Reflections on flurbiprofen eyedrops
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CHAPTER 6

CONSTITUTIVE CYCLOOXYGENASE-1 AND INDUCED CYCLOOXYGENASE-2 IN ISOLATED HUMAN IRIS INHIBITED BY S(+) FLURBIPROFEN

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ABSTRACT

The purpose of the present study was to characterize the isoforms of cyclooxygenase (COX) in the human iris before and after stimulation with lipopolysaccharide (LPS) and to determine the selectivity of the nonsteroidal anti-inflammatory drug (NSAID,) S(+) flurbiprofen, for inhibition of COX-1 and COX-2 in homogenates of this tissue. Spotblots were made of extracts of human iris in the absence and presence of LPS plus acetylsalicylic acid (aspirin). After reacting with anti-COX-1 and anti-COX-2 immunoglobulin G, the presence of both immunoreactive COX enzymes was substantiated using an indirect immunoperoxidase method. Authentic COX-1 and COX-2 were used as controls. Using an enzyme immune assay (EIA), the production of prostaglandin E2 (PGE2) was quantified in tissue homogenates of human iris under the same conditions as described above. S(+) flurbiprofen was added to tissue homogenates in order to determine the inhibitory effect on PGE2 production. Half maximal inhibitory concentrations (IC50) of S(+) flurbiprofen for the PGE2 production in the tissue homogenates were determined from concentration inhibition curves.

The selectivity of S(+) flurbiprofen for inhibition of COX-1 was expressed as the ratio of IC50 for COX-2 / COX-1. Spotblots of non-stimulated iris-extracts showed positive staining for COX-1 immunoreactivity (ir) only. After incubation with LPS plus acetylsalicylic acid, positive staining was observed for both COX-1-ir and COX-2-ir. Concentrations of PGE2, released from homogenates of untreated iris varied from 1.5-4 ng/ml and of LPS-stimulated tissue from 10-20 ng/ml of assay mixture.

S(+) flurbiprofen inhibited PGE2 production of untreated tissue homogenates at an IC50 of 8x10^-10 M, whereas in the stimulated tissue IC50 was found to be 3x10^-6 M. The selectivity of S(+) flurbiprofen for inhibition of constitutively present COX-1, relative to the inhibition of induced COX-2, was 3,600.

Our results indicate that specific expression of COX isoforms in normal human iris was substantiated at the protein level by immunoreaction on spotblots. COX-1 represents the constitutively present enzyme, and COX-2 appears after stimulation with LPS. At the functional level, S(+) flurbiprofen possesses a specificity for COX-1 in inhibiting PGE2 production.

INTRODUCTION

The iris is the major site for prostaglandin (PG) formation in the eye. These prostanoids act through prostanoid receptors (1) to regulate smooth muscle contraction, blood-aqueous-barrier penetration and intraocular pressure. PGs are synthesized by a multistep pathway from arachidonic acid, which is either released...
from membrane phospholipids by phospholipase A₂ (phosphatide 2-acylhydrolase, EC 3.1.1.4) (2) or from intracellular triacylglycerols by triacylglycerol acylhydrolase (EC 3.1.1.3) (3). Their formation is catalyzed by the cyclooxygenase (COX) and glutathione-dependent peroxidase activities of PG endoperoxide synthase (EC 1.14.99.1). Two isoforms of COX have been characterized. COX-1 was initially isolated from sheep seminal vesicles (4) and is constitutively expressed in a variety of normal tissues (5) such as stomach, kidney, platelets, and in iris/ciliary body (6). However, it has been reported, that also COX-1 can make an important contribution to inflammatory responses (7). COX-2 is essentially expressed only following cell activation (8); however one exception to the low constitutive expression of COX-2-ir is the brain (9). Expression is time dependent and induced by various mediators of inflammation and bioactive agents such as lipopolysaccharides (10), cytokines (11), tumor necrosis factor (TNF) (11) and platelet activating factor (PAF) (12). COX-2 induction has been described in a variety of cells including migratory cells such as monocytes and macrophages. Also in ocular rabbit models of inflammation, COX-2 activity is found in endothelium (13) and epithelium (14) of the cornea and in iris/ciliary body (15).

The elicited, immediate, production of PGs by the non-inflamed iris in vivo has been described following several experimental manipulations including paracentesis (16), mechanical stimulation (17), alkali burns (18), arachidonic acid administration (19), laser photocoagulation (20), and homogenization in vitro (21). Time related PG production has been observed after induction of uveitis with bovine serum albumin (22) or endotoxin (23). The PG synthesis in the normal iris/ciliary body has been suggested to be due to COX-1 (24) activity, being dependent solely on the availability of arachidonic acid.

Induction of COX-2 may be an explanation for increased prostaglandin formation by the inflamed iris/ciliary body during long term consequences of eye injury, infection or intra-vitreal injection of endotoxin. Microsomes of rabbit iris/ciliary body synthesize increased amounts of cyclooxygenase products after intravitreal injection of endotoxin (25). The induction of COX-2 mRNA in the rabbit iris has been demonstrated within three hours following surgery of the iris (15). In contrast paracentesis failed to induce COX-2 mRNA (15). This suggests that, at least, trauma or resection of the iris is required for the appearance of COX-2 mRNA.

The therapeutic use of non steroidal anti-inflammatory drugs (NSAIDs) is, in general, thought to be based on their ability to inhibit the induced COX-2 activity, being responsible for the signs of inflammation. Inhibition of COX-1 may explain the undesired side-effects, such as gastric and renal toxicity and bleeding disorders. However, in ophthalmology, during cataract extraction and laser treatment of the anterior eye, inhibition of constitutively present COX-1 accounts for the therapeutic effect of NSAIDs as prophylactic treatment to prevent miosis and ocular irritation.
Although data are available on the potency and/or selectivity of several NSAIDs on COX-1 and COX-2 in human blood (26), of bromfenac and nimesulide on the rabbit iris/ciliary body (27), and of flurbiprofen on the bovine iris/ciliary body (24), no study has been performed on human ocular tissue. In the present study, we report the induction of COX-2 by stimulation with LPS in the human iris and dose effect inhibition curves of S(+) flurbiprofen on COX-1 and COX-2 in homogenates of human iris.

MATERIALS AND METHODS

Materials

Human iris tissue with a post mortem time varying from 24-30 hrs was provided by the Cornea Bank Amsterdam. Lipopolysaccharide (LPS), derived from E.Coli 0.111:B4, was purchased from Sigma (St Louis, MO, USA). COX-1 peptide (sc-1752 P), COX-2 peptide (sc-1745 P), goat anti-COX-1 immunoglobulin G (sc-1752), goat anti-COX-2 immunoglobulin G (sc-1745), and horseradish peroxidase-labeled polyclonal anti-goat immunoglobulin G (anti-goat IgGHRP; sc 2020) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, USA). S(+) flurbiprofen was obtained from Duchefa Pharma bv (Haarlem, The Netherlands). Assay kits for enzyme immune analysis of PGE2 and enzyme activity of phospholipase A2 were from Cayman Chemical Co. (Ann Arbor MI, U.S.A.). A solution, containing 3,3'diaminobenzidine (DAB) tetrahydrochloride and H2O2 was purchased from ICN Biomedicals Inc. (Amsterdam, The Netherlands).

Stimulation of Iris Tissue with LPS

For the induction of COX-2 the isolated iris, in a ratio of one tissue per 300 µl PBS, was incubated with 1 µl of a solution of LPS containing 5 mg/ml in DMSO and with 10 µl of acetylsalicylic acid (10 mg/ml in PBS) during 24 hrs at 37ºC.

Immunoprecipitation and Identification of COX-immunoreactivity Isoforms

Human iris tissue was homogenized in sodium dodecyl sulfate sample buffer (125 mM Tris HCl, 4% sodium dodecyl sulfate, 20% glycerol, 1% dithiothreitol, pH6.8), boiled for 5 min and centrifuged for 5 min at 16,000 g. Nitrocellulose membrane was spotblotted with 15 µl of the supernatant and 3 µl of the control COX-1- and COX-2 peptide, treated with blocking buffer (50 mM Tris, 0.15 M NaCl, 0.5% Tween-20, 2% non-fat dry milk, 0.02% sodium azide, pH 10) for 90 min and probed using goat anti-COX-1 or anti-COX-2 at a 1:500 dilution in blocking buffer during 90 min.

After three washings of 5 min in washing buffer (10 mM Tris, 0.15 M NaCl, 0.05% Tween-20, pH 8) the blot was treated with anti-goat IgGHRP at a 1:1000 dilution in blocking buffer during 30 min.
After three washings of 5 min in washing buffer and one washing in buffer containing 10 mM Tris and 0.15 M NaCl staining was developed with a solution, containing DAB tetrahydrochloride and H₂O₂.

Measurement of Prostaglandin E₂ Production

The assay was performed in a modified form according to Van Sorge (24). Human iris tissue was homogenized in Tris HCl 0.05 M, pH 7.4, containing 1 mM phenylmethylsulfonylfluoride (PMSF), in a ratio of 1 iris per 400 µl buffer solution, using a Potter-Elvehjem glass in glass homogeniser in melting ice. For determination of COX activity 100 µl of homogenate was incubated with 25 µl of phosphate buffered saline (PBS) in a 1-ml Eppendorf tube at 37°C during 60 min. The effect of S(+) flurbiprofen was studied by addition of 25 µl of an appropriate dilution of the stock solution in PBS. The enzyme reaction was stopped by heating for 3 min in boiling water. Blanks were prepared by heating the tubes in boiling water, without previous incubation. In the supernatant of centrifugation (30 min at 16,000 g) released PGE₂ was determined. The assay proved linear for the incubation time used and proportional to the amount of tissue-homogenate with, on average, 96% recovery. Inhibition of PGE₂ synthesis by S(+) flurbiprofen was calculated as a percentage of the activity in the presence versus the activity in the absence of the drug. The potency of the drug for COX was assessed by calculating the concentration of the drug causing 50% inhibition (IC₅₀) of the maximal activity. The selectivity of S(+) flurbiprofen for the two COX isoforms is expressed as the ratio of the IC₅₀ for COX-2 versus COX-1. The higher the ratio, the more potently S(+) flurbiprofen inhibits COX-1 relative to COX-2.

Measurement of Phospholipase A₂ Activity

Phospholipase A₂ activity was measured in human iris tissue homogenate, prepared as described above. In a kinetic assay using diheptanoyl-thio-phosphatidylcholine as substrate and 5,5″-dithiobis(2-nitrobenzoic acid) (DTNB) as color reagent the enzyme activity was measured at 414 nm and calculated in µmol/min/ml of homogenate, using the DTNB extinction coefficient of 10.66 mM⁻¹.

RESULTS

The presence of COX-1 immunoreactivity (-ir) and COX-2-ir was substantiated in extracts of human iris, either untreated or following stimulation with LPS, using in vitro incubation experiments. Acetylsalicylate was added together with LPS to eliminate any COX-1 activity in the iris by irreversible binding to the enzyme. To detect the two isoforms of COX in the homogenates of the differently treated tissues, spotblots were treated with anti-COX-1 antibody on the blotting paper, followed by
immunoperoxidase labeling. Representative immunoblots with positive staining for COX-1-ir or COX-2-ir in both untreated and LPS-incubated iris ciliary body tissue are shown in figure 1. Controls of authentic COX-1 peptide but not COX-2 peptide showed positive staining. Treatment with anti-COX-2 produced staining of COX-2-ir only with LPS-incubated tissue and not with untreated iris tissue extract. Positive staining was detected using control COX-2 peptide but not COX-1 peptide.

Figure 1. Detection of cyclooxygenase in human iris extracts. Authentic COX-1 and COX-2 peptide and 15 µl of tissue extract were spotblotted on nitrocellulose membrane. Cyclooxygenase was detected using immunoglobulin G specific for COX-1 or for COX-2. With anti-COX-1, spots were observed in untreated iris, LPS-treated iris and with COX-1 peptide. With anti-COX-2, spots were observed in LPS-treated iris only and with COX-2 peptide.

To quantitate the functional activity of the COX isoenzymes, PGE$_2$ production was measured. The concentration of PGE$_2$ produced by homogenates of normal iris tissue varied from 1.5-4 ng/ml assay mixture/hr and of iris treated with LPS from 10-20 ng/ml of assay mixture/hr. The synthesis of PGE$_2$, produced under these circumstances was inhibited differentially by S(+) flurbiprofen as shown in figure 2. S(+) flurbiprofen inhibits the PGE$_2$ production of untreated iris tissue at an IC$_{50}$ of 8 x 10$^{-10}$ M, whereas the IC$_{50}$ after LPS stimulation was found to be 3 x 10$^{-6}$M.

The phospholipase activity in iris homogenate (43 ± 5 µmol/min/ml) was not significantly influenced in the presence of S(+) flurbiprofen in a final concentration of 10$^{-9}$M.
The iris is the major site for prostaglandin (PG) formation in the eye. These PGs are produced after experimental manipulations but also following induction of uveitis. Whereas PG synthesis in the iris/ciliary body has been ascribed to COX-1 activity, induction of COX-2 may be an explanation for increased prostaglandin formation by the inflamed human iris such as in uveitis. In this study, not only were the involved COX-isofoms detected at the protein level, but also their functional activity was characterized using the NSAID S(+) flurbiprofen. With homogenates of untreated tissue, positive spot blot-staining for COX-1 and substantial production of PGE$_2$ could be demonstrated. Next to the usual positive staining for COX-1-ir additional positive staining for COX-2-ir was achieved after stimulation with LPS, concomitant with greater production of PGE$_2$.

In porcine ciliary body it has been demonstrated that cytochrome P450 dependent $\omega/\omega$-1 hydroxylase activity can inactivate accumulated prostaglandins (28). In polymorphonuclear leukocytes hydrolysis of triacylglycerols by triacylglycerol hydrolase may also provide arachidonate as a source of fatty acid for COX. In our assay we obviously measured the net result of production of PGE$_2$ by COX-1 or COX-2 from endogenous arachidonate mobilized by activation of specific hydrolases.
The selectivity of S(+) flurbiprofen for inhibition of COX-1 with a COX-2/COX-1 ratio of 3,600 is greater than the value of 32 found using human blood as assay system (29). This discrepancy between COX-2 inhibition for S(+) flurbiprofen in ocular tissue and blood was unexpected and not readily explained. Carabaza et al. (30) found a similar great ratio for S(+) flurbiprofen, using ram seminal vesicles as source for COX-1, reporting an IC$_{50}$ of 2X10$^{-9}$ M, as compared to 0.47X10$^{-6}$ M on COX-2, using the whole blood assay. Considering the complexity of the dynamics involved in inhibition of COX-1 and COX-2 by flurbiprofen, it is conceivable that intra-species differences are possibly due to differences in tissue, protein binding and assay protocols (31).

Inhibition of PG production for some NSAIDs, like indomethacin and sulindac, may also be caused by inhibition of phospholipase A$_2$ (32,33), but not of triacylglycerolhydrolases (32). However, flurbiprofen has been shown not to inhibit human synovial or rat peritoneal phospholipase A$_2$ (33) and our results show the same for the enzyme in the iris. Therefore the observed inhibition of PGE$_2$ production in this study might be ascribed solely to inhibitory effects on COX-1 and COX-2 and not to inhibition of phospholipase A$_2$.

The clinical use of flurbiprofen in the inhibition of intraoperative miosis, of disruption of the blood-aqueous barrier and of cystoid macular edema is based on inhibition of the "housekeeping" enzyme, COX-1, present in the iris/ciliary body. Flurbiprofen's activity directed against inflammatory signs generated by induced COX-2, present hours after surgery or laser treatment is minor in degree (15,27). The relative contribution of other eicosanoid or non-eicosanoid pathways in mechanical blood-aqueous barrier disruption has been estimated from experiments using flurbiprofen to be less than 5% (34).

We used the S(+) enantiomer of flurbiprofen, because it is the most potent isomer, like in many other enantiomeric pairs of NSAIDs. In experiments using bovine iris/ciliary body the R(-) enantiomer of flurbiprofen showed 1% of the inhibitory activity of the S(+) enantiomer (24) and in the human whole blood assay 0.3 and 2% on COX-1 and COX-2 respectively (29). With respect to the demands for ballastfree stereo specific drugs providing a reduction in metabolic load to the patient, S(+) flurbiprofen in the racemic mixture can be designated as the active agent and R(-) flurbiprofen as unnecessary ballast. Outside the field of ophthalmology, a report is available on the clinical use of S(+) flurbiprofen in dentistry (35). The NSAID S(+) ibuprofen proved pharmacologically active in the prevention of leakage of protein over the blood aqueous barrier in rabbits (36). There is no indication (37) of a pro-drug property which could be based on metabolic inversion of the less active R(-) isomer into the more active S(+) isomer, as has been observed in other species than man (38). The S(+) flurbiprofen has been formulated into a stereoselective ballast-free eyedrop solution in a concentration of 0.015% (free acid form), which is approximately half the usual
concentration of commercial available specialties containing racemic flurbiprofen in the sodium salt form (39).

After systemic administration, R(-) flurbiprofen shows about one third of the antinociceptive activity of the S(+) form, indicating a central site of action independent of prostaglandin synthesis inhibition and in this respect the use of racemic flurbiprofen may be reserved for analgesic applications (40). In eye conditions resulting from prostaglandin production caused by induced COX-2, such as chronic uveitis, the concentration of S(+) flurbiprofen as selective COX-1 inhibitor in the regular eyedrops may be too low to give sufficient inhibition. The concentration of flurbiprofen in human aqueous humor has been measured as 60 ng/ml two hours after instillation of a single drop (41), which corresponds to 0.25 µM, being much lower than the IC_{50} of 3 µM for COX-2 as measured in our study.

The use of selective topical COX-2 inhibitors, such as meloxicam for the treatment of all forms of ocular inflammation, as advocated by Masferrer and Kulkarni (42) seems not always justified. Only the management of inflammation caused by induced COX-2 activity (15,27) may be reserved for selective COX-2 inhibitors. Meloxicam appears to be suitable for administration as eyedrops (43). The IC_{50} of about 0.5 µM (26,29) for COX-2 as measured in human blood might suffice for intraocular inhibition, depending on the concentration of meloxicam reached in the aqueous humor.

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