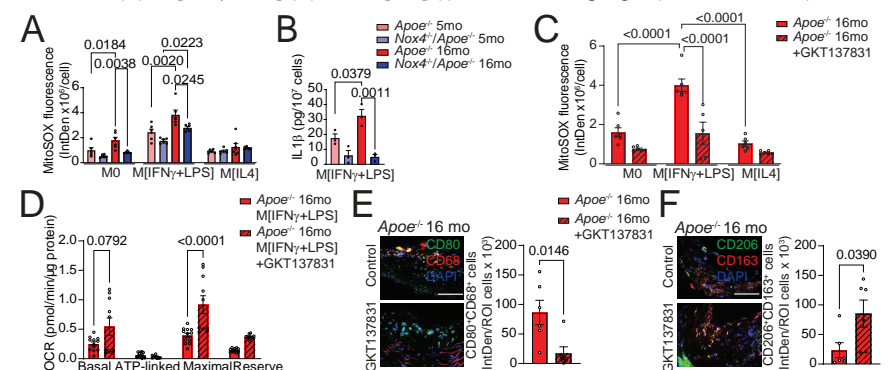

Figure 6. **Inhibition of NOX4 improves mitochondrial function inducing pro-resolving phenotype in atherosclerotic lesion macrophages in aged _Apoe⁻/⁻_ mice.** (A) Quantification of MitoSOX fluorescence in control M0, M[IFNγ+LPS]- and M[IL4] macrophages isolated from young and aged $Apoe^{-/-}$ and $Nox4^{-/-}/Apoe^{-/-}$ mice (mean±SEM, n=6). (B) Concentration of IL1β in conditional media from M[IFNγ+LPS] macrophages (mean±SEM, n=4). (C) Quantification of MitoSOX fluorescence in cultured control M0, M[IFNγ+LPS] or M[IL4] macrophages pre-treated with vehicle or GKT137831 (mean±SEM, n=6). (D) Mitochondrial bioenergetic parameters were derived from OCR in aged $Apoe^{-/-}$ M[IFNγ+LPS] macrophages pre-treated with vehicle or GKT137831 (mean±SEM, n=12). (E-F) Representative fluorescence microscopy images and quantification of CD80+CD68+ (E) and CD206+CD163+ (F) macrophages in brachiocephalic artery sections counterstained with DAPI from aged $Apoe^{-/-}$ mice treated with GKT137831 (mean±SEM, n=6).

CONCLUSIONS

- Aging-associated increase in NOX4 expression/activity leads to mitochondrial dysfunction in macrophages, a metabolic shift towards glycolysis, and a proinflammatory phenotype
- An inflammatory plaque microenvironment causes lesion expansion in aging
- Reducing the expression/activity of NOX4 or improving mitochondrial function may help alleviate vascular inflammation and atherosclerosis