

Cellular and Molecular Mechanisms of Retinal Ganglion Cell Death in Hypoxic-Ischemic Injuries

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Received: May 13, 2015

Accepted: Aug 06, 2015

Published: Aug 11, 2015

Citation: Kaur C, Rathnasamy G, Foulds WS, Ling EA. 2015. Cellular and Molecular Mechanisms of Retinal Ganglion Cell Death in Hypoxic-Ischemic Injuries. *J Neurol Exp Neurosci* 1(1): 10-19.

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Abstract

Loss of retinal ganglion cells (RGCs) occurs in retinal degenerative diseases, such as glaucoma, age-related macular degeneration, diabetic retinopathy, central retinal artery occlusion and ischemic central retinal vein thrombosis in adults and in retinopathy of prematurity in infants. A critical role of hypoxia, which underlies most of the above disorders, has been reported in causing RGC death. Disruption of blood-retinal barrier (BRB) occurs due to increased production of vascular endothelial growth factor and nitric oxide in response to hypoxia resulting in extravasation of serum derived substances and retinal edema and this may be one of important factors mediating RGC death. Activation of microglial cells in response to hypoxia and subsequent increased release of proinflammatory cytokines by them such as tumor-necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) target the TNF-receptor 1 and IL-receptor 1 on RGCs. Excess release of glutamate in retinal tissue under hypoxia activates the ionotropic glutamate receptors causing excitotoxicity through increased influx of calcium into the RGCs. Increased production of TNF- α , IL-1 β and enhanced intracellular calcium results in RGC death through activating several pathways such as caspase signaling, mitochondrial dysfunction and oxidative stress. In our investigations, melatonin, an antioxidant, was shown to reduce RGC death in the adult and neonatal hypoxic retina, through suppression of TNF- α , IL-1 β and glutamate levels as well as oxidative stress. Moreover, the function and structure of BRB was well preserved in these animals along with reduction in retinal edema. In view of our observations, we suggest that melatonin could be considered as a therapeutic agent to reduce RGC death in various retinal pathologies, in addition to other potential drugs/molecules of therapeutic interest that could render protection to RGCs. However, future in-depth research on the effects of melatonin on the retina under hypoxic conditions needs to be undertaken to explore its full potential in preventing/ameliorating RGC death.

Keywords

Retina, Retinal ganglion cell death, Hypoxia, Microglia, Inflammatory cytokines and other mediators, Oxidative stress

Abbreviations

AMPA: Amino-methyl-propionic-acid; BRB: Blood-retinal barrier; eNOS: Endothelial NOS; GSH: Glutathione; HRP: Horseradish peroxidase; HIF-1 α : Hypoxia inducible factor-1 α ; iNOS: Inducible NOS; IL-1R1: Interleukin receptor 1; IL-1 β : Interleukin-1 β ; LPO: Lipid peroxidation; MCP-1: Monocyte chemoattractant protein-1; nNOS: Neuronal NOS;

NOS: Nitric oxide synthase; NMDA: *N*-methyl-d-aspartate; NO: Nitric oxide; NF- κ B: Nuclear factor kappa B; ROS: Reactive oxygen species; RGC or RGCs: Retina ganglion cell or Retina ganglion cells; RhIC: Rhodamine isothiocyanate; TJ: Tight junctions; TNF-R1: Tumor necrosis factor receptor 1; TNF- α : Tumor necrosis factor- α ; VEGFR1 (Flt-1): Vascular endothelial growth factor receptor 1; VEGFR2 (KDR/Flk-1): Vascular endothelial growth factor receptor 2; VEGF: Vascular endothelial growth factor; ZO-1: Zonula occludens-1

Introduction

The retina is one of the most metabolically active tissues and its demand for oxygen is higher than many other tissues including the brain [1-3]. Proper function of the retina depends on a continuous supply of oxygen which diffuses into it from circulation. The inner retina is supplied by capillary plexuses formed by branches of the central artery while the outer layers of the retina are nourished by the choriocapillaries. Deficient oxygen supply to the retina results in tissue hypoxia which is a critical factor implicated in retinal ganglion cell (RGC) death resulting in loss of vision in many ocular conditions such as retinal artery occlusion, ischemic central retinal vein thrombosis, glaucoma, diabetes [4, 5] and experimental branch retinal vein occlusion [6]. It may also result from systemic factors such as cardiorespiratory diseases. Chronic obstructive disease that is common among smokers is a significant cause of systemic and retinal hypoxia. In conditions such as the diabetes, occlusion of retinal capillaries by leukocytes and increased levels of endothelial cell/leukocyte adhesion molecules [7] reduce the retinal blood flow [8] and result in tissue hypoxia.

Normal development and maturation of many tissues including the retina are affected by either birth asphyxia (lack of oxygen in the blood stream), hypoxia (reduction in the available oxygen), ischemia (reduction in blood flow that could lead to hypoxia) or hypoxia-ischemia (decreased blood flow combined with a reduction in the supply of oxygen). Prenatal or fetal hypoxia can occur in association with many maternal causes such as diabetes, asthma, anemia, smoking and the use of alcohol or drugs. A reduction in utero-placental blood flow, premature onset of labour or prolonged labour may also compromise fetal oxygenation. Hypoxia may also result from respiratory distress syndrome in the premature new-born that may lead to development of retinopathy of prematurity. RGC death is known to occur following hypoxic injuries in the developing retina [9].

Several factors such as vascular endothelial growth factor (VEGF), nitric oxide (NO), inflammatory cytokines, glutamate and oxidative stress are up regulated or induced in response to hypoxia thus adversely affecting the structure and function of the retina. Additionally, vascular permeability is increased and the blood-retinal barrier (BRB) is disrupted resulting in leakage of fluid into the retinal tissue. In diabetes, this may result in the development of diabetic macular edema, a leading cause of visual loss in diabetes [10]. Although various cells in the retina can be adversely affected by hypoxia, this review focuses on the death of the RGCs in hypoxia.

Factors Mediating RGC Death

Several factors induced or upregulated by hypoxia may be involved in causing RGC death. These factors include, among others, disruption of the blood-retinal barrier, activation of microglial cells and the accompanying enhanced release of inflammatory cytokines and excess release and accumulation of glutamate in the retinal tissues.

Hypoxia and the blood-retinal barrier

The BRB regulates the passage of molecules from blood into the retina [11, 12]. It consists of an inner BRB formed by the tight junctions (TJ) between capillary endothelial cells and an outer barrier formed by TJ between retinal pigment epithelial cells [12-16]. The TJ are made up of transmembrane proteins such as occludin, claudin-5 and cytoplasmic proteins such as zona occludens (ZO)-1 that are structurally and functionally important for maintaining the integrity of the BRB. The proper functioning of the inner BRB also requires the collective contribution of pericytes, Müller cells and astrocytes that are in close apposition to the capillary endothelial cells [16-19]. Moreover, disruption of the inner BRB results in vascular leakage resulting in the formation of edema [16, 20, 21] a well-recognized and early feature of human diabetic retinopathy [22, 23]. BRB disruption due to hypoxia has also been reported to cause RGC swelling [6], endothelial cell death [24-27] and leukocyte plugging of vessels [28, 29]. Associated with BRB disruption there is increased RGC death [30-32].

Hypoxic-ischemic insult to the adult retina, has been shown to affect the integrity of TJ by reducing the expression of ZO-1 and occludin. This alteration in the expression of TJ molecules was associated with increased BRB permeability and subsequent vasogenic edema [33]. Similar changes in the expression of TJ molecules were observed in the neonatal retina following a hypoxic injury [34]. Concomitant to the alterations in the expression of TJ molecules, the vascular permeability of the retinal vessels was enhanced, and was established by administering tracers such as horseradish peroxidase (HRP) intravenously or rhodamine isothiocyanate (RhIC) intraperitoneally in hypoxic rats. An increased leakage of these tracers was observed through the blood vessels in the inner retina inundating the retinal tissues thereafter. These findings strongly imply that hypoxia results in BRB breakdown. In addition, the hyaloid vessels in the neonatal vitreous also showed increased permeability following hypoxic/ischemic injury [9].

Hypoxia induced disruption of inner BRB was further evident with the ultrastructural analysis of both neonatal and adult hypoxic retina. In neonatal hypoxic retina, the basal lamina was disintegrated and the endothelial cells exhibited cytoplasmic vacuoles and swollen mitochondria. The capillaries were often surrounded by empty perivascular spaces suggesting the presence of edema [34]. The Müller cell and astrocyte processes surrounding the capillaries in the inner retina were found to be hypertrophic. Taken together all these structural changes indicate impairment in the integrity of BRB in hypoxic retina. Moreover, the expression of aquaporin 4, a water-transporting protein, was found to be increased in Müller cells and astrocytes indicating

impairment in water and ion homeostasis that could lead to retinal edema [21]. The increased BRB permeability has been documented to alter the biochemical composition of retinal interstitial fluid [35] rendering the retinal microenvironment unbecoming for retinal cells. This could also compromise the neuron-glia communication [36] that serves to maintain retinal homeostasis. Of note, in transient retinal ischemia, the perturbation in Müller cell K^+ conductance could lead to retinal detachment [37] that could account for neuronal death in the retina. Although the molecular mechanisms underlying the BRB disruption and associated RGCs death has not been elucidated entirely, a few studies have highlighted the involvement of vasoactive substances such as VEGF and nitric oxide synthases (NOS) in these processes [29, 36, 38, 39].

The expression of hypoxia inducible factor-1 α (HIF-1 α), a master regulator of cellular and developmental O_2 homeostasis that is induced by hypoxia, is known to induce the production of VEGF and nitric oxide synthase (NOS) in the neonatal and adult retina. In response to hypoxic injury, we have documented the increased production of VEGF and NO in the developing and adult retina [21, 9]. The increased expression of VEGF in the retinal vessels is reported to cause the disruption of BRB following hypoxia [40-42], and an inhibition of VEGF has been shown to preserve the integrity of BRB [21]. In response to hypoxic injury, in adult and developing retina, VEGF was predominantly expressed in the astrocytes and Müller cells [21, 9]. VEGF exerts its action by binding to two tyrosine kinase receptors, VEGFR1 (Flt-1) and VEGFR2 (KDR/Flk-1). In neonatal hypoxic retina, the increased expression of Flt-1 and Flk-1 was localized on the vascular endothelium [43]. Activation of these receptors is known to cause disruption of TJ by decreasing the expression of occludin [44] and ZO-1 [45], and may be important in the pathogenesis of BRB dysfunction [44]. VEGF induced activation of Flk-1 is implicated in the increased vascular permeability/BRB breakdown, that occurs in diabetic retina [46]. Activation of Flt-1 has been shown to mediate pericyte loss in the retinal vasculature resulting in increased vascular leakage and retinal damage [47]. Moreover, VEGF is also known to induce fenestrations in endothelial cells [48-50] and degenerative changes in the basement membranes thus increasing the vascular permeability [51]. It enhances the adhesion of leucocytes to vascular wall by increasing intercellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-expression [29, 52] on the endothelial cells resulting in capillary occlusion by adherent leukocytes, endothelial cell apoptosis and BRB breakdown [26, 29, 53]. Occlusion of blood vessels could further lead to increased production of angiogenic factors that could lead to neovascularisation and subsequent retinal detachment [38, 54]. Parallel to the BRB breakdown, recruitment of leukocytes and death of RGCs is frequently documented following an ischemic insult to the retina [30-32]. The entry of circulating leukocytes into the retina following BRB dysfunction is considered to play a critical role in RGC death [36]. The infiltrating leukocytes may activate the resident microglia that could result in death of RGCs and exacerbation of injury. Neufeld et al. [39] have documented that the infiltrating leukocytes could trigger RGC death through production of nitric oxide (NO). We have also documented an increased incidence of apoptotic RGCs and increased NO in the retinas

of hypoxic animals [34].

NO is also reported to be involved in the breakdown of the BRB [55, 56]. NO is primarily synthesized from L-arginine by the NOS enzymes and is known to mediate neuronal communication as well vasodilatation. NOS from neurons (nNOS) and endothelial cells (eNOS) are constitutively expressed enzymes, the activities of which are calcium dependent, while inducible NOS (iNOS) is calcium-independent and NO generated from this isoform is known to mediate immune functions. Vasodilatation after hypoxic-ischaemic episodes is known to be caused by eNOS [57] resulting in increased blood flow in neural tissues. Investigations in retinas affected with diabetic retinopathy have thrown light on the mechanisms through which NOS could mediate BRB disruption. It has been suggested that NO from eNOS is involved in mediating VEGF-induced vascular hyperpermeability and NO produced by iNOS has an additive effect [58]. Jousseaume et al. [29] demonstrated that the increased production of VEGF could induce eNOS expression in the retina which could result in increased NO. NO derived from iNOS and eNOS was further found to facilitate BRB disruption by increasing ICAM-1 expression in the retinal endothelial cells which could promote leukocyte adhesion [55, 29]. The potential of NO in mediating BRB breakdown was further evident with its ability to reduce the expression of TJ molecules such as ZO-1 and occludin [55]. NO is also known to interact with superoxides to form peroxynitrite which are highly toxic to all retinal cells including endothelial cells [55]. Our studies in the adult and neonatal retina following hypoxic injury have shown that eNOS expression in the retinal endothelial cells was significantly increased after hypoxic exposure [21, 9]. We also showed an induction of iNOS expression by hypoxia in the retinal blood vessels as well as in the RGCs. Concomitant to this, there was increased NO production in the hypoxic retina [9]. As discussed above the disruption of BRB could facilitate the infiltration of blood borne substances which could trigger microglial activation in the retina leading to excess production pro-inflammatory cytokines and excitatory amino acids [36] that could result in death of RGCs. Moreover, *in vitro* studies have shown that NO can disrupt TJ between retinal pigment epithelial cells [59]. However, in our studies in the adult and neonatal hypoxic animals, there was no evidence of disruption of the outer BRB.

Although VEGF has detrimental effects, it has also been reported to be important for the survival of RGCs in diabetic retina [60]. In cultured RGCs, the activation of VEGFR2 by VEGF-A conferred neuroprotection against free radicals through activation of AKT pathway [61]. VEGF-A is also documented to protect retinal neurons during the adaptive response to acute ischemia [62]. VEGF mediated cytoprotection is documented to involve glutathione, an antioxidant [63]. Given the complex contribution of VEGF in retinal diseases and injuries, and in protecting RGCs, future studies are warranted to delineate the isotypes mediating BRB disruption and cytoprotection. This could favour a holistic approach to the treatment of retinal diseases by preserving the retinal neurons.

Hypoxia and microglial activation

Microglial cells are the resident immune effector cells in the retina. Under normal circumstances, they are confined to the nerve fibre layer, ganglion cell layer, inner and outer plexiform and inner nuclear layers in the adult retina [64, 65]. However, under pathological conditions, they have been detected in the outer nuclear layer and in the subretinal space [66]. In the retina of newborns, microglial cells are found mostly in the nerve fibre and ganglion cell layers [67, 68]. Microglial cells have a phagocytic function during the early development of the retina where they remove cellular debris derived from spontaneous degenerating elements. They also act as sensors to detect any disturbances in the normal microenvironment. They have both neuroprotective and detrimental roles in the immature and mature retina. During development, microglia play an important role in scavenging cellular debris in the neural tissues [69, 70]. However, on activation in many pathological conditions they show increased production of noxious substances such as reactive oxygen species (ROS) and inflammatory cytokines [71].

Production of proinflammatory cytokines such as tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) is known to be stimulated by hypoxia-ischemia in the retina [67, 72]. It has been well documented that these cytokines are derived from microglial cells and participate in the breakdown of the BRB [73, 74] and in RGC death [67]. Activated microglia have been linked to diabetic and ischemic retinopathies in the adult [66, 75, 76] and their numbers have been shown to increase in such conditions [77]. Microglial cells in the developing retina were the main source of TNF- α and IL-1 β , the expression levels of which were increased drastically in response to hypoxic exposures [67]. It is to be noted that RGCs were the target of these cytokines as they vigorously expressed TNF receptor 1 (TNF-R1) and IL receptor 1 (IL-1R1). TNF- α has been shown to mediate RGC death by binding with its receptor TNF-R1 in experimental elevation of intraocular pressure and in ocular conditions such as retinal ischemia, optic nerve crush and glaucoma [78-80] RGC apoptosis occurs through this binding as a result of activation of caspase signaling pathways, mitochondrial dysfunction and oxidative damage [81].

Microglia derived IL-1 β has been reported to augment brain damage in ischemic, traumatic or excitotoxic insults [82-84] through depolarizing neurons. Our recent investigations have shown that increased release of IL-1 β by retinal microglia and enhanced expression of IL-1R1 on RGCs occurred concurrently in the retina of hypoxic neonatal rats. IL-1 β can damage the RGCs by binding to its receptor IL-1R1 and initiating mechanisms such as excitotoxicity and increased iNOS production through IL-1R1 signalling [80].

Retinal hypoxia-ischemia is also known to induce the expression of monocyte chemoattractant protein-1 (MCP-1) that attracts microglia and macrophages to the hypoxic areas resulting in aggravation of the inflammatory response [67]. MCP-1 expression has been suggested to attract resident macrophages/microglia, vitreous macrophages, and/or circulating monocytes through the BRB in the ischemic retina [76]. We have shown that MCP-1 expression was enhanced in

the microglial cells in the hypoxic neonatal retina and this may be involved in attracting macrophages and inducing migration of microglia to the vicinity of RGCs. Increased numbers of microglia were indeed observed in the ganglion cell layer in the retinas of adult rats subjected to hypoxia. *In vitro* studies have lent further support to the involvement of microglia in hypoxia-induced RGC death. A significant reduction in RGC apoptosis was observed when they were treated with conditioned medium derived from primary microglia after neutralization with TNF- α and IL-1 β antibodies. This suggests that hypoxia initiates retinal inflammation by direct activation of microglia with subsequent damage to RGCs [67]. In addition to the microglia, the hyalocytes, hyaloid and the retinal vessels showed enhanced expression of TNF- α and IL-1 β in the developing retina. It has also been reported that the vascular walls are a source of TNF- α production in the retina of diabetic patients [85] thus amplifying the inflammatory response of the microglial cells. TNF- α and other cytokines have also been documented to play a role in the development of vasogenic edema by inducing TJ disruption [86] and resulting in damage to the RGCs. Increased vascular permeability causes increased leakage of fluid and other potentially harmful molecules into the retina that would otherwise be excluded by an intact inner BRB.

Hypoxia and glutamate

Glutamate is the major excitatory neurotransmitter in the retina and is released by photoreceptors, bipolar neurons and RGCs [87, 88]. Glutamate exerts its action through ionotropic [amino-methyl-propionic-acid (AMPA), *N*-methyl-d-aspartate (NMDA) and kainate GluRs] and metabotropic receptors [89,90]. It is also a potent neurotoxin involved in RGC death in hypoxic-ischemic conditions [91, 92] where increased accumulation of glutamate occurs in the extracellular spaces in the retinal tissue. Glutamate has been shown to affect the ganglion cell layer and also cause necrosis of cells in the inner retina [93]. Exposure of RGCs derived from newborn rats to glutamate resulted in induction of neurotoxicity [94]. Excess extracellular release of glutamate is known to kill neurons by excitotoxic mechanisms through activation the NMDA and AMPA receptors. Excitotoxicity has been suggested as a major mechanism underlying RGC death in many ocular pathologies including diabetic retinopathy, retinal and choroidal vessels occlusion and glaucoma [95-100]. Excitotoxic neuronal death involves calcium influx into the cells [101] that has been reported as an essential component of glutamate neurotoxicity [102]. Stimulation of glutamate receptors in chick RGCs *in vitro* caused calcium influx through NMDA receptor-associated channels and subsequently their death. Increased calcium influx in the neurons causes mitochondrial dysfunction and nNOS generation resulting in neuronal cell death [103, 104]. We have shown that expression of nNOS in the RGCs is enhanced in hypoxic injury and that hypoxic induction of nNOS is mediated by nuclear factor kappa B (NF- κ B). The increased amount of NO released subsequently, by RGCs, results in their apoptosis through caspase-3 activation [105]. Activation of NF- κ B was demonstrated in the RGCs and was implicated in their apoptosis in the adult retina following optic nerve transection [106] and in hypoxia [107]; apoptosis of RGCs was prevented by administration of inhibitors for

NF- κ B, such as parthenolide and sulfasalazine [108].

Recent studies in the hypoxic neonatal retina have shown upregulation of expression of various subunits of the NMDA receptor i.e. NR1 and NR2A-D and NR3A localized in the RGCs [109] and increased calcium influx in the hypoxic RGCs along with their augmented apoptosis. Apart from NMDA receptors, involvement of AMPA GluRs in hypoxic-ischemic cell death in the adult retina and neonatal retina has been reported [110]. AMPA receptors are composed of various combinations of four subunits (GluR 1-4), GluR1, GluR3 and GluR4 being calcium permeable, whereas the GluR2 subunit is calcium impermeable [111]. Accumulation of calcium in the mitochondria occurs with an increase in intracellular calcium and this calcium overload causes opening of the permeability transition pore resulting in massive swelling of the mitochondria and release of apoptotic mediators such as cytochrome *c* [101, 112, 113]. Mitochondrial failure results in energy depletion and free radical production [114].

Calcium influx through AMPA receptors and subsequent death of chick RGCs was shown to occur through stimulation of these receptors [115] and generation of free radicals such as superoxide through increased intracellular calcium has been reported. Activation of NOS leading to excess production of NO are also initiated by increased intracellular calcium and in ischemic conditions NO has been reported to produce peroxynitrite, which generates hydroxyl radical. The involvement of the NMDA and AMPA receptors in RGC death in hypoxic conditions is evidenced by significant attenuation in the intracellular calcium, cytochrome *c*, caspase-3 and ROS in the hypoxic RGCs treated with NMDA receptor antagonist MK-801 [109] or AMPA receptor antagonist, 6, 7-dinitroquinoxaline-2,3-dione as reported by us [110].

Hypoxia and oxidative stress

The generation of free radicals derived from oxygen [ROS] is balanced in health as any excess is eliminated by endogenous antioxidants such as superoxide dismutase, catalase, glutathione peroxidase, glutathione (GSH) and others. Oxidative stress occurs in pathological conditions when the balance between free radical formation and the ability of endogenous antioxidants to eliminate them is disturbed. The disturbance in this balance damages the lipids, proteins, and DNA resulting in their oxidation and causing cell death by necrosis or apoptosis. It is well documented that hypoxia induces oxidative stress that plays a pivotal role in damaging the neurons in the adult and developing neural tissues [116-120]. ROS has been reported to be involved in signaling RGC death by acting as a second messenger and/or modulating protein function in conditions such as glaucoma [121-123] and ischemic injuries of the retina [107, 124]. Hypoxia and oxidative stress are also involved in the development of diabetic retinopathy [125] resulting in RGC death [126]. Increased lipid peroxidation (LPO) and depletion of antioxidants such as GSH in response to hypoxia/reoxygenation have been reported by us to cause damage to RGCs [43]. Oxidative stress induces structural, biochemical, and functional abnormalities of mitochondria such as mitochondrial permeability transition pore opening and leakage of cytochrome *c* from mitochondria into the cytosol in hypoxic conditions [109] contributing to

cell injury or apoptosis and necrotic cell death [127-129]. Our studies on the neonatal retina have shown unequivocally that LPO was increased in the hypoxic retina while conversely, GSH levels were decreased along with increased apoptosis of RGCs thus underscoring the role of oxidative stress in RGC death [43].

Besides causing damage to the RGCs directly, oxidative stress has also been linked to the disruption of BRB in hypoxic-ischemic injuries and diabetic retinopathy [130]. ROS has been thought to contribute to the increased vascular permeability by causing TJ alterations through the enhanced production of inflammatory cytokines such as IL-1 β and TNF- α and VEGF [130].

Hypoxia and purinergic receptors

The extracellular signalling by purine nucleotides and nucleosides, such as adenosine and adenosine tri phosphate (ATP) are now appreciated and the receptors through which they exert their action are called purinergic receptors. The purinergic receptors are further classified into three distinct classes P1 (A_1 , A_{2A} , A_{2B} and A_3 for adenosine), P2X (P2X₁₋₇, ligand gated ion channel receptors) and P2Y receptors (P2Y₁₋₁₄, G-protein coupled purinoceptors) [131]. Purines along with their metabolites are known to contribute to a large number of functions in the eye including daily maintenance of ocular tissue and their dysregulated stimulation could contribute to ocular/retinal diseases involving death of RGCs [132]. The RGCs are known to express both P1 and P2 receptors [132]. While the information on the role of these receptors in RGCs are emerging, studies have focused on the activation of P2X₇ receptor and associated RGC death. P2X₇ receptors expressed on RGCs are essential for modulating their synaptic responses [133]. While a short term stimulation of P2X₇ receptors induces physiological calcium response, prolonged stimulation is shown to mediate RGC death [134]. Under hypoxic/ischemic conditions, activation of P2X receptors, specifically the P2X₇ receptor, has been reported to cause death of RGCs [134, 135] by enhancing intracellular calcium levels. However, inhibition of P2X₇ receptor prevented the death of RGCs following ischemic insult [135]. Taken together these studies provide compelling evidence for the involvement of P2X₇ in RGCs death. Conversely, adenosine, that is accumulated following hypoxia, has been shown to confer protection to the RGCs by acting via the A_1 [136, 137] and A_3 [138] receptors. Adenosine could also stimulate the A_{2A} receptors expressed on retinal vascular cells and induce VEGF production through cAMP-dependent protein kinase A pathway [139]. Given the complex role of VEGF in mediating BRB disruption and protecting RGC, in hypoxic conditions, it would be justifiable to state that purinergic receptors are also involved in this process and modulating their response would be of therapeutic interest.

Protective effect of melatonin in hypoxia

Melatonin, an indoleamine, is produced mainly by the pineal gland with some synthesis occurring in the retina also [140]. It is well established that melatonin has antioxidant properties [141] and has the ability to scavenge free radicals, induce expression of antioxidant enzymes and reduce lipid peroxidation to mitigate oxidative stress in neural tissues

[142]. Hypoxia induced increase in lipid peroxidation in the retina was diminished and the reduced content of glutathione was increased in hypoxic rats treated with melatonin [43]. In addition to mitigating the oxidative stress, melatonin treatment was effective in significantly suppressing the TNF- α and IL-1 β levels in the retina of hypoxic rats thus mitigating hypoxia-induced inflammation. Suppression of the inflammatory response in ischemic conditions [143] and apoptosis in the developing hypoxic brain and retina [142, 43] by melatonin has been demonstrated. Very interestingly, melatonin was also effective in reducing the production of VEGF and NO and hypoxia-induced enhanced vascular permeability [9]. Reduced vascular permeability was evidenced by a reduction in extravasation of intraperitoneally or intravenously administered RhIC and HRP into the retinas of hypoxic adult and neonatal rats [21, 9].

Mitochondrial damage is induced by hypoxia in a variety of cells in the retina including RGCs and Müller cells [109]. We have shown recently that hypoxia resulted in an increased release of cytochrome *c* into the cytosol of RGCs as a result of opening of mitochondrial transition pores which activates cytosolic caspases such as caspase-3 [109]. Cytochrome *c* release and subsequent activation of caspase-3, which leads to apoptosis, were reduced in the RGCs following melatonin administration in the hypoxic retinas [43] and the occurrence of apoptotic RGCs was significantly reduced.

Conclusion

There is voluminous evidence that hypoxia is associated with many ocular pathologies and is a primary cause of death of RGCs. This may be mediated by different factors such as a disrupted BRB, excitotoxicity through increased extracellular glutamate levels and activation of NMDA and AMPA receptors, increased inflammation and oxidative stress (Figure 1). Therefore, mitigating RGC death would require therapies targeting the suppression of these factors.

We have shown that melatonin treatment of hypoxic animals has promising results in protecting the RGCs through suppression of inflammation, oxidative stress and

excitotoxic mediators as well as preserving the BRB. In view of its beneficial effects, we propose that melatonin could be considered as a potential therapeutic agent for the treatment of retinal pathologies in children and adults, in addition to other drugs/molecules of therapeutic interest. Future research on other beneficial properties of melatonin to identify its full neuroprotective potential in addition to its effectiveness in amelioration of oxidative stress and inflammation is certainly warranted.

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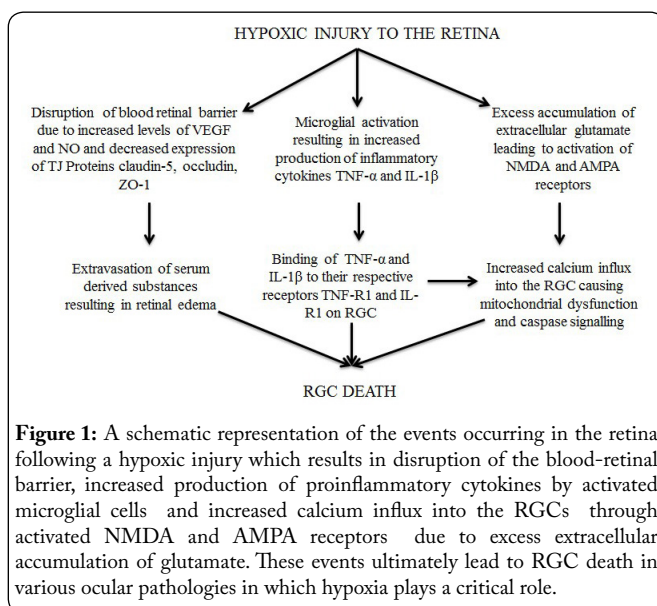


Figure 1: A schematic representation of the events occurring in the retina following a hypoxic injury which results in disruption of the blood-retinal barrier, increased production of proinflammatory cytokines by activated microglial cells and increased calcium influx into the RGCs through activated NMDA and AMPA receptors due to excess extracellular accumulation of glutamate. These events ultimately lead to RGC death in various ocular pathologies in which hypoxia plays a critical role.

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