2. MATERIALS AND METHODS

2.1 MATERIALS

Fatty acids and eicosanoids:-

HXA_3 - Calbiochem (Germany) and Biomol (Germany); 12(*S*)-HETE - Cascade Biochem (United Kingdom); 5(*S*)-HpETE, 12(*S*)-HpETE, 13-HODE and ETYA - Cayman Chemical Company (U.S.A); arachidonic acid and PAPC - Sigma (Germany).

Metabolic inhibitors, Receptor agonists and antagonists:-

PMA, fMLP, pertussis toxin, staurosporine – Sigma (Germany).

Radiochemicals:-

^{14}C-Arachidonic acid (55 mCi/mmol) and ^{3}H-Thymidine (2.0 Ci/mmol) - Amersham (England) and NEN (Boston, USA) respectively.

Kits:-

RNeasy Mini Kit and QIAshredder - Qiagen (Germany); LTB_4 and cAMP ELISA - Cayman Chemical Company (U.S.A), Cell Death Detection ELISA - Boehringer (Germany) respectively; Diff-Quik staining set - DADE (Germany), Total protein kit – Sigma (Germany), ECL Detection kit – Santa Cruz (California, USA).

Cell culturing media:-

FCS, DMEM, IMEM, Trypsin/EDTA, Streptomycin/Penicillin and GM-CSF – Seromed (Germany).

Enzymes:-

Purified platelet and leukocyte type 12-LOX - Oxford Biomedical Research (Germany), soybean lipoxygenase – Cayman, Proteinase K and Superoxide dismutase - Sigma (Germany), Expand reverse transcriptase and Taq DNA polymerase – Boehringer (Germany).

Separation media:-

Dextran T500, Ficoll-Paque Plus and Sephadex G-100 (SF) – Pharmacia (Germany), Agarose – Boehringer (Germany), Silica gel-60 TLC plates – Merck (Germany), Rotiphorese Gel 30 – ROTH (Germany).

Primers:-

All primers were purchased from TIB Biomol (Germany).

All organic solvents used in the experiments analysed by HPLC and GC-MS were of LiChrosolv or SupraSolv quality; all other reagents were of analytical grade.

2.2 METHODS

2.2.1 Cell preparation and pretreatments

Peripheral blood was withdrawn from healthy volunteers by vein puncture into citrated tubes. Neutrophils were isolated according to Böyum (1968). Briefly, Ficoll-Hypaque gradient centrifugation followed by dextran sedimentation was employed. Contaminating erythrocytes were eliminated by hypotonic lysis in water for 20 - 30 s. The isolated cells were suspended in PBS/BSA/glc without $CaCl_2$, in a final concentration of 20×10^6 cells/ml.

Platelets were isolated according to the technique of Hamberg *et al*. (1974). Briefly, whole blood was centrifuged at 200 × g for 15 min and the platelet-rich plasma (300 000 – 400 000 platelets/µl) was further centrifuged at 650 × g for 15 min. The platelet pellet was suspended in 0.15 M NaCl, 0.15 M Tris-Cl (pH 7.4), 0.077 M Na EDTA (90:8:2, v/v/v), equivalent to half the volume of the platelet-rich plasma, centrifuged and suspended at a concentration of 1×10^9 platelets/ml.

Human megakaryocytes were isolated from bone marrow aspirates according to the technique of Tanaka *et al*. (1989). Briefly, 6 ml of bone marrow aspirate from healthy volunteers was diluted with an equal volume of 'MK medium'. The cell suspension was layered on a Percoll (Pharmacia) density gradient. The megakaryocytes were purified using monoclonal anti-glycoprotein IIb/IIIa antibody and magnetic beads coated with anti-mouse IgG antibody. A biotinylated secondary antibody was used to coat the streptavadin-conjugated magnetic beads. Separation was performed using a Dynal magnetic particle concentrator, Dynal MPC 1 (Dynal A.S., Oslo, Norway).

For arachidonic acid-labelling of the membrane lipids, the neutrophils were incubated at 37°C for 20 min in the presence of 0.25 µCi [1-^{14}C]-arachidonic acid. The cells were washed twice with PBS/BSA/glc to remove unesterified fatty acids and finally suspended in the same medium to a concentration of 5×10^6 cells/ml.

For kinetic measurements of intracellular calcium concentration the neutrophils were incubated with 1 µM Fura-2/AM at 37°C for 40 min. Excess Fura-2/AM was removed by centrifugation, the cells were suspended in PBS/BSA/glc (5×10^6 cells/ml) and kept on ice.

2.2.2 Cell lines and culturing

The cell lines A431, HeLa and UT-7 were grown at 37°C (5% CO_2, 100% humidity). A431 and HeLa were cultured in Dulbeco's modified Eagles medium supplemented with 10% fetal bovine serum (FCS), 100 µg/ml streptomycin and 100 U/ml penicillin. Cells were grown to

100% confluence for enzyme activity experiments and ~70% confluence for gene expression experiments. The UT-7 cells were cultured in Iscove's medium supplemented with 10% FCS and 10 ng/ml GM-CSF. To produce selenium deficient cells, the cells were kept for 20 days in medium supplemented with 0.75% FCS.

2.2.3 Preparation of 1-Palmitoyl-2-[(15*S*)-hydroperoxy-(5*Z*,8*Z*,11*Z*,13*E*)-eicosatetra-enoyl]-phosphatidylcholine] (PAPC-OOH)

PAPC-OOH was prepared by the use of soybean lipoxygenase (Brash *et al*., 1987; Chaitidis *et al*., 1998). Briefly, 780 µg of PAPC was dissolved in 6 ml 10 mM sodium deoxycholate and 13 ml 0.2 M sodium borate (pH 9.0) by sonification for 5 min. The reaction was started by the addition of 500 µg soybean lipoxygenase per 10 ml sample at room temperature. The reaction was stopped after 10 min by the addition of methanol:chloroform:water (2:1:1, v/v/v). The products were separated by reverse-phase HPLC on a Nucleosil 1005 C_8 column using the solvent system methanol:acetonitrile:water (90:6:4, v/v/v) containing 20 mM choline chloride.

2.2.4 Assay of enzymatic activity

PHGPx and GPx-1 activity were measured according to Imai *et al*. (1998) with minor modifications. Platelets, A431, RINm5F and UT-7 cells were lysed by sonication in 10 mM Tris-HCl buffer (pH 7.4) containing 10 µg/ml leupeptin and 0.1 mM PMSF. The homogenate was centrifuged at 10 000 × g for 10 min at 4°C. The reaction mixture contained 0.1 M Tris-HCl (pH 7.4), 5 mM EDTA, 1.5 mM sodium azide, 3 mM glutathione, 0.25 mM NADPH, 1U glutathione reductase and 0.1% Triton X-100. GPx-1 activity was measured by the addition of the substrates 0.25 mM hydrogen peroxide or 0.25 mM *tert*-butylhydroperoxide. PHGPx activity was measured by the addition of 20 µM PAPC-OOH. 12-HpETE reductase activity was measured with 20 µM 12-HpETE. Carboxymethylation of the selenocysteine of peroxidases was carried out by incubation with 2 mM iodoacetate (10 min, 37°C). The reaction was monitored spectrophotometrically by measuring the decrease in absorbance at 340 nm (UV max kinetic microplate reader, Molecular Devices, CA), corrected for the blank (reaction mixture + substrate) and activity calculated from the rate of NADPH oxidation.

2.2.5 Rate of breakdown of PHGPx mRNA in UT-7 cells

To determine the breakdown rate of PHGPx mRNA in differentiated UT-7 cells, the cells (10^6/ml) were treated with 10 µM PMA for 72 h to induce differentiation to megakaryocytes

in a thrombopoietin-like manner (Madoiwa *et al*., 1999). Thereafter the cells were treated with 10 ng/ml actinomycin D and harvested at various time intervals. The amount of mRNA was quantified by semi-quantitative RT-PCR and normalised using β-actin.

2.2.6 AA metabolism in platelets, A431 and UT-7 cells

Human platelets were incubated in the presence and absence of either ETYA (10 μM), iodoacetate (2 mM) or Na cyanide (2 mM) for 10 min and $CaCl_2$/$MgCl_2$ for 5 min before the addition of either arachidonic acid or 12-HpETE. For GC-MS analysis of Trioxilin A_3/HXB_3 formation, the reactions were incubated for 30 min and 10 min. The reactions were stopped by the addition of diethylether-saturated 10 mM HCl, allowed to stand at room temperature for 30 min and extracted 3× with ice-cold ether and 300 μl brine. The ether was removed and added to a vial containing Na_2SO_4. The ether was transferred to a clean vial, dried under nitrogen gas and the samples prepared as described under GC-MS analysis of eicosanoids. For HPLC analysis of 12-HpETE/12-HETE formation, the samples were incubated for 10 min in the presence of both substrates. The samples were extracted, firstly with ether/methanol/1 M citrate (135:15:1, by vol.) containing 5 ppm butylated hydroxytoluene followed by a further 2 extractions with ice-cold diethylether. For HPLC analysis of 12-HETE, 12-HpETE and 12-HHTrE formation, 13-HODE was added as internal standard before extraction.

The A431 cells were washed, trypsinated and rewashed. The cells were suspended at a concentration of 8-10 × 10^6/ml in PBS/BSA/glc and 500 μl used for each determination as for the platelet experiments. The UT-7 cells were harvested, washed and resuspended at a concentration of 4-8 × 10^6 cells/ml. All other conditions were the same as described for the platelet experiments.

2.2.7 HXA_3 formation by mammalian 12-Lipoxygenases

Pure human recombinant 12-lipoxygenase and porcine leukocyte 12-lipoxygenase were incubated with the substrates arachidonic acid (50 nmoles) or 12-HpETE (20 nmoles) in an Oxygen Meter Model 781 chamber (Strathkelvin Instruments, Glasgow, UK). Inactivated 12-lipoxygenases, either by pre-incubation with 10 μM ETYA (5 min at 37°C) or heat-inactivation (10 min at 65°C) were also used. The reaction was stopped after 30 min (platelet enzyme) or 4.5 min (leukocyte enzyme) as described under Measurement of Oxygen Uptake (section 2.2.21) and further analysed by GC-MS as described below.

2.2.8 GC-MS analysis of eicosanoids

For analysis, the acid-hydrolysed, evaporated, dried extracts from the biological samples were converted to their methyl esters by the addition of 50 µl methanol and 300 µl ethereal diazomethane for 15 min in the dark (Jones *et al*., 1978; Bryant *et al*., 1982). Thereafter the samples were evaporated to dryness, 30 µl trimethylsilylimidazole in dry pyridine (TriSil-Z®, Pierce) was added and the sample heated at 60°C for 5 min. Finally the samples were dissolved in 10 µl dodecane.

Gas chromatography-mass spectrometry (GC-MS) was performed by means of a Varian Saturn 4D GC-MS-MS system equipped with a Supelco DB5-MS column (30 m x 0.25 mm; 0.25 µM d_f). The temperature program was started at 150°C increasing to 250°C within 10 min with a rate of 10°C/min. The temp. of injector and transfer line were 230°C and 220°C respectively.

Calibration curves were obtained using authentic standards of the hepoxilins. Trioxilin A_3 was quantified from the peak areas of the mass fragments m/e 243 and 213 characteristic for 8,9,12-TriHETrE and 8,11,12-TriHETrE, respectively. HXB_3 was quantified using the peak areas of the mass fragments m/e 119 and 269.

2.2.9 HPLC analysis of eicosanoids

Platelet extracts, extracted as described under section 2.2.5, were evaporated and suspended in eluent. High-pressure liquid chromatography (HPLC) was performed using a Shimadzu pump LC-10AT and diode-array detector SPD-M10A. The samples were separated on a straight phase Supelcosil LC-S1 (250 x 46 mm; 5 µm particle size) column in n-hexane:i-propanol:acetic acid (100:2:0.1, by vol.) at a flow rate of 1 ml/min. Standard curves were prepared with authentic standards of AA and 12-HETE.

2.2.10 Detection of PHGPx and 12-LOX by Reverse Transcription-PCR

To detect the presence of PHGPx and 12-LOX mRNA, total RNA was isolated from the different cells using QIAGEN RNeasy mini-columns according to the manufacturers recommendations. Reverse transcription of the total RNA (5 µg) was performed using Expand reverse transcriptase at 42°C for 50 min. For amplification of PHGPx-cDNA, the primers 5'-TGT GCG CGC TCC ATG CAC GAG T-3' and 5'-AAA TAG TGG GGC AGG TCC TTC TCT-3' were used. Platelet-type 12-LOX was amplified using the primers 5'-CTG GCC CCA GAA GAT CTG ATC-3' and 5'-GAT GAT CTA CCT CCA AAT ATG-3'. β-actin cDNA was amplified using various primer sets supplied by Stratagene (La Jolla, CA).

The PCR was performed with 1 U Taq polymerase using a Biometra UNOBLOCK thermal cycler (Biometra). The PCR conditions used were 30 cycles at 95°C for 30 s, 68°C (PHGPx and β-actin) and 60°C (12-LOX) for 30 s and 72°C for 60 s. PCR products were separated on a 2% agarose gel and stained with ethidium bromide.

2.2.11 Quantification of PHGPx expression

HeLa and A431 cells were grown to ~70% confluence in DMEM/10% FCS. The cells were washed twice with PBS before the addition of fresh medium containing the test compounds and incubated at 37°C (5% CO_2, 100% humidity) for the indicated times. At the appropriate times the medium was aspirated, the cells washed with PBS, lysed and the total RNA extracted using QIAGEN RNeasy Mini Kit according to manufacturers' recommendations. The RNA was quantified and 5 µg utilised for reverse transcription using Expand Reverse Transcriptase (50 U/µl, Boehringer Mannheim) and 100 pM oligo-dT_{18}. PCR was performed using Taq DNA polymerase (Boehringer Mannheim) according to the manufacturers recommendations and the PHGPx specific primers 5'-TGTGCGCGCTCCATGCACGAGT-3' and 5'-AAATAGTGGGGCAGGTCCTTCTCT-3' (TIB MOLBIOL, Germany). The annealing temp used was 67°C. β-Actin (annealing temp. 58°C, no. of cycles 30) was used as control. The products were separated on a 1% agarose gel, photographed, scanned and the relative intensities quantified using the software TINA (version 2.09g).

2.2.12 Measurement of chemotaxis

Chemotaxis was performed using a Neuro Probe Standard 48 well Chemotaxis Chamber (Neuro Probe, Inc.). Polycarbonate filters (Costar) with 3 µm pore size were used. The neutrophils were suspended in PBS/BSA/$CaCl_2$ (2×10^5 cells/ml). Stock solutions of HXA_3 and fMLP were diluted in the same buffer and applied to the bottom wells (27 µl). 50 µl of the neutrophil suspension was applied to the top wells and incubated at 37°C (5% CO_2, 100% humidity) for 2 h. The cells were visualised using either acridine-orange staining or Dade® Diff-Quik® staining set and counted. Each estimate was performed in duplicate and three squares of equal size/well were randomly counted.

2.2.13 Arachidonic acid release

The [1-^{14}C]-arachidonic acid-labelled cells were incubated at 37°C for 5 min in the presence of 1.2 mM $CaCl_2$/1 mM $MgCl_2$ before the addition of fMLP or HXA_3. The kinetic effect of

HXA_3 on fMLP induced arachidonic acid release was determined by incubating the cells with HXA_3 for 5 min before the addition of fMLP. Incubations were performed in duplicate and terminated at the denoted intervals by the addition of 3.5 ml chloroform/methanol (2:5 v/v) and extracted as described earlier (Nigam *et al*., 1990a). Samples were applied on heat-activated silica gel-60 TLC plates and separated in the solvent n-hexane/diethyl ether/acetic acid (50:50:1; v/v/v). The plates were developed in an iodene chamber, the relevant areas scratched and added to 10 ml scintillation fluid (Ultima Gold, Packard) and the radioactivity was quantified by scintillation counting.

2.2.14 LTB_4 release

LTB_4 release was measured by two techniques, namely ELISA (CAYMAN) and TLC separation of the 5-LOX products extracted from [1-^{14}C]-arachidonic acid-labelled cells. The ELISA was performed according to the manufacturers recommendations. To study the effects of HXA_3 on 5-lipoxygenase products (5-HETE, LTB_4 and LTB_4 isomers), the same procedure was used as for arachidonic acid release except that the extracts were separated in the solvent system chloroform/methanol/water/acetic acid (90:7:0.7:1, by vol.). The 5-LOX products were detected using an automatic TLC-linear analyser (Tracemaster 40, Berthold) and quantified by liquid scintillation counting.

2.2.15 Cyclic-adenosine monophosphate (cAMP) release

The effect of 10, 100 and 1000 nM HXA_3 on cAMP release and fMLP-induced cAMP release was investigated by the use of ELISA (Cayman Chemical Company, U.S.A). Human neutrophils (10×10^6 cells/ml) were incubated at 37^{o}C for 5 min in the presence of 1.2 mM $CaCl_2$/1 mM $MgCl_2$ before the addition of HXA_3. To examine the effect of HXA_3 on fMLP-induced cAMP release, the HXA_3 was added 15 s before the addition of fMLP. At times 30, 60, 120 and 500 s, the reactions were stopped by the addition of four volumes ice-cold methanol and further analysed according to the manufacturer's recommendations. All experiments were performed in duplicate from two different blood donors. The basal level of expression of cAMP was expressed as that detected in cells incubated for 7 min in the presence of 1.2 mM $CaCl_2$/1 mM $MgCl_2$.

2.2.16 Intracellular Ca^{2+} measurement

The release of intracellular calcium was measured as described by Nigam *et al*. (1990a) with minor modifications. The cells were incubated with 1μM FURA 2/AM for 40 min, washed,

and incubated at 37°C for 5 min in the presence of 1.2 mM $CaCl_2$/1 mM $MgCl_2$ before the addition of fMLP or HXA_3. The change in fluorescence was measured using a Fluorescence Spectrophotometer F-4500 (Hitachi) equipped with continuous stirring, a beam splitter and two excitation monochrometers.

2.2.17 PMN aggregation

The aggregation of human neutrophils was determined as according to Nigam *et al*. (1990a). The cells were suspended at a concentration of 5×10^6 cells/ml, kept on ice and 200 µl utilised per experiment. A LAbor APACT aggregometer and software were used.

2.2.18 Phospholipid remodelling

Phospholipid remodelling analysis was performed using [1-^{14}C] arachidonic acid labelled cells and two-dimensional TLC as previously described by Serhan *et al*. (1982). The cells were incubated at 37^{o}C for 5 min in the presence of $CaCl_2$/$MgCl_2$ before the addition of fMLP or HXA_3. In some cases, the cells were incubated with HXA_3 for 5 min before the addition of fMLP. The reaction was stopped by the addition of methanol/chloroform/acetic acid (50:25:1, v/v/v) and extracted using the method of Bligh and Dyer (1959). The phospholipids were separated in the first dimension using chloroform/methanol/ammonium hydroxide (65:25:6, v/v/v) and chloroform/acetone/methanol/acetic acid/water (3:4:1:1:0.5, by vol.) in the second dimension. The TLC plates were developed and the radioactivity measured as described earlier.

2.2.19 Cell proliferation

Cell proliferation was quantified by measuring thymidine-(methyl-^{3}H) incorporation. The A431 and HeLa cells were washed, harvested by trypsination, rewashed and suspended at a concentration of between 60 000 – 80 000/ml in modified DMEM supplemented with 10% FCS. This was used to seed 6 well (3.8 ml diameter) plates (Nunclon, Nunc). Cells were grown to ~70% confluence and synchronised by the addition of DMEM/1% FCS for 2-5 days. The test compounds were either added (i) in DMEM/1% FCS for four hours before the addition of DMEM/20% FCS plus labelled thymidine (0.5 µCi) or (ii) in DMEM/20% FCS plus labelled thymidine (0.5 µCi) at time 0 or after 24 h. At the appropriate times, the medium was aspirated, the cells washed three times, trypsinated and the incorporated radioactivity measured in a Beckmann Scintillation Counter.

2.2.20 Superoxide anion generation

Superoxide anion generation was measured spectrophotometrically by super oxide dismutase (SOD) reduction of cytochrome C at 550 nm with a UV_{max} Kinetic® (Molecular Devices) microtitre plate reader (Pick *et al*., 1981). The neutrophils were suspended at a concentration of 5×10^6 cells/ml and 100 µl utilised for each determination.

2.2.21 Measurement of oxygen uptake

Oxygen uptake was measured at 37°C with an Oxygen Meter Model 781 (Strathkelvin Instruments, Glasgow, UK) fitted with a micro Clark electrode 1302 in a closed chamber of 500µl. Human neutrophils (10×10^6 cells/ml) were equilibrated until a constant base line was achieved before the addition of HXA_3, fMLP, genistein or staurosporine and oxygen uptake measured.

Assays with pure enzyme were started by the addition of 12-LOX (either platelet- or porcine leukocyte-type) to the reaction chamber containing the substrate. The reaction was stopped by the addition of diethylether-saturated 10 mM hydrochloric acid and allowed to stand for 30 min to ensure complete hydrolysis of the hepoxilins formed. Samples were further analysed as described in section 2.2.7.

2.2.22 Western blotting

To detect PHGPx protein; human platelets, A431 cells (positive control) and red blood cells (negative control) were lysed in modified RIPA buffer (1 x PBS, 1% NP-40, 10% glycerol, 145 mM NaCl, 10 mg/ml PMSF, 10 µg/ml aprotinin, pepstatin, antipain and leupeptin). The protein (50 µg) was separated on a 15% SDS-polyacrylamide gel and Western blotting performed. Briefly, the samples were boiled for 5 min, separated by polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane using a semi-dry electroblotter (Biometra, Germany). The membrane was blocked with 5% BSA for 1 h and primary detection performed using (i) a rabbit polyclonal antibody raised against a peptide fragment of human PHGPx (amino acids 116-130) obtained from FZB Biotechnik GmbH (Berlin, Germany) and kindly donated by Dr. H. Kühn (Berlin, Germany) and (ii) a rabbit polyclonal antibody against porcine PHGPx kindly donated by Dr. F. Ursini (Padova, Italy). Detection was performed using enhanced chemiluminescence (Santa Cruz Biotechnology, Germany).