Forestalling age-impaired angiogenesis and blood flow by targeting NOX: Interplay of NOX1, IL-6, and SASP in propagating cell senescence

Yao Li[a,b], Damir Kračun[a,b], Christopher M. Dustin[a,b], Mohamed El Massry[a,b], Shuai Yuan[a,b,c], Christian J. Goossen[a,b], Evan R. DeVallance[a,b], Sanghamitra Sahoo[a,b], Cynthia St. Hilaire[a,d,e], Aditi U. Gurkar[f,g,h], Toren Finkel[f], Adam C. Straub[a,b,c], Eugenia Cifuentes-Pagano[a,b], and Patrick J. Pagano[a,b,1]

[a]Pittsburgh Heart, Lung, Blood and Vascular Medicine Institute, University of Pittsburgh, Pittsburgh, PA 15261; [b]Department of Pharmacology and Chemical Biology, University of Pittsburgh, Pittsburgh, PA 15261; [c]Center for Microvascular Research, University of Pittsburgh, Pittsburgh, PA 15261; [d]Division of Cardiology, Department of Medicine, University of Pittsburgh, Pittsburgh, PA 15213; [e]Department of Bioengineering, University of Pittsburgh, Pittsburgh, PA 15260; [f]Aging Institute of University of Pittsburgh Medical Center, University of Pittsburgh School of Medicine, Pittsburgh, PA 15219; [g]Division of Geriatric Medicine, Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213; and [h]Geriatric Research, Education and Clinical Center, Veterans Affairs Pittsburgh Healthcare System, Pittsburgh, PA 15240

Edited by Judith Campisi, Buck Institute for Research on Aging/Lawrence Berkeley National Laboratory, Novato, CA, and approved August 20, 2021 (received for review August 4, 2020)

In an aging population, intense interest has shifted toward prolonging health span. Mounting evidence suggests that cellular reactive species are propagators of cell damage, inflammation, and cellular senescence. Thus, such species have emerged as putative provocateurs and targets for senolysis, and a clearer understanding of their molecular origin and regulation is of paramount importance. In an inquiry into signaling triggered by aging and proxy instigator, hyperglycemia, we show that NADPH Oxidase (NOX) drives cell DNA damage and alters nuclear envelope integrity, inflammation, tissue dysfunction, and cellular senescence in mice and humans with similar causality. Most notably, selective NOX1 inhibition rescues age-impaired blood flow and angiogenesis, vasodilation, and the endothelial cell wound response. Indeed, NOX1i delivery in vivo completely reversed age-impaired hind-limb blood flow and angiogenesis while disrupting a NOX1-IL-6 senescence-associated secretory phenotype (SASP) proinflammatory signaling loop. Relevant to its comorbidity with age, clinical samples from diabetic versus nondiabetic subjects reveal as operant this NOX1-mediated vascular senescence and inflammation in humans. On a mechanistic level, our findings support a previously unidentified role for IL-6 in this feedforward inflammatory loop and peroxisome proliferator–activated receptor gamma (PPARγ) down-regulation as inversely modulating p65-mediated NOX1 transcription. Targeting this previously unidentified NOX1-SASP signaling axis in aging is predicted to be an effective strategy for mitigating senescence in the vasculature and other organ systems.

endothelium | senescence | aging | NADPH oxidase | IL-6

As the US and world population ages and prevalence of diseases that plague the elderly grows, there has been an ever-burgeoning interest in the aging process, with a goal toward prolonging life and health span. Notably, decreased lifespan and disease comorbidities are strongly associated, a pattern typified in cardiopulmonary diseases (1). However, specific targeted therapies combating descent of vascular cells into senescence and the overall vascular aging process are still in their infancy. Senescence is an irreversible form of cell cycle arrest initiated by various stimuli including genotoxic and oxidative stress (2), leading to DNA damage, oncogene activation, and telomere shortening (3). Augmented senescence is deleterious to organ function vis-à-vis interference with self-renewal.

Aging is a major risk factor to chronic disease, of which Type II diabetes is a major comorbidity (4). As such, diabetes is a recognized cause of accelerated aging through impaired glucose tolerance and hyperglycemia (5). It is widely acknowledged that aging with or without diabetes is a risk factor for the development of cardiovascular pathologies in the elderly, including hypertension, atherosclerosis, stroke, and peripheral vascular disease. The latter remain the leading causes of morbidity and mortality in modern societies (5). A common pathway shared by aging and hyperglycemia is cellular senescence (5).

As first reported by Harman (6), oxidative stress was introduced as one driver of the aging process, and subsequent studies inferred a link between it and senescence. Primary sources of reactive oxygen species (ROS) across cells and organ systems are NADPH oxidases (NOXs) (7–12). Inasmuch as endothelial cells (ECs) are essential to normal health and their dysfunction pivotal to disease, we examined young versus aged ECs and interrogated mechanisms linking NOX, senescence, and vascular dysfunction.

Revelatory of the clinical significance of diabetes in the aged population, hyperglycemia was recapitulated in mouse and human ECs as a compounding factor for senescence and EC and tissue dysfunction. Indeed, we postulated that hyperglycemia would act as "proxy" stimulator of EC senescence (13, 14). Herein, we present data indicating NADPH oxidase-1 (NOX1) promotes autoamplification of NOX1 and senescence-associated secretory phenotypes (SASP) in driving inflammation and

Significance

In the current study, a feedforward signaling loop is revealed involving inflammation, NADPH oxidase 1, cell cycle arrest, DNA damage, loss in nuclear integrity, and senescence. Selective NOX1 inhibition rescues age-impaired blood flow and angiogenesis, abrogates senescence, and restores vascular function. NF-κB and peroxisome proliferator–activated receptor gamma counter-regulate NOX1; and IL-6 and NOX1 are central to a degenerative spiral. Clinical samples from aged diabetic human subjects corroborate this NOX1-SASP signaling nexus in humans and authenticate the potential for NOX1i as therapy.

Author contributions: Y.L., D.K., and P.J.P. designed research; Y.L., D.K., C.M.D., M.E.M., S.Y., C.J.G., E.R.D., and S.S. performed research; C.S.H. contributed new reagents/analytic tools; Y.L., C.M.D., M.E.M., S.Y., C.J.G., E.R.D., E.C.-P., and P.J.P. analyzed data; Y.L., D.K., and P.J.P. wrote the paper; and A.U.G., T.F., A.C.S., and E.C.-P. contributed conceptually and helped design the study.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

[1]To whom correspondence may be addressed. Email: pagano@pitt.edu.

This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2015666118/-/DCSupplemental.

Published October 15, 2021.

senescence. More specifically, we provide key insight into a role for NOX1 in exacerbated NOX1 up-regulation, increased ROS, $p21^{cip}$, p53, and $p16^{INK4a}$, SASP and γ-H2AX, decreased phospho-Rb and nuclear lamin B1, and a senescent and dysfunctional phenotype reversed by selective NOX1 blockade in vitro and in vivo. Remarkably, targeting NOX1 rescued age-impaired angiogenesis ex vivo and peripheral blood flow in vivo and nullified aging-induced senescence and inflammation. Additionally, blockade

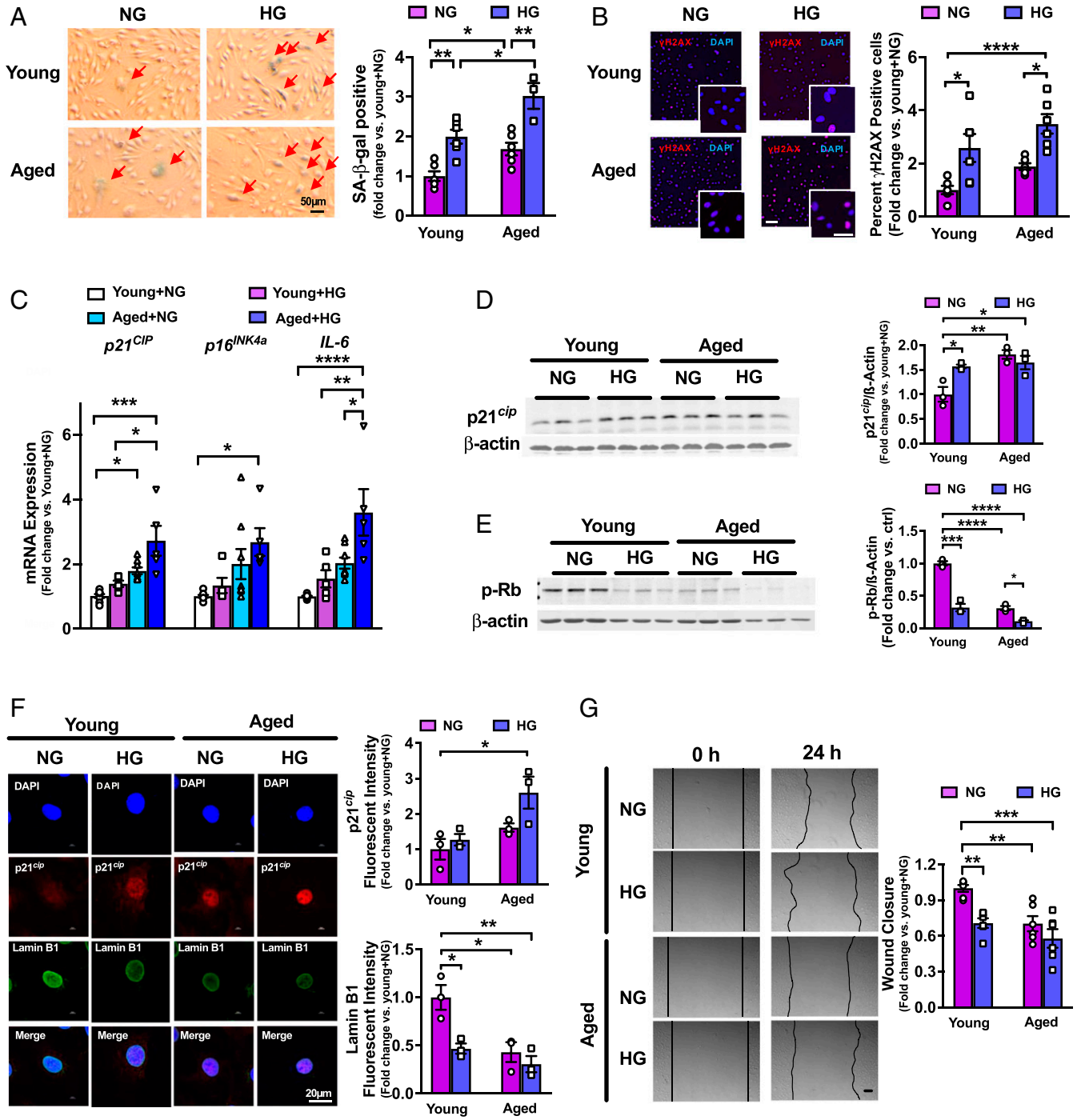

Fig. 1. Advanced-stage aging and HG induce senescence and inhibit proliferation/migration in MAECs. (*A*) SA-β-gal staining in MAECs from young (10-wk-old) and aged (58-wk-old) mice, grown in NG and HG EC growth media for 72 h ($n = 6$). (*B*) γ-H2AX (pink) immunofluorescent staining [20× (Scale bar, 100 μm) and 120× (Scale bar, 50 μm) magnification] of young and aged MAECs grown in NG and HG media. Nuclei were stained with DAPI (blue). Fluorescence intensity was quantified from three images/slide, $n = 6$ independent experiments. (*C*) mRNA levels of $p21^{cip}$, $p16^{INK4A}$, and IL-6 of young and aged MAECs treated with NG and HG EC media ($n = 5$ to 8). (*D* and *E*) Western blots and cumulative data showing the effects of aging and HG on the levels of $p21^{cip}$ and phospho-Rb. The levels of $p21^{cip}$ and p-Rb were normalized to the level of β-actin in the same sample ($n = 3$). (*F*) Immunofluorescent detection (200× magnification) of $p21^{cip}$ (red) and lamin B1 (green) grown in NG and HG EC media. Nuclei were stained with DAPI (blue). Fluorescence intensity was quantified from three images/slide, $n = 3$ independent experiments. (*G*) MAEC wound healing affected by age and HG treatment ($n = 6$). All data were analyzed with two-way ANOVA followed by Tukey's test. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

of IL-6 disrupted this cycle. Coming full-circle, data from human diabetic patients corroborate these findings. Our studies proffer a previously unidentified, tightly controlled signaling axis for drug intervention. With this broader and in-depth view comes opportunities to define and target a seminal and central cause of aging with curative consequences to multiple organ systems and an improved quality of life (15).

Results

Aging and Comorbid Hyperglycemic Stress Synergistically Induce IL-6 and Potentiate Indicators of DNA Damage and EC Senescence. Mouse aortic ECs (MAECs) from young (8 to 10 wk) and aged (58 wk) mice were treated with normal glucose (NG, 5 mM glucose + 20 mM mannitol for osmolarity control) or high glucose (HG, 25 mM glucose) for 72 h and contrasted for senescent markers, DNA damage, and proliferation/migration. Significantly more senescence-associated β-galactosidase (SA-β-gal)-positive cells were found in aged MAECs versus young under NG conditions (1.7 ± 0.2-fold). HG stress increased SA-β-gal by 2.0 ± 0.2-fold versus NG in young cells (NG – young) and age independently enhanced SA-β-gal compared to NG – young (1.5 ± 0.2-fold; Fig. 1*A*). Moreover, HG and age compounded levels compared to NG – aged (1.8 ± 0.2-fold; Fig. 1*A*). Staining for phosphorylated gamma histone 2AX (γ-H2AX; indicative of DNA damage and cellular senescence) revealed increased numbers of γ-H2AX–positive cells in response to HG in both groups with the most pronounced effect in HG-treated aged cells (Fig. 1*B*). The data are consistent with aging and hyperglycemia potentiating senescence and DNA damage. In support of this, cell cycle inhibitor $p21^{cip}$ messenger RNA (mRNA) (Fig. 1*C*) and protein (Fig. 1*D*) markedly increased with age, with the trascriptional effect potentiated in HG MAECs. This was followed in HG – aged MAECs by an increased nuclear $p21^{cip}$ translocation (Fig. 1*F*). Nuclear envelope protein lamin B1 markedly decreased with age and HG (Fig. 1*F*), demonstrative of a robust senescent phenotype by either stressor. Senescence modulator $p16^{INK4A}$ was significantly augmented, and a principle senescence-associated secretory phenotype (SASP) cytokine, interleukin 6 (IL-6), rose synergistically in HG – aged MAECs (Fig. 1*C*). These changes were accompanied by virtual ablation of phospho-Rb in each group (Fig. 1*E*). Taken together, the data point to decreased cell cycle progression and a more pronounced senescent state. Functionally, we compared proliferative and migratory function in young versus aged ECs in response to NG and HG with scratch wound healing. Wound closure (24- versus 0-h) was inhibited in aged versus young cells and by exposure to HG (Fig. 1*G*). In aggregate, our data are consistent with HG being a proxy instigator of age-induced senescence as postulated.

Selective NOX1 Inhibition Restores to Normal EC Senescence, Impaired Angiogenesis, and Endothelial Dysfunction. Postulating a role for NOX1 in the mediation of senescence in response to age and hyperglycemia, we showed that hydrogen peroxide (H_2O_2) production rates reached a statistical difference in HG – aged versus NG – young and NG – aged MAECs (*SI Appendix*, Fig. 1). Moreover, staining for ROS/lipid peroxidation marker 4-hydroxynonenal (4-HNE) as well as the footprint indicator of oxidation 3-nitrotyrosine (3-NT) revealed higher adduct formation with age or HG and a synergistic rise in response to both factors combined (Fig. 2 *A* and *B*).

In line with this, we found that mRNA levels of NOX1 and its canonical NOXO1 and NOXA1 cytosolic subunits all increased with age (Fig. 2*C*). Furthermore, core subunit NOX1 protein was sharply amplified both with age (Fig. 2*D*) or HG (Fig. 2*E*). Treatment with a selective NOX1 inhibitor [NOXA1ds (16–19), 10 μmol/L] abolished HG-induced protein nitration as shown by 3-NT staining (Fig. 2*F*) and H_2O_2 production in aged ECs supplemented with HG (HG – aged, Fig. 2*G*).

A role for NOX1 in aging/HG-induced senescence was further illustrated wherein NOXA1ds eradicated a more than six- and fivefold increase in the number of SA-β-gal–positive cells in response to aging and HG, respectively (Fig. 3 *A* and *B*). Consistent with these observations, NOXA1ds suppressed (by 59.3 ± 8.8%) γ-H2AX that was augmented approximately threefold in aged MAECs + HG compared to young NG cells (Fig. 3*C*). In addition, HG induced cell-cycle inhibitor $p21^{cip}$ and a loss in lamin B1, both of which were wholly reversed by NOXA1ds, reinforcing NOX1's role in senescence (Fig. 3*D*). Phenotypically, NOXA1ds rescued the 24- versus 0-h wound closure change of aged MAECs in HG media compared to young NG-treated cells (Fig. 3*E*), indicating that NOX1-mediated senescence is responsible for functional debilitation (reduced proliferation and migration). Furthermore, aortas from aged (18 mo) mice displayed suppressed endothelial sprouting compared to aortas from young mice (3 mo) (approximately threefold at 21 d). The sprouting response in aged arteries treated with NOXA1ds was restored ($P < 0.01$) and ostensibly surpassed that of young arteries, though the latter did not reach statistical significance (Fig. 3*F*). NOXA1ds reinstated endothelial sprouting in HG media back to normal growth (Fig. 3*G*). Additionally, aortas presented with endothelial dysfunction; that is, maximal dilatation of aortas from aged mice to endothelium-dependent vasodilator acetylcholine decreased by 62.9 ± 4.9% compared to young. In contrast, NOXA1ds markedly improved maximal dilatory capacity (from 37.0 ± 4.9 to 74.7 ± 7.8%; Fig. 3*H*).

Human ECs Mirror Senescence in Murine Cells. Cross-Validation of NOX1-Selective Inhibitor (NOXA1ds) with NOX1 Knockdown. Human aortic ECs (HAECs) were employed to cross-examine foundational experiments in the mouse. NOXA1ds ameliorated HG-induced reduction in 24-h HAEC wound closure (Fig. 4*A*). In addition, $p21^{cip}$ and p53 up-regulation by HG was blocked by NOXA1ds (Fig. 4*B*). NOXA1ds is highly selective for NOX1 (16). All the same, we corroborated NOXA1ds-elicited changes with NOX1 small interfering RNA (siRNA). In line with the results in MAECs and HAECs, NOX1 siRNA not only normalized SA-β-gal staining (Fig. 4*C*) but also $p21^{cip}$ and p53 levels (Fig. 4*D*) in cells in HG. In aggregate, these findings are strongly supportive of NOX1 playing a critical role in senescence in both mouse and human ECs.

Selective NOX1 Inhibition Restores Blood Flow and Suppresses NOX1, Senescent Markers, and SASP. To investigate in vivo the role of NOX1 in aging-induced senescence and functional decline, young and aged mice were treated with scrambled peptide (Scrm control) or NOX1-selective inhibitor (NOXA1ds; 20 mg/kg/d; intraperitoneal osmotic pumps) for 28 d. In accordance with in vitro findings, RT-qPCR detected increased NOX1 and SASP indicators IL-6 and monocyte chemoattractant protein 1 (MCP1) in vessels with age (Fig. 5*A*). NOXA1ds abolished NOX1 and IL-6, whereas MCP1 displayed a tendency toward a decrease (Fig. 5*A*). Elevated $p21^{cip}$ and $p16^{ink4a}$ were suppressed by NOXA1ds (Fig. 5*A*).

Furthermore, immunofluorescence imaging (IF) of tissue taken ex vivo from these animals revealed increased NOX1 coinciding with EC marker von Willebrand Factor (vWF) in aged aortic intima compared with young Scrm, a signal abrogated in aged mouse vessels treated with NOXA1ds (Fig. 5*B*). Finally, to assess the contribution of NOX1 to age-related blood flow, we utilized laser Doppler imaging to determine the rate of hind-limb femoral artery flow in aged versus young mice, with or without NOXA1ds. Hind-limb femoral artery blood flow was sharply reduced with aging (~50%, aged versus young Scrm) and rescued in mice treated with NOXA1ds (Fig. 5*C*).

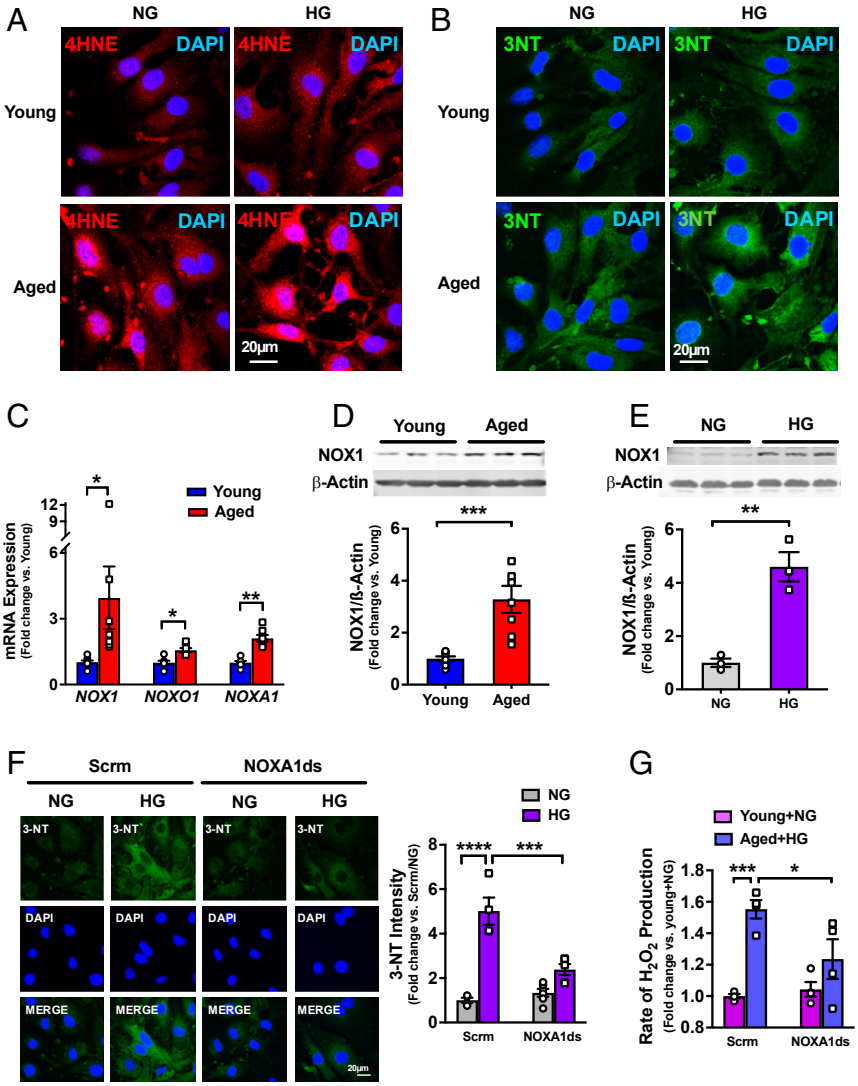

Fig. 2. NOX1-mediated ROS production is potentiated by aging and HG. (*A*) IF microscopy (120× magnification) detection of 4-hydroxynonenal (4-HNE) adducts (red) affected by aging and HG (representative of three biological replicates). Nuclei were labeled with DAPI (blue). (*B*) IF microscopy (120× magnification) detection of 3-NT formation (green) affected by aging and HG. Nuclei were labeled with DAPI (blue). (*C*) Effects of aging on the mRNA levels of NOX1, NOXO1, and NOXA1 ($n = 7$ to 8). (*D* and *E*) Effects of aging (*D*) and HG (*E*) on the protein levels of NOX1. Level of NOX1 is normalized to the level of β-actin in the same samples ($n = 3$ to 9). (*F*) Immunofluorescent microscopy (120× magnification) detection of 3-NT (green) upon HG stimulation of MAECs treated with NOX1-selective inhibitor NOXA1ds (10 μmol/L) or Scrm (10 μmol/L). Nuclei were stained with DAPI (blue). Fluorescence intensity was quantified from three images/slide, $n = 4$ to 6 independent experiments. (*G*) Effects of NOXA1ds (10 μmol/L) on aging plus HG-induced H_2O_2 production in MAECs measured by CBA assay ($n = 4$). Student's *t* test was used in panels *C*–*E*. Two-way ANOVA followed by Tukey's test was used in panels *F* and *G*. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

Corroboration in Tissue from Human Subjects. In aortas of anonymized diabetic versus sex- and age-matched human control subjects (*SI Appendix*, Table 1), we detected sharply increased protein expression of NOX1 and γ-H2AX, while metabolic regulator PPAR-γ was decreased (Fig. 5*D*). In addition, human transcripts corroborated higher NOX1 (approximately sixfold) and increased $p21^{cip}$, $p16^{ink4a}$ ($P = 0.056$), SASP, IL-6, and MCP1 in diabetic samples, indicative of increased senescence and inflammation converging with NOX1 (Fig. 5*E*).

Interplay of PPAR-γ, NF-κB, and IL-6 in Heightened Proinflammatory Effect of Age and Hyperglycemia. A unique aspect of this study was the ability to cross-examine senescence modulators from both the singular and combined effects of comorbid phenotype instigators. Inasmuch as aging and HG might stimulate NF-κB vis-à-vis oxidation, we examined whether its subunit p65 is activated in response to each stimulus alone or combined. Active, nuclear phospho-p65 (p-p65) rose sharply (approximately threefold) in human ECs in response to HG (*SI Appendix*, Fig. 2). This strong effect was substantiated in response to both age and HG. Indeed, with HG or age, we observed a robust increase in active p-65 (phospho-p65, *SI Appendix*, Fig. 3*A*) and its concomitant nuclear translocation (*SI Appendix*, Fig. 3*B*; white arrows, second column). The latter was reversed by PPAR-γ agonist rosiglitazone Rsg (white arrows indicating p65 return to cytosol, fourth column, *SI Appendix*, Fig. 3*B*).

Furthermore, PPAR-γ decreased sharply in response to HG (~50%), a response compounded by age (~90%, Fig. 6*A*). Indeed, Rsg partially suppressed γ-H2AX (20) and ameliorated wound closure (Fig. 6 *B* and *C*). Accordingly, Rsg was highly effective at reverting to normal age-related $p21^{cip}$ and HG-perturbed $p21^{cip}$ and lamin B1 (*SI Appendix*, Fig. 4 *A* and *B*), while PPAR-γ

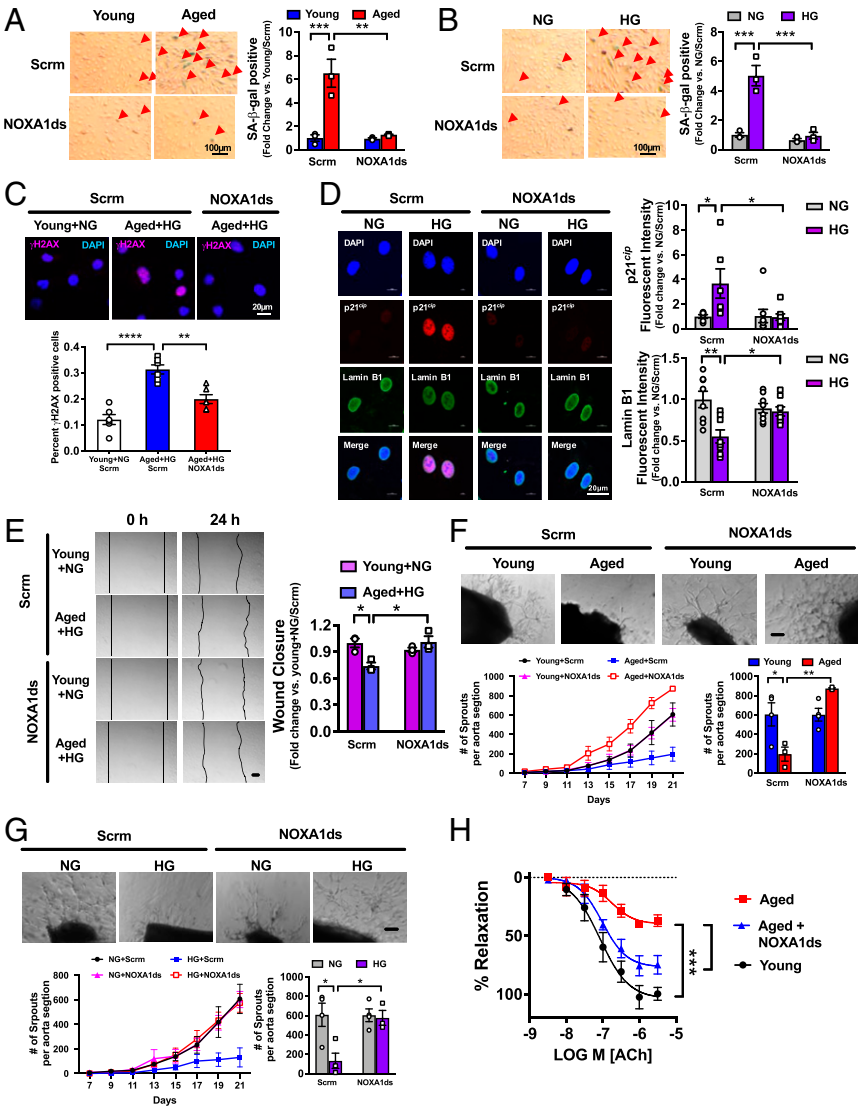

Fig. 3. NOX1 is involved in aging and HG-induced EC senescence, inhibition of wound healing, impaired angiogenesis, and dysfunction. (*A*) Effects of a NOX1-selective inhibitor NOXA1ds (10 μmol/L) on aging-induced increase in SA-β-gal staining in MAECs ($n = 3$). (*B*) Effects of NOXA1ds (10 μmol/L) on HG-induced increase in SA-β-gal staining in MAECs ($n = 3$). (*C*) Immunofluorescent microscopy (120× magnification) detection of γ-H2AX (pink) staining in MAEC nuclei elicited by aging plus HG and inhibited by NOXA1ds (10 μmol/L). Nuclei were stained with DAPI (blue), three images/slide, $n = 6$. (*D*) Immunofluorescent microscopy (200× magnification) detection of lamin B1 (green, nuclear envelope) and $p21^{cip}$ (red) staining showing the effects of NOXA1ds (10 μmol/L) on HG-induced $p21^{cip}$ up-regulation and lamin B1 suppression. Nuclei were stained with DAPI (blue). Merge with blue nuclear DAPI, purple indicates nuclear $p21^{cip}$, three images/slide, $n = 3$. (*E*) Effects of NOXA1ds (10 μmol/L) on 24-h wound healing response of aged cells treated with HG compared to young cells cultured in NG media ($n = 3$) (Scale bar, 100 μm). (*F*) Effects of NOXA1ds (10 μmol/L) on aging (18-mo-old)-induced reduction in sprouting angiogenesis from mouse aortas compared to the young (3-mo-old) mouse aortas ($n = 3$ to 4) (Scale bar, 100 μm). (*G*) Effects of NOXA1ds (10 μmol/L) on HG-induced reduction in sprouting angiogenesis from aortas of young (3-mo-old) mice ($n = 4$) (Scale bar, 100 μm). (*H*) Acetylcholine (Ach)-induced endothelium-dependent vasodilation was impaired in aortas from aged (18-mo-old) mice compared to the young (3-mo-old) mice, and NOXA1ds (30 μmol/L, 1 h) ameliorated endothelial dysfunction in aortas from aged mice ($n = 3$–4 for each group). One-way ANOVA followed by Tukey's test was used in panels *C* and *H*. Two-way ANOVA followed by Tukey's test was used in all the other panels. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

knockdown in normal cells increased p53, β-galactosidase expression, and decreased wound closure (*SI Appendix*, Fig. 4 *C*–*E*). Our data showing Rsg suppresses p65 translocation to the nucleus and NOX1 expression (*SI Appendix*, Figs. 3 and 4) authenticates PPAR-γ as a "brake" for NF-κB and NOX1 (21, 22). Thus, in our hands, in young cells when PPAR-γ levels are high or in aged/HG cells when PPAR-γ is activated, p65 translocation to the nucleus is prevented (22), thus limiting NF-κB–triggered NOX1 transcription (vide infra, Fig. 6*D*) (23) and senescence (*SI Appendix*, Figs. 3 *A* and *B*, 4 *A* and *B*, and 5 and Fig. 6 *A*–*C*). In the same vein, under conditions of aging and/or HG, PPAR-γ is markedly decreased (Fig. 6*A*). This is consistent with a) our findings shown in *SI Appendix*, Fig. 5, wherein HG stimulates NOX1 expression and Rsg eradicates NOX1; and b) an inverse countervailing relationship of PPAR-γ with NOX1 in the aging process.

Harking back to data presented in Fig. 5*A*, NOX1 inhibition in vivo disrupts NOX1 expression, senescence indicators, and suppresses SASP (IL-6 and MCP1), placing NOX1 at the center of the inflammatory cytokine response (i.e., SASP). We expanded on SASP analysis in vitro by examining not only IL-6 and MCP1 but also transforming growth factor beta (TGF-β), tumor necrosis factor alpha (TNF-α), and plasminogen activator inhibitor 1 (PAI1) in aged – HG cells, all of which mirrored in vivo changes in IL-6 and MCP1 (Fig. 5) and were sharply up-regulated

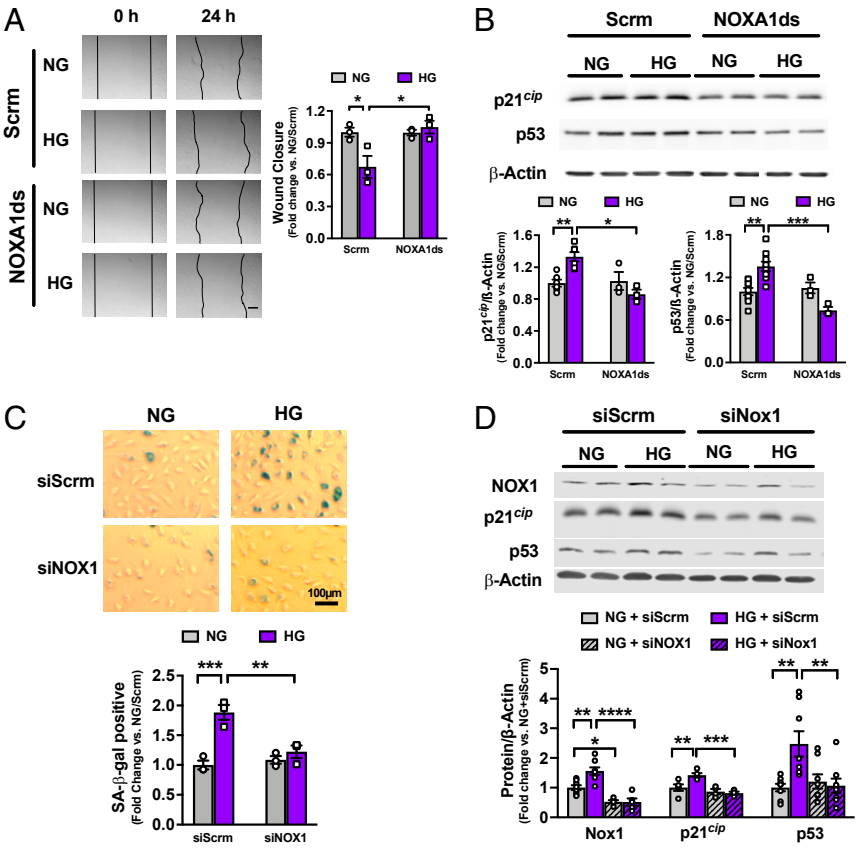

Fig. 4. NOXA1ds restores wound closure; NOXA1ds and NOX1 siRNA suppress NOX1 and senescent markers in HAECs. (*A*) Representative images (*Left*) and cumulative data (*Right*) showing the effects of NOXA1ds (10 μmol/L) on 24-h wound healing response of HAECs treated with HG compared to HAECs cultured in NG media (Scale bar, 100 μm) ($n = 3$). (*B*) Representative Western blots (*Upper*) and cumulative results (*Lower*) showing the effects of NOXA1ds (10 μmol/L) on HG-induced changes in $p21^{cip}$ and p53 levels in HAECs. The bands were normalized to the level of β-actin in the same samples ($n = 3$). (*C*) Effects of NOX1 siRNA (10 nmol/L) on HG-induced increase in SA-β-gal staining in HAECs ($n = 3$). (*D*) Effects of NOX1 siRNA (10 nmol/L) on HG-induced changes in various protein levels in HAECs. Bands were normalized to the level of β-actin in the same samples ($n = 5$ to 8). All data were analyzed with two-way ANOVA followed by Tukey's test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

(Fig. 6*E*). Moreover, the increase in NOX1 in vivo that drove senescence, tissue malfunction, and IL-6 and MCP1 (Fig. 5) was quelled in vitro by pretreatment with a selective IKK-1 and -2 inhibitor BMS 345541 [a selective inhibitor of NF-κB complex (24)] pointing to the primacy of regulation by NF-κB in this pathway (Fig. 6*D*). Furthermore, we asked the question of whether IL-6 can control NOX1 in age- plus HG-evoked inflammation. To that point, pretreatment with blocking IL-6 antibody (25 ng/mL) decreased NOX1 mRNA in aged – HG ECs (Fig. 6*F*). In aggregate, these data support a vicious cycle of NF-κB–mediated and IL-6–induced NOX1 as propagatory of the senescence phenotype.

Discussion

One of the hallmarks of aging is a decline in cardiovascular health. Indisputably, cardiovascular disease (CVD) is a significant health problem as it is the major cause of death in the United States and worldwide. It is increasingly being appreciated that a common factor across CVDs and a myriad of other pathologies is oxidative stress instigated by NOXs (10, 25). It should also be noted that NOXs and their metabolic products are emerging as signaling moieties in their own right, which over time and in response to various stimuli are up-regulated transcriptionally and translationally, transitioning the cell from homeostatic to injurious states.

Despite an enduring interest in the contribution of redox signaling to aging, the role of NOX in senescence modulation and its clinical implications remain unknown. Herein, our studies employing cell culture of murine and human cells, in vivo translational experiments, and human tissue may be considered groundbreaking in that they reveal a prominent functional role for NOX1 in EC senescence and dysfunction in response to two comorbid factors in humans and provide evidence for the clinical utility of a NOX1 inhibitor to stave off cardiovascular degeneration with aging and hyperglycemia. The salient findings of the current study are that 1) NOX1 plays a prominent role in murine and human senescence, aging-related vascular dysfunction, as well as impaired angiogenesis and blood flow (i.e., a NOX1-selective inhibitor restores these impairments to normal); 2) NOX1 is promoted by and propagates DNA damage, senescence, and SASP expression; 3) NOX1 is at least partially induced by IL-6; 4) this signaling nexus is corroborated in tissue from human diabetic patients. Moreover, the studies are demonstrative of 5) a mechanism by which NOX1 is derepressed with aging (and hyperglycemia) of mouse and human cells; and 6) a counterregulatory interplay between NF-κB and SASP/IL-6 in this pathway. The findings underscore the basic, translational, and potential clinical significance of selective NOX1 inhibition in the preemption of senescence and tissue dysfunction. Indeed, we expect our findings herein to provide insight into NOX1's role in cellular senescence. Moreover, as ECs are found in all vascularized organ systems, the direct or paracrine implications for this objective cannot be overestimated.

Our findings suggest that in young and healthy ECs, PPAR-γ suppresses NOX1-derived ROS via inhibition of NF-κB, maintaining

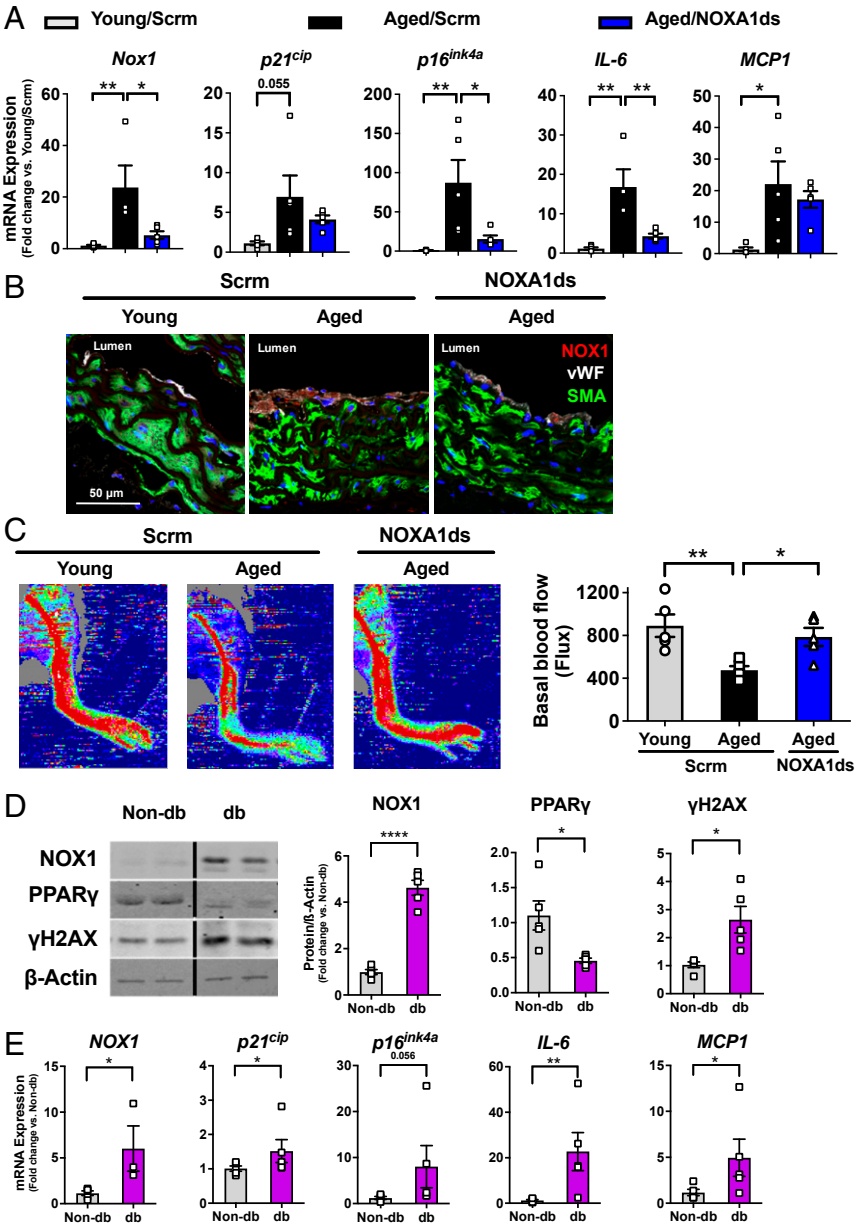

Fig. 5. In vivo administration of NOX1-selective inhibitor NOXA1ds decreases NOX1 and SASP, ameliorates senescence, and improves blood flow in aged (18-mo) mice. Comparison of NOX1, PPAR-γ, senescent markers, and SASP expression in human aortas from diabetic (db) and nondiabetic (Non-db) patients. (*A*) Effects of in vivo administration of NOXA1ds (20 mg/kg/d, 28 d) compared to Scrm on expression of NOX1, senescent markers, and SASP genes in aortas from aged (18-mo) mice compared to young (3-mo) mice ($n = 4$ to 5). (*B*) IF detection (40×) of NOX1 in mouse aortas showing the effects of in vivo administration of NOXA1ds (20 mg/kg/d) on aging-induced changes in NOX1 ($n = 3$ for each group). (*C*) Hind-limb blood flow was reduced in aged mice compared to young as measured by laser Doppler. NOXA1ds (20 mg/kg/d) improved hind limb blood flow in aged mice ($n = 5$ for each group). (*D*) Comparison of NOX1, PPAR-γ, and γ-H2AX protein expression between human aortas from nondiabetic (Non-db) and diabetic (db) subjects ($n = 5$ for each group). (*E*) Comparison of various gene products between human aortas from nondiabetic (Non-db) and diabetic (db) ($n = 3$ to 5 for each group). One-way ANOVA followed by Tukey's test was used for comparisons in panels *A* and *C*. Student's *t* test was used for comparisons in panels *D* and *E*. $^*P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$.

low-grade inflammation and bestowing on ECs their physiological integrity and normal migratory and dilatory functions. In contrast, in aged senescent ECs, our findings support a previously unidentified role for diminished PPAR-γ releasing the brake on NF-κB (p65), permitting p65 to translocate to the nucleus and for NOX1 transcription to rise unabatedly. Based on our findings, we propose that this and its attendant ROS induce secretion of inflammatory cytokines (SASP), leading to transcriptional activation of NOX1 and ensuing ROS, a senescent signature leading to impaired angiogenesis, vasodilator function, and blood flow (Fig. 7).

Aging and diabetes are well-established risk factors for CVD (5). And epidemiological studies have demonstrated an association among the age-related diseases and diabetes (4, 5). Additionally, both aging and diabetes were shown to correlate with chronic inflammation (26). Moreover, studies implicate both as culprits for cellular senescence in cardiovascular aging (5, 27–30). In support of those findings are studies showing that suppression of cellular senescence or elimination of senescent cells reverses phenotypic changes of aging in cell and animal models (31). However, drivers of oxidative stress that can be targeted to proactively halt the senescence and ensuing inflammatory process are

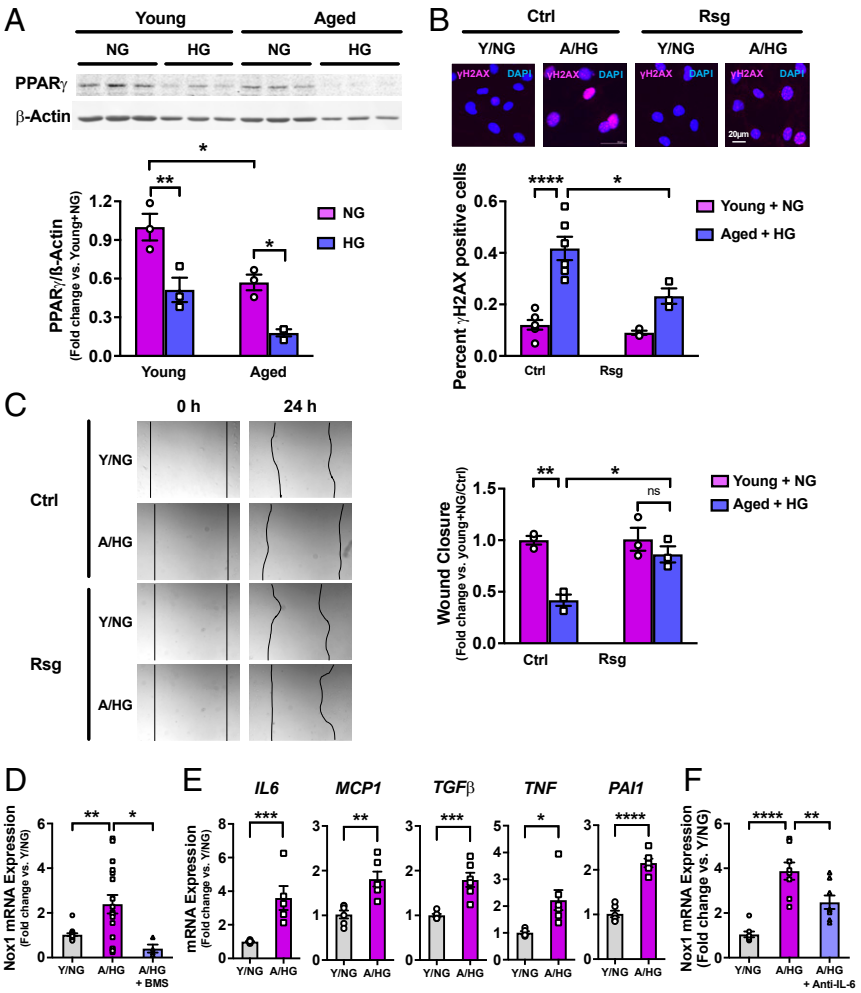

Fig. 6. Counterregulatory roles of PPAR-γ, NF-κB, and IL-6 in a compounded senescence phenotype of age and hyperglycemia. (*A*) Representative Western blots (*Upper*) and cumulative data (*Lower*) showing the effects of age and HG on PPAR-γ expression level in MAEC whole-cell lysate ($n = 3$). (*B*) IF microscopy (120× magnification) of γ-H2AX (pink) showing age plus HG (A/HG) increased the level of γ-H2AX, which was suppressed by the Rsg (10 μmol/L) treatment. Nuclei were counterstained with DAPI (blue). Merge of γ-H2AX with DAPI (purple) reveals nuclear γ-H2AX, three images/slide, $n = 3$ to 6 independent experiments. (*C*) Effects of Rsg (10 μmol/L) on the 24-h wound healing response of MAECs affected by age plus HG (A/HG) versus young cells in NG media (Y/NG) ($n = 3$). (*D*) Effects of IKK-1 and IKK-2 inhibitor (BMS, 10 μmol/L) on age plus HG-induced elevation in NOX1 expression ($n = 4$ to 18). (*E*) Effects of aging plus HG (A/HG) on the expression of various SASP and inflammatory genes in MAECs compared to young cells cultured in NG media (Y/NG) ($n = 6$). (*F*) Effects of anti–IL-6 neutralizing antibody (25 ng/mL) on NOX1 expression ($n = 8$). Two-way ANOVA followed by Tukey's test was used in panels *A*–*C*. Student's *t* test was used in panels *D*–*F*. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

unknown. Thus, identifying mechanisms underlying aging- as well as hyperglycemia-stimulated oxidative stress and senescence in vascular ECs appears essential to revealing targets that can be manipulated in patients suffering from multiple age-related cardiovascular and metabolic pathologies.

In the current study, we illustrated the mechanisms by which an inverse relation between PPAR-γ and NOX/ROS with age effects declining EC health and function. Studies have shown that human aging is associated with increased endothelial senescence as evidenced by elevated senescent indicators (32), halted angiogenesis (33, 34), and exacerbated endothelial dysfunction (32). Notably, employing human ECs, we observed that senescence aligns with those in mouse cells. In particular, aged MAECs displayed increased SA-β-gal (35) activity. We went a step further and in our hands observed enhanced γ-H2AX (marker for DNA double strand breaks and senescence) (36) staining (Fig. 1). Moreover, lamin B1, a structural protein located on the nuclear envelope, the loss of which is a reported biomarker for chromatin disorganization and senescence (37, 38), was significantly reduced in aged ECs (Fig. 1). Senescence relies on two main pathways: p53-$p21^{cip}$-Rb and $p16^{INK4a}$-Rb signaling pathways, both of which converge at preventing Rb phosphorylation/activation by CDK (cyclin D kinase), thus limiting E2F-dependent cell cycle progression (39–41). Herein, hypophosphorylation of Rb, together with enhanced expression of $p21^{cip}$ and $p16^{INK4a}$, are indicative of increased cell cycle arrest and senescence (Fig. 1). In support of a senescent phenotype, one of the major SASPs, IL-6 (42), displayed a trend toward a significant increase in response to aging and was in fact synergistically and significantly elevated by HG and age (Fig. 1). This is quite noteworthy in and of itself, in that it points to IL-6 as a possible driver of the phenotype vis-à-vis its ability to promote NOX1 (vide infra).

Functional impairment of ECs by aging was also observed, including weakened wound healing capability (Fig. 1) and blunted dilatory responses (Fig. 3*H*), both of which are demonstrative of established, aging-associated impairments in the human vasculature (34, 43). Specifically, diminished migratory and proliferative capacity of vascular ECs witnessed in wound healing response experiments could be expected to culminate in impaired angiogenesis, giving way to age-related decline in vessel

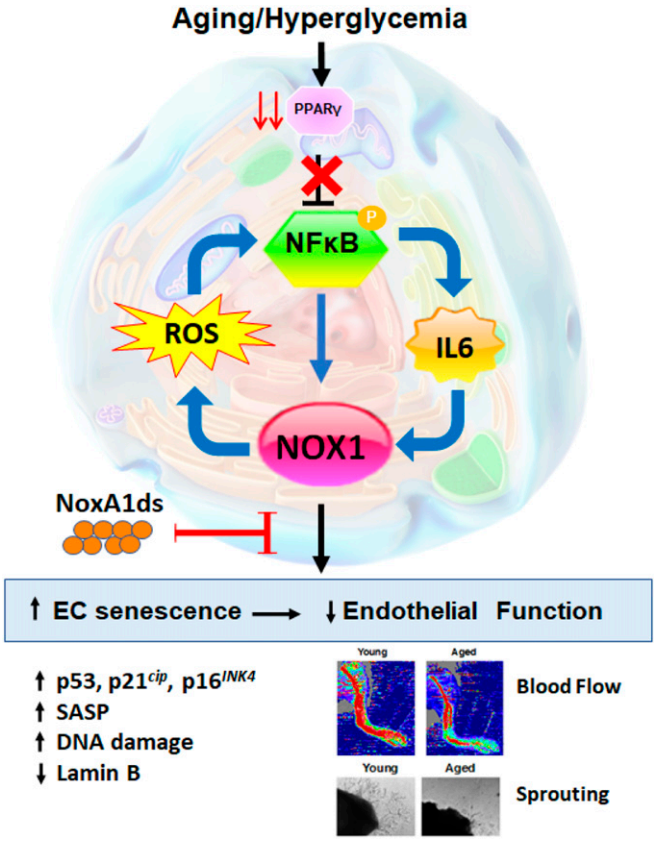

Fig. 7. Proposed propagation of aging- and hyperglycemia-induced EC senescence and dysfunction, reduced blood flow, and impaired angiogenesis via NF-kB, NOX1, and IL-6; Inverse modulation by PPAR-γ and targeting NOX1. In aged ECs or ECs exposed to proxy hyperglycemic stress, diminished PPAR-γ unleashes NF-κB (p65) for nuclear translocation and NOX1 transcription. IL-6 propagates NOX1-driven ROS, resulting in EC senescence signaling and organ dysfunction. Selective NOX1 inhibitor NOXA1ds halts senescence, dysfunction, and restores tissue to normal.

density, exacerbated ischemic tissue injury, and compromised recovery from ischemic insults (34, 43). In fact, our findings reveal an astonishing ability of NOX1-selective inhibitor (NOXA1ds) to completely restore endothelium-dependent angiogenesis (Fig. 3 *F* and *G*). These salient results underscore the significance of proactively suppressing NOX1 to stave off senescence and cardiovascular decline. That notwithstanding, endothelial dysfunction, per se, is an early indicator of atherosclerosis, impaired microvascular perfusion, and human peripheral vascular disease (43), and any attempt to preserve EC function could be seen as a means to avert or halt the disease process. In the current study, NOX1-selective inhibitor effectively restored endothelial function and vasodilatation (Fig. 3*H*). As ECs are found in all organ systems, the implications for this result cannot be overstated.

Hyperglycemia, a hallmark of diabetes and prevalent in a substantial cohort of aged patients (19.3%, 2019) (26, 44, 45) with diabetes, recapitulated most changes we observed with aging. Indeed, we employed it both as an established modality in the field to instigate senescence and as a second, clinically relevant insult to ECs. Consistent with previous publications reporting induction of senescence in ECs by HG (13, 14), we herein confirmed that senescence indicators are up-regulated (Fig. 1), oxidative stress is potentiated (Fig. 2 *A* and *B*), and cell proliferation/migration are suppressed (Fig. 1) in mouse ECs cultured in a hyperglycemic versus normoglycemic environment. Perhaps more importantly, effectors and markers investigated in mouse mirrored those in human ECs (Figs. 3–6 and *SI Appendix*, Fig. 1), and combining HG and age as factors potentiated and in some cases synergized in their responses, allowing for an optimal milieu in which to study advanced senescence in the context of oxidative stress. It is worth noting that even though aging and hyperglycemia both have, in our in vitro as well as ex vivo models, stimulated NOX1-depenendent endothelial senescence and dysfunction, they are considered two independent instigators for EC senescence in vivo. In particular, as young and aged mice have similar levels of blood glucose concentration as reported by others (46, 47), NOX1-mediated senescence and impaired blood flow observed in the aged animals (Fig. 5 *A*–*C*) should be attributed to advanced age rather than hyperglycemia. That said, in humans, increasing incidence of diabetes with age is reported. Specifically, incidence grows from below 5% in young adults (20 to 30 y) to 12% in middle-aged adults (40 to 55 y) and reaches 20% in the aged (65 to 99 y) population (48). Moreover, our data comparing aortas from nondiabetic and diabetic age-matched patients (Fig. 5 *D* and *E*) as well as in vitro findings (Figs. 1 and 2 *A* and *B*) all have shown that hyperglycemia compounds the vascular damage triggered by aging. Therefore, although hyperglycemia might not be the major factor in age-induced vascular senescence and dysfunction, it is nevertheless clinically relevant to study the effects of HG on senescent signaling.

The free radical theory of aging, first proposed by Harman (6) postulated that age-related physiological deterioration is determined by the endogenous production of ROS in cells. Although broad in its claims and at times controversial, studies have inferred ROS participating in vascular aging by promoting endothelial dysfunction in both animals (49) and humans (50, 51). Notably, NOXs of late have emerged as proximal upstream signaling nidi that produce tightly regulated ROS levels, educing signaling involved in cellular phenotypic modulation. From our perspective, this newfound appreciation of NOXs sheds light on this theory. Indeed, with NOXs being major generators of ROS production in the vasculature, involvement of NOX1 was inferred in the aging process and organ dysfunction in vessels from rats (52) and in lung biopsies from human subjects (53). In another report, a pan-NOXi ameliorated vascular function in aged rats (54).

In the current study, we came at this with the hypothesis that NOX1 is central to a unique and more-complex process and that targeting it in vitro and in vivo would have profound salutary effects on the aging process. Indeed, NOX1 appears to serve as the proximal stimulus for ECs senescence in that inhibiting NOX1 effectively 1) suppressed aging $^{+/-}$ HG-induced elevation in ROS, SA-β-gal, γ-H2AX, and $p21^{cip}$ levels and 2) rescued the loss of nuclear integrity indicator lamin B1 (Figs. 2 *F* and *G* and 3 *A*–*D*). Consequently, NOX1-selective inhibitor NOXA1ds demonstrated significant salubrious effects for ECs (i.e., ROS and SASP suppression), which predictably converged on the NOXi's ability to ameliorate impaired functional wound healing response and to confer an extraordinary permissive effect for angiogenesis to flourish. Furthermore, corroborating this notable ability of the NOX1-selective inhibitor, NOX1 siRNA mitigated senescence in human ECs, illustrated by abolishment of elevated SA-β-gal, $p21^{cip}$, and p53 (Fig. 4 *C* and *D*). All told, our current and previous findings (53) advance the notion that NOX1 is a proximal orchestrator of regulatory signaling pathways controlling cell senescence and declining organ function.

Quite strikingly in this regard, we show here that aging-impaired endothelial dysfunction, angiogenesis, and blood flow is ameliorated by selective NOX1 inhibition. NOX1 inhibition ameliorated endothelial dysfunction (Fig. 3*H*) and improved endothelial sprouting (Fig. 3 *F* and *G*). In line with this effect and improvements in vascular function, we observed a profound rescue of diminished blood flow with NOXA1ds delivered to aged mice (Fig. 5*C*). On broader examination of these findings, it

could be that in vessels exposed to NOXA1ds, other cell types might be expected to exhibit suppressed NOX1 activity as well as ROS production and thereby exert some influence. That being said, multiple reports have shown that vasodilation exerted by an endothelial-independent vasodilator, sodium nitroprusside, is not altered by age in various vascular beds across multiple different species (55–61). Thus, it appears that aging and ROS do not affect the ability of smooth muscle to dilate. Together with effects of NOXA1ds on endothelial function and endothelium-dependent angiogenesis, we can assert with a considerable level of confidence that NOXA1ds conferred protection largely via its impact on ECs, per se. In aggregate, our data in murine and human ECs indicate that EC NOX1 inhibition and amelioration of senescence is at the center of the improved blood flow in vivo.

In addition to NOXs, an abundant source of ROS in vascular cells is clearly mitochondria, the dysfunction of which has been associated with aging and senescence (62–64). Apart from altering the metabolic profile of cells that triggers cellular senescence (62), mitochondrial electron transport dysfunction with aging would be expected to potentiate NOX-induced ROS levels and aggravate cellular senescence by enhancing DNA damage (65). Interestingly, studies have suggested crosstalk between NOXs and mitochondria, which purportedly represent a feed-forward vicious cycle of ROS overproduction under varied pathophysiological conditions, such as hypertension (66) and cancer (67). For example, angiotensin II (AngII)-stimulated NOXs can elicit mitochondrial dysfunction, as a NOX inhibitor as well as knocking down $p22^{phox}$ (an essential component of NOXs 1–4) have both decreased ROS production in mitochondria isolated from AngII-treated cells (68). It is therefore conceivable that such a feed-forward interaction between NOXs and mitochondria is extant in our models and perhaps even bolstered during the aging process. A more in-depth investigation outside of the current scope of this study is warranted.

More mechanistically, herein we proffer that senescence depends on liberation of p65 from PPAR-γ in the cytosol (and p65 nuclear translocation) as a consequence of PPAR-γ's diminution with age (vide supra). Indeed, we showed that p65/NF-κB activation is instrumental to NOX1 up-regulation (Fig. 6*D*). To this point, we know that NF-κB activation has been shown to be a driver of NOX1 transcription in other settings. Going a step further, our data support a surprising but remarkable feed-forward redox activation of NOX1 by NOX1 (Fig. 5*A*). More to the point, NOX inhibition in vivo significantly suppresses NOX1. It also appears to attenuate salient indicators of proinflammatory SASP IL-6 and MCP1 (Fig. 5*A*).

Expanding on this, we wanted to know whether combined HG and aging would similarly effect an increase in an array of SASP, which it did. Intriguingly, a neutralizing antibody for IL-6 caused a reduction in NOX1 illustrating an induction of NOX1 via inflammatory cytokines in age/hyperglycemia. Thus, in aggregate our findings implicate NOX1 as a central driver of SASP and suggest a propagative deleterious cycle at play in aged, hyperglycemic cells and tissue.

Analysis of aortas from diabetic versus age-matched control human subjects showed NOX1 and γ-H2AX increased, while PPAR-γ was decreased (Fig. 5*D*). Accordingly, mRNA transcripts displayed higher levels of $p21^{cip}$, IL-6, and MCP1, indicative of increased senescence and inflammation in diabetic patient samples (Fig. 5*E*). These data uniformly parallel and corroborate our observations in murine and human cells.

Collectively, these findings provide exciting evidence that aging and HG increase the NF-κB–driven NOX1-dependent release of inflammatory cytokines in ECs and vasculature. The consequent ROS release predictably fuels the cycle by the additional induction of NF-κB and NOX1. Moreover, the data appear to show that IL-6 plays its part in further advancing this cycle. In turn, our data align with NOX1-derived ROS propagating endothelial senescence and dysfunction part and parcel with inflammation, markedly reduced wound healing, angiogenesis, and blood flow. Finally, these findings underscore the potential clinical implications of a NOX1 inhibitor such as ours to stave off "inflammaging" and senescence and, in doing so, forestall the development of aging-related cardiovascular decline.

Methods

All methods for this study are described in detail in the *SI Appendix, Methods and Materials*, including reagents, cell culture and siRNA transfection, ROS assays, SA-β-gal staining, quantitative real-time RT-PCR, Western blotting, fluorescent imaging, in vitro wound scratch assay, blood flow, angiogenesis, and vessel myography. All animal experiments were approved by the Institutional Animal Care and Use Committee, University of Pittsburgh, and are in accordance with NIH guidelines.

Data Availability. Original Western blots, IF images, PCR data, data analysis, and so on have been deposited in Figshare (10.6084/m9.figshare.12724664).

ACKNOWLEDGMENTS. Funds were provided by NIH Grant Nos. R01HL142248, R01HL079207, R01HL112914, T32GM008424, and American Heart Association 18TPA34170069 (to P.J.P.), AHA fellowship 17POST33660330 (to Y.L.), R01 HL 133864, R01 HL 128304, R01 HL 149825, R01 HL 128304-S1, R01 HL153532, AHA Established Investigator Award No. 19EIA34770095 (to A.C.S.), R01HL142932, and K22HL117917 (to C.S.H.), Institute for Transfusion Medicine and the Hemophilia Center of Western Pennsylvania and Vitalant (to P.J.P. and A.C.S.). Imaging facilities provided by the Center for Biologic Imaging at University of Pittsburgh are gratefully acknowledged.

1. R. M. Tuder, I. Petrache, Pathogenesis of chronic obstructive pulmonary disease. *J. Clin. Invest.* **122**, 2749–2755 (2012).
2. D. J. Kurz *et al.*, Chronic oxidative stress compromises telomere integrity and accelerates the onset of senescence in human endothelial cells. *J. Cell Sci.* **117**, 2417–2426 (2004).
3. F. Fyhrquist, O. Saijonmaa, T. Strandberg, The roles of senescence and telomere shortening in cardiovascular disease. *Nat. Rev. Cardiol.* **10**, 274–283 (2013).
4. Centers for Disease Control and Prevention, National diabetes statistics report: Estimates of diabetes and its burden in the United States (Centers for Disease Control and Prevention, U.S. Dept of Health and Human Services, Atlanta, GA, 2020).
5. C. W. Chia, J. M. Egan, L. Ferrucci, Age-related changes in glucose metabolism, hyperglycemia, and cardiovascular risk. *Circ. Res.* **123**, 886–904 (2018).
6. D. Harman, The aging process. *Proc. Natl. Acad. Sci. U.S.A.* **78**, 7124–7128 (1981).
7. G. Csányi, W. R. Taylor, P. J. Pagano, NOX and inflammation in the vascular adventitia. *Free Radic. Biol. Med.* **47**, 1254–1266 (2009).
8. I. Al Ghouleh *et al.*, Oxidases and peroxidases in cardiovascular and lung disease: New concepts in reactive oxygen species signaling. *Free Radic. Biol. Med.* **51**, 1271–1288 (2011).
9. S. Sahoo, D. N. Meijles, P. J. Pagano, NADPH oxidases: Key modulators in aging and age-related cardiovascular diseases? *Clin. Sci. (Lond.)* **130**, 317–335 (2016).
10. Y. Li, P. J. Pagano, Microvascular NADPH oxidase in health and disease. *Free Radic. Biol. Med.* **109**, 33–47 (2017).
11. Y. Zhang, P. Murugesan, K. Huang, H. Cai, NADPH oxidases and oxidase crosstalk in cardiovascular diseases: Novel therapeutic targets. *Nat. Rev. Cardiol.* **17**, 170–194 (2020).
12. G. A. Knock, NADPH oxidase in the vasculature: Expression, regulation and signalling pathways; role in normal cardiovascular physiology and its dysregulation in hypertension. *Free Radic. Biol. Med.* **145**, 385–427 (2019).
13. S. C. Rogers, X. Zhang, G. Azhar, S. Luo, J. Y. Wei, Exposure to high or low glucose levels accelerates the appearance of markers of endothelial cell senescence and induces dysregulation of nitric oxide synthase. *J. Gerontol. A Biol. Sci. Med. Sci.* **68**, 1469–1481 (2013).
14. R. Mortuza, S. Chen, B. Feng, S. Sen, S. Chakrabarti, High glucose induced alteration of SIRTs in endothelial cells causes rapid aging in a p300 and FOXO regulated pathway. *PLoS One* **8**, e54514 (2013).
15. A. Benetos *et al.*, Polypharmacy in the aging patient: Management of hypertension in octogenarians. *JAMA* **314**, 170–180 (2015).
16. D. J. Ranayhossaini *et al.*, Selective recapitulation of conserved and nonconserved regions of putative NOXA1 protein activation domain confers isoform-specific inhibition of Nox1 oxidase and attenuation of endothelial cell migration. *J. Biol. Chem.* **288**, 36437–36450 (2013).
17. A. Anagnostopoulou, L. L. Camargo, D. Rodrigues, A. C. Montezano, R. M. Touyz, Importance of cholesterol-rich microdomains in the regulation of Nox isoforms and redox signaling in human vascular smooth muscle cells. *Sci. Rep.* **10**, 17818 (2020).

18. E. Cifuentes-Pagano, P. J. Pagano, Rational design and delivery of NOX-inhibitory peptides. *Methods Mol. Biol.* **1982**, 417–428 (2019).
19. L. L. Camargo *et al*., Vascular Nox (NADPH Oxidase) compartmentalization, protein hyperoxidation, and endoplasmic reticulum stress response in hypertension. *Hypertension* **72**, 235–246 (2018).
20. L. J. Mah, A. El-Osta, T. C. Karagiannis, GammaH2AX as a molecular marker of aging and disease. *Epigenetics* **5**, 129–136 (2010).
21. G. Pascual *et al*., A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR-gamma. *Nature* **437**, 759–763 (2005).
22. Y. Hou, F. Moreau, K. Chadee, PPARγ is an E3 ligase that induces the degradation of NFκB/p65. *Nat. Commun.* **3**, 1300 (2012).
23. A. Manea, L. I. Tanase, M. Raicu, M. Simionescu, Transcriptional regulation of NADPH oxidase isoforms, Nox1 and Nox4, by nuclear factor-kappaB in human aortic smooth muscle cells. *Biochem. Biophys. Res. Commun.* **396**, 901–907 (2010).
24. J. R. Burke *et al*., BMS-345541 is a highly selective inhibitor of I kappa B kinase that binds at an allosteric site of the enzyme and blocks NF-kappa B-dependent transcription in mice. *J. Biol. Chem.* **278**, 1450–1456 (2003).
25. K. K. Griendling *et al*., Oxidative stress and hypertension. *Circ. Res.* **128**, 993–1020 (2021).
26. N. de Rekeneire *et al*., Diabetes, hyperglycemia, and inflammation in older individuals: The health, aging and body composition study. *Diabetes Care* **29**, 1902–1908 (2006).
27. N. Farhat *et al*., Stress-induced senescence predominates in endothelial cells isolated from atherosclerotic chronic smokers. *Can. J. Physiol. Pharmacol.* **86**, 761–769 (2008).
28. G. Voghel *et al*., Chronic treatment with N-acetyl-cystein delays cellular senescence in endothelial cells isolated from a subgroup of atherosclerotic patients. *Mech. Ageing Dev.* **129**, 261–270 (2008).
29. B. G. Childs *et al*., Senescent intimal foam cells are deleterious at all stages of atherosclerosis. *Science* **354**, 472–477 (2016).
30. Y. Yin *et al*., Vascular endothelial cells senescence is associated with NOD-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome activation via reactive oxygen species (ROS)/thioredoxin-interacting protein (TXNIP) pathway. *Int. J. Biochem. Cell Biol.* **84**, 22–34 (2017).
31. G. Katsuumi, I. Shimizu, Y. Yoshida, T. Minamino, Vascular senescence in cardiovascular and metabolic diseases. *Front. Cardiovasc. Med.* **5**, 18 (2018).
32. M. J. Rossman *et al*., Endothelial cell senescence with aging in healthy humans: Prevention by habitual exercise and relation to vascular endothelial function. *Am. J. Physiol. Heart Circ. Physiol.* **313**, H890–H895 (2017).
33. R. P. Brandes, I. Fleming, R. Busse, Endothelial aging. *Cardiovasc. Res.* **66**, 286–294 (2005).
34. J. Lähteenvuo, A. Rosenzweig, Effects of aging on angiogenesis. *Circ. Res.* **110**, 1252–1264 (2012).
35. D. J. Kurz, S. Decary, Y. Hong, J. D. Erusalimsky, Senescence-associated (beta)-galactosidase reflects an increase in lysosomal mass during replicative ageing of human endothelial cells. *J. Cell Sci.* **113**, 3613–3622 (2000).
36. F. d'Adda di Fagagna *et al*., A DNA damage checkpoint response in telomere-initiated senescence. *Nature* **426**, 194–198 (2003).
37. A. Freund, R. M. Laberge, M. Demaria, J. Campisi, Lamin B1 loss is a senescence-associated biomarker. *Mol. Biol. Cell* **23**, 2066–2075 (2012).
38. A. S. Wang, P. F. Ong, A. Chojnowski, C. Clavel, O. Dreesen, Loss of lamin B1 is a biomarker to quantify cellular senescence in photoaged skin. *Sci. Rep.* **7**, 15678 (2017).
39. C. J. Sherr, J. M. Roberts, CDK inhibitors: Positive and negative regulators of G1-phase progression. *Genes Dev.* **13**, 1501–1512 (1999).
40. K. M. Latham, S. W. Eastman, A. Wong, P. W. Hinds, Inhibition of p53-mediated growth arrest by overexpression of cyclin-dependent kinases. *Mol. Cell. Biol.* **16**, 4445–4455 (1996).
41. J. Chen *et al*., Contribution of p16INK4a and p21CIP1 pathways to induction of premature senescence of human endothelial cells: Permissive role of p53. *Am. J. Physiol. Heart Circ. Physiol.* **290**, H1575–H1586 (2006).
42. J. P. Coppé, P. Y. Desprez, A. Krtolica, J. Campisi, The senescence-associated secretory phenotype: The dark side of tumor suppression. *Annu. Rev. Pathol.* **5**, 99–118 (2010).
43. Z. Ungvari *et al*., Endothelial dysfunction and angiogenesis impairment in the ageing vasculature. *Nat. Rev. Cardiol.* **15**, 555–565 (2018).
44. P. G. Lee, J. B. Halter, The pathophysiology of hyperglycemia in older adults: Clinical considerations. *Diabetes Care* **40**, 444–452 (2017).
45. A. Sinclair *et al*., Diabetes and global ageing among 65-99-year-old adults: Findings from the international diabetes federation diabetes Atlas, 9th edition. *Diabetes Res. Clin. Pract.* **162**, 108078 (2020).
46. J. Zhao *et al*., Evaluation of organ glucose metabolism by ^{18}F-FDG accumulation with insulin loading in aged mice compared with young normal mice. *Sci. Rep.* **11**, 7421 (2021).
47. N. Ehrhardt *et al*., Adiposity-independent effects of aging on insulin sensitivity and clearance in mice and humans. *Obesity (Silver Spring)* **27**, 434–443 (2019).
48. International Diabetes Federation, *IDF Diabetes Atlas*, ed. 9 (International Diabetes Federation, 2019).
49. A. Csiszar *et al*., Aging-induced phenotypic changes and oxidative stress impair coronary arteriolar function. *Circ. Res.* **90**, 1159–1166 (2002).
50. K. L. Jablonski, D. R. Seals, I. Eskurza, K. D. Monahan, A. J.Donato, High-dose ascorbic acid infusion abolishes chronic vasoconstriction and restores resting leg blood flow in healthy older men. *J. Appl Physiol (1985)* **103**, 1715–1721 (2007).
51. A. J. Donato *et al*., Direct evidence of endothelial oxidative stress with aging in humans: Relation to impaired endothelium-dependent dilation and upregulation of nuclear factor-kappaB. *Circ. Res.* **100**, 1659–1666 (2007).
52. S. Dal-Ros *et al*., Chronic intake of red wine polyphenols by young rats prevents aging-induced endothelial dysfunction and decline in physical performance: Role of NADPH oxidase. *Biochem. Biophys. Res. Commun.* **404**, 743–749 (2011).
53. D. N. Meijles *et al*., The matricellular protein TSP1 promotes human and mouse endothelial cell senescence through CD47 and Nox1. *Sci. Signal.* **10**, eaaj1784 (2017).
54. S. Wind *et al*., Oxidative stress and endothelial dysfunction in aortas of aged spontaneously hypertensive rats by NOX1/2 is reversed by NADPH oxidase inhibition. *Hypertension* **56**, 490–497 (2010).
55. C. R. Woodman, E. M. Price, M. H. Laughlin, Selected contribution: Aging impairs nitric oxide and prostacyclin mediation of endothelium-dependent dilation in soleus feed arteries. *J. Appl. Physiol. (1985)* **95**, 2164–2170 (2003).
56. C. R. Woodman, E. M.Price, M. H. Laughlin, Aging induces muscle-specific impairment of endothelium-dependent dilation in skeletal muscle feed arteries. *J. Appl. Physiol. (1985)* **93**, 1685–1690 (2002).
57. R. Feng *et al*., Stem cell-derived extracellular vesicles mitigate ageing-associated arterial stiffness and hypertension. *J. Extracell. Vesicles* **9**, 1783869 (2020).
58. J. Tao *et al*., Reduced arterial elasticity is associated with endothelial dysfunction in persons of advancing age: Comparative study of noninvasive pulse wave analysis and laser Doppler blood flow measurement. *Am. J. Hypertens.* **17**, 654–659 (2004).
59. X. Guo, G. S. Kassab, Increased serum Klotho with age-related aortic stiffness and peripheral vascular resistance in young and middle-aged swine. *Front. Physiol.* **11**, 591 (2020).
60. B. van der Loo *et al*., Enhanced peroxynitrite formation is associated with vascular aging. *J. Exp. Med.* **192**, 1731–1744 (2000).
61. S. Novella *et al*., Aging-related endothelial dysfunction in the aorta from female senescence-accelerated mice is associated with decreased nitric oxide synthase expression. *Exp. Gerontol.* **48**, 1329–1337 (2013).
62. N. Sun, R. J. Youle, T. Finkel, The mitochondrial basis of aging. *Mol. Cell* **61**, 654–666 (2016).
63. S. Dikalov, Cross talk between mitochondria and NADPH oxidases. *Free Radic. Biol. Med.* **51**, 1289–1301 (2011).
64. P. V. S. Vasileiou *et al*., Mitochondrial homeostasis and cellular senescence. *Cells* **8**, E686 (2019).
65. J. F. Passos *et al*., Feedback between p21 and reactive oxygen production is necessary for cell senescence. *Mol. Syst. Biol.* **6**, 347 (2010).
66. A. E. Dikalova *et al*., Therapeutic targeting of mitochondrial superoxide in hypertension. *Circ. Res.* **107**, 106–116 (2010).
67. M. M. Desouki, M. Kulawiec, S. Bansal, G. M. Das, K. K. Singh, Cross talk between mitochondria and superoxide generating NADPH oxidase in breast and ovarian tumors. *Cancer Biol. Ther.* **4**, 1367–1373 (2005).
68. A. K. Doughan, D. G. Harrison, S. I. Dikalov, Molecular mechanisms of angiotensin II-mediated mitochondrial dysfunction: Linking mitochondrial oxidative damage and vascular endothelial dysfunction. *Circ. Res.* **102**, 488–496 (2008).