Reflections on flurbiprofen eyedrops
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CHAPTER 9

ALTERNATIVE SPLICING OF CYCLOOXYGENASE-1 mRNA IN THE HUMAN IRIS

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ABSTRACT

In homogenates of the human iris, the non-steroidal anti-inflammatory drug (NSAID) S(+)-flurbiprofen has been reported to inhibit cyclooxygenase-1 (COX-1) 70-fold more potently than in human whole blood. We hypothesized that this difference may be due to alternative splicing of COX-1 mRNA in the human iris or in whole blood. In this study, we have identified a similar COX-1 splice variant (COX-1SV) in both tissues with comparable COX-1/COX-1SV expression ratios. Therefore, we conclude that the difference in IC50 values of S(+)-flurbiprofen towards COX-1 in the human iris and human whole blood is not related to differences in the occurrence of spliced COX-1.

INTRODUCTION

Prostaglandin G/H synthase (PGHS), cyclooxygenase (COX) (EC 1.14.99.1) is a membrane bound homodimer of two 70 kDa polypeptides, catalyzing the first two steps in prostaglandin, thromboxane and prostacyclin synthesis (1). Two PGHS isoforms, referred to as COX-1 and COX-2, have now been identified and cloned, and characterized as hemoproteins possessing both cyclooxygenase and hydroperoxidase activity (2-4). Inhibition of the cyclooxygenase activity of PGHS is responsible for the anti-inflammatory activity of non-steroidal anti-inflammatory drugs (NSAIDs). The peroxidase activity of PGHS catalyzes oxidation of a broad range of substrates.

COX-1 is constitutively expressed in various tissues (5), such as kidney, stomach, platelets, and the iris/ciliary body (6), whereas COX-2 is induced after cell activation by various mediators of inflammation and bioactive agents (7). In the human eye, the iris is the major site for producing prostaglandins, which regulate smooth muscle contraction, blood-aqueous-barrier penetration and intra-ocular pressure (8). We have previously shown in human iris homogenates that COX-1 is indeed constitutively expressed whereas COX-2 could be detected after stimulating with lipopolysaccharide (LPS) (9).

Inhibition of the COX activity towards prostaglandin E2 production could be achieved with the NSAID S(+)-flurbiprofen in human iris homogenates. Remarkably, a 3600 fold stronger inhibition of COX-1 over COX-2 was observed (9). On the other hand, in separate experiments with human whole blood, S(+)-flurbiprofen inhibited COX-1 and COX-2 rather similarly with a ratio of 32 (10). We hypothesized that the differential effects of S(+)-flurbiprofen could be due to the presence of an alternative splice variant of the COX-1 enzyme in the iris as compared to whole blood. This seems reasonable since Diaz and coworkers reported that a splice variant of COX-1 is present in human lung fibroblasts (11). Indeed, comparison of the IC50 values (half maximal inhibitory concentrations) for the inhibition of COX-1 by...
S(+) flurbiprofen in human iris tissue and whole blood revealed a 70-fold higher potency towards COX-1 present in human iris tissue (9-10).

MATERIALS AND METHODS

Tissue collection
Tissue of human irides was obtained immediately after surgery from eyes scheduled for enucleation due to melanoma formation. After enucleation, the dissected iris tissue was snap-frozen in liquid nitrogen and stored at -80°C for subsequent RNA isolation. Human whole blood was collected in the presence of ethylenediaminetetraacetic acid (EDTA) and RNA extraction was performed subsequently.

RNA isolation
Total RNA was isolated from iris and whole blood samples using the RNeasy mini kit and QIAamp RNA blood mini kit, respectively (Qiagen) (12). Small amounts of iris tissue (<50 mg) were homogenized in 1 ml of lysis solution using a motorized rotor-stator homogenizer. The homogenates were repeatedly centrifuged to remove tissue debris before proceeding to filtration. Further extraction steps were performed according to the manufacturer's protocol. RNA derived from this procedure was treated with DNAseI (Ambion) to remove contaminating DNA. RNA was quantified by measuring the optical density at 260 nm in triplicate.

Oligonucleotides
Primers were designed as described by Diaz and coworkers (11). In short, a set of primers was designed to specifically span the COX-1SV junction: COX-1SVREV1: 5'-TTC ATG CCA AAC CTC TTG-3'; COX-1SVFOR1: 5'-GGA GAC CAT CAA GAT TGT-3' (Life Technologies). The primers amplified 405 (COX-1 mRNA) or 294 (COX-1SV mRNA) base pairs, respectively.

Reverse transcription
Total RNA (1.0 µg) was reverse transcribed in a volume of 75 µl containing: 1 x Reverse transcription buffer, 10 mM MgCl₂, 1 mM dNTPs, 60 units RNasin ribonuclease inhibitor, 30 units AMV reverse transcriptase and 1.25 µg random hexamer primers (all from Promega). Control reactions, containing neither reverse transcriptase nor RNA were run in parallel. Each subsequent PCR contained 3 µl cDNA template. Therefore, the amount of amplified PCR product was relative to a constant amount of starting RNA. To evaluate RNA quality, we performed a RT-PCR with 18S primers (18Suni1: 5'-CTG AAC GCC ACT TGT CCC TC-3'; 18Suni2: 5'-CTG AAC GCC ACT TGT CCC TC-3'; Eurogentec)
PCR
The COX-1 gene fragment was amplified using the primers COX-1SVFOR and COX-SVREV. All PCRs were performed using Pfu DNA polymerase (Stratagene, La Jolla, CA, U.S.A.). Amplification of the cDNA was performed by 40 cycles of PCR in 50 µl of Pfu DNA polymerase mixture, containing 25 pm of the COX-1SVREV1 and COX-1SVFOR1 primers. The PCR protocol had low annealing and extension temperatures: 4 min. at 94°C, followed by 10 cycles of 30 s at 94°C, 1 min 37°C and 10 min at 50°C, followed by 30 cycles in which the extension time was at first 8 min, but increased by 15 s in each cycle (first cycle: 30 s 94°C, 1 min 55°C and then 8 min 72°C). At the end, DNA production was finished with 10 min at 72°C. The PCR products were analyzed by 1.5% agarose gel electrophoresis stained with ethidium bromide. DNA size markers were from Fermentas (GeneRuler™ 100bp DNA Ladder). Restriction analysis using Ncol verified the sequence of the constructs.

RESULTS AND DISCUSSION

The COX-1 gene is localized on the human chromosome 9q32-q33.3. The COX-1 protein is expressed constitutively in almost all mammalian tissues and is described as a housekeeping enzyme, responsible for cell-to-cell signaling, tissue homeostasis, and cytoprotection.

More recently, Diaz et al have reported the expression of two COX-1 isoforms in human lung fibroblasts. By cloning of the cDNA, they demonstrated that the corresponding mRNA can be spliced in such a way that 111 base pairs are eliminated from exon 9, resulting in a COX-1 isoform that lacks residues 396-432 (11). Since this splicing occurs in-frame, it is expected that it will be translated into an identical protein. However, the alternative splicing results in the elimination of one of the four functionally required N-glycosylation sites at residue 409, providing a possible mechanism for differential regulation of enzymatic activity under physiological or pathological conditions (11,13).

In this respect, we hypothesized that different expression levels of COX-1 and its shorter isoform could account for the difference in IC₅₀ values of S(+) flurbiprofen observed with human iris and human whole blood. Therefore, three human iris samples were analysed for the presence of the two transcripts variants. In addition, human whole blood (corresponding to iris 1) was screened for the presence of both transcripts as well as human blood samples from four healthy volunteers. Total mRNA was extracted from the human iris and blood samples and RT-PCR was performed subsequently. To assess whether the transcripts corresponded to COX-1 or the alternatively spliced COX-1, the cDNA was PCR amplified using a set of primers that specifically spanned the COX-1SV junction: a fragment of 405 base pairs.
should be obtained from transcripts with an intact exon 9, and a fragment of 294 base pairs if exon 9 was lacking. PCR amplification revealed that both transcripts were present in the human iris tissues as well as in the whole blood samples (figure 1 (A)) as judged by the appearance of two major bands of approximately 400 and 300 base pairs, respectively.

The gene fragments were further characterized by restriction analysis using Ncol. Restriction analysis would yield fragments of 285 and 120 base pairs (from the transcript of 405 base pairs corresponding to an intact exon 9) and 174 and 120 base pairs (from the transcript of 294 base pairs corresponding to the spliced variant). Figure 1 (B) illustrates that all fragments were indeed obtained, thereby confirming the correct identity of both transcripts. No significant dissimilarities were observed between the various iris and whole blood samples. Furthermore, the majority of the total COX-1 mRNA is expressed as the fully intact COX-1, whereas only a minor amount is present as the alternatively spliced variant COX-1SV. In addition, relative expression levels of COX-1 and COX-1SV in the human iris and plasma are similar.

Figure 1: (A) Analysis of mRNA from human iris tissue and whole human whole blood. 1 µg of total RNA was reverse-transcribed and amplified by PCR as described in the material and methods. The PCR product were analysed on a 1.5% agarose gel stained with ethidium bromide. Lane 1 - 3: human iris tissue; lane 4 human whole blood (corresponding to iris 1); lane 5 - 7: human whole blood references.
In conclusion, we have detected a splice variant of COX-1 in human irides and whole blood for the first time. Comparison of the expression levels of COX-1 and COX-1SV in the human iris and blood showed no striking differences. These findings indicate that the observed difference in IC$_{50}$ values of S(+) flurbiprofen towards the human iris and human whole blood does not result from an alternatively spliced COX-1 transcript. An alternative explanation may be that the iris COX-1 recognition site of S(+) flurbiprofen is able to adopt a different conformation than the blood enzyme.

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