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Caloric restriction confers persistent anti-oxidative, pro-angiogenic, and anti-inflammatory effects and promotes anti-aging miRNA expression profile in cerebromicrovascular endothelial cells of aged rats

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Csiszar A, Gautam T, Sosnowska D, Tarantini S, Banki E, Tucsek Z, Toth P, Losonczy G, Koller A, Reglodi D, Giles CB, Wren JD, Sonntag WE, Ungvari Z. Caloric restriction confers persistent anti-oxidative, pro-angiogenic, and anti-inflammatory effects and promotes anti-aging miRNA expression profile in cerebromicrovascular endothelial cells of aged rats. *Am J Physiol Heart Circ Physiol* 307: H292–H306, 2014. First published June 6, 2014; doi:10.1152/ajpheart.00307.2014.—In rodents, moderate caloric restriction (CR) without malnutrition exerts significant cerebrovascular protective effects, improving cortical microvascular density and endothelium-dependent vasodilation, but the underlying cellular mechanisms remain elusive. To elucidate the persisting effects of CR on cerebromicrovascular endothelial cells (CMVECs), primary CMVECs were isolated from young (3 mo old) and aged (24 mo old) ad libitum-fed and aged CR F344xBN rats. We found an age-related increase in cellular and mitochondrial oxidative stress, which is prevented by CR. Expression and transcriptional activity of Nrf2 are both significantly reduced in aged CMVECs, whereas CR prevents age-related Nrf2 dysfunction. Expression of miR-144 was upregulated in aged CMVECs, and overexpression of miR-144 significantly decreased expression of Nrf2 in cells derived from both young animals and aged CR rats. Overexpression of a miR-144 antagomir in aged CMVECs significantly decreases expression of miR-144 and upregulates Nrf2. We found that CR prevents age-related impairment of angiogenic processes, including cell proliferation, adhesion to collagen, and formation of capillary-like structures and inhibits apoptosis in CMVECs. CR also exerts significant anti-inflammatory effects, preventing age-related increases in the transcriptional activity of NF- κ B and age-associated pro-inflammatory shift in the endothelial secretome. Characterization of CR-induced changes in miRNA expression suggests that they likely affect several critical functions in endothelial cell homeostasis. The predicted regulatory effects of CR-related differentially expressed miRNAs in aged CMVECs are consistent with the anti-aging endothelial effects of CR observed in

vivo. Collectively, we find that CR confers persisting anti-oxidative, pro-angiogenic, and anti-inflammatory cellular effects, preserving a youthful phenotype in rat cerebromicrovascular endothelial cells, suggesting that through these effects CR may improve cerebrovascular function and prevent vascular cognitive impairment.

aging; angiogenesis; caloric restriction; dietary restriction; senescence

NUMEROUS STUDIES DEMONSTRATE that aging promotes oxidative stress and pro-inflammatory phenotypic changes in cerebromicrovascular endothelial cells (CMVECs), which likely contribute to neurovascular uncoupling (61), disruption of the blood-brain barrier (100), and neuroinflammation (74). There is also evidence that aging impairs endothelial angiogenic capacity (87), leading to cerebromicrovascular rarefaction and a decline in cerebral blood flow (70). All of these age-related phenotypic alterations in CMVECs are thought to contribute to the development of both vascular cognitive impairment (VCI) and Alzheimer's disease (31, 50, 100). Our long-term goal is to identify effective therapeutic interventions to promote endothelial health in the cerebral microcirculation for the prevention of VCI in the elderly.

Moderate caloric restriction (CR) without malnutrition is a dietary regimen recognized to delay aging and extend lifespan in many evolutionary distant organisms (reviewed in Ref. 83). Importantly, in laboratory rodents CR was shown to exert significant cerebrovascular protective effects, improving cortical microvascular density (53) and endothelial function (93). The existing evidence suggests that CR may improve vascular health by eliciting changes in circulating neuroendocrine factors. In support of this concept we have demonstrated that circulating factors present in the sera of CR F344 rats confer significant anti-oxidative and anti-inflammatory effects in cultured endothelial cells (14). More recently, we demonstrated that circulating factors induced by CR in the nonhuman pri-

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mate *Macaca mulatta* also confer pro-angiogenic effects in cultured endothelial cells (19). Yet, the long-lasting protective effects of CR on oxidative and inflammatory status and angiogenic potential of aged CMVECs remain poorly understood.

The present study was designed to test the hypothesis that in response to lifelong CR, CMVECs retain a youthful phenotype, which does not depend on the continuing presence of circulating factors induced by CR. To test this hypothesis using cultured primary CMVECs derived from young and aged ad libitum (AL)-fed rats and CR rats as a model system, we determined whether lifelong CR elicits anti-oxidative, pro-angiogenic, and anti-inflammatory changes in the endothelial phenotype, which persists in culture. As endpoints, cellular reactive oxygen species (ROS) production (dichlorofluorescein and MitoSox fluorescence), transcriptional activity of Nrf2, endothelial angiogenic capacity (tube formation, proliferation, and adhesion capacity), apoptosis, transcriptional activity of NF- κ B, and pro-inflammatory cytokine secretion were assessed.

METHODS

Animals and diet. Male Fischer 344 $\times$ Brown Norway (F344xBN) rats were used as a model of aging, since this strain has a lower incidence of age-specific pathology than other rat strains. Thus in F344xBN rats the primary effects of aging can be studied uncomplicated by compensatory effects caused by age-related pathology. The following experimental groups were used: 1) 3 mo old ("young") AL fed, 2) 24 mo old ("aged") AL fed, and 3) 24 mo old with lifelong 40% CR (CR). All animals ($n = 5$ in each group) were disease free with no signs of systemic inflammation and/or neoplastic diseases. The rats were housed in an environmentally controlled vivarium under pathogen-free conditions with unlimited access to food and water and a controlled photoperiod (12-h:12-h light/dark). All rats were maintained according to National Institutes of Health guidelines, and all animal use protocols were approved by the Institutional Animal Care and Use Committees of the University of Oklahoma Health Sciences Center. The animals were euthanized with CO₂. The brains were rapidly dissected to establish primary CMVEC cultures.

Establishment and characterization of primary CMVECs. Primary CMVEC cultures were established as previously described (84, 87). In brief, brains were removed aseptically, rinsed in ice-cold PBS, and minced into ≈ 1 -mm squares. The tissue was washed twice in ice-cold 1 $\times$ PBS by low-speed centrifugation (50 g , 2 to 3 min). The diced tissue was digested in a solution of collagenase (800 U/g tissue), hyaluronidase (2.5 U/g tissue), and elastase (3 U/g tissue) in 1 ml PBS/100 mg tissue for 45 min at 37°C in rotating humid incubator. The digested tissue was passed through a 100 μ m cell strainer to remove undigested blocks. The single cell lysate was centrifuged for 2 min at 70 g . After the supernatant was carefully removed, the pellet was washed twice in cold PBS supplemented with 2.5% FCS, and the suspension centrifuged at 300 g , for 5 min at 4°C.

To create an endothelial cell enriched fraction, the cell suspension was gradient centrifuged by using OptiPrep solution (Axi-Shield, PoC, Norway). Briefly, the cell pellet was resuspended in HBSS and mixed with 40% iodixanol thoroughly [final concentration: 17% (wt/vol) iodixanol solution; $\rho = 1.096$ g/ml]. HBSS (2 ml) was layered on top and centrifuged at 400 g for 15 min at 20°C. Endothelial cells, which banded at the interface between HBSS and the 17% iodixanol layer, were collected. The endothelial cell enriched fraction was incubated for 30 min at 4°C in dark with anti-CD31/PE, anti-MCAM/FITC (BD Biosciences, San Jose, CA). After the cells were washed twice with MACS Buffer (Milltenyi Biotech, Cambridge, MA), anti-FITC magnetic bead-labeled and anti-PE magnetic bead-labeled secondary antibodies were used for 15 min at room

temperature. Endothelial cells were collected by magnetic separation using the MACS LD magnetic separation columns according to the manufacturer's guidelines (Milltenyi Biotech). The endothelial fraction was cultured on fibronectin-coated plates in Endothelial Growth Medium (Cell Application, San Diego, CA) for 10 days. Endothelial cells were phenotypically characterized by flow cytometry (GUAVA 8HT; Merck Millipore, Billerica, MA). Briefly, antibodies against five different endothelial specific markers were used (anti-CD31-PE, anti-erythropoietin receptor-APC, anti-VEGF R2-PerCP, anti-ICAM-fluorescein, anti-CD146-PE) and isotype-specific antibody-labeled fractions served as negative controls. All antibodies were purchased from R&D Systems (R&D Systems, Minneapolis, MN). All other reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

To study age-related and CR-induced changes in endothelial phenotype, primary CMVECs were initially cultured in MesoEndo Endothelial Cell Growth Medium (Cell Applications) followed by Endothelial Basal Medium supplemented with 10% FCS. Cells after passage 3 were used. To induce angiogenic processes, CMVECs were treated with recombinant human VEGF (100 ng/ml; R&D Systems).

Measurement of cellular ROS production. Despite recent advances in redox biology and aging research (9, 21, 27, 33, 37, 49, 51, 52, 55, 59, 65, 72, 97), the role of ROS in the cerebrovascular effects of CR remains elusive. To assess cellular peroxide production, we used the cell-permeant oxidative fluorescent indicator dye CM-H₂DCFDA [5 (and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate-acetyl ester; Invitrogen, Carlsbad, CA] as we previously reported (16, 86). In brief, cells were washed with warm PBS and incubated with CM-H₂DCFDA (10 μ M, at 37°C, for 30 min). CM-H₂DCFDA fluorescence was assessed by flow cytometry (16, 86). In separate experiments, mitochondrial O₂⁻ production in CMVECs was measured using MitoSOX Red (Invitrogen), a mitochondrion-specific hydroethidine-derivative fluorescent dye, as previously reported (16, 20, 81, 82, 84). Cell debris (low forward and side scatter) and dead cells (Sytox Green) were gated out for analysis.

Quantitative real-time RT-PCR and miRNA expression profiling. A quantitative real time RT-PCR technique was used to analyze the effect of CR on mRNA expression of *Nox4*, *Nfe2l2* (Nrf2), and *Keap1*, as well as the reference genes *Hprt*, *Gapdh*, and *Actb*, as previously reported (84, 87). In brief, total RNA was isolated using a TaqMan Cells-to-CT Kit (Applied Biosystems, Foster City, CA) and was reverse transcribed using Superscript III RT (Invitrogen) as described previously (17, 22). A real-time RT-PCR technique was used to analyze mRNA expression using a Stratagen MX3000 device, as reported (17). Amplification efficiencies were determined using a dilution series of a standard vascular sample. Quantification was performed using the efficiency-corrected $\Delta\Delta$ Cq method. The relative quantities of the reference genes were determined, and a normalization factor was calculated based on the geometric mean for internal normalization. Oligonucleotides used for quantitative real-time RT-PCR are listed in Table 1.

The expression profile of 373 unique rat miRNAs in CMVECs derived from young and aged rats was analyzed using the TaqMan Array Rodent MicroRNA A Cards Set v3.0 (Applied Biosystems, Life Technologies, Carlsbad, CA). Of the 384 probes on the array, 194 were mappable to rat miRNAs using the probe mapping tables provided by the manufacturer. Only probes with at least one nonundetermined value in both control and CR groups were included, leaving 124 unique miRNAs for further analysis. The remaining miRNAs were normalized to $\Delta\Delta$ Ct values using the average of the four replicated probes of MammU6, and the resulting expression values were then quantile normalized. Differential expression raw P values were determined using a Student's t -test and corrected using Benjamini-Hochberg multiple hypothesis correction at a q -value (FDR) cutoff of 0.1, resulting in 17 upregulated and 27 downregulated miRNAs. Validated miRNA target sites within mRNA transcripts were obtained from the MirWalk database (28).

Assessment of the effects of CR on the transcriptional activity of *Nrf2* in CMVECs. The effect of CR on Nrf2 activity in CMVECs was assessed using a reporter gene assay as described (5, 19, 20, 76, 77). To pharmacologically stimulate Nrf2 activity, we treated the cells with either resveratrol (3 $\mu\text{mol/l}$) or the prototypical Nrf2 activator sulforaphane (SFN; 5 $\mu\text{mol/l}$). We used an ARE reporter comprising tandem repeats of the ARE transcriptional response element upstream of firefly luciferase (SA Biosciences, Frederick, MD) and a renilla luciferase plasmid under the control of the CMV promoter (as an internal control). Transfections in CMVECs were performed using the Amaxa Nucleofector technology (Amaxa, Gaithersburg, MD), as we have previously reported (75, 76, 77). Firefly and renilla luciferase activities were assessed after 24 h using the Dual Luciferase Reporter Assay Kit (Promega, Madison, WI) and a Tecan Infinite M200 plate reader.

Cell proliferation assay. Cell proliferation capacity was assessed in CMVECs using the flow cytometry based Guava CellGrowth assay (Guava Technologies, Hayward, CA), as reported (84, 87, 91). Briefly, cells were collected, resuspended in PBS containing 0.1% BSA, and stained with 16 $\mu\text{mol/l}$ carboxyfluorescein diacetate succinimidyl ester (CFSE) for 15 min at 37°C. This dye diffuses into cells and is cleaved by intracellular esterases to form an amine-reactive product that produces a detectable fluorescence and binds covalently to intracellular lysine residues and other amine sources. Upon cell division, CFSE divides equally into the daughter cells halving the CFSE concentration of the mother cell; therefore, there is an inverse correlation between the fluorescence intensity and the proliferation capacity of the cells. After incubation, unbound dye was quenched with serum-containing medium. Cells were then washed three times and incubated for 24 h with 100 ng/ml VEGF. Finally, cells were collected, washed, stained with propidium iodide (to gate out dead cells), and analyzed with a flow cytometer (Guava EasyCyte 8HT; Millipore). The inverse of the fluorescence intensity was used as an index of proliferation.

Apoptosis assay. To determine whether CR exerts persistent anti-apoptotic effects, apoptotic cell death was assessed by measuring caspase activities using the Caspase-Glo 3/7 assay kit (Promega, Madison, WI) as previously reported (6, 85). In 96-well plates, 50 μl sample was mixed for 30 s with 50 μl Caspase-Glo 3/7 reagent and incubated for 2 h at room temperature. Lyses buffer with the reagent served as blank. Luminescence of the samples was measured using an Infinite M200 plate reader (Tecan, Research Triangle Park, NC). Luminescent intensity values were normalized to the sample protein concentration.

Cell adhesion assays. Angiogenesis is a multistep process involving cell adhesion, proliferation, migration, and morphogenesis (11). To determine the effects of CR on the adhesion capacity of endothelial cells, we used electric cell-substrate impedance sensing (ECIS) technology (Applied Biophysics, Troy, NY) to monitor adhesion of CMVECs to collagen, as reported (84, 87, 91). Briefly, the cells were collected and counted. VEGF (100 ng/ml)-stimulated cells were seeded in collagen-coated (50 $\mu\text{g/ml}$) 96-well array culture dishes containing gold film surface electrodes (ECIS 96W1E; in each well 1 active electrode and a large counter electrode). The same numbers of cells were added to each well (2.5×10^5 cells/well). The arrays were placed in an incubator, and the time course for changes of capacitance (measured at 60 kHz) due to the adhesion of cells to the active electrode was obtained. Time to reach 50% cell adhesion was used as an index of adhesiveness (100% change corresponds to the maximum level of cell coverage reached on the active electrode). To assay barrier function, the time course of changes in capacitance (measured at 32 kHz) and resistance (measured at 1,000 Hz) were monitored in parallel.

Tube formation assay. To investigate the influence of CR on tube formation ability, CMVECs were plated on Geltrex Reduced Growth Factor Basement Membrane Matrix (Invitrogen) in Medium 200PRF (Invitrogen). Briefly, 150 μl /well of Geltrex was distributed in ice-cold 24-well plates. The gel was allowed to solidify while incubating the plates for 30 min at 37°C. CMVECs were then seeded at a density of 5×10^4 cells/well and placed in the incubator for 24 h. Microscopic

images were captured using a Nikon Eclipse Ti microscope equipped with a 10 $\times$ phase-contrast objective (Nikon Instruments, Melville, NY). The extent of tube formation was quantified by measuring total tube length in five random fields per well using NIS-Elements microscope imaging software (Nikon Instruments), as recently reported (19, 84, 87, 91). The mean of the total tube length per total area imaged (in μm tube/ mm^2) was calculated for each well. Experiments were run in quadruplicate. The experimenter was blinded to the groups throughout the period of analysis.

NF- κB reporter gene assay. Transcriptional activity of NF- κB was tested in CMVECs by a reporter gene assay as described (75–77). We used a NF- κB reporter comprising an NF- κB response element upstream of firefly luciferase (NF- κB -Luc; Stratagene) and a renilla

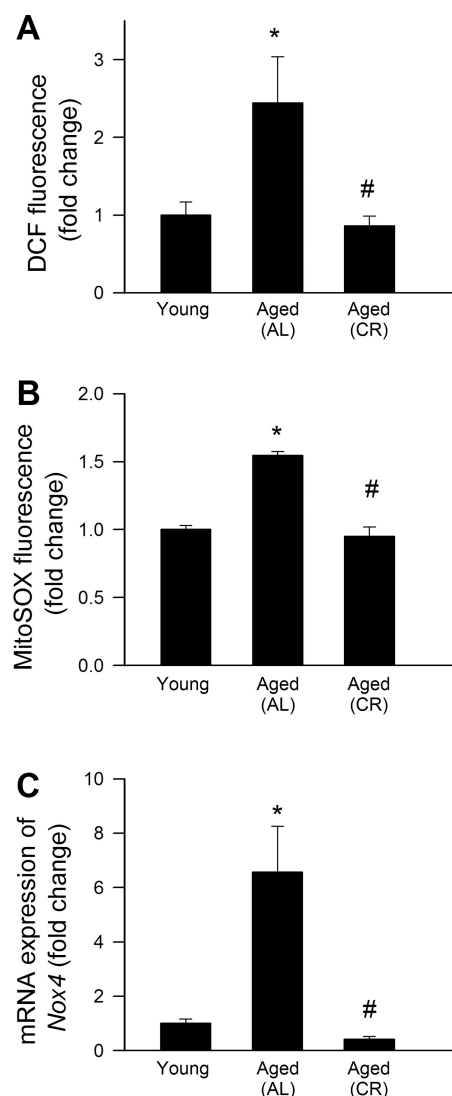


Fig. 1. Caloric restriction (CR) significantly decreases oxidative stress in aged cerebrovascular endothelial cells (CMVECs). *A* and *B*: effect of CR on cellular oxidative stress (*A*) and mitochondrial reactive oxygen species production (*B*) in CMVECs. Cellular peroxide production and mitochondrial $\text{O}_2^{\cdot-}$ production were assessed in cultured primary CMVECs derived from young and ad libitum (AL)- and CR-fed aged rats using the flow cytometry-based CM-H₂DCFDA fluorescence (*A*) and MitoSOX Red (*B*) method, respectively. Data are means $\pm$ SE ($n = 5$ for each group). * $P < 0.05$ vs. control; # $P < 0.05$ vs. aged (AL). *C*: quantitative real time RT-PCR data showing the effect of aging and CR on mRNA expression of *Nox4* in CMVECs. Data are means $\pm$ SE ($n = 5$ in each group). * $P < 0.05$ vs. control; # $P < 0.05$ vs. aged (AL). DCF, dichlorofluorescein.

luciferase plasmid under the control of the CMV promoter. All transfections in CMVECs were performed using Amaxa Nucleofector technology (Amaxa, Gaithersburg, MD), as we have previously reported (13, 18, 24). Firefly and renilla luciferase activities were assessed after 24 h using the Dual Luciferase Reporter Assay Kit (Promega) and a Tecan Infinite M200 plate reader.

Analysis of secreted cytokines in conditioned media. Profiling of cytokines secreted by cultured CMVECs was conducted using a magnetic multiplex bead array (MILLIPLEX Cytokine Magnetic Bead Panel; Millipore). The concentration of a range of cytokines and growth factors involved in vascular physiology and pathophysiology [monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1 α , TNF- α , IL-6, IL-1 β , GM-CSF, and MIP-2] was measured in the conditioned media collected after 48 h of culture. The cells were harvested, and sample protein content was determined by a spectrophotometric quantitation method using BCA reagent (Pierce Chemical, Rockford, IL).

Data analysis. Statistical analyses were performed using one-way ANOVA followed by Tukey's post hoc test. $P < 0.05$ was considered statistically significant. Data are expressed as means $\pm$ SE.

RESULTS

CR attenuates age-related oxidative stress in aged CMVECs. Using CM-H₂DCFDA and MitoSox fluorescence-based methods, we demonstrated that aging is associated with increased

cellular peroxide production and increased mitochondrial oxidative stress in CMVECs (Fig. 1, *A* and *B*, respectively). We found that CR resulted in a significant decline in dichlorodihydrofluorescein and MitoSox fluorescence, indicating a decreased cellular and mitochondrial ROS production (Fig. 1, *A* and *B*). Aging significantly upregulated mRNA expression of *Nox4* in CMVECs (Fig. 1*C*). In CMVECs derived from CR rats, when compared with cells derived from AL-fed aged rats, mRNA expression of *Nox4* was significantly decreased (Fig. 1*C*).

CR increases transcriptional activity of Nrf2 in aged CMVECs. Previous studies reported that CR in mice is associated with activation of Nrf2-driven antioxidant defense mechanisms (64) and that Nrf2 activation confers important anti-oxidative, pro-angiogenic, anti-apoptotic, and anti-inflammatory effects in endothelial cells (91). We determined the effects of aging and lifelong CR in rats on the transcriptional activity of Nrf2 in CMVECs. With the use of a reporter gene assay, a significant decline in Nrf2 activation was noted in CMVECs derived from aged rats (Fig. 2*A*). In contrast, CR resulted in preservation of endothelial Nrf2 activity (Fig. 2*A*). Both resveratrol and SFN, which are pharmacological activators of Nrf2, elicited similar increases in transcriptional activity of Nrf2 in each group of cells (data not shown). In CMVECs

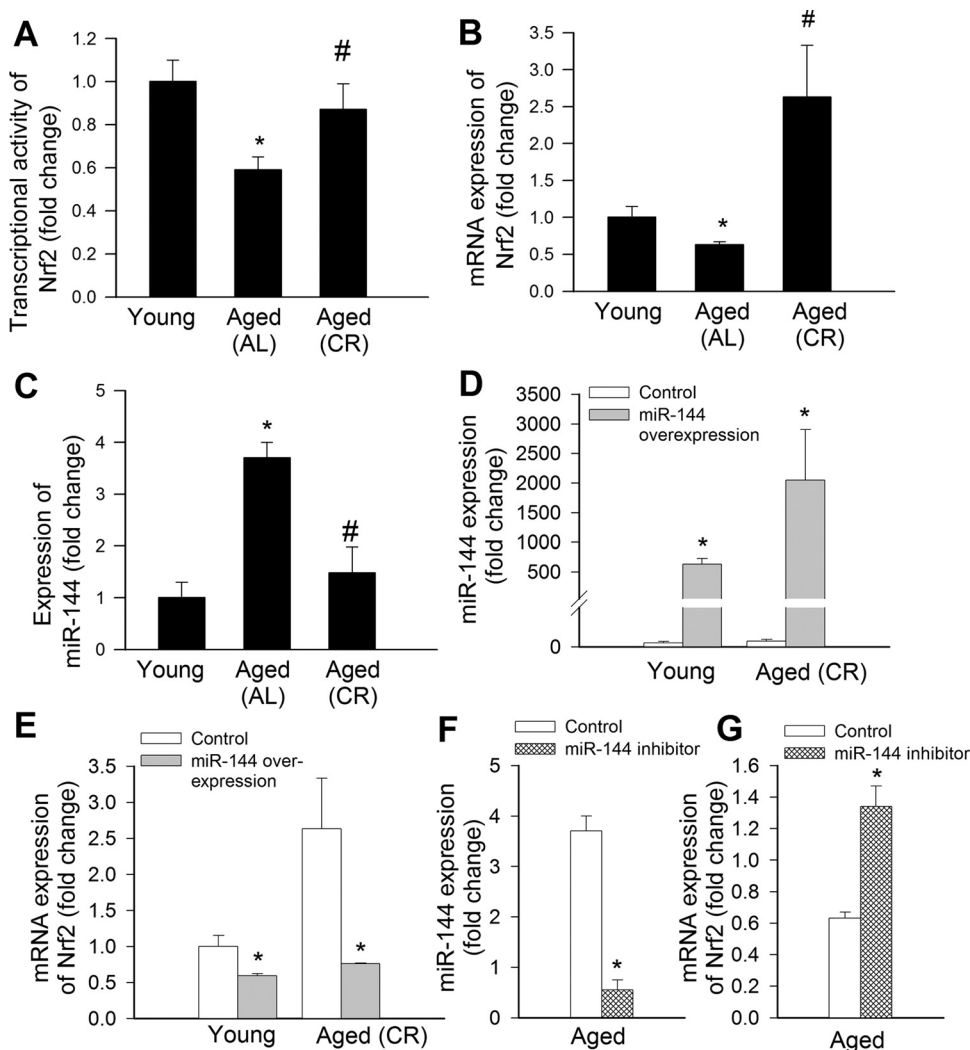


Fig. 2. CR prevents age-related Nrf2 dysfunction in CMVECs. *A*: reporter gene assay showing the effects of CR on Nrf2/ARE reporter activity in cultured primary CMVECs. Cells derived from young and AL- and CR-fed aged rats were transiently cotransfected with ARE-driven firefly luciferase and CMV-driven renilla luciferase constructs. The cells were then lysed and subjected to luciferase activity assay. Data are means $\pm$ SE ($n = 5$ for each group). * $P < 0.05$ vs. control; # $P < 0.05$ vs. aged (AL). *B* and *C*: quantitative real time RT-PCR data showing the effect of CR on mRNA expression of *Nfe2l2* (Nrf2; *B*) and expression of miR-144 (*C*) in cultured primary CMVECs. Data are means $\pm$ SE ($n = 5$ in each group). * $P < 0.05$ vs. control; # $P < 0.05$ vs. aged (AL). *D*: to assess the effect of miR-144 on Nrf2 expression, miR-144 was overexpressed in CMVECs derived from young and aged CR rats. In *E*, overexpression of miR-144 significantly decreased expression of Nrf2 in both group. Data are means $\pm$ SE. * $P < 0.05$ vs. respective controls. *F* and *G*: overexpression of a miR-144 antagonist in CMVECs derived from aged AL-fed rats significantly decreases expression of miR-144 (*F*) and upregulates Nrf2 (*G*). Data are means $\pm$ SE. * $P < 0.05$ vs. control.

derived from CR rats, when compared with cells derived from AL-fed aged rats, mRNA expression of Nrf2 significantly increased (Fig. 2B). miR-144 is a known regulator of Nrf2 expression (58, 68). Because miR-144 was not among the targets present on the TaqMan Array Rodent MicroRNA A Cards Set v3.0, its expression was analyzed by QRT-PCR separately. We found that expression of miR-144 significantly increased in CMVECs derived from AL-fed aged rats compared with that in young cells (Fig. 2C). In contrast, in CMVECs derived from aged CR rats, when compared with cells derived from AL-fed aged rats, expression of miR-144 significantly decreased (Fig. 2C). To assess the effect of miR-144 on Nrf2 expression, miR-144 was overexpressed in CMVECs derived from young and aged CR rats (Fig. 2D). Figure 2E shows that overexpression of miR-144 significantly decreased expression of Nrf2 in both groups. In agreement with our predictions, overexpression of a miR-144 antagonist in CMVECs derived from aged AL-fed rats significantly decreased expression of miR-144 (Fig. 2F) and upregulated Nrf2 (Fig. 2G).

CR increases proliferative capacity of aged CMVECs. Increased mitochondrial oxidative stress has been shown to impair angiogenic functions (90) and promote apoptosis in endothelial cells. We tested the hypothesis that the marked anti-oxidative action of CR is associated with pro-angiogenic and anti-apoptotic endothelial effects.

Proliferation represents a key step in angiogenesis. We found that aged CMVECs exhibit impaired proliferative capacity (Fig. 3A), extending our recent findings (87). In CMVECs derived from aged CR-fed rats, there was a significantly decreased CFSE fluorescence (resulting in an increased proliferation index), indicating that proliferation capacity is significantly increased by CR in a cell-autonomous manner (Fig. 3A).

CR inhibits programmed cell death in aged CMVECs. Induction of endothelial apoptosis is an important mechanism that

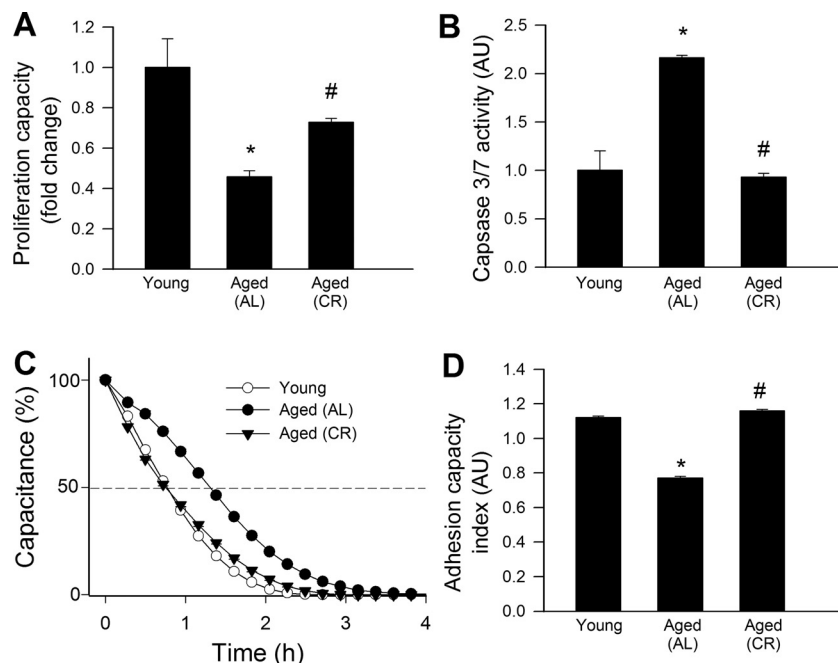
inhibits angiogenesis promoting microvascular rarefaction. We found that in CMVECs derived from aged AL-fed rats apoptosis was increased (Fig. 3B). CR significantly inhibited endothelial apoptosis as shown by the decreased caspase 3/7 activity, restoring it to levels observed in young cells (Fig. 3B).

CR improves adhesion of aged CMVECs to collagen. Endothelial cell adhesion events are known to have an important role in angiogenesis. We used ECIS technology to monitor changes of capacitance (at 60 kHz) due to the adhesion of VEGF (100 ng/ml)-stimulated cells to the collagen-coated active electrode (Fig. 3C). The inverse of the time constant (τ), calculated from an exponential curve fitting, was used as an index of adhesiveness. Aged CMVECs exhibited impaired adhesiveness to collagen (shown as an increase in the time needed to reach 50% cell adhesion) compared with young cells. CR significantly increased endothelial adhesiveness, restoring it to levels observed in young cells (Fig. 3, C and D).

CR promotes formation of capillary-like structures by aged CMVECs. We performed an in vitro tube formation assay to model the reorganization stage of angiogenesis. In vitro tube formation is a multi-step process involving cell adhesion, migration, differentiation, and growth. We assessed the effect of CR on the ability of aged endothelial cells to form capillary-like structures. When seeded onto Geltrex matrices, young CMVECs formed elaborated capillary networks in the presence of VEGF, and this response was significantly impaired in aged CMVECs (Fig. 4, A and B). We found that CR significantly increased the formation of capillary-like structures by aged CMVECs (Fig. 4C). Summary data (expressed as tube length per area in $\mu\text{m}/\text{mm}^2$) are shown in Fig. 4D.

CR inhibits transcriptional activity of NF- κ B in aged CMVECs. Previously, we have found that age-related vascular inflammation in laboratory rodents and nonhuman primates is associated with increased NF- κ B activation (25, 82). We found that in CMVECs derived from AL-fed rats aged, transcriptional activity of NF- κ B was significantly increased compared

Fig. 3. CR significantly increases proliferation and adhesion capacity and inhibits apoptosis in aged CMVECs. **A:** cell proliferation capacity was assessed in primary CMVECs, derived from young and AL- and CR-fed aged rats, stimulated with VEGF (100 ng/ml) using the flow cytometry based Guava CellGrowth assay (see METHODS). The inverse of the fluorescence intensity of the indicator dye carboxyfluorescein diacetate succinimidyl ester was used as an index of proliferation capacity of the cells. Data are means $\pm$ SE ($n = 5$ in each group). * $P < 0.05$ vs. control; # $P < 0.05$ vs. aged (AL). **B:** CR significantly inhibits apoptosis in aged CMVECs. Apoptotic cell death was assessed by measuring caspase 3/7 activity in cell lysates. * $P < 0.05$ vs. control; # $P < 0.05$ vs. aged (AL). Data are means $\pm$ SE ($n = 5$ for each group). **C and D:** effect of CR on VEGF-induced adhesion of CMVECs. VEGF (100 ng/ml)-stimulated cell adhesion was monitored by electric cell-substrate impedance sensing technology (see METHODS). **C:** time course of changes of capacitance (at 32 kHz) after addition of CMVECs to collagen-coated wells. One-hundred percent change corresponds to the maximum level of cell coverage reached on the active electrode. Data are means $\pm$ SE ($n = 5$ in each group). We calculated the time constant from each individual dataset, the inverse of which was used as an index of adhesiveness. In **D**, summary data for cell adhesion index in CMVECs from each experimental group are shown. Data are means $\pm$ SE ($n = 5$ in each group). * $P < 0.05$ vs. control; # $P < 0.05$ vs. aged (AL). AU, arbitrary units.



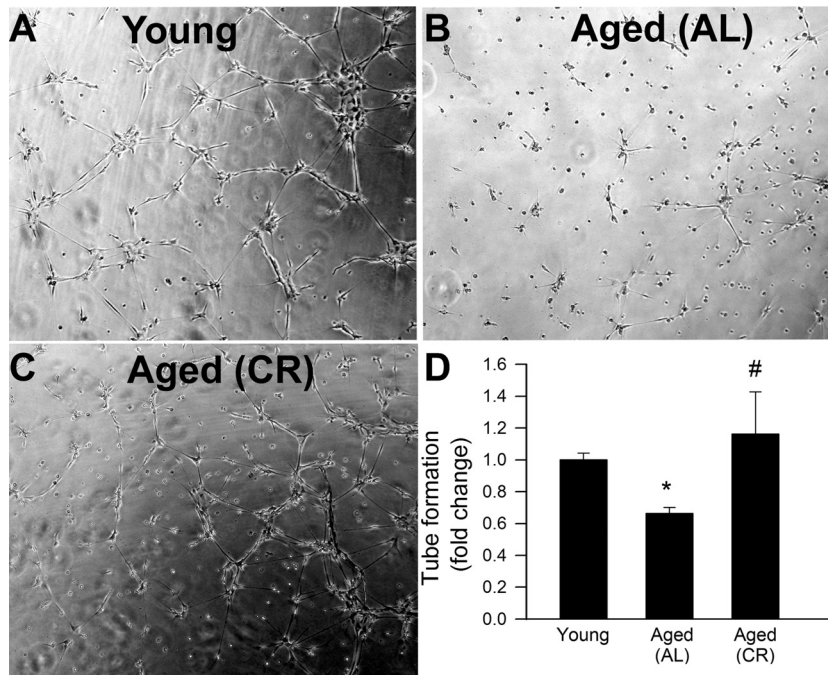


Fig. 4. CR significantly increases angiogenic capacity in aged CMVECs. A–C: ability to form capillary-like structures by primary CMVECs, derived from young and AL- and CR-fed aged rats, was assessed. CMVECs were plated on Geltrex-coated wells, and tube formation was induced by treatment of the cells with VEGF (100 ng/ml, for 24 h). Representative examples of capillary-like structures are shown in A–C. Summary data, expressed as total tube length per total area scanned (in μm tube/ mm^2), are shown in D. Data are means $\pm$ SE ($n = 5$ in each group). * $P < 0.05$ vs. control; # $P < 0.05$ vs. aged (AL).

with CMVECs from young animals (Fig. 5A). NF- κ B activation was significantly inhibited in CMVECs from CR-aged animals (Fig. 5A).

CR inhibits secretion of pro-inflammatory cytokines in aged CMVECs. We have demonstrated previously that aging in laboratory rodents and nonhuman primates is associated with a pro-inflammatory shift in the vascular cytokine expression profile (20, 23). To determine the persisting effect of CR on the CMVEC secretome, we used a multiplex bead array to simultaneously measure the level of a range of cytokines, chemo-

kines, and growth factors in the conditioned media. Of the cytokines tested, secretion of MCP-1, MIP-1 α , TNF- α , IL-6, IL-1 β , GM-CSF, and MIP-2 (Fig. 5B) was significantly increased in aged CMVECs compared with young CMVECs. Here we report that CR reverses many of the age-related changes in the secretory phenotype of these cells, significantly decreasing secretion of MCP-1, TNF- α , IL-6, IL-1 β , GM-CSF, and MIP-2 (Fig. 5B).

CR-induced changes in miRNA expression profile. Figure 6A shows the changes in miRNA expression across experimental

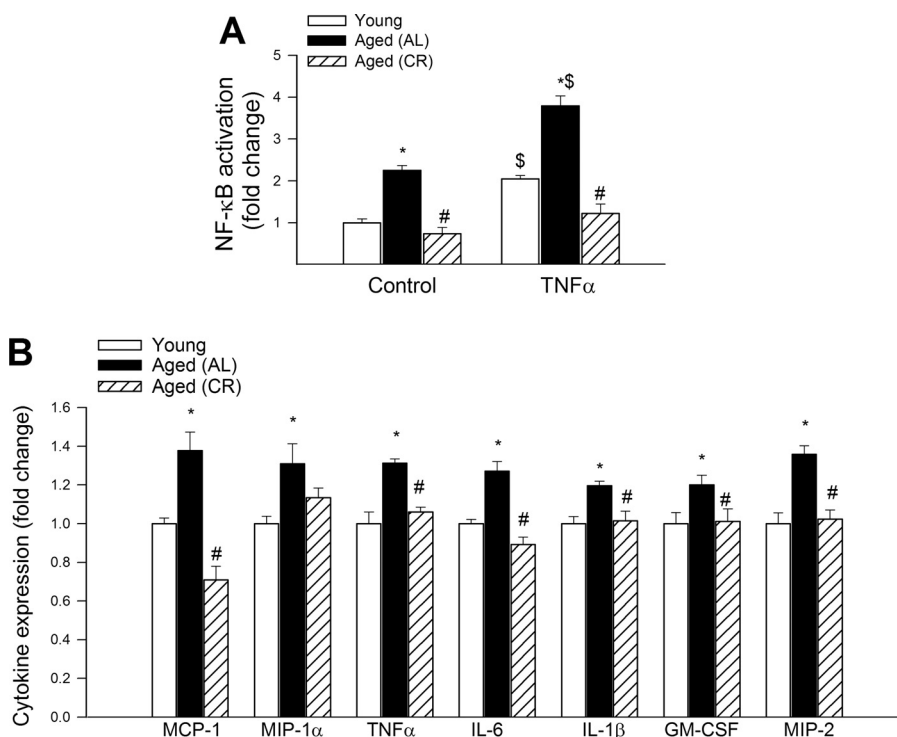


Fig. 5. CR exerts anti-inflammatory effects. A: reporter gene assay showing that aging significantly increases transcriptional activity NF- κ B in cultured primary CMVECs. Cells derived from CR-fed aged rats exhibited significantly decreased NF- κ B activity compared with CMVECs derived from AL-fed animals. The inhibitory effect of CR on NF- κ B activation was evident also after TNF- α stimulation. CMVECs were transiently cotransfected with NF- κ B-driven firefly luciferase and CMV-driven renilla luciferase constructs. The cells were then lysed and subjected to luciferase activity assay. Data are means $\pm$ SE ($n = 5$ for each group). * $P < 0.05$ vs. control; # $P < 0.05$ vs. aged (AL); \$ $P < 0.05$ vs. no TNF- α . B: secretory profiles of CMVECs derived from young and AL- and CR-fed aged rats. Soluble monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1 α , TNF- α , IL-6, IL-1 β , granulocyte-macrophage colony-stimulating factor (GM-CSF), and MIP-2 secreted by these cells were detected by a magnetic multiplex bead array (see METHODS). Data are means $\pm$ SE. * $P < 0.05$ vs. control; # $P < 0.05$ vs. aged (AL).

replicates. Upon analysis of group-wide changes of significance, there were 18 miRNAs upregulated and 29 miRNAs downregulated when comparing AL versus calorie-restricted rodents (Tables 1 and 2, respectively). We cross-referenced these differentially expressed miRNAs against the miRwalk database (28) to identify genes they targeted (Table 3). Because miRNAs typically target multiple genes, we calculated whether any of the genes with miRNA binding sites were disproportionately targeted by multiple up- or downregulated miRNAs using Fisher's Exact test (Table 3). Figure 6B shows changes in miRNA expression and how these miRNAs interact with a subset of significantly overrepresented target genes in this dataset. Analysis of the differentially expressed miRNAs on the array indicated that a statistically significant number of the upregulated miRNAs had target sites within genes associated with the TGF- β pathway, suggesting CR tends to repress or restrict TGF- β signaling within CMVECs, specifically through the SMAD4 pathway. TGF- β is a multifunctional cytokine, but potential repression of TGF- β signaling is consistent with the other observations thus far in CR rodents.

TGF- β can promote programmed cell death (43) and regulates NF- κ B activation (92).

Number of differentially regulated miRNAs targeting at least one gene annotated with specific gene ontology (GO) categories, and the significance by Fisher's Exact test is shown in Table 4. We attempted to predict the regulatory effects of the differentially expressed miRNAs by first narrowing GO terms to only those with both "positive regulation of X" and "negative regulation of X" categories. Second, miRNAs were associated with genes (via miRwalk). Third, genes were associated with these regulatory GO categories. Relationships between miRNAs and regulatory categories were weighted by the number of miRNA-gene-GO relationships for that miRNA-regulatory pair. Net directionality of the regulatory category was calculated by taking the average signed t-statistic for each miRNA-regulation pair. *P* values were obtained by permuting the sample labels 100 times and observing how many times a more extreme t-statistic was obtained (similar to the limma "roast" function). The results are consistent with several already established age-related changes. For example, between

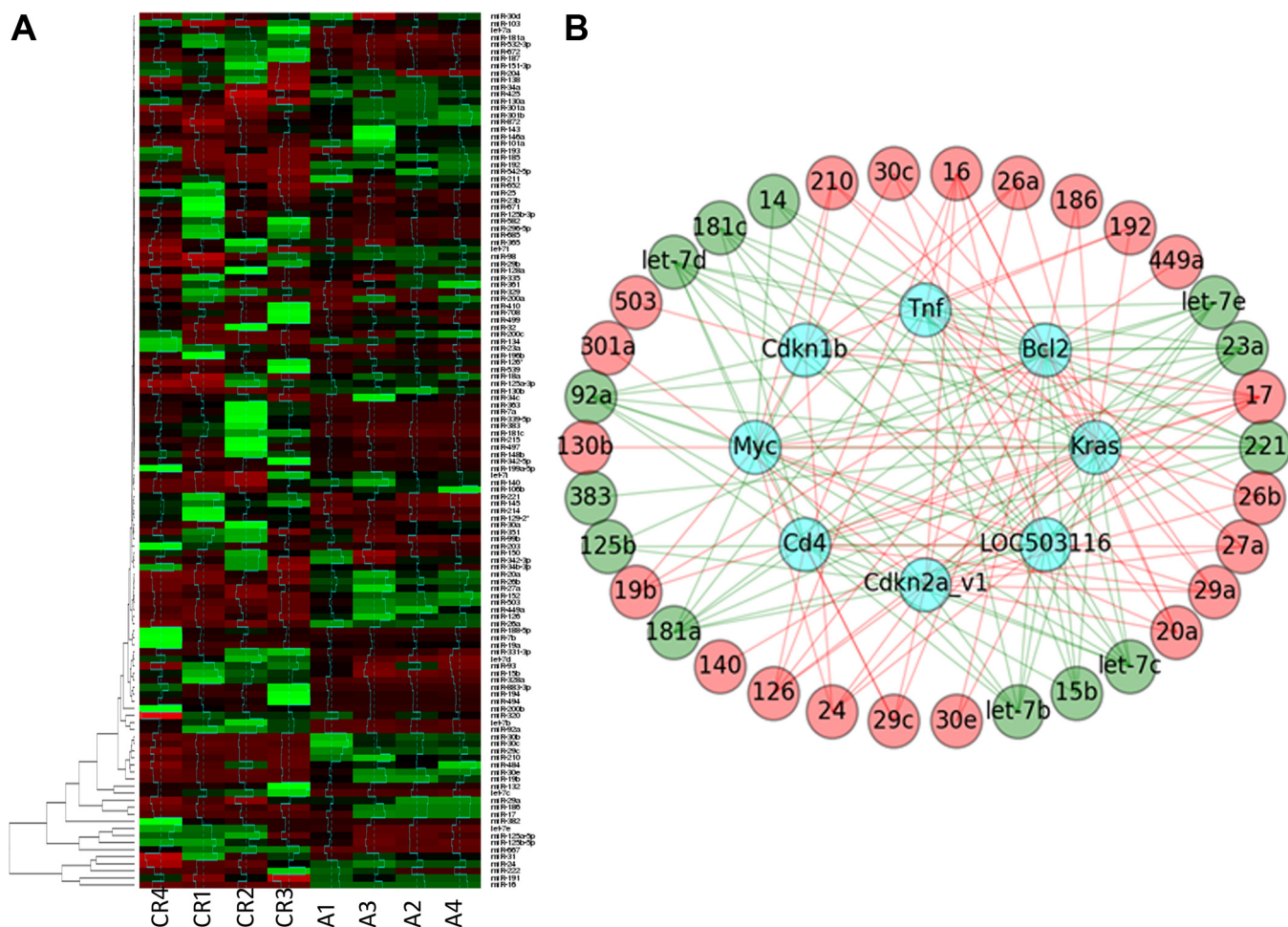


Fig. 6. CR-induced changes in miRNA expression profile in CMVECs. A: heat map is a graphic representation of normalized miRNA expression values in CMVECs derived from aged CR and AL-fed rats, depicted by color intensity, from highest (bright red) to lowest (bright green) expression. Values represent average miRNA expression levels [$\log_2$ (fold change, normalized to the respective control mean value)] in cells derived from 4 different animals in each group. B: key genes (blue) predicted to be regulated by the differentially expressed miRNAs. In green are upregulated miRNA in CMVECs derived from CR rats. In red are downregulated miRNA in CMVECs derived from CR rats. Note that CR-induced changes in TNF- α expression are predicted to be causally linked to changes in miRNA expression.

Table 1. Summary of miRNAs upregulated by CR in CMVECs (log₂ fold-changes)

miRNA Name	Fold Change	P Value	Number of Targets
rno-miR-667	5.01	0.002	0
rno-miR-383	4.82	0.027	39
rno-miR-328a	4.42	0.030	0
rno-let-7b	3.18	0.017	121
rno-miR-92a	3.09	0.009	104
rno-miR-532-3p	2.96	0.004	1
rno-miR-181c	2.76	0.006	23
rno-miR-145	2.73	0.001	57
rno-let-7c	2.52	0.025	118
rno-miR-329	2.49	0.015	0
rno-miR-23a	2.49	0.026	74
rno-miR-214	2.47	0.049	61
rno-miR-125b-5p	2.21	0.017	27
rno-miR-15b	1.86	0.007	44
rno-let-7e	1.59	0.012	118
rno-miR-181a	1.34	0.001	51
rno-miR-221	1.12	0.032	61
rno-let-7d	0.96	0.008	119

Positive values are miRNAs upregulated in caloric restriction (CR) relative to ad libitum (AL). Also shown are the significance of the fold-changes (determined by *t*-test) and number of genes that are experimentally validated to target according to the miRwalk database. CMVECs, cerebromicrovascular endothelial cells.

CMVECs derived from CR versus AL rats, there is predicted to be increased cell proliferation, decreased apoptosis, and decreased inflammatory processes, including decreased IL-6, and IL-1 β secretion and NF- κ B activation (Table 5).

Table 2. Summary of miRNAs downregulated by CR in CMVECs (fold-changes)

miRNA Name	Fold Change	P Value	Number of Targets
rno-miR-34a	-0.99	0.041	102
rno-miR-24	-1.14	0.003	80
rno-miR-872	-1.26	0.014	4
rno-miR-29a	-1.38	0.020	104
rno-miR-140	-1.79	0.016	7
rno-miR-301a	-1.85	0.019	40
rno-miR-30c	-1.99	0.007	71
rno-miR-30b	-2.05	0.001	0
rno-miR-27a	-2.13	0.013	71
rno-miR-26a	-2.20	0.000	28
rno-miR-542-5p	-2.25	0.006	3
rno-miR-152	-2.38	0.004	14
rno-miR-26b	-2.39	0.000	57
rno-miR-186	-2.44	0.003	7
rno-miR-20a	-2.46	0.005	36
rno-miR-126	-2.47	0.007	46
rno-miR-29c	-2.55	0.000	88
rno-miR-192	-2.66	0.002	30
rno-miR-30e	-2.72	0.005	66
rno-miR-301b	-2.78	0.003	0
rno-miR-101a	-2.78	0.004	41
rno-miR-210	-2.82	0.012	35
rno-miR-106b	-2.90	0.037	21
rno-miR-449a	-3.13	0.016	10
rno-miR-17	-3.33	0.016	72
rno-miR-130b	-3.57	0.001	16
rno-miR-16	-4.02	0.000	69
rno-miR-19b	-4.22	0.004	20
rno-miR-503	-6.18	0.004	13

Also shown are the significance of the fold-changes (determined by *t*-test) and number of genes that are experimentally validated to target according to the miRwalk database.

DISCUSSION

This is the first study to demonstrate the persisting protective effects of CR on the function and phenotype of CMVECs in a rodent model of aging that recapitulates cerebrovascular alterations and deficits present in elderly humans at risk for VCI.

The oxidative stress hypothesis of aging postulates that increased production of ROS induces a variety of macromolecular oxidative modifications and that accumulation of such oxidative damage gradually leads to cellular dysfunction, which is a primary causal factor in the aging process. A prediction based on the oxidative stress hypothesis of aging is that increased ROS production contributes to the development of age-related diseases as well. In agreement with this prediction previous studies in laboratory rodents and nonhuman primates provided ample evidence that oxidative stress develops with age in the vascular system (7, 20, 22, 73, 78, 82), which impairs endothelial function and ability to activate inflammatory processes promoting the development of atherosclerosis, stroke, and vascular dementias (for a recent review see Ref. 25). CR increases healthspan in many species, including nonhuman primates, yet its effects on the cerebral microvasculature are not well understood (53). Here we have devised an in vitro approach to determine the persistent effects of CR on age-related endothelial oxidative stress, angiogenic capacity, and secretory phenotype.

Our data demonstrate for the first time that CR significantly attenuates ROS production in CMVECs, at least in part, by downregulating NADPH oxidases (Fig. 1). These findings extend the results of previous studies showing attenuation of ROS production in conduit arteries of CR-fed laboratory rodents and nonhuman primates (35, 36, 98). It is likely that reduction of endothelial oxidative stress contributes to the documented anti-atherogenic (35) and endothelial protective (14) effects of CR.

There is growing evidence to suggest that the evolutionarily conserved stress-activated “cap’n’collar” transcription factor Nrf2 plays an important role in vasoprotection and regulating the aging process by orchestrating the transcriptional response of cells to oxidative stress (48, 62, 64, 77, 78). Here we demonstrate, for the first time, that development of oxidative stress in aged rodents CMVECs is also associated with a significant decline in transcriptional activity and expression of Nrf2 (Fig. 2). Our findings extend the results of recent studies showing that in the conduit arteries of rodents (12, 78) and nonhuman primates (77) Nrf2-dependent cytoprotection against oxidative stress diminishes with aging. Recent studies have demonstrated that in young animals activation of Nrf2 and upregulation of its downstream target enzymes provide vascular protection in oxidative stress by conferring important anti-oxidative and anti-inflammatory effects (10, 30, 45, 76, 96, 99). Because dysregulation of Nrf2 likely promotes the development of cerebrovascular pathologies in aging, the findings that CR restores Nrf2 expression and activity in aged CMVECs have important physiological relevance. Importantly, previous studies have attributed the anti-oxidative effects of CR, at least in part, to Nrf2 activation and upregulation of Nrf2-driven antioxidant systems, including NQO1 (38–40, 83). There are also studies extant demonstrating that activation of the Nrf2-driven antioxidant system contributes to the anticancer effects of CR (54, 64). The pathways by which aging leads to persist-

Table 3. *Selected genes predicted to be targeted by differentially expressed miRNAs*

Entrez ID	Gene Name	Number of miRNAs Targeting	%Upregulated	% Downregulated	<i>P</i>		
					Up miRNAs	Down miRNAs	Up vs. Down miRNAs
50689	Mapk3	22	53	7	0.000	0.156	0.001
50554	Smad4	20	59	11	0.000	0.561	0.002
500562	LOC503116	28	65	19	0.000	0.795	0.003
24498	Il6	14	29	0	0.025	0.039	0.006
60371	Birc2	10	29	0	0.004	0.116	0.006
25325	Il10	10	29	0	0.004	0.116	0.006
310756	NP_001101181.1	12	29	0	0.012	0.067	0.006
246097	Fas	21	35	4	0.041	0.043	0.009
81504	Grb2	7	24	0	0.007	0.345	0.018
299602	Cdc34_predicted	7	24	0	0.007	0.345	0.018
114515	Lanc1	7	24	0	0.007	0.345	0.018
171054	Ccr4	7	24	0	0.007	0.345	0.018
313264	Trim32	7	24	0	0.007	0.345	0.018
25434	Sparcl1	7	24	0	0.007	0.345	0.018
25648	Slc7a1	7	24	0	0.007	0.345	0.018
29139	Dcn	7	24	0	0.007	0.345	0.018
304809	NP_001100647.1	8	24	0	0.012	0.199	0.018
24864	Pgc	8	24	0	0.012	0.199	0.018
25661	Fn1	8	24	0	0.012	0.199	0.018
24854	Clu	8	24	0	0.012	0.199	0.018
170943	Slc25a10	8	24	0	0.012	0.199	0.018
24932	Cd4	39	59	22	0.021	0.349	0.024
81707	Mmp14	10	29	4	0.004	0.689	0.025
24547	MBP_RAT	10	29	4	0.004	0.689	0.025
246325	Kcnh8	21	41	11	0.010	0.562	0.030
60628	Cxcr4	22	41	11	0.013	0.401	0.030
29527	Ptgs2	13	0	26	0.212	0.008	0.032
315655	Rdx	16	47	15	0.000	0.749	0.035
252971	Socs1	23	47	15	0.004	0.781	0.035
298894	Mycn	17	35	7	0.013	0.359	0.040
25172	Gata1	3	18	0	0.002	1.000	0.051
25112	Gadd45a	4	18	0	0.008	0.576	0.051
29184	LOC685953	8	24	4	0.012	1.000	0.065
25235	Ghr	8	24	4	0.012	1.000	0.065
297480	Foxp1	18	35	11	0.018	0.761	0.068
60447	Clock	8	29	7	0.001	1.000	0.089
291966	RGD1305061	8	29	7	0.001	1.000	0.089
361789	RGD1303066	8	29	7	0.001	1.000	0.089
25729	Ccne1	12	29	7	0.012	1.000	0.089
362686	NP_001102171.1	13	29	7	0.017	0.732	0.089
59112	Hand1	13	29	7	0.017	0.732	0.089
83516	Ppargc1a	13	29	7	0.017	0.732	0.089
59328	Smad5	14	29	7	0.025	0.733	0.089
24835	Tnf	28	47	22	0.024	1.000	0.107
266603	Aldh1a3	2	12	0	0.018	1.000	0.144
116724	Epb4.113	2	12	0	0.018	1.000	0.144
114108	Pdlim3	2	12	0	0.018	1.000	0.144
24884	Yes1	2	12	0	0.018	1.000	0.144
25402	Casp3	2	12	0	0.018	1.000	0.144
25617	Hspa5	2	12	0	0.018	1.000	0.144
305066	NP_001100671.1	2	12	0	0.018	1.000	0.144
116636	Eif4ebp1	3	12	0	0.049	1.000	0.144
57027	Adam17	3	12	0	0.049	1.000	0.144
25671	Smad1	6	0	15	1.000	0.020	0.147
24253	Cebpb	13	35	15	0.003	0.477	0.150
25705	Zeb1	25	41	19	0.044	1.000	0.164
29152	Gdf8	9	24	7	0.020	1.000	0.186
117062	Hmga1	10	24	7	0.031	1.000	0.186
60427	Kitl	10	24	7	0.031	1.000	0.186
89829	Socs3	10	24	7	0.031	1.000	0.186
25735	Hnf4a	11	24	7	0.044	1.000	0.186
24224	BclII	54	71	48	0.019	0.663	0.213
362432	Clec4d	9	6	22	1.000	0.003	0.220
29467	Ddit3	11	29	11	0.007	0.703	0.227
501157	MGC124653	13	29	11	0.017	1.000	0.227
24566	Mst1	13	29	11	0.017	1.000	0.227
365748	NP_001102410.1	3	0	11	1.000	0.009	0.272

Continued

Table 3.—Continued

Entrez ID	Gene Name	Number of miRNAs Targeting	%Upregulated	% Downregulated	<i>P</i>		
					Up miRNAs	Down miRNAs	Up vs. Down miRNAs
303614	NP_001100531.1	4	0	11	1.000	0.032	0.272
24602	Nppa	4	0	11	1.000	0.032	0.272
408230	Pi3	16	29	15	0.045	0.749	0.275
24881	NP_001099184.1	6	18	4	0.033	1.000	0.282
83571	Cdkn1b	26	41	22	0.049	1.000	0.309
24525	Kras	55	71	52	0.033	0.390	0.346
444984	Dnmt3a	10	6	19	1.000	0.039	0.380
29263	Acvr2a	14	29	19	0.025	0.184	0.473
288734	Chfr	2	0	7	1.000	0.046	0.515
25579	Map3k12	2	0	7	1.000	0.046	0.515
24617	Serpine1	2	0	7	1.000	0.046	0.515
24577	Myc	30	47	33	0.030	0.215	0.526
25163	Cdkn2a_v1	35	53	41	0.021	0.146	0.539
50683	Sftpc	3	12	4	0.049	0.525	0.549
24772	Cxcl12	10	12	19	0.626	0.039	0.689
50662	Runx1	16	18	26	0.460	0.045	0.716

The %upregulated and %downregulated miRNAs targeting the gene are in CR relative to AL. *P* values for up and down miRNAs indicate whether the number of targeting miRNAs is significant (Fisher's Exact test). The up vs. down miRNAs is the uncorrected *P* value for the proposition that the proportion of up miRNAs and down miRNAs targeting this gene significantly differs.

tent downregulation of Nrf2 in CMVECs likely involve post-transcriptional regulation of Nrf2 expression. There is growing evidence that miRNAs, which are important for the posttranscriptional regulation of numerous biological processes, are deregulated during aging (42, 87), and some miRNAs have been implicated in age-associated decline of organ functions and development of age-related diseases (56). Analysis of age-related changes in miRNA expression profile in CMVECs (87) and existing data from the literature (56) identified miRNAs, such as miR-144, whose expression is upregulated in aging and whose predicted targets include Nrf2. Here we demonstrate for the first time that expression of miR-144 is upregulated in aged CMVECs and is reduced by CR. Interestingly, similar pattern of aging- and CR-induced changes in miR-144 expression has been observed recently in the skeletal muscle from rhesus monkeys (56). The findings that overexpression of miR-144 in young CMVECs and cells derived from aged CR rats significantly reduces Nrf2 expression, whereas expression of an antagomiR-144 upregulates Nrf2 in aged CMVECs, provide further evidence in support of the concept that dysregulation of miR-144 and Nrf2 in aging are causally linked. The association of increased miR-144 with downregulation of Nrf2 can also be expected based on the existing data obtained in nonvascular cells (68).

The process of angiogenesis is critical for maintenance of the cerebrovasculature. Previous studies demonstrate that advanced aging is associated with a progressive deterioration of microvascular homeostasis due to age-related impairment of angiogenic processes (2, 4, 66, 71, 80). It is assumed that these changes have a key role in age-related microvascular rarefaction (79), decreasing tissue blood supply and impairing adaptation to hypoxia (8, 41, 57). Our present findings extend the results of previous studies (87) showing that aging impairs angiogenic processes in CMVECs, including endothelial cell proliferation, endothelial adhesiveness, and capillary morphogenesis (Figs. 3 and 4). Our data show for the first time that CR restores angiogenic capacity of aged CMVECs, promoting endothelial cell proliferation and capillary morphogenesis. These findings support the concept that CR elicits persisting

changes in the endothelial phenotype, which preserve structural integrity of the microcirculation, at least in part, by stimulating angiogenesis. The pro-angiogenic effects of CR likely contribute to CR-induced increases in microvascular density in the brain (1, 53) and heart (46) that underlie progressive improvements in tissue blood supply and organ function. Interestingly, our recent studies demonstrate that treatment of endothelial cells with sera derived from CR-fed *M. mulatta* also stimulates endothelial cell proliferation and tube forming capacity (19). It is predicted that chronic presence of circulating factors induced by CR reprograms endothelial cells and thus some of the phenotypic changes induced by CR will persist even in the absence of continuing presence of these factors.

It has been proposed that increased apoptotic cell death contributes to the age-related microvascular rarefaction (80). Accordingly, histological studies show that both in laboratory rodents and nonhuman primates the percentage of apoptotic endothelial cells significantly increases in aging (3, 15, 24, 63). Another potentially important finding of our study is CR exerts persistent anti-apoptotic effects in endothelial cells (Fig. 3B), extending earlier ex vivo observations (29, 69). Importantly, short-term treatment with sera from caloric-restricted rats and nonhuman primates also confers significant anti-apoptotic effects in endothelial cells in vitro (14, 19). We propose that anti-apoptotic effects of CR will increase the angiogenic capacity of endothelial cells in vivo and importantly contribute to the microvascular protective effects of CR in aging. The cellular mechanism(s) that mediate the pro-angiogenic and anti-apoptotic effects of CR are presently unknown. On the basis of the existing data we predict that increased Nrf2 activity contributes to pro-angiogenic effects of CR. In support of this concept previous studies show that the Nrf2 pathway regulates the endothelial angiogenic response (91). Accordingly, all the major steps of the angiogenic process (e.g., adhesion, proliferation, migration, and capillary morphogenesis) are compromised by disruption of Nrf2 signaling in endothelial cells (91). Furthermore, Nrf2 blockade was reported to suppress angiogenesis in mouse models in vivo (47). Moreover, the Nrf2

Table 4. Number of differentially regulated miRNAs targeting at least one gene annotated with the Gene Ontology category listed, and the significance by Fisher's Exact test

Gene Ontology Terms Enriched Among Upregulated miRNAs	Number Associated	P Value
Transforming growth factor- β receptor, common-partner cytoplasmic mediator activity	10	1.21E-05
Endothelial cell activation	10	1.21E-05
miRNA metabolic process	11	6.34E-05
RNA 3'-end processing	11	6.34E-05
miRNA catabolic process	11	6.34E-05
pre-miRNA processing	11	6.34E-05
Regulation of gene silencing by miRNA	11	6.34E-05
Cytoplasmic mRNA processing body	11	9.65E-05
mRNA binding	12	0.000112
Translation initiation factor binding	11	0.000144
Positive regulation of transforming growth factor- β receptor signaling pathway	11	0.000144
DNA damage induced protein phosphorylation	9	0.000353
IL-1-mediated signaling pathway	9	0.000353
Misfolded protein binding	6	0.000416
IL-6 receptor binding	8	0.000462
SMAD protein signal transduction	11	0.000593
RNA polymerase II core promoter proximal region sequence-specific DNA binding	8	0.000739
Arachidonic acid metabolic process	9	0.000805
Positive regulation of histone acetylation	10	0.001034
RNA polymerase II transcription factor binding	10	0.001034
Transcription factor activity	4	0.001159
Transcriptional repressor complex	5	0.001181
Histone acetyltransferase activity	10	0.001939
Protein binding transcription factor activity	3	0.002193
Transcriptional activation by promoter-enhancer looping	3	0.002193
Enhancer sequence-specific DNA binding	7	0.002256
Ligand-dependent nuclear receptor binding	5	0.002432
RNA polymerase II core promoter proximal region sequence-specific DNA binding transcription factor activity	6	0.002628
Regulation of IL-6 biosynthetic process	9	0.003169
Response to endoplasmic reticulum stress	8	0.003506
Regulation of RNA biosynthetic process	8	0.003506
Kinase inhibitor activity	13	0.003933
Cellular response to cAMP	11	0.004036
Negative regulation of ubiquitin-protein ligase activity	6	0.004218
mRNA transcription from RNA polymerase II promoter	5	0.004447
Negative regulation of IL-12 production	5	0.004447
Positive regulation of protein monoubiquitination	5	0.004447
Negative regulation of nitric oxide biosynthetic process	5	0.004447
Negative regulation of TNF biosynthetic process	5	0.004447
miRNA binding	11	0.004813
Positive regulation of IL-12 production	8	0.004857
IGF receptor binding	9	0.005647
Positive regulation of TNF production	9	0.005647
RNA polymerase II transcription factor binding	11	0.005829
transcription factor activity involved in positive regulation of transcription	6	0.006443
IL-6 receptor complex	10	0.006673
Positive regulation of cytokine secretion	4	0.00676
Negative regulation of cAMP-mediated signaling	7	0.007001
Proteasome-mediated ubiquitin-dependent protein catabolic process	5	0.007453
Negative regulation of cytokine production	5	0.007453
Base-excision repair	5	0.007453
Response to unfolded protein	5	0.007453
Negative regulation of transcription regulatory region DNA binding	5	0.007453

Continued

Table 4.—Continued

Gene Ontology Terms Enriched Among Upregulated miRNAs	Number Associated	P Value
Negative regulation of IL-8 production	3	0.00801
Regulation of transforming growth factor β 2 production	10	0.008802
Negative regulation of insulin receptor signaling pathway	10	0.008802
mRNA cleavage involved in gene silencing by miRNA	5	0.011675
Growth hormone receptor complex	4	0.012338
Histone H3-K4 demethylation	4	0.012338
Positive regulation of cell-matrix adhesion	4	0.012338
Cellular response to hydroperoxide	4	0.012338
Response to growth hormone	4	0.012338
Growth hormone receptor activity	4	0.012338
Regulation of removal of superoxide radicals	4	0.012338
Negative regulation of transcription factor import into nucleus	4	0.012338
Response to misfolded protein	4	0.012338
Apoptotic cell clearance	4	0.012338
Regulation of reactive oxygen species metabolic process	9	0.01237
Cellular response to oxidative stress	7	0.012997
Positive regulation of chemokine production	9	0.015419
Cell-cell adherens junction	9	0.015419
Response to growth factor	12	0.015694
RNA polymerase II distal enhancer sequence-specific DNA binding transcription factor activity	12	0.015694
Cell redox homeostasis	7	0.017149
Endothelial cell proliferation	7	0.017149
Regulation of cell death	5	0.017329
Positive regulation of cellular metabolic process	5	0.017329
Positive regulation of energy homeostasis	5	0.017329
Positive regulation of mitochondrion organization	5	0.017329
Positive regulation of mitochondrial DNA metabolic process	5	0.017329
Venous blood vessel morphogenesis	2	0.017834
Positive regulation of cell cycle checkpoint	2	0.017834
Positive regulation of IL-6 production	9	0.017914
Intrinsic apoptotic signaling pathway in response to oxidative stress	12	0.018949
rRNA processing	9	0.021117
Regulation of apoptotic DNA fragmentation	9	0.021117
Positive regulation of DNA damage response, signal transduction by p53 class mediator	9	0.021117
Positive regulation of cellular senescence	9	0.021117
IL-6-mediated signaling pathway	7	0.022211
Negative regulation of cell-matrix adhesion	10	0.022557
Positive regulation of IL-18 production	8	0.023869
Positive regulation of chemokine biosynthetic process	8	0.023869
Negative regulation of cytokine secretion involved in immune response	8	0.023869
Regulation of I- κ B kinase/NF- κ B signaling	8	0.023869
Regulation of protein secretion	8	0.023869
Cellular response to hormone stimulus	8	0.023869
DNA damage checkpoint	8	0.023869
Inner mitochondrial membrane organization	8	0.029779
Cytokine production	11	0.031742
Endoplasmic reticulum unfolded protein response	6	0.032042
Negative regulation of translational initiation	6	0.032042
Regulation of mitochondrial membrane permeability	12	0.033172
Positive regulation of IGF receptor signaling pathway	3	0.033399
Negative regulation of intrinsic apoptotic signaling pathway	12	0.034401
Regulation of mitochondrial membrane potential	12	0.034401
Regulation of programmed cell death	12	0.039572
Positive regulation of ATP biosynthetic process	8	0.040052
TNF-activated receptor activity	6	0.040999
Positive regulation of IL-1 β secretion	5	0.044725

Table 5. Predicted regulatory effects of differentially expressed miRNAs

Regulatory Category	Number	t-Statistic	P	
			Up miRNAs	Down miRNAs
IL-6 secretion	8	9.39	1	0.01
Endothelial cell apoptotic process	13	7.18	1	0.01
NF- κ B import into nucleus	33	5.30	1	0.01
Cytokine secretion	32	4.86	1	0.01
Transforming growth factor- β production	44	4.82	1	0.01
Reactive oxygen species metabolic process	71	3.44	1	0.01
Cell aging	52	3.12	1	0.01
Chemokine production	38	2.45	1	0.01
Cellular senescence	37	2.25	1	0.01
Inflammatory response	60	2.16	1	0.01
IL-1 β production	33	2.15	1	0.01
Superoxide anion generation	39	2.04	1	0.01
I κ B kinase/NF- κ B signaling	70	1.88	1	0.01
Apoptotic cell clearance	8	1.86	1	0.01
Transcription regulatory region DNA binding	45	1.82	1	0.01
TNF biosynthetic process	10	1.80	1	0.01
Response to reactive oxygen species	22	-1.21	0.01	1
DNA biosynthetic process	53	-1.84	0.01	1
DNA repair	36	-1.86	0.01	1
Protein monoubiquitination	10	-2.10	0.01	1
Protein acetylation	46	-2.37	0.01	1
Endothelial cell chemotaxis	31	-2.37	0.01	1
Ubiquitin-protein ligase activity	38	-2.63	0.01	1
Cyclin-dependent protein serine/threonine kinase activity	71	-2.64	0.01	1
Histone acetylation	34	-3.35	0.01	1
Cell cycle	81	-3.50	0.01	1
Histone phosphorylation	27	-3.73	0.01	1
GTPase activity	18	-3.88	0.01	1
Cell growth	77	-4.14	0.01	1
Cell cycle phase transition	69	-4.30	0.01	1
Mitotic cell cycle phase transition	69	-4.30	0.01	1
Transcription elongation from RNA polymerase II promoter	17	-4.88	0.01	1
DNA-templated transcription, elongation	17	-4.88	0.01	1
CREB transcription factor activity	20	-6.71	0.01	1
Gene expression, epigenetic	15	-6.94	0.01	1
Peroxisome proliferator activated receptor signaling pathway	7	-10.89	0.01	1
Double-strand break repair	7	-10.89	0.01	1

The t-statistic indicates selected processes affected by CR. Where the sign is positive, the process is predicted to be positively affected in AL-fed rodents and negatively affected by CR. Where the sign is negative, the process is predicted to be positively affected by CR.

targets, heme oxygenase-1 (26, 34) and thioredoxin (67), were shown to confer pro-angiogenic effects in animal disease models, and Nrf2 activation was shown to confer significant anti-apoptotic effects in endothelial cells (75, 76, 77, 78, 91). Furthermore, treatment with a pharmacological activator of Nrf2 (resveratrol) was shown to both increase capillary density (60) and improve neurovascular coupling (73) in the aged brain, which were associated with improved cognitive function (60).

Current views of vascular aging are consistent with chronic low-grade vascular inflammation, which promotes the development of cerebrovascular diseases and VCI (20, 80, 83). An important new finding of this study is that aging promotes

NF- κ B activation and significantly alters the secretome of CMVECs (Fig. 5). Characterization of this secretome highlights an increased production of pro-inflammatory cytokines, including several factors (i.e., IL-6, IL-1 β , TNF- α) previously identified in the cytokine expression profile in blood vessels from aged rodents (14, 15, 22, 23). These observations support the concept that the age-associated changes in the secretome of vascular cells [previously described as Age-Associated Arterial Secretory Phenotype or AAASP (94)] are, at least in part, due to cell-autonomous mechanisms. Similar conclusions have been reached previously with the use of VSMCs obtained from aged rodents (44) and nonhuman primates (20). Importantly, CR exerts significant anti-inflammatory effects in CMVECs, inhibiting NF- κ B activation and decreasing secretion of pro-inflammatory cytokines (Fig. 5). Increasing evidence supports a key role for elevated levels of ROS in activation of NF- κ B and induction of vascular pro-inflammatory phenotypic alterations during aging (reviewed in Ref. 80). This concept is also supported by the findings that knockdown of Nrf2 results in a secretory profile in CMVECs that closely mimic the aging phenotype (Csiszar and Ungvari, unpublished observation 2014) and that pharmacological activation of Nrf2 can reverse age-related pro-inflammatory phenotypic alterations in vascular cells (20). Thus we propose that the anti-oxidative effects of CR observed in the present study significantly contribute to its anti-inflammatory effects in CMVECs.

Recent studies demonstrate that both endothelial angiogenic capacity (87) and activation of inflammatory processes in endothelial cells (reviewed in Ref. 95) are regulated by miRNAs. Moreover, age-related dysregulation of miRNA expression has been causally linked to functional changes of aged CMVECs, including age-related impairment of endothelial angiogenic capacity (87). In the present study we characterized CR-related miRNAs and their predicted targets (Fig. 6 and Tables 1–3). A single miRNA can target up to several hundred mRNAs, thus capable of significantly altering gene expression regulatory networks. Characterization of CR-induced changes in miRNA expression suggests that they likely affect several critical functions in endothelial cell homeostasis (Table 4). The predicted regulatory effects of CR-related differentially expressed miRNAs in aged CMVECs (Table 5) are consistent with the anti-aging endothelial effects of CR observed in vivo (14, 53, 83). Importantly, the experimental findings obtained in the present CMVEC model presented in Figs. 1–5 (showing increased cell proliferation, decreased apoptosis, and decreased inflammatory processes, including decreased IL-6, and IL-1 β secretion and NF- κ B activation) functionally validate many of the predicted roles of miRNAs (Table 5) in the endothelial protective effects of CR. We hope that our findings will facilitate future endeavor of uncovering the mechanistic role of miRNA gene expression regulatory networks in the anti-aging effects of CR. Future studies should also investigate the links between miRNAs regulated by CR and miRNAs known to act in the conserved pathways of aging and major aging-related diseases.

Conclusions

In conclusion, CR activates endogenous anti-oxidative, pro-angiogenic, anti-apoptotic, and anti-inflammatory mechanisms, retaining a youthful phenotype in aged CMVECs. These

changes in endothelial cell function and responsiveness may contribute to the cerebrovascular protective effects of CR in aging. In addition, CR also improves several risk factors for cerebrovascular disease, stroke, and VCI, including a reduction in blood pressure and increased insulin sensitivity (83). Potentially, the mechanisms of CR could be harnessed for development of new pharmacological approaches for the prevention and treatment of VCI in elderly patients.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: A.C., C.B.G., W.E.S., and Z.I.U. conception and design of research; A.C., T.G., D.S., S.T., E.B., Z.T., C.B.G., J.W., and Z.I.U. performed experiments; A.C., T.G., D.S., Z.T., C.B.G., J.W., and Z.I.U. analyzed data; A.C., S.T., P.T., C.B.G., J.W., W.E.S., and Z.I.U. interpreted results of experiments; A.C., C.B.G., J.W., and Z.I.U. prepared figures; A.C. and Z.I.U. drafted manuscript; A.C., P.T., G.L., A.K., D.R., C.B.G., J.W., W.E.S., and Z.I.U. edited and revised manuscript; A.C. and Z.I.U. approved final version of manuscript.

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