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Role of adenosine in diabetic retinopathy

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Abstract In diabetic retinopathy (DR), abnormalities in vascular and neuronal function are closely related to the local production of inflammatory mediators whose potential source is microglia. Adenosine and its receptors have been shown to possess anti-inflammatory properties that have only recently been studied in DR. Here, we review recent studies that determined the roles of adenosine and its associated proteins, including equilibrative nucleoside transporters, adenosine receptors, and underlying signaling pathways in retinal complications associated with diabetes.

Keywords Diabetic retinopathy · Adenosine · Microglia · Cannabinoids · Adenosine receptors

Introduction

Diabetic retinopathy (DR) is a leading cause of blindness among working-age adults [1]. Despite many years of research, treatment options for DR, including photocoagulation, vitrectomy, and repeated intraocular injections of steroids and anti-VEGF, remain limited and with adverse effects. Discovery of new molecular entities with adequate clinical activity for DR remains one of the key research priorities in ophthalmology.

Activation of retinal microglial cells in early diabetes is critical in causing the major complications in DR, including losses of blood–retinal barrier (BRB) function and retinal neurons [2, 3]. Although these losses may be a major vision-threatening complication in diabetes, by the time they become easily demonstrable, the progress of DR is already irreversible. The preceding microglial activation and other changes that cause the development of vascular and neuronal changes are highly significant to the understanding and treatment of DR.

Activation of retinal microglial cells is most likely associated with oxidative stress and inflammation. Tissue inflammation is modulated by extracellular adenosine via adenosine receptors. Our research in DR has focused on delineating the inflammatory processes involved. We have identified new noninvasive receptor-based therapies for mitigating microglial activation associated with diabetes. This review is focused on the therapeutic effects of cannabidiol (which are linked with adenosine) and adenosine receptor agonists on animal models of DR. Special emphasis is placed on novel mechanisms described in recent studies of retinal models which help to explain some of the pharmacological effects observed with these therapies.

Diabetic retinopathy

DR is a chronic ocular disorder that will lead to blindness if untreated. In the USA, over 20 million, or 10% of the total population, currently have diabetes. Of this group, over 12,000 patients will be diagnosed with new-onset blindness annually, making it one of the leading causes of legal blindness in Americans within the age group of 20–74 [4]. Type 1 diabetics usually have high incidence of DR, and it occurs in

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almost all patients with diabetes for 20 years or more [1]. The earliest detectable signs of DR are categorized as non-proliferative diabetic retinopathy (NPDR). NPDR is clinically subdivided into mild, moderate, and severe categories. Loss of retinal pericytes and alterations in retinal blood flow are preclinical changes that are often non-detectable by physical exam [5, 6]. Retinal venous dilation and microaneurysms are the first alterations detectable by ophthalmoscopy. Following these alterations, intraretinal hemorrhage and exudation may occur. These may then lead to macular edema, which may lead to blindness if untreated. As hyperglycemia persists, the disease progresses which presents with hemorrhages and venous beading, suggesting decreased retinal circulation and dilated capillaries [7]. Proliferative diabetic retinopathy (PDR) is the next stage when proliferation of new blood vessels begins. Approximately 50% of patients with severe NPDR progress to PDR within 1 year [8]. This stage is characterized by the onset of ischemia-induced new vessel proliferation from the optic nerve head as well as in the retina. These new vessels are fragile and tend to bleed easily resulting in vitreous hemorrhage. If untreated, the neovascularization will undergo fibrosis and contraction leading to traction retinal detachments.

The early signs of DR in experimental diabetic models include vascular inflammatory reactions due to glycated albumin, oxidative stress, pro-inflammatory cytokines, and the consequent binding of leukocyte adhesion molecules CD18 and intercellular adhesion molecule 1 (ICAM-1) [9]. These reactions lead to breakdown of the BRB function, vascular occlusion, and tissue ischemia, which in turn leads to neuronal cell death. However, diabetes could also directly affect metabolism within the neural retina leading to neuronal cell death [9–14]. Whether diabetes affects vascular or neural retina first, both microglial and macroglial cells are activated [15]. The function of activated macroglia in transporting [16] and metabolizing glutamate may be impaired [16, 17]. This leads to glutamate accumulation [18–21]. Glutamate excitotoxicity occurs via activation of *N*-methyl-D-aspartic acid (NMDA) and non-NMDA receptors, to directly or indirectly induce calcium influx and the release of superoxides, leading to neuronal cell death [21]. This is followed by neuroinflammation, during which activated microglial cells migrate toward dying neurons and release inflammatory cytokines to further exacerbate the damage [22]. These findings suggest that pharmacological interventions that reduce oxidative stress and inflammation might be effective neuroprotectants for DR [20, 23].

Microglia in DR

Microglia are very sensitive to small changes in their environment, and they can be activated by a variety of factors including: pro-inflammatory cytokines, lipopolysaccharide, damaged cells, or any immune-stimulatory agents [24]. Activated microglia have phagocytic and cytotoxic ability to destroy foreign materials by secreting cytokines and other signaling molecules. However, if microglia remain in a sustained activated state, the secreted cytokines can affect other cell types in the proximity, particularly neuronal and vascular cells [25]. Recently, overwhelming evidence has sculpted the concept of activated microglia as an important player in the pathogenesis of DR. This input has originated partly from histopathologic studies that showed clustering of apparently activated microglia in the diabetic rat retina [2, 3]. These initial observations have been supported in postmortem human retinas [26] and reinforced by additional histopathological studies showing that many inflammatory molecules, such as tumor necrosis factor alpha (TNF-α), can be detected in the diabetic retina, often in association with microglia [27–29]. The retinal expression of TNF-α has been reported to be associated with neuronal and endothelial cell death, hallmark features of the disease [12, 30], and inhibition of TNF-α has demonstrated beneficial effects in the prevention of early DR [31]. Moreover, the in vitro studies on co-cultured retinal neurons R28 with activated microglia have shown that microglia produce cytotoxins that kill retinal neuronal cells [32]. It remains unclear why diabetes would incite microglia activation in the retina to release inflammatory cytokines. However, recent studies from our group have recognized Amadori-glycated albumin (AGA)/inflammation cascade as a potential culprit mechanism contributing to microglia activation and their secretion of inflammatory cytokines [33]. AGA is present in the retinal capillaries of patients with DR [34] and in the retina of STZ-induced diabetic rats [33, 35] in regions of microglial distribution. Treatment of diabetic rats with A717, a specific AGA-neutralizing antibody, significantly attenuated overexpression of both Iba1, a microglial marker, and TNF-α mRNAs. These observations, together with the finding that intravitreal injection of AGA per se in normal rats induced Iba-1 expression as well as TNF-α release, have strengthened the notion that increased levels of AGA in the diabetic retina is an important contributor to microglial activation and thereby inflammation [33]. Accordingly, the direct relationship between microglia and AGA has been explored through in vitro study. The results showed that formation of reactive oxygen species (ROS) with subsequent activation of extracellular signal-regulated kinase (ERK) and P38, but not JNK, are molecular events underpinning retinal microglial TNF-α release during AGA treatment [33]. Therefore, treatment of cultured microglia with glycated proteins has been used as an in vitro model to simulate inflammation during diabetes [33, 36–38].

Roles of adenosine receptors in DR

After having shown the contribution of microglia to retinal inflammation and DR, the next question is how the protective, anti-inflammatory actions are being harnessed to develop new drug targets for DR control. Adenosine has shown a non-redundant role in the attenuation of inflammation in other tissues through interaction with its receptors. Extracellular adenosine can activate transmembrane adenosine receptors (ARs), which are classified as A_1, A_{2A}, A_{2B}, and A_3 subtypes [39]. These receptors are classified based on their mechanism of signal transduction. A_1 and A_3 receptors interact with G proteins of the Gi and Go family to inhibit adenylate cyclase and stimulate phospholipases. The A_{2A} receptor stimulates adenylate cyclase through Gs coupling [40]. In addition to interaction with Gs, A_{2B} receptor also stimulates phospholipase C activity through Gq [41]. The increased adenosine at inflamed sites exhibits anti-inflammatory effects to protect against excessive cellular damage through A_{2A}AR [42, 43]. Subthreshold doses of an inflammatory stimulus that caused minimal tissue damage in wild-type mice were sufficient to induce extensive tissue damage and more prolonged and higher levels of pro-inflammatory cytokines in $A_{2A}AR^{-/-}$ mice [44]. Moreover, A_{2A}AR agonist treatment blocks the inflammation, functional and histological changes associated with diabetic nephropathy in wild-type diabetic mice but not in the $A_{2A}AR^{-/-}$ diabetic mice [45]. Activation of the A_{2A}AR in the LPS-stressed retinal microglial cells is the most efficient in mediating TNF-α inhibition [46]. In the DR context, $A_{2A}AR^{-/-}$ mice had significantly more retinal terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells, TNF-α release, and ICAM-1 expression compared with diabetic wild type [47]. Furthermore, knockout of A_{2A}AR altered microglia phenotype in unison with TUNEL and cytokine expression profiles during diabetes. This was manifested by the finding that when microglia encountered diabetic milieu, they transformed from their ramified resting state into an amoeboid shape, the activated and cytokine-releasing state, and this phenotypic configuration became more obvious in $A_{2A}AR^{-/-}$ diabetic mice than in diabetic wild-type mouse retina. Furthermore, treatment with the A_{2A}AR agonist resulted in marked decreases in diabetes-induced retinal cell death and TNF-α release [47]. Following this further, we have addressed an interesting feature, acquisition of reactive microglial phenotype, that could be an important determinant for understanding the mechanisms by which A_{2A}AR agonist affects TNF-α release. In this regard, we have noted that treatment of diabetic mice with A_{2A}AR agonist attenuated the morphological transformation of ramified microglia into an activated ameboid microglia. Taken together, these results suggest that A_{2A}AR plays a crucial role in limiting retinal inflammation, microglial activation, and neuronal cell injury associated with diabetes. Little, however, is known about how these receptors regulate inflammation in DR. Additional studies from our group have shown that activation of A_{2A}AR inhibits Raf activation and TNF-α release in AGA-treated retinal microglial cells. These data suggest an important crosstalk regulation between adenosine and inflammation signaling (Ras/Raf/MEK/MAPK) through A_{2A}AR-cAMP regulator. However, this remarkable regulator appears to both activate and inhibit MAPK activity in different cell line. The opposite effects of cAMP on MAPK activity in different cell line could be explained by dissimilar involvement of RAF isoform (C-Raf or B-Raf). Originally, it was assumed that cAMP-triggered inhibition of MAPK seemed to be mediated by C-RAf, while induction of MAPK by cAMP in another cell type involved B-Raf. In AGA-treated microglia, activation of A_{2A}AR inhibits C-Raf activation without affecting B-Raf phosphorylation, suggesting that the mechanism of anti-inflammation involving negative crosstalk between A_{2A}AR-cAMP and Ras/Raf/MEK/MAPK is operative. Furthermore, the observed specificity in the cAMP signaling that is PKA-independent and EPAC-dependent suggests a novel mode in controlling the inflammatory events associated with microglia activation [47].

Adenosine reuptake and degradation

The therapeutic application of adenosine and its agonists is limited by systemic side effects, such as hypotension, bradycardia, and sedation [48]. Adenosine disappears rapidly in physiological or inflammatory conditions due to rapid reuptake via nucleoside transporters (NTs) and subsequent intracellular metabolism [49]. Since endogenous adenosine levels are increased at inflamed sites, prevention of adenosine reuptake into the cells and its subsequent metabolism can selectively enhance extracellular levels of adenosine at the inflamed sites, resulting in a site-specific anti-inflammatory effect. There are two subtypes of NTs: concentrative NTs, which are dependent on the presence of

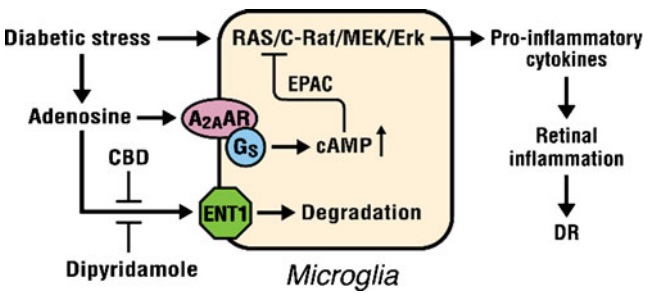

Fig. 1 Regulation of inflammation by adenosine in diabetic retinopathy

extracellular sodium, and equilibrative NT (ENTs). In the microglial cells, the majority of adenosine transport is not affected by sodium removal, suggesting ENTs are the primary transporters functioning in these cells [50]. ENTs are classified into two subtypes on the basis of their sensitivities to inhibition by the drug *S*-(4-nitrobenzyl)-6-thioinosine (nitrobenzylmercaptopurine riboside, NBMPR). NBMPR-sensitive ENTs bind NBMPR with high affinity and have the functional designation equilibrative sensitive (ENT1). NBMPR-insensitive transporters are designated ENT2. Dipyridamole, an inhibitor for both ENT1 and ENT2 [51], is used clinically as a coronary vasodilator and a platelet aggregation inhibitor [52, 53]. Dipyridamole plus aspirin improves retinal vasculature patterns in experimental diabetes [54].

Role of ENT1 in adenosine function in diabetes

ENT1 plays an integral role in adenosine function in diabetes by regulating adenosine levels in the vicinity of adenosine receptors [55]. In this study, *V*max of adenosine transport in high glucose (HG)-treated human aortic smooth muscle cells (HASMCs) was increased by 40% without affecting *K*m. Similarly, *B*max of high-affinity [3H]NBMPR binding was increased without affecting *K*d. Consistent with these observations, HG increased mRNA and protein expression of ENT1. Treatment of cells with the selective inhibitors of ERK, PD98059, and U0126 abolished the effect of HG on ENT1. These results suggest that HG upregulates the expression and functional activity of ENT1 in HASMCs via ERK-dependent pathways. Pathologically, the increase in ENT1 activity in diabetes may affect the availability of adenosine in the vicinity of adenosine receptors and thus alter vascular functions in diabetes. Because diabetes-induced changes in ENT1 expression vary depending on cell types [56], how diabetes alters retinal ENT1 is relevant for its role in the regulation of retinal inflammation.

Cannabinoids as neuroprotectant therapeutics

The marijuana-derived cannabinoids (−)-Δ9–tetrahydrocannabinol (THC) and (−)-cannabidiol (CBD) each has antioxidative and immunosuppressive effects [57]. THC is neuroprotective in a rat model of glaucoma [58]. The psychotropic and anti-inflammatory effects of THC are, at least in part, mediated by CB1 and CB2 cannabinoid receptors, respectively. CBD, though, does not bind well to these receptors, resulting in the inability of CBD to produce the subjective "high" and cognitive effects [59]. The antioxidative effect of CBD (≧1 μM) [60] is due to its ability to scavenge ROS. CBD decreases inflammation in arthritis [61] and uveitis [62], prevents cerebral damage in cerebral ischemia [63] and cerebral infarction [64], reduces hyperglycemia-induced endothelial cell inflammation and barrier disruption [65], decreases the incidence of diabetes in non-obese diabetic mice [66], and is neuroprotective and BRB-preserving in diabetic rats [12]. CBD is well tolerated when chronically administered to humans and has been approved for the treatment of inflammation and spasticity associated with multiple sclerosis in humans [67]. Nanomolar concentrations of CBD inhibit uptake of adenosine by ENT1 in microglia and macrophages [50]. In vivo treatment with a low dose of CBD decreases TNF-α production in serum in LPS-treated mice; this effect is reversed with an $A_{2A}AR$ antagonist and abolished in $A_{2A}AR^{-/-}$ mice [50]. Similar results were obtained in the retinas of LPS-treated rats and retinal microglial cells [46]. These studies suggest that CBD has the ability to enhance adenosine signaling through inhibition of reuptake via ENT1, thus using a mechanism that is not cannabinoid receptor-mediated. The above results are summarized in a diagram (Fig. 1).

Conclusion

This study is important for the development of adenosine receptor agonists or adenosine reuptake inhibitors as a potentially novel and effective therapy for DR. The effect of these therapies is based on their ability to attenuate microglial activation, which precedes the irreversible vascular and neuronal losses in DR. However, the therapeutic values of these agents should be confirmed by clinical trials. Furthermore, depending on the difference in the genetic makeups for the metabolism and pharmacological target of the $A_{2A}AR$ agonists, CBD, or dipyridamole, it may be important to consider these agents as a personalized medicine, i.e., adjusted dosages according to individual's genetic makeups, to offer significant advantages over traditional clinical approaches [68].

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