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Am J Physiol Heart Circ Physiol 293:37-47, 2007. First published Apr 6, 2007;
doi:10.1152/ajpheart.01346.2006

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Increased mitochondrial H₂O₂ production promotes endothelial NF-κB activation in aged rat arteries

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Submitted 10 December 2006; accepted in final form 8 March 2007

Ungvari Z, Orosz Z, Labinskyy N, Rivera A, Xiangmin Z, Smith K, Csiszar A. Increased mitochondrial H₂O₂ production promotes endothelial NF-κB activation in aged rat arteries. *Am J Physiol Heart Circ Physiol* 293: H37–H47, 2007. First published April 6, 2007; doi:10.1152/ajpheart.01346.2006.—Previous studies have shown that the aging vascular system undergoes pro-atherogenic phenotypic changes, including increased oxidative stress and a pro-inflammatory shift in endothelial gene expression profile. To elucidate the link between increased oxidative stress and vascular inflammation in aging, we compared the carotid arteries and aortas of young and aged (24 mo old) Fisher 344 rats. In aged vessels there was an increased NF-κB activity (assessed by luciferase reporter gene assay and NF-κB binding assay), which was attenuated by scavenging H₂O₂. Aging did not alter the vascular mRNA and protein expression of p65 and p50 subunits of NF-κB. In endothelial cells of aged vessels there was an increased production of H₂O₂ (assessed by 5,6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate-acetyl ester fluorescence), which was attenuated by the mitochondrial uncoupler FCCP. In young arteries and cultured endothelial cells, antimycin A plus succinate significantly increased FCCP-sensitive mitochondrial H₂O₂ generation, which was associated with activation of NF-κB. In aged vessels inhibition of NF-κB (by pyrrolidinedithiocarbamate, resveratrol) significantly attenuated inflammatory gene expression and inhibited monocyte adhesiveness. Thus increased mitochondrial oxidative stress contributes to endothelial NF-κB activation, which contributes to the pro-inflammatory phenotypic alterations in the aged vasculature. Our model predicts that by reducing mitochondrial H₂O₂ production and/or directly inhibiting NF-κB novel anti-aging pharmacological treatments (e.g., calorie restriction mimetics) will exert significant anti-inflammatory and vasoprotective effects.

inflammation; endothelial cell; senescence; aging; resveratrol

EPIDEMIOLOGICAL STUDIES showed that even “healthy” aging is an independent risk factor for cardiovascular disease, which is the number one cause for mortality and morbidity in persons aged ≥65 yr in the Western world. Previous studies by this and other laboratories have shown that the aging vascular system undergoes pro-atherogenic phenotypic changes, including increased oxidative stress and a pro-inflammatory shift in endothelial gene expression profile (10–12, 15). Also, there is increasing evidence that chronic inflammation in aging renders endothelial cells susceptible to damage and is associated with an exacerbated response to vascular injury (7, 12, 41). Yet, the underlying mechanisms responsible for pro-inflammatory al-

terations in vascular phenotype and the link between increased oxidative stress and vascular inflammation in aging remain unclear.

NF-κB is a redox-sensitive transcription factor expressed in both endothelial and smooth muscle cells (5, 9). There is increasing evidence that endothelial oxidative stress can lead to NF-κB activation and vascular pro-inflammatory gene expression in various pathophysiological conditions (9, 35). NF-κB activation can induce the transcription of a large range of genes implicated in vascular inflammation, including cytokines, chemokines, and adhesion molecules, and it is generally believed that chronic activation of NF-κB predisposes arteries to atherosclerosis (16). However, the role of NF-κB in the aged vasculature remains unexplored.

Since Harman (20) originally proposed the free radical theory of aging, considerable evidence has been published that increased production of reactive oxygen species (ROS) underlies cellular dysfunction in various organ systems of aged humans and laboratory animals (7, 10, 15, 18, 39, 44). The mitochondrial theory of aging (21) postulates that mitochondria-derived H₂O₂ diffuses readily through cellular membranes and contributes to a variety of macromolecular oxidative modifications. Accumulation of such oxidative damage has been proposed to be a primary causal factor in the aging process (21). However, there is an emerging view that mitochondria-derived H₂O₂, in addition to causing oxidative damage, plays important signaling roles as well. On the basis of the aforementioned studies, we hypothesized that mitochondrial ROS production increases in the aged blood vessels, and H₂O₂ produced by mitochondria is implicated in the inflammatory process by acting as a second messenger activating NF-κB.

Thus, in the present study, we tested the hypotheses that in aged arteries 1) there is an increased NF-κB activity in the endothelial cells, 2) NF-κB activation is responsible, at least in part, for upregulation of pro-inflammatory gene expression, and 3) increased NF-κB activation is due, at least in part, to an increased vascular production of H₂O₂ by mitochondrial sources.

METHODS

Animal models. All animal-use protocols were approved by the Institutional Animal Care and Use Committee of the New York Medical College, Valhalla, NY. Male Fisher 344 rats [ages: 3 mo old

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("young"), 18 mo old, and 24 mo old ("aged"), $n = 70$, purchased from the National Institute of Aging; kept under pathogen-free conditions, were used as previously described (11, 12). All animals were disease free with no signs of systemic inflammation and/or neoplastic alterations. The animals were euthanized by an overdose of pentobarbital sodium as described (11, 12), and the carotid arteries and aorta were isolated from subsequent studies.

Measurement of NF- κ B activity in aged endothelial cells: organoid culture, conditions of electroporation, and reporter gene assay. Effect of aging on NF- κ B activity in the endothelial cells of aged rat aortas was tested by a reporter gene assay as described (8). We used a NF- κ B reporter composed of an NF- κ B response element upstream of firefly luciferase (NF- κ B-Luc, Stratagene) and a renilla luciferase plasmid under the control of the cytomegalovirus promoter (as an internal control). Isolated aortic segments were transfected with the reporter gene construct as described previously (8). In brief, square-wave electric pulses were delivered to the vessels with a cylindrical external electrode and an intraluminal electrode (1 cm long, 1-mm fixed distance between the electrodes) by using an electric pulse generator (model CUY 201 BTX; Protech International, San Antonio, TX), and then the vessels segments were maintained in organoid culture for 24 h (6, 8, 9). The electric pulse was regulated as follows: voltage: 20 V, pulse-on time (P_{on}): 10 ms, interval time (P_{off}): 990 ms, and number of pulses: 10. These optimized parameters were determined in previous studies by measuring luciferase activity 1 day after electroporation of a cytomegalovirus-driven renilla luciferase construct at various electrode voltages, pulse numbers, and pulse durations (8). Previously, electroporation with a vector that expresses the red fluorescent DsRed-monomer fluorescent protein was used to assess the efficiency of the endothelial transfection by this method (8). The concentration of the plasmid DNA solution used for transfection was adjusted to 1.5 μ g/ μ l. In separate experiments, vessel segments from young animals were incubated with H_2O_2 (10^{-4} mol/l, for 4 h). After the culture period all vessels were homogenized in 500 μ l of dual luciferase assay lysis reagent (Promega). The firefly and renilla luciferase activities in homogenates of transfected aortas were measured by a procedure described previously (6, 8, 9).

Nuclear extraction and NF- κ B binding activity assay. Nuclei were isolated from freshly isolated carotid arteries using the Nuclear Extraction kit from Active Motif (Carlsbad, CA) as reported (9). In brief, carotid arteries of young and aged rats were homogenized with a dounce tissue homogenizer in 500 ml ice-cold hypotonic lysis buffer followed by two centrifugation steps (500 g , for 30 s, $4^\circ C$) to exclude tissue debris. Nuclear proteins (~ 10 μ g/vessel segment) were then extracted according to the manufacturer's protocol. Protein concentrations in samples were equalized using a Bradford protein assay (Bio-Rad). With the use of obtained nuclear extract, NF- κ B binding

activity was assayed using the TransAM NF- κ B ELISA kit (Active Motif) according to the manufacturer's guidelines.

In separate experiments, carotid arteries isolated from young and aged rats were maintained in organoid culture (for 24 h). Some vessels were treated with the H_2O_2 scavenger PEG-catalase (200 U/ml), the NAD(P)H oxidase inhibitor apocynin (10^{-4} mol/l), or FCCP (1 μ mol/l, an uncoupler of oxidative phosphorylation; Invitrogen), which effectively decreases mitochondrial H_2O_2 generation. At the end of the culture period, nuclear NF- κ B binding activity was assayed as described above.

mRNA and protein expression of NF- κ B subunits in aged arteries: quantitative RT-PCR and Western blot analysis. To analyze protein expression of NF- κ B subunits, Western blot analysis was performed as described (10, 11), using primary antibodies, which recognize the p65 and p50 subunits of NF- κ B (Cell Signaling, Danvers, MA). Anti- β -actin (Novus Biologicals, Littleton, CO) was used for normalization purposes.

We have used a quantitative real-time RT-PCR (QRT-PCR) technique to analyze mRNA expression of NF- κ B subunits and other regulatory factors that modulate NF- κ B activity (Table 1) in carotid arteries of 3-, 18-, and 24-mo-old rats, using the Strategen MX3000 as reported (6, 9–11). Total RNA was isolated with Mini RNA Isolation Kit (Zymo Research, Orange, CA) and was reverse transcribed using Superscript III RT (Invitrogen). Efficiency of the PCR reaction was determined by using dilution series of a standard vascular sample. Quantification was performed using the $\Delta\Delta$ -cycle threshold method. The housekeeping gene β -actin was used for internal normalization. Oligonucleotides used for real-time QRT-PCR are listed in Table 1. Fidelity of the PCR reaction was determined by melting temperature analysis and visualization of product on a 2% agarose gel.

Measurement of mitochondrial $O_2^{\cdot-}$ and H_2O_2 production. MitoSox (Invitrogen, Carlsbad, CA), a mitochondrion-specific hydroethidine-derivative fluorescent dye, was used to assess mitochondrial $O_2^{\cdot-}$ production in situ. In brief, segments of the carotid arteries and aortas of young and aged rats ($n = 5-6$ in each group) were incubated with MitoSox (10^{-6} mol/l; at $37^\circ C$ for 60 min). SYTOX green was used for nuclear staining. In some experiments, vessels coincubated with FCCP or apocynin. The vessels were then washed three times, embedded in OCT medium, and cryosectioned. Images were captured using a Bio-Rad confocal microscope. In separate experiments optical section of the endothelium of en face vessel preparations were obtained. Average perinuclear MitoSox fluorescence intensities were quantified. Ten to fifteen entire fields per vessel segment were analyzed with one image per field. The mean fluorescent intensities for each vessel in each group were averaged.

In other experiments, the cell-permeant oxidative fluorescent indicator dye 5,6-chloromethyl-2',7'-dichlorodihydrofluorescein diac-

Table 1. Oligonucleotides for real-time RT-PCR

mRNA Targets	Description/Alternative Names	Sense	Antisense
p65	NF- κ B p65 subunit; v-rel	ATGGACGATCTGTTTCCC	GTCTTAGTGGTATCTGTGCT
p50	NF- κ B p50 subunit; NF- κ B p105 subunit	CAAGACAGCACATAGATGAG	TGTTCCAGAGATAGCAGTGG
p100	NF- κ B p100 subunit; Oncogene Lyt-10	CGCCTGAGACCCGAGACAAG	CTGCCTCCTGCTCCACTGAC
c-rel	v-rel avian reticuloendotheliosis viral oncogene homolog	CCCAAGAGACCCGAGACAAGT	TTAGGATGATGTGCGCTCGTTTGC
I κ B α	NF- κ B inhibitor α	GTGGAACCGCCATAGACTGTAGC	AAGCACATACCCTGAACACCTCG
I κ B β	NF- κ B inhibitor β	GCAGCAACAGCAGCAGT	GGTCATCAGGAAGAGGTTTGG
I κ B-like-1	NF- κ B inhibitor-like protein 1	GAATGAGCAAGAACCCTGTG	CGTTAGCCATCCCATATCC
I κ B kinase β	Inhibitor of NF- κ B kinase β -subunit	CCACCACAGAAGCACAACAATG	CCCGCAGCAGTCCAAAGG
I κ B kinase ϵ	Inhibitor of NF- κ B kinase ϵ subunit	CCTACCACTGCCTCATTG	CCACTTATCCAACCTCCTG
Nkiras-1	NF- κ B inhibitor interacting Ras-like 1	TGAACAAGATTGCCAGACCTATGC	GAGCCAGCCACAGTTGAAAGC
Nkiras-2	NF- κ B inhibitor interacting Ras-like 2	TGCTCCAACCTCTTCAAG	ACAACCAAGAAACAACCAG
ICAM-1		CACAGCCTGGAGTCTC	CCCTTCTAAGTGGTTGGAA
iNOS		TCCGAAACGCTACACT	CAATCCACAACCTCGCT
β -Actin		GAAGTGTGACGTTGACAT	ACATCTGCTGGAAGGTG

Nkiras-1, NF- κ B inhibitor interacting Ras-like 1; iNOS, inducible nitric oxide synthase.

etate-acetyl ester (DCF or CM-H₂DCFDA, Invitrogen) was used to assess H₂O₂ production in isolated arterial segments, as previously reported (26, 35). In brief, the arteries were pretreated with apocynin (3×10^{-4} mol/l), 250 μ mol/l α -cyano-4-hydroxycinnamic acid (4-OHCA; to inhibit pyruvate transport across the mitochondrial membrane), and 1 μ mol/l FCCP or high-dose DPI [100 μ mol/l, which inhibits mitochondrial O₂^{•-} production probably through inhibiting complex I (30)]. The vessel segments were then treated with DCF (10^{-5} mol/l; at 37°C for 60 min). Untreated arteries were used as controls. The arteries were then washed three times. Fluorescent images of the endothelial layer of en face preparations were captured and analyzed using the Axiovision software (Carl Zeiss, Gottingen, Germany). Each experiment was performed in quadruplicates. Ten to fifteen entire fields per vessel were analyzed with one image per field. The background-corrected mean fluorescent intensities of the images were averaged. In some experiments, vessels coincubated with catalase were used as positive controls.

In other experiments we tested whether treatment of aged carotid arteries with the nitric oxide (NO) synthase inhibitor nitro-L-arginine methyl ester (L-NAME, 3×10^{-4} mol/l, for 2 h) attenuates endothelial DCF fluorescence or MitoSox fluorescence.

In separate experiments, we demonstrated that in aortic segments from young rats mitochondrial ROS production can be increased by coadministration of antimycin A (AA; 10^{-6} mol/l, which inhibits complex III by binding to the UQI site, blocking electron transfer from haem b_H to ubiquinone) (27, 32) plus succinate (10 mmol/l, a substrate of complex II). To demonstrate that AA + succinate selectively increase mitochondrial O₂^{•-} production, the time course of increases in endothelial MitoSox fluorescence was recorded in the presence and absence of FCCP. To demonstrate the effect of AA + succinate on cellular H₂O₂ levels, the time course of DCF fluorescence was also obtained in the presence and absence of FCCP. In separate experiments, rat coronary arterial endothelial cells (CAEC; Celprogen, San Pedro, CA) were grown in a 96-well plate (6) and loaded with 10 μ mol/l DCF, and the effect of AA + succinate was assessed using a Tecan Infinite 200 plate reader. The blue fluorescent DNA binding dye Hoechst 33342 was used for normalization purposes.

In separate experiments DCF fluorescence was compared in senescent (passage 13) CAECs and passage 3 CAECs.

Detection of senescent endothelial cells in aged carotid arteries. Senescent cells were identified in isolated carotid arteries (en face) by staining for senescence-associated β -galactosidase using the Senescence Cells Histochemical Staining Kit (Sigma-Aldrich). In brief, arteries were fixed in 2% formaldehyde for 3 min at room temperature, washed twice with PBS, and stained for β -galactosidase activity at pH 6.0. Under these conditions, β -galactosidase is a biomarker specific for senescent cells.

Measurement of endothelial NF- κ B activation induced by mitochondria-derived H₂O₂. Effect of mitochondria-derived H₂O₂ on NF- κ B activity was tested by a reporter gene assay in rat CAECs (Celprogen) as described (6, 8, 9). Transfection of the NF- κ B-Luc vector in CAECs was performed using the Amaxa Nucleofector technology (Amaxa, Gaithersburg, MD), as we have previously reported (6, 9, 12). To test the role of mitochondria-derived H₂O₂ on endothelial NF- κ B activity, CAECs were preincubated with AA (10^{-6} mol/l) plus succinate (for 4 h), which significantly increases mitochondrial H₂O₂ generation. Exogenous H₂O₂ was used as positive control. Firefly and renilla luciferase activities were assessed after 24 h using the Dual Luciferase Reporter Assay Kit (Promega).

Effect of NF- κ B inhibition on inflammatory gene expression in aged carotid arteries. To assess the effects of chronic in vivo NF- κ B inhibition on inflammatory gene expression, aged rats ($n = 6$ in each group) were treated with pyrrolidinedithiocarbamate [PDTC; intraperitoneally, 80 mg·kg⁻¹·day⁻¹; for 1 wk (29)] or resveratrol [10 mg·kg⁻¹·day⁻¹, in the drinking water (1), for 1 wk], which are potent inhibitors of NF- κ B activation in endothelial cells (8). Expres-

sion of ICAM-1 mRNA in the carotid arteries was analyzed by QRT-PCR.

To test the role of H₂O₂ in regulation of ICAM-1 expression, carotid arteries of aged rats were maintained in organoid culture for 24 h (6, 8, 9) in the presence or absence of resveratrol (10 μ mol/l), PDTC (10 μ mol/l), PEG-catalase (200 U/ml), or *N*-acetylcysteine (1 mmol/l), apocynin (3×10^{-4} mol/l), or FCCP (1 μ mol/l). At the end of the culture period, mRNA expression of ICAM-1 and inducible NO synthase (iNOS) was analyzed by QRT-PCR.

Monocyte adhesion assay and functional studies. To assess the functional consequence of NF- κ B activation, monocyte adhesion assay was performed as reported (6, 35). In brief, carotid arteries from aged rats were treated in organoid culture with PDTC (10^{-5} mol/l, for 24 h). Untreated vessels from young and aged rats and young arteries treated with tumor necrosis factor- α (TNF- α , 10 ng/ml) were used for comparison. After the treatment period, the vessels were cut open (en face) and incubated with fluorescently labeled PMA (10^{-6} mol/l)-pretreated human monocytic (THP-1) cells. After a 1-h incubation at 37°C, unbound monocytes were washed out. Bound monocytes were quantified by counting the cells under a fluorescent microscope.

To elucidate the consequence of NF- κ B inhibition on endothelial vasodilator function, ring preparations of aged carotid arteries were treated with PDTC (10^{-5} mol/l) or FCCP (1 μ mol/l) in organoid culture (for 24 h). Acetylcholine-induced relaxations were assessed as reported (6, 35). In brief, arterial ring segments 2 mm in length were mounted on 40- μ m stainless steel wires in the myographs chambers (Danish Myo Technology A/S, Atlanta, GA) containing Krebs buffer solution (in mM: 118 NaCl, 4.7 KCl, 1.5 CaCl₂, 25 NaHCO₃, 1.1 MgSO₄, 1.2 KH₂PO₄, and 5.6 glucose; at 37°C; gassed with 95% air-5% CO₂) for measurement of isometric tension. After an equilibration period of 1 h during which an optimal passive tension of 0.5 g was applied to the rings (as determined from the vascular length-tension relationship), the vessels were contracted by phenylephrine (10^{-6} mol/l) and relaxations to acetylcholine (from 10^{-9} to 10^{-4} mol/l) were obtained.

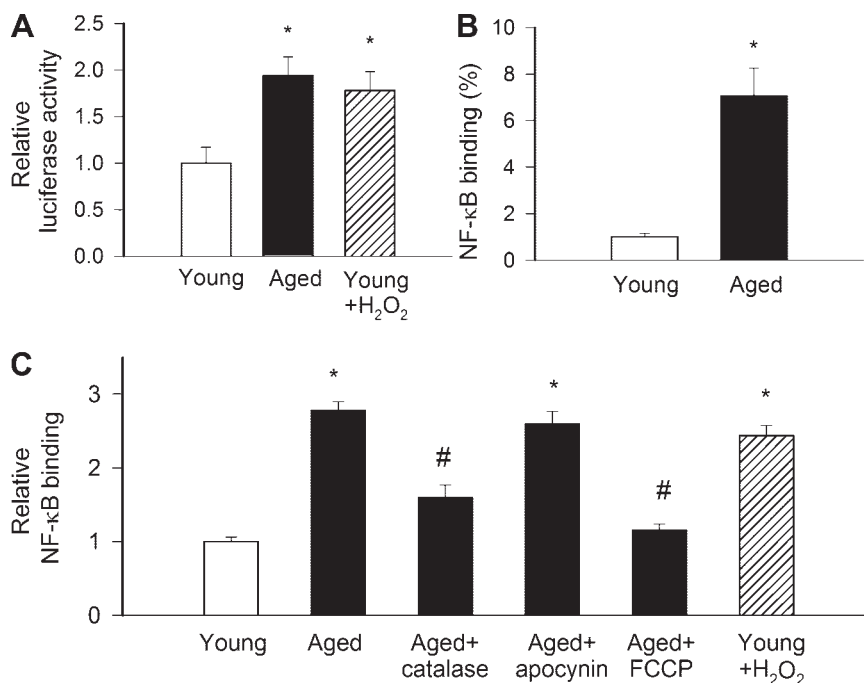
Data analysis. Data were normalized to the respective control mean values and are expressed as means \pm SE. Statistical analyses of data were performed by Student's *t*-test or by two-way ANOVA followed by the Tukey post hoc test, as appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

Increased NF- κ B activation in aged arteries. To determine the effect of aging on endothelial NF- κ B activation, we transiently transfected endothelial cells in aged rat aortas with a NF- κ B-driven reporter gene construct. We found that luciferase activity was significantly greater in aged vessels compared with young ones (Fig. 1A). In young vessels administration of H₂O₂ elicited significant increases in luciferase activity (Fig. 1A). NF- κ B binding activity was also increased in nuclei extracted from freshly isolated aged carotid arteries compared with those isolated from young rats (Fig. 1B). Increased NF- κ B activity was present in aged vessels after 24 h in organoid culture (Fig. 1C). Pretreatment of cultured aged vessels with PEG-catalase and FCCP, but not apocynin, significantly attenuated NF- κ B binding activity in aged carotid arteries (Fig. 1C). Treatment of young vessels with H₂O₂ significantly increased NF- κ B binding activity (Fig. 1C).

Expression of NF- κ B subunits in aged arteries. Protein expression of p50 and p65 subunits of NF- κ B also did not show age-dependent changes (Fig. 2, A and B). QRT-PCR data showed that the mRNA expression of p50, p65, and p100 subunits of NF- κ B did not change with age in rat carotid arteries (Fig. 3, A-C). Expression of c-Rel, I κ B α , and I κ B-

Fig. 1. *A*: increased NF- κ B reporter activity in the endothelial cells of aged rat aortas electroporated with the reporter gene construct. H₂O₂ treatment also increased NF- κ B reporter activity in young vessels. Data are means \pm SE. * P < 0.05. vs. young control. *B*: ELISA-based demonstration of increased NF- κ B binding activity in nuclear extracts from aged rat carotid arteries. Data are means \pm SE. * P < 0.05. *C*: effect of treatment with PEG-catalase (200 U/ml), apocynin (10⁻⁴ mol/l), and FCCP (1 μ mol/l) on NF- κ B binding activity in cultured carotid arteries of aged rats. H₂O₂ treatment increased NF- κ B activation in cultured arteries from young rats. Data are means \pm SE. * P < 0.05 vs. young, # P < 0.05 vs. untreated.



like-1 increased in aged vessels (Fig. 3, *D*, *E*, and *G*), whereas changes in I κ B β expression did not reach statistical significance (Fig. 3*F*). Expression of both I κ B kinases investigated tended to decrease with age (Fig. 3, *H*–*I*). NF- κ B inhibitor interacting Ras-like (Nkiras) 1 and Nkiras2 also showed age-related downregulation (Fig. 3, *J* and *K*).

Increased mitochondrial O₂^{•-} and H₂O₂ production in aged arteries. In sections of aged carotid arteries and aortas there was an increased MitoSox fluorescence (Fig. 4, *A* and *B*), which was unaffected by apocynin and abolished by FCCP (not

shown). Similar results were obtained when endothelial MitoSox fluorescence was measured in en face aorta preparations (arbitrary fluorescence units; young: 1.0 \pm 0.2, aged: 3.8 \pm 0.3; P < 0.05). In both aged aortas and carotid arteries (Fig. 4, *C* and *D*) there was an increased DCF fluorescence, which was significantly attenuated by pretreatment with FCCP, OHCA, or DPI (Fig. 4*D*). Apocynin elicited a small (~10%) decline in DCF fluorescence, which did not reach statistical significance. The DCF signal was also abolished by pretreatment with PEG-catalase and substantially increased by administration of H₂O₂ (10⁻⁶ mol/l for 1 h), showing the specificity of the assay to H₂O₂. Treatment of aged carotid arteries with L-NAME did not affect DCF or MitoSox fluorescence (Fig. 4, *E* and *F*).

Increased presence of senescent endothelial cells does not explain increased H₂O₂ production in aged arteries. In senescent CAECs in culture there was an increased DCF fluorescence compared with passage 3 cells (Fig. 5*A*). In aged carotid arteries staining for senescence-associated β -galactosidase revealed that the absolute number of senescent endothelial cells is increased in aged vessels (Fig. 5, *B* and *C*). However, senescent cells represented only a small fraction of the total cell population (Fig. 5, *B* and *C*). Interestingly, β -gal-positive adventitial fibroblasts were more abundant in aged arteries (Fig. 5*B*, inset).

Increased mitochondrial H₂O₂ production elicits NF- κ B activation in endothelial cells. In the endothelial cells of en face preparations of aortic segments, AA + succinate significantly increased mitochondrial ROS production (assessed by MitoSox fluorescence; Fig. 6*A*) and cellular H₂O₂ production (measured by DCF fluorescence; Fig. 6*B*), which could be prevented by pretreatment with FCCP. Similar results were obtained in cultured CAECs as well (Fig. 6*C*). We evaluated the effect of mitochondrial H₂O₂ on NF- κ B activation by assaying the effect of AA + succinate on NF- κ B-driven luciferase expression. We found that AA + succinate signifi-

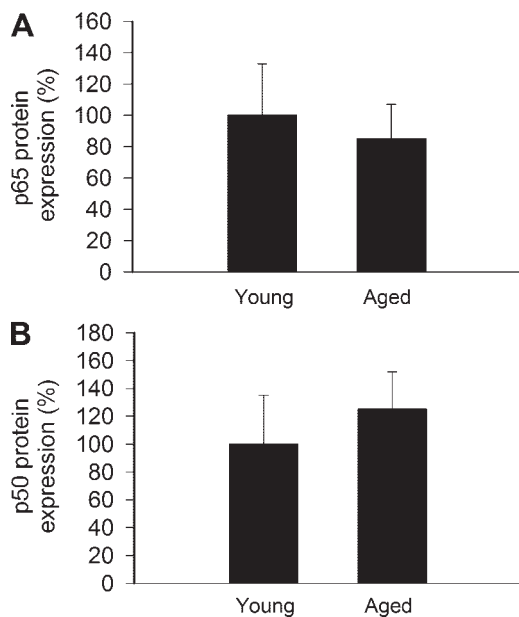


Fig. 2. Protein expression of the p65 (*A*) and p50 (*B*) subunit of NF- κ B in carotid arteries of young and aged rats. Protein expression was assayed by Western blot analysis. β -Actin was used for normalization. Data are means \pm SE (n = 5 for each group).

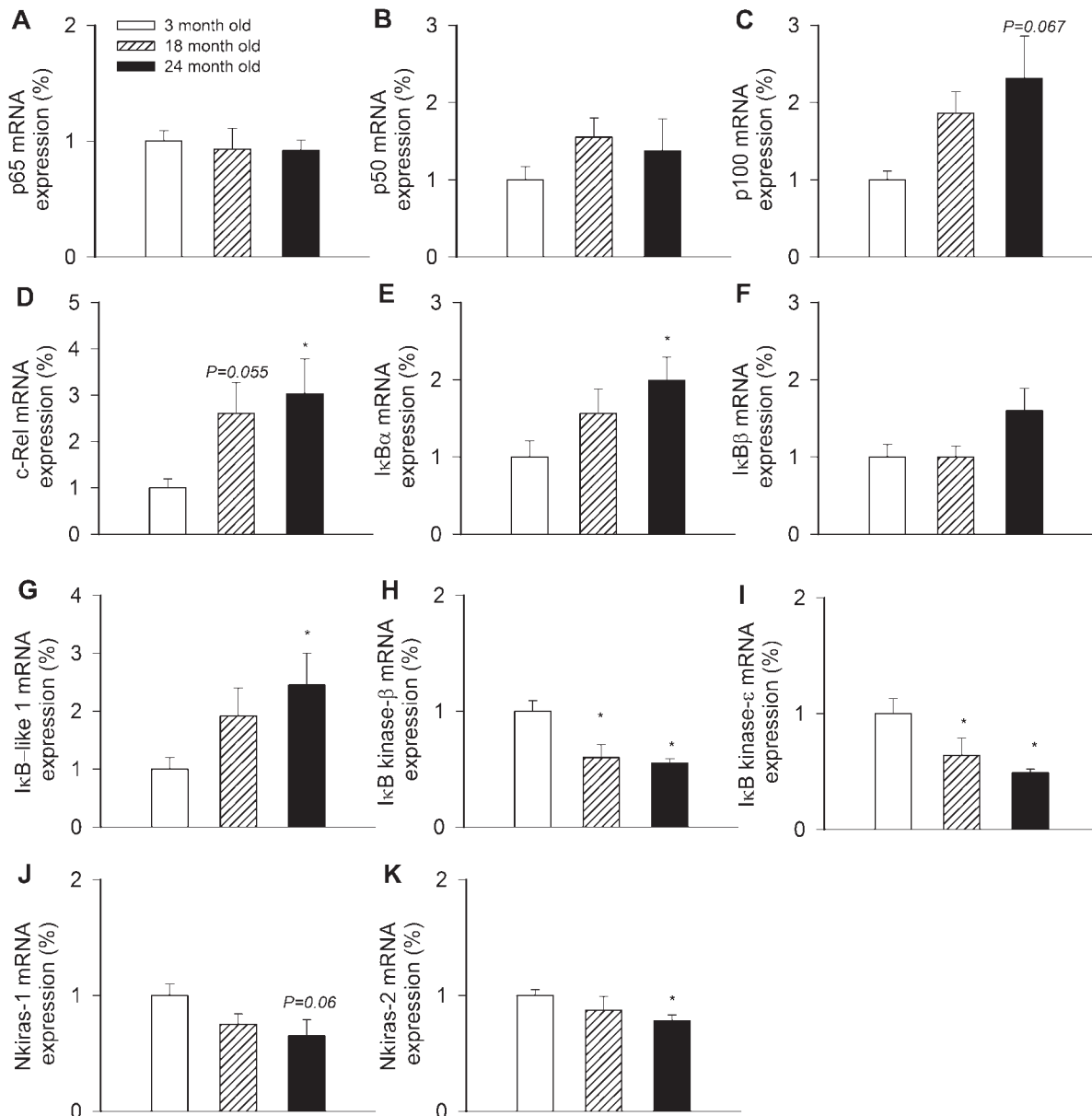


Fig. 3. Analysis of mRNA expression of NF- κ B subunits (P65, p50, and p100), c-rel, NF- κ B inhibitors (I κ B α , I κ B β , and I κ B-like-1), and other regulators of NF- κ B activation (I κ B kinase β and ϵ , Nkiras-1 and -2) in carotid arteries of 3-, 18-, and 24-mo-old rats. Analysis of mRNA expression was performed by real-time quantitative RT-PCR. β -Actin was used for normalization. Data are means \pm SE. * P < 0.05 vs. young (n = 4–6 for each group).

cantly increased NF- κ B activity in CAECs, which could be prevented by administration of FCCP or PEG-catalase (Fig. 6D). NF- κ B activity in CAECs was also substantially increased by pretreatment with H₂O₂ (Fig. 6E). These observations suggest that mitochondria-derived H₂O₂ can activate NF- κ B in endothelial cells.

Increased NF- κ B activity contributes to pro-inflammatory gene expression in aged carotid arteries. In carotid arteries of aged rats there was an increased mRNA expression of ICAM-1 compared with vessels of young rats (Fig. 7A). In vivo treatment of aged rats with resveratrol or PDTC elicited significant decreases in vascular ICAM-1 expression (Fig. 7A). Resveratrol and PDTC also decreased ICAM-1 expression when administered in vitro to cultured aged arteries (Fig. 7B). PEG-catalase, *N*-acetyl cysteine, and

FCCP elicited similar decreases in ICAM-1 expression in aged vessels, whereas apocynin did not affect ICAM-1 significantly (Fig. 7B). We also found that expression of iNOS was significantly increased in aged carotid vessels (by ~72%) when compared with young ones. Treatment of the aged arteries with resveratrol, PDTC, and PEG-catalase significantly decreased iNOS mRNA expression (untreated: 100 \pm 18%, resveratrol: 51 \pm 17%, PDTC: 50 \pm 8%, PEG-catalase: 65 \pm 11%).

Monocyte adhesion assay and functional studies. Adhesiveness of activated THP-1 monocytic cells to the endothelial surface of aged carotid arteries was significantly increased, which could be inhibited by PDTC treatment (Fig. 7C). Increased monocyte adhesiveness induced by TNF- α is shown as positive control in Fig. 7C.

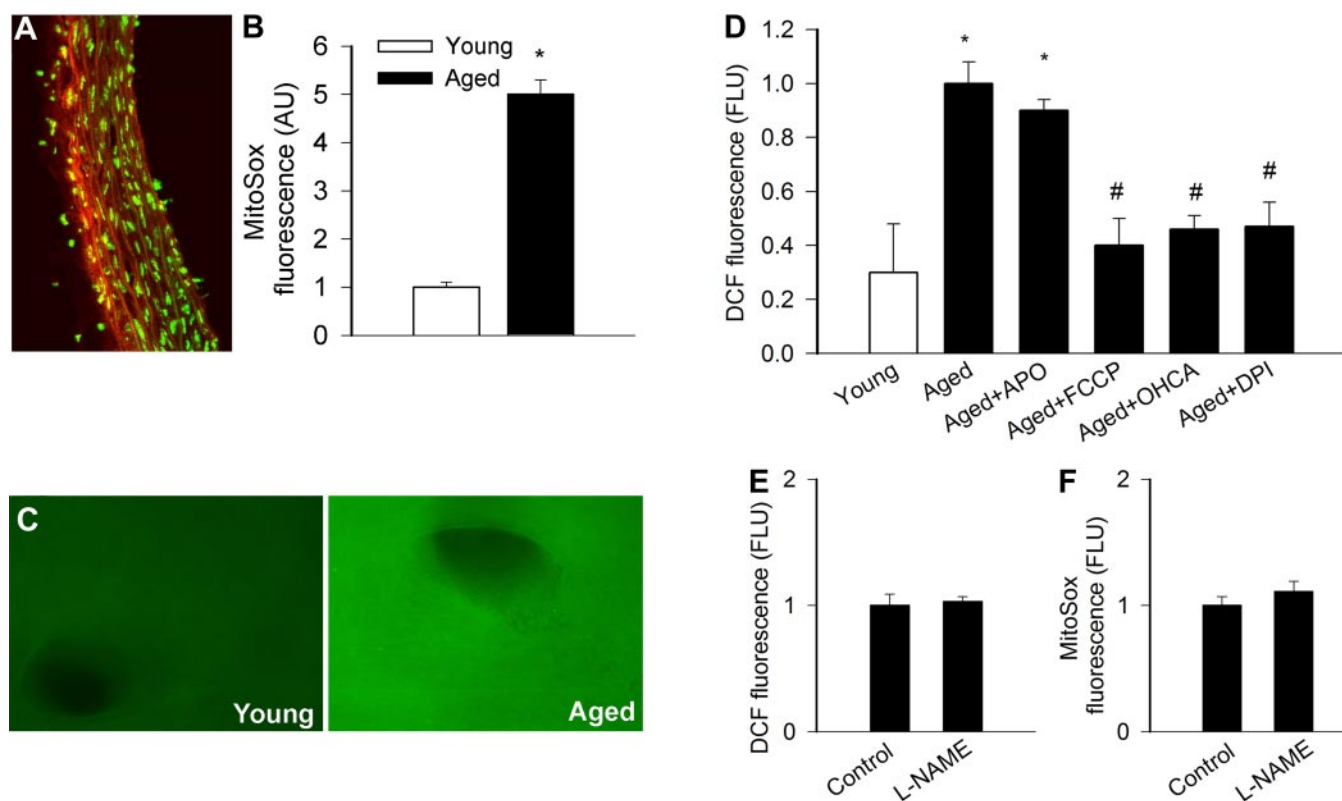


Fig. 4. *A*: representative confocal image showing MitoSox staining (red fluorescence) in a cross section of an aged rat artery. SYTOX green (green fluorescence) was used for nuclear staining. *B*: summary data for perinuclear MitoSox fluorescence in aged and young carotid arteries. Data are means \pm SE ($n = 5$ for each group). *C*: representative images showing DCF fluorescence in the endothelial cells of en face preparations of young and aged aortic segments. The opening of an intercostal artery is shown in both images for orientation purposes. Similar differences in DCF fluorescence were detected in carotid arteries as well. *D*: summary data for endothelial DCF fluorescence in aged and young carotid arteries. The effects of pretreatment with apocynin (3×10^{-4} mol/l), FCCP ($1 \mu\text{mol/l}$), OHCA 250 ($\mu\text{mol/l}$), and DPI ($100 \mu\text{mol/l}$) are also shown. Data are means \pm SE ($n = 4-6$ for each group). * $P < 0.05$ vs. young, # $P < 0.05$ vs. untreated aged. *E* and *F*: Treatment of aged carotid arteries with the nitric oxide (NO) synthase inhibitor N^G -nitro-L-arginine methyl ester (L-NAME, 3×10^{-4} mol/L, for 2 h) did not affect endothelial DCF fluorescence (*E*) or MitoSox fluorescence (*F*).

In aged carotid arteries, relaxations to acetylcholine were impaired when compared with responses of young vessels (Fig. 7*D*). Treatment of the aged arteries with PDTC or FCCP did not significantly change acetylcholine-induced relaxation (Fig. 7*D*).

DISCUSSION

There are three salient findings in this study. First, we have shown that in arteries of aged rats there is an increased NF- κ B activity, which is due, at least in part, to the elevated cellular H_2O_2 levels. Second, vascular aging is associated with increased mitochondrial H_2O_2 production in the endothelial cells. Third, NF- κ B activation seems to contribute to the upregulation of inflammatory gene expression in aged arteries.

Here we report a significant age-related increase in nuclear NF- κ B binding activity in aged vessels (Fig. 1, *A* and *B*). We propose that NF- κ B activation and chronic inflammation is a generalized phenomenon in aging, because increases in NF- κ B activity have been observed recently in the aged rat skeletal muscle, liver, brain, and cardiac muscle (22, 25, 36, 46). Our results indicate that the protein expression level of the NF- κ B subunits p50 and p65 is not significantly affected by aging (Fig. 2). The expression of NF- κ B mRNAs (P50, p65, and p100) with the exception of c-rel also did not show any statistically significant age-related changes (Fig. 3, *A-D*) (23).

We found age-related changes in the mRNA expression of NF- κ B inhibitors ($\text{I}\kappa\text{B}\alpha$ and $\text{I}\kappa\text{B}$ -like 1) and $\text{I}\kappa\text{B}$ kinases (Fig. 3, *E-J*), which may represent a negative feedback loop regulating constitutive NF- κ B activation in aging. Interestingly, we found an age-dependent downregulation of Nkiras-1 and Nkiras-2, which are known to interact with the $\text{I}\kappa\text{B}\alpha$ and $\text{I}\kappa\text{B}\beta$, decreasing their rate of degradation (14).

Because scavenging of H_2O_2 attenuated NF- κ B activation in aged vessels (Fig. 1*B*), we propose a role for H_2O_2 in regulation of endothelial NF- κ B activity in aging. This view is in line with the finding that exogenous H_2O_2 substantially increased NF- κ B activation in vessels of young rats, mimicking the aging phenotype (Fig. 1, *A-B*). Because administration of SOD (not shown) or inhibition of NAD(P)H oxidase (Fig. 1*C*), which is responsible for a significant portion of cytoplasmic $\text{O}_2^{\cdot-}$ generation in aged arteries (7, 10, 18), did not significantly attenuate NF- κ B activation and NF- κ B-dependent gene expression, it is less likely that $\text{O}_2^{\cdot-}$ plays a direct role in NF- κ B activation in aged endothelial cells.

Several lines of evidence suggest that mitochondria are a major source of H_2O_2 in aged blood vessels. First, we found that mitochondrial $\text{O}_2^{\cdot-}$ production significantly increases in aged arteries (Fig. 4, *A* and *B*). Because MnSOD is abundant in the mitochondria ($\sim 10^{-5}$ mol/l) (10, 39), we predicted that a significant portion of mitochondria-derived $\text{O}_2^{\cdot-}$ is dismutated

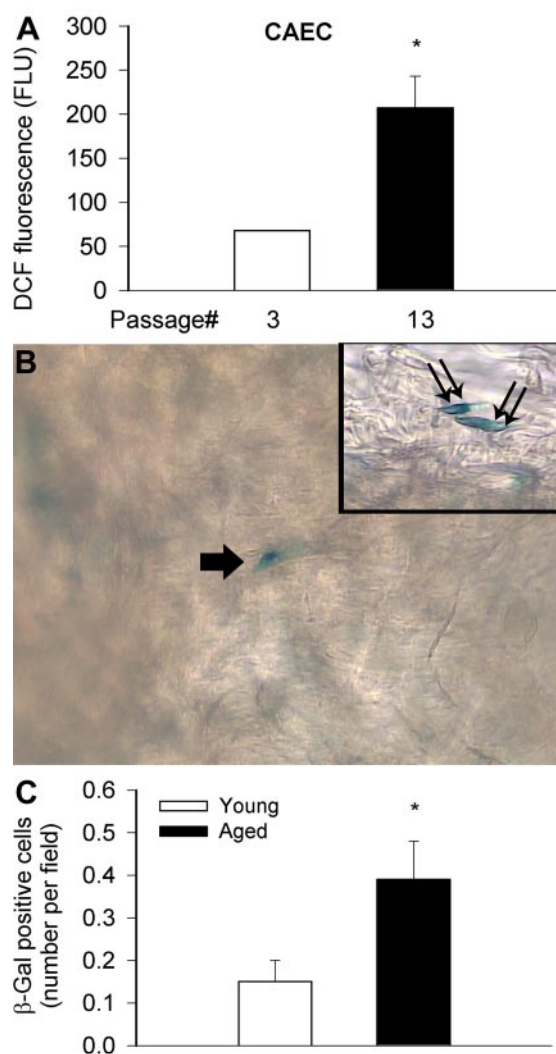


Fig. 5. A: cellular replicative senescence in cultured coronary arterial endothelial cells (CAECs; after passage 13) is associated with an increased cellular H_2O_2 production (assessed by DCF fluorescence) compared with primary CAECs after low number of passages. $*P < 0.05$. B: representative light microscopic image showing staining for senescence-associated β -galactosidase in an endothelial cell (arrow) in en face preparation of an aged rat carotid artery. Inset: fibroblasts with positive staining for senescence-associated β -galactosidase (double arrows) in the adventitia of an aged rat carotid artery. Original magnification: $\times 20$. C: summary data for the frequency of β -galactosidase-positive endothelial cells in young and aged rat carotid arteries (en face preparations). $*P < 0.05$.

increasing also H_2O_2 levels (19). Indeed, measurements of DCF fluorescence showed that endothelial H_2O_2 production is significantly increased in aged arteries (Fig. 4, C and D). Analysis of the effects of pharmacological inhibitors confirmed that the primary source of H_2O_2 is likely the mitochondrial electron transport chain (Fig. 4D). Although one could hypothesize that uncoupled endothelial and/or mitochondrial NO synthase may also produce significant amount of $O_2^{\bullet-}$, and consequently H_2O_2 , in aging, the findings that L-NAME did not affect mitochondrial $O_2^{\bullet-}$ and endothelial H_2O_2 production in aged arteries (Fig. 4, E and F) renders this hypothesis unlikely. This finding also accords with earlier observations that release of H_2O_2 from mitochondria isolated from mouse skeletal muscle significantly increases with age (31). Based on the

findings that endothelial H_2O_2 production was sensitive to inhibition of complex I (Fig. 4D) (30), we hypothesize that complex I is an important source of ROS in the aged endothelium. Whether high rates of $O_2^{\bullet-}$ production from complex I occur in endothelial cells likely depends on the supply of electrons from NADH and ubiquinone, on the current value of ΔpH (which is modulated by uncoupling proteins) and on other cellular regulators of complex I function (37, 40). In isolated mitochondria, $O_2^{\bullet-}$ generation on the matrix side of the membrane is particularly high during reversed electron transport to complex I driven by oxidation of succinate (3, 27, 28). It is also important to note that other sites of the electron transport chain are also capable of producing $O_2^{\bullet-}$, particularly when the electron transport is inhibited. The observation that antimycin increases H_2O_2 production in endothelial cells (Fig. 6, A and B), as well as in isolated mitochondria (3, 19), is in line with the notion that complex III can also be a major source of ROS production. Mitochondrial $O_2^{\bullet-}$ production is very sensitive to proton motive force and can be strongly decreased by mild uncoupling of oxidative phosphorylation (3, 4). It is significant that the pharmacological uncoupler FCCP substantially decreased H_2O_2 production in aged vessels (Fig. 4D). The oxidative stress hypothesis of aging predicts that if mitochondrial ROS production is important in determining the rate of aging, then cells of long-lived animals should produce less. Our recent findings showing that H_2O_2 production in the arterial endothelial cells of aged naked mole rats (*Heterocephalus glaber*, maximum lifespan potential >28.3 yr) is significantly lower than that of senescent shorter-living rodents support this premise (Csizsar A, Buffenstein R, Ungvari Z, unpublished observation, 2006). A central role for mitochondrial H_2O_2 production in organismal aging is also suggested by the findings that overexpression of mitochondrial catalase may extend lifespan in mice (38). Further studies are definitely needed to determine whether decreasing mitochondrial H_2O_2 production (e.g., by caloric restriction mimetics) would delay vascular aging and development of atherosclerotic vascular disease in the elderly.

Replicative senescence is a growth-arrest state associated with loss of division potential, changes in cell morphology, shape and physical appearance, and the pattern of gene expression in cells. Because replicative senescence in cultured endothelial cells was associated with increased H_2O_2 production (Fig. 5A), we hypothesized that increased presence of senescent cells may explain the increased H_2O_2 production in the aged arterial endothelium. However, this hypothesis could be refuted because the number of senescent [β -galactosidase positive (43)] endothelial cells in aged arteries was very low (Fig. 5, B and C). Interestingly, the number of senescent adventitial fibroblasts significantly increased with age (Fig. 5B). An age-related increase in senescent skin fibroblasts was previously reported in elderly humans (13).

There are several lines of evidence supporting the view that $O_2^{\bullet-}$ in the mitochondria is dismutated to H_2O_2 , and it is the increased release of H_2O_2 , which is responsible for activating NF- κ B in the cytoplasm of aged cells. First, $O_2^{\bullet-}$ is membrane impermeable (except in the protonated perhydroxyl radical form, which represents only a small fraction of total $O_2^{\bullet-}$ produced), whereas H_2O_2 easily penetrates the mitochondrial membranes. Second, AA + succinate increased mitochondrial $O_2^{\bullet-}$ production in endothelial cells, which was associated

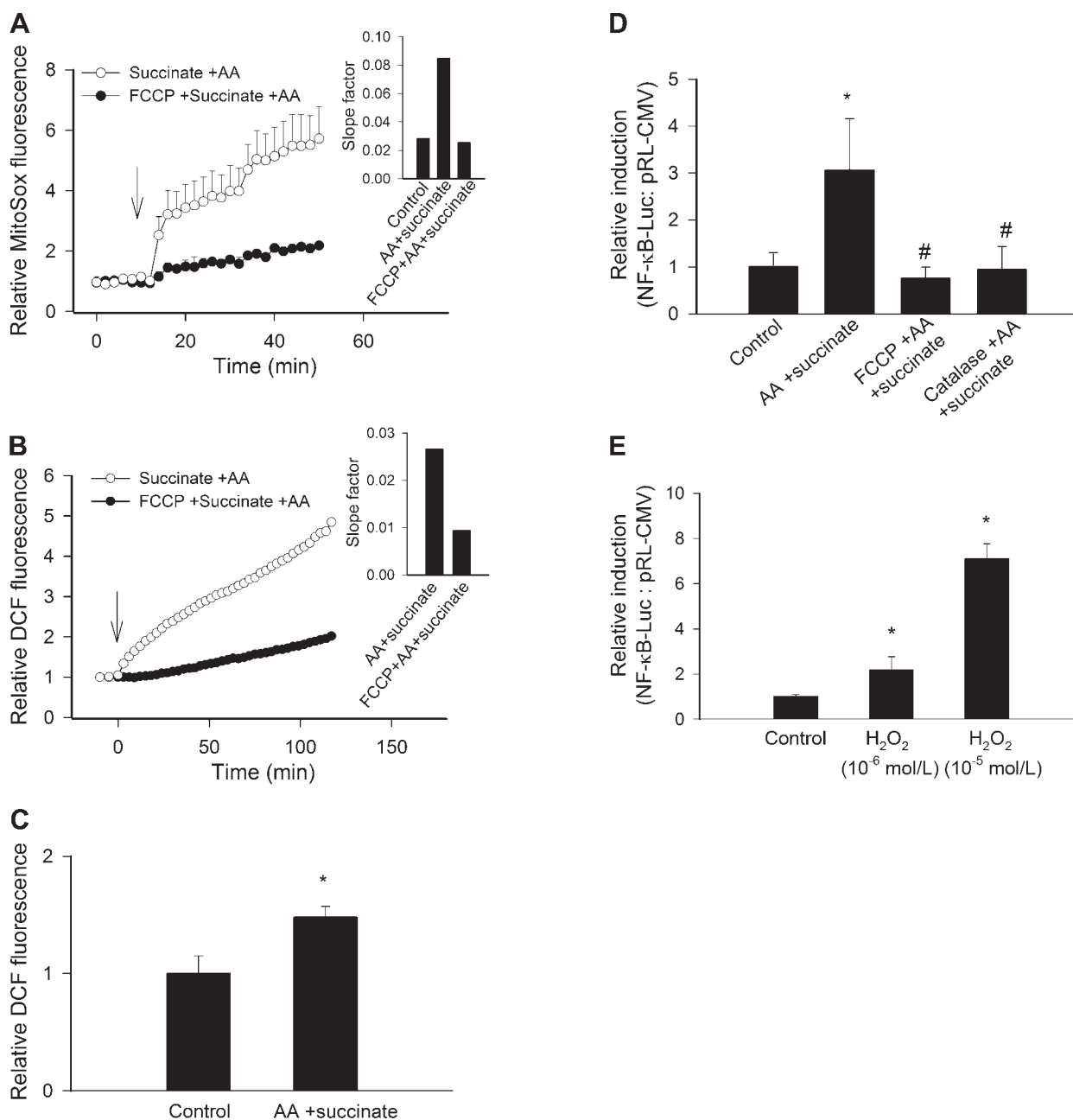


Fig. 6. *A* and *B*: time course of increases in MitoSox (*A*) and DCF (*B*) fluorescence in response to antimycin A (1 μ mol/l) plus succinate (10 mmol/l) in en face preparations of aortic segments from young rats in the presence or absence of FCCP (1 μ mol/l). *Insets*: slope factors derived from a best-fit tangent to the time course curves. *C*: in cultured primary rat coronary arterial endothelial cells (CAECs) antimycin A (1 μ mol/l) plus succinate (10 mmol/l) elicited significant increases in H₂O₂ production (assayed as increases in DCF fluorescence). Data are means \pm SE ($n = 6$ for each group). * $P < 0.05$ vs. untreated. *D*: reporter gene assay showing that antimycin A plus succinate also increased NF- κ B reporter activity in CAECs. FCCP (1 μ mol/l) and PEG-catalase (200 U/ml) abolished NF- κ B activation by antimycin A plus succinate. Data are means \pm SE ($n = 6$ for each group). * $P < 0.05$ vs. untreated, # $P < 0.05$ vs. antimycin A plus succinate only. *E*: in CAECs H₂O₂ also increased NF- κ B reporter activity. Data are means \pm SE ($n = 6$ for each group). * $P < 0.05$ vs. untreated.

with increased H₂O₂ levels in the cytoplasm (Fig. 6, *A–C*). Importantly, mitochondrial oxidative stress (similar to exogenous H₂O₂) elicited endothelial NF- κ B activation, which was prevented by scavenging of H₂O₂ (Fig. 6, *D* and *E*). Third, scavenging of intracellular H₂O₂, similar to mitochondrial uncoupling, attenuated both NF- κ B activation (Fig. 1*B*) and pro-inflammatory gene expression (Fig. 7*B*) in aged arteries.

In aged arteries, an increased constitutive nuclear NF- κ B binding activity is likely to upregulate the expression of NF- κ B

target genes. Indeed, studies by this and other laboratories revealed that vascular expression of ICAM-1 (Fig. 7), iNOS, and various pro-inflammatory cytokines are upregulated both in aged rats and mice (7, 10, 15). To establish a direct link between NF- κ B activation and vascular inflammatory gene expression, we have used PDTC and resveratrol, which effectively inhibit NF- κ B activation in endothelial cells (8). We found that both inhibitors of NF- κ B activation attenuated inflammatory gene expression in aged vessels when adminis-

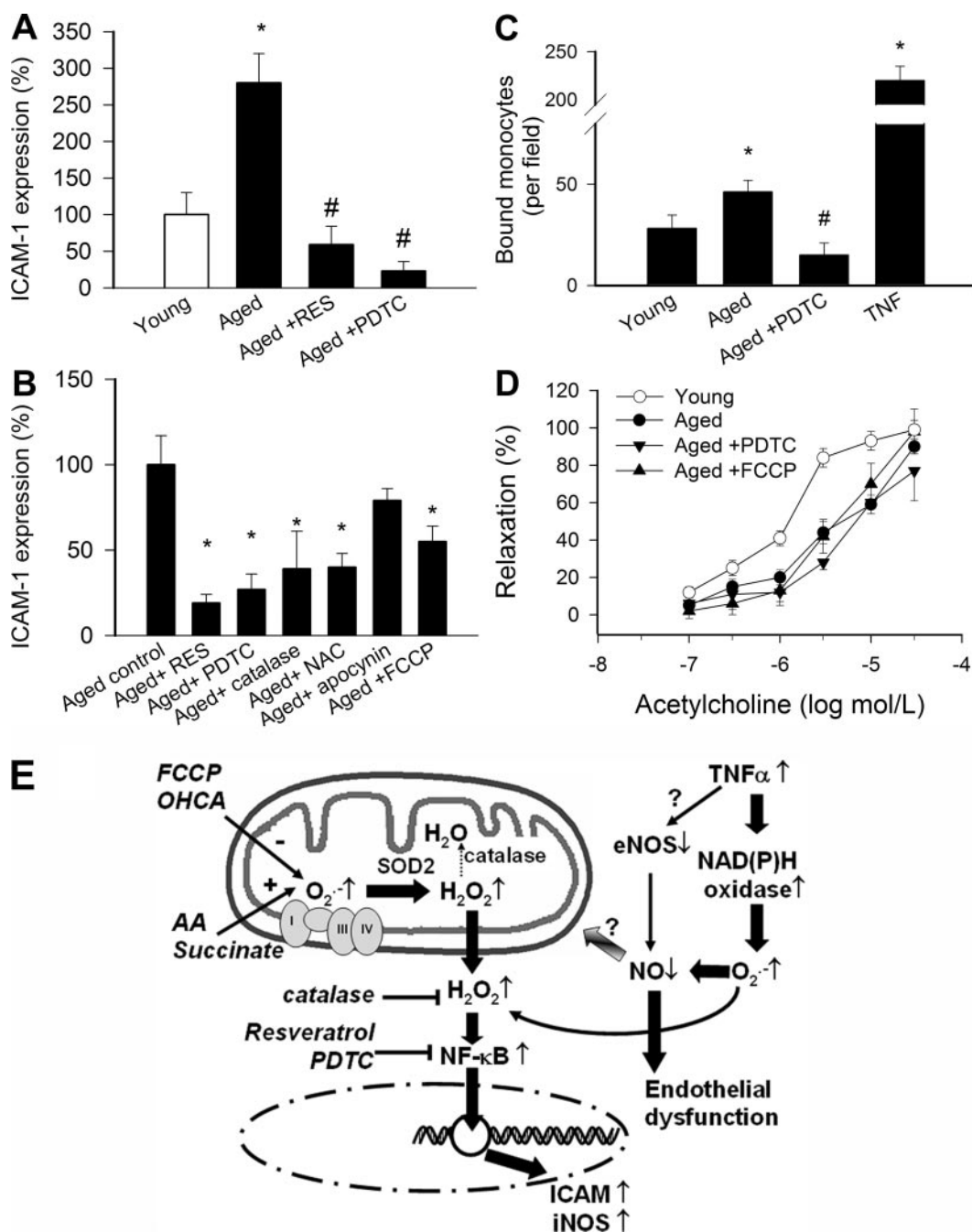


Fig. 7. A: expression of ICAM-1 mRNA in carotid arteries of young and aged rats. Treatment of aged rats with the NF-κB inhibitors resveratrol ($10 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$; for 1 wk) and PDTC ($80 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$; for 1 wk) significantly attenuated vascular ICAM-1 expression. Analysis of mRNA expression was performed by real-time quantitative RT-PCR. β -Actin was used for normalization. Data are means \pm SE. ($n = 5-6$ for each group). * $P < 0.05$ vs. young, # $P < 0.05$ vs. untreated aged. B: effects of in vitro treatment of aged carotid arteries (for 24 h) with resveratrol ($10 \mu\text{mol/l}$), PDTC ($10 \mu\text{mol/l}$), PEG-catalase (200 U/ml), l-acetylcysteine (NAC; 1 mmol/l), apocynin ($3 \times 10^{-4} \text{ mol/l}$), or FCCP ($1 \mu\text{mol/l}$) on the mRNA expression of ICAM-1. Data are means \pm SE ($n = 5-6$ for each group). * $P < 0.05$ vs. untreated. C: results of monocyte adhesion assay (see METHODS). Bar graphs are summary data of fluorescently labeled PMA-stimulated THP-1 monocytes bound to the endothelial surface of young and aged arteries. Treatment of aged arteries with PDTC significantly decreased monocyte adhesiveness. The effect of TNF α (10 ng/ml) is shown for comparison. Data are means \pm SE. * $P < 0.05$ vs. young control. # $P < 0.05$ vs. untreated aged. D: effects of in vitro treatment of aged carotid arteries (for 24 h) with PDTC ($10 \mu\text{mol/l}$) or FCCP ($1 \mu\text{mol/l}$) on relaxations to acetylcholine. Responses of cultured young carotid arteries are shown for comparison. Data are means \pm SE ($n = 4$ for each group). E: proposed scheme for the link between oxidative stress and vascular inflammation in aging: in aged endothelial cells increased levels of $O_2^{\cdot-}$ generated by the electron transport chain are dismutated to H_2O_2 , which can penetrate the mitochondrial membranes increasing cytoplasmic H_2O_2 levels. H_2O_2 contributes to the activation of NF-κB, resulting in a pro-inflammatory shift in endothelial gene expression profile. Increased $O_2^{\cdot-}$ production by the NAD(P)H oxidase (stimulated, at least in part, by TNF- α) and/or downregulation of eNOS is responsible for the impaired bioavailability of NO and endothelial vasodilator dysfunction in aged arteries. The model predicts that upregulation of TNF- α and/or impaired NO bioavailability may contribute to the development of mitochondrial oxidative stress in aging.

tered in vivo and in vitro (Fig. 7, A and B). The functional relevance of upregulation of NF- κ B-dependent genes is suggested by the increased adhesiveness of activated monocytes to the aged endothelium (Fig. 7C). Previous studies demonstrated that endothelial dysfunction in aging is predominantly due to an increased production of O₂^{•-} [predominantly by the NAD(P)H oxidase] (10, 17). The finding that attenuation of mitochondrial oxidative stress did not improve endothelial function in aged arteries supports the view that aged mitochondria predominantly release H₂O₂ rather than O₂^{•-} in the cytoplasm (Fig. 7D).

On the basis of the present findings and previous results, we propose a model for the link between oxidative stress and vascular inflammation in aging (Fig. 7E). Accordingly, in aged endothelial cells increased levels of O₂^{•-} generated by the electron transport chain are dismutated to H₂O₂, which can penetrate the mitochondrial membranes increasing cytoplasmic H₂O₂ levels. H₂O₂ contributes to the activation of NF- κ B, resulting in a pro-inflammatory shift in endothelial gene expression profile. Increased O₂^{•-} production by the NAD(P)H oxidase (stimulated, at least in part, by TNF- α) and/or down-regulation of endothelial NO synthase is responsible for the impaired bioavailability of NO and endothelial vasodilator dysfunction in aged arteries. Because recent studies demonstrated that NO and TNF- α (by decreasing NO bioavailability) can regulate mitochondrial biogenesis and the expression of electron transport chain components (33, 34, 42), future studies should explore whether upregulation of TNF- α and/or impaired NO bioavailability contribute to the development of mitochondrial oxidative stress in aging. Finally, because resveratrol was shown to exert anti-aging activity extending the lifespan of lower organisms (24, 45), future studies should determine whether chronic NF- κ B inhibition contributes to the putative anti-aging, lifespan-extending effects of resveratrol in mammalian systems (1, 2).

GRANTS

This work was supported by grants from the American Heart Association (0430108N, 0435140N) and the National Heart, Lung, and Blood Institute (HL-077256 and HL-43023) and by Philip Morris USA and Philip Morris International.

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