Esterase activity in porcine and albino rabbit ocular tissues

Emma M. Heikkinen⁎, Eva M. del Amo, Veli-Pekka Ranta, Arto Urtti⁎, Kati-Sisko Vellonen, Marika Ruponen

A School of Pharmacy, Faculty of Health Sciences, University of Eastern Finland, FI-70211 Kuopio, Finland
B Drug Research Program, Division of Pharmaceutical Biosciences, Faculty of Pharmacy, University of Helsinki, P.O. Box 56, FI-00014 Helsinki, Finland
C Institute of Chemistry, St Petersburg State University, 198504 Petergof, Russia

ARTICLE INFO
Keywords:
Esterases
Ocular metabolism
Ocular ADME
Ocular prodrugs
Ocular drug bioavailability

ABSTRACT
Corneal esterases are utilized in the activation of topicaly applied ester prodrugs. Esterases may also be involved in the metabolism of drugs in posterior eye tissues, but their physiological activity is unknown. Furthermore, extrapolation of the esterase activity from protein level to the tissues is missing.

The aims of the current study were to determine esterase activities in porcine and albino rabbit ocular tissues, calculate the activities for whole tissues and compare esterase activity between the species. We conducted a hydrolysis study with ocular tissue homogenates using an esterase probe substrate 4-nitrophenyl acetate. The hydrolysis rates were first normalized to protein content and then scaled to whole tissues.

The hydrolytic rate normalized to protein content was high in the cornea and iris-ciliary body and low in the lens and aqueous humor, and in general, the rabbit tissues had higher hydrolytic rates than the porcine ones. Esterase activity scaled to whole tissue was high in cornea and iris-ciliary body and low in aqueous humor and retinal pigment epithelium in both species.

The current study revealed differences in esterase activities among the ocular tissues and the species. This basic knowledge on ocular esterases provides background information particularly for posterior segment drug development.

1. Introduction
Ocular ADME processes, such as permeability, active transport (Vellonen et al., 2017), melanin binding (Pelkonen et al., 2017; Rimpelä et al., 2017) and metabolism, are important aspects for ocular drug delivery (Argikar et al., 2017). Drug-metabolizing enzymes in the eye are not yet well characterized, even though ocular metabolism is known to affect pharmacokinetics of topical drugs (Argikar et al., 2017). For example, metabolic capacity of the cornea activates topicaly applied prodrugs, releasing parent compounds that are active in the inner eye tissues.

Several drug metabolizing enzymes, such as esterases, peptidases, alcohol and aldehyde reductases and cytochrome P450s have been detected in the eye (Duvvuri et al., 2004). Interestingly, whilst cytochrome P450s are the most important drug metabolizing enzymes in the liver, in the eye their expression levels are low and their functional role in drug metabolism and pharmacokinetics of the eye is unclear (Nakano et al., 2014). Esterases, a class of phase I hydrolytic enzymes cleaving ester bonds, in contrast have been shown to affect ocular pharmacokinetics of topical drugs (Anderson et al., 1980; Chang and Lee, 1982; Chang et al., 1987; Dias et al., 2002; Hellberg et al., 2003; Lee et al., 1982b; Lee et al., 1982a; Lee, 1983; Redell et al., 1983; Sjöquist et al., 1998). This hydrolytic reaction is employed in the design of ester prodrugs that have higher lipophilicity and permeation through biological membranes than their parent drug. For example the hydrolysis of latanoprost (Sjöquist et al., 1998), tafufrost (Fukano and Kawazu, 2009) and dipivefrin (Anderson et al., 1980; Mandell et al., 1978) to their active parent drugs by corneal esterases is utilized in glaucoma treatment.

The interest in ester prodrug approach has stimulated studies on esterase activity in ocular tissues (Lee, 1983). These studies have focused on anterior tissues, such as cornea, iris-ciliary body and aqueous humor. A single study with ganciclovir ester prodrugs (Dias et al., 2002) showed prodrug hydrolysis in posterior tissues, such as choroid, but the report did not clarify whether the prodrugs were cleaved by esterases. Esterase activity in the posterior segment is important since it may affect the drug delivery to the posterior eye.

In the present study, we investigated hydrolysis of enzyme substrate 4-nitrophenyl acetate (NPA) in porcine and albino rabbit ocular tissues. The study aims were to 1) determine the esterase activities in anterior...
and posterior ocular tissues (conjunctiva, cornea, aqueous humor, iris-ciliary body, lens, vitreous, neural retina, retinal pigment epithelium (RPE), choroid and sclera) dissected from pig and albino rabbit; 2) establish hydrolytic activities for whole ocular tissues; 3) compare esterase activities in the rabbit and porcine eye. To our knowledge, this is the first comprehensive report on esterase activities in the ocular tissues.

2. Materials and Methods

All chemicals were purchased from Sigma-Aldrich (Espoo, Finland).

2.1. Tissue Collection

2.1.1. Porcine Eye Tissues

Enucleated porcine eyes were received from a local slaughterhouse and transported to the laboratory on ice in phosphate-buffered saline (PBS) within 5 h after the slaughter. The dissection of the eye was started by collecting a piece of bulbar conjunctiva. Then, the extraocular tissue was removed. Aqueous humor was collected by aspirating through limbus with a needle and a syringe. The eyeball was cut open from limbus and lens, iris-ciliary body and cornea were collected from the anterior part. Vitreous was collected from the posterior eye cup and traces of pigment and neural retina were removed. The neural retina was collected. One milliliter of PBS was pipetted to the eyecup and retinal pigment epithelium was suspended into the buffer by scratching with a small brush. RPE suspension was collected and, thereafter, the procedure was repeated twice. The total RPE suspension was centrifuged at 6000 g for 5 min at +4 °C and the supernatant was discarded to obtain a RPE pellet. Choroid was collected from the remaining posterior eye cup, the outer surface of sclera was cleaned from any remaining extraocular tissues, optic nerve was cut off and the sclera was collected. All tissues were weighted, stored at −80 °C and thawed on ice before the experiment. Tissues were collected in triplicate, except for conjunctiva n = 6 and choroid n = 4.

2.1.2. Rabbit Eye Tissues

Eyes of adult female albino New Zealand White rabbits, with body mass of 3.5–5.0 kg, were frozen after enucleation and stored at −80 °C. On day of the experiment, the eyes were thawed at room temperature until unfrozen and dissected in the same manner as the porcine eyes. The isolated tissues were weighted and stored on ice. For aqueous humor, choroid, conjunctiva and RPE n = 3; for cornea, neural retina, sclera and vitreous n = 4; for iris-ciliary body n = 5 and for lens n = 6.

2.1.3. Rat Liver

An adult male albino Wistar Han rat was euthanized with carbon dioxide and perfused intra-cardially with 0.9% sodium chloride solution. Liver (n = 1) was collected, rinsed with PBS, homogenized (see 2.2) and stored as cell homogenate at −80 °C until the experiment day.

2.2. Tissue Homogenization and Dilution

2.2.1. Porcine and Rabbit Eye Tissue Homogenates

On the day of the experiment, all ocular tissues except the aqueous humor were first homogenized with Dounce homogenizer in PBS (200 μl/tissue for rabbit choroid, RPE and vitreous; 400 μl/tissue all the rest; tissue/buffer ratio 0.1–7.6 g/ml). Then, they were sonicated with an ultrasonic processor and microtips (Vibra-Cell VCX750 and four-element probe; Sonics & Materials Inc., Newton, CT) on ice 4 times for 45 s with 15 s pause in between. The tissue homogenates were centrifuged at 12000 g for 2 min at +4 °C and the supernatants were collected for further use.

2.2.2. Rat Liver Homogenate

Rat liver homogenate was prepared after liver isolation by adding 4 ml of PBS to 1 g of liver and homogenizing on ice with a dispersing device (Ultra-Turrax T8; IKA Works, Inc., Wilmington, NC) in 10 s bursts 3 times, and stored at −80 °C. On the day of the experiment, liver homogenate was thawed on ice and centrifuged at 10000 g for 20 min at +4 °C. Supernatant was then collected.

2.2.3. Dilution of Tissue Homogenate Supernatants for Incubations

Protein concentrations of the collected supernatants were quantified with Bradford assay (Bradford, 1976) and bovine serum albumin standards. The tissue homogenates were then diluted with PBS to protein concentrations of 0.1, 0.3 and 0.5 μg/μl. This dilution was done to decrease sample background absorbance and standardize the protein binding of the hydrolysis substrate NPA, and the product 4-nitrophenol (NP) in the sample.

2.3. Hydrolysis of 4-Nitrophenyl Acetate

2.3.1. Hydrolysis Study with Tissue Homogenates

On the day of experiment, 20 mM stock solutions of NPA and NP in ethanol were prepared and NPA stock was further diluted to 200 μM with PBS. Tissue homogenate dilutions of 80 μl were pipetted to clear 96-well plates as triplicates and pre-incubated at +37 °C for 10 min. Equal volume of 200 μM NPA was added to samples with a multi-channel pipet and the sample absorbance was immediately measured at 405 nm every 90 s for 45 min in +37 °C. In sample wells, the initial NPA concentration was 100 μM and protein concentrations were 0.05, 0.15 and 0.25 μg/μl. Ethanol concentration in the incubation was <0.5%.

2.3.2. 4-Nitrophenol Standards

NP stock solution at 20 mM was diluted with PBS to 200 μM, from which 5, 20, 40, 60, 80, 100 and 120 μM NP standards were prepared with PBS. NP standards (160 μl/well) were included to all plates.

2.3.3. Chemical Hydrolysis of 4-Nitrophenyl Acetate

Chemical hydrolysis of NPA was studied in PBS and 5 M sodium hydroxide similar to Section 2.2. NPA hydrolysis in PBS was negligible. Furthermore, in the presence of sodium hydroxide, NPA yielded equal molarity of NP, indicating that NPA is fully converted to NP. To further clarify the non-enzymatic hydrolysis of NPA, ocular tissue homogenates (n = 2) were prepared as in Section 2.2, heated in +80 °C for 30 min to denature the enzymes and cooled before incubation. The homogenates (80 μl), were incubated with 80 μl of 200 μM NPA as described above. The experiment showed that NPA hydrolysis was negligible in all tissues except for the porcine vitreous and the rabbit aqueous humor (Supplementary Fig. 1). Therefore, we excluded the results for these tissues.

2.4. Calculation of Esterase Activity

2.4.1. Normalization to Protein Concentrations

NPA hydrolysis rate was calculated on the slope of linear part of NP concentration-time curve, that starts at 0 and typically ends from 1.5 to 20 min depending on the tissue and the homogenate protein concentration.

2.4.2. Scaling to Whole Tissues

From NPA hydrolysis rates in tissue homogenates, we calculated the reaction rates for the whole ocular tissues (Fig. 1). NPA hydrolysis rates from incubations at 0.15 μg/μl protein concentration (homogenate protein concentration 0.3 μg/μl) were used in the calculation of esterase activity for whole tissue. First, tissue protein yield was calculated from total tissue mass and tissue protein content. Tissue mass in incubation was calculated from tissue protein yield and protein concentration in the incubation. Then, NPA hydrolysis rate was divided by the tissue mass in incubation to get reaction rate per gram of tissue, which was
multiplied by whole tissue mass to obtain hydrolysis rate in whole tissue. For conjunctiva, isolating the whole tissue was unfeasible, and the total mass has not been reported in the literature, thus conjunctival esterase activity could not be scaled to whole tissue.

2.4.3. Statistical Analysis

Esterase activities were compared among tissues with Kruskal–Wallis test and Dunn’s post hoc-test and between species with Mann–Whitney U test. Significance was set at 0.05.

3. Results

3.1. Hydrolysis Rate Normalized to Protein Concentrations

For ocular tissues, the NPA hydrolysis was similar regardless of the sample protein concentration (Supplementary Figs. 2 and 3) and thus, for clarity, all results shown in this report correspond to 0.15 μg/μl protein concentration (from homogenate protein concentration of 0.3 μg/μl). In rat liver the rate was 103 ± 2 pmol/min/μg protein (mean ± S.D.).

3.1.1. Porcine Tissues

Cornea, sclera, choroid, conjunctiva and neural retina had the highest hydrolysis rates normalized to the protein content (Fig. 2). Iris-ciliary body presented two-fold and RPE three-fold lower hydrolysis rates than cornea. Aqueous humor and lens showed the lowest hydrolysis rates, 6- and 66-fold lower than the cornea.

3.1.2. Rabbit Tissues

Cornea, vitreous, iris-ciliary body, conjunctiva, choroid, neural retina and RPE (Fig. 2) showed the highest esterase activities. Lower hydrolysis rates were seen in sclera and lens.

Between the species, the hydrolysis rates were higher in the rabbit than in the porcine tissues. Conjunctiva, RPE, iris-ciliary body and lens had 1.5- to 8-fold higher hydrolysis rates in rabbit than in pig (Mann Whitney p < 0.001). In contrast, sclera had 10-fold, choroid 8-fold and neural retina 2-fold higher hydrolysis rate in the pig than in the rabbit.

3.2. Tissue Masses and Tissue Protein Content

For pig, vitreous and sclera had the highest masses (45% and 18% of total eye mass) (Supplementary Table 1). The highest tissue masses for rabbit were vitreous and lens (26% and 17% of total eye mass). The other tissues constituted 0.44–8.3% of the total weight of the eye in both species. Tissue protein content ranged over very wide range from 0.600 to 297 mg/g, lens having the highest and vitreous the lowest levels in both species (Supplementary table 2).

3.3. Hydrolysis Rates Scaled to Whole Tissues

3.3.1. Porcine Tissues

The highest hydrolysis rates in whole tissues were in the sclera, neural retina, iris-ciliary body and choroid (Fig. 3). Cornea and lens presented two- to six-fold lower rates than sclera. The lowest rates were in aqueous humor and RPE; 55- and 75-fold lower than in the sclera, respectively.

3.3.2. Rabbit Tissues

The highest hydrolysis rates were in the lens, iris-ciliary body and cornea (Fig. 3). Vitreous, neural retina, sclera, choroid and RPE showed low hydrolysis rates (9–295 times less than the lens).

Comparing the two species, lens had ten-fold and RPE three-fold higher hydrolysis rate in the rabbit than in the pig (Mann Whitney p < 0.001). In contrast, sclera had 10-fold, choroid 8-fold and neural retina 2-fold higher hydrolysis rate in the pig than in the rabbit.
(p < 0.01). There were no significant differences between the species in corneal and iris-ciliary body hydrolysis rates. These results were mostly in line with hydrolysis rates per gram of tissue (Supplementary Fig. 4).

4. Discussion

In the current study, we characterized the esterase activity in the ocular tissues of albino rabbit and pig by using NPA that is a substrate for esterases, such as carboxylesterase (EC 3.1.1.1) (Landowski et al., 2006; Nakamura et al., 1993), acetyl esterase (EC 3.1.1.6) (Nakamura et al., 1993) and arylesterase (EC 3.1.1.2) (Nakamura et al., 1993). The information from porcine eye is useful because it is a feasible preclinical contribution of ocular tissues in the metabolism of esters. Drlysis activity showed the highest levels among the porcine tissues, we scaled the esterase activities to the whole tissue level with total homogenates and reported as reaction rates related to protein amount in incubation. This approach alone does not inform about the overall homogenates could be compared. Lee (1983) have studied esterase activity in the anterior rabbit eye with naphthyl produgs showing that the highest esterase activity was in iris-ciliary body > cornea > aqueous humor. Furthermore, they found butyrylcholinesterase from conjunctiva (Lee et al., 1985). In our study, in both rabbit and pig, esterase activity was high in cornea and iris-ciliary body, and we detected activity in conjunctiva. These findings were in line with the previous reports (Lee, 1983; Lee et al., 1985).

Another hydrolysis experiment with homogenized tissues showed that ganciclovir ester produg hydrolysis was fastest in iris-ciliary body = retina-choroid ≈ cornea > lens > aqueous humor > vitreous (Dias et al., 2002). Our results were in line with these observations. In sclera, bimatoprost hydrolysis catalyzed by esterases was similar to the levels in the cornea and iris-ciliary body (Hellberg et al., 2003). Likewise, we observed that the scleral esterase activity was in the same order as the corneal and iris-ciliary body activities.

In the current study, rabbit tissues had higher absolute hydrolysis rates (normalized to protein concentration) than porcine tissues. Similarly, an investigation on naphthyl esters showed 2-50 fold higher esterase activities in rabbit than in bovine eye (Lee et al., 1982a, 1982b). Unfortunately, information on the esterase activities in human ocular tissues is scarce, thus comparison to the current results is difficult. A histochemical study of anterior eye hydrolases (Coupland et al., 1994) suggests that esterase activities in human and pig eye were similar, though the study was only semi-quantitative.

4.2. Esterase Activities at Whole Tissue Level

Previously, esterase activities in the eye have been studied in tissue homogenates and reported as reaction rates related to protein amount in incubation. This approach alone does not inform about the overall quantitative roles of the tissues in ocular drug metabolism. Therefore, we scaled the esterase activities to the whole tissue level with total tissue mass and protein content (Fig. 1). Interestingly, the scleral hydrolysis activity showed the highest levels among the porcine tissues, whereas lens had the highest activity in the rabbit eye. These are surprising results but can be rationalized. Porcine sclera has high mass, thus esterase activity scaled to whole tissue is high. Similarly, rabbit lens mass (related to the total eye mass) is substantial, and its protein content is exceptionally high, which results in high esterase activity at the whole tissue level. The lowest esterase activities for whole tissues were in the aqueous humor (available only for the pig), which is expected because the acellular aqueous humor has low protein content. Similarly, RPE possessed low esterase activity in both species, since the RPE is a single cell layer and has low total mass.

Since the scaling was based on total tissue mass and protein content, variability in tissue isolation and homogenization could affect the results. For albino rabbit, separating the neural retina from the unpigmented RPE and choroid was challenging. Therefore, the purity of the isolated tissues was uncertain. The esterase activities normalized to protein concentrations were, however, similar to previous reports (Lee, 1983, Lee et al., 1985, Hellberg et al., 2003, Dias et al., 2002) and the tissue masses (related to eye mass) were in line with Struble et al. (2014). Thus, the tissue isolation seems to have been successful. Homogenization of tough tissues such as cornea and sclera proved difficult, and their protein yield (relative to total protein content) was presumably lower than for softer tissues. Esterase activity for whole cornea was still among the highest ones, so the difficulties in tissue homogenization did not seem to affect the order of tissue activities.

4.3. Relevance of Ocular Esterases on Drug Pharmacokinetics

Previous studies on ocular ester produgs, such as latanoprost (Sjöquist et al., 1998) and dipivefrin (Anderson et al., 1980; Fukano and Kawazu, 2009), have clearly demonstrated that corneal esterases affect the pharmacokinetics of topical drugs and are a feasible strategy to improve drug delivery to the eye. However, since most ocular tissues seem to possess esterase activity, the ester produg approach could be feasible for other routes of administration than topical. For intravitreal drugs, esterases in the vitreous may contribute to drug hydrolysis. This has been demonstrated in an in vivo study with rabbits after intravitreal administration of ganciclovir ester produgs (Macha et al., 2004), suggesting that vitreal esterases could be utilized for intravitreal produgs and also for controlled release delivery systems (Borke et al., 2018). The drug can diffuse from the vitreous to iris-ciliary body or to neural retina, RPE and choroid. In the current study, esterase activities were detected in iris-ciliary body, neural retina and choroid, thus they can be considered relevant metabolic sites for intravitreal drugs. Esterase activity in the RPE appeared weak, but the RPE is an important barrier between blood circulation and posterior eye segment and it has relatively strong metabolic activity in general. For example, peptidases, such as cathepsin D (Rakoczty et al., 1997), are expressed in the RPE and these enzymes can be used to cleave peptide linkers in drug delivery systems (Bhatattacharya et al., 2017). In periocular drug administration, scleral esterases might affect drug pharmacokinetics and could potentially be utilized for subconjunctival produgs.

The pharmacokinetic role of lens is poorly understood, yet the lens seemed to possess some esterase activity, which could be utilized for antiglare drugs (Abdelkader et al., 2015). In general, development of targeted ester produgs that would release the parent compound in specific eye tissue seems difficult, since most of the ocular tissues presented some esterase activity. Still, the tissue targeting could be facilitated to some extent by choosing an optimal drug administration site.

Ocular esterases have conventionally been utilized in the design of produgs, but they are feasible for other applications as well. For instance, the cleavage of ester bonds in a polymer-based drug delivery system is an interesting concept that could enable prolonged and controlled drug release (Borke et al., 2018).

To conclude, the current study revealed differences in esterase activities among different ocular tissues and between albino rabbit and
pig. The results are applicable in ocular drug development and pharmacokinetic modeling and simulation.

Acknowledgements

This work was supported by the Doctoral Programme in Drug Research (University of Eastern Finland), Business Finland (grant number 637/31/2015 TEKES) and Academy of Finland (grant numbers 311122 and 268868). The authors acknowledge Lea Pirskanen, Laura Hellinen, Mika Reinisalo and Elisa Toropainen for the assistance with tissue isolation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejps.2018.07.034.

References

Struble, C., Howard, S., Rebl, J., 2014. Comparison of ocular tissue weights (volumes) and tissue collection techniques in commonly used preclinical animal species. Acta Ophthalmol. 92, 0.