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Mechanisms underlying caloric restriction and life span regulation: implications for vascular aging

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Abstract

This review focuses on the emerging evidence that attenuation of the production of reactive oxygen species (ROS) and inhibition of inflammatory pathways play a central role in the anti-aging cardiovascular effects of caloric restriction (CR). Particular emphasis is placed on the potential role of the plasma membrane redox system in CR-induced pathways responsible for sensing oxidative stress and increasing cellular oxidative stress resistance. We propose that CR increases bioavailability of NO, decreases vascular ROS generation, activates the Nrf2/ARE pathway inducing ROS detoxification systems, exerts anti-inflammatory effects and, thereby, suppresses initiation/progression of vascular disease that accompany aging.

Historical perspective

Almost a century ago Moreschi and Rous published separately their observations on the impact of underfeeding laboratory animals on transplanted and induced tumors [1,2]. Two decades later, McCay and colleagues first observed lifespan extension in laboratory rats maintained on a CR diet [3]. Since then, CR has been studied intensively with consistent results showing its beneficial effects on longevity, age-associated diseases, attenuation of functional declines, and carcinogenesis across a broad variety of species and diet formulations [4–5]. Despite these observations the precise mechanism(s) underlying the effects of CR protection and lifespan extension remain unknown. It is safe to say that, calorie restriction reduces metabolic rate and oxidative damage, improves markers of diabetes such as insulin sensitivity.

CR decreases the incidence of cardiovascular disease and has been shown to alter neuroendocrine and sympathetic nervous system in laboratory animals and some of these are replicating now in ongoing human studies. In particular, the National Institute on Aging through its program, CALERIE (Comprehensive Assessment of Long-Term Effects of Reducing Intake of Energy, initiated in 2002) endeavors to fund clinical trials address the feasibility of using CR as therapeutical tool as well as its effects and mechanisms in disease prevention. CALERIE studies examine the delay of aging-related comorbidities, particularly those associated with metabolic rate and biomarkers of aging, studying those that predict age-related diseases such as cardiovascular disease and type 2 diabetes [6–13].

Oxidative stress, aging and the plasma membrane

Mitochondria are the main source of ATP production. During mitochondrial oxidative phosphorylation, reactive oxygen species [ROS] are produced. ROS are associated with damage to DNA, lipids and proteins [14–16]. The pathology of aging and age-related diseases involves oxidative stress as an early stage in its development [17–19] as confirmed by a decrease in antioxidant defenses and an increase in oxidative damage [20, 21]. Aging is also associated

with changes in levels of antioxidant capacity and oxidative damage ostensibly leading to mitochondrial impairment. These changes have been coupled to increased oxidative damage to DNA [22–25], lipids [26, 27] and proteins [23, 28–30]. Accumulation of mitochondrial DNA mutations, commonly identified in age-related diseases, induce impairments of mitochondrial complexes [31–33], including mitochondrial complex III activity in aged heart [34]. Impaired mitochondrial function causes shortage of ATP supply, resulting in induction of further problems in biochemical pathways [31].

The free radical theory of aging [35, 36] has generated considerable interest regarding the search for possible biochemical bases of aging processes. Many past studies have shown that CR decreases production of reactive oxygen species (ROS) production thus minimizing oxidative damage [37, 38]. These studies have lead collectively to the hypothesis that CR by reducing oxidative stress extends the lifespan. The mitochondrial [39] and plasma [40] membranes are sites of active and abundant ROS production and thus are at high risk of ROS damage. Therefore, it follows that a central mechanism for the actions of CR may involve membrane alterations that either reduce ROS production or resist oxidative damage.

It has been proposed that life span is inversely related to the degree of membrane phospholipid unsaturation [41, 42] and that elucidation of this relationship can provide insight on the mechanism for life span extension with CR[43]. Modulation of membrane susceptibility to peroxidation, however, may be too simplistic to explain aging processes since this hypothesis, for the most part, does not consider other membrane-associated processes. Such processes include changes in cellular signaling, leakage of protons (and other ions) [44], production of ROS [39], induction of apoptosis [45], and maintenance of antioxidant systems [46–49]. Membrane-induced alterations in any of these processes could have major consequences that influence oxidative stress and life span.

CR Increases CoQ-Dependent Reductases in Plasma Membranes in vivo and in vitro

Coenzyme-Q (CoQ) contributes to stabilize plasma membrane, regenerates antioxidants such as ascorbate and α–tocopherol, and regulates the extracellulary-induced ceramide-dependent apoptosis pathway[49, 50]. NAD(P)H-dependent reductases act at the plasma membrane to regenerate $CoQH_2$, contributing to maintain its antioxidant properties. As a whole, both CoQ and its reductases (Fig. 1) constitute a trans-plasma membrane antioxidant redox system responsible of the above described functions [51–53].

The aforementioned antioxidants are maintained in their reduced forms at the plasma membrane by different CoQ-dependent reductases, NADH-dependent *cytochrome* b_5-reductase [54] and NAD(P)H:Quinone-oxidoreductase-1 (NQO1) [55]. Different dietary modifications can modulate these enzyme activities to protect the plasma membrane [56–58]. Our previous work has shown that these two enzyme activities are increased in plasma membranes from rat and mouse tissues under long term CR compared to ad libitum conditions [46–48]. Increases in the activities of these enzymes are due to enhanced concentration of these proteins at the plasma membrane [46, 47]. Both enzyme activities are known to be present in the cardiovascular system [59–62] and we posit that they are regulated by CR in a similar manner. Data from our laboratories and others, provide support that the plasma membrane redox system is, at least in part, responsible of the maintenance of the antioxidant capacity during oxidative stress challenges induced by the diet and aging. The up-regulation of the plasma membrane redox system that occurs during CR decreases the levels of oxidative stress in aged membranes [46, 48, 63, 64]. CR modifies composition of fatty acid in the plasma membrane, resulting in decreased oxidative damage including lipid peroxidation [65 66]. More importantly, plasma membrane redox activities and also the content of CoQ, which decline with age, are enhanced by CR providing protection to phospholipids and preventing the lipid peroxidation reaction progression [46, 48, 63, 64].

The plasma membrane also contributes to the regulation of the cellular redox homeostasis through the maintenance of $NAD(P)^+$/NAD(P)H ratio [67]. This function is driven in cooperation with mitochondria, an interaction particularly observed in ρ° cells [68, 69][48]. The ratio of pyridine nucleotides is considered an important regulator of yeast life span, as well as the establishment of respiration [70]. The ratio of NAD^+/NADH is also an important regulator of the deacetylase activity of Sir2, an enzyme involved in the regulation of life span in yeast. We and others have shown that expression of mammalian Sir2 (SIRT1) is induced under CR in laboratory animals and humans, as well as in cells in culture that are treated with serum from CR animals[11, 47, 71–73]. As we have indicated above, CR increases the activity of NAD(P)H-dependent reductases in the plasma membrane and CoQ, which likely contributes to the regulation of $NAD(P)^+$/NAD(P)H ratio. Since NADH and NADPH are substrates for NAD(P)H oxidases, the availability of these electron donors also influences the generation of ROS by these enzymes[38]. There is increasing evidence for age-related up-regulation of NAD(P)H oxidases in the cardiovascular system[74, 75], however, neither the role of CR-induced alterations in $NAD(P)^+$/NAD(P)H ratio in modulation of NAD(P)H oxidase activity nor the role of the plasma membrane redox system in this process are well understood. Plasma membrane-associated redox system and mitochondria are the major source of ROS in cells, which are generated mainly when CoQ-dependent electron transport is disrupted [37, 76]. Aging is associated with increased rates of stress-induced apoptosis in multiple organs[77], including an increased rate of endothelial apoptosis[75, 78]. CR promotes the activation of stress response genes and attenuates the stress-induced apoptosis by inducing *SIRT1* [72, 79]. Ceramide is a major signal molecule that mediates stress responses [80], and induces apoptosis through the activation of caspases [81]. We have previously shown that CoQ within plasma membranes prevent the cytosolic accumulation of ceramide by inhibiting the neutral sphingomyelinase present in membranes [50, 82]. It is conceivable that changes in CoQ concentration observed in liver plasma membrane induced by CR (see above) modulates the activity of neutral sphingomyelinase. We have studied this activity in plasma membrane-enriched fractions of rat liver and brain and observed that the activity of neutral sphingomyelinase decreases significantly after long-term CR [47, 46, 48]

CR Induces SIRT1 Protein Levels In Vivo and In Vitro

SIRT1 is distributed in all mammalian tissues studied and modulates cellular and tissue homeostasis interacting with metabolic and stress response proteins and factors. Mounting evidence suggests that SIRT1 regulates energy metabolism, endocrine signaling and some stress responses [83]. SIRT1 is also inducible by a broad variety of signals, in response to CR [79] or fasting [84], suggesting a broad role in mammalian physiology. It is becoming clear that sirtuins are regulated by stress and nutritional status in yeast, worms, flies and mammals [79, 85–87]. Endocrine and energy metabolism pathways coordinate organismal development and physiology, and are intrinsic to pathologies such as cancer, neurodegeneration and diabetes. These systems respond to a variety of external signals, as diverse as environment, stress and nutrients. Sir2 regulates, in opposite ways, both replicative [88] and chronological life span in yeast [89]. Extra copies of sirtuin genes extend the life spans of multicellular organisms such as worms, flies and fish [86, 90, 91]. In principle, understanding how these pathways respond to environmental and nutritional factors could enable us to better understanding to develop successful therapies.

SIRT1 regulates several transcription factors that regulate stress responses, energy metabolism and endocrine signaling, including peroxisome proliferator-activated receptor γ (PPARγ), PPARγ-coactivator 1α (PGC1-α), forkhead-box transcription factors (FOXOs), LXR and p53[92–98]. There is mounting data supporting that SIRT1 regulates energy metabolism, endocrine signaling and some stress responses [83, 99]. The biological effects identified for

sirtuins have fueled speculation that sirtuins modulate processes that affect longevity, age-related disease, diabetes and tumorigenesis [100].

CR animals and humans have significantly higher levels of SIRT1 protein in most tissues including brain, kidney, muscle, visceral fat pads, and liver [11, 79, 101]. Up regulation of SIRT1 by CR is also observed in cultured cell models that recapitulate the key *in vivo* proliferative and phenotypic features of CR [72]. Increasing the resistance of cells to apoptosis is beneficial if a cell is not critically damaged and is difficult to replace. However, this situation is clearly not always desirable if, for example, a cell is mutated or otherwise irreparably damaged. Under conditions of severe stress or pro-apoptotic signals such as TNF, SIRT1 can switch into a pro-apoptotic mode [79]. A recent study by Alt et al. [102, 103] found that mouse embryonic cells lacking the SIRT1 gene continue to divide long after they should have senesced due to chronic cell stress, indicating that SIRT1 is able to suppress the proliferation of damaged cells. SIRT1 regulates several transcription factors that regulate stress responses, energy metabolism and endocrine signaling, including peroxisome proliferator-activated receptor γ (PPARγ) [97], PPARγ-coactivator 1α (PGC1-α) [98], forkhead-box transcription factors (FOXOs)[92–96], LXR[104] and p53. There is mounting data supporting that SIRT1 regulates energy metabolism, endocrine signaling and some stress responses[83, 99]. Recent reports associate SIRT1 with the regulation of apoptosis, senescence and proliferation [79, 105–107].

Vasoprotective effects of CR

CR was shown to attenuate atherogenesis in rodents[108]. The cardiovascular effects of CR observed so far are consistent with the view that CR may confer vasoprotection in humans, although the effects of CR on progression of atherosclerosis and plaque composition in elderly humans or aged primates[109] are still not well documented. In general, CR may affect vascular health both by improving systemic risk factors for coronary artery disease (CAD) (e.g. plasma lipid and glucose levels, blood pressure) and by modulating cellular functions and gene expression in endothelial and smooth muscle cells that create a microenvironment in the vascular wall, which does not favor atherogenesis (e.g. attenuation of ROS production, anti-inflammatory effects).

Caloric restriction improves cardiovascular risk factor profile

Most current knowledge on the effects of CR on cardiovascular risk factors in humans emanates from studies in which obese individuals were treated with some form of relatively short-term dietary restriction to loose weight. High calorie diets and the resulting obesity are major risk factors for hypertension and coronary artery disease, In addition, weight loss has been associated with significant improvement in the cardiovascular risk factor profile in these individuals (including a decreased weight, body mass index, waist circumference, hip circumference, waist-to-hip ratio, total body fat, total cholesterol, serum triglyceride) [110, 111]. CR exerts beneficial effects on risk factors of atherosclerosis in non-obese individuals as well. This effect has also been shown both in studies on the eight individuals (including Dr. Roy Walford, an early proponent of CR) sealed inside Biosphere 2 for two years, who had to restrict their calorie intake due to a technical problem[112] and on 18 individuals who had been on voluntary CR for an average of 6 years[6]. Accordingly, CR in non-obese individuals elicits significant decreases in serum cholesterol, triglycerides, fasting glucose and fasting insulin levels as well as in systolic and diastolic blood pressure[6, 10, 112]. Studies of the effects of CR in rhesus monkeys have also shown reductions in serum triglyceride[113], Lp(a) in males[114] and fasting plasma glucose and insulin levels[115], which likely contribute to the cardioprotective effect of CR. The available rodent data seem to corroborate this conclusion[116–118].

CR increases bioavailability of NO and improves endothelial function

The direct effects of CR on vascular function and phenotype in aging are not well characterized. It is generally accepted that tonic release of NO from the endothelium exerts vasculoprotective and cardioprotective effects, such as maintenance of normal coronary blood flow, inhibition of platelet aggregation and inflammatory cell adhesion to endothelial cells and disruption of pro-inflammatory cytokine-induced signaling pathways. Abundant experimental and clinical data show that aging impairs endothelial NO production (recently reviewed elsewhere[119], which has been suggested to play a role in atherogenesis. The severe impairment of NO bioavailability in aging also limits cardiac blood supply and alters myocardial O_2 consumption and cardiac contractility[120]. Our recent data suggest that lifelong CR in rats prevents aging-induced endothelial dysfunction. Accordingly, CR elicited significant improvement of both agonist- and flow-induced, NO-mediated dilation of resistance arteries from the skeletal muscle of aged F344 rats (Fig. 2A–B), suggesting that CR increases bioavailability of NO. Available data also suggest that weight reduction with very low calorie diets improves flow-mediated vasodilation in obese individuals[121, 122]. It is yet to be determined whether CR can also improve endothelial function in non-obese aged monkeys[109] and elderly humans independent of weight reduction.

The mechanisms by which CR increases bioavailability of NO improving endothelial function in aged rodents likely include up-regulation of eNOS (Fig. 2C–D). Although the upstream mediator(s) of the vascular effects of CR are not well understood, there is data suggesting that CR may regulate both eNOS activity and expression via activation of SIRT-1. An interesting study recently reported that SIRT1 and eNOS colocalize in endothelial cells, and SIRT1 deacetylates eNOS, stimulating eNOS activity and increasing endothelial nitric oxide[123]. Moreover, CR in mice leads to deacetylation of eNOS[123], whereas SIRT1 overexpression or SIRT1 activators were shown to induce eNOS expression in endothelial cells[124]. Further studies are definitely needed to elucidate whether SIRT-1 activation results in increased NO bioavailability improving endothelial function in aged CR individuals.

CR may attenuate vascular inflammation in aging

Atherosclerotic vascular disease is now recognized as a chronic inflammatory disease[125]. There is abundant evidence showing that aging is associated with vascular inflammation promoting atherogenesis (reviewed recently elsewhere [119, 126, 127]). For example, aging promotes endothelial activation, increasing the expression of adhesion molecules[75, 124, 128, 129] and enhancing leukocyte adhesion to the endothelial cells[124, 129, 130]. Previous studies by this and other laboratories have shown that endothelial activation in aging is mediated, at least in part, by oxidative stress-induced increased NF-κB activation[124, 129]. In this regard it is important that CR seems to attenuate vascular NF-κB induction and endothelial activation in aged rats[128, 129]. CR also protected against the age-associated increase of JNK and P38 activities in aged rat aortas[131]. Moreover, CR similarly reversed the age-related increase of AP-1 DNA binding activity[131]. In aging a pro-inflammatory shift develops in the vascular cytokine expression profile (including up-regulation of TNFα, IL-1β and IL-6)[74, 78, 132]. Aging is also associated with increased plasma levels of inflammatory mediators (e.g. TNFα, IL-6 and CRP), both in humans and rodents[7, 133, 134]. In studies of CR in rats and mice, it was found that CR results in marked decreases in these inflammatory markers[135, 136]. The observation that CR in humans also seem to decrease serum CRP and TNFα[137] provides preliminary evidence that CR may also reduce vascular inflammation in humans.

CR attenuates oxidative stress in the vasculature

Advanced age is associated with endothelial oxidative stress, which leads to functional inactivation of NO by high concentrations of $O_2^{\cdot -}$ resulting in an enhanced $ONOO^-$ formation[74, 120, 138, 139]. The role of increased oxidative and nitrosative stress in eliciting

endothelial dysfunction and activation of proatherogenic inflammatory processes in aging has been recently reviewed[119, 126]. In 1996 Dr. Richard Weindruch's group[38] proposed that the anti-aging action of CR stems from the attenuation of the age-associated increase in oxidative stress[140]. Indeed, it has been amply demonstrated that CR decreases the age-associated accumulation of oxidatively damaged lipids, proteins, and nucleic acids in multiple organ systems, including the liver and skeletal muscle[141–143]. Our findings suggest that CR in aged rats significantly decreases vascular $O_2^{\cdot -}$ production (Fig. 2E). This data is in line with the findings that endothelial cells obtained from CR mice exhibit decreased $O_2^{\cdot -}$ and H_2O_2 production as compared with those obtained from mice fed ad libitum[130]. CR also significantly attenuates oxidative DNA damage[144] and normalizes the tissue content of lipid peroxidation-derived aldehydes (HNE, MDA) in aortas of aged rats[131]. There are studies extant suggesting that reduction of oxidative stress in the arterial wall may contribute to the anti-atherogenic effect of CR in $ApoE^{-/-}$ mice[108]. In parenchymal tissues of experimental animals CR modulate the expression of various antioxidant enzymes, however, at present it is unclear whether this is the case in the vasculature as well. Previous studies have identified vascular NAD(P)H oxidases as an important source of ROS production in small coronary arteries, aorta and carotid arteries of aged rodents[74, 75, 119]. In addition, aging also increases mitochondrial ROS generation in the endothelial cells[124]. Future studies should elucidate how CR affects NAD(P)H oxidase activity/expression and mitochondrion-derived ROS generation[145, 146] in the aged blood vessels.

There is data in the literature attributing some of the effects of CR to a decreased insulin-like signaling. Studies in *Caenorhabditis elegans* provided the first evidence that reduced insulin-like signaling may actually promote longevity in lower organisms. By now it is well established that insulin-like signals promote the phosphorylation and deactivation of DAF-16, a forkhead transcription factor, which is a key regulator of oxidative stress resistance and metabolism in *C. elegans* (reviewed in Ref.147). There is also evidence that loss of IGF-like signaling contributes to longevity response to CR in *Drosophila*[148]. The first evidence to support a role of insulin-like signals in regulation of mammalian longevity came from the observation that mice with hereditary dwarfism (Ames dwarf) have low circulating IGF-1, extended longevity and exhibit many symptoms of delayed aging[149]. However, the link between IGF signaling and vascular oxidative stress is likely complex. In Ames dwarf aortas endothelial ROS generation are more than in vessels of wild type mice (Ungvari, submitted 2008). Moreover, in cultured coronary arterial endothelial cells treatment with IGF significantly reduces cellular O_2^- and H_2O_2 production and ROS generation by mitochondria and up-regulates expression of antioxidant enzymes and eNOS (Ungvari, submitted 2008). These in vitro findings accord with the observations that in humans GH and IGF-I deficiency is associated with premature atherosclerosis and elevated cardiovascular disease mortality[150]. Recent evidence suggests that cardiovascular disease risk may also be elevated among apparently healthy individuals who have serum IGF-1 levels in the low-normal range[151]. There is also increasing evidence that IGF-1 may exert vasculoprotective effects in aging[152, 153]. By now it has been firmly established that IGF-1 protects myocardiocytes from apoptotic cell death[154–156]. Cardiac stem cells and early committed cells were also demonstrated to express IGF-1 receptors and secrete IGF-1[157] and IGF-1 was shown to promote cardiac stem cell survival and proliferation[157, 158]. The findings that cardiac overexpression of IGF-1 significantly improved cardiomyocyte contractile function in old mice[159] support the view that IGF-1 signaling plays protective role in the cardiovascular system and that loss of IGF-1 contributes to cardiac aging. Thus, low IGF-1 levels are less likely to be the cause of reduced ROS production and increased bioavailability of NO in the vasculature in CR.

Nrf2: a novel pathway for vasoprotection

Nrf2 (NF-E2-related factor 2) is a transcription factor that binds to the antioxidant response element (ARE) of target genes and increases the transcription of a variety of antioxidant proteins. Kelch-like ECH-associated protein-1 (Keap1) normally sequesters Nrf2 in the cytoplasm, but upon oxidation of cysteine residues Nrf2 dissociates from Keap1, translocates to the nucleus and binds to ARE sequences leading to transcriptional activation of phase II detoxifying genes (such as glutathione-S-transferase and NQO1 and antioxidant enzymes (such as glutathione reductase, glutathione peroxidase and catalase). In parenchymal tissues of the aged rat there is a significant decline in transcriptional activity of Nrf2, which causes age-related loss of glutathione synthesis[160] likely promoting cellular oxidative stress. In a series of studies currently we are testing the hypothesis whether Nrf2 induction plays a role in attenuation of cellular oxidative stress in aged tissues. In this context our recent studies demonstrated that induction of Nrf2 is responsible for the anti-carcinogenic effects of CR, but is dispensable for increased insulin sensitivity. Accordingly, Nrf2 deficient mice developed tumors more readily in response to carcinogen exposure than did wild-type mice, and CR was ineffective in suppressing tumors in the Nrf2-deficient mice (Pearson K and de Cabo R, in press). The aforementioned Nrf2-dependent ROS detoxification systems are expressed in endothelial cells and previous studies have provided solid evidence that the ARE-mediated genes are regulated by atheroprotective laminar flow through a Nrf2-dependent mechanism[103, 161–163]. Also, induction of Nrf2 in cultured endothelial cells results in a marked increase in ARE-driven transcriptional activity and protected the cells from H_2O_2 -mediated cytotoxicity[103]. Nrf2 also suppresses TNFα-induced endothelial activation and inhibits monocyte adhesiveness to the endothelial cells[103]. Although presently it is unknown how aging affects Nrf2 transcriptional activity in the vascular endothelial and smooth muscle cell, we have strong evidence for an age-dependent decline in glutathione synthesis in aged rat aortas, which is prevented by CR (Csiszar A, Ungvari Z, Pinto J, unpublished data 2008). Further studies are evidently needed to test the hypothesis that the Nrf2/ARE pathway is induced in aged arteries, which acts as an endogenous atheroprotective system for antioxidant protection and suppression of redox-sensitive vascular inflammation.

Conclusions and perspectives

Oxidative stress plays an important role in the pathogenesis of CAD by mediating expression of inflammatory genes and eliciting oxidative modification of lipoprotein particles. CR seems to attenuate both vascular oxidative stress and exert anti-inflammatory effects in aged animals. We posit that CR activates the Nrf2/ARE pathway, which may serve as an endogenous antioxidant system within the vasculature increasing cellular oxidative stress tolerance. CR also increases bioavailability of anti-atherogenic NO and augments endothelial function. In addition, CR exerts beneficial effects on a range of systemic cardiovascular risk factors. There is a great deal of effort on dissecting the pathways that invoke CR benefits in order to develop pharmacological agents that would act as CR mimetics[164–166]. Several of the currently proposed CR mimetics are phytochemicals (resveratrol, quercetin and curcumin) that act, at least in part, through the activation of Nrf2 pathway [167 168–170]. Importantly, newly identified CR mimetics, such as resveratrol, exert cardiovascular effects that are remarkably similar to those of CR. Accordingly, resveratrol increases vascular oxidative stress resistance[171], up-regulate eNOS[171], inhibit endothelial activation[172] and vascular inflammatory gene expression[171] and activates both SIRT1 and the Nrf2/ARE pathways providing a pharmacological alternative for CR for the prevention of CAD in the elderly.

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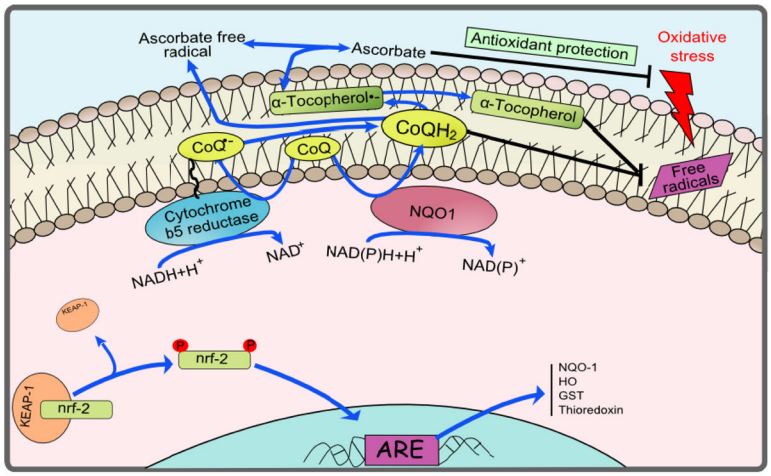

Figure 1.

A diagram of the plasma membrane redox system. The redox cycle is shown in blue. CoQ, oxidized form of coenzyme Q; $CoQ^{\cdot -}$,semiquinone radical; $CoQH_2$, reduced form of coenzyme Q; NQO1, NADH-quinone oxidoreductase. Modified from Hyun et al. (2006a).

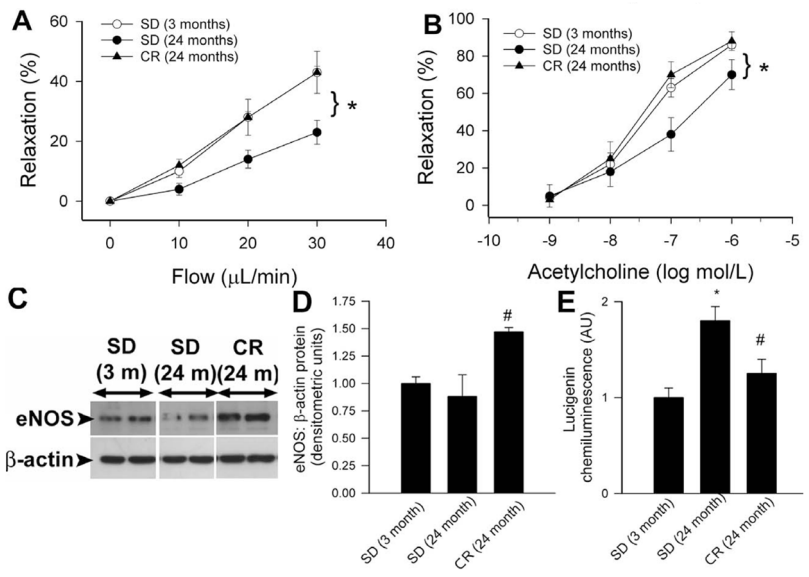

Figure 2.

Dilations in response to step increases in intraluminal flow (Panel A) or administration of acetylcholine (Panel B) in isolated, cannulated, first order gracilis muscle arterioles (d: ~100 μm; pressurized to 80 mmHg[74]) of aged (24 month old) F344 rats fed a standard diet (SD) are impaired, as compared to young vessels. Lifelong caloric restriction (CR) preserved microvascular endothelial function. *P<0.05 vs. aged SD. Data are mean±S.D. (n=4 in each group). Panel C: Original Western blots showing that expression of eNOS is up-regulated in carotid arteries of aged CR rats. Bar graphs (Panel D) are summary densitometry data. #P<0.05 vs. SD. Panel E: Lucigenin chemiluminescence measurements revealed that age-related increases in O_2.- production in the aorta of F344 rats are prevented by lifelong caloric restriction (CR). *P<0.05 vs. young, #P<0.05 vs. standard diet (SD)-fed rats.