The RNA-binding Protein HuR Regulates the Expression of Cyclooxygenase-2*

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The cyclooxygenase-2 (COX-2) gene encodes the inducible prostaglandin synthase enzyme implicated in inflammation, cell growth, and tumorigenesis. Regulation of the COX-2 gene expression at the post-transcriptional level is poorly understood. For example, protein factors that regulate the post-transcriptional mRNA metabolism of COX-2 have not been fully characterized. In this study, we demonstrate that the RNA-binding protein HuR binds to COX-2 mRNA and regulates its expression. We show that there are three binding sites for HuR in the 3′-untranslated region of human COX-2. These sites are located at the following positions in the COX-2 3′-untranslated region: 39–84 nucleotides (nt), 1155–1187 nt, and 1244–1256 nt (hereinafter referred to as Sites I, II and III, respectively). Although all three sites are present in the 4.6-kb COX-2 mRNA, only site I is present in the shorter 2.8-kb isoform. HuR in MDA-MB-231 cell extracts associated with COX-2 mRNA at the identified sites. Further, HuR location in the cytoplasm is induced by serum withdrawal, a stimulus known to induce COX-2 mRNA. Down-regulation of HuR by two independent methods, namely RNA interference as well as antisense RNA expression, significantly attenuated serum withdrawal-induced increase in COX-2 mRNA (both the 4.6- and 2.8-kb isoforms) and protein levels. These data suggest that HuR binding to COX-2 is critical for its post-transcriptional mRNA stabilization.

Messenger RNA stability is an important determinant of gene expression (1, 2). It is the rate-limiting step in regulating expression of immediate-early genes in response to various extracellular signals. One such immediate-early gene is COX-2, the inducible cyclooxygenase (COX)3 enzyme (3). COX-1 and -2 are enzymes that catalyze the rate-limiting steps in prostanoid biosynthesis (3–5). Although COX-1 is expressed in a constitutive manner, COX-2 levels are low under basal conditions but are highly induced in response to hormones, tumor promoters, growth factors, and pro-inflammatory cytokines (6). Exaggerated expression of COX-2 is observed in various pathological conditions such as cancer and rheumatoid arthritis (6, 7). A better understanding of the molecular basis of COX-2 expression in health as well as disease will require studies to define the factors/mechanisms that regulate COX-2 expression. An increase in COX-2 gene transcription is not sufficient to account for the fold increase in COX-2 expression observed in response to stimuli such as IL-1 (8). Indeed, cytokine induction and glucocorticoid repression of COX-2 is achieved, at least in part, by the modulation of the mRNA stability of COX-2 (8, 9).

In early studies, it was shown that IL-1α induces COX-2 and that blocking transcription by actinomycin D at later times had no effect on IL-1α-induced COX-2 levels (8), thus implying the involvement of post-transcriptional mechanisms. Furthermore, cytokine-induced COX-2 was down-regulated by the anti-inflammatory steroid dexamethasone, whereby the COX-2 transcript isoforms, 4.6 and 2.8 kb, decayed with different half-lives (9), suggesting that sequences in the 3′-UTR are critical for mRNA stability. To define the cis-acting stability-regulating elements, segments of the 3′-UTR of human COX-2 were fused downstream of the luciferase gene, and their influence on the stability of the chimeric transcript was assessed. Using this approach, a proximal stability element (−1–100 bp of the 3′-UTR) and a distal instability element (that extends from the first canonical poly(A) signal to the end of the 3′-UTR) were identified (10). The 2.8-kb COX-2 isoform lacks the distal instability element, which could explain its increased half-life in response to dexamethasone-induced destabilization of cytokine induced COX-2 (9). Another deletion analysis using a similar strategy in human lung fibroblasts identified the proximal 116-nt segment of COX-2 3′-UTR as a prime regulator of COX-2 expression (via translational repression) (11). Using tetracycline-suppressible luciferase chimeric mRNAs in rat vascular smooth cells, Murphy and colleagues (12) identified a distal 130-base AU-rich region in the murine COX-2 3′-UTR that under basal conditions serves as an instability determinant regulated by the p42/44 MAPKs. This 130-nucleotide, although highly conserved between rat and mouse COX-2 3′-UTRs, aligns poorly with its human counterpart. Deletion analysis of murine COX-2 3′-UTR (by fusing different 3′-UTR segments downstream of luciferase) identified two translational and stability control elements and two stability control elements, as shown in Fig. 1A (13). Although these 3′-UTR constructs responded to IL-1-dependent COX-2 induction, they failed to respond to dexamethasone, suggesting that elements within the COX-2 3′-UTR may not be sufficient for its responsiveness.

Several groups have identified the p38 stress-activated protein kinase (SAPK) pathway as an important regulator of tran-
scriptional regulation as well as mRNA stability in response to stimuli such as IL-1β (9–11), bacterial lipopolysaccharide (14), serum deprivation in MDA-MB-231 cells (15), and dexamethasone (16). Using a tetracycline-regulated system, a short 1–123-nt segment of the human COX-2 3′-UTR was identified as the minimal element required for regulation of COX-2 mRNA stability by the p38 (stabilizes COX-2) cascade (17) as well as by dexamethasone, which is known to destabilize COX-2 mRNA (16). Furthermore, protein kinase B (Akt/PKB) has also been implicated in K-Ras-mediated COX-2 expression (18). HuR, a member of the ELAV (embryonic lethal abnormal vision) family of mRNA-binding proteins, is known to stabilize several inducible mRNAs such as c-Fos and tumor necrosis factor-α (19–23, 27). Although the elements that regulate the stability of COX-2 mRNA have been defined (Fig. 1A), the cis-acting factors that mediate this regulation have not been identified unequivocally. Therefore we investigated whether HuR could bind to and stabilize the COX-2 transcript.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Cell Lines**—The HuR antisense clone was made in the vector backbone provided by pcDNA3.1 (a gift from Dr. Gordon Carmichael, University of Connecticut Health Center). The hammerhead ribozyme was then inserted into the 5415-bp vector backbone provided by pcDNA3.1 (−) that had been digested with BamHI and KpnI to generate pSS1. We then cut out the poly(A) signal from pSS1 by digesting it with AflIII and BssH1, purified the 5323-bp fragment, generated blunt ends using Klenow, and allowed the fragment to religate, thus generating pSS2. The 1.6-kb HuR (antisense) fragment was cut out from pSS2 and cloned in the vector backbone provided by pSS2 that had been linearized with EcoRV. We used HuR antisense clone 5 (HuRAS#5) to transfect MDA-MB-231 cells and generated stable clones that demonstrated reduced levels of HuR compared with the vector control (MDA-MB-231 cells transfected with pSS2). Our HuR antisense clones maintain their phenotype even in late passage numbers.

We used the human breast carcinoma cell line MDA-MB-231 (ATCC HTB-26) for all of our experiments. Growth conditions and other manipulations were done as described previously (15).

**Immunofluorescence**—Cells were grown in the absence or presence of serum for 0, 4, 8, and 24 h. Because the cytoplasmic localization of HuR is known to be increased by various agents such as UV light or actinomycin D (24), cells were also treated with actinomycin D (2 μg/ml) for 2 h as a control. At each time point, cells were fixed in 4% formaldehyde, permeabilized with 0.2% Triton X-100 in phosphate-buffered saline, and incubated with anti-HuR monoclonal antibody in phosphate-buffered saline (1:100) for 1 h followed by Alexa 488 goat anti-mouse IgG (1:500, Molecular Probes). Confocal images were obtained using a Zeiss LSM410 confocal microscope. Monoclonal antibody against HuR (19F12) was a kind gift of Clonetics, LLC (Hartford, CT).

**Preparation of RNA Transcripts**—Plasmid DNA digested with the appropriate restriction enzymes or PCR fragments served as templates for *in vitro* transcription of the desired RNA. To transcribe full-length COX-2 3′-UTR, we used the pCRICOCO-2-3′-UTR plasmid linearized with BglII as the template for *in vitro* transcription. To map the HuR binding site precisely, we subdivided the COX-2 3′-UTR into six fragments (1 through 6). These stretches were amplified by PCR using primers that contained the T7 polymerase promoter (refer to Table I for details). For a positive control we used the region between residues 568 and 781 of the c-Fos 3′-UTR, which is known to contain a high affinity HuR binding site (25). For a negative control we used the 3′-end of human γ-globin RNA (165 nt), which does not bind HuR (25). pAUFL was digested with HindIII, whereas pSP65H (γ-globin) was digested with Sau3A1. c-Fos (AU-rich element) was transcribed using T3 RNA polymerase, γ-globin, and full-length COX-2 3′-UTR with SP6 RNA polymerase and the subfragments of COX-2 3′-UTR (fragments 1–6) with T7 RNA polymerase. Briefly, the RNA transcripts were synthesized *in vitro*, wherein the template was incubated in reactions (0.02 ml) containing 50 mM Tris-HCl, pH 7.0, ATP/GTP/CTP/UTP, 0.5 μM each, 1 μl of RNasin, 2 μl of 10× reaction buffer, and [32P]UTP (Amersham Biosciences) at 37 °C for 30–60 min (specific activity, 0.12 × 10^6 cpm/μmol). After synthesis all the transcripts were gel-purified. RNA oligonucleotides corresponding to the HuR binding sites in fragments 1 and 4 as well as the binding site mutants were synthesized from Integrated DNA Technologies. These RNA oligonucleotides were labeled at the 5′-end (specific activity, 1 × 10^6 cpm/μmol) with T4 polynucleotide kinase using [γ-32P]ATP and gel-purified thereafter.

**RNA Complex Assay**—RNA-protein binding reactions were carried out using radiolabeled RNA and protein as described above. After 10 min at 37 °C, 5 μl of gel loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol) was mixed therein and one-fifth of the reaction was analyzed on a 1% agarose gel (in 1× Tris-acetate-EDTA) at 40–45 V for 2.5–3 h. After electrophoresis the agarose gel was dried on DE81 paper and exposed to film.

**Nitrocellulose Filter Binding Assay**—For the nitrocellulose filter binding assay, radiolabeled RNA (4–8 fmol; specific activity, 0.12 × 10^6 cpm/μmol) was incubated with GST or GST-HuR in reaction mixtures containing 50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 0.25 mg/ml tRNA, and 0.25 mg/ml bovine serum albumin at 37 °C for 10 min. Then the reactions were diluted 1:6 with buffer F (20 mM Tris-HCl, pH 7.0, and 0.05 mg/ml hRNA) and filtered through nitrocellulose (BAS5, Schleicher & Schuell). After being washed with 2 ml of buffer F, the nitrocellulose filters containing RNA complexed with protein were placed in scintillation vials and counted.

**Apparent Kd Determination**—Radiolabeled RNA was incubated with increasing concentrations of recombinant GST-HuR, and binding was quantified using a nitrocellulose filter binding assay. The fraction of RNA bound by the protein was calculated and plotted against the HuR protein concentration. The protein concentration at which half-maximal RNA bound by the protein was calculated and plotted against the HuR protein concentration. The protein concentration at which half-maximal binding is attained is defined as the apparent Kd value (34).

**Electrophoretic Mobility Shift Assay (EMSA)**—Nuclear extracts were prepared from MDA-MB-231 cells grown in complete medium containing 10% FBS. Subcellular fractionation, EMSA, and supershift experiments were done as described elsewhere (24). The COX-2 3′-UTR fragments were uniformly labeled with [32P]UTP, whereas the Site I and Site III mutant RNA oligonucleotides were end-labeled at the 5′-end with [γ-32P]ATP and gel-purified thereafter.

**RNAase T1 Selection Assay**—Reaction mixtures (0.2 ml; as described above) containing 4–8 fmol radiolabeled RNA and purified GST-HuR were incubated as described above and then treated with RNAase T1 (1 μl of 5 units/μl) for an additional 10 min. The reactions were then filtered through nitrocellulose as before. After the nitrocellulose filter was washed with 2 ml of buffer F, the bound RNA was eluted with phenol-chloroform extraction, denatured in formamide buffer (1× 10°C...
for 3 min), and analyzed on 12% polyacrylamide/urea gel electrophoresis. The gels were fixed in 10% acetic acid and dried on DE81 paper, and autoradiograms were obtained (25).

**Immunoblot Analysis**—After the appropriate treatment, the MDA-MB-231 cells were trypsinized and centrifuged, and the cell pellets were lysed in 1× SDS sample buffer, boiled at 100 °C for 10–15 min, and then stored frozen at −80 °C. Equal amounts of whole cell extracts were loaded on 10% SDS-PAGE at around 100 V. The proteins were transferred onto nitrocellulose membrane (Protran; Schleicher & Schuell). For HuR Western blot we used 1× TBS-T (Tris-buffered saline with 0.1% Tween 20) containing 0.2 M NaCl, whereas for COX-2 β-actin immunoblot we used the protocol described earlier (15). The membranes were blocked in 5% milk (in the respective 1× TBS-T buffers) and then incubated with mouse anti-human HuR antibody at a concentration of 0.1 μg/ml. After washing, the membrane was further incubated with secondary antibodies coupled to horseradish peroxidase and developed using ECL detection reagents (Amersham Biosciences).

**Total RNA Isolation and Northern Blots**—Total RNA was isolated from MDA-MB231 cells using RNA STAT-60 reagent (Tel-Test Inc.) according to the manufacturer’s instructions. The Northern hybridization was performed as described earlier (15).

**Inhibition of HuR Expression by Small Interfering RNAs (SiRNAs)**—The protocol followed was essentially the same as outlined in Tuschel and co-workers (26). The HuR siRNA duplex was synthesized by Dharmacon Inc., and the sequences were: HuR sense, 5′-AAC AUG ACC GAG UUA dTdT-3′, HuR antisense, 5′-UAU CUC AUC CUG GGU CAU GUU dTdT-3′. Transfection of the siRNA/Oligo-lectin (Invitrogen) duplexes was carried out according to the manufacturer’s instructions.

**RESULTS**

**Purified HuR Binds to Multiple Sites in the COX-2 3′-UTR**—Because HuR is a ubiquitous regulator of mRNA stability, we tested whether it interacted with the COX-2 mRNA. We incubated purified HuR with labeled COX-2 3′-UTR RNA and determined the binding sites using the RNase T1 selection assay (25). The principle of the assay is that the interaction of RNA and protein would select the specific interacting fragment on nitrocellulose filters. Because this enzyme cleaves only after G residues, the precise location of the interaction can be ascertained by gel electrophoresis. The c-Fos 3′-UTR served as a positive control, whereas the β-globin 3′-UTR constituted a negative control. HuR-COX-2 RNA complexes were allowed to form and were then digested with RNase T1. RNA fragments bound to HuR were isolated by adsorption to nitrocellulose followed by elution with phenol-chloroform. As shown in Fig. 1B, purified HuR selected three RNase T1 fragments, 45, 33, and 22 nt from the full-length (FL) COX-2 3′-UTR. In contrast, no fragments are selected from the negative control (β-globin) mRNA. This suggests that there are three HuR binding sites in the COX-2 3′-UTR.

To confirm these data we assayed HuR binding on six subfragments (F1–F6) of the COX-2 3′-UTR (Fig. 1, A–C). These three binding sites are located at nucleotides 39–84, 1155–1187, and 1244–1265 and will be referred to as Sites I, II, and III, respectively (Fig. 1C). The location of these sites was deciphered from the length of the RNase T1-selected fragments and the known RNA sequence for the COX-2 3′-UTR. The specific nature of this interaction of HuR with the three sites on the COX-2 3′-UTR is evident because fragments F2, F5, and F6 failed to bind in this assay.

**High Affinity Interaction of HuR with COX-2 mRNA**—To test the relative affinities of the three HuR binding sites, we incubated different concentrations of GST-HuR with the respective transcripts and then analyzed complex formation by agarose gel electrophoresis. We used c-Fos as our positive control and GST (at the highest concentration) as our negative control. We observed discernible complex formation at HuR concentration of 10 nM for all three fragments containing HuR binding sites as well as the c-Fos control (Fig. 2A), although the Site I-containing fragment interacted somewhat less at lower concentrations. This interaction is specific for HuR because the 3′-UTR fragments did not bind to GST alone at the highest concentration (400 nM). This assay is a measure of both the on-rate and the off-rate.

Another independent method to measure the affinity of the three COX-2 3′-UTR binding sites for HuR was utilized. Using the nitrocellulose filter binding assay (25), which measures the on-rate, we find that the HuR binding constant (apparent Kd) is 10 nM for c-Fos, 20 nM for Site I, 18 nM for Site II, and 20 nM for Site III (Fig. 2B). Thus all the three sites in COX-2 3′-UTR are high affinity HuR binding sites with apparent Kd values comparable with HuR binding to the AU-rich element of the c-Fos mRNA (25).

**Precise Identification of HuR Binding Sites I and III**—The T1 selection assay provided us with the putative binding sites (I to III) within each of the 3′-UTR fragments F1 and F4 (Fig. 1B). Highly conserved sequence elements were identified in all
three fragments (Fig. 3A). In particular, U-rich elements in Sites I and III conform to the previously identified HuR binding site (25). To confirm that HuR binds to conserved elements of Sites I and III, we designed synthetic RNA oligonucleotides spanning the putative binding sites in which the critical U residues have been mutated to Cs and Gs. We end-labeled the mutant oligonucleotides and tested their ability to bind HuR in the RNA complex assay. As shown in Fig. 3B, neither of the mutants showed any HuR binding even at 400 nM of HuR. In contrast, mutant sequences bound *Escherichia coli* single-stranded binding protein effectively. It is known that at high concentrations single-stranded DNA-binding protein can interact with RNA (28). These data show that HuR binds specifically to multiple sites on the COX-2 3′-UTR.

**HuR Regulates Expression of COX-2**

*HuR Is Necessary for COX-2 Induction*—MDA-MB231 cells were used in functional studies of HuR interaction with COX-2 mRNA because we had shown previously that serum deprivation induces RNA stabilization of COX-2 mRNA via the p38 stress-activated protein kinase pathway in these cells (15). HuR protein was abundantly expressed in these cells as determined by immunoblot analysis (see below). Immunofluorescence microscopy was conducted to determine the effect of serum deprivation on HuR expression and localization. As shown in Fig. 4, HuR is localized primarily in the nucleus. As expected, actinomycin D treatment, which is known to induce HuR translocation into cytosol, resulted in the appearance of HuR primarily in the cytosol. Serum deprivation induced a fraction of HuR to translocate into the cytosol at the 8-h time point. These data suggest that HuR is abundantly expressed in MDA-MB231 cells and that stimuli that induce COX-2 stabilization, namely serum deprivation, induce transient translocation of HuR into the cytosol.

Next, we determined whether HuR from MDA-MB-231 cells is capable of binding to COX-2 mRNA. Cell extracts were incubated with radiolabeled RNA, which resulted in the formation of RNA-protein complexes. Incubation of specific monoclonal antibody against HuR (19F12) would result in the formation of "supershift" if HuR was present in the complex. As shown in Fig. 5A, HuR is present in the c-Fos mRNA-protein complex (positive control) and not in the γ-globin mRNA-
protein complex (negative control). In vitro transcript derived from the COX-2 fragment 1 (which contains the HuR binding site 1) was also supershifted by the HuR antibody, whereas the transcript from fragment 6 was not. To directly test whether the HuR binding site is important, we performed an RNA-protein binding assay and a supershift experiment with anti-HuR antibody using end-labeled RNA oligonucleotides corresponding to the wild-type HuR binding site or the mutant (Fig. 5B). As can be seen, a HuR supershift was seen only in the wild-type RNA oligonucleotide. In none of the cases did the control anti-CDC6 monoclonal antibody cause a supershift. These data suggest that HuR in the lysates of MDA-MB231 cells is capable of interacting with COX-2 mRNA at the identified HuR binding sites.

To test the requirement for HuR in COX-2 mRNA induction, we used two approaches, namely RNA interference (RNAi) and antisense RNA expression. siRNA (26) directed against HuR specifically reduced HuR levels in MDA-MB-231 cells. Such cells, in which RNAi was used to lower HuR levels, were impaired in their ability to induce COX-2 upon serum starvation (Fig. 6A). Down-regulation of an irrelevant gene (N-cadherin) by siRNA did not affect the induction of COX-2 expression. Down-regulation of endogenous HuR levels via RNAi did not decrease the basal expression of COX-2. However, a reduction in HuR levels abrogated the ability of these cells (both antisense as well as RNAi-treated) to up-regulate COX-2 in the presence of the appropriate signal (in this case serum starvation).

In the second approach we generated a stable antisense HuR clone expressing antisense HuR upstream of the self-cleaving hammerhead ribozyme. As shown in Fig. 6B, HuR levels in the antisense clones were reduced compared with the vector control. Decreasing HuR attenuates serum deprivation induced levels of COX-2 in the HuRAS#5 when compared with the corresponding COX-2 levels in the vector control (Fig. 6B, Vector#2). However, we also observed a decrease in the basal level of COX-2 protein in the HuR antisense clone, HuRAS#5. A Northern blot of total RNA from the HuRAS#5 cells shows a...
reduction in COX-2 transcript levels as well, compared with the vector control (Fig. 6C).

DISCUSSION

Previous work (20, 29) has identified RNA binding proteins that bind to the mRNA and either stabilize or destabilize the transcript in question. Thus these RNA-binding proteins have a profound impact on protein expression from such mRNAs. For example, AUF1 and HuR bind to AU-rich elements (AREs) within the 3'-UTR to regulate mRNA stability. Although AUF1 binding accelerates message decay (29), binding of the ELAV family member HuR impedes decay of the cognate message (20). Recent work has shown that HuR regulates expression of several inducible genes such as tumor necrosis factor-

We tested whether HuR binds to COX-2 in cells. Our work shows that HuR binds to three high affinity HuR binding sites that lie in COX-2 3'-UTR subfragments F1, F3, and F4, named herein Sites I, II, and III. Site III is essentially a U-tract, whereas Site I site contains numerous AREs. This ARE-rich proximal portion of F1 is also known as CR1 (conserved region 1) because this sequence is evolutionarily conserved. Moreover this region has also been identified as the minimal element required for COX-2 regulation in response to p38 MAPK (stabilizes the COX-2 mRNA) and dexamethasone (accelerates COX-2 mRNA decay). Therefore, our finding that this 3'-UTR fragment that plays key role in COX-2 regulation also contains a binding site for the message stability factor, HuR, suggests that HuR may be involved in regulation of COX-2 mRNA stability induced by various factors. Furthermore it provides a basis for altering COX-2 message stability and thus COX-2 levels in response to a wide variety of signals.

We find that the residues that are critical for HuR-COX-2 3'-UTR interaction are mostly U residues. This agrees with the consensus binding sequence for HuR (25), which is also pyrimidine-rich and shows a strong requirement for the U residues. It also agrees with the critical residues depicted by the RNA-protein co-crystal of the closely related HuD protein (31). Altering these critical residues abolishes the ability of Sites I and III to bind HuR, thus reiterating their importance in this interaction.

We tested whether HuR in cell extracts can bind to COX-2 3'-UTR. We have previously shown that MDA-MB-231 mammary carcinoma cells exhibit a serum withdrawal-induced post-transcriptional stabilization of COX-2 mRNA (15). This event did not require transcription but required the activity of the p38 stress-activated protein kinase (15). EMSA analysis of nuclear extracts of MDA-MB-231 cells with COX-2 3'-UTR fragments indicated the existence of RNA-protein complexes, which were specifically supershifted by the anti-HuR antibody. This suggests that naturally expressed HuR in MDA-MB-231 cells binds to the high affinity site on the COX-2 3'-UTR. Importantly, serum deprivation induced the cytosolic appearance of HuR, an event that is known to stabilize the transcripts for immediate-early genes such as c-fos (32).

Because the binding data were suggestive of an interaction between HuR and COX-2, we checked whether there was a correlation between the cellular levels of HuR and COX-2. HuR binds to ARE-containing messages and stabilizes them; hence a decrease in HuR levels should bring about a concomitant decline in COX-2 levels. Our data show that reduction of HuR levels by antisense expression of HuR cDNA or siRNA-medi-
HuR, which may be an effector molecule in regulating COX-2 expression in response to a wide range of stimuli. One of the ways by which factors that enhance COX-2 expression could act would be by simply enhancing the binding of HuR to the COX-2 message, whereas destabilizing agents could bring about the opposite effect. This binding occurs in vivo as well, as evidenced by our experiments with MDA-MB-231 cells. In addition, HuR could be involved in the proper trafficking of COX-2 mRNA, without which degradation might occur. Moreover, suppression of HuR expression resulted in the inhibition of COX-2 induction. Therefore, the interaction of COX-2 and HuR could be an important event in pathological conditions in which enhanced expression of COX-2 is observed.

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