3. MATERIALS AND METHODS

3.1. CELL CULTURES

Materials

-Sebomed^{TM} Basal Medium (Biochrom, Germany).

-Dulbecco's MEM (Biochrom, Germany).

-RPMI 1640 (Biochrom, Germany).

-Fetal Bovine Serum, (FBS) (Biochrom, Germany).

-Penicillin, 10000 U/ml – Streptomycin, 10000 µg/ml (P/S) (Biochrom, Germany).

-Human recombinant Epidermal Growth Factor, (EGF) (Sigma, Germany).

-Trypsin 0.05% - EDTA 0.02% (Biochrom, Germany).

-Dulbecco's Phosphate Buffered Saline (PBS), w/o Calcium and Magnesium (PAA Laboratories, Austria).

-NUNCLON^{TM} Surface Cultured Flasks (25 and 75 cm^2) (Nalge NUNC, Germany).

Cultures

-SZ95 sebocytes were cultured in Sebomed^{TM} containing 10% heat inactivated FBS, 5 ng/ml EGF, 1 mM $CaCl_2$ and P/S.

-HL60 cells were cultured in RPMI 1640 containing 10 % heat inactivated FBS and P/S.

-MCF7 cells were cultured in DMEM with glutamine containing 10% heat inactivated FBS and P/S.

-A549 cells were cultured in DMEM containing 10% heat inactivated FBS and P/S.

All the cells were cultured at 37 ^{o}C in a humidified atmosphere containing 5% CO_2.

3.2. ZILEUTON EXTRACTION

Since the substance zileuton was not available for experimental purposes, we extracted the active substance from an available oral drug preparation. The molecule is soluble in ethanol.

Material

-Zyflo Filmtab 600 mg (Abbott, USA).

Procedure

1. Coated film was removed from Zyflo tablet.
2. The tablet was pulverized and homogenized in a mortar.
3. The powder was weished and the mass of zileuton was estimated.
4. Ethanol was added to the powder and mixed well.

5. The mixture was centrifuged for 20 minutes at 4000 rpm.
6. The supernatant was transferred to a new tube and the pellet was discarded.
7. The supernatant containing zileuton was dissolved in ethanol and stored as a stock solution.

3.3. LDH CYTOTOXICITY ASSAY

This assay is based on the measurement of the cytoplasmic enzyme lactate dehydrogenase (LDH), which is present in all cells. When plasma membranes are damaged, the enzyme is released to the cell culture supernatants. The LDH activity is determined enzymatically. In a first step, NAD^+ is reduced to NADH/H^+ by the LDH catalyzed conversion of lactate to pyruvate. In a second step, the catalyst diaphorase transfers H/H^+ from NADH/H^+ to the tetrazolium salt, which is reduced to formazan. The increase in the amount of LDH in the supernatant correlates directly with the amount of formazan formed during a limited period of time. The intensity of formed color in the assay is proportional to the number of lysed cells. Formazan dye (red) shows an absorption at 500 nm, whereas tetrazolium does not show significant absorption at this wavelength.

Materials

-Arachidonic acid (Sigma, Germany).

-Calcium ionophore A23187 (Sigma, Germany).

-Caffeic acid (Sigma, Germany).

-Extracted zileuton.

-Dimethyl sulfoxide (DMSO) (Sigma, Germany). Arachidonic acid, calcium ionophore and caffeic acid were dissolved in DMSO and redissolved in culture medium at a final maximum DMSO concentration of 0.1% (v/v).

-96-Wells Tissue Culture Plate (TPP, Switzerland)

-LDH Cytotoxicity Detection Kit (Roche, Germany) consists of:

- Tetrazolium catalyst solution.
- Dye solution.

Procedure

A. Cells culture and treatment

1. SZ95 sebocytes (20000 cells per well) were cultured in 96-well plates for 2 days.
2. The third day, the sebocytes were treated with:
   a) 10^{-4} M AA
   b) $5x10^{-6}$, 10^{-5}, $5x10^{-5}$, 10^{-4} M Caf

c) $5x10^{-8}$, 10^{-7}, $5x10^{-7}$, 10^{-6}, $5x10^{-6}$ M CaI

d) $5x10^{-7}$, 10^{-6}, $5x10^{-6}$, 10^{-5}, $5x10^{-5}$ M Zil

One 96-well plate was incubated with these molecules for 4 hours and a second one for 24 hours. Every detected concentration was assayed in 10-fold.

B. Cytotoxicity assay

1. After the appropriate time, 100 µl of culture supernatant of each sample were collected and centrifuged.
2. 100 µl of each cell free supernatant was transfered into a clear 96-well plate.
3. Catalyst (250 µl) and dye solutions (11250 µl) were mixed shortly before use.
4. 100 µl of the reaction mixture was added to each well.
5. The plates were covered and incubated up to 30 minutes in the dark at room temperature on a plate shaker.
6. The samples of the plates were read at 490 nm on a Dynatec MR 500 plate reader.

3.4. PROTEIN DETECTION

3.4.1. Cells culture, treatment and protein extraction

SZ95 sebocytes were used for the protein detection of 5-LOX, 15-LOX-1 and LTA_4 hydrolase. HL60, MCF7 and A549 cells were cultured and treated simultaneously to verify the positive signals.

Materials

-M-PER™ Mammalian Protein Extraction Reagent (Pierce, USA).

Procedure

1. A. SZ95 cells (1000000 cells per 75 cm^2 flask) were incubated for 4 days. On the 5th day, the sebocytes were treated as following:

- for 30 and 60 minutes, with:
  a. 100 µM AA,
  b. 0.1 µM CaI,
  c. 100 µM AA + 0.1 µM CaI,
  d. 1 µM CaI,
  e. 100 µM AA + 1 µM AA,
- for 6, 12 and 24 hours, with:
  a. 100 µM AA,
  b. 0.1 µM CaI,

c. 100 μM AA + 0.1 μM CaI.

At all points in time, untreated sebocytes were used as controls.

B. HL60 cells (500000 cells per 75 cm^2 flask) were incubated for 5 days in the presence of 1.3% DMSO. The next day, the cells were treated for 6 hours with 100 μM AA.

C. MCF7 cells (500000 cells per 75 cm^2 flask) were incubated for 5 days. The next day, the cells were treated for 6 hours with 100 μM AA.

D. A549 cells (500000 cells per 25 cm^2 flask) were incubated for 1 day. The next day, the cells were treated for 24 hours with 10 ng/ml IL-4.

2. After the appropriate incubation time, the cells were washed twice with PBS.
3. 1000 μl ofM-PER™ reagent was adding to each flask. The cells were incubated for 15 minutes on ice.
4. The lysates were collected with a policeman rubber and transfered to microcentrifuge tubes (on ice).
5. The samples were centrifuged at 18000 g (Z233 MK, Hermle, Germany) for 10 minutes at 4 °C to pellet the cell debris.
6. The supernatants were transfered to clean tubes for further analysis or stored at –20°C.

In the case of SZ95 sebocytes treated for 30 minutes, a parallel procedure was performed. After the adding of M-PER™ reagent, the procedure was modified as follow:

5a. The lysates were sonicated for 10 seconds.

6a. The lysates, without centrifugation, were processed for further analysis.

3.4.2. Protein quantitation

The BCA Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. The method combines the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium with the colorimetric detection of the cuprous cation (Cu^{1+}) using a reagent containing bicinchoninic acid. The purple color of the reaction is formed by the chelation of two BCA molecules with one Cu^{1+}. This complex exhibits a strong absorption at 562 nm (73).

Materials

-BCA Protein Assay Reagent (Pierce, USA) contains:

- Reagent A, consists of sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide.
- Reagent B, consists of 4% cupric sulfate.

-Albumin Standard, Bovine Serum Albumin, Fraction V (Pierce, USA).

Procedure

1. A fresh protein standard was prepared by diluting the 2 mg/ml BSA stock standard in serial dilutions. PBS was used as diluent. 8 points of standard were applied (1500, 1000, 750, 500, 250, 125, 25, 0 mg/ml).
2. The wells of a 96-well plate were determined as standards, samples or blanks.
3. The samples were diluted 10x in PBS.
4. 10 μl of each standard, sample or blank was added to each well.
5. Reagents A and B were mixed together in a ratio of 196/4.
6. 200 μl of reagent's mixture was added to each standard, sample or blank.
7. The solutions were mixed for 30 seconds on a plate shaker.
8. The plate was incubated for 30 minutes at 37 ^{o}C.
7. The absorptions were read at 550 nm on a Dynatec MR 500 plate reader.
8. A standard curve was prepared and the protein concentrations were determined using the standard curve.

3.4.3. Western blot

Western blot is a method to detect a certain protein in a sample by using an antibody specific to that protein. It also gives information about the size of that protein. The procedure comprises in 3 steps:

-Gel electrophoresis. The proteins from an homogenized tissues or cells samples are separated according to size on a gel (usually Sodium Dodecyl Sulfate poly-acrylamide) using SDS poly-acrylamide gel electrophoresis (SDS-PAGE). The gel has several lanes so that several samples can be tested simultaneously and each lane contains one or more protein bands.

-Blotting. The proteins in the gel are then transfered onto a membrane made of nitrocellulose or polyvinylidene fluoride (PVDF), by pressure or by applying a electric voltage. This is the actual blotting process and is necessary to expose the proteins. The membrane binds all proteins, non-specifically.

-Blocking. The membrane is then blocked, suppressing non-specific protein interactions. This is done by a solution of BSA or dry milk. The first antibody is applied. This antibody recognizes only the protein of interest and does not bind any other protein on the membrane. When the second antibody is applied, it binds to the first antibody. This second antibody is conjugated to a chemical signal that can visually identify where on the membrane the antibody is bound. This chemical signal is often an enzyme such as horseradish peroxidase, which can produce

fluorescence or chemiluminescence in its substrate. (The enzyme conjugated to the secondary antibody catalyzes a colorimetric reaction, resulting in the deposition of colored substrate on the membrane at the reaction site.) Free antibody is washed away and a substance is added to the membrane, so that the second antibody will become visible.

Materials

-The NuPAGE® Tris-acetate Electrophoresis System (Invitrogen, USA) is a pre-cast polyacrylamide minigel system consisting of gels and buffers:

- NuPAGE® Novex Tris-acetate gels. While the tris-acetate buffered (ph 7.0) polyacrylamide (3-8%) gels do not contain SDS, they can be used for denaturing gel electrophoresis, when they are used with the recommended tris-acetate SDS running buffer. A 3-8% NuPAGE® Novex Tris-acetate gel provides a separation and resolution range of proteins between 40-400 kDa. The gels are pocketed in styrene cassettes.
- NuPAGE® Loading Dye Solution (LDS) Sample Buffer (4x) consists of: 4 g glycerol (40% w/v), 0.682 g tris-base (564 mM), 0.666 g tris-HCl (424 mM), 0.8 g LDS (8% w/v), 0.006 g EDTA (2.04 mM), 0.0075 g serva blue G250 (0.88 mM), 0.0025 g phenol blue (0.7 mM) in 10 ml ultrapure water (ph 8.5).
- NuPAGE® Tris-acetate SDS Running Buffer (20x) consists of: 89.5 g tricine (1 M), 60.5 g tris-base (1 M), 10 g SDS (2% w/v) in 500 ml ultrapure water (ph 8.5).
- NuPAGE® Reducing Sample (0.5 M dithiothreitol).
- NuPAGE® Antioxidant.

Other materials:

-5-Lipoxygenase (mouse) monoclonal antiserum (BD, Germany).

-15-Lipoxygenase (rabbit) polyclonal antiserum (Cayman, USA).

-Leukotriene A_4 Hydrolase (sheep) polyclonal antiserum (Cayman, USA).

-B-Actin (mouse) monoclonal antiserum (Sigma, Germany).

-Anti-mouse (rabbit) IgG (H+L) horseradish peroxidase (Jackson, USA).

-Anti-rabbit (mouse) IgG (H+L) horseradish peroxidase (Jackson, USA).

-Anti-sheep (donkey) IgG peroxidase conjugate (Sigma, USA).

-Full range rainbow protein molecular weight marker RPN 800 (Amersham, UK).

-Transfer buffer. A 5x solution consists of: 58.15 g tris (Sigma, Germany), 21.75 g glycine (Sigma, Germany), 3.7 g SDS (Sigma, Germany) in 1.6 L ddH_2O. An 1x transfer buffer is diluted in methanol.

-Gel Blotting Paper GB 003 (Schleicher-Schuell, Germany).

-Immobilon-P™ (Millipore, USA).

-Blocking solution, consists of: PBS, 0.1% Tween 20 (ICI, USA) and 0.2% (w/v) I-Block™ (Tropix, USA). I-Block™ is a highly purified casein-based blocking reagent.

-ECL Western Blotting Detection Kit (Amersham-Pharmacia, Sweden).

-Hyperfilm ECL (Amersham-Pharmacia, Sweden).

Procedure

A. Preparing samples

1. 30 µg of each sample (50 µg for 15-LOX-1), dissolved in lysis buffer, was evaporated by Speed Vac® Plus SC110A (Savant, USA).
2. The sample solutions were reconstructed adding 15.6 µl ddH_2O, 6 µl NuPAGE sample buffer (4x) and 2.4 µl NuPAGE reducing agent (10%). The sample solutions were mixed well.
3. The sample solutions were heated for 10 minutes at 70 ^oC. Then they were vortexed.

B. Gel electrophoresis

1. The NuPAGE gels were placed into Mini Protean™ II (Biorad, Germany) electrophoresis cell.
2. The inner electrophoresis chamber (cathode) was filled with 1x NuPAGE Tris-acetate SDS running buffer containing NuPAGE antioxidant (500 µl in 200 ml) and the outer chamber was filled with 1x NuPAGE tris-acetate SDS running buffer.
3. The samples solutions and a weight molecular marker were loaded into the wells of the gels.
4. The gels were run for 1 hour at 150 V (constant) at room temperature.
5. After the electrophoresis procedure, the cassettes of the gels were removed from the mini-cell. The gels were liberated from the cassettes and kept wet in transfer buffer before blotting.

C. Blotting

1. The filter papers and the PVDF membranes were cut to the size of the gels.
2. The pads and the filter papers were soaked in transfer buffer.
3. The PVDF membranes were briefly soaked in methanol and then in transfer buffer.
4. The "sandwiches" were assembled containing components in the following order: pad, 2 sheets of filter paper, gel, PVDF membrane, 2 sheets of filter paper and pad. The "sandwiches" were constructed in a dish containing cold transfer buffer.

5. The “sandwiches” were placed in the transfer chamber (Biorad, Germany), which were already filled with cold transfer buffer, so that the membrane in the “sandwich” was closest to the anode.
6. The proteins were transferred for 135 minutes at 100 V and 4°C.
7. After blotting, the PVDF membranes were removed from the “sandwiches”. The sites of the molecular weight markers were marked with a pencil.
8. The membranes were transfered immediately to a dish containing blocking solution and were incubated overnight at 4°C.

D. Blocking

1. The next day, the PVDF membranes were removed from the blocking solution.
2. The membranes were incubated with primary antibodies for 1 hour at room temperature. The appropriate antibodies were diluted in blocking solution. The dilutions were: 1/250 (5-LOX), 1/1000 (15-LOX-1) and 1/2000 (LTA_4 hydrolase). The diluted antibodies after the incubation were stored at –20 °C and reused. In the case of 15-LOX-1, the membrane was incubated for 1 hour with blocking solution at room temperature, followed immediately by blotting. The membrane was incubated overnight with the primary antibody at 4 °C. Washing as described below followed the next day. This modification resulted in an enhanced signal for 15-LOX on the membrane.
3. After the application of the primary antibody, the membranes were washed with PBS/ Tween 20 (0.1%) for 10 minutes. The wash solution was discarded and the membranes were washed again twice.
4. The membranes were incubated with the secondary antibodies for 30 minutes at room temperature. The propriate antibodies were diluted in blocking solution. The dilutions were: 1/5000 (anti-mouse IgG), 1/5000 (anti-rabbit IgG) and 1/2000 (anti-sheep IgG).
5. After the incubation with secondary antibodies, the membranes were washed with PBS/Tween 20 (0.1%) for 10 minutes. The wash solution was discarded and the membranes were washed again twice. In all steps, incubation was followed by agitation of the membranes.
6. Prior to use, reagents A and B of ECL™ detection kit were mixed (1/1).
7. The membranes were removed to a clean tray containing the mixed ECL reagents. The tray was rotated to allow the mixed solution to cover the surface of the membranes for 1 minute.
8. The membranes were removed and placed between 2 pieces of write-on transparent leaves. The leaves were softly pressed in order to remove air bubbles.

9. The membranes were placed in an X-ray cassette.
10. The membranes were exposed to X-ray film for various lengths of time until the exposure was optimal.

3.4.4. Membrane stripping and immunoblocking with β-actin

The blotted PVDF membranes, which were used for 5-LOX or 15-LOX-1 or LTA_4 hydrolase protein detection, were re-used for the detection of β-actin protein, too. This was possible by stripping the PVDF membrane. The only limitation in this technique is when the molecular weights of the former and latter proteins coincidentally fit in. Herein, 5-LOX (79 kDa), 15-LOX-1 (75 kDa) and LTA4 hydrolase (69 kDa) were diverse from β-actin (42 kDa).

<u>Reagent</u>

-Citric acid (Sigma, Germany).

<u>Procedure</u>

1. The blotted PVDF membranes were washed for 5 minutes in PBS (3x) after the end of blocking.
2. The membranes were incubated in an agitated dish containing citric acid (1 M, ph 1.5) for 30 minutes at room temperature.
3. The membranes were washed for 10 minutes in PBS (3x).
4. The membranes were transfered to a dish containing blocking solution and incubated overnight at 4 ^{o}C.
5. The next day, the blocking with β-actin followed as described above (blocking section). The dilution of β-actin was 1/10000 in blocking solution.

3.5. RNA DETECTION

3.5.1. Cells culture and treatment

<u>Procedure</u>

1. SZ95 sebocytes (1000000 cells per 75 cm^2 flask) were incubated for 4 days.
2. The 5th day, the sebocytes were treated for an hour with:
    a. 100 μM AA,
    b. 0.1 μM CaI
    c. 100 μM AA + 0.1 μM CaI,
    d. 1 μM CaI,
    e. 100 μM AA + 1 μM CaI.

3. After the incubation, the cells were washed twice with PBS and proceeded to RNA isolation.

3.5.2. Total RNA isolation

The RNeasy Mini Kit was used for total RNA isolation. This technique combines the selective binding properties of a silica-based membrane with the speed of microspin technology. A specialized high salt buffer system allows up to 100 µg of RNA longer than 200 bases to bind to RNeasy silica gel membrane. Biological samples are lysed and then homogenized in the presence of a highly denaturing guanidium isothio-cyanate containing buffer, which immediately inactivates RNases. Therefore, RNA remains intact. Ethanol is added to provide appropriate binding conditions. Then the samples are applied to RNeasy mini spin columns, where the total RNA binds to the membrane and contaminants are washed away. In the end, RNA was eluted from the column with RNase free water.

Materials

RNeasy Mini Kit (Qiagen, Germany) consists of:

-RNeasy Mini Spin Columns.

-QIAshredder Spin Columns.

-Buffer RLT. 1% β-mercaptoethanol must be added to RLT buffer before use.

-Buffer RW1.

-Buffer RPE (4x). The working solution is diluted in 100% ethanol.

-RNase free water.

Procedure

1. 1000 µl RLT buffer were added to each flask (75 cm^2) containing SZ95 sebocytes.
2. Homogenization was started after a few minutes and the flasks were stored at -70^oC before proceeding to next step.
3. The frozen lysates were allowed to warm up at room temperature.
4. The lysates were collected with a policeman rubber and transfered into QIAshredder columns sitting in 2-ml microcentrifuge tubes.
5. The complex column-tube was centrifuged for 2 minutes at ≥8000 g in order to homogenize the lysates.
6. QIAshredder columns were discarded.
7. 1000 µl of 70% ethanol was added to each microcentrifuge tube. The samples were mixed well by pipetting.

8. 700 µl of each mixture were applied to an RNeasy mini spin column sitting in a new 2-ml microcentrifuge tube.
9. The complex column-tube was centrifuged for 15 seconds at ≥8000 g.
10. The last step was repeated with the rest of the mixture using the same mini spin column and tube, but discarding every time the flow-through of the microcentrifuge tube.
11. 700 µl of RW1 buffer was added to each RNeasy column.
12. The complex column-tube was centrifuged for 15 seconds at ≥8000 g.
13. Each RNeasy mini spin column was placed in a new 2-ml microcentrifuge tube.
14. 500 µl of RPE buffer was added to each RNeasy mini column.
15. The complex column-tube was centrifuged for 15 seconds at ≥8000 g.
16. Flow-through was discarded from each microcentrifuged tube.
17. 500 µl RPE buffer were re-added to each column.
18. The complex column-tube was centrifuged for 2 minutes at ≥8000 g.
19. Each RNeasy mini column was placed in a 1.5-ml microcentrifuge tube.
20. 50 µl RNase free water were added to each column.
21. The complex column-tube was centrifuged for 1 minute at ≥8000 g.
22. Another 50 µl RNase free water were added to each column and RNA was eluted in a second centrifugation.
23. Eluted total RNA was stored at –70 ^{o}C.

3.5.3. Quantitation of total RNA

The concentration and purity of RNA were determined by measuring the absorption at 260 nm and 280 nm in an Ultraspec 2000 spectrophotometer (Pharmacia Biotech, USA). Absorption at 260 nm measures RNA concentration. An absorption of 1 unit at 260 nm corresponds to 40 µg/ml of RNA with water as reference. The ratio between 260 nm and 280 nm absorption values gives an estimation of RNA purity.

3.5.4. Reverse transcription

Ready-To-Go^{TM} T-primed First Strand Kit was used to generate full length first strand cDNA from total RNA templates. An oligo-(dT) primer was used for the transcription of RNA by a Moloney murine leukemia virus (M-MuLV) reverse transcriptase. Single strand complementary DNA (cDNA) generated from this kit was used as template for amplification by RT-PCR.

Materials

-Ready-To-Go^{TM} T-primed First Strand Kit (Amersham-Pharmacia, USA) consists of vials containing First-Strand Reaction Mix: dATP, dCTP, dGTP, dTTP, M-MuLV reverse transcriptase, RNase/DNase free BSA and Not I-d(T)$_{18}$ primer.
-Diethyl pyrocarbonate (DEPC)-treated water.
Procedure

1. 5 μg of total RNA of each sample were transfered into a RNase free microcentrifuge tube.
2. DEPC-treated water was added up to 33 μl total volume including RNA sample.
3. The solution was heated for 5 minutes at 65 ^{o}C.
4. The microcentrifuge tubes were transferred to a 37 ^{o}C bath and incubated for 5 minutes.
5. The vials containing the reaction mix were incubated for 5 minutes at 37 ^{o}C.
6. The RNA solutions were transferred from the microcentrifuge tubes to the mix-containing vials.
7. The vials were incubated for 5 minutes at 37 ^{o}C.
8. The contents of the vials were vortexed gently and centrifuged briefly.
9. The vials were incubated for 1 hour at 37 ^{o}C.
10. The ready single-strand cDNAs were stored at –20 ^{o}C, before proceeding to RT-PCR.

3.5.5. RT-PCR

Reverse transcriptase polymerase chain reaction (RT-PCR) is a simple method, which a cDNA template is amplified by a factor of many thousands or millions quickly and reliably. A single-strand cDNA template generated in a former step by reverse transcriptase is applied to a thermal cycler. A typical amplification solution includes a cDNA diluted in buffer, a thermostable DNA polymerase, 2 oligonucleotides primers, deoxynucleotide triphosphates (dNTPs) and salts. This solution passes through a series of different temperatures and times. Each RT-PCR procedure has many series termed as cycles. Each cycle comprises of 3 steps: a) denaturation, where cDNA is heating in order to be separated in single strands; b) annealing, where primers can be bound to the separated single-strands; c) elongation, where the extension of the primers occurs by DNA polymerase catalysis. A preincubation is performed usually in order to inactivate proteases/nucleases, reverse transcriptase and to denature cDNA. In addition, a final extension step is performed to fill-in the protruding ends of the newly synthesized PCR product.

Materials
-Ampli-Taq DNA Polymerase, 5 U/μl (Perkin-Elmer, Roche, USA).

-RT-PCR Buffer (10x) containing 15 mM $MaCl_2$, 100 mM Tris-HCl, 500 mM KCl (Perkin-Elmer, Roche, USA).

-dNTPs, 10 mM per nucleotide.

-Primers, 10 pmol/µl (Metabion, Germany):

- 5-LOX (Locus: NM_000698), 488bp:
  forward (5’ 1794-1819 3’): 5’-ACC ATT GAG CAG ATC GTG GAC ACG C-3’
  reverse (5’ 2257-2282 3’): 5’-GCA GTC CTG CTC TGT GTA GAA TGG G-3’
- 15-LOX-1 (Locus: U88317), 521 bp:
  forward (5’ 1375-1397 3’): 5’-TTC TAT GCC CAA GAT GCG CTG CG-3’
  reverse (5’ 1873-1895 3’): 5’-GCA GCC AGC TCC TCC CTG AAC TT-3’
- LTA_4 hydrolase (Locus: NM_000895), 378 bp:
  forward (5’ 1335-1356 3’): 5’-GAT GAC TGG AAG GAT TTC C-3’
  reverse (5’ 1691-1712 3’): 5’-CCA CTT GGA TTG AAT GCA GAG C-3’
- β-glucuronidase (Locus: NM_000181), 336 bp:
  forward (5’ 1411-1432 3’): 5’-ACT TGA AGA TGG TGA TGG CTC A-3’
  reverse (5’ 1726-1746 3’): 5’-CCA GAC CCA GAT GGT ACT GCT-3’

-DEPC-treated water.

Procedure

1. A mastermix was prepared, containing x-fold the following reagents:
   a. 5 µl RT-PCR buffer,
   b. 1 µl mixture of dNTPs,
   c. 2.5 µl forward primer,
   d. 2.5 µl reverse primer,
   e. 36.75 µl DEPC-treated water.
   f. At the end before use, 0.25 µl Taq-polymerase were added.

   The solution, where x was the number of the samples was mixed well.
2. 2 µl single-strand cDNA of each sample were added to RT-PCR tubes.
3. 2 µl DEPC-treated water were added to a tube and used as negative control.
4. 48 µl of the mastermix were added to each tube.
5. The tubes were transfered to thermal cycler (Mastecycler Gradient®, Eppendorf, Germany).
6. The PCR amplification was performed as following:

- 5-LOX: Preincubation, 94 ^{o}C / 5 minutes; denaturation, 94 ^{o}C / 30 seconds; annealing, 64 ^{o}C / 30 seconds; elongation, 72 ^{o}C / 1 minute; final extension, 72 ^{o}C / 7 minutes.
- 15-LOX-1: Preincubation, 94 ^{o}C / 5 minutes; denaturation, 94 ^{o}C / 1 minute; annealing, 58 ^{o}C / 30 seconds; elongation, 72 ^{o}C / 45 seconds; final extension, 72 ^{o}C / 7 minutes.
- LTA_4 hydrolase: Preincubation, 94 ^{o}C / 5 minutes; denaturation, 94 ^{o}C / 45 seconds; annealing, 56 ^{o}C / 45 seconds; elongation, 72 ^{o}C / 1 minute; final extension, 72 ^{o}C / 7 minutes.
- β-glucuronidase: Preincubation, 95 ^{o}C / 5 minutes; denaturation, 95 ^{o}C / 1 minute; annealing, 67 ^{o}C / 1 minute; elongation, 72 ^{o}C / 1 minute; final extension, 72 ^{o}C / 10 minutes.

3.5.6. Agarose gel electrophoresis

Materials

-DNA Molecular Weight Marker VIII, 16-1114 bp (Roche, USA).

-Bromophenol Blue Dye Solution (5x) (Biorad, Germany).

-Agarose (Eurogentec, Belgium).

-DNA Typing Grade TAE Buffer (50x), 2 M Tris-acetate, 50 mM EDTA (Gibcol-BRL, USA).

-Ethidium Bromide, 10 mg/ml (Sigma, Germany).

Procedure

1. A 2% agarose (w/v in ddH_2O) solution was prepared and heated. Then 50 µg/ml ethidium bromide were added. The solution was transfered into a gel tray and kept to cold.
2. The gel tray was placed in a clean gel tank (Biometra, Germany). The tank was filled with TAE buffer (1x).
3. 20 µl of each sample of 5-LOX / 15-LOX-1 / negative control were mixed with 5 µl dye solution. 8 µl of each sample of LTA4 hydrolase / β-glucuronidase / negative control were added to 2 µl dye solution. Then 1.5 µl of the DNA molecular weight was mixed with 8 µl dye solution.
4. Samples, negative control and molecular marker were loaded into individual wells of the gel.
5. Electrophoresis was performed at 80 V.
6. After electrophoresis, photos were taken of the gel (Kodak EDAS 290 photo camera) on a UV field.

3.6. LTB_4 ENZYME IMMUNO ASSAY (EIA)

The assay is based on the competition between LTB_4 and an LTB_4-acetylcholinesterase (AChE) conjugate (LTB_4 tracer) for a limited amount of LTB_4 antiserum. Since the concentration of the LTB_4 tracer remains constant, while the concentration of LTB_4 varies, the amount of LTB_4 tracer that is able to bind to LTB_4 antiserum will be inversely proportional to the concentration of LTB_4 in the well. This antibody-LTB_4 complex binds to a rabbit IgG mouse monoclonal antibody that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's reagent (which contains the substrate to AChE) is added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 402 nm. The intensity of the color, determined spectrophotometrically, is proportional to the amount of LTB_4 tracer bound to the well, which is inversely proportional to the amount of free LTB_4 present in the well during the incubation (74).

Materials

LTB_4 EIA kit (Cayman, USA) contains:

-96-well strip plate.

-LTB_4 EIA antibody.

-LTB_4 AChE tracer.

-LTB_4 EIA standard.

-EIA buffer.

-Wash buffer.

-Tween 20.

-Ellman's reagent.

Procedure

A. Cells culture and treatment

1. SZ95 sebocytes (500000 per 25 cm^2 flask) were cultured for 5 days.
2. The day after, sebocytes were treated for an hour with:
    a) 100 µM AA
    b) 1 µM CaI
    c) 10 µM Caf
    d) 5 µM Zil
    e) 100 µM AA + 1 µM CaI
    f) 100 µM AA + 1 µM CaI + 10 µM Caf
    g) 100 µM AA + 1 µM CaI + 20 µM Caf
    h) 100 µM AA + 1 µM CaI + 5 µM Zil.

SZ95 sebocytes were pre-incubated for 15 minutes with Zil before additional treatment with AA/CaI (191). In the case of Caf, no pre-incubation was required (188). For the time of incubation with various molecules, PBS (w/o Ca^{+2}) was used instead of culture medium.

3. After incubation, PBS was collected and stored at –80°C before proceeding to EIA.

B. EIA

1. 50 µl of each sample was added to the wells, in duplicates.
2. 50 µl of LTB_4 AChE tracer was added to each well.
3. 50 µl of LTB_4 antiserum was added to each well.
4. The plate was covered and incubated overnight at 4°C.
5. The next day, reagents were removed from the wells and the wells were washed 5 times with wash buffer.
6. 200 µl of fresh Ellman's reagent were added to each well.
7. The plate was covered and incubated 90 minutes in the dark at room temperature on a plate shaker.
8. The samples were read at 420 nm on a Dynatec MR 500 plate reader.
9. For the evaluation of LTB_4, the ratio 1/absorption was calculated for each sample.

3.7. CYTOKINES DETECTION

Cytokines were detected from SZ95 sebocytes supernatants using the Beadlyte^{TM} Human Multi-Cytokine Detection System. This is a homogeneous no-wash kit for rapidly and stimultaneously measuring of 6 cytokines from one 50 µl sample. Each 96-well strip plate is suitable for 96 assay points per cytokine (IL-1β, IL-6, IL-8, IL-10, $IL\text{-}12_{(p7O)}$ and TNF-α). The kit has greater sensitivity (8-2500 pg/ml) and a larger linear range than standard ELISA assays. Beadlyte^{TM} reagents are designed to be used by Bioplex^{TM} (Biorad, Germany). This instrument measures multiple analytes in a single reaction vessel. The assay is based on fluorescent microbeads and reporter molecules that can detect multiple cytokines in the same assay well. This is a sandwich immunoassay in which cytokine-specific capture antibodies are linked to unique fluorescent bead sets. The microsphere bead sets are classified by precise ratios of 2 fluorophors. A reporter molecule coupled to a third fluorophore quantifies the biomolecular interaction. Using microfluidics to align the beads in a single file, the Bioplex^{TM} instrument employs two lasers: one for the detection of the fluorescent bead itself and the other for the fluorescent reporter. The color signatures are captured optically and processed digitally into binding data.

Materials

Beadlyte^{TM} human Multi-cytokine Detection System 2 contains:

-96-well strip plate.

-Beadlyte^{TM} human multi-cytokine standards. Each standard contains 5000 pg of each cytokine, lyophilized from 100 µl PBS, ph 7.4, 10 mg/ml trehalose. Each multi-standard must be rehydrated.

-Beadlyte^{TM} assay buffer (20 ml) containing PBS, 1% BSA, 0.05% Tween 20, 0.05% sodium azide (ph=7.4).

-Beadlyte^{TM} anti-human multi-cytokine beads 2 containing IL-1β, IL-6, IL-8, IL-10, IL-$12_{(p7O)}$ and TNF-α mouse and rat monoclonal IgGs conjugated to beads. Each 2.5 ml of bead solution contains 100 beads per monoclonal cytokine antibody per µl dissolved in PBS, 1% BSA, 0.05% Tween 20, 0.05% sodium azide (ph=7.4).

-Beadlyte^{TM} biotinylated anti-human multi-cytokine reporter containing 2.5 ml of biotin-conjugated anti-IL-1β, -IL-6, -IL-8, -IL-10, -IL-$12_{(p7O)}$ and –TNF-α mouse and rat IgGs in PBS, 1% BSA, 0.05% Tween 20, 0.05% sodium azide (ph=7.4).

-Beadlyte^{TM} streptavidin-phycoerythrin containing 1 mg/ml streptavidin-phycoerythrin solution, 15 mM Tris, 150 mM NaCl, 0.05% sodium azide solution (ph=7.8).

-Beadlyte^{TM} stop solution containing 0.2% (v/v) formaldehyde in PBS (ph=7.4).

Procedure

A. Cells culture and treatment

1. SZ95 sebocytes (1000000 per 25 cm^2 flask) were cultured for 3 days.
2. The day after, sebocytes were treated for an hour with:
   a) 100 µM AA
   b) 1 µM CaI
   c) 10 µM Caf
   d) 5 µM Zil
   e) 100 µM AA + 1 µM CaI + 10 µM Caf
   f) 100 µM AA + 1 µM CaI + 5 µM Zil. Sebocytes were pre-incubated for 15 minutes with Zil before additional treatment with AA/CaI. In addition, all sebocytes were incubated in the presence of 1% FBS.
3. After 1 hour of treatment, the medium was replaced with normal medium containing 10% FBS. The samples were further incubated for 23 hours.
4. The next day, media were collected and stored at –80°C before proceeding to multi-cytokines detection.

B. Multi-cytokines detection

1. The multi-cytokine standards were serial diluted to make 7 points of concentration standard (1000, 250, 125, 62.5, 31.25, 15.6, 0 pg/ml).
2. 50 µl of each point of standard was added to wells, determined as standards.
3. 50 µl of each sample was added to each well, determined as sample. The samples were diluted 20-folds.
4. Multi-cytokine beads solution was vortexed at high speed for 15 seconds and then sonicated for an additional 15 seconds using a sonication bath (RM6, MGW, Lauda, USA).
5. 25 µl of multi-cytokine beads solution was added to each well of standard or sample.
6. The 96-well plate was covered and vortexed gently at low speed.
7. The cover was removed and the plate was incubated for 2 hours in the dark at room temperature on a plate shaker.
8. After incubation, 25 µl of multi-cytokine reporter solution was added to each well of standard or sample.
9. The 96-well plate was covered and vortexed gently at low speed.
10. The cover was removed and the plate incubated for 1.5 hours in the dark at room temperature on a plate shaker.
11. Streptavidin-phycoerythrin solution was diluted in assay buffer (1/25). From the new solution, 25 µl were added to each well of standard or sample.
12. The 96-well plate was covered and vortexed gently at low speed.
13. The cover was removed and the plate was incubated 30 minutes in the dark at room temperature on a plate shaker.
14. After incubation, 25 µl of stop solution were added to each well of standard or sample.
15. The 96-well plate was covered and vortexed gently at low speed.
16. The cover was removed and the plate was incubated 5 minutes in the dark at room temperature. The samples of the plate were read on Bioplex^{TM} instrument.

3.8. LIPID DETECTION

3.8.1. Nile-red staining

The dye nile-red (9-diethylamino-5H-benzo[alpha]phenoxazine-5-one) is an excellent vital stain for the detection of intracellular lipid droplets by fluorescence microscopy. The dye is very soluble in the lipids that it is intended to show and it does not interact with any tissue constituent except by solution. Nile-red, like oil-red dye, stains neutral lipids, but also non-esterified

cholesterol. When the cells are viewed for yellow-gold fluorescence (excitation, 450-500 nm; emission greater than 528 nm), neutral lipids such triglycerides are detected and when the cells are wieved for red fluorescence (excitation, 515-560 nm; emission greater than 590 nm) polar lipids such as phospholipids are detected (75).

Material

9-diethylamino-5H-benzo[alpha]phenoxazine-5-one (Kodak, USA) was dissolved in acetone (100 µg/ml).

Procedure

(see MUH test).

3.8.2. MUH test

The MUH test is a simple, fast method to evaluate the proliferation of adherent cultured cells. The assay is based on the emission of fluorescence after hydrolysis of 4-methylumbelliferyl heptanoate (MUH) by esterases in the cytoplasm of live cells. The emitted fluorescence is correlated with the number of live cells when the cells are in logarithmic proliferative phase (76).

Material

4-methylumbelliferyl heptanoate (Sigma, Germany) was dissolved in DMSO (10 mg/ml).

Procedure

The 96-well plates, which were used for the LDH cytotoxicity assay, were used also for MUH and nile-red staining evaluation. The plates after 24 hours of treatment with various concentrations of AA, CaI, Caf, Zil and combinations (see LDH cytotoxicity assay procedure) were processed as follows:

1. The medium of each well were discarded.
2. The wells were rinsed in PBS twice.
3. The 96 wells were divided in 2 groups. The first group of wells (60% of total wells) was performed for nile-red staining evaluation and the second group of wells (40%) was performed for MUH test.
4. 100 µl of nile-red solution (1 µg/ml nile-red dissolved in PBS) were added to each well of the first group and 100 µl MUH solution (100 µg/ml MUH dissolved in PBS) were added to each well of the second group.
5. The plates were incubated for 20 minutes and 30 minutes (37^oC, 5% CO_2) for nile-red and MUH assays, respectively.

6. The samples of the 96-well plates were read by Spectra Max Gemini plate reader. The excitation and emission wavelengths for non-polar lipids were 485 nm and 565 nm and these of polar lipids, 540 nm and 620 nm, respectively. The excitation and emission wavelengths for MUH were 355 nm and 460 nm, respectively. The data were analyzed with Max Pro 3.1.2 software and values were given in absolute fluorescence units (AFU).

3.9. IMMUNOCYTOCHEMICAL STUDIES

3.9.1. Cytocentrifugation

Cytocentrifugation is a technique to separate the cells from their suspension medium and recover them on a microscope slide. It was performed in a specially designed bench-top centrifuge (Cytospin 2, Shandon, Germany).

Procedure

1. SZ95 sebocytes (1000000 cells per well) cultured in 25 cm^2 flasks were treated for an hour with: a) 100 µM AA, b) 1 µM CaI, c) 100 µM AA and 1 µM CaI. In addition, untreated cells were used as control.
2. After 1 hour incubation (37°C, 5% H_2O), cells were trypsinized as was above. The cells were suspended in PBS and counted. 40000 cells of each sample were transferred to 100 µl of PBS.
3. The centrifugation equipments were assembled. Sample suspensions were loaded in centrifuge adaptors and centrifuged at 700 rpm for 4 minutes.
4. After centrifugation, the specimens were air dried for some minutes.
5. Cells on slides were fixated for 5 minutes in ice-cold acetone. Then samples were stored at –20°C until their usage or processed immediately using the APAAP technique.

3.9.2. APAAP

The alkaline phosphatase anti-alkaline phosphatase (APAAP) technique is an immunohistochemical method. It is an unlabelled antibody bridge technique for labelling monoclonal antibodies. The first antibody binds to our specific antigen. The secondary antibody forms a bridge between primary and third antibody. First, a mouse monoclonal antibody is applied, then a polyclonal bridge antibody and finally a soluble complex of alkaline phosphatase and monoclonal mouse anti-alkaline phosphatase. After applying these antibodies, an alkaline phosphatase substrate containing a naphthol salt and new fuchsin as dye is added. Endogenous alkaline phosphatase is inhibited by adding levamisole to the substrate solution. The specific reaction produces a vivid red color. The specimens are counterstained with hematoxylin. The

intensity of the APAAP labelling reaction can be enhanced by repeating the bridge and APAAP complex incubation steps (77, 78).

Materials

-Primary antibody: Mouse anti-human 5-LOX monoclonal antibody (B&D Biosciences, USA).

-Secondary antibody: Rabbit anti-mouse (DAKO, Germany).

-APAAP complex: Mouse, AP/7/6/7, monoclonal (DAKO, Germany).

-Modified RPMI medium (ph=7.4-7.6): 50 ml RPMI (Seromed, Germany) + 450 ml ddH$_2$O + 50 ml inactivated FBS (Biochrom, Germany).

-Development solution: (87.5 ml NaCl + 1.5 g tris-HCl + 4.9 g tris-base) + 21 g propandiol in 1000 ml ddH$_2$O.

-Tris buffered saline (ph=7.4-7.6): 4.5 g tris-base (Sigma, Germany) + 34.25 g tris-HCl (Sigma, Germany) + 43.90 NaCl (Sigma, Germany) in 5000 ml ddH$_2$O.

-Human serum protein (BIOSEKO-Biotest, Germany).

-Sodium nitrite (Merck, Germany).

-Levamisol (Sigma, Germany).

-Naphthol-As-Bi-phosphate (Sigma, Germany).

-Dimethyl-formamide (Merck, Germany).

-New fuchsin (Sigma, Germany).

-Mayer's hematoxylin counterstain (Sigma, Germany).

Procedure

1. Centrifuged preparations were incubated for 30 minutes with primary antibody (1/150 diluted in modified RPMI) at room temperature. Some samples, which served as negative controls, were incubated with modified RPMI in the absence of 5-LOX antibody.
2. The slides were washed with tris buffered saline (2x5 minutes).
3. The preparations including the negative controls, were incubated for 30 minutes with secondary antibody (1/20 diluted in modified RPMI containing 1/8 human serum) at room temperature.
4. The slides were washed with tris buffered saline (2x5 minutes).
5. The preparations were incubated for 30 minutes with APAAP complex (1/50 diluted in modified RPMI containing 1/8 human serum) at room temperature.
6. The slides were washed with tris buffered saline (2x5 minutes).
7. The incubation with secondary antibody was repeated for 10 minutes.
8. The slides were washed with tris buffered saline (2x5 minutes).
9. The incubation with APAAP complex was repeated for 10 minutes.

10. The slides were washed with tris buffered saline (2x5 minutes).
11. Alkaline phosphatase substrate was prepared immediately before the use as follows: 50 mg of sodium nitrite was dissolved in 1250 µl ddH_2O and 500 µl new fuchsin. The solution was allowed to react for 1 minute. A second solution was prepared containing 125 mg naphthol-As-Bi-phosphate in 1500 ml dimethyl formamide. Then both solutions were added to 237.5 ml development buffer. The buffer (ph=8.8) was filtrated.
12. The slides were incubated in alkaline phosphatase substrate-chromogen solution for 20 minutes at room temperature.
13. The slides were washed with tris buffered saline (3x5 minutes).
14. The preparations were stained with Mayer's hematoxylin for 1 minute.
15. The slides were washed with ddH_2O many times and kept to air dry.
16. The slides were covered with slips.

3.10. STATISTICAL ANALYSIS

Statistical significance was determined by using the Student's t-test. The significance of each sample was referred always in relation with control, except other was reported. The values were defined as significant when $P \leq 0.05$ (*$P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

4. RESULTS

4.1. CYTOTOXICITY ASSAY

First of all, we examined the bioability of SZ95 sebocytes when they were treated with various concentrations of the tested substances. The concentrations tested were based on literature studies. Treated sebocytes as well controls were incubated for 4 and 24 hours. Then LDH released from damaged cells were measured using the cytotoxicity detection kit (Roche). This investigation was done in order to detect the non-toxic concentration rate of Caf, AA, CaI and Zil. We have defined as toxic concentration a concentration, which the LDH value was higher than 15% of the baseline (control cells).

In SZ95 sebocytes treated for 4 hours with Caf concentrations equal to or higher than 50 µM were toxic. Lower concentrations were non-toxic. After 24 hours of incubation, 100 µM Caf were also toxic (Fig. 1a). Therefore, we used 20 and 10 µM of Caf for 1 hour of treatment in our experiments.

100 µM AA were non-toxic at both points in time (Fig. 1a). We used 100 µM AA for up to 24 hours treatment.