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FOCUS SEMINAR: OXIDATIVE STRESS AND CARDIOVASCULAR DISEASE

STATE-OF-THE-ART REVIEW

# Basic Biology of Oxidative Stress and the Cardiovascular System

## Part 1 of a 3-Part Series

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**ABSTRACT**

The generation of reactive oxygen species (ROS) is a fundamental aspect of normal human biology. However, when ROS generation exceeds endogenous antioxidant capacity, oxidative stress arises. If unchecked, ROS production and oxidative stress mediate tissue and cell damage that can spiral in a cycle of inflammation and more oxidative stress. This article is part 1 of a 3-part series covering the role of oxidative stress in cardiovascular disease. The broad theme of this first paper is the mechanisms and biology of oxidative stress. Specifically, the authors review the basic biology of oxidative stress, relevant aspects of mitochondrial function, and stress-related cell death pathways (apoptosis and necrosis) as they relate to the heart and cardiovascular system. They then explore telomere biology and cell senescence. As important regulators and sensors of oxidative stress, telomeres are segments of repetitive nucleotide sequence at each end of a chromosome that protect the chromosome ends from deterioration. (J Am Coll Cardiol 2017;70:196–211)


Inflammation and oxidative stress contribute to the pathobiology of almost every human disease. Reactive oxygen species (ROS), which may be generated by both enzymatic and nonenzymatic systems, lie at the core of the biology of oxidative stress. Under homeostatic nonpathological conditions, ROS in low to modest amounts play a major role in routine cellular and mitochondrial signaling and functionality. However, if unchecked, ROS may mediate oxidative tissue and cell damage that can spiral in a cycle of inflammation and more oxidative stress.

In this 3-part review series, we cover and review the full breadth of oxidative stress and the cardiovascular system under 6 subsections (Table 1), with each subsection having been written by a dedicated authorship team with acknowledged expertise in this field. As reviewed across these 3 papers (Table 1), oxidative stress is fundamental to cardiovascular biology and disease, and is a fast-moving topic of research that has presented several early opportunities for therapeutic intervention. This review series should serve as a benchmark and resource for the field moving forward.

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## BASIC BIOLOGY OF OXIDATIVE STRESS, ROS, AND MITOCHONDRIAL FUNCTION

### BASIC MITOCHONDRIAL BIOLOGY AND FUNCTION.

Mitochondria are pivotal intracellular mediators of oxidative stress throughout the body, and are the logical starting point to begin this review of oxidative stress in the cardiovascular system. Mitochondria function to sustain numerous homeostatic processes in the heart and vasculature, including generation of energy and control of calcium and redox homeostasis. Mitochondria also play a role in regulating susceptibility to injury and to injury-initiated programmed cell death. Although we now recognize that mitochondria are specifically programmed for distinct functions in different cell types and organs (1), for the purpose of this review, in this initial section we focus on those functions relevant to the myocardium.

**Energy production.** The heart is required to generate a vast amount of high-energy adenosine triphosphate (ATP) on a continuous basis (2). To sustain this energy production and cardiac contractile function, mitochondria constitute almost one-quarter of the volume of cardiomyocytes (3). Notably, the heart functions as an omnivore and consumes all available fuel substrates. Mitochondrial fatty acid β-oxidation is the most efficient and predominant substrate for energy production in the normal adult human heart, although glucose, lactate, amino acid and ketone oxidation, and glycolysis also contribute toward overall myocardial ATP production (4). The regulation of the use of different energy substrates in cardiac metabolism has been extensively explored (5,6), and adaptative or maladaptative metabolic changes in the heart are proposed to contribute to different disease states, including diabetic cardiomyopathy, cardiac hypertrophy, heart failure, and myocardial ischemia (5–7).

The final common pathway for mitochondrial oxidative metabolism to generate ATP is through the sequential passage of substrate oxidation reducing equivalents (nicotinamide adenine dinucleotide or flavin adenine dinucleotide), which then donate electrons down the electron transfer chain (ETC) with the concurrent utilization of oxygen to generate ATP. The complete process within mitochondria is termed *oxidative phosphorylation*, and a schematic illustration of the biochemical reactions that generate ATP production in the ETC is shown in **Figure 1**. The genetic disruption of oxidative phosphorylation pathway enzymes has been linked to cardiac pathology that usually manifests with cardiomyopathic features (8). At the same time, acquired cardiac pathologies can also disrupt oxidative phosphorylation and, most notably, the disruption of the final common pathway (i.e., the ETC) not only affects ATP production, but also can additionally impair intracellular calcium homeostasis, increase the generation of ROS, and alter redox balance. These aspects of mitochondrial biology are expanded on in the following sections.

**Mitochondrial calcium handling.** Cardiac contraction is regulated, in part, by increasing cytosolic calcium transients. Although the sarcoplasmic reticulum is the central organelle in the myocardial regulation of calcium transients, cytosolic calcium is also transmitted to mitochondria, leading to activation of enzymes involved in oxidative phosphorylation for ATP production to sustain cardiac work (9). Among the mitochondrial enzymes regulated in this fashion, the best characterized include calcium-mediated activation of pyruvate dehydrogenase, isocitrate dehydrogenase, and oxoglutarate (or α-ketoglutarate) dehydrogenase (10–12). Under pathological conditions of high cytosolic calcium (calcium overload), mitochondria are capable of taking up large amounts of calcium. Here, the mitochondria can function as a calcium reservoir or sink. Calcium also functions as an intracellular signaling intermediate and orchestrates many cellular processes, including cardiac hypertrophy (13,14). At the same time, when mitochondrial calcium levels are excessive, they lead to mitochondrial production of ROS and mitochondrial-triggered cardiomyocyte death pathways (discussed later) (15). It is of potential therapeutic interest that, until recently, the ability to manipulate mitochondrial calcium uptake and efflux was not possible, given the lack of information about the mechanisms underpinning these processes. Recently, however, the structure and molecular details of the mitochondrial calcium uniporter were identified and partially characterized (16,17). In parallel, greater clarity on cardiac mitochondrial calcium efflux has come to the fore, with the characterization of mitochondrial sodium-calcium exchange components (18). Whether the proton-calcium antiporter is operational in the myocardium is less certain (12). Finally, the mitochondrial permeability transition pore (PTP) may play a regulatory role in fast calcium release from mitochondria (12). This concept may be better understood with our advancing understanding of the composition and function of the PTP. The major function of the PTP currently being explored is in the promulgation of cell death pathways, and these are discussed later.

### ABBREVIATIONS AND ACRONYMS

**ATP** = adenosine triphosphate
**ETC** = electron transfer chain
**$H_2O_2$** = hydrogen peroxide
**IMM** = inner mitochondrial membrane
**LTL** = leukocyte telomere length
**OMM** = outer mitochondrial membrane
**PTP** = permeability transition pore
**ROS** = reactive oxygen species
**SASP** = senescence-associated secretory phenotype
**SIRT** = sirtuin

**TABLE 1 Summary of 3-Part Review Series: The Role of Oxidative Stress in Cardiovascular Disease**

| |
|---|
| Part 1 of 3: Basic Biology of Oxidative Stress and the Cardiovascular System |
| 1.1 Basic Biology of Oxidative Stress, Reactive Oxygen Species, and Mitochondrial Function |
| 1.2 Oxidative Stress, Telomeres, and Cell Senescence |
| Part 2 of 3: Impact of Oxidative Stress on the Heart and Vasculature |
| 2.1 Pathophysiological Role of Oxidative Stress in Heart Failure |
| 2.2 Oxidative Stress in the Vasculature |
| Part 3 of 3: Oxidative Stress and Cardiovascular Risk: Obesity, Diabetes, Smoking, and Pollution |
| 3.1 Obesity, Diabetes, and Oxidative Stress |
| 3.2 Role of Oxidative Stress in the Cardiovascular Effects of Air Pollution and Smoking |

**Mitochondrial role in ROS biology.** The transfer of electrons down the ETC is usually not completely coupled between oxygen consumption and ATP production. Thus, a small minority (<0.1%) of electrons can dissociate (leak) from the ETC and cause the partial reduction of oxygen ($O_2$) into superoxide ($O_2^-$) and subsequent ROS intermediates such as hydrogen peroxide ($H_2O_2$) and hydroxyl radicals (19). In parallel, other enzymes within mitochondria can also generate $H_2O_2$ as direct and obligatory products of catalytic reactions (20). Examples of mitochondrial enzymes that generate $H_2O_2$ include monoamine oxidase and nicotinamide adenine dinucleotide phosphate oxidase 4. The relative contribution of the ETC versus other mitochondrial sources in generating $O_2^-$ and $H_2O_2$ is not well established, although it is interesting that in human atrial biopsies the expression of monoamine oxidase correlates with more ROS production and a higher incidence of post-operative atrial fibrillation (21). An additional complexity is that different ROS may confer different functions within mitochondria, and also more broadly within the cell, by retrograde signaling out of the mitochondria. For example, $O_2^-$ is proposed to remain within the inner matrix of the mitochondrion and $H_2O_2$ can diffuse out to mediate effects within the cytosol (22). The combination of these mechanisms makes mitochondria a prominent source of cardiomyocyte ROS.

Mitochondria also contain antioxidant mechanisms to remove ROS. An example of this system is superoxide dismutase enzymes that dismutate $O_2^-$ to $H_2O_2$. Mitochondrial $H_2O_2$ is then catabolized by additional enzymes, including glutathione peroxidase I and peroxiredoxin III (23,24), and nonenzymatic scavengers, with its ultimate reduction to water. The majority of experimental studies related to excess mitochondrial ROS formation or disruption of ROS breakdown support their role in exacerbating ischemia or reperfusion injury, heart failure, and diabetic cardiomyopathy (7,25,26), and the augmentation of ROS-ameliorating pathways in experimental models blunts these same pathophysiologies (27–30). Here, excess ROS can lead to irreversible damage to mitochondria, and to cardiomyocytes becoming significant contributors to the development of cardiovascular disease. These maladaptive consequences of excessive ROS, with specific references to mitochondrial consequences and amplification of cardiac stressors, are discussed in the following sections.

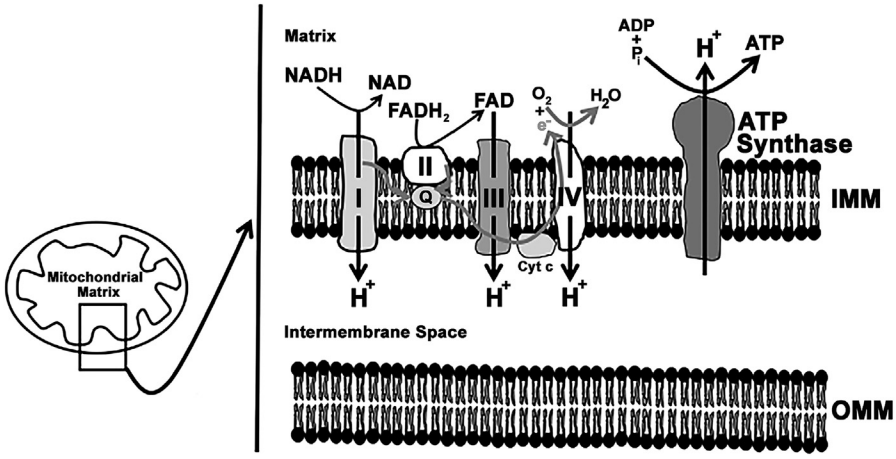
In contrast, physiological levels of ROS act as signaling molecules, to modify proteins or lipid species within mitochondria, and to modulate mitochondrial and cardiomyocyte function (31,32). This role of mitochondrial ROS is linked both to their role in the oxidative modification of cysteine amino acid residues on multiple proteins (33) and to the modification of the function of transcription factors that regulate target gene transcription and the less well-characterized modifications of lipid signaling intermediates (34,35).

## ROS-ACTIVATED MITOCHONDRIAL PROGRAMS ORCHESTRATING CARDIAC PATHOLOGY.

**Cell death programs: apoptosis and necrosis.** In the early decades of the molecular and cellular exploration of cell death, it was postulated that pathology-related cell death was unregulated and chaotic. However, by the mid- to late 1990s, it was recognized that pathology-related cell death was highly regulated and that mitochondria play an important role in cell death programs. The major forms of cell death in the heart divide into 2 distinctly regulated molecular pathways defined as apoptosis and necrosis (**Figure 2**). Despite their differences, both death pathways can be activated by evoking cell surface “death” receptors, or through ROS and calcium overload effects on mitochondria (36–38). Furthermore, irrespective of the involvement of signals through death receptors, mitochondria are often part of a critical amplification loop orchestrating these cell death programs. Additionally, the extent and rate of ATP depletion is 1 factor in the determination as to whether necrosis or apoptosis is the predominant death pathway (39).

Apoptosis is a tightly regulated death pathway, where the integrity of the cell membrane is maintained until the fragmented cellular components are engulfed and eliminated by phagocytosis. Plasma membrane receptor-initiated apoptosis is through activation of cytokine receptors, with subsequent activation of intracellular protease enzymes. The triggering event in mitochondrial-mediated apoptosis

**FIGURE 1 Schematic Illustration of the Mitochondrial Electron Transfer Chain**

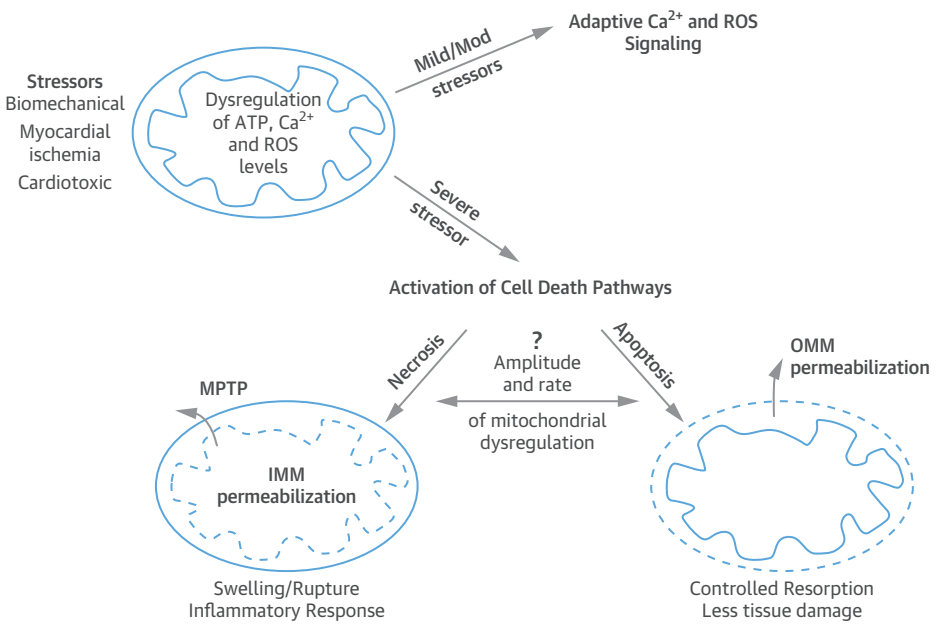



The **rectangular box on the left panel** illustrates the structure of the inner mitochondrial membrane (IMM) and outer mitochondrial membrane (OMM), and the **expanded inset (right panel)** shows the arrangements of the protein complexes (I though IV) that make up the ETC. The final common pathway for oxidative metabolism, which generates the bulk of cardiac adenosine triphosphate (ATP), is the sequential passage of electrons from high (NADH or $FADH_2$) to low (molecular oxygen) redox potentials down complexes I through IV of the electron transfer chain. This stepwise electron transfer results in the active pumping of hydrogen ions out of the mitochondrial matrix into the intermembranous space. The electrons travel down the complexes shown as the **gray arrow** and ultimately react with molecular oxygen to form water at complex IV. The "leak" of electrons along the chain provides substrate for reactive oxygen species (ROS) production. The ensuing electrochemical gradient generated across in the IMM facilitates the translocation of protons from the intermembranous space through the ATP-synthase complex back into the mitochondrial matrix. This proton translocation is coupled to the phosphorylation of adenosine diphosphate (ADP) to generate ATP. Also note cytochrome c (Cyt c) on the IMM. The release of this IMM protein following permeabilization of the OMM plays an important role in the initiation of apoptosis as described in the cell death section. $FAD/FADH_2$ = flavin adenine dinucleotide; $H^+$ = proton; $H_2O$ = water; NAD/NADH = nicotinamide adenine dinucleotide; $O_2$ = oxygen.

results in the permeabilization of the outer mitochondrial membrane (OMM), allowing the release of apoptosis-triggering molecules termed *apoptogens* (38). A shared feature of these mitochondrial-extruded proteins is that within mitochondria, they function to sustain healthy organelle and cellular function. However, when released, these same molecules are toxic within the cytosolic compartment. A classic example of an apoptogen is cytochrome c, which normally participates in electron transport as part of the ETC within mitochondria; however, once released into the cytosol it triggers apoptosis. Regardless of the initiating pathway, apoptosis activates caspases, a class of cystinyl proteases, which cleave proteins at aspartic acid residues. Caspase-mediated proteolysis of multiple cellular substrates then brings about the demise of the cell. The controlled resorption is aided by the cessation of ATP-requiring functions, including DNA repair, translation, and proteasome function (40-42), but with a more moderate reduction in ATP synthesis that maintains cellular membrane integrity functions during apoptosis.

In contrast to controlled cellular resorption during apoptosis, cellular and organelle membrane breakdown is a defining feature of necrosis, resulting in the extrusion of inflammatory mediators that cause both surrounding tissue damage and leukocyte recruitment. Induction of necrosis is either through the death receptor pathway (necroptosis) that activates receptor-interacting protein homologous serine or threonine kinases, or due to cellular injury that evokes sustained permeabilization of the inner mitochondrial membrane (IMM). The IMM is usually impermeable to the extrusion of mitochondrial proteins. This permeabilization process is induced by calcium overload or by excessive ROS signaling (43). This mitochondrial permeability transition event, if sustained, results in the rapid dissipation of the proton gradient across the IMM. As the IMM proton gradient is essential for ATP synthesis, this loss in membrane potential depresses ATP production. To further compound this energetic deficit and in contrast to apoptosis, ATP consumption at other intracellular sites continues largely unabated during necrosis (44). At the same time, the osmotic gradient

**FIGURE 2 Schematic of the Role of Mitochondria in Cell Death Pathways**




Multiple stressors on the heart disrupt mitochondrial bioenergetic, calcium, and ROS homeostasis. If the stressors are not excessive, calcium- and ROS-mediated signaling can activate adaptive programs to enable the heart to tolerate and overcome the stressors. Excessive stress with major perturbations in bioenergetics, calcium, or ROS leads to programmed cell death pathway activation or amplification by mitochondria. It is postulated that the amplitude and rate of onset of the stressors play a role in preferential activation of the necrotic or apoptotic pathways. Necrosis is linked to the mitochondrial permeability transition pore (MPTP) with leaking of the IMM, a more destructive cell death program. In contrast, permeabilization of the OMM initiates apoptosis, with the release of apoptogens leading to controlled resorption of the cell. $Ca^{2+}$ = calcium ion; other abbreviations as in **Figure 1**.

into the solute-rich mitochondrial matrix results in the influx of water, which can exacerbate the injury by swelling the mitochondrial matrix, leading to mitochondrial rupture (45).

The molecular aspects of the permeabilization of the IMM have been extensively studied over the last few decades, given that this phenomenon can be blunted with the concomitant attenuation of necrosis by the pharmacological administration of cyclosporin A and derivatives (15,46). Although numerous candidate proteins in the IMM and OMM had been implicated as components of mitochondrial PTP, these have mostly been dismissed as being of critical importance for permeabilization, following genetic depletion studies showing the lack of necessity of these proteins. The target of cyclosporin A, namely cyclophilin D, although not necessary for permeability transition, was found to regulate the PTP. More recently, a component of the ETC itself, namely the $F_1$-$F_o$ ATP synthase, was identified as a core component of the PTP (47). The biochemical data supporting the role of the $F_1$-$F_o$ ATP synthase is convincing, although its genetic validation in vivo will be complex, given the integral role of this ETC component in mitochondrial ATP synthesis. Additional components of the multimolecular ATP synthase complex are also being studied as potential regulators of the PTP, and hence as potential targets for antinecrotic drug development (48).

It should be noted that the mitochondrial roles in cell death pathways are not completely distinct during necrosis and apoptosis. This is illustrated by the apoptotic OMM proteins BAX and BAK, which appear to be necessary for some forms of cardiac necrosis (38,49). This pathophysiological overlap between apoptosis and necrosis is also evident when visualizing histological consequences of cardiac disease. This is most clearly evident following myocardial infarction, where the central ischemic zone shows necrosis and the peri-infarct area shows evidence of apoptosis (50,51). In heart failure, apoptosis is implicated as the major pathophysiology resulting in cardiomyocyte dropout, although evidence of necrosis has also been reported (37,52).

Ongoing research into the molecular programs governing both these forms of cell death and their role in disease will be important in developing new agents to blunt programmed cell death linked to cardiac pathology. As mitochondria play a central role in these programs, further research into understanding the quality control pathways that sustain mitochondrial integrity, including mitochondrial dynamics, mitochondrial autophagy (mitophagy), and the mitochondrial protein folding and unfolding responses, will be of importance so as to reveal additional targets to attenuate or prevent cell death going forward (53,54). These homeostatic programs are probably regulated, in part, through ROS signaling, although these regulatory links are less well characterized and not discussed here further. Nevertheless, the role of these mitochondrial quality control programs and their links to cardiac pathology are increasingly appreciated as important components in cardiovascular disease. More recently, evidence has emerged showing that mitochondrial damage can lead to retrograde signaling to the cell-autonomous nucleus, and to cell-nonautonomous signaling with systemic effects (55). Here, mitochondrial injury signaling evokes the induction of mitokines to relay these systemic effects (56,57). Cardiac mitochondria can also contribute to this program by initiating the production of cardiac fibroblast growth factor-21 as a systemic-functioning mitokine (58).

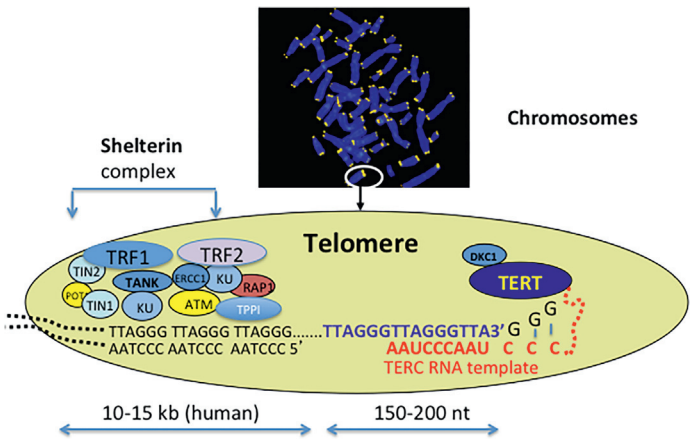
**Mitochondrial damage and inflammation.** Emerging evidence also suggests that mitochondrial damage can directly give rise to immune activation in numerous organs, including the heart (59). Here, immune activation due to loss of mitochondrial integrity is thought to partly arise from the bacterial origins of mitochondria. The working hypothesis is that approximately 1.5 billion years ago, a primitive bacterial cell entered into a more complex bacterial (archaeal) cell. The incorporation of an efficient energy-conversion system (primitive bacteria) into the archaeal cell has been termed *endosymbiosis*. During subsequent evolution, the majority of the engulfed bacterial genes were transferred to the nuclear genome (60). The residual bacterial genome became the mitochondrial genome and still harbored bacterial features. A consequence of this evolutionary occurrence is that the mitochondrial genome is seen as foreign material by mammalian intracellular and extracellular immune surveillance systems. The mitochondrial genomic DNA is, in a sense, "immune privileged" by being isolated from the cytosol and outside environment by its encasement within a unique mitochondrial double membrane. Experimental studies have shown that when mitophagy, the mitochondrial recycling program to remove damaged mitochondria, is disrupted, the cardiac response to pressure overload includes the activation of cardiomyocyte-autonomous inflammation, with the exacerbation of myocarditis and dilated cardiomyopathy (59). Conversely, following myocardial infarction and necrosis, mitochondrial DNA can be released into the circulation, which then activates neutrophils and initiates systemic inflammation (61,62). Additional components of the mitochondria, including cardiolipin from within the mitochondrial membranes, can also activate the immune system (63,64), although the role of these in cardiovascular diseases has been less well established.

## CONCLUDING REMARKS ON THE BASIC BIOLOGY OF OXIDATIVE STRESS, ROS, AND MITOCHONDRIAL FUNCTION.

The advances in our understanding of mitochondrial biology in recent decades have been substantial, with a parallel increase in our understanding of their role in disease pathophysiology. Mitochondrial signaling through the control of calcium and ROS can have adaptive effects, and when these "signaling intermediates" are in excess, they overwhelm the system and promote mitochondrial-orchestrated cell death pathways. Emerging evidence supports that both metabolic intermediates (22) and mitokines may expand the repertoire of mechanisms whereby the modulation of mitochondrial function and integrity can initiate both intracellular signaling responses and systemic effects. At the same time, our understanding of the regulatory control of mitochondrial quality control programs has expanded. These programs may also control retrograde signaling from the mitochondria to the cell and beyond, and the augmentation of the fidelity of mitochondrial quality control programs is being explored as an approach to prevent or blunt the consequences of cardiovascular disease (53,65,66). These strategies include lifestyle changes (caloric restriction and exercise training) and therapeutic agents (67,68), and, at least in experimental systems, are found to work, in part, by controlling mitochondrial ROS production or breakdown, controlling mitochondrial homeostasis, and preventing apoptosis, necrosis, and inflammation.

As is evident from the breadth of this review series, there are many facets to oxidative stress, both in terms of how it influences cardiovascular biology and also in its varied clinical manifestations. From this outline provided in the preceding sections on oxidative stress, ROS, and mitochondrial function, we now turn to another fundamental biological aspect of oxidative stress in the cardiovascular system:

**FIGURE 3 Human Chromosomes and Simplified Telomere Structure**




Telomeres can be visualized under the microscope and are shown in **yellow** in the top panel at the ends of metaphase chromosomes **(blue)**. They are composed of thousands of tandem DNA repeats: TTAGGG in the leading 3′ strand, and CCCTAA in the lagging 5′ strand. Associated protective proteins that form the shelterin complex include protection of telomere protein 1 (POT1), repressor/activator protein 1 (RAP1), TERF1-interacting protein (TIN1), adrenocortical dysplasia protein homologue (TPP1), telomere repeat-binding factor 1 (TRF1), and telomere repeat-binding factor 2 (TRF2). The 3′ end of the telomeric leading strand is a single-strand overhang, which is incompletely reproduced at cell division leading to telomere shortening. Adapted with permission from Fyhrquist et al. (101). ATM = serine protein kinase; DKC1 = H/ACA ribonucleoprotein complex subunit 4 (dyskerin); ERCC1 = DNA excision repair unit; KU = K70 protein involved in telomere maintenance; TANK = tankyrase; TERC = telomerase RNA component; TERT = telomerase reverse transcriptase.

telomeres and telomere function (**Central Illustration**). It is important to note that apart from mitochondrial function and telomeres, which are covered here in depth, there are many other broad biological areas that are affected by oxidative stress, such as altered cellular epigenetics, that will be covered only in passing, where relevant to the remainder of this review series. This is not any reflection on their importance, but merely that the body of published reports implicating these additional basic biological aspects of oxidative stress in cardiovascular disease is less developed than it is for mitochondrial and telomere function.

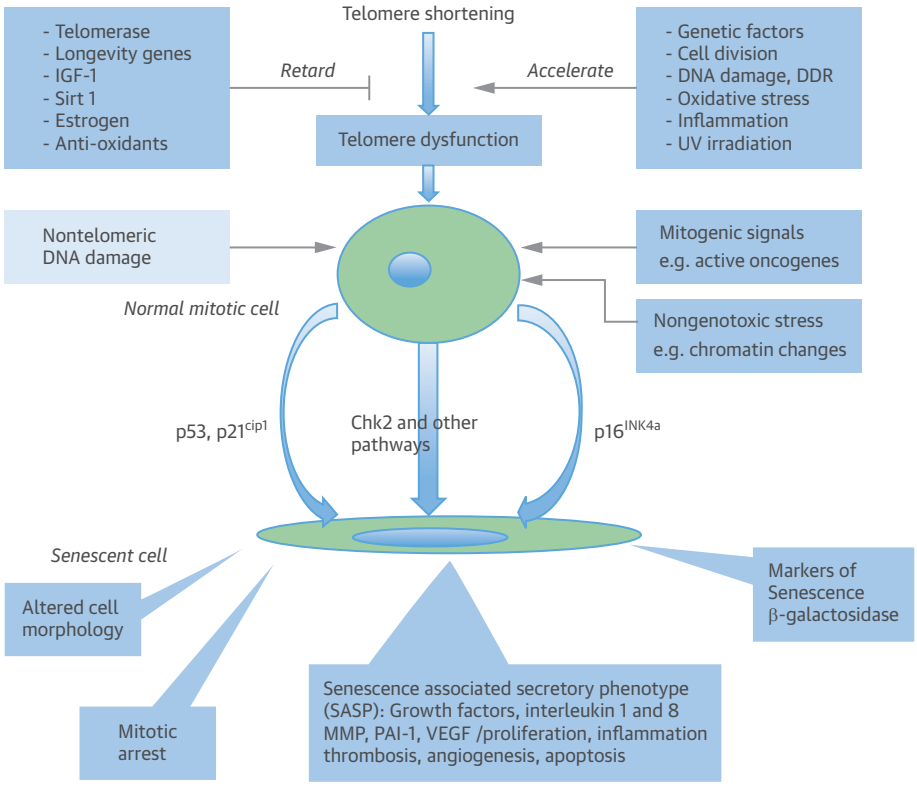
## OXIDATIVE STRESS, TELOMERES, AND CELL SENESCENCE

The connections between oxidative stress, telomere biology, and cell senescence are complex and incompletely understood. Cell division and aging are the most important causes of telomere shortening. Oxidative stress is well known to promote telomere shortening and dysfunction (69), which, among other factors, drives cell senescence and apoptosis. Cell senescence, characterized by mitotic arrest and, as described later, leads to a senescence-associated secretory phenotype (SASP) of a large number of proinflammatory and growth factors, leading to an increased burden of tissue inflammation and oxidative stress (70). This inflammatory milieu induces further oxidative stress and telomere shortening, and hence promotes cell senescence. Telomere shortening is strongly associated with aging and related to cardiovascular disease. Whether telomere shortening is just a consequence of aging, oxidative stress, inflammation, and so on, or is also a cause of cardiovascular disease is an intriguing and as yet unsolved question.

**TELOMERE STRUCTURE AND FUNCTION.** Telomeres are regions of DNA and associated proteins located at the ends of chromosomes, and consisting of tandem repeats of the TTAGGG sequence and related nucleoproteins forming the shelterin complex (**Figure 3**) (71). Telomeres participate in the maintenance of genomic and cellular stability and replication. They protect the genome from degradation, unwanted recombination, and chromosomal fusion (71). Telomeres shorten by 30 to 150 base pair (bp) with each cell division (72). When a critical telomere length is reached, shelterin proteins can no longer maintain the protective nucleotide T-loop, and the DNA damage repair system and cell-cycle inhibitors become activated (**Figure 4**). The cell enters replicative senescence, followed by apoptosis (73). Also, disruption of components and interactions within the shelterin complex can initiate telomere dysfunction (74,75). Therefore, telomere shortening is not necessary for telomere dysfunction.

When only a few telomeres are critically shortened, they form end associations, leading to a DNA-damage signal that results in replicative senescence. This is called the M1 stage (76). In the absence of cell-cycle checkpoint pathways (e.g., p53 and p16 [a regulator of cell division and a tumor suppressor]), cells bypass the M1 stage of senescence. Telomeres then continue to shorten, resulting in a crisis, also called the M2 stage. The M2 stage is characterized by chromosome end fusions, mitotic catastrophe, and cell apoptosis (76). Telomerase, associated with the telomere complex, catalyzes DNA synthesis to maintain telomere length. Germ cells, stem cells, and cancer cells have high telomerase activity to avoid senescence, whereas somatic cells have low or undetectable telomerase activity. Human telomerase consists of the telomerase RNA component and a catalytic subunit, termed telomerase reverse transcriptase, which generate new telomeric TTAGGG repeats (73,77).

**FIGURE 4 Factors Affecting Telomere Attrition and Cellular Senescence**




Factors not inducing telomere dysfunction (nontelomeric DNA damage, oncogenic signals, and chromatin changes) are also depicted. These factors trigger the tumor suppressing pathways p16, p21, and p53. Senescent cells are characterized by mitotic arrest and altered morphology. Cells with a senescence-associated secretory phenotype (SASP) display autocrine and endocrine activities involved in tissue repair, proliferation, inflammation, and apoptosis. Checkpoint signaling kinase 2 (Chk2) is activated by DNA damage and involved in cell cycle arrest. Adapted with permission from Fyhrquist et al. (101). DDR = DNA damage response; IGF = insulin-like growth factor; MMP = matrix metalloproteinase; PAI = plasminogen activator inhibitor; Sirt 1 = sirtuin 1; UV = ultraviolet; VEGF = vascular endothelial growth factor.

Telomerase, similar to telomere length, is under both genetic (78) and environmental control (79). In addition to telomerase, other enzymes are also implicated in telomere regulation and protection. For example, dyskerin pseudouridine synthase-1 plays a role in telomerase stabilization and maintenance, and mutations of the gene encoding dyskerin pseudouridine synthase-1 are associated with bone marrow failure (80).

**MEASUREMENT OF TELOMERE LENGTH.** In clinical and epidemiological studies, telomere length is mostly measured in DNA from circulating leukocytes and reported as mean leukocyte telomere length (LTL). Telomere length may also be measured in various tissues, vascular wall samples, saliva, and so on. Analysis of the terminal restriction fragment by Southern blot has been considered the gold-standard assay (81,82). Alternatively, an increasingly used assay to measure LTL is the quantitative polymerase chain reaction (81). Other methods for measurement of telomere length include fluorescence in situ hybridization and single-telomere analysis (83). A reported synchrony between LTL and telomere length in somatic cells of individuals (84) suggests that LTL can serve as a proxy for telomere length in other cell types, such as vascular or cardiac cells.

**REGULATION OF TELOMERE LENGTH.** LTL displays high interindividual variability at birth and throughout life. LTL is at its longest at birth, then shortens rapidly during adolescence and more slowly during the rest of life (85). Genetic factors are involved in the regulation of telomere length (86,87), which partly explains individual variation in LTL. Loci associated with telomere length have been

identified by genome-wide association studies near the telomerase RNA component and a component of the telomere-maintenance complex (88,89). However, only a minor proportion (1.6%) of the variation in telomere length is explained by currently known loci (86,90,91). Individuals with Werner syndrome, having mutations in the *WRN* gene that is involved in telomere maintenance, show premature aging, myocardial infarction, and cancer at young age (92).

The sirtuin (SIRT) 1 to 7 family promotes survival, stress resistance, and longevity (93,94). SIRT1 is the most extensively studied (95), although SIRT6 has emerged as a key modulator of telomere structure, DNA repair, and nuclear factor kappa B pathway regulation in aging (93,95). Of note, a single nucleotide polymorphism located in the SIRT1 gene was associated with both long LTL and longevity in the Louisiana Healthy Aging Study (96), suggesting a link among SIRT1, telomere length, and longevity.

Short LTL has been associated with the D allele of the angiotensin-converting enzyme I/D polymorphism in 1,249 patients from the LIFE (Losartan Intervention For Endpoint Reduction in Hypertension) Study (97), which accords with the association of high plasma renin activity with short LTL in the Framingham Heart Study (98). Oxidative stress induced by high tissue angiotensin II levels might explain this relationship. Interestingly, angiotensin II also accelerates cellular senescence via telomere shortening (93).

Endogenous factors causing telomere attrition are listed in **Table 2**. Foremost among these is that, due to the need for DNA replication machinery to bind at the terminal ends of chromosomal DNA before the initiation of DNA strand replication, there is obligatory telomere shortening in somatic cells with each cell division (72,99). Oxidative stress and inflammation are important additional causes of telomere shortening, and are implicated in aging processes (100–103). Conversely, among endogenous factors promoting telomere maintenance (**Table 2**), telomerase is the most important. Telomerase counteracts telomere shortening in stem and germ cells by replacing telomere repeats. Most cancer cells also have active telomerase (90%), allowing continuous proliferation (76). Estrogen activates telomerase (104), probably explaining why women have longer telomeres than men. Endogenous antioxidants are also thought to inhibit telomere shortening (69).

Environmental factors shortening LTL (**Table 3**) are mostly related to lifestyle and cardiovascular risk factors. Smoking, obesity, and alcohol abuse may shorten telomeres by promoting inflammation and oxidative stress (105–108). Recently, air pollution has been suggested to promote oxidative stress and inflammation, and was associated with telomere attrition (109). Even mental stress (110) and childhood abuse (111) are reportedly associated with telomere attrition, although possible causality remains an open question.

**TABLE 2 Endogenous Factors Affecting Telomere Length**

| |
|---|
| Promoting telomere shortening |
| Age |
| Cell division |
| Genetic factors |
| Oxidative stress |
| Inflammation |
| Renin-angiotensin system activation |
| Counteracting telomere shortening |
| Telomerase |
| Genetic factors (i.e., sirtuins 1 and 6) |
| Estrogen |
| Antioxidants |

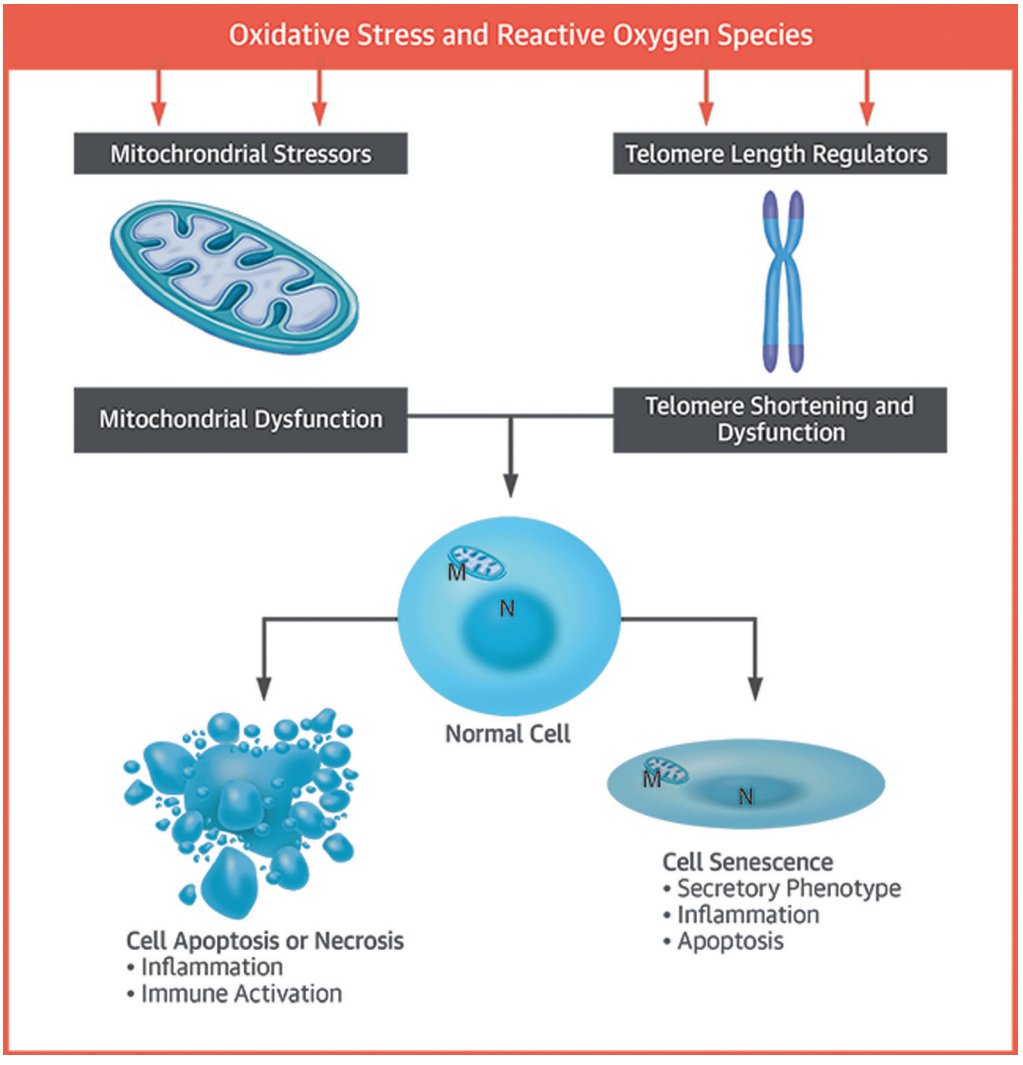
Environmental factors inhibiting shortening of LTL (**Table 3**) include a healthy lifestyle (112–114), estrogen treatment, and vitamins C, D, and E. Additional therapies associated with longer LTL include omega 3 fatty acids (115), statins (116), and metformin (117). High levels of physical activity are also associated with longer LTL (118). Of particular note is the recent observation that LTL increased after weight loss induced by bariatric surgery in a 10-year follow-up study in 142 grossly obese subjects (119).

Interestingly, spontaneous elongation of telomeres during follow-up (up to 25%) has been reported in some studies (120,121). The great variety of factors that regulate telomere length (**Tables 2 and 3**) may explain this seeming paradox. Methodological differences, individual variations in leukocyte composition, the cross-sectional nature of most studies on

**TABLE 3 Environmental Factors Affecting Telomere Length**

| |
|---|
| Promoting telomere shortening |
| Smoking |
| Alcohol abuse |
| Obesity |
| Air pollution |
| Mental stress |
| Counteracting telomere shortening |
| Healthy lifestyle |
| Estrogen treatment |
| Vitamins C, D, and E |
| Omega-3 fatty acids |
| Statin treatment |
| Metformin |
| Barosurgery |

**CENTRAL ILLUSTRATION** **Basic Biology of Oxidative Stress**




**Sack, M.N. et al. J Am Coll Cardiol. 2017;70(2):196–211.**

Simplified scheme of the effect of oxidative stress and reactive oxygen species, and the interaction between mitochondrial and telomere dysfunction, in the aging (degenerative) pathway from normal cell to senescent, apoptotic cell or cellular necrosis. M = mitochondria; N = nucleus.

LTL, and the limited number of individuals involved, call for caution when interpreting results.

Telomere attrition is considered by some as being 1 of 9 biological "hallmarks of aging." As summarized in 2 excellent review articles (122,123), these 9 aging hallmarks are considered to be genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication. Although telomere attrition is considered a "primary hallmark" causing damage, cellular senescence is considered an "antagonistic hallmark" that arises as a secondary response to damage and protects against malignant transformation.

**Genetic mutations with very short telomeres and strategies for telomere lengthening.** Several mutations in genes responsible for telomere repair and maintenance have been identified. These mutations are often associated with critically short

telomere length, and include mutations in the *TERT*, *TERC*, *DKC1*, and *RTEL1* (regulator of telomere elongation helicase 1) genes. Such mutations often result in critically short telomeres, which subsequently lead to organ dysfunction, including bone marrow failure, liver cirrhosis, and pulmonary fibrosis, and also increased risk of cancer (124). Interestingly, male hormones have been successfully used to treat bone marrow failure for decades (125), and additional evidence exists indicating that sex hormones regulate telomerase (126). Therefore, on the basis of these observations, investigators at the National Institutes of Health recently undertook an open-label phase I/II clinical study to investigate if LTL can be influenced by sex hormone therapy with danazol, a derivative of ethisterone previously used to treat endometriosis, but that has significant masculinizing effects. Enrollment criteria were age adjusted LTL at or below the first percentile or an identified mutation in a telomere maintenance or repair gene, plus evidence of bone marrow failure (124). Heralding a likely breakthrough for the field, the study was halted early due to a strong efficacy signal. Of the 12 patients who reached the pre-specified final 2-year time point, a mean increase in LTL was observed of 386 bp (95% confidence interval: 178 to 593 bp). Furthermore, 10 of these 12 patients experienced a hematologic response to danazol therapy. Similar trends were seen in 12 additional participants who were evaluated after the study was prematurely halted (124). Although further studies are required to assess the effect of danazol on survival and progression of bone marrow failure to myelodysplastic syndrome or acute myeloid leukemia in such patients, this landmark clinical study has opened the door to the use of targeted therapeutics aimed specifically at enhancing telomere length. However, this study was limited in subject number and focused on "telomere diseases" not associated with cardiovascular disease. It does not support treatment of telomere length in cardiovascular disease with male steroids.

**OXIDATIVE STRESS AND TELOMERES.** Telomeres are rich in guanine nucleotides, making them particularly sensitive to oxidative stress by causing single-strand damage to telomeric DNA (127). Several studies have shown that ROS can accelerate telomere shortening (100) and can damage DNA and thus induce a DNA damage response and senescence (100). For instance, oxidants cause telomere attrition in cultured human endothelial cells (128), whereas antioxidants reduce telomere shortening. Senescence, in turn, leads to further ROS generation via a SASP with activation of p53, mitogen-activated protein kinase, nuclear factor kappa B, and mitochondrial dysfunction (34). As mentioned previously, telomere shortening and dysfunction can be promoted by a variety of factors not necessarily associated with oxidative stress, including genetic and genomic perturbation, cell division, mitogenic signals, and nontelomeric damage (**Figure 4**).

**CELLULAR SENESCENCE.** Cellular senescence is characterized by proliferative arrest. There are 2 types of cellular senescence: replicative and stress-induced premature senescence. Replicative senescence is driven by cell division with telomere shortening and, consequently, dysfunction of telomeres (**Figure 4**). In addition, replicative senescence is further induced by factors that accelerate telomere shortening, including genes, cell division, DNA damage, and oxidative stress (**Tables 2 and 3**). Stress-induced premature senescence is induced by a number of factors, such as oxidative stress, mitogens, oncogenes, and irradiation (129,130). This form of premature senescence is not usually associated with telomere attrition. Collectively, these factors trigger DNA damage response signals from detectable nuclear foci called DNA-SCARS (DNA segments with chromatin alterations reinforcing senescence) (131–133), and activate the tumor-suppressing p16 and p53 pathways (134). Besides irreversible proliferative arrest, senescent cells display morphological changes, including flattening, vacuolization, and cellular enlargement (135). Senescent cells express increased β-galactosidase, the tumor suppressor kinase inhibitor 2a, and p16ink4a. The complex role of cell senescence in aging and age-related disease has been reviewed in considerable detail elsewhere (130).

Increased amounts of senescent cells have been found among vascular smooth muscle cells, endothelial cells, and macrophages from aged arteries and atherosclerotic plaques (87,136,137). Interestingly, short telomeres have been found in endothelial and tissue specimens from human vasculature (138–140), notably in atherosclerosis-prone vessels, such as carotid arteries (141,142). Such findings may be seen as indication of a link between cellular senescence and atherosclerosis. However, the exact nature of this postulated link remains to be clarified.

Senescent cells carrying DNA-SCARS of the SASP (**Figure 4**) have important autocrine and paracrine activities. Thus, they secrete inflammatory mediators, interleukin 6 and interleukin 8, metalloproteases, monocyte attractants, plasminogen activator inhibitor 1, and a number of growth factors

(87,131). Due to these active factors, cells of the SASP category contribute to degenerative and proliferative age-related tissue alterations causing a state of chronic inflammation, remodeling, and tissue repair. This promotes further oxidative stress and telomere shortening, driving more cells into premature senescence. Nitric oxide production is reduced in senescent vascular endothelial cells (135,143,144), causing endothelial dysfunction. Thus, vascular accumulation of senescent cells is likely to contribute to the development of atherosclerosis.

**TELOMERE LENGTH IN CARDIOVASCULAR DISEASE.** In cultured human endothelial cells, introduction of the telomere repeat-binding factor-2 (TRF2) extended cellular lifespan and inhibited cell senescence, indicating a role for telomere dysfunction in the triggering of senescence in vascular endothelial cells (135). Telomere dysfunction and vascular senescence are related to increased formation of ROS, adhesion molecules and inflammation, and β-galactosidase (145,146).

In human vascular tissues, telomere shortening is associated with atherosclerosis, with longer telomeres found in vessels that are less prone to atherosclerosis, such as saphenous veins and mammary arteries, and with shorter telomeres found in atherosclerosis-prone vessels, such as the aorta (140,142,147). Furthermore, endothelial cells from atherosclerotic plaques have shorter telomeres and express more β-galactosidase than do control cells (135,145). Moreover, regions of increased hemodynamic stress have short telomeres (139,140,142), with telomere shortening and senescence of vascular endothelium likely being related to local hemodynamics. A recent study (148) reported that plaque vascular smooth muscle cell senescence in atherosclerosis is associated with loss of TRF2, suggesting a major role for TRF2 in the regulation of smooth muscle cell senescence.

The importance of telomere function and cellular senescence in cardiac disease has also been emphasized by several lines of evidence (102,149,150). Thus, the aging heart contains increased numbers of senescent cardiomyocytes, as defined by the expression of β-galactosidase, p16, p21, and p53 (151). Also, cardiac biopsies from patients with heart failure have revealed shortened telomeres and increased cell senescence (152). Mitochondrial dysfunction, including compromised oxidative phosphorylation, may also be causally related to heart failure (153,154). In addition, aging mitochondria produce increasing numbers of oxidative radicals (155), which may contribute to telomere shortening and cellular senescence. Overall, therefore, mitochondrial and telomere dysfunction appear to exert a complex interactive role in heart disease.

Clinical studies have clearly shown an association between shortened LTL and both atherosclerosis (141,156–159) and coronary artery disease (159–161). In turn, LTL shortening is related to cardiovascular risk factors, including smoking, high cholesterol levels, and obesity (**Table 3**). Although association does not prove causality, cardiovascular risk factors generally cause increased oxidative stress (69,105) and concurrently promote telomere attrition. Consistent with this, a healthy lifestyle is associated with slowing of telomere shortening (101,162,163). We endorse the unifying hypothesis that telomere shortening, which is primarily brought about by aging and thus a progressive increase in overall cell division, is accelerated by cardiovascular risk factors and disease. Thus, telomere length reflects the total cumulative burden of inflammatory, oxidative, and mechanical stress on the cardiovascular system.

**CONCLUDING REMARKS ON OXIDATIVE STRESS, TELOMERES, AND CELL SENESCENCE.** Cell division and telomeric DNA damage are major factors driving telomere shortening and dysfunction. Oxidative stress and inflammation strongly contribute to telomere attrition, thereby promoting cellular senescence. Conversely, in a vicious cycle, cellular senescence triggers inflammation and generation of oxidative radicals.

It appears reasonably well documented that telomere shortening and cellular senescence are involved in aging and the development of age-related diseases, but to what extent this is causal or secondary has not been fully clarified. Telomere shortening and dysfunction are affected by a number of factors independently of age. Of these factors, some are modifiable (**Table 3**), but most are nonmodifiable (**Table 2**). That healthy lifestyle is related to delayed telomere attrition and less to age-related diseases, and vice versa, is well known, but does not prove a causal relation. Regardless of causality, a healthy lifestyle remains a cornerstone of preventing age-related cardiovascular disease. How does protection of telomeres prevent premature cardiovascular disease? Can antioxidants or certain drugs, for instance statins, metformin, or renin-angiotensin system inhibitors, be clinically beneficial by protecting telomeres? Will new compounds, for instance antioxidants that are presently being intensively studied and developed (123,128), result in the development of clinically useful drugs to prevent age-related diseases and to promote healthy aging?

Future research on telomere biology and cellular senescence will hopefully bring answers to such key questions.

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