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EXPRESSION OF CYCLOOXYGENASE ENZYMES IN THE EYE OF DOGS

A. M. HARITOVA[1], S. ZH. KRASTEV[2], R. R. SANTOS[3] & J. FINK-GREMMELS[3]

[1]Department of Pharmacology, Physiology of Animals and Physiological Chemistry, [2]Department of Veterinary Surgery, Faculty of Veterinary Medicine, Stara Zagora, Bulgaria; [3] Division of Veterinary Pharmacology, Pharmacotherapy and Toxicology, Institute for Risk Assessment Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

Summary

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The aim of the present study was to measure mRNA levels of cyclooxygenase-1 (COX1), cyclooxygenase-2 (COX2) and nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (NFIkBα) in eye tissues of dogs by real time polymerase chain reaction (RT-PCR). These factors are involved in the maintenance of homeostasis and in inflammation. Additionally, they are target for anti-inflammatory drugs. Six dogs from different breeds and without ocular diseases were included in the study. COX1, COX2 and NFIkBα mRNA were found in the conjunctiva, the cornea and the retina. Expression of mRNA levels of COX1, COX2 and NFIkBα suggest a constitutive expression and indicate a role in canine ocular homeostasis. These data can be used in the light of proper management of inflammation in eye tissues as well as anti-inflammatory drug application.

Key words: COX enzymes, dog, eye, NFIkBα

INTRODUCTION

The inflammatory process in the eye involves activation of two isoenzymes: cyclooxygenase-1 (COX1) and cyclooxygenase-2 (COX2). While COX1 is constitutively expressed in most tissues, including the eye and plays a role in keeping its integrity, by contrast, COX2 is rapidly inducible and tightly regulated (Kim *et al.*, 2010). These enzymes are involved in prostaglandin production. It is aknowledged that apart from COX isoenzymes, a transcription factor, called nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (NFIkB), plays an important role in regulation of immune and inflammatory responses in the eye (Kimura *et al.*, 2008). It is involved in the regulation of nuclear factor kappa B (NFkB) activation, which controls inflammatory process through expression of a wide variety of genes that encode cytokines, chemokines, the inducible nitric-oxide synthase (iNOS) isoform and COX2 (Oeckinghaus & Ghosh, 2009; Srivastava & Ramana, 2009). The inflammatory response in the eye results in alteration of vascular permeability, retinal and corneal angiogenesis, and disruption of the blood-ocular barrier. Therefore, comprehension of the level of expression of factors

involved in inflammation, such as COX enzymes and NFIkBα, is necessary. It will allow the regulation of their expression and function and contribute to understanding of the pharmacodynamic response to therapy with antibacterial and anti-inflammatory drugs as well as predicting the efficacy of applied drug combinations in treatment of eye diseases. Regardless of existing data about the expression and modulation of COX1 and COX2 in ciliary body of human eye, COX2 in canine ciliary body and NFkB in Sprague-Dawley rats, the expression pattern of these factors in eye compartments is so far not well characterised and data about NFIkBα are virtually lacking (Maihofner *et al.*, 2001; Zhang *et al.*, 2006; Paglia *et al.*, 2009). Moreover, published literature is controversial and interspecies differences have been identified (Radi & Render, 2008).

The current study aimed to evaluate mRNA levels of expression of NFIkBα and COX enzymes in the conjunctiva, the cornea and the retina of dogs because of their role in ocular homeostasis and inflammation and their role as targets for anti-inflammatory drugs.

MATERIALS AND METHODS

Animals

The subjects of the study were dogs of different breeds (Deutsch Kurzhaar, Staffordshire terrier, Pinscher, Collie, Rottweiler and mixed-breed from a dog shelter) presented to the Small Animal Clinic at the Faculty of Veterinary Medicine, Trakia University. The collection of post-mortem tissue samples was approved by the Ethical Committee of Faculty of Veterinary Medicine, Trakia University and was performed after owner's consent. The selected animals had no ocular diseases and were euthanised due to various diseases or car accidents. Euthanasia was performed with thiopental sodium (Thiopental, flacon of 1 g, Sandoz), administered intravenously as 5% solution prepared *ex tempore*. Tissue samples from the cornea, the conjunctiva and the retina were quickly collected from both eyes and snap frozen in liquid nitrogen. They were stored at –70 ^{o}C till analysis.

RT-PCR analysis

Total RNA was isolated using a combination of TRIzol Reagent (Invitrogen Life Science Technologies) and Promega method (SV Total RNA Isolation System, Promega). Total RNA concentration was measured with the RiboGreen RNA Quantitation Kit (R-11490, Molecular Probes) and a fluorescence microplate reader (FLUOstar Optima F, BMG Labtech), using fluorescein excitation (480 nm) and emission (520 nm) wavelengths. First-strand cDNA was synthesised using the iScript™cDNA Synthesis Kit (Cat. No. 170-8891, Bio-Rad, Herculus, CA) according to the manufacturer's instructions. Specific primers for COX1, COX2, NFIkBα, HPRT and RPS5 were used (Table 1). They were taken from the literature (Brinkhof *et al.*, 2006; Gropp *et al.*, 2006; Kowalewski *et al.*, 2006; Fang *et al.*, 2010). The efficiency (100±5%) of the primers (Table 1), the optimal annealing temperatures were obtained by real-time PCR analysis of a dilution series of cDNA samples. Sybr Green technology was applied for the real-time PCR analysis by using iQ™ Sybr Green Supermix (Cat. No. 170-8885, Bio-Rad, Hercules, CA), conducted according to the instructions of the manufacturer. The reaction was performed with an

Table 1. Specific gene primers used in the study

Gene	NCBI accession number	Forward primer 5′→ 3′	Reverse primer 5′→ 3′	Ta (°C)
COX1	AF_535139	GCAAAGCCGCATAACCAT	CTGCTTTGGGGGTATCTC	61.3
COX2	AY_044905	ACAGGAGAGAAGGAAATGGC	GGATTGAGGCAGTGTTGATG	61.3
NFIkBα	XM_537413.2	CCAGCACCTCTACTCCATCC	CATCAGCACCCAAAGACACC	58.7
HPRT	AY_283372	AGCTTGCTGGTGAAAAGGAC	TTATAGTCAAGGGCATATCC	56.0
RPS5	XM_533568	TCACTGGTGAGAACCCCCT	CCTGATTCACACGGCGTAG	62.5

cyclooxygenase-1 (COX1); cyclooxygenase-2 (COX2); nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IkBα); hypoxanthine phosphoribosyltransferase (HPRT); ribosomal protein S5 (RPS5); NCBI – the National Centre for Biotechnology Information; Ta – optimal annealing temperature.

iCycler iQ PCR system (Bio-Rad, Hercules, CA) and analysed using MyiQ System Software, Version 1.0.410 (Bio-Rad Laboratories Inc.). Each reaction went through a PCR cycle with a denaturation step at 95 °C for 20 s, an annealing step specific for each set of primers for 30 s and an elongation step at 72 °C for 30 s. After 35 cycles a melting curve was obtained by increasing the temperature with 0.5 °C every 10 s from 65 °C to 95 °C demonstrating the formation of only one product.

Gene expression data were presented using the algorithms outlined by Vandesompele *et al.* (2002) and the geNorm software (http://medgen.ugent.be/~jvdesomp/genorm). Relative mRNA expression levels of genes of interest were normalised against hypoxanthine phosphoribosyltransferase (HPRT) and ribosomal protein S5 (RPS5). Among the tested reference genes, HPRT and RPS5 were the most stably expressed gene products in the tested tissues and they were used in further analysis. Efficiencies for each reaction were estimated by LinRegPCR 7.0 software.

Statistical analysis

Results were presented as mean±SD for six animals (Statistica 6.1, Statistica for Windows, StatSoft, Inc., USA, 1984-2002). Mean values of data from both eyes were considered for analysis. Statistical analysis was performed with Friedman-ANOVA test and Wilcoxon *post hoc* test at level of significance $P<0.05$.

RESULTS

COX1, COX2 and NFIkBα mRNA levels were detected in all investigated tissues (Fig. 1). Expression levels of COX1, COX2 and NFIkBα mRNA were similar in the cornea and in the retina. COX1 and NFIkBα mRNA levels were significantly higher from COX2 mRNA in the conjunc-

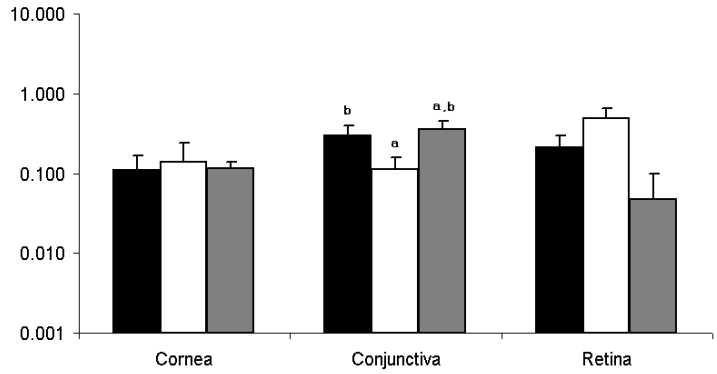

Fig. 1. Mean relative levels of mRNA expression of COX1 (■), COX2 (□) enzymes and NFIkBα (▩) in eye compartments of dog (mean ± SD; n=6). a – statistically significant differences between the levels of expression of COX1 mRNA and other two genes of interest; b – statistically significant differences between the levels of expression of COX2 mRNA and other two genes of interest.

tiva. NFIkBα mRNA was expressed at higher levels ($P<0.05$) than COX1 mRNA in the same tissue.

DISCUSSION

The role of COX enzymes in the inflammation of the eye and their involvement in the pharmacodynamic responses to the non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids commonly used in treatment of inflammation, including after topical administration are reported (Giuliano, 2004). Their levels of expression in the eye tissues however are not consistently described. It is difficult to extrapolate data from one species to another due to species differences, the use of cell lines or different methods for determination of expression pattern. Our study describes for the first time mRNA levels of expression of genes involved in inflammation and in tissue homeostasis in canine conjunctiva, cornea and retina.

Attention has been paid to conjunctiva and cornea as part of physiological barriers of the eye and as barriers which topically administered drugs must pass in order to reach the underlying ocular tissues (Kompella *et al.*, 1993). In rabbits, COX1 is normally expressed throughout all layers of the cornea similarly to what we observed in the dog (Amico *et al.*, 2004). Expression of COX1 mRNA in the cornea can be discussed in the light of its function for maintaining homeostasis and cytoprotection in the eye. Among the factors, involved in inflammation, mRNA expression levels of COX2 have been studied in more details than COX1. This could be explained by the application of NSAIDs in ophthalmology, which are mainly COX2 inhibitors. mRNA levels of the inducible enzyme COX2 have been found in the cornea of the dog. COX2 mRNA has been also found in mouse cornea (Biswas *et al.*, 2005). So far, one immunohistochemical study has published results about COX2 protein localisation in dogs with normal and glaucomatous eyes (Marshall *et al.*, 2004). Minimal staining for COX2 protein has been found in the

ciliary body epithelium but it was absent in cornea of healthy dogs (Marshall *et al.*, 2004; Sellers *et al.*, 2004). These results are not controversial to our data for expression of mRNA levels of this enzyme in healthy eyes. These levels could be easily induced in pathological conditions and translated into COX2 protein.

COX1 mRNA and COX2 mRNA are found in the conjunctiva of rats, rabbits as well as of dogs (Oka *et al.*, 2004; Biswas *et al.*, 2005; Cruz *et al.*, 2008). The levels of COX1 mRNA in the conjunctiva of dogs were higher than the expression of COX2 mRNA which can be related to the role of the former enzyme in maintaining the integrity of this tissue.

Different cellular localisations of COX1 and COX2 have been found by immunohistochemistry in the retina of rats, mice and humans (Ju *et al.*, 2002). Detected expression levels of COX1 mRNA in the dog retina can be characterised as constitutive. COX2 has been detected in human retinal pigment epithelial cells by RT-PCR and it is the predominant COX isoform in these cells as observed in our study in dogs (Chin *et al.*, 2001; Hooks *et al.*, 2006). Normal COX2 expression in the retina, determined by immunohistochemistry, varies among species and is detectable in the retina of newborn pigs, but not in the adult rat and monkey (Degi *et al.*, 2001). These species variations can be a reason for differences in inflammatory response in the eye. Considering the expression pattern of COX2 in the tissues of the dogs' eye, clinical benefits from NSAIDs and corticosteroids can be expected. Application of these drugs with antibiotics can be recommended as a suitable treatment of ocular inflammation.

In addition to COX isoforms, a transcription factor that plays an important role in transcriptional regulation of inflammatory proteins NFIkBα was investigated in conjunctiva, cornea and retina of dogs. NFkB activation, regulated by the inhibitory subunits such as NFIkBα, has been observed in almost all eye tissues including lens, retina, cornea and iris under various oxidative stress conditions, but their levels of expression have not been described so far (Srivastava & Ramana, 2009). Expression levels of NFIkBα mRNA were found in the conjunctiva, the cornea and the retina of dogs. These results indicate that this transcription factor plays a role in inflammation of the studied canine tissues, especially in the conjunctiva where NFIkBα mRNA was expressed at significantly higher levels than COX1 and COX2 mRNAs. These data allow us suggesting that co-administration of anti-inflammatory drugs for treatment of conjunctivitis could be advantageous over single use of antibiotics. Interaction of antibacterial drugs and NSAIDs during inflammation of the eye is more complex and not only of pharmacodynamic significance, but it plays a role in the disposition of drug molecules and inflammatory factors. Since functional studies have not been performed in the current research, additional investigations are necessary to clarify the pharmacokinetic-pharmacodynamic interactions between these drugs.

In conclusion, our study described for first time mRNA levels of expression of important factors of inflammation in canine eye tissues. Expression of COX enzymes and NFIkBα advocates topical application of anti-inflammatory agents. This field of drug application in veterinary ophthalmology is not well studied yet. Therefore, further investigations are needed with regard to proper decision making for use of drugs and drug combinations.

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Correspondence:

Assoc. Prof. Anelia M. Haritova
Department of Pharmacology, Physiology of Animals and Physiological Chemistry,
Faculty of Veterinary Medicine,
6000 Stara Zagora, Bulgaria
tel: 00359 42 699 696
e-mail: haritova@uni-sz.bg