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RESEARCH ARTICLE

Angiogenesis is VEGF-independent in the aged striatum of male rats exposed to acute hypoxia

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Abstract Brain hypoxia is involved in many diseases. The activation of angiogenesis is one of the major adaptive mechanisms to counteract the adverse effects of hypoxia. In a previous work, we have shown that the adult rat striatum promotes angiogenesis in response to hypoxia via upregulation of the most important proangiogenic factor, the vascular endothelial growth factor (VEGF). However, the effects of hypoxia on angiogenesis in the aged striatum remain unknown and constitute our aim. Here we show the upregulation of hypoxia-inducible factor-1α in the striatum of aged (24–25 months old) Wistar rats exposed to acute hypoxia and analysed during a reoxygenation period ranging from 0 h to 5 days. While the mRNA expression of the proangiogenic factors VEGF, transforming growth factor-β1 (TGF-β1), and adrenomedullin dropped at 0 h post-hypoxia compared to normoxic control, no changes were detected at the protein level, showing an impaired response of these proangiogenic factors to hypoxia in the aged striatum. However, the striatal blood vessel network increased at 24 h of reoxygenation, suggesting that mechanisms independent from these proangiogenic factors may be involved in hypoxia-induced angiogenesis in the striatum of aged rats. A thorough understanding of the factors involved in the response to hypoxia is essential to guide the design of therapies for hypoxia-related diseases in the aged brain.

Keywords Angiogenesis · Aging · Hypoxia · Reoxygenation · Brain

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Introduction

Brain hypoxia is considered a critical factor in many diseases of the central nervous system, including neurodegenerative diseases, stroke, and tumours. Angiogenesis is induced in response to hypoxia to avoid the deleterious effects of oxygen deficiency by ensuring an appropriate blood supply to living cells (Fong 2008). The main regulator of angiogenesis under conditions of hypoxia is the hypoxia inducible factor-1 (HIF-1), composed of a stable beta subunit (HIF-1β) and one of two oxygen-regulated α-subunits (HIF-1α or HIF-2α). Under hypoxia, the α/β hetero-dimeric HIF complex is stabilized and induces

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transcription through the interaction with the hypoxia-response elements (HREs) located in the promoter regions of the target genes (Fong 2008). HIF activates the expression of the most important proangiogenic factor, the vascular endothelial growth factor-A (VEGF-A). VEGF-A, normally termed as VEGF, is a selective endothelial mitogen that promotes growth of vascular endothelial cells in vivo and in vitro models (reviewed in Ferrara et al. 2003). However, angiogenesis is a highly complex and coordinated process that is modulated by a multitude of pro- and antiangiogenic factors. Some of these other proangiogenic factors are adrenomedullin and transforming growth factor-β1 (TGF-β1). Adrenomedullin is a HIF-1-dependent vasoactive target gene that modifies tissue perfusion by increasing VEGF expression (Iimuro et al. 2004) and modulating the action of TGF-β1 (Wang et al. 2006). TGF-β1, another HIF-1 target gene (Hung et al. 2013), is a cytokine that mediates vascular development, regulates VEGF expression (Donovan et al. 1997), and stabilizes HIF-1 protein (McMahon et al. 2006).

A maladaptive response of the brain vasculature to hypoxia may trigger several neurological disorders and neurodegenerative diseases (Correia et al. 2013). It has been reported that the vascular response to hypoxia varies among brain areas and during aging (Patt et al. 1997; Brown and Thore 2011). The striatum, the largest brain basal ganglion, is particularly vulnerable to hypoxia (Pisani et al. 1997). This vulnerability may be related to a membrane depolarization associated with a massive raise in calcium levels, which may lead to cell death in striatal neurons (Pisani et al. 1997). The striatum is also sensible to neurodegenerative processes related to aging (Gardoni and Bellone 2015). In a previous work, we have found that the adult striatum promotes angiogenesis via VEGF in response to hypoxia as a neuroprotective mechanism (Molina et al. 2013). In the present study, we have investigated the effects of hypoxia on the angiogenic pathway in the striatum of 24–25 months old rats subjected to acute hypoxia and analysed during a reoxygenation period ranging from 0 h to 5 days. A fuller understanding of the factors involved in the response to hypoxia in the brain of aged individuals, which are more frequently exposed to conditions of oxygen deficiency, is critical for identifying new ways of managing hypoxia-related diseases.

Methods

Experimental procedure

The acute hypobaric hypoxia was carried out as previously published by our group (Molina et al. 2013). Briefly, animals were placed in a hypoxic chamber, and acute hypobaric hypoxia was induced by downregulating the environmental oxygen pressure to a final barometric pressure of 225 mm Hg ($\approx$ 30.000 feet), resulting in a maximum of 48 mmHg oxygen partial pressure. These conditions were maintained for 20 min. After the acute hypobaric hypoxia period, animals were kept under normobaric normoxic conditions for different reoxygenation times (0 h, 24 h, and 5 days). Control animals were sacrificed after being maintained for 20 min in the chamber under normobaric normoxic conditions.

Animals

The study was performed on aged (24–25 months old) male albino Wistar rats kept under standard conditions of light and temperature and allowed ad libitum access to food and water. All the experiments were conducted according to E.U. guidelines on the use of animals for biochemical research (86/609/EU). The study was approved by the Ethics Committee of the University of Jaén (Spain). Twenty Wistar rats (five animals per group) were used for the biochemical experiments. The rats were sacrificed by cervical dislocation and the striatum was immediately manually removed and stored at −80 °C until used. For histochemistry, 20 rats (five animals per group) were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB), and the whole brain was removed and postfixed in the same fixative.

Semi-quantitative real-time polymerase chain reaction (RT-PCR)

Total RNA isolation and cDNA synthesis were performed from aged striatum as previously described (Molina et al. 2013). FAM labeled rat Hif-1α (Assay ID: Rn00577560_m1), Vegf-a (Assay ID: Rn00582935_m1), Adrenomedullin (Assay ID: Rn00562327_m1), and Tgf-β1 (Assay ID: Rn99999016_m1)

TaqMan gene expression assays were purchased from Thermo Fisher Scientific. VIC labeled endogenous reference gene 18S ribosomal RNA (Assay ID: Hs99999901_s1) TaqMan gene expression assay was also purchased from Thermo Fisher Scientific. RT-PCR reactions were carried out in the CFX-96^{TM} thermal cycler (Bio-Rad) according to amplification conditions following the manufacturer's protocol. Expression levels were normalized to that of the endogenous 18S ribosomal RNA, and data were calculated as fold expression relative to the average of control group. The relative expression of each gene was calculated by the 2[$-\Delta\Delta$C(T)] method (Li et al. 2006).

Enzyme-linked immunosorbent assay (ELISA)

The striatum was homogenized in PBS buffer (1:3 w/v) with a homogenator (Pellet Pestle Motor Cordless, Kontes, USA), and then centrifuged at 40.000 rpm for 30 min. Protein concentration of VEGF-A, TGF-β1, and Adrenomedullin was determined in the supernatants using Rat VEGF-A ELISA kit (Ab Frontier, Cat. No. LF-EK50417), Rat TGF-β1 ELISA kit (Ab Frontier, Cat. No. LF-EK50357), and ELISA kit for rat Adrenomedullin (Uscn Life, Cat. No. E90220Ra) according to the manufacturer's protocol. Total protein concentrations were determined by the Bradford method, using bovine serum albumin as the standard. Protein values were referred to the total protein concentration in the initial supernatants.

Histological procedures for blood vessel labelling

The histological protocol for blood vessel labelling was performed as previously described (Molina et al. 2013). Briefly, the postfixed brain was cryoprotected and sectioned following a rostrocaudal direction using a cryostat (Leica CM1950, Germany). Sections of aged striatum (15 μm thickening) were incubated in 1:100 Lycopersicon esculentum lectin (10 μg/mL, Sigma-Aldrich, Ref. L0651), and then in Cy3-Streptavidin solution (Sigma-Aldrich, Ref. S6402). No labelling was detected in negative controls when lectin was omitted. Striatum sections were digitally captured from a fluorescence microscope (Olympus BX51, Olympus Optical, Tokyo, Japan).

Quantification of vascular complexity by fractal dimension analysis

One random 1.56 mm^2 field (image 10×) on each section, and five random sections (from rostral to caudal striatum) for each rat were transformed into black and white images, and then inverted using ImageJ (an NIH image analysis and processing software downloaded free from http://rsbweb.nih.gov/ij/). The vascular complexity was quantified by fractal dimension, which was estimated using the box-counting method as previously described (Di Ieva et al. 2007). Data were expressed as arbitrary units ranging from 1 (absence of lectin labelling) to 2 (total lectin labelling).

Statistical analysis

Data were expressed as mean ± SD (standard deviation). The statistical treatment was performed with IBM SPSS Statistics version 19. The data that followed a normal distribution (tested with the Kolmogorov–Smirnov test) and the principle of homoscedasticity of variances (tested with the Levene test) were tested by one-way ANOVA (Analysis of Variance). The statistical significance was established by applying the Dunnett test to compare differences versus control group. The data that followed neither a normal distribution, nor the principle of homoscedasticity were tested using the Kruskal–Wallis test (mRNA levels of HIF-1α, VEGF-A, adrenomedullin, and TGF-β1, as well as quantification of vascular complexity). The degree of statistical significance was established by applying the U Mann–Whitney test to compare differences between means. The statistically significant differences versus control group were expressed as $*p < 0.05$. Microsoft Excel 2013 and Adobe Photoshop CS4 were used to create the figures.

Results

mRNA expression of HIF-1α in the striatum of 24–25 months old rats exposed to hypoxia/reoxygenation

RT-PCR was used to analyse the mRNA expression of the main regulator of the molecular adaptive responses to hypoxia, HIF-1α. Hif-1α mRNA levels (Fig. 1) increased more than fourfolds at 0 h post-hypoxia
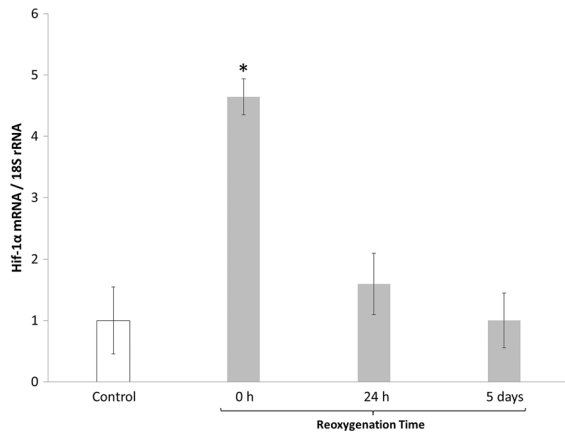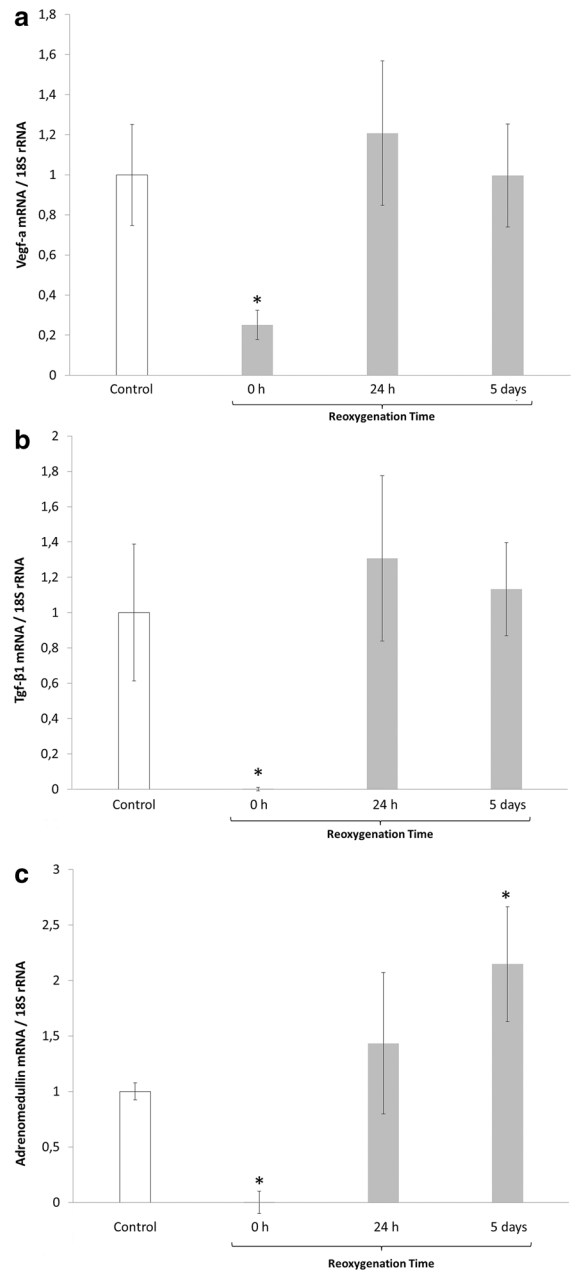
Fig. 1 mRNA expression of HIF-1α in the striatum of 24–25 months old rats exposed to hypoxia/reoxygenation. *White bar* control animals were maintained for 20 min in the hypobaric hypoxic chamber under normobaric normoxic conditions, and then were sacrificed. *Grey bars* animals were exposed to hypobaric hypoxia for 20 min, and then were kept under normobaric normoxic conditions for different reoxygenation times (0 h, 24 h, and 5 days). Data, expressed as arbitrary units, are mean ± SD of three independent experiments and five animals per group. All experiments were performed in triplicate, and the values were used to calculate the ratio of Hif-1α to 18S ribosomal RNA, with a value of 1 used as control. The statistically significant differences versus control group were expressed as *p < 0.05

(4.64 ± 0.29) in comparison to control group (p < 0.05), and returned to control levels after 24 h.

mRNA and protein expression of proangiogenic factors in the striatum of 24–25 months old rats exposed to hypoxia/reoxygenation

The mRNA levels of the proangiogenic factors Vegf-a (Fig. 2a), Tgf-β1 (Fig. 2b), and Adrenomedullin (Fig. 2c) dramatically decreased just after hypoxia (Vegf-a = 0.025 ± 0.02; Tgf-β1 = 0.0002 ± 0.009; Adrenomedullin = 0.0003 ± 0.1) when compared to control (all p < 0.05). At 24 h post-hypoxia, the mRNA expression of the three genes returned to control values. At 5 days of reoxygenation, only Adrenomedullin (Fig. 2c) showed increased mRNA levels (2.15 ± 0.51) in comparison to normoxic control (p < 0.05). These proangiogenic factors were also analysed at the protein level using ELISA. The protein levels of VEGF-A (Fig. 3a), TGF-β1 (Fig. 3b), and Adrenomedullin (Fig. 3c) remained unchanged after hypoxia vs. control group.

Distribution and quantification of the vascular complexity in the striatum of 24–25 months old rats exposed to hypoxia/reoxygenation

Biotinylated lectin from Lycopersicon esculentum specific for N-acetyl-glucosamine and N-acetyl-poly-lactosamine oligomers is widely used as the best marker for brain endothelium. Figure 4a shows a representative

◀**Fig. 2** mRNA expression of proangiogenic factors in the striatum of 24–25 months old rats exposed to hypoxia/reoxygenation. mRNA levels of Vegf-a (**a**), Tgf-β1 (**b**), and Adrenomedullin (**c**). *White bar* control animals were maintained for 20 min in the hypobaric hypoxic chamber under normobaric normoxic conditions, and then were sacrificed. *Grey bars* animals were exposed to hypobaric hypoxia for 20 min, and then were kept under normobaric normoxic conditions for different reoxygenation times (0 h, 24 h, and 5 days). Data, expressed as arbitrary units, are mean ± SD of three independent experiments and five animals per group. All experiments were performed in triplicate, and the values were used to calculate the ratio of each gene to 18S ribosomal RNA, with a value of 1 used as control. The statistically significant differences versus control group were expressed as *p < 0.05

image of lectin-labelled blood vessels in the aged striatum of each experimental group. The striatal blood vessels of control and hypoxic animals (Fig. 4a) were homogeneously distributed throughout the aged striatum, and showed an irregular lumen and tortuous path (see inserts). Lectin labelling was quantified using the box-counting method for estimation of the fractal dimension. The fractal dimension analysis (Fig. 4b) for lectin-positive area showed a significant increase in the complexity of the blood vessel network at 24 h post-hypoxia (1.46 ± 0.03) compared to normoxic control (1.31 ± 0.02; p < 0.05). At 5 days of reoxygenation (1.34 ± 0.02), the blood vessel complexity recovered control values.

Discussion

Angiogenesis is an important component of the brain homeostatic mechanisms that try to avoid the deleterious effects of hypoxia. The influence of hypoxia on angiogenesis remains unknown in the aged striatum and constitutes our aim.

The master regulator of the adaptation to hypoxia is HIF-1. Our results showed an over-expression of HIF-1α at the mRNA level in the aged striatum immediately after hypoxia, proposing that aged individuals may quickly respond to conditions of oxygen deficiency. In contrast, the mRNA levels of HIF-1α did not change either in hypoxic vascular smooth muscle cells of aged animals (Rivard et al. 2000), or in muscle tissue from aged mice subjected to ischaemia (Lam et al. 2016). These contradictory results could be explained taking into account the tissue analysed and
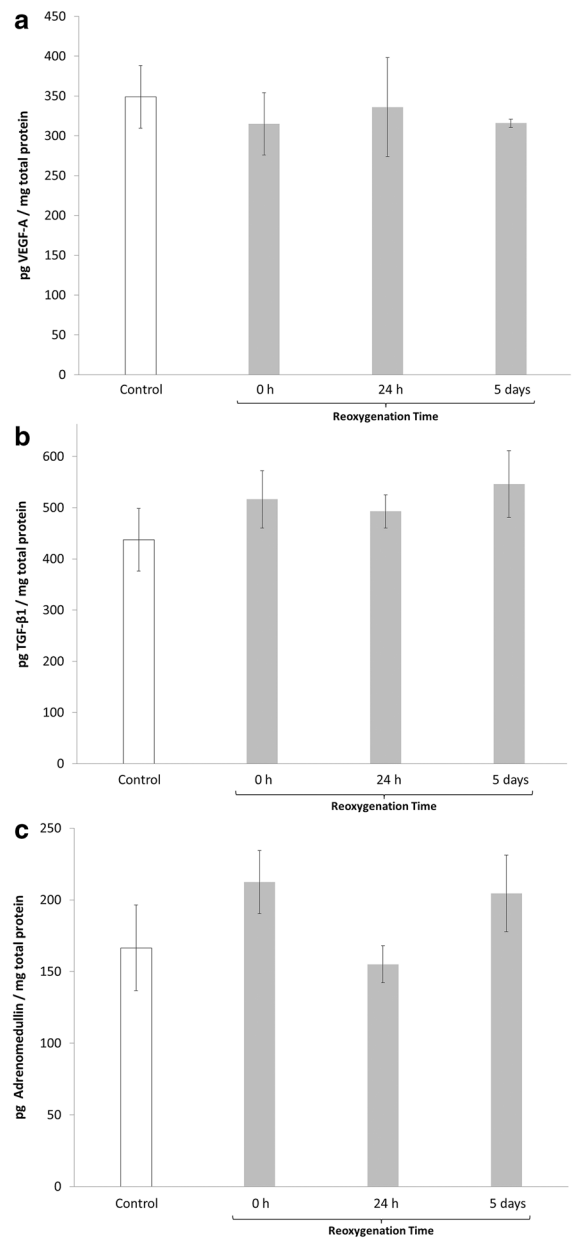
Fig. 3 Protein expression of proangiogenic factors in the striatum of 24–25 months old rats exposed to hypoxia/reoxygenation. Protein levels of VEGF (**a**), TGF-β1 (**b**), and Adrenomedullin (**c**). *White bar* control animals were maintained for 20 min in the hypobaric hypoxic chamber under normobaric normoxic conditions, and then were sacrificed. *Grey bars* animals were exposed to hypobaric hypoxia for 20 min, and then were kept under normobaric normoxic conditions for different reoxygenation times (0 h, 24 h, and 5 days). Data are mean ± SD of three independent experiments with five animals per group. All experiments were performed in duplicate

Fig. 4 Distribution and quantification of the vascular complexity in the striatum of 24–25 months old rats exposed to hypoxia/reoxygenation. *White bar* control animals were maintained for 20 min in the hypobaric hypoxic chamber under normobaric normoxic conditions, and then were sacrificed. *Grey bars* animals were exposed to hypobaric hypoxia for 20 min, and then were kept under normobaric normoxic conditions for different reoxygenation times (0 h, 24 h, and 5 days). **a** Representative microphotographs of striatal sections stained for lectin histochemistry. *Scale bars* 100 μm. **b** Fractal dimension analysis of vascular surface area from histological sections stained with lectin. Data are mean ± SD of 25 microphotographs (five microphotographs per animal and five animals per group). The statistically significant differences versus control group were expressed as *p < 0.05
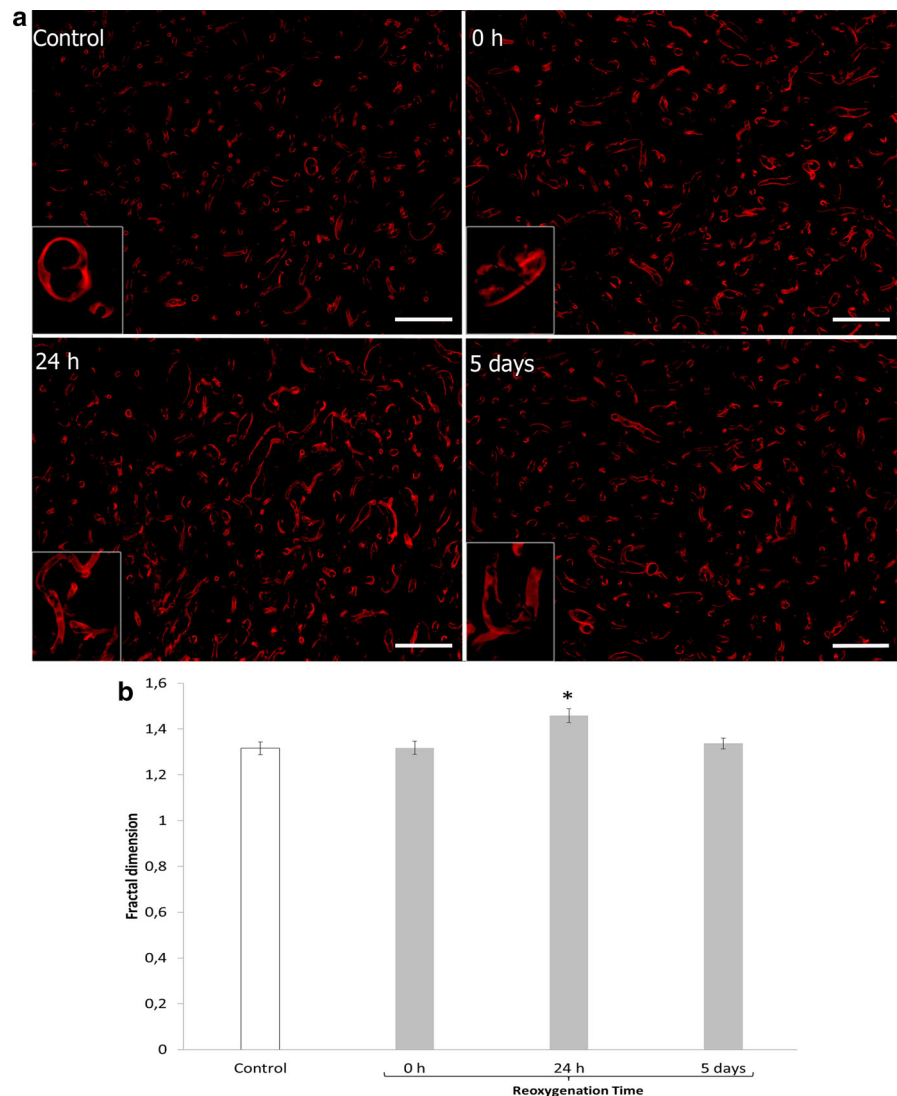
the different experimental models used. HIF-1 is induced by hypoxia in order to promote an adequate oxygen delivery to tissues by initiating angiogenesis, among other actions. However, our results showed that the mRNA levels of the proangiogenic factors VEGF, TGF-β1, and adrenomedullin drastically dropped just after hypoxia in the aged striatum, returning to control values after 24 h. In contrast, we have previously reported that hypoxia upregulated, not only HIF-1α, but also VEGF, TGF-β1, and adrenomedullin in the adult striatum, suggesting that aging may alter the response of these proangiogenic factors to hypoxia (Molina et al. 2013). An explanation for these differences between adult and aged animals may be

related to the mode of action of HIF-1α. HIF-1α translocates to the nucleus under hypoxia to activate gene transcription using a specific importin. However, aging may reduce the translocation of HIF-1α to the nucleus (Ahluwalia and Tarnawski 2012), preventing the transcription of target genes such as VEGF, TGF-β1, and adrenomedullin. In the same line, the expression of the prolyl hydroxylases (PDHs), the enzymes that degrade HIF-1α, increased with age (Ndubuizu et al. 2009), which is in concordance with the lack of activation of the HIF-1 target genes in the hypoxic aged striatum. The decline found in mRNA levels immediately after hypoxia agrees with our previous results on nitric oxide synthases (NOS) mRNA levels

in the hypoxic aged striatum (Molina et al. 2016). NOS isoforms can be induced by hypoxia to increase the levels of the potent vasodilator nitric oxide (NO) in order to restore oxygen supply. Accordingly, it has been reported that hypoxia strongly diminished gene transcription due to changes in histone acetylation and methylation (Johnson et al. 2008), and altered the activity of RNA polymerase II (Ignacak et al. 2009). Previous studies using different experimental models have shown contradictory results on mRNA levels of the proangiogenic factors. Contrarily to our results, VEGF mRNA expression increased in the aged brain exposed to chronic hypoxia (Ndubuizu et al. 2010). Buga et al. (2014a) performed a detailed transcriptomic analysis of post-stroke angiogenesis in aged and young rats. Similar to our results of VEGF mRNA levels at 24 h post-hypoxia, they did not find significant differences in VEGF mRNA expression in aged rats at 3 days post-stroke versus control aged rats. While Efimenko and coworkers described decreased TGF-β mRNA expression in cultured mesenchymal cells from aged mice after hypoxia (Efimenko et al. 2011), Buga et al. (2014b) reported a 5.97 fold increase in TGF-β1 mRNA levels in the ischemic brain of aged rats at 3 days post-stroke versus control aged rats. Both ischaemic and chronic hypoxic insults may be more aggressive than acute hypoxic events, resulting in a different expression of VEGF and TGF-β1 in each case. Cerebral ischemia produces a focal injury that may lead to massive cell death and tissue damage. Tissue damage activates the wound healing process, which leads to the reorganization of the extracellular matrix (ECM), including mainly ECM degradation and scar tissue formation. TGF-β1 has long been known as a key factor in scar tissue development in the central nervous system (Logan et al. 1994). Moreover, it has been found increased expression of the matrix-degrading encoding proteases genes Adam 17 and Mmp14 in aged rats at 3 days post-stroke (Buga et al. 2012). On the other hand, few studies are focused on acute hypoxia and even fewer analyse the reoxygenation period, as we have done, which is important because when oxygen level is restored after hypoxia, an increase in reactive oxygen species occurs that may result in tissue damage (Prabhakar 2001).

Our data showed that protein levels of the proangiogenic factors VEGF, TGF-β1, and adrenomedullin remained unaltered after hypoxia in comparison to normoxic control, showing the absence of activation of the VEGF-related angiogenic pathway in the aged striatum in response to acute hypoxia. The unchanged protein levels of VEGF are in concordance with previous results found in the aged brain subjected to chronic hypobaric hypoxia (Benderro and Lamanna 2011). It is noteworthy that the mRNA levels of these proangiogenic factors dropped after hypoxia in the aged striatum without parallel changes in protein expression. We previously found a similar expression pattern for NOS isoforms in the hypoxic aged striatum (Molina et al. 2016). Hypoxia may diminish the activity of the proteasome (Gozal et al. 2003), preventing the degradation of the proangiogenic proteins under hypoxia, and allowing detecting similar protein expression than that of normoxic control. Hence, the reduced function of the proteasome may lead to sustained protein levels despite the decreased mRNA expression. Our results also showed divergent patterns in adrenomedullin gene and protein expression at 5 days post-hypoxia, since mRNA levels increased, but protein levels remained unchanged. In the aged lung exposed to acute hypoxia, increased adrenomedullin mRNA levels were found, although no changes were reported in protein expression (Hwang et al. 2007). The same authors found that the reason may be the hypoxia-induced decrease in the percentage of translatable adrenomedullin mRNA in aged animals (Hwang et al. 2007).

The quantification of the vascular network by fractal dimension analysis showed an increase at 24 h post-hypoxia in the aged striatum. In agreement, chronic hypoxia augmented the microvascular density in the striatum of aged rats (Ndubuizu et al. 2010). The same authors found that both young and aged animals increased angiogenesis in response to hypoxia. We have previously reported that the blood vessel network augmented at 24 h post-hypoxia in the adult striatum (Molina et al. 2013), results similar to those found in aged animals. Moreover, morphological differences may be described in the striatal vasculature of aged rats versus the adult ones. The blood vessels of the aged striatum showed an irregular lumen and tortuous path when compared to the adult vasculature (Molina et al. 2013), which agrees with previous results in brain capillaries of aged individuals (Wang et al. 2004). Despite the increase detected in the blood vessel complexity in the aged striatum in response to hypoxia, the protein

levels of the proangiogenic factors VEGF, TGF-β1, and adrenomedullin remained unchanged throughout the post-hypoxia period, suggesting that other factors are ultimately able to allow the aged striatum adapting to acute hypoxia by increasing microvascular angiogenesis. Many molecules, apart from those analysed in the present work, have been implicated in angiogenesis, such as angiopoietins, basic fibroblast growth factor (bFGF), tumor necrosis factor-α (TNF-α), angiogenin, and IL-8 (reviewed in Ferrara et al. 2003). Buga et al. (2012) performed a Genome-Wide Analysis to investigate the whole-gene transcriptome in a model of cerebral stroke in aged and young rats. They found that some genes involved in angiogenesis and vascular remodelling were increased in aged rats, either on day 3 post-stroke (Adam17, Gpc3, Mmp14, Nid2, Tagln, Wnt5b) or on day 14 after stroke (Col4a2, Col8a1, Cthrc1, Cxcl1, Gpc3, Tgfbr2). Some of these genes, including Mmp14, Gpc3 and Tgfbr2, are part of the TGF-β signalling pathway involved in angiogenesis in the central nervous system. In a more recent study, the same authors (Buga et al. 2014a) performed a detailed transcriptomic analysis of post-stroke angiogenesis in aged and young rats. They found that the majority of genes involved in sprouting angiogenesis (Angpt2, Angptl4, Cib1, Nrp1, Rac2, Runx1), reconstruction of a new basal lamina (Lamc1, Plod2), or tube formation and maturation (Angpt1, Gpc3, Igfbp7, Sparc, Tie2, Tnfsf10) were more increased in the aged rats at 14 days post-stroke than at 3 days post-stroke. Investigators of the same research group (Sandu et al. 2016) showed that hypothermia increased vascular density in the brain of ischemic aged rats at 14 days post-stroke. The temporal difference in detection of angiogenesis in such study and in the present one may be due to several reasons. On one hand, the authors did not analyse the vascular density before 14 days post-stroke, making it impossible to know if shorter post-stroke periods may change the vascular density. On the other hand, the method used for analysing the vessel network in both studies is different. In the study of Sandu et al. (2016) the microvascular density was quantified using the "hot spot" analysis, while we have examined the complexity of the vessel network by fractal dimension analysis. The fractal dimension analysis has been widely used for the study of angiogenesis because is an automatized and objective method that avoids subjective bias in the quantification of the blood vessel network (Mancardi et al. 2008). Moreover, the fractal dimension analysis does not only quantify the marked surface area in the microphotographs, but also the distribution and organization of the marked area (Grizzi et al. 2005). Recent findings in an in vitro model have shown a slight vessel sprouting and an increased VEGF protein expression in endothelial cells at 12 h of exposure to hypoxia in comparison to normoxic control cells (Wang et al. 2015). Such a slight sprouting, beginning of angiogenesis, might also occur in our aged rats at 24 h post-hypoxia and could be detected by fractal dimension analysis. In our study, we did not find increased protein levels of VEGF, TGF-β1 or Adrenomedullin, but other proangiogenic factors may be involved in this process (Buga et al. 2012; Buga et al. 2014b). Therefore, because of the high sensitivity of the fractal dimension analysis and the high consistency of the method, small changes (for example, due to a slight vessel sprouting after a short post-hypoxia period) in the complexity of the blood vessel network could be detected. This transitory change in the vascular network detected in our aged rats may appear soon after hypoxia, but might be sufficient to respond to short-term hypoxia, which may involve a moderate damage in comparison to the ischemic processes. To conclude, although our data show that both the adult and aged striatum increased the microvascular network at 24 h of reoxygenation in response to acute hypoxia, angiogenesis was mediated by VEGF in the adult striatum (Molina et al. 2013), but was VEGF-independent in the aged striatum.

In the present study, we included only male rats in order to avoid the possible influence of the ovarian hormone reduction in aged female rats on the angiogenic response to hypoxia. Not including female animals in research has traditionally been justified due to the variable nature of female data caused by hormonal fluctuations associated with the female reproductive cycle (Rich-Edwards et al. 1995; Sullivan and Fowlkes 1996; Lawlor et al. 2002). However, recent meta-analyses have reported similar results in different traits, including neuroscience-related traits, in male and female animals and humans (Prendergast et al. 2014; Itoh and Arnold 2015; Becker et al. 2016). Despite there are no similar reviews available analysing sex differences in aged animals or humans, based on the previous findings it may be expected not

to find significant differences in the results of our study between aged male and female rats.

In conclusion, our findings reveal that the aged striatum of male rats may upregulate HIF-1α in response to acute hypoxia, although the protein expression of the proangiogenic factors VEGF, TGF-β1, and adrenomedullin remained unchanged. However, the vascular network increased after hypoxia, suggesting that compensatory mechanisms independent from these proangiogenic factors may be involved in hypoxia-induced angiogenesis in the striatum of aged rats. Further research may be needed to identify the factors implicated in the angiogenic response to hypoxia in the aged brain.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in animals were in accordance with the ethical standards of the University of Jaén (Spain) at which the study was conducted.

References

Ahluwalia A, Tarnawski AS (2012) Critical role of hypoxia sensor-HIF-1α in VEGF gene activation. Implications for angiogenesis and tissue injury healing. Curr Med Chem 19(1):90–97

Becker JB, Prendergast BJ, Liang JW (2016) Female rats are not more variable than male rats: a meta-analysis of neuroscience studies. Biol Sex Differ 26(7):34. doi:10.1186/s13293-016-0087-5

Benderro GF, Lamanna JC (2011) Hypoxia-induced angiogenesis is delayed in aging mouse brain. Brain Res 1389:50–60. doi:10.1016/j.brainres.2011.03.016

Brown WR, Thore CR (2011) Review: cerebral microvascular pathology in ageing and neurodegeneration. NeuropatholApplNeurobiol 37(1):56–74. doi:10.1111/j.1365-2990.2010.01139.x

Buga AM, Scholz CJ, Kumar S, Herndon JG, Alexandru D, Cojocaru GR, Dandekar T, Popa-Wagner A (2012) Identification of new therapeutic targets by genome-wide analysis of gene expression in the ipsilateral cortex of aged rats after stroke. PLoS ONE 7(12):e50985. doi:10.1371/journal.pone.0050985

Buga AM, Margaritescu C, Scholz CJ, Radu E, Zelenak C, Popa-Wagner A (2014a) Transcriptomics of post-stroke angiogenesis in the aged brain. Front Aging Neurosci 18(6):44. doi:10.3389/fnagi.2014.00044

Buga AM, Margaritescu C, Scholz CJ, Radu E, Zelenak C, Popa-Wagner A (2014b) Transcriptomics of post-stroke angiogenesis in the aged brain. Front Aging Neurosci 6:44. doi:10.3389/fnagi.2014.00044

Correia SC, Carvalho C, Cardoso S, Santos RX, Plácido AI, Candeias E, Duarte AI, Moreira PI (2013) Defective HIF signaling pathway and brain response to hypoxia in neurodegenerative diseases: not an "iffy" question! Curr Pharm Des 19(38):6809–6822

Di Ieva A, Grizzi F, Ceva-Grimaldi G, Russo C, Gaetani P, Aimar E, Levi D, Pisano P, Tancioni F, Nicola G, Tschabitscher M, Dioguardi N, Baena RR (2007) Fractal dimension as a quantitator of the microvasculature of normal and adenomatous pituitary tissue. J Anat 211(5):673–680

Donovan D, Harmey JH, Toomey D, Osborne DH, Redmond HP, Bouchier-Hayes DJ (1997) TGF beta-1 regulation of VEGF production by breast cancer cells. Ann SurgOncol 4(8):621–627

Efimenko A, Starostina E, Kalinina N, Stolzing A (2011) Angiogenic properties of aged adipose derived mesenchymal stem cells after hypoxic conditioning. J Transl Med 9:10. doi:10.1186/1479-5876-9-10

Ferrara N, Gerber HP, LeCouter J (2003) The biology of VEGF and its receptors. Nat Med 9(6):669–676

Fong GH (2008) Mechanisms of adaptive angiogenesis to tissue hypoxia. Angiogenesis 11(2):121–140. doi:10.1007/s10456-008-9107-3

Gardoni F, Bellone C (2015) Modulation of the glutamatergic transmission by Dopamine: a focus on Parkinson, Huntington and Addiction diseases. Front Cell Neurosci 9:25. doi:10.3389/fncel.2015.00025

Gozal D, Row BW, Kheirandish L, Liu R, Guo SZ, Qiang F, Brittian KR (2003) Increased susceptibility to intermittent hypoxia in aging rats: changes in proteasomal activity, neuronal apoptosis and spatial function. J Neurochem 86(6):1545–1552

Grizzi F, Russo C, Colombo P, Franceschini B, Frezza EE, Cobos E, Chiriva-Internati M (2005) Quantitative evaluation and modeling of two-dimensional neovascular network complexity: the surface fractal dimension. BMC Cancer 8(5):14

Hung SP, Yang MH, Tseng KF, Lee OK (2013) Hypoxia-induced secretion of TGF-β1 in mesenchymal stem cell promotes breast cancer cell progression. Cell Transplant 22(10):1869–1882. doi:10.3727/096368912X657954

Hwang IS, Fung ML, Liong EC, Tipoe GL, Tang F (2007) Age-related changes in adrenomedullin expression and hypoxia-inducible factor-1 activity in the rat lung and their responses to hypoxia. J Gerontol A BiolSci Med Sci 62(1):41–49

Ignacak ML, Harbaugh SV, Dayyat E, Row BW, Gozal D, Czyzyk-Krzeska MF (2009) Intermittent hypoxia regulates RNA polymerase II in hippocampus and prefrontal cortex. Neuroscience 158(4):1436–1445. doi:10.1016/j.neuroscience.2008.11.025

Iimuro S, Shindo T, Moriyama N, Amaki T, Niu P, Takeda N, Iwata H, Zhang Y, Ebihara A, Nagai R (2004) Angiogenic

effects of adrenomedullin in ischemia and tumor growth. Circ Res 95(4):415–423

Itoh Y, Arnold AP (2015) Are females more variable than males in gene expression? Meta-analysis of microarray datasets. Biol Sex Differ 6:18

Johnson AB, Denko N, Barton MC (2008) Hypoxia induces a novel signature of chromatin modifications and global repression of transcription. Mutat Res 640(1–2):174–179. doi:10.1016/j.mrfmmm.2008.01.001

Lam YT, Lecce L, Clayton Z, Simpson P, Karas R, Ng M (2016) Aging impairs ischemia-induced neovascularization by attenuating the mobilization of bone marrow-derived angiogenic cells. IJC MetabEndocr 12:19–29

Lawlor DA, Ebrahim S, Davey-Smith G (2002) Role of endogenous oestrogen in aetiology of coronary heart disease. BMJ 325:311–312

Li H, Witte K, August M, Brausch I, Godtel-Armbrust U, Habermeier A, Closs EI, Oelze M, Munzel T, Forstermann U (2006) Reversal of endothelial nitric oxide synthase uncoupling and up-regulation of endothelial nitric oxide synthase expression lowers blood pressure in hypertensive rats. J Am CollCardiol 47:2536–2544

Logan A, Berry M, Gonzalez AM, Frautschy SA, Sporn MB, Baird A (1994) Effects of transforming growth factor beta 1 on scar production in the injured central nervous system of the rat. Eur J Neurosci 6(3):355–363

Mancardi D, Varetto G, Bucci E, Maniero F, Guiot C (2008) Fractal parameters and vascular networks: facts & artifacts. Theor Biol Med Model 17(5):12. doi:10.1186/1742-4682-5-12

McMahon S, Charbonneau M, Grandmont S, Richard DE, Dubois CM (2006) Transforming growth factor beta1 induces hypoxia-inducible factor-1 stabilization through selective inhibition of PHD2 expression. J BiolChem 281(34):24171–24181

Molina F, Rus A, Peinado MA, Del Moral ML (2013) Short-term hypoxia/reoxygenation activates the angiogenic pathway in rat caudate putamen. J Biosci 38(2):363–371

Molina F, Peinado MA, del Moral ML, Rus A (2016) Response of Nitric Oxide system to hypobaric hypoxia in the aged striatum. Gerontology. doi:10.1159/000450607

Ndubuizu OI, Chavez JC, LaManna JC (2009) Increased prolyl 4-hydroxylase expression and differential regulation of hypoxia-inducible factors in the aged rat brain. Am J PhysiolRegulIntegr Comp Physiol 297(1):R158–R165. doi:10.1152/ajpregu.90829.2008

Ndubuizu OI, Tsipis CP, Li A, LaManna JC (2010) Hypoxia-inducible factor-1 (HIF-1)-independent microvascular angiogenesis in the aged rat brain. BrainRes 1366:101–109. doi:10.1016/j.brainres.2010.09.064

Patt S, Sampaolo S, Théallier-Jankó A, Tschairkin I, Cervós-Navarro J (1997) Cerebral angiogenesis triggered by severe chronic hypoxia displays regional differences. J Cereb Blood Flow Metab 17(7):801–806

Pisani A, Calabresi P, Bernardi G (1997) Hypoxia in striatal and cortical neurones: membrane potential and Ca2 + measurements. NeuroReport 8(5):1143–1147

Prabhakar NR (2001) Oxygen sensing during intermittent hypoxia: cellular and molecular mechanisms. J ApplPhysiol 90:1986–1994

Prendergast BJ, Onishi KG, Zucker I (2014) Female mice liberated for inclusion in neuroscience and biomedical research. Neurosci Biobehav Rev 40:1–5

Rich-Edwards JW, Manson JE, Hennekens CH, Buring JE (1995) The primary prevention of coronary heart disease in women. N Engl J Med 332:1758–1766

Rivard A, Berthou-Soulie L, Principe N, Kearney M, Curry C, Branellec D, Semenza GL, Isner JM (2000) Age-dependent defect in vascular endothelial growth factor expression is associated with reduced hypoxia-inducible factor 1 activity. J BiolChem 275(38):29643–29647

Sandu RE, Uzoni A, Ciobanu O, Moldovan M, Anghel A, Radu E, Coogan AN, Popa-Wagner A (2016) Post-stroke gaseous hypothermia increases vascular density but not neurogenesis in the ischemic penumbra of aged rats. Restor Neurol Neurosci 34(3):401–414. doi:10.3233/RNN-150600

Sullivan JM, Fowlkes LP (1996) The clinical aspects of estrogen and the cardiovascular system. Obstet Gynecol 87:36S–43S

Wang LN, Xu D, Gui QP, Zhu MW, Zhang HH, Hu YZ (2004) Morphological and quantitative capillary changes in aging human brain. Zhongguo Yi XueKeXue Yuan XueBao 26(2):104–107

Wang Y, Zhang JS, Qian J, Huang GC, Chen Q (2006) Adrenomedullin regulates expressions of transforming growth factor-beta1 and beta1-induced matrix metalloproteinase-2 in hepatic stellate cells. Int J ExpPathol 87(3):177–184

Wang L, Bhatta A, Toque HA, Rojas M, Yao L, Xu Z, Patel C, Caldwell RB, Caldwell RW (2015) Arginase inhibition enhances angiogenesis in endothelial cells exposed to hypoxia. Microvasc Res 98:1–8. doi:10.1016/j.mvr.2014.11.002