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Cardiovascular Research 66 (2005) 286–294

Cardiovascular Research

www.elsevier.com/locate/cardiores

Review

Endothelial aging

Ralf P. Brandes*, Ingrid Fleming, Rudi Busse

Institut für Kardiovaskuläre Physiologie, J.W. Goethe-Universität, Theodor-Stern-Kai 7, D-60596 Frankfurt am Main, Germany

Received 2 November 2004; received in revised form 20 December 2004; accepted 29 December 2004
Available online 1 February 2005
Time for primary review 14 days

Abstract

Aging is considered to be the major risk factor for the development of atherosclerosis and, therefore, for coronary artery disease. Apart from age-associated remodeling of the vascular wall, which includes luminal enlargement, intimal and medial thickening, and increased vascular stiffness, endothelial function declines with age. This is most obvious from the attenuation of endothelium-dependent dilator responses, which is a consequence of the alteration in the expression and/or activity of the endothelial NO synthase, upregulation of the inducible NO synthase, and increased formation of reactive oxygen species. Aging is also associated with a reduction in the regenerative capacity of the endothelium and endothelial senescence, which is characterized by an increased rate of endothelial cell apoptosis.

Keywords: Nitric oxide; Vasodilatation; Atherosclerosis; Oxidative stress; Superoxide; Regeneration

1. Introduction

The vascular endothelium is situated at the interface between the blood and the vascular wall/tissue and is more than a protective barrier since it possesses anticoagulatory properties and generates a number of autacoids that regulate vascular tone and homeostasis. Nitric oxide (NO) is frequently described as the primary endothelium-derived autacoid [1] and anti-atherosclerotic principle [2]. Apart from its vasodilator property, NO exerts inhibitory effects on leukocyte adhesion [3], thrombocyte aggregation [4] and smooth muscle cell proliferation [5]. Moreover, although the radical NO acts as an antioxidant and terminates lipid peroxidation chain reactions, it also possesses pro-oxidant effects by the formation of peroxynitrite from its reaction with superoxide anions [6].

Early, but reversible manifestations of atherosclerosis, such as fatty streaks, are already found in utero [7], and frequently lead to full blown disease in early adulthood [8]. The slowly progressing atherosclerotic process eventually results in clinical events such as ischemia and myocardial infarction. Although atherosclerosis is a disease which requires a certain amount of time to become apparent, vascular aging in itself is not synonymous with atherosclerosis. It is however exceedingly difficult to differentiate between the two, especially with the limited data available relating to human subjects.

2. Aging and endothelium-dependent vasodilator responses

Endothelial function is usually clinically assessed by determining changes in blood flow or arterial diameter in response to endothelial stimulation, which are estimates of vascular NO bio-availability. The outcome of such tests is not only dependent on endothelial autacoid release (mainly NO versus superoxide anions), but also on smooth muscle function and the extent of neo-intima formation.

Several clinical studies have shown that endothelium-dependent vasodilatation progressively declines with age. This observation has consistently been made in conduit arteries, such as coronary arteries [9], the brachial artery in vivo [10] and the basilar artery ex vivo [11], but also in

* Corresponding author. Tel.: +49 69 6301 6995; fax: +49 69 6301 7668.
E-mail address: r.brandes@em.uni-frankfurt.de (R.P. Brandes).

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doi:10.1016/j.cardiores.2004.12.027

resistance vessels [12,13], and occurs earlier in men than in women [14]. Thus, there is evidence indicating that aging per se leads to an attenuated generation and release or enhanced breakdown of endothelial autacoids. These observations in humans are supported by many studies determining endothelium-dependent vasomotor responses in animals. In particular, in rat conduit [15–17] and resistance vessels [18,19], but also in arteries from pigs [20], rabbits [21] and mice [22], endothelium-dependent vasodilator responses decrease with age, independently of structural changes of the vascular wall. Endothelium-independent relaxations to sodium nitroprusside in contrast are unaffected by aging. One consequence of the aging-associated impairment of endothelial function is an enhanced reactivity to vasoconstrictors [23]. This impairment affects three major endothelium-derived vasodilators, NO, prostacyclin and the endothelium-derived hyperpolarizing factor (EDHF) [24]. For example, in rats the contribution of K^+ channels to endothelium-dependent relaxation progressively declines with age [25], and in aged spontaneously hypertensive rats, EDHF responses are usually completely absent and vasodilator responses are exclusively mediated by NO [26]. In humans, aging is also associated with a decreased urinary excretion of 6-keto-prostaglandin F1α, the stable metabolite of prostacyclin [27,28], and similar observations have been made in blood vessels from aged male but not female rats [29]. In contrast to the latter studies, an increased generation of prostacyclin was observed in the aorta of aged Wistar rats. This phenomenon was attributed to an age-induced endothelial expression of cyclooxygenase-2, and partially compensated for the age-associated lack of NO-mediated relaxation [30]. Accordingly, the expression of prostaglandin H synthase-1 and prostacyclin synthase was reported to increase with age in the aorta of Wistar–Kyoto rats [31]. The decrease in endothelium-dependent relaxation exhibits heterogeneity throughout the vascular system; with the large conduit vessels, such as the aorta, being most affected [32]. The reason underlying this observation might be that the contribution of the endothelial factors to the overall response varies between vessels as well as the ratio of endothelial cells to smooth muscle cells [32].

3. Mechanisms of aging-induced endothelial dysfunction

The mechanism underlying the aging-induced attenuation of endothelium-dependent dilatation is almost impossible to assess in humans, as it necessitates studies in isolated vessels performed on a large cohort of samples. The specimens available from human material; segments of the internal mammary artery from coronary bypass-grafting, coronary artery segments from explants during cardiac transplantation and peripheral arteries from amputation due to angiopathy, are usually heavily affected by the underlying disease. Consequently, more specific time-course studies have to rely on data obtained in animal experiments, traditionally performed in rats. In the rat aorta, the endothelium-dependent relaxation is primarily an indicator of NO bio-availability, which is determined by the rate of NO production, as well as by its scavenging by superoxide anions (O_2^-) [33]. Several studies report that endothelial NO synthase (eNOS) expression and NO production decline with age [19,32,34,35], whereas other authors have observed eNOS expression to be increased during aging [36,37]. Several studies also reported an increased vascular formation of O_2^- [19,22,38] and O_2^- is known to contribute to impaired relaxation since scavenging of radicals improves endothelium-dependent responses [19]. A concomitant increase in O_2^- and NO production leads to the generation of peroxynitrite [19,36] and the uncoupling of the eNOS [19]. Some reports also suggest that the cellular antioxidative defense system is attenuated during the aging process. For example, the plasma concentration of superoxide dismutase (SOD), but not the cellular SOD content in rats decreases with age [32]. Although the biological relevance of this observation is uncertain, altered plasma SOD activity is most likely a consequence of the attenuated NO bio-availability, as plasma SOD reflects extracellular SOD (ecSOD) activity in these animals [39] and ecSOD expression is induced by NO [40]. The increased formation of peroxynitrite during aging may also inactivate antioxidative enzymes, and this has been demonstrated for manganese SOD (MnSOD) in mitochondria [36] (Fig. 1).

The mechanism which leads to attenuated eNOS expression in aging is unknown. In vivo, the most important stimulus for the expression of eNOS is the shear stress generated by the flowing blood on the endothelial surface [41], which increases eNOS mRNA expression and stability [42], a fact which may explain why physical training improves eNOS expression in older humans [43,44] and animals [35]. Nevertheless, several other factors/stimuli can increase eNOS expression including estrogens [45], growth factors [46] and hydrogen peroxide [47]. The secretion of many growth factors and hormones declines with age, and this, in particular, has been shown for human growth hormone [48]. Therapy with growth hormone improves endothelial function in aged rats [49] and in humans [50]. The plasma concentration of several steroid hormones also decreases with aging (for review see [51,52]). Of these, estrogens and dehydroepiandrosterone (DHEA) [52] have gained most attention and DHEA has become a widely used "anti-aging" drug. Although hard scientific evidence justifying therapy has not been presented, animal experiments suggest that higher levels of DHEA may protect against the development of atherosclerosis [53–55]. Indeed, treatment with DHEA has been shown to improve endothelium-dependent flow-mediated dilator responses in middle-aged men with hypercholesterolemia [56]. In cultured human endothelial cells, DHEA stimulates NO release [57,58], and, treatment with DHEA increases aortic eNOS expression in ovariectomized Wistar rats via a pathway independent of the sex steroid receptor [59]. Whether, or not, chronic supple-

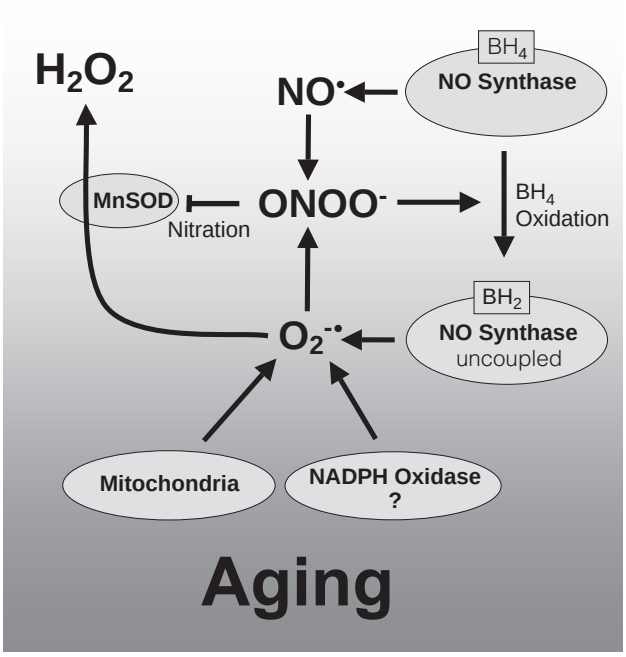

Fig. 1. Potential mechanisms of aging-induced oxidative stress in endothelial cells. Aging leads to mitochondrial dysfunction resulting from cumulative DNA damage. The shortage of enzymes of the respiratory burst promotes mitochondrial formation of superoxide (O_2^-), which is usually rapidly detoxified to H_2O_2 by mitochondrial manganese superoxide dismutase (MnSOD). Particularly in endothelial cells, nitric oxide (NO) is present in high concentrations and reacts with O_2^- to form peroxynitrite ($ONOO^-$). $ONOO^-$ can inactivate MnSOD by tyrosine nitration and can switch the NO synthase via the oxidation of tetrahydrobiopterin (BH_4) from an NO- to an O_2^--generating enzyme (NO synthase uncoupling). Both reactions lead to an increase in the concentration of O_2^-, in terms of a vicious circle, further promoting DNA damage and $ONOO^-$ formation.

mentation with DHEA is able to prevent aging-induced endothelial dysfunction is still unknown.

Until very recently estrogen supplementation has been advocated as atheroprotective therapy. Each year of delay in the onset of the menopause reduces cardiovascular mortality risk by 2% [60], and epidemiological studies suggest that estrogen replacement in postmenopausal women is associated with a 50% reduction in the incidence of cardiac events [61]. It should however be mentioned that recent large clinical trials using conjugated equine estrogens, which also contain testosterone, and not only 17β-estradiol have challenged these observations [62–65]. Estrogens acutely improve the reduced endothelium-dependent dilator response in postmenopausal women [66] and prolonged estrogen therapy has been shown in humans to restore the endothelial function to the premenopausal level [66,67]. In animal experiments, natural 17β-estradiol increases eNOS expression and NO production, enhances the generation of prostacyclin and augments the EDHF-mediated relaxation and hyperpolarization (for review see [68,69]).

Despite an attenuated generation of endothelial NO, an excessive constitutive generation of NO by smooth muscle cells might be relevant to endothelial aging. Increased expression of the inducible NO synthase (iNOS) has been reported in aged rats [19,37]. This enzyme generates high amounts of NO in a calcium-independent manner and is dependent mainly on its level of expression. The latter is in turn determined, at least in part by the activity of the transcription factor NFκB and by inflammatory cytokines [70]. Indeed, there is evidence linking aging with increased inflammatory burden, since aging is associated with a proinflammatory shift in gene expression, e.g. endothelial expression of interleukin 1β and interleukin 6 in aged rats [71]. Oxidative stress and inflammation are the main stimuli determining the activity of NFκB. The induction of iNOS within the vascular wall would be expected to further promote oxidative stress via two mechanisms; (1) the peroxynitrite formed from the reaction of iNOS-derived NO with O_2^- is a much stronger oxidizing agent than the two radicals on their own [72]. (2) iNOS, because of its high turnover rate, also generates O_2^- following uncoupling or the depletion of its substrate L-arginine and subsequent transfer of electrons to O_2 [73]. In this context it is interesting to note that the expression of arginase, which also metabolizes arginine, is increased with age in rabbits [74] and inhibition of arginase in rats improves endothelium-dependent relaxation [75].

4. Endothelial aging and oxidative stress

There is little doubt that aging is associated with increased oxidative stress and oxidative damage [76,77] and the endothelium appears to be an important source of O_2^- in the vascular wall [38,78–80]. This effect seems to increase with age and leads to an endothelium-dependent attenuation of nitrovasodilator reactivity in aortic and mesenteric rings from rats [23]. Removal of the endothelium, as well as inhibition of the NADPH oxidase and eNO synthase reduce vascular O_2^- generation in the aorta of aged Wistar–Kyoto rats [38].

There is controversy regarding the enzymatic sources of radical generation. It is widely believed that leakage of O_2^- and particular of H_2O_2 from mitochondria and mitochondrial dysfunction increase with age. Mitochondria continuously produce O_2^- and mitochondrial DNA is continuously exposed to oxidative stress during the life span, which in turn results in an ever increasing amount of DNA damage [76,77,81]. The consequence of this is a decrease in the number of mitochondria [82] and impaired expression of mitochondrial proteins as well as the formation of dysfunctional proteins, which leads to cellular energy depletion and further radical formation. Indeed, it has been shown that a senescence-induced lack of the mitochondrial cytochrome *c* oxidase (complex IV) leads to oxidative stress in endothelial cells [83]. In the vasculature, peroxynitrite-dependent inactivation of the mitochondrial MnSOD occurs, which further promotes mitochondrial O_2^- formation [36] (Fig. 1).

DNA damage, however, is not restricted to the mitochondria and one enzyme family involved in the labeling of damaged DNA—the poly(ADP-ribose) polymerases

(PARP) has been implicated in the aging-induced attenuation of endothelium-dependent relaxation [84,85]. Indeed, inhibition of PARP restored the acetylcholine-induced relaxation in the aorta of aged rats [86].

Several other enzymes are thought to be involved in aging-induced radical formation. As mentioned above, NO synthases can be transformed into radical generating enzymes [87]. In rodents, a role for xanthine oxidase has been suggested [88]. Although direct evidence is lacking, the NADPH oxidase might be a source of radical generation as activation of small GTPases [89] and potentially the NADPH oxidase subunit Nox4 [90] leads to O_2^- formation and senescence. That the NADPH oxidase may contribute to the aging phenomenon can also be derived from the observation that the stimulation of cultured cells with angiotensin II, a potent inducer and activator of the enzyme in vascular cells, leads to DNA fragmentation [91]. Indeed, treatment with angiotensin converting enzyme (ACE) inhibitors prevents the aging-induced endothelial dysfunction in rats [92]. Hydroxymethylglutaryl-CoA reductase inhibitors (statins), in contrast, had no effect on aging-induced vascular dysfunction, whereas in the rat cycloxygenase-2 (COX-2) inhibition was just as effective as ACE inhibition [93]. The real contribution of the NADPH oxidase, however, requires further investigation, as the classic, protein kinase C-dependent isoform of this enzyme, is apparently not involved in aging-induced O_2^- formation [94].

5. Aging, endothelial senescence and regeneration

As with most other mammalian cells, the capacity of endothelial cells to divide is limited and ultimately the cells enter a state of irreversible growth arrest, termed senescence [51]. Senescent cells are metabolically active but morphologically altered and express senescence-associated enzymes such as the acidic β-galactosidase (SA-β-gal). Under normal conditions endothelial cells rarely divide and exhibit a turnover rate estimated at approximately 3 years. Several processes, such as endothelial injury, wound healing or angiogenesis initiate endothelial proliferation [51], and consequently, the impaired wound healing and angiogenesis that is typically observed in the elderly, has been attributed to endothelial senescence [95]. Nevertheless, it is uncertain, whether or not endothelial cell senescence is an aging-associated or a vascular disease-associated phenomenon. An increased SA-β-gal activity has been observed in the endothelial cells within atherosclerotic plaques, but not in human coronary artery explants without signs of atherosclerosis [96]. Another widely used index of senescence is telomere length. Telomeres are critical for chromosomal integrity and during each DNA duplication, approximately 50–200 bp of telomeric DNA fails to replicate. Senescence is reached when the telomeres are shortened below a critical length [97] (for review on telomeres and cardiovascular disease, see [98]). Indeed, telomere length is inversely correlated to age in endothelial cells in vivo [99–101]. The process of telomere shortening is counteracted by the telomerase reverse transcriptase (TERT). TERT is not expressed in most human somatic cells but in germline cells and most tumor cells, preventing senescence in these lines [97]. Interestingly, cultured endothelial cells possess TERT activity, which potentially delays the onset of senescence [102,103]. Passaging during the cell culture process leads to a decrease in NO formation, which results in a loss of TERT activity. Treatment with NO-donors can however restore TERT activity and prevent the onset of senescence in cultured endothelial cells [102]. Recently, it has been reported that oxidative stress is a central regulator of endothelial nuclear TERT activity. Increased radical production and the subsequent activation of the tyrosine kinase Src leads to the export of TERT from the nucleus [104] (Fig. 2). Indeed, the aging-induced loss in nuclear TERT activity can be prevented by antioxidants and by statins, which also inhibit cellular radical generation [105].

Senescent endothelial cells are more prone to pro-apoptotic stimuli and this is largely a consequence of an

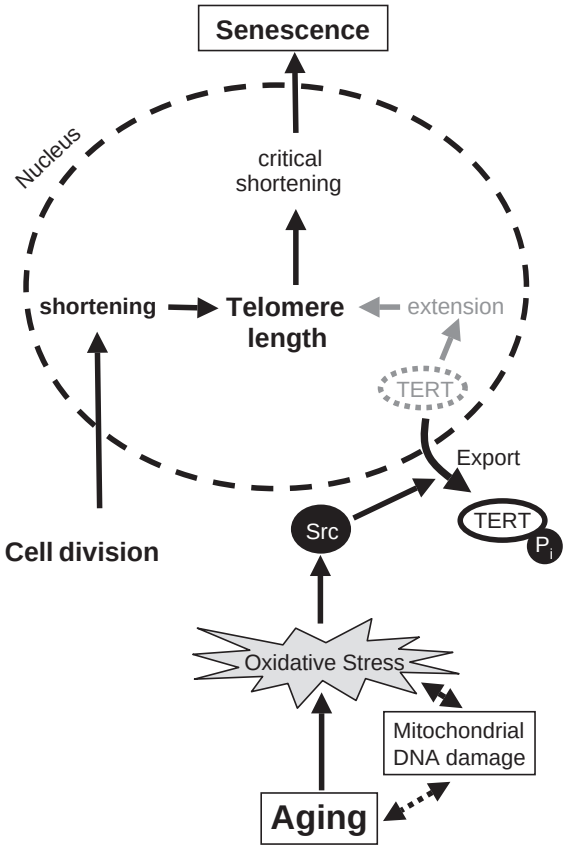

Fig. 2. Model of endothelial senescence in aging. Aging and the accumulation of mitochondrial damage lead to oxidative stress, which activates the tyrosine kinase Src. Src phosphorylates the telomerase reverse transcriptase (TERT) resulting in an export of TERT from the nucleus. Each DNA replication during cell division shortens the telomeres of the chromosomes, a process which is usually compensated by TERT. The aging-induced lack of nuclear TERT activity finally leads to cellular senescence due to telomere shortening.

attenuated NO production [106]. However, passaging of endothelial cells per se renders them susceptible to apoptosis [107] and thus it is uncertain whether the senescence of endothelial cells is a relevant phenomenon in vivo. There is, however, little doubt that endothelial cell apoptosis can occur in vivo. Various stimuli, such as inflammatory cytokines, infection, oxidized lipids and turbulent blood flow seem to promote this process [108]. Indeed, telomere length, as a marker of repetitive cell division, is reduced in endothelial cells localized at sites exposed to high mechanical stress [99–101].

The gap in the endothelial monolayer resulting from endothelial cell injury or apoptosis has to be filled, and this may occur via three different mechanisms: spreading of adjacent endothelial cells, hyperplasia of existing endothelial cells and engraftment of circulating endothelial progenitor cells. Certainly, the contribution of each of these processes to endothelial regeneration is difficult to judge. However, there is evidence that aging is associated with an attenuated capacity of the endothelium to regenerate, which is partially a consequence of an impaired secretion of and/or sensitivity to growth factors [109–111]. Recently, the regeneration of the endothelium by bone marrow-derived circulating progenitor cells has gained particular attention [112]. The number of circulating endothelial progenitor cells (EPCs) decreases with age and is thought to reflect the attenuated mobilization of these cells from the bone marrow [113]. Moreover, EPCs from older subjects have a reduced capacity to engraft. Some studies suggest that the regenerated endothelium is functionally impaired: regenerated endothelium is morphologically different, is dysfunctional [114,115] and exhibits an increased uptake of modified low-density lipoprotein (LDL) and decreased NO production [116]. Aging further impairs endothelial regeneration after injury in rats and this is associated with a decreased expression and phosphorylation of eNOS [116]. Interestingly, arginine supplementation partially restores the "juvenile" phenotype, suggesting that substrate depletion may play a role in this process [86]. The aging-induced loss of eNOS phosphorylation in the aorta from aged rats, in contrast, appears to be a consequence of an attenuated activity of the protein kinase Akt [117].

There is also evidence that angiogenesis is reduced with age. Vascular endothelial growth factor (VEGF)-induced angiogenesis in aged rats [74] and rabbits [95] is attenuated and even angiogenesis-dependent tumor growth is retarded with age [118]. Several reports have demonstrated that wound healing is delayed in aged subjects and this is to some extent attributed to an impaired angiogenic process [110,119]. Apart from the attenuated proliferative and regenerative capacity of the endothelium with age (as discussed above), changes in endothelial gene expression may also contribute to this effect. The inflammation-induced expression of intercellular adhesion molecule 1 (ICAM-1) is retarded and reduced in endothelial cells from aged subjects [119]. In aged mice and in cultured human microvascular endothelial cells aged by progressive passaging, the expression of the tissue inhibitor of metalloproteinase-2 (TIMP-2) is increased. Enhanced TIMP-2 expression would be expected to correlate with an attenuated capacity of endothelial cells to degrade extracellular matrix, a process required for angiogenesis [120]. Moreover, in mice, the angiogenesis-associated inflammation and matrix deposition as well as the expression of VEGF and transforming growth factor β1 (TGF β1) are reduced with age whereas the expression of thrombospondin-2 increases [121,122]. VEGF promotes endothelial proliferation and migration, whereas TGF β1 is involved in matrix formation. Thrombospondin-2, in contrast, inhibits angiogenesis [123], by reducing endothelial cell migration [123] and proliferation [124].

6. Aging and pro-atherosclerotic endothelial phenotype

VEGF is not the only growth factor whose expression is altered by aging. Aging increases the release of endothelin from endothelial cells in man and animals [32,37,125–127]. Endothelin is a potent vascular growth factor and endothelin receptor blockers have been shown to prevent the development of atherosclerosis in ApoE−/− mice [128,129]. Moreover, endothelial overexpression of endothelin in mice results in vascular remodeling and endothelial dysfunction [130]. The vascular reactivity to endothelin is also differentially affected by aging. In the rat aorta and femoral artery, aging impairs the vasoconstrictor response to this peptide [32,131], whereas in rat coronary arteries, it is increased [132,133]. Recently, endothelin has even been suggested to be involved in down-regulation of eNOS in fetal porcine pulmonary artery endothelial cells [134]. The differential expression of endothelin during aging might be a consequence of age-related differences in transcription factor activity particularly that of AP-1, NFκB, CRES, TFIID, CTF and AP-2 but not Sp1 [125,135]. Given the large number of transcription factors altered by aging, it is not surprising that also the expression of a plethora of growth factors and endothelial cell adhesion molecules is altered with aging. In rats, aging leads to an enhanced expression of adhesion molecules in the aorta [136] and in rabbits vascular monocyte adherence is increased with age [137]. A similar process also appears to occur in humans, where the level of serum-soluble adhesion molecules directly correlates with age [138].

7. Conclusion

Aging elicits several changes in the vascular endothelium gradually altering its phenotype from an anti- to a pro-atherosclerotic one. Reactive oxygen species and the concomitant oxidative and nitrosative stress may play an important role in the process of endothelial aging, affecting vascular function as well as endothelial gene expression and monolayer integrity.

Although senescence is an attractive concept to account for the attenuated angiogenic and regenerative capacity of endothelial cells with aging, future studies are needed to elucidate whether senescence, as well as alterations in the circulating level of endothelial progenitor cells are of clinical relevance.

Acknowledgment

Work performed in the authors own laboratory was supported by the DFG (BR 1839/2-1 und 2-2).

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