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Increased protein expression of VEGF-A, VEGF-B, VEGF-C and their receptors in the temporal neocortex of pharmacoresistant temporal lobe epilepsy patients

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ABSTRACT

The vascular endothelial growth factor (VEGF) system has been shown to play a crucial role in several neuropathological processes. Temporal lobe epilepsy (TLE) is the most common focal epilepsy type in adult humans. We assessed the protein expression levels of VEGF-A, VEGF-B, and VEGF-C, their specific receptors VEGFR-2 and -3, their accessory receptors neuropilins 1 and 2, and PI3 and Akt kinases, in temporal neocortex from pharmacoresistant TLE (PR-TLE) patients and control subjects by western blotting. All proteins were found to be significantly overexpressed in samples of PR-TLE patients, indicating that the VEGF system contributes to PR-TLE pathogenesis and should be further studied.

1. Introduction

Vascular endothelial growth factor (VEGF), initially identified as a neovascularization promoter, is now recognized by its multifunctional roles as an essential component of a signalling system that includes six extracellular activators: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (PlGF); three primary tyrosine-kinase (TK) receptors: VEGFR-1, VEGFR-2 and VEGFR-3; and two non-TK coreceptors: Neuropilins (NRP) 1 and 2, which are also sensitive to semaphorins (Djordjevic and Driscoll, 2013; Shibuya, 2014). VEGFR-1–3 activation triggers several intracellular phosphorylation pathways, where phosphatidylinositol 3-kinase (PI3K) and the serine-threonine kinase Akt (Akt) seem to be common components of the cell signalling mediated by all these receptors (Ruiz de Almodóvar et al., 2009; Claesson-Welsh, 2016). Classical VEGF effects include angiogenesis, endothelial cell proliferation and migration, and increased vascular permeability. Then, high VEGF levels can promote an excessive formation of vessels and bleeding, in particular during inflammatory processes, vascular malformations and tumour growth into the brain (Ruiz de Almodóvar et al., 2009). In addition, it has been proposed that VEGF could have a neuroprotective role in neurological illnesses such as amyotrophic lateral sclerosis, Alzheimer's disease, Huntington's disease, Parkinson's disease, peripheral neuropathies, demyelinating diseases and epilepsy (Ruiz de Almodóvar et al., 2009; Lange et al., 2016).

Temporal lobe epilepsy (TLE) is the most common focal convulsive epilepsy type in adult humans, characterized by spontaneous recurrent seizures whose occurrence tends to be progressively more frequent, implying significant modifications in neuronal networks of both mesial and cortical structures that promote prevalent excitatory neuronal activity (Zhang et al., 2002; Ben-Ari and Dudek, 2010). In addition, more than 30% of TLE patients develop pharmacoresistant (PR) seizures, and they are commonly submitted to surgical interventions to improve seizure control (De Vries et al., 2016). Among the events that mediate pharmacoresistance in epilepsy, cerebrovascular network remodelling

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seems to play an essential role, implying changes in both cell transport mechanisms and blood-brain barrier (BBB) permeability, conditions that could modify antiepileptic drug delivery to the brain and its retention there to control seizures (Morin-Brureau et al., 2011; Marchi and Lerner-Natoli, 2013; Vazana et al., 2016). In addition, BBB capillary damage triggers an inflammatory response that could promote to aberrant neurovascular remodelling, formation of scar tissue and neuronal dysfunction (Giannoni et al., 2018).

Activation of the VEGF system has been observed in several models of experimental seizures (Rigau et al., 2007; Castañeda-Cabral et al., 2017; Tawfick et al., 2018) and in brain tissues resected from patients with PR focal epilepsies (Rigau et al., 2007; Boer et al., 2008; Sun et al., 2016); however, most of the evidence is related to the upregulation of VEGF-A and VEGFR-2 expression as predominant mediators of the VEGF system. Recently, it was found that both VEGF-C and VEGFR-3 expression are also upregulated in TLE human samples (Sun et al., 2016), but changes in other ligands and receptors of the VEGF system remain to be established. The aim of this study was to evaluate the expression levels of VEGF-A, VEGF-B, VEGF-C, VEGFR-2, VEGFR-3, NRP-1, NPR-2, PI3K and Akt proteins in surgically resected temporal neocortex from PR-TLE patients to contribute to a better characterization of the VEGF system as a putative therapeutic target for this neurological disease.

2. Materials and methods

2.1. Samples and subjects

Human temporal neocortex (T2-T3 gyri) samples from 10 PR-TLE patients with hippocampal sclerosis and 6 control subjects were assessed. The pre-surgical evaluation and epilepsy surgery were performed in the National Institute of Neurology and Neurosurgery "Manuel Velasco Suárez" (INNNMVS) in Mexico City (for details, review Rocha et al., 2015). PR-TLE samples associated to any kind of tumour growth or vascular malformation were not included in this study. All procedures were performed following the ethical principles of the Declaration of Helsinki for human research, and informed consent was signed for each patient. In addition, full research was approved by the Ethics Committee in Research of the INNNMVS (Agreement No. CEI/058/16).

The Forensic Medical Service of Mexico City provided us with samples obtained from autopsies of people who died by diverse causes but without apparent clinical data of neurologic disease as controls. The clinical characteristics of the PR-TLE patients and control subjects are summarized in Table 1.

2.2. Total protein extracts

Samples from patients and control subjects were homogenized by sonication in lysis buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 20 mM NaF, 0.5 mM Na_3VO_4, and 1% NP-40) with a protease inhibitor cocktail (Cat. sc-29130, Santa Cruz Biotechnology, USA) in a cold bath at 4 °C. Homogenates were centrifuged at 16,060 x *g* for 30 min at 4 °C, and then the supernatant was recovered, aliquoted and frozen at −20 °C. The protein concentration in the supernatants was determined by the Lowry method (DC Protein Assay kit, Cat. 5000116, Bio-Rad Laboratories, USA) using bovine serum albumin (Cat. 5000007, Bio-Rad Laboratories, USA) as an external standard.

2.3. Western blotting

Total protein (40 µg) was denatured in 5 µl of Laemmli buffer (500 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 10% beta-mercaptoethanol, 0.1% bromophenol blue) by boiling the mix for 5 min at 95 °C. Proteins were electrophoresed in 12% SDS-PAGE and electrotransferred onto nitrocellulose membranes (Cat. 1620115, Bio-Rad Laboratories, Germany) using a Mini-Protean Tetra Cell (Cat. 1658005, Bio-Rad Laboratories, USA) and a PowerPac HC (Cat. 1645052, Bio-Rad Laboratories, USA) as a power supply (for methodological details, review Castañeda-Cabral et al., 2017).

Membranes with blotted proteins were incubated in 3% BLOT-QuickBlocker Reagent (Cat. WB57, EMD Millipore, USA) in PBS-0.1% Tween 20 (PBST) for 30 min, then washed 5 times in PBST for 3 min each, followed by incubation in primary antibodies: rabbit (Rb)-anti-VEGF-A (1:1500; Cat. ab46154, Lot. GR118654-1, Abcam, USA), Rb-anti-VEGF-B (1:250; Cat. ab185696, Lot. GR286730-3, Abcam, USA), mouse (Ms)-anti-VEGF-C (1:1000; Cat. sc-374628, Lot. H1417, Santa Cruz Biotechnology, USA), Ms-anti-NRP-1 (1:500; Cat. sc-5307, Lot. I1216, Santa Cruz Biotechnology, USA), Ms-anti-NRP-2 (1:500; Cat. sc-13117, Lot. L0716, Santa Cruz Biotechnology, USA), Rb-anti-VEGFR-2 (1:500; Cat. ab39256, Lot. GR145584-1, Abcam, USA), Ms-anti-VEGFR-3 (1:500; Cat. sc-28297, Lot. H1017, Santa Cruz Biotechnology, USA), Ms-anti-PI3K (1:1000; Cat. 4249, Lot. 9, Cell Signaling Technology, USA), Ms-anti-Akt (1:100; Cat. 4691, Lot. 20, Cell Signaling Technology, USA) and Rb-anti-β-actin (1:6000; Cat. ab8227, Lot. GR124009–1, Abcam, USA). All primary antibodies were diluted in PBS and 0.05% sodium azide and incubated for 18 h at 4 °C. Then, the membranes were washed as described above and incubated in the respective PBS-diluted secondary antibody: HRP-goat anti-Rb-IgG (1:5000; Cat. 926-80011, Lot. C30118-03, LI-COR Bioscience, USA) or HRP-goat anti-Ms-IgG (1:5000; Cat. 926-80010, Lot. C50814-01, LI-COR Bioscience, USA) for 2 h at 4 °C. After this incubation, the membranes were washed in PBS 5 times for 3 min each. Finally, membranes were incubated in Western Sure Premium Chemiluminescent Substrate (Cat. 92695000, LI-COR Bioscience, USA) at room temperature for 5 min. All incubations and washings were done with continuous shaking.

The chemiluminescent signal was acquired through a C-DiGit Blot Scanner (Cat. 6536-030, LI-COR Bioscience, USA) and analysed using free Image Studio Lite Software 3.1.4 (LI-COR Bioscience, USA). The expression ratio for each studied protein was calculated by dividing the chemiluminescent signal corresponding to VEGF-A, VEGF-B, VEGF-C, NRP-1, NRP-2, VEGFR-2, VEGFR-3, PI3K and Akt over the chemiluminescent signal corresponding to its respective β-actin. All samples were evaluated at least in triplicate for each protein.

Table 1
Demographic and clinical data of PR-TLE patients and control subjects.

Patient	Gender	Age	Seizure onset age	AED treatment before surgery	Side focus
P171	F	65	19	CBZ, LMG, PHE, VPA,	Left
P158	M	25	1	LMG, PHE, TPM, VPA	Left
P412	F	48	28	CBZ, CLB, ETX, PHB, PHE	Right
P156	F	25	21	CBZ,VPA	Left
P414	M	31	9	LMG, LVT, PHE, TPM	Right
P419	F	26	3	CBZ, LMG, VPA	Right
P538	F	29	27	OXC, VPA,	Left
P528	M	23	20	LVT, PHE, VPA	Right
P546	F	20	4	VPA	Left
P534	F	30	6	CBZ, PHE, VPA	Left

Control	Gender	Age	*Post mortem* elapsed time (h)	Cause of death
C1	M	29	18	Severely bruised
C2	F	21	14	Suffocation
C3	M	36	12	Thoracic trauma
C4	M	73	15	Diabetes mellitus
C5	F	12	14	Suffocation
C6	M	16	18	Suffocation

Abbrevations: F: Female; M: Male; AED: Antiepileptic drug; CBZ: carbamazepine; CLB: clobazam; ETX: ethosuximide; LMG: lamotrigine; LVT: levetiracetam; OXC: oxcarbazepine; PHB: phenobarbital; PHE: Phenytoin; TPM: topiramate; VPA: valproic acid.

2.4. Statistical analysis

Data are expressed as the mean ± SD and were analysed using Student's *t*-test with the Holm-Sidak *post hoc* test. Differences with $p \leq .05$ were considered significant. Statistical analysis and graph construction were achieved using GraphPad Prism Software ver 6.01 (GraphPad Software Inc., USA).

3. Results

The immunlabelling for VEGF-A, VEGF-B and VEGF-C corresponded to one band located between 40 and 45 kDa; for VEGFR-2 and VEGFR-3, the band was located near 200 kDa; and for NRP-1, it was close to 120 kDa. For NRP-2, two bands near 120 kDa were observed, but only the upper band was analysed, according to the description of the primary antibody manufacturer. With respect to the immunolabelling for PI3K, the band was located near 110 kDa; while for Akt, it was close to 60 kDa. Finally, for β-actin, one band near 43 kDa was observed.

In general, the results showed that all ligands and receptors of the VEGF system evaluated here had significantly increased protein expression levels in the temporal neocortex of PR-TLE patients compared with controls. Specifically, the increase in VEGF-B (> 150%) was greater than that observed in VEGF-C (> 100%) and VEGF-A (> 50%) (Fig. 1), while the increase in VEGFR-3 (> 200%) was more pronounced than that observed for VEGFR-2 (> 150%), NRP-1 (> 75%) and NRP-2 (> 50%) (Fig. 2). In addition, it should be noted that VEGF forms showed a similar expression level, fluctuating at an expression ratio of 0.125 to 0.15 and with VEGF-C slightly above VEGF-B and

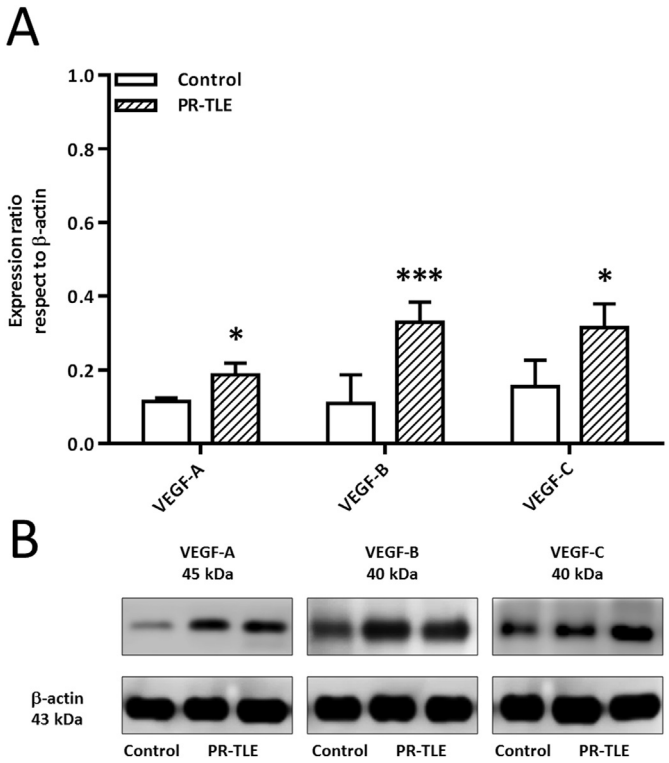

Fig. 1. The expression levels of VEGF-A, VEGF-B and VEGF-C proteins in the temporal neocortex of control subjects and PR-TLE patients. A) Expression ratio of VEGF-A (control $n = 6$; PR-TLE $n = 10$), VEGF-B (control n = 6; PR-TLE n = 10) and VEGF-C (control $n = 3$; PR-TLE $n = 4$) with respect to β-actin for all analysed proteins. B) Representative images of western blot detections for VEGF-A, VEGF-B and VEGF-C and its respective loading control protein β-actin. Data represent the mean ± SDM, and each sample was assessed at least in triplicate. Statistically significant differences between PR-TLE patients and control subjects are represented by $*p < .05$ and $***p < .001$. Student's *t*-test with Holm-Sidak *post hoc* and alpha = 5.00% was used.

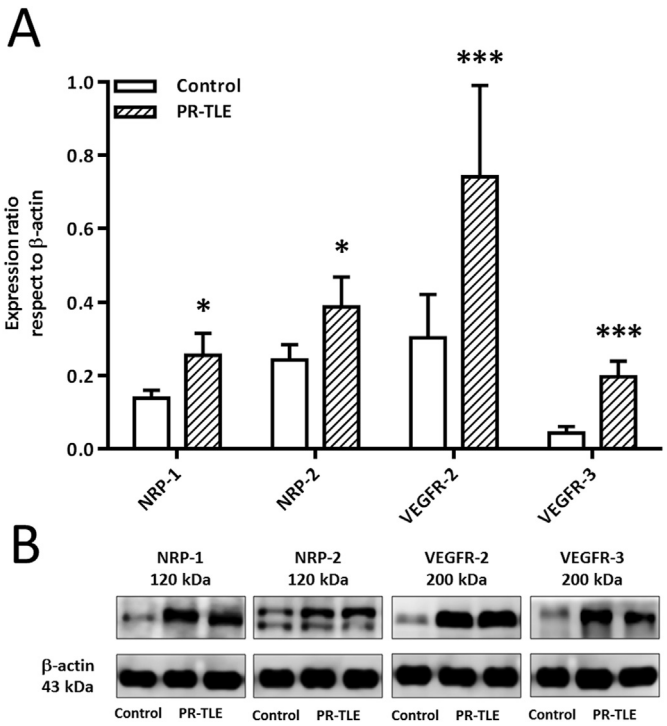

Fig. 2. The expression levels of NRP-1, NRP-2, VEGFR-2, and VEGFR-3 proteins in the temporal neocortex of control subjects and PR-TLE patients. A) Expression ratio of NRP-1 (control n = 3; PR-TLE n = 4), NRP-2 (control n = 3; PR-TLE n = 4), VEGFR-2 (control n = 6; PR-TLE n = 10) and VEGFR-3 (control n = 3; PR-TLE n = 10) with respect to β-actin for all analysed proteins. B) Representative images of western blot detections for NRP-1, NRP-2, VEGFR-2, and VEGFR-3 and its respective loading control protein, β-actin. Data represent the mean ± SDM, and each sample was assessed at least in triplicate. Statistically significant differences between PR-TLE patients and control subjects are represented by $*p < .05$ and $***p < .001$. Student's t-test with Holm-Sidak *post hoc* and alpha = 5.00% was used.

VEGF-A, while in the PR-TLE group, the protein expression level of VEGF-B was greater than VEGF-C followed by VEGF-A (Fig. 1). Otherwise, the protein expression levels of the receptors maintained the same relationship in both the control and PR-TLE groups, with the highest level for VEGFR-2, followed by NRP-2, NRP-1 and VEGFR-3 (Fig. 2).

Furthermore, with respect to the intracellular signals triggered by VEGF system activation, although no significant change was observed in the PI3K protein expression level, the Akt protein expression level was significantly higher in the PR-TLE group, reaching an expression ratio of 2.3 in comparison to the control group expression ratio of 1.6 (Fig. 3).

4. Discussion

Our results demonstrated that the protein expression levels of VEGF-A, VEGF-B, VEGF-C and its receptors NRP-1, NRP-2, VEGFR-2, and VEGFR-3, as well as Akt, increased in the temporal neocortex of PR-TLE patients. We also found that in the temporal neocortex of the control subjects, the three VEGF forms are expressed at similar levels, but VEGFR-2 and NRP-2 are expressed at higher levels than the other receptors. Our results confirmed that the VEGF system is involved in the pathogenesis of PR-TLE.

Several studies have suggested that prolonged and repetitive seizures lead to a progressive and permanent modification of cortical neuronal networks (Ben-Ari and Dudek, 2010). In addition, recent evidences have shown that both the increased vascular density and altered BBB permeability are part of epileptogenesis (Morin-Brureau et al., 2011; Marchi and Lerner-Natoli, 2013), and BBB dysfunction is

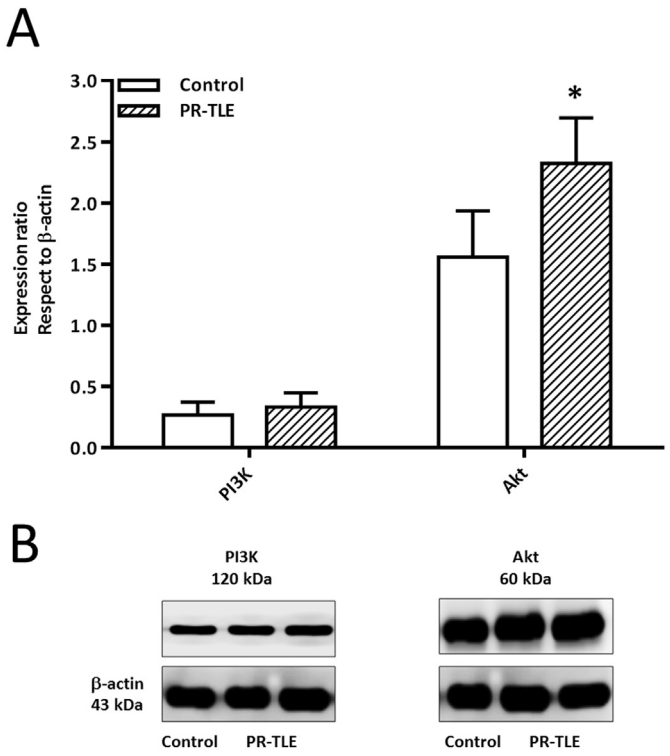

Fig. 3. The expression levels of PI3K and Akt protein in the temporal neocortex of control subjects and PR-TLE patients. A) Expression ratio of PI3K (control n = 3; PR-TLE n = 4) and Akt (control n = 3; PR-TLE n = 4) with respect to β-actin for all analysed proteins. B) Representative images of western blot detections for PI3K and Akt and its respective loading control protein β-actin. Data represent the mean ± SDM, and each sample was assessed at least in triplicate. Statistically differences between PR-TLE patients and control subjects are represented by $*p < .05$. Student's t-test with Holm-Sidak *post hoc* and alpha = 5.00% was used.

temporally and anatomically associated with epileptic seizures (Rüber et al., 2018). Epilepsy-associated cerebrovascular remodelling commonly includes increases in VEGF-A and VEGFR-2 expression levels, which have been observed in neurons, astrocytes and vascular endothelial cells (Rigau et al., 2007; Marchi and Lerner-Natoli, 2013). Because these modifications have been observed in other pathological states, it has been suggested that cerebrovascular remodelling could be a common endpoint of the events triggered by several stimuli that damage the nervous system (Marchi and Lerner-Natoli, 2013). In this sense, it has been recently demonstrated that not only VEGFR-2 but also VEGF-C and VEGFR-3 are overexpressed in both the hippocampus and neocortex of mesial TLE patients (Sun et al., 2016), findings that were replicated by our results, where even the expression levels for these three proteins were very similar to previously reported in the neocortex of both control subjects and mesial TLE patients. The increases in the expression levels of VEGF-C, VEGFR-2 and VEGFR-3 proteins reported in mesial TLE were found in neurons and astrocytes but not in granular cells or microglia (Sun et al., 2016). In addition, it has also been shown that VEGF-A, VEGF-B, and its receptors VEGFR-1 and VEGFR-2 are highly expressed in dysplastic neurons from patients with focal cortical dysplasia type IIB and intractable epilepsy (Boer et al., 2008). Functionally and in neuropathological conditions, VEGF-B acting through VEGFR-2 has been mainly related to neuroprotection, VEGF-A with angiogenesis and inflammatory processes, and VEGF-C with lymphangiogenesis (Ruiz de Almodóvar et al., 2009). However, in epilepsy, the functional implications of each signalling pathway triggered by each form of VEGF or VEGFR remain to be clarified. Furthermore, NRPs have been poorly studied in human neurological diseases, including epilepsy. However, in murine models, it was shown that NRP-2 increases after seizures (Shimakawa et al., 2002), which is consistent with our results, but the role of NRPs should be studied more extensively. In this sense, it is known that NRP-1 interacts with VEGF-A, VEGF-B and VEGF-E, and NRP-2 interacts with VEGF-A, VEGF-C and VEGF-D (Djordjevic and Driscoll, 2013). Both NRPs have a synergistic effect on VEGF signalling and add complexity to the ligand-receptor interactions in the VEGF system, and even though a depth characterization of they is still needed, NRPs could provide a target to regulate VEGF signalling in epilepsy (Ruiz de Almodóvar et al., 2009).

Regarding the functional implications of the VEGF system, some studies have indicated that it contributes to the pathogenesis of epilepsy by remodelling the cerebrovascular network surrounding epileptic foci (Rigau et al., 2007; Marchi and Lerner-Natoli, 2013) through inflammatory mechanisms, which is a hypothesis supported by several studies indicating that neuroinflammation is involved in epilepsy (Bañuelos-Cabrera et al., 2014; Klement et al., 2018; Lorigados Pedre et al., 2018) and that seizure-mediated excitotoxic neuronal death promotes neuroinflammation and VEGF secretion (Croll et al., 2004; De Vries et al., 2016; Castañeda-Cabral et al., 2017; Salehi et al., 2017). A recent study, showed that cytokines pro-inflammatory such as IL-1β, promotes pericyte modifications and pericyte-microglia clustering, modifying BBB permeability and exacerbating the epileptic pathology (Klement et al., 2018). However, the VEGF system has also been involved in endothelial and neural stem cell proliferation (Ruiz de Almodóvar et al., 2009; Lange et al., 2016), as well as in neuroprotection (Schoch et al., 2002; Lee and Agoston, 2010; Zhang et al., 2015), but in epilepsy, these VEGF system roles have been poorly studied.

The activation of VEGFRs triggers the PI3K/Akt intracellular pathway, stimulating cell proliferation (Shibuya, 2014; Claesson-Welsh, 2016), and it has recently been reported that the expression level of the phosphorylated form of Akt (p-Akt) is increased in both the hippocampus and neocortex of TLE patients (Talos et al., 2018). Furthermore, in a pilocarpine TLE animal model, it has been observed that the increase in p-Akt protein is directly related to the increase in VEGF protein (Shu et al., 2016). Akt pathway activation inhibition and reduction in p-Akt expression levels have been proposed as mechanisms to control seizures and epileptogenic process (Shu et al., 2016; Zhu et al., 2018). In this sense, although we did not measure p-Akt, the increase in Akt found in our PR-TLE samples also suggests an important participation of this kinase in epileptogenesis, which should be more broadly studied.

5. Conclusions

The increases shown here in the temporal neocortex of PR-TLE patients on VEGF-A, VEGF-B, VEGF-C, VEGFR-2, VEGFR-3, NRP-1, NRP-2, and Akt proteins clearly indicate that the VEGF system has an important role in the pathogenesis of epilepsy, either as a proinflammatory factor or as a neuroprotective agent. Therefore, more studies on the functional implications of the VEGF system should be performed to design new therapeutic strategies that contribute to better epilepsy control.

Contributions

Conception and design of the experiments: M.E.U.G., L.R.A., and J.L.C.C. Reagents and samples: M.E.U.G., C.B.Z., L.R.A, S.O.S., M.A.V., and R.G.G. Execution of experiments: J.L.C.C. Data analysis: J.L.C.C. and M.E.U.G. Writing of manuscript: J.L.C.C. and M.E.U.G. All authors have approved the final article.

Conflict of interest

The authors have no conflict of interest to report.

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