Oxygenation by COX-2 (cyclo-oxygenase-2) of 3-HETE (3-hydroxyeicosatetraenoic acid), a fungal mimetic of arachidonic acid, produces a cascade of novel bioactive 3-hydroxyeicosanoids

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Cyclo-oxygenases-1/2 (COX-1/2) catalyse the oxygenation of AA (arachidonic acid) and related polyunsaturated fatty acids to endoperoxide precursors of prostanooids. COX-1 is referred to as a constitutive enzyme involved in haemostasis, whereas COX-2 is an inducible enzyme expressed in inflammatory diseases and cancer. The fungus *Dipodascopsis uninucleata* has been shown by us to convert exogenous AA into 3(*R*)-HETE [3(*R*)-hydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid]. 3*R*-HETE is stereochemically identical with AA, except that a hydroxy group is attached at its C-3 position. Molecular modelling studies with 3-HETE and COX-1/2 revealed a similar enzyme–substrate structure as reported for AA and COX-1/2. Here, we report that 3-HETE is an appropriate substrate for COX-1 and -2, albeit with a lower activity of oxygenation than AA. Oxygenation of 3-HETE by COX-2 produced a novel cascade of 3-hydroxyeicosanoids, as identified with EI (electron impact)–GC–MS, LC–MS–ES (electrospray) and LC–MS–API (atmospheric pressure ionization) methods. Evidence for *in vitro* production of 3-hydroxy-PGE₂ (3-hydroxy-prostaglandin E₂) was obtained upon infection of HeLa cells

with *Candida albicans* at an MOI (multiplicity of infection) of 100. Analogous to interaction of AA and aspirin-treated COX-2, 3-HETE was transformed by acetylated COX-2 to 3,15-di-HETE (3,15-dihydroxy-HETE), whereby C-15 showed the (*R*)-stereochemistry. 3-Hydroxy-PGs are potent biologically active compounds. Thus 3-hydroxy-PGE₂ induced interleukin-6 gene expression via the EP₃ receptor (PGE₂ receptor 3) in A549 cells, and raised cAMP levels via the EP₄ receptor in Jurkat cells. Moreover, 3*R*,15*S*-di-HETE triggered the opening of the K⁺ channel in HTM (human trabecular meshwork) cells, as measured by the patch-clamp technique. Since many fatty acid disorders are associated with an ‘escape’ of 3-hydroxy fatty acids from the β -oxidation cycle, the production of 3-hydroxyeicosanoids may be critical in modulation of effects of endogenously produced eicosanoids.

Key words: cyclo-oxygenase 2 (COX-2), 3-hydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid (3-HETE), 3-hydroxyeicosanoid, interleukin 6 (IL-6), K⁺ channel.

INTRODUCTION

More than a decade ago, we, together with Dr Kock’s group in South Africa, discovered that a South African fungus of the Lipomycetaceae family, called *Dipodascopsis uninucleata*, produced a novel metabolite of AA (arachidonic acid), 3*R*-HETE [3(*R*)-hydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid], when fed with exogenous AA. The compound carried a hydroxy group at C-3 position [1]. Unlike lipoxygenase-derived eicosanoids occurring in mammalian and plant cells, such as 5-, 9-, 11-, 12-, and 15-HETEs, 3*R*-HETE resulted from an incomplete β -oxidation of AA [2,3]. Without utilizing any exogenous substrate, *D. uninucleata* was capable of synthesizing a 3-hydroxy fatty acid, identified as 3*R*-HTDE (3*R*-hydroxy-5Z,8Z-tetradecadienoic acid), from the endogenous linoleic acid after 3 cycles of β -oxidation [2]. Formation of 3*R*-HTDE was also observed in AA-fed *Mucor genevensis*, whereby AA underwent a retroconversion into linoleic acid prior to β -oxidation [4]. Interestingly, the formation of 3*R*-HETE was suppressed by aspirin. As salicylates

also suppressed 3*R*-HETE formation, COX (cyclo-oxygenase)-mediated formation of 3*R*-HETE could be ruled out. Since polyclonal antibodies raised against 3*R*-HETE cross-reacted with all 3-hydroxylated fatty acids [5], we termed all short- or long-chain 3-hydroxy fatty acids, in general, as 3-hydroxyoxylipins. Moreover, we uncovered 3-hydroxyoxylipins of different chain lengths in many lipomycetaceous species, such as *Lipomyces doorenjongii*, *Lipomyces yarrowi*, *Lipomyces starkeyi* etc. [6]. Immunofluorescence microscopic studies of the life cycle of *D. uninucleata* revealed that 3-hydroxyoxylipins serve as growth regulators during the sexual phase [5], and that aspirin prevented the onset of the reproductive cycle. 3-Hydroxyoxylipins have also been found to be associated with the ascospore ornamentation in *D. uninucleata* [7] and as a flocculating agent in *Saccharomyces cerevisiae* [8]. Recently, we showed the presence of 3-hydroxyoxylipin, identified as 3,18-di-HETE (3,18-dihydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid), in *Candida albicans* isolated from the vaginas of patients suffering from recurrent candidosis [9,10].

Abbreviations used: AA, arachidonic acid; 2-AG, 2-arachidonoylglycerol; API, atmospheric pressure ionization; COX, cyclo-oxygenase; ACD, AA–COX complex; BSTFA, bis-(trimethylsilyl)-trifluoroacetamide; DMEM, Dulbecco’s modified Eagle’s medium; EI, electron impact; ES, electrospray; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 3(*R*)-HETE, 3(*R*)-hydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid; 3*R*-HTDE, 3*R*-hydroxy-5Z,8Z-tetradecadienoic acid; 3,15-di-HETE, etc., 3,15-dihydroxy-HETE, etc; HTM, human trabecular meshwork; HTR, 3*R*-HETE when in a complex with COX; HTS, 3*S*-HETE when in a complex with COX; IBMX, isobutylmethylxanthine; IL-6, interleukin 6; LC, liquid chromatography; LDH, lactate dehydrogenase; MOI, multiplicity of infection; PG, prostaglandin; EP_{1/2/3/4}, PGE₂ receptor 1, 2, 3 or 4 respectively; RP, reverse phase; RT-PCR, reverse transcriptase-PCR; TCA, trichloroacetic acid; TMS, trimethylsilyl.

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COX-1 and COX-2 [also known as PG (prostaglandin) endoperoxide synthases 1 and 2] have been shown to react with AA consecutively via two distinct activities: (i) an oxygenase activity, which is responsible for oxygenation and cyclization of AA within the COX active site to produce PGG₂; and (ii) a peroxidase activity, which causes reduction of the released PGG₂ to PGH₂ at the peroxidase active site [11]. PGH₂ is the essential precursor of various eicosanoids, including PGs, thromboxane and prostacyclin [12]. Although COX-1 and COX-2 have 60% identical amino acid sequences, the amino acids constituting the active sites of both isoforms are fully conserved [13] and their X-ray crystallographic structures are almost superimposable. COX-1 is a constitutive enzyme responsible for PG and thromboxane production in different cells and tissues to maintain homeostasis [14], whereas COX-2 is an inducible enzyme predominantly expressed in hyperalgesia, inflammation and cancer [15]. COX-2 is stimulated in response to various stimuli, including growth factors, cytokines and other lipid mediators [16–18]. Recently, we reported the selective up-regulation of COX-2 by *C. albicans* [19]. Homozygous COX-1 mutant mice exhibit, in general, reduced platelet aggregation, AA-induced inflammatory responses and indomethacin-induced gastric ulceration [20] as compared with homozygous COX-2 mutant mice, which have been shown to possess a normal inflammatory reaction to AA and phorbol esters, but develop severe nephropathy [21].

The distinct biochemical actions of COX-1 and COX-2 prompted several investigators to perform the structural analysis of both enzyme isoforms with the help of different COX inhibitors [22–25]. Recently, several groups have used site-directed mutagenesis and supplied significant information for a deeper understanding of AA–COX interactions. It was reported that Arg-120 in COX-1 or COX-2 binds to the carboxy group of AA [26–29], with subsequent abstraction of the 13-*pro-S* hydrogen by Tyr-385 in the oxygenation step [30,31]. Here, the ω -end of AA was positioned in a 'kinked' or 'L'-form above Ser-530, in a so-called 'top channel region' [32]. Moreover, it was found that the top channel does not function as a product exit route, and that the oxygenated substrate leaves the COX active site in a direction proximal to the membrane [32]. In a recent report, Marnett and co-workers [33] showed that 2-AG (2-arachidonoylglycerol) is also an appropriate substrate for COX-2, and that its metabolism is similar to that of AA.

Since the structure of 3-HETE, although basically different from that of mammalian lipooxygenase- or COX-derived monohydroxyeicosatetraenoic acids [2], is stereochemically almost identical with AA, we report in the present study that 3-HETE is an excellent substrate for COX-1 and COX-2, and leads to the formation of a cascade of novel bioactive 3-hydroxyeicosanoids. Based on the observations of biological actions of 3-hydroxy-PGE₂ in the present study, it can be proposed without reservation that these 3-hydroxyeicosanoids do exhibit strong biological activities comparable with that of endogenous eicosanoids in various organs and tissues. The reaction of 3-HETE with COX-2 was found to be slightly stronger than with COX-1, but weaker than that of AA with COX-2 and COX-1. Since the activities of 3*R*- and 3*S*-HETE isomers towards COXs hardly differed, we performed all experiments in the present study with racemic 3-HETE, unless stated otherwise.

MATERIALS AND METHODS

Reagents

Synthetic 3*R*- and 3*S*-HETEs were synthesized according to procedures described previously [34,35]. Ovine COX-1 (specific activity >40 000 units/mg) and COX-2 (specific activity

>3000 units/mg) and AA were purchased from Cayman Chemicals (Ann Arbor, MI, U.S.A.). Human COX-2 (specific activity >10 000 units/mg) and Gly-533 mutant COX-2 proteins (G533A, G533V and G533L) were kindly given by Dr Lawrence J. Marnett (Department of Biochemistry, Vanderbilt University Medical School, Nashville, TN, U.S.A.). [¹⁴C]AA (specific radioactivity 40–60 mCi, or 1.48–2.22 GBq/mmol) was supplied by NEN (Dreieich, Germany). Chemicals for making methyl- and silyl-derivatives of products obtained from 3-HETE–COX-1- and 3-HETE–COX-2 interactions were purchased from Sigma (Munich, Germany). Blocking peptides for EP receptors (PGE₂ receptors) were purchased from Cayman Chemicals. Primers for IL-6 (interleukin 6) gene analysis were synthesized by TIB Biomol (Berlin, Germany). All other chemicals were supplied by Sigma, unless otherwise stated.

Molecular modelling

The modelling of 3*R*- and 3*S*-HETE isomers with COX-1 and COX-2 was performed analogously to that of AA and COX enzymes, as described previously by others [22–29,32,33]. After conversion of Cartesian co-ordinates into internal co-ordinates, the C-3 hydroxy group was added to create 3*R*- and 3*S*-HETE using MODEL software [36]. HTR and HTS (3*R*-HETE and 3*S*-HETE when in complexes with COX respectively) were placed in active cavities of COX-1 and COX-2 with C-13 in close proximity (within 4 Å) from Tyr-385 using MOLMOL software [37]. The 3-HETEs were allowed to translate and rotate around the cavity axis, and simultaneously translate along the *x*, *y* and *z* directions. Thus we obtained four models: HTR–COX-2 (HTR2), HTS–COX-2 (HTS2), HTR–COX-1 (HTR1) and HTS–COX-1 (HTS1). Data obtained for complexes of AA with COX-1 (ACD1) and with COX-2 (ACD2) were used for purposes of comparison. In all models of 3-HETE isomers with COXs, Tyr-385 was found in close proximity to the C-13 group to facilitate the abstraction of the 13-*pro-S* hydrogen, which is a prerequisite for the oxygenation of AA to PGG₂.

Cell culturing

A549 lung adenocarcinoma cells were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% fetal calf serum. Cells were treated with trypsin, and 2 × 10⁵ cells were seeded into each well of a 24-well plate in the same medium and allowed to adhere overnight. Jurkat T-cells were cultivated in RPMI 1640 medium supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin.

COX activity

Oxygen consumption was measured at 37°C with a Strathkelvin Oxymeter 781 (Edinburgh, Scotland, U.K.) equipped with an electrode and a thermostat-controlled respiration chamber. Aliquots of COX enzymes (20 µg) were added to 100 mM Tris/HCl buffer, pH 8.0, containing 500 µM phenol and 1 µM haematin in a final volume of 1.2 ml. Oxygen uptake was triggered by the addition of 100 µM 3-HETE or AA. The initial reaction rate was determined from the linear part of the oxygen-uptake curve.

LDH (lactate dehydrogenase) activity assay

Following incubations of Jurkat and A549 cells with 3-hydroxy-PGE₂, supernatants were collected at various time points and assayed for LDH using a commercial kit (Sigma) according to the manufacturer's instructions. LDH released in the supernatant was used as an index of necrotic death.

Peroxidase activity assay

Peroxidase activity of wild-type and mutant COX-1 and COX-2 proteins was assayed spectrophotometrically (Shimadzu UV 160U; Kyoto, Japan) by measuring the initial rates of guaiacol oxidation at 436 nm. Aliquots of enzymes were added to 100 mM Tris/HCl, pH 8.0, containing 1 μ M haematin and 500 μ M guaiacol in a total volume of 1 ml. The reaction was started by the addition of 400 μ M H₂O₂.

Assay of COX products

Aliquots of COX enzymes containing 1 μ M haematin and 500 μ M phenol were incubated for 2 min at 37 °C with 100 μ M AA or 3-HETE. After termination of the reaction, products were extracted as described previously [38]. The extracted compounds were separated by RP (reverse-phase)-HPLC (Shimadzu) and analysed by GC–MS (Varian, Darmstadt, Germany).

For assaying production of PGE₂ in intact cells, HeLa cells were incubated with AA or 3-HETE, as described in the Figure legends. Products were extracted as described in [38], and subjected to HPLC and GC–MS analyses [39].

RP-HPLC system

HPLC separation was performed on a Shimadzu system equipped with two pumps coupled with a Novapack column (C₁₈ column, 250 mm $\times$ 4 mm, 4 μ m particle size; Macherey-Nagel, Germany) using a gradient system of acetonitrile/water/0.05 % acetic acid. The acetonitrile concentration was increased from an initial concentration of 20 % to 90 % (final) in 20 min, and was then kept stable for a further 15 min. The flow rate was 1.0 ml/min. UV detection was performed on a diode-array detector (Shimadzu SPD-M10A) using 195 nm for PGs, 235 nm for HETEs and 278 nm for PGB₂. The latter served as a standard. The fractions showing relevant peaks were collected and subjected to GC–MS or LC–MS analysis.

Chiral-phase HPLC analysis

Chiral-phase analysis of HETEs and dihydroxy-HETEs was performed on a Daicel Chiralpak AD column (Daicel, Nagoya, Japan; 4.6 mm $\times$ 250 mm, 5 μ m) using a solvent system of n-hexane:propan-2-ol (96:4, v/v). Authentic standards of 3*R*- and 3*S*-HETE, 11*R*- and 11*S*-HETE and 15*R*- and 15*S*-HETE were used as reference compounds.

GC–MS analysis

GC–MS analysis was performed on a Varian tandem apparatus SATURN 2000 as described previously [9]. Briefly, fractions containing relevant compounds in HPLC were dried under a nitrogen stream and reconstituted in methanol. The products were first methylated with freshly prepared diazomethane and then silylated with BSTFA [bis-(trimethylsilyl)-trifluoroacetamide] at 50 °C for 10 min. The sample was then reconstituted in n-hexane and analysed by GC–MS. For locating double bonds, methylated compounds were first hydrogenated by hydrogen gas on platinum oxide as a catalyst, and then silylated for GC–MS analysis.

LC–MS analysis

LC–MS analysis was performed on a Waters/Micromass Masslynx instrument using a Novapack C₁₈ column (4 μ m, 150 mm $\times$ 4 mm; Macherey-Nagel) using the gradient mobile phase described above at a flow rate of 50 μ l/min. API (atmospheric pressure ionization) was performed using nitrogen as the desolvation gas (flow rate 215 l/h) and cone gas (flow rate 212 l/h). The cone

temperature was kept at 520 °C; the cone voltage was kept at 46 V. The desolvation temperature was 450 °C, and the extractor voltage was 10 V. For LC–MS–ES (electrospray) experiments, the spray voltage was –2800 V, with the nebulizer kept at 0.6 ml/min. The orifice voltage was kept at –50 V, with a collisional offset potential of 15 eV. Argon was used in the collision cell at a pressure equivalent to 150 $\times$ 10¹³ molecules/cm².

RT (reverse transcriptase)-PCR

IL-6 cDNA was reverse-transcribed from the total RNA (1 μ g) extracted using the RNeasy extraction kit (Qiagen, Hilden, Germany) from A549 cells without or with pre-treatment of 50 nM PGE₂ or 3-hydroxy-PGE₂ for 24 h. PCR amplification was performed for 35 cycles using the human IL-6-gene-specific primers 5'-ATGACTTCCAAGCTGGCCGTGGCT-3' (sense) and 5'-GAAGAGCCCTCAGGCTGGACTG-3' (antisense). The cycling parameters were as follows: initial denaturation for 4 min at 95 °C; denaturation for 30 s at 94 °C; annealing for 60 s at 60 °C; extension for 60 s at 75 °C; and a final extension for 5 min at 75 °C. For normalization of IL-6 mRNA, amplification of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was performed using the specific primers 5'-TCGGAGTCAACGGATTGGTCGTA-3' (sense) and 5'-ATGGACTGTGGTCATGAGTCCTTC-3' (antisense). The PCR products were separated on a 2 % (w/v) agarose gel containing ethidium bromide, and visualized. Blocking peptide for the EP₃ receptor was used to selectively antagonize the IL-6 gene expression. PCR data are representative of two separate experiments.

The density of the bands of RT-PCR was measured by scanning densitometry, and expressed as normalized values relative to GAPDH. Values represent the means of two separate PCR experiments.

Assay of cAMP release by 3-hydroxy-PGE₂ from Jurkat cells

For cAMP assay, Jurkat T-cells without or with modulators for the EP₂ (agonist butaprost) or EP₄ (inhibitor L161982) receptor were pre-incubated in a six-well plate (10⁶ cells/well) with 50 μ M IBMX (isobutylmethylxanthine) for 30 min, and then with increasing concentrations of 3-hydroxy-PGE₂ and PGE₂ for 5 min. The reaction was stopped by addition of 100 μ l of 70 % TCA (trichloroacetic acid). Cells were then sonicated for 10 s, followed by centrifugation at 14 000 rev./min (17 500 g) for 10 min. The supernatant subsequently collected was extracted three times with 5 ml of ethyl ether (saturated previously with water) to remove TCA. Aliquots (50 μ l) of supernatant after dilution (1:10) with PBS were used for assay with a commercial ELISA kit (Cayman Chemicals), according to the manufacturer's instructions.

Measurement of current induced by 3-HETE, 3,15-di-HETE (3,15-dihydroxy-HETE) and AA using the patch-clamp technique in the HTM (human trabecular meshwork) of the eye

HTM cells were isolated from the tissue obtained from donor eyes or from eyes enucleated for melanoma. Cells were cultured in Dulbecco's modification of DMEM supplemented with 20 % fetal-calf serum. Patch-clamp recordings were made at room temperature, as described previously [40]. Briefly, a coverslip with cells was introduced under continuous superfusion with Ringer's solution at 37 °C under an inverted microscope (Axiovert 35; Zeiss, Oberkochen, Germany). Superfusion with calcium-free solution was achieved using EGTA solution containing 1 μ M thapsigargin, a Ca²⁺-ATPase inhibitor. Iberiotoxin (100 nM) was used as a blocker of 'maxi-K' channels (high-conductance, calcium-activated K⁺ channels). The input resistance was 4–5 Ω .

Figure 1 Conformational aspects of HTR and HTS with COX-2 and COX-1 in an EM model

For comparison, also shown are conformations of AA in complex with COX-1 designated as ACD1 in crystal structure IDIY, and AA in complex with COX-2 designated as ACD2 in crystal structure ICVU. Four models for 3-HETEs were thus obtained: HTR-COX-1 (HTR1), HTS-COX-1 (HTS1), HTR-COX-2 (HTR2) and HTS-COX-2 (HTS2). In all cases, atoms C-1, C-2 and C-3 of each model are overlapped using the juxtaposition algorithm of IMF1. Significant conformational differences between 3-HETEs-COX-1 complexes and 3-HETEs-COX-2 complexes can be seen.

Potentials were referenced to the bath, so that a negative potential corresponds to a negative pipette potential. Membrane currents with and without AA, 3-HETE, 3,15- or 3,9-di-HETE were recorded using an EPC-9 (Heka Electronic, Lambrecht, Germany) patch-clamp amplifier. TIDA software for Windows was employed for electrical stimulation, data storage and data processing. Values of increases in outward current are given as multiplication (fold) of the initial current level.

RESULTS

Molecular modelling

The energy minimization of complexes HTR-COX-2, HTS-COX-2, HTR-COX-1 and HTS-COX-1 revealed that HTR and HTS complexes with COX-2 have an energetically more favourable orientation than those with COX-1 (−5740 and −5719 kcal/mol for HTR-COX-2 and HTS-COX-2 compared with −5621 and −5463 kcal/mol for HTR-COX-1 and HTS-COX-1 respectively; 1 cal = 4.184 J). This energy is indicative of the stability of the complex.

As far as conformational features of 3-HETEs are concerned, Figures 1(A) and 1(B) describe HTR and HTS in complex with COX-1 and COX-2 in an EM model. For comparison, complexes of both enzymes with AA, i.e. ACD1 and ACD2, have also been shown. C-1, C-2 and C-3 atoms in complexes of HTR and HTS with COX-1 and COX-2 were found to overlap, as in the case of AA (Figures 1A and 1B). At the ω -end, however, significant differences between 3-HETE isomers and AA were observed.

Oxygenation of 3-HETE by purified COX-2 enzymes

Uptake of oxygen for the oxygenation of 3-HETE by ovine COX-1 and COX-2 was measured using a Clark electrode and compared with AA oxygenation. The rate of O_2 uptake for the 3-HETE/COX-2 reaction was a little bit higher than that for 3-HETE/COX-1, but much less than that observed with AA as substrate (Figure 2, inset). Taking the values of O_2 consumption for reactions of AA with human COX-2, ovine COX-2 and ovine COX-1 as 100%, the oxygenation rates of 3-HETE with the respective COX enzymes were comparatively lower, being 40 %,

Figure 2 Oxygenation of 3-HETE by purified ovine COX-2 and COX-1 and human COX-2 mutants

Aliquots of COXs (20 μ g) were added to 100 mM Tris/HCl buffer containing phenol (500 μ M) and haematin (1 μ M) in a final volume of 1.2 ml. Oxygen uptake was measured by the addition of 3-HETE or AA at 37 °C in a thermostat-controlled respiration chamber. The linear part of the oxygen consumption curve was used for calculating the initial reaction rate. Data for human COX-2 and ovine COX-1/2 represent three independent experiments, each performed in duplicate. The values are expressed as means $\pm$ S.E.M. For human COX-2 mutants, the values are expressed as means of two independent experiments. Inset: representative original tracings of oxygen uptake in experiments of AA or 3-HETE reacting with COX-1 and COX-2 are shown. The horizontal bar represents 10 s; the vertical bar represents 2 μ M O_2 .

61 % and 33 % respectively (Figure 2). Previously, Rowlinson et al. [32] have shown that Gly-533 is crucial for conversion of AA into PGG₂, which in turn is converted into PGH₂ at the peroxidase active site. Thus incubation of AA with a G533A mutant enzyme led to reduced AA binding and PGG₂ formation, whereas G533L and G533V mutants were totally unable to convert AA into PGG₂. Analogous reactions of these mutants with 3-HETE displayed comparable activities, albeit to a far less extent (Figure 2). The peroxidase activity of the wild-type enzyme and the G533A mutant for 3-HETE as substrate was, however, similar to that for AA [32] (results not shown). Chiral-phase analysis of the

Table 1 Kinetic analysis of AA and 3*R*-HETE interactions with COX-2 and COX-1

K_m and V_{max} values were calculated from Lineweaver–Burk plot analysis ($1/[S]$ against $1/V$). Parameters used for both experiments (COX-1 and COX-2): the temperature was 37 °C; reactions were allowed to proceed in a volume of 610 μ l (600 μ l of 100 mM Tris/HCl, pH 8, and 10 μ l of enzyme corresponding to 15 μ g). Four concentrations were used for AA and 3-HETE: 1.7 μ M (0.1 μ l); 4.1 μ M (0.25 μ l); 7.0 μ M (0.425 μ l); and 12.3 μ M (0.75 μ l). Each concentration was assayed in duplicate.

Enzyme used/ substrate	Kinetic parameters			
	K_m (μ M)	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ · μ M ⁻¹)	V_{max} (μ M O ₂ · min ⁻¹ · mg protein ⁻¹)
COX-2/AA	2.1 ± 0.2	14.7 ± 1	7	12.2 ± 0.8
COX-2/3-HETE	1.9 ± 0.2	1.5 ± 0.2	0.8	1.2 ± 0.2
COX-1/AA	8.3 ± 0.2	15 ± 2	1.8	13 ± 2
COX-1/3-HETE	11.0 ± 1.1	2.1 ± 0.2	0.2	1.8 ± 0.6

oxygenated products of 3-HETE showed that both *R*- and *S*-isomers of 3-HETE were equally active as substrates (results not shown). Also, oxygenation of 3-HETE was abrogated when the incubation mixture was pre-treated with 1 mM aspirin (results not shown). Kinetic analyses of 3-HETE with ovine COXs, using 100 μ M substrate, revealed K_m values that were comparable to those of AA, albeit the V_{max} values were only approx. 10% those of AA (Table 1). The k_{cat}/K_m values for oxygenation of 3-HETE by COX-2 were higher than for COX-1. Moreover, these were 9-fold higher for AA than for 3-HETE. Owing to a lack of double bonds in their structures, saturated 3-hydroxy-16:0 and 3-hydroxy-14:0 were not appropriate substrates for COXs (results not shown). Introduction of a methoxy group in place of the hydroxy group at the C-3 position of AA rendered 3-methoxy-AA an inappropriate substrate for oxygenation (results not shown).

Analysis of oxygenated products of the 3-HETE/COX-2 reaction

Products obtained from the oxygenation of 3-HETE with COX-2 were characterized by HPLC, EI–GC–MS–MS, LC–MS–ES and LC–MS–API methods. Purified wild-type COX-2, and mutant enzymes with or without pre-treatment of 1 mM aspirin (for 1 h), were incubated for 30 min at 37 °C with 100 μ M AA and processed as described in the Materials and methods section. Samples were analysed on HPLC for eicosanoids, with standard prostanoids serving as controls. Relevant fractions of peaks related to parent compounds were collected and analysed by GC–MS or LC–MS methods. Since the hydroxy group at the C-3 position is not a phenolic hydroxy group, we found that the transformation of 3-HETE by COX-2 was identical with that of AA, except that the eicosanoids produced were now 3-hydroxyeicosanoids. Figures 3(A)–3(C) describe the HPLC profiles of spiked eicosanoids PGE₂, PGF_{2 α} and PGD₂, and novel eicosanoids 3-hydroxy-PGE₂, 3-hydroxy-PGF_{2 α} , 3-hydroxy-PGD₂, 3,11-di-HETE and 3,15-di-HETE obtained from the incubation of 3-HETE with ovine COX-2 (left panels). The ratio of 3-hydroxy-PGs to di-HETEs as measured by HPLC peak quantification was approx. 0.3. Being more polar due to the additional hydroxy group, 3-hydroxyeicosanoids displayed shorter retention times on the RP-HPLC column than the parent eicosanoids. Alkaline hydrolysis of 3-hydroxy-PGE₂ with NaOH resulted in the dehydration/isomerization product 3-hydroxy-PGB₂, which could be easily detected due to its UV absorption at 280 nm (Figure 3B). The corresponding EI–GC–MS and LC–MS–ES/API spectra, together with characteristic mass fragments of

490 ($M^+ - 2 \times \text{OTMSi}$), 407 ($M^+ - \text{OTMSi-CH} \cdot \text{OTMSi} \cdot \text{C}_5\text{H}_{11}$) and 317 ($M^+ - 2 \times \text{OTMSi-CH} \cdot \text{OTMSi} \cdot \text{C}_5\text{H}_{11}$) (where OTMSi represents the trimethylsilyl ester) are shown in Figures 3(D)–3(F), and in Table 2. Being an unstable compound, 3-hydroxy-PGH₂, an intermediate and precursor of the aforementioned prostanoids, could not be detected. However, when treated with endoperoxide-reducing agents, such as sodium thiosulphate, 3-hydroxy-PGH₂ was converted into 3-hydroxy-PGF_{2 α} , which was identified by LC–MS–ES analysis. Characteristic mass fragments were 420 ($M^+ + 1$), 400 ($M^+ - 1 - \text{H}_2\text{O}$), 384 ($M^+ + 1 - 2\text{H}_2\text{O}$) and 442 ($M^+ + \text{Na}^+$), as shown in Table 2.

Furthermore, LC–MS–ES and LC–MS–API spectra revealed the formation of two major products of the 3-HETE/COX-2 incubations, i.e. 3,15- and 3,11-di-HETEs (Table 2), whereby 3,11-di-HETE was predominantly formed. The ratio of 3,11-di-HETE to 3,15-di-HETE was 0.6. However, aspirin-treated COX-2 altered the ratio of formation of 3,15- to 3,11-di-HETEs to 3.0, indicating an increase of 500% in favour of 3,15-di-HETE. Since acetylated mouse or human COX-2 has been shown previously to convert AA into 15*R*-HETE [41], we might have expected, from chiral-phase analysis of 3,11- and 3,15-di-HETEs without or with aspirin-treated COX-2, resultant (*R*)-stereochemistry for these compounds (results not shown). This would have been in line with several reports [41–43] in which conversion of AA by wild-type human COX-2 delivered 99% of 11-HETE in the (*R*)-configuration and approx. 85% of 15-HETE in the (*S*)-configuration. However, the conversion of AA by aspirin-treated COX-2 (acetylation of Ser-530) yielded 13% of 11-HETE and 14–99% 15-HETE in the (*R*)-configuration, the latter depending on mutations of human COX-2 of S530T or S530V [41]. However, no formation of 11*S*-HETE was observed.

Scheme 1 summarizes the cascade of bioactive prostanoids obtained so far from the conversion of 3-HETE by ovine COX-2.

Production of 3-hydroxy-PGE₂ by *C. albicans*-infected HeLa cells in the presence of AA

C. albicans has been reported to produce 3-HETE when incubated with exogenous AA [9]. This led us to investigate whether co-incubation of HeLa cells and *C. albicans*, which simulates the host–pathogen interaction in vulvovaginal candidiasis, would produce 3-hydroxyeicosanoids *in vivo*. Thus HeLa cells were treated with 30 μ M AA for 5 min prior to infection with *C. albicans* for 6 h at an MOI (multiplicity of infection) of 100. After separation from *C. albicans*, cells were lysed and extracted according to the method of Bligh and Dyer [44]. The lipid fraction was reconstituted in *n*-hexane and subjected to HPLC analysis. Fractions containing 3-hydroxy-PGE₂ were collected, re-extracted and treated with diazomethane and BSTFA to convert 3-hydroxy-PGE₂ into its Me-TMS derivative. Upon analysis with GC–MS, we could clearly identify 3-hydroxy-PGE₂ by comparing the mass spectrum with that of the parent PGE₂ (Figure 4) and the characteristic mass fragments (Table 2). Further confirmation was achieved by HPLC analysis of 3-hydroxy-PGB₂ obtained after alkaline hydrolysis of 3-hydroxy-PGE₂, as described above (results not shown).

Biological activities of 3-hydroxy-PGE₂, 3-HETE and 3,15-di-HETE

Activation of IL-6 gene expression by 3-hydroxy-PGE₂ from lung adenocarcinoma A549 cells

The biological actions of PGE₂ are mediated by four different G-protein-coupled receptors: EP₁, EP₂, EP₃ and EP₄ [45]. Whereas EP₂ and EP₄ receptors signal through coupling to G_{as} proteins and increasing intracellular cAMP levels, the signal transduction

Figure 3 Characterization by HPLC and GC- and LC-MS of 3-hydroxyeicosanoids formed following 3R-HETE-ovine-COX-2 interaction

Aliquots of COX enzyme (20 μ g) containing 1 μ M haematin and 300 μ M phenol were incubated for 2 min at 37 °C with 100 μ M 3R-HETE. After termination of the reaction with methanol/acetic acid (3:2, v/v) on ice, products were extracted as described in the Materials and methods section. The extracted compounds were separated by RP-HPLC. The peaks of interest were collected and analysed by GC-MS. Similar experiments were performed with AA as substrate to check the enzyme activity. For LC-MS, the extracted compounds after 3R-HETE-COX-2 interaction were analysed

Table 2 Characteristic mass fragments of various 3-hydroxyeicosanoids obtained from the oxygenation of 3*R*-HETE by COX-2

OTMSi, trimethylsilyl ester.

3-Hydroxyeicosanoid	Ionization mode	Characteristic mass fragment
3 <i>R</i> -HETE	API	367 ($M^+ - 1$); 349 ($M^+ - 1 - H_2O$); 333 ($M^+ + 1 - 2H_2O$); 315 ($M^+ + 1 - 3H_2O$); 391 ($M^+ + Na^+$); 415 ($M^+ + 2Na^+$)
3-Hydroxy-PGE ₂	EI	580 [$M^+ - (OTMSi)$]; 490 [$M^+ - 2(OTMSi)$]; 407 [$M^+ - (OTMSi) - CH \cdot (OTMSi) \cdot C_5H_{11}$]; 317 [$M^+ - 2(OTMSi) - CH \cdot (OTMSi) \cdot C_5H_{11}$]
3-Hydroxy-PGD ₂	API	369 ($M^+ + 1$); 351 ($M^+ + 1 - H_2O$); 333 ($M^+ + 1 - 2H_2O$); 315 ($M^+ + 1 - 3H_2O$); 391 ($M^+ + Na^+$)
3-Hydroxy-PGB ₂	API	351 ($M^+ + 1$); 333 ($M^+ + 1 - H_2O$); 315 ($M^+ + 1 - 2H_2O$); 297 ($M^+ + 1 - 3H_2O$); 373 ($M^+ + Na^+$)
3-Hydroxy-PGF _{2α}	API	420 ($M^+ + 1$); 400 ($M^+ - 1 - H_2O$); 384 ($M^+ + 1 - 2H_2O$); 442 ($M^+ + Na^+$)
3,15-Di-HETE	EI	479 ($M^+ - CH_3$); 389 [$M^+ - CH_3 - (OTMSi)$]; 225 [$C_4H_4 \cdot CH(OTMSi) \cdot C_5H_{11}$] ⁺ ; 175 [$CH(OTMSi) \cdot CH_2COOCH_3$] ⁺ ; 173 [$CH(OTMSi) \cdot C_5H_{11}$] ⁺
3,11-Di-HETE	EI	479 ($M^+ - CH_3$); 225 [$C_4H_4 \cdot CH(OTMSi) \cdot C_5H_{11}$] ⁺ ; 175 [$CH(OTMSi) \cdot CH_2COOCH_3$] ⁺

Scheme 1 Cascade of 3-hydroxyeicosanoids produced from the 3-HETE–COX-2 reaction

The compound shown in square brackets was not detected.

pathway for the EP₃ receptor is mediated by G_{αi} protein, leading to decreased intracellular cAMP levels [46]. Human A549 lung adenocarcinoma cells express EP₃ as well as EP₄ receptors, whereas alveolar type II cells express only the EP₄ receptor [47]. Activation of A549 cells by PGE₂ also stimulates cytokine release. Thus we next determined whether 3-hydroxy-PGE₂ could modify IL-6 gene expression, and whether this effect was mediated by the EP₃ or the EP₄ receptor. As described above, A549 cells were incubated with either vehicle or 50 nM PGE₂ or 50 nM 3-hydroxy-PGE₂. After a further 24 h, RNA was extracted and IL-6 mRNA expression was determined by RT-PCR. IL-6 mRNA expression was significantly enhanced by 3-hydroxy-PGE₂, whereas PGE₂-

mediated IL-6 activation was insignificantly altered (Figures 5A and 5B). No further significant enhancement of IL-6 mRNA was observed when higher concentrations of 3-hydroxy-PGE₂ (1 μM) were applied (results not shown). To identify the EP receptor subtype that mediates the IL-6 activation, we compared the effects of PGE₂ or 3-hydroxy-PGE₂ in cells pre-treated with EP₃- or EP₄-blocking peptide. Blocking peptides for the EP₃ and EP₄ receptors were used because, in separate immunohistochemical experiments, these peptides were found to inhibit immunostaining of the EP₃ and EP₄ receptors respectively (results not shown). Whereas the enhanced response to 3-hydroxy-PGE₂ and feeble response to PGE₂ was completely abrogated in EP₃-blocked cells (Figures 5A and 5B), no difference in the response of EP₄-blocked cells to PGE₂ or 3-hydroxy-PGE₂ was observed (results not shown). These results demonstrate that IL-6 activation by 3-hydroxy-PGE₂ is mediated by EP₃ receptors.

Release of cAMP by 3-hydroxy-PGE₂ from Jurkat cells

In a recent study, PGE₂ has been shown to stimulate cAMP in Jurkat cells via EP₄ receptors [48]. To investigate whether 3-hydroxy-PGE₂ also enhances the release of cAMP by EP₄ receptors, Jurkat cells were incubated with increasing concentrations of PGE₂ and 3-hydroxy-PGE₂. Although the release of cAMP was hardly affected by 10 nM 3-hydroxy-PGE₂, in comparison with a 5-fold augmentation by 10 nM PGE₂, a concentration of 100 nM 3-hydroxy-PGE₂ raised the cAMP level almost to the same extent as did PGE₂ (Figure 6). This accumulation of cAMP, however, was significantly inhibited by 100 nM EP₄ inhibitor L161982 (5.44 compared with 1.76 pmol/ml for PGE₂ and 6.60 compared with 2.51 pmol/ml for 3-hydroxy-PGE₂), whereas stimulation of the EP₂ receptor with 100 nM butaprost, or blocking of the receptor with EP₂-specific peptide, did not lead to any alterations in the cAMP level (R. Ciccoli, S. Sahi, R. Deva, G. Ishdorj, S. Singh, M. P. Zafiriou and S. Nigam, unpublished work). This suggests that EP₄ receptors are responsible for G_{αs} stimulation of adenylate cyclase and cAMP up-regulation.

by LC–API- or LC–ES–MS. (A) HPLC chromatogram taken with a DAD (diode array detector) at a wavelength of 195 nm of 'spiked' eicosanoids (1 μg) PGE₂ and PGD₂, as well as of transformed 3-hydroxy-PGE₂ and 3-hydroxy-PGD₂. (B) HPLC chromatogram taken with DAD at a wavelength of 278 nm of spiked eicosanoid (1 μg) PGB₂, the hydrolysis product of PGE₂, as well as of 3-hydroxy-PGB₂. (C) HPLC chromatogram taken with DAD at 235 nm wavelength of spiked eicosanoids (1 μg) 11-HETE and 5,15-di-HETE, as well as of transformed 3,11- and 3,15-di-HETEs. (D, E) LC–MS–APCI spectra of 3-hydroxy-PGE₂ (D) with $M^+ + 1$ at 367 and 3-hydroxy-PGB₂ (E) with $M^+ + 1$ at 351. (F, G) GC–MS spectra of 3,15-di-HETE (F) and 3,11-di-HETE (G) with $M^+ - 15$ at 479.

Figure 4 Evidence for the conversion of AA into 3-hydroxy-PGE₂ by *C. albicans*-infected HeLa cells

HeLa cells (1×10^7) were treated with 30 μ M AA for 5 min, before infecting with *C. albicans* for 6 h at a MOI of 100. Cell lysates were then prepared and lipids were extracted according to the method of Bligh and Dyer [43]. Extracted compounds were separated by RP-HPLC. The peak corresponding to 3-hydroxy-PGE₂ was collected and analysed by GC-MS, as described in the Materials and methods section. Upper panel: EI-mass spectrum of 3-hydroxy-PGE₂ as obtained from *C. albicans*-infected HeLa cells. For characteristic mass fragments, see Table 2. Lower panel: EI-mass spectrum of synthetic PGE₂. Characteristic mass fragments are 567 ($M^+ - 15$), 492 ($M^+ - 90$), 409 ($M^+ - 173$) and 319 ($M^+ - 90 - 173$). OTMS, trimethylsilyl ether.

Effects of 3-HETE and 3,15-di-HETE on maxi-K channels in HTM cells

To investigate the effect of 3-HETE metabolites on ion channels using the patch-clamp technique, we applied 1 μ M 3,15-di-HETE or 3-HETE or 3,9-di-HETE to HTM cells. Both 3,15-di-HETE and 3-HETE were potent, and stimulated maxi-K channels. The increase in outward current was almost 8-fold for 3,15-di-HETE and 5-fold for 3-HETE (Figure 7), the latter being comparable with that elicited by 1 μ M AA. This effect could be abrogated by 100 nM iberiotoxin, a specific inhibitor of maxi-K channels. To determine whether the stimulation of maxi-K channels by 3-HETE or 3,15-di-HETE is caused by a rise in cytosolic calcium, cells were superfused with EGTA solution containing 1 μ M thapsigargin (a Ca^{2+} -ATPase inhibitor) for depletion of intra- and extra-cellular calcium. However, depletion of calcium had no effect on the stimulated outward current (results not shown). Surprisingly, 3,9-di-HETE, a product obtained by the interaction of 3-HETE and 5-lipoxygenase, did not activate maxi-K channels (Figure 7).

DISCUSSION

The present study describes the oxygenation by COX-2 of 3-HETE, a mimetic of AA obtained by incomplete β -oxidation of AA, discovered by us in the fungus *D. uninucleata* [1]. Considering the stereochemical structure of 3-HETE, which is almost identical to that of AA, it was not surprising that 3-HETE served as an excellent substrate for COX-2, but less so for COX-1. This finding was in line with the enzyme/substrate oxygenation pattern

described earlier for AA/COX-2 or 2-AG/COX-2 [32,33,49]. The molecular modelling studies revealed that 3-HETE also binds in an L-shaped configuration as shown for AA, leading to the smallest C-1–C-20 and C-10–C-20 distances and ϕ angle, which demonstrates the strong folding at C-10. All complexes of 3-HETE with COX-2 demonstrated that Arg-120 and Tyr-355 in COX-2 interact with the carboxy-group end of 3R-HETE (Figure 1) by placing the 13-*pro-S* hydrogen next to Tyr-385 in a way similar to that described for AA [32], albeit the latter is much closer in the AA–COX-2 complex.

Analogously to previous studies of COX-2 interaction with AA or 2-AG, we found that 3-HETE was oxygenated by various COXs, albeit to a lesser extent than AA (Figure 2). Although it is also a substrate for COX-1, the activity for transformation to 3-hydroxyprostanoids was significantly diminished (results not shown). Since the hydroxy group at the C-3 position is not a phenolic hydroxy group, we found the transformation of 3-HETE by COX-2 to occur in an identical manner as of AA, except that the eicosanoids produced were now 3-hydroxy-eicosanoids. The conversion of the endoperoxide product of the 3-HETE–COX-2 interaction into various prostaglandins (Figure 3, Table 2 and Scheme 1) results in the production of a novel family of eicosanoids, which are as diverse as those produced from AA. The production of 3-hydroxy-PGE₂ in the lysate of *C. albicans*-infected HeLa cells pre-treated with AA (Figure 4) gives clear-cut evidence of the following mechanism in candidiasis: AA released from the infected or inflamed host tissue/cell is converted by *C. albicans* into 3R-HETE [9,10], which serves as substrate for *C. albicans*-stimulated COX-2 [19] in the host tissue/cell to

Figure 5 Effect of 3-hydroxy-PGE₂ on IL-6 mRNA expression in A549 cells

(A) A549 cells were treated with 50 nM PGE₂ or 3-hydroxy-PGE₂ for 24 h. Total RNA (1 µg) was extracted from A549 cells without or with pre-treatment of 50 nM PGE₂ or 3-hydroxy-PGE₂ for 24 h. PCR amplification using the human IL-6-gene-specific primers was performed as described in the Materials and methods section. Amplification of GAPDH was performed as a control to ensure that RNA amounts were equal. PCR data are representative of two separate experiments. (B) The density of the bands of RT-PCR was measured by scanning densitometry and expressed as normalized values to GAPDH. Values represent the means of two separate PCR experiments.

Figure 6 Effect of 3-hydroxy-PGE₂ on cAMP release from Jurkat cells

Jurkat T-cells (10⁶ cells/well) were pre-incubated with 50 µM IBMX for 30 min, followed by treatment (5 min) with 0.01 µM 3-hydroxy-PGE₂ or 1 µM PGE₂. After stopping the reaction with TCA, cells were sonicated for 10 s and then centrifuged at 14000 rev/min (17500 g) for 10 min. After removing the TCA by diethyl ether treatment, the supernatant (50 µl) was assayed for cAMP using a commercial ELISA kit. The values are expressed as means ± S.E.M. for three independent experiments, each performed in triplicate. cAMP production by 3-hydroxy-PGE₂ at a concentration of 1 µM was identical with that observed for PGE₂, and highly significant over the control value (**P* < 0.001).

produce pro-inflammatory 3-hydroxy-PGE₂. Strikingly, our preliminary experiments with 3-HETE and human platelets or human neutrophils have shown, instead of 3-hydroxy-thromboxane A₂

Figure 7 Enhancement by 3-hydroxyeicosanoids of current through maxi-K channels of HTM cells as measured by the patch-clamp technique

Dispersed cells were allowed to settle on coverslips, which were then superfused with Ringer's solution. AA, 3-HETE, 3,15- or 3,9-diHETE (all at 1 µM concentration) were added without or with the maxi-K channel blocker ibiritoxin (100 nM), and outward current was recorded as described in the Materials and methods section. An initial outward current at 80 mV was taken as 100 %. Results are expressed as means ± S.D. of *n* = 7 measurements. Significance: for 3-HETE compared with the control, **P* < 0.001; and for 3,15-diHETE compared with AA, ***P* < 0.01.

and 3-hydroxy-leukotriene B₄ respectively, different novel di- and trihydroxy-metabolites of 3-HETE (R. Ciccoli, R. Deva, S. Singh, M. P. Zafiriou, S. Sahi, R. Roux, G. Ishdorj and S. Nigam, unpublished work). Whether the hydroxy group at the C-3 position near the carboxy group influenced the product pattern is unclear. Molecular modelling experiments showing alignment of C-1, C-2 and C-3 groups in the large side pocket of the COX-2 enzyme argue against this assumption. Reaction of 3-HETE with COX-2 pre-treated with aspirin produced largely 3,15R-diHETE and a minor quantity of 3,11-diHETE. The mechanism for a shift in the product pattern 3,11-diHETE:3,15S-diHETE (ratio 0.6) to 3,15R-diHETE:3,11R-diHETE (ratio 3) is related to one described by Rowlinson et al. [32] for an AA-acetylated-COX-2 interaction. Thus 3-HETE, with a carboxy group, should bind to Arg-120 of acetylated COX-2, and the rest of the carbon chain then slips sinuously ('like a snake') into the enzyme cavity, having a kink at the C-13 position for *pro*-hydrogen abstraction by Tyr-385. As a consequence, the conformation of 3-HETE should change in order to access the top channel of acetylated COX-2. Owing to an increased volume near Arg-513, a stable conformation of the active site is developed, which facilitates 3-HETE binding to produce favourably 3,15R-diHETE.

To investigate the biological activities of these novel eicosanoids, we isolated and purified 3-hydroxy-PGE₂ as a representative compound for its biological actions on mammalian cells. RT-PCR studies on the induction of IL-6 mRNA in 24 h showed that 3-hydroxy-PGE₂ is more potent in stimulating the IL-6 mRNA expression than PGE₂, which hardly affects the IL-6 mRNA expression (Figures 5A and 5B). A high basal level of IL-6 found in these experiments may be due to the action of some leakage of endogenous PGE₂ during the long incubation time of 24 h, albeit that no LDH was detected in the supernatant of A549 cells up to 18 h (results not shown). The

differential activity of PGE₂ and 3-hydroxy-PGE₂ with respect to EP₃ receptors, as shown by utilizing the EP₃-specific blocking peptide, is in agreement with a recent study [50] that has highlighted the different affinities of PGE₂ receptors for PGE₂ and PGE₂ metabolites in signalling pathways located downstream of adenylate cyclase up-regulation. Thus we consider that 3-hydroxy-PGE₂ desensitizes the EP₃ receptor to a lesser extent than does PGE₂, and that the rapid desensitization by PGE₂ of the EP₃ receptor may thus account for significantly lower release of PGE₂-stimulated IL-6 expression.

Furthermore, the stimulatory effect of 3-hydroxy-PGE₂ on cAMP via EP₄, but not via EP₂, receptors in Jurkat cells demonstrated that this hydroxyeicosanoid imitates the action of PGE₂. However, the difference in action of PGE₂ and 3-hydroxy-PGE₂ with respect to receptor subtype may be attributed to the pattern and level of expression of these two receptors. These differences can vary dramatically, as seen above in the case of EP₃ receptor, which underwent apparently rapid desensitization. Nevertheless, it remains to be established whether the cAMP activation is accompanied by a rise in intracellular calcium, another indispensable second messenger, for eliciting biological effects of PGE₂-like compounds. The strong activity of 3-hydroxy-PGE₂ with respect to IL-6 and cAMP release from A549 and Jurkat cells respectively pinpoints that, in a large number of patients with genetically distinct inherited mitochondrial fatty acid β -oxidation disorders [51,52], 3-hydroxyeicosanoids may aggravate the effect of endogenous eicosanoids. We believe that the *in vitro* synthesis of 3-hydroxyeicosanoids shown in the present study may reflect the real situation in patients suffering from recurrent candidosis. A marked effect of a 3-HETE metabolite (3,18-di-HETE) produced by *C. albicans* isolated from patients with vaginal candidiasis has recently been shown to enhance the production of PGE₂ in HeLa cells, which in turn up-regulates the proliferation of *C. albicans* [10]. However, it should be stressed that the biological effects of a large number of possible 3,n-HETEs, exemplarily shown for 3,15-di-HETE on maxi-K channels of HTM cells in the present study (Figure 7), may have diverse effects in various organisms. Moreover, 3-hydroxyeicosanoids may also compete effectively for endogenous eicosanoids in eliciting different effects.

In conclusion, we have shown that 3-HETE is an appropriate substrate for COX-2, which is almost as effective as AA and produces novel 3-hydroxyeicosanoids (Scheme 1). In addition, we have shown that 3-hydroxyeicosanoids, especially 3-hydroxy-PGE₂, possess strong biological activities comparable with endogenous eicosanoids, in some cases even more potent than parent compounds. The molecular structures of (*R*)- and (*S*)-isomers of 3-HETE with COX-1 and COX-2 in modelling studies showed hardly any preference for 3*R*-HETE over 3*S*-HETE with COX-2. These studies may, therefore, be effectively used for designing and developing selective COX-2 inhibitors.

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