Stimulation of Leukotriene Synthesis in Intact Polymorphonuclear Cells by the 5-Lipoxygenase Inhibitor 3-oxo-Tirucallic Acid

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ABSTRACT

Commercially available extracts from *Boswellia serrata* resin used as anti-inflammatory drugs or phytonutrients show paradoxical concentration-dependent potentiating and inhibitory actions on 5-lipoxygenase (5-LO) product synthesis in stimulated PMNs. In our attempt to characterize the stimulating constituents, we identified the tetracyclic triterpene 3-oxo-tirucallic acid (3-oxo-TA), which, in the range from 2.5 to 15 μM, enhanced 5-LO product formation in ionophore-challenged polymorphonuclear cells (PMNs) (e.g., from 1981 ± 177 to 3042 ± 208 pmol at 10 μM 3-oxo-TA), and initiated Ca^{2+} mobilization, MEK-1/2 phosphorylation, 5-LO translocation, and 5-LO product formation in resting cells (534 ± 394 pmol/ 5×10^6 PMNs). In cell-free 5-LO assays, 3-oxo-TA acted only inhibitory (IC_{50} value of about 3 μM), demonstrating the pivotal role of intact cell structure for its activating property. In 3-oxo-TA-challenged PMNs, the mitogen-activated protein kinase kinase (MEK)-1/2 inhibitor PD098059 abolished 5-LO product formation, along with inhibition of MEK-1/2 phosphorylation and 5-LO translocation. The 3-acetoxy derivative of 3-oxo-TA acted like 3-oxo-TA in intact PMNs, whereas 3-hydroxy-TA barely stimulated MEK phosphorylation in resting cells and showed only inhibition on ionophore-induced 5-LO product synthesis. Steroid-type tetracycles neither induced 5-LO activation nor had enhancing or inhibitory effects. In summary, defined natural tetracyclic triterpenes, which act as inhibitors of the 5-LO in the cell-free assay, initiate 5-LO activation by a MEK-inhibitor sensitive mechanism and potentiate stimulated product synthesis in intact cells. Because TAs contribute significantly to the overall biological effects of *B. serrata* resin extracts, special precaution for standardization is recommended when using *B. serrata* preparations as drugs or dietary supplements.

5-Lipoxygenase (5-LO; EC 1.13.11.34) catalyzes the first two steps in the biosynthesis of leukotrienes and 5(*S*)-HETE from arachidonic acid. Leukotrienes and 5-oxo-eicosa-tetraenoic acid, a final metabolite from 5(*S*)-HETE (Powell et al., 1992), are potent mediators of inflammatory processes (Samuelsson et al., 1987; Powell et al., 1995). Recent observations suggest that 5-LO products and/or the 5-LO protein itself participate in both intra-and transcellular signaling governing cell growth and functions. Intranuclear and cell surface receptors for 5-LO products (Coleman et al., 1994; Devchand et al., 1996; O'Flaherty et al., 1998), multitranscript forms of 5-LO (Boado et al., 1992), a variety of protein-protein interactions of the Src homology-3 binding motif of 5-LO (Lepley and Fitzpatrick, 1994; Provost et al., 1999), and euchromatin-associated 5-LO (Brock et al., 1994) might be molecular correlates for the pleiotropic actions of the 5-LO protein and its products.

The enzymatic activity of 5-LO, as well as its binding to other macromolecules, is regulated in a highly complex manner (for concise reviews on many aspects of 5-LO, products, and receptors, see Ford-Hutchinson and Jakobsson, 1998; Dahlen 1998; Peters-Golden, 1998). The compartmentalization of 5-LO (cytosolic, membrane bound, or intranuclear) is cell-type–specific and is a dynamic process. Subcellular distribution and redistribution with or without product formation vary in dependence of stimulator and duration of stimulation. In intact cells, enzymatic activity of 5-LO requires an increase in $[Ca^{2+}]_I$; substrate release by cytosolic and/or secretory PLA_2; ATP; translocation of 5-LO to membranes; the presence of the arachidonic acid-binding, 18-kDa membrane protein FLAP; and is prone to modulation by the peroxide tonus/redox status of the cell. Endogenous and exogenous ligands of its allosteric site can further modify 5-LO actions

ABBREVIATIONS: 5-LO, 5-lipoxygenase; PLA_2, phospholipase A_2; TA, tirucallic acid; HPLC, high-performance liquid chromatography; BA, boswellic acid; AKBA, 3-*O*-acetyl-11-keto-β-boswellic acid; KBA, 11-keto-β-boswellic acid; PD098059, 2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one; HETE, hydroxyeicosatetraenoic acid; HpETE, hydroperoxyeicosatetraenoic acid; PMN, polymorphonuclear leukocyte; LT, leukotriene; PBS, phosphate-buffered saline; MEK, mitogen-activated protein kinase kinase; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine.

(Ahorony and Stein, 1986; Safayhi et al., 1995; Sailer et al., 1998). Upon stimulation, 5-LO and cPLA2 are phosphorylated by tyrosine and mitogen-activated protein kinase-mediated mechanisms (Lin et al., 1993; Durstin et al., 1994; Lepley et al., 1996). There is increasing evidence that MEK-1/2 and/or p38 signaling pathways in particular directly participate in 5-LO activation in neutrophils (Boden et al., 2000; Werz et al., 2000) and that 5-LO phosphorylation has an impact on the regulation of its protein-protein interactions and compartmentalization, and thus on 5-LO's catalytic and signaling activities.

For screening of 5-LO inhibitors, mainly two assay systems are used: 1) the measurement of 5-LO product formation from endogenous substrate in ionophore or receptor ligand-stimulated intact leukocytes identifies compounds, which interact with any of the steps from substrate release to 5-LO action and 2) cell-free 5-LO assays in the presence of exogenously added arachidonate substrate and calcium characterize compounds that interfere directly with 5-LO catalysis. For the first time, we report on two natural, tetracyclic, triterpene compounds that inhibit cell-free 5-LO activity but induce 5-LO product formation in intact cells and potentiate product synthesis in stimulated cells. The impact of our observation for the clinical use of crude *Boswellia serrata* resin preparations as anti-inflammatory drugs or phytonutrients is briefly reflected under *Discussion*.

Experimental Procedures

Materials. 3-Acetoxy-TA, 3-hydroxy-TA, and 3-oxo-TA were isolated from the polycyclic triterpene acid fraction of *B. serrata* resin, purified by rechromatography on a reversed-phase HPLC-system and characterized by electron ionization-mass spectrometry, HPLC-electrospray ionization, IR, ^{1}H-NMR, and ^{13}C-NMR (data not shown). The isolation, characterization, and content analyses for pentacyclic triterpene acids including AKBA, KBA and acetyl-β-boswellic acid were described elsewhere (Safayhi et al., 1992, 2000; Schweizer et al., 2000). PD098059 was obtained from Calbiochem Novabiochem (Bad Soden, Germany), Percoll was obtained from Amersham Pharmacia Biotech (Freiburg, Germany), arachidonic acid was from Cayman Chemical (Ann Arbor, MI), Fura-2/AM was from Molecular Probes (Eugene, OR), and the Complete R protease inhibitors cocktail from Roche Diagnostics (Mannheim, Germany). "Standardized Boswellia Extract" tablets from Nature's Way Products Inc. (Springville, UT), declared to contain 65% boswellic acids and suggested to "support joint health and motility", were purchased in 1999 in Cleveland, Ohio. One tablet (656.5 mg), which, according to the product label, was made from 307 mg of *B. serrata* resin dried extract, contained 20.78 ± 4.73 mg AKBA and 16.09 ± 4.73 mg KBA (i.e., complete 5-LO inhibitors) and 23.88 ± 4.73 mg Ac-β-BA (i.e., a partial inhibitor of the enzyme) along with many nonquantified polycyclic terpenes, including TAs and further BA derivatives (Fig. 9). The tablet's extract fraction had an estimated 25 to 30% total of boswellic acid derivatives. Antibodies were from Cayman Chemical, Jackson Immunoresearch, Inc (West Grove, PA), or New England Biolabs, Inc. (Beverly, MA), and all other chemicals were from Sigma-Aldrich Chemie (Taufkirchen, Germany), Merck (Darmstadt, Germany), or Serva (Heidelberg, Germany) in the highest available analytical grade.

PMN Isolation. Buffy coat fractions from venous blood of healthy volunteers were obtained from the Universitätsklinikum Tübingen. PMNs were purified by dextran sedimentation, centrifugation through Percoll, and subsequent lysis of erythrocytes (Roos and de Boer, 1986). Final PMN preparations were > 95% pure and viability exceeded 98%, as determined by Trypan Blue exclusion. All solutions used for PMN isolation were nominally Ca^{2+}-free. The PMNs were resuspended in Ca^{2+}-free PBS, pH 7.3, supplemented with 5.5 mM glucose at a cell density of 5×10^6 cells/ml for incubation.

Assays of 5-LO Activity. 5-LO product formation from endogenous substrate (5-min incubations at 37°C) was studied in ionophore A23187 (2 μM) and Ca^{2+} (1.8 mM) stimulated intact PMNs. The standard system (2-min preincubation with ionophore and start with Ca^{2+}) had been optimized for maximal LTB$_4$ synthesis: arachidonate release and reduction of 5(*S*)-HpETE to 5(*S*)-HETE are not rate limiting, the capacity of LTA$_4$ hydrolase is slightly exceeded, and the 20-hydroxylation of LTB$_4$ is not substantial. Tirucallic acids or analogs and the MEK inhibitor PD098059 were added 5 and 30 min, respectively, before stimulation. The dimethyl sulfoxide concentration was 0.5% in all incubations, including control samples. Reactions were terminated by adding 1 ml of an ice-cold mixture of methanol/1 N HCl [97:3 (v/v)]. C18 solid phase extraction, reversed phase-HPLC separation, detection (at 280 and 235 nm), and quantification of 5-LO products were as described previously (Safayhi et al., 1995; Sailer et al., 1996). Cell-free 5-LO activity assay was performed with homogenates of nitrogen-cavitated PMNs in the presence of arachidonate substrate (10 μM). The 5-LO products of intact cells were 20-OH-LTB$_4$, LTB$_4$, 6-*trans*-LTB$_4$, 6-*trans*-12-*epi*-LTB$_4$, and 5(*S*)-HETE. LTB$_4$ and 5(*S*)-HETE represented about 95% of the 5-LO products formed under the conditions of our setting. In the homogenate assay, substantial amounts of 5(*S*)-HpETE were formed as well. PGB$_2$ (500 pmol) was used as an internal standard for the calculation of extraction efficiency in each sample. 13(*S*)-HODE (500 pmol) was added in addition to PGB$_2$ as a second internal standard to incubations with PD098059, because PD098059 and PGB$_2$ were coeluted with comparable retention times and showed UV-absorbance at 280 nm. The molar extinction coefficients, which were used for the correction of differences in UV absorbance, were 28,680 for PGB$_2$, 23,000 for 13(*S*)-HODE, 39,500 for 20-OH-LTB$_4$ and LTB$_4$, 44,000 for 6-*trans*-LTB$_4$ and 6-*trans*-12-*epi*-LTB$_4$, and 30,500 for 5(*S*)-HETE and 5(*S*)-HpETE (Powell, 1987).

5-LO Translocation Assay. At the end of reactions, cells were placed on ice, pelleted by centrifugation at 600*g* for 5 min (4°C), washed in ice-cold PBS buffer, resuspended in ice-cold PBS containing 1 mM EDTA and a cocktail of protease inhibitors, and subjected to nitrogen cavitation (500 psi, 5 min). From the cavitates, 100,000*g* supernatants were obtained by sequential centrifugation. Protein content was determined according to the method of Bradford and equal amounts of protein were loaded on SDS/9%-polyacrylamide gels. Electrophoresis was followed by a transfer to polyvinylidene difluoride membranes, which were then probed with rabbit anti-h5-LO polyclonal antiserum (amino acids 130–149; 1:1,000) from Cayman and alkaline phosphatase-conjugated donkey anti-rabbit IgG (1:2,500) from Jackson Immunoresearch, Inc.

MEK Phosphorylation Assay. Reactions were carried out according to the protocol described above except that 4×10^6 cells were incubated in a test volume of 100 μl. The reactions were quenched by the addition of 5× concentrated SDS-electrophoresis sample buffer (final concentrations, 0.4% SDS, 2.9% 2-mercaptoethanol, 5% glycerol, and 0.1% bromphenol blue in 12 mM Tris buffer, pH 6.8) and the samples were immediately heated at 95°C for 5 min. Proteins were electrophoresed on 10% SDS-polyacrylamide gels and then transferred to polyvinylidene difluoride membranes. The membranes were probed with dual-phosphospecific (Ser217/221) rabbit anti-MEK-1/2 antibody (1:1000) and goat alkaline phosphatase-linked anti-rabbit IgG antibody (1:1000) from New England Biolabs, Inc.

Determination of [Ca^{2+}]$_i$ in Single Cells. PMNs were loaded with 2 μM Fura-2/AM for 30 min at 37°C, washed twice, and resuspended in PBS supplemented with 5.5 mM glucose and 1.8 mM Ca^{2+}. In experiments where intracellular calcium stores were depleted, the preincubation buffer also contained 1 μM thapsigargin. Fluorescence intensities (F) were measured with an LP 515 nm emission wavelength filter (TILL Photonics, Planegg, Germany). Changes in

$[Ca^{2+}]_i$ are presented as the calculated ratio of the emitted light intensities at the alternating excitation wavelengths 340 and 380 nm for 20 ms, each (Grynkiewicz et al., 1985).

Data. Amounts of 5-LO product formation in absolute values (pmol) or as percentage of products in controls. Data are shown as means ± S.D. throughout for (n) independent experiments. Statistical evaluation was performed by Student's t test for unpaired data. In Figs. 2 and 7, which show effects in percent, the presented p values were calculated with nontransformed absolute values.

Results

Stimulation of intact PMNs by 1.8 mM Ca^{2+} and 2 μM ionophore induced the de novo synthesis of substantial amounts of 5-LO products from endogenous arachidonic acid. In contrast to nonstimulated cells, which produced neither LTB_4 nor 5(S)-HETE in detectable amounts (detection limits about 10 pmol for either product), stimulated cells produced 1019 ± 191 pmol LTB_4 and 777 ± 249 pmol 5(S)-HETE ($n = 29$) as main products. The presence of 10 μM 3-oxo-TA, the prominent tetracyclic triterpene acid of *B. serrata* resin, increased ionophore-mediated 5-LO product formation by 54% (1981 ± 177 versus 3042 ± 208 pmol; $n = 10$; $p < 0.001$), roughly doubled 5(S)-HETE formation (725 ± 84 versus 1477 ± 144 pmol; $n = 10$; $p < 0.001$) but only moderately enhanced the synthesis of LTB_4 (957 ± 96 versus 1094 ± 97 pmol; $n = 10$; $p < 0.01$) and its all-*trans*-isomers (Fig. 1 for molecule structures; Figs. 2 and 3). To address the question whether the potentiating action of 3-oxo-TA was caused by a calcium ionophore property of this compound, which might have increased calcium import in an additive manner, we increased the ionophore concentration from 2 to 3 μM in the absence of 3-oxo-TA: compared with the stimulation with 2 μM A23187, the increase in ionophore addition did not significantly enhance 5-LO product formation (105 ± 5%, $n = 3$). The enhancement of A23187-induced 5-HETE synthesis by 3-oxo-TA had a bell-shaped concentration-action relation and an optimum in the range of 2.5 to 10 μM (Fig. 3). A comparable action was also observed by 3-acetoxy-TA, which is present in *B. serrata* resins as a natural analog in more scarce amounts: 3-acetoxy-TA at 2.5 μM increased the ionophore-stimulated LTB_4 synthesis in intact cells to 135%

(from 1113 ± 260 to 1500 ± 302 pmol; not significant), the 5(S)-HETE formation to 186% (from 996 ± 334 to 1850 ± 473 pmol; $p < 0.01$) and the total of 5-LO product synthesis to 160% (from 2442 ± 654 to 3916 ± 902 pmol; $p < 0.05$). 3-Hydroxy-TA, a third minor constituent of the resins, acted inhibitory only in ionophore-stimulated cells with an IC_{50} value of about 5 μM (Fig. 2). In contrast to TAs, a panel of steroid-type tetracyclics in comparable concentrations from 1 to 20 μM (i.e., lanosterol, cholesterol, cortisone, testosterone), which were previously shown not to bind to the allosteric site of 5-LO (Safayhi et al., 1995), neither increased ionophore-mediated 5-LO product formation nor acted inhibitory (not shown).

In the absence of ionophore, 3-oxo-TA and 3-acetoxy-TA both initiated 5-LO product formation in substantial amounts (Fig. 4). The stimulatory actions of these two TAs required a threshold concentration of about 250 to 500 μM extracellular calcium (not shown). As suggested by the TA-mediated activation of 5-LO product formation, we observed the concomitant induction of 5-LO translocation by the two stimulatory TAs: Fig. 5 illustrates the disappearance of 5-LO protein from the soluble fraction and its enrichment in the membrane fraction in 3-oxo-TA-challenged cells. Comparable changes in the subcellular 5-LO distribution were observed in ionophore-stimulated cells, along with an enhancement of the ionophore-induced translocation by 3-oxo-TA. In contrast, the pentacyclic triterpene acid AKBA, which is a nonredox, noncompetitive inhibitor of 5-LO that acts by binding to the allosteric site of the enzyme (Safayhi et al., 1992, 1995, 2000; Sailer et al., 1996,1998), neither induced 5-LO translocation in resting cells nor modulated the ionophore-initiated translocation.

As suggested by its potential to stimulate 5-LO product formation in intact cells and as illustrated in Fig. 6, 3-oxo-TA initiated a moderate increase in $[Ca^{2+}]_i$ in about 40% of Fura-2 loaded cells in Ca^{2+}-supplemented media (9 PMNs of a total of 22 single-cell experiments). In 3-oxo-TA-sensitive PMNs the subsequent fMLP addition had no effect. 3-oxo-TA (alone or with fMLP) was ineffective in 13 cells, whereas the subsequent stimulation by 2 μM ionophore caused a marked increase in $[Ca^{2+}]_i$ in all tested PMNs. In thapsigargin-pre-

3-oxo-TA

R = H,   3-hydroxy-TA
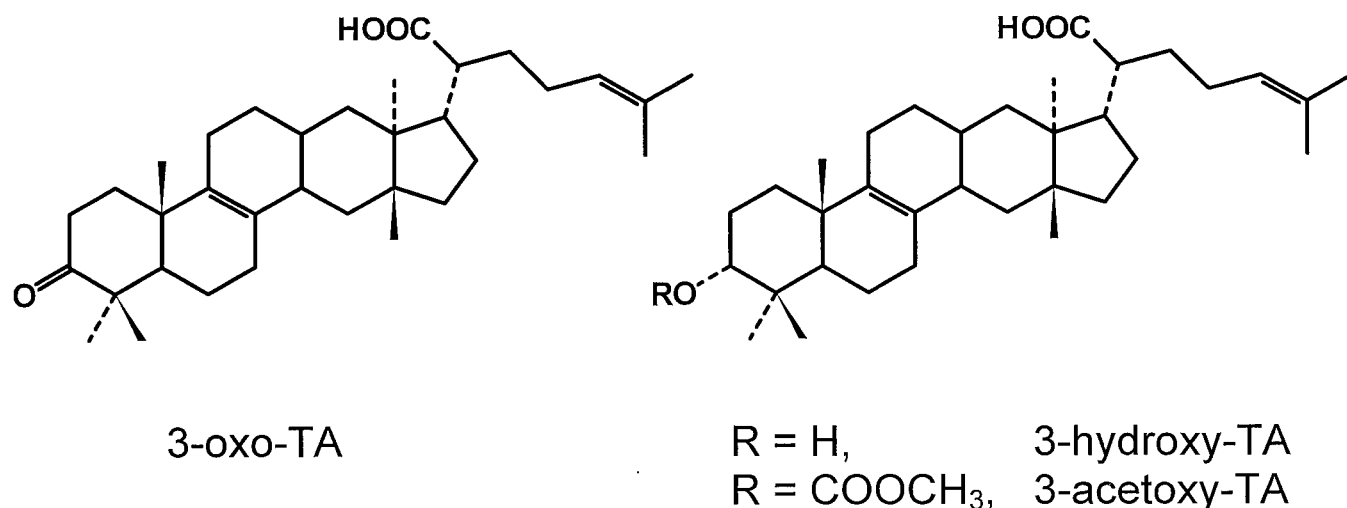R = $COOCH_3$,   3-acetoxy-TA

Fig. 1. Chemical structures of genuine tirucallic acids from *B. serrata* resin; 3-oxo-TA (3-oxo-tirucall-8,24-dien-21-oic acid), 3-acetoxy-TA (3-acetoxy-tirucall-8.24-dien-21-oic acid) and 3-hydroxy-TA (3-hydroxy-tirucall-8.24-dien-21-oic acid).

incubated PMNs, 3-oxo-TA did not mediate Ca^{2+} mobilization in any of the PMNs assayed ($n = 12$). All thapsigargin-preincubated PMNs answered to a subsequent A23187 stimulation with a substantial increase in $[Ca^{2+}]_i$ (not shown).

Strikingly, in the cell-free 5-LO assay in the presence of exogenously added substrate (10 μM arachidonic acid), all three TAs (3-oxo-TAs, 3-acetoxy-TA, and 3-hydroxy-TA) acted inhibitory only on 5-LO product formation (Fig. 7). The contradictory actions of 3-oxo-TA and 3-acetoxy-TA (stimulatory in intact cells and inhibitory in cell-free assay) prompted us to study the MEK-1/2 signaling pathway, which was

shown to be pivotal for the chemotactic *N*-formyl-peptide fMLP-induced translocation of 5-LO (Boden et al., 2000). Figure 8A shows that 3-oxo-TA and 3-acetoxy-TA both, as well as fMLP, initiated MEK-1/2 phosphorylation. In contrast, the pentacyclic triterpene amyrin, which is a non-stimulatory, noninhibitory ligand of 5-LO's allosteric site, and the steroid-type tetracyclic cortisone, which does not bind to the allosteric site of 5-LO (Safayhi et al., 1995; Sailer et al., 1998), induced no MEK-1/2 stimulation. 3-Hydroxy-TA, which acts as a 5-LO inhibitor in both intact cell and cell-free assays, failed also to mediate a substantial MEK-1/2 phosphorylation. The actions of 3-oxo-TA and 3-acetoxy-TA on MEK-1/2 phosphorylation were sensitive to MEK-1/2 inhibition by 50 μM PD098059 (Fig. 8B). In line with this observation, PD098059 totally abolished 3-oxo-TA-mediated 5-LO product formation and reduced 3-oxo-TA-induced 5-LO translocation (Fig. 9). In contrast, the 3-acetoxy-TA-mediated 5-LO product synthesis was not completely blocked by PD098059 (1497 ± 834 versus 320 ± 223 pmol), which correlates well with the less pronounced inhibition of 3-acetoxy-TA-mediated MEK-1/2 phosphorylation (Fig. 8B).

Fig. 2. Effects of 3-oxo-TA and 3-hydroxy-TA on Ca^{2+} and ionophore-mediated 5-LO product formation in intact PMNs. Cells (5 × 10⁶ PMNs/ml) were stimulated by 1.8 mM Ca^{2+} and 2 μM ionophore for 5 min in the absence or presence of 3-oxo-TA (■) and 3-hydroxy-TA (●), respectively. Data are shown as mean percentages (± S.D.) of 5-LO product formation from endogenous substrate in control cells (positive control cells stimulated by Ca^{2+} and ionophore in the absence of TAs) ($n = 3–10$).
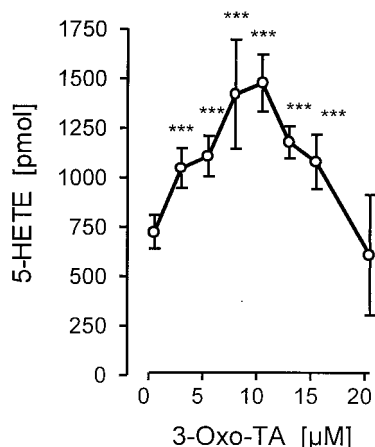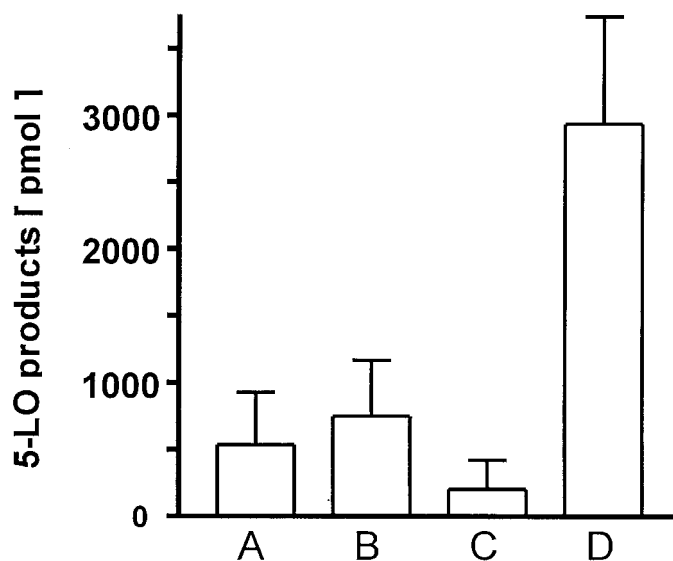

Fig. 4. Stimulation of 5-LO product synthesis in PMNs. PMNs were stimulated in the presence of 1.8 mM Ca^{2+} by 10 μM 3-oxo-TA (A), 5 μM 3-acetoxy-TA (B), 1 μM fMLP (C), or 2 μM A23187 (D) for 5 min. Sums of 5-LO products from endogenous substrate are shown as mean picomoles/5 × 10⁶ PMNs (± S.D.; $n = 9–22$).

Fig. 3. Concentration-action relation of 3-oxo-TA effect on 5(*S*)-HETE formation in stimulated PMNs. PMNs (5 × 10⁶ cells/ml) were stimulated by 2 μM ionophore and 1.8 mM Ca^{2+} in the absence or presence of different concentrations of 3-oxo-TA. 5(*S*)-HETE amounts formed in 5-min incubations are shown as mean picomoles/5 × 10⁶ PMNs (± S.D; $n = 3–10$). ***$p < 0.001$; significantly different from stimulated controls incubated in the absence of 3-oxo-TA.
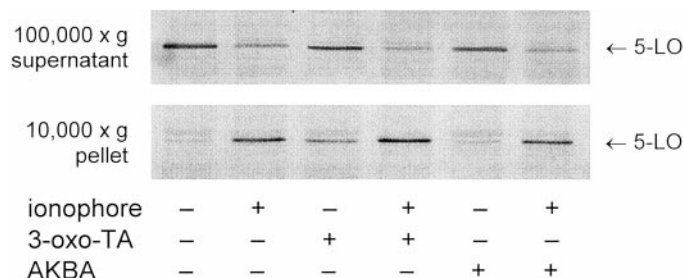

Fig. 5. Compartmentalization of 5-LO in resting and stimulated PMNs. PMNs were stimulated in the presence of 1.8 mM Ca^{2+} by 2 μM ionophore (A23187), 10 μM 3-oxo-TA, 10 μM AKBA, or combinations of these compounds. 100,000*g* supernatants (top*)* and membrane fractions obtained as 10,000*g* pellets (bottom) were analyzed by Western blot using anti-human 5LO polyclonal antiserum. A representative blot from four independent experiments is shown.

Finally, the practical impact of our observation is illustrated in Fig. 10: at low concentrations, a *B. serrata* resin product from the US market, which contains substantial amounts of TA-derivatives, significantly potentiated 5-LO product formation in ionophore-stimulated intact cells. However, the addition of higher concentrations of this product reduced ionophore-stimulated 5-LO product synthesis, as observed with other crude extracts from *B. serrata* resins (Safayhi et al., 2000).

Discussion

In nonprimed resting PMNs, 3-oxo-TA initiated MEK-1/2 phosphorylation and 5-LO translocation as the early and crucial step of 5-LO activation, which in turn consistently resulted in substantial 5-LO product synthesis from endogenous substrate. The 3-oxo-TA-mediated product formation was about 15% of the product synthesis obtained by ionophore/calcium-challenge and more than two-times higher than the fMLP-initiated production. The stimulatory actions of 3-oxo-TA were completely sensitive to inhibition of MEK-signaling by 50 μM PD098059, as it was previously reported for the fMLP stimulation of human PMNs (Boden et al.,

Fig. 6. Effects of 3-oxo-TA, fMLP, and ionophore on $[Ca^{2+}]_i$. 3-Oxo-TA (10 μM), fMLP (1 μM), and A23187 (2 μM) were added sequentially to PMNs in 1.8 mM Ca^{2+}-supplemented buffer, as indicated by the arrows. $[Ca^{2+}]_i$ was measured in Fura-2-loaded single cells. The figure shows a representative recording from observations with 3-oxo-TA sensitive PMNs (9 of a total of 22 tested PMNs).

Fig. 7. Inhibitory actions of TAs on 5-LO product formation from exogenous substrate in the cell-free assay. 3-Oxo-TA (A), 3-acetoxy-TA (B), or 3-hydroxy-TA (C) was added to PMN homogenates, and 5-LO product formation was initiated by 1.8 mM Ca^{2+} and 10 μM arachidonate substrate. Data are shown as mean percentage ($\pm$ S.D.) of 5-LO product formation from exogenous substrate in stimulated controls in the absence of TAs ($n = 3$).

2000). In this context, it is worth noting that PD098059 in the test concentration used, in contrast to many other inhibitors of signaling kinases, does not directly inhibit cell-free 5-LO (Boden et al., 2000). The 3-acetoxy analog exerted qualitatively comparable stimulatory actions on MEK phosphorylation, 5-LO translocation, and product formation. However, the 3-acetoxy-TA effects were sensitive to PD098059 to a lesser extent, as illustrated by the limited inhibitions of 3-acetoxy-TA-initiated MEK phosphorylation and 5-LO product synthesis. In contrast to the former TA derivatives, 3-hydroxy-TA did not stimulate MEK phosphorylation and 5-LO product synthesis in resting cells substantially. Although the stimulatory actions of 3-oxo-TA and 3-acetoxy-TA required a threshold extracellular calcium concentration of about 250 μM, the limited maximal action of ionophore A23187 in our setting suggests that the rational for the additional stimulatory effects of 3-oxo-TA and 3-acetoxy-TA is not solely a nonrecognized calcium ionophore property of TAs. The critical role of calcium influx for TA-induced 5-LO product synthesis is comparable with calcium requirements observed previously with fMLP-challenged cells (Boden et al., 2000). In line with this interpretation, the inhibition of 3-oxo-TA induced increase in $[Ca^{2+}]_i$ by thapsigargin directly indicates that Ca^{2+} mobilization from intracellular stores is a crucial step of the stimulatory action of 3-oxo-TA in intact cells.
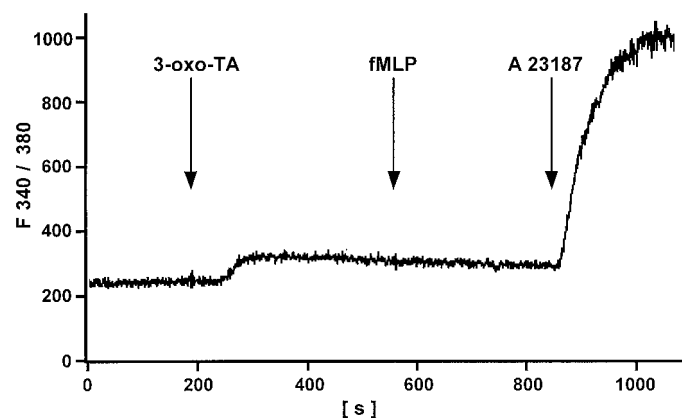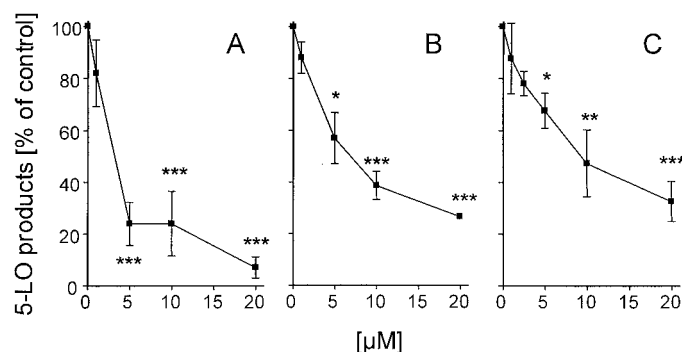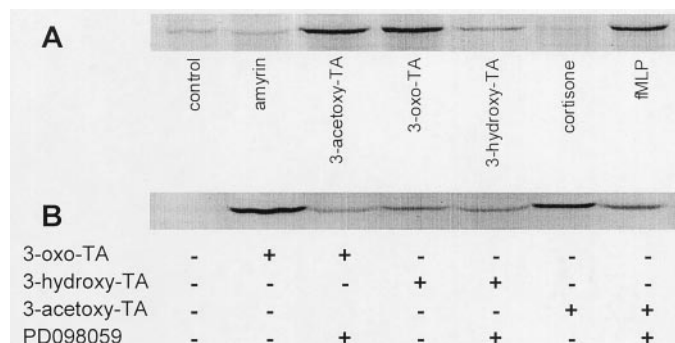

Fig. 8. Stimulation of MEK-1/2 phosphorylation by 3-oxo-TA and 3-acetoxy-TA in PMNs. A, tetracyclic and pentacyclic compounds were added in final concentrations of 10 μM in the presence of 1.8 mM Ca^{2+}. The concentration of fMLP was 1 μM. B, 3-oxo-TA (10 μM), 3-acetoxy-TA (5 μM), or 3-hydroxy-TA (10 μM) were added either in the absence or in the presence of 50 μM PD098059. Proteins from cell lysates were analyzed by Western blot using a dual phosphospecific anti-MEK-1/2 antibody. The numbers of independent observations with a compound, concentration, or combination were as indicated in parentheses: amyrin (5), 10 μM (11) and 5 μM (8) 3-acetoxy-TA, 3-oxo-TA (19), 3-hydroxy-TA (14), cortisone (3), fMLP (25), and TAs with PD098059 (3–4).
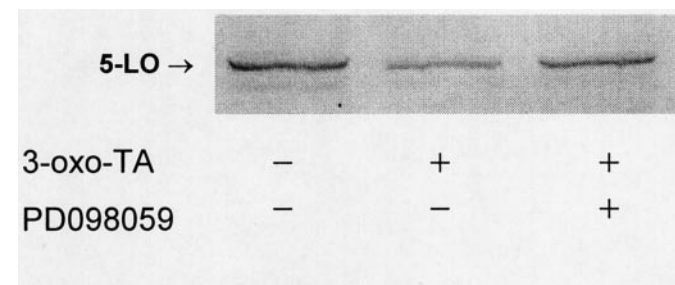

Fig. 9. Inhibition of 3-oxo-TA-mediated 5-LO translocation by PD098059. 100,000g supernatants of resting PMNs and 3-oxo-TA-challenged PMNs, which were incubated for 5 min in the absence or presence of 50 μM PD098059, were analyzed by Western blot using anti-human 5LO polyclonal antiserum. A blot from five independent experiments is shown.

In line with our observations with resting cells, 3-oxo-TA and 3-acetoxy-TA, but not 3-hydroxy-TA, further increased ionophore-challenged 5-LO product formation in intact cells. Upon ionophore stimulation, the activating TAs mainly increased 5(S)-HETE formation but had less pronounced effects on the synthesis of di-HETEs (i.e., LTB_4, 20-OH-LTB_4, 6-*trans*-LTB_4, and 6-*trans*-12-*epi*-LTB_4). Under conditions of moderate 5-LO stimulation, however, we also observed a significant increase in LTB_4 and further diHETEs (not shown). The potentiation of the ionophore-stimulated 5(S)-HpETE formation by the first catalytic step of 5-LO action (i.e., the dioxygenation of arachidonic acid) and a facilitated dissociation of 5(S)-HpETE from the enzyme in the presence of 3-oxo-TA best fit kinetic data: 3-oxo-TA presence both increased the V_{max} value of 5-HETE synthesis and delayed turnover-dependent irreversible enzyme deactivation without affecting the lag-time of activation (not shown).

In the cell-free 5-LO assay, all three TA derivatives unexpectedly inhibited the exogenous substrate-stimulated 5-LO activity, demonstrating that 3-oxo-TA and 3-acetoxy-TA require intact cell structures for their stimulatory actions. The inhibition of cell-free 5-LO activity by TAs suggests a direct interaction of the TA series tetracyclics with the 5-LO protein. However, a binding of TAs onto 5-LO protein, as was previously documented for the binding of the pentacyclic triterpene acid AKBA onto the allosteric site of the enzyme (Sailer et al., 1998), has not yet been shown.
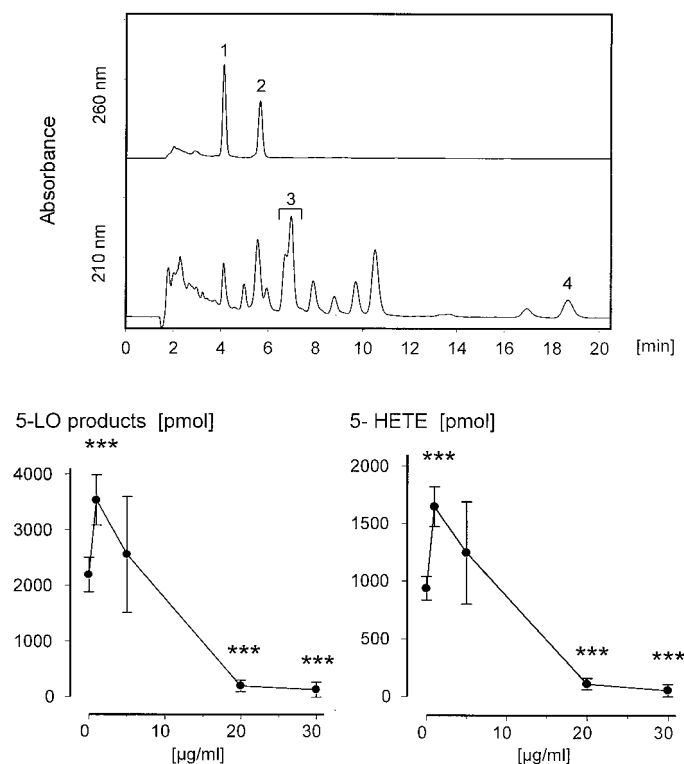
Reports stating that defined pentacyclic triterpenes from the BA series (e.g., AKBA and KBA) inhibit 5-LO product formation by a unique mechanism (Safayhi et al., 1992, 1995) and positive actions of *B. serrata* resin preparations in pilot trials in patients with inflammatory bowel diseases (Gupta et al., 1997; Gerhardt et al., 2001), asthma (Gupta et al., 1998) and intracranial peritumoral edema without severe side effects (Böker and Winking, 1997; Janßen et al., 2000; Weller, 2000), promoted the commercial availability of a panel of *B. serrata* resin products of varying quality as drugs and dietary supplements. Most of these products in high concentrations reduce 5-LO activity in vitro. However, because the potentiation of 5-LO product formation by low concentrations is not unique to the one commercial product tested in the present study but was also observed with low concentrations of crude extracts from *B. serrata* resins from different regions (Safayhi et al., 2000), our data underline the urgency of standardization of *B. serrata* resin products by appropriate chemical and biological methods to provide the basis for a rational and safe use of such extracts in general.

Fig. 10. Rp-HPLC-UV elution profiles of a commercial *B. serrata* resin product and its effects on 5-LO product formation in ionophore-challenged PMNs. Elution profiles at 210 and 260 nm (top) of an ethanolic extract from tablets illustrate the elution positions of the following biologically active polycyclic triterpene derivatives: KBA (1), AKBA (2), TAs (3), and 3-*O*-acetyl-β-BA (4). Concentration-action relation diagrams show the paradox effects of tablet extracts on ionophore-challenged 5-LO product and 5(S)-HETE formations from endogenous substrate. Data are presented as mean picomoles/5×10^6 PMNs ($\pm$ S.D.; $n = 3$; ***$p < 0.001$).

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