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Original Article

Age-Related Decline of Autocrine Pituitary Adenylate Cyclase-Activating Polypeptide Impairs Angiogenic Capacity of Rat Cerebromicrovascular Endothelial Cells

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Abstract

Aging impairs angiogenic capacity of cerebromicrovascular endothelial cells (CMVECs) promoting microvascular rarefaction, but the underlying mechanisms remain elusive. PACAP is an evolutionarily conserved neuropeptide secreted by endothelial cells and neurons, which confers important antiaging effects. To test the hypothesis that age-related changes in autocrine PACAP signaling contributes to dysregulation of endothelial angiogenic capacity, primary CMVECs were isolated from 3-month-old (young) and 24-month-old (aged) Fischer 344 x Brown Norway rats. In aged CMVECs, expression of PACAP was decreased, which was associated with impaired capacity to form capillary-like structures, impaired adhesiveness to collagen (assessed using electric cell-substrate impedance sensing [ECIS] technology), and increased apoptosis (caspase3 activity) when compared with young cells. Overexpression of PACAP in aged CMVECs resulted in increased formation of capillary-like structures, whereas it did not affect cell adhesion. Treatment with recombinant PACAP also significantly increased endothelial tube formation and inhibited apoptosis in aged CMVECs. In young CMVECs shRNA knockdown of autocrine PACAP expression significantly impaired tube formation capacity, mimicking the aging phenotype. Cellular and mitochondrial reactive oxygen species production (dihydroethidium and MitoSox fluorescence, respectively) were increased in aged CMVECs and were unaffected by PACAP. Collectively, PACAP exerts proangiogenic effects and age-related dysregulation of autocrine PACAP signaling may contribute to impaired angiogenic capacity of CMVECs in aging.

Key Words: Capillary density—Senescence—Vascular cognitive impairment—Vasculardementia—Ischemia.

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Several studies analyzing cerebrovascular architecture, assessing capillary density, and measuring cerebral blood flow in elderly humans and aged rodents suggest that angiogenesis in the brain is compromised by aging (1–3), which might contribute to a range of age-related central nervous system disorders with microvascular involvement, including vascular cognitive impairment and Alzheimer's disease. The age-related loss of microvascular plasticity and rarefaction in the brain has significance for impaired metabolic support for the neuronal tissue and for impaired recovery after cerebral ischemia. Microvascular rarefaction is also related to increased inflammatory signals that may negatively regulate the stem cell population (4). Although age-related changes in expression of pro and antiangiogenic factors in the brain parenchyma likely have a role in dysregulation of brain capillarization in aging, there is increasing evidence that aging impairs the intrinsic angiogenic capacity of cerebromicrovascular endothelial cells (CMVECs) (5). Despite provocative evidence linking age-related microvascular rarefaction with diminished endothelial angiogenic capacity, the underlying mechanisms remain elusive.

PACAP is an evolutionarily conserved C-terminally α-amidated peptide that was first isolated 25 years ago from an ovine hypothalamic extract on the basis of its ability to stimulate cAMP formation in anterior pituitary cells (6). It exists in two forms, consisting of either 27 or 38 amino acids, the longer form being dominant in mammalian tissues (7). The PACAP belongs to the vasoactive intestinal polypeptide (VIP)-secretin-growth hormone-releasing hormone-glucagon superfamily. Although PACAP is a multifunctional peptide and shows wide tissue distribution (7), its expression is highest in the brain and several diverse functions of PACAP have been described in the central nervous system (8). Recent studies suggest that alterations in PACAP signaling may play an important role in age-related cognitive decline and development of age-related diseases of the CNS (9,10). Accordingly, PACAP has remarkable protective effects in animal models of age-related neurodegenerative diseases, including Parkinson's disease and Alzheimer's disease (9). The PACAP knockout mice exhibit symptoms of accelerated neurocognitive aging, including impairment of learning and memory (11–13), increased oxidative stress (14), and accelerated aging of the retina (15). The antiaging and neuroprotective effects of PACAP have been attributed, at least in part, to its antiapoptotic, anti-inflammatory, and antioxidant actions (9,10). Treatment with PACAP was also shown to exert anti-apoptotic effects (16) and reverse age-related learning impairment in molluscan models of aging (17) suggesting that the antiaging actions of PACAP are evolutionarily conserved. Recent studies demonstrate that endothelial cells are not only important sources of PACAP secretion (18) but also express receptors for PACAP (19) and their function is regulated by PACAP (20–23). Importantly, PACAP was also suggested to exert proangiogenic effects in tumor models (reviewed in Ref. (24)). Furthermore, a recent study demonstrated that secretion of PACAP by cerebral microvessels progressively declines with age in laboratory rodents (18). Nevertheless, the specific role of PACAP in age-related impairment of endothelial angiogenic capacity of CMVECs remains poorly understood.

The present study was designed to test the hypothesis that the age-related decline in autocrine PACAP expression contributes to age-related impairment of endothelial angiogenic capacity. A prediction based on this hypothesis is that increases in autocrine PACAP should restore a youthful endothelial phenotype improving endothelial angiogenic capacity. To test this hypothesis using cultured primary CMVECs derived from aged rats as a model system, we determined whether overexpression of PACAP or treatment with exogenous PACAP-38 elicits proangiogenic, antioxidative, and antiapoptotic changes in the endothelial phenotype. We also tested whether down-regulation of PACAP (shRNA) in cultured primary CMVECs derived from young animals confers antiangiogenic effects, mimicking the aging phenotype. As endpoints, endothelial angiogenic capacity (tube formation, adhesion, and migration capacity), apoptosis, and cellular reactive oxygen species (ROS) production (dihydroethidium [DHE] and MitoSox fluorescence) were assessed.

Materials and Methods

Establishment and Characterization of Primary Cerebromicrovascular Endothelial Cell Cultures

Primary CMVEC cultures were established from the brains of 3-month old ("young") and 24-month old ("aged") male Fischer 344 x Brown Norway rats as described previously (5,25). All animals (*n* = 5 in each group) were disease free with no signs of systemic inflammation and/or neoplastic diseases. All rats were maintained according to National Institutes of Health guidelines and all animal use protocols were approved by the Institutional Lyses buffer Animal Care and Use Committees of the University of Oklahoma Health Sciences Center. The animals were euthanized with CO_2. The brains were removed aseptically, rinsed in ice-cold PBS, and minced into ≈1 mm². The tissue was washed twice in ice cold 1× PBS by low-speed centrifugation (50*g*, 2–3 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 minutes at 37°C in rotating humid incubator. The digested tissue was passed through a 100-μm cell strainer to remove undigested blocks. The single cell lysate was centrifuged for 2 min at 70*g*. After removing the supernatant carefully the pellet was washed twice in cold PBS supplemented with 2.5 % fetal calf serum and the suspension centrifuged at 300g, 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 Hanks' balanced salt solution and thoroughly mixed with 40% iodixanol (final concentration: 17% (w/v) iodixanol solution; ρ = 1.096 g/mL). 2 mL of Hanks' balanced salt solution was layered on top and centrifuged at 400*g* for 15 min at 20°C. Endothelial cells, which banded at the interface between Hanks' balanced salt solution and the 17% iodixanol layer, were collected. The endothelial cell enriched fraction was incubated for 30 min at 4°C in the dark with anti-CD31/PE (BD BD Biosciences, San Jose, CA), anti-MCAM/FITC (BD Biosciences, San Jose, CA). After washing the cells 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 minutes at room temperature. Endothelial cells were collected using the MACS LD magnetic separation columns according to the manufacturer's guidelines (Milltenyi Biotech, Cambridge, MA). 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). In brief, antibodies against five different endothelial specific markers were used (anti-CD31-PE, antierythropoietin receptor-APC, anti-VEGF R2-PerCP, anti-ICAM-fluorescein, and 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 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% fetal calf serum.

Quantitative Real-Time RT–PCR

Quantitative real time RT–PCR was performed to analyze the mRNA expression of PACAP, PAC1R (Adcyap1r1), VPAC1 (Vipr1), VPAC2R (Vipr2), VEGF and VEGFR2, as well as the reference genes *Hprt, Ywhaz,* and *B2m,* as reported previously (5,25,26). In brief, total RNA was isolated using a TaqMan Cells-to-CT Kit (Applied Biosystems, Foster City, California) and was reverse transcribed using Superscript III RT (Invitrogen, Carlsbad, California) as described previously (27,28). A real time RT–PCR technique was used to analyze mRNA expression using a Stratagen MX3000, as reported (28). 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 geneswere determined and a normalization factor was calculated based on the geometric mean for internal normalization.

Knockdown and Overexpression of PACAP in CMVECS

To investigate the role of autocrine PACAP signaling in regulation of the endothelial phenotype, downregulation of PACAP in young CMVECs was achieved by RNA interference using proprietary shRNA sequences (OriGene Technologies, Rockville, MD). Overexpression of PACAP in aged CMVECs was achieved by using a full-length PACAP cDNA encoding plasmid (OriGene). Transfection was performed using the electroporation-based LonzaNucleofector technology (Amaxa, Gaithersburg, Maryland), as described earlier (29–32). The success of transfection was confirmed by quantitative real-time RT–PCR. Negative controls included transfection with scrambled shRNA (OriGene) or a GFP expressing vector (33).

Tube Formation Assay

To investigate the influence of autocrine PACAP signaling on tube formation ability, young CMVECs with or without shRNA knockdown of PACAP and aged CMVECs with or without overexpression of PACAP were plated on Geltrex Reduced Growth Factor Basement Membrane Matrix (Invitrogen, Carlsbad, California) in Medium 200PRF (Invitrogen). In separate experiments, the effect of treatment with PACAP-38 (10^{-7} mol/L) on endothelial tube formation was assessed. In brief, 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 minutes at 37ºC. The CMVECs were then seeded at a density of 5×10^4 cells/well and placed in the incubator for 24 hours. Microscopic images were captured using a Nikon Eclipse Ti microscope equipped with a 10× phase-contrast objective (Nikon Instruments, Melville, New York). 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 Inc), as recently reported (5,25,33,34). The mean of the total tube length per total area imaged (µm tube/mm^2) was calculated for each well. Experiments were run in quadruplicate. The experimenter was blinded to the experimental groups throughout the period of analysis.

Cell Adhesion Assay

Cell adhesion plays an important role in the multistep process of angiogenesis (35). To determine the effect of autocrine PACAP signaling on the adhesion capacity of endothelial cells, we used ECIS technology (Applied Biophysics, Troy, New York). Adhesion of young CMVECs (transfected with PACAP shRNA or scrambled shRNA) and aged CMVECs (transfected with PACAP cDNA or control plasmid) to collagen was monitored as reported (5,25,33). In a separate experiment, cells were treated with PACAP-38 (from 10^{-8} mol/l to 10^{-6} mol/l; for 48 h). Cells were seeded at a density of 2.5×10^5 cells/well in collagen coated (50 µg/mL) 96-well array culture dishes containing gold film surface electrodes (ECIS 96W1E; in each well one active electrode and a large counter electrode). 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).

Assessment of Cell Migration by ECIS-based Wound-Healing Assay

The ECIS technology was used to monitor migration of young CMVECs (transfected with PACAP shRNA or scrambled shRNA) and aged CMVECs (transfected with PACAP cDNA or control plasmid), as reported (33). In a separate experiment, cells were treated with PACAP-38 (from 10^{-8} mol/l to 10^{-6} mol/l). In brief, CMVECs (2.5×10^5 cells/well) were seeded in 96-well array culture dishes (ECIS 96W1E), placed in an incubator (37ºC), and changes in resistance and impedance were continuously monitored. When impedance reached a plateau, cells in each well were subjected to an elevated field pulse ("wounding") of 5 mA applied for 20 seconds at 100 kHz, which killed the cells present on the small active electrode due to severe electroporation. The detachment of the dead cells was immediately evident as a sudden drop in resistance (monitored at 4000 Hz) and a parallel increase in conductance. CMVECs surrounding the active electrode that had not been subjected to the wounding then migrated inward to replace the detached dead cells resulting in resistance recovery (continuously monitored at 4000 Hz for up to 24 h). Time to reach 50% resistance recovery (corresponding to 50% confluence on the active electrode) was determined and this parameter and the known physical dimensions of the electrode were used to calculate the migration rate (expressed as µm/h).

Apoptosis Assay

To determine whether PACAP exerts antiapoptotic effects in aged CMVECs, apoptotic cell death was assessed by measuring caspase activities using the Caspase-Glo3/7 assay kit (Promega, Madison, Wisconsin) as reported earlier (36, 37). Young and aged CMVECs were seeded at a density of 2×10^4 cells/well in white-walled 96-well plates. Aged CMVECs were treated with PACAP-38 (10^{-7} mol/L). To measure caspase3/7 activity, 50 µL of sample was mixed for 30 seconds with of 50 µL Caspase-Glo3/7 reagent and incubated for 2 hours at room temperature. Lysis buffer with the reagent served as blank. Luminescence of the samples was measured using an Infinite M200 plate reader (Tecan, Research Triangle Park, North Carolina). Luminescent intensity values were normalized to the sample protein concentration.

Measurement of Cellular Reactive Oxygen Species Production

To assess the effect of PACAP on cellular production of ROS we used DHE (Invitrogen, Carlsbad, California), an oxidative

fluorescent dye, as we reported earlier (38–40). In brief, 2×10^4 cells/well were seeded to a transparent 96-well plate and were treated with different concentrations of PACAP-38 (from 10^{-8} to 10^{-6} mol/l, for 24 h). Then the cells were washed with warm PBS and incubated with DHE (41,42) (3×10^{-6} mol/L; at 37°C for 30 minutes). The DHE fluorescence was assessed by flow cytometry (38,39). In separate experiments, mitochondrial O_2^- production in CMVECs was measured using MitoSOX Red (Invitrogen, Carlsbad, California), a mitochondrion-specific hydroethidine-derivative fluorescent dye, as reported earlier (25,38,43–45). Cell debris (low forward and side scatter) and dead cells (Sytox Green) were gated out for analysis.

Data Analysis

Statistical analyses were performed using one-way ANOVA followed by Tukey post hoc tests. $p < .05$ was considered statistically significant. Data are expressed as means ± S.E.M.

Results

Age-Related Changes in Expression of PACAP and PACAP Receptors in CMVECs

Expression of PACAP was significantly decreased in aged CMVECs when compared with that in young cells (Figure 1A). Compared to young CMVECs, mRNA expression of PAC1R, VPAC1, and VPAC2R was increased in aged cells (Figure 1A), perhaps as a partial compensation for the age-related decline in PACAP production.

Autocrine PACAP Promotes Formation of Capillary-Like Structures by CMVECs

We performed an in vitro tube formation assay to model the reorganization stage of angiogenesis. When seeded onto Geltrex matrices, young CMVECs formed elaborated capillary networks and this response was significantly impaired in aged CMVECs (Figure 2C–D). We found that knockdown of PACAP impaired the ability of young CMVECs to form capillary-like structures (Figure 2D). Overexpression of PACAP in aged CMVECs (Figure 2D) and treatment with PACAP-38 (Figure 2E) increased tube formation by endothelial cells.

Effects of PACAP on Adhesion of 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 cells to the collagen-coated active electrode (Figure 3A). 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) as compared to young cells. Knockdown of PACAP in young CMVECs was without effect on endothelial adhesiveness (Figure 3A and B). Overexpression of PACAP in aged CMVECs (Figure 3A and B) or treatment with PACAP-38 (not shown) decreased endothelial adhesiveness to collagen.
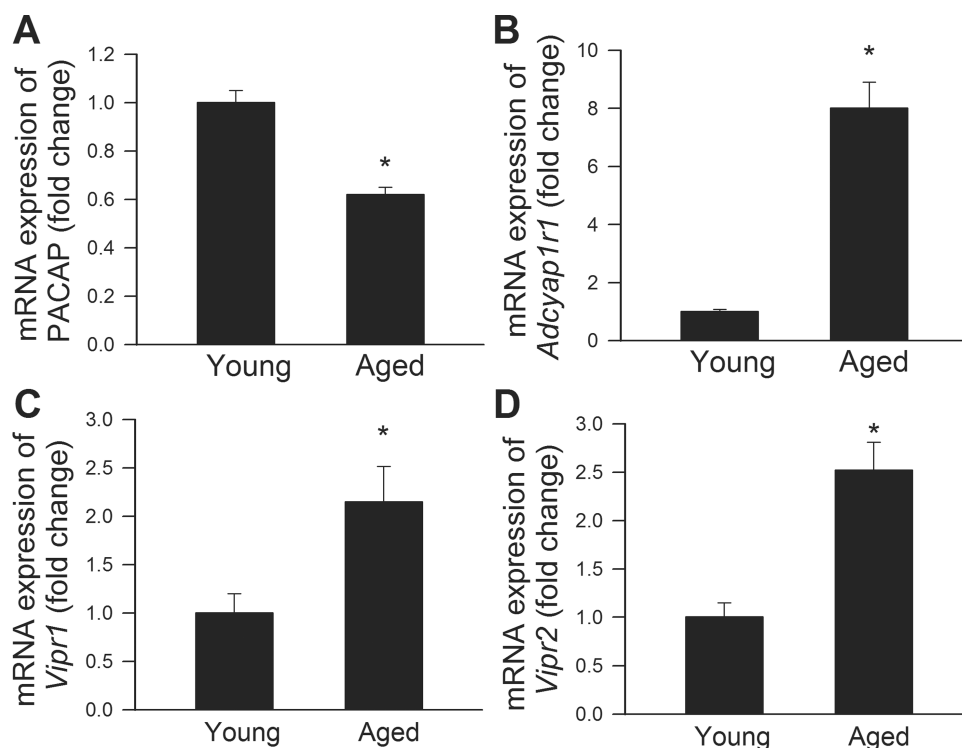

Figure 1. Downregulation of PACAP expression in aged cerebromicrovascular endothelial cells (CMVECs). (**A–D**) Quantitative real time RT–PCR data showing mRNA expression of PACAP (Panel A) and the PACAP receptors PAC1R (Adcyap1r1, Panel B), VPAC1 (Vipr1, Panel C), and VPAC2R (Vipr2, Panel D) in young and aged CMVECs. Data are means ± S.E.M. ($n = 5$ in each group), *$p < .05$ vs. young control.
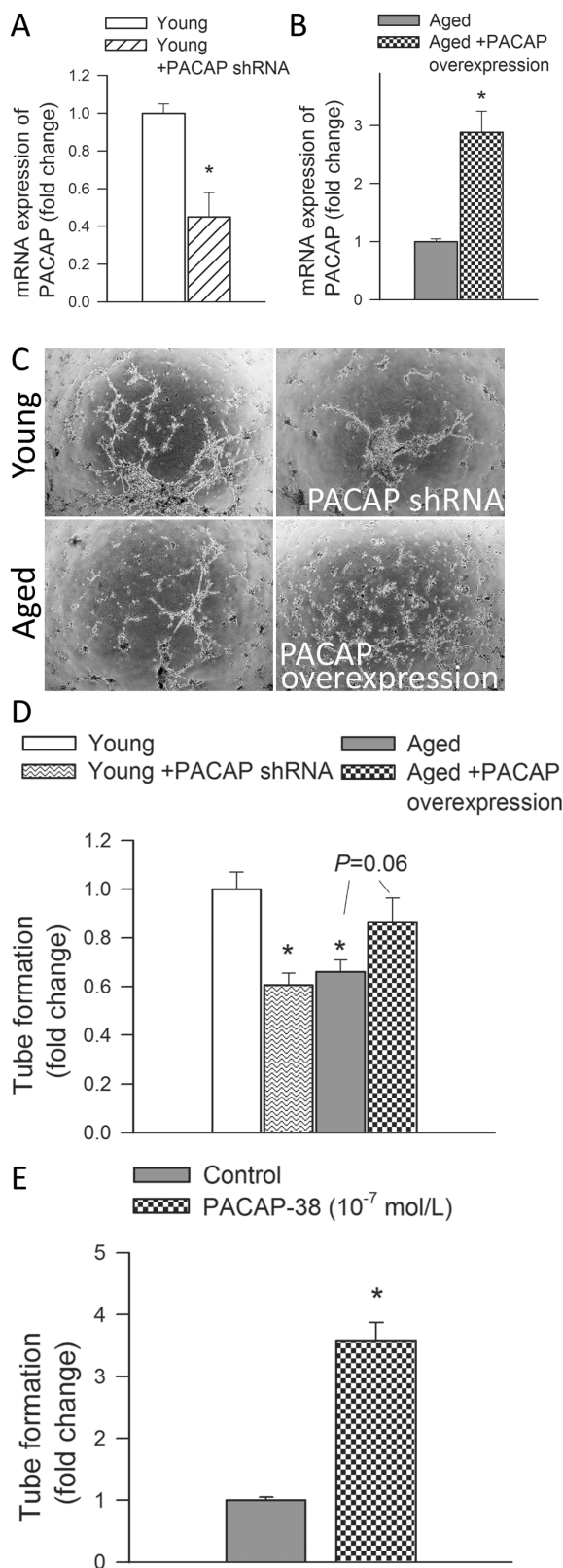
Figure 2. PACAP increases angiogenic capacity in cerebromicrovascular endothelial cells (CMVECs). (**A–B**) Quantitative real time RT–PCR data showing mRNA expression of PACAP in young CMVECs with or without transfection with PACAP shRNA and aged CMVECs with or without overexpression of PACAP. (**C**) The ability to form capillary-like structures by young CMVECs with or without shRNA knockdown of PACAP and aged CMVECs with or without overexpression of PACAP was assessed. Representative examples of capillary-like structures are shown on Panel C. (**D**) Summary data, expressed as relative changes in total tube length per area scanned, are shown in Panel D. (**E**) Changes in angiogenic capacity in CMVECs induced by PACAP-38 (10^{-7} mol/l) treatment. Data are means ± standard error of the mean (*n* = 5 in each group), *p < .05 vs. respective control.

Effect of PACAP on the Migratory Capability of CMVECs

The migratory capability of vascular endothelial cells has a pivotal role in the maintenance of microvascular integrity and angiogenesis. An ECIS-based wound-healing assay was used to assess the effect of PACAP on migratory capability of CMVECs. We found that aged CMVECs exhibited impaired migratory capability as compared to young CMVECs (Figure 3C and D). The PACAP treatment was without effect on migratory capability of young CMVECs (Figure 3C and D). Treatment with PACAP tended to decrease the time for aged CMVECs to reach 50% of the maximum confluence (Figure 3C). Figure 3D indicates that the increase in the calculated migration rate in aged CMVECs with PACAP treatment did not reach statistical significance.

PACAP 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 rats apoptosis was increased (Figure 4). The PACAP-38 significantly inhibited endothelial apoptosis as shown by the decreased caspase3/7 activity, restoring it to levels observed in young cells (Figure 4).

Effect of PACAP on Expression of VEGF and VEGFR2

Quantitative real time RT–PCR technique was performed to elucidate the effect of PACAP on the expression of factors involved in VEGF signaling. Neither overexpression of PACAP in CMVECs derived from aged rats (Figure 5A) nor downregulation of the peptide in young cells (not shown) influenced the expression of VEGF (not shown). The mRNA expression of VEGFR2, the receptor which is mainly responsible for mediating the proangiogenic effects of VEGF, was upregulated by increased expression of PACAP in aged cells (Figure 5B).

Effect of PACAP on Cellular and Mitochondrial ROS Production in CMVECs

Aging is associated with increased cellular ROS production (46), which contributes to cerebromicrovascular endothelial dysfunction (47). As shown by the DHE and MitoSox fluorescence-based measurements, PACAP-38 treatment exerted no significant effect on cellular and mitochondrial ROS production in CMVECs (Figure 6).

Discussion

This is the first study to demonstrate the protective effects of PACAP on function and phenotype of CMVECs derived from a rodent model of aging that recapitulates cerebrovascular alterations and deficits present in elderly humans at risk for vascular coginitive impairment. There is increasing evidence in support of the hypothesis that the composition of the age-associated vascular secretory phenotype is a key determinant in the development of age-related diseases. In support of this hypothesis previous studies demonstrate that aging in endothelial cells is associated with a proinflammatory
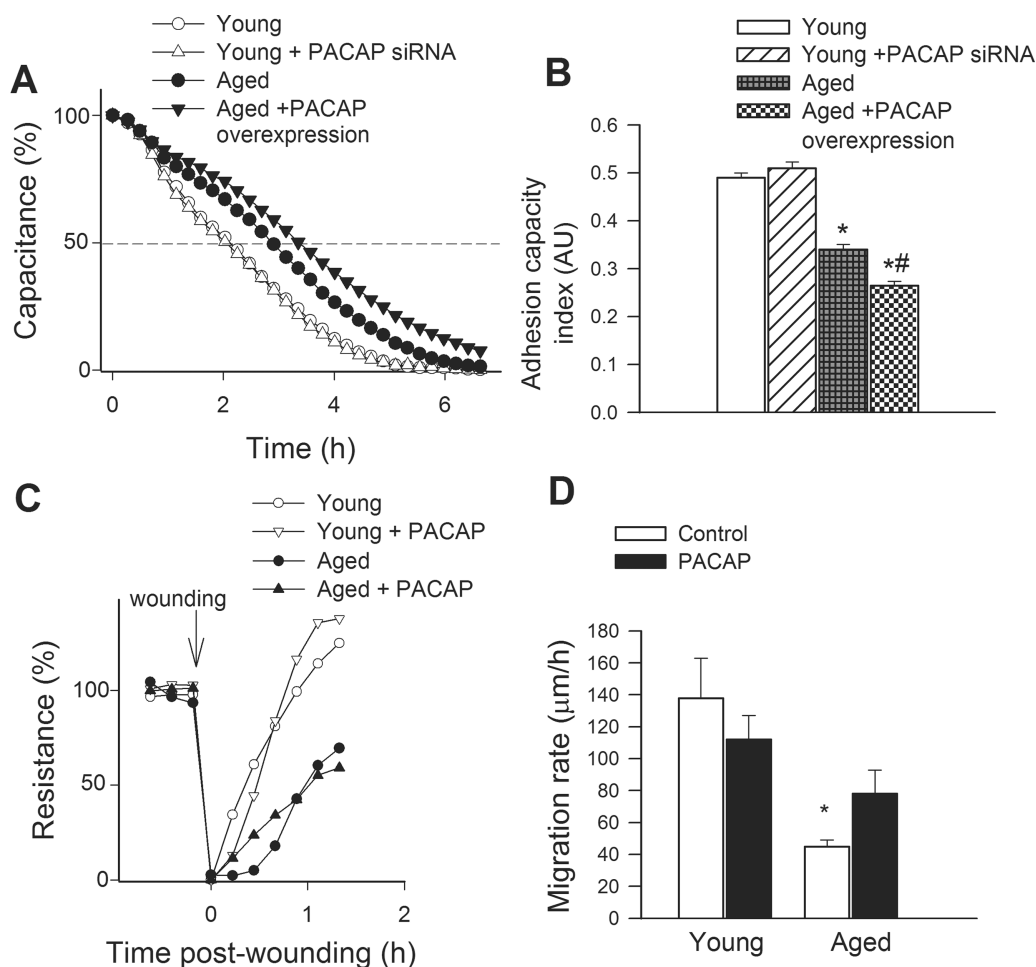
Figure 3. Effects of PACAP on adhesion capacity and migration capacity of cerebromicrovascular endothelial cells (CMVECs). (**A**) In young CMVECs with or without transfection with PACAP shRNA and aged CMVECs with or without overexpression of PACAP adhesion to collagen was monitored by electric cell-substrate impedance sensing (ECIS) technology (see Methods section). Time course of changes of capacitance (at 32 kHz) after addition of CMVECs to collagen-coated wells is depicted in Panel A. An 100% change corresponds to the maximum level of cell coverage reached on the active electrode. We calculated the time constant from each individual dataset, the inverse of which was used as an index of adhesiveness. (**B**) The summary data for cell adhesion index in CMVECs from each experimental group. Data are means ± standard error of the mean ($n = 5$ in each group). *$p < .05$ vs. young control, #$p < .05$ vs. aged control. (**C**) Cell migration was monitored by electric cell-substrate impedance sensing (ECIS) technology in a wound-healing assay (see Methods section). The time course of resistance recovery after wounding (electric pulse of 5 mA for 20 seconds at 60 kHz; 100% represents prewounding levels) in cultures of young and aged CMVECs with or without treatment with PACAP-38 is clearly shown. Time to reach 50% resistance recovery (corresponding to 50% confluence on the active electrode) was determined for each group and this parameter and the known physical dimensions of the electrode were used to calculate the migration rate (expressed as μm/h). (**D**) The summary data for migration rate in each group. Data are means ± S.E.M. ($n = 5$ in each group), *$p < .05$ vs. young control.

shift in cytokine expression profile (48) and alterations in the secretion of trophic factors and autocrine/paracrine growth factors (for example, BDNF, TGFβ). Recent studies demonstrate that CMVECs are an important source of the trophic factor PACAP (18), which is known to regulate the function of a range of cell types in the brain. Here, we demonstrate that aging leads to downregulation of PACAP in CMVECs (Figure 1), extending earlier findings showing that secretion of PACAP by cerebral microvessels significantly declines with age (18).

We found that CMVECs express both PACAP and PACAP receptors (Figure 1). The findings that knockdown of PACAP in young CMVECs impairs capillary morphogenesis and overexpression of PACAP or treatment with PACAP improves the ability of aged CMVECs to form capillary-like structures (Figure 2) suggest that dysfunction of a PACAP/PACAP receptor autocrine system contributes to the age-related impairment of endothelial angiogenic

capacity. Although the proangiogenic effects of PACAP are strongly supported by observations in tumor models [reviewed in ref. (24)], further studies are needed to determine whether genetic depletion of PACAP results in cerebromicrovascular rarefaction in mice, mimicking the aging phenotype. Future studies should also determine whether restoration of PACAP signaling in aging can improve endothelial angiogenic capacity in vivo, increasing cerebromicrovascular density. Interestingly, not every step (for example, adhesion and migration) in the multistep process of angiogenesis appears to be regulated by PACAP (Figure 3), suggesting that its regulatory role is different from that of VEGF and other proangiogenic growth factors.

Several lines of evidence indicate that endothelial cell apoptosis may play a major role in regulation of angiogenesis [reviewed in Ref. (49)]. Previously aging was shown to be associated with significantly increased endothelial apoptosis (50–52). Counteracting proliferation, age-related excessive apoptosis likely limits angiogenesis
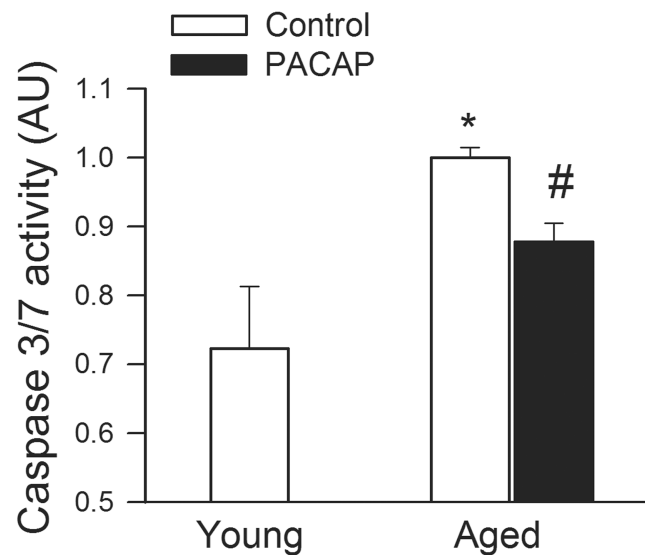
Figure 4. PACAP significantly inhibits apoptosis in aged cerebromicrovascular endothelial cells (CMVECs). Apoptotic cell death was assessed by measuring caspase 3/7 activity in cell lysates. *$p < .05$ vs. control, #$p < .05$ vs. aged. Data are mean ± S.E.M. ($n = 5$ for each group).
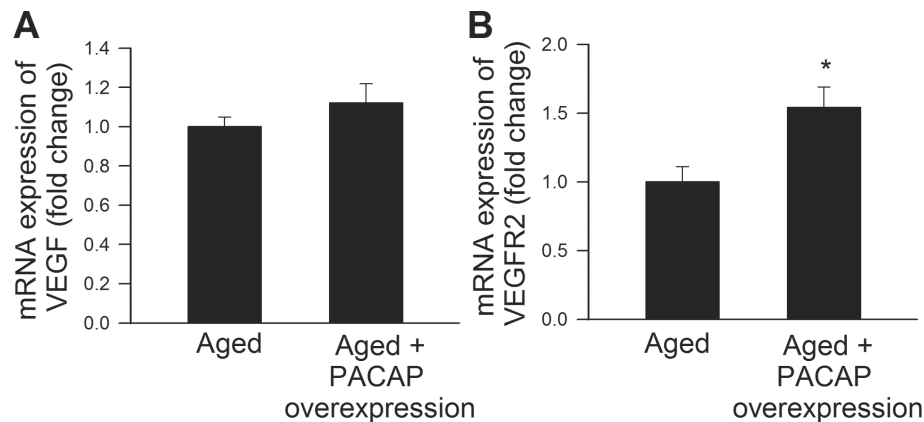

Figure 5. Quantitative real time RT–PCR data showing the effect of overexpression of PACAP on mRNA expression of VEGF (**A**) and VEGFR2 (**B**) in aged cerebromicrovascular endothelial cells (CMVECs). Data are means ± standard error of the mean ($n = 5$ in each group), *$p < .05$ vs. control.

and may actively lead to regression of cerebral microvessels (49). Growth factors, which confer antiaging vascular effects, not only stimulate endothelial cell proliferation and migration but also concomitantly inhibit endothelial cell apoptosis (49). In agreement with its putative role as a proangiogenic and antiaging autocrine mediator, PACAP was found to attenuate apoptotic cell death in aged CMVECs (Figure 4). Previous studies also demonstrate that PACAP inhibits H_2O_2-induced apoptosis in young endothelial cells (23). The antiapoptotic action of PACAP has also been demonstrated in several other cell types, including human hemangioendothelioma cells, kidney cells, and retinal pigment epithelial cells, and attributed to phosphorylation of the antiapoptotic ERK1/2 and/or decreases the proapoptotic activation of JNK and p38MAPK (23,53–55). In the present study, we show that overexpression of PACAP upregulates the expression of VEGFR2 in aged CMVECs, suggesting that a crosstalk between autocrine PACAP and VEGF signaling may contribute to the proangiogenic and antiapoptotic effects of PACAP (Figure 5). This concept is supported by previous studies in a variety of cell types showing that PACAP/VIP and VEGF signaling are functionally linked (56–59). Further studies are warranted

to experimentally test this hypothesis in CMVECs. Increased oxidative stress was suggested to contribute to impairment of endothelial angiogenic capacity, endothelial apoptosis, and thereby to capillary rarefaction (60). Although previous studies suggested that PACAP may increase cellular resistance against oxidative stress (14,55), in this study PACAP treatment had no effect on increased ROS production in aged CMVECs (Figure 6).

Findings from previous investigations suggest that in addition to regulation of angiogenesis PACAP also regulates other aspects of cerebromicrovascular endothelial function. Accordingly, we have recently provided evidence that PACAP increases transendothelial electrical resistance and reduces disassembly of tight and adherens junctions in pathological conditions (20). Because there is evidence that aging is associated with dysregulation of tight junctions and disruption of the blood–brain barrier (61–63), further studies are warranted to elucidate the role of PACAP deficiency in these alterations. In addition to its autocrine endothelial effect, endothelium-derived PACAP is also likely to cross the blood–brain barrier and regulate the function of neurons (9,10,12,13), pericytes (64), astrocytes (65,66), and microglia (67) as well. The PACAP receptors are
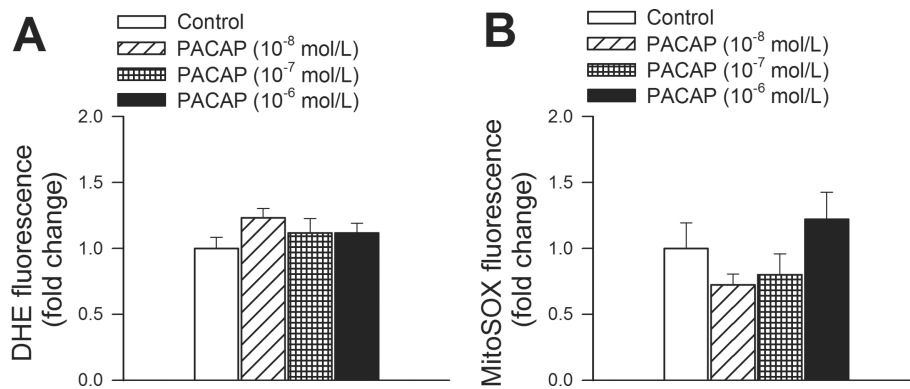
Figure 6. PACAP decreases oxidative stress in aged cerebromicrovascular endothelial cells (CMVECs). (**A** and **B**) Flow cytometric analysis of DHE- (A) and MitoSox- (B) fluorescence (indicating cellular and mitochondrial ROS production, respectively) in primary CMVECs derived from aged Fischer 344 x Brown Norway rats. The effect of PACAP treatment (10^{-8} to 10^{-6} mol/l, for 24 h) on ROS production by aged CMVECs. Data are mean ± standard error of the mean ($n = 8$ in each group).

widely distributed in the central nervous system, including the hypothalamus, cerebral cortex, hippocampus, amygdala, substantia nigra, dentate gyrus, cerebellum, and pons (7,68–70) and its neuromodulator and neurotrophic actions have been well characterized [reviewed in Ref. (9)]. Because PACAP was shown to exert multifaceted neuroprotective actions in several neuropathological disorders (9,71–74), further studies are warranted to elucidate the role of impaired microvascular production of PACAP in development of age-related neurodegenerative diseases.

The PACAP and VIP belong to the same superfamily of neuropeptides which exert their effects by activating G-protein-coupled receptors. Importantly, VIP participates in the pathophysiology of several neurological disorders, inhibiting cell death and confering anti-inflammatory and neuroprotective effects. Because microvascular endothelial cells express PACAP, VIP, and PACAP receptors (PAC1R, VPACR-1, and VPACR-2), the existence of a complex microvascular autocrine regulatory mechanism seems to be very likely. Because there are data suggesting that aging may suppress VIP expression in the brain, age-related decline in PACAP and VIP may exert synergistic effects (75–77).

In conclusion, autocrine PACAP activates endogenous proangiogenic and antiapoptotic mechanisms in CMVECs. We propose that dysregulation of PACAP signaling contributes to aging-induced impairment of angiogenic capacity of CMVECs, which may contribute to cerebromicrovascular rarefaction in aging. Aging-induced phenotypic alterations of CMVECs were also shown to contribute to neurovascular uncoupling (78), disruption of the blood–brain barrier (79), and neuroinflammation (62), which are thought to contribute to the development of both vascular cognitive impairment and Alzheimer's disease (79–81). Further studies are warranted to determine whether autocrine PACAP signaling also exerts protective effects against the aforementioned aging-induced phenotypic and functional endothelial alterations as well.

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