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Authors

Ahluwalia, A
Narula, J
Jones, M K
et al.

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A. AHLUWALIA, J. NARULA, M.K. JONES, X. DENG, A.S. TARNAWSKI

IMPAIRED ANGIOGENESIS IN AGING MYOCARDIAL MICROVASCULAR ENDOTHELIAL CELLS IS ASSOCIATED WITH REDUCED IMPORTIN α AND DECREASED NUCLEAR TRANSPORT OF HIF1α: MECHANISTIC IMPLICATIONS

VA Long Beach Healthcare System, Long Beach, CA, USA, University of California, Irvine, CA, USA

Aging is associated with increased incidence of myocardial infarctions and impaired angiogenesis - new capillary blood vessel formation from preexisting vessels. The molecular mechanism(s) of aging-related impairment of angiogenesis are unknown. In the present study we focused on the mechanism of activation of the gene for vascular endothelial growth factor (VEGF - the most potent stimulator of angiogenesis) in young and aging myocardial microvascular endothelial cells (MMEC). Activation of VEGF gene in the cell nucleus is mediated in part by the transcription factor hypoxia-inducible factor 1α (HIF1α). In order to activate VEGF gene, HIF1α must first be transported to the nucleus, but the mechanisms of this transport are unknown. We hypothesized that reduced VEGF gene activation and impaired angiogenesis in myocardium during aging can result from downregulation of the nuclear transport receptor - importin α that leads to decreased transport of HIF1α to the nucleus. We examined in MMEC isolated from young (3 months of age) and aging (24 months old) Fisher F-344 rats: 1) *in vitro* angiogenesis; and 2) the expression of VEGF, importin α and HIF1α. Aging MMEC exhibited a 3.7-fold reduction in angiogenesis and a corresponding reduction in VEGF (by 3-fold) and importin α (by 1.9-fold) levels compared to young MMEC. Aging MMEC also exhibited cytoplasmic accumulation (by 1.8-fold) of HIF1α protein, reduced HIF1α transport to the nucleus and decreased binding of HIF1α protein to the VEGF gene promoter. This study is the first demonstration of the downregulation of importin α in aging MMEC and reduced nuclear transport of HIF1α, which likely lead to decreased VEGF gene activation and impaired angiogenesis.

Key words: *aging, angiogenesis, myocardial microvascular endothelial cells, importin α, hypoxia-inducible factor 1α, vascular endothelial growth factor*

INTRODUCTION

Aging is associated with increased incidence of cardiovascular diseases, including myocardial infarctions (1) and reduced tolerance to ischemia (2). Compared with younger patients, myocardial infarction in the elderly is associated with less favorable clinical outcomes, which may be attributed to a decline in the angiogenic capacity of the aging heart (3). Angiogenesis - new capillary blood vessel formation from preexisting vessels, is essential for the healing of myocardial infarctions (4). However, angiogenesis is reduced and delayed in aging, which impairs revascularization of ischemic tissues and healing of tissue injury. The aging-associated decrease in angiogenesis is also associated with reduced expression of vascular endothelial growth factor (VEGF) and contributes to ischemia in the heart (5, 6). VEGF is an endothelial-specific angiogenic growth factor that initiates and regulates angiogenesis in injured tissues by promoting endothelial cell migration, proliferation, survival and tube and capillary blood vessel formation (7). VEGF gene activation is mediated by the transcription factor hypoxia-inducible factor 1α (HIF1α) (8, 9). In order to activate the VEGF gene, HIF1α must be transported to the nucleus; however the mechanism of this transport in endothelial cells is not known. Importin α and β are nuclear transport receptors (10) which bind to nuclear localization signals on target proteins like retinoblastoma protein and can promote their transport into the nucleus (11). We hypothesized that importin α is the key molecule in transport of HIF1α to the nucleus. In addition, the mechanisms of aging-related impairment of angiogenesis in myocardial microvascular endothelial cells (MMEC), and the roles of importin α in angiogenesis and in aging have not been examined before. We hypothesized that impaired angiogenesis and reduced VEGF expression in aging MMEC is due to reduced importin α expression leading to impaired nuclear transport of HIF1α into the nucleus and thus reduced VEGF gene activation. The aims of the present study were to examine in aging MMEC the mechanism of impaired angiogenesis with a focus on novel targets and mechanisms of VEGF gene activation - downregulation of importin α and impaired nuclear transport of HIF1α.

MATERIALS AND METHODS

Experimental animals

All animal studies described here were conducted with the approval of the subcommittee on animal studies (IACUC) of the

VA Healthcare System, Long Beach, CA. We used 3 months of age (young) and 24 months of age (aging) Fisher F-344 (*H. pylori* and viral free) rats purchased from the National Institute on Aging for the isolation of MMEC. This strain of rats has been frequently used in studies on aging.

Isolation and cell culture of MMEC from young and aging rats

Young and aging MMEC were isolated from Fisher F-344 rats using a protocol similar to that used in our previous study (12). Rats were euthanized and hearts removed, cut into small pieces and incubated with collagenase II. Following trypsinization, cells were passed through polypropylene meshes. Cells were then PECAM-1 selected using anti-PECAM-1 (anti-CD31) antibody (Chemicon, Temecula, CA) by magnetic bead separation (Miltenyi Biotec Inc., Auburn, CA). MMEC were grown on collagen coated dishes in endothelial cell growth media containing 10% FBS, heparin and endothelial cell growth supplements. The isolated MMEC 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.

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

An *in vitro* capillary tube formation assay was performed as described in our previous studies (13). Briefly, 2×10^4 MMEC were plated on a Matrigel (BD Biosciences, Mountain View, CA) coated plate with either medium alone (control) or with VEGF (20 ng/ml). After 24 hours the plates were examined for capillary tube formation under an inverted Nikon microscope, photographed and quantified using MetaMorph 7.0 videoimage 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 x magnification.

Real-time RT-PCR

We examined expression levels of VEGF, importin α1 and importin α3 genes by a reverse transcription real-time PCR method as described in our previous study (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 mRNA were normalized to the levels of 18S rRNA.

Protein extraction and immunoblotting

Proteins were extracted from cultured MMEC as previously described (15). Cell lysates were subjected to SDS-PAGE and immunoblotting using specific antibodies against VEGF (1:250, Santa Cruz Biotechnology, Santa Cruz, CA, HIF1α (1:250, Novus Biologicals, Littleton, CO) or importin α (1:500, Sigma-Aldrich, St. Louis, MO) 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 videoimage analysis system (Molecular Devices, Downington, PA) that also allows detection and quantitation of bands with relatively low density, which are not apparent visually.

Immunocytochemistry (ICC)

Protein expression and localization in MMEC were analyzed by ICC staining as previously described (13). Briefly, MMEC were cultured on collagen- coated coverslips, fixed in paraformaldehyde and then permeabilized with acetone. Cells were incubated with antibodies against HIF1α (1:100, Novus Biologicals, Littleton, CO), or importin α (1:100, Sigma-Aldrich, St. Louis, MO) followed by incubation with Alexa 488-conjugated secondary antibody (1:200; Invitrogen, Carlsbad, CA). Slides were then examined under a Nikon epifluorescence microscope, photographed and the immunofluorescence intensity quantified using MetaMorph 7.0 videoimage analysis system (Molecular Devices, Downington, PA).

Immunoprecipitation

Immunoprecipitation was carried out using HIF1α antibody (Novus Biologicals, Littleton, CO) followed by affinity purification using protein G-agarose beads (Roche Applied Science, Indianapolis, IN). Immunoprecipitates were separated on 10% SDS-polyacrylamide gels and probed with importin α antibody.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed using 50 μg of nuclear protein and a ^{32}P labeled probe containing the HIF1α consensus binding sequence 5'- TACGTGGG -3' (Operon Biotechnologies, Huntsville, AL) as described in a previous study (9). Nuclear extracts of PC12 cells cultured under hypoxic conditions (GeneTex Inc., San Antonio, TX) that express high levels of HIF1α were used as a positive control for HIF1α binding activity. A competition reaction in the presence of 200-fold excess unlabeled probe was used as a control. The protein-DNA complexes were subjected to polyacrylamide gel electrophoresis, subsequently dried and exposed to X-ray film.

Statistical analysis

Results were expressed as means±standard deviation (SD). 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

Angiogenesis in aging rat MMEC is impaired

We compared angiogenesis *in vitro* on Matrigel in young and aging MMEC and quantified tube formation by measuring the total length of the capillary tubes in randomly selected fields, under 200 x magnification. MMEC isolated from hearts of aging rats demonstrated a significant 3.7-fold ($P<0.001$) reduction in angiogenic capability compared to cells isolated from young rats (*Fig. 1*).

VEGF gene expression is reduced in aging rat MMEC

Since VEGF is the most potent angiogenic growth factor, we determined the levels of VEGF mRNA and protein using quantitative RT-PCR and Western blotting, respectively in young and aging MMEC. There was a 4.6-fold ($P<0.01$) reduction in VEGF mRNA and a 3-fold ($P<0.05$) decrease in VEGF protein in aging *vs*. young MMEC (*Fig. 2*).

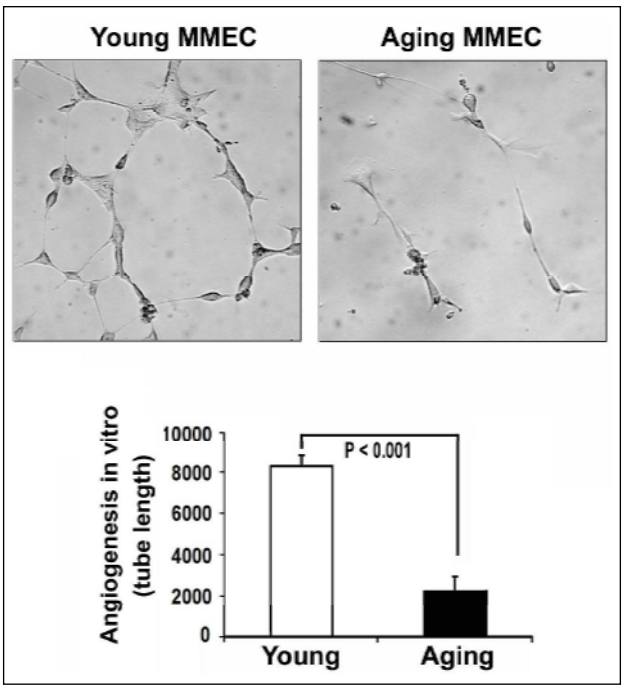

Fig. 1. Decreased *in vitro* angiogenesis in aging myocardial microvascular endothelial cells (MMEC). (A) *In vitro* angiogenesis in young and aging MMEC was determined at baseline on growth factor-reduced matrigel. Photographs were taken after 24 h (magnification 100×). The data are expressed as capillary tube length in pixels (mean±SD) and represent three experiments performed in triplicate (n=9).

Aging MMEC have reduced importin α levels

We examined the expression of importin α nuclear transport receptor that mediates transport of target proteins to the nucleus. Quantitative RT-PCR studies demonstrated a significant decrease in importin α1 and importin α3 mRNA expression in aging MMEC (*Fig. 3A*). Western blotting showed that aging MMEC have a 1.9-fold (P<0.05) lower level of importin α protein than young MMEC (*Fig. 3B*). Immunofluorescence staining also confirmed that aging MMEC have reduced importin α protein expression compared to young MMEC (*Fig. 3C*).

Aging MMEC exhibit cytoplasmic accumulation of HIF1α likely due to reduced nuclear transport by importin α.

Since HIF1α transcription factor is known to activate the VEGF gene in several tissues and cell lines, we examined if in aging MMEC downregulation of HIF1α protein is the mechanism for reduced VEGF gene expression. Aging MMEC exhibited a significant cytoplasmic accumulation (1.8-fold; P<0.05) of HIF1α protein and absence of nuclear HIF1α (*Fig. 4A*) as determined by Western blotting studies using cytoplasmic and nuclear protein extracts of young and aging MMEC. Immunofluorescence studies confirmed the absence of HIF1α protein in the nucleus and its concurrent cytoplasmic accumulation in aging MMEC (*Fig. 4B*).

To determine the binding of HIF1α protein to importin α, we examined HIF1α-importin α interaction in nuclear protein extracts isolated from young and aging MMEC. Co-immunoprecipitation studies demonstrated that the binding of HIF1α to importin α was 1.7-fold lower in nuclear extracts from

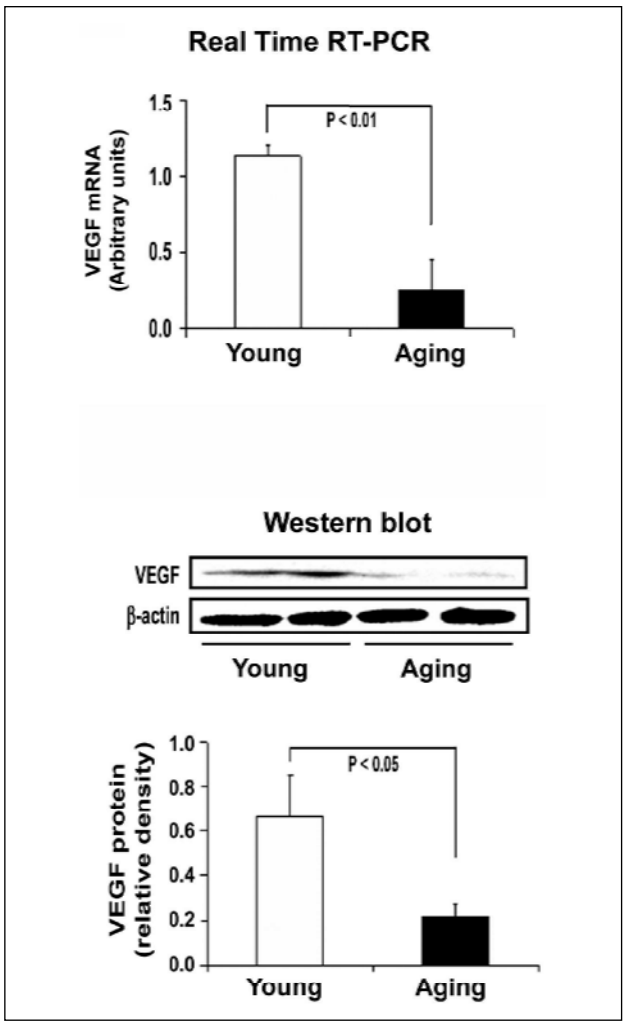

Fig. 2. Aging MMEC exhibit reduced VEGF levels. Expression of VEGF mRNA and protein levels in young and aging MMEC using quantitative RT-PCR and Western blotting, respectively. Both VEGF mRNA and protein are significantly downregulated in aging MMEC compared to young MMEC. The values are expressed as mean±SD and represent two experiments done in duplicate (n=4).

aging MMEC than from young MMEC (*Fig. 4C*). EMSA demonstrated reduced binding of HIF1α to VEGF promoter in MMEC. (*Fig. 4D*). These findings indicate that reduced importin α and decreased nuclear transport of HIF1α may be responsible for reduced VEGF expression in aging MMEC.

DISCUSSION

This study demonstrated for the first time in aging MMEC: impaired angiogenesis and reduced VEGF gene activation associated with the reduced expression of the nuclear transport protein importin α, reduced binding of importin α to HIF1α and impaired nuclear transport of HIF1α compared to young MMEC.

Although a decline in the angiogenic potential of the aging heart has been reported and its contribution to consequences of ischemic syndromes well described (16, 17), the mechanisms of aging-related impairment of angiogenesis are not clearly defined. We recently demonstrated reduced ghrelin levels and impaired angiogenesis in aging human dermal microvascular

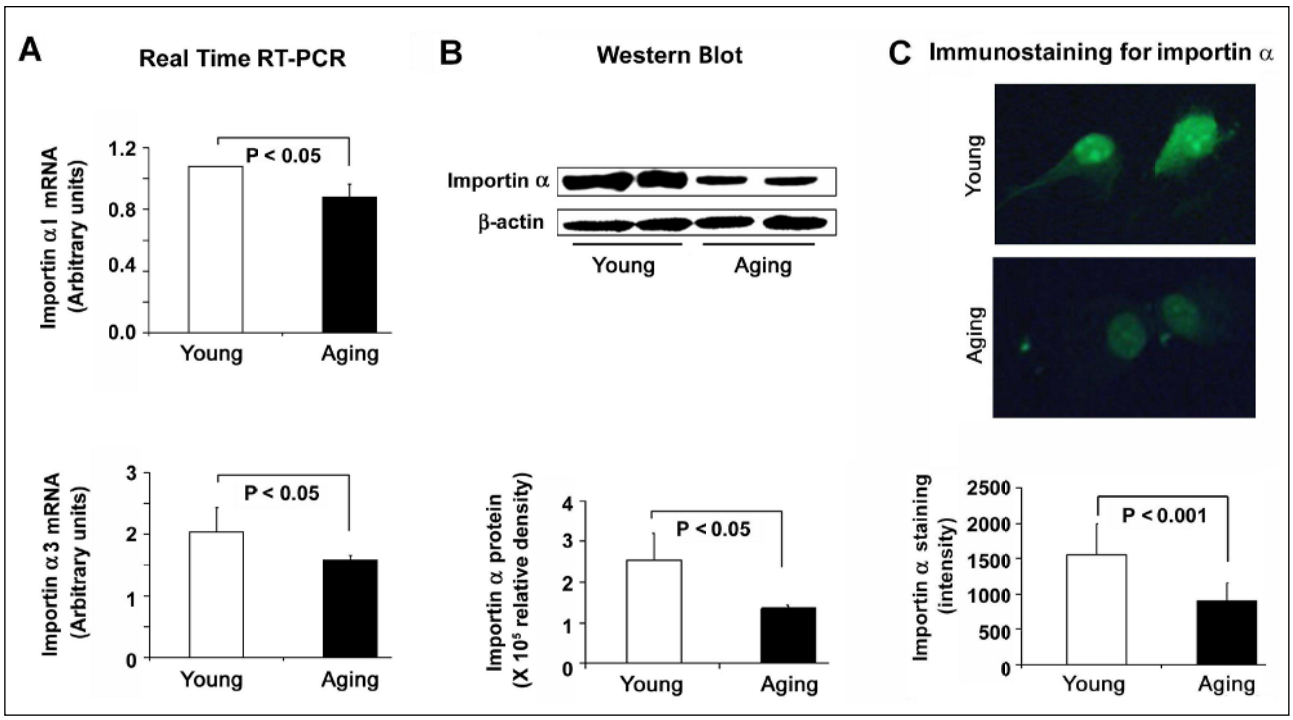

Fig. 3. (A) Importin α mRNA expression by real time RT-PCR. In aging MMEC importin α1 and importin α3 mRNA is significantly downregulated *vs*. young MMEC. (B) Representative Western blots demonstrate significantly reduced importin α protein expression in aging (*vs*. young) MMEC. The values are expressed as mean±SD and represent two experiments performed in duplicate (n=4). (C) Immunofluorescence staining of young and aging MMEC for importin α shows a decrease in importin α level in aging MMEC *vs*. young MMEC (magnification 100×). Insets represent images at higher magnification (magnification 500×). The data are expressed as immunofluorescence intensity (mean±SD) in six randomly selected fields per group.

endothelial cells and also showed the restoration of angiogenesis by ghrelin (18).

We show here for the first time that endothelial cells isolated from hearts of aging rats have a significantly reduced angiogenic capability and reduced expression of VEGF, the most potent angiogenic growth factor (16, 19, 20) compared to those isolated from young rats.

Transcriptional regulation of the VEGF gene involves several transcription factors including hypoxia inducible factor-1 (HIF-1) (8, 9). HIF-1 is a dimeric protein complex consisting of two subunits, HIF1α and HIF1β. HIF1β is a hypoxia independent, constitutively expressed protein, while HIF1α is continually generated in cells, but under normoxia conditions, it is rapidly degraded in proteasomes by von-Hippel-Lindau tumor suppressor protein (VHL)-mediated ubiquitination (8, 21). In contrast, under hypoxia conditions, HIF1α protein rapidly accumulates in some cells following stabilization by the heat shock protein HSP90 and after dimerization with HIF1β, it is translocated to the nucleus (9, 21). In the nucleus the HIF1 complex binds to the hypoxia response elements (HRE) within the VEGF gene promoter, recruits additional transcriptional factors like phosphorylated cAMP response element binding protein (P-CREB) and phosphorylated signal transducer and activator of transcription 3 (P-STAT3) to the VEGF gene promoter and initiates VEGF transcription (9). Thus the transport of HIF1α to the nucleus is critical for VEGF gene activation. However, the precise mechanisms of HIF1α transport to the nucleus are not known forming the basis for this study.

Our study demonstrated in aging MMEC the absence of HIF1α protein in the nucleus concurrent with its cytoplasmic accumulation. The HIF1α levels in the nuclear fraction of both young and aging cells were not detectable by Western blotting. However; the co-immunoprecipitation studies and the EMSA results summarized in *Fig. 4C* and *Fig. 4D*, respectively, do reflect that there is HIF1α protein in the nucleus of young cells and aging cells but the levels are possibly too low to be detected by Western blotting. Our findings are consistent with the fact that under the normoxia conditions for culture of these cells, we do not expect (based on literature) large amounts of HIF1α protein, since it is promptly degraded in proteasomes (8, 21). Our studies in these cells cultured under hypoxia condition demonstrate detectable by Western blotting HIF1α protein levels in the nuclear fraction of young and aging endothelial cells (unpublished data).

The increased cytoplasmic accumulation of HIF1α in aging MMEC can likely be attributed to impaired transport of HIF1α from the cytoplasm into the nucleus due to decreased expression and/or impaired function of importins that are required for the nuclear transport of proteins. In general, import of large proteins (>40-60 kDa) into the nucleus takes place through nuclear pore complexes within the nuclear membrane and requires the presence in the cytoplasm of specific carrier proteins, importins (also referred to as karyopherins) (10, 11). Importins consist of two subunits, importin α and importin β that form heterodimers. Of these, importin α acts as an adaptor protein that binds to the the nuclear localization signal (NLS) of the protein to be imported to the nucleus, whereas importin β mediates interactions with the pore complex and helps in the docking of the importin heterodimer-bound protein to the nuclear pore complex. However, the possible role of importin α in nuclear transport of HIF1α has not been examined before. We demonstrated that aging MMEC have reduced levels of importin α compared to young MMEC. Decrease in importin α expression in aging MMEC in our study is consistent with a recently reported aging-associated reduction in importin α but

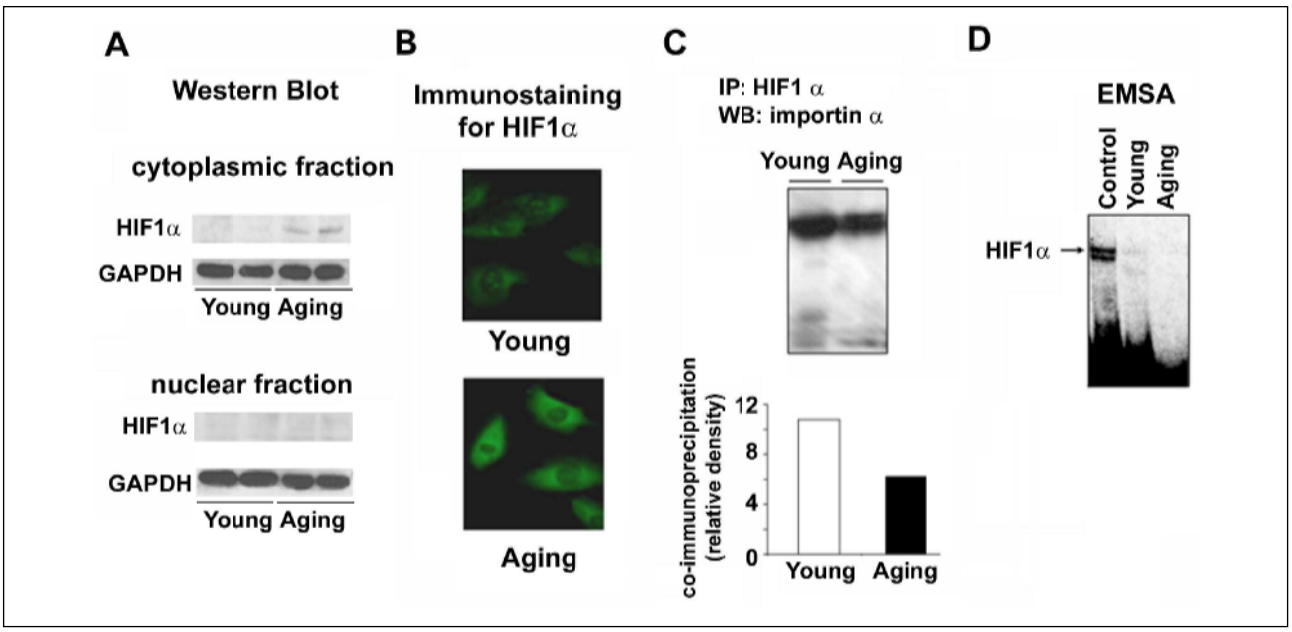

Fig. 4. Cytoplasmic accumulation and reduced nuclear expression of HIF1α in aging (*vs*. young) MMEC leads to its reduced binding VEGF gene promoter. (A). Representative Western blots demonstrate that HIF1α protein expression in aging (*vs*. young) MMEC is significantly increased in the cytoplasm. The values are expressed as mean±SD and represent two experiments performed in duplicate (n=4). (B) Immunofluorescence staining for HIF1α shows an increased cytoplasmic accumulation of HIF1α in aging (*vs*. young) MMEC (magnification 100×). (C) Binding of HIF1α to importin α in nuclear extracts from young and aging MMEC was determined using co-immunoprecipitation studies. Nuclear extracts were subjected to immunoprecipitation with anti-HIF1α antibody. Samples were then reduced, evaluated *via* 10% SDS-PAGE and immunoblotted with anti- importin α antibody. Binding of HIF1α to importin α was higher in nuclear extracts from young MMEC than from aging MMEC. (D) Aging (*vs.* young) MMEC exhibit reduced HIF1α DNA binding activity. Nuclear extracts were incubated with ^{32}P labeled probe containing the HIF1α binding site. Resulting complexes were resolved by electrophoretic mobility shift assay (EMSA). Lane 1 represents PC12 cells cultured under hypoxic conditions (that express high levels of HIF1α) used as a positive control for HIF1α binding activity and lanes 2 and 3 represent young and aging MMEC cultured under normoxia.

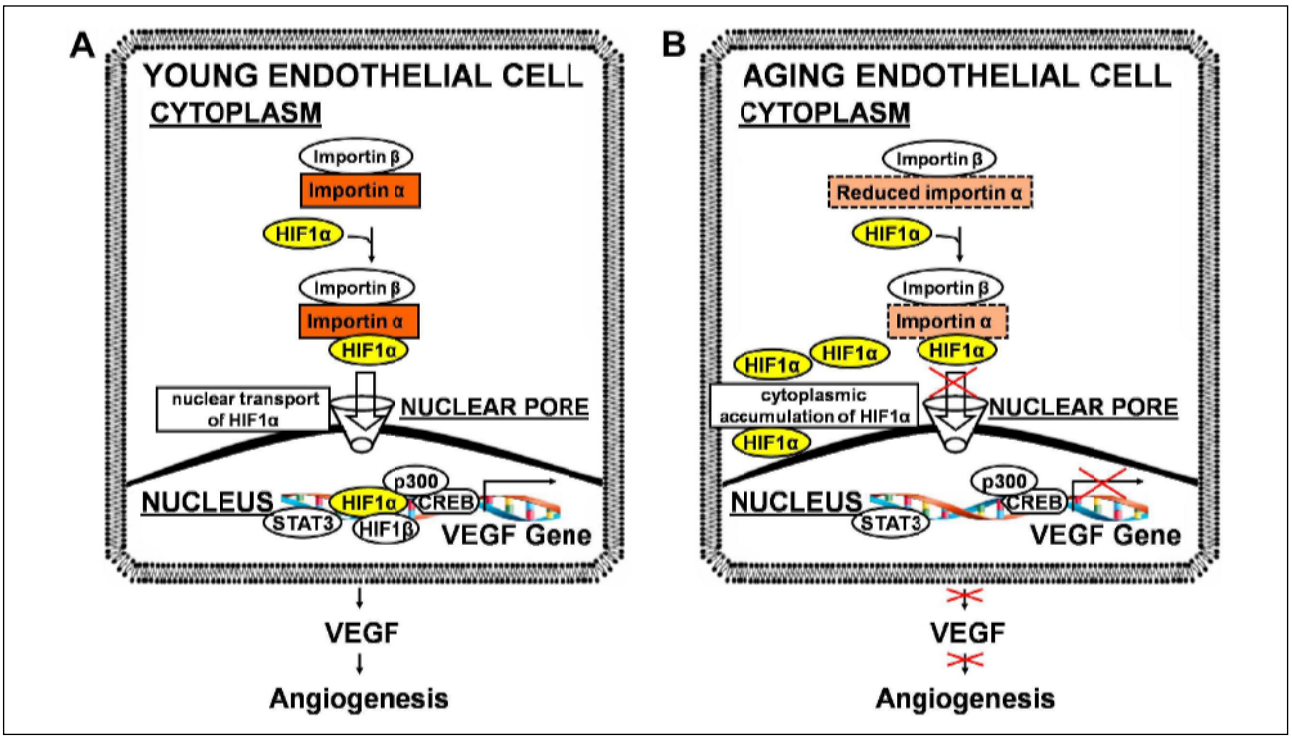

Fig. 5. Novel players and new mechanism of VEGF gene activation and angiogenesis in MMEC. Diagrammatic representation of proposed mechanism of aging-related impairment of angiogenesis. (A) In young MMEC importin α binds to and transports the transcription factor HIF1α to the nucleus where HIF1α activates VEGF gene leading to expression of pro-angiogenic VEGF protein that promotes angiogenesis. (B) On the contrary, in aging MMEC reduced levels of importin α lead to impairment of nuclear transport and hence cytoplasmic accumulation of HIF1α resulting in reduced VEGF protein levels and impairment of angiogenesis.

not importin β expression levels in human skin fibroblasts (22). Only one previous study demonstrated that *in vitro* synthesized HIF1α protein interacts with the nuclear transport receptor importin α (23), and substantiates our findings of the binding of HIF1α protein with importin α protein. The dysregulation of this process in aging MMEC may explain decreased nuclear expression and transcription factor function of HIF1α leading to reduced activation of the VEGF gene and hence impairment of angiogenesis.

In order to determine whether the mechanism demonstrated here - reduced importin α and reduced VEGF may apply to aging endothelial cells from other tissues and to human cells, we performed similar preliminary studies in human dermal microvascular cells and also in rat gastric mucosal microvascular endothelial cells. Both these cell types showed aging-related decrease in importin α and VEGF protein expression and impaired *in vitro* angiogenesis. Therefore, these studies in cells from other species and tissues indicate the fundamental paradigm found in our present study in aging endothelial cells may be generally applicable.

Our findings may be relevant to other non-aging- related angiogenesis for example in cancer and during exercise. Angiogenesis is also required for cancer growth, as shown first by Dr. Folkman (24). A recent article reviewed the role of HIF1α inhibition in reducing angiogenesis and decreasing tumor survival and identified potential therapeutic agents that inhibit HIF1α (25). A recent study reviewed the adaptations of ischemic muscle to exercise showing that the increase in collateral blood flow observed with exercise progresses over time of training and enhances the responses observed with angiogenic growth factors such as VEGF (26). It examined several of the proteins that were also examined in our study including HIF1α and VEGF proteins and described their role in angiogenesis during exercise. However it did not address aging-related impairment of angiogenesis.

In summary, the present study has identified several novel cellular and molecular events and mechanisms that mediate the impairment of angiogenesis in aging MMEC (*Fig. 5*). These include reduced importin α, increased cytoplasmic accumulation of HIF1α protein (but its absence in the nucleus - indicating impaired nuclear transport) and decreased transcriptional activation of the VEGF gene. We demonstrated that importin α binds to HIF1α and therefore is involved in the transport of HIF1α to the nucleus of endothelial cells and that during aging, importin α expression is reduced. In aging MMEC, the decreased importin α level likely impairs transport of HIF1α to the nucleus, resulting in reduced VEGF gene activation and thereby significantly diminished angiogenic capability of the aging MMEC. Our findings demonstrate a potential mechanistic role of importin α as one of the major players in myocardial angiogenesis and also as a potential therapeutic target.

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Author's address: Andrzej Tarnawski, M.D., D.Sc., University of California, Irvine, VA Long Beach Healthcare System; Phone: (562) 826-5437; Fax: (562) 826-8016; E-mail: atarnawski@yahoo.com