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COX-1 and -2 Expressions in Sex-Related Organs of Neonatally Estrogen-Treated Rats and in Activated and Nonactivated Macrophage RAW264.7 Cells with Phytoestrogen

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Cyclooxygenase (COX)-2 is an inducible isoform, expressed in inflamed leukocytes and cancer cells. It is known that estrogen causes prostate dysplasia, but little is known about COX-2 expression and its influence on male reproductivity. In this study, we show that COX-2 was abolished in the distal end of the vas deferens in neonatally estrogenized (diethylstilbestrol, NeoDES) Sprague–Dawley (SD) rats at age of 15 mo, but the control normal rats were found to remain constitutive expression at the same age, while the levels of COX-1 in these rats remained intact. Furthermore, BAX, an indicator of sperm quality, was observed in the endothelium of vas deferens and sperm of the aged rats. However, COX-2 was not detected in the inflamed lesions of NeoDES rat's prostate by immunohistochemistry. In addition to estrogen, hydroxymatairesinol (HMR), a phytoestrogen, was analyzed in vitro for possible regulation on COX-2. Through Western blot analysis, HMR was shown to have no inhibitory affect on COX-2 expression. These results indicated that estrogen treatment strongly influences the expression of COX-2 that is associated with fertility, but no induction of COX-2 by estrogen may not exclude COX-2's role in prostatitis, and the anti-tumor mechanism of HMR largely remains elusive.

Key Words: COX-2; estrogen; hydroxymatairesinol (HMR); lipopolysaccharide (LPS); RAW264.7 cell line; vas deferens.

Introduction

Cyclooxygenase (COX) is a key enzyme in the conversion of polyunsaturated fatty acids and arachidonic acid to prostaglandin (PG) H_2, which is further converted into various prostanoids (PGs, prostacyclins, and thromboxanes). COX-1 is constitutively expressed in most tissues, where it synthesizes physiological amounts of prostaglandins. COX-2 is an inducible isoform, expressed in activated macrophages, and is strongly upregulated after exposure to growth factors or inflammatory stimuli, but it is also constitutively and physiologically expressed in some organs or tissues including the distal end of the vas deferens, the glomerulus of the kidneys, and the cortex of the brain. The elevation of COX-2 expression has been repeatedly reported in malignancies including human pancreatic carcinomas, suggesting its contribution to carcinogenesis by promoting angiogenesis and hampering apoptosis in malignant cells *(1–3)*. For instance, the stromal fibroblasts, and the endothelial cells in malignant mammary tissues exhibit strong staining of COX-2 *(4)*. Estrogen has been shown to regulate COX-2 through estrogen receptor (ER) and mitogen-activated protein (MAP) kinase, but final effects vary as other factors and organs differ. One direct evidence is that COX-2 inhibitor, celecoxib, stopped the vasodilation induced by estrogen *(5)*. Recently, cardiovascular risk and other severe side effects of selective COX-2 inhibitors have been brought up in debates and have led a Food and Drug Agency (FDA) advisory panel to re-evaluate the wisdom of using synthetic COX-2 inhibitors *(6,7)*. Thus, COX-2 inhibitors are still controversial, challenging both pharmacologists and the pharmaceutical industry to develop "safe" and improved painkilling and anti-inflammatory drugs. One possibility is to screen a dual-functional compound that is able to selectively increase the conversion of COX-2–dependent PGH_2 into PGI_2, or able to inhibit TXA2 synthase in the cardiovascular endothelium for anticoagulation in arteries while inhibiting COX-2. PGI_2 maintains vaso-homeostasis, and inhibition of PGI_2 is one of the reasons that may cause the infarct and stroke in the long-term users of COX-2 inhibitors. Synthetic COX-2 inhibitors have high selectivity and the advantage of irreversible inhibition, whereas naturally derived COX-2 inhibitors have lower selectivity and fewer side effects, with the medical effects in general not being as striking as those achieved using synthetic inhibitors, but they do have a wider safety margin.

Lignans, a type of mild phytoestrogen because of its structural similarities to the estrogens, are found in many sources as glycosidic conjugates associated with fiber components. Plant lignans hydroxymatairesinol (HMR) and secoisolari-

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ciresinol are converted by gut microflora to mammalian lignans enterolactone and enterodiol, respectively, which have been suggested as acting as cancer chemopreventive agents. Lignan-rich diets (flax or rye), for example, were shown experimentally to protect against prostate and colon tumors *(8–10)*. Furthermore, the urine and serum concentrations of enterolactone have been shown to be lower in women who have been diagnosed with breast cancer *(11)*. Enterolactone and enterodiol have been hypothesized to modulate the development and growth of hormone-related cancers such as breast cancer and benign prostatic hyperplasia (BPH) in men. Possible anti-tumor effects have been associated with inhibition of aromatase enzyme, resulting in reduced estrogen formation *(12)*. The potential antioxidant activity of lignans could also represent a mechanism associated with the preventive action of lignans in the development of cancers. Furthermore, mammalian lignans (in concentrations achievable in humans) inhibit the conversion of testosterone to 5-dihydrotestosterone (DHT) in vitro *(13)*. Reduction of the intracellular androgen would modify the risks of prostate cancer. Thus, one of action mechanism of HMR anti-tumor could be COX-2 inhibition and/or regulation.

Estrogen and COX-2's effects on male reproductive biology and physiology are largely unknown, but have been the subject of intensive investigation. One of these effects is human sperm quality that has been declining worldwide *(14,15)* and much of the attention in this debate has been focused on the hypothesis that environmental estrogens might be responsible. Environmental estrogens have been blamed for breast cancer in women, and prostate cancer and infertility in men. In the case of the latter, prostate and vas deferens are the major organs usually affected. Infertility due to physical abnormality of the organs has largely been clarified. However, the quality of sperm, such as motility, sperm counts, etc., remains unknown, but recent findings have shed some light on the matter like PGE_2's role in sexual behavior *(16)* and the discovery of COX-2 in the distal end of the vas deferens *(17)*. Also recently, it has been shown that the apoptosis-like phenotype is prevalent in ejaculated sperms. Apoptotic phenotypes have been significant indicators of human sperm quality. One of the pre-apoptotic factors is BAX, a member of the Bcl-2 gene family, has been linked to sperm activity *(18)*. BAX encodes a 21-kDa protein whose association with Bcl-2 is believed to play a critical role in apoptosis. BAX promotes apoptosis possibly by countering/inhibiting the effect of Bcl-2 on cell survival through heterodimer interaction regulated by p53. Lowered expression of BAX gene causes male infertility, suggesting that a balance of apoptotic/anti-apoptotic factors is necessary for normal spermatogenesis *(19,20)*.

It is clear that induction of COX-2 is involved in inflammation and carcinogenesis. However, the complexity of COX-2's expression remains in the inflammation, some of its downstream products may possibly resolve the inflammation *(21)* and keep genomic integrity, which may eventually prevent the tumorigenesis. COX-2 has not been detected in estrogen-induced hyperplasia except for one example of canine squamous epithelial cells of a prostate treated by estrogen *(22)*. We had observed the basal expression of COX-2 in the bladder and urethra of NeoDES rats by RT-PCR *(23)*. In this study, we analyzed neonatally estrogen-treated aged male rats to observe the possible differential expression of COX-2 in the genital organs, the vas deferens, the prostate gland, and the possible enhanced COX-2 expression in estrogen-induced hyperplasia of the prostate in these rats, and furthermore, to test in vitro, whether phytoestrogen HMR inhibits or regulates COX-2 with the RAW264.7 cell line.

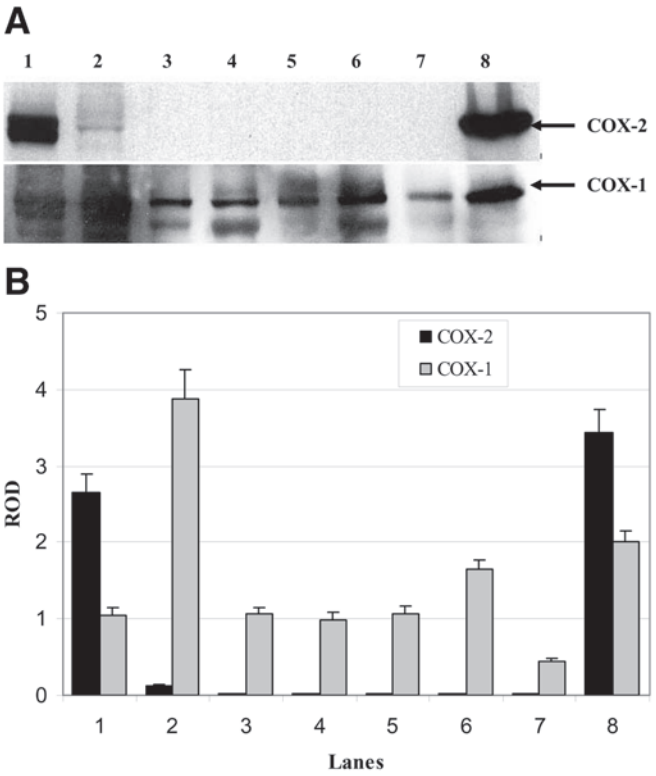

Fig. 1. (**A**) The representive Western blots for COX-1 and -2 detections in different parts of vas deferens (VD) of NeoDES and normal Sprague-Dawley rats. (Lane 1) 10 µg/mL of LPS-induced RAW264.7 cells. (Lane 2) 0.0 µg/mL of LPS-induced RAW264.7 cells. (Lane 3) NeoDes rat-I (15 mo) VD, distal end. (Lane 4) NeoDes rat-II (15 mo) VD, distal end. (Lane 5) NeoDes rat-I (15 mo) VD, proximal end. (Lane 6) NeoDes rat-II (15 mo) VD, proximal end. (Lane 7) Normal rat (15 mo) VD, one-quarter proximal end. (Lane 8) Normal rat (15 mo) VD, one-quarter distal end. (**B**) ROD: relative optical density.

Results

Long-Term Effects of DES on COX-2 Expression in the Vas Deferens of Aged Rats

The LPS-induced RAW264.7 cells were used as control for COX-2 expression levels (Fig. 1, lane 1). The RAW264.7 cells without LPS induction were used as control for COX-

1 expression levels (Fig. 1, lane 2). COX-2 was constitutively expressed in the distal end of the vas deferens in control rats (Fig. 1, lane 8). COX-2 disappeared in the distal end of the vas deferens in the 15-mo-old neonatal estrogenized rat (Fig. 1A,B, lane 3, 4). COX-2 was not present at the proximal end of the vas deferens as always (Fig. 1A,B, lane 5, 6), but COX-1 was expressed at basal levels throughout the vas deferens. COX-1 levels of both the proximal and distal portions of the vas deferens of NeoDES aged mice were approximately the same, even though COX-1 levels of distal ends were slightly lower than that in proximal end (Fig. 1A,B, lane 3, 4, 5, 6), but all are slightly higher than proximal end of nontreated rats (Fig. 1, lane 7). Both COX-1 and -2 remain at substantial levels, but COX-2 is more dominant than COX-1 in the distal end of the control rat at the age of 15 mo (Fig. 1, lane 8). In general, COX-1 is supposed to be able to substitute for COX-2's function if it is not present or deficient. We can reasonably suggest that COX-1 is also possibly involved in fertility in the early age, while COX-1 may be the sole provider of prostaglandins in the estrogenized aged rat.

COX-2 Expression in the Prostate of NeoDES Rats

COX-2 was not detected in hyperplasia and the inflamed prostate of NeoDES aged male rats by immunohistochemistry (data not shown). The prostate of NeoDES Sprague–Dawley rat at 10 mo age normally showed signs of inflammation characterized by increased expression and deposition of extracellular matrix components, such as fibronectin and collagen, and a large amount of emerged lymphocytes in the tissues (Fig. 2A). The epithelial dysplasia was sporadically observed, which was indicated by loss of normal cellular organization forming a multilayered and disordered structure instead. Furthermore, the morphology of the nuclei was changed (Fig. 2B). The dysplastic lesions morphologically resemble alterations seen earlier in NeoDES rats, which is close to prostatic intraepithelia neoplasia (PIN), a precursor lesion of human adenocarcinoma.

Expression of COX-2 and BAX in Vas Deferens

At no point up to 10 mo were there any significant differences of COX-2 immune staining between control and NeoDES Sprague–Dawley rats in distal end of vas deferens, a site of high constitutive expression. COX-2 disappeared at 15 mo in the NeoDES rat, while COX-2 remained expressed in the distal end of the vas deferens in the control rats at the same age (Fig. 3A). BAX, one of male reproductive markers for sperm quality, was also detected in the sperm and endothelium of vas deferens of the same rats (Fig. 3B), which both indicate that the aged control rats were fertile.

HMR Had No Effect on COX-2 Expression in RAW264.7 Cell Line

Analysis with Western blots revealed that COX-2 expression in LPS-induced RAW264.7 cells treated with different concentrations of HMR kept unchanged whether there was simultaneous addition of LPS and HMR (Fig. 4A) as well as when HMR was added 5 h prior to LPS (Fig. 4B). This further provides the evidence that anti-tumor roles of HMR are probably related to enterolactone, the metabolites of HMR by intestinal microflora.

Discussion

To our knowledge, this is the first time that we demonstrated the long-term effects of estrogen on COX-2 expression that COX-2 was selectively repressed by neonatal estrogenization and that COX-1 remained intact in the distal end of the vas deferens in aged neonatally estrogenized rats. We also demonstrated that COX-2 remained normal in untreated aged rats. Correspondingly, it is known that COX-2 is not present in the vas deferens of a human fetus at 9 wk, but starts to be expressed at 11 wk *(26)*. It indicates there is a mechanism of on and off for COX-2 expression. COX-2 is androgen dependent because castration depletes COX-2, but can be restored with androgen replacement. In this case, neonatal estrogenization likely led to the reduction of androgen, or testosterone. The progress of COX-2 depletion is slow and the complete depletion occurred at age of 15 mo, so COX-2 is, in general, present in the NeoDES rat at the age of 10 mo *(23)*, or longer, but no longer than 15 mo. The disappearance of COX-2 in neonatal estrogenized aged rats may suggest that the early accumulation of estrogen impairs sexual function and that COX-1 has a different signaling pathway and different transcription factors, which were not affected by estrogen. Thus, COX-1 can possibly play COX-2's role to sustain sexual function. This is probably one of the mechanisms of how male reproductive capability can be sustained even though it becomes reduced with age. PGE_2 has been regarded as major effective molecule that influences the sperm counts in semen *(27)*. The newly discovered PGE_2 synthase-1 (PGES-1) has been shown to be present in the epithelial cells of the vas deferens as well, and the binding of cytosolic PGES (cPGES-1) with COX-1 has been observed, and microsomal PGES (mPGES) has also been observed associated with COX-2 in the uterus of monkeys *(28)*.

We have studied the effect of neonatal estrogenization on the responsiveness of COX-2 expression in reproductive organs, especially in the prostate and the vas deferens. Estrogen DES is one of the most potent carcinogenic estrogens. NeoDES rats normally develop severe inflammation and dysplasia after "middle age." COX-2 was not detected in the sites of inflammation or dysplasia of prostate of neonatally estrogenized aged rats by immunohistochemistry. One possible explanation is that the integration of genomic action remains in these samples. Our results of COX-2 expression are consistent with a similar experiment wherein the transcription level of COX-2 was not increased when male rats were exposed to estradiol *(29)*. However, this does not exclude the possibility of COX-2 induction as was

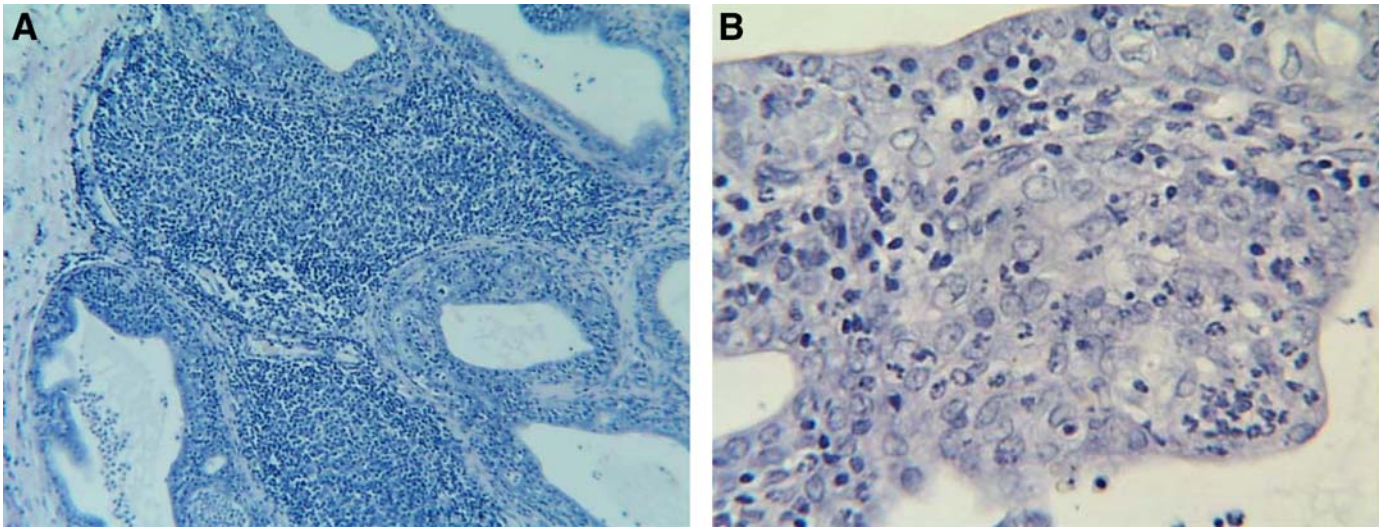

Fig. 2. (**A**) A representive image of inflamed rat prostate in estrogen caused prostatitis, mainly indicated by abound emerged lymphocytes by H&E stain, original magnification 200×. (**B**) A representive image of loss of normal organization (glandular architecture), aberration of nuclear (size, shape, staining), original magnification 400×.

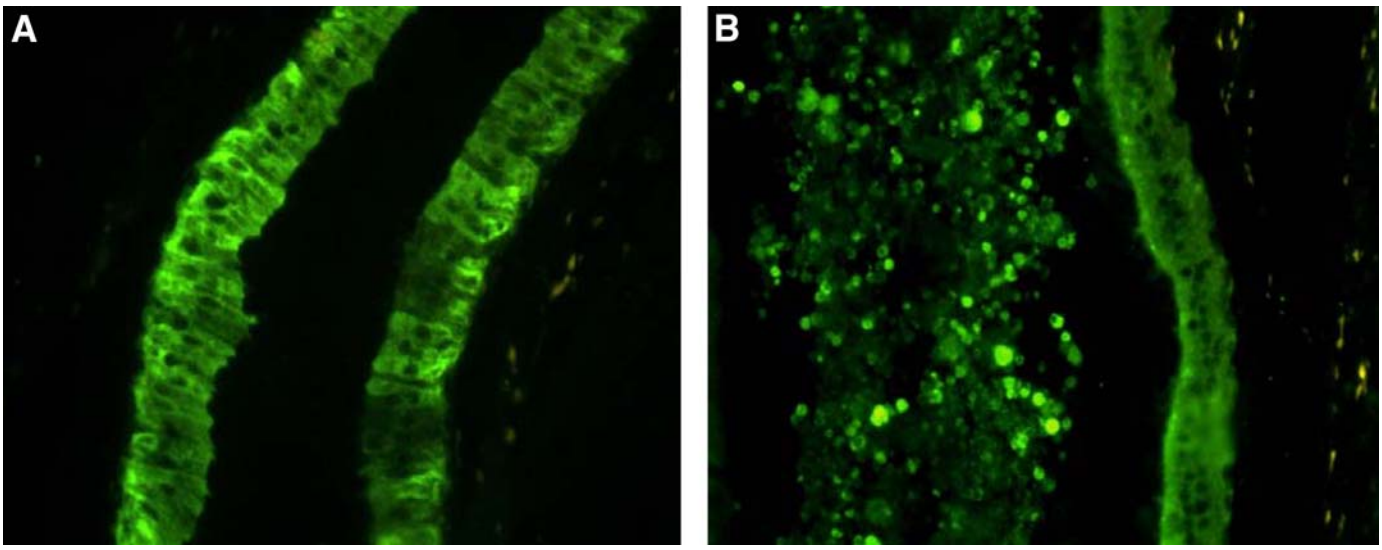

Fig. 3. (**A**) A representive image of distal vas deferens of 15-mo-old normal SD rat, shows that COX-2 is intensively expressed in the nuclear membrane, and extends to cytosol, original magnification 400X. (**B**) A representive image of vas deferens of 15-mo-old normal SD rat, shows that BAX is expressed in some of sperms and endothelium of vas deferens (200×).

reported in the case of the estrogen-dependent induction of COX-2 in the dog prostate *(22)*. Estrogen was believed to have cardiovascular protective effects with some risk of carcinogenesis and may not promote COX-2 expression under a threshold dosage without other mutations. It is likely that COX-2 expression is only indirectly controlled by estrogen even though estrogen receptors are involved in COX-2 induction *(30)*. However, estrogen was also reported to increase COX-2 expression in vascular endothelial cells in vitro and rapidly stimulated those cells to secrete PGI_2 and PGE_2 *(31)*. Atheroprotective effects of prostacyclin in female mice are mediated, at least in part, through estrogen receptor alpha induced upregulation of COX-2, and orientally increased PGI_2 *(32)*. Estrogen attenuates ischemia-reperfusion-related cerebral injury in experimental animal models of stroke. Similar evidence showed that estrogen suppresses the IL-1 mediated COX-2 dependent PGE_2, but not prostacyclin PGI_2. In contrast, commercially available COX-2 selective inhibitors have no such selection. This is one of the strongest pieces of evidence of increased cardiovascular events associated with COX-2 inhibitors *(6,33,34)*. The converted action mechanism of COX-2's downstream products by estrogen seems to be attributed to cardiovascular disease (CVD) prevention. The present study does not exclude the possibility that COX-2 expression would be involved in the progression of dysplastic lesions, as elevated levels of COX-2 could be confined to those regions. However, we do not really know if COX-2 is transiently induced in the

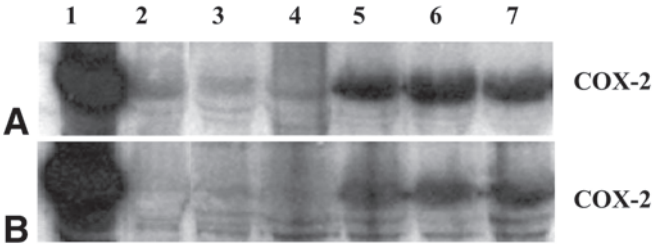

Fig. 4. The representive Western blot for COX-2 detection. (1) LPS 10 μg/mL, HMR 0.0 m*M*. (2) LPS 0.0 μg/mL, HMR 0.0 m*M*. (3) 0.1 μg/mL LPS, 0.01 m*M* HMR. (4) LPS 1.0 μg/mL and HMR 0.1 m*M*. (5) LPS 2.0 μg/mL and HMR 1 m*M*. (6) LPS 2.0 μg/mL and HMR 5.0 m*M*. (7) LPS 2.0 μg/mL and HMR 10 m*M*. (**A**) Simultaneously addition of LPS and HMR. (**B**) HMR addition 5 h earlier than LPS.

certain stage of the development of dysplastic lesions. COX-2 is a relatively transiently expressing gene, for example, COX-2 expression in endometrium is affected with menstrual cycle and in early pregnancy *(35)*. Furthermore, recent findings have shown that COX-2 expression is organ and cell type specific, as well as being species specific *(36)*.

Analyzed by Western blots, COX-2 did not respond to HMR in LPS-induced RAW264.7 cells in transcription and translation levels at different concentrations of LPS and HMR, or with different timing points of the induction and inhibition. This indicated that HMR does not affect COX-2 expression. HMRs anti-tumour mechanism is probably independent of COX-2 *(37)*. Estrogen has the tendency to suppress or induce COX-2 expression, where the suppression of COX-2 leads to conversion of PGI_2 as an end product with cardioprotective effects. Conversely, androgen mainly increases COX-2 expression to form PGE_2, a product that normally causes vasoconstriction. Thus, the balance between estrogen and androgren is carefully controlled in nature. It has recently been revealed in clinical trials that COX-2 chemotherapy for arthritis and colon cancer had increased myocardial infarction and stroke leading to a setback of COX-2 inhibitor development. Thus we believe that, even if HMR is not a COX-2 regulator, and its anti-tumour mechanism remains largely elusive, screening estrogen-like compounds as COX-2 inhibitor or regulator can be a potential preventive chemotherapy for cancers sparing the CVD risk. BAX is largely independent from estrogen, but has a significant role in testicular germ cell apoptosis, which has been regarded as normal human spermatogenesis in testes. The strong expression of BAX in endothelium of vas deferens and sperm indicate the fertility of the aged rats. However, the correlations for COX-2 and estrogen or BAX and sperm quality are needed to be studied further.

Materials and Methods

Animals

All Sprague–Dawley rats (SD) 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 experiments involving estrogen's influence on COX-2 expression in sex-related organs, the male rats were treated subcutaneously with a daily dose of 10 μg of diethylstilbestrol (DES) *(24)* on d 1–5 of postnatal life. For long-term effects of DES on COX-2 expression in neonatally DES estrogenized (NeoDES) rats, test and control groups of rats were followed for 15 mo, and isoflavone-free synthetic and balanced C-1000 diet (Altromin GmbH, Lage, Germany) were provided. Six of NeoDES rats and normal rats for control were used. Test protocols were approved by the Committee of the Laboratory Animal Center of Turku University.

Antibodies

Affinity-purified rabbit anti-rat COX-1 monoclonal and COX-2 polyclonal antibodies and the COX-2 antigenic block-ing peptide were purchased from Cayman Chemical (Ann Arbor, MI, USA). Anti-BAX polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Biotin-conjugated goat anti-rabbit or anti-mouse immunoglobin was used as the secondary antibody. Horseradish peroxidase (HRP)–streptavidin and diaminobenzidine were purchased from Zymed (Burlingame, CA, USA). FITC-conjugated goat anti-rabbit IgG was obtained from Molecular Probe (Eugene, Oregon, USA).

Western Blotting

For immunodetection, the tissue specimens of the one-quarter most distal ends and the most proximal ends of three rats were placed in the protein extraction buffer (10 m*M* CHAPS, 2 m*M* EDTA, 4 m*M* iodoacetate, 100 μ*M* PMSF in PBS, pH 7.4) in a wt/vol ratio of approx 200 mg/mL and gently homogenized. The protein concentration in the supernatant was determined using Bio-Rad assays with bovine serum albumin as a standard. A total of 100 μg protein was resolved in 12% SDS-PAGE. The SDS-PAGE resolved proteins were transferred onto nitrocellulose membrane (Bio-Rad) using a Bio-Rad protein transfer apparatus. Immunodetection of COX-2 was carried out using 1:2500 dilution of rabbit anti-COX-2 (murine) polyclonal antibody (Cayman Chemical). The secondary antibody was biotin-goat anti-rabbit Ig G (1:5000), the complex was detected with horseradish peroxidase–streptavidin (Zymed). Peroxidase activity was visualized using ECL fluorescence reagents (Amersham Pharmacia, Buckinghamshire, UK), and exposed on highly sensitive BioMax MS-1 film (Kodak, New York, USA). The same blot used to detect COX-2 was stripped with beta-mercaptoethanol-SDS solution, and was reprobed with COX-1 antibody. COX-2 expression in HMR-treated, LPS-induced RAW264.7 cells were detected with same method except the loading of protein that was 50 μg/well.

Immunohistochemistry and Immunofluorescence Histochemistry

Formalin-fixed and paraffin-embedded samples were prepared according to standard protocol *(25)*. The sections were deparaffinized in xylene and then hydrated. The slides were then boiled in PBS buffer in a microwave oven for 10 min to retrieve the antigen. The endogenous peroxidase was depleted by 3% H_2O_2 for 30 min. After two washes, 10% normal goat serum was used to block nonspecific antigens. The sections were incubated with primary antibody 1:400 diluted in 3% bovine serum albumin in PBS for 4 h at room temperature. After two more washes, the specimens were incubated with 1:800 dilution of the secondary antibody, biotin-conjugated goat anti-rabbit IgG at room temperature for 30 min, and then with 1:800 dilution of the HRP-streptavidin for 15 min at room temperature. The specimens were exposed to brown diaminobenzidine for 3 min and counterstained with hematoxylin, dehydrated in ethanol and xylene, and finally mounted under coverslips with permanent Mountex (BDH Laboratory, England, UK). For immunofluorescence histochemistry, the secondary antibody conjugated with FITC was used *(25)*.

HMR on COX-2 Expression in RAW264.7 Cell Lines

7-Hydroxymatairesinol (HMR), a dibenzylbutyrolactone plant lignan containing a 2,3-dibenzylbutane skeleton, is chemically a close relative to matairesinol, a lignan present at high concentration in flaxseed. HMR is found at relatively high concentrations in the heartwood of branches and knots of Norway spruce (*Picea abies*) trees. In addition, detectable levels are present in sesame seeds. HMR is a near white powder. Because of the chiral carbon at position 7, HMR is composed of two stereoisomers (allo-HMR and HMR) that are present in an approximate 1:9 ratio within the HMR potassium acetate preparation. HMR and its potassium acetate complex are slightly soluble in water and soluble in acetone, aqueous ethanol, methanol, acetonitrile, DMSO, PEG, and corn oil. COX-2 can be rapidly induced in macrophage cells by lipopolysaccharide (LPS). A mouse macrophage cell line, RAW264.7, was grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/mL penicillin, and 100 µg/mL streptomycin in 5% CO_2 at 37°C. Exponentially growing cells were used in all experiments. The cells were incubated with HMR, or LPS induced 3 d after seeding. HMR was dissolved in ethanol, and diluted in PBS, the final volume of ethanol was less than 1% (v/v). The final HMR concentrations were 0.0 m*M*, 0.01 m*M*, 0.1 m*M*, 5.0 m*M*, and 10 m*M*, while LPS final concentrations was 0.0, 1, 2, and 10 µg/mL, which was applied to analyze the inhibition of COX-2 expression in RAW264.7 cells before or after addition of LPS. The cells were collected for Western blotting analysis with a spade-like tool after 24 h incubation.

Image Analysis

The exposed X-ray films of Western blotting filters were transferred into computer using software KDS1D2.0, DC120 image program system from KODAK Digital Science package and saved in TIFF format. Immunohistochemistry and immunofluorescence images were taken by a Zeiss research microscope. The images were directly transmitted to a computer. The relative optical density (ROD) was measured and analyzed with Analysis Image System (AIS) version 4.0 software (AIS, Ontario, Canada). All images were edited using Adobe Photoshop 6.01 without adding any artifacts.

Statistical Analysis

Number of each experiment, $n = 2–4$, statistical significance was calculated with Student's t test of Excel 2002, and values of $p < 0.05$ were considered as statistically significant. All results are shown as means ± SE.

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