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Expression of mRNA for cyclooxygenase-1 and cyclooxygenase-2 in human tissues

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The rate-limiting step in the formation of prostanoids is the conversion of arachidonic acid to prostaglandin H_2 by cyclooxygenase, also known as prostaglandin G/H synthase/cyclooxygenase. Two forms of cyclooxygenase have been characterized: a ubiquitously expressed form (COX-1) and a recently described second form (COX-2) inducible by various factors including mitogens, hormones, serum and cytokines. Here we quantitate by the reverse transcriptase-polymerase chain reaction (RT-PCR) the expression of COX-1 and COX-2 mRNA in human tissues including lung, uterus, testis, brain, pancreas, kidney, liver, thymus, prostate, mammary gland, stomach and small intestine. All tissues examined contained both COX-1 and COX-2 mRNA and could be grouped according to the level of COX mRNA expression. The highest levels of COX mRNAs were detected in the prostate where approximately equal levels of COX-1 and COX-2 transcripts were present. In the lung high levels of COX-2 were observed whereas COX-1 mRNA levels were about 2-fold lower. An intermediate level of expression of both COX-1 and COX-2 mRNA was observed in the mammary gland, stomach, small intestine, and uterus. The lowest levels of COX-1 and COX-2 mRNA were observed in the testis, pancreas, kidney, liver, thymus, and brain.

Cyclooxygenase; Prostaglandin synthase; Prostaglandin; Thromboxane

1. INTRODUCTION

The conversion of arachidonic acid to prostaglandin H_2 is catalyzed by cyclooxygenase (COX; prostaglandin H synthase/cyclooxygenase; EC 1.14.99.1) which has recently been shown to exist in at least two isoforms, termed COX-1 and COX-2 [1–9]. The COX-1 enzyme was originally characterized from ovine and bovine vesicular glands (reviewed in [10,11]) and the cDNA and gene encoding a human platelet/erythroleukemia cell COX-1 have been isolated [1,12]. Several groups have observed that a second isoform of COX (termed COX-2) is inducible by a variety of agents including mitogens, cytokines, phorbol esters and serum in murine, chicken, and human cell lines [2,3,5–9,13–17]. Comparison of the recently cloned human COX-2 cDNA to the human COX-1 cDNA revealed that it has 64% overall amino acid sequence identity to human COX-1 [1,2]. Both human COX-1 and COX-2 cDNAs encode enzymatically active cyclooxygenases which are inhibited by nonsteroidal anti-inflammatory drugs such as indomethacin [1,2].

COX-1 protein and mRNA has been demonstrated in many cell lines and in virtually all mammalian tissues (reviewed in [10]). The distribution of COX-2 mRNA appears to be much more restricted with its expression detected in a limited number of cell lines [2,3,5–9,13–17]. Since there is no data available on the tissue distribution of COX-2 in man, we have undertaken an analysis of the tissue distribution of human COX-2 mRNA. We have used both Northern and reverse transcriptase-polymerase chain reaction (RT-PCR [18,19]) techniques since previous studies have demonstrated the difficulty of detecting COX mRNA by Northern analysis [20–24]. Our analyses suggest that both COX-1 and COX-2 mRNAs are normally co-expressed at low but detectable levels in most human tissues.

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2. EXPERIMENTAL

2.1. *Materials*

The human COX-1 (hCOX-1) cDNA was a gift from Dr. C. Funk [1]. The human COX-2 (hCOX-2) cDNA was kindly provided by Dr. B. Kennedy (Merck Frosst Centre for Therapeutic Research, Montreal, Que., Canada) and had been generated by RT-PCR using oligonucleotides based on the published hCOX-2 cDNA sequence [2]. A 1.1 kb cDNA coding for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; [25]) was purchased from Clontech Laboratories (Palo Alto, CA). Human poly(A)$^+$ RNA samples were prepared by Clontech from the following sources: adult female was the source for lung, uterus, liver, stomach and small intestine RNAs; mammary gland RNA was from a pool of 2 nonlactating females; male adult was the source for brain, pancreas, kidney and liver RNA; prostate RNA was a pool of five adult males; thymus RNA was from a three year old male. Single stranded RNA molecular weight markers were purchased from GIBCO-BRL (Burlington, Ont., Canada).

2.2. *Northern blot analysis*

Three micrograms of poly(A)$^+$ RNA isolated from human stomach

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and small intestine (Clontech) were separated in a 0.8% agarose gel containing 6.6% formaldehyde and transferred to a nitrocellulose membrane by overnight capillary blotting in 20 × SSC (1 × SSC is 0.15 M NaCl, 0.015 sodium citrate, pH 7.0). The blots were baked at 80°C for 2 h and then prehybridized for 5 h at 42°C in 4 × SSPE (1× SSPE is 0.15 M NaCl, 0.01 M sodium phosphate, and 0.001 M EDTA, pH 7.4), 8 × Denhardt's solution (1 × Denhardt's is 0.02% Ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone), 100 μg/ml sonicated calf thymus DNA, 50% formamide, and 2% SDS. The blot was hybridized for 18 h at 42°C in fresh prehybridization solution containing 1×10^6 cpm/ml of ^{32}P-labeled DNA probe. The probes were prepared by labeling purified DNA fragments using a random hexamer priming kit ([26]; United States Biochemical, Cleveland, OH). The hCOX-1 and hCOX-2 probes were 1.8 kbp DNA fragments containing only the respective coding sequences but not the 5′ or 3′ untranslated flanking sequences [1,2]. Following hybridization the blots were washed three times for 20 min at 55°C in 0.2 × SSC containing 0.1% SDS and then exposed to Kodak XAR-5 film, with two intensifying screens at −80°C for 1–6 days.

2.3. *RT-PCR quantitation of mRNA*

To identify tissues expressing mRNA for COX-1 and COX-2, exact primers were synthesized based on an analytical RT-PCR procedure developed for human cyclooxygenases [2,20]. For hCOX-1, primers were 5′-TGCCCAGCTCCTGGCCCGCCGCTT-3′ (a 24-mer sense oligonucleotide at position 516) and 5′-GTGCATCAACACAGGCGCCTCTTC-3′ (a 24-mer antisense oligonucleotide at position 819), giving rise to a 303 bp PCR product [1,2]. For hCOX-2, primers were 5′-TTCAAATGAGATTGTGGGAAAATTGCT-3′ (a 27-mer sense, oligonucleotide at position 573) and 5′-AGATCATCTCTGCCTGAGTATCTT-3′ (a 24-mer antisense oligonucleotide at position 878), giving rise to a 305 bp PCR product [1,2]. Primers were also synthesized to amplify the cDNA encoding GAPDH, a constitutively expressed gene, as control. For GAPDH, primers were 5′-CCACCCATGGCAAATTCCATGGCA-3′ (a 24-mer sense oligonucleotide at position 216) and 5′-TCTAGACGGCAGGTCAGGTCCACC-3′ (a 24-mer antisense oligonucleotide at position 809), giving rise to a 593 bp PCR product [25]. Poly(A)$^+$ RNA (1 μg) from each of twelve human tissues was converted to single-stranded cDNA using Moloney murine leukemia virus reverse transcriptase and 2.5 μM of random hexamers according to the manufacturer's protocol (GeneAmp RNA PCR kit, Perkin Elmer Cetus, Norwalk, CT). The cDNA samples were then split into three equal aliquots for amplification by the specific primers for COX-1, COX-2 and GAPDH [2]. PCR reactions were carried out in a buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM deoxynucleotides triphosphates and 0.5 μM primers using a cycling program of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min in a Perkin Elmer Cetus 9600 thermal cycler. The RT-PCR quantitation was perfomed using two protocols established for the estimation of COX mRNA levels [2,20,22,24]. In the first RT-PCR protocol, following the twentieth, twenty-fifth, and thirtieth cycle an aliquot of each reaction was removed and stopped by the addition of EDTA to 10 mM [2]. In the second RT-PCR protocol, PCR was performed on two-fold serial dilutions of the reverse transcribed RNA [20]. In order to establish a standard curve and the limits of sensitivity, PCR was preformed on serial dilutions of known amounts of plasmids containing either hCOX-1 or hCOX-2 cDNA. PCR products were separated by electrophoresis in 1.2% agarose gels, visualized by ethidium bromide staining, blotted to nitrocellulose membranes by overnight capillary transfer in 10 × SSC [27].

2.4. *Southern hybridizations*

Southern blot hybridizations were performed at 42°C for 6 h in a solution containing 6 × SSC, 5 × Denhardt's, 100 μg/ml denatured calf thymus DNA, 0.1% SDS, 50% formamide, and 1×10^6 cpm/ml of hybridization fluid of a ^{32}P-labeled probe. The ^{32}P-labeled probes (specific activities of $1–3 \times 10^9$ cpm/μg DNA) were prepared by random hexamer priming [26] of the following DNA fragments: the COX-1 probe was a cDNA fragment coding for amino acids 173–271 of hCOX-1 [1]; the COX-2 probe was a cDNA fragment spanning amino acids 160–260 of hCOX-2 [2]; and the GAPDH probe was a cDNA fragment coding for amino acids 52–250 of hGAPDH [25]. The blots were washed three times in 0.2 × SSC, 0.2% SDS for 20 min at 55°C and then exposed to Kodak XAR-5 film for 3–120 min at 23°C. The mRNA levels were quantitated by cutting the portion of the nitrocellulose membrane corresponding to the autoradiographic bands and quantifying the bound radioactivity by liquid scintillation counting. To normalize the signals obtained from different RNA samples, the signal for GAPDH was used as an internal control for each sample with the signals for COX-1 and COX-2 normalized with respect to GAPDH.

3. RESULTS

3.1. *Northern blot analysis*

In order to evaluate the sensitivity of Northern blot analysis for COX mRNAs, stomach and small intestine RNAs were examined (Fig. 1). Under high stringency hybridization conditions COX-1 and COX-2 transcripts were detected in stomach and small intestine RNA using 3 μg poly(A)$^+$ RNA and a 3 day autoradiographic exposure. In both stomach and small intestine, two COX-1 transcripts of approximately 3 kb and 5 kb were detected (Fig. 1A), whereas a single 5 kb COX-2 transcript was observed. Under the hybridization conditions we employed (6 × SSC, 50% formamide at 42°C) the COX-1 and COX-2 probes should not cross-hybridize as their cDNA coding sequences are only 64% identical [1,2]. Our results are in agreement with the size and number of human COX-1 and COX-2 transcripts previously observed [1,2]. The 3 kb COX-1 transcript has been cloned but the 5 kb COX-1 transcript remains

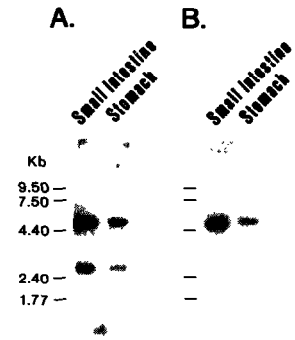


Fig. 1 Northern blot analysis of poly(A)$^+$ RNA isolated from human stomach and small intestine for COX-1 and COX-2. Three μg of poly(A)$^+$ RNA were loaded per lane, electrophoresed in a 0.8% agarose-6.6% formaldehyde gel, and transferred to a nylon membrane. The membranes were probed at high stringency (42°C, 50% formamide) with a ^{32}P-labeled COX-1 cDNA probe (panel A) or a ^{32}P-labeled COX-2 cDNA probe (panel B). The positions of single stranded RNA molecular weight standards are indicated at the left in kb. The autoradiogram was developed after a three day exposure.

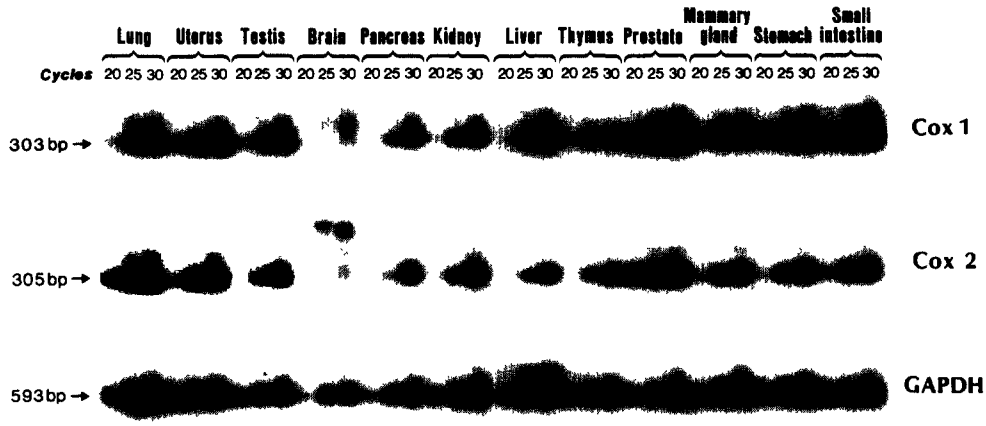


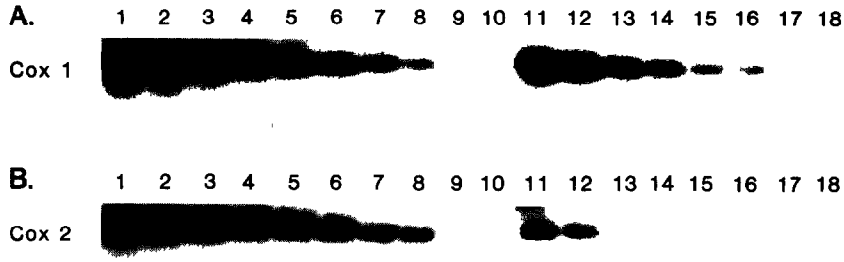
Fig. 2 RT-PCR analysis of mRNA for COX-1 and COX-2 in human tissues. Detection of PCR products by Southern blot hybridization. Single-stranded cDNA converted from a variety of poly(A)$^+$ RNA's (250 ng; see Section 2 for sources of RNA) was used for PCR amplification with COX-1, COX-2 and GAPDH specific primers for 20, 25, and 30 cycles. The PCR products were electrophoresed on 2.0% agarose gels, capillary blotted onto nitrocellulose membranes and probed with ^{32}P-random primer-labeled human COX-1, COX-2 or GAPDH probes using high stringency hybridization conditions (42°C, 50% formamide). The expected fragments of 303, 305, and 593 base pairs for COX-1, COX-2, and GAPDH, respectively, are indicated. The higher molecular weight signals amplified from brain RNA samples result from genomic DNA contamination (see text for details). The relative amounts of each signal were determined by scintillation counting of the radioactivity of the excised bands, followed by normalization of each sample using expression of GAPDH as the standard [2,22,24].

uncharacterized [1,2]. It has been suggested that the 5 kb transcript is a partially spliced COX-1 mRNA [1].

3.2. *RT-PCR analysis*

Due to the relatively low abundance of COX RNAs in stomach and small intestine we used the more sensitive RT-PCR technique [2,20] to quantitate COX sequences in mRNA from twelve different human tissues. As a control, the distribution of the constitutively expressed GAPDH was also examined. Two methods to quantitate COX mRNA by RT-PCR have been developed [2,20]. In the limited-cycle method, samples are removed from the PCR reaction at the twentieth, twenty-fifth, and thirtieth cycles to ensure that quantitation is done during the exponential phase of amplification [22,24]. In a second method described for the RTPCR quantitation of COX mRNA, the PCR is performed on serial dilutions of the cDNA reaction mixture with all samples removed at the same cycle number [20]. As shown in Fig. 2 for the limited-cycle PCR method, COX-1 and COX-2 PCR products of the expected size were amplified from all tissues examined, including lung, uterus, testis, pancreas, kidney, liver, thymus, prostate, mammary gland, stomach, and small intestine, after 25 or 30 cycles of amplification. All RNA samples yielded a GAPDH-specific PCR product demonstrating that all RNA's were sufficiently intact to support RT-PCR (Fig. 2). Faint signals for COX-1 and COX-2 were seen in brain only after 30 cycles of amplification and a 10-fold over-exposure of the autoradiograph shown in Fig. 2. Brain RNA samples also gave rise to an additional COX-1 and COX-2-hybridizing product of approximately 1.0 kb (Fig. 2). These signals result from genomic DNA contamination of the poly (A)$^+$ RNA preparation since hybridizing products of 1.0 kb were also observed in PCR samples using equivalent samples of brain poly(A)$^+$ RNA that were not treated with reverse transcriptase as template. The higher molecular weight PCR products were also observed when purified human genomic DNA was used as the template (data not shown). The primer sets for COX-1 and COX-2 are all located in different exons in order to distinguish the amplification of RNA from DNA templates. Amplification of the genomic COX-1 and COX-2 intron-containing sequences yield higher molecular weight PCR products [1,2,12].

In order to more accurately quantitate the absolute level of COX mRNA, the RT-PCR quantitation was repeated using a serial dilution RT-PCR method [20]. For each tissue, poly(A)$^+$ RNA was reverse transcribed and COX-1 and COX-2 specific PCR's were performed on serial dilutions of the cDNA. In parallel, PCR amplification was carried out for 25 cycles on serial dilutions of known quantities of cloned COX-1 and COX-2 cDNAs to establish standard amplification curves and sensitivity limits. An example of quantitation of COX mRNAs in kidney is shown in Fig. 3. The amplification curves for the diluted COX-1 and COX-2 cDNAs showed that at twenty-five cycles of PCR the logarithmic phases of the amplifications were obtained when up to 10,000 cDNA molecules were used as the starting template and that both COX-1 and COX-2 sequences were amplified with about the same efficiency (Fig. 3C). The lower limit of senstitvity for 25 cycles of PCR was about 10 molecules of input COX-1 or COX-2 cDNA template; Approximately 6,000 COX-1 and 2,000 COX-2 transcripts were present in 100 ng of the kidney poly(A)$^+$ RNA sample (Fig. 3). Quantitative RT-PCR



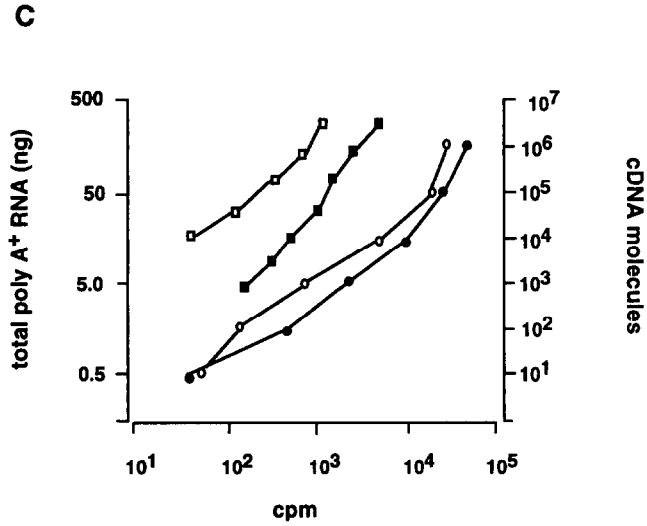


Fig. 3 Quantitative analysis by RT-PCR of COX-1 and COX-2 mRNA sequences in serially diluted human kidney RNA. (A, B) Autoradiograms of COX-1 and COX-2 PCR products following amplification for 25 cycles of serial 1:2 dilutions of 1×10^6 plasmids containing either COX-1 (panel A , lanes 1–8) or COX-2 (panel B, lanes 1–8) cDNAs and amplification of serial 2-fold dilutions of 250 ng of reverse transcribed kidney poly $(A)^+$ RNA to obtain COX-1 (panel A, lanes 11–17) and COX-2 (panel B, lanes 11–17) specific PCR products. Lanes 9 and 18 in panels A and B are control reactions carried out in the absence of added template. Samples of the PCR were separated on a 2% agarose gel, Southern blotted with COX-1 or COX-2 radiolabeled cDNA probes, and then analyzed by autoradiography. (C) PCR amplification curves of serial dilutions of known quantities of plasmids containing either COX-1 (●) or COX-2 (○) cDNAs or kidney poly$(A)^+$ RNA using specific COX-1 (■) and COX-2 (□) oligonucleotide primers. The autoradiograms from panels A and B were used to locate the positions of the bands on the nitrocellulose filters, the respective areas were cut out, and the radioactivity was quantitated by liquid scintillation counting. The radioactivity was plotted against the amount of input RNA or the number of input plasmid cDNA molecules. The standard amplification curves for the plasmid-encoded COX-1 and COX-2 cDNAs represents four experiments using 1:2 and 1:10 serial dilutions of the template.

using serial dilution of the reverse transcribed poly$(A)^+$ RNA was performed for eleven other tissues; the results of the quantitative analysis are shown in Table I. COX-1 and COX-2 mRNA s were both co-expressed to high levels in the prostate, whereas high levels of COX-2 but not COX-1 mRNAs were present in the lung. Intermediate and approximately equivalent levels of both COX-1 and COX-2 mRNA were present in stomach, small intestine, uterus, and mammary gland. Lower but approximately equivalent levels of both COX-1 and COX-2 were observed in the thymus, liver, testis, kidney, and pancreas with the lowest levels of COX-1 and COX-2 observed in the brain.

4. DISCUSSION

Prostaglandins and thromboxane A_2 have been implicated as important mediators of various pathological processes including pain, inflammation, fever and uterine contractions. In addition prostaglandins are thought to have important physiological roles, for example in the maintenance of normal gastric function and renal blood flow. The mechanism of action of non-steroidal anti-inflammatory drugs is through inhibition of cyclooxygenase and because of this such compounds have important therapeutic effects as well as mechanism-based side effects (e.g. gastric ulceration). It appears that conventional cyclooxygenase inhibitors, such as indomethacin, have little selectivity for either human COX-1 or COX-2 (P. Vickers unpublished results). There has thus been speculation as to whether selective inhibitors of either COX-1 or COX-2 might have useful therapeutic effects with a diminished ability to induce mechanism-based side effects. In particular the discovery that the expression of COX-2 is highly inducible and selective suggests that this enzyme may be of particular importance in, for example, either hormonally-induced uterine contractions, as seen in dysmenorrhea, or cytokine-induced prostaglandin production, as seen in inflammatory joint diseases. It is thus of considerable interest to investigate the distribution of COX-1 and COX-2 in human tissues.

The present study used human tissues, mainly obtained from trauma victims, which presumably are unstimulated and not subject to induction. Although these studies did not address the inducibility of COX mRNA in human tissues, it appears that all the tissues tested contained constitutively expressed levels of both COX-1 and COX-2 mRNA . Of particular interest was the presence of both forms of mRNA in stomach and small intestine in view of the importance of gastric damage as the major mechanism-based side effect of non-steroidal anti-inflammatory drugs. It should be noted, however, that as observed in a variety of mammalian cell lines, the level of COX-2 may be selectively increased over

Table I

COX-1 and COX-2 mRNA levels in human tissues

Molecules mRNA/100 ng poly(A)$^+$ RNA	mRNA species	
	COX-1	COX-2
> 50,000	prostate	prostate
25,000–50,000		lung
12,500–25,000	uterus	uterus
	small intestine	small intestine
	mammary gland	mammary gland
	stomach	stomach
	lung	
1000–12,500	thymus, liver	thymus, liver
	kidney, testis	kidney, testis
	pancreas	pancreas
100–1000	brain	brain

20-fold by exposure of the cells to mitogens and cytokines with little effect on COX-1 mRNA levels [2,3,5–8,11,13–17]. For example, in human umbilical vein endothelial cells, monocytes, vascular smooth muscle cells and foreskin fibroblasts, low levels of both COX-1 and COX-2 transcripts are expressed, while after stimulation of the cells with phorbol myristic acid the level of COX-2 mRNA was selectively and dramatically increased over 20-fold [2].

There is limited information available on the tissue distribution of COX-2. The expression of COX-2 mRNA in mice is reported to be both tissue-specific and developmentally regulated [28]. Murine COX-2 was detectable in the newborn thymus and kidney and in the adult brain, lung and testis; COX-2 mRNA was not observed in either the murine heart, spleen, muscle or liver [28]. Our studies are in agreement with the mouse tissue distribution study [28] except we detect COX-2 in the human adult liver and very low levels of COX-2 mRNA prepared from human whole brain. The low levels of COX-1 and COX-2 mRNA we observed in RNA extracted from whole brain suggests that the COX protein is also present at low levels or that COX expression is not uniform in the brain. Immunohistochemical studies of monkey and ovine brain tissue have demonstrated cyclooxygenase-immunoreactive protein expressed only in specific cell types and areas of the brain [29,30]. The highest levels detected in the present study were observed in the prostate, consistent with the high levels of prostaglandins present in seminal fluid.

In conclusion these studies show that in a variety of human tissues both COX-1 and COX-2 mRNA are present. It will clearly be important to relate these levels to the expression of the protein and active enzyme in the various tissues and it will also be of considerable interest to see if these ratios are dramatically disturbed in vivo following, for example, hormonal or inflammatory stimuli.

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