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Alternate COX-2 Transcripts Are Differentially Regulated: Implications for Post-Transcriptional Control

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Prostaglandin (PG) synthesis during inflammation occurs mainly via the transcriptionally regulated cyclooxygenase, COX-2. In pulmonary type II A549 cells, Northern analysis identified multiple IL-1β-inducible COX-2 mRNA transcripts. Amplification of 3′-cDNA ends by anchored PCR revealed products corresponding to the predominant 4.5 and 2.7 kb transcripts. Sequence analysis of amplification products indicated that these transcripts arose by alternate consensus and non-consensus polyadenylation site usage. The predominant 4.5 kb transcript showed a half-life in excess of two hours that was further stabilized by IL-1β. In addition, the COX-2 3′-untranslated region (UTR), which contains 22 copies of the putative RNA instability motif, AUUUA, when cloned downstream of a constitutively expressed luciferase gene, was found to confer partial IL-1β responsiveness in LA-4 cells. Finally, *in vivo* in LPS-treated rats, differential expression of similar COX-2 mRNA isoforms was also observed. Taken together these data suggest a functional role for post-transcriptional mechanisms, including alternate polyadenylation, in the control of COX-2.

Prostaglandins (PGs) are paracrine and autocrine lipid mediators that are implicated in many cellular processes, including inflammation, regulation of smooth muscle tone and pain perception. They are produced by inflammatory cells as well as fibroblasts, endothelial and epithelial cells (1, 2). The first steps in prostanoid synthesis are the release of arachidonic acid from membrane phospholipids by phospholipases and conversion to PGH_2 by the two cyclooxygenase (COX) enzymes (1, 2). COX-1 is constitutively expressed (2, 3), whilst COX-2 is induced by pro-inflammatory stimuli and is required for mitogen-dependent PG synthesis (2-4). These enzymes are the main targets for non-steroidal anti-inflammatory drugs (NSAIDs) (1, 2), whose anti-inflammatory effects are thought to derive from inhibition of COX-2, whilst many of the undesirable side effects are due to COX-1 inhibition (1-3). Use of selective COX-2 inhibitors confirms this and highlights the role of COX-2 in inflammation (5, 6).

The human COX-2 3′-untranslated region (UTR), in common with many cytokines genes (7), contains multiple copies of a sequence determinant, AUUUA (8, 9), thought to play a role in mRNA destabilization (10). It is increasingly clear that induction of many cytokine genes occurs, at least partially, from post-transcriptional mechanisms that stabilize the mRNA as well of, or even instead of, transcriptional activation (11, 12). Indeed such post-transcriptional mechanisms have recently been demonstrated for COX-2 (13). Here we describe the identification, cloning and characterization of alternate COX-2 transcripts in pulmonary type II epithelial cells and report differential expression of multiple COX-2 mRNA isoforms *in vivo* in lipopolysaccharide treated rats.

MATERIALS AND METHODS

Cell culture. Human A549 and mouse LA-4 cells were grown as described (14, 15). Confluent 6-well plates were incubated over-night in serum-free media (SFM) before changing to new SFM containing 1ng/ml IL-1β (2×10^5U/μg) (Genzyme, MA, USA) or vehicle.

RNA extraction, anchored PCR, and Southern hybridization. Cytoplasmic RNA was extracted (16). RNA (1μg) was reverse transcribed in a 20μl volume at 54°C using 0.5μg poly-T Adaptor Primer (AP), 5′-GGC CAC GCG TCG ACT AGT AC $(T)_{17}$-3′ (GIBCO, Paisley, UK) and 200U Superscript II reverse transcriptase (GIBCO) as recommended by the manufacturers. Aliquots (2μl) were used for anchored polymerase chain reaction (PCR) in a 50μl reaction with 5U *Taq* polymerase (Bioline, London, UK) plus 5U *Taq* Extender (Stratagene, Cambridge, UK)

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Abbreviations used: COX, cyclooxygenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; PG, prostaglandin; RT-PCR, reverse transcriptase-polymerase chain reaction; SFM, serum free media; UTR, untranslated region.

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using *Taq*Start antibody (Clontec, Cambridge, UK) for "hot" start as recommended by the manufacturers. Primers were: C2Bs 5′-GCT GGA ACA TGG AAT TAC CCA-3′, and C2s, 5′-TTC AAA TGA GAT TGT GGG AAA ATT GCT-3′ (17) and an Anchored Amplification Primer (AAP) homologous to the AP adaptor region 5′-GGC CAC GCG TCG ACT AGT AC-3′. Cycling parameters were: 94°C, 15 seconds; 58°C, 1 minute; 72°C, 6 minutes, for 28 cycles then 10 minutes at 72°C. Major products were cloned into pGEM5z (Promega) and Sequenase II (Amersham) sequencing performed to verify identity. Semi-quantitative RT-PCR was performed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using 20 cycles on 2μl of cDNA in a 50μl reaction (16). Products (10μl) were run on agarose gels and Southern hybridization carried out as described (16).

Northern blotting and hybridization. RNA (10μg) was fractionated by 1.0% agarose-formaldehyde gel electrophoresis in 1× MOPS. Capillary blotting and hybridizations were performed as described (18). The GAPDH cDNA hybridization probe was as described (16). The COX-2 hybridization probe was a 515 bp fragment of COX-2 as indicated (Fig. 1).

COX-2.3′UTR reporter construct. A 2.6 kb *ApaI*/*SacI* fragment containing the 3′-UTR was excised from pGEM5z and the ends blunted with T4 polymerase. 12mer *XbaI* linkers (Promega) were added and the fragment subcloned into the pUC19 *XbaI* site. One recombinant was recut with *XbaI* and the fragment cloned into the *XbaI* site of pGL3control (Promega) to give pGL3.C2.3UT.

Transient transfections. DNA, 1μg reporter vector + 1μg pSV-β-galactosidase control vector (Promega), was incubated with 5μl Tfx50 reagent (Promega) in 1ml SFM for 15 minutes. Cells at 60% confluency in 6 well plates were washed in SFM and transfected with 1ml of DNA/Tfx50 mix for 2 hours before incubation in 1ml SFM. Cells were treated with IL-1β (1ng/ml) and harvested at 24 hours in Reporter Lysis Buffer (Promega). Luciferase and β-galactosidase assays were performed using Luciferase Assay Reagent (Promega) and a β-galactosidase assay kit (Promega). Luminescence was measured on a TD 20/20 Luminometer (Turner Designs, Stevenage, UK). Relative luminescence readings were normalized to β-galactosidase activity and expressed as percent activation relative to control ± SEM.

Animal treatment and analysis. Male Wistar rats were injected intraperitoneally with either saline (1ml/kg) or *Salmonella enteritidis* LPS (10mg/kg). Four hours later animals were sacrificed and lungs and hearts were removed for poly-A$^+$ RNA extraction and northern blot analysis using rat COX-2 and GAPDH probes as described (19).

RESULTS

Induction of multiple COX-2 transcripts by IL-1β. In response to IL-1β, human A549 alveolar type II cells produce multiple COX-2 transcripts with predominant forms at ~4.5 and ~2.7 kb (Fig. 1A). Using anchored PCR with the gene specific primer, C2Bs, multiple IL-1β-inducible amplification products were obtained with major products of 3.1 and 1.2 kb and minor products of 2.6, 2.1 1.1 and 0.9 kb (Fig.1B-E). Assuming no alternate mRNA splicing 5′ to primer C2Bs, these amplification products correspond to major transcripts of 4.4 and 2.5 kb and minor transcripts of 3.9, 3.4, 2.4 and 2.2 kb. Allowing for the poly A tail, this correlates very well with the transcripts detected by Northern analysis and suggests the existence of further transcripts below the detection limit. Southern hybridization using probe AS confirmed that these products were COX-2 specific and were induced in a time-dependent manner (Fig. 1C & 1D). Southern hybridization of anchored PCR products using primer C2s gave an identical banding pattern except that products were, as expected, 724 bp larger than for primer C2Bs (data not shown). Since the hybridization probe, BS, was internal to the amplification primers this excludes the possibility of primer-dependent artefacts. The two major fragments, a and d (Fig. 1B-E), were cloned into pGEM5z (Promega) and partial sequencing of three independent clones performed. This showed that the full-length transcript arose from polyadenylation at a site 25 bp downstream from a canonical polyadenylation signal (AAUAAA) (20), whilst the minor, 2.7 kb, transcript seemed to result from polyadenylation 15 bp downstream of the less common non-canonical polyadenylation signal (AUUAAA) (21). Interestingly, the minor 2.1 and 1.1 kb products also terminate just downstream of further non-canonical polyadenylation signals, whilst the 2.6 kb product stops after a canonical site. Northern hybridization using probe BS revealed only the 4.5kb transcript supporting differential polyadenylation rather than alternate splicing to generate these transcripts (data not shown).

Stability of COX-2 transcripts in A549 cells. The stability of COX-2 transcripts in A549 cells, left untreated and following IL-1β treatment for various times, was examined after addition of actinomycin D to stop transcription (Fig. 2). In untreated cells, the half-life of the 4.5 kb COX-2 mRNA was in excess of 2 hours whilst that of the less abundant 2.7 kb transcript could not be measured due to its low expression. Following IL-1β treatment, modest time-dependent increases in the stability of both transcripts were observed.

Effect of the COX-2 3′UTR on expression of a constitutively transcribed luciferase reporter. Transfection of A549 cells with pGL3-control resulted in high levels of luciferase expression. However, ligation of the COX-2 3′UTR downstream of the luciferase gene resulted in an approximate 5-10 fold drop in luciferase activity that was unaffected by IL-1β treatment (data not shown). Likewise in LA-4 cells, pGL3.C2.3UT showed a 5 fold lower level of luciferase expression than pGL3-control (Fig. 3A). However, in this case IL-1β caused a reproducible twofold increase in luciferase expression from pGL3.C2.3UT (Fig. 3B). Since there was no effect on pGL3-control expression possible effects of IL-1β on the SV40 promoter or other intrinsic properties of the luciferase gene are ruled out.

Multiple COX-2 transcripts in LPS-treated rats. Male Wistar rats were treated with bacterial LPS for 4 hours and the hearts and lungs excised for RNA extraction and northern analysis. Similar results were found for both heart and lungs. However since hearts expressed COX-2 most highly only these data are pre-

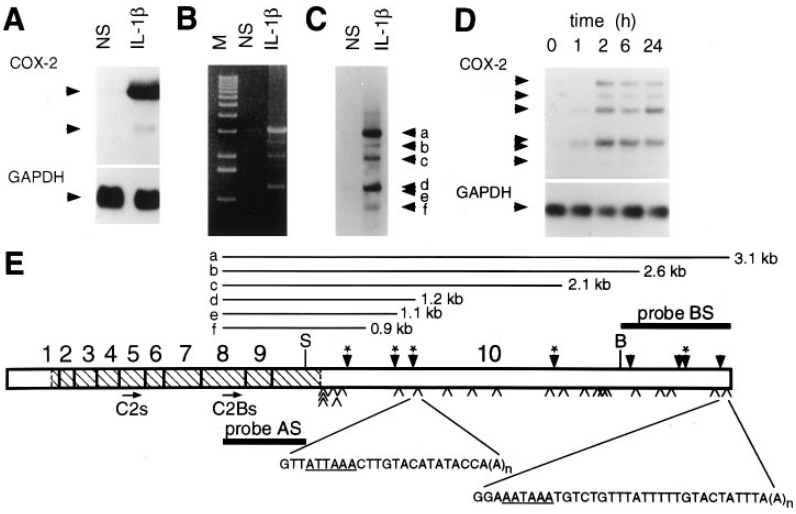

FIG. 1. Induction of multiple COX-2 transcripts by IL-1β in A549 cells. (A) Northern blot showing COX-2 and GAPDH expression in total RNA harvested form IL-1β and non-stimulated (NS) cells after 6 hours. (B) Ethidium bromide stained 0.8% agarose gel showing anchored PCR amplification products performed using primer C2Bs on RNA from IL-1β and non-stimulated cells after 2 hours. (C) Autoradiograph after Southern hybridization analysis of the gel shown in (B) using the probe AS. (D) Autoradiographs after Southern hybridization showing COX-2 anchored PCR products using primer C2Bs and semi-quantitative RT-PCR products for GAPDH from IL-1β-treated cells harvested at the time points indicated. (E) Schematic diagram of the full-length COX-2 cDNA showing alternate transcripts identified. Shaded regions represent open reading frame. Solid lines separate the exons, 1-10, which are numbered above. Anchored PCR products, a-f, are represented above the cDNA. The positions of PCR primers and hybridization probes AS and BS are indicated. The *Sac* I and *Bst EII* sites used in cloning and probe generation are shown as S and B, respectively. Open arrowheads represent AUUUA motifs, solid arrowheads represent canonical AAUAAA polyadenylation sites, and those with asterisks indicate non-canonical AUUAAA sites. Sequence data from the 3′-ends of fragments a and d are shown.

sented (19). In untreated animals, multiple COX-2 transcripts were observed (Fig 4). LPS treatment substantially induced the 4.4 kb transcript (transcript I). Transcripts II and III were also inducible, but due to proximity to the major 4.4 kb transcript reliable densitometric analysis could not be performed. The minor 2.6 kb transcript (transcript IV) was also induced although to a lesser extent than transcript I. However, the second major transcript (transcript V) at ~2.0 kb was uninducible as was the lower molecular weight smear (transcript VI). These transcripts do not result from cross-hybridization with COX-1 since these blots were also probed with COX-1, which produced a band at 2.8 kb migrating just above the 2.6 kb transcript III (19). Additionally, the smearing observed was not due to poor RNA quality since hybridization with iNOS, GAPDH and COX-1 all gave discrete bands (19 and data not shown).

DISCUSSION

The proinflammatory cytokine, IL-1β, induces COX-2 and PG synthesis in a number of systems including A549 cells, which in common with human bronchial epithelial cells produce PGE_2 via *de novo* COX-2 synthesis (2, 14, 16). Multiple COX-2 transcripts have been observed *in vitro* in cells lines (22, 23) and *ex vivo* in mouse and human cells (22, 24, 25). In A549 cells, we found major COX-2 transcripts of ~4.5 kb and ~2.7 kb that were induced by IL-1β and seemed to result from alternate polyadenylation. Since these transcripts have 22 and 7 copies respectively of the instability motif, AUUUA, they may be expected to show different stabilities. Half-life studies using actinomycin D to prevent further transcription indicated that both transcripts showed half-lives in excess of 2 hours. In both cases a modest, but reproducible increase in stability was observed following IL-1β treatment. Stabilization of the major 4.5 kb transcript occurred more rapidly than for the 2.7 kb transcript, indicating possible differential regulation of these transcripts. However, the small increase in stability and the fact that the hybrid luciferase/COX-2 3′UTR reporter was not responsive to IL-1β suggests that transcriptional events are primarily responsible for induction of COX-2 following IL-1β treatment in these cells. In contrast, stabilisation of COX-2 mRNA has been reported in other models (13). However in LA-4 type II alveolar cells, the luciferase/COX-2 3′UTR construct was IL-1β-inducible indicative of a functional post-transcriptional role for the 3′UTR.

Finally, *in vivo* in LPS-treated rats seemingly equivalent COX-2 mRNA isoforms were found to be differentially regulated. Two major and various minor transcripts were detected that showed remarkably similar hybridization patterns to the anchored PCR products from A549 cells. By analogy with the human,

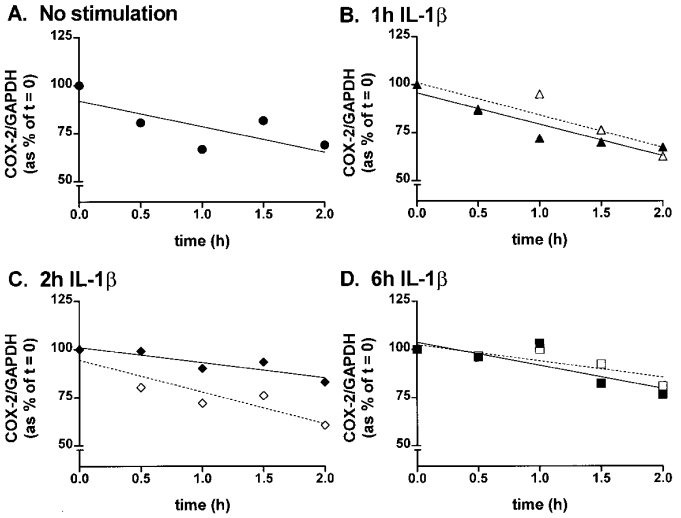

FIG. 2. Stability of alternate COX-2 transcripts after (A) 0 hour, (B) 1 hour, (C) 2 hours, and (D) 6 hours of IL-1β treatment. Cells were either not stimulated (circles) (A) or treated with IL-1β (1ng/ml) for 1 hour (triangles) (B), 2 hours (diamonds) (C), or 6 hours (squares) (D). After addition of actinomycin D (10μg/ml), cells were harvested at the time points indicated and RNA prepared for Northern analysis using probes for COX-2 and GAPDH. After laser densitometry data were normalised to GAPDH and expressed as a percentage of t = 0. All data points represent at least 3 separate determinations. Solid symbols indicate the 4.5 kb COX-2 mRNA isoform and open symbols represent the 2.7 kb isoform.

it is likely that these mRNA isoforms arise by alternate polyadenylation and therefore give rise to identical COX-2 proteins. A similar situation was found with E-selectin where alternate transcripts produced

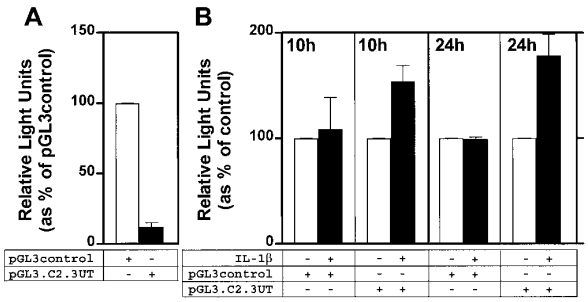

FIG. 3. Effect of the COX-2 3′-UTR on luciferase expression in a heterologous reporter system driven by a constitutive SV-40 promoter. (A) Effect of the COX-2 3′-UTR on a constitutive luciferase reporter. Cells were transfected with 1μg of pGL3 control or pGL3.C2.3UT plus 1μg of pSV-β-galactosidase control and incubated in SFM for a further 24 hours before harvesting. Luminescence was measured and normalized to β-galactosidase activity and data expressed as a percentage of pGL3 control expression. Data are from three separate determinations performed in duplicate. (B) Effect of IL-1β on luciferase expression from the luciferase/COX-2 3′UTR construct. Transfections were carried out as above. Cells were transferred to SFM and treated with IL-1β (1ng/ml) or left untreated for the times indicated. Data are expressed as a percentage of control (untreated) as means ± SEM of at least two experiments in duplicate.

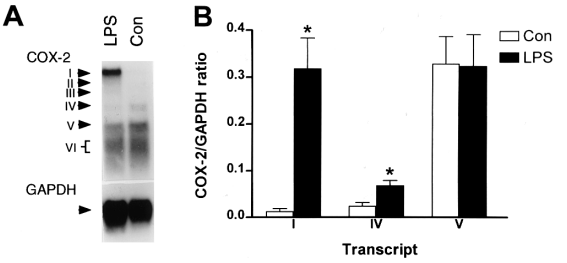

FIG. 4. Differential regulation of multiple COX-2 transcripts *in vivo* in LPS-treated rats. (A) Autoradiographs showing COX-2 and GAPDH expression in heart tissue of control (Con) and LPS-treated rats after four hours. (B) Following laser densitometry of bands from 7 such experiments data were normalized to GAPDH expression and plotted in arbitrary units as means ± SEM. *$P<0.05$ compared with control using Mann-Whitney U-test.

by differential polyadenylation were expressed in a tissue specific manner (26). The assumption that these COX-2 mRNA isoforms give rise to identical functional proteins in both humans and rodents raises questions as to their functional significance. The fact that over half the COX-2 mRNA is 3′UTR is suggestive of some important role. Here, we have shown differential stabilization, albeit to a small degree, of COX-2 mRNA isoforms that arose by alternate polyadenylation. These isoforms have also been shown to destabilize at different rates (27). In addition our data suggest a role, at least in some systems, for the COX-2 3′UTR in gene induction. Thus COX-2 may be post-transcriptionally regulated by alternate polyadenylation site usage giving rise to a number of distinct transcripts which vary in the length of their 3′UTR. These in turn may also be differentially regulated via differential stabilization, destabilization or possible effects on translation. Since in the rat, LPS predominantly induced the full-length transcript, this mRNA isoform may be primarily involved in the rapid induction of COX-2. However, the shorter transcript, transcript V, appeared almost constitutively expressed and may therefore contribute to basal levels of COX-2 in an almost housekeeping fashion.

In conclusion we have identified multiple COX-2 mRNA isoforms that appear to arise by alternate polyadenylation. These mRNA isoforms are differentially expressed and may be expected to play different functional roles in the regulation of COX-2.

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