Aging Enhances the Sensitivity of Endothelial Cells Toward Apoptotic Stimuli
Important Role of Nitric Oxide

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Abstract—Advanced aging leads to impaired endothelial NO synthesis and enhanced endothelial cell apoptosis; therefore, we investigated the sensitivity of aged endothelial cells toward apoptotic stimuli and determined the role of NO. Human umbilical vein endothelial cells (HUVECs) were cultured until 14th passage. In aged cells, oxLDL and tumor necrosis factor-α–induced apoptosis and caspase-3–like activity were significantly enhanced more than 3-fold compared with young cells (passage 3). Because NO contributes to protection against endothelial cell death via S-nitrosylation of caspases, we determined endothelial NO synthase (eNOS) protein expression and the content of S-nitrosylated proteins. Aged HUVECs showed significantly reduced eNOS expression (35 ± 10%) and a decrease in the overall S-NO content (33 ± 3%), suggesting that eNOS downregulation may be involved in age-dependent increase of apoptosis sensitivity. Indeed, eNOS knockout endothelial cells showed a significantly enhanced apoptosis induction. Exogenous NO donors abolished increased apoptosis and caspase-3–like activity. In contrast, the application of shear stress, which exerts a profound apoptosis inhibitory effect via upregulation of NO synthesis in young cells, failed to inhibit apoptosis in aged cells. Moreover, no upregulation of eNOS protein expression and S-NO content in response to shear stress was detected in aged cells. Overexpression of wild-type eNOS completely restored the antiapoptotic effect of shear stress, whereas only a partial inhibitory effect was detected under steady conditions. Strikingly, transfection of constitutively active phosphomimetic eNOS (S1177D) further abrogated apoptosis in aged HUVECs. Thus, aging of endothelial cells is associated with decreased NO synthesis and concomitantly increased sensitivity of apoptosis, which may contribute to functional impairment of the endothelial monolayer. (Circ Res. 2001;89:709-715.)

Key Words: apoptosis ■ endothelial cells ■ nitric oxide ■ S-nitrosylation

The endothelium is located in a strategic anatomical position within the blood vessel wall and thereby acts as a barrier between the blood and the vascular smooth muscle cells. Therefore, the functional integrity of the endothelium monolayer is essential to prevent vascular leakage and the formation of atherosclerotic lesion.1 Apoptosis of endothelial cells may critically disturb the integrity of the endothelial monolayer and may thereby contribute to vascular injury and atherosclerosis (for review see Dimmeler and Zeiher2). Indeed, all classical proatherosclerotic factors such as oxLDL, proinflammatory cytokines and reactive oxygen species induce endothelial cell apoptosis.3,4 Consistent with the findings in various cell types, endothelial cell apoptosis is executed via activation of the cysteine protease family, the caspases.5,6 However, in contrast to other cell types, endothelial cells are more resistant to apoptosis. Endothelial cells are protected against apoptosis by antiapoptotic proteins such as FLICE-inhibitory protein, which specifically interferes with the Fas-induced apoptotic pathways.7 In addition, the endothelial synthesis of NO inhibits apoptosis induced by various apoptotic stimuli.4,6,8,9 NO interferes with the apoptosis signal-transduction pathway by several mechanisms.9–11 Importantly, the execution of apoptosis is blocked by inhibition of caspases via S-nitrosylation of the essential cysteine residue.6,12–14

Aging is one of the major risk factors for the development of cardiovascular disease. On a cellular level, advanced age leads to impaired endothelial NO synthesis and endothelial dysfunction.15–17 A recent study demonstrates that endothelial cell apoptosis is enhanced in old monkeys, suggesting a link between aging and apoptotic cell death.18 Moreover, an age-related increase of apoptosis was found in superoxide dismutase knockout mice.19 Therefore, we investigated the sensitivity of aged endothelial cells toward proapoptotic stimuli and determined the underlying mechanism. The present study demonstrates that

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aging is associated with increased sensitivity of endothelial cells to apoptotic stimuli and a reduction of endothelial NO synthase (eNOS). The increased apoptosis sensitivity was prevented by exogenous NO donors. In contrast, exposure of endothelial cells to shear stress did not rescue aged endothelial cells from being driven into apoptosis. The impaired apoptosis inhibitory capacity of shear stress was related to the inability to upregulate the eNOS in aged cells.

Materials and Methods

Cell Culture
HUVECs were cultured as previously described until the 14th passage.20 Population doublings (PDLs) were calculated using the following formula: PDL=(log10F-log10I)/0.301 (F indicates number of cells at the end of the passage; I, number of cells when seeded). HUVECs were stained with von Willebrand factor to exclude dedifferentiation. Shear stress exposure was performed with a cone-and-plate apparatus, as described previously.21

Isolation of Primary Endothelial Cells From Mouse Aorta
Isolation of endothelial cells from mouse aorta was mainly performed as previously described for rats.22 Six-month-old, male wild-type and eNOS knockout mice (Jackson Laboratories) were killed and the aorta was removed. The vessel was cleaned of periadventitial fat and connective tissue, opened longitudinally, and placed with the aortic intimal side down into collagen-matrix–coated 24-well plates (collagen-matrix components: 3 mg/mL collagen, 5×DMEM, and 100× penicillin/streptomycin). The filled collagen-matrix was overlaid with RPMI medium with supplements and after an additional 4 days the medium and the aorta were removed from the collagen-matrix. The matrix was digested with 1 mg/mL collagenase (Serva) for 10 minutes at 37°C and 10 mL EBM added. After centrifugation at room temperature for 5 minutes at 800g, the supernatant was removed and the resulting cell pellet was suspended in EBM. Cells were seeded on 24-well plates. After growing to confluence in EBM, cells were passaged once (1:6) and used for the experiments. About 70% to 80% of the cells showed positive for CD31 or von Willebrand factor as assessed by fluorescence-activated cell sorter (FACS) analysis.

Detection of Apoptosis
Cells were washed with PBS and fixed in 4% formaldehyde. Cells were stained with 4’,6-diamidino-phenylidole (DAPI; 0.2 μg/mL in 10 mmol/L Tris-HCl [pH 7.0], 10 mmol/L EDTA, and 100 mmol/L NaCl) for 30 minutes. Then, cells were washed with PBS and nuclei were analyzed by fluorescence microscopy.

Caspase Activity
For detection of caspase-3–like activity, protein was isolated and caspase activity was detected in resulting supernatants using the fluorogenic substrate 7-amino-4-coumarin (AMC)-DEVD, as described previously.6

Western Blot
After stimulation for the indicated times, HUVECs were scraped off the plates and lysed in RIPA buffer (50 mmol/L Tris-HCl [pH 8.0], 150 mmol/L NaCl, 1% Nonidet-P40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate). 60 μg protein/slot were resolved on SDS-polyacrylamide gels and were blotted on polyvinylidene difluoride membranes. Membranes were incubated with antibodies against eNOS (1:1000, Cell Signaling), phospho-eNOS Ser1177 (gift from Dr Fleming),23 Institution für Kardiokardiovaskuläre Physiologie, Frankfurt, Germany) (1:1000), Akt (1:500, Cell Signaling), phospho-Akt Ser473 (1:500, Pharmingen), p21 Cip-1/Waf-1 (1:500, Pharmingen), ERK1/2 (1:1000, New England Biolabs), or actin (1:5000, Sigma). After incubation, the corresponding secondary antibody signals were detected by the enhanced chemiluminescence system (Amersham).

S-NO Content
S-NO content was measured using the Saville-Griess assay, as described.24 In brief, HUVECs were lysed in Griess-lysiss buffer (50 mmol/L Tris-HCl [pH 8.0], 150 mmol/L NaCl, 5 mmol/L KCl, 1% Nonidet-P40), 1 mmol/L phenethylsulfonyl fluoride, 1 mmol/L bathocuproinedisulfonic acid, 1 mmol/L diethylenetriaminepenta-acetic acid, and 10 mmol/L N-ethylmaleimide), and 80 μg of cell lysate was incubated with 1% sulfanilamide and 0.1% N-(1-naphthyl)ethylenediamine (NED) in the presence or absence of 3.75 mmol/L p-chloromercuribenzenesulfonic acid (PCMBs) for 20 minutes. S-NO content was measured photometrically at 540 nm. The amount was calculated using defined S-nitrosoglutathione concentrations as a standard.

FACS Analysis
HUVECs were trypsinized from the cell-culture dish and stained with anti-integrin-receptor subunits α1 and β1 antibodies for FACS analysis, as described previously.25 Apoptosis of mouse aortic endothelial cells was determined by double-staining with FITC-labeled antibodies against CD31 and with phenylephrine (PE)-conjugated annexin (15 minutes of labeling) using isotype antibodies as controls (Becton Dickinson).

Transfection
The plasmid encoding eNOS was a kind gift from Dr Nakane (Abbott Laboratories, Abbott Park, Ill) and was subcloned and mutated, as previously described.26 HUVECs were cotransfected with 2.25 μg plasmid and 0.75 μg lacZ and 25 μL Superfect, as described previously.27 Apoptosis was detected by counting the morphological changes of the cells after β-galactosidase staining, as described.27

Statistics
Statistical analysis was performed with ANOVA followed by modified Bonferroni least-significant difference test (SPSS Software).

Results
Aging Is Associated With Enhanced Apoptosis Induction
HUVECs were cultured until the 14th passage (corresponds to 42 PDLs) and apoptosis was induced in each passage by oxLDL or tumor necrosis factor-α (TNFα). As shown in Figure 1A, stimulation of endothelial cells with TNFα leads to a drastically enhanced apoptosis induction in higher passages. Similar results were achieved when using oxLDL (Figure 1A) or serum deprivation (PDL 9, 9.8±1.3% compared with PDL 37, 24.5±3.2%; n=3) as proapoptotic stimuli. The increase of apoptotic cell death in aged HUVECs correlates with a more pronounced activation of the caspase cascade, as shown by elevated caspase-3–like activity (Figure 1B).

However, neither basal apoptosis nor caspase activity was elevated in untreated cells during the observed cultivation period (Figure 1A), indicating that aging of endothelial cells does not directly stimulate the apoptotic cell death program but enhances the sensitivity of the cells toward proapoptotic stimuli.
Aging Leads to a Reduction in eNOS and Akt Phosphorylation and Protein Levels

Next, we investigated the mechanism by which aging leads to an increased sensitivity to apoptosis. Endothelial NO protects against apoptosis. Moreover, endothelial NO generation was shown to be reduced with increased age. Therefore, the expression of eNOS and its phosphorylation status was determined by Western blot analysis. As illustrated in Figure 2A, eNOS phosphorylation at Ser1177 and protein levels are reduced in aged cells (Figures 2A and 2B). Moreover, the expression and phosphorylation of the serine/threonin protein kinase Akt, which is known to upregulate eNOS activity, is also significantly diminished in aged cells (Figures 2A and 2B) with a similar kinetic as shown for the eNOS. However, the ratios of phosphorylated eNOS to total eNOS and phosphorylated Akt to total Akt did not change with age (P-eNOS/eNOS PDL 9, 0.68±0.09 versus PDL 37, 0.81±0.11; P-Akt:Akt PDL 9, 0.62±0.1 versus PDL 37, 0.76±0.13; NS, n=3), suggesting that the decreased basal phosphorylation is mainly a consequence of reduced protein levels. To ensure that the reduction of eNOS and Akt is not due to unspecific downregulation of proteins, we determined expression of the cell-cycle inhibitor p21Cip-1/Waf-1, which is known to be increased in aged cells. As expected, expression of p21Cip-1/Waf-1 is increased in aged HUVECs (Figure 2A). To demonstrate that the reduction of eNOS protein levels lead to a reduction of NO synthesis, we determined the content of S-nitrosylated proteins as a marker for the bioactivity of NO. As shown in Figure 2C, the S-NO content was significantly reduced in aged cells.

Endothelial Cells From eNOS−/− Mice Show Enhanced Apoptosis Induction

To further investigate the causal role of eNOS to protect against apoptosis induction, we isolated primary endothelial cells from the aorta of wild-type and eNOS−/− mice and induced apoptosis by serum deprivation. Serum deprivation
induced significantly more apoptosis in endothelial cells derived from eNOS\textsuperscript{−/−} mice, as measured by FACS analysis with annexin staining, compared with cells from age- and gender-matched wild-type mice (Figure 3). To exclude any contaminating other cell types, only cells positive for the endothelial surface marker protein CD31 were analyzed.

Exogenous NO Donors Prevent Age-Related Increases in Apoptosis

To test whether the lack of NO is indeed involved in enhancing the sensitivity of aged cells to apoptosis induction, we investigated the effect of exogenous NO. The NO donor sodium nitroprusside (SNP) abrogated TNF\textsubscript{α} and oxLDL-induced apoptosis in aged cells (Figure 4A and data not shown). Similarly, S-nitroso-N-acetylpenicillamine (SNAP) also prevented apoptosis induction in aged HUVECs (TNF\textsubscript{α}, 15.3±2.3 versus TNF\textsubscript{α}+SNAP, 3.2±0.9 for PDL 42). Moreover, TNF\textsubscript{α}-induced caspase activation was abolished in the presence of NO donors (Figure 4B and data not shown).

Shear Stress Does Not Inhibit Age-Related Increases in Apoptosis Sensitivity

Shear stress stimulates the endogenous NO synthesis by upregulation of eNOS mRNA and posttranscriptional activation of the eNOS\textsuperscript{26,30,31} and potently blocks apoptosis induced by various factors in young endothelial cells.\textsuperscript{21} Therefore, we determined whether shear stress prevented apoptosis in aged cells. However, in contrast to young cells, in aged cells no protective effect of shear stress was achieved (Figure 5A). Moreover, shear stress could not reverse the pronounced TNF\textsubscript{α}-stimulated caspase-3–like activity in aged HUVECs (Figure 5B). Likewise, upregulation of eNOS was significantly attenuated in aged cells (Figure 5C) and reduction of the S-NO content in aged cells (9.2±0.9 \textmu mol/L S-NO) was not reversed by shear stress exposure (9.6±1.1 \textmu mol/L S-NO).

Because shear stress did not upregulate eNOS protein expression in aged endothelial cells, we next investigated whether aging inhibited the sensitivity of endothelial cells to responses induced by shear stress. However, the typical phenotypic changes induced by shear stress were still observed when aged HUVECs were exposed to shear stress (Figure 5D). Moreover, the fibronectin-receptor subunits \(\alpha\) and \(\beta\), which are required for cell adhesion and cell survival, were expressed to the same extent at the cell surface in young as well as in aged HUVECs (Figure 5E). Furthermore, we investigated whether the activation of the MAPK ERK 1/2, a downstream target of the integrin signaling pathway,\textsuperscript{32} is impaired in aged cells. As shown in Figure 4F, ERK1/2 was activated in young and aged HUVECs to a similar extent. Taken together, although the integrin signaling pathway seems to be functionally active, shear-stress exposure failed to increase eNOS expression in aged cells.

Overexpression of eNOS Prevents Age-Related Increase in Apoptosis

To underscore the hypothesis that age-dependent increase of apoptosis sensitivity is because of reduced eNOS expression in old HUVECs, we overexpressed eNOS wild-type (eNOS\textsuperscript{wt}) and assessed the effect on apoptosis signaling. Overexpression of eNOS\textsuperscript{wt} partially reversed the proapoptotic activity of TNF\textsubscript{α} and oxLDL (Figure 6 and data not shown). In addition, shear stress abolished apoptosis of

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\caption{Endothelial cells derived from eNOS\textsuperscript{−/−} mice show enhanced sensitivity toward apoptosis. Endothelial cells were isolated from wild-type and eNOS\textsuperscript{−/−} mice as described under Materials and Methods. Apoptosis was induced by serum deprivation for 18 hours, and cells were stained with FITC-labeled anti–CD31-antibody and PE-labeled annexin. CD31/annexin double positive cells were counted by FACS analysis (\(P<0.05\) vs wild-type serum-deprived cells, \(n=6\)).}
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\caption{A, Exogenous NO inhibits apoptosis in aged HUVECs. Young (PDL 9) and aged HUVECs (PDLs 39 to 42) were incubated with TNF\textsubscript{α} in the presence or absence of SNP for 18 hours, and apoptosis was detected using DAPI staining. SNP significantly reduced apoptosis induction in young and aged HUVECs. Data are mean±SEM, \(n=3\) to 5 (\(P<0.05\) vs TNF\textsubscript{α} in young cells; **\(P<0.05\) vs TNF\textsubscript{α} in aged cells). B, Exogenous NO inhibits caspase activity in aged HUVECs. Young (PDL 9) and aged (PDLs 39 to 42) HUVECs were incubated with TNF\textsubscript{α} in the presence or absence of SNP for 18 hours, and caspase-3–like activity was measured. SNP significantly reduced caspase-3–like activity in young and in aged HUVECs. Data are mean±SEM, \(n=3\) to 5 (\(P<0.05\) vs TNF\textsubscript{α} in young cells; **\(P<0.05\) vs TNF\textsubscript{α} in aged cells).}
\end{figure}
eNOSwt overexpressing cells and showed an enhanced S-NO content (Figure 6 and data not shown). Finally, overexpression of an active eNOS construct (S1177D), which is known to exert a 2-fold higher activity compared with the wild-type enzyme, inhibited apoptosis even in the absence of shear stress and prevented the reduction of the S-NO content induced by TNFα completely (Figure 6 and data not shown).

**Discussion**

The present study demonstrates that aged HUVECs show enhanced apoptosis sensitivity and dramatically increased caspase-3-like activity in response to TNFα and oxLDL when compared with young cells. Moreover, enhanced apoptosis induction was also found in endothelial cells derived from eNOS−/− mice when compared with wild-type. The increased apoptosis induction is paralleled by a reduction of eNOS expression and intracellular S-NO content, suggesting an important role of NO. Indeed, transfection of endothelial cells with eNOS wild-type restored apoptosis suppression by shear stress and the phosphomimetic-activated enzyme eNOS (S1177D) abrogated apoptosis. These data provide evidence for a causal role of NO for age-dependent increased sensitivity to apoptotic stimuli.

We and others showed previously that apoptosis induction in endothelial cells was dependent on the caspase cascade and that predominantly caspase-3 activity was inhibited by direct S-nitrosylation of the cysteine 163 in the active center of the catalytic subunit p17.6,14 In this study, we provide evidence that impaired bioavailability of NO caused enhanced apoptosis induction in aged endothelial cells. Under basal conditions, we could not detect any apoptosis induction in aged HUVECs. HUVECs at PDL 42 were exposed to shear stress for 18 hours and morphological changes were visualized by phase-contrast microscopy. Expression of integrin-receptor subunits α5 and β1 in young (PDL 9) and aged (PDLs 35 to 42) HUVECs by FACS analysis. Arrows indicate isotype control, young, and aged HUVECs. A representative FACS analysis is shown for young and aged HUVECs in the top panel (FL1 indicates fluorescence intensity). Data are mean ± SEM (bottom panel; n = 3). F, Induction of ERK1/2 activation by serum in young and aged HUVECs. Young (PDL 9) and aged (PDLs 35 to 42) HUVECs were serum-deprived for 6 hours and ERK1/2 activation was stimulated with serum for 10 minutes. Phosphorylation of ERK1/2 was detected using a phospho-specific ERK1/2 antibody (top panel). The membrane was stripped and reprobed using an anti-actin antibody (bottom panel). A representative Western blot is shown (n = 3).
endothelial cells are unable to recruit enough NO for S-nitrosylation of caspases, which facilitates the activation of these enzymes and enhances apoptosis induction. Indeed, the S-NO content was reduced in aged endothelial cells. The finding that about 95% of the S-NO detected in our endothelial cells is bound to proteins as demonstrated by gel filtration using Sephadex G-25 columns to separate protein and low-mass S-NO fractions suggests that protein S-nitrosylation is indeed reduced in aged endothelial cells.

The reduction of bioavailable NO and the content of S-nitrosylated proteins in aged cells may be explained by a downregulation of the phosphorylation and protein expression of eNOS. This is in accordance with previous in vivo studies that demonstrated downregulation of eNOS expression in aging and in atherosclerotic vessels. Moreover, it should be noted that the loss of eNOS protein expression is paralleled by a reduced Akt protein expression in aged endothelial cells, which may further reduce NO synthesis because Akt is required for eNOS activation.

In addition to downregulation of eNOS expression, an increase in superoxide production may decrease NO bioavailability. Indeed, there is growing evidence that an accumulation of age-related damage to mitochondria occurs, leading to reactive-oxygen species formation. Moreover, vascular peroxynitrite formation has been shown to increase with age. Therefore, the rapid formation of peroxynitrite from superoxide and NO could be another possible reason for the reduced S-NO content in aged endothelial cells. However, apoptosis and caspase-3–like activity were inhibited by exogenous NO in old endothelial cells, indicating that peroxynitrite formation may play a minor role for the age-associated increase in apoptosis sensitivity.

Shear stress is one of the most important physiological stimuli for endothelial NO synthesis. Shear stress increases protein expression of eNOS, further posttranscriptionally activates the enzymatic activity, and potently prevents apoptosis of endothelial cells in vitro and in vivo. Interestingly, the data of the present study indicate that shear stress–induced upregulation of eNOS expression was impaired in aged cells. Consistent with this finding, apoptosis and caspase activity were not inhibited by shear stress in aged cells. Because shear stress requires an intact integrin signaling to prevent apoptosis induction, we investigated whether the failure of shear stress to prevent apoptosis in aged cells might be caused by impairment of integrin expression. However, cell-surface expression of the fibronectin-receptor subunits α5 and β1 were similar comparing young and aged endothelial cells. Furthermore, ERK1/2 phosphorylation, a prototype downstream pathway activated by integrin-receptor activation, was intact in aged endothelial cells. Finally, the phenotypic changes initiated by shear-stress application were still observed in aged cells, suggesting that aging negatively influences specific pathway(s) that regulate shear stress–induced eNOS expression and, therefore, cannot provide the antiapoptotic signal. Indeed, overexpression of wild-type eNOS restored the ability of shear stress to prevent endothelial cell apoptosis in aged cells.

Taken together, the maintenance of an intact endothelial monolayer and an intact endothelial function is necessary to protect against the initiation of atherogenesis. Taking into account that aging is known to be an independent risk factor for atherosclerosis, this study provides new evidence that aging of endothelial cells leads to enhanced apoptosis induction because of the loss of eNOS expression and, therefore, reduction of intracellular S-NO content. Recent studies further demonstrate that senescent endothelial cells are detectable in reendothelialized areas of the vascular wall after repeated balloon injury. In accordance with the data of the present study, these regenerated endothelial cells were characterized by a reduced NO synthesis. Therefore, one may hypothesize that the reendothelialized regions are predisposed to apoptosis induction, which may accelerate endothelial cell aging and further reduce NO synthesis. Because the endothelial NO synthesis potently blocks vascular smooth muscle cell proliferation, this vicious cycle may have further implications for the development of restenosis after balloon injury.

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