Regular Article

Influence of Endogenous Substances on Site-II to Site-I Displacement of Diclofenac Bound to Albumin in the Aqueous Humor of Patients with Cataract

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Diclofenac instillation is useful in preventing intraoperative miosis and macular edema caused by postoperative inflammation in cataract surgery; however, optimum efficacy is not attained when the instilled diclofenac strongly binds to albumin in patients' aqueous humor. Therefore, a method that inhibits diclofenac binding and increases the concentration of its free fraction is needed. We conducted a basic study regarding the effects of inhibitors on the binding of instilled diclofenac to albumin and endogenous substances in aqueous humor. Aqueous humor samples from 16 patients were pooled together for analysis. The free fraction of diclofenac was measured using ultrafiltration methods in various experiments with pooled and mimic aqueous humor. Free fraction of diclofenac, a site II drug, in pooled aqueous humor was 0.363 ± 0.013. The binding of diclofenac in the presence of phenylbutazone (PB), a site I inhibitor, was significantly inhibited (free fraction = 0.496 ± 0.013); however, no significant inhibition by ibuprofen, a site II inhibitor, (free fraction = 0.379 ± 0.004), was observed. The unexpected result was due to free fatty acids (FFAs; palmitic acid (PA)) and L-tryptophan (Trp). The inhibition of diclofenac binding by PB in the mimic aqueous humor containing these endogenous substances revealed significant binding inhibition in the presence of PA and Trp. Diclofenac is strongly rebound from site II to site I in the presence of FFAs and Trp in the aqueous humor because FFAs and Trp induce a conformational change in albumin. Therefore, PB significantly inhibits the binding of diclofenac to albumin.

Key words diclofenac ophthalmic solution, albumin, conformational change, aqueous humor, fatty acid

INTRODUCTION

Diclofenac instillation is widely used in cataract surgery to prevent intraoperative miosis[1] and macular edema caused by postoperative inflammation.[2,3] However, instilled diclofenac binds to albumin[4] in the aqueous humor.[5] If diclofenac is strongly bound to albumin, its effect cannot be optimally exerted intraocularly, and it is excreted into the venous system through Schlemm's canal. We previously showed that >80% of instilled diclofenac was bound to albumin in the aqueous humor in some patients.[5] Thus, such patients require an effective method of administration to inhibit this binding and increase the free (unbound) concentration of diclofenac. However, to the best of our knowledge, no other studies have explored the albumin binding of instilled diclofenac in the aqueous humor. We have successfully achieved pain relief by increasing the free concentration of diclofenac through the inhibition of the albumin binding of diclofenac by 6-methoxy-2-naphthylacetic acid, the primary active metabolite of nabumetone, and enhancing the analgesic effect of diclofenac after the administration of diclofenac suppositories to patients with rheumatoid arthritis[6] (a method to increase diclofenac migration to target tissues—extravascular inflammation sites—by inhibiting the albumin binding of diclofenac in blood vessels).

In this context, using pooled aqueous humor, we previously studied the inhibition of the albumin binding of diclofenac, a site II drug,[5,6] with site II inhibitors. Since the volume of aqueous humor collected from one patient is insufficient to perform inhibition experiments, various binding-inhibition experiments were performed using pooled aqueous humor from approximately 15 samples. However, site II inhibitors did not significantly inhibit albumin binding to diclofenac in the pooled aqueous humor.[5] In the early stage of this study, we assumed that this result is due to the weak binding of diclofenac to albumin in the pooled aqueous humor. In other words, although some patients show strong diclofenac binding in the aqueous humor, this binding is averaged out and weakened in pooled aqueous humor and site II inhibitors reduced the binding-inhibition effect.[5] However, we hypothesized that the effects of endogenous substances on albumin sites I and II vary greatly as the microenvironment surrounding albumin differs significantly in the aqueous humor, where the concentration and composition of both albumin and endogenous substances differ from those in the blood.[4] This hypothesis was based on previous findings of unique binding-inhibition phenomena, such as the cascade effect, where albumin binding of furosemide—a site I drug—was synergistically inhibited by the presence of uremic toxins and free fatty acids (FFAs) in the blood of patients with chronic kidney failure,[7] as well as remarkable changes in the binding capacity of albumin sites I and II in the special environment in blood immediately before and after hemodialysis[8] and immediately before and during the operation using an artificial heart–lung machine.[9] These changes resulted from the combination of competitive (same site) and allosteric (different site) inhibition. Hence, the unique inhibition phenomena at the binding sites of albumin

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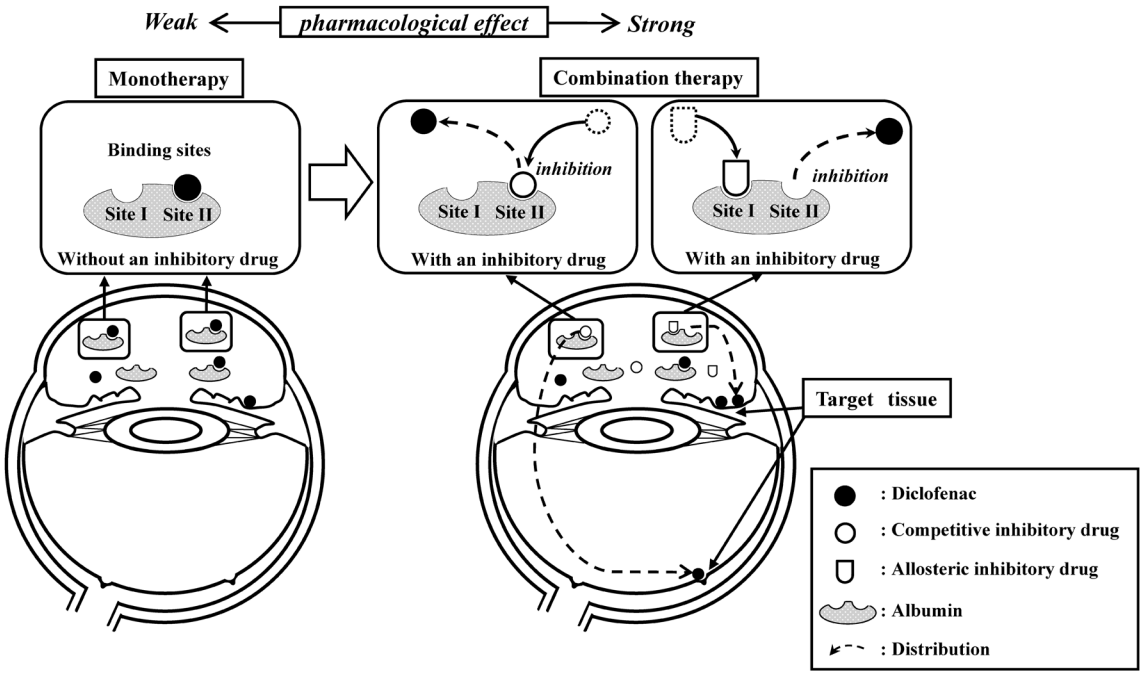

Fig. 1. Intraocular Distribution of Free Diclofenac by an Inhibitory Drug

Monotherapy: binding of diclofenac to albumin in the aqueous humor in the absence of an inhibitory drug. Combination therapy: inhibition of diclofenac binding to albumin or a competitive or an allosteric inhibitory drug in the aqueous humor.

in aqueous humor are not unusual. Thus, we focused not only on the competitive inhibition, which inhibits site II drugs targeting the same site as does diclofenac but also an allosteric inhibition, which inhibits site I drugs targeting a different site (Fig. 1).

Therefore, we expanded our study on the binding inhibitors involved in the albumin binding of diclofenac in the aqueous humor from site II to site I. We also explored the endogenous substances affecting the binding sites of albumin in the aqueous humor and elucidated the mechanism associated with the inhibition of diclofenac binding in the presence of these endogenous substances.

MATERIALS AND METHODS

Materials Each chemical was obtained from the following sources: diclofenac sodium (Novartis Pharma, Tokyo, Japan); 0.1% diclofenac ophthalmic solution (Wakamoto, Tokyo, Japan); ibuprofen (IP) and palmitic acid (PA) (Tokyo Chemical Industry, Tokyo, Japan); urea, D-(+)-glucose, and L-tryptophan (Trp) (Nakalai Tesque Inc., Kyoto, Japan); (+)-ascorbic acid (Wako Pure Chemical Corporation, Osaka, Japan); L-(+)-lactic acid, (MP Biomedicals, Illkirch-Graffenstaden, France); human serum albumin (essentially fatty acid free) and phenylbutazone (PB) (Sigma-Aldrich, MO, U.S.A.). All chemicals were of analytical grade.

Collection and Preparation of Pooled Aqueous Humor Samples from Patients with Cataract This study was approved by the ethics committees of Miyazaki University (Approval Number: 2019-509) and Kyushu University of Health and Welfare (Approval Number: 15-027). The study was performed according to the tenets of the Declaration of Helsinki. Informed consent was obtained from patients at the Ozaki Eye Hospital, and one drop of diclofenac ophthalmic solution was instilled in patients at 3, 2, 1, and 0.5 h before cataract surgery. After the initiation of surgery, the aqueous humor was collected from the anterior chamber of the eye using a 30-gauge needle and cryopreserved at −80 °C until further use. The aqueous humor collected from 16 patients with cataract was mixed to prepare pooled aqueous humor.

Preparation of the Mimic Aqueous Humor The mimic aqueous humor samples were prepared by adding albumin powder to 0.067 M phosphate buffer (pH 7.4), ensuring a concentration of 2.66 μM and diclofenac sodium to a concentration of 0.47 μM, similar to the concentrations in the pooled aqueous humor sample. Further, ascorbic acid, lactic acid, glucose, urea, Trp, and PA (the most abundant among the FFAs) were added as endogenous substances to the mimic aqueous humor sample, varying as per the purpose of each experiment. Final concentrations of ascorbic acid, lactic acid, glucose, urea, Trp, and PA were 1334, 6439, 3907, 4712, 32.4, and 4.8 μM, respectively.

Measurement of the Total Concentration of Diclofenac in the Pooled Aqueous Humor Calibration curve samples were prepared by adding diclofenac to 25 μL of diluted serum (with 2.0 μM albumin prepared in 67 mM phosphate buffer, pH 7.4) to prepare samples of 0.25, 0.5, and 0.75 μM. Subsequently, 200 μL of 3.0 M hydrochloric acid and 2.5 mL of cyclohexane were added to 25 μL of the calibration curve and pooled aqueous humor samples. Next, hexyl 4-hydroxybenzoate [internal standard (IS)] was added, ensuring a concentration of 0.25 μM. This mixture was shaken for 10 min to extract diclofenac and then centrifuged for 10 min at 1650 × $\boldsymbol{g}$ (25 °C). The organic layer (1.5 mL) was collected and dried by suction under reduced pressure. Next, the dried samples were dissolved in the mobile phase and used as samples for ultra-high performance LC (UHPLC).

Measurement of the Free Concentration of Diclofenac in Pooled and Mimic Aqueous Humor In the pooled aqueous humor, calibration curve samples were prepared by adding

diclofenac to 0.067 M phosphate buffer (pH 7.4) to obtain final diclofenac concentrations of 0.1, 0.2, and 0.3 μM. Calibration curve samples (55 μL) and pooled aqueous humor (55 μL) were added to Sartorius plastic ultrafiltration apparatus (Vivacon® 500 10000 MWCO HY; Sartorius, Goettingen, Germany). Alternatively, in the mimic aqueous humor, calibration curve samples were prepared by adding diclofenac to 0.067 M phosphate buffer (pH 7.4) to attain final diclofenac concentrations of 0.1, 0.2, and 0.3 μM. A mimic aqueous humor sample was prepared by adding various endogenous substances corresponding to the experimental purposes. These samples (110–220 μL) were added to Sartorius plastic ultrafiltration apparatus (Vivacon® 500 10000 MWCO HY; Sartorius). All samples were centrifuged for 10 min at 2250 $\times$ ***g***. Thereafter, the concentration of diclofenac (free fraction) in filtrates was measured by UHPLC.

Measurement of the Concentrations of Albumin, Glucose, and Urea in the Pooled Human Aqueous Humor The concentrations of albumin, glucose, and urea in the pooled human aqueous humor samples were measured *via* immune nephelometry using a BiOLiS 24i premium system (Tokyo Boeki Medical System, Tokyo, Japan). The following assay kits were used: albumin, Cias ALB-M (Kanto Chemical Co., Inc., Tokyo, Japan); glucose, CicaLiquid GLU reagents 1 and 2 (Kanto Chemical Co., Inc.); and urea, CicaLiquid–NUN reagents 1 and 2 (Kanto Chemical Co., Inc.). For the measurements, samples were obtained by diluting the pooled aqueous humor 20 times with physiological saline.

Measurement of the Concentrations of FFAs in the Pooled Human Aqueous Humor A total of 10 μL of heneicosanoic acid (2 μg/mL) was added to a vial as the IS and dried by spraying nitrogen gas. Next, 40 μL of aqueous humor was added, and dried by spraying nitrogen gas. Then, to prompt FFA methylation in the first time, 50 μL of methanol, 200 μL of benzene, and 50 μL of trimethylsilyldiazomethane were added and stirred (1500 rpm, 60 min). Subsequently, the mixture was dried by spraying nitrogen gas, and 200 μL of hexane was added for washing. FFA methylation was induced three times, following the same protocol. This hexane was used for the determination of FFA by GC-MS (auto-injector: AOC-20i + s, Shimadzu, Kyoto, Japan, CAS chromatograph: GC-2010 plus, Shimadzu, mass spectrometer: GC-MS-QP2010 Ultra, Shimadzu, column: Rtx-2330, GL Sciences, Tokyo, Japan). The blank was similarly measured using a solution, prepared as described above, without the addition of aqueous humor.

Measurement of the Concentrations of Ascorbic, Lactic, and Amino Acids in Pooled Human Aqueous Humor Ascorbic,[10] lactic,[11] and amino[12] acids were measured using methods suitable for each substance in samples prepared by the addition of physiological saline to the pooled aqueous humor sample.

Conditions of Drug Measurements Using UHPLC and Calculation of the Free Fraction The concentration of diclofenac was determined using a UHPLC system (Shimadzu) comprising an SPD-20A UV/VIS detector, an LC-30AD pump, a SIL-30AC auto-injector, a CBM-20A system controller, and a CTO-10ACvp column oven equipped with an Inert Sustain® C18 column (2 μm; GL Sciences Inc.). UHPLC was performed at a flow rate of 1.0 mL/min at 40 °C. The eluent was monitored at a wavelength of 270 nm. The mobile phase comprised acetonitrile, distilled water, methanol, and acetic acid at a ratio of 108: 91 : 20 : 1 (v/v).

The free fraction of diclofenac was calculated as follows:

$$\text{Free fraction} = \frac{[D_f]}{[D_{total}]} \quad (1)$$

where $[D_f]$ and $[D_{total}]$ are the unbound and total (unbound + bound) drug concentrations, respectively.

Influence of PB and IP on Diclofenac Binding to Albumin Sites in the Pooled Aqueous Humor and Various Types of Mimic Aqueous Humor The following experiments were performed to investigate the effects of PB and IP on the binding site of diclofenac in pooled aqueous humor. Solutions containing no PB and IP were added to the pooled aqueous humor samples (Experiments were performed with 55 μL of the pooled aqueous humor sample and 55–220 μL of the mimic aqueous humor sample). Solutions containing PB and IP (5.0 μM each) were added to the Sartorius plastic ultrafiltration apparatus and centrifuged for 10 min at 2250 $\times$ ***g***. The filtrates were then analyzed by UHPLC. Additionally, when investigating the effects of endogenous substances in the mimic aqueous humor, experiments on the inhibition (displacement) of diclofenac binding by PB (5.0 μM) and IP (5.0 μM) were performed with samples containing ascorbic acid, glucose, urea, lactic acid, Trp, and PA. Concentrations of the endogenous substances and diclofenac were used measured values (refer to "Preparation of the Mimic Aqueous Humor"). These samples were added to Sartorius plastic ultrafiltration apparatus and centrifuged for 10 min at 2250 $\times$ ***g***, with the filtrates analyzed by UHPLC. Since the albumin concentration is 2.66 μM, PB or IP concentration that is approximately twice the albumin concentration is required to bind majority of the PB or IP to site I or II of the albumin. Therefore, PB and IP concentrations were set at 5 μM.

Statistical Analysis ANOVA was used to analyze the differences among three groups, Bonferroni's tests were used to evaluate the significance of differences between two means in these groups.

RESULTS

Effects of PB and IP on Albumin Binding of Diclofenac in the Pooled Aqueous Humor To evaluate whether albumin binding of diclofenac in pooled aqueous humor could be inhibited by inhibitors other than site II inhibitors, we examined the degree of inhibition of diclofenac binding using PB (binding constant: approximately $1.2 \times 10^6\,M^{-1}$),[13] a site I binding inhibitor, in addition to IP (binding constant: $3.3 \times 10^6\,M^{-1}$),[13] a site II binding inhibitor. As a result, the free fraction of diclofenac significantly increased in the presence of PB but not IP (Fig. 2). This result was unexpected, as diclofenac, which was supposed to bind to site II, was inhibited by PB, a site I inhibitor.

Endogenous Substances in the Pooled Aqueous Humor It was considered that various endogenous substances in the aqueous humor affect albumin binding sites. Therefore, the unexpected phenomenon that we observed the inhibition of diclofenac binding by PB might be due to the presence of endogenous substances in the pooled aqueous humor sample. Thus, we selected the endogenous substances in the pooled

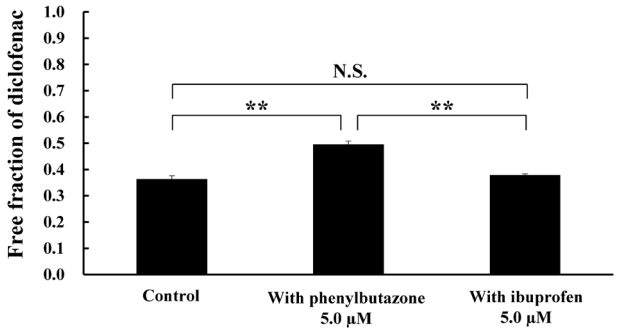

Fig. 2. Influence on the Binding of Diclofenac to Sites of Albumin by Phenylbutazone/Ibuprofen in the Pooled Aqueous Humor

The following concentrations were used: [pooled aqueous humor] (as albumin), 2.66 μM; [diclofenac], 0.47 μM; [phenylbutazone], 5.0 μM; and [ibuprofen], 5.0 μM. Each column is the mean of three experiments ± standard deviation. $^{**}p < 0.01$: significantly different for each group comparison; N.S.: not significantly different for each group comparison.

aqueous humor based on the following perspectives and measured their concentration: 1) the inhibition of diclofenac binding by FFAs,[14] amino acid,[15] and lactic acid[16]; 2) conformational change induced by FFAs[17]; 3) antioxidation by ascorbic acid[18]; 4) degeneration by urea[19]; and 5) glycosylation by glucose[20]; these affect the binding sites of albumin. Concentrations of the endogenous substances are shown in Table 1. The main component of FFAs was long-chain fatty acids; among these, PA was the most abundant. Thus, we used the PA as a representative FFA in this study. The FFA value of 4.8 μM represents the total concentration of all the fatty acids. The concentration of endogenous substances in the aqueous humor was compared with albumin as the reference; the aqueous humor albumin concentration at 2.66 μM was significantly lower, or approximately 1/225, than the blood albumin concentration of approximately 600 μM. Therefore, the concentrations of ascorbic acid, glucose, urea, lactic acid, and Trp (except FFA) were significantly higher than the concentration of albumin in the aqueous humor (2.66 μM) (Table 1), the microenvironment surrounding albumin in the aqueous humor differed significantly from that surrounding blood albumin.

Effects of PB and IP on the Binding of Diclofenac in the Mimic Aqueous Humor Containing Various Endogenous Substances PB (a site I inhibitor) but not IP (a site II inhibitor) inhibited the albumin binding of diclofenac in the pooled aqueous humor. Thus, we examined the endogenous substances that led to this unexpected phenomenon. We added various endogenous substances (shown in Table 1) to the mimic aqueous humor at the same concentrations equal to the pooled aqueous humor sample, and investigated the inhibitory effects of PB and IP to albumin binding of diclofenac. When PB and IP were added to the mimic aqueous humor without any endogenous substances, the inhibitory effects on the albumin binding of diclofenac by IP were greater than that by PB (Fig. 3A). This inhibition pattern was the same when ascorbic acid, glucose, urea, and lactic acid were added to the mimic aqueous humor sample (Figs. 3B–E). However, when PB and IP were added to the mimic aqueous humor containing PA and Trp, the observations were the opposite; PB more significantly inhibited diclofenac binding compared to IP (Figs. 3F, G).

Effects of PA or Trp on the Albumin Binding of Diclofenac in the Mimic Aqueous Humor Although there were differences in the degrees of inhibition of diclofenac albumin binding by PB and IP in the mimic aqueous humor containing PA or Trp, the pattern was similar to that observed in the pooled aqueous humor. This indicates that the concentrations of PA or Trp in the aqueous humor varied across patients. Thus, we investigated the effects of PA and Trp concentrations on the PB- and IP-mediated inhibition of diclofenac binding.

Table 1. Concentration of Drug and Endogenous Substances in the Pooled Aqueous Humor Sample

Drug and endogenous substance	Concentration (μM)
Diclofenac	0.47
Albumin	2.66
Ascorbic acid	1334
Lactic acid	6439
Glucose	3907
Urea	4712
Free fatty acid (mainly long-chain fatty acid)	4.8
L-Tryptophan	32.4

1) Effects of PA on the Albumin Binding of Diclofenac Assuming a range of FFA concentrations in the aqueous humor of each patient, an experiment was performed in which diclofenac binding was inhibited by PB and IP in the mimic aqueous humor samples containing 0, 1.0, 2.0, 3.0, and 5.0 μM PA. The free fraction of diclofenac in the presence of IP was higher than that in the presence of PB in the mimic aqueous humor without PA (Fig. 4A) and containing 1.0 μM PA (Fig. 4B). However, inhibition of diclofenac binding in a mimic aqueous humor containing 2.0 μM PA showed a similar free fraction of diclofenac in the presence of PB and IP (Fig. 4C). When diclofenac binding was inhibited in the mimic aqueous humor sample containing 3.0 μM PA, the free fraction of diclofenac was higher in the presence of PB compared to that in the presence of IP (Fig. 4D). Although the free fraction of diclofenac in the aqueous humor sample containing 5.0 μM PA showed the same pattern as that for 3.0 μM PA (Fig. 4E).

2) Effects of Trp on the Albumin Binding of Diclofenac Assuming a range of concentrations of Trp in the aqueous humor of patients, diclofenac binding was inhibited by PB and IP in the mimic aqueous humor samples containing 0, 1, 2, 3, 5, 10, 20, 30, and 40 μM Trp. In the mimic aqueous humor that did not contain Trp, the free fraction of diclofenac was higher in the presence of IP than that in the presence of PB (Fig. 5A). In addition, although the free fraction of diclofenac in 1, 2, 3, 5, and 10 μM (Figs. 5B–F) of Trp gradually increased in the presence of PB, the inhibition pattern was similar to that shown in Fig. 5A. However, the free fraction of diclofenac in a mimic sample containing 20 μM Trp was similar to that in the presence of PB and IP (Fig. 5G). Moreover, the free fraction of diclofenac in 30 and 40 μM Trp was higher in the presence of PB than that in the presence of IP (Figs. 5H, I).

DISCUSSION

We conducted a basic study to investigate the effective inhibition of albumin binding of diclofenac, a site II drug, in the pooled aqueous humor sample and observed an unexpected phenomenon that was not explained by only the allosteric inhibition by PB. We observed that PB (a site I inhibitor) but not IP (a site II inhibitor) significantly inhibited diclofenac

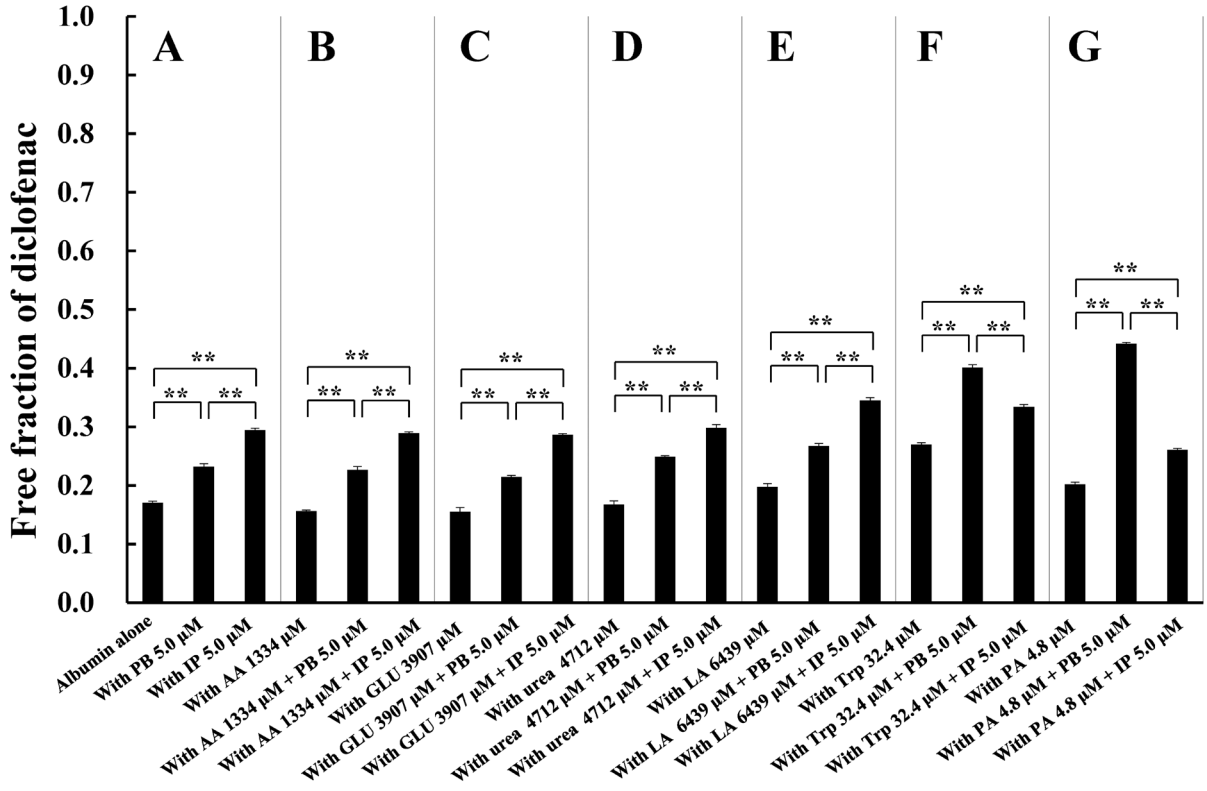

Fig. 3. Influence of Endogenous Substances (Ascorbic Acid, Glucose, Urea, Lactic Acid, L-Tryptophan, and Palmitic Acid) on the Free Fraction of Diclofenac in the Presence of Phenylbutazone/Ibuprofen in Mimic Aqueous Humor

The following concentrations were used: [albumin of mimic aqueous humor], 2.66 μM; [diclofenac], 0.47 μM; [ascorbic acid (AA)], 1,334 μM; [glucose (GLU)], 3,907 μM; [urea], 4,712 μM; [lactic acid (LA)], 6,439 μM; [L-tryptophan (Trp)], 32.4 μM; [palmitic acid (PA)], 4.8 μM; [phenylbutazone (PB)], 5.0 μM; and [ibuprofen (IP)], 5.0 μM. Each column shows the mean of three experiments ± standard deviation. $^{**}p < 0.01$: significantly different for each group comparison.

binding. To investigate this phenomenon, we performed the diclofenac binding-inhibition experiments by IP and PB in the mimic aqueous humor samples containing one of the endogenous substrates at the same concentrations as those measured in the pooled aqueous humor sample. We found that inhibition of diclofenac binding, similar to that noted in the pooled aqueous humor, in the mimic aqueous humor to which PA and Trp had been added. We further clarified that this phenomenon did not occur unless the PA concentration was >1-fold the albumin concentration in the aqueous humor and the Trp concentration was >10-fold the albumin concentration in the aqueous humor.

As shown in Fig. 4, we explored the inhibitory mechanism of PB and IP on the albumin binding of diclofenac in aqueous humor. IP inhibited diclofenac binding more strongly than PB in the absence of PA (Fig. 4A). This inhibition mechanism in the absence of an FFA is shown in Fig. 6 (without FFA system). When diclofenac is bound to site II of albumin (Fig. 6A), the binding of PB to site I causes allosteric inhibition at site II to weaken the binding of diclofenac (Fig. 6B). In contrast, the binding of IP to site II competitively inhibits the binding of diclofenac to the same site (displaced; Fig. 6C). Therefore, it is considered that the inhibitory effect on diclofenac binding was high in the presence of IP and low in the presence of PB.

Albumin binding of FFAs results in a conformational change in albumin, leading to changed binding modes for several drugs and endogenous substances.[17,21,22] Additionally, the primary binding site of FFAs on albumin exists close to site II; FFAs also bind to site II at a higher concentration,[23,24] competitively displacing drugs that bind to this site.[22,25] A reversal phenomenon was also observed in which PB more strongly inhibited the diclofenac binding of albumin compared to IP in the presence of PA concentrations of 3 and 5 μM (Figs. 4D, E), it appears that FFAs bind strongly to site II of albumin, making it difficult for diclofenac to bind to site II. FFAs also induce a conformational change in albumin, causing diclofenac to tend to rebind to site I (Fig. 6D). These findings are supported by those reported in the study by Chamouard *et al.*, where site II is the high-affinity site and site I is the low-affinity site in the albumin binding of diclofenac in the absence of FFAs.[26] Yamasaki *et al.* also reported that in the absence of FFAs, inhibition of the albumin binding of diclofenac by IP leads to a transition of diclofenac from site II to site I.[13] Thus, diclofenac can rebind to site I when the binding to site II has been inhibited. In addition, increased FFA concentrations cause conformational changes in albumin and enhance the binding capacity of drugs to site I.[13,22,27–30] Thus, it is possible that the binding capacity of diclofenac rebound to site I was enhanced. Therefore, as shown in Fig. 6 (with FFA system), site I would be drawn deeper in the presence of FFAs than in the absence of FFAs to indicate the enhanced rebound binding capacity of diclofenac. To the best of our knowledge, no previous report has described that site I binding of PB and site II binding of IP results in a shift from high-affinity to low-affinity sites, similar to that observed with diclofenac, in the presence of various drugs and endogenous substances. Therefore, it is suggested that PB competitively inhibited diclofenac, which was strongly rebound to site I because of PA (Fig. 6E). On the other hand, the inhibition of diclofenac binding by IP was weak because diclofenac had already been

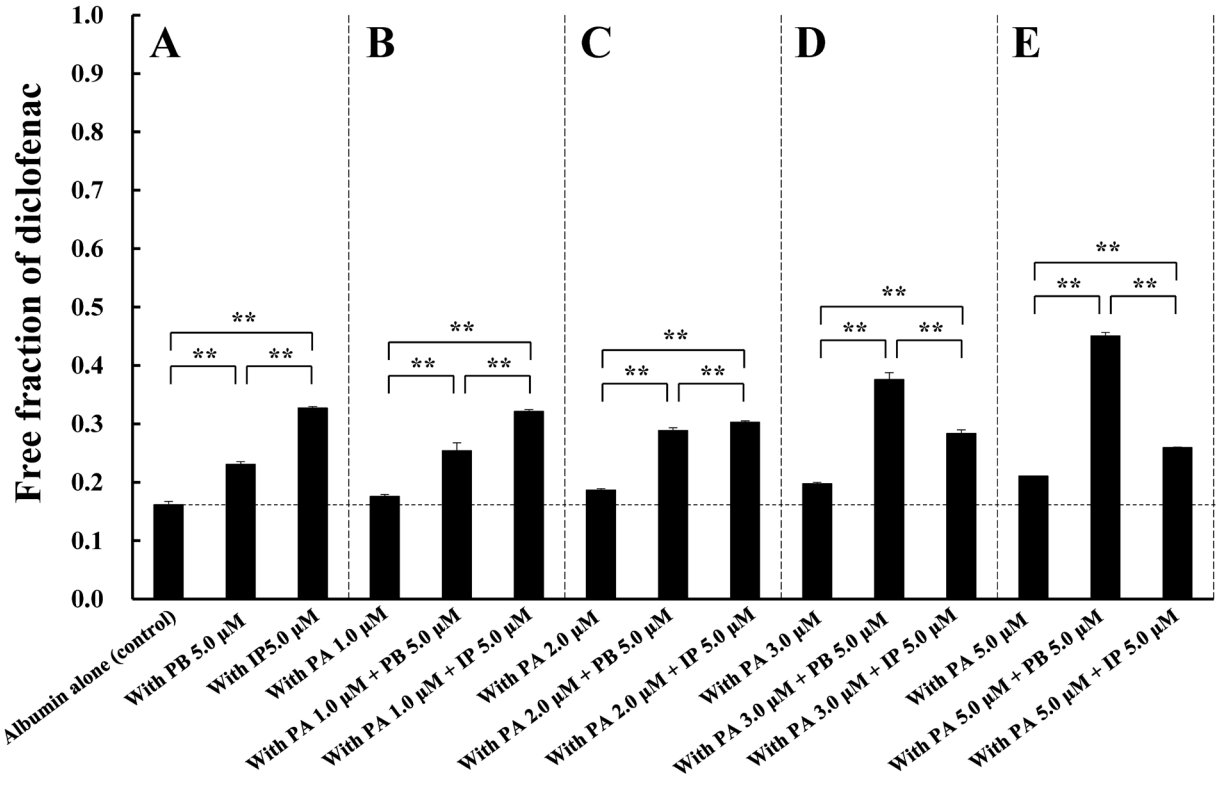

Fig. 4. Degree of Inhibition of Albumin Binding of Diclofenac by Various Concentrations of Palmitic Acid (0, 1.0, 2.0, 3.0, and 5.0 μM) in the Presence of Phenylbutazone/Ibuprofen in Mimic Aqueous Humor

The following concentrations were used: [albumin of mimic aqueous humor], 2.66 μM; [diclofenac], 0.47 μM; [palmitic acid (PA)], 0, 1.0, 2.0, 3.0, and 5.0 μM; [phenylbutazone (PB)], 5.0 μM; and [ibuprofen (IP)], 5.0 μM. Each column is the mean of three experiments ± standard deviation. $^{**}p < 0.01$: significantly different for each group comparison.

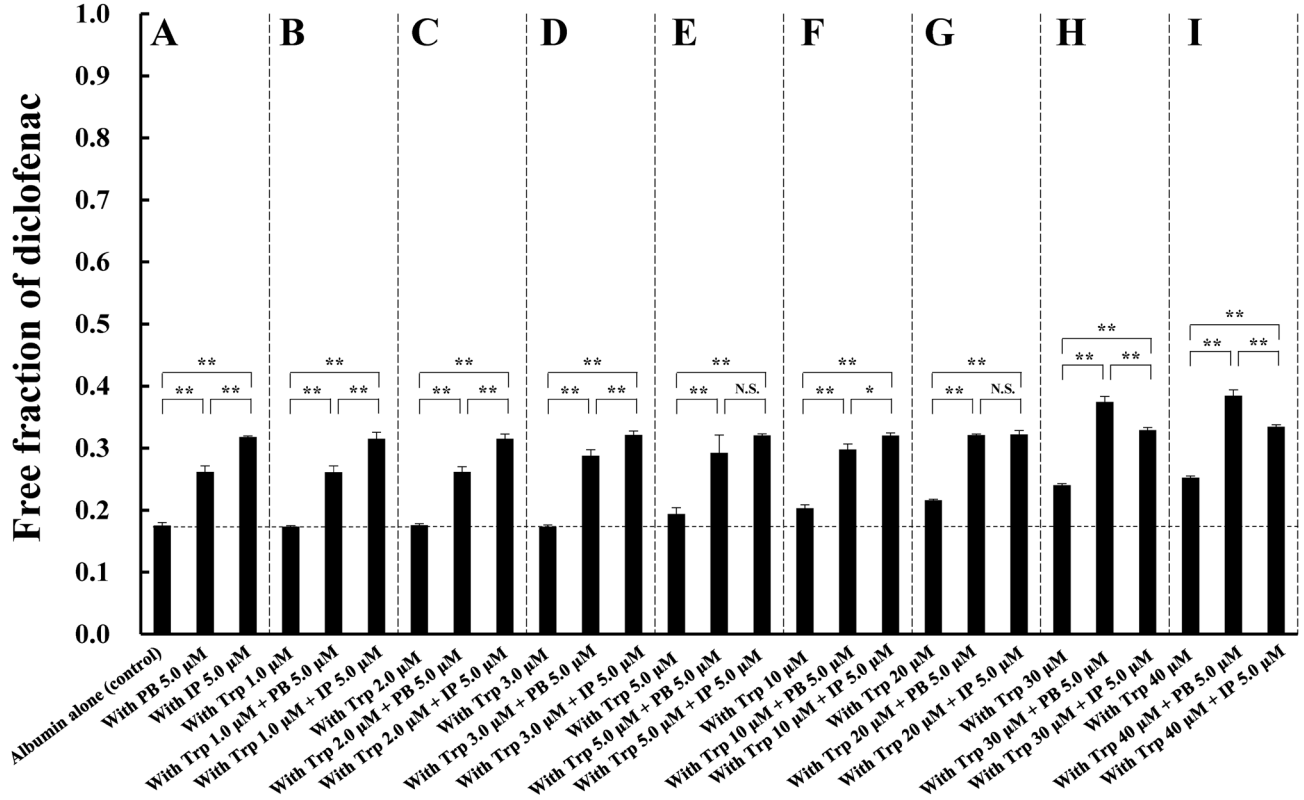

Fig. 5. Degree of Inhibition of Albumin Binding of Diclofenac by Various Concentrations of L-Tryptophan (0, 1.0, 2.0, 3.0, 5.0, 10.0, 20.0, 30.0, and 40.0 μM) in the Presence of Phenylbutazone/Ibuprofen in Mimic Aqueous Humor

The following concentrations were used: [albumin of mimic aqueous humor], 2.66 μM; [diclofenac], 0.47 μM; [L-tryptophan (Trp)], 0, 1.0, 2.0, 3.0, 5.0, 10.0, 20.0, 30.0, and 40.0 μM; [phenylbutazone (PB)], 5.0 μM; and [ibuprofen (IP)], 5.0 μM. Each column is the mean of three experiments ± standard deviation. $^{**}p < 0.01$ and $^{*}p < 0.05$: significantly different for each group comparison; N.S.: not significantly different for each group comparison.

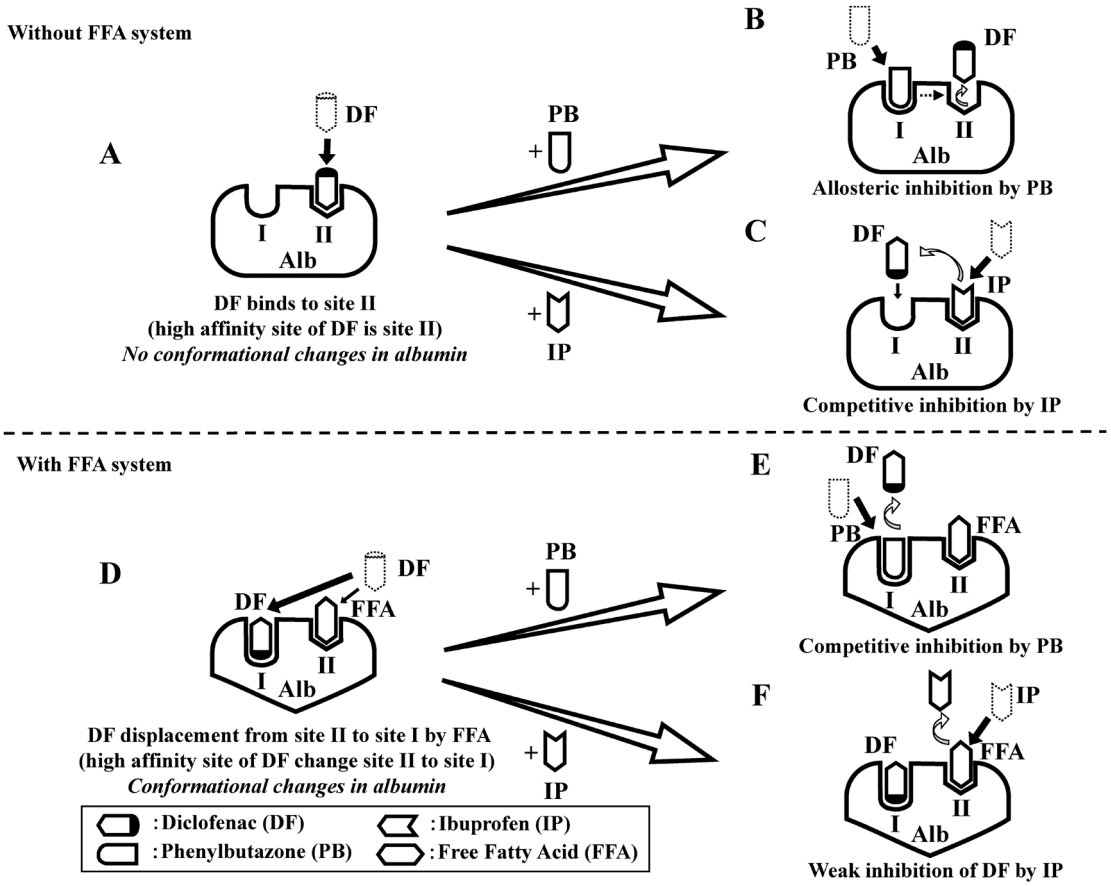

Fig. 6. Proposed Inhibitory Mechanism Models of the Diclofenac–Albumin–FFA Interactions in the Presence or Absence of Phenylbutazone/Ibuprofen

Heavy arrows: drugs binding to its high-affinity site; thin arrows: drugs binding to its low-affinity site; white arched arrows: displacement from site I or site II by inhibition; broken-line arrows: allosteric inhibition.

rebound to site I (Fig. 6F). Thus, the unexpected phenomenon of diclofenac binding-inhibition in the pooled aqueous humor could be explained.

Given that the binding-inhibition of diclofenac by PB in the presence of Trp was greater than that by IP with the increase in Trp concentration (Figs. 5H, I), the inhibition mechanism is assumed to be similar to that of PA. Similar to PA, Trp has also been reported to bind to site II.[31] Additionally, 30 μM of Trp was needed to induce this phenomenon compared to 3 μM of PA, consistent with the significantly smaller binding constant of Trp ($1.6 \times 10^4\,M^{-1}$)[15] than that of PA ($6.2 \times 10^7\,M^{-1}$).[14] In a preliminary study, we investigated whether glutamine, valine, and alanine, which were abundant in the pooled aqueous humor, showed the same effect as Trp on diclofenac binding. We found no rebinding of diclofenac bound to site II to site I in the presence of these three amino acids.

Our previous study demonstrated that some patients in which the albumin binding of diclofenac in the aqueous humor is strong,[5] which likely significantly reduces the pharmaceutical effect of diclofenac in these patients. However, at present, the concentration of FFAs in the aqueous humor of these patients remains unknown during medical examinations; thus, it will also be difficult to determine whether site I or site II binding inhibitors that effectively inhibit albumin binding of diclofenac. Nevertheless, as site I binding inhibitors inhibited significantly albumin binding of diclofenac more than site II binding inhibitors in the pooled aqueous humor prepared by mixing the aqueous humor collected from 16 patients with cataract in this study, it is clear that site I binding inhibitors are effective in many patients. The same phenomenon has been observed in multiple pooled aqueous humors prepared from different patients, thus supporting the effect of site I binding inhibitors.

In summary, the unexpected phenomenon of the inhibition of diclofenac binding by PB (a site I inhibitor) but not IP (a site II inhibitor) in the pooled aqueous humor occurred owing to the inhibition of diclofenac binding to site II (the high-affinity binding site on albumin) with increased FFA concentrations and significant conformational change in albumin, which increased the binding capacity of site I (the low-affinity binding site), resulting in the strong rebinding of diclofenac for site I. Additionally, Trp may similarly affect the binding-inhibition of diclofenac, although more weakly than FFAs. As the albumin binding of diclofenac in the pooled aqueous humor was inhibited more strongly by site I inhibitors than by site II inhibitors, an effective administration method may be established by instilling site I inhibitors before diclofenac instillation.

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Conflict of Interest The authors declare no conflict of interest.

Data Availability The data that support the findings of this study are available from the corresponding author upon request.

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