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Enhanced Ca^{2+} response and stimulation of prostaglandin release by the bradykinin B_2 receptor in human retinal pigment epithelial cells primed with proinflammatory cytokines

Rose-Marie Catalioto*, Claudio Valenti, Carlo Alberto Maggi, Sandro Giuliani

Pharmacology Department, Menarini Ricerche SpA, Via Rismondo 12A, 50131 Florence, Italy

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ABSTRACT

Kallikrein, kininogen and kinin receptors are present in human ocular tissues including the retinal pigment epithelium (RPE), suggesting a possible role of bradykinin (BK) in physiological and/or pathological conditions. To test this hypothesis, kinin receptors expression and function was investigated for the first time in human fetal RPE cells, a model close to native RPE, in both control conditions and after treatment with proinflammatory cytokines. Results showed that BK evoked intracellular Ca^{2+} transients in human RPE cells by activating the kinin B_2 receptor. Pretreatment of the cells with TNF-α and/or IL-1β enhanced Ca^{2+} response in a time- and concentration-dependent additive manner, whereas the potency of BK and that of the selective B_2 receptor antagonist, fasitibant chloride, both in the nanomolar range, remained unaffected. Cytokines have no significant effect on cell number and viability and on the activity of other GPCRs such as the kinin B_1, acetylcholine, ATP and thrombin receptors. Immunoblot analysis and immunofluorescence studies revealed that cytokines treatment was associated with an increase in both kinin B_2 receptor and COX-2 expression and with the secretion of prostaglandin E_1 and E_2 into the extracellular medium. BK, through activation of the kinin B_2 receptor, potentiated the COX-2 mediated prostaglandin release in cytokines-primed RPE cells while new protein synthesis and prostaglandin production contribute to the potentiating effect of cytokines on BK-induced Ca^{2+} response. In conclusion, overall data revealed a cross-talk between the kinin B_2 receptor and cytokines in human RPE in promoting inflammation, a key feature in retinal pathologies including diabetic retinopathy and macular edema.

1. Introduction

Human retinal pigment epithelial (RPE) cells are highly differentiated polarized cells, situated between the choroidal vasculature and the neural retina, that constitute the outer blood–retinal barrier (BRB) [1]. RPE forms a multifunctional monolayer that provides structural support to the photoreceptors but also metabolic and neurotrophic sustain to the neural layer and choriocapillaris through the polarized secretion of growth factors including VEGF [2]. It regulates the transport of ions, water and metabolites from subretinal space to the choroid and provides nutrients to the neural retina. RPE is involved in photoreceptor renewal and visual cycle, and synthesizes melanin to absorb stray light.

Because of the intimate relationship between photoreceptors, RPE cells and choroid, RPE dysfunction can result in secondary degeneration of the adjacent photoreceptors and choriocapillaris, leading to vision loss and blindness [3,4]. Indeed severe and/or persistent retinal injury caused by inflammation, ageing or diabetes, results in BRB breakdown as observed in ocular diseases such as diabetic retinopathy (DR) and age-related macular degeneration (AMD) [5,6]. BRB dysfunction may lead to increased permeability to serum components that gain access to neuroretinal tissue. Interaction of plasma proteins with retinal cells may be associated with leukocytes infiltration, activation of a number of inflammatory pathways including the complement and the kallikrein–kinin pathway on the

Abbreviations: $[Ca^{2+}]i$, intracellular calcium ion concentration; BRB, blood–retinal barrier; CHX, cycloheximide; DAKD, lys-[des-Arg9]-BK; DALKD, lys-[Leu8][des-Arg9]-BK; GPCR, G-protein coupled receptor; hfRPE, human fetal retinal pigment epithelium; HI-FBS, heat-inactivated fetal bovine serum; IL, interleukin; MEM, minimum essential medium eagle; PBS, phosphate buffered saline; PG, prostaglandin; RPE, retinal pigment epithelium; VEGF, vascular endothelial growth factor; WST-1, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate.

* Corresponding author. Fax: +39 55 56809954.
E-mail address: rcatalioto@menarini-ricerche.it (R.-M. Catalioto).

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retinal/choroidal interface and increased cytokine/chemokine production [7,8,3].

Indeed, an important element of AMD and DR is a low grade, subclinical inflammatory process and the interaction of multiple factors including proinflammatory cytokines, such as interleukin-1-beta (IL-1β) and tumor necrosis factor-alpha (TNF-α), and prostaglandins (PGE series) have been implicated in outer BRB breakdown, increased leukostasis and the pathogenesis of these ocular diseases [9]. Inflammatory cells but also retinal glial cells, including astrocytes and Müller cells, are a major source of cytokine/chemokine induction after retinal detachment, including TNF-α and IL-1β [10]. The RPE itself is a significant source of cytokines and chemokines that may in turn induce progressive pathologic changes in the retina and RPE [2]. The expression of both TNF-α and IL-1β is upregulated by intravitreal injection of amyloid-beta, an important constituent of Drusen, in the RPE/choroid and the neuroretina of non-diabetic rats [11]. In turn TNF-α and IL-1β are the major inducer of the production of chemokines by both retinal microvascular endothelial cells and RPE [12]. Moreover it has been demonstrated that increased levels of IL-1β and TNF-α in retinas from diabetic animals are involved in the development of clinically recognized lesions of diabetic retinopathy, including capillary degeneration, pericyte loss and permeability while the inhibition of cytokine signaling using receptor knockout mice protected the animals from diabetes-induced retinal pathology [9].

Among the inflammatory mediators, bradykinin (BK) is one of the most important. BK is a member of the kallikrein–kinin system (KKS) and is produced during inflammatory response in plasma and tissues from the cleavage of kininogen precursors. BK exerts its effects through the selective binding and activation of the kinin B_2 receptor, a G-protein coupled receptor constitutively expressed in a variety of different cell types and tissues [13]. By difference the kinin B_1 receptor, which exhibits greater selectivity for BK metabolites lacking the C-terminal Arg, is weakly expressed under physiological conditions but is up-regulated during tissue injury and inflammation. BK acts as a mediator of a wide variety of physiological and pathophysiological responses including vasodilation, increased vascular permeability and inflammatory cell recruitment, leading to tissue edema, perpetuation of inflammation and pain. The KKS including mRNAs for kinin B_1 and B_2 receptors is endogenously expressed in human ocular tissues, and a complete system is expressed in human retina and in human RPE [14,15]. In addition, increased levels of components of the KKS have been detected in the vitreous of patients with diabetic retinopathy and in a rat model of carbonic anhydrase-I-induced retinopathy [16] and a role of the KKS in the development of diabetes-induced vascular dysfunction in the retina has been contemplated [17,18].

Although there is functional evidence of the presence of both kinin B_1 and B_2 receptors in the RPE cell line, ARPE-19, where they mediate BK-induced glutamate uptake and a transient reduction of cell monolayer resistance [19,20], a possible role of BK and of kinin B_2 receptors in human fetal RPE, a culture model closely related to native tissue, has not yet been described. Moreover the effect of proinflammatory cytokines on BK response and kinin B_2 receptor has not been evaluated to date. The aim of the present study was therefore to characterize the effect of BK in human fetal RPE cells and to characterize the receptors involved in control conditions and after TNF-α and IL-1β treatment. The effect of BK has been investigated by measuring intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) since Ca^{2+} is a tightly regulated second messenger that integrates numerous stimuli and, in the RPE, it is essential for the maintenance of normal retinal function. At a cellular level, we determined the effect of BK on cell number, cell viability by WST-1 assay and the release of prostaglandins by specific ELISAs. The receptors mediating BK effects have been characterized pharmacologically, by using both kinin B_1 and B_2 selective agonists and antagonists and by Western blot and immunofluorescence studies.

2. Materials and methods

2.1. Drugs

BK, Lys-[des-Arg^9]-BK, and Lys-[Leu^8][des-Arg^9]-BK were obtained from PolyPeptide (Strasbourg, France). Fasitibant chloride bis hydrochloride (fasitibant chloride, 4-(*S*)-amino-5-(4-{4-[2,4-dichloro-3-(2,4-dimethyl-8-quinolyloxymethyl) phenylsulfonamido]-tetrahydro-2H-4-pyranylcarbonyl}piperazino)-5-oxopentyl](trimethyl)ammonium chloride hydrochloride) was synthesized at the Chemical Development Department of Menarini Ricerche in Pisa, Italy. Thrombin from human plasma (EC: 3.4.21.5, ≥2800 NIH units/mg protein), ATP (adenosine 5′-triphosphate), TNF-α, lisinopril and cycloheximide (CHX) have been obtained from Sigma (St. Louis, MO) while acetylcholine chloride was from Tocris Bioscience (Bristol, UK) and IL-1β from R&D systems (Abingdon, UK). Highly cross-adsorbed Alexa Fluor 488-labelled secondary antibody, Image-iT^{TM} FX signal enhancer and SlowFade Gold antifade reagent were from Molecular Probes (Eugene, OR, USA).

Primaria cell culture flasks, 40-μm nylon meshfilter and fibronectin from human plasma were from BD Biosciences. 96 well special optics, flat clear bottom black polystyrene TC-treated microplates, and 6-well plates were obtained from Corning Costar (Mainz, Germany). MEM alpha modification, N1 supplement 100×, L-glutamine–penicillin–streptomycin solution, taurine, hydrocortisone, triiodo-thyronin, and MEM non-essential amino acid solution (100×) have been obtained from Sigma (St. Louis, MO). Fetal bovine serum, heat inactivated, was from Gibco Invitrogen.

2.2. Human fetal RPE cell culture

Human retinal pigment epithelial cells were obtained from Lonza (Walkersville, MD), cultured and differentiated as previously described [21,22]. Briefly, human RPE cells (donated from healthy donors, 19–22 weeks of gestation, after obtaining permission for their use in research applications by informed consent or legal authorization, lot number 3F3017, 0000319368, 0000418984) were seeded into BD primaria 25 cm^2 flasks at a density of 10^4 cells/cm^2 in MEM-alpha modification medium containing N1 supplement 1:100 ml/ml, Glutamine–penicillin–streptomycin 1:100 ml/ml, and nonessential amino acid solution 1:100 ml/ml, and a mixture of hydrocortisone (20 μg/l), taurine (250 mg/l), and triiodo-thyronin (0.013 μg/l) (RPE culture medium) to whom 15% HI-FBS has been added. Cells were maintained at 37 °C in an atmosphere of 5% CO_2/95% air and 90% relative humidity until confluence with medium change to 10% HI-FBS the day after seeding, and to 5% HI-FBS every other day thereafter. RPE cells were subcultured once using the subculture reagents pack from Lonza (Walkersville, MD) and plated at a density of 10^5 cells/cm^2 into human fibronecting-coated 96-well special optics microplates ($[Ca^{2+}]i$ measurements, ELISA test), human fibronectin-coated 6-well plates (western blot analysis) or human fibronecting-coated 4-well Nunc Lab-Tek chamber slides (immunofluorescence studies) in the presence of 10% HI-FBS (plating medium) and transferred after 18 h in fresh medium supplemented with 5% HI-FBS (growth medium). The day after, cells are transferred in fresh medium containing 1% HI-FBS (differentiation medium) and maintained in this medium until assay. The phenotypic changes of primary human RPE cells were determined by light and phase-contrast microscopy at different time interval after seeding and induction of differentiation. Images were collected with a Leitz