Cyclosporin A Down-regulates the LTA$_4$ Hydrolase Level in Human Keratinocyte Cultures

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Leukotriene A$_4$ hydrolase is a key enzyme in the biosynthesis of leukotriene B$_4$, a potent pro-inflammatory compound. The purpose of this study was to determine the capacity of anti-inflammatory and anti-proliferative compounds to regulate the levels and activity of leukotriene A$_4$ hydrolase in cultured human keratinocytes. The content of leukotriene A$_4$ hydrolase was determined by Western blot analysis, and the activity of leukotriene A$_4$ hydrolase was expressed as the leukotriene B$_4$ formation after incubation of keratinocyte cultures with leukotriene A$_4$. Leukotriene B$_4$ was measured by reversed-phase high performance liquid chromatography. Preincubation for 10 min of the cultured keratinocytes with the leukotriene A$_4$ hydrolase inhibitor RP 64699 (0.1–10 μM) caused a significant dose-dependent inhibition of leukotriene B$_4$ formation (IC$_{50}$=0.7 μM). Cyclosporin A (0.1 μg/ml and 1.0 μg/ml) had no direct effect on leukotriene A$_4$ hydrolase activity, but after incubation for 72 h there was a decrease in the mean leukotriene B$_4$ formation per culture dish (35% and 48%, respectively). The decreased leukotriene B$_4$ formation was caused mainly by a decrease in the mean leukotriene A$_4$ hydrolase content per mg protein (30.1% at 0.1 μg/ml cyclosporin A and 40.0% at 1.0 μg/ml cyclosporin A), although keratinocyte proliferation was also slightly decreased. Incubations with 1,25-dihydroxyvitamin D$_3$ (10$^{-7}$–10$^{-10}$ M), all-trans retinoic acid (10$^{-8}$–10$^{-10}$ M), eicosatrienoic acid (10$^{-6}$–10$^{-8}$ M), dexamethasone (10$^{-5}$–10$^{-7}$ M), interferon-γ (10 and 100 units/ml) or methotrexate (0.1–10 μg/ml) had no effect on either the leukotriene B$_4$ formation or the amount of leukotriene A$_4$ hydrolase in keratinocyte cultures. These results show that cyclosporin A, in contrast to other anti-inflammatory and anti-proliferative compounds, inhibits the level of leukotriene A$_4$ hydrolase in keratinocyte cultures. Since similar cyclosporin A concentrations are obtained during treatment of psoriasis with cyclosporin A, the effect on leukotriene A$_4$ hydrolase may play a role in the anti-inflammatory action of cyclosporin A. Key words: leukotriene B$_4$; inflammation; psoriasis.

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Leukotriene B$_4$ (LTB$_4$) is present in biologically active amounts in both acute cutaneous lesions (1) and in chronic plaque psoriasis (2, 3). Furthermore, topical application as well as intradermal injections of LTB$_4$ induce skin inflammation (4, 5) and epidermal hyperproliferation (6). LTA$_4$ is formed from arachidonic acid (AA) by a two-step reaction of the 5-lipoxygenase enzyme (5-LO) and then further converted into LTB$_4$ by the LTA$_4$ hydrolase (7), which is the rate-limiting enzyme in LTB$_4$ formation (8, 9). While it is controversial whether 5-LO activity is present in the epidermis, we and others have demonstrated significant LTA$_4$ hydrolase activity in human epidermis (10) and cultured human keratinocytes (11, 12). Keratinocyte LTA$_4$ hydrolase may play an important role in LTB$_4$ formation, because transcellular LTB$_4$ formation, in which LTA$_4$ is formed in one cell type and converted by the LTA$_4$ hydrolase in another cell type, has been demonstrated between human neutrophils and cultured human keratinocytes (11, 12) and between human neutrophils and human epidermis in vitro (10). Furthermore, the epidermal LTA$_4$ hydrolase undergoes catalytic inactivation when transforming LTA$_4$ into LTB$_4$ (13). Catalytic inactivation of the LTA$_4$ hydrolase has also been demonstrated to occur in other cell types (14–16). Our recent results (17) have shown a decreased LTA$_4$ hydrolase activity in involved psoriatic skin compared to uninvolved psoriatic skin. Because of the catalytic inactivation of the enzyme, this observation is compatible with the idea of transcellular LTB$_4$ formation in psoriatic plaques. The epidermal LTA$_4$ hydrolase is, therefore, supposed to play a key role in LTB$_4$ formation in inflammatory skin diseases, and inhibition of this enzyme may have therapeutic implications. Previously, several compounds, including RP 64699 (18), bestatin (10, 19) and captopril (10, 20), have been shown to inhibit the activity of recombinant LTA$_4$ hydrolase and LTA$_4$ hydrolase in human neutrophils, human erythrocytes, human epidermis and porcine leukocytes.

The purpose of the present study was to investigate some anti-inflammatory and anti-proliferative compounds for their capacity to regulate the activity and/or the content of LTA$_4$ hydrolase in cultured human keratinocytes. The novel compound RP 64699 (18) inhibited the LTA$_4$ hydrolase activity, and cyclosporin A (CsA) down-regulated the amount of the keratinocyte LTA$_4$ hydrolase at concentrations similar to those found in the epidermis during CsA treatment of psoriasis.

MATERIAL AND METHODS

Materials
The LTA$_4$-methyl ester and the authentic LTB$_4$ were obtained from Cascade Biochem Limited, Reading, UK. The organic solvents were all HPLC-grade from Merck, Darmstadt, Germany. Octadeclsilyle C18-SEP-PAK cartridges were from Waters (USA). Human serum albumin was obtained from Nordisk Gentofte, Bagsværd, Denmark. Culture dishes, RPMI 1640 medium and the low-calcium, fatty acid and serum-free keratinocyte growth medium were from Life Technologies, European Division, Paisley, UK. Fetal calf serum was from AH Diagnostics, Aarhus, Denmark. The protein assay kit was purchased from Bio-Rad, Munich, Germany. Hybont enhanced chemiluminescence (ECL) nitrocellulose-membrane, ECL detection system and hyperfilm-ECL were all obtained from Amersham, Denmark. Trypsin, MTT (3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) methotrexate, all-trans retinoic acid and dexamethasone were purchased from Sigma Chemical, St. Louis, USA. 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$) was a kind gift from LEO, Ballerup, Denmark. CsA was obtained from Sandoz AS, Denmark. Eicosatrienoic acid was from Cayman Chemical, European Division, Paris, France. Human IFN-γ was obtained from R&D Systems Europe.
LTA₄ hydrolyase level in human keratinocyte cultures

Cultures of human keratinocytes

Human keratinocytes were cultured in serum-free keratinocyte growth medium as previously described (12). At subconfluence first passage cell cultures were incubated with 1,25(OH)₂D₃ (10⁻⁷ - 10⁻¹⁰ M), all-trans retinoic acid (10⁻⁸ - 10⁻¹⁰ M), 1,25(OH)₂D₃ (10⁻⁷ - 10⁻⁸ M), demethasone (10⁻⁵ - 10⁻⁷ M), IFN-γ (10 and 100 units/ml), RP64966 (10⁻⁵ - 10⁻⁷ M), methotrexate (0.1 - 10 µg/ml) or CsA (0.1 and 1.0 µg/ml) for 24 or 72 h. Then, incubation with LTA₄ for determination of the LTA₄ hydrolyase activity was carried out (see below) or the keratinocytes were scraped off in Tris-HCl buffer (pH 7.4) for quantitation of LTA₄ hydrolyase.

Detection and quantitation of LTA₄ hydrolyase by Western blot analysis

The released keratinolysates were sonicated 3 × 10 s on ice and then centrifuged 8,000 g for 15 min. The protein content in the supernatant was determined according to Bradford (21). The method used for quantitation of LTA₄ hydrolyase was Western blot analysis followed by immunological detection using an affinity-purified antibody. This method has previously been described in detail (17).

LTA₄ hydrolyse activity

LTA₄-free acid was prepared from LTA₄-methyl ester, as previously described (12, 22), and the concentration was estimated by UV-absorption at 280 nm. Subconfluent keratinocytes grown with the various compounds (see above) were equilibrated in the culture dishes in 3 ml of keratinocyte growth medium supplemented with 1 mg/ml human serum albumin for 10 min at 37°C before incubation with LTA₄ (60 nM) for 5 min at 37°C. In separate experiments preincubation with RP64999 (A (0.1, 1.0, 10 µM)) or CsA (0.1, 1.0 µg/ml) was carried out for 10 min before incubation with LTA₄. All incubations were terminated by the addition of 2 vol of ice-cold MeOH. Because LTA₄ was dissolved in a mixture of EtOH/NaOH, a pH of 7.8 was obtained in the incubation medium while LTA₄ was added.

Lipid extraction and reversed-phase high performance liquid chromatography (RP-HPLC)

The reaction mixture was kept at -20°C for 20 min. Cells were released from the culture dishes by scraping and then centrifuged (1,500 x g) for 10 min at 4°C to precipitate cellular debris and denatured protein. Lipids were then extracted from the collected supernatant as previously described (23). Identification of LTB₄ was by chromatographic comparison to authentic LTB₄ and by characteristic UV absorption (12).

Authentic LTB₄ was used to construct a calibration curve expressing the absorption as a function of the amount of standard LTB₄. Quantification of the LTB₄ was by integrated optical density and comparison to the calibration curve. Because incubations were carried out in the culture dishes, LTB₄ formation was expressed as ng LTB₄/culture dish/5 min.

Cell viability and proliferation

MTT (tetrazolium) assay. The tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) is used in a colorimetric assay for determination of living cells. MTT is cleaved in active mitochondria and can therefore be used to measure cytotoxicity and proliferation (24). Briefly, keratinocytes were grown in 96-well plates and then incubated for either 24 or 72 h with the various inhibitors investigated. Then 25 µl MTT stock solution (5 mg/ml in phosphate-buffered saline) per well was added to culture medium. The plates were shaken for 5 min at room temperature and then incubated for 4 h at 37°C before the medium was removed. Then the plates were frozen at -20°C for 30 min and thawed for 30 min at room temperature. Finally, 0.2 ml fixative (ethanol/aceton, 60:40, v/v) per well was added and the plate was shaken for 30 min at 4°C before the optical density was measured at 570 nm using an ELISA reader. A reference wavelength of 630 nm was also measured.

Trypan blue exclusion. Keratinocytes were grown in culture dishes and then incubated for 24 or 72 h with CsA (0.1, 1.0 µg/ml) or RP64999 (10⁻⁵ - 10⁻¹⁰ M). To assess cell viability incubations with trypan blue were carried out for 12 min. Then cells were counted under the microscope in three different fields in each culture dish. The total number of cells counted per field was 100.

Statistical methods

Results were expressed as mean ± standard error of the mean (SEM). Statistical significance was assessed by Wilcoxon’s rank sum test.

RESULTS

The LTA₄ hydrolyase activity in keratinocyte cultures, expressed as ng LTB₄ formed/culture dish/5 min, was 63.7 ± 7.6 (mean ± SEM, n = 6). Preincubation of cultured keratinocytes for 10 min with RP64966, a known inhibitor of LTA₄ hydrolyase activity, resulted in a significant and dose-dependent inhibition of the conversion of LTA₄ into LTB₄ (Fig. 1). CsA had no direct effect on the LTA₄ hydrolyase activity (Fig. 1). However, incubation for 72 h resulted in a decrease in the mean LTB₄ formation of 35% at 0.1 µg/ml CsA (p < 0.05 vs. control) and 48% at 1.0 µg/ml CsA (p < 0.05 vs. control) (Fig. 2). Also, keratinocyte proliferation was inhibited by CsA, although to a smaller degree than LTB₄ formation (Fig. 2).

To determine whether the reduced conversion of LTA₄ into LTB₄ was due to an inhibition of the activity or the amount of LTA₄ hydrolyase, Western analysis was performed. Analysis of protein extracts from keratinocyte cultures with an affinity
purified anti-LTA₄ hydrolase antibody showed a single band (Fig. 3). Incubation of keratinocyte cultures with CsA for 24 h did not change the amount of LTA₄ hydrolase (Fig. 3), but after 72 h there was a dose-dependent decrease in the amount of LTA₄ hydrolase per mg protein (30.1% at 0.1 μg/ml and 40.0% at 1.0 μg/ml) (Figs. 3 and 4). As assessed by trypan blue exclusion, the decreased amount of LTA₄ hydrolase was not due to a cytotoxic effect of CsA. The percentage of viable cells was 98%±1%, 97%±1%, and 98%±1% after 72 h incubation with vehicle, 0.1 μg/ml CsA and 1.0 μg/ml CsA, respectively (mean±SEM, n=4). Also, the decrease in keratinocyte proliferation cannot explain the reduced LTA₄ hydrolase content, because this was expressed per mg protein.

Normal levels of LTA₄ hydrolase were found in cultures treated with other anti-inflammatory and anti-proliferative compounds. Thus, incubations of keratinocyte cultures with 1,25(OH)₂D₃ (10⁻¹⁰, 10⁻⁸, 10⁻⁶ M), all-trans retinoic acid (10⁻⁶, 10⁻⁸, 10⁻₁⁰ M), eicosatetraenoic acid (10⁻⁶, 10⁻⁸, 10⁻₁⁰ M), dexamethasone (10⁻³, 10⁻⁵, 10⁻⁷ M), IFN-γ (10 and 100 units/ml) or methotrexate (0.1, 1.0, 10 μg/ml) for 24 or 72 h did not result in any significant changes in the amount of LTA₄ hydrolase per mg protein (data not shown). Furthermore, the activity of the LTA₄ hydrolase (ng LTB₄/culture dish) was not changed after 24 h incubation with these compounds (data not shown).

**DISCUSSION**

This study shows that non-toxic concentrations of CsA inhibit the capacity of cultured keratinocytes to form LTB₄ by inhibiting the amount of the LTA₄ hydrolase enzyme, whereas CsA has no direct effect on LTA₄ hydrolase activity. The decrease in LTA₄ hydrolase was expressed per mg protein and, therefore, not secondary to the decreased keratinocyte proliferation induced by CsA. Accordingly, the anti-proliferative compounds 1,25(OH)₂D₃, all-trans retinoic acid, dexamethasone and methotrexate did not change the LTA₄ hydrolase levels in...
keratinocyte cultures. The effect of CsA on LTA₄ hydrolase levels was only detectable after prolonged incubation, but it remains to be determined whether it is caused by decreased synthesis and/or accelerated degradation of the enzyme. At a CsA concentration of 1.0 μg/ml the LTA₄ hydrolase content was inhibited by 40%. It is therefore, of interest that treatment with CsA 14 mg/kg body weight/day results in a CsA concentration of 2.8 μg/ml in involved psoriatic epithelium (25). Although lower CsA doses (2.5–5.0 mg/kg/day) are usually used for the treatment of psoriasis, these results indicate that the tissue concentrations of CsA obtained during CsA treatment can inhibit the levels of keratinocyte LTA₄ hydrolase. Accordingly, treatment with CsA 14 mg/kg body weight/day for 7 days decreases the LTB₄ content by 64% in lesional psoriasis (26). Taken together, these findings are compatible with the idea that CsA may owe part of its anti-psoriatic effect to a decrease of LTA₄ hydrolase levels and thereby the LTB₄ synthesis in the epidermis. Even though a slight inhibition of keratinocyte LTA₄ hydrolase levels was detected at a CsA concentration of only 0.1 μg/ml, lymphocytes are apparently more sensitive to CsA (27, 28). Therefore our results do not argue against the notion that CsA acts primarily as an immunosuppressant in psoriasis.

RP 64699 has previously been demonstrated to be a LTA₄ hydrolase inhibitor in porcine leukocytes, with an IC₅₀ of 1.5 μM (18). This is in accordance with our finding of an IC₅₀ of 0.7 μM in cultured keratinocytes. It was also demonstrated that incubation with 1.0 and 10 μM RP 64699 for 24 h resulted in a marked decrease in cell viability and proliferation, as evaluated by trypan blue exclusion and the MTT assay (data not shown). Therefore, RP 64699 appears not to be a candidate for the treatment of inflammatory skin diseases.

All other compounds tested in this study did not modulate the expression or activity of keratinocyte LTA₄ hydrolase. The lack of effect of all-trans retinoic acid on the LTA₄ hydrolase activity after 24 h incubation is in accordance with a previous study by Hamasaki et al. (29) who showed that RA induced LTC₄ synthase activity, but not LTA₄ hydrolase activity in rat basophilic leukemia-1 cells. Methotrexate has previously been suggested to suppress neutrophil LTA₄ hydrolase activity (30, 31). The inhibitory effect of methotrexate in neutrophils was demonstrated in freshly isolated neutrophils obtained from patients with rheumatoid arthritis treated with a single dose of methotrexate. 1,25(OH)₂-vitamin D₃ can in combination with transforming growth factor β1 upregulate the S-LO activity and expression in the human monocyte cell line Mono Mac 6, while 1,25(OH)₂-vitamin D₃ alone only had a minor effect (32). In the present study, 1,25(OH)₂-vitamin D₃ had no effect on either the amount or the activity of LTA₄ hydrolase. Dexamethasone has been shown to inhibit LTA₄ hydrolase activity in differentiated HL-60 cells, but not in nondifferentiated HL-60 cells (33). This difference between differentiated and nondifferentiated cells may explain the lack of effect of dexamethasone in subconfluent keratinocyte cultures, which mainly consist of undifferentiated cells. IFN-γ failed to modulate the LTA₄ hydrolase activity and content in cultured keratinocytes, which is in contrast to endothelial cells. After pretreatment for 72 h IFN-γ (100 U/ml) increased their LTA₄ hydrolase activity by approximately 100% (34). Dietary supplementation with eicosapentaenoic acid and eicosatrienoic acid has also been shown to inhibit LTB₄ formation in human and rat neutrophils. An inhibition of the LTA₄ hydrolase has been suggested to be their mechanism of action (35, 36). This inhibition is probably mediated via the two S-LO products of eicosapentaenoic acid and eicosatrienoic acid, LTA₄ and LTA₃, by covalent coupling to the LTA₄ hydrolase (37-39). The absence of detectable S-LO activity in undifferentiated cultured keratinocytes (11, 12, 40) may explain why eicosatrienoic acid had no effect on keratinocyte LTA₄ hydrolase.

The anti-psoriatic effect of CsA is believed to be a result of immuno-suppression. In the present study CsA, in contrast to other anti-inflammatory and anti-proliferative compounds, inhibited keratinocyte LTA₄ hydrolase at doses obtained in the epidermis during treatment of psoriasis. Since LTA₄ hydrolase activity results in the formation of pro-inflammatory LTB₄, part of the anti-psoriatic effect of CsA may be due to an anti-inflammatory effect.

REFERENCES


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