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Tolerance of high and low amounts of PLGA microspheres loaded with mineralocorticoid receptor antagonist in retinal target site

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ABSTRACT

Mineralocorticoid receptor (MR) contributes to retinal/choroidal homeostasis. Excess MR activation has been shown to be involved in pathogenesis of central serous chorioretinopathy (CSCR). Systemic administration of MR antagonist (MRA) reduces subretinal fluid and choroidal vasodilation, and improves the visual acuity in CSCR patients. To achieve long term beneficial effects in the eye while avoiding systemic side-effects, we propose the use of biodegradable spironolactone-loaded poly-lactic-*co*-glycolic acid (PLGA) microspheres (MSs). In this work we have evaluated the ocular tolerance of MSs containing spironolactone in rat' eyes. As previous step, we have also studied the tolerance of the commercial solution of canrenoate salt, active metabolite of spironolactone. PLGA MSs allowed *in vitro* sustained release of spironolactone for 30 days. Rat eyes injected with high intravitreous concentration of PLGA MSs (10 mg/mL) unloaded and loaded with spironolactone maintained intact retinal lamination at 1 month. However enhanced glial fibrillary acidic protein immunostaining and activated microglia/macrophages witness retinal stress were observed. ERG also showed impaired photoreceptor function. Intravitreous PLGA MSs concentration of 2 mg/mL unloaded and loaded with spironolactone resulted well tolerated. We observed reduced microglial/macrophage activation in rat retina compared to high concentration of MSs with normal retinal function according to ERG. Spironolactone released from low concentration of MSs was active in the rat retina. Low concentration of spironolactone-loaded PLGA MSs could be a safe therapeutic choice for chorioretinal disorders in which illicit MR activation could be pathogenic.

1. Introduction

Mineralocorticoid receptor (MR), the ancestor corticoid receptor is expressed in the distal nephron where it regulates sodium and fluid reabsorption, modulating blood volume and arterial pressure. More recently, MR was evidenced in heart, blood vessels, brain, adipose tissue, skin and macrophages, recognized as non-classical mineralocorticoid targets [1,2]. Illicit activation of MR has been demonstrated to induce inflammation, oxidative stress, fibrosis, hypertrophic remodelling and endothelial dysfunction and increasing evidences suggest the MR antagonism has therapeutic value for endothelial dysfunction, atherosclerosis, hypertension, heart failure and chronic kidney disease [3–5].

Spironolactone has been the first steroidal competitive MR antagonist drug (MRA) approved for the treatment of hypertension half a century ago. It is a potent but low-specific MRA, interacting at high dose with androgen receptors, and causing subsequent dose-dependent hormonal side-effects [6]. Eplerenone, less potent but more specific

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MRA, designed to reduce the hormonal effects of spironolactone, was approved by the Food and Drug Administration in 2002 for the prevention of cardiac complications after myocardial infarction and ventricular dysfunction [7,8]. Both MRA can potentially induce hyperkalemia, particularly with impaired renal function.

The eye is also a mineralocorticoid sensitive organ, expressing MR and pre-receptoral enzyme 11β-hydroxysteroid dehydrogenase type 2 in the iris/ciliary body, neuroretina, retinal pigment epithelium and endothelial cells [9–13]. MR activation contributes to retinal fluid homeostasis through regulation of specific ion and water channels in retinal macroglial cells (Kir4.1 and AQP4) [9,14]. An over-activation of MR by excess glucocorticoid or endogenous activation has been hypothesized in the pathogenesis of central serous chorioretinopathy (CSCR) [11]. Systemic administration of MRA has effect on subretinal fluid and choroidal vasodilation, and improves the visual acuity in CSCR patients [11,15–18]. Since our first studies, > 15 clinical studies have confirmed that MRA reduces subretinal fluid associated with CSCR. However, to achieve and to further maintain therapeutic effect, long-term use of MRA may be required [18], increasing the risk of side-effects. Spironolactone, 40-fold more potent MRA than eplerenone, is associated on the long-term with hormonal side effects such as gynecomastia, erectile dysfunction and menstrual irregularities [6], being particularly bad tolerated in the CSCR population consisting mostly of middle-aged men. Although the ocular bioavailability of spironolactone and eplerenone after systemic administration has not been specifically studied, it is known that spironolactone induces the expression of the P-glycoprotein, a drug efflux protein that regulates the blood-brain barrier, and is one of its ligands [19], which may limit its ocular bioavailability.

In order to avoid systemic undesirable effects and ensure optimized MR blockade, intraocular local MRA delivery is desired and sustained release drug delivery system is mandatory to avoid repeated intraocular injections. For preclinical development in animal models and for potential clinical development, we have chosen to prepare biodegradable microspheres (MSs) using poly-lactic-*co*-glycolic acid (PLGA) to provide a controlled delivery of the drug in a long fashion. Particle size can be reduced to allow injection of a suspension through a regular 30G needle and provide the sustained release of active products [20–22]. PLGA, approved by FDA and the European Medical Agency for intraocular use [23,24], is progressively hydrolysed in its monomers (lactic acid and glycolic acid), and subsequently converted into CO_2 and water *via* Kreb's cycle. Biodegradation in PLGA microspheres is described to occur in the polymeric matrix through a homogeneous hydrolytic chain cleavage mechanism leading to a sustained release of the active compounds included in the polymeric matrix [23,24].

Intraocular tolerance of controlled drug delivery systems is critical as they are proposed for long term treatments. As the device is formed by a biomaterial and the active substance, it is also important to evaluate the *in vivo* response of the unloaded system. In the present work, spironolactone-loaded MSs releasing the drug *in vitro* for at least 30 days, were screened for anatomical and functional tolerance in normal rat eyes. *In vivo* and *ex vivo* tolerance analyses were performed 4 weeks after intravitreal injection of low and high amounts of microspheres in rats (0.1 mg and 0.5 mg, final concentration in the rats vitreous of 2 and 10 mg/mL respectively). As the development of a novel controlled release also requires tolerance studies of the active molecule itself in the target tissue, we also evaluated the intraocular tolerance of an intravenous commercial solution of canrenoate salt (Soludactone), one of the active metabolites of spironolactone. To our knowledge the intraocular tolerance of spironolactone and its metabolites has not been previously studied.

2. Materials and methods

2.1. Animals

All experiments were performed in accordance with the European Communities Council Directive 86/609/EEC and approved by local ethical committees (Ce5/2012/113 and Approval VD2928). Adult male Wistar rats (8 weeks old) from Janvier breeding Center (Le Genest-Saint-Isle, France) or Charles-River (L'Arbresle, France) were used. Animals were kept in pathogen-free conditions with food, water and litter and housed in a 12-hour light/12-hour dark cycle. Anesthesia was induced by intramuscular ketamine (40 mg/kg) and xylazine (4 mg/kg). Animals were sacrificed by carbon dioxide inhalation.

2.2. Intravitreous injection of soludactone

Intravitreous injections (IVT) were performed using microfine (300 μL) syringes with 30G needles under topical anesthesia (tetracaine 1%, Aldrich, Lyon, France). Five microlitres of the Soludactone® solution (Pfizer, Paris, France) at 37 μg/μL (final concentration in the vitreous: 10 μM) was injected in the rat eyes. Control rats received 5 μL of 0.9% NaCl. Soludactone® contains trometamol and water as excipients. Trometamol containing preparations have already been injected in the vitreous of animals and humans without any detectable toxicity [25,26].

2.3. In vivo and histological analyses for rats injected with soludactone

One week after intravitreous Soludactone injection, rat retinal morphology was examined *in vivo* using a spectral domain optical coherence tomography (SD-OCT) system adapted for rat eyes (Bioptigen, Leica Microsystems, Buffalo Grove, IL, USA), and a retinal camera specially designed for mouse/rat retinal imaging (Micron III, Phoenix Research Labs, USA).

Visual function of rat eyes was also evaluated 1 week after IVT using electro-retinography (ERG). ERGs were performed using Espion E2 system from Diagnosys LLC (Lowell, MA, USA). Animals were dark adapted overnight. Scotopic ERGs were performed in the dark with light intensities of flashes ranging from 0.0001 to 30 cd·s/m^2. For photopic recordings, animals were light adapted for 5 min and then the response to light intensities of flashes ranging from 0.1 to 30 cd·s/m^2 was recorded. ERG records were analyzed using 2-way ANOVA and multiple *t*-tests. Statistical significance was determined using the Holm-Sidak method, with alpha = 5%. Each row was analyzed individually, without assuming a consistent SD.

Rats were then sacrificed, each eye was dipped in Bouin's solution for 24 h, embedded in paraffin, cut in 4.5 μm thick sections, and stained with haematoxylin - eosin. Sections through the optic nerve and through the injection site were analyzed under a microscope (Zeiss, Oberkochen, Germany) associated with a digital camera.

2.4. In vitro stability of spironolactone in the presence of human vitreous

An isotonic formulation of spironolactone was prepared using 0.9% NaCl at a concentration of 6.2 μM (2.6 μg/mL). To determine whether spironolactone is stable when in contact with vitreous, 100 μL of spironolactone-containing formulation were added and mixed with 500 μL human vitreous and kept in room temperature. Fifty microlitres of vitreous sample were harvested at each time point at 0, 5, 10, 30 and 60 min, then diluted with 25 μL acetonitrile containing ISTD, filtrated and analyzed using Waters Acquity UPLC system (Saint-Quentin-en-Yvelines, France). Experiments were performed in triplicate. Kinetics of spironolactone concentration over time was established with reference to time 0 considered as 100%.