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.

CaI was toxic after 4 hours' incubation at a concentration of 5 μM. Concentrations between 0.05 and 1 μM were non-toxic. 24 hours' treatment exhibited toxic effects at concentrations of 1 μM and higher (Fig. 1b). Therefore, 1μM CaI was applied for 1 hour and 0.1 μM for over 1 hour treatment in our experiments.

Zil was non-toxic at any investigated concentration (0.5-50 μM) and at both points in time (Fig. 1c). We used a concentration of 5 μM.

4.2. WESTERN BLOT ANALYSIS

Multiple exposure times were used for all blots in order to ensure that band densities were within the linear range of exposure. The X-ray films were photographed with Kodak EDAS 290 photo-camera and then the photographed bands were analyzed by densitometry using the Kodak 1D Image Analysis Software. For each individual sample, the area under the peak from the densitometer tracing that corresponded to the protein band was used as a measure of the amount of protein in each sample. Although an indirect measurement of the absolute quantity of enzyme was performed, this method provided a reliable estimation of the protein quantities. In order to limit possible variations of the amount of each loaded protein, we calculated the ratio between measured density of each sample and the corresponding β-actin signal of each individual sample. Hence, relative differences of the enzyme expression among the various samples could be estimated. Moreover, the values of each sample were calculated in such way as they were referred as a percentage of the controls. The mean ±SD of the values was also calculated.

4.2.1. Beta-actin

Beta-actin is a housekeeping protein, which is constitutively expressed in all almost cells. The protein is also expressed in SZ95 sebocytes and its expression remains stable. The antibody we used, targeted a 42 kDa protein. In our blotting, the β-actin band was located at a point, that corresponded to this exact molecular weight.

4.2.2. 5-Lipoxygenase

Protein lysates were centrifugated at 18000 g and the supernatants were used in Western Blotting (experiment performed 5 times). At that centrifugation speed, the pellet is certain to contain the nuclei. According to the manufacturer reference, the molecular weight of 5-LOX is 79 kDa. When a 3-8% tris-acetate gel was used, multiple bands occurred after blotting with the 5-LOX antibody (Fig. 2). A band corresponding to 79 kDa was also present in both treated SZ95 sebocytes and controls. The other bands were unspecific and were due to interactions of the 5-

LOX antibody with cytoskeletal proteins, as confirmed by the manufacturer. The human mammary carcinoma cell line MCF7 was used as positive control for the protein expression of 5-LOX (79, 80) and was found to express the 79 kDa. In our studies, MCF7 cells were treated for 6 hours with 100 μM AA. The human promyelocytic leukemia HL60 line was used as second positive control, since HL60 cells undergo differentiation into neutrophils in the presence of DMSO and express 5-LOX (81, 82). HL60 cells were also treated for 6 hours with 100 μM AA but no expression of a 79-kDa band was identified.

After 30 minutes' incubation time with AA and/or CaI, the quantity of 5-LOX was reduced (Fig. 2b). AA caused a slight decrease of the protein. However, the reduction was not significant. On the other hand, 0.1 μM CaI and in combination with 100 μM AA decreased 40% the protein amount. A weaker reduction was observed in samples treated with 100 μM AA and 1 μM CaI. These results were statistically significant as shown in figure 5b. Although stimulation with 1μM CaI down-regulated 5-LOX expression remained not significant. The fact that 5-LOX expression in supernatants was down-regulated in the presence of AA and CaI suggests that 5-LOX was translocated to the nucleus.

At the second point time (1 hour), AA and 0.1 μM CaI induced strongly the protein expression. Less vigorous was the induction in the presence of 1 μM CaI or combined treatment (AA and CaI). However, the differences were not significant (Figs. 2c, 5c). It is known that in resting cells, 5-LOX activity is present in cytosol (22). In addition, when exogenous AA is present, leukotrienes generation occurs independently of the 5-LOX translocation to the nuclear membrane (5). It is possible that after a long period of incubation, AA and CaI could not cause new translocation of 5-LOX to nucleus. Moreover, when mast cells and macrophages are activated with CaI for a long time, membrane binding leads to irreversible enzyme inactivation (83). We supposed that after long period of AA and CaI incubation, only the 5-LOX cytosolic pool is active and the part of 5-LOX bound to the nuclear envelope remains inactivated.

At 6 hours of incubation, 5-LOX was slightly up-regulated in the presence of AA and/or CaI. Only 100 μM AA increased significantly the expression of 5-LOX (Figs. 2b, 5d).

Figures 2c and 5e show that a new reduction of the protein occurred at 12 hours incubation. The motive was similar to that of the 30 minutes point. CaI and AA acted synergically (significant reduce). AA caused less protein loss from the cytosol than CaI (not significant). Probably a translocation occurs a second time.

Finally, at 24 hours, no significant differences were observed in 5-LOX expression (Figs. 2d, 5f).

In order to investigate the expression of 5-LOX and 15-LOX in whole lysates of the SZ95 sebocytes, we used the lysates without proceeding in centrifugation. The lysates were only sonicated for 10 seconds for the purpose of homogenization. Here, the procedure was performed twice and only for the 30 minutes' point in time. The treatment of the cells with AA and CaI together resulted in an upregulation of 5-LOX (Fig. 2a). The expression was stronger with 1μM CaI than 0.1 μM CaI. The combined treatment was also more effective than mono-treatment and CaI dose-dependent. Nevertheless the results were no significant due to few numbers of statistical data (Fig. 5a). In conclusion, Western Blot analysis shows that 5-LOX protein is present in sebocytes, its production is upregulated by AA and CaI and a translocation of the enzyme to the nucleus occurs in the first minutes of treatment.

4.2.3. 15-Lipoxygenase

A 75 kDa band corresponding to the molecular mass of 15-LOX was detected. Control and treated cells expressed this protein at all points in time. As reported for 5-LOX detection, multiple bands occured on the 15-LOX blocked membrane, too. These bands were unspecific. A549 human lung carcinoma cell line, was used as positive control for 15-LOX-1 (49). A549 cells were treated for 24 hours with IL-4 in order to express 15-LOX-1. The 75 kDa specific band was present in these cells.

The expression of 15-LOX-1 follows a similar profile with 5-LOX. In 30 minutes, AA and CaI reduce the quantity of the protein by about 40%. Also here it seems that the enzyme was translocated to membrane fractions. In this effect, CaI was more important than AA. CaI induced reduction of 15-LOX-1 was significant at both concentrations. We noted also a synergic effect in combined treatment, significant at concentrations of 100 μM AA and 0.1 μM CaI (Figs 3b, 6b).

At 1 hour incubation time, densitometry showed at least 50% elevation of protein expression after AA and 0.1 μM CaI stimulation. The differences were found to be not significant. In other samples no differences were present (Figs 3c, 6c).

At the points of 6, 12 and 24 hours, no remarkable alterations were observed (Figs 3b-d and 6d-f). Only 0.1 μM CaI increased the protein expression, but without being significant.

As the last point, we will report the western blot analysis of whole lysates at 30 minutes' incubation time. Here, densitometry showed small differences with trends to down-regulate the protein expression. However, the results were not significant (Fig.3a, 6a). We speculate that AA and CaI do not affect the expression of 15-LOX-1. They could only participate in the process of the enzyme translocation to membranes. This translocation is notable during the first 30 minutes. Our data did not show which of membrane was the new location of the enzyme. According to

other studies, this would be the cellular membrane and not the nuclear envelope. Another important observation was the weak expression of 15-LOX-1 in SZ95 sebocytes. When we compared the bands between 15-LOX-1 and 5-LOX, the latter was stronger. In addition, 50 μg of total protein were loaded to each well of 15-LOX gel instead of 30 μg used in 5-LOX gel.

4.2.4. LTA_4 hydrolase

LTA_4 hydrolase as expected, was present in SZ95 sebocytes. Human LTA_4 hydrolase has a molecular weight of 69 kDa. In our western blot analysis, a 69 kDa band was visible in all samples and points in time. HL60 and MCF7 lysates expressed also this band. AA or CaI in any concentrations did not affect the protein expression of the enzyme. Only at 30 minutes in the presence of CaI and AA, a small reduction was observed, without being significant (Figs. 4a. 7a). At other points of time, no remarkable differences were observed (Figs. 4a-c and 7b-e). Thus, neither translocation nor AA and CaI induction might be necessary for the LTA_4 hydrolase expression.

4.3. RT-PCR ANALYSIS

To investigate the expression of the enzymes of interest, total RNA was isolated from resting and treated samples. In these studies, mRNA expression was determined by RT-PCR. RT-PCR analysis of β-glucuronidase (β-Gus), a housekeeping gene expressed constitutively in SZ95 sebocytes was used to define the lower limit of detection for the assay and provided a control for cDNA fidelity. The visualization using UV light showed a 336 bp band encoding the β-Gus cluster. As it is shown in figure 8d, all samples used contained equal amounts of cDNA.

4.3.1. 5-Lipoxygenase

In RT-PCR with 5-LOX specific primers, a 488 bp band corresponding to the expected 5-LOX fragment was found in all samples. Controls as well treated SZ95 sebocytes expressed 5-LOX mRNA after 1 hour of incubation. Although the RT-PCR was not quantitative, with respect to β-glucuronidase, a higher expression was noted in the cells treated with AA and CaI in combination. Thus, a combination of 100 μM AA and 0.1 μM CaI as well a combination of 100 μM AA and 1 μM CaI stimulated the expression of 5-LOX mRNA. No visual differences were found between controls and AA-treated cells or between controls and CaI-treated cells (Fig. 8a).

4.3.2. 15-Lipoxygenase

A 521 bp band identical to 15-LOX-1 was detected in SZ95 sebocytes. Resting cells had a positive signal for 15-LOX-1. After 1 hour' treatment with AA and CaI, the sebocytes expressed 15-LOX-1 mRNA, too. All bands looked similar (Fig. 8b). When we compared the intensity of the bands between 5- and 15-LOX-1, we observed that 5-LOX signaling was stronger than that of 15-LOX-1. In both cases, the conditions of the procedure remained stable: 20 μl of each sample were mixed with 5 μl loading dye (5X) and then the samples were run in 2% agarose gel. Samples for 5-LOX, 15-LOX-1 and LTA_4 hydrolase amplification were derived from the same cDNA source. Thus, 15-LOX-1 mRNA expression was weaker than that of 5-LOX in SZ95 sebocytes.

4.3.3. LTA_4 hydrolase

The third investigated enzyme, LTA_4 hydrolase, was also present in SZ95 sebocytes. A detectable signal coding a LTA_4 hydrolase fragment appeared in electrophoresis gel. A 378 bp band was detected in control and in treated cells with 100 μM AA, 0.1 or 1μM CaI. The intensities of the detected bands indicated that the LTA_4 hydrolase was expressed equally in all samples. Confrontations between LTA_4 hydrolase and 5- and 15-LOX-1 visualized bands showed that the signals from the first were much stronger (Fig. 8c). Moreover, only 8 μl of each LTA_4 hydrolase sample were added to 2 μl dye and then loaded in 2% agarose gel. Thus, LTA_4 hydrolase occurred at mRNA level in SZ95 sebocytes. The enzyme seems to be expressed constitutionally since controls expressed LTA_4 hydrolase and the signal was very strong.

4.4. 5-LIPOXYGENASE ACTIVITY

We have investigated the 5-LOX activity as generation of LTB_4 by SZ95 sebocytes. The LTB_4 formation was determined by enzyme immuno assay (EIA) detecting the release of LTB_4 in cell supernatants. SZ95 sebocytes produced detectable amounts (ng/ml) of this eicosanoid after 1 hour' treatment with AA and CaI. The results were evaluated in units defined as 1/ absorption values. Absorption values were inversely proportional to the amount of free LTB_4. AA (100 μM) up-stimulated significantly the production of LTB_4, whereas 1μM CaI had no stimulatory effect on the production. Furthermore, a significant increase of leukotriene generation was noted after combined treatment with AA and CaI. LTB_4 remained at the baseline when 5-LOX inhibitors Caf and Zil were added alone. Combined treatment of the cells with AA, CaI and 5 μM Zil or 20 μM Caf did not inhibit LTB_4 formation. An inhibitory effect was only seen in the presence of 10 μM Caf. Here, the reduction of LTB_4 was significant (Fig. 9). It is

strange that CaI could not induce LTB_4 production of sebocytes since AA could. We will try to give an explanation based on studies performed on mast cells, macrophages and leucocytes and we have previously reported (5, 22, 83). Long incubation with CaI causes a 5-LOX translocation, inactivation of the enzyme and loss of leukotriene synthetic capacity. However, a part of 5-LOX activity is still present in cytoplasm and dependent of exogenous AA. This activity is Ca^{2+} independent. Therefore, exogenously administrated AA can induce the LTB_4 synthesis in sebocytes. In contrast, CaI does not induce LTB_4, since 5-LOX is inactivated in nuclear envelope. The stimulation of LTB_4 after AA and CaI incubation is only through AA. Zil inability to inhibit LTB_4 formation in our study differs from published data. The presence of Zil in briefly (up to 5 min) CaI treated rat basophilic leukaemia cells blocks 5-LOX inactivation and enhances LTB_4 formation. However, with longer (15 min) CaI incubation, Zil does not block enzyme inactivation or reduce LTB_4 production (83). Probably the extract of Zil might play a role.

4.5. CYTOKINES RELEASE

SZ95 sebocytes are capable of producing cytokines. IL-1β, IL-6, IL-8, IL-10, IL-12(p70) and TNF-α were investigated. The molecules were detected by ELISA 24 hours after various stimulation.

4.5.1. IL-1β

This interleukine was released by sebocytes. When cells were treated with AA (100 μM) and CaI (1 μM) for an hour, no alterations of the IL-1β release were noted after 24 hours. 5-LOX inhibitors, Caf (10 μM) and Zil (5 μM) did not modulate the IL-1β release (Fig. 10).

4.5.2. IL-6

IL-6 was detected in sebocyte supernatants. AA and CaI enhanced significantly IL-6 production. AA (100 μM) stimulation on IL-6 was greater than CaI (1 μM). Combined treatment resulted in moderate stimulation. On the other hand, we did not observe any effect of Caf (10 μM) and Zil (5 μM) on IL-6 (Fig. 11).

4.5.3. IL-8

This cytokine was also present in SZ95 cells supernatants. Here CaI (1 μM) up-regulated significantly IL-8 generation. AA (100 μM) did not result in a cytokine elevation. The increased amount of cytokine after combined treatment was due to CaI administration. 5-LOX inhibitors did not show any significant change (Fig. 12).

4.5.4. TNF-α

TNF-α was produced by SZ95 sebocytes. However, neither 5-LOX stimulators nor 5-LOX inhibitors affect the cytokine release (Fig. 13).

4.5.5. IL-10, IL-12(p70)

None of these interleukines were detected in SZ95 sebocytes.

4.6. LIPID SYNTHESIS

The effect of 5-LOX stimulators and inhibitors on lipids synthesis was investigated in SZ95 sebocytes. Cells were treated for 24 hours with CaI ($5x10^{-8}$ M – $5x10^{-6}$ M), Caf ($5x10^{-6}$ M - 10^{-4} M) and AA (10^{-4} M). In a second step, we treated the cells with fixed concentration of AA (10-4 M) and CaI (5x10-7 M) and various concentrations of Caf ($5x10^{-6}$ M - 10^{-5} M) or Zil ($5x10^{-6}$ M - $5x10^{-5}$ M). Then the accumulation of non-polar and polar lipids was visualized using nile-red staining on cells. In addition, the effect of the examined molecules on cells proliferation was tested using the MUH test.

4.6.1. Non-polar lipids

CaI does not practically succeed in inducing the formation of non-polar lipids. A small significant elevation was noted in the concentration of 10^{-7} M. As the MUH test demonstrated, concentrations of 10^{-6} M and higher were cytotoxic after 24 hours' treatment. This was in accord with LDH cytotoxicity results. It was obvious that 10^{-4} M AA significantly accelerated the production of non-polar lipids (Fig. 14a). When a combined treatment was applied, AA and CaI together significantly stimulated lipids, too (Fig. 16a). Mono-treatment with Caf did not reduce any lipid generation (Fig. 14a). Furthermore, we did not observe significant differences between control and Zil mono-treated cells (Fig. 15a). Only $2x10^{-5}$ M Caf significantly inhibited the lipids formation after stimulation with AA and CaI. Similar effect was shown when AA/CaI stimulated cells were incubated with 10^{-5} M Zil. Other concentrations failed to reduce non-polar lipids (Fig. 16a).

4.6.2. Polar lipids

5-LOX stimulators, CaI and AA did not affect polar lipid synthesis at any concentration tested. On the other hand, Caf showed a slight inhibition of lipid accumulation at all concentrations, but it was not significant (Fig. 14c). Application of Zil also did not cause any inhibition (Fig. 15c). Combined treatment (AA/CaI) had no effect on polar lipid synthesis. When

we tried to inhibit their synthesis with various concentration of Caf, no decrease was noted in stimulated cells. Furthermore, a strange observation was made when AA/CaI treated cells were incubated with Zil. Zil ($5x10^{-5}$ M - 10^{-5} M) seemed to induce significantly polar lipid generation (Fig. 16c). Probably this effect was due to the purification state of Zil.

We conclude that AA and in less manner CaI stimulated non-polar lipids synthesis. By contrast, no effect was noted in polar lipids. 5-LOX inhibitors had an inhibitory effect at particular concentrations when they were administrated stimultaneously with AA/CaI. This occured only in non-polar lipids.

4.7. 5-LIPOXYGENASE IMMUNOSTAINING

The APAAP technique was applied in SZ95 sebocytes. This method was found to give good labelling in cytocentrifuged cells. The vivid red reaction color was easily identified even, when the staining reaction was relatively weak or only few positive cells were present in each sample. In our study, 5-LOX was present in cytocentrifuged preparation of sebocytes. The enzyme was present even in control and treated cells (Fig. 17). Positive stained SZ95 cells accounted for less than 10% of the total cell population. We could not find any differences in immunostaining between controls and sebocytes treated for 1 hour. Moreover, we did not observe any differences between cells treated with 100 μM AA, cells treated with 1 μM CaI and those treated with both molecules (100 μM AA and 1 μM CaI). Among all samples, 5-LOX staining was stronger in bigger well differentiated SZ95 cells than in smaller undifferentiated ones. In large sebocytes, cytosol possesses large part of the cell. The well staining of 5-LOX in sebocytes cytoplasm is evidence that the enzyme has mainly cytoplasmic allocation. This is in accord with our western blot analysis results, where 1-hour incubation with AA and/or CaI enhanced 5-LOX protein in cytosol. Probably a new translocation of the enzyme to the nucleus did not occur. However, translocation occured earlier within the first minutes of stimulation.

5. DISCUSSION

We have demonstrated the presence of LOXs in SZ95 sebocytes. The AA pathway in SZ95 sebocytes in vitro does exist and is active. The following findings support this statement:

a) 5-LOX, 15-LOX-1 and LTA_4 hydrolase are expressed in SZ95 sebocytes at protein and mRNA levels.
b) 5-LOX is translocated from the cytoplasm to the membranes, incl. the nucleal membrane, after stimulation.