Meeting report

New functions for Cox-2 in health and disease: Report of "The Third International Workshop on Cox-2", Ka'upulehu, Kona, Hawaii, USA, 30 August to 2 September 1999

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Introduction

A year ago, the second workshop in this series presented early clinical experiences with a new group of inhibitors of Cox-2. Two of these, celecoxib and rofecoxib, are now marketed in some 30 countries worldwide. Just like last year's meeting, this one was organised by Peter Lipsky, just appointed to the post of Scientific Director of the National Institute of Musculoskeletal and Skin Diseases at NIH, Bethesda, MD, USA, together with an international study group. This workshop was again very informative, presenting the latest research results from leading experts in several fields.

Structure and function of Cox enzymes

Although the crystal structure of the Cox enzymes has been known in principle for a few years, several functionally important features still remain to be explored. Ravi Kurumbail, working at Searle in St Louis, MO, reported new crystallographic details from an improved model with a resolution of 2.15 Å. The Cox-2 enzymatic channel is wider than that of Cox-1, and it has an open side pocket where the sulphonamide group of the selective inhibitors can bind. In the region corresponding to the side pocket the human isoenzymes have four amino acid differences, the most essential being the 523 position, where Cox-1 has a bulky tyrosine and Cox-2 a valine, which requires less space. In Cox-2 Kurumbail could show the formation of five different hydrogen bonds between the selective inhibitors and the pocket, leading to strong binding. This reaction requires some time, however, and explains the time-dependent inhibition observed when testing Cox-2 inhibition with selective inhibitors. He demonstrated that site-directed mutations in the pocket region resulted in weaker binding and fewer hydrogen bonds. He also showed results with a second generation Cox-2 inhibitor now in phase II/III of development, called valecoxib. This compound has a Cox-2 selectivity that is more than 10–20 times that of celecoxib, marketed as Celebrex in the US. After binding the inhibitors are dissociated and the "off" speed determines the degree of selectivity. All these experiments are done in enzyme-substrate systems, and their relevance for *in vivo* conditions is not proven.

William Smith (Michigan State University, East Lansing, MI, USA) reported work on the interaction of arachidonic and related fatty acids with the Cox enzymes. He showed that substitution of Arg120 with glutamine in Cox-1 reduced its potency by a factor of 1000, whereas the same substitution in Cox-2 did not affect enzyme activity. Arg120 anchors arachidonic acid in the Cox-1 channel, but does not have this function in Cox-2. Eighteen further sites in Cox-1 bind arachidonic acid, and serve to optimize its position. Three products are formed from arachidonic acid: prostaglandin (Pg)G_2, 11-HETE and 15-HETE. This is evidence that arachidonic acid can bind in at least three different shapes. The usually depicted hairpin shape is less likely to occur than an L-shape. Smith also addressed the interesting question of how the metabolized prostanoid leaves the enzyme, and he favored escape through a bent continuation of the catalytic channel, for which he showed crystallographic evidence.

Cellular regulation of Cox synthesis

Harvey Herschman (University of California, Los Angeles, CA, USA) reported recent progress in the understanding of the molecular basis for activation of the Cox-2 gene, which he cloned in 1991. It was shown that c-Jun but not Erk was involved. In other experiments he used osteoblasts from Cox-2-mutated animals transfected with the promoter element. Activation involved always the CRE region of the promoter but the IL-6 site was also involved. This was tested with a number of activators, such as bFGF (basic fibroblast growth factor), PDGF (platelet-derived growth factor), PgG_2, tumour necrosis factor

(TNF)α and IL-1β. The fundamental question of why the cells have two Cox isozymes is still enigmatic. Thus mast cells where Cox-2 is inhibited produce no PgE_2, despite having functional Cox-1. Another line of experiments in progress has aimed at visualizing Cox-2 activation *in vivo* in rodents using positron emission tomography (PET) scanning. For this to work it was necessary to improve resolution from 5 mm to 1.8 mm. This had been achieved by using Fluorogancyclivir, and Herschman hoped to report results to this workshop next year.

Timothy Hla (University of Connecticut School of Medicine, Farmington, CT, USA) focused on Cox-2 regulation at the 3′ end of the gene. This area has 22 repeats of an AUUUA motif. These repeats determine the stability of the mRNA. Glucocorticoids destabilize and IL-1 stabilizes Cox-2 mRNA. Overexpression of Cox-2, as it occurs in the rheumatoid synovium, leads to angiogenesis by complex mechanisms involving both Pg-dependent and other pathways and mediated by G-protein-coupled nuclear PPAR (peroxisome proliferator-activated receptor) family of receptors. Thus overexpression of Cox-2 is a critical step in the pathogenesis of tissue damage in rheumatoid arthritis (RA). Similar mechanisms are involved in invasiveness of malignant tumours.

The mechanism by which Cox-2 might be induced during tumorigenesis was further investigated by Dan Beauchamp (Vanderbilt University Medical Center, Nashville, TN, USA). The key role of the oncogene Ras and of TGFβ1 was demonstrated. TGFβ1 is synergistic with Ras, and stabilizes Cox-2 mRNA, resulting in 20–50-fold increases in induction, compared to only 3–5-fold increases by each stimulator. A paradox is that, in tumour cells, TGFβ1 fails to inhibit cell growth. One effect of the increased Pg production in tumour cells is plasmin activation, which also requires urokinase plasminogen activator (uPA). Celecoxib has been shown to inhibit uPA. This illustrates how elucidation of complex cellular events can help in devising therapeutic targets.

Biology of arachidonic acid metabolites

Two talks dealt with biological effects of the arachidonic acid metabolite PGE_2. Richard M Breyer (Vanderbilt University Medical Center, Nashville, TN, USA) worked with PG receptors. Four receptors have been described, EPs 1–4. These mediate different partly overlapping biological functions. The function of EP2 was studied by using mice with the EP2 gene knocked out ($EP^{-/-}$). Such mice develop normally, but have a slightly elevated blood pressure and produce small litters. They also develop hypertension when exposed to high salt intake, which does not occur in wild-type mice, and they fail to react with hypotension to PGE_2. The small litter size is linked to a pre-implantation defect. These results indicate that EP2 could be a putative target for new anti-hypertensive drug treatment. Thomas M Coffman (Duke University Medical Center, Durham, NC, USA) further explored the different tissue distribution of these receptors and how the receptors explain the variety of biological functions of PGE_2. These experiments used the technique of mutating one or more of the receptors and differing but partly overlapping functions emerged.

Cox-2 expression in the eye

Kay Brune (University of Erlangen, Germany) is studying the role of Cox-2 in the eye. He reminded us of the pressure-lowering effect of topical PGs, and of the fact that 30% of patients on chronic glucocorticoid therapy develop elevated intraocular pressure. In the normal eye Cox-2 is strongly expressed in the ciliary body and the enzyme is mostly membrane-associated. Patients with primary open angle glaucoma (POAG) have subnormal concentrations of PG in the aqueous humour. In familial glaucoma a gene defect was located to chromosome 1, to which the Cox-2 gene is also mapped. No Cox-2 gene defect has, however, been detected in such individuals. Administration of Celebrex to two normal individuals resulted in moderate but significant increases of the intraocular pressure, and in less diurnal variation. In the discussion Johannes Bijlsma (Utrecht University, The Netherlands) asked an important question: why do patients treated with classical NSAIDs (nonsteroidal anti-inflammatory drugs) not develop glaucoma. Brune pointed out that, due to the non-acidic nature of the new Cox-2 inhibitors, which is in contrast to the acidic nature of NSAIDs, the Cox-2 drugs may not penetrate into inflammatory tissue with the same affinity as the NSAIDs. This may cause a weaker antiinflammatory action. The involvement of iNOS (inducible nitric oxide synthase) was also of possible importance.

Cox-2 and pregnancy

Three speakers addressed the role of Cox-2 in ovulation and pregnancy. Kumano Dey (University of Kansas Medical Center, Kansas City, KS, USA), who co-authored the *Cell* paper on Cox-2 knockout mice in 1997 (which among other defects showed infertility in females), has explored the role of Cox-2 in baboon, skunk, pig and mink embryos. There is a uniformity in so far as Cox-2 is involved in ovulation and implantation of the egg and not least in decidualization. In Cox-2-deficient mice it was also shown that Cox-2-derived PGI_2 was essential for this process. PGI_2 signals via the PPARδ receptor family. Angiogenesis is involved in the process.

Jean Sirois (University of Montreal, Saint-Hyacinthe, Canada) focused on the molecular control of Cox-2 expression in mammalian ovulation. The so-called Ebox in the promoter region is essential and ovulation occurs 10 h after Cox-2 expression in all species. There is a delay in expression, however, which differs markedly between the rat (2–4 h), bovine (18 h), and equine (30 h) follicles,

suggesting that Cox-2 plays a role in the control of the mammalian ovulatory clock.

Finally, James M Trzaskos (Dupont Pharmaceuticals, Wilmington, DE, USA) studied the intrauterine kinases after induced decidualization and showed that p38 and not ERK was necessary for Cox-2 expression in the deeper stromal cells. Thus the physiologic process of decidualization is now known to involve p38, Cox-2, prostacyclin and PPARδ.

Cox-2 and the kidney

The role of Cox-2 in the kidney was addressed by two investigators, Raymond C Harris (Vanderbilt University School of Medicine, Nashville, TN, USA) and Marina Noris (Mario Negri Institute for Pharmacological Research). Cox-2 expression in the macula densa and its vicinity is of interest in physiology and pathophysiology as addressed by Harris. He proposed a working model for macula densa regulatory interactions where neuronal (n)NOS, renin, angiotensin II and Cox-2 are the main players. Partial renal ablation leads to increased expression of Cox-2 and renin; nNOS also increases Cox-2 and renin. Renin inhibits angiotensin II, which attenuates Cox-2 expression and augments renin. Captopril dramatically increases renin formation and plasma renin levels and celecoxib reduces this effect. High salt intake induces Cox-2 expression in renal papillary cells. This increased expression may lead to renal damage, and both Cox-2 inhibition and p38 inhibition have beneficial effects. Thromboxane (TX) A_2 is a likely mediator inducing increased collagen deposition and fibrosis.

Noris studies the lupus nephritis model of NZB/W mice who develop renal disease in a predictable fashion. Disease onset is accompanied by increased Cox-2 expression and TX formation. She presented data indicating that Cox-2 inhibition as well as TXA_2 antagonists could ameliorate or postpone renal damage in these models. These results are not uncontroversial, as Noris pointed out. One caveat is that Cox-2 inhibitors also inhibit the formation of prostacyclin, which may compromise renal circulation. Another is that TX antagonists do not work in practice. Time of administration may be of critical importance. The subject will no doubt continue to be of central interest in the near future.

Cox-2 in the lung

In the lung, prostanoids are important in conditions such as pulmonary hypertension and idiopathic pulmonary fibrosis. Continuos infusion of a prostacyclin analogue is now standard therapy in severe pulmonary hypertension. Marc Peters-Golden (University of Michigan Medical Center, Ann Arbor, MI, USA) reviewed the field and concluded that Cox-2 expression in the lung may have more beneficial than adverse effects.

Cox-2 in the pancreas

R Paul Robertson (University of Washington, Seattle, WA, USA) dealt with the pancreas, where Cox-2 is constitutively expressed in the β-cells. Interleukin (IL)-1 is known to be important in the pathogenesis of diabetes and its effect seems to be mediated by PGE_2. Selective inhibitors of Cox-2 can partly ameliorate the deleterious effects of IL-1 on β-cell function, indicating that it is mediated by prostanoids via Cox-2.

Cox-2 gastrointestinal tolerance

A novel role for Cox-2 in the gut was proposed by William F Stenson (Washington University School of Medicine, St Louis, MO, USA). The gut is abundant with microorganisms of potentially harmful nature, yet no inflammatory reactions occur normally. This is sometimes termed 'tolerance'. To study the mechanism of this tolerance Stenson and his colleagues had utilized mice with ablated TCRα chains and transfected them with TCRα chains specific for the antigen hen egg-white lysozyme peptide 46–61. When fed this antigen the mice developed no symptoms. However if a classical NSAID or the Cox-2 selective celecoxib were administered concomitantly with the antigen, an inflammatory reaction resulting in villous atrophy and crypt proliferation was elicited. This could be overcome by administering PGE_2. It was concluded that the lamina propria mononuclear cells produce Cox-2 and the resulting prostanoids inhibit the activation of pathogenic T-cells in the gut wall. An argument against this hypothesis is the fact that exposure to nonselective NSAIDs, which inhibit both Cox isozymes, usually does not lead to inflammation.

MAP kinases as putative targets

An interesting approach was presented by Phyllis Whiteley (Roche Bioscience, Palo Alto, CA, USA). The stress-activated mitogen-activated protein (MAP) kinase p38 (here called MKp38) is involved in a signal transduction pathway leading to TNF, IL-1 and prostanoid formation. It is thus upstream from Cox-2. The inhibition of MKp38 showed similar levels of suppression of inflammation and pain as did Cox-2 inhibitors, but in addition MKp38 inhibition was effective in treating collagen-induced arthritis, which does not respond to Cox inhibition. MKp38 is an obvious therapeutic target that will be tested in the near future.

Clinical impact of Cox-2 inhibition

Relatively little new information on the two recently marketed selective Cox-2 inhibitors emerged. Gregory Bell (Merck & Co) presented rofecoxib experiences. Its efficacy was comparable to that of strong classical NSAIDs; gastrointestinal symptoms were close to but not identical to those induced by placebo. Discontinuation of therapy in trials was also intermediate. It is known that close to 5% of patients develop edema and this has led to careful monitoring of renal function. No change in glomerular filtra-

tion rate occurred over 12 months of exposure in clinical trials, mostly in osteoarthritis (OA). Radiographic data showed no difference between diclofenac and rofecoxib. There were no data on any influence on progression of spur formation in OA. Importantly, low dose aspirin for cardiovascular disease was excluded in these trials.

Celecoxib data were reported by John Fort (Searle, Skokie, IL, USA). This drug has been dispensed in 9 million prescriptions in the USA, corresponding to perhaps 30% of new NSAID prescriptions, so far without major reports regarding adverse reactions. The dose of 100 mg twice daily was not superior to 200 mg daily. It also appears that a dose of 400 mg daily was not more potent than 200 mg; however, the higher dose caused more adverse reactions.

Perry Halushka (Medical University of South Carolina, Charleston, SC, USA) drew attention to the potential risk of TX-related vascular occlusion after selective Cox-2 inhibition, and illustrated this with a couple of case reports. One patient suffering from scleroderma had developed pulmonary embolism after 2–4 doses of a Cox-2 inhibitor and her TXA_2 concentration was four times normal. Patients with an antiphospholipid syndrome may also be at risk, and vascular occlusions have been seen in connection with celecoxib therapy. On the other hand, patients with a tendency to bleed, such as those with hemophilia, have not been exposed to selective Cox-2 inhibitors.

Addressing the question of the cardiovascular safety of celecoxib, Robert Makuch (Yale University, New Haven, CT, USA) had analysed data in Searle's database of 13 000 patient records from 2–24 weeks of placebo controlled trials as well as records for 6000 patients in a two year open label trial of celecoxib safety. No increased risk for myocardial infarct or any serious vascular event emerged from this investigation.

Is Cox-2 expressed in platelets?

Karsten Schör (Heinrich Heine Universität, Düsseldorf, Germany) provoked the meeting by claiming that platelets — against all previous evidence — contained Cox-2. He showed western blots using three different monoclonal antisera to Cox-2. The majority of experts were sceptical.

Cost effectiveness

James Scheiman (University of Michigan Medical Center, Ann Arbor, MI, USA) addressed the burning question of cost effectiveness of the new drugs. Both misoprostol and omeprazole have been shown to lower but not eliminate the risk for serious gastrointestinal complications, but cost effectiveness only occurs if administration can be limited to high risk patients, and it is doubtful whether this ever can be achieved. Similar reasoning can be applied to the new Cox-2 inhibitors when used to treat pain.

Cox-isoenzymes and cancer

Cox-2 is expressed in several cancer forms and the striking effects of Cox-2 inhibition in experimental familial adenomatous polyposis (FAP) was reported at the meeting last year. Although Cox-2 is firmly linked to tumour pathogenesis, a contribution from Cox-1 cannot be excluded.

Luis García Rodríguez (Spanish Center for Pharmacoepidemiologic Research, Madrid, Spain) reported a large new study on the putative protective role of aspirin and NSAIDs in relation to dose and time of exposure. He had utilised data from the United Kingdom General Practice Research Database related to cases of colorectal adenoma and cancer in patients between the age of 40 and 79. Out of a population of 900 000, 1869 patients were identified with adenomas and 2002 with cancer, in the period January 1994 to September 1997. This corresponds to an incidence of 6.8 and 7.3 per 10 000 person years, respectively. The technique used was a prospective population based nested case control study. Long term users of NSAIDs were relatively protected. The age adjusted relative risks ranged between 0.4 and 1.0 and were on average 0.6. Protection was similar with all NSAIDs and occurred only after 12 months use or longer. Aspirin was in general less effective, and only if given in doses of 300 mg/day or higher. Glucocorticoids and paracetamol had no protective influence. It was estimated that 1000 patient years of exposure would prevent one case of cancer; this exposure would on the other hand induce 10 gastrointestinal bleeding events. Extensive search of the literature confirmed that aspirin in doses of 100 mg/day or less was not protective for gastrointestinal cancer (Michael Thun, American Cancer Society, Atlanta, GA, USA).

Subbaramaich K Dannenberg (Cornell University, Ithaca, NY, USA) studied the mechanism of tumour induction by the oncogene HER-2/neu, which is present in 20–30% of human breast cancer. Cells from 29 patients were transfected with HER-2/neu and half of them responded with 10-fold or more increase in Cox-2 expression. These cells also overexpressed the upstream c-Jun product and the MKp38 mentioned above. MKp38 phosphorylates c-Jun which then induces Cox-2 expression via the CRE site on the Cox-2 promoter.

Robert Langenbach (National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA) had studied skin tumour formation in mice with mutated Cox-1 and Cox-2 genes. Both deletions inhibited tumour formation but by different mechanisms.

Concluding remarks

This meeting was another impressive testimony to the exciting evolving biological significance of the cyclooxygenases. The impatient reader may wonder why there were not more data on clinical effects in rheumatological

situations. This is probably because the real positioning of the new agents will require longer periods of exposure. Safety data, with the exception of anecdotal reports to the contrary, so far seem encouraging. Tissue penetration of the non-acidic Cox-2 inhibitors may differ from that of classical NSAIDs, which may be of significance with regard to both efficacy and adverse reactions. Cox-2 inhibition may have a place in tumour prevention, in particular in the gastrointestinal system, but the data are still vague. No new information regarding the important field of central nervous system disease, in particular Alzheimer's, was given. This may, in the end, become the most important of all indications for Cox-2 inhibition, as the age groups at risk for Alzheimer's disease are growing at a frightening rate. The renal role of Cox-2 in health and disease is another important question, in particular whether Cox-2 inhibition may be beneficial in lupus nephritis. Will there be a fourth workshop? The preliminary commercial success as well as the identification of many fields of unfinished business do indicate that this would be a good idea.

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