Effects of Cytochrome P450 Inducers on the Gene Expression of Ocular Xenobiotic Metabolizing Enzymes in Rats

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In our current study, we investigated the expression profiles of the cytochromes P450 (CYPs) and transporters of the ocular tissues in Sprague-Dawley (SD) rats. Extensive expression of CYP1A1 in the cornea and CYP2E1 in the iris was observed whereas the expression of CYP2B1 and transporters was mostly ubiquitous throughout the ocular tissues. To further understand the regulation of these genes in ocular tissues, we investigated the effects of CYP inducers on the expression of the CYP and other xenobiotic-metabolizing enzyme genes in the cornea and lens. The administration of β-naphthoflavone (BNF), an agonist for aryl hydrocarbon receptor (AhR), induced CYP1A1 gene expression in the cornea, lens and liver of the rats, although the levels of induction were greatest in the liver. An AhR-sensitive UDP-glucuronosyl transferase (UGT) 1A6 gene was also induced in the cornea and the lens by BNF. Phenobarbital (PB) is a known inducer of the CYP2B genes, the expression of which is mediated by constitutive activated receptor (CAR), but did not induce CYP2B1 in the cornea or lens. This insensitivity to PB may be due to the lack of CAR expression in the ocular tissues as revealed in our present study. Pregnenolone-16α-carbonitrile (PCN) is known to induce CYP3A gene expression in the liver via the activation of pregnane X receptor (PXR). However, although PCN was found to induce the CYP3A1 gene in the rat cornea and liver, it failed to do so in the lens. In addition, another of the PXR-mediated genes, multidrug resistance-associated protein 3 (Mrp3), was not induced by PCN in either ocular region. Since the expression of the PXR gene was not detected in the rat ocular tissues, an unknown mechanism for the inducible regulation of CYP3A1 gene expression by PCN in the cornea is suggested.

Key words —— aryl hydrocarbon receptor, cytochrome P450, nuclear receptor, ocular, UDP-glucuronosyl transferase

INTRODUCTION

The environmental and occupational exposure to toxic chemicals, gases, and vapors, in addition to side effects resulting from therapeutic drugs, frequently results in structural and functional alterations in the eyes and central visual system.[1] Xenobiotic biotransformation catalyzed by phase I [mainly cytochrome P450s (CYPs)] and/or phase II (mainly conjugation) enzymes often enhances the toxicity of such chemicals. For instance, the metabolic transformation of acetaminophen to *N*-acetyl-*p*-benzoquinone imine by CYP1A enzymes is a prerequisite for acetaminophen-induced cataract formation in mice.[2] Most of the CYPs and conjugation enzymes are located in the liver, but some are expressed in extrahepatic tissues, such as the small intestine, lungs and kidneys. Several studies have demonstrated in the livers and other organs, the coordinated regulation of xenobiotic metabolizing enzymes such as the CYPs, phase II conjugation enzymes and transporters has been well established.[3] Nuclear receptors comprise a superfamily of ligand-activated transcriptional factors that are involved in diverse physiological, developmental and metabolic processes. Aryl hydrocarbon receptor (AhR), pregnane X receptor (PXR) and constitutive active receptor (CAR) are the members of the 'xenobiotic-sensing' nuclear receptors.[4,5] Several studies have demonstrated the presence of CYP enzymes and other metabolizing enzymes such as aldehyde oxidase in the ocular tissues, especially in the ciliary body of bovine and other experimental animals.[6–8] Previously, we have reported the characterization of

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CYP expression in rat ocular tissues and also reported the age- and gender-related expression patterns of the CYPs and phase II conjugation enzymes.[9, 10] We have further reported the changes in the gene expression of drug metabolizing enzymes in Shumiya cataract rats (SCR) and selenite-induced cataract rats.[11] To investigate the regulation of xenobiotic metabolizing enzymes by these nuclear receptors in rat ocular tissues in our present study, we treated male rats with several classic CYP inducers, *i.e.* phenobarbital (PB), β-naphthoflavone (BNF), and pregnenolone-16α-carbonitrile (PCN), and then determined the gene expression levels of CYPs and other xenobiotic metabolizing enzymes.

MATERIALS AND METHODS

Materials —— PB, BNF and PCN were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Primers for polymerase chain reaction (PCR) were designed and purchased from Sigma Genomics (Sapporo, Japan). Reagents for reverse transcription (RT)-PCR were obtained from Stratagene (La Jolla, CA, U.S.A.). Reagents for RNA preparation were from WAKO Chemicals (Tokyo, Japan).

Animals and Treatment —— Male Sprague-Dawley (SD) rats (Sankyo Laboratories, Shizuoka, Japan) were obtained and supplied with ordinary diet and tap water *ad libitum*. Animals were acclimatized for 1 week prior to each treatment and each group consisted of at least three rats. With the exception of PB, which was dissolved in saline, all other compounds were dissolved in corn-oil. Each of the groups received the following regimens intraperitoneal (*i.p.*) once daily between 9:00 and 11:00 a.m. for four days: vehicle control, BNF (100 mg/kg), PB (100 mg/kg) in saline, and PCN (50 mg/kg), respectively. All animals were killed 24 hr after the last treatment. Their livers were dissected and frozen immediately and then stored at −80°C until needed for RNA extraction. The enucleated eyes were immediately used without freezing for dissection and RNA extraction.

RNA Isolation and RT-PCR —— Freshly enucleated rat eyes were briefly immersed in saline, and the lens, cornea, and other tissues were excised. Total RNA was then isolated from the tissues using Isogen reagent according to the manufacturer's protocol. RT using 10 μg total RNA and amplification reactions were performed using a standard protocol. PCR was performed for 30 (liver) or 35 (ocular) cycles in 25 μl of a 1.0 min incubation at 94°C, 1.5 min incubation at 56°C, and 1.5 min incubation at 72°C. Quantitative real-time PCR was performed with an ABI-Prism 7700 thermal cycler using a SYBR green PCR core reagent kit (Applied Biosystems Inc., Warrington, U.K.). Calculations of the initial amounts of mRNA were performed according to the cycle threshold method.[12] The mRNA levels were normalized using the 18S rRNA levels, which had been quantified by real-time PCR. The sequences of the primers used are as follows (forward and reverse, 5′ to 3′); 18S rRNA, TGGTTGCAAAGCTGAAACTTAAAG and AGTCAAATTAAGCCGCAGGC; CYP1A1, TGGGGAGGTTACTGGTTCTG and GTGGACATTGGCATTCTCG; CYP2B1, TCATCGACACTTACCTTCTGC and GTGTATGGCATTTTACTGCGG; CYP2C11, CTGCTGCTGCTGAAACACGTG and GGATGACAGCGATACTATCAC; CYP2E1, GGGTTGTGGGTCTTTCCGT and GCCATGCAGGACCACGAT; CYP3A1, GATGTTGAAATCAATGGTGTGT and TTCAGAGGTATCTGTGTTTCC; UDP-glucuronosyl transferase (UGT) 1A6, TTGCCTTCTTCCTGCTGC and TCTGAAGAGGTAGATGGAAGGC; organic anion transporting polypeptide (Oatp) 1, GAGGACAAGCCAGAGAGGAA and CCATGTGTTCGTTGAGCAGC; Oatp3, AAGCCAACGCAAGACCCAGC and CCGCACAGATCAAAAAGCCG; multidrug resistance protein (Mdr) 1a, TCCACCAGTTCATCGACTCA and GTAAGATGTGAGGCTGTCTGA; Mdr1b, AGTCTATGGCTGGCAGCTTACA and CATGAGTTCTCGTGCCACCAAGTA; multidrug resistance-associated protein (Mrp) 1, ATGCTCAAGTGGACATGTTTCG and TCGATTCTGGACATGGATTCG; Mrp2, CAGGCAATGGTGTGTACGAAA and AGGAGTGCTCGTATCAGAGTCTGA; breast cancer resistance protein (Bcrp), CTGACCCTTCCATCCTCTTC and CGAGGCTGGTGAATGGAGAA; AhR, TTCCCTTATGAGTGCCTTGA and TTCGAAATCGATCCCTAGGT; AhR nuclear translocator (Arnt), CAGGACAGAGATCCGAGGTT and GTCTTGGCTGTAGCCTGAGT; CAR, TTCCATGCCCTGACTTGTGA and GCAGTGCCAGGGCTTCTG; PXR, GTTGGCCTTGTACAACGTGA and CTGTGAAACACCGCAGGTAG.

Statistics —— Data were analyzed by the Student's *t*-test. *P*-values below 0.05 were considered to be statistically significant.

RESULTS

Basal CYP Expression in Rat Ocular Tissues

Previously, we have reported the CYP expression patterns in the lens and extralenticular tissues.[10] In our present report, we have furthered this analysis as shown in Fig. 1. It was difficult to dissect and separate iris and ciliary body, so that the mixture of both regions was analyzed as "iris." The expression levels of CYP1A1 were high in the cornea, and in fact greater than those in the liver. A weak but significant expression of CYP1A1 was also detectable in the remaining ocular tissues. The ubiquitous expression of CYP2B1 was observed throughout the ocular tissues and was found to be particularly high in the cornea, choroid and sclera. No expression of CYP2C11 was detectable, however, in any ocular tissue. The exclusive expression of CYP2E1 was observed in the iris, although weak expression of this gene was observed in the sclera. A low level of expression of CYP3A1 was observed in the iris, lens and choroids.

Expression of Transporter Genes in Rat Ocular Tissues

Transporters are major barriers to chemical toxicants in various tissues.[13–15] As a first step in further investigating the role of the transporter genes in rat ocular tissues, we analyzed the expression of the major transporters, such as Oatp, Mdr, Mpr and Bcrp. Many of these genes were found to be expressed in almost all regions of the ocular tissues in rat (Fig. 2). In addition, these expression levels are almost equivalent to those of the liver. In particular, strong expression of the Oatp3 and Mdr1a genes was observed throughout the ocular tissues and of the Bcrp gene in the iris and the choroid. A lack of expression of the Oatp1, Mdr1 and Mdr2 genes in the rat lens was also characteristic.

Effects of CYP Inducers on the Expression of CYPs and Other Xenobiotic Metabolizing Enzyme Genes

To better understand the regulation of ocular xenobiotic metabolizing enzyme genes, we analyzed the effects of CYP inducers on CYP gene expression in rat ocular tissues, particularly the cornea and lens. These two parts play essential roles for ocular functions and are easy to be quickly isolated without contamination of other ocular parts; this avoids degradation of mRNAs during manipulations. BNF (100 mg/kg per day), PB (100 mg/kg per day) or PCN (50 mg/kg per day) was intraperitoneally administered to male SD rats for four consecutive days and the CYP1A1 (BNF), CYP2B1 (PB) and CYP3A1 (PCN) genes in the cornea, lens and liver were analyzed the next day by quantitative real-time PCR.

BNF is an agonist for aromatic (aryl) hydrocarbon receptor (AhR) and induces CYP1A1 gene expression in various tissues.[16] As shown in Fig. 3A, BNF induced the expression of CYP1A1 by 25-fold in the cornea, 180-fold in the liver, but no induction was observed in the lens. The UGT1A6 gene, which is also known to be regulated by AhR, showed this same induction profile (Fig. 3B). The regulatory proteins AhR and Arnt are known to mediate the induction of CYP1A1 in the liver and other tissues.[16] To elucidate the differences in their in-

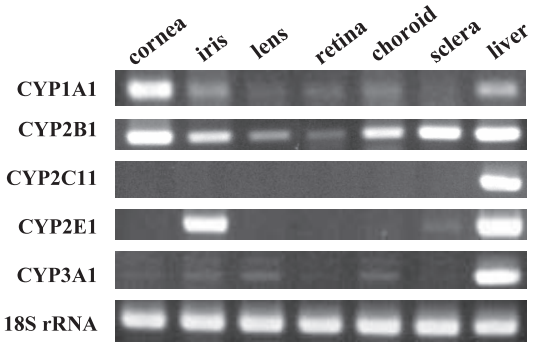

Fig. 1. CYP Gene Expression in the Ocular Tissues of Male SD Rats

Total RNAs were isolated from the tissues of enucleated rat eyes, and subjected to isoform-specific RT-PCR analyses. The product sizes in base pairs are 332 (CYP1A1), 256 (CYP2B1), 248 (CYP2C11), 474 (CYP2E1), 581 (CYP3A1) and 345 (GAPDH), respectively.

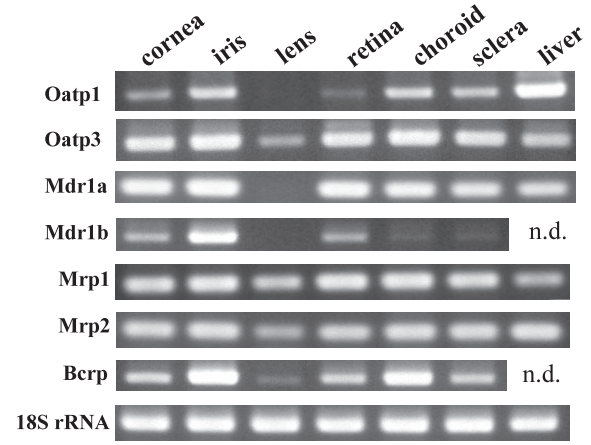

Fig. 2. Expression Profile of Transporter Genes in Rat Ocular Tissues

The expression of each transporter gene indicated was analyzed by RT-PCR using total RNA from rat ocular tissues as described in Fig. 1. n.d.: not determined.

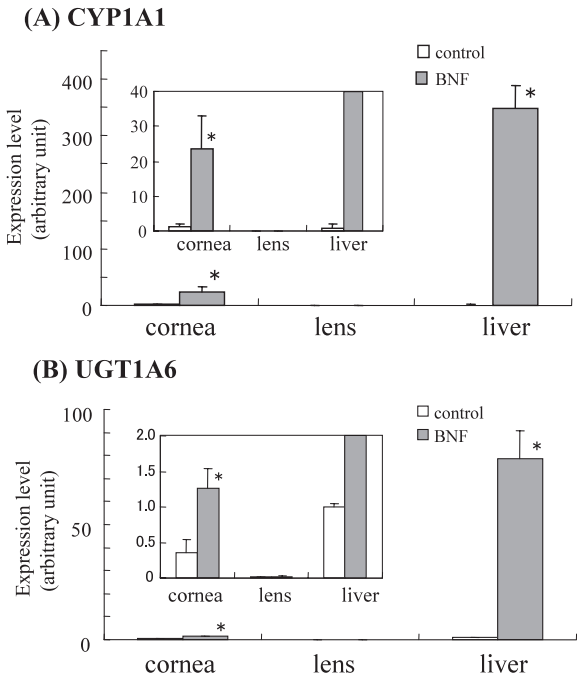

Fig. 3. Effects of BNF on the Expression of the CYP1A1 and UGT1A6 Genes

Rats were administered intraperitoneally with 100 mg/kg BNF for four days, and the expression levels of the CYP1A1 and UGT1A6 genes in the cornea, lens and liver were determined by quantitative real-time PCR. The values shown are expressed as arbitrary units, with the value in the liver set as 1. Data are the means + S.D. from three experiments. *, $p < 0.05$.

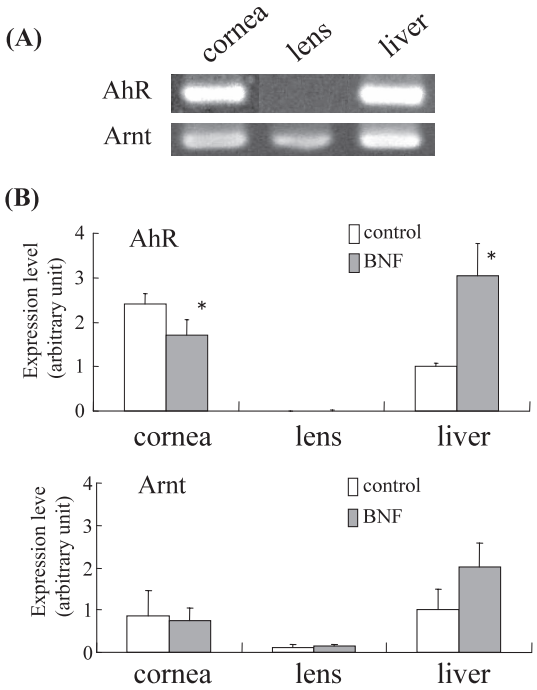

Fig. 4. Effects of BNF on the Expression of the AhR and Arnt Genes

(A) Expression analyses of the AhR and Arnt genes in the cornea, lens and liver of the rat by RT-PCR. (B) Effects of BNF on the expression of AhR and Arnt genes in the cornea, lens and liver. Quantitative real-time PCR analyses were performed as described in Fig. 3. The values are expressed as arbitrary units with the value in the liver set as 1. Data are the means + S.D. from three experiments. *, $p < 0.05$.

duction indexes between the cornea, lens and liver in rat, we measured for the AhR and Arnt transcript levels. As shown in Fig. 4, the expression of the AhR and arnt genes in the liver was induced by 3-fold and 2-fold, respectively, but was unaltered in the cornea. Furthermore, no expression of AhR gene was observed in the lens.

Although the precise mechanism remains unclear, PB is known to initiate the nuclear translocation of CAR and thereby promote subsequent CYP2B gene activation.[17] However, the expression of CYP2B1 in the cornea and the lens was found in our analyses to be unaffected by PB administration, whereas in the liver was induced by 170-fold by PB (Fig. 5A). Furthermore, CAR expression was not detectable in the cornea or lens following PB exposure (Fig. 5B).

It is now well established that PXR is a key regulator of xenobiotic-inducible CYP3A gene expression in the liver.[18] PCN is a ligand for PXR and induces PXR-mediated gene expression in the liver and other tissues. Following PCN administration into rats, an almost 10-fold induction of the CYP3A1 gene was observed in the cornea as well

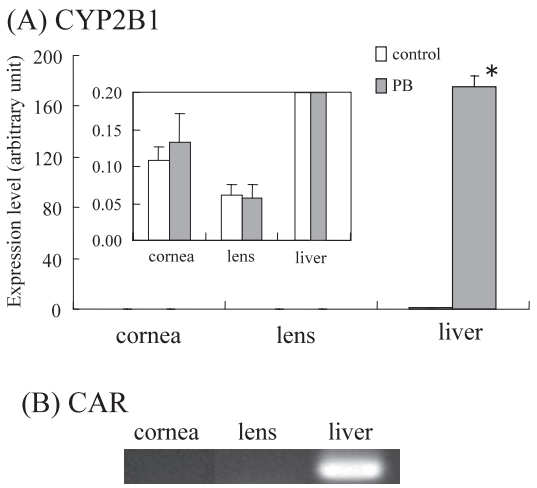

Fig. 5. Effects of PB on the Expression of the CYP2B1 Gene

(A) Rats were administered intraperitoneally with 100 mg/kg PB for four days, and the expression levels of the CYP2B1 gene in the cornea, lens and liver were determined by quantitative real-time PCR. The values are expressed as arbitrary units with the value in the liver set as 1. Data are the means + S.D. from three experiments. *, $p < 0.05$. (B) Expression of the CAR gene in the cornea, lens and liver analyzed by RT-PCR.

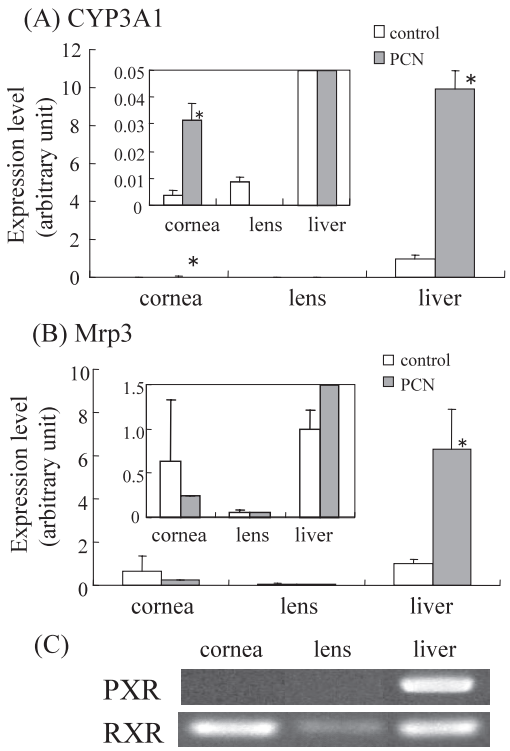

Fig. 6. Effects of PCN on the Expression of the CYP3A1 and Mrp3 Genes

Rats were administered intraperitoneally with 50 mg/kg PCN for four days, and the expression levels of the (A) CYP3A1 and (B) Mrp3 genes in the cornea, lens and liver of the rat were determined by quantitative real-time PCR. The values are expressed as arbitrary units with the value in the liver set as 1. Data are the means + S.D. from three experiments. *, $p < 0.05$. (C) Expression of the PXR and retinoid X receptor (RXR) genes in the cornea, lens and liver analyzed by RT-PCR.

as the liver, but not in the lens where the expression was in fact reduced (Fig. 6A). PXR-target genes also encode key hepatic drug transporter proteins such as Oatp2, Mdr1/P-glycoprotein, Mrp2, and Mrp3. To elucidate the possibility that PXR-independent CYP3A gene induction is initiated by PCN in ocular tissues, we examined the Mrp3 gene expression profile in the rat cornea and lens. As shown in Fig. 6B, no induction of Mrp3 gene expression in the cornea and the lens by PCN was observed, whereas a 7-fold activation of this Mrp3 in the liver was detected following PCN treatment. Analysis of the PXR gene revealed no expression in the cornea or lens, but expression was evident in the liver whereas expression of retinoid X receptor (RXR) is evident in the cornea and lens as well as in the liver (Fig. 6C).

DISCUSSION

In our current study, we have analyzed the expression profiles of the CYP and transporter genes in rat ocular tissues and characterized the effects of CYP inducers on these expression patterns. Region-specific CYP expression, most notably CYP1A1 in the cornea and CYP2E1 in the iris, was observed in our experiments, whereas the expression of transporter genes was ubiquitous in ocular tissues. A lack of expression of several transporter genes such as Mdr1 and Bcrp was seen only in the lens. These data suggest the existence of a region-specific regulation mechanism for xenobiotic metabolizing enzyme genes in rat ocular tissues. To investigate further the regulation of these enzymes in ocular tissues by nuclear receptors, the effects of CYP inducers were examined in the cornea and the lens of SD rats.

BNF, an AhR agonist, was found to induce the CYP1A1 and UGT1A6 genes in the cornea and liver, but not in the lens of SD rats. This lack of induction by BNF in the lens might be due to the lack of AhR expression in this tissue as shown in Fig. 4A. The differences in the induction indexes of the CYP1A1 and UGT1A6 genes between the cornea and the liver might be partly explained by the fact the AhR and Arnt genes are induced only by BNF in the liver (Fig. 4B). Although the concentration of BNF in ocular tissues is not known, differences in this concentration might be another determinant of its induction index.

PB is a known CYP2B inducer but failed to induce CYP2B1 gene expression in the cornea and the lens of SD rats, although a very high induction (180-fold) was observed in the liver (Fig. 5). We also found no CAR expression in either the cornea or lens (Fig. 5B) which might underlie the lack of induction of the CYP2B1 gene in these tissues. Our preliminary experiments also showed the absence of CAR gene expression throughout the ocular tissues whereas expression of RXR, which forms a heterodimer with CAR to activate the PB-responsible enhancer module (PBREM), was observed (Fig. 6C). The regulatory mechanism underlying this suppression of CAR expression in ocular tissues remains to be elucidated.

We detected no PXR gene expression in rat ocular tissues in our current analyses (Fig. 6C). In mammals, the expression of PXR is reported to be abundant in the liver and to a lesser extent in other tissues.[14] It has been suggested also that PXR gene expression is regulated by a hepatic nuclear factor, HNF-4α which may be absent from ocular tissues.[19] This remains to be clarified in future studies. Despite the absence of PXR expression, PCN

was found to induce the expression of CYP3A1 in the cornea but not Mrp3 (Fig. 6A and 6B). We speculate from this that PCN may induce an unknown transcription factor that activates CYP3A genes, or may modulate, directly or indirectly, the degradation of CYP3A1 mRNA in the cornea. The difference between corneal and lens tissue in terms of the induction of CYP3A1 by PCN is another issue to be resolved in the future. Differences in the concentration of PCN between the two regions may be the reason for this difference. Administered PCN would be delivered to the aqueous humor across the ciliary epithelium and iris capillaries. The endothelial cells of the cornea and the epithelial cells of the lens might face the aqueous humor in equal measure. Tears may contain a higher dose of PCN than aqueous humor and may directly interact with corneal epithelial cells to induce CYP3A genes. These possibilities will need to be tested in the future.

Here we reported the effects of CYP-inducers administered intraperitoneally on the regulation of xenobiotic metabolizing enzyme genes, however, many kinds of drugs such as antibiotics or anticataract are administered topically. The effects of eye drugs on the regulation of xenobiotic metabolizing enzyme genes in the ocular tissues should be investigated for the safety use of these drugs.

Many kinds of cells exist in each ocular region, including epithelial and endothelial cells, but in our current experiments we analyzed the gene expression patterns in mixed populations of these cells. Further precise analyses for each subtype of CYP and transporter must be carried out using immunohistochemistry or *in situ* hybridization to better understand the regulation of xenobiotic metabolizing enzyme genes in ocular tissues.

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