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Aging differentially alters the expression of angiogenic genes in a tissue-dependent manner

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ABSTRACT

Organ functions are altered and impaired during aging, thereby resulting in increased morbidity of age-related diseases such as Alzheimer's disease, diabetes, and heart failure in the elderly. Angiogenesis plays a crucial role in the maintenance of tissue homeostasis, and aging is known to reduce the angiogenic capacity in many tissues. Here, we report the differential effects of aging on the expression of angiogenic factors in different tissues, representing a potentially causes for age-related metabolic disorders. PCR-array analysis revealed that many of angiogenic genes were down-regulated in the white adipose tissue (WAT) of aged mice, whereas they were largely up-regulated in the skeletal muscle (SM) of aged mice compared to that in young mice. Consistently, blood vessel density was substantially reduced and hypoxia was exacerbated in WAT of aged mice compared to that in young mice. In contrast, blood vessel density in SM of aged mice was well preserved and was not different from that in young mice. Moreover, we identified that endoplasmic reticulum (ER) stress was strongly induced in both WAT and SM during aging *in vivo*. We also found that ER stress significantly reduced the expression of angiogenic genes in 3T3-L1 adipocytes, whereas it increased their expression in C2C12 myotubes *in vitro*. These results collectively indicate that aging differentially affects the expression of angiogenic genes in different tissues, and that aging-associated down-regulation of angiogenic genes in WAT, at least in part through ER stress, is potentially involved in the age-related adipose tissue dysfunction.

1. Introduction

With advancing age, the structure and function of many organs such as the brain, heart, pancreas, and bone is altered, and such changes are strongly associated with age-related organ dysfunction. Such organ dysfunction includes cognitive decline in the aging brain, reduced insulin secretion in the aging pancreas, dysregulated immune function, and reduced left ventricular diastolic function, thereby leading to increased morbidity of age-related diseases such as Alzheimer's disease, diabetes, infectious disease, and heart failure in the elderly [1–4]. Therefore, elucidating the molecular mechanisms underlying the organ dysfunction that occurs during aging is important for treating and/or preventing age-related diseases.

Blood vessels are essential routes for delivering oxygen and nutrients throughout the body, and play a crucial role in the maintenance of organ function. Moreover, endothelial cells support the homeostasis and regeneration of stem and progenitor cells *in situ* after tissue injury [5,6]. These roles establish angiogenesis as a distinctive and indispensable factor in the regulation of organ function. Aging also affects angiogenesis, and impaired angiogenesis is closely associated with a higher incidence and morbidity of ischemic diseases in the elderly [7–12]. Therefore, age-related changes in angiogenesis may play a unique and important role in age-related organ dysfunction.

Here, we investigated changes in the expression of angiogenic genes during aging in various tissues. We found that changes in the expression of angiogenic genes considerably differed between organs, whereas inflammatory gene expression largely increased in all tissues examined. Notably, many angiogenic genes were considerably down-regulated in the white adipose tissue (WAT) of aged mice, and such down-regulation was found to be associated with reduced vascularity and exacerbated hypoxia in the aged adipose tissue. In contrast, angiogenic genes were largely up-regulated in the skeletal muscle (SM) of aged mice, consistent with the preserved vasculatures observed in the aged SM. A potential causal role of endoplasmic reticulum (ER) stress in tissue-dependent changes in the expression of angiogenic genes during aging was also revealed.

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2. Material and methods

2.1. Materials

The CHOP antibody (L63F7) was purchased from Cell Signaling Technology, and the GAPDH antibody was purchased from Merck Millipore.

2.2. PCR array

Three separate samples of WAT and SM isolated from young (7-week-old) or aged (70-week-old) mice were mixed in individual tubes, and analyzed for the expression of 96 angiogenic genes using the RT^2 Profiler PCR Array (QIAGEN) according to the manufacturer's instruction. Gene expression heat maps were generated using the web-based software package provided by QIAGEN.

2.3. Cell culture

3T3-L1 pre-adipocytes were purchased from the Health Science Research Resources Bank (Japan) and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% calf serum (Gibco). Adipogenesis was induced by treatment with insulin, dexamethasone, and isobutylmethylxanthine as described previously [13].

The C2C12 mouse skeletal myoblast cell line was cultured in DMEM supplemented with 10% fetal bovine serum (FBS). Myogenesis was induced as described previously [14]. In brief, when myoblasts reached 70–80% confluence, the growth medium was replaced with the differentiation medium (DMEM supplemented with 2% horse serum). Fresh differentiation medium was given every other day.

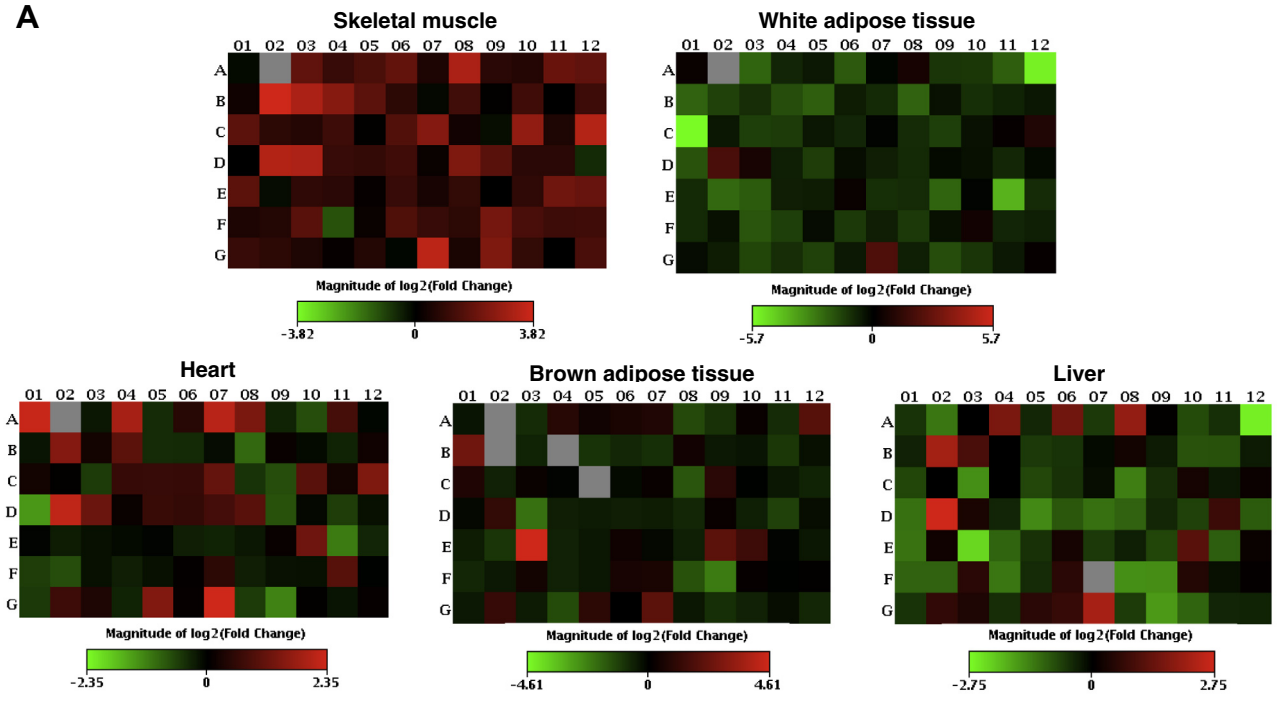
Myotubes and 3T3-L1 adipocytes were treated with hydrogen peroxide (50 or 200 μM) for 2 days or 7 days, tunicamycin (10 μg/ml) for 24 h, or thapsigargin (0.5 μM) for 24 h.

2.4. Animal study

All experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Kyoto Prefectural University of Medicine. C57/BL6 mice were used for all experiments.

2.5. Immunohistochemistry

Target tissues were excised following perfusion fixation with 4% paraformaldehyde. Specimens were further fixed with 4%



	1	2	3	4	5	6	7	8	9	10	11	12
A	Akt1	Ang	Angpt1	Angpt2	Anpep	Bai1	Ccl11	Ccl2	Cdh5	Col18a1	Col4a3	Csf3
B	Ctgf	Cxcl1	Cxcl2	Cxcl5	Edn1	Efna1	Efnb2	Egf	Eng	Epas1	Ephb4	Erbb2
C	F2	F3	Fgf1	Fgf2	Fgf6	Fgfr3	Figf	Flt1	Fn1	Hgf	Hif1a	Ifng
D	Igf1	Il1b	Il6	Itgav	Itgb3	Jag1	Kdr	Lect1	Lep	Mapk14	Mdk	Mmp14
E	Mmp19	Mmp2	Mmp9	Nos3	Nrp1	Nrp2	Pdgfa	Pecam1	Pgf	Plau	Plg	Ptgs1
F	Ptk2	S1pr1	Serpine1	Serpinf1	Smad5	Sphk1	Tbx1	Tek	Tgfa	Tgfb1	Tgfb2	Tgfb3
G	Tgfbr1	Thbs1	Thbs2	Tie1	Timp1	Timp2	Tnf	Tnfsf12	Tymp	Vegfa	Vegfb	Vegfc

Fig. 1. Changes in the expression of angiogenic genes in different tissues during aging. (A) The expression of angiogenic genes was analyzed by PCR-array. Heat maps display the relative expression of each gene in target tissues isolated from aged (70-week-old) versus young (7-week-old) mice. The expression levels of genes were normalized to those of β-actin expression, and gene expression is displayed as higher (red) or lower (green) in aged mice in comparison to young mice. Gene names for each panel are shown in the bottom table. (B) The expression levels of representative inflammatory and angiogenic genes in SM and WAT of aged mice in comparison to young controls. (C) The expression of representative inflammatory genes was analyzed by quantitative PCR in young and aged mice (n = 5–7 each). The expression of inflammatory genes was largely enhanced in both aged SM and WAT compared to that in young SM and WAT ($^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.005$ versus young controls). (D) The expression of representative angiogenic genes was analyzed by quantitative PCR in young and aged mice (n = 5–7 each). The expression of angiogenic genes was largely enhanced in aged SM compared to that in young SM ($^*P < 0.05$). In contrast, expression of these genes was mostly down-regulated in aged WAT ($^*P < 0.05$ and $^{****}P < 0.001$).

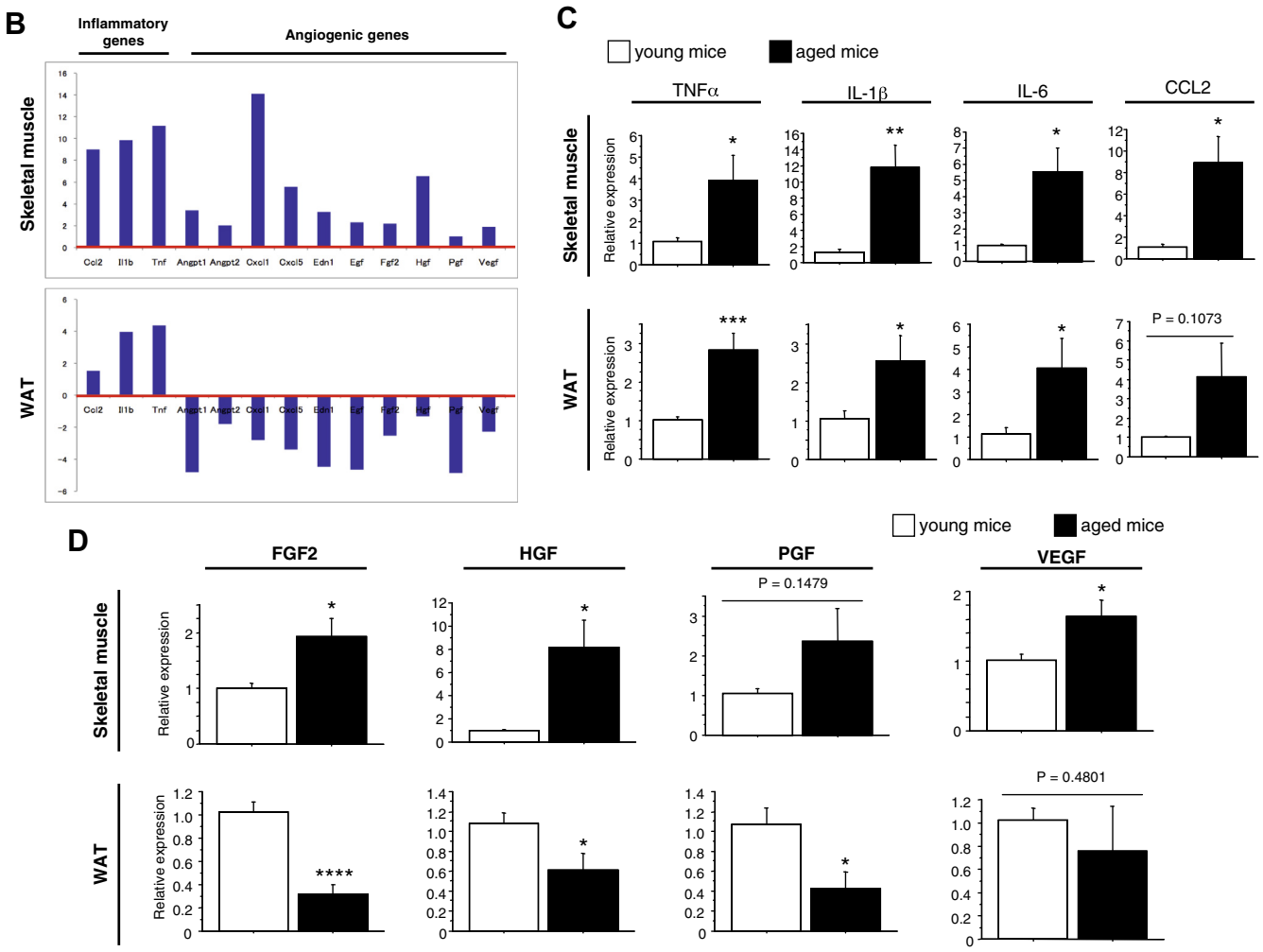


Fig. 1 (*continued*)

paraformaldehyde and then embedded in paraffin. Sections were stained with isolectin GS-IB4 from Griffonia Simplicifolia (Invitrogen) to detect microvessels as described previously [15]. In brief, sections were incubated with isolectin-IB4 (1:200 dilution) at 4 °C overnight after blocking in PBS containing 5% normal goat serum for 30 min. Isolectin-positive microvessels were quantified in two randomly chosen, independent fields per section by using the NIH imageJ software as described previously [16].

2.6. Hypoxyprobe staining

Hypoxia in WAT and SM was detected using the Hypoxyprobe-1 Kit (Hypoxyprobe, Inc.) according to a standard protocol as described previously [17]. In brief, the Hypoxyprobe was administered by intra-peritoneal injection. After 1 h, target tissues were excised and fixed with 4% paraformaldehyde, followed by embedding in paraffin. Hypoxia was detected by immunohistochemistry using the anti-Hypoxyprobe antibody.

2.7. Immunoblotting

Cell lysates were prepared in RIPA buffer, and then immunoblotted as described previously [18]. In brief, lysates containing the same amount of proteins were subjected to SDS–PAGE, followed by transferring onto the nitrocellulose membranes. The membranes were blocked with 5% non-fat milk in TBS containing 0.05% Tween20 at room temperature for 1 h. Membranes were then incubated with specific antibody to detect target molecules (1:1000 for anti-CHOP antibody or 1:2000 for anti-GAPDH antibody) at 4 °C overnight. Subsequently, membranes were incubated with HRP-conjugated secondary antibody, followed by detection with the ECL reagent (Amersham).

2.8. Quantitative RT-PCR

Total RNA of adipose tissues and 3T3-L1 adipocytes was isolated using Qiazol (Invitrogen) followed by purification with the RNeasy Lipid Tissue Mini Kit (QIAGEN). Total RNA of SM and C2C12 myotubes was isolated using Trizol (Invitrogen) followed by purification with the NucleoSpin RNA Clean-up Kit (MACHEREY–NAGEL).

Quantitative PCR was performed as described previously [18]. In brief, cDNA was synthesized from 0.5 to 1.0 μg of total RNA using the PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa). PCR reactions were prepared using SYBR Premix Ex TaqII (TaKaRa) followed by quantitative PCR on Thermal Cycler Dice (TaKaRa). The nucleotide sequence of each primer is listed in the Supplementary Table. mRNA levels of target genes relative to that of β-actin are shown for all experiments.

2.9. Statistical analysis

All data are presented as mean ± S.E. Differences between groups were analyzed by Student's *t*-test or one-way ANOVA with

post hoc multiple comparison by Bonferroni/Dunn test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Expression of angiogenic factors is differentially regulated during aging in different tissues

To investigate the changes in the expression of angiogenic genes during aging, we performed PCR-array analysis using RNAs isolated from various tissues of young (7-week-old) and aged (70-week-old) mice. Inflammatory genes such as chemokine ligand 2 (CCL2; MCP-1), interleukin (IL)-1β, and tumor necrosis factor (TNF)-α were generally increased in every organ of aged mice compared to those in young mice (Fig. 1A). In contrast, the expression of angiogenic genes was differentially altered in different tissues. In SM, angiogenic genes were largely up-regulated in aged mice; similar results were observed in the heart. In contrast, the expression of angiogenic genes was mostly down-regulated in WAT of aged mice, whereas mixed changes were detected in aged brown adipose tissue and liver compared to that in young mice (Fig. 1A).

We focused on SM and WAT for further analysis because they showed marked contrasts with regard to age-related changes in the expression of angiogenic genes (Fig. 1B). We first performed quantitative PCR analysis for representative angiogenic and inflammatory genes using individual samples. Inflammatory genes such as CCL-2, TNF-α, IL-1β, and IL-6 were largely up-regulated in both SM and WAT of aged mice (Fig. 1C). Consistent with the results of PCR-array, gene expression of angiogenic factors such as fibroblast growth factor-2, hepatic growth factor, placental growth factor, and vascular endothelial growth factor were also up-regulated in SM of aged mice (Fig. 1D). In contrast, the expression of these angiogenic genes was mostly down-regulated in WAT of aged mice (Fig. 1D). These results suggest that angiogenic gene expression is differentially regulated during aging in different tissues.

3.2. Angiogenesis was reduced in WAT during aging

Next, we investigated whether angiogenesis is indeed altered during aging. The blood vessel density in WAT of aged mice was substantially reduced compared to that in young mice (Fig. 2A), whereas the capillary density in SM did not differ between young

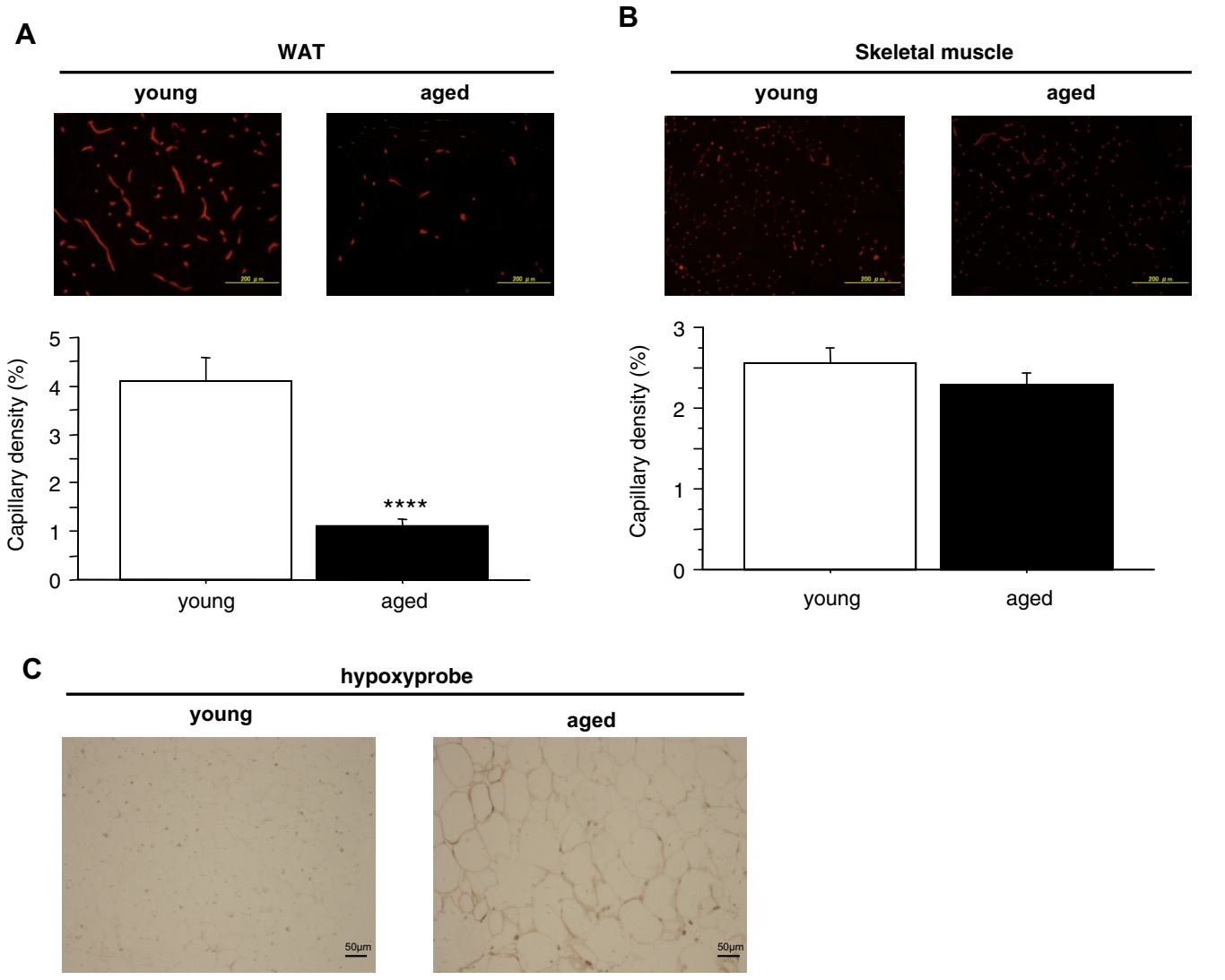


Fig. 2. Vascularity in WAT was substantially reduced during aging. (A) Blood vessels were detected by isolectin-staining in WAT isolated from young and aged mice ($n = 6$ each). Bar: 200 μM. Blood vessel density was significantly reduced in aged WAT (****$P < 0.001$ versus young controls). (B) Blood vessels were detected by isolectin-staining in SM isolated from young and aged mice ($n = 6$ each). Bar: 200 μM. Blood vessel density was not different between young and aged SM. (C) The presence of hypoxia was investigated by Hypoxyprobe staining in WAT of young and aged mice. Hypoxic areas are stained in brown. Bar: 50 μM.

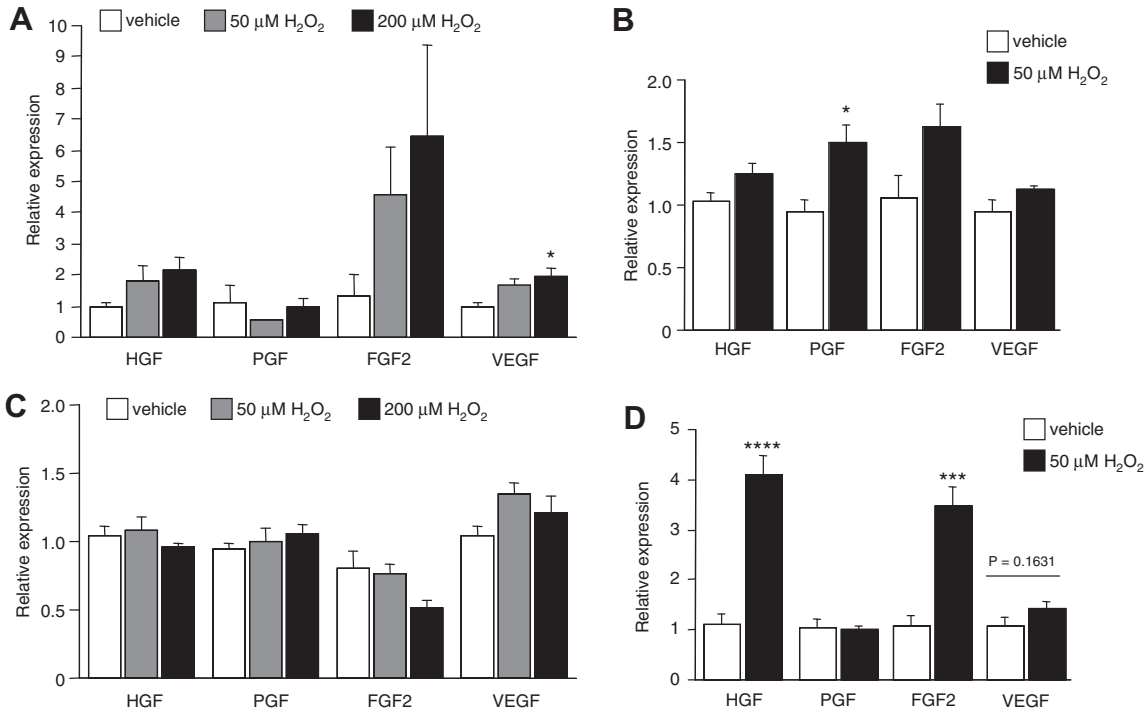


Fig. 3. The effect of oxidative stress on the expression of angiogenesis-related genes. (A) 3T3-L1 adipocytes were treated with hydrogen peroxide for 2 days, and the expression of angiogenic genes was analyzed ($n = 3$ each). The expression of angiogenic genes tended to increase with hydrogen peroxide treatment ($^{*}P < 0.05$ versus vehicle control). (B) 3T3-L1 adipocytes were treated with hydrogen peroxide for 7 days, and the expression of angiogenic genes was analyzed ($n = 3$ each). The expression of angiogenic genes tended to increase with hydrogen peroxide treatment ($^{*}P < 0.05$). (C) C2C12 myotubes were treated with hydrogen peroxide for 2 days, and the expression of angiogenic genes was analyzed ($n = 4$ each). (D) C2C12 myotubes were treated with hydrogen peroxide for 7 days, and the expression of angiogenic genes was analyzed ($n = 4$ each). The expression of angiogenic genes was increased by chronic treatment with hydrogen peroxide ($^{***}P < 0.005$ and $^{****}P < 0.001$).

and aged mice (Fig. 2B). Consistent with reduced vascularity, adipose tissue hypoxia was considerably exacerbated in aged mice as detected by Hypoxyprobe-staining (Fig. 2C), while hypoxia was barely detected in SM of both young and aged mice (data not shown). These results are consistent with the changes in the expression of angiogenic genes in SM and WAT during aging, suggesting that angiogenic factors play a crucial role in the regulation of angiogenesis during aging.

3.3. ER stress reproduces the age-related changes of angiogenic gene expression in WAT and SM

To clarify the molecular mechanisms involved in the age-related changes in the expression of angiogenic genes, we examined the effect of oxidative and ER stresses on the expression of angiogenic genes in 3T3-L1 adipocytes and C2C12 myotubes *in vitro*. Treatment with hydrogen peroxide tended to increase the expression of angiogenic genes in 3T3-L1 adipocytes, which was different from the changes observed in aged WAT *in vivo* (Fig. 3A and B). These results suggest that oxidative stress during aging probably does not play a causative role in the age-related changes in the expression of angiogenic genes in WAT *in vivo*. On the other hand, chronic stimulation with hydrogen peroxide up-regulated the expression of angiogenic genes in C2C12 myotubes, which was similar to the changes detected in aged SM *in vivo* (Fig. 3C and D).

In contrast, ER stress induced by tunicamycin largely decreased the expression of angiogenic genes in 3T3-L1 adipocytes, which was similar to the changes observed in aged WAT *in vivo* (Fig. 4A). Induction of ER stress was confirmed by the considerable expression of C/EBP homologue protein (CHOP) in tunicamycin-treated cells (Fig. 4B). Also, treatment with thapsigargin, another ER stress inducer, reduced the expression of angiogenic genes in 3T3-L1 adipocytes (Fig. 4C). On the other hand, tunicamycin treatment increased the expression of angiogenic genes in C2C12 myotubes, which is also consistent with changes in the expression of these genes in SM *in vivo* (Fig. 4D and E).

3.4. ER stress is dramatically increased in SM and WAT during aging

We finally examined whether ER stress is indeed increased in SM and WAT during aging *in vivo*. The expression of CHOP was substantially enhanced in both SM and WAT of aged mice compared to that in young mice, indicating that ER stress is significantly increased in SM and WAT during aging (Fig. 4F). These results together with the results of our *in vitro* experiments indicate that ER stress plays an important role in the age-related changes in angiogenesis in both WAT and SM, whereas oxidative stress may contribute to these changes only in SM. Given that ER stress is crucially involved in age-related adipose tissue dysfunction via deterioration of adipose tissue hypoxia, reducing ER stress is a potential therapeutic approach to prevent and treat age-related metabolic disorders.

4. Discussion

Angiogenesis plays a crucial role in the maintenance of organ function by regulating blood flow and secretion of angiocrine factors from endothelial cells [5,6]. Angiogenesis is also affected by aging, and impaired angiogenesis in the ischemic limbs and the tumor grafts has been shown in aged animals [10,11]. Therefore, changes in angiogenic capacity may play a significant role in the age-related alteration of organ function. The reduced expression of angiogenic factors, impaired vasodilation, and reduced angiogenic capacity in endothelial cells have been reported as possible

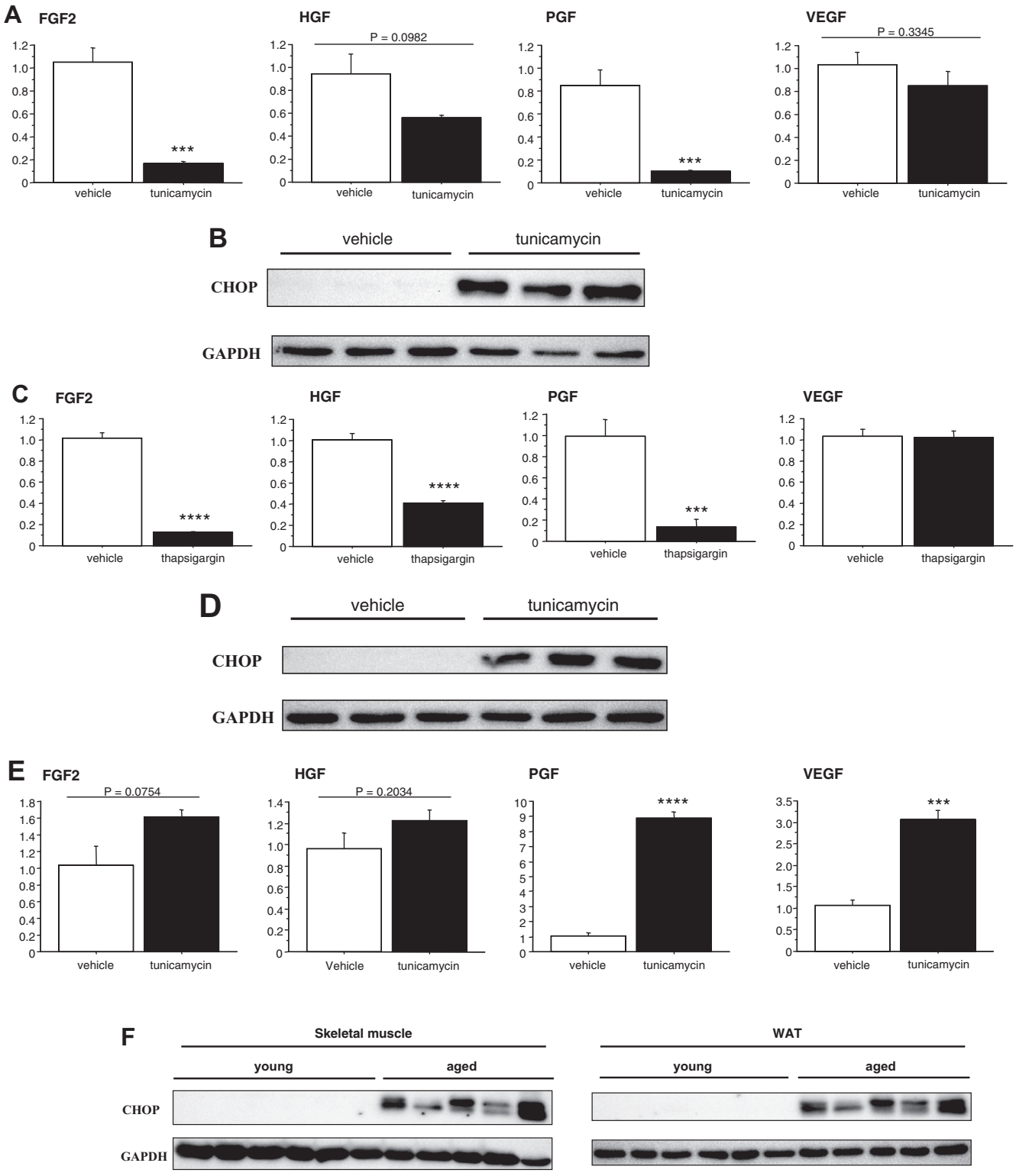


Fig. 4. ER stress reproduced the age-related changes in the expression of angiogenic genes. (A) 3T3-L1 adipocytes were treated with tunicamycin, and the expression of angiogenic genes was analyzed ($n = 3$ each). The expression of angiogenic genes was largely decreased by tunicamycin treatment ($^{***}P < 0.005$ versus vehicle control). (B) The expression of CHOP in 3T3-L1 adipocytes treated with either vehicle or tunicamycin was analyzed by immunoblotting. (C) 3T3-L1 adipocytes were treated with thapsigargin, and the expression of angiogenic genes was analyzed ($n = 4$ each). The expression of angiogenic genes was largely decreased by thapsigargin treatment ($^{***}P < 0.005$ and $^{****}P < 0.001$). (D) The expression of CHOP in C2C12 myotubes treated with either vehicle or tunicamycin was analyzed by immunoblotting. (E) C2C12 myotubes were treated with tunicamycin, and the expression of angiogenic genes was analyzed ($n = 3$ each). The expression of angiogenic genes was largely increased by tunicamycin treatment ($^{***}P < 0.005$ and $^{****}P < 0.001$). (F) The expression of CHOP in SM and WAT isolated from young or aged mice was analyzed by immunoblotting.

causative factors leading to defective angiogenesis associated with aging [10,11]. However, the molecular mechanisms involved in the regulation of the expression of angiogenic genes during aging remain to be elucidated. In this study, we demonstrated for the first

time that the expression of angiogenic genes is differentially regulated during aging in different tissues.

Aging is closely associated with metabolic disorders, including obesity, impaired glucose tolerance, and reduced insulin sensitivity [19,20]. Fat tissue is crucially involved in age-related metabolic dysfunction, and aging is strongly associated with adipose tissue hypoxia [21,22]. It is noteworthy that the expression of many angiogenic factors was down-regulated in WAT of aged mice compared to that in WAT of young mice. In combination with the reduced endothelial angiogenic capacity [12], this age-related reduction in angiogenic factors should reduce angiogenesis, and consequently, induce a decrease in blood vessel density in WAT. In fact, aged mice exhibited substantially reduced vascularity in WAT in association with significant adipose tissue hypoxia. Adipose tissue angiogenesis is an emerging factor for glucose homeostasis and energy metabolism, because hypoxia triggers adipose tissue inflammation and impairs the secretion of the adipokines regulating glucose homeostasis [23,24]. Therefore, an age-related reduction in angiogenic factors in WAT may play a unique and important role in the pathogenesis of metabolic disorders associated with aging.

We found that ER stress is a potential causative factor for age-related dichotomous changes in the expression of angiogenic genes in SM and WAT. ER is the organelle where newly synthesized proteins are folded and post-translationally modified for their proper functioning. ER contains many chaperones and processing enzymes to assist proper protein folding. Aging causes reduction in expression and activity of key chaperones and enzymes, resulting in ER dysfunction [25,26]. Prolonged ER stress leads to inflammatory signaling, and excessive stress leads to apoptosis largely through the activation of CHOP [27]. It has been reported that the expression of CHOP is increased in various tissues including the brain, lung, liver, kidney, heart, and spleen with aging [28]. In this study, we showed that the expression of CHOP was also increased in SM and WAT of aged mice compared to that in young mice. ER stress plays crucial roles in age-related diseases such as neurodegenerative diseases, metabolic syndrome, and atherosclerosis [26,29,30]. Our results newly reveal that ER-stress modulates organ function partly by differentially altering angiogenesis in different tissues during aging, and that this tissue-dependent alteration of angiogenesis may play a significant role in the morbidity of age-related diseases including metabolic disorders.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.098.

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