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ORIGINAL ARTICLE

Circulating IGF-1 deficiency exacerbates hypertension-induced microvascular rarefaction in the mouse hippocampus and retrosplenial cortex: implications for cerebromicrovascular and brain aging

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Abstract Strong epidemiological and experimental evidence indicate that both age and hypertension lead to significant functional and structural impairment of the cerebral microcirculation, predisposing to the development of vascular cognitive impairment (VCI) and Alzheimer's disease. Preclinical studies establish a causal link between cognitive decline and microvascular rarefaction in the hippocampus, an area of brain important for learning and memory. Age-related decline in circulating IGF-1 levels results in functional impairment of the cerebral microvessels; however, the mechanistic role of IGF-1 deficiency in impaired hippocampal microvascularization remains elusive. The present study was designed to characterize the additive/synergistic effects of IGF-1 deficiency and hypertension on microvascular density and expression of genes involved in angiogenesis and microvascular

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regression in the hippocampus. To achieve that goal, we induced hypertension in control and IGF-1 deficient mice ($Igf1^{f/f}$ + TBG-Cre-AAV8) by chronic infusion of angiotensin II. We found that circulating IGF-1 deficiency is associated with decreased microvascular density and exacerbates hypertension-induced microvascular rarefaction both in the hippocampus and the neocortex. The anti-angiogenic hippocampal gene expression signature observed in hypertensive IGF-1 deficient mice in the present study provides important clues for subsequent studies to elucidate mechanisms by which hypertension may contribute to the pathogenesis and clinical manifestation of VCI. In conclusion, adult-onset, isolated endocrine IGF-1 deficiency exerts deleterious effects on the cerebral microcirculation, leading to a significant decline in cortical and hippocampal capillarity and exacerbating hypertension-induced cerebromicrovascular rarefaction. The morphological impairment of the cerebral microvasculature induced by IGF-1 deficiency and hypertension reported here, in combination with neurovascular uncoupling, increased blood-brain barrier disruption and neuroinflammation reported in previous studies likely contribute to the pathogenesis of vascular cognitive impairment in elderly hypertensive humans.

Keywords Cerebral blood flow · Mild cognitive impairment · Ischemia · Brain aging · Vascular aging · Dementia · Alzheimer's disease

Introduction

There is growing evidence that alterations of the cerebral microcirculation play a key role in age-related decline in higher brain function (Toth et al. 2013a; Tucsek et al. 2003; Tucsek et al. 2014; Zlokovic 2011). Normal brain function is critically dependent on a continuous, tightly-controlled supply of oxygen and nutrients through adequate cerebral blood flow. The human brain receives almost 15 % of the cardiac output through a network of over 600 km of capillaries. In the brain, the number of endothelial cells is very similar to that of neurons (Garcia-Amado and Prensa 2012) and nearly every neuron is supplied by its own capillary, with an average distance of 8–20 μm between the neuron and the microvessels. Importantly, there is strong evidence that aging is associated with a decline in cerebral capillary density ("microvascular rarefaction") and that decreases in cerebromicrovascular density contribute to the age-related decline in regional cerebral blood flow (Mitschelen et al. 2009; Riddle et al. 2003; Khan et al. 2001; Lynch et al. 1999; Sonntag et al. 1997; Martin et al. 1991; Moeller et al. 1996; Farkas and Luiten 2001; Kawamura et al. 1993; Krejza et al. 1999; Schultz et al. 1999; Bentourkia et al. 2000; Hagstadius and Risberg 1989; Pagani et al. 2002). The resulting mismatch between energy supply and demand has been causally linked to significant cognitive impairment (reviewed in (Khan et al. 2001; Sonntag et al. 1997; Ingraham et al. 2008; Sonntag et al. 2000; Warrington et al. 2011; Warrington et al. 2012). Recent research substantiates that endocrine mechanisms play a critical role in age-related cerebromicrovascular pathology (Sonntag et al. 2013; Ungvari and Csiszar 2012). Principally, the age-related decline in circulating levels of insulin-like growth factor-1 (IGF-1) has been linked to microvascular aging and cognitive decline (reviewed recently in (Sonntag et al. 2013). IGF-1, which is secreted mainly by the liver and mediates the actions of growth hormone, is a pleiotropic growth factor which confers multifaceted pro-angiogenic effects, regulating microvascular remodeling (Lopez-Lopez et al. 2004) and protection against endothelial injury (Bailey-Downs et al. 2012a; Bailey-Downs et al. 2012b). We have recently demonstrated that mice with isolated decreases of circulating IGF-1 levels exhibit aging-like microvascular phenotypes, including impaired neurovascular coupling and cerebromicrovascular autoregulatory dysfunction (Lopez-Lopez et al. 2004; Toth et al. 2015; Toth et al. 2014a). Despite these advances, the role of circulating IGF-1 deficiency in age-related cerebromicrovascular rarefaction remains elusive.

Hypertension substantially contributes to cerebromicrovascular damage and promotes the development of vascular cognitive impairment. Hypertension-induced microvascular rarefaction has been observed both in humans (Wolf et al. 1994) and in laboratory rodents (Sokolova et al. 1985). Recent evidence shows that old age exacerbates hypertension-induced cerebromicrovascular rarefaction (Toth et al. 2013a). Further, low circulating IGF-1 levels increase the risk for hypertension-induced microvascular brain damage in elderly patients (Angelini et al. 2009), findings which have been also replicated in laboratory animals (Toth et al. 2014a). Despite these advances, the synergistic/additive effects of circulating IGF-1 deficiency and hypertension on cerebral microvascular density have not been explored.

The present study was designed to test the hypotheses that isolated circulating IGF-1 deficiency promotes

microvascular rarefaction in the hippocampus and the neocortex and leads to structural maladaptation of the cerebral microcirculation to hypertension, mimicking the aging phenotype. To test our hypotheses, we used a novel mouse model of adult-onset, isolated endocrine IGF-1 deficiency induced by adeno-associated viral knockdown of IGF-1 specifically in the liver of post-pubertal mice using Cre-lox technology (*Igf1*$^{f/f}$ + TBG-Cre-AAV8) (Bailey-Downs et al. 2012a; Toth et al. 2015). Hypertension was induced in control and IGF-1 deficient mice by chronic infusion of angiotensin II and changes in microvascular density and expression of genes involved in regulation of angiogenesis and microvascular regression in the hippocampus were assessed.

Materials and methods

Post-developmental liver-specific knockdown of Igf1 in mice

Male mice homozygous for a floxed exon 4 of the *Igf1* gene (*Igf1*$^{f/f}$) (Liu et al. 1998) in a C57BL/6 background were purchased from Jackson Laboratories. These mice have the entirety of exon 4 of the *Igf1* gene flanked by loxP sites, which allows for genomic excision of this exon when exposed to Cre recombinase. Transcripts of the altered *Igf1* gene yield a protein upon translation that fails to bind the IGF receptor. Animals were housed in the Rodent Barrier Facility at OUHSC, on a 12 h light/12 h dark cycle, with access to standard rodent chow (Purina Mills, Richmond, IN) and water ad libitum.

To target hepatocytes, adeno-associated viruses (AAVs) were purchased from the University of Pennsylvania Vector Core (Philadelphia, PA). At 4 months of age, approximately 1.3×10^{10} viral particles (as assayed by genome content at the University of Pennsylvania) of AAV8.TBG.PI.Cre.rBG or AAV8.TBG.PI.eGFP.WPRE.bGH were administered to *Igf1*$^{f/f}$ mice to knockdown IGF-1 or as a control, respectively. Mice were anesthetized with ketamine/xylazine (100 and 15 mg/kg, respectively), and given retroorbital injections of virus diluted to the appropriate concentration in 100 μl 0.9 % saline. While AAV8 is effective at transducing multiple tissues after i.v. delivery, including liver, the thyroxine binding globulin (TBG) promoter restricts expression solely to hepatocytes (Toth et al. 2015; Toth et al. 2014a). Dosages were determined empirically in preliminary studies. All procedures were approved by and followed the guidelines of the Institutional Animal Care and Use Committee of OUHSC in accordance with the ARRIVE guidelines.

Measurement of serum IGF-1 levels

Submandibular venous blood was collected into microcentrifuge tubes using a sterile lancet (Medipoint, Mineola, NY) according to the manufacturer's instructions. Whole blood was centrifuged at 2500×*g* for 20 min at 4 °C to collect serum, which was then stored at −80 °C. IGF-1 concentration in the serum samples was measured by ELISA (R&D Systems, Minneapolis, MN) as reported (Toth et al. 2015; Toth et al. 2014a). An IGF-1 control sample, with aliquots stored at −80 °C, was included on each plate. Serum IGF-1 levels are reported in nanogram/milliliter.

Infusion of angiotensin II

To induce hypertension, Alzet mini-osmotic pumps (Model 2006, 0.15 μl/h, 42 days; Durect Co, Cupertino, CA) were implanted into *Igf1*$^{f/f}$ + TBG-Cre-AAV8 and control mice (2-month post-AAV injection), as previously described (Toth et al. 2013a). Pumps were filled either with saline vehicle or solutions of angiotensin II (Sigma Chemical Co., St. Louis, MO, USA) that delivered (subcutaneously) 1000 ng/min/kg of angiotensin II for 28 days (Toth et al. 2013a). Pumps were placed into the subcutaneous space of ketamine/xylazine anesthetized mice through a small incision in the back of the neck that was closed with surgical sutures. All incision sites healed rapidly without the need for additional medication.

Blood pressure measurements

Systolic blood pressure of mice in each experimental group was measured by the tail cuff method (CODA Non-Invasive Blood Pressure System, Kent Scientific Co., Torrington, CT) before and 2 weeks (to check for pump failure) and 4 weeks (before the terminal experiments) after the minipump implantation (Toth et al. 2013a).

Analysis of microvascular density: immunofluorescent labeling and confocal microscopy

Mice were transcardially perfused with PBS; the brains were removed and hemisected. From the right hemispheres, the hippocampi were isolated and frozen for subsequent analysis. The left hemispheres were fixed overnight in 4 % paraformaldehyde, then they were cryoprotected in a series of graded sucrose solutions (10, 20, and 30 % overnight), and frozen in Cryo-Gel (Electron Microscopy Sciences, Hatfield, PA) as described (Toth et al. 2014a). Coronal sections of 70 μm were cut through the hippocampus and stored free-floating in cryopreservative solution (25 % glycerol, 25 % ethylene glycol, 25 % 0.2 M phosphate buffer, 25 % distilled water) at −20 °C. Selected sections were ~1.6 mm caudal to Bregma, representing the more rostral hippocampus. After washing (3 × 5 min with TBS plus 3 × 5 min with 1× TBS + 0.25 % TritonX-100), sections were treated with 1 % of sodium-borohydride solution for 5 min. After a second washing step (3 × 5 min with distilled water plus 3 × 5 min with 1× TBS) and blocking in 5 % BSA/TBS (with 0.5 % Triton X-100, 0.3 M glycin and 1 % fish gelatin; for 3 h), sections were immunostained using a mouse anti-CD31 (1: 50; Cat N: 550274, BD Pharmingen, San Jose, CA) primary antibody (for two nights at 4 °C) to label endothelial cells. Sections were washed for 3 × 5 min with TBS plus 3 × 5 min with 1× TBS + 0.25 % TritonX-100. For nuclear counterstaining, Hoechst 33342 was used. Then, the sections were transferred to slides and coverslipped. Confocal images were captured using a Leica SP2 MP confocal laser scanning microscope.

Immunofluorescent labeling for CD31 was used to identify microvessels in the brain as we previously described (Toth et al. 2013a; Tucsek et al. 2014; Warrington et al. 2011). Capillary density in the CA1 region of the hippocampus was quantified, using stereological methods, as the length of blood vessels <10 μm in diameter per volume of tissue using Neurolucida with AutoNeuron (MicroBrightField, Williston, VT). Brain regions were identified based on reference (Hof et al. 2000). The total length of capillaries (mm) was divided by the volume of brain tissue scanned (mm^3) to obtain capillary density (length per tissue volume) within the CA1 region for each animal. The experimenter was blinded to the groups and treatments of the animals throughout the period of blood vessel staining and analysis.

Targeted qPCRarray to analyze mRNA expression of pro- and anti-angiogenic factors

A quantitative real time RT-PCR technique was used to analyze mRNA expression of genes known to be involved in regulation of angiogenesis and microvascular regression in hippocampi of mice from each experimental group as reported (Toth et al. 2013a; Tucsek et al. 2003; Tucsek et al. 2014; Csiszar et al. 2013). In brief, total RNA was isolated with a Mini RNA Isolation Kit (Zymo Research, Orange, CA) and was reverse transcribed using Superscript III RT (Invitrogen) as described previously (Bailey-Downs et al. 2012a). Hippocampal mRNA expression of pro- and anti-angiogenic genes was analyzed using validated TaqMan probes (Applied Biosystems) and a Strategen MX3000 platform, as previously reported (Tucsek et al. 2014). Quantification was performed using the efficiency-corrected ΔΔCt method. Using the Bioconductor HTqPCR package (Dvinge and Bertone 2009), arrays were first rescaled to ΔCt values using *Ywhaz*, *B2m*, and *Hprt* as a reference, and then quantile normalized. "Undetermined" Ct values were replaced with the global maximum Ct value. Differential expression significance was determined using the Bioconductor limma package, and the directionality and magnitude of the angiogenic signature was assessed using both the *t* statistic and Gene Set Enrichment Analysis (Subramanian et al. 2005) as implemented in the Bioconductor GSA package.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc tests. All statistical comparisons were performed using Prism 5.0 for Windows (Graphpad Software, La Jolla, CA), and were considered significant at $p < 0.05$. Data are expressed as mean ± S.E.M.

Results

Circulating IGF-1 levels and blood pressure measurements

Although the factors responsible for the deleterious effects of aging on behavior and neuronal function (Baciu et al. 2016; Berghuis et al. 2015; Campbell et al. 2014; Doi et al. 2015; Haider et al. 2014;

Hofmann et al. 2014; Kumar and Thakur 2015; Lopez et al. 2014; Manich et al. 2014; Salminen et al. 2014; Samaras et al. 2014; Sarubbo et al. 2015; Loprinzi 2016; Wallis et al. 2016) are not completely understood, there is strong evidence that in elderly humans a decline in circulating IGF-1 levels (Franco et al. 2014) plays a critical pathophysiological role. To understand the effects of IGF-1 deficiency on the cerebral microcirculation in the present study, we used a novel mouse model of adult-onset isolated endocrine IGF-1 deficiency, which phenotypically better mimics age-related IGF-1 deficiency observed in humans that most other available rodent models of GH/IGF-1 deficiency (Arum et al. 2014a; Hill et al. 2015; Rojanathammanee et al. 2014; Wiesenborn et al. 2014; Arum et al. 2014b; Schneider et al. 2014). Serum IGF-1 levels and physiological parameters obtained in the same experimental cohorts of animals used for the present study have been recently reported (Toth et al. 2014a). Accordingly, mice receiving TBG-Cre-AAV8 had significantly decreased (by ~75 %) serum IGF-1 levels compared with control mice receiving TBG-eGFP-AAV8. Both groups had similar serum IGF-1 levels prior to administration of liver-targeted viruses. Previously, we reported that knockdown of IGF-1 in this model does not lead to change in body weight, serum insulin, IGFBP-1 and −2, adiponectin and glucose levels (Toth et al. 2015). To induce hypertension, we used angiotensin II, which has special relevance for aging research (Salminen et al. 2014; Mellor et al. 2014; Schuch et al. 2014; Simon et al. 2015). We found that blood pressure was significantly increased (by ~50 %) in both control and IGF-1 deficient mice receiving Ang II infusion (Toth et al. 2014a). Although previous studies reported that in mice a~80 % reduction in serum IGF-1 levels may be associated with a~5 mmHg increase in blood pressure (Tivesten et al. 2002), and in our studies, no significant interaction between IGF-1 levels and blood pressure was noted.

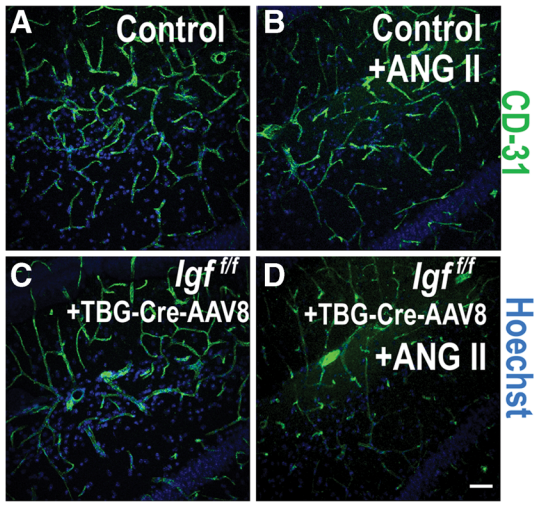
IGF-1 deficiency exacerbates hypertension-induced cerebromicrovascular rarefaction

We quantified the effects of IGF-1 deficiency and hypertension on brain capillary density in the hippocampus of mice. Capillaries were identified by their expression of CD31 (Fig. 1a–e), using a lumen diameter of 10 μm or less as a standard identifier. We found that IGF-1 deficiency was associated with significant decreases in capillary density in the CA1 region (Fig. 1) and the dentate gyrus (Fig. 2) of the hippocampus, indicating significant cerebromicrovascular rarefaction. To determine whether the microvascular rarefaction could be observed in brain regions outside of the hippocampus, we next measured capillary density in the retrosplenial cortex of the control and IGF-1 deficient mice. We found that within this region IGF-1 deficiency also reduced capillary density by ~20 % (Fig. 3).

Previous studies demonstrate that IGF-1 deficiency impairs functional adaptation of the cerebral circulation to hypertension (Toth et al. 2014a). Here we demonstrate that IGF-1 deficiency exacerbates hypertension-induced cerebromicrovascular rarefaction, as indicated by the substantial decline in CD31-positive capillaries both in the hippocampus and the retrosplenial cortex of hypertensive *Igf1*$^{f/f}$ + TBG-iCre-AAV8 mice (Fig. 1, Fig. 2, and Fig. 3, respectively).

Effects of circulating IGF-1deficiency and hypertension on hippocampal angiogenic gene expression signature

To elucidate the mechanisms contributing to cerebromicrovascular rarefaction, we detected and analyzed the mRNA expression of 90 angiogenesis- and rarefaction-associated genes in the mouse hippocampi (Tucsek et al. 2014). The effects of IGF-1 deficiency and hypertension on the expression of cytokines regulating angiogenesis (*Vegfa*, *Vegfb*, *Vegfc*, *Figf*, *Pdgfb*, *Fgf1*, *Fgf2*, and *Ctgf*) are shown in Fig. 4. Expression of vascular endothelial growth factor A (*Vegfa*), a potent inducer of angiogenesis, which prevents microvascular rarefaction (Steeghs et al. 2010), was unaltered. Importantly, both hypertension and IGF-1 deficiency were associated with down-regulation of *Vegfb*. VEGF-B is a pro-angiogenic growth factor, which is constitutively expressed in cerebral microvessels and plays a role only in the maintenance of the microcirculatory network (Nag et al. 2002) and adaptation to ischemic challenges (Bellomo et al. 2000). Among other members of the VEGF, family expression of *Vegfc* (Witzenbichler et al. 1998) was unchanged, whereas expression of *Vegfd* (C-fos-induced growth factor/FIGF) (Debinski et al. 2001) was down-regulated by IGF-1 deficiency. Expression of platelet-derived growth factor (*Pdgfβ*) (He et al. 2015) and fibroblast growth factors-1 and −2 (*Fgf-1*, *Fgf-2*) was unaltered by either IGF-1 deficiency and hypertension, whereas expression of connective tissue growth



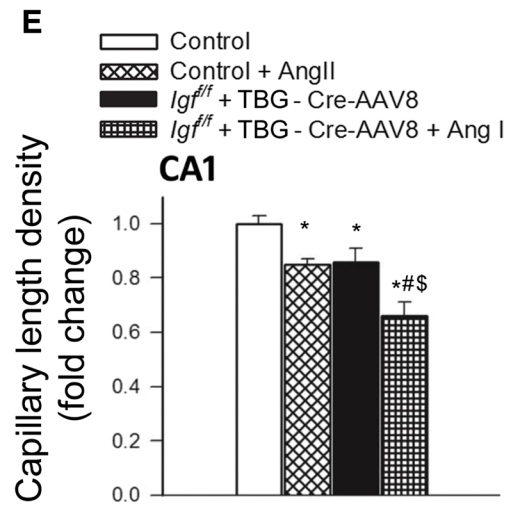
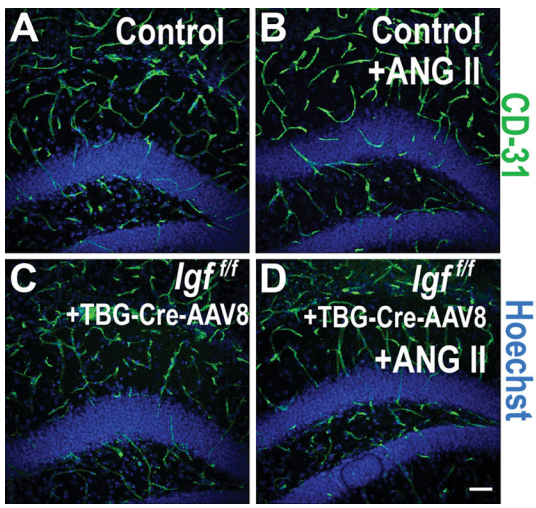


Fig. 1 IGF-1 deficiency exacerbates hypertension-induced microvascular rarefaction in the CA1 region of the mouse hippocampus. **a**–**d** Representative confocal images showing CD31-positive capillary endothelial cells (*green*) in the CA1 region of the hippocampi of in normotensive control (*Igf1*$^{f/f}$ + TBG-eGFP-AAV8), hypertensive control (*Igf1*$^{f/f}$ + TBG-eGFP-AAV8 + Ang II), normotensive IGF-1 deficient (*Igf1*$^{f/f}$ + TBG-Cre-AAV8), and hypertensive IGF-1 deficient mice (*Igf1*$^{f/f}$ + TBG-Cre-AAV8 + AngII). Hoechst 33342 was used for nuclear counterstaining (scale bar: 100 μm). **e** Summary data for hypertension-induced changes of capillary length density in the CA1 region of the hippocampus in control and IGF-1 deficient mice. Data are mean ± S.E.M. *$p < 0.05$ vs. normotensive control, # $p < 0.05$ vs. normotensive IGF-1 deficient; $ $p < 0.05$ vs. hypertensive control mice

factor (*Ctgf*) was up-regulated in hypertensive IGF-1 deficient mice.

The effects of IGF-1 deficiency and hypertension on the expression of the pro-angiogenic factor *Angpt1*, the closely related factors *Angptl1* and *Angptl2*, the anti-angiogenic factor *Angpt2*, and the angiopoietin receptors *Tek* and *Tie1* are shown in Fig. 5. The up-regulation of *Angpt2* (an antagonist of the pro-angiogenic



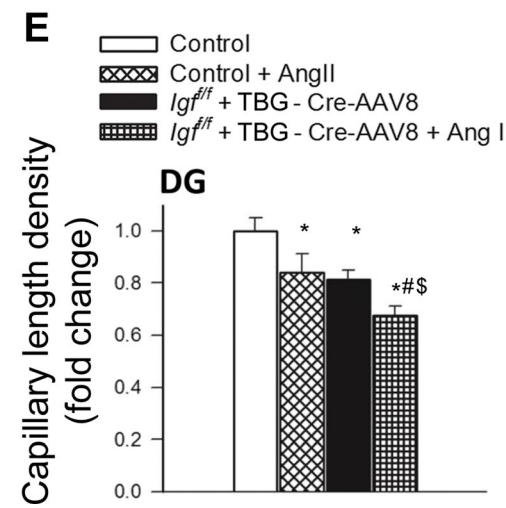


Fig. 2 IGF-1 deficiency exacerbates hypertension-induced microvascular rarefaction in the dentate gyrus of the mouse hippocampus. **a**–**d** Representative confocal images showing CD31-positive capillary endothelial cells (*green*) in the dentate gyrus (*DG*) of the hippocampi of in normotensive control (*Igf1*$^{f/f}$ + TBG-eGFP-AAV8), hypertensive control (*Igf1*$^{f/f}$ + TBG-eGFP-AAV8 + Ang II), normotensive IGF-1 deficient (*Igf1*$^{f/f}$ + TBG-Cre-AAV8), and hypertensive IGF-1 deficient mice (*Igf1*$^{f/f}$ + TBG-Cre-AAV8 + AngII). Hoechst 33342 was used for nuclear counterstaining (scale bar: 100 μm). **e** Summary data for hypertension-induced changes of capillary length density in the dentate gyrus of the hippocampi in control and IGF-1 deficient mice. Data are mean ± S.E.M. *$p < 0.05$ vs. normotensive control, # $p < 0.05$ vs. normotensive IGF-1 deficient; $ $p < 0.05$ vs. hypertensive control mice

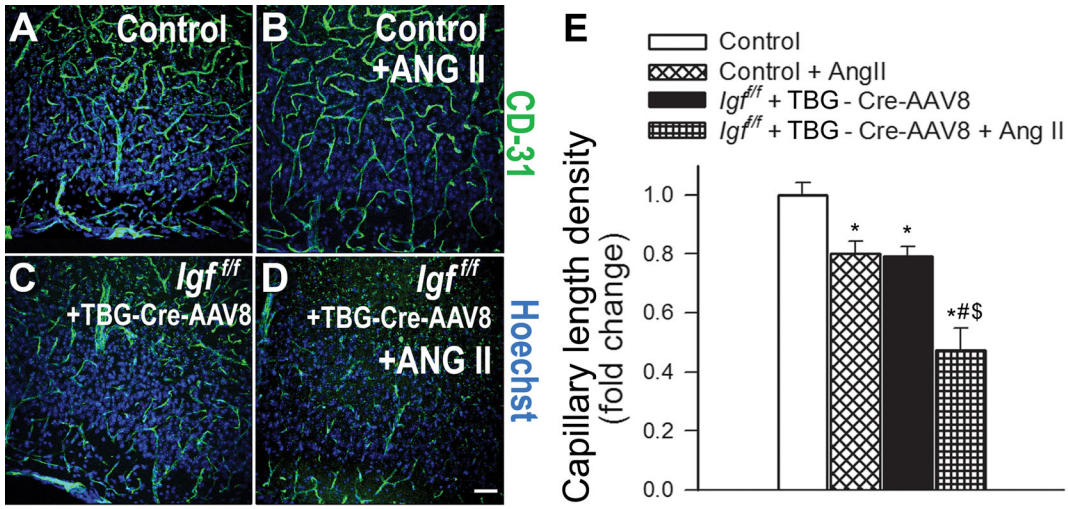


Fig. 3 IGF-1 deficiency exacerbates hypertension-induced microvascular rarefaction in the retrosplenial cortex of mice. **a**–**d** Representative confocal images showing CD31-positive capillary endothelial cells (*green*) in the retrosplenial cortex of normotensive control (*Igf1*$^{f/f}$ + TBG-eGFP-AAV8), hypertensive control (*Igf1*$^{f/f}$ + TBG-eGFP-AAV8 + Ang II), normotensive IGF-1 deficient (*Igf1*$^{f/f}$ + TBG-Cre-AAV8), and hypertensive IGF-1 deficient mice (*Igf1*$^{f/f}$ + TBG-Cre-AAV8 + AngII). Hoechst 33342 was used for nuclear counterstaining (scale bar: 100 μm). **e** Summary data for hypertension-induced changes of capillary length density in the retrosplenial cortex in control and IGF-1 deficient mice. Data are mean ± S.E.M. *$p < 0.05$ vs. normotensive control, # $p < 0.05$ vs. normotensive IGF-1 deficient; $ $p < 0.05$ vs. hypertensive control mice

angiopoetin-1) in hypertensive IGF-1 deficient mice is significant, as angiopoetin 2 expression has been causally linked to capillary rarefaction (Lobov et al. 2002; Cao et al. 2007).

Figure 6 shows the effects of IGF-1 deficiency and hypertension on the hippocampal expression of the angiogenesis inhibitors *Serpinf1* (*PEDF*), fibulin-5 (*Fbln5*) (Sullivan et al. 2007), thromhospondin-1 (*Thbs1*) (Lawler 2002), *Thbs2* (Volpert et al. 1995), the potent anti-angiogenic chemokine platelet factor 4 (*Pf4*) (Bikfalvi 2004); vasohibin-1 (*Vash1*), which is a newly recognized

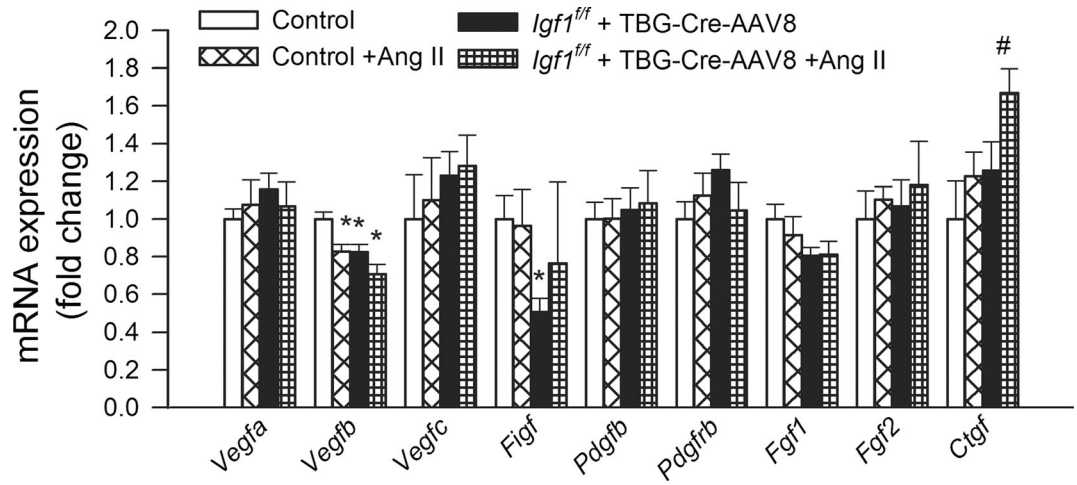


Fig. 4 Effects of IGF-1 deficiency and hypertension on the hippocampal expression of cytokines regulating angiogenesis. QRT-PCR data showing mRNA expression of *Vegfa*, *Vegfb*, *Vegfc*, *Figf*, *Pdgfb*, *Pdgfrb*, *Fgf1*, *Fgf2,* and *Ctgf* in hippocampi of normotensive control (*Igf1*$^{f/f}$ + TBG-eGFP-AAV8), hypertensive control (*Igf1*$^{f/f}$ + TBG-eGFP-AAV8 + Ang II), normotensive IGF-1 deficient (*Igf1*$^{f/f}$ + TBG-Cre-AAV8), and hypertensive IGF-1 deficient mice (*Igf1*$^{f/f}$ + TBG-Cre-AAV8 + AngII). Data are mean ± S.E.M. (n = 5–8 in each group). *$p < 0.05$ vs. normotensive control, # $p < 0.05$ vs. normotensive IGF-1 deficient; $ $p < 0.05$ vs. hypertensive control mice

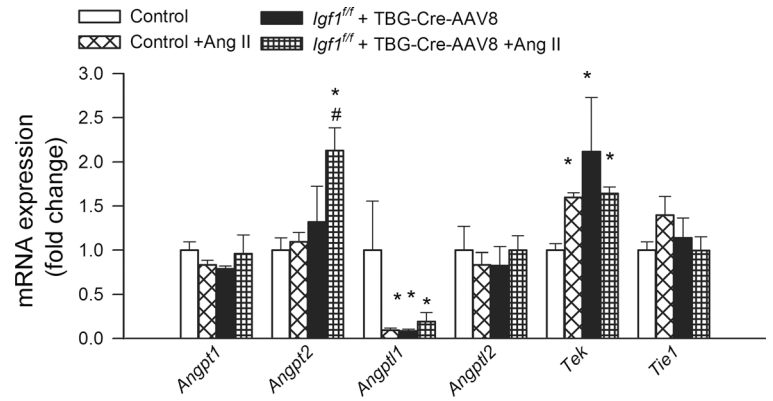


Fig. 5 Effects of IGF-1 deficiency and hypertension on the hippocampal expression of angiopoietins and related factors. QRT-PCR data showing mRNA expression of the pro-angiogenic factor *Angpt1*, the closely related factors *Angptl1* and *Angptl2*, the anti-angiogenic factor *Angpt2* and the angiopoietin receptors *Tek* and *Tie1* in hippocampi of normotensive control (*Igf1*$^{f/f}$ + TBG-eGFP-AAV8), hypertensive control (*Igf1*$^{f/f}$ + TBG-eGFP-AAV8 + Ang II), normotensive IGF-1 deficient (*Igf1*$^{f/f}$ + TBG-Cre-AAV8), and hypertensive IGF-1 deficient mice (*Igf1*$^{f/f}$ + TBG-Cre-AAV8 + AngII). Data are mean ± S.E.M. (n = 5–8 in each group). *$p < 0.05$ vs. normotensive control, # $p < 0.05$ vs. normotensive IGF-1 deficient; $ $p < 0.05$ vs. hypertensive control mice

negative regulator of angiogenesis produced by endothelial cells (Sato 2013); interferon-β (*Ifnb1*) (Takano et al. 2014), *Adamts1* ("a disintegrin and metalloproteinase with thrombospondin motifs 1"), which inhibits angiogenesis (Lee et al. 2006) by suppressing endothelial cell proliferation; *Col18a1*, whose expression level impacts endostatin signaling and endothelial angiogenic capacity (Li and Olsen 2004) (endostatin, a potent inhibitor of angiogenesis, is a 20-kDa C-terminal fragment derived from type XVIII collagen); semaphorin-3F (*Sema3f*) (Ungvari et al. 2011b*;* Frisbee et al. 2007*)*; tenomodulin (*Tnmd*) (Oshima et al. 2003); brain-specific angiogenesis inhibitor 1 (*Bai1*; also known as adhesion G protein-coupled receptor B1 [ADGRB1]) (Nishimori et al. 1997); chromogranin A (*Chga*), which encodes the precursor to several angiogenesis inhibitor

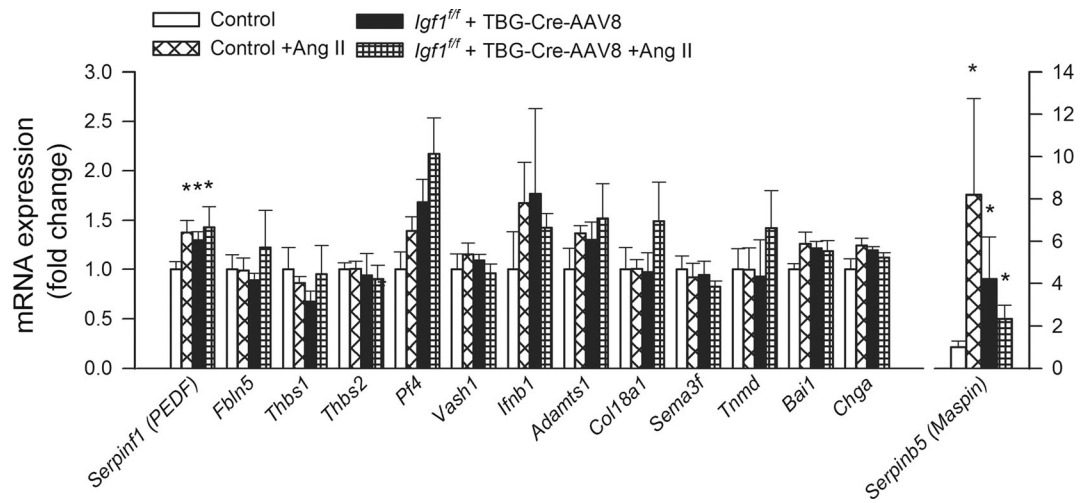


Fig. 6 Effects of IGF-1 deficiency and hypertension on the hippocampal expression of angiogenesis inhibitors. QRT-PCR data showing mRNA expression of *Serpinf1 (PEDF)*, *Fbln5*, *Thbs1*, *Thbs2*, *Pf4*, *Vash1*, *Ifnb1*, *Adamts1*, *Col18a1*, *Sema3f*, *Tnmd*, *Bai1*, *Chga*, and *Serpinb5*, in hippocampi of normotensive control (*Igf1*$^{f/f}$ + TBG-eGFP-AAV8), hypertensive control (*Igf1*$^{f/f}$ + TBG-eGFP-AAV8 + Ang II), normotensive IGF-1 deficient (*Igf1*$^{f/f}$ + TBG-Cre-AAV8), and hypertensive IGF-1 deficient mice (*Igf1*$^{f/f}$ + TBG-Cre-AAV8 + AngII). Data are mean ± S.E.M. (n = 5–8 in each group). *$p < 0.05$ vs. normotensive control, # $p < 0.05$ vs. normotensive IGF-1 deficient; $ $p < 0.05$ vs. hypertensive control mice

peptides including vasostatin-1 and vasostatin-2 (Helle and Corti 2015) and maspin ("mammary serine protease inhibitor"; encoded by the *Serpinb5* gene (Qin and Zhang 2010).

Figure 7 shows the expression of *Tnfa*, whose overproduction has been causally linked to microvascular rarefaction (Frisbee et al. 2014); *Tgfb1*, which regulates multiple aspects of the angiogenic process and contributes to hypertension-induced microvascular rarefaction in the heart (Koitabashi et al. 2011); *Tgfa;* angiogenin (*Ang*, also known as ribonuclease 5), which is a potent stimulator of angiogenesis and an inhibitor of endothelial apoptosis; *Edil3* (EGF-like repeats and discoidin I-like domains 3), which encodes a glycoprotein secreted by endothelial cells that regulates apoptosis, cell migration (Zhong et al. 2003) and induces cerebral angiogenesis in mice (Fan et al. 2008); midkine (*Mdk*, also known as neurite growth-promoting factor 2 or NEGF2), which is a pleiotropic growth factor regulating cell proliferation, cell migration and promoting angiogenesis (Mashour et al. 2001); pleiotrophin (*Ptn*; also known as heparin-binding brain mitogen [HBBM], heparin-binding growth factor 8 [HBGF-8], neurite growth-promoting factor 1 [NEGF1], heparin affinity regulatory peptide [HARP] or heparin-binding growth associated molecule [HB-GAM]), which is a pro-angiogenic growth factor that is structurally related to midkine and whose expression in the adult brain is induced by ischemia; *Tymp* (thymidine phosphorylase, also known as platelet-derived endothelial cell growth factor [ECGF1], which stimulates endothelial cell proliferation and induces angiogenesis in the brain (Hayashi et al. 2007); platelet endothelial cell adhesion molecule (*Pecam1*; also known as CD31), which confers pro-angiogenic effects (Park et al. 2015); angiomotin (*Amot*), which regulates endothelial cell migration and angiogenic capacity; the growth factor granulin (*Grn*), which plays a role in regulation of blood vessel formation; carcinoembryonic antigen-related cell adhesion molecule 1 (*Ceacam1*; also known as CD66a), which confers pro-angiogenic effects (Wagener and Ergun 2000); the basic helix-loop-helix-type transcriptional repressor *Hey1*, which is a primary target gene of the Notch signaling pathway and is thought to confer pro-angiogenic effects (Wang et al. 2014); neuropilin-1 (*Nrp1*), which modulates VEGF- and semaphorin signaling, regulating multiple aspects of angiogenesis (Fantin et al. 2014); sphingosine-1-phosphate receptor 1 (*S1pr1*, also known as endothelial differentiation gene 1 [EDG1]) (Fujii et al. 2012); and *Hif1a*, which also

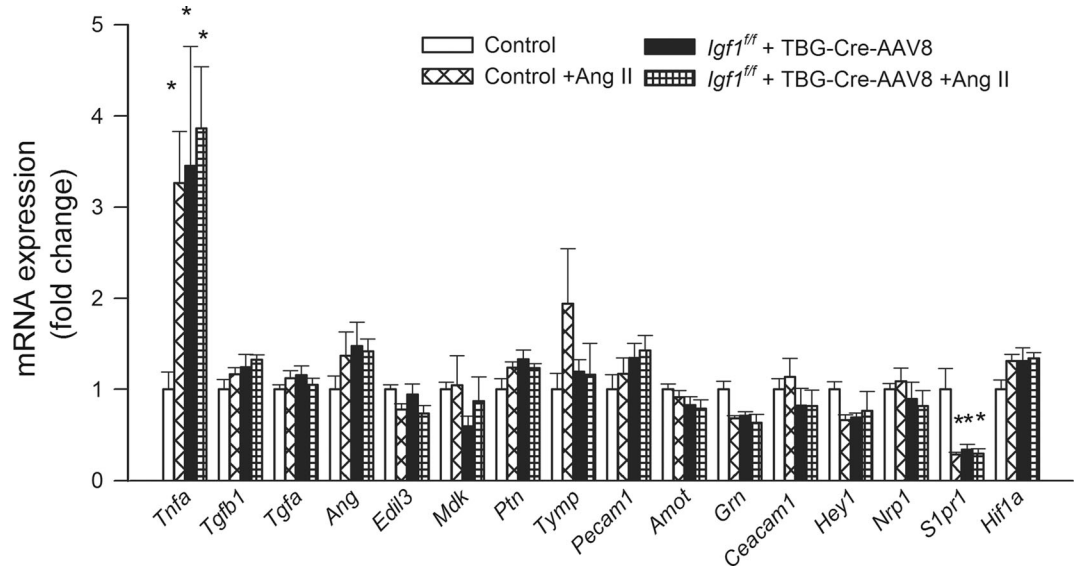


Fig. 7 Effects of IGF-1 deficiency and hypertension on the hippocampal expression of factors regulating processes underlying angiogenesis and/or microvascular rarefaction. QRT-PCR data showing mRNA expression of *Tnfa*, *Tgfb1*, *Tgfa*, *Ang*, *Edil3*, *Mdk*, *Ptn*, *Tymp*, *Pecam1*, *Amot*, *Grn*, *Ceacam1*, *Hey1*, *Nrp1*, *S1pr1*, and *Hif1a* in hippocampi of normotensive control (*Igf1*$^{f/f}$ + TBG-eGFP-AAV8), hypertensive control (*Igf1*$^{f/f}$ + TBG-eGFP-AAV8 + Ang II), normotensive IGF-1 deficient (*Igf1*$^{f/f}$ + TBG-Cre-AAV8), and hypertensive IGF-1 deficient mice (*Igf1*$^{f/f}$ + TBG-Cre-AAV8 + AngII). Data are mean ± S.E.M. (n = 5–8 in each group). *$p < 0.05$ vs. normotensive control, # $p < 0.05$ vs. normotensive IGF-1 deficient; $ $p < 0.05$ vs. hypertensive control mice

regulate processes underlying angiogenesis and microvascular rarefaction.

To determine whether the array data suggests a positive or negative angiogenesis signature for various conditions, genes annotated with the Gene Ontology terms for "positive regulation of angiogenesis" (GO:0045766; example: Vegfa) and "negative regulation of angiogenesis" (GO:0016525; example: Thbs) were obtained and intersected with the genes on the array. Genes annotated with both terms were removed. To assess the directionality of the angiogenesis signature, *t* statistic values were calculated. Figure 8a depicts mean *t* statistic for positive and negative angiogenesis regulators, and the signed aggregate of both groups. To interpret the gene expression data, Gene Set Enrichment Analysis (GSEA) scores were also calculated for positive regulators of angiogenesis (Fig. 8b). Both hypertension and IGF-1 deficiency appear to decrease the angiogenesis signature. The interaction of IGF-1 deficiency and hypertension appear to have significant negative effect, implying that IGF-1 deficiency and hypertension act in a strongly synergistic manner toward altering the angiogenic gene expression signature.

Discussion

The results of this study suggest that adult-onset, isolated endocrine IGF-1 deficiency is associated with a significant decline in microvascular density both in the hippocampus and the neocortex, mimicking the aging phenotype (Khan et al. 2001; Sonntag et al. 1997; Amenta et al. 1995; Bell and Ball 1981; Hicks et al. 1983). Our findings, taken together with results of previous studies obtained in mice with developmental liver-specific knockdown of IGF-1 (Lopez-Lopez et al. 2004), suggest that circulating level of IGF-1 is a critical regulator of brain capillarity. These findings are clinically relevant as circulating IGF-1 levels significantly decrease with age in humans (Franco et al. 2014; O'Connor et al. 1998), and restoration of circulating IGF-1 in older laboratory animals was shown to significantly increase cerebromicrovascular density (Sonntag et al. 1997).

It is generally considered that cerebromicrovascular rarefaction contributes to a decline in cerebral blood flow (CBF) that reduces metabolic support for neural signaling, thereby promoting neuronal dysfunction (Riddle et al. 2003; Sonntag et al. 1997; Khan et al. 2002; Troen et al. 2008). The hippocampus is a key brain region involved in learning and memory, one of the main regions affected in Alzheimer's disease and it is known to be particularly vulnerable to ischemia. The retrosplenial cortex is also implicated in a wide range of cognitive functions including episodic memory and navigation. Importantly, previous studies demonstrate that rarefaction of the hippocampal microvasculature and/or the retrosplenial cortex in various pathophysiological conditions predicts cognitive dysfunction and impaired performance on spatial memory tests in the absence of or preceding neurodegeneration (Tucsek et al. 2014; Warrington et al. 2012; Troen et al. 2008).

Previous studies showed that hypertension promotes microvascular rarefaction in the brain and this effect is significantly exacerbated in old age (Toth et al. 2013a). This is the first study to demonstrate that circulating IGF-1 deficiency exacerbates the deleterious effects of hypertension on hippocampal microvascular density (Fig. 1–3), mimicking the aging phenotype. Importantly, the available human evidence suggest that hypertension in IGF-1 deficient patients also significantly compromise higher brain function (Angelini et al. 2009). Early studies on decreased capillary density in the peripheral circulation of hypertensive experimental animals and human patients proposed that rarefaction can be either structural (capillary attrition) or functional, associated with impaired recruitment of nonperfused capillaries (Chen et al. 1981; Hashimoto et al. 1987; Ono et al. 1989; Prewitt et al. 1986; Prewitt et al. 1982; Prewitt et al. 1984; Stacy and Prewitt 1989; Sullivan et al. 1983). Our studies provide additional evidence that interaction of IGF-1 deficiency and hypertension promote structural rarefaction in the mouse brain, extending previous findings obtained in hypertensive patients (Wolf et al. 1994; Bell and Ball 1981; Bell and Ball 1990; Abernethy et al. 1993; Mann et al. 1986) and animal models of aging (Sonntag et al. 1997; Wiesenborn et al. 2014), hypertension (Toth et al. 2013a) and IGF-1 deficiency (Lopez-Lopez et al. 2004), but further studies are needed to understand their synergistic effects on the cerebral microvasculature.

The mechanisms underlying IGF-1 deficiency-related structural microvascular rarefaction are likely multifaceted. There is substantial evidence that the cerebral microcirculation is subject to continuous dynamic structural adaptation, a concept that implies a high plasticity of the cerebral microvascular network (Riddle et al. 2003). Accordingly, there is a dynamic balance between capillary regression and growth, which is regulated by various paracrine factors produced by brain cells. It is

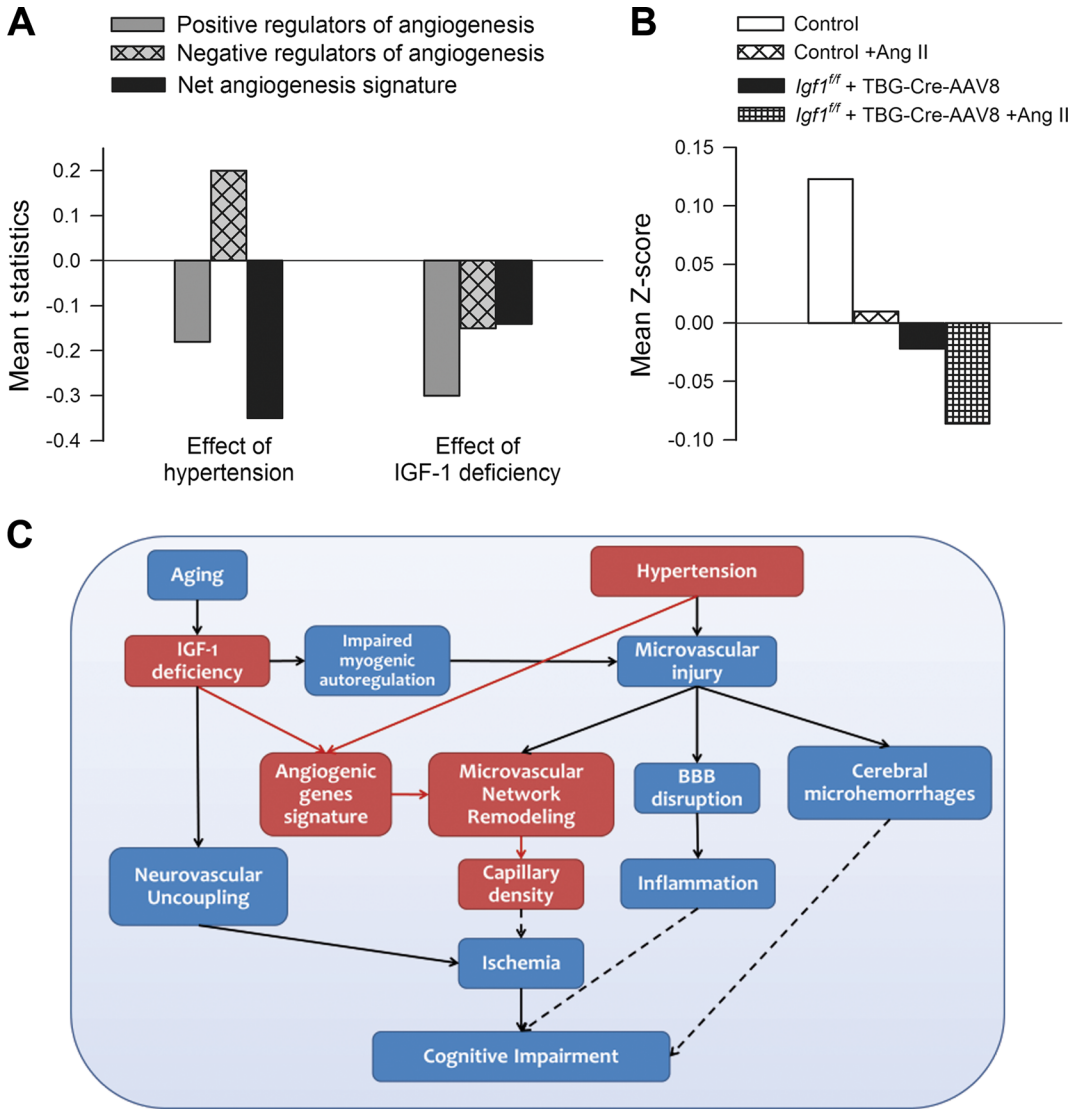


Fig. 8 Hypertension in IGF-1 deficiency is associated with a gene expression signature in the mouse hippocampus that favors microvascular rarefaction. **a** Mean *t* statistic for positive and negative angiogenesis regulators, and the signed aggregate of both groups. "effect of hypertension" refers to hypertensive vs. normotensive groups, "effect of IGF-1 deficiency" refers to IGF-1 deficient vs control groups. **b** Gene set enrichment analysis (GSEA) scores for positive regulators of angiogenesis. Both hypertension and IGF-1 deficiency decreases the angiogenesis signature. Exacerbation of the effects of hypertension by IGF-1 deficiency is evident. **c** Proposed scheme depicting the mechanisms by which IGF-1 deficiency may exacerbate hypertension-induced microvascular damage and promote the pathogenesis of vascular cognitive impairment. The model predicts that IGF-1 deficiency impairs both functional and structural adaptation of the cerebral microcirculation to hypertension. Solid lines represent links supported by existing experimental evidence (see Discussion for details). *Dashed lines* represent proposed mechanistic links among microvascular injury, regional ischemia, microhemorrhages, neuroinflammation, and cognitive impairment, which should be tested experimentally by future studies

generally accepted that impaired angiogenesis, due to dysregulated production of autocrine/paracrine regulators of angiogenic processes, is a critical mechanism involved in cerebromicrovascular rarefaction (Tucsek et al. 2014; Ungvari et al. 2010). Here, we demonstrate that in the mouse hippocampus IGF-1 deficiency decrease the angiogenesis gene expression signature (e.g., decreasing the expression of pro-angiogenic genes and up-regulating several anti-angiogenic factors). In accordance with our findings, there is strong in vitro evidence

that IGF-1 regulates multiple aspects of the angiogenic process, including induction of endothelial cell proliferation, migration, and tube formation (Viana et al. 2015). Furthermore, previous studies demonstrate that treatment of mice with IGF-1 results in formation of new capillaries in the mouse brain (Lopez-Lopez et al. 2004). Our data suggest that hypertension also results in dysregulated expression of pro- and anti-angiogenic genes, and these effects tend to be exacerbated by IGF-1 deficiency. The IGF-1 dependent mechanisms responsible for impairment of endothelial angiogenic capacity likely also include dysregulation of Nrf2, a newly discovered regulator of endothelial angiogenic processes (Valcarcel-Ares et al. 2012). This concept is supported by the findings that aging is associated with Nrf2 dysfunction in endothelial cells (Ungvari et al. 2011a; Ungvari et al. 2011b) and that IGF-1 deficiency exacerbates Nrf2 dysfunction (Bailey-Downs et al. 2012a), mimicking the aging phenotype.

Previous studies have established a causal link among decreased bioavailability of NO, impaired angiogenesis and microvascular rarefaction (Frisbee et al. 2007). Endothelium-derived NO is both a downstream mediator of VEGF signaling and a critical regulator of microvascular endothelial cell viability. Previous studies have shown that IGF-1 deficiency may impair endothelial NO bioavailability by increasing breakdown of NO due to elevated cellular production of ROS (Toth et al. 2015) and/or by down-regulating eNOS (Csiszar et al. 2008). Thus, it is possible that impaired microvascular NO mediation contributes to microvascular rarefaction associated with circulating IGF-1 deficiency. Collectively, on the basis of our recent and previous findings, we propose that a decline in pro-angiogenic stimuli and up-regulation of anti-angiogenic factors is an important cause for both cerebromicrovascular rarefaction associated with IGF-1 deficiency and the exacerbation of hypertension-induced decreases in capillary density in IGF-1 deficient animals.

It is also likely that a significant part of IGF-1 deficiency-related and hypertension-induced microvascular rarefaction can be ascribed to endothelial injury and apoptosis. IGF-1 is known to exert diverse anti-apoptotic effects (Higashi et al. 2012), including regulation of Nrf2 activity (Bailey-Downs et al. 2012a; Bailey-Downs et al. 2012b) in endothelial cells. Importantly, our previous studies provide evidence that circulating IGF-1 deficiency impairs cellular stress resistance pathways in the vasculature, exacerbating oxidative stress-mediated endothelial apoptosis (Bailey-Downs et al. 2012a; Bailey-Downs et al. 2012b). Further studies are warranted to determine the role for increased microvascular apoptosis in hypertension-induced microvascular rarefaction in IGF-1 deficient mice.

Several additional mechanisms may also be considered to contribute to structural microvascular rarefaction in IGF-1 deficiency, including pericyte damage (Toth et al. 2013a), increased precapillary arteriolar constriction and cessation of capillary blood flow, increased susceptibility to microemboli, platelet adhesion and macrophage activation, and formation of string vessels (Brown 2010). Further, the mechanisms underlying the exacerbation of hypertension-induced microvascular injury in IGF-1 deficiency are also likely to include hemodynamic factors (Fig. 8c). There is strong evidence that in healthy young animals pressure-induced myogenic constriction of proximal arterial branches of the cerebrovascular tree acts as a critical homeostatic mechanism that assures that increased systemic arterial pressure cannot penetrate the distal portion of the cerebral microcirculation and cause damage to the thin-walled arteriolar and capillary microvessels (Toth et al. 2013a; Toth et al. 2014a; Kontos et al. 1978; Harper and Bohlen 1984). In cerebral resistance arteries isolated from hypertensive young control animals (Toth et al. 2013a; Toth et al. 2013b), the myogenic constriction at high pressures is augmented, suggesting that the pressure range for autoregulatory cerebrovascular protection is extended. The aforementioned functional adaptation of cerebral arteries to higher systemic blood pressure is believed to protect the cerebral microcirculation from pressure-induced injury (Toth et al. 2013a; Toth et al. 2014a). Our recent studies demonstrate that cerebral arteries of IGF-1 deficient mice do not exhibit a hypertension-induced adaptive increase in myogenic tone observed in mice with normal IGF-1 levels (Toth et al. 2014a), which mimics the aging phenotype (Toth et al. 2013a; Toth et al. 2013c; Springo et al. 2015). Pathological loss of autoregulatory protection in IGF-1 deficiency likely allows high blood pressure to penetrate the distal, injury-prone portion of the cerebral microcirculation, leading to significant downstream damage.

In addition to microvascular rarefaction, the impairment of microvascular dilator mechanisms is also likely to contribute to dysregulation of CBF and cognitive decline in IGF-1 deficiency (Toth et al. 2015) (Fig. 8c). Our recent studies demonstrate that IGF-1 deficiency leads to profound neurovascular dysregulation, characterized by impaired CBF responses induced by synaptic activity (Toth

et al. 2015), which recapitulates cerebromicrovascular alterations present in aged mice (Toth et al. 2014b). Circulating IGF-1 deficiency also appears to compromise the barrier function of the cerebromicrovascular endothelial cells (Toth et al. 2014a) and exacerbate hypertension-induced disruption of the blood-brain barrier, promoting low-grade neuroinflammation (Toth et al. 2014a), mimicking the aging phenotype (Toth et al. 2013a). This was an important finding, as there is growing evidence causally linking blood-brain barrier disruption and neuroinflammation to age-related cognitive decline (Montagne et al. 2015; Carnevale et al. 2012).

In conclusion, adult-onset, isolated endocrine IGF-1 deficiency exerts multifaceted deleterious effects of the cerebral microcirculation, leading to a significant decline in cortical and hippocampal capillarity and exacerbating hypertension-induced cerebromicrovascular rarefaction. The morphological impairment of the cerebral microvasculature induced by IGF-1 deficiency and hypertension, in association with neurovascular uncoupling, increased blood-brain barrier disruption and neuroinflammation reported in previous studies (Toth et al. 2015; Toth et al. 2014a) likely contribute to the pathogenesis of vascular cognitive impairment in the elderly.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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