Anti-Aging Medicine: Molecular Basis for Endothelial Cell-Targeted Strategies – A Mini-Review

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Aging is an inevitable part of life. Human life expectancy has risen impressively over the last centuries due to significant improvements in hygiene and health care. Consequently, the proportion of aged people is steadily increasing. With aging, some physiological functions are altered and resemble those occurring in disease conditions such as hypertension, chronic coronary disease and diabetes. Thus, there is the urge to better understand molecular and cellular mechanisms underlying aging and aging-related diseases. In rodents and possibly primates, calorie restriction is an effective approach to extend lifespan by reducing free radical-induced damage. Increased production of oxygen-derived free radicals plays an important role in the process of aging. Reactive oxygen species are generated by different intracellular molecular pathways principally located in the cytoplasm and in the mitochondria. The mitochondrial protein p66Shc is considered a longevity assurance gene since its genetic deletion extends the lifespan of rodents and displays protective effects in several models of cardiovascular disease. Silent mating type information regulation 2 homolog 1 Saccharomyces cerevisiae (SIRT1) is a nicotinamide adenine dinucleotide (NAD+)-dependent histone deacetylase that may also be involved in aging and diseases. SIRT1 also deacetylates a number of nonhistone target proteins, including p53, endothelial nitric oxide synthase and forkhead box protein. This review focuses on the latest scientific advances in understanding aging as well as delineates the possible therapeutic implications of p66Shc and SIRT1 in this process.

Key Words
Calorie restriction · Free radical-induced damage · Reactive oxygen species

Abstract
Due to improvements in lifestyle and healthcare, the proportion of aged people is rising steadily, especially in developed countries. With aging, some physiological functions are altered and resemble those occurring in disease conditions such as hypertension, chronic coronary disease and diabetes. Thus, there is the urge to better understand molecular and cellular mechanisms underlying aging and aging-related diseases. In rodents and possibly primates, calorie restriction is an effective approach to extend lifespan by reducing free radical-induced damage. Increased production of oxygen-derived free radicals plays an important role in the process of aging. Reactive oxygen species are generated by different intracellular molecular pathways principally located in the cytoplasm and in the mitochondria. The mitochondrial protein p66Shc is considered a longevity assurance gene since its genetic deletion extends the lifespan of rodents and displays protective effects in several models of cardiovascular disease. Silent mating type information regulation 2 homolog 1 Saccharomyces cerevisiae (SIRT1) is a nicotinamide adenine dinucleotide (NAD+)-dependent histone deacetylase that may also be involved in aging and diseases. SIRT1 also deacetylates a number of nonhistone target proteins, including p53, endothelial nitric oxide synthase and forkhead box protein. This review focuses on the latest scientific advances in understanding aging as well as delineates the possible therapeutic implications of p66Shc and SIRT1 in this process.
dependent risk factor for the development of cardiovascular disorders. Therefore, aging and aging-related diseases currently represent an important research topic.

Calorie restriction is a widely used protocol for life span studies in experimental animals (rodents and primates) [2]. It was first reported in 1935 that white rats fed with a low-calorie diet have an extended life span, lower body weight, lower heart weight and smaller bone size [3]. However, there are still unanswered questions concerning the global effects of calorie restriction. Indeed, as spontaneous cancer develops with aging in rodents, the real role of calorie restriction in animals is often confused with a reduction in the incidence of cancer, extension of life span, or both. Moreover, it is not fully understood whether the beneficial effects of calorie restriction also extend to humans; however, if they do, the underlying mechanisms have yet to be elucidated.

Interestingly, in a 20-year longitudinal adult-onset calorie restriction study in rhesus monkeys, 30% calorie restriction significantly reduces aging-related deaths [4]. Monkeys in the calorie-restricted group conserved glucose homeostasis, indicating a beneficial effect of calorie restriction on diabetes. The incidence of cancer increases with aging in rhesus monkeys, confirming the spontaneous development of cancer in aged rodents and indicating that cancer is an aging-related disease as well. However, monkeys under calorie restriction have a lower incidence of cancer compared with control animals. Calorie restriction also reduces the incidence of cardiovascular diseases in the same group. In addition, aged monkeys have been shown to develop brain atrophy in the bilateral frontal and temporal cortex in both control and diet-treated groups, suggesting a pathophysiological change observed with aging which is independent of calorie intake. However, monkeys on a low-calorie diet maintain grey matter volume in subcortical regions [4], supporting the finding that calorie restriction preserves locomotory activity in aged monkeys [5]. These results underscore the beneficial effects of calorie restriction on aging and aging-related diseases in primates.

In humans, Okinawan populations consuming 20% less calories compared to the average caloric consumption of the Japanese population display reduced mortality in elderly people and also decreased death rates of 30–40% due to lower incidences of cancer, cerebrovascular and heart diseases. Furthermore, Okinawans who switch to a Western diet are shown to have similar mortality and morbidity as people from Western countries. These data confirm the beneficial effects of calorie restriction in humans, although it cannot be excluded that a normal diet may adversely affect longevity independently of its caloric content. Strikingly, the data from members of the Calorie Restriction Society show consistent and positive effects of calorie restriction in adults. Compared with subjects on normal Western diets, subjects on a low-calorie diet have less risk factors for atherosclerosis, including reduced body mass index and blood pressure (both systolic and diastolic), lower levels of glucose, insulin, tumor necrosis factor-α and C-reactive protein, decreased levels of triglycerides and low-density lipoprotein (LDL)-cholesterol, and higher levels of high-density lipoprotein (HDL)-cholesterol. These beneficial effects are shown after one year of calorie restriction and improve further after six years of restriction [6]. Likewise, a Mediterranean diet characterized by large quantities of olive oil also has protective effects on cardiovascular risk factors [7].

The Free Radical Theory of Aging

The free radical theory of aging was first proposed by Denham Harman in the 1950s and states that production of reactive oxygen species (ROS), occurring as part of normal cellular metabolism, increases with aging and further contributes to the process of aging. Hydroxyl radicals and hydrogen peroxide (H₂O₂) are considered to play an important role in the process of aging [8]. However, the exact source of increased ROS observed in aging is still unclear. Mitochondria are one of the main sources of intracellular superoxide anion radical (O₂⁻) and H₂O₂, thus they are thought to experience heavy oxidative damage with aging. The life-time exposure of mitochondria to ROS damage in turn enhances ROS production, which ultimately and negatively affects life span. Enhanced production of ROS in aging is also associated with upregulated expression of inducible nitric oxide synthase, and nicotinamide adenine dinucleotide phosphate-oxidase and/or downregulation of antioxidants, such as extracellular superoxide dismutase, glutathione peroxide and decreased catalase activity [9].

The increased production of ROS observed with aging contributes to oxidizing lipids, consequently modifying membrane lipid composition and reducing its fluidity. The mitochondrial membrane is particularly susceptible to peroxidation because of its high composition of unsaturated lipids. Aging and aging-related ROS production increase mitochondrial membrane rigidity and further reduce efficiency of mitochondrial oxidative phosphorylation, resulting in cell apoptosis [10]. Calorie
reduction and a Mediterranean-style diet maintain normal membrane composition by reducing the index of unsaturation/saturation of lipids, thus blunting ROS-mediated damage of cell membranes [11].

Protein carbonyl, a protein oxidation marker, has been shown to increase with aging in the mouse brain and skeletal muscle mitochondria [12]. However, data concerning the effect of calorie restriction on protein carbonyl content are not consistent. Indeed, mitochondrial carbonyl concentration was not significantly changed in mice under calorie restriction [13].

Aging-related ROS also damage mitochondrial DNA (mtDNA) as confirmed by the elevated frequency of deletion occurring in mtDNA. An increased level of deletion frequency of mtDNA is observed in the brain, liver, heart and skeletal muscle of old rats and a calorie restriction diet decreases the higher level of deletion frequency in liver of aged mice [14]. Hydroxytyrosol, an important polyphenolic compound in olive oil, stimulates mitochondrial transcription factor A and increases the amount of mitochondria inside of cells, therefore maintaining mitochondrial functions [15].

**The Effect of Calorie Restriction on ROS Generation**

It is generally believed that the effect of calorie restriction on life span is due to a reduced metabolic rate and a decreased mitochondrial oxygen consumption. Nevertheless, pigeons have a comparable metabolic rate and body mass to rats, but have an eightfold longer life span. More importantly, the rate of H2O2 generation in mitochondria is lower in long-lived rather than short-lived animals. Thus, it is logical to correlate the rate of aging and maximum longevity to the rate of mitochondrial ROS generation [16].

In mitochondria, aging-related ROS production is mainly due to reduced activity of mitochondrial electron chain complexes [17]. The activity of the four electron transport systems is decreased with aging in human as well as in rodent skeletal muscles. Calorie restriction maintains the activity of the four electron complexes, thus reducing ROS production in mitochondria [17]. In addition, calorie restriction has a direct effect on antioxidant enzymes. In rat liver, calorie restriction is shown to restore catalase and glutathione peroxidase activity, as well as upregulating the expression of Cu-Zn superoxide dismutase [18]. It is also reported that calorie restriction prevents the decrease of glutathione and glutathione transferase activity in rat skeletal muscle [19].

On the whole, calorie restriction reduces the generation of free radicals due to attenuation of mitochondrial damage and upregulation of defense enzymes. Strict control of calorie intake is, however, an unpleasant and tough experience. Severe long-term restriction could induce malnutrition, especially in aged people. Meanwhile, in large prospective clinical trials, neither the separate nor the combined antioxidant treatment with vitamins C and E significantly reduced the incidence of major adverse cardiovascular events. This could be explained by several factors: (1) natural antioxidants are commonly found in normal human daily diet and, thus supplements may have no additional effects; (2) additional antioxidant treatment may depress endogenous antioxidant defense systems, thus preventing the expected positive effects of antioxidant therapy; and (3) the intracellular pathway may be sequestered in organelles (for instance mitochondria) and antioxidants are not reachable for the compound. Therefore, there is a call for new therapeutics other than dieting and antioxidant treatment to prolong life span and to improve life quality.

**Mammalian Longevity Assurance Gene Sirtuins**

Silent mating type information regulation 2 homolog (Sir2) family proteins, sirtuins, are considered to be crucial regulators of life span extension in the setting of calorie restriction. Calorie restriction activates Sir2 protein in yeast *Saccharomyces cerevisiae* and flies. SIRT1, the mammalian Sir2, is consistently upregulated in mice under calorie restriction. SIRT1 protein is a nicotinamide adenine dinucleotide (NAD+)-dependent histone deacetylase, which also deacetylates a number of non-histone target protein, including tumor suppressor p53 protein, members of the forkhead transcription factor family (FoxO), stress response proteins Ku70 and nuclear factor κ-light-chain-enhancer of activated B cells, and metabolic regulator α-subunit of the G protein (PCG-1α) [20, 21]. Starvation enhances the expression of SIRT1 protein in mouse liver and in human peripheral blood mononuclear cells, which returns to a normal level upon feeding [22], indicating that SIRT1 is sensitive to nutrition and metabolic changes. The upregulated SIRT1 protein deacetylates PGC-1α, a transcriptional coactivator that controls energy state in a NAD+-dependent manner in hepatic cells from both vivo and vitro experiments, suggesting a regulatory role of SIRT1 in mitochondrial biogenesis and energy metabolism [22]. Thus, SIRT1 is an important mediator of the beneficial effects observed...
with calorie restriction, and is probably involved in life span control.

The presence of SIRT1 protein is also observed in pancreatic β-cells [23]. In cell culture experiments, inhibition of SIRT1 reduces insulin secretion, suggesting a role of SIRT1 in insulin secretion [23]. In the aorta of streptozotocin-induced type I diabetes mice, the expression of SIRT1 protein is reduced while acetylated p53 and p21 proteins are upregulated [24]. Resveratrol, a small-molecule activator of SIRT1, deacetylates p53 and p21 in the aorta, thereby improving endothelial dysfunction by downregulating intercellular adhesion molecule-1 expression and inhibiting leukocyte rolling, which suggests a protective role for SIRT1 in diabetes-induced vascular dysfunction [24]. In type II diabetes mice, genetic upregulation of SIRT1 [24] or treatment with resveratrol [25] consistently enhances insulin secretion and improves glucose tolerance. These data suggest that modulation of SIRT1 expression and/or activity would be of therapeutic interest against diabetes and diabetes-related cardiovascular complications.

SIRT1 also has beneficial effects on endothelial cells. In rat aorta, inhibition of SIRT1 ex vivo causes an impaired endothelium-dependent and nitric oxide (NO)-mediated relaxation. Restoration of SIRT1 increases endothelial nitric oxide synthase (eNOS) expression by deacetylation of both lysine 496 and 506, indicating that SIRT1 directly regulates eNOS deacetylation of both lysine 496 and 506, indicating that dothelial nitric oxide synthase (eNOS) expression by mediated relaxation. Restoration of SIRT1 increases en paired endothelium-dependent and nitric oxide (NO)-mediated relaxation of the aorta, thereby improving endothelial dysfunction by downregulating intercellular adhesion molecule-1 expression and inhibiting leukocyte rolling, which suggests a protective role for SIRT1 in diabetes-induced vascular dysfunction [24]. In type II diabetes mice, genetic upregulation of SIRT1 [24] or treatment with resveratrol [25] consistently enhances insulin secretion and improves glucose tolerance. These data suggest that modulation of SIRT1 expression and/or activity would be of therapeutic interest against diabetes and diabetes-related cardiovascular complications.

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In transgenic mice with heart-specific overexpression of SIRT1, beneficial effects are observed only in animals with a slight or moderate upregulation of SIRT1 mediated by an increased catalase expression through the FoxO-dependent signaling pathway. Furthermore, heart-specific overexpression of SIRT1 retards aging of the heart and protects the heart from paraquat-induced oxidative stress [29].

Mammalian Longevity Assurance Gene p66Shc

Shc protein is considered to be a regulator of longevity in mammals [30]. The mammalian Shc adaptor protein has three isoforms of 46, 52 and 66 kDa (p46Shc, p52Shc, and p66Shc). p66Shc is the only isoform to play an important role as a redox enzyme implicated in mitochondrial ROS generation and translation of oxidative signals into apoptosis. Genetic deletion of p66Shc in the mouse leads to an extended life span which is correlated with a reduced production of intracellular oxidants and a decreased level of mtDNA content [9, 30]. p66Shc predominantly localizes in the cytoplasm, with a small fraction (10–40%) in the mitochondria. Ultraviolet light irradiation as well as oxidants such as H₂O₂ phosphate p66Shc on serine 36, which in turn induces translocation of p66Shc to the mitochondria [31]. Activated p66Shc increases mitochondrial Ca²⁺ sensitivity and thus interacts with reduced cytochrome c to increase the production of H₂O₂ which promotes the opening of the mitochondrial permeability transition pore and leads to apoptosis [31]. p66Shc is also involved in H₂O₂-induced forkhead inhibition by increasing the phosphorylation of forkhead (Drosophila) homolog (rhabdomyosarcoma) like 1 (FKHRL1, also known as FoxO3a) [32]. Furthermore, according to cell type and stimulation, this ROS-sensitive protein plays an important role in regulating apoptosis by both p53-dependent and p53-independent mechanisms. In light of its pivotal role in ROS generation, many efforts have been made to investigate the pathophysiological role of p66Shc in aging and aging-related ROS-mediated diseases (fig. 1) [33].

It is reported that p66Shc is highly expressed in fibroblasts from centenarians and in peripheral blood monocytes from diabetic patients. In line with the above, it is widely accepted that p66Shc is linked to aging and/or ROS-related diseases in humans. Genetic deletion is a useful tool to study the role of p66Shc protein under pathophysiological conditions since identical Shc genomic organization, transcript assembly and a high degree of amino acid identity have been reported in mice and humans [33].

In line with this hypothesis, p66Shc–/– mice are protected from aging-dependent endothelial dysfunction [34]. Wild-type mice display aging-dependent loss of NO-mediated vasorelaxation, while p66Shc–/– mice show a maintained ability to relax by virtue of an increased NO bioavailability, lower aortic O₂ levels and reduced aortic 3-nitrotyrosine content (fig. 2). In endothelial cell culture experiments, inhibition of p66Shc increases NO production by phosphorylating eNOS on serine 1177 [35]. There-
Fig. 1. Schematic representation of the endothelium and some of its key derived vasoactive substances. p66Shc is principally located in the mitochondria, where it contributes to the production of superoxide anion (O$_2^-$), radicals which scavenge NO to form peroxynitrite (ONOO$^-$). Different stress stimuli (e.g. glucose, oxLDL, hypertension, shear stress) increase in number and occurrence with age and enhance the production of O$_2^-$, thus exacerbating endothelial dysfunction. oxLDL = Oxidized low-density lipoprotein; ET-1 = endothelin-1; NOS = NO synthase; ONOO = peroxynitrite; MCP-1 = monocyte chemotactic protein-1; ICAM = intercellular adhesion molecule; VCAM = vascular cells adhesion molecule; Nf$\kappa$B = nuclear factor-kappa B; IkB = inhibitor to Nf$\kappa$B; NADPH = nicotinamide adenine dinucleotide phosphate-oxidase; PKC1 = protein kinase C-1; GPx = glutathione peroxidase.

Fig. 2. a Age-dependent changes in endothelium-dependent relaxation of wild-type (WT) and p66Shc$^{-/-}$ aortas. Line graphs show concentration-response curves to acetylcholine. Results are presented as means ± SEM; n = 7 in each group. b Bar graph showing peak concentrations of NO in young (gray bars) and old (black bars) WT and p66Shc$^{-/-}$ mice, respectively. Results are presented as means ± SEM; n = 4 to 6 in each group. * p < 0.05 vs. young WT [34].
**Fig. 3.** a Bar graphs show ONOO\(^-\) levels determined by chemiluminescence. Results are presented as means ± SEM; n = 6 in each group. Immunostaining of nitrotyrosine residues in aortas from control and diabetic WT and p66\(^{Shc-/-}\) mice is shown. * p < 0.05 for p66\(^{Shc-/-}\) control vs. WT control; ** p < 0.05 for WT diabetic vs. WT control. b TBARS levels. Bar graphs show TBARS levels in aortas from control and diabetic WT and p66\(^{Shc-/-}\) mice. Results are presented as means ± SEM; n = 4 in each group. * p < 0.05 for p66\(^{Shc-/-}\) control vs. WT control; ** p < 0.05 for WT diabetic vs. WT control [37].

**Fig. 4.** Schematic representation of effects of p66\(^{Shc}\) and SIRT1 in anti-aging treatments. The production of ROS increase with aging. p66\(^{Shc}\) activates eNOS by phosphorylation while SIRT1 maintains eNOS protein by deacetylation. ROS-induced apoptosis goes through FoxO3 or the p53 pathway. Oxidative stress activates FoxO3 by a combination of acetylation and phosphorylation events. p66\(^{Shc}\) mediates in H\(_2\)O\(_2\)-induced phosphorylation, whereas SIRT1 directly deacetylates effect on FoxO3. p66\(^{Shc}\) is a down-streaming transcriptional target of p53, while SIRT1 negatively regulates the ability of p53 by protein deacetylation.
fore, p66Shc adaptor protein is regarded as a key player in aging-dependent loss of endothelial integrity and NO bioavailability.

Furthermore, p66Shc−/− apoE−/− mice fed with a high-fat diet display lower levels of lipid peroxidation compared to control mice, thus suggesting a possible role of p66Shc in the oxidative modification of lipoprotein. Compared with control littermates fed high-fat diets, p66Shc−/− apoE−/− mice are shown to have less aortic lesion formation accompanied by a decrease in intimal foam cell formation and lower levels of systemic plasma isoprostanes [36]. Additional studies confirm a tight link between plasma levels of LDL-cholesterol and p66Shc in pacemaker-implanted patients, thus underscoring that p66Shc plays a role in translating the effects of different aging-related risk factors into arterial dysfunction and early atherosclerotic lesion [33].

High glucose induces a cascade of cellular events which increase the production of free radicals, thus decreasing NO bioavailability and eventually leading to vascular dysfunction. In conditions of raised glucose plasma levels, p66Shc is known to oxidize cytochrome c and, in turn, generate proapoptotic ROS through a protein kinase c-β dependent pathway [31]. In line with the above, p66Shc protein expression is increased in aortas of type I diabetes mice [37]. p66Shc−/− mice have comparable glucose levels as control mice [37, 38], indicating that knocking out p66Shc protein does not alter glycemic levels. However, they are protected from diabetic glomerulopathy as demonstrated by reduced glomerular cell apoptosis and decreased extracellular matrix deposition, indicating the pivotal role of p66Shc in translating ROS-mediated insults into apoptosis [38]. Furthermore, streptozotocin-induced diabetic p66Shc−/− mice are protected against endothelial dysfunction via a blunted ROS production and the consequently preserved NO bioavailability (fig. 3). This finding reinforces the concept of a causal relationship between high glucose, ROS, p66Shc and vascular dysfunction [37].

Notably, a definite balance between ROS damage and endothelial progenitor cell (EPC)-mediated repair exists and guarantees endothelial integrity. In keeping with this theory, the increased ROS production observed in aged mice or mice with a pathophysiological condition, such as hyperglycemia, could explain both impairment of endothelial function and loss of EPC-mediated repair, which ultimately leads to vascular dysfunction [39]. These changes, however, are not observed in p66Shc knockout mice which present lower levels of ROS and a preserved endothelial function. Therefore, the mitochondrial adaptor protein p66Shc represents a promising target protein for slowing the process of aging and blunting aging-related ROS generation [33].

Clinical Perspectives and Conclusions

The signaling pathways relevant to aging discussed here are currently under intensive investigation as potential targets for new anti-aging treatments based either on reducing oxidative damage or enhancing cell defenses. Indeed, p66Shc and SIRT1 share multiple molecular targets (fig. 4). First, both proteins enhance NO bioavailability through eNOS upregulation/activation and/or reduction of NO scavenging. Indeed, p66Shc activates eNOS by phosphorylating serine 1177 [35], while SIRT1 regulates eNOS activity by deacetylating both lysine 496 and 506 [26]. Second, upregulation of FoxO3, which is important for increasing activity of antioxidant systems such as catalase and superoxide dismutases, is mediated by both p66Shc and SIRT1 through phosphorylation and acetylation, respectively. In fact, p66Shc plays a role in H2O2-induced phosphorylation of FoxO3 [32], whereas SIRT1 has a direct effect on deacetylation of FoxO3 [20]. Third, p66Shc is a downstream target of p53 [30], whereas SIRT1 negatively regulates p53 by protein deacetylation [24].

It may seem optimistic to assume that simply down-regulating p66Shc, upregulating SIRT1, or a combination of the two will effectively slow down aging in humans and shift the pathophysiological imbalance towards a normalized condition. SIRT1 exerts multiple protective effects. Nevertheless, physical training is reported to restore SIRT1 protein in both heart and adipose tissue of aged mice, supporting previous evidence showing beneficial effects of exercise training [40]. Furthermore, resveratrol, one of the polyphenolic compounds in grapes and wine, is reported to be a small-molecule activator of SIRT1 [21, 25], which may be worth investigating as a potential treatment for aging-related human diseases. p66Shc is a mitochondrial protein mediating oxidative stress [9, 33]. Genetic deletion of p66Shc protein has shown multiple beneficial effects in the process of atherosclerosis and diabetes in experimental animal models. Therefore, it is believed that the inhibition of p66Shc is the more attractive therapeutic option to reduce oxidative stress in human rather than antioxidant treatment as previously discussed [33]. The target proteins described in this review may offer new insights to better characterize underlying mechanisms of aging and may potentially represent novel therapeutic targets to prevent aging-related disease.
References


