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Proinflammatory phenotype of coronary arteries promotes endothelial apoptosis in aging

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Csiszar, Anna, Zoltan Ungvari, Akos Koller, John G. Edwards, and Gabor Kaley. Proinflammatory phenotype of coronary arteries promotes endothelial apoptosis in aging. *Physiol Genomics* 17: 21–30, 2004; 10.1152/physiolgenomics.00136.2003.—Previously we demonstrated that aging in coronary arteries is associated with proinflammatory phenotypic changes and decreased NO bioavailability, which, we hypothesized, promotes vascular disease by enhancing endothelial apoptosis. To test this hypothesis we characterized proapoptotic alterations in the phenotype of coronary arteries of aged (26 mo old) and young (3 mo old) F344 rats. DNA fragmentation analysis and TUNEL assay showed that in aged vessels there was an approximately fivefold increase in the number of apoptotic endothelial cells. In aged coronary arteries there was an increased expression of TNFα, TNFβ, and caspase 9 (microarray, real-time PCR), as well as increased caspase 9 and caspase 3 activity, whereas expression of TNFR1, TNFα-converting enzyme (TACE), Bcl-2, Bcl-X(L), Bid, Bax, caspase 8, and caspase 3 were unchanged. In vessel culture (18 h) incubation of aged coronary arteries with a TNF blocking antibody or the NO donor *S*-nitroso-penicillamine (SNAP) decreased apoptotic cell death. Incubation of young arteries with exogenous TNFα increased caspase 9 activity and elicited endothelial apoptosis, which was attenuated by SNAP. Inhibition of NO synthesis in cultured young coronary arteries also induced apoptotic cell death and potentiated the apoptotic effect of TNFα. Thus we propose that age-related upregulation of TNFα and caspase 9 and decreased bioavailability of NO promote endothelial apoptosis in coronary arteries that may lead to impaired endothelial function and ischemic heart disease in the elderly.

TACE activity; caspase 9; small inhibitory RNA; senescence; cytokine; inflammation

PREVIOUS STUDIES SUGGEST that endothelial cell injury due to the activation of the cellular apoptotic pathways is an initial step in the development of coronary artery disease (CAD) (6). CAD is the leading cause of mortality among the elderly, yet the role of apoptosis in the aging process in the coronary circulation has not been elucidated.

With advancing age, coronary arteries were shown to undergo complex phenotypic and functional alterations (7, 8), including endothelial vasodilator dysfunction (11), downregulation of eNOS and decreased NO production, increased activity of NAD(P)H oxidases, and upregulation of proinflammatory cytokines (7, 8), which likely affect the survival of vascular cells (13, 22). Recent studies suggest that advanced age itself, even in the absence of other disease-related proapoptotic factors (e.g., ox-LDL; Ref. 18) may promote apoptotic cell death in various tissues, including peripheral arteries (4). Also, accelerated aging in different mutant mouse models is associated with an imbalanced apoptotic cell loss (30). High number of passages in vitro was also shown to enhance the sensitivity of endothelial cells toward apoptotic stimuli (13).

The regulation and execution of apoptosis in endothelial cells is a complex process involving paracrine factors, membrane receptors, interaction of pro- and anti-apoptotic factors and cysteinyl aspartate-specific proteases (caspases). Recent studies suggest that in aging there is an imbalance in the expression of pro- and anti-apoptotic genes resulting in an enhanced apoptosis in the myocardium (19), central nervous system (24), skeletal muscle (10), lung (33), and liver (2, 33). Yet, age-related alterations in the expression of pro- and anti-apoptotic genes in coronary arteries have not been elucidated.

On the basis of the aforementioned findings we hypothesized that age-related changes in gene expression create a proapoptotic microenvironment, which results in enhanced endothelial cell death in coronary arteries.

METHODS

Animals. Young adult (age, 3 mo; $n = 15$) and aged (age, 26 mo; $n = 15$) male Fischer 344 rats kept under pathogen-free conditions were used, as described previously (8). In two aged rats subcutaneous adenoid tumor and splenomegaly of unknown origin were diagnosed upon autopsy, and thus the vessels isolated from these animals were discarded. All other aged rats were disease free with no signs of systemic inflammation and/or neoplastic alterations. All animal use protocols were approved by the Institutional Animal Care and Use Committee of the New York Medical College, Valhalla, NY.

Isolation of coronary vessels. Coronary arteries were isolated from the left ventricle of young and aged rat hearts as previously described (7, 8). Samples were cleaned of adhering tissue and snap-frozen in liquid nitrogen.

Detection of apoptotic cell death by ELISA. Vessels were lysed and cytoplasmic histone-associated DNA fragments, which indicate apoptotic cell death, were quantified by the Cell Death Detection ELISA^{Plus} kit (**http://www.roche-applied-science.com**). Results are reported as arbitrary optical density (OD) units normalized to protein concentration.

TUNEL assay. DNA fragmentation was detected in coronary arteries in situ by using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) on cryosections (thickness: 5 μm) of the heart of young and aged rats. After proteinase K (2.5 μg/ml) treatment, DNA fragments in the sections were labeled with 2 nmol/l digoxigenin-conjugated dUTP and 0.1 U/μl TdT for 2 h at 37°C. The incorporation of digoxigenin-16-dUTP into DNA was determined by incubating the sections with FITC-anti-digoxigenin (1:50, Sigma Chemical) at room temperature for 60 min. The sections were immunolabeled for smooth muscle α-actin, and the nuclei were stained with propidium iodide.

RNA preparation and analysis of mRNA expression with microarrays and real-time PCR. Total RNA from coronary arteries was isolated with Mini RNA Isolation Kit (Zymo Research, Orange, CA)

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and was reverse transcribed using SuperScript II RT (Life Technologies, Gaithersburg, MD) as described previously (7, 8).

Real-time RT-PCR technique was used to analyze mRNA expression, as previously described (7, 8). PCR reactions were performed in the Roche Molecular Biochemicals LightCycler System. The housekeeping gene β-actin was used for internal normalization. Oligonucleotides used for real-time quantitative RT-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.

Expression of 96 apoptosis-related genes in coronary arterial samples from aged ($n = 5$) and young ($n = 5$) animals was also analyzed by the GEArray Q series nonradioactive apoptosis gene array (list of genes is available at **http://www.superarray.com**) as previously described (8). Analysis of data was performed with an image analysis software (Scanalyze, by Michael Eisen; **http://www.microarrays.org/software.html**) followed by significance analysis of microarrays (SAM) using an Excel add-in program. Pairwise comparisons were made between individual samples in each group (1.6 or greater was used as the cutoff for significant differences in gene expression) as reported (8). The reproducibility of our procedures was tested by hybridizing identical samples from one vessel to two arrays yielding a correlation coefficient of $r = 0.98$.

Microdissection of coronary arterial endothelium. Microdissection was performed using a P.A.L.M. Microlaser Technologies system (Bernried, Germany) on frozen sections (thickness: 10 μm, stored on −80°C) of aged and young hearts stained by hematoxylin. For RNA analyses, the endothelial layer of multiple coronary arteries was dissected and catapulted into 20 μl of cDNA reaction mixture and stored on ice until cDNA synthesis. RNA was extracted Mini RNA Isolation Kit (Zymo Research) as described.

Western blotting. Using coronary arterial samples from young and aged rats, we performed Western blotting as described (7, 8) using primary antibodies directed against cleaved caspase 9, cleaved caspase 3 (Cell Signaling, 1:100), truncated Bid (Oncogene, 1:100), TRADD, and TNFR1 (Santa Cruz Biotechnology, 1:100). Anti-β-actin (Novus Biologicals, 1:5,000) was used for normalization.

Vascular caspase activities. Isolated coronary arteries from aged and young rats were homogenized in lysis buffer, and caspase activities were measured using the caspase 9, caspase 3, and caspase 8 Colorimetric Activity Assay kits, according to the manufacturer's instruction (Chemicon International). Optical density values were normalized to the sample protein concentration.

Vascular TACE activity. TNFα-converting enzyme (TACE) is a membrane-bound metalloprotease-disintegrin in the ADAM family (ADAM-17). To measure TACE activity, vessel samples were homogenized in Tris buffer (10 mmol/l, pH 7.5, 0°C), containing 300 mmol/l sucrose, 1 mmol/l DTT, and protease inhibitors (10 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml soybean trypsin inhibitor, 0.2 mg/ml α1-antitrypsin, 10 μmol/l pepstatin A). Crude membranes were separated by precipitation at 15,000 g for 20 min after a 15-min incubation period on ice after addition of $CaCl_2$ (10 mmol/l, in Tris). The resulting pellet was dissolved in Tris buffer containing 1% Nonidet P-40 and protease inhibitors followed by centrifugation at 15,000 g_{av} for 5 min. The supernatant (referred to in the following as detergent extract) was used for the oligopeptide hydrolysis assay. Detergent extract, 50 μl (protein concentration ~5 mg/ml), was added to the incubation medium consisting of Tris buffer (10 mmol/l; pH 7.5), $MgCl_2$ (1 mmol/l), $CaCl_2$ (0.2 mmol/l), and protease inhibitors. Samples incubated with EDTA (5 mmol/l) were used as negative controls. The reaction was started by addition of the substrate peptide Abz-LAQA-VRSSSR-Dpa (10 μg/ml) to the incubation medium. After the incubation (37°C, for 60 min) the reaction was stopped by addition of HCl (0.1 mol/l). Specific peptide breakdown was determined by the System Gold HPLC system (Beckman Coulter) equipped with C_{18} guard and 150 × 2.0 mm MiniBore Ultrasphere 5μ C_{18} column with a 20–100% acetonitrile [0.09% trifluoracetic acid (TFA), vol/vol]/H_2O (0.1% TFA, vol/vol) gradient. Separation was monitored at fluorescence excitation = 320 nm, emission = 420 nm. Data are expressed as arbitrary units (AU/mg) corresponding to the peak area of the breakdown product in the chromatogram.

Vascular TACE expression. TACE mRNA expression was assessed in young and aged coronary arteries using RT-PCR. To localize TACE expression in sections of coronary arteries, immunolabeling was carried out as described previously (7, 8) with a primary antibody against TACE (Novus Biologicals, 1:80, overnight, at 4°C), smooth muscle α-actin (Sigma, 1:1,000, 1 h, at room temperature), and CD31 (PECAM, an endothelium-specific marker; 1:50, Pharmingen) using the Zenon rabbit IgG labeling Kit (Molecular Probes, Eugene, OR). Images were captured using an Olympus BX61 fluorescent microscope. TACE mRNA expression was also assessed in cultured rat coronary arterial endothelial cells (CAEC, see below) and vascular smooth muscle cells (VSMC, SV40LT-SMC clone HEP-SA; American Type Culture Collection, Manassas, VA).

Vessel culture studies. Coronary arteries of young and aged rats were incubated for 18 h (at 37°C) in a vessel culture system as previously described (28) in the absence or presence of recombinant TNFα (1–100 ng/ml). Coronary arteries from aged rats were cultured (for 18 h) in the absence and presence of the NO donor *S*-nitroso-penicillamine (SNAP, 10^{-6} mol/l) or the NO synthesis inhibitor N^{ω}-nitro-L-arginine methylester (L-NAME, 3×10^{-4} mol/l) or a monoclonal anti-rat TNFα antibody (R&D Systems) to neutralize bioactivity of endogenous TNFα (28). In separate experiments young coronary arteries were incubated with TNFα in the absence and presence of L-NAME or SNAP. After the culture period apoptotic cell death and/or caspase 9 activity were detected as described.

Table 1. *Oligonucleotides for real-time quantitative RT-PCR*

mRNA Targets	Sense	Tm	Antisense	Tm
TNFα	TCGTAGCAAACCAAG	60.8	CTGCGGTGTGGGTGA	61.2
TNFβ	GTCGGGTGACAACTAGG	59.6	GGGATTCACGGATGGT	59.8
TNFRI	AAAGCCAGGAGAGGTG	59.9	CTTAGGGAGTTCAACCGT	59.9
Bcl-2	TGATTTCTCCTGGCTGT	59.2	TTTGACCATTTGCCTGAATG	60.0
Bcl-X(L)	TGCGTGGAAAGCGTAG	60.5	CCGACTGAAGAGTGAGC	60.4
Bax	GCGAATTGGCGATGAAC	60.6	CGAAGTAGGAAAGGAGGC	60.1
Bad	GGGAGAAGAGCTGACG	60.5	GTCTCGGTTTACCAGGAC	60.5
Bid	CCAACGGAACTGTGAC	60.2	GCCTGCTTGTAGGTTTAATT	60.3
TACE	GCAATGTGCTCACCAG	60.2	ACCCCACTCACCTTAG	60.1
TRADD	GCCAGACTTTTCTGTTCC	59.1	CTCGTATAGCCATCACGG	60.6
FADD	ACACGAAAGCAAGTGC	59.6	TTATTTGGCCGCCCAG	59.7
Caspase 9	CTGAGTATTTCTCTGTGTTCCA	59.9	CATGTCACTGTTGCCC	59.8
Caspase 8	ACTGTGTTTCCTACCGAG	59.7	AGCTTCTTCCGTAGTGT	59.6
Caspase 3	GTCTCGGTTTACCAGGAC	59.5	ACTGTCAGGGAGACTTT	59.1
β-Actin	GAAGTGTGACGTTGACAT	59.7	ACATCTGCTGGAAGGTG	60.4

Cell culture studies. Primary rat CAECs (Celprogen, San Pedro, CA) were maintained in culture according to the vendor's guidelines and harvested after passage 3 and passage 9 (referred to as "young" and "aged" CAEC, respectively). Activity and expression of caspase 9 were measured as described.

To assess the effect of TNFα, young CAEC were incubated (18 h) in the absence or presence of recombinant TNFα (10–100 ng/ml). Apoptotic cells were detected by the ApoAlert Annexin V Apoptosis Kit (**http://www.clontech.com**). This assay takes advantage of the fact that phosphatidylserine is translocated to the outer plasma membrane after the induction of apoptosis and that the annexin V protein has a strong, specific affinity for phosphatidylserine. Images were captured using an Olympus confocal microscope. TNFα-induced changes in caspase 9 activity and expression were also determined.

Downregulation of caspase 9 in CAEC using RNAi. Downregulation of caspase 9 in CAEC was achieved by RNA interference (RNAi,

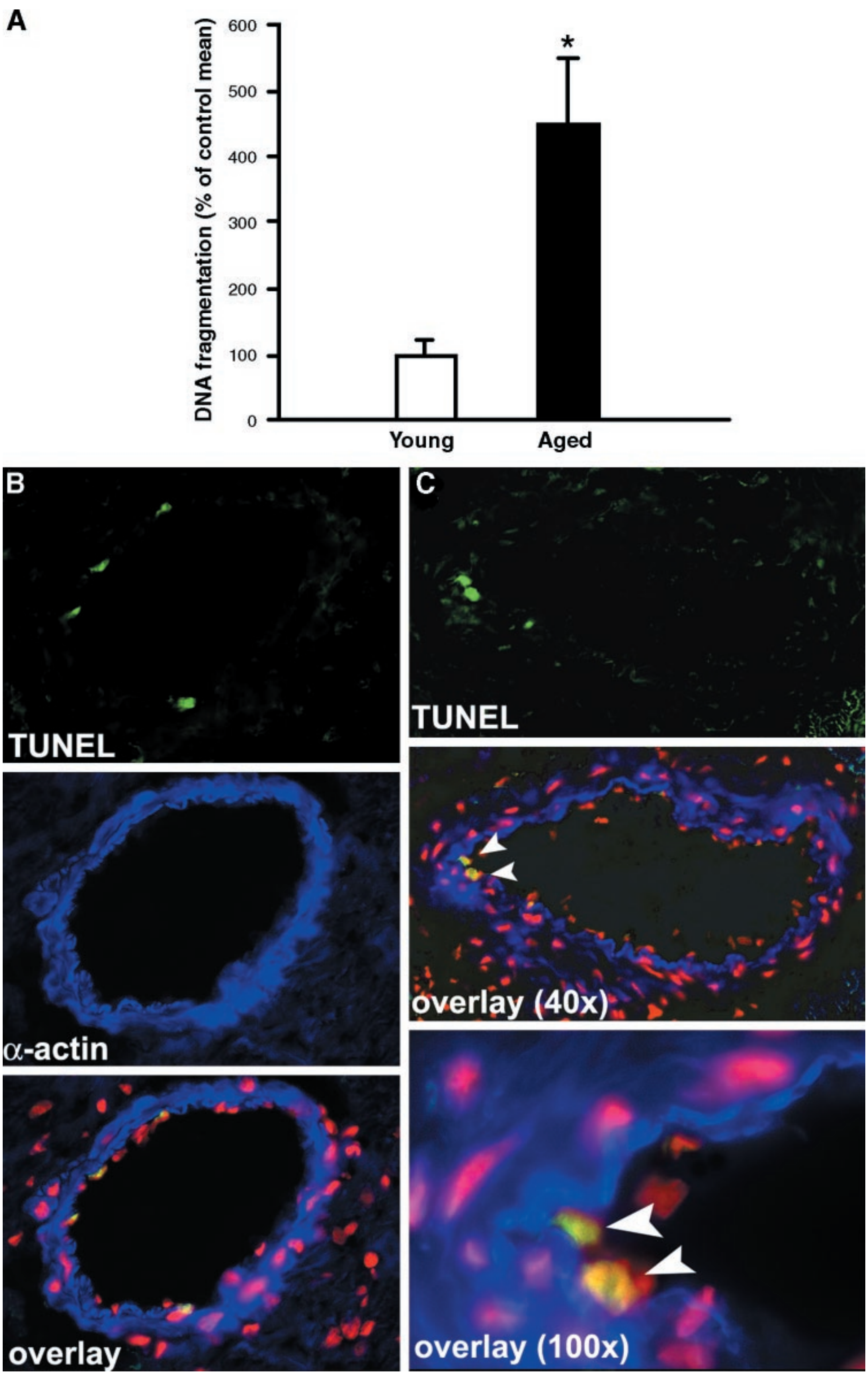

Fig. 1. *A*: DNA fragmentation (an indicator of apoptotic cell death) in coronary arteries of young and aged rats. Data are means ± SE ($n = 5$ for each group, one vessel from each animal was used). $^*P < 0.05$. *B* and *C*: representative images of coronary arteries of aged rats showing TUNEL-positive endothelial cell nuclei (green fluorescence). Blue fluorescence indicates immunolabeling for smooth muscle α-actin. Red fluorescence indicates nuclear staining with propidium iodide Original magnifications are ×40, except *bottom right*, which is ×100.

a phenomenon in which double-stranded RNA specifically suppresses expression of a target protein by inducing the degradation of the target RNA) using the siLentGene U6 Cassette RNA Interference System according to the manufacturer's guidelines (Promega, Madison, WI). This system allows production and transfection of small inhibitory RNA (siRNA) sequences under control of an engineered U6 promoter sequence coupled with a specific U6 polymerase terminator. Three different siRNA sequences were screened for optimal gene silencing. The following downstream primer was used in the experiments: 5′-GCCCAAGCTGTTCTTCATC-3′. CAEC cell density at transfection was 30%. Specific gene silencing was verified by Western blotting. CAEC transfected with anti-caspase 9 siRNA or scrambled siRNA on *day 4* after the transfection were incubated with TNFα (10 ng/ml, for 0, 2, 4, or 6 h). Then, DNA fragmentation assay was performed as described.

Data analysis. Data are expressed as means ± SE. Densitometric ratios and enzyme activity values were normalized to the respective control mean values. 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. If two data sets had different variances, then the Welch modified *t*-test was used. The array data was analyzed by the pairwise comparisons method as described (8). $P < 0.05$ was considered statistically significant.

RESULTS

Detection of apoptotic cell death by ELISA. In coronary arteries of aged rats there was a significantly increased level of mono- and oligonucleosomes, indicating enhanced apoptotic cell death (Fig. 1*A*).

TUNEL assay. Quantification of TUNEL-positive cells showed that there was a higher incidence of apoptotic cells in coronary arteries of aged rats (∼2.5%) compared with vessels of young rats (∼0.5%). TUNEL-positive cells were present predominantly in the endothelium of aged coronary arteries (Fig. 1, *B* and *C*). An increased number of TUNEL-positive cells were also observed in the myocardium of aged rats (not shown), confirming previous reports (14).

Table 2. *Expression profile of apoptosis-related genes in coronary arteries*

Gene Name	Change in Expression in Aging: Microarray	QRT-PCR
TNF ligands		
TNFα	↑	↑
TNFβ (Lta)	↑	↑
TNF receptor family		
TNFR-1	→	→
TNFRSF11A	→	
TNFRSF12 (DR3)	→	
Bcl-2 family		
Bcl-2	→	→
Bcl2ald (Bfl-1)	→	
Bik	→	
Bcl-X(L)		→
Bid		→
Bax		→
Bad		→
Caspase family		
caspase 2	→	
caspase 3		→
caspase 8	→	→
caspase 9	↑	↑
Other related genes		
TRAF1	→	
TRADD		→
FADD		→
Gadd45	→	

mRNA expression of apoptosis-related genes in coronary arteries of young and aged Fisher 344 detected by microarray (Apoptosis GEArray; SuperArray, Frederick, MD) or by real-time RT-PCR, as described in the METHODS. Arrows indicate unchanged (→) or increased (↑) gene expression in aging. QRT-PCR, quantitative RT-PCR.

Table 3. *Western blot analysis of cleaved caspases in coronary arteries of young and aged rats*

	Densitometric Ratio, % of young mean: Young	Aged
Cleaved caspase 9	100±20	197±32*
Cleaved caspase 3	100±5	246±60*

Data are means ± SE. Densitometric data are normalized to β-actin content (n = 4–5 for each group). *$P < 0.05$.

mRNA expression. Experiments using the Apoptosis Gene Arrays indicated an increased expression of TNFα, TNFβ, and caspase 9 in aged coronary arteries, whereas expression of other apoptosis-related gene transcripts were similar to that in control vessels by this method (Table 2). Using real-time PCR, we detected an increased presence of caspase 9 mRNA in intact aged coronary arteries (Fig. 2*A*), as well as in the endothelium of aged coronary arteries obtained by laser capture microdissection (Fig. 2*B*). Increased caspase 9 mRNA content was also present in aged CAEC (Fig. 2*D*). In coronary arteries of aged rats a significantly increased mRNA expression of TNFα and TNFβ (Fig. 3, *A* and *B*) was detected, confirming our recent findings (7, 8). The vascular mRNA expressions of caspase 8 and caspase 3, Bcl-2, Bcl-X(L), Bid, Bax, TNFR1, TRADD, and FADD were unaffected by aging (Table 2).

Western blotting. In coronary arteries of aged rats there was an increased presence of cleaved caspase 9, cleaved caspase 3 (Table 3), and truncated Bid (young, 100 ± 21%; aged, 400 ± 120%; $P < 0.05$). Aging did not affect protein expression of TNFR1 (young, 100 ± 30%; aged, 117 ± 16%; not significant) and that of TRADD (young, 100 ± 21%; aged, 91 ± 18%; not significant).

Caspase activities. In aged coronary arterial samples there was a significantly increased caspase 9 (Fig. 2*C*), caspase 8, and caspase 3 activity (Table 4). Increased caspase 9 activity was also present in aged cultured CAEC (Fig. 2*E*).

TACE activity and expression. In coronary arterial samples there was a significant TACE activity, which could be inhibited by EDTA and by HCl. TACE activity and expression (RT-PCR) was completely preserved in aged vessels (Fig. 4, *A* and *B*). Immunofluorescent labeling for TACE in aged coronary arteries was present both in the endothelium (arrowheads) and smooth muscle (arrows, Fig. 4, *D–F*). In addition, mRNA expression of TACE was demonstrated both in cultured CAEC and VSMC by RT-PCR (Fig. 4*C*).

Vessel culture studies. In cultured aged coronary arteries there was an increased DNA fragmentation, compared with young vessels that could be significantly decreased by the NO donor SNAP and an anti-TNFα blocking antibody, but was unaffected by L-NAME (Fig. 5*A*). In cultured coronary arteries of young and aged rats incubation with recombinant TNFα (18 h incubation) induced apoptosis in a concentration-dependent manner (Fig. 5*B*). TUNEL assay showed that in TNFα-treated coronary arteries apoptotic cells (green) were predominantly

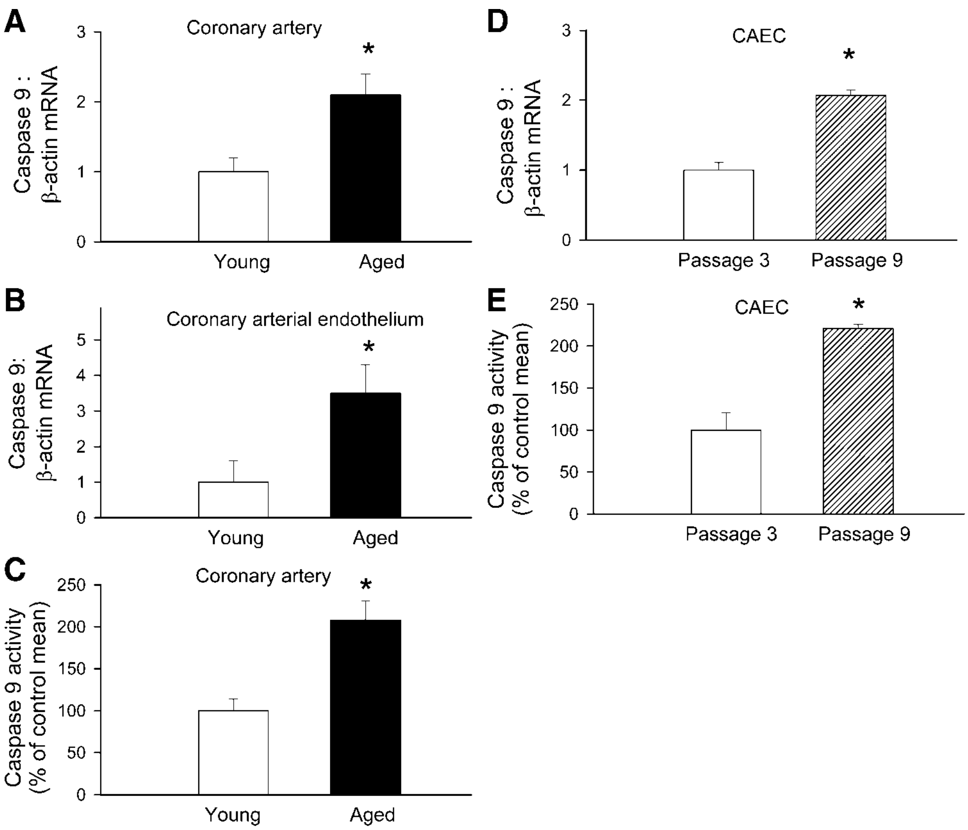

Fig. 2. Expression of caspase 9 mRNA in coronary arteries of young and aged rats (*A*), in endothelium of young and aged coronary arteries obtained by laser capture microdissection (*B*), and (*D*) in cultured primary rat coronary arterial endothelial cells (CAEC) after passage 3 and 9. Analysis of mRNA expression was performed by real-time PCR with the LightCycler System and was normalized to β-actin mRNA. *C*: caspase 9 activity in coronary arteries of young and aged rats. *E*: caspase 9 activity in young and aged CAEC. Data are means ± SE ($n = 5$–6 for each group, one vessel from each animal was used). $^*P < 0.05$.

localized to the endothelium (Fig. 5*C*, arrowheads), whereas the media (blue, smooth muscle α-actin staining) was relatively free from TUNEL-positive cells. In cultured coronary arteries of young rats inhibition of NO synthesis also induced apoptosis and potentiated the apoptotic effects of TNFα (Fig. 6*A*). In cultured coronary arteries of young rats TNFα-induced apoptosis was reduced by the NO donor SNAP (Fig. 6*B*). In young coronary arteries caspase 9 activity was significantly increased by TNFα, whereas it was unaffected by SNAP or L-NAME (Fig. 7*A*).

Cell culture studies. TNFα induced apoptosis in cultured CAEC, as shown by annexin V staining (Fig. 5*D*). Annexin V-positive cells did not exhibit significant propidium iodide staining, indicating that TNFα induces primarily apoptosis, not necrosis, in endothelial cells (not shown). In cultured CAEC TNFα significantly increased caspase 9 activity (Fig. 7*B*) and caspase 9 expression [caspase 9, β-actin mRNA: control, 100 ± 11%; TNFα treated (10 ng/ml, 18 h), 188 ± 18%]. Downregulation of caspase 9 protein in CAEC was achieved by RNAi (by ~80% on *day 4* after siRNA treatment) as illustrated in Fig. 7*C*. Downregulation of caspase 9 levels resulted in partial protection of CAEC against TNF-induced apoptosis (Fig. 7*D*).

DISCUSSION

The main finding of the present study is that in rats with a biological age corresponding to that of 70- to 75-yr-old humans (26) there is an enhanced endothelial apoptosis in the coronary arteries (Fig. 1). Importantly, this biological age in both rats (7) and humans (9) is associated with significant coronary arterial endothelial dysfunction leading to a reduced cardiac perfor-

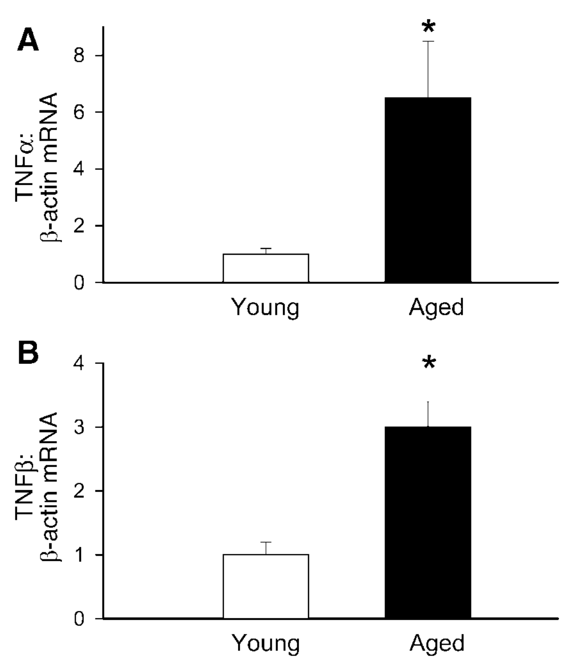

Fig. 3. Expression of TNFα (*A*) and TNFβ (*B*) mRNA in coronary arteries of young and aged rats. Analysis of mRNA expression was performed by real-time PCR with the LightCycler System and was normalized to β-actin mRNA. Data are means ± SE ($n = 5$–6 for each group, one vessel from each animal was used). $^*P < 0.05$.

Table 4. *Caspase activities in coronary arteries of young and aged rats*

	Enzyme Activity, % of young mean	
	Young	Aged
Caspase 8	100±11	165±20*
Caspase 3	100±12	251±53*

Values are means ± SE; n = 4–5 for each group. *$P < 0.05$.

mance (3, 12) and an increased risk for development of CAD. Enhanced endothelial apoptosis was also reported in peripheral arteries of primates of similar biological age (4).

The coronary arterial endothelium in healthy vessels has vasodilative, anticoagulant, and angiogenic functions (6). An age-related increased loss of coronary endothelial cells may impair cardiac blood supply and create a procoagulant environment, favoring platelet adhesion and thrombus formation promoting the development of CAD (6). Endothelial apoptosis may also contribute to the age-related structural remodeling of the coronary vasculature (3, 27).

Recent studies suggest that age-related functional alterations in the cardiovascular system are due to alterations in gene expression profile (7, 8, 15). To elucidate the underlying mechanisms of enhanced endothelial apoptosis in aged coronary arteries, we analyzed the expression of apoptosis-related genes that are involved in induction, execution, and regulation of programmed cell death. Among the apoptosis-related signaling molecules, the expression of caspase 9 was significantly upregulated in the endothelium (Table 2; Fig. 2, *A* and *B*), whereas expression of other caspases (e.g., caspase 3, caspase 8) seems to be unaffected by aging (Table 2). Increased presence of active caspase 9 and the downstream effector caspase 3 (Table 3) and increased caspase 9 (Fig. 2*C*) and caspase 3 activities (Table 4) were present in aged vessels. Upregulated caspase 9 expression and activity was also present in aged CAEC (Fig. 2, *D* and *E*), suggesting that upregulation/activation of caspase 9 may contribute to age-related endothe-

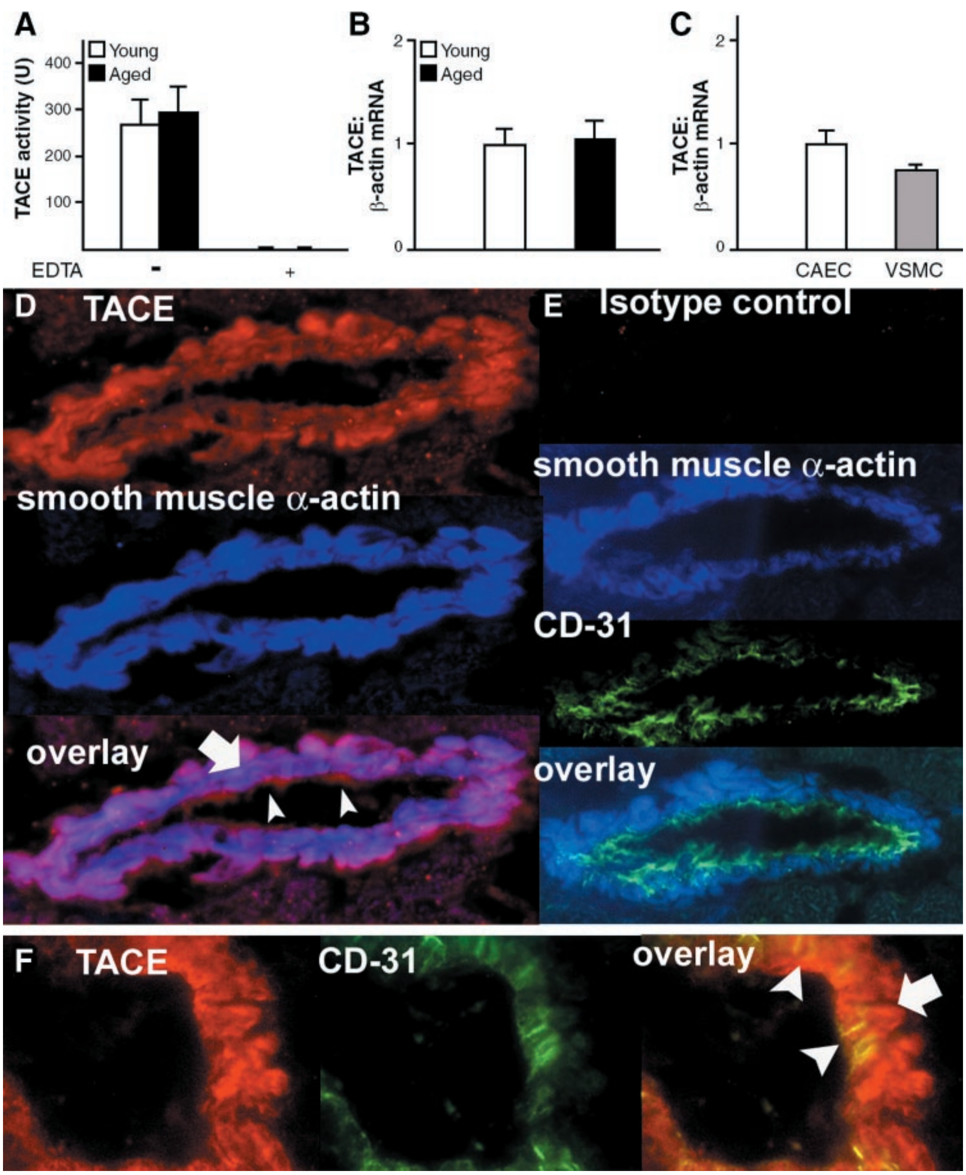

Fig. 4. *A*: TNFα-converting enzyme (TACE)-like activity (measured by a HPLC-based oligopeptide hydrolysis assay) in homogenates of coronary arteries from young and aged rats. *B*: expression of TACE mRNA in coronary arteries of young and aged rats. Analysis of mRNA expression was performed by real-time PCR with the LightCycler System and was normalized to β-actin mRNA. Data are means ± SE (n = 5–6 for each group, one vessel from each animal was used). *C*: RT-PCR demonstrated TACE expression in cultured primary rat CAEC and smooth muscle cells (VSMC). *D*: representative images showing immunofluorescent labeling for TACE (red) in the endothelium (arrowheads) and smooth muscle (arrow) in aged coronary arteries. Blue fluorescence indicates immunolabeling for smooth muscle α-actin (original magnification, ×40) *E*: lack of red immunostaining (*top*) with an isotype control antibody shows the specificity of the labeling reaction (blue is α-actin; green is endothelium-specific marker CD31). *F*: overlaying images showing immunofluorescent labeling for TACE (red) and CD31 (green) demonstrates that TACE is present both in the endothelium (arrowheads) and the smooth muscle of coronary arteries (arrow) (original magnification, ×100).

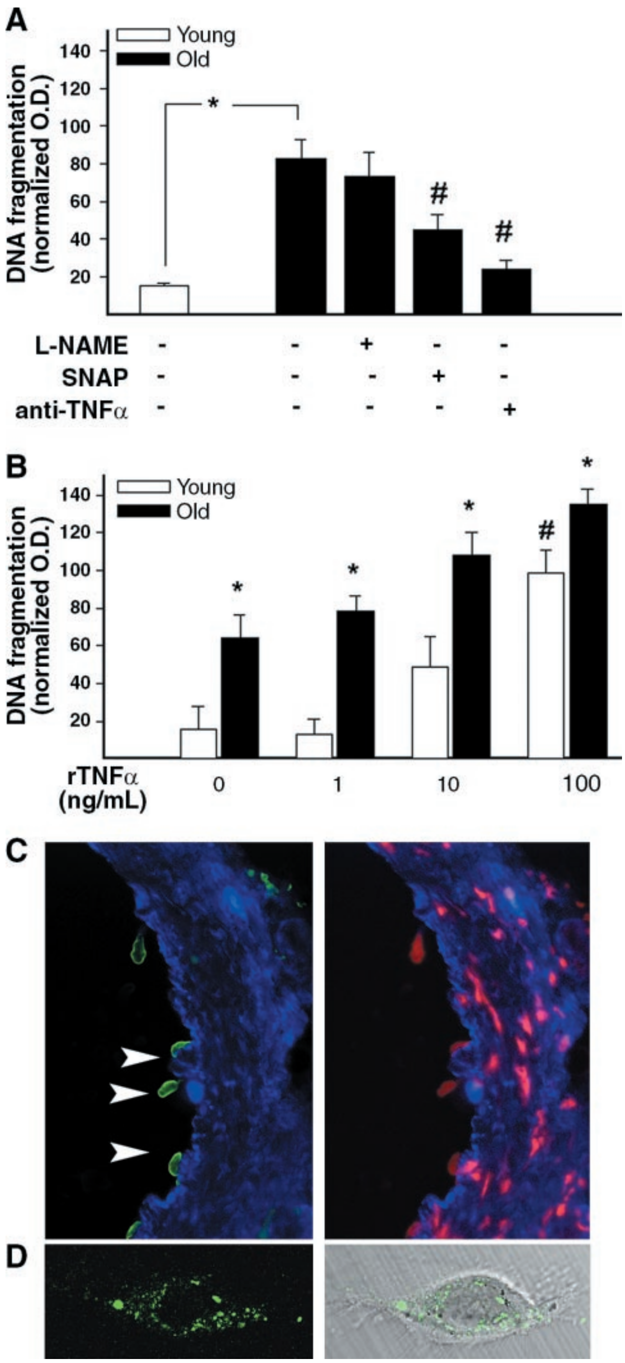

Fig. 5. *A*: DNA fragmentation (an indicator of apoptotic cell death) in cultured coronary arteries of young rats and in vessels of aged rats after incubation (18 h) with the NO synthesis inhibitor L-NAME (3×10^{-4} mol/l), the NO donor SNAP (10^{-6} mol/l), or TNFα blocking antibody. **P* < 0.05 vs. young. #*P* < 0.05 vs. untreated. *B*: DNA fragmentation in cultured coronary arteries of young and aged rats after incubation (18 h) with recombinant TNFα (rTNFα). **P* < 0.05 vs. young. #*P* < 0.05 vs. untreated. *C*: fluorescent photomicrographs of a young coronary artery after in vitro incubation (18 h) with TNFα (100 ng/ml). *Left*: large number of TUNEL-positive cells (green) were observed in the endothelium (arrowheads), whereas cells stained for smooth muscle α-actin (blue) were predominantly free from TUNEL staining. *Right*: propidium iodide (red) was used for nuclear staining to help orientation (original magnification, ×20). *D*: representative confocal image (*right*: overlaying with bright-field image) showing TNFα-induced annexin V staining in a cultured primary rat CAEC (original magnification, ×40).

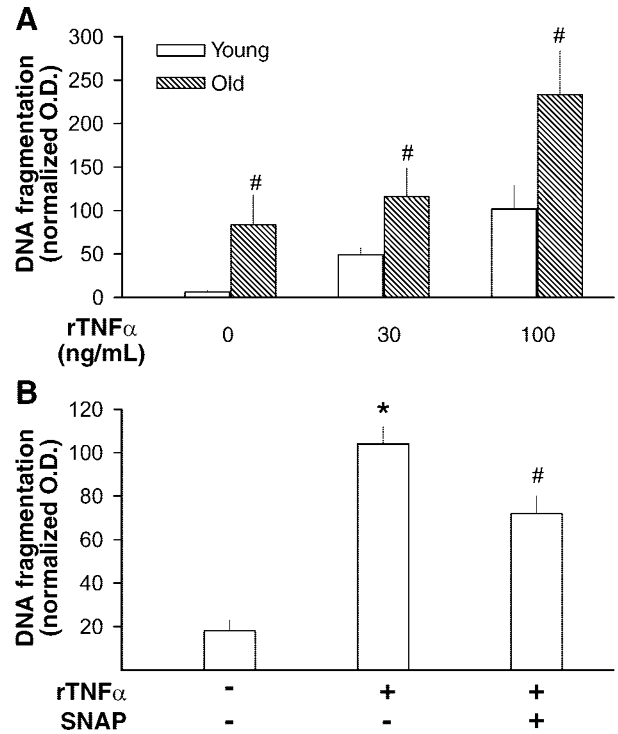

Fig. 6. DNA fragmentation in cultured coronary arteries of young rats induced by TNFα (18 h incubation) in the absence and presence of the NO synthesis inhibitor L-NAME (3×10^{-4} mol/l, *A*) or the NO donor SNAP (10^{-6} mol/l, *B*). #*P* < 0.05 vs. untreated. Data are means ± SE (*n* = 5–7 for each group). O.D., optical density units.

lial apoptosis. Increased activity of caspase 3 and/or caspase 9 is also present in the liver (33), brain (24), and skeletal muscle (10) of aged rats and lymphocytes of aged humans (1). Expressions of the protooncogene Bcl-2, which is inversely related to apoptotic cell death (6), and that of the proapoptotic homolog Bax, Bad, and Bid or the Bcl family member Bcl-X(L) were unaltered in aged coronary vessels (Table 2). Similar results were obtained in cardiomyocytes (19, 21), skeletal muscle (10), and liver (33) of aged F344 rats, despite the presence of enhanced apoptosis in these organs. The increased presence of truncated Bid found in aged coronary arteries may be related to the increased activity of caspase 8 (32) (Table 4).

Our present and previous findings (7, 8) showed that expression of the proinflammatory cytokine TNFα and that of TNFβ (which acts on the same receptor) significantly increased in aged coronary arteries (Table 2, Fig. 3). Previously we demonstrated that it is the VSMC that abundantly express TNFα in aged coronary arteries (8). Previous studies have also reported age-related increases in plasma TNFα concentrations in humans (5) and experimental animals (31). Since the early work of Robaye and coworkers (22) TNFα has been recognized as one of the most potent inducers of programmed cell death in endothelial cells. However, TNFα in the smooth muscle cells is synthesized as a membrane-bound precursor that has to be cleaved to generate secreted TNFα, which acts as a paracrine mediator. This is the first study to demonstrate that coronary arteries abundantly express TACE and have significant TACE activity (Fig. 4). Importantly, we found that in aging TACE expression and activity is unchanged (Fig. 4, *A* and *B*), whereas expression of TNFα is substantially upregulated. Thus we hypothesize that preserved TACE activity in aging is an important permissive factor that likely contributes to increased levels of bioactive TNFα in the aged vascular wall. This view is also in line with recent observations showing increased serum levels of TNFα in aged experimental animals (25) and elderly patients (20).

Increased vascular tissue levels of TNFα likely contribute to the enhanced endothelial apoptosis in aged coronary arteries, because TNFα blocking antibody significantly decreased apoptotic cell death in these vessels (Fig. 5*A*). Furthermore, exogenous TNFα significantly enhanced apoptosis in the endothelium of coronary arteries of young and aged rats (Fig. 5, *B* and *C*) and in cultured rat coronary endothelial cells (Fig. 5*D*). These findings, taken together with results of previous studies, suggest that coronary endothelial cells are especially vulnerable to a proinflammatory microenvironment (6, 16). Age-related upregulation of TNFα and increased sensitivity toward TNFα-induced apoptosis were also reported in lymphocytes of aged humans (1). Moreover, decreased rate of apoptosis in various organs of aged rats due to caloric restriction (2), the only known procedure that increases maximum life span, is associated with a reduced TNFα production (25).

Recent studies suggest that the sensitivity of endothelial cells to apoptosis is regulated in part by NO (13), which can inhibit various steps of TNFα-induced apoptosis signaling via modification of proteins (13) and can induce telomerase activity (29). These effects of NO qualify it as an important anti-apoptotic and anti-aging factor that contributes to the maintenance of a youthful vascular phenotype. Indeed, pharmacological inhibition of NO synthesis in young coronary arteries substantially increased apoptotic cell death and potentiated the proapoptotic effect of TNFα (Fig. 6*A*). Also, TNFα-induced apoptosis in young coronary arteries was attenuated by an exogenous NO donor (Fig. 6*B*). Previously we demonstrated that bioavailability of NO is significantly decreased in aged coronary arteries due to an age-dependent decrease in eNOS expression (7) and an increase in superoxide production resulting in enhanced peroxynitrite formation (7). In addition, Akt-dependent eNOS activation may also decrease in aging (13). Because apoptotic cell death in aged coronary arteries in culture could be reduced by an exogenous NO donor, but inhibition of endogenous NO synthesis did not further increase apoptotic cell death (Fig. 5*A*), it is likely that a decreased bioavailability of NO in these vessels promotes apoptosis and potentiates the apoptotic effect of increased levels of TNFα. This idea is supported by the findings that genetic lack of NO resulted in a substantially increased apoptosis in vessels of aged male eNOS knockout mice (unpublished observation) and promoted apoptosis in cultured endothelial cells (13). In senescent endothelial cells in culture there is also a downregulation of eNOS and the eNOS activator Akt, associated with an increased sensitivity toward TNFα-induced apoptosis (13).

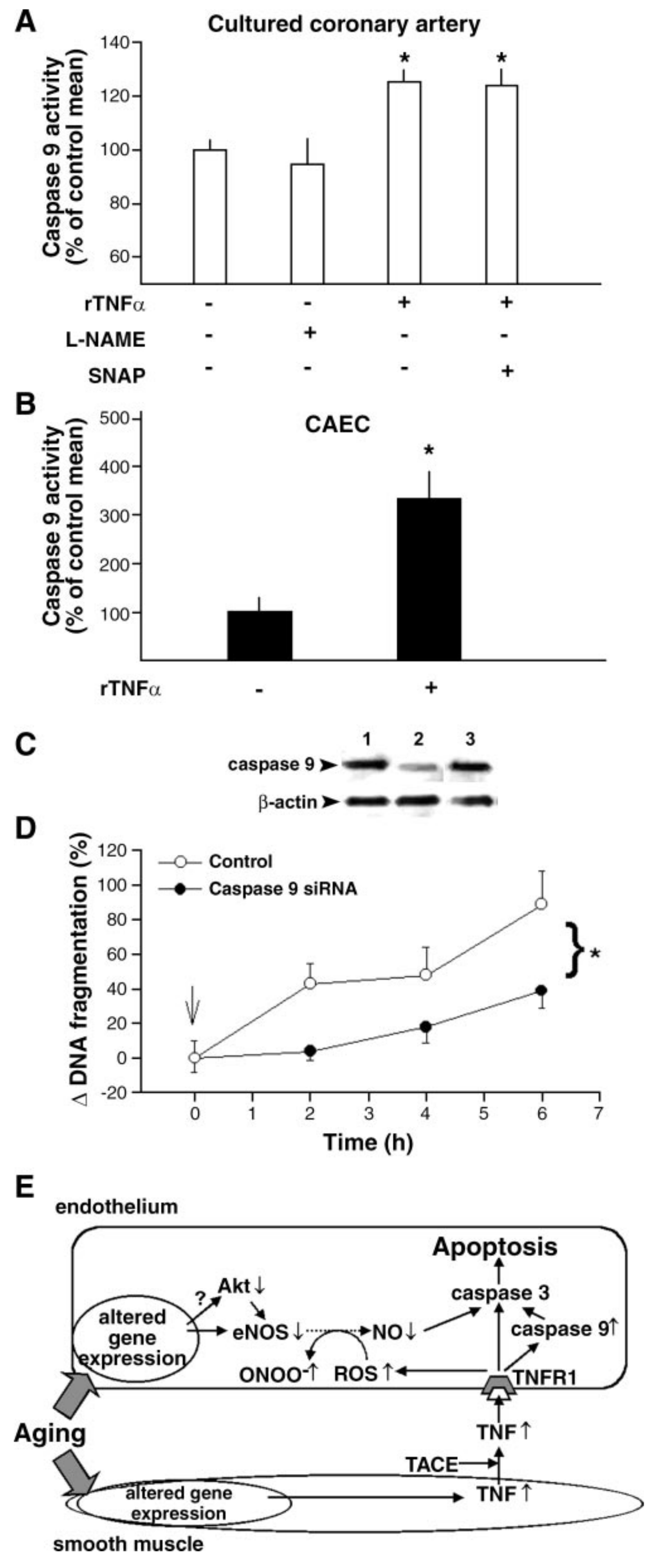

Fig. 7. *A*: effects of recombinant TNFα (10 ng/ml), the NO synthesis inhibitor L-NAME (3×10^{-4} mol/l), or the NO donor SNAP (10^{-6} mol/l) on caspase 9 activity in cultured coronary arteries of young rats. *$P < 0.05$ vs. without TNFα. *B*: TNFα-induced increased caspase 9 activity in primary rat CAEC in culture (18 h). *C*: representative Western blot showing downregulation of caspase 9 by RNA interference (RNAi) in cultured CAEC (on *day 4* after siRNA treatment). *Lane 1*, untreated control; *lane 2*, anti-caspase 9 siRNA; and *lane 3*, scrambled siRNA. *D*: effect of downregulation of caspase 9 by RNAi on the time course of TNFα-induced changes in DNA fragmentation indicating apoptotic cell death in CAEC. *$P < 0.05$ vs. untreated. Data are means ± SE (n = 4–6 for each group). *E*: proposed scheme for age-related phenotypic changes leading to a decreased NO bioavailability, increased vascular TNF levels, and increased caspase 9 activity that promote endothelial apoptosis in the coronary arteries.

The mechanisms by which TNFα induces apoptosis include binding of TNFα to the death receptor TNFR1 and activation of a death-inducing signaling complex via the receptor death domain (6). Because administration of TNFα activated caspase 9 both in young coronary arteries (Fig. 7*A*) and in cultured endothelial cells (Fig. 7*B*), it is logical to hypothesize that the increased tissue TNFα levels contribute to increased caspase 9 activation in aged coronary arteries. Caspase 9 is involved in the mitochondrial proapoptotic pathway, and it can be assumed that its upregulation may enhance cellular sensitivity toward apoptotic stimuli. Indeed, downregulation of caspase 9 in CAEC (Fig. 7*C*) resulted in partial protection of these cells against TNF-induced apoptosis (Fig. 7*D*). Our results are in line with the findings of McDonnell et al. (17) showing TNFα-induced early processing of caspase 9 and delaying the progression of apoptosis by a caspase-9-specific inhibitor. Importantly, a recent study found that caloric restriction, which decreases apoptosis in the heart of aged mice (15), downregulates the expression of caspase 9 in addition to TNFα (25). It is likely that NO inhibits cellular apoptotic pathways downstream of caspase 9 (23), because administration of L-NAME or SNAP did not affect TNFα-induced caspase 9 activity (Fig. 7*A*).

On the basis of the aforementioned findings, we propose (Fig. 7*E*) that aging alters gene expression in coronary arteries, resulting in upregulation of TNFα and caspase 9 and decreased bioavailability of NO. These alterations promote endothelial apoptosis, which is likely to contribute to coronary arterial endothelial dysfunction and the development of ischemic heart disease in the elderly.

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