0026-895X/06/6903-796–804$20.00
MOLECULAR PHARMACOLOGY
Vol. 69, No. 3
20529/3090359
Mol Pharmacol 69:796–804, 2006
Printed in U.S.A.

Novel Targeting of Cyclooxygenase-2 (COX-2) Pre-mRNA Using Antisense Morpholino Oligonucleotides Directed to the 3′ Acceptor and 5′ Donor Splice Sites of Exon 4: Suppression of COX-2 Activity in Human Amnion-Derived WISH and Myometrial Cells

Alison J. Tyson-Capper and G. Nicholas Europe-Finner

School of Surgical and Reproductive Sciences, The Medical School, University of Newcastle upon Tyne, Newcastle upon Tyne, United Kingdom

Received November 2, 2005; accepted December 13, 2005

ABSTRACT

Increased expression of cyclooxygenase-2 (COX-2) has been implicated in the onset of both term and preterm labor. In this context, both selective and nonselective COX-2 inhibitors have been used in clinical trials to determine their efficacy in delaying preterm labor. However, recent evidence indicates that these tocolytics may have potentially adverse fetal and maternal side effects. Therefore, the development of more specific and nontoxic agents to inhibit COX-2 needs to be considered. We have evaluated whether antisense morpholino oligonucleotides have therapeutic potential in inhibiting COX-2 by specifically targeting both the 3′ and 5′ acceptor and donor sites of exon 4 of COX-2's pre-mRNA sequence. Confocal microscopy on "live" cells illustrated high levels of penetrance of antisense morpholino oligonucleotides using the Endo-Porter formula (GeneTools, LLC, Philomath, OR), with delivery efficiencies of 82 and 78%, respectively, in amnion-derived WISH and myometrial cells. Substantial inhibition by the morpholino oligonucleotides of COX-2 expression, induced by lipopolysaccharide administration, was observed at both the mRNA and protein levels. Loss of enzymic activity of COX-2 was confirmed using a sensitive COX enzyme activity assay, which reflects the rate of conversion of arachidonic acid to prostaglandin H_2. Our results indicate that antisense morpholino oligonucleotides significantly inhibit expression and activity of this enzyme in in vitro cultures of amnion-WISH and myometrial cells. The potential thus exists that a similar approach can be mimicked in vivo to produce a highly specific and nontoxic strategy to inhibit COX-2 activity with its subsequent effects on the better management of preterm labor and other inflammatory conditions.

Cyclooxygenase-2 (COX-2) is a key regulatory enzyme responsible for the catalysis of arachidonic acid to prostaglandins and other prostanoids. Prostaglandins participate in various biological processes, including immune function and cancer, as well as the reproductive processes of ovulation, implantation, and both term and preterm labor (Chakraborty at al, 1996; Lim et al., 1997; Slater et al., 1999; Loudon et al., 2003; Olson, 2005). Under normal conditions, expression of COX-2 is undetectable in most tissues. Induction of COX-2 occurs rapidly and transiently in response to a variety of stimuli, including lipopolysaccharides (LPS), cytokines, growth factors, and tumor promoters, signifying that COX-2 is responsible for the biosynthesis of prostaglandins under acute inflammatory conditions (Fu et al., 1990; Coyne et al., 1992; Dubois et al., 1998; Williams et al., 1999).

With respect to human pregnancy, considerable effort has been expended in unraveling the molecular events that regulate the activity of the uterus during gestation and parturition, and several key genes have so far been identified whose inappropriate expression may underlie the initiation of preterm delivery. In this context, there is now growing evidence to indicate that both normal healthy term and preterm labor may involve an inflammatory mechanism by which increased production of cy-

This study was supported by grant 066148 made available by the Wellcome Trust.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.105.020529.

ABBREVIATIONS: COX, cyclooxygenase; LPS, lipopolysaccharide; IL, interleukin; DMEM, Dulbecco's modified Eagle's medium; EPEI, ethoxylated polyethylenimine; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; bp, base pair(s); TMPD, *N,N,N*^{1},*N*^{1}-tetra-methyl-*p*-phenylenediamine; PG, prostaglandin; SC-560, 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1*H*-pyrazole; DuP-697, 5-bromo-2-(4-fluorophenyl)-3-(4-methylsulfonyl)phenyl)-thiophene; IL-5Rα, interleukin-5 receptor-α.

tokines such as tumor necrosis factor α, IL-1α, IL-1β, IL-6, and IL-8 and up-regulation of COX-2 occurs within the uterus (Challis et al., 2000). This increase in COX-2 expression is initially observed in the fetal membranes (Slater et al., 1995), resulting in increased prostaglandin production, which is also augmented by the concurrent down-regulation of prostaglandin dehydrogenase, which inactivates prostaglandins. In normal pregnancies, both cytokines and prostaglandins promote cervical ripening and coordinate uterine contractions to expel the neonate at term (Challis et al., 2000). However, bacterial infection leading to stimulation of Toll-like receptors (Elovitz et al., 2003) may trigger their early increased synthesis in uterine tissues resulting in premature labor as well as the associated effects of cytokines on normal fetal cerebral development. Evidence has also accrued to indicate the differential expression of specific prostaglandin receptors as the activity of the myometrium switches from a quiescent state (expressing predominantly EP_2 and EP_4 receptors) to an active contractile state (expressing prostaglandin $F_{2\alpha}$ receptors) at term (Myatt et al., 2004).

Various nonsteroidal anti-inflammatory drugs have been used to selectively and nonselectively inhibit COX-2 at sites of inflammation. However, evidence proposes that some of these COX-2 inhibitory drugs, such as rofecoxib (Vioxx) and celecoxib (Celebrex) may, in the long term, increase the risk of cardiovascular events (Psaty and Furberg, 2005; Warner and Mitchell, 2005). Nonselective COX-2 inhibitors, such as indomethacin and sulindac, have been used clinically in an attempt to delay preterm birth (Loudon et al., 2003; Olson, 2005); however, clinical evidence indicates that these tocolytics have potentially adverse fetal side effects, including closure of the ductus arteriosus, high blood pressure in the lungs, bleeding in the brain or heart, and impaired renal function (reviewed in Loudon et al., 2003; Groom et al., 2005). Rofecoxib has been tested in a double-blind randomized controlled trial to assess its safety and efficacy in delaying preterm delivery in women at high risk (Groom et al., 2005). However, the outcome from this study indicated that the use of rofecoxib also results in adverse fetal side effects, which although reversible with discontinuation of treatment, does not reduce the incidence of preterm delivery at early gestation ages (<30 weeks), and moreover, its usage is also associated with an increased risk of premature delivery in women at high risk. These studies highlight that there are no effective therapeutic strategies for preventing early gestational deliveries or even later preterm labor not associated with infection. Therefore, the development of novel, specific, and nontoxic approaches to inhibit proinflammatory genes, such as COX-2, needs to be considered.

Antisense oligonucleotides to specific cellular RNAs have shown great promise in both research and clinical studies as sequence-specific agents able to modulate the expression of targeted genes. In principle, the most important feature of antisense oligonucleotides is their ability to base-pair with the target RNA to either block its translation or, primarily, to mediate its destruction by RNase H, an enzyme that destroys RNA in a DNA/RNA duplex (Sazani and Kole, 2003). However, many studies have also indicated that these oligonucleotides can have toxic side effects and may exert their effects nonspecifically by binding directly to a number of proteins in vivo in a sequence-dependent rather than a sequence-specific manner (Sazani and Kole, 2003). This nonantisense mechanism of binding was shown to occur particularly with the most commonly used 2′-oligodeoxynucleoside phosphorothioate species, which also seem to be sensitive to degradation by RNase H. Moreover, in vivo cellular RNAs are nearly always complexed with proteins that may block the sites and/or change the secondary/tertiary structure of the targeted RNA such that oligonucleotide targeting is frequently a trial-and-error process (Sazani and Kole, 2003). A new application of antisense oligonucleotides has been developed whereby these agents have been used to modify the splicing pattern of pre-mRNA in contrast to down-regulation of gene expression by targeting mRNA (Schmajuk et al., 1999; Sierakowska et al., 1999). Morpholino oligonucleotides have been designed to bind to specific *cis* elements within target precursor mRNA to correct aberrant splicing brought about by disease mutations, an example of which is the β-globin gene in thalassemic patients (Sierakowska et al., 1996). In contrast, the potential also exists to use this method to selectively delete individual exons of specific pre-mRNA species and thus increase production of variant mRNAs, which are translated into proteins devoid of functional domains. In light of this, a similar strategy could be adopted to knock out specific exons within pre-mRNA of uterine pro-labor genes, such as COX-2 (which we report here), resulting in decreased expression of functionally active protein species.

Materials and Methods

Morpholino Oligonucleotides and Delivery Systems. All morpholino antisense oligonucleotides used in this study were synthesized by Gene Tools, LLC (Philomath, OR). Two delivery systems were applied: the EPEI system that delivers morpholinos using ethyoxylated polyethylenimine complexed ionically with morpholino oligonucleotides preannealed with DNA, and the recently developed Endo-Porter system that delivers "bare"oligonucleotides also by an endocytosis-mediated process. Both systems deliver oligonucleotides to the nucleus to affect pre-mRNA splicing as alluded to in the introduction. The morpholino oligonucleotides were designed to splice out exon 4 (which encodes for part of the catalytic domain) of the pre-mRNA transcript of COX-2 by targeting the splice site boundaries flanking exon 4 (shown in Fig. 1). The sequences for the 3′ acceptor site and 5′ donor site blocking morpholino oligonucleotides were TGTGATCTGGCTGAAATTTTCAAAG and GATTCTTCTTACTCACCTTTCACAC, respectively. As a control, invert antisense morpholino oligonucleotides with the same sequences but in a reverse orientation were used. Special delivery fluoresceinated standard control morpholino oligonucleotides and bare fluoresceinated standard control morpholino oligonucleotides were included in this study to determine and optimize the transfection efficiency of the two delivery systems in both cell types. The sequence for the FSC oligonucleotides was CCTCTTACCTCAGTTACAATTTATA. Confirmation of delivery was measured by confocal microscopy using a Leica TCS SP2 UV with a 40×, numerical aperture 0.8, HCX water-dipping lens (Leica, Wetzlar, Germany). Delivery efficiencies were determined by calculating the number of positive cells with a fluorescence signal within defined fields. Live, unfixed cells were used as fixing cells, which may give a false-positive result.

Cell Culture and Delivery of Morpholino Oligonucleotides. Both human amnion-derived WISH cells and myometrial cells were used in this study. WISH cells (clone CCL-25) purchased from American Type Culture Collection-LGC Promochem (Teddington, Middlesex, UK), and liquid nitrogen-frozen stocks of human pregnant myometrial cells (prepared from samples collected from women undergoing elective caesarean sections with local research ethical committee approval from the Newcastle upon Tyne Hospital Trust at the

Royal Victoria Infirmary, Newcastle upon Tyne, UK) were cultured in DMEM-Glutamax medium (Invitrogen, Carlsbad, CA) containing 10% fetal calf serum, penicillin (1 U/ml), and streptomycin (1 ng/ml). WISH and myometrial cells were transfected at 80 to 90% subconfluence in OptiMEM medium (Invitrogen), in the absence of antibiotics, and the effect of individual concentrations of morpholino oligonucleotides was assayed 24 or 48 h after nuclear delivery using the two systems detailed above. In brief, 1 ml of tissue-culture grade water was combined with 33.6 μl of Morpholino/DNA stock (0.5 mM), 33.6 μl of EPEI and 5.4 ml of serum-free OptiMEM and 1.5 ml of the above EPEI/morpholino complex solution applied to cell cultures in six-well plates in the presence of LPS (1 μg/ml) for 24 or 48 h. After 3 h, the medium was removed and replaced with complete DMEM (in the absence of antibiotics). Experiments applying the Endo-Porter delivery system used 6 to 8 μl of Endo-Porter solution per ml of OptiMEM medium (in the absence of antibiotics) with 3 to 10% fetal calf serum and 3 to 10 μl of the 0.5 mM bare morpholino preparations. Note that all transfection experiments were carried out in triplicate and repeated three times.

RT-PCR Analysis. Confirmation of a COX-2 mRNA spliced variant, with exon 4 skipped because of targeting with antisense morpholino oligonucleotides, was achieved by RT-PCR using COX-2-specific sense and antisense primers spanning exon 4. This procedure provides a quick and simple assay to both confirm and quantify the level of inhibition. RT-PCR was performed using total RNA extracted from individual experiments using the SV total RNA isolation kits as recommended by the manufacturer (Promega, Madison, WI) and first-strand cDNA synthesized from 1 μg of RNA using 20 units of Superscript III reverse transcriptase (Invitrogen) with 100 ng of oligo(dT)$_{16}$ as primer. PCR amplification was carried out with 2 μl of cDNA using COX-2-specific sense and antisense oligonucleotide primers, which amplify COX-2 mRNA-spliced variants with and without the exon 4 sequence. DNA sequences for the PCR primers were CTACATACTTACCCACTTCAAGG (sense exon 3) and GTAGATCATCTCTGCCTGAGTATC (antisense exon 6). PCR was performed under standard conditions with an initial hot start cycle at 94°C (4 min), 55°C (30 s), and 72°C (1 min) followed by 25 to 28 cycles at 94°C (1 min), 55°C (30 s), and 72°C (1 min). PCR products representing the spliced COX-2 mRNA variants (472 bp with exon 4 and 328 bp without exon 4) were then analyzed by gel electrophoresis followed by densitometric scanning using a UMAX scanner coupled to the intelligent quantifier software from BioImage (Soeborg, Denmark). GAPDH primers were also included as control primers for use in RT-PCR with each cDNA sample. DNA sequences for the GAPDH primers were CTGCCGTCTAGAAAAACC (sense) and CCACCTTCGTTGTCATACC (antisense).

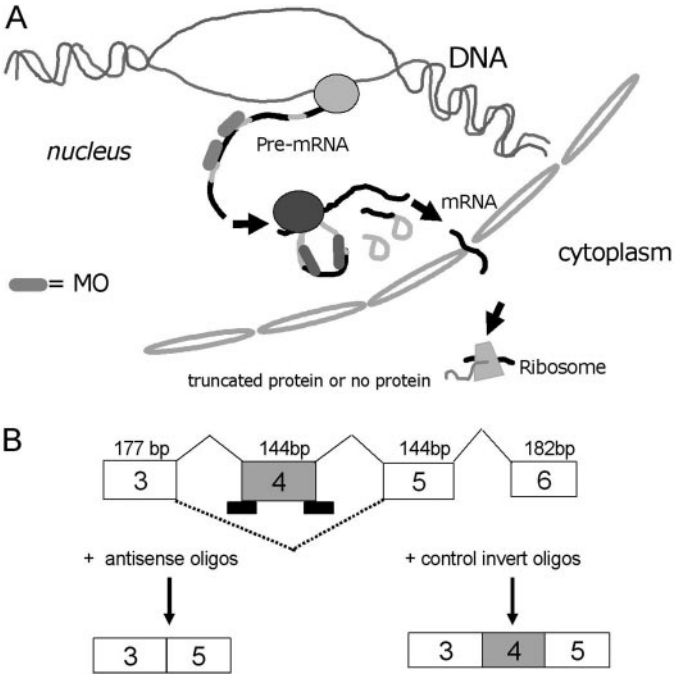

Fig. 1. Targeting pre-mRNA of COX-2 by antisense morpholino oligonucleotides. A, diagram to show mechanism of action of morpholino oligonucleotides in targeting nuclear pre-mRNA. Binding of morpholino oligonucleotides to the splice sites blocks assess to the splice sites by splicing factors and redirects the splicing machinery to skip out the exon. This results in a shorter mRNA and a truncated protein or no protein. B, two splice-site blocking antisense morpholino oligonucleotides were designed to target the 3′ acceptor and 5′ donor sites of exon 4 of the precursor mRNA sequence of COX-2 resulting in skipping of exon 4.

Western Immunoblotting. The effectiveness of the morpholino oligonucleotides in repressing functional protein expression was determined by immunoblotting. Protein lysates from morpholino oligonucleotide and LPS-treated WISH and myometrial cells were prepared and resolved by 10% SDS-polyacrylamide gel electrophoresis as described previously (Pollard et al., 2000). Recombinant COX-2 protein was also included as a positive control. Immunoblotting was then performed using a monoclonal COX-2 antibody (Upstate Biotechnology, Lake Placid, NY) at 1:1000 dilution overnight at 4°C. All membranes were reprobed with a Gβ control antibody to confirm equal loading. Immunoreactive bands were detected by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) followed by densitometric scanning using a UMAX scanner coupled to the intelligent quantifier software from BioImage.

COX-2 Enzyme Activity Assay. Loss of enzymic activity of COX-2 because of morpholino inhibition was measured using a COX enzyme activity assay (Cayman Chemical, Ann Arbor, MI). This procedure measures COX-2 activity by oxidation of the peroxidase cosubstrate TMPD (*N,N,N*^{1},*N*^{1}-tetra-methyl-*p*-phenylenediamine) in 96-well plates and has been shown to accurately reflect the rate of conversion of arachidonic acid to PGH_2. In brief, WISH and myometrial cells were cultured to 90% confluence in T75cm flasks and treated with LPS/morpholino oligonucleotides as described in the figure legends. Cells were then rinsed twice in phosphate-buffered saline to remove all traces of DMEM, sonicated in 400 μl of ice-cold 0.1 M Tris, pH 7.8, containing 1 mM EDTA, and freeze-dried to 200 μl. The assay mixture containing 140 μl of assay buffer,10 μl of heme, 10 μl of sample (or protein standard or inactivated protein prepared by boiling lysates for 5 min), and 10 μl of the COX-1 inhibitor SC-560 to eliminate COX-1 activity (or as a control reaction the COX-2 inhibitor DuP-697 to eliminate COX-2 activity) was incubated for 5 min at 25°C, and 20 μl of TMPD was added. The reactions were then initiated by adding 20 μl of arachidonic acid to all wells, and plates gently were shaken and incubated for 5 min at 25°C. The absorbance was then read at 590 nM using a Spectra MAX 190 plate reader (Molecular Devices, Sunnyvale, CA). COX-2 activity was then calculated using the following formula whereby 1 unit is defined as the amount of enzyme to oxidize 1 nmol of TMPD per min at 25°C: COX-2 activity = $[(\Delta_{590}/5\text{ min}/0.00826\ \mu M^{-1}) \times (0.21\text{ ml}/0.01\text{ ml})]/2$ (it takes two molecules of TMPD to reduce PGG_2 to PGH_2) = nanomoles per minute per milliliter (units per milliliter). Inhibition of COX-2 activity, because of morpholino inhibition, was then measured and normalized to transfection efficiency.

Statistical Analysis. Data were compared using an unpaired, two-tailed *t* test; $P < 0.05$ was considered statistically significant. All experiments were performed three times in triplicate, and results are expressed as the mean ± S.E.M.

Results

Delivery of Morpholino Oligonucleotides within Live Amnion-Derived WISH and Myometrial Cells. To first determine the optimal conditions for delivery of the oligonucleotides, we compared the relative efficacy of the

EPEI and Endo-Porter delivery systems under different experimental conditions, as described, using fluorescein-tagged morpholino oligonucleotides. Delivery efficiencies were determined by confocal microscopy, calculating the number of positive cells in several fields with a fluorescence signal. Note that live, unfixed cells were used. Results clearly indicated that the most effective delivery method in our test system was the Endo-Porter formula. Cell cultures treated with the morpholino oligonucleotides (5 μM) and Endo-Porter (8 μM) for 48 h resulted in a transfection efficiency of 82% for WISH cells and 78% for myometrial cells (Fig. 2), whereas using the EPEI formula, transfection efficiencies were 46 and 42% for WISH cells and myometrial cells, respectively (data not shown). When cells were treated for 24 h, the delivery efficiencies using Endo-Porter were 66% for WISH cells and 60% for myometrial cell cultures (data not shown).

Twenty-Four-Hour Effect of Morpholino Oligonucleotides on LPS-Induced COX-2 mRNA Expression. Morpholino oligonucleotides were designed to target the 3′ acceptor and 5′ donor splice site boundaries flanking exon 4 of the COX pre-mRNA sequence (Fig. 1). The steric blocking of these splice site sequences in theory blocks the binding of proteins that regulate splice-site selection and prevents inclusion of exon 4 into COX-2 mRNA transcripts (Fig. 1). Confirmation of a COX-2 spliced variant with exon 4 deleted as a consequence of morpholino inhibition was observed by RT-PCR using COX-2-specific primers spanning exons 3 and 6, respectively. As shown in Fig. 3A, COX-2 expression was induced by LPS in both cell types, as described under *Materials and Methods*. GAPDH mRNAs were also included as internal controls. Treating WISH and myometrial cells with the two antisense morpholino oligonucleotides for 24 h, in the presence of LPS, resulted in a significant reduction in the expression of COX-2 mRNAs containing exon 4, as reflected by the decrease in the intensity of the 472-bp PCR product and the appearance of a new PCR band of smaller size, 328 bp, representing COX-2 spliced variants with the 144-bp exon 4 skipped out. Note that only the 472-bp PCR product was observed when the invert morpholino oligonucleotides were used, hence confirming that the control oligonucleotides have no effect. Levels of inhibition were normalized to the calculated delivery efficiencies of 66% for WISH cells and 60% for myometrial cells. The residual 472-bp band observed in antisense morpholino oligonucleotide-treated cells represents those cells that have not been transfected.

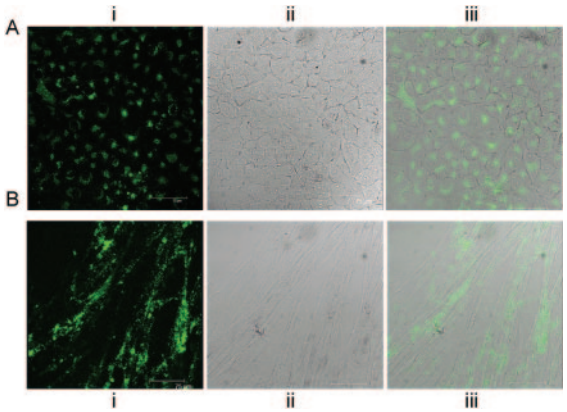

Fig. 2. Endo-Porter delivery of fluorescein-tagged morpholino oligonucleotide(s) in human amnion-derived WISH and myometrial cells. Endo-Porter delivery of morpholino oligonucleotides in cells was as described under *Materials and Methods*. Delivery efficiencies were determined by confocal microscopy using a Leica TCS SP2 UV with the 40×, numerical aperture 0.8, HCX water-dipping lens. A, human amnion-derived WISH and (B) myometrial cells. Fluorescence signal showing delivery of the oligonucleotides inside live nonfixed cells and also the high level of transfection efficiency for both cell types after 48 h (82% for WISH cells and 78% for myometrial cells), 24-h incubations resulted in efficiencies of 66% for WISH and 60% for myometrial cells (data not shown). i, fluorescence; ii, bright field same slide; and iii, overlay field.

Twenty-Four-Hour Effect of Morpholino Oligonucleotides on LPS-Induced COX-2 Protein Expression. The ability of morpholino oligonucleotides to inhibit the production of full-length COX-2 protein was then assessed by Western immunoblotting using protein lysates prepared from WISH and myometrial cells treated with the two morpholino oligonucleotides for 24 h. Quantification demonstrated that levels of 72-kDa COX-2 were significantly lower ($p < 0.01$) in samples treated with the inhibitory COX-2 morpholino oligonucleotides compared with the intensity of the 72-kDa protein bands generated from the cells treated with LPS and the control morpholino oligonucleotides (Fig. 4). The appearance of a novel band of smaller size (less than 65 kDa) was observed only in samples treated with the morpholino oligonucleotides, indicating translation of a truncated COX-2 protein. The predicted molecular mass of a COX-2 protein with the 144-bp exon 4 spliced out, based on its amino acid content, was calculated to be 63 kDa (http://www.bioinformatics.org/sms/prot_mw.html). Note that the reduction in 72-kDa COX-2 protein levels, as a consequence of morpholino oligonucleotide inhibition, correlated with the preceding decrease in full-length COX-2 mRNA (Fig. 3). The residual 72-kDa band observed in antisense morpholino oligonucleotide-treated cells represents those cells that have not been transfected.

Forty-Eight-Hour Effect of Morpholino Oligonucleotides on LPS-Induced COX-2 mRNA and Protein Expression. We then evaluated whether the activity of the morpholino oligonucleotides could be prolonged to further reduce the levels of LPS-induced COX-2 expression. WISH and myometrial cell cultures were incubated for 48 h, with the morpholino oligonucleotides and the RT-PCR and Western immunoblotting analyses repeated as described previously. We observed that using the two morpholino oligonucleotides targeted to both the 3′ acceptor and 5′ donor splice sites of COX-2 pre-mRNA for this extended time period further increased the inhibitory effect of these oligonucleotides on COX-2 expression at both mRNA and protein levels (see Fig. 5), which correlates with the calculated delivery efficiencies of 82% for WISH cells and 78% for myometrial cells observed at this time.

Antisense Morpholino Oligonucleotides Suppress COX-2 Enzyme Activity. An important aspect of this present study was to ascertain whether morpholino oligonucleotides could significantly suppress COX-2 activity. To address this, we tested the effect of the morpholino oligonucleotides after 48 h on the enzymic activity of COX-2 by using a sensitive COX enzyme assay that accurately reflects the rate of conversion of arachidonic acid to PGH_2. SC-560, a potent and selective inhibitor of COX-1, was also included in the assay to eliminate all COX-1 activity and ensure that the enzymic activity was specifically that of COX-2. Note that each reaction was carried out in triplicate, and the assay was repeated three times. As shown in Fig. 6, antisense morpho-

lino oligonucleotides targeted to COX-2 pre-mRNA significantly inhibited the activity of C0X-2 in cultures of amnion-WISH and myometrial cells.

Discussion

In this study, we have taken the novel approach of evaluating the use of two antisense morpholino oligonucleotides directed to both the 3′ acceptor and 5′ donor splice sites of exon 4 of COX-2 pre-mRNA in suppressing expression and activity of COX-2 in human amnion-derived WISH and myometrial cells. Our results indicate that these antisense morpholino oligonucleotides can significantly inhibit expression and activity of COX-2 in in vitro cultures of both cell types. Initial confocal microscopy on live unfixed amnion-WISH and myometrial cells illustrated high levels of penetrance of antisense morpholino oligonucleotides using the recently developed nontoxic Endo-Porter system from Gene Tools LLC, with delivery efficiencies of 66 and 60% after 24 h and 82 and 78% after 48 h, respectively, in WISH and myometrial cells. Substantial inhibition by the morpholino oligonucleotides of COX-2 expression, induced by LPS administration, was observed at both the mRNA and protein levels using RT-PCR and Western immunoblotting. Loss of enzymic activity of COX-2 was also confirmed using a highly sensitive COX enzyme activity assay, which accurately reflects the rate of conversion of arachidonic acid to PGH_2.

Evidence from previous studies show that antisense oligonucleotides targeted to pre-mRNA splice sites redirect the splicing machinery to adjacent consensus splice sites or cryptic splice sites and alter the splicing pattern of the pre-mRNA

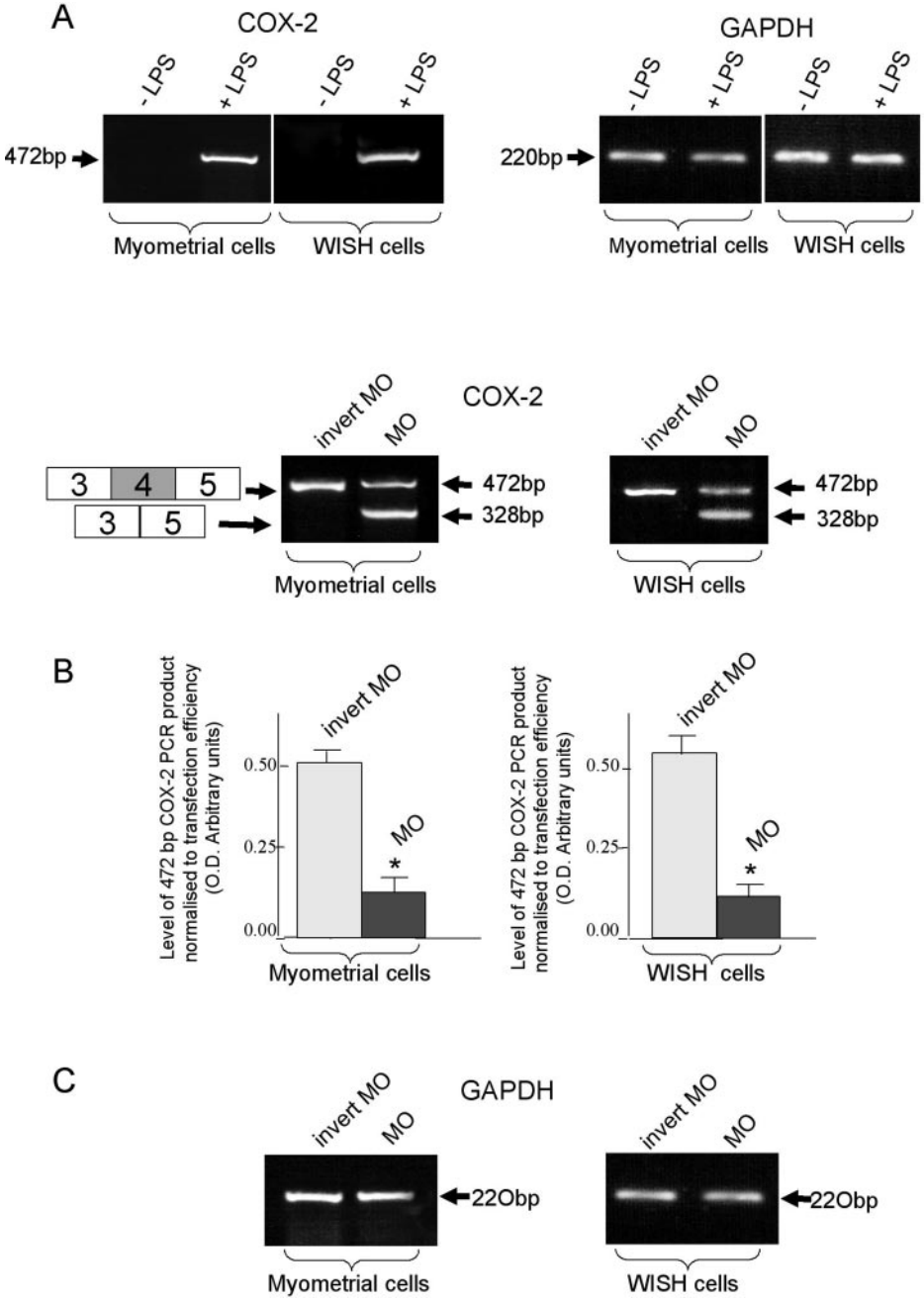

Fig. 3. Twenty-four-hour effect of antisense morpholino oligonucleotides on LPS-induced COX-2 mRNA expression. A, control LPS induction of COX-2 mRNA expression in amnion-derived WISH and primary myometrial cell cultures. Cells were stimulated with LPS for 24 h (1 μg/ml) to induce COX-2 mRNA expression. RT-PCR using COX-2-specific primers spanning exons 3 and 6 and total RNA isolated from cells treated with LPS resulted in a PCR product of 472 bp, representing untruncated full-length COX-2 compared with untreated cells. Two further experiments gave similar results. B, amnion-derived WISH and myometrial cells were administered antisense and control invert morpholino oligonucleotides for 24 h in the presence of LPS. RT-PCR using COX-2-specific primers spanning exons 3 and 6 resulted in PCR products of 472 and 328 bp, representing COX-2 mRNAs with exon 4 included or skipped out, respectively. The 472-bp bands representing full-length COX-2 were scanned and quantified using a UMAX scanner coupled to the intelligent quantifier software from BioImage. Data were normalized to delivery efficiencies for each cell type. Note that the residual 472-bp band in antisense-treated cells represents COX-2 expression in untransfected cells. Data are means ± S.E.M. ($n = 6$). *, $P < 0.01$ (t test) in antisense compared with invert morpholino oligonucleotide-treated cells. C, GAPDH housekeeping RT-PCR was used in each case to confirm equal loading.

(Schmajik et al., 1999; Sierakowska et al., 1999; Sazani et al., 2002). Oligonucleotides used to modify splicing result in products that are readily detectable with null or very low background contamination, whereas the effects of oligonucleotides directed to mRNA may easily be overlooked because of high background of the pre-existing mRNA. Moreover, because splicing takes place in the nucleus, the shift in the pattern of splicing of the target gene can only be due to the intranuclear activity of the oligonucleotides (Schmajik et al., 1999; Sierakowska et al., 1999). The requirements for oligonucleotides that shift splicing are different to those required for down-regulation of mRNA. In essence, they must not activate RNase H, which would destroy the pre-mRNA target before splicing, and must be able to effectively compete with splicing factors for access to target pre-mRNA (Sierakowska et al., 1999). Synthetic morpholino oligonucleotides having phosphordiamidate internucleotide linkages fit these requirements because they are RNase H-inactive with a high affinity for target pre-mRNA and are nuclease-resistant, and they have the ability to cross cell membranes relatively easily (Schmajik et al., 1999; Heasman, 2002; Sazani et al., 2002). In several in vitro test systems, antisense oligonucleotides have proven to be highly successful in correcting aberrant splicing brought about by β-globin gene in thalassemia and the cystic fibrosis transmembrane conductance regulator gene (Friedman et al., 1999). Until now only two published studies have used this antisense approach to target pre-mRNA and selectively splice out specific exons (Karras et al., 2000; Ittig et al., 2004). The Karras et al. (2000) study reported that constitutive/alternative splicing of murine interleukin-5 receptor-α (IL-5Rα) chain pre-mRNA can be modulated in cells using specific antisense morpholino oligo-

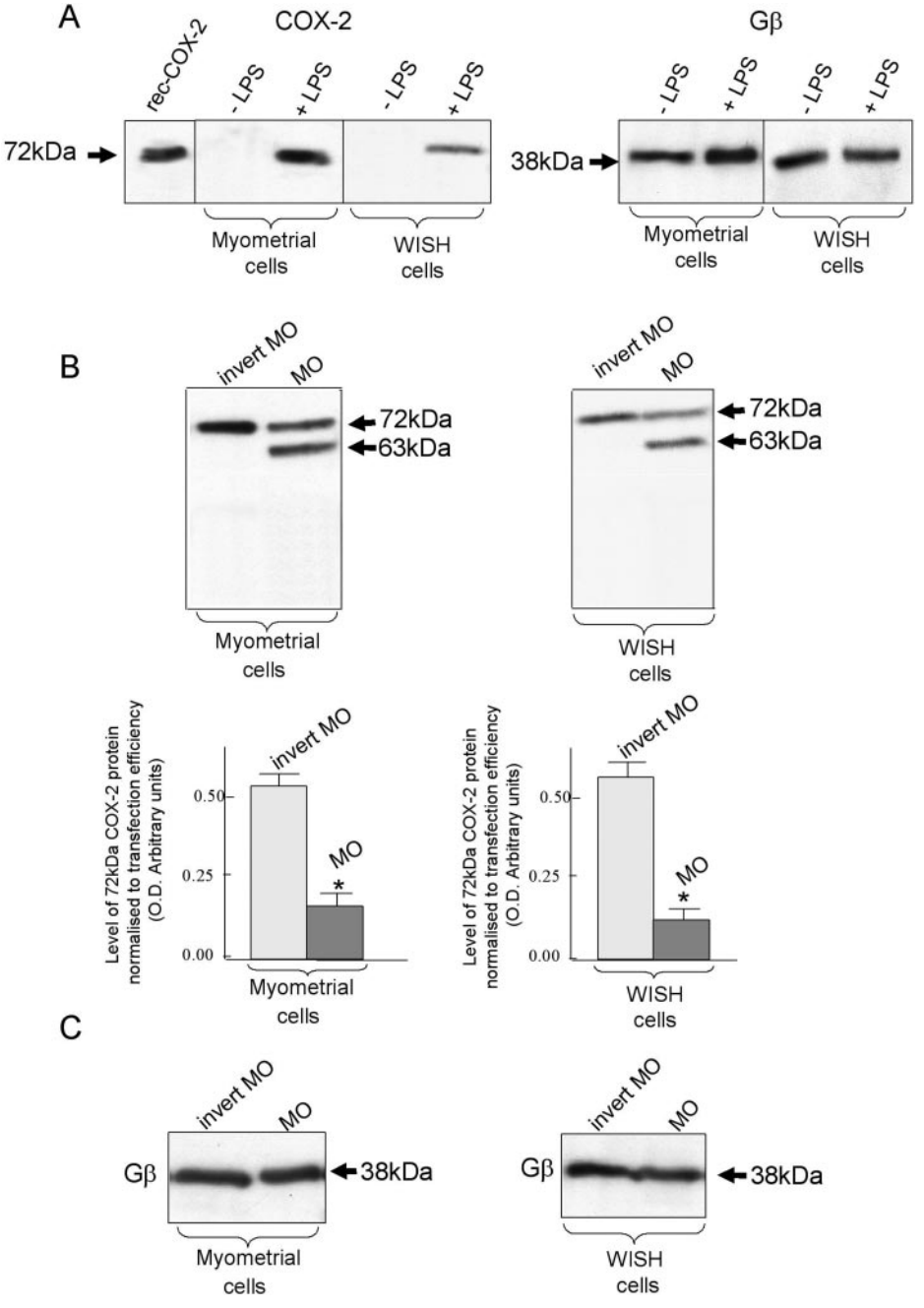

Fig. 4. Twenty-four-hour effect of antisense morpholino oligonucleotides on LPS-induced COX-2 protein expression. A, control LPS induction of COX-2 protein expression in amnion-derived WISH and primary myometrial cell cultures. Cells were stimulated with LPS for 24 h (1 μg/ml) to induce COX-2 protein expression. LPS treatment resulted in a 72-kDa protein as detected by Western blotting compared with untreated cells. Recombinant COX-2 protein (rec-COX-2) was used as a positive control. Two further experiments gave similar results. B, amnion-derived WISH and myometrial cells were administered antisense and control invert morpholino oligonucleotides for 24 h in the presence of LPS. The 72-kDa bands representing full-length COX-2 were scanned and quantified using a UMAX scanner coupled to the Intelligent quantifier software from BioImage. Data were normalized to delivery efficiencies for each cell type. Note that the residual 72-kDa band in antisense-treated cells represents COX-2 expression in untransfected cells. Data are means ± S.E.M. (n = 6). *, $P < 0.01$ (t test) in antisense compared with invert morpholino oligonucleotide-treated cells. C, all membranes were re-probed with a Gβ control antibody to confirm equal loading.

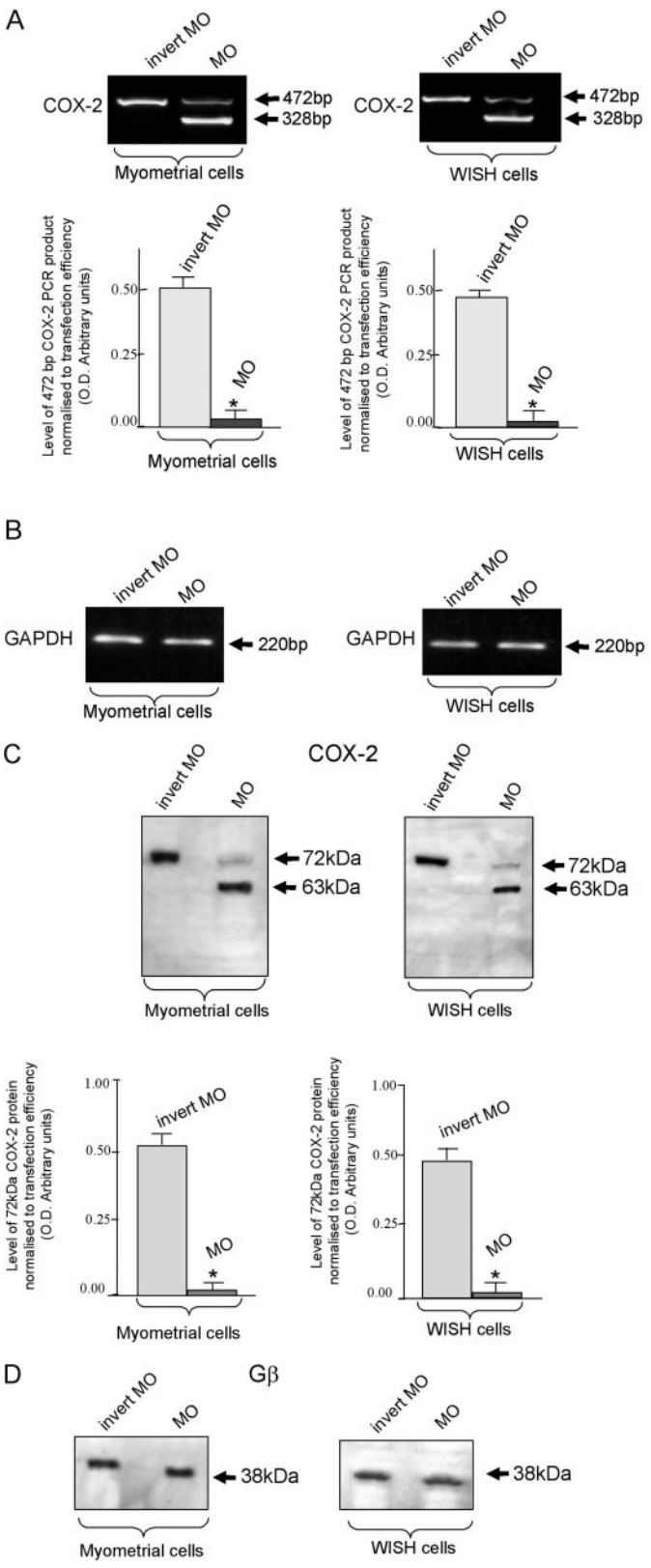

Fig. 5. Forty-eight-hour effect of morpholino oligonucleotides on LPS-induced COX-2 mRNA and protein expression. A, amnion-derived WISH and myometrial cells were administered antisense and control invert morpholino oligonucleotides for 48 h in the presence of LPS. RT-PCR using COX-2-specific primers spanning exons 3 and 6 resulted in PCR products of 472 and 328 bp, representing COX-2 mRNAs with exon 4 included or skipped out, respectively. The 472-bp bands representing full-length COX-2 were scanned and quantified using a UMAX scanner coupled to the intelligent quantifier software from BioImage. Data were normalized to delivery efficiencies for each cell type. Note that the residual 472-bp band in antisense-treated cells represents COX-2 expression in untransfected cells. Data are means ± S.E.M. ($n = 6$). ∗, $P < 0.001$ (t test) in antisense compared with invert morpholino oligonucleotide-treated cells. B, GAPDH housekeeping RT-PCR was used in each case to confirm and normalize equal loading. C, amnion-derived WISH and myometrial cells were administered antisense and control invert morpholino oligonucleotides for 48 h in the presence of LPS. The 72-kDa bands, as detected by Western blotting, representing full-length COX-2 were scanned and quantified using a UMAX scanner coupled to the intelligent quantifier software from BioImage. Data were normalized to delivery efficiencies for each cell type. Note that the residual 72-kDa band in antisense-treated cells represents COX-2 expression in untransfected cells. Data are means ± S.E.M. ($n = 6$). ∗, $P < 0.001$ (t test) in antisense compared with invert morpholino oligonucleotide-treated cells. D, all membranes were reprobed with a Gβ control antibody to confirm equal loading.

nucleotides directed to specific 3′/5′ splice sites such that individual exons may be selectively deleted from mature transcripts. This study used a range of different morpholino oligonucleotides designed to target either 3′ acceptor or 5′ donor sites of the IL-5Rα chain pre-mRNA. It is noteworthy that in some cases, specific oligonucleotides redirected splicing events to nearby cryptic splice site, resulting in novel IL-5Rα chain mRNA transcripts. The use of two morpholino oligonucleotides targeted to both the 3′ acceptor and 5′ donor sites of the pre-mRNA sequence of COX-2 seemed to be effective in our in vitro test system.

The results from this present study provide the first evidence to indicate that antisense morpholino oligonucleotides can be used, in vitro, in amnion-WISH and myometrial cell cultures to produce a functionally inactive protein and that this approach may therefore have therapeutic potential in the better management of preterm labor if it can be reproduced in vivo. In this respect, a recent study by Luu et al. (2004) using the mouse model provides direct evidence to show the efficacy of morpholino oligonucleotides in vivo. This study reported that morpholino oligonucleotides were successful in down-regulating calbindins in the mouse uterus and that the effect of the morpholinos remained localized within the uterus of the mouse. Moreover, the study highlighted that administration of morpholino oligonucleotides by intrauterine injection using the less potent EPEI delivery system (Gene Tools, LLC) provided a highly effective technique in specifically targeting uterine genes in vivo.

Antisense morpholino oligonucleotides have major advantages over other antisense gene silencing systems in that: 1) they are DNA analogs that are not susceptible to enzymatic degradation and thus have increased biological stability (Hudziak et al., 1996); 2) whereas targeted mRNA with conventional oligonucleotides is continually being replaced by new transcription requiring continued treatment, morpholino oligonucleotide targeting of pre-mRNA requires a single dose; 3) they have been shown to have greater specificity than siRNA and other phosphorothioate-based oligonucleotides (Summerton, 1999) and hence have no off-target toxic antisense effects; and 4) they have a high "loss-of-function" effect, which has been shown to last up to 4 days (Braat et al., 2001; Dutton et al., 2001). Because antisense morpholino oligonucleotides provide greater stability, nuclease resistance, long-term activity, low toxicity, and excellent specificity compared with alternative gene-silencing reagents, they therefore may represent potential therapeutic tools within

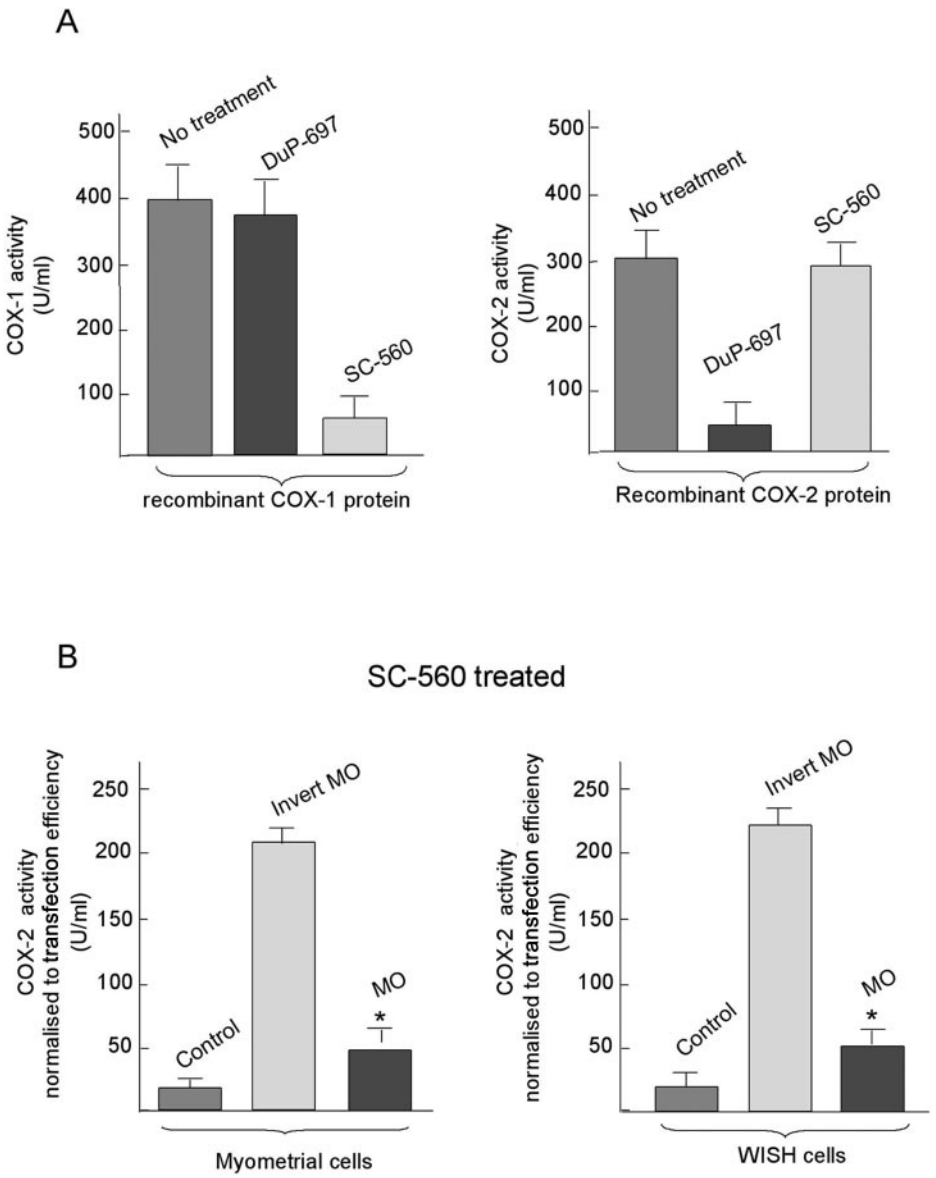

Fig. 6. Forty-eight-hour effect of antisense morpholino oligonucleotides on COX-2 enzyme activity. Loss of enzymic activity of COX-2, because of antisense morpholino inhibition, was measured using a COX enzyme activity assay. This procedure measures COX activity by oxidation of the peroxidase cosubstrate TMPD and reflects the rate of conversion of arachidonic acid to PGH_2. A, reactions using recombinant COX-1 and COX-2 protein with and without specific COX inhibitors SC-560 or DuP-697 to eliminate COX-1 and COX-2 activity, respectively, were used as controls. B, amnion-derived WISH and myometrial cells were administered antisense and control invert morpholino oligonucleotides for 48 h in the presence of LPS. SC-560 was used to eliminate all COX-1 activity, and inactivated protein from transfected cell lysates was also used as a control. The absorbance was read at 590 nM using a Molecular Devices Spectra MAX 190 plate reader. COX-2 activity was calculated using the following formula: COX-2 activity = $[(\Delta_{590}/5 \text{ min}/0.00826 \text{ mM}^{-1}) \times (0.21 \text{ ml}/0.01 \text{ ml})]/2$ = nanomoles per minute per milliliter (units per milliliter), and the percentage inhibition was then measured. Data were normalized to delivery efficiencies for each cell type. Data are means ± S.E.M. (n = 6). *, $P < 0.01$ (t test) in antisense compared with invert morpholino oligonucleotide-treated cells.

many fields of medicine, including obstetrics. In this context, there is extensive evidence to indicate increased expression of COX-2 at sites of inflammation and disease (Williams et al., 1999; Warner and Mitchell, 2005). For example, expression of COX-2 is greatly increased in rheumatoid arthritic joints (Warner and Mitchell, 2005), plus clinical and experimental evidence suggests that COX-2 contributes to lesion formation in atherosclerosis (Paramo et al., 2005). COX-2 overexpression seems to be particularly prevalent in different cancers, including gastric cancers (Saukkonen et al., 2001), esophageal cancer (Kaur and Triadafilopoulos, 2002), pancreatic cancer (Tucker et al.,1999), lung adenocarcinoma (Wolff et al., 1998), and colon carcinomas (Gupta et al., 2001). In this light, suppression of COX-2 activity by antisense morpholino oligonucleotides may also have potential in the better treatment inflammatory diseases and cancer.

In conclusion, this study has shown that antisense morpholino oligonucleotides designed to target both the 3′ acceptor and 5′ donor sites of exon 4 of the pre-mRNA sequence of COX-2 results in a substantial suppression of COX-2 activity in cultured human amnion-derived WISH and myometrial cells. Therefore, the possibility exists that a similar approach can be mimicked in vivo to produce a highly specific and nontoxic strategy to inhibit COX-2 activity.

Acknowledgments

We thank Dr. Trevor Booth (Bio-Imaging, Medical School, University of Newcastle upon Tyne, UK) and Dr. Jon Moulton (Gene Tools, LLC) for invaluable technical assistance.

References

Braat AK, van De Water S, Korving J, and Zivkovic D (2001) A zebrafish vasa morphant abolishes vasa protein but does not affect the establishment of the germline. *Genesis* **30:**183–185.

Chakraborty I, Das SK, Wang J, and Dey SK (1996) Developmental expression of the cyclo-oxygenase-1 and cyclo-oxygenase-2 genes in the peri-implantation mouse uterus and their differential regulation by the blastocyst and ovarian steroids. *J Mol Endocrinol* **16:**107–122.

Challis JRG, Matthews SG, Gibb W, and Lye SJ (2000) Endocrine and paracrine regulation of birth at term and preterm. *Endocrine Rev* **21:**514–550.

Coyne DW, Nickols M, Bertrand W, and Morrison AR (1992) Regulation of mesangial cell cyclooxygenase synthesis by cytokines and glucocorticoids. *Am J Physiol* **263:**F97–F102.

Dubois RN, Abramson SB, Crofford L, Gupta RA, Simon LS, Van De Putte LB, and Lipsky PE (1998) Cyclooxygenase in biology and disease. *FASEB J* **12**:1063–1073.

Dutton K, Dutton JR, Pauliny A, and Kelsh RN (2001) A morpholino phenocopy of the colourless mutant. *Genesis* **30:**188–189.

Elovitz MA, Wang Z, Chien EK, Rychlik M, and Phillippe M (2003) A new model for inflammation induced preterm birth: the role of platelet-activating factor and Toll-like receptor-4. *Am J Pathol* **163:**2103–2111.

Friedman KJ, Kole J, Cohn JA, Knowles MR, Silverman LM, and Kole R (1999) Correction of aberrant splicing of the cystic fibrosis transmembrane conductance regulator (CFTR) gene by antisense oligonucleotides. *J Biol Chem* **274:**36193–36199.

Fu JY, Masferrer, Seibert K, Raz A, and Needleman P (1990) The induction and suppression of prostaglandin H2 synthase (cyclooxygenase) in human monocytes. *J Biol Chem* **265:**16737–16740.

Groom KM, Shennan AH, Jones BA, Seed P, and Bennett PR (2005) TOCOX–a randomised, double-blind, placebo-controlled trial of rofecoxib (a COX-2-specific prostaglandin inhibitor) for the prevention of preterm delivery in women at high risk. *Br J Obstet Gynaecol* **112:**725–730.

Gupta DC and Dubois RN (2001) Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. *Nat Rev Cancer* **1:**11–21.

Heasman J (2002) Morpholino oligos: making sense of antisense. *Dev Biol* **243:**209–214.

Hudziak R, Barofsky E, Barofsky D, Weller DL, Huang S, and Weller DD (1996) Resistance of morpholino phosphorodiamidate oligomers to enzymatic degradation. *Antisense Nucleic Acid Drug Dev* **6**:267–272.

Ittig D, Songkai L, Renneberg D, Schümperli D, and Leumann CJ (2004) Nuclear antisense effects in cyclophilin A pre-mRNA splicing by oligonucleotides: a comparison of tricyclo-DNA with LNA. *Nucleic Acids Res* **32:**346–353.

Karras JG, McKay RA, Dean NM, and Monia BP (2000) Deletion of individual exons and induction of soluble interleukin-5 receptor-a chain expression through antisense oligonucleotide-mediated redirection of pre-mRNA splicing. *Mol Pharmacol* **58:**380–387.

Kaur BS and Triadafilopoulos G (2002) Acid- and bile-induced PGE(2) release and hyperproliferation in Barrett's esophagus are COX-2 and PKC-ϵ dependent. *Am J Physiol* **283:**G327–G334.

Lim H, Paria BC, Das SK, Dinchuk JE, Langenbach R, Trzaskos JM, and Dey SK (1997) Multiple female reproductive failures in cyclooxygenase 2-deficient mice. *Cell* **91:**197–208.

Loudon JA, Groom KM, and Bennett PR (2003) Prostaglandin inhibitors in preterm labour. *Best Pract Res Clin Obstet Gynaecol* **17:**731–744.

Luu K, Nie GY, and Salamonsen LA (2004) Endometrial calbindins are critical for embryo implantation: evidence from *in vivo* use of morpholino antisense oligonucleotides *Proc Natl Acad Sci USA* **101:**8028–8033.

Myatt L and Lye SJ (2004) Expression, localization and function of prostaglandin receptors in myometrium. *Prostaglandins Leukot Essent Fatty Acids* **70:**137–148.

Olson DM (2005) The promise of prostaglandins: have they fulfilled their potential as therapeutic targets for the delay of preterm birth? *J Soc Gynecol Investig* **12:**466–478.

Paramo JA, Rodriguez JA, Beloqui O, and Orbe J (2005) Monocyte cyclooxygenase-2 activity: a new therapeutic target for atherosclerosis? *Curr Drug Targets Cardiovasc Haematol Disord* **5:**303–311.

Pollard AJ, Sparey C, Robson SC, Krainer AR, and Europe-Finner GN (2000) Spatio-temporal expression of the *trans* acting splicing factors SF2/ASF and hnRNPA1/A1B in the myometrium of the pregnant human uterus: a molecular mechanism for regulating regional protein isoform expression in vivo. *J Clin Endocrinol Metab* **85:**1928–1936.

Psaty BM and Furberg CD (2005) COX-2 inhibitors—lessons in drug safety. *N Engl J Med* **352:**1133–1135.

Saukkonen K, Nieminen O, van Rees B, Vilkki S, Harkonen M, Juhola M, Mecklin JP, Sipponen P, and Ristimaki A (2001) Expression of cyclooxygenase-2 in dysplasia of the stomach and in intestinal-type gastric adenocarcinoma. *Clin Cancer Res* **7:**1923–1931.

Sazani P and Kole R (2003) Therapeutic potential of antisense oligonucleotides as modulators of alternative splicing. *J Clin Investig* **112:**481–486.

Sazani P, Vacek MM, and Kole R (2002) Short-term and long-term modulation of gene expression by antisense therapeutics. *Curr Opin Biotech* **13:**468–472.

Schmajuk G, Sierakowska H, and Kole R (1999) Antisense oligonucleotides with different backbones. Modification of splicing pathways and efficacy of uptake. *J Biol Chem* **274:**21783–21789.

Sierakowska H, Sambade MJ, Agrawal S, and Kole R (1996) Repair of thalassemic human β-globin mRNA in mammalian cells by antisense oligonucleotides. *Proc Natl Acad Sci USA* **93:**12480–12844.

Sierakowska H, Sambade MJ, Schumperli D, and Kole R (1999) Sensitivity of splice sites to antisense oligonucleotides in vivo. *RNA* **5:**369–377.

Slater D, Dennes W, Sawdy R, Allport V, and Bennett P (1999) Expression of cyclo-oxygenase types-1 and -2 in human myometrium throughout pregnancy. *J Mol Endocrinol* **22:**11125–11130.

Slater DM, Berger L, Newton R, Moore GE, and Bennett PR (1995) Expression of cyclooxygenase types 1 and 2 in human fetal membranes at term. *Am J Obstet Gynecol* **172:**77–82.

Summerton J (1999) Morpholino antisense oligomers: the case for an RNase H-independent structural type. *Biochem Biophys Acta* **1489:**141–158.

Tucker ON, Dannenberg AJ, Yang EK, Zhang F, Teng L, Daly JM, Soslow RA, Masferrer JL, Woerner BM, Koki AT, et al. (1999) Cyclooxygenase-2 expression is up-regulated in human pancreatic cancer. *Cancer Res* **59:**987–990.

Warner TD and Mitchell JA (2005) Cyclooxygenases: new forms, new inhibitors and lessons from the clinic. *FASEB J* **18:**790–804.

Williams CS, Mann M, and DuBois RN (1999) The role of cyclooxygenases in inflammation, cancer and development. *Oncogene* **18:**7908–7916.

Wolff H, Saukkonen K, Anttila S, Karjalainen A, Vainio H, and Ristimaki A (1998) Expression of cyclooxygenase-2 in human lung carcinoma. *Cancer Res* **58:**4997–5001.

Address correspondence to: Dr. Alison J. Tyson-Capper, School of Surgical and Reproductive Sciences, 3rd Floor, William Leech Building, The Medical School, University of Newcastle upon Tyne, NE2 4HH, UK. E-mail: a.j.tyson-capper@ncl.ac.uk