Constant Expression of Cyclooxygenase–2 Gene in Prostate and the Lower Urinary Tract of Estrogen-Treated Male Rats

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Expression of cyclooxygenase–2 (E.C. 1.14.99.1) in prostate and the lower urinary tract (LUT) of the neonatally estrogenized male rat has been studied by using a COX–2’s PCR fragment of 724 nt spanning 3 introns and a 478nt internal standard for quantitative RT-PCR. The same fragment of 724 nt was used for RNA probe in Northern hybridization. Neonatal estrogenization (10 μg/day of diethylstilbestrol on days 1–5) had no effect on COX–2 expression in prostatic urethra, prostatic lobes, or bladder. Acute estrogen treatment of castrated animals did not induce COX–2 expression, either. In addition the differential expression of basal level of COX–2 in the different lobes of prostate in normal rat was demonstrated. Our results suggest a constant expression of COX–2 gene in prostate and the lower urinary tract of the neonatally estrogenized (neoDES) rats. The present study indicates that the increased expression of COX–2 is probably not essential for the estrogen-driven development of stromal inflammation or hyperplastic and dysplastic alterations in the prostate of neoDES rats.

Introduction

Cyclooxygenase (COX) is a key enzyme in the conversion of poly-unsaturated fatty acid, arachidonic acid, to prostaglandin (PG) H2, which is then converted to various prostanooids (PGs, prostaacyclins and thromboxanes). Two isozymes, COX–1 and COX–2, have been described, which share 60% homology in amino acid sequence (O'Banion MK, et al., 1991; 1992), are encoded by two separate genes (Hla et al., 1992). They have different tissue localization, promoter structures and NSAID (non steroid anti-inflammatory drug) -binding sites (Morham et al., 1995; Smith et al., 1996). COX–1 is constitutively expressed in most tissues, where it synthesizes prostaglandins at low levels to maintain physiological functions. COX–2, on the other hand, becomes upregulated in response to growth factors and inflammatory stimuli. The time course of COX–2 transcription induction is rapid and consistent with the expression patterns of the immediate early genes. The elevation of COX–2 expression has repeatedly been reported in malignancies suggesting a contribution to carcinogenesis (Tucker et al., 1999; Ristimäki et al., 1997; Gilhooly and Rose, 1999). The human prostate cancer cell lines LnCaP and PC–3 exhibit relatively high basal levels of COX–2 mRNA with RT-PCR analysis (Tjandrawinata et al., 1997). COX–2 expression is also up-regulated in cells involved in inflammatory processes and diseases. The level of the 4.1-kb COX–2 mRNA was rapidly increased by serum or interleukine (IL–1) β in mouse fibroblasts or human monocyes (Ristimäki et al., 1996; Tsuji et al., 1998), and decreased by glucocorticoids (Newton et al., 1997). In addition, COX–2 has been induced in response to UV-B light, cytokines, such as interleukine (IL–1) α, tumor necrosis factor–α, interferon–γ, lipo polysaccharides (LPS) and phorbol myristate acetate (Buckman et al., 1998; Barrios-Rodiles et al., 1996).

Neonatal estrogenization of rat or mouse with diethylstilbestrol (DES), a potent nonsteroidal estrogen, results in prostatic inflammation, infravesical obstruction and epithelial dysplasia, morphologically resembling prostatic intraepithelial neoplasia (PIN), a precursor lesion of human...
prostatic adenocarcinoma. The Han:NMRI mice developmentally exposed to diethylstilbestrol, have shown permanent overexpression of protooncogenes c-myc and c-fos in the prostate (Pylkkänen et al., 1993: Salo et al., 1997). As an additional sign of increased growth potential of prostatic epithelial cells, DES promotes epithelial hyperplasia and dysplasia in the posterior periurethral region (Pylkkänen et al., 1993). Histologically, the dysplastic lesions closely resemble the PIN-lesions in the human prostate, which are considered to be precursor lesions for some adenocarcinomas. The prostate of neonatally estrogenized animals also shows stromal inflammation which becomes more extensive with age (Pylkkänen et al., 1991). Chronic inflammation is recognized as a risk factor for epithelial carcinogenesis. In theory, stromal cells under the influence of inflammatory mediators could contribute to the neoplastic growth and consequently to the maintenance of the malignant phenotype of prostatic epithelial cells as recently suggested for prostate (Tjandrawinata et al., 1997), colon cancer (Kargman et al., 1993; Salo et al., 1997; Norrish et al., 1998), which could make it a potential target for therapy.

Materials and Methods

Animals

All Sprague-Dawley male rats used in the study were produced in the Animal Quarters, Institute of Biomedicine, University of Turku, Turku, Finland. Animals were kept in 12–12 h light-dark cycle, and had free access to food and water. For studying the COX–2 distribution and regulation by estrogen, normal rats weighing 520–550 grams were sacrificed at the age of 9–12 month. For neonatal estrogenization, the rats were treated with 10 µg of diethylstilbestrol daily subcutaneously (Rajfer and Coffey, 1978) on days 1–5 of the postnatal life. For the analysis of acute COX–2 expression the rats at 4–6 months of age were changed to isoflavone-free synthetic and balanced C–1000 diet (Altromin GmbH, Lage, Germany) two weeks before sacrifice. The rats were castrated under barbiturate anesthesia, and 7 days later they were injected s.c. with 17 β-estradiol (25 µg/kg, 250 µg/kg or 2500 µg/kg of body weight in 200 µl of DMSO) or with the vehicle only. Animals were sacrificed 5 h after the injection. The tissues for different experiments were collected after animal sacrifice, immediately frozen in liquid nitrogen and stored at −70 °C after dissection for quantitative RT-PCR analysis. Test protocols were approved by the Committee of the Laboratory Animal Center of Turku University.

RNA isolation

Total RNA was isolated from tissue samples of 100–200 mg by one-step method (Chomczynski and Sacchi, 1987) for different tissues, and all processes were handled with 0.1% diethyl-pyrocarbonate (DEPC)-treated water.

RT-PCR and cloning of 724bp COX–2 fragment

A 724 bp COX–2 fragment was cloned using a sense primer COX–2-rat-F1 (nucleotide 841–863, 5'-GTGCCCCGCTCTGATGATGCTG-3') and an antisense primer COX–2-rat R2 (nucleotide1541–1564, 5'-CCATAAGGCC TTTCAGGAC AATG-3'). The primers were based on sequence Accession No. L20085 of GenBank, designed with default setting by program Primer3 (Whitehead Institute for Biomedical Research, Boston, USA). RT-PCR was performed using a commercial kit (AMV and Tfl, Promega, USA) with 1 µg of total RNA in 20 µl reaction buffer containing 1 mM MgSO₄, 0.15 mM dNTP, 300 nmol of both sense and antisense primers, 2.5 U AMV reverse transcriptase, 2.5 U thermal polymerase Tfl and 8 U rRNasin (RNase inhibitor, Promega, USA). In Perkin Elmer Cetus thermal cycler RT-PCR was carried out as follows: 48 °C, 45 min; 94 °C, 3 min; 35 cycles with a denaturation step (94 °C, 35s); an annealing step (60 °C, 35s); an elongation step (72 °C, 35s); and finally an additional extension step (72 °C, 5 min.). RT-PCR products were electrophoresed in 1.7 or 2% agarose gel, and 5 µl of PCR products were always loaded throughout the work. The positive (vas deferens
total RNA) and negative controls (neither reverse transcriptase nor template) were used in all RT-PCR reactions and visualization of PCR products throughout the work. The PCR fragments were purified using low melting agarose and Promega PCR fragment purification kit, and cloned in pGEM-T-easy TA cloning vector (Promega, Madison, USA). The correct clone, selected by mini-preparation of plasmid and DNA sequencing, was used for antisense RNA probe preparation in Northern hybridization. For the confirmation of specific PCR products in the non quantitative, semiquantitative and quantitative RT-PCR, the purified DNA fragments from low melting agarose gel were directly sequenced. RT-PCR reaction without RNA or cDNA was used as a negative control.

**Multiplex quantitative RT-PCR**

For β-actin controlled RT-PCR the normal RT-PCR (non quantitative) as described above, using 750 nmol of COX–2 sense and antisense primers (COX–2-rat-F1 and COX–2-rat R2) and 150 nmol of sense and antisense β-actin primers, was carried out in the same RT-PCR reaction as an internal control for the efficiency of RT and to determine the amount of RNA. The primed sequences and PCR product sizes were as follows: for COX–2 the same oligonucleotides were used as described above for the 724 bp fragment; for β-actin it is a fragment of 417 bp referred to the sequence accession No. J00691 of GenBank, with sense primer, 5’-CCAGAGCAAGAGAGGCA TC C-3’ (β-actin-rat-F3, 1502-1521) and antisense primer, 5’-CTG TGGTGGTGAGCTGCTAG-3’ (-actin-rat-R4, 2402-2383). The PCR products were sized on 2% agarose gels with 0.5 ng/ml of ethidium bromide, and visualized by image analysis.

For competitive quantitative RT-PCR the construction of COX–2 competitor template containing a deletion of nucleotide fragment was basically followed with a described method (Tucker et al., 1999). A competitive RT-PCR deletion construct (mimic) for COX–2 was synthesized using a mutant sense primer, 5’-GTGCCGGGTCTGATGTTATGACCTGCAGTGATGGCTATTCAAGCTGCATTCCCATGCAGGCTGCTGCAG-3’ (nt 841–863 attached to nts 1110–1129); and antisense primer, 5’-CCATAAGGCC TTTCAG-GAG AATG-3’ (nt 1541–1564), producing a 478 bp PCR product. The mutant sense primer contains the primer-binding sequence of endogenous target (from nucleotide 841–863) attached to the end of the intervening DNA sequence (a 247 bp deletion from nucleotide 864 to 1109). Thus, the mimic DNA has primer binding sequence identical to the target cDNA. The 478 bp mimic was further amplified using the sense primer (5’-TCCA-GATGCT ATCTTTGGG–3’) and the antisense primer (5’-ATGGTGAGCTGCTTTGAG-3’) in a 20 µl reaction with the same condition as described above, purified, and quantified to be essentially used as an internal standard (IS). cDNA was synthesized with oligonucleotide dT15 in the standard avian myeloblastosis virus reverse transcriptase condition with AMV-RT kit (Promega, Madison, USA), and the cDNA correspondent 1 µg RNA and a five concentration in linear range of IS (6 pg, 1.2 pg 0.24 pg, 0.12 pg, 0.06 pg, in the range of 10^4–10^7 copies of IS), respectively, was applied in all quantitative competitive RT-PCR.

**Northern blots**

A total of 20 µg aliquots of total RNA were size-fractionated in 1% agarose/formaldehyde gels and transferred onto nylon membranes (Genescreen, DuPont, NEN, Boston, MA) by micro-capillary methods (Eimert et al., 1997). The membranes were hybridized in buffer as suggested by the manufacturer. After hybridization, the membranes were washed twice for twenty minutes with 2 X SSC, 0.1% SDS at 60 °C; twice for twenty minutes with 0.1 X SSC, 0.1% SDS at 60 °C, and then exposed to X-OMAT AR film (Kodak Rochester, NY) for about 1 week in -70 °C. The [32P] (Amer-sham) UTP-labeled COX–2 antisense RNA probe was synthesized according to the manufacturer’s directions, using T7 RNA polymerase, from the insert of Sall linearized pGEM-T-E-cl–1 (Promega, USA). The probe for COX–2 was made by cloning the same RT-PCR fragment in this work into pGEM-T vector, and the orientation was confirmed by sequencing. The probe for c-fos was originally obtained by digestion and subcloning of the mouse pc-fos–3 clone as previous described (Loose-Mitchell et al., 1988).

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**Image analysis**

The images of UV light-illuminated gels were transferred into computer using software KDS1D2.0, DC120 image program system of KODAK Digital Science package, saved in TIFF format. The relative optical density (ROD) was measured and analyzed with Analysis Image System (AIS) version 4.0 software (AIS, Ontario, Canada).

**Statistical analysis**

Number of each experiment, n = 2–4, Statistical significance was calculated with the Students t-test of Excel 97 SR–1, and values of \( P < 0.05 \) were considered as statistically significant. All results are shown as means ± SE.

**Results**

**COX-2 expression in the tissues of control and neoDES rats**

The prostate of neoDES Sprague-Dawley rat normally showed signs of inflammation characterized by increased expression and deposition of extracellular matrix components, such as fibronectin and collagen, and emerged nucleus in the destroyed tissues. The epithelial dysplasia was sporadically observed, which is indicated by loss of normal cellular organization including multilayered and disordered structure, and also the normal morphology of nuclei changed. The dysplastic lesions resemble morphologically alterations seen earlier in neoDES mice (Pylkkanen et al., 1996) and prostatic intraepithelial neoplasia (PIN), a precursor lesion of human adenocarcinoma. The long-term effects of diethylstilbestrol on COX-2 gene expression were analyzed by using RT-PCR, semi-quantitative and quantitative RT-PCR, and Northern and Western blottings. There were no significant differences in gene expression between control and neoDES Sprague-Dawley rats including at the site of highly constitutively expressing vas deferens. The expression levels of COX-2 gene in the bladder and prostatic lobes of normal rat and neoDES rat were almost equal (Fig. 1). Further, there was no obvious difference in the constitutively overexpressed COX-2 in the distal end of vas deferens between normal rat and neoDES rat (Fig. 2A), but approximately 50–100 fold difference between distal and proximal ends was demonstrated in normal Sprague-Dawley rats (Fig. 2B). This finding was confirmed by immunohistochemical staining (data not shown). The distal part of vas deferens was consequently used as a positive control in our analysis for all experiments. To confirm the RT-PCR data, we performed Northern blot analysis with an RNA probe, which was cloned from the same PCR fragment (see Materials and Methods) for RT-PCR. No transcripts for COX-2 could be detected in control rats and neoDES rats except in 1/4 distal end of vas deferens using Northern blot analysis. As examples, Northern blots of the 1/4 distal end of vas deferens
Fig. 2. Gene expression of COX-2 in vas deferens. A: Constitutive overexpression of COX-2 in 1/4 distal end of vas deferens in normal and neoDES Sprague-Dawley rats. Lane 1, 0.5 ng total RNA, lane 2, 0.25 ng total RNA. B: RT-PCR for RNA in non-excessive condition with normal Sprague-Dawley rats. 1–25 ng of total RNA of distal and proximal ends of vas deferens was applied in the RT-PCR. 1.7% agarose gel was used. ROD: relative optical density.

from normal Sprague-Dawley rats and those of some lower urinary tract and prostate tissues from normal and neoDES rats are shown (Fig. 3).

Acute estrogen-treatment fails to induce COX-2 expression

Because COX-2 expression level in most tissues of rat is so low that it can not be detected by Northern and Western blottings, quantitative RT-PCR becomes a crucial technique to detect and measure the basal level of expression or down/up regulation of COX-2 gene. The effects of acute estrogen treatment on COX-2 expression in castrated Sprague-Dawley rats were demonstrated by competitive quantitative RT-PCR using an internal standard (Fig. 4). If the relative optical density (ROD) of the bands for amplified endogenous COX-2 mRNA and internal standard is close or equal, as in this case, it gives the amount of endogenous COX-2 mRNA in 1 µg of total RNA. Different doses of 17β-estradiol were tested, but none of the doses induced the COX-2 expression in 5 hours of acute treatment. The equal density of COX-2 and internal standard in the test panels is achieved in the same dilution. Fig. 4A only shows the panels of control, doses of 2.5 µg/kg and 250 µg/kg with the samples of ventral prostate. The ROD in lane 3 for 3 groups is equal and presents 0.24 pg of COX-2 mRNA in 1 µg of total RNA, in control and in rat treated with 2.5 µg/kg or 2.5 mg/kg of 17β-estradiol. On the contrary, c-fos proto-oncogene was up-regulated by estrogen in the same samples indicating that the estrogen treatment of animals had been successful (Fig. 4B).

Relative basal level of COX-2 in prostate and lower urinary tract: uneven distribution of COX-2 in different tissues and different lobes of the same tissue

Dorsal prostate, urethra, kidney, ventral prostate and bladder gave the ROD of β-actin in semiquantitative RT-PCR: 0.79, 0.74, 0.77, 0.69, 0.75,
Fig. 4. Quantitative competitive RT-PCR for ventral prostate of acute estrogen treatment. **A**: Total initial RNA was 1 µg in each lane, internal standard was linearly loaded. Panel a, castrated with only vehicle DMSO; panel b, castrated and injected with 25 µg/kg of 17β-estradiol; panel c, castrated and injected with 250 µg/kg of 17β-estradiol. Lane 1, 6 pg; lane 2, 1.2 pg; lane 3, 0.24 pg; lane 4, 0.12 pg; lane 5, 0.06 pg. Filled arrow points to 724 bp fragment of COX−2, open arrow points to internal standard (IS), 478 bp. 1.7% agarose gel was used. **B**: The c-fos gene is clearly induced by 17β-estradiol. The c-fos gene expression by Northern blotting, 28S rRNA was detected by own probe without c-fos probe. Lanes 1 and 2, control with DMSO; lanes 3 and 4, 25 µg/kg of 17β-estradiol; lanes 5 and 6, 250 µg/kg of 17β-estradiol. 1% agarose/formaldehyde gel was used.

Discussion

There were no differences between neoDES and control rats in the expression of COX−2 gene indicated by semiquantitative and quantitative RT-PCR, Northern blotting, and immunohistochemistry (data not shown) even though inflammation and sporadic dysplastic lesions in affected regions of the prostate of neoDES Sprague-Dawley rat were observed. There was no detectable COX−2 protein in neoDES and control rats except vas deferens by Western blotting (data not shown). This was a surprise because the induction of COX−2 has been regarded as a mechanism by which cells increase the capacity to synthesize prostaglandins in inflammation and carcinogenesis in excess of COX−1 (Karim et al., 1996). COX−2 expression is known to be stimulated by bladder outlet obstruction induced by ligation (Park et al., 1997), which neoDES male rats and mice have the similar infravesical outlet obstruction even though the causes are different.

There are only a few studies on the premalignant changes in the prostate of laboratory animals (Pylkkänen et al., 1996; Prins and Birch, 1997). This may be due to the fact that premalignant...
changes in the prostate have not been widely recognized until recently. Prostatic carcinoma and its possible precursors develop spontaneously in certain animal species and strains, and can be induced by different chemical and hormonal manipulations. The neoDES rats developed both chronic prostatitis and dysplastic alterations had been seen earlier in neoDES mice (Pylkkänen et al., 1993). In the androgen/estrogen treated Noble rat the development of induced atypical epithelial hyperplasia was carefully described as well (Leav et al., 1989). In addition to the emergence of nuclear anaplasia, the architectural pattern of the glands was disturbed e.g. layering of enlarged acinar-lining cells with pale cytoplasm form intraluminal glands within the acini. No increase in the overall expression of COX-2 was found in prostate of the neoDES rats. This indicates that constitutively expressed COX-2 is perhaps not involved in the inflammatory development, and not in the development of dysplastic lesions, either. However, the present study does not exclude the possibility that COX-2 expression would be involved in the progression of dysplastic lesions since infiltraion of leukocytes has not been shown in these neoDES rats. The elevated COX-2 could be confined to the region of the dysplastic lesions as we do not really know if COX-2 is induced in the certain stage of the development of dysplastic lesions, and COX-2 is relatively transient expressing gene. It is pertinent to mention that increased levels of COX-2 have been detected in premalignant intestinal tumors (Williams et al., 1996).

Diethylstilbestrol given neonatally increased the expression of the protooncogenes, c-myc and c-fos in the mouse prostate but in the present study it had no observable effect on the expression of COX-2 in the rat. Regardless of that, overexpression of COX-2 triggered by growth factors, tumor promoters, cytokines and other inflammatory mediators may still be one of the essential steps in the progression of these presumed cancer precursors to carcinomas. Activated oncogenes or mutated suppressor genes may initially or concomitantly activate a major step toward carcinogenesis before COX-2 induction (Howe et al., 1999). To date, no overt metastasizing carcinoma has been found in animals treated neonatally with diethylstilbestrol or 17β-estradiol. It has been reported that COX-2 is the end product of in the c-jun signal transduction pathway triggered by LPS or IL-1. Although c-fos expression was increased as a response to neonatal estrogen treatment or acute treatment of the castrated adult animal (Salo et al., 1997), the induction of COX-2 did not correlate with that of c-fos and was not up-regulated by neonatal estrogen treatment. This is consistent with the suggested signal transduction of COX-2 by the pathway of c-jun/ERK (extracellular regulated kinase), or p38 mitogen-activated protein kinase (Matsuoka et al., 1999; Reddy et al., 2000; Bartlett et al., 1999).

Bladder wall distension results in a marked and prompt stimulation of COX-2 expression at both mRNA and protein levels suggesting a role for prostaglandins in the regulation of bladder contractions. Both COX-1 and COX-2 transcripts are expressed at low levels in adult bladder of the mouse but COX-1 expression remained unaltered in obstruction (Park et al., 1997). Enlarged bladder and thickened bladder wall, occasionally with bladder stones, have been seen in neonatally estrogenized mice and rats suggesting the presence of infravesical obstruction. In accordance with this, neonatally estrogenized mice had lower voided urine volumes, higher voiding frequencies and decreased ratio of the urinary flow rate to the bladder pressure (Lehtimäki et al., 1996). Regardless of the infravesical obstruction no changes were seen in the COX-2. Induction of COX-2 occurred at 3 h of complete mechanical obstruction, and thereafter, a gradual decline in COX-2 expression took place (Park et al., 1997). This may explain why no increase in COX-2 gene expression was seen in the bladder of the neoDES rat known to develop a partial infravesical obstruction. The mechanism of downregulation in chronic obstruction is not known. The differences of COX-2 expression in the prostatic lobes, prostatic urethra and bladder of the control animal may suggest that COX-2 plays a role in their individual physiological functions.

The present study suggests a constant expression of COX-2 that is probably not under estrogenic control in neoDES Sprague-Dawley rats, and confirmed that COX-2 is highly constitutively expressed in the distal 25% of normal vas deferens of the rat (Mckanna et al., 1998). Furthermore, we detected low basal level of expression of COX-2 in the prostate of the adult control rat. There were
regional differences in the prostate, the dorsal lobe had 4.5-fold higher expression level than the ventral lobe (Fig. 5). The biological significance of uneven distribution of COX-2 expression in prostate lobes remains unknown.


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