Impairment of angiogenesis - new capillary blood vessel formation from pre-existing vessels, is frequent in aging tissues and cells. Reduced angiogenesis in aging individuals is associated with increased incidence of myocardial infarctions and other cardiovascular diseases. Therefore there is a need to develop novel strategies to enhance angiogenesis in aging individuals. Our previous study demonstrated aging-related impairment of angiogenesis in aging (vs. young) rat myocardial microvascular endothelial cells (MMEC), and identified reduced activation of the vascular endothelial growth factor (VEGF, the most potent stimulator of angiogenesis) gene as the main underlying mechanism. In the present study we examined the possibility of increasing angiogenesis and activating VEGF gene expression in aging MMECs using a chemical activator of the metabolic sensor - AMP activated protein kinase (AMPK). We hypothesized that activation of VEGF gene in aging MMECs by AMPK would stimulate angiogenesis and reverse the impairment in angiogenesis seen in these cells. We used MMECs isolated from aging (24 months old) Fisher F-344 rats and treated them with 5-amino-imidazole-4-carboxamide ribonucleotide (AICAR), a specific pharmacological stimulator of AMPK. We examined: 1) in vitro angiogenesis; and 2) the expression of phosphorylated AMPK, VEGF, and P-MAPK/Erk1/2. Treatment of aging MMECs with AICAR increased in vitro angiogenesis and VEGF mRNA expression by 2.1-fold and 3.7-fold, respectively. Furthermore, AICAR treatment resulted in phosphorylation of MAPK/Erk1/2. This study demonstrated the successful use AICAR to reverse aging-related impairment of angiogenesis in aging MMECs by enhancing VEGF gene expression and also identified phosphorylation of MAPK/Erk1/2 as a likely mechanism of these changes.

Key words: aging, AMP activated protein kinase, angiogenesis, myocardial microvascular endothelial cells, vascular endothelial growth factor
from the National Institute on Aging for the isolation of MMECs. This strain of rats has been frequently used in studies on aging.

Isolation and cell culture of MMECs from young and aging rats

Aging MMECs were isolated from Fisher F-344 rats as described in our previous study (6). MMECs were characterized by positive staining for endothelial markers - von Willebrand’s factor (Factor VIII - related antigen) and PECAM-1 (CD31) and by absence of staining for the myofibroblast marker - smooth muscle α-actin. MMECs were grown on collagen coated dishes in endothelial cell growth media containing 10% FBS, heparin and endothelial cell growth supplements.

Assessment of in vitro angiogenesis by capillary-like tube formation on matrigel

An in vitro capillary tube formation assay was performed similar to our previous study (6, 13). Briefly, 2×10⁴ aging MMECs were cultured on a Matrigel (BD Biosciences, Mountain View, CA) coated plate with either medium alone (control) or with AICAR (1 mM; Sigma-Aldrich, St. Louis, MO). After 24 hours the plates were examined for capillary tube formation under an inverted Nikon microscope, photographed and quantified using MetaMorph 7.0 videomage analysis system (Molecular Devices, Downington, PA). Tube formation was quantified by measuring the total length of the capillary tubes in randomly selected fields, under 200× magnification.

Real-time RT-PCR

We examined VEGF mRNA levels by a reverse transcription real-time PCR method as described in our previous studies (6, 14). Briefly, total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) and 1 µg of total RNA was treated with deoxyribonuclease I and reverse transcribed using the GeneAmp RNA-PCR kit (Applied Biosystems, Foster City, CA). Quantitative PCR on 2.5 µl cDNA was performed with the iCycler real-time PCR detection system (Bio-Rad, Hercules, CA) with prevalidated QuantiTect assays (Qiagen, Valencia, CA). Levels of VEGF mRNA were normalized to the levels of 18S rRNA.

Protein extraction and immunoblotting

Proteins were extracted from cultured MMECs as previously described (6, 15). Cell lysates were subjected to SDS-PAGE and immunoblotting using specific antibodies against P-AMPK and P-AMPK/Erk1/2 (both 1:500, Cell Signaling Technology, MA) followed by peroxidase-conjugated secondary antibodies and chemiluminescence detection. The density of the band in Western blotting studies was quantified using a highly sensitive MetaMorph 7.0 videomage analysis system (Molecular Devices, Downington, PA).

Statistical analysis

Results were expressed as means ± standard deviation (S.D.). Comparisons were made using one-way analysis of variance (ANOVA) followed by Student’s t test. Student’s t test was used to determine statistical significance between each group. A P value of <0.05 was considered statistically significant.

RESULTS

AICAR activates AMP activated protein kinase enzyme in aging myocardial microvascular endothelial cells

AICAR (5-amino-imidazole-4-carboxamide ribonucleotide) is a cell-permeable strong and highly specific activator of AMPK. We treated MMECs with AICAR (1 mM; Sigma-Aldrich, St. Louis, MO) and examined AMPK activation (phosphorylation) by Western blotting using specific P-AMPK antibody (1:500, Cell Signaling Technology, MA). AICAR activated (phosphorylated) AMPK in aging MMECs 2.1-fold (P<0.01) vs. untreated cells (Fig. 1).

AIACR treatment reverse impairment of angiogenesis in aging rat myocardial microvascular endothelial cells

We compared angiogenesis in vitro on Matrigel in aging MMECs at baseline and after treatment with AICAR. We quantified tube formation by measuring the total length of the capillary tubes in randomly selected fields, under 200× magnification for 5 minutes.

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Results were expressed as means ± standard deviation (S.D.). Comparisons were made using one-way analysis of variance (ANOVA) followed by Student’s t test. Student’s t test was used to determine statistical significance between each group. A P value of <0.05 was considered statistically significant.

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magnification. AICAR significantly increased angiogenesis in aging MMECs by 2.1-fold (P<0.05) compared to untreated cells (Fig. 2).

**Fig. 2.** AICAR treatment increases in vitro angiogenesis in aging MMECs. (A) In vitro angiogenesis in aging MMECs was determined on growth factor-reduced matrigel at baseline and following AICAR treatment. Photographs were taken after 24 h (100× magnification). (B) Quantitative analysis of in vitro angiogenesis. Treatment with AICAR significantly increases angiogenesis in aging MMECs. The data are expressed as capillary tube length in pixels (mean ±S.D.) and represent three experiments performed in triplicate (n=9).

**Activation of AMP activated protein kinase by AICAR increases vascular endothelial growth factor mRNA expression in aging myocardial microvascular endothelial cells**

We determined the levels of VEGF mRNA using quantitative RT-PCR in aging MMECs following treatment with or without AICAR. In aging MMECs, AICAR treatment significantly increased expression of VEGF mRNA by 3.7-fold (P<0.05) as compared to untreated cells (Fig. 3).

**Fig. 3.** AICAR treatment increases VEGF levels in aging MMECs. Expression of VEGF mRNA in aging MMECs using quantitative RT-PCR. VEGF mRNA expression is significantly increased following AICAR treatment in aging MMECs. The values are expressed as mean ±S.D. and represent two experiments done in duplicate (n=4).

**DISCUSSION**

This study demonstrated, for the first time that the impairment of angiogenesis and reduction in VEGF gene expression in aging myocardial microvascular endothelial cells can be reversed by activating the AMP-activated protein kinase using a pharmacological stimulator, AICAR.

We previously demonstrated reduced angiogenesis and VEGF levels in aging myocardial microvascular endothelial...
cells (6). We show here for the first time that the AMPK signaling cascade regulates VEGF expression and angiogenesis, in myocardial microvascular endothelial cells. The role of AMPK in angiogenesis in the myocardium and in aging-related cardiovascular diseases has not been examined before. A previous study showed that activation of the AMPK signaling cascade by AICAR in mouse myoblasts increases VEGF production in these cells and can promote angiogenesis (16). However, that study did not examine the role of AMPK signaling in aging cells. Our present findings indicate that the function and/or sensitivity of the metabolic sensor, AMPK, are impaired in the aging myocardium and that treatment with AICAR can reverse these abnormalities.

We examined the mechanisms mediating AICAR induced increase in angiogenesis and VEGF gene expression in aging MMECs. AICAR treatment triggered the activation (phosphorylation) of MAPK/Erk1/2 in aging MMECs. Our previous study demonstrated that the MAPK/Erk1/2 signaling cascade is critical in human dermal microvascular endothelial cells for stimulation of angiogenesis by the peptide hormone ghrelin (17). Taken together these findings indicate a potential critical requirement of MAPK-Erk1/2 signaling, in endothelial cells in general, for angiogenesis.

In summary, the present study has identified novel pathways that regulate angiogenesis in aging MMECs. These include activation of P-AMPK, phosphorylation of MAPK/Erk1/2 and increased VEGF expression. Our findings demonstrate a crucial role of AMPK in angiogenesis and VEGF expression in the myocardium, and identify AMPK as a new therapeutic target for enhancing angiogenesis in the aging heart. Our findings may be relevant in other pathological conditions such as ischemic limb disease. VEGF treatment has been shown to improve healing of ischemic lesions by the formation of new collateral vessels in patients with severe chronic lower limb ischemia (18). AICAR, a pharmacological stimulator of AMPK induces VEGF expression and offers significant potential as a novel therapy for improving angiogenesis in cardiovascular disease and other pathological conditions.

Acknowledgments: This research was supported by the AHA grant-in-aid (AA).

Conflict of interests: None declared.

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Received: August 26, 2011
Accepted: September 16, 2011

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