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REVIEW

Erythropoietin regulates signaling pathways associated with neuroprotective events

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Abstract

Erythropoietin is a cytokine that binds to the Erythropoietin receptor and regulates the formation of erythroid cells during erythropoiesis in the bone marrow. However, many other organs and tissues express Erythropoietin and its receptor, such as the Nervous System, which principally regulates tissue protection. In the Central Nervous System, Erythropoietin is principally expressed by astrocytes, while neurons mainly express Erythropoietin receptors. Moreover, Erythropoietin acts as a pleiotropic molecule with neuroprotective effects, and its mechanisms of signal transduction pathways are defined, and there is a growing interest in its therapeutic potential. This review focuses on the role of Erythropoietin and its relationship with HIF1, PI3/Akt, GSK3B, JAK/STAT, and MAPKs signaling pathways that leads to cell survival after injury in the Central Nervous System. Knowledge of these signaling systems comprehensively could better guide EPO treatment to restoring different SNC alterations mediated by different insults.

Keywords Erythropoietin · Erythropoietin receptor · Signaling pathway · Neuroprotection · Central nervous system · Hypoxic Inducible factor 1

Introduction

Erythropoietin (Epo) is a cytokine essential for erythropoiesis from development through birth and adulthood. Besides, Epo is also associated with other phenomena, including differentiation, survival, and proliferation (Wang et al. 2004). The expression of Epo is identified in several organs such as the retina, testes, muscles, bones, lungs, heart, spleen, and the Central Nervous System (CNS), which principally mediate tissue protection (Arcasoy 2008). Specifically, in CNS, Epo promotes neuroprotection against many kinds of insults such as hypoxic-ischemic injury, ethanol-induced neurodegeneration, epilepsy, Alzheimer's, and Parkinson's diseases (Sargin et al. 2010). Epo mediates neuroprotection by binding to Erythropoietin receptor (EpoR), a cytokine receptor type I that lacks tyrosine kinase activity but is constitutively bound to Janus Kinase-2 (JAK2), through two binding sites located in its cytoplasmic domain known as Box-1 and Box-2. Additionally, EpoR has eight tyrosine residues in the cytoplasmic domain, which JAK2 can phosphorylate. The binding of Epo to its receptor induces the junction of two EpoR subunits and its reciprocal phosphorylation by JAK2 (Watowich 2011; Castañeda-Arellano et al. 2014). Continuously, phosphorylated EpoR cause the activation of the JAK/STAT, phosphoinositide 3-kinase (PI3K) Akt (PI3K/Akt), and p42/44 mitogen-activated protein kinase (MAPK) pathways (Kuhrt and Wojchowski 2015). The activation of those pathways by Epo induces different physiological mechanisms, including the prevention of apoptosis, excitotoxicity, inflammation, oxidative stress, induction of astrocyte formation, neurite outgrowth, and neurogenesis (Ureña-Guerrero et al. 2020). However, the precise contribution of each signaling pathway to neuroprotection was not well characterized. The present review is focused on the integration and organization of the information available so far on the mechanism of the regulation of Epo and EpoR expression, the signaling cascades activated by Epo/EpoR

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during neuroprotection, and the mechanisms induced against different insults in the CNS.

Mechanism of the regulation of Epo and EpoR expression

HIF-1, Epo, and neuroprotection

The expression of Epo is principally regulated by hypoxia-inducible factors 1 (HIF-1) in an oxygen-dependent manner (Chikuma et al. 2000). Moreover, HIF-1 is expressed in the CNS, including the spinal cord and brain (Acker and Acker 2004). HIF-1 factor is composed of an oxygen-regulated one known as HIF-1α and a constitutively expressed subunit known as HIF-1β. Under normoxic conditions, prolyl hydroxylase domain protein 2 (PHD2) hydroxylates HIF-1α at proline residues, promoting its polyubiquitination and degradation by the 26S proteasome. Hypoxia inhibits HIF-1α hydroxylation, which accumulates in the cytoplasm, travels to the nucleus, and joins the HIF-1β subunit inducing the formation of the HIF-1 factor (Chilov et al. 1999). After that, HIF-1 binds to the cis-acting hypoxia response elements (HRE), driving the transcription of target genes involved in hypoxia adaptation like Epo (Schofield and Ratcliffe 2004; Semenza 2007). In the neocortex and hippocampus of newborn rats and the retina of adult mice, hypoxia increases the expression of HIF-1α protein principally in the cell nucleus, which is correlated with the augmentation of Epo mRNA expression (Grimm et al. 2002; Kletkiewicz et al. 2018). Meanwhile, in the brain of adult mice, the application of IOX3, an inhibitor of PHD2, mediated the upregulation of HIF-1α protein and Epo mRNA leading to neuroprotection (Chen et al. 2014). In addition, a study performed in primary neuron-rich cultures containing only a limit number of astrocytes revealed that hypoxic/ischemic damage upregulated protein and mRNA expression of HIF-1α from 30 min to 24 h and Epo from 3 to 24 h demonstrating HIF-1α is expressed before Epo (Liu et al. 2006). However, mixed neuronal/astrocyte cultures showed a more significant increase of HIF-1α and Epo expression after hypoxic/ischemic damage. The same study also showed that hypoxia significantly reduced neuronal survival in primary neuronal-rich cultures, but not in neuronal/astrocytes mixed cultures indicating astrocytes are the principal source of Epo and essential for neuroprotection (Liu et al. 2006).

EpoR expression and HIF-1

It has been reported hypoxia-induced upregulation of EpoR mRNA in the cerebrum of adult mice and neuronal cell cultures triggering neuroprotection (Chikuma et al. 2000; Chin et al. 2000). Likewise, ischemic damage, which results in decreased oxygen levels, to cerebral cortices of mice increased EpoR protein 1–3 days after damage, implying in CNS EpoR is induced by low oxygen concentration (Bernaudin et al. 1999, 2000). In sciatic nerves of rats, hypoxic damage induced upregulation of HIF-1α and EpoR proteins and mRNAs. Whereas the treatment of damaged rats with YC-1, an inhibitor of HIF-1, suppressed the induction of EpoR (Luo et al. 2013). Thereby, these data support the idea that the HIF-1 factor plays a crucial role in controlling the expression of EpoR in the nervous system.

Additionally, in vitro evaluation of EpoR protein and mRNA showed an upregulation from 1 to 24 h after hypoxia exposure, reaching a peak at 6 h, but it was further increased in neutron-rich cultures compared to mixed neuronal/astrocytic cultures (Liu et al. 2006). Accordingly, administration of recombinant human (rhEpo), Epo produce in cell cultures, to spinal cord primary cell cultures damaged by excitotoxicity, increased the expression of EpoR principally by neurons and triggers neuroprotection (Won et al. 2007). Moreover, excitotoxic damage to mix neuronal-glial cultures upregulated the extended processes of astrocytes and Glial fibrillary acidic protein (GFAP) expression, a well-determined astrogliosis parameter, as well as instantaneous neuronal death. Whereas rhEpo treatment after excitotoxic damage prevented neuronal death but caused almost any reduction of GFAP expression by astrocytes, specifying Epo neuroprotection is performed by direct effects in neurons instead of astrocytes (Yoo et al. 2009). Other studies reported that Epo and EpoR are constitutively expressed in neurons and astrocytes (Bernaudin et al. 1999, 2000; Chong et al. 2003). However, higher Epo concentrations have been identified in the cytoplasm of astrocytes, while EpoR is principally expressed all over the surface of neurons (Liu et al. 2006; Yoo et al. 2009). Based on the data, it seems that astrocytes principally express Epo while neurons mainly express EpoR this way; astrocytes are essential to prompt protection of neurons against different kinds of insults.

Epo regulates the expression of EpoR

Hypoxia increases the expression of Epo and EpoR in the nervous system, which then mediates neuroprotection (Sirén et al. 2001). However, other insults such as excitotoxicity and oxidative stress decrease its expression leading to neuronal death (Yoo et al. 2009; Li et al. 2016). Many reports have demonstrated that the administration of exogenous Epo against different insults protects neurons and increases neuronal survival (Won et al. 2007; Simon et al. 2008; Li et al. 2016). Therefore, we wonder if applied Epo interacts with preformed EpoR, or it increased its expression allowing a greater number of interactions and a significant neuroprotection effect. Spinal GABAergic neuron cultures showed that Kainic Acid (KA) administration decreased the levels

of EpoR protein and the number of cells. On the other hand, rhEpo application restored the amount of EpoR protein and prevented GABAergic neuron loss (Won et al. 2007). Similarly, the administration of rhEpo to neonatal rats with excitotoxic damage increased EpoR mRNA and protein expression in the hippocampus (Rivera-Cervantes et al. 2019). These data provided evidence about the induction of EpoR protein and mRNA expression by exogenous Epo application in the nervous system. In addition, an in vitro study conducted in spinal cord primary cell cultures reported that excitotoxicity decreased the expression of EpoR protein in neurons and glial cells, which triggers neuronal death. However, rhEpo treatment after excitotoxic damage increased EpoR expression primarily by neurons and mediated neuroprotection, while co-application of rhEpo with anti-EpoR antibodies completely abolished EpoR upregulation and prevented neuroprotection (Yoo et al. 2009). This implies that interaction between applied Epo and preformed EpoR directly upregulates the expression of EpoR, which potentiates the neuroprotective effect, but the pathways involved in EpoR upregulation have not been elucidated. Several studies, including clinical trials, have evaluated the administration of Epo against brain pathologies, psychiatric disorders, spinal cord injury, optic neuritis, and peripheral neuropathies (Simon et al. 2019; Hemani et al. 2021). Some of these studies revealed that a number of patients treated with Epo developed minor adverse effects like nausea, pyrexia, headache, generalized weakness, and superficial phlebitis. In comparison, a limited number of patients developed severe adverse effects such as intracerebral hemorrhage, brain edema, thromboembolic events, and even death, principally those treated with Epo plus tissue plasminogen activator (tPA) (Hemani et al. 2021). In addition, it has been reported that high doses of Epo, 100,000–200,000 UI, within 3–5 days after CNS injury increase the risk of severe adverse effects. In contrast, several experimental models and clinical trials have demonstrated that the administration of low doses of Epo, 300–500 UI/kg, is safe and does not increase the risk for serious adverse effects (Simon et al. 2019; Hemani et al. 2021). New compounds derived from Epo such as Carbamylated erythropoietin (CEPO), asialo erythropoietin (AsialoEPO), and Neuro-Epo which maintain neuroprotective effect but lost the ability to induce hematocrit, are being developed and studied (Mofidi et al. 2011; Simon et al. 2011, 2016; Lagarto et al. 2012; Rosenzweig et al. 2004; Torup 2007). However, more studies are needed to compare the therapeutic against adverse effects of Epo and its derivatives to better guide neuroprotection. Also, other receptors expressed in CNS that binds to Epo has been described, such as β-common receptor (βcR) and the transmembrane receptor Ephrin type-B4 (EphB4), but the signaling pathway activated by these receptors have not been well characterized as the pathways activated by the classical EpoR (Vittori et al. 2021). For this reason, in the present work, we aim to summarize and integrate the information available so far about the signaling pathways and mechanisms activated by Epo and its receptor during neuroprotection to guide better the therapeutic effect induced by Epo.

The signaling pathways activated by Epo/EpoR during neuroprotection

Epo and JAK2/STAT signaling

Initially, Epo mediates the formation of the homodimer EpoR2, which facilitates its reciprocal phosphorylation and activation at the tyrosine residues by JAK2 as well as the activation of signal transducer and activator of transcription (STAT) proteins (Kretz et al. 2005; Zhao et al. 2011; Cheng et al. 2015; Zhang et al. 2020). The family of STAT proteins comprises of seven subtypes called STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6, which contain a DNA-binding domain in the C-terminal region involved in DNA transcription as well as SRC homology 2 (SH2) domains required for its dimerization and activation (Stark and Darnell 2012). During the interaction of Epo with EpoR, STAT proteins are recruited and binding by phosphorylated tyrosine residues of EpoR. Then, activated JAK2 phosphorylate and activate STAT proteins which dimerized, released from EpoR, and travels to the nucleus, where induce the expression of different genes (Kuhrt and Wojchowski 2015). In CNS, many works have been described the binding of Epo to EpoR activate STAT3 and STAT5 through JAK2, which then mediated neuroprotection by the induction of neurite outgrowth, neurogenesis, and anti-apoptosis (Digicaylioglu and Lipton 2001; Zhang et al. 2006; Yoo et al. 2009).

Epo activates JAK2/STAT5

It observed a drastic reduction of the phosphorylated levels of Tyr-1007 and Tyr-1008 of the JAK2 kinase and Tyr-694 of the SATA5 protein in the hippocampus of adult rats with hypoxic/ischemic damage (Ma et al. 2018). Otherwise, the administration of rhEpo restored the phosphorylated levels of tyrosine residues and triggered neuroprotection, while EpoR silencing by lenti-Epo-R-shRNA-GFP abolished the restoration of phosphotyrosine residues and prevented neuroprotection (Ma et al. 2018). Similarly, an in vitro excitotoxic model conveyed an upregulation of phosphorylated JAK2 and STAT5 generated by Epo application which correlated with a decrement of the activated caspase-3 immunoreactivity and an increment in the survival rate of damage hippocampal cells (Cheng et al. 2015). It demonstrates that Epo activates the JAK2/STAT5 pathway against damage in the hippocampus of CNS and mediates neuroprotection

through the inhibition of the apoptotic molecule caspase-3. Another in vitro study conducted with cerebrocortical neurons showed that rhEpo administration upregulated the anti-apoptotic proteins X-linked inhibitor of apoptosis (XIAP) and inhibitor of apoptosis proteins (c-IAP2) as well as depleted neuronal apoptosis caused by *N*-methyl-D-aspartic acid (NMDA) and nitric oxide (NO) treatment. While anti-EpoR monoclonal antibody application coprecipitated EpoR and JAK2 proteins extracted from cerebrocortical neuronal cultures, it precipitated neither JAK1 nor Non-receptor tyrosine-protein kinase (TKY2). Moreover, rhEpo treatment increases EpoR and JAK2 association (Digicaylioglu and Lipton 2001). Additionally, in vivo study of global ischemia–reperfusion carried out on adult rats, the increment of the phosphorylation levels of STAT5 and its translocation from the cytoplasm to the nucleus observed in hippocampal neurons of the CA1 region. Nonetheless, intracerebroventricular (ICV) administration of rhEpo 20 h before the induction of ischemia caused a further upregulation of p-STAT5a and p-STAT5, breaching a pick three h after ischemia. Also, it was an upregulation of the anti-apoptotic B-cell lymphoma-extra-large proteins (Bcl-xL) and XIAP at 6 and 24 h, respectively, after ischemia–reperfusion and downregulation of caspase-9 in rhEpo treated animals, which led to neuronal survival. Contrastingly, ICV application of Tyrphostin AG490, an inhibitor of the JAK2, after rhEpo treatment inhibited phosphorylation of STAT5 and prevented neuronal survival (Zhang et al. 2007). Other works have reported that XIAP and c-IAP2 bind and inhibit the activation of pro-apoptotic molecules caspase-3, caspase-7, and caspase-9 (Deveraux and Reed 1999; Kasof and Gomes 2001), whereas Bcl-xL is involved in the regulation of the integrity of the mitochondria membrane (Gross et al. 1999). Based on the data, it could be possible that Epo activated the JAK2/STAT5 signaling pathway, which increased the expression of anti-apoptotic molecules Bcl-xL, c-IAP2, and XIAP. After that, c-IAP2 and XIAP inhibited the activation of caspase-3 and caspase-9, while Bcl-xL prevents mitochondria permeability culminating in anti-apoptosis and protection of the neuron cells in the CNS (Fig. 1).

Epo activates JAK2/STAT3

An in vivo model of traumatic brain injury performed in adult male rats showed rhEPO administration increased phosphorylated levels of JAK2 and STAT3 together with anti-apoptotic proteins Bcl-xL and B-cell lymphoma 2 (Bcl-2) triggering the anti-apoptotic effect in the cerebral cortex. Contrary, AG490 administration with rhEpo abolished the increased phosphorylation levels of JAK2 and STAT3 and abrogated the induction of Bcl-xL and Bcl-2, which led to apoptosis of cortical neurons (Zhao et al. 2011). It proves that in the CNS, Epo also mediates anti-apoptosis through the activation of JAK2/STAT3 signaling. Furthermore, incubation of damaged cerebrocortical neurons by excitotoxicity with rhEpo provoked IκBα (an inhibitor of NF-κB) serine phosphorylation, promoting the release of the transcription nuclear factor kappa B (NF-κB), which then translocate from the cytoplasm to the nucleus and prevented neuronal apoptosis (Digicaylioglu and Lipton 2001). In contrast, either the transfection with a plasmid containing a mutant JAK2 gene (pRK5/JAK2) and the application of AG490 to damaged

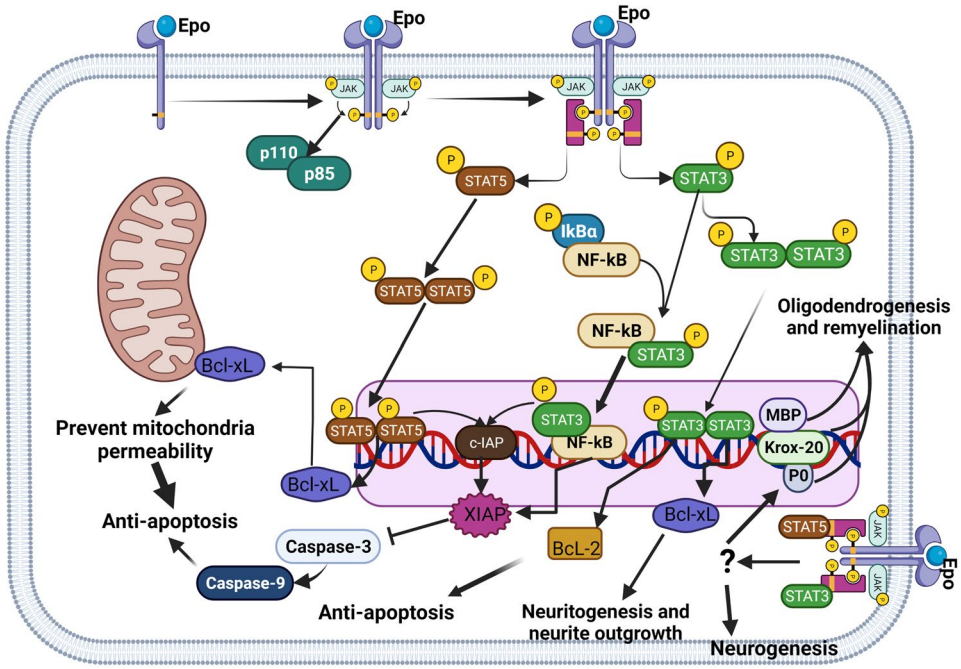

Fig. 1 Epo and EpoR activate JAK2, STAT3, and STAT5 pathways during the neuroprotection. The JAK2/STAT5 mediates the expression of anti-apoptotic molecules Bcl-xL, c-IAP2, and XIAP. JAK2/STAT3 activates NF-κB, which travels to the nucleus. Also, activated STAT3 travels to the nucleus and forms a heterodimer with NF-κB (STAT3:NF-κB), which induces the expression of c-IAP2 and Bcl-xL. Then c-IAP2 and XIAP inhibited the activation of caspase-3 and caspase-9 while Bcl-xL prevents mitochondria permeability, mediating anti-apoptosis. Moreover, Bcl-xL induces neurite outgrowth and neurogenesis. Created with BioRender.com

cerebrocortical neurons treated with rhEpo repressed NF-κB nuclear translocation and blocked rhEpo anti-apoptotic effect (Digicaylioglu and Lipton 2001). Other studies reported that JAK2/STAT3 signaling mediates the activation of NF-κB (Nadiminty et al. 2006; Kong et al. 2019). These data suggest that activated STAT3 induces the phosphorylation of IκBα, which releases NF-κB, however, more studies are needed to elucidate the complete mechanism. Bittorf et al. (2001) observed in hematopoietic cell cultures that activated EpoR enhanced NF-κB DNA binding. While Lee et al. 2009, demonstrated in cancer cells that activated STAT3 directly joins and retains activated NF-κB in the cell nucleus, triggering the formation of the complex STAT3:NF-κB, which then binds to its DNA target sequences (Lee et al. 2009). Additionally, in primary cell liver cultures extracted from adult male rats, the inhibition of NF-κB by a construct mutant IκBα gene which binds irreversibly to NF-κB resulted in the induction of caspase-3 activity and apoptosis. Meanwhile, activated NF-κB was able to induce the expression of c-IAP2, and Bcl-xL further c-IAP2 inhibited the activation of caspase-3 and prevented apoptosis (Schoemaker et al. 2002). The available data suggest that Epo induced the JAK2/STAT3 signaling activation, then activated STAT3 travels to the nucleus and binds to NF-κB, causing the formation of the complex STAT3:NF-κB, which induces the expression of c-IAP2 and Bcl-xL. Finally, c-IAP2 inhibits the activation of caspase three and potentiates neuroprotection (Fig. 1). However, more studies are required to confirm this hypothesis.

Epo, JAK2/STAT3 and neurogenesis

In the last decades, it demonstrated that Epo induces neurogenesis, a process by which new neurons are formed during development and in the adult brain of animals. However, the signaling pathways involved in Epo neurogenesis in the adult brain have not been described. For this reason, we summarize the available data so far. The in vitro excitotoxic model performed in primary spinal neurons and glial cells mix cultures showed that KA administration induces a reduction of the number and length of neurites, whereas rhEpo treatment prevented neurites damage. Interestingly, the application of rhEpo alone to neuronal and glial mix cultures increases the number and length of neurites and the number of neurons compared to untreated cultures, demonstrating that Epo induced neuritogenesis, neurite outgrowth, and neurogenesis (Yoo et al. 2009). Besides, there was a reduction of JAK2 protein mediated by excitotoxicity, although rhEpo treatment restored JAK2 levels.

Moreover, when an anti-EpoR antibody was added in conjugation with rhEpo to damaged cells, JAK2 protein expression was decreased, which prevented the increase of new neurons and neurite number and length. According to this data, in CNS, Epo can exert neurite outgrowth, neuritogenesis, and neurotrophic effects through the activation of the JAK/STAT signaling pathway (Yoo et al. 2009). In addition, rhEpo treatment caused the upregulation of phosphorylated STAT3 and anti-apoptotic protein Bcl-XL and extended neurites and axons of retinal ganglion cells (RGCs) derived from adult female rats with optic nerve lesions. The inhibition of the JAK/STAT signaling by AG490 completely abolished neurites and axon growth stimulation (Kretz et al. 2005). Another in vitro study reported that Bcl-XL overexpression in RGCs increased the number and length of neurites and axons (Kretz et al. 2004). While in primary hippocampal neurons extracted from rat fetuses, Bcl-xL depletion by RNAi gene silencing decreased the length and bifurcation of neurites compared to hippocampal neurons without gene silencing, demonstrating the importance of Bcl-xL in neurite outgrowth (Park et al. 2015). Based on the above data, Epo promotes the expression of Bcl-XL through JAK2/STAT3 pathway, which then mediates neuritogenesis and neurite outgrowth; however, more studies are needed.

Additionally, it observed that RSC96 Schwann cultured cells damaged with hydroperoxide developed apoptosis. In contrast, rhEpo treatment inhibited apoptosis, promoted cell proliferation, and increased the expression of myelin-associated protein P0, myelin basic protein MBP, and Krox-20, a transcription factor that regulates myelination. At the same time, the application of AG490 abolished RSC96 Schwann cells proliferation mediated by rhEpo. Therefore, this suggests that Epo could also induce remyelination of the nervous system through the activation of the JAK/STAT pathway (Fig. 1) (Luo et al. 2013).

Epo and PI3K/Akt signaling pathway

Protein Inositol 3 kinases (PI3K) are a large family of enzymes that mediate lipid and serine/threonine phosphorylation. (Martini et al. 2014). The canonical PI3K class 1A consists of two different subunits, an inhibitor/adaptor (p85), also known as a regulatory subunit, and a catalytic one (p110) (Cully et al. 2006). Activated PI3K phosphorylate phosphatidylinositol-4,5-bisphosphate (PIP2) producing phosphatidylinositol-3,4,5-trisphosphate (PIP3). Subsequently, PIP3 recruited phosphatidylinositol-dependent kinase 1 (PDK1) to the cell membrane and induced its activation, then activated PDK1 phosphorylate protein kinase B (PKB, also known as Akt), which phosphorylate downstream substrates mediating the activation of different mechanisms (Manning and Toker 2017). Many reports demonstrated in CNS activated Akt by Epo phosphorylate downstream substrates that mediate neurite outgrowth and anti-apoptosis of neurons and brain endothelial cells (Maggioni et al. 2010; Hou et al. 2011; Wenker et al. 2013). In addition, reported JAK2 is required to activate PI3Kin response to Growth

Hormone, while γ2A/GHR cells that lack JAK2 were unable to activate PI3K (Yamauchi et al. 1998). Meanwhile, the CNS activated JAK2 mediated the phosphorylation of Akt while the inhibition of JAK2 by AG490 prevented Akt phosphorylation and activation. On the other hand, the inhibition of PI3K/Akt by LY294002 (PI3K inhibitor) does not affect JAK2 activation, indicating activated JAK2 mediates the activation of the PI3K/Akt pathway (Hou et al. 2018). Based on the previous data, it could be possible that CNS Epo and EpoR mediate the activation of PI3K by JAK2 producing PIP3, which then recruit and activate Akt kinase. However, more studies are required to prove this hypothesis.

Epo, PI3K/Akt and neurite outgrowth

It documented that cisplatin, a chemotherapeutic treatment to dorsal root ganglia primary cultures, caused an increment of neuronal death and reduced neurite outgrowth and about 60% of phosphorylated Akt (p-Akt) levels. Although, Epo co-application with cisplatin induced a partial but significant recovery of neurite elongation and diminished neuronal death (Maggioni et al. 2010). While the administration of wortmannin, an inhibitor of PI3K, to neuronal cultures exposed to cisplatin and Epo prevented neurite outgrowth and completely abrogated neuronal survival (Maggioni et al. 2010). Another study observed that hypoxic/ischemic damage decreased dendritic spine density and the amount of PI3K and Akt proteins in the hippocampus of adult rats. Instead, Epo treatment after hypoxia restored protein levels, dendritic spine density, and the percentage of mushroom-type spines to normal levels (Ma et al. 2018). Likewise, a study performed in RGCs cultures extracted from adult rats with optic nerved lesions showed that rhEpo treatment increases Akt phosphorylation and induces axonal growth.

In contrast, inhibition of the PI3K/Akt pathway by LY294002 abrogated it (Kretz et al. 2005). Accordingly, these data indicate in CNS Epo-mediated neurite outgrowth together with neuronal survival through the activation of PI3K/Akt signaling pathway (Fig. 1). An idea supported by other studies which demonstrate the crucial role of the PI3K/Akt pathway in the induction of neurite outgrowth (Read and Gorman 2009; Zheng et al. 2011). Nonetheless, the intermediaries between PI3K/Akt and neurite outgrowth are not described, and more studies are necessary to elucidate the complete mechanism.

Epo and SIRT1 pathway

Oxygen glucose deprivation (OGD) exposure of primary microvascular endothelial cell cultures derived from adult rat brain cerebra led to a downregulation of silent mating type information regulation two homologs (SIRT1) protein, a histone deacetylase, and cellular dead by apoptosis. Moreover, inhibition of SIRT1 by a siRNA or EX527, a selective inhibitor of SIRT1, resulted in decreased phosphorylated Akt1 levels and increased mitochondria depolarization and cytochrome C release, which suggest SIRT1 increases the phosphorylation of Akt and prevents apoptosis (Hou et al. 2011). In breast cancer tissue, high SIRT1 protein expression upregulated p-Akt, whereas knockdown of SIRT1 decreased p-Akt protein levels, indicating a positive correlation between SIRT1 and Akt (Jin et al. 2018). Interestingly, the administration of Epo to primary microvascular endothelial cells before OGD exposure prevented downregulation of SIRT1, inducing its translocation from the cytoplasm to the nucleus and resulting in the inhibition of apoptosis. It indicates Epo prevented apoptosis at least in part by inducing the SIRT1/p-Akt pathway (Hou et al. 2011).

Moreover, upregulated p-Akt1 by Epo mediated the phosphorylation of Forkhead box O3 (FoxO3a) and inhibited its translocation from the cytoplasm to the nucleus while SIRT1 inhibition increased the transference of FoxO3a to the cell nucleus even more than OGD (Hou et al. 2011).

Furthermore, Epo pretreatment of primary cell cultures with OGD augmented the phosphorylated BCL2 Associated Agonist of Cell Death (p-Bad) protein level and decreased cleaved caspase three and caspase 1. Otherwise, the inhibition of SIRT1 in the presence of Epo downregulated p-Bad and upregulated cleaved caspase three and caspase 1 (Hou et al. 2011). Other works have demonstrated that unphosphorylated FoxO3a translocates from the cytoplasm to the nucleus, inducing the expression of pro-apoptotic Bcl-2-like protein 11 (Bim) and triggers apoptosis (Sunters et al. 2003; Chong and Maiese 2007). Active Akt1 phosphorylate and sequestered FoxO3a in the cytoplasm by its association with 14-3-3 proteins and inhibiting apoptosis (Maiese et al. 2008). Also, p-Akt inactivates the apoptotic protein Bad, localized in the outer membrane of mitochondria attached to Bcl-xL, mediating the release of Bcl-xL and preventing mitochondria dysfunction (Yang et al. 1995; Chong et al. 2005). Following these results, Epo prevents SIRT1 loss after OGD and mediates the activation of Akt1, which triggers anti-apoptosis of brain microvascular endothelial cells by the phosphorylation of FoxO3a as Bid and the inhibition of caspase-1 and caspase-3 (Fig. 1). A rat model of aging induced by D-galactose administration depicted that rhEpo treatment increased SIRT1 and Bcl-2 expression, which inhibited neuronal apoptosis mediated by aging in the hippocampus, indicating that the induction of SIRT1 by Epo in the CNS could mediate similar mechanisms in endothelial cells and neurons (Wu et al. 2015). Nevertheless, more studies are needed to determine the similarities and differences in the mechanisms mediated by Epo and SIRT1 interaction in neurons, endothelial cells as well as glial cells.

Epo regulates GSK-3β

SH-SY5Y neuronal cells were cultured in a conditioned medium (CM) from the EOC-2 microglia cells activated by hypoxia, characterized by an increment in reactive oxygen species (ROS), NO, and Tumoral necrosis factor α (TNF-α) production. Meanwhile, Epo application to SH-SY5Y neuronal cells treated with CM reverted apoptosis, although the addition of Ly294002 with Epo abolished the anti-apoptotic effect evidencing Epo-mediated anti-apoptosis through PI3K/Akt pathway (Wenker et al. 2013). Furthermore, rhEpo administration to PC-12 neural crest cells with neurotoxic damage counteracted caspase-3 like activity and apoptosis mediated by damage and induced an increment of p-Akt from 30 min to 12 h (Wu et al. 2007). Subsequently, p-Akt phosphorylates and inactivate GSK-3β (Wu et al. 2007), which is related to the development of neuronal apoptosis (Li et al. 2000). In contrast, the addition of LY294002 to damaged PC-12 cellular cultures pretreated with rhEpo decreased p-Akt, inhibited the phosphorylation of GSK-3β, prevented caspase-3 inactivation, and triggered apoptosis (Wu et al. 2007). Also has been reported that ICV administration of rhEpo to rats with ischemic damage increased Akt and GSK-3β phosphorylation levels in the CA1 region of the hippocampus and prevented neural apoptosis. Whereas ICV infusion of LY294002 after rhEpo treatment reverted p-Akt concentration to the levels observed in ischemic animals without treatment and abolished the CA1 hippocampal region (Zhang et al. 2006). Likewise, primary cortical neuronal cultures treated with Ketamine, a noncompetitive anesthetic blocker of the N-methyl-D-aspartate (NMDA) receptor, exhibited downregulation of the phosphorylation of Akt and GSK-3β as well as an upregulation of caspase-3-like proteinase activity, which prompted apoptosis. While rhEpo application to primary cortical neurons treated with Ketamine increased the phosphorylation levels of Akt and GSK-3β, prevented caspase-3-like activity, and stimulated the anti-apoptotic effect. However, all those effects reverted by LY294002 (Shang et al. 2007). Other works reported that activated GSK-3β mediated the activation of caspase-3, which induced the development of apoptosis (Zaheer et al. 2008; Quintavalle et al. 2010). The currently available data provide that in CNS, apoptosis is triggered at least in part by GSK-3β, which mediated caspase-3 like activity and apoptosis. In contrast, Epo activates the PI3K/Akt signaling pathway, which inactivated GSK-3β by phosphorylation, preventing the activation of caspase-3 and the induction of neuronal apoptosis (Fig. 1).

Epo and MAPK signaling

The mitogen-activated protein kinases pathway (MAPK) comprises different signaling families, which included the canonical MAPK/ERK pathway, the MAP kinase-1 (BMK-1), c-Jun N-terminal kinase (JNK), and p38 signaling families (Burotto et al. 2014). The activation of the MAPK signaling pathway is performs by activated receptor tyrosine kinases such as epidermal growth factor receptor, c-KIT, platelet-derived growth factor receptor, vascular endothelial growth factor receptor, fibroblast growth factor receptor, FMS-related tyrosine kinase-3 (FLT-3), and EpoR. The binding of extracellular signaling molecules to these receptors induces activation that consecutively activates Rat sarcoma virus protein (Ras), a membrane-bound GTPase. After that, activated Ras mediate the activation of MAPK signaling (Burotto et al. 2014). Interestingly, Ras can also induce the PI3K/Akt pathway; thereby, there is a cross-talk between MAPK and PI3K/Akt pathways (Cully et al. 2006). The classical MAPK/ERK signaling comprises three different MAKKKS known as A-RAF, B-RAF, and RAF-1 or C-RAF; two different MAPKKS, MEK1, and MEK2 and finally two more different MAPKs named as ERK1 and ERK2, which are the final effectors of the pathway (Burotto et al. 2014). During erythropoiesis, the binding of Epo to EpoR mediates the activation of GTPase Ras proteins, which then activate MAPK signaling, which is essential for the formation of erythroid cells (Kuhrt and Wojchowski 2015). At the same time, another study revealed that JAK2 directly mediates the activation of the MAPK pathway by Ras (Winston and Hunters 1995). Moreover, in the CNS, the activation of EpoR by Epo mediates the activation of the MAPK pathway and triggers anti-apoptosis, anti-oxidative stress, anti-excitotoxicity, and astrocyte formation (Lee et al. 2004; Zheng et al. 2013; Li et al. 2017). This way, it could be possible in CNS that the interaction of Epo with EpoR activates JAK2, which then induces the activation MAPK pathway through Ras. However, studies are needed to evaluate the interaction of JAK2, Ras, and MAPK signaling in CNS after EpoR activation by Epo.

In primary cortical cells extracted from newborn rats, sevoflurane exposure increased the expression of pro-apoptotic cleaved caspase-1 and caspase-3 and triggered apoptosis. Otherwise, Epo treatment increased phosphorylated ERK1 and ERK2 proteins and Nfr2 mRNA levels, a transcription factor that regulated the expression of antioxidants, as well as decreased cleaved caspase-1 and caspase-3, promoting neuronal survival. Also, the administration of Epo alone or in combination with sevoflurane increased the Nfr2/Bach1 ratio and heme-oxygenase-1 (HO-1) expression (Li et al. 2017; Zhang et al. 2020). Nevertheless, PD98059, an inhibitor for MAPK/ERK pathway, plus Epo application increased cleaved caspase-1 and caspase-3 and blocked the induction of Nfr2 and HO-1 expression culminating in apoptosis (Li et al. 2017). It is well-known HO-1 plays an essential role during cytoprotection against oxidative stress (Turkseven et al. 2005),

and it reported that Nfr2 induced its expression (Krantic et al. 2007), whereas CNC homology 1 (Bach1) represses it (Fuse et al. 2015). Likewise, another study performed on primary cortical cultures with neurotoxic damage showed sevoflurane exposure-induced neuronal apoptosis. Contrary, rhEpo treatment upregulated phosphorylated ERK1 and ERK2, Nrf2/Bach1 ratio, and HO-1 expression, which prevented apoptosis of neurons, while PD98059 plus rhEpo application blocked all those effects (Zhang et al. 2020). Previous data indicate that Epo induced the expression of Nfr2 by the MAPK/ERK pathway activation, then Nfr2 promotes the synthesis of HO-1, which triggers antioxidative stress, while Bach1 prevents the transcription of HO-1 during neurotoxic damage and inhibits protection. Moreover, Epo triggered an anti-apoptotic effect by inhibiting the cleavage of caspase-1 and caspase-3 by MAPK/ERK signaling pathway activation (Fig. 1).

Epo-mediated astrocyte formation by MAPK/ERK

It observed in primary cortical cultures derived from newborn SD rats that Epo application increased the expression of EpoR by neuronal progenitor cells (NPCs) and promoted their morphological differentiation into astrocytes and upregulated the expression of GFAP in a dose-dependent manner. The investigation of the MAPK kinase family in the regulation of differentiation of NPCs to astrocytes by Epo revealed that ERK2 protein was activated while the activation of JNK1 occurred at three days. Furthermore, it observed that Epo induced the activation of NFκB during differentiation, reaching a peak at 5 days after starting the administration (Lee et al. 2004). On the contrary, after EPO administration, the inhibition of ERK2 and JNK1 by PD98059 and salicylic acid, repressed astrocyte differentiation and decreased GFAP expression. Meanwhile, the application of Epo and EpoR antibodies to NPCs cultures treated with Epo prevented the activation of ERK2 and NFκB, decreased GFAP expression, and abolished astrocyte differentiation (Lee et al. 2004). Other studies have reported that activated ERK2 influences astrocyte proliferation (Bajetto et al. 2001; Barbero et al. 2002), whereas NFκB identified as an essential factor during differentiation of neuronal progenitors (O'Neill and Kaltschmidt 1997).

Additionally, there is evidence that IκBα is phosphorylated by ERK kinases promoting the release of NFκB and its binding to DNA target sequences (Song et al. 2005; Diomede et al. 2017). Thus, Epo could also mediate the phosphorylation of IκBα by MPAK/ERK2 and JNK signaling pathways and the deliberation of NFκB, which travels to the nucleus and induces NPCs' differentiation to astrocytes. Although, with the performance of more studies, the complete mechanism will be clarified in more detail.

Epo interacts with PI3K/Akt and MAPK pathways

Since Ras protein interacts with MAPK and PI3K/Akt signaling, it could be cross-talk between both pathways that trigger neuroprotection. In this chapter, we resumed the principal works that have reported the interaction between MAPK and PI3K/Akt pathways and the triggered mechanisms during Epo protection in the CNS. The treatment of neuronal progenitor cells (NPC) extracted from the Subventricular Zone of male mice with rhEpo increased phosphorylated Akt at 5 min and ERK1/2 at 10 min, although it does not alter the total levels of proteins. It demonstrates that Epo mediates the activation of both pathways immediately after its interaction with EpoR. Moreover, rhEpo treatment to NPC upregulated the expression of Vascular endothelial growth factor (VEGF) mRNA and protein, whereas cells derived from EpoR (ΔEpoR) null male mice treated with rhEpo did not increase the total levels of VEGF. In addition, the inhibition of PI3K/Akt and MAPK/ERK signaling abolished the induction of VEGF by neuronal progenitor cells showing that Epo mediates the activation of both pathways, which increases the expression VEGF (Wang et al. 2008). VEGF is the principal mediator of new vessel formation (Simon et al. 2020). The application of supernatant extracted from NPCs treated with rhEpo to mouse brain endothelial cells (MBEC) also increased the expression of VGEF and induced angiogenesis which is observed as the increment in the number and length of capillary tubes.

Meanwhile, MBECs treated with supernatant harvested from neuronal cultures with inhibitors for PI3K/Akt and ERK1/2 pathways induced neither VGEF expression nor angiogenesis. Together, these data indicate that in the CNS, Epo activates MAPK/ERK and PI3K/Akt pathways, which induces the expression of VEGF by NPCs and MBECs (Fig. 2). After that, VEGF induces proliferation of MBECs and angiogenesis; nonetheless, since these studies are performed in vitro, studies conducted in vivo are required to confirm this hypothesis (Wang et al. 2008).

Another in vivo study revealed that ICV application of KA increased the average population spike amplitude (PS) in the CA3 region of the hippocampus. While intraperitoneal administration of recombinant rat Epo (rEpo) before excitotoxicity decreased PS amplitude. Interestingly, the administration of soluble EpoR to damaged animals without Epo treatment induced a more considerable increase of average PS, indicating endogenous Epo downregulate hyperexcitability of damaged CA3 hippocampal neurons. Also, Patch-clamp recordings showed that KA application in cultured hippocampal neurons induced an increment in the voltage-gated calcium current (ICa) which plays an essential role during the development of epilepsy (Raza et al. 2004; Zheng et al. 2013). Moreover, EPO silencing

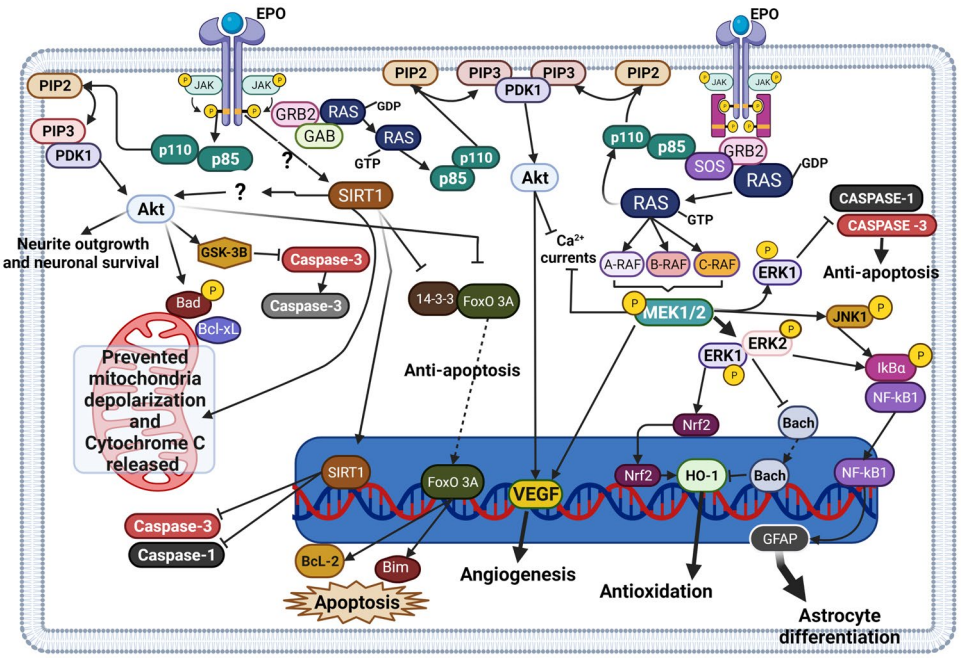

Fig.2 Epo activated JAK2, PI3K/Akt, and MAPK/ERK pathways during neuroprotection. The junction of Epo to its receptor mediates the activation of JAK2, which phosphorylates and activates PI3K and Ras. After that, PI3K activates Akt by PDK1. Meanwhile, Ras activates MAPK/ERK as well as PI3K. Activated Akt phosphorylate FoxO3a and repressed its translocation to the nucleus, which inhibited Bid, caspase-1, and caspase-3. Also, Akt prevents the induction of caspase-3 and caspase-9 by the activation of GSK-3β. MAPK/ERK induces the expression of Nfr2, which promotes anti-oxidative stress by the synthesis of OH-1. Moreover, MAPK/ERK mediates the translocation of NFκB to the nucleus and induces astrocyte formation. Finally, the interaction between PI3K/Akt and MAPK/ERK promotes angiogenesis by expressing VEGF in MBECs and preventing calcium influx and excitotoxic damage in neurons. Created with BioRender.com

by siRNA transfection to damaged animals without Epo treatment further enhanced ICa and hyperexcitability. By contrast, the administration of rEPO to animals treated with KA reverts the increment of calcium currents and excitotoxic damage. The endogenous Epo down-regulates voltage-gated calcium currents, hyperexcitability, and exogenous rEpo induced a bigger downregulation of calcium currents and excitotoxicity, enhancing neuroprotection (Zheng et al. 2013). In addition, Epo application to primary hippocampal cultures upregulated the amount of pPI3K and pERK1/2. While Epo siRNA markedly down-regulated pPI3K as well as pERK1/2 levels. The inhibition of PI3K/Akt and MAPK/ERK pathways by LY294002 and U0126 respectably in hippocampal cells treated with rEpo increased the voltage-gated calcium currents. Together this data indicate Epo prevented at least in part calcium influx in hippocampal neurons damaged by excitotoxicity through the activation of PI3K/Akt and MAPK/ERK pathways and protects neurons against excitotoxicity (Fig. 2) (Zheng et al. 2013). Notwithstanding, studies are needed to clarify the contribution of each pathway and the precise mechanism involved in cellular protection against damage mediated by Ca++ influx in the CNS.

Conclusion

It is known that Epo induces neuroprotection in the CNS through its interaction with EpoR. Moreover, EpoR is principally expressed by neurons, while astrocytes are the principal source of Epo that explains the importance of the interaction between neurons and astrocytes during neuroprotection. At the molecular level, the expression of EpoR and Epo regulate by the HIF-1 factor, and it is well known that hypoxia mediates HI1-1α protein stabilization leading to HIF-1 formation and the induction of its target genes. Importantly, exogenous Epo also induced the expression of Epo and EpoR, which could potentiate neuroprotection, but the mechanism of the Epo and EpoR induction by exogenous Epo application was not characterized. In addition, applied and endogenous Epo induced the activation of PI3K/Akt, JAK/STAT, and MAPK pathways which mediate different protection mechanisms in CNS. The activation of PI3K/Akt by Epo mediates neurite outgrowth and phosphorylate FoxO3a and GSK-3β, which prevents apoptosis of neurons and microvascular endothelial cells. While activated JAK2/STAT5 by Epo mediates anti-apoptosis of neurons through the induction of the expression of pro-apoptotic and anti-apoptotic genes.

Furthermore, JAK2/STAT3 also induces anti-apoptosis of neurons and neurite outgrowth and the proliferation of Schwann cells, indicating Epo could mediate remyelination of neurons. Besides, MAPK/ERK signaling pathway protects neurons against oxidative stress and inhibits apoptosis. Finally, it observes that the interaction between PI3K/Akt and MAPK pathways mediate VEGF expression in neurons and endothelial cells, leading to angiogenesis and neuroprotection. In addition, PI3K/Akt and MAPK/ERK interaction prevent calcium influx in hippocampal neurons and protect them against oxidative damage. The present work describes all the signaling pathways activated during neuroprotection reported. Furthermore, more studies are required to elucidate all the mechanisms in detail.

Author contributions Cornelio-Martinez S: writing—original draft preparation, investigation, term; Castañeda-Arellano R: conceptualization, writing, project supervision, final manuscript approval, funding acquisition, project administration.

Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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